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Jaanus Harro *Editor*

Psychiatric Vulnerability, Mood, and Anxiety Disorders

Tests and Models in Mice and Rats

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Psychiatric Vulnerability, Mood, and Anxiety Disorders

Tests and Models in Mice and Rats

Edited by

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Preface to the Series

Experimental life sciences have two basic foundations: concepts and tools. The Neuromethods series focuses on the tools and techniques unique to the investigation of the nervous system and excitable cells. It will not, however, shortchange the concept side of things as care has been taken to integrate these tools within the context of the concepts and questions under investigation. In this way, the series is unique in that it not only collects protocols but also includes theoretical background information and critiques which led to the methods and their development. Thus, it gives the reader a better understanding of the origin of the techniques and their potential future development. The Neuromethods publishing program strikes a balance between recent and exciting developments like those concerning new animal models of disease, imaging, *in vivo* methods, and more established techniques, including, for example, immunocytochemistry and electrophysiological technologies. New trainees in neurosciences still need a sound footing in these older methods in order to apply a critical approach to their results.

Under the guidance of its founders, Alan Boulton and Glen Baker, the Neuromethods series has been a success since its first volume published through Humana Press in 1985. The series continues to flourish through many changes over the years. It is now published under the umbrella of Springer Protocols. While methods involving brain research have changed a lot since the series started, the publishing environment and technology have changed even more radically. Neuromethods has the distinct layout and style of the Springer Protocols program, designed specifically for readability and ease of reference in a laboratory setting.

The careful application of methods is potentially the most important step in the process of scientific inquiry. In the past, new methodologies led the way in developing new disciplines in the biological and medical sciences. For example, physiology emerged out of anatomy in the nineteenth century by harnessing new methods based on the newly discovered phenomenon of electricity. Nowadays, the relationships between disciplines and methods are more complex. Methods are now widely shared between disciplines and research areas. New developments in electronic publishing make it possible for scientists that encounter new methods to quickly find sources of information electronically. The design of individual volumes and chapters in this series takes this new access technology into account. Springer Protocols makes it possible to download single protocols separately. In addition, Springer makes its print-on-demand technology available globally. A print copy can therefore be acquired quickly and for a competitive price anywhere in the world.

Saskatoon, SK, Canada *Wolfgang Walz*

Preface

Worldwide, mood and anxiety disorders place the largest burden on mental health, and in turn, disorders of the brain are the largest source of health loss across all diseases. Our ability to combat these psychiatric disorders has grown with each decade, and our understanding of the underlying genetics and neurobiology is improving and much facilitated by recent technological advances. Nonetheless, there is a major perceived unmet need in the management of mood and anxiety disorders and consequently a massive research effort, as reflected in the accelerating growth of the number of scientific publications. Such an expansive growth is also well observable in animal modelling literature. It is a both gratifying and worrisome trend, the former because animal models provide the necessary molecular and cellular precision, the latter because expansion can go with reduction of rigour in methodology. The appropriate use of methods is however ever important lest the disappointment of lay public, and drug regulation authorities with the perceived slow growth of innovation were attributed to animal models.

Additionally to the continued use of many traditional animal tests and models, technological conceptual advances have taken place since the two previous successful volumes edited by Todd Gould for Neuromethods. Several novel techniques now becoming commonplace in neuroscience will permit to elicit and monitor behaviour with high precision. These have also helped to raise the role of the rat as an experimental animal closer to the range it occupied before the rise of the mighty mouse. This also makes it necessary to reconsider the classic tests and models that have helped us to where we are now. Genetic studies in human patients and careful observation of animals in mood and anxiety models converge in the suggestion that vulnerability and resilience factors should be included into modeling. Thus, this collection of chapters will refresh the view on classic tests and models and proceed to include novel methods likely to aid in understanding mood and anxiety disorders and foster precision medicine. The authors are world-renowned scientists who have made important discoveries by means of using the models they describe in detail. Behaviour is complex, so the background sections bear particular importance. It is nevertheless the careful description of how to plan and conduct the experiments and how to interpret the results that distinguishes this book. God is in the detail.

Tartu, Estonia ***Jaanus Harro***

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Chapter 1

Animal Models of Mood and Anxiety Disorders: The Pursuit of Standardization and Recognition of the Complex Neurobiology of Human Mental Health

Jaanus Harro

Abstract

The research field of psychiatric animal modeling has ever been striving toward better validation of tests and models, and this includes attempts to standardize them. Nevertheless, animal models of mood and anxiety are used in many modifications. This is the outcome of a variety of objective reasons, ranging from the sensitivity of behavior to a large number of variables to the vagueness of constructs in clinical psychiatry. Animal modeling must maintain emphasis on reproducible experimenting, but that is likely to be aided by better understanding of dimensionality of symptoms, hierarchical nature of disorders of the brain, and their common roots that can be explicitly addressed by using vulnerability models.

Key words Mood and anxiety disorders, Tests and models, Animal behavior, Vulnerability, Neurobiology

1 Introduction

The *Neuromethods* series of books is serving the research community by providing detailed protocols for the most salient techniques for the study of the CNS and its disorders, often with insider notes that help to avoid pitfalls between the lines of text in the materials and methods section of the journal papers. Detailed methodological description and rigorous application of established good practice is quite similarly of paramount significance across all fields of science. Applying this standard to animal models of psychiatric disorders does present specific challenges, however, and the recognition of these challenges itself may not only make our predictions from mice to men more precise but also reveal important clues how to proceed in clinically based psychiatric research.

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There is no doubt that animal models have been most helpful to psychiatry in revealing the mechanism of action of the earliest modern psychotropic medicines, screening for novel lead molecules, and helping to establish the likely dose–effect curves. Yet, in recent years, the use of animal models in psychiatry has been criticized along several lines. These criticisms have ranged from doubts on the validity of often-used tests to the question of whether a complex human mental condition can be modeled in lower animals [1]. There are other viewpoints that can shape public attitudes, such as that the concept of psychiatric disorder is a fundamental fallacy or that all kinds of animals have mental inner life similar to humans, this rendering almost any kind of animal experiments unethical. Only by persistent professional approach to methods of behavioral neuroscience can the concerns that the most extreme ideas may bring about in lay public be mitigated. Let us address a few that are also pertinent to this collection of chapters on models of mood and anxiety disorders.

It should go without saying that, in science, tests and models must be valid. Validity of animal models has been a long-standing objective [2–5], and major advances have been made in the development of tests and models that are valuable in making predictions on the mental processes and efficacy of interventions in humans. Standardization of such tools obviously is a key issue, and usually papers reporting the results of animal studies respectfully cite the work of the original developers of the method and, if applicable, previous work from their own laboratories. At closer inspection it appears that methods that have obtained status are being used in many modifications, under unconventional conditions and with amendments that often are idiosyncratic [6]. Behavioral tests of mood are fairly complex in terms of design parameters that provide immense variability, and then, minor modifications have been reported to change the outcome [7]. Therefore, careful following of the established procedures in great detail is strongly suggested.

However, the research community is well aware of, and does not question, the principles of validation and methodological stringency, only it appears not to adhere to them faithfully. Why this is so? There may be real problems in two major categories. One burns down to feasibility: Not all materials and technologies that had been originally used are available and accessible at any laboratory and can be replaced with the expectation that one detail or another does not matter. Such a belief is often standing on a very weak ground but is fueled by the limitations that can be inherent to validation of standard models [8]. Notwithstanding the natural limitations to validations and standardization that make behavioral methods unique, it is advisable to proceed with careful following of the best described protocols. If these fail to work, it is prudent to ask why, and if a valid answer is found, it could even advance the field qualitatively.

There is a different issue with validation that is inherent to the psychiatric disorders themselves. Psychiatric disorders, including mood and anxiety disorders, are an unsteady target. The diagnostic systems do not include any objective record of physical symptoms (if not consider body weight changes). The disorder classification systems have been rewritten repeatedly. As these have increased in sophistication, discussions on the dimensional nature or hierarchical organization of symptoms and, thus, the underlying neurobiology are increasingly casting doubt on the feasibility of breakthrough discoveries if pursuing the present-day classification. Approaches to psychiatric disorders and their underlying neurobiology are shifting toward consideration of similarities at the symptom and also symptom group level between diagnostic entities and dynamics of the emergence of the disorder. Such influential reconceptualizations include the Hierarchical Taxonomy of Psychopathology (HiTOP) that seeks to cross what is seen as arbitrary boundaries between psychopathology and normality or between disorders, and remedy the issues with comorbidity, heterogeneity within disorders and limited reliability of making the diagnosis [9]. Studies using animal models must take these developments into consideration in facing the challenge to "tie the complex and poorly understood symptomatology in humans to observable behaviors in animals" [10].

Another major development that is bound to have implications to animal models, and their validation and standardization, is the developmental aspect of psychiatric disorders that has high complexity. A myriad of gene–environment interactions have been identified in the development of psychiatric disorders that may vary over life course. This dynamics is the likely source of mental health/illness trajectories not conforming to the categorical diagnoses that depend on real-time symptom-based criteria. Of note, the path toward mood disorders receives independent major contributions of early and recent life events [11], its neurobiology at the molecular level also being sex/gender sensitive to a highly important degree [12, 13]. Similarly, in animal studies, adverse events have profound sex-specific effects at the transcriptomic level [14]. This further emphasizes the importance of the sex factor to consider in animal behavior-based models [15], as the accumulating evidence suggests substantial differences between males and females in behavioral and neurobiological adaptive mechanisms to meet environmental demands [16]. Their origin appears to go far beyond the direct regulatory effects of gonadal hormones and involve trajectories shaped by the social environment.

So what and how can we learn from this for better animal models? Some of the suggestions may sound trivial, but the state of the art necessitates reiteration. For the beginning, we should not oversell the tests we conduct in laboratory animals as sufficient models of any complex human disorder of the brain. We can do

better in explaining what each test or model can do and what they cannot. (The latter may be an important thing to keep in mind if speaking to an overzealous science journalist.) Then, we must be precise on terminology within the community, too, regarding drug screening tests, assessment of (endo)phenotypes, as well as models with ambition to higher complexity. Here, higher precision should also be exercised regarding what is the specific object in psychology or psychiatry and what is the question about it that is being addressed by the selected technique. Next comes the standardization of methods, acknowledging that it begins with animal breeding and husbandry. While good care of laboratory animals is essential and ethical conduct in research is the very minimum of standard, we should also recognize that details of the handling and the whole environment in the animal house contribute to the animal model, this being critically important for the science of the organ with the highest complexity of organization. In behavior the sensitivity of the adaptive capacity is reflected in a highly dynamic way, and it is often forgotten that what we observe is ephemeral and situation bound, and, even if the aim is description of phenotype of, say, in a genetic model, what we record is the apparent characteristics of the so-called dramatype [17, 18]. Dramatypes are instable and may feed into irreproducible results if conditions vary, which is what they, across laboratories, inevitably do.

In recent critical overviews of the state of the art that acknowledge the inconsistencies in results obtained with animal models, suggestions for mending abound, such as to use behavioral test batteries, consider individual differences, sex, strain and stock, control for early life conditions, incorporate biomarkers, implement stringent success criteria for drug development, make use of technological advances, and carry out cross-species comparisons [19]. The near future of animal models in psychiatry will certainly hinge on the success of such considerations that are, however, challenging in their multitude, given that any additional stratification or comparison makes additional resources necessary. Therefore, strategic prioritization becomes increasingly vital. One promising strategy is offered by systematic endophenotype-based approach explicitly correlating research in animals and humans [20]. An animal model is likely to represent one or a few endophenotypes of the psychiatric disorder, and it is unlikely to recapitulate the whole psychopathology [21]. Endophenotypes support consideration of the dimensional aspects of psychopathology, and the affect-related endophenotypes of the Research Domain Criteria have animal equivalents owing to evolutionary continuity [22].

The search for endophenotypes that would be informative in animal models can benefit from the concepts of transdiagnostic and trans-syndromal approaches, addressing the continuum aspects in psychiatric disorders. The former has contributed the view that disorders of mental health comprise microphenotypes that ebb

and flow, until those remaining stable will take together the form of a syndrome or, more often than not, syndromes [23]. The readily changing and more persistent alterations also require separation in animal behavior and neurobiology. Endophenotypes that are being modelled must be persistent, adaptively meaningful entities.

At another dimension of categorization that appears helpful is stratification into vulnerability models and pathogenetic models and screening tests [1]. The concept of models of predisposition of depression or vulnerability models was introduced to the discussion on animal models of depression by Willner and Mitchell [24], suggesting models that "increase the ease with which an analogue of major depression may be evoked, or a presentation analogous to dysthymia (chronic mild depression)." Whether a specific model such as congenital learned helplessness or olfactory bulbectomy would be better labeled as a model of depression itself or vulnerability to depression would be a matter of debate, but in the light of the trans-syndromal nature of psychopathology in psychiatry, and especially so for mood and anxiety disorders, the vulnerability concept, while in need of clear definition, will be desirable. It will, however, also need reinforcement by using supportive translational evidence at the neurobiological level [25].

Focus on vulnerability in diathesis–stress models could help us in understanding how coping with the everyday "slings and arrows" fails in neurobiological terms. One hypothesis is that vulnerability may correspond to lower ability for complexity of the response of the brain. One whole-brain mapping study of neuronal activity by c-fos expression in mice demonstrated distinct patterns in helpless and resilient animals if submitted to the learned helplessness paradigm [26]. In these mice as well as in a separate positron emission tomography experiment in rats, a higher similarity between individual responses was observed in helpless animals. This evidence agrees with the network theory of psychopathology, positing causally connected psychopathological symptoms that can become self-sustaining and form a disorderly brain state [27]. A weakly connected symptom (and underlying neurobiology node) network is resilient and responds adaptively to external events, while a strongly connected network can sustain pathological activity if the environmental pressure is strong and overwhelming. Vulnerability models correspond to the requirements emerging from clinical evidence: genetic and developmental predisposition to the disorder that is precipitated by adverse life events, whereas the risk factors are not specific to any diagnostic entity and neither are the predispositions at the molecular level. The vulnerability concept is also well compatible with the hierarchical models of psychopathology that propose, e.g., a general factor of psychopathology and, at lower levels, several specific factors predictive of specific functional outcomes [28]. The general factor of psychopathology appears like the general vulnerability, and while, in the absence of agreement of

the preferred statistical model for the hierarchy of phenotypes and causal factors, the definitive psychopathological hierarchy trees are not yet available, it can be among the most legitimate targets in animal modeling.

Experimental induction of vulnerability can range from targeted genetic modifications [24] to attempts to achieve a certain neurobiological state, such as facilitating the depletion of monoamine stores or diminishing the capacity for replenishment of monoamines [29]; another vulnerability candidate mechanism appears to be the BDNF–trkB system [30]. It does, however, matter that every case of vulnerability is not an absolutely persistent trait. Windows of vulnerability, with reduced function of defensive measures, can occur in a dynamic manner. In humans, time frames associated with adolescence and young adulthood may represent this type of vulnerabilities.

Neurobiological findings in clinical disorders have often been examined in animal models, and recent larger-scale gene expression pattern data analyses suggest that such studies can reinforce our animal models [31]. A lesson from the early neuropsychopharmacology is how neuroscientific studies in clinical psychiatry were successfully guided by discoveries in animal experiments performed with the first serendipitously discovered medicines. Certainly translational approach can only benefit from becoming again truly bidirectional, so that neurobiological hypotheses derived from novel neurobiological findings in animal models were explicitly tested in humans.

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Chapter 2

The Open Field Test

Vootele Võikar and S. Clare Stanford

Abstract

The open field test (OFT) is the most widely used method for observing the behavior of mice and rats under laboratory conditions. It is used to evaluate the exploratory behavior, general locomotor activity and emotionality of rats and mice. However, it has to be kept in mind that these behaviors are not independent; they interact and so a change in one will affect another. Despite being a seemingly simple test, several caveats need to be acknowledged when selecting the variables for analysis and interpreting the data with regard to the aforementioned domains. The observed behaviors can depend on a number of procedural, environmental, and biological factors, which should be carefully considered when planning the experiment, as well as during the analysis and interpretation of the results. This review provides a critical overview of these factors, followed by some warnings and practical tips for conducting the OFT.

Key words Open field, Mouse, Rat, Anxiety, Exploration, Emotionality, Ambulation, Locomotor activity

1 Introduction

1.1 Background and Short Historical Overview

The open field test (OFT) is the most commonly used method for evaluating the spontaneous locomotor activity, emotionality, and exploratory behavior of rats and mice in a novel environment. The history of the OFT dates back to 1930s when Calvin Hall reported this method in a series of papers [1–3]. In Hall's experiments, the rat was exposed to a large, brightly-lit circular arena for 2 min, during which time the animal's ambulation was scored and fecal pellets counted. The test was repeated over several consecutive days, and these measures were interpreted as an index of the animals' emotionality, although what aspect of emotionality was being evaluated was not specified.

Since then, the OFT and other "novel environments" have been used extensively for studying emotionality and/or ambulatory activity of rats and mice. A comprehensive set of experiments was conducted by Peter L. Broadhurst for standardizing and further

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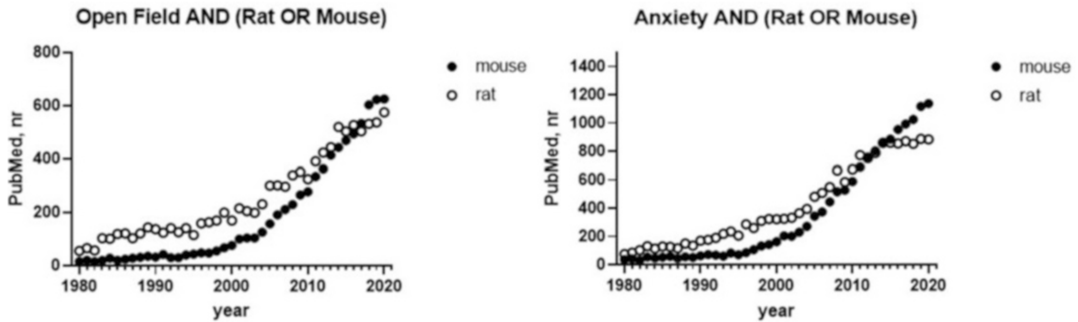


Fig. 1 The number of publications per year revealed in PubMed search (run on January 12, 2021) with the following keywords: "open field," "anxiety," "rat OR rats," "mouse OR mice." Note the prevalence of "rat" until the mid-1990s and the sharp increase for "mouse" since then (especially for "anxiety")

understanding of rat behavior in the open field [4–6]. He highlighted the importance of environmental variables, genetic differences, and the pretest history of animals. In 1954, Broadhurst started a selective breeding program, which resulted in the generation of the Maudsley reactive and nonreactive rat strains. The strain designations (reactive and nonreactive) were related to their defecation scores in the OFT [7]. In addition to studies of emotionality, these strains have been investigated extensively for many other behavioral domains and their underlying physiology [8, 9].

Since the 1950s, several decades of research have provided a wealth of material that have invigorated debates about what aspects of animals' behavior and emotionality are actually evaluated in the OFT [10–13], and the number of publications that have reported using the "open field test" has grown steadily, year on year (Fig. 1). This increased output, particularly over the last 25 years, largely reflects the use of the OFT in experiments aimed at characterizing the phenotype of genetically modified mice and as a high-throughput screen for the effects of pharmacological compounds [14]. In such studies, the test is often used to evaluate *either* spontaneous locomotor activity *or* emotional behavior [15–17]. However, that is problematic because locomotor activity and emotional behavior are not independent variables, and a change in one of these aspects of behavior will inevitably affect the other (see below).

In her book, "What's wrong with my mouse," Jacqueline Crawley strongly recommended a close collaboration with experts in behavioral neuroscience for carrying out behavioral phenotyping of knockout and genetically modified mice [18]. Despite that warning, concerns have been expressed about potential "misuse" of the test, particularly through oversimplification and misinterpretation of the results, which arises from ignoring important fundamental factors that have been identified over decades of research [19–22]. Moreover, it has been argued even more strongly that

"widely used assays such as the open-field test are performed at the wrong time, for inadequate durations and using inappropriate mouse strains" [23].

1.2 Ethogram in the Open Field

When studying changes in animals' behavior following an experimental challenge (such as drug administration, neuronal lesion, or genetic alteration), an important first step is to be confident about what counts as normal (baseline) behavior of the test species in the context of the experiment. That rests on the assembly of a profile of the full behavioral repertoire of the animal (ethogram)—not simply focusing on the single aspect of behavior that is of particular interest.

As with many other behavioral tests, the OFT was initially developed and validated for the rat. Yet, as has often been commented before, mice are not little rats. Indeed, there are important differences between the two species [24], and many of these differences depend on the test procedure, the environment, and the behavioral parameters that are measured [25, 26]. Unfortunately, the richness of species-specific behavior is often ignored, but consideration of the animals' full behavioral repertoire is essential, if the aim of the experiment is to draw conclusions about its emotional status, locomotor activity, or exploratory motivation [27, 28]. In short, extreme caution is needed when adjusting any procedure that was developed using rats for studies of the mouse. Regardless of any differences in the innate behavior of mice and rats, the basic procedure(s) for measuring exploratory and approach-avoidance (anxiety-like) behavior in the OFT and other novel arenas are basically similar for both species [29, 30].

Tests for exploratory activity can be broadly divided into two main categories [31]. The forced exploration paradigm is the more common and involves exposing the animal to the novel arena, from the beginning of the experiment, without any possibility of escape. By contrast, in a "free exploration" protocol, the animal has the option to enter the arena from its home cage, or other safe and familiar "container," which is attached to, or placed within, the test arena. Some experimenters combine the OFT with object exploration tests, by placing an unfamiliar object in the center of the arena and measuring the latency of the animal to approach the novel object and the time spent investigating it.

Whichever approach is adopted, the duration of the OFT is highly variable—from 2–5 min up to 60–90 min. Yet, animals typically stop moving around the open field after about 20 min. However, there are exceptions: some strains exhibit specific behavioral profiles, which become evident with prolonged exposure. For instance, the BALB/c mice show high avoidance in exploring the large open field arena during the initial 30 min of the test, followed by increased activity, by comparison with first-generation, captive wild (feral) mice [23, 32].

Longer periods of testing are usually applied when measuring drug effects (e.g., sedation, arousal) but, again, different mouse or rat strains exhibit different response profiles [33–35]. A longer duration of single exposure, or repeated testing, is usually interpreted as leading to habituation to the novel arena: i.e., it is assumed that the environment becomes familiar and no longer provokes exploration. However, there is rarely a confirmation of that assumption, which must be assessed empirically, such as by monitoring changes in plasma corticosteroid or catecholamine concentrations. It should be borne in mind that, without that information, a reduction in exploration of the arena could indicate sensitization (quasi-freezing) rather than habituation.

Paradoxically, the major trap for measuring and interpreting the behavior of a mouse or rat in the OFT lies in the perceived simplicity of the test. The most commonly reported parameters in the OFT are locomotor activity (ambulation, measured as total distance travelled) and time spent in the center of the arena. Avoidance of the center and increased time spent close to the wall (thigmotaxis) are used as an index of "anxiety-like" behavior [36]. This is despite evidence that drugs with established antianxiety effects in humans have inconsistent effects on rodent behavior in the OFT, *especially* in respect of their activity in the center of the field (e.g., [37]). This inconsistency was underlined by a comprehensive review, which revealed that only 56% of published studies (i.e., barely more than chance) reported an increase in animals' activity in the center of the field after treatment with antianxiety drug [14]. These reports call into question the validity of the assumption that an increase in center-field activity (or a reduction in thigmotaxis) reflects a reduction in animals' anxiety status.

In connection with this, a key point for interpretation of the behavioral measures in the OFT is the problem that locomotor activity and emotionality are not independent variables: a change in one will affect the other. This leads to the problem that center-field activity will depend on the animals' total activity in the OFT. The key parameter is the proportion of the animals' activity that is directed toward the center of the field [38], which is often not taken into account. Similarly, some aspects of the animals' behavior can be mutually exclusive: for instance, animals cannot move around the arena while grooming, scratching, or rearing. This confound can have important consequences because some experimental challenges induce changes in some aspects of animals' behavior, but not others [39, 40].

Another factor to consider is that ambulation in the environment is not always exploratory, whereas other behaviors (e.g., sniffing and rearing) can certainly involve an exploratory component, even though the animal is not moving around the arena. Also, both mice and rats can establish a home base in novel environment: a specific location from where the exploratory trips or excursions are made into the remaining environment [41, 42].

Many of these problems can be ameliorated by measuring multiple ethological parameters, to build up a full profile of the animals' behavior in the OFT. Detailed attention to the recorded pattern of all the animals' movements in the novel arena (not just ambulation) can greatly inform inferences about the structure and interpretation of exploratory behavior. This profile would include behaviors such as follows: rearing [43–46], grooming [47–52], sniffing, risk assessment (stretched attend postures and flat-back approach), tail rattling, freezing and jumping—all of which are interpreted as providing information on animal behavior—their emotional states, exploratory activity and motivation, curiosity, and fear [53, 54].

Observing and recording a comprehensive ethogram of animal's behavior with score-sheets is the most important and valuable approach for its characterization [55–57]. However, manual scoring of behavior is a time-consuming, labor-intensive work, which is prone to errors and bias. With the increasing number of experiments, there has been progressively more pressure to automate the process through video recording and computerized evaluation of the behavior(s). Even so, errors in detection can arise due to improper calibration of the equipment for animal size or light conditions (reviewed in [58]), for example, and so the raw data and videos should be always checked for the quality of recording.

A current limitation of automated recording is that it is not ideal for counting multiple ethological parameters. To date, the behavioral parameters that are scored are typically restricted to the distance travelled by the animal and time spent in different zones of the arena. This precludes the possibility of taking into account other aspects of the animals' behavior that could confound interpretation of the results (as explained above).

A major breakthrough in recent years has been the development of artificial intelligence and deep learning methods as promising solutions for improved scoring of behavior expressed by animals in test situations [59–63]. Machine learning approaches bring enormous potential for automated, precise, and unbiased scoring (notwithstanding the limitation that the (supervised) learning requires annotation of behaviors by a human) or the exploration and detection of novel behavioral sequences and patterns (unsupervised learning). The technology is growing and developing rapidly, but it is not without challenges. Indeed, the large number of recorded variables definitely complicates the analysis and interpretation of the data. Large amounts of complex data will require advanced expertise in the dedicated areas of data mining. For an interesting example of a research project that involves gathering extensive data on the spontaneous behavior of group-housed mice in the home cage, see [64] and https://www.zooniverse.org/projects/r-dot-bains/rodent-little-brother-secret-lives-of-mice/about/research. The successful development of this

approach will also depend on sharing the codes and algorithms, validation, and standardization of behavioral annotations (for different strains, test environments etc.), followed by uptake of these novel and complex methodologies by behavioral neuroscientists. These challenges are discussed comprehensively in [62].

In short, novel methods and advances in the technology for recording animal behavior are most welcome, but there is much work to do in terms of developing the meaningful ethological analysis that is needed for a valid interpretation of the data-sets. Moreover, these should incorporate not only fine movements that involve specific body points but also complex behaviors that are affected by animals' motor function, exploratory motivation, and emotional status.

1.3 Biological Factors: Sex and Genetic Background

For a long time, it has been common practice to use only male rodents for behavioral experiments, because of the belief that the estrous cycle in females increases the variability of their behavior [65, 66]. This view was reinforced by many studies that consistently demonstrated sex differences in the behavior of male and female rats in the open field test (and other behavioral tests), with females being more active than males [4, 67–69].

However, it appears that the sex differences in mice are not so robust and may depend on many different factors (including strain, age, test conditions, etc.) [33, 70–72]. Importantly, several recent studies, including meta-analysis of published reports, cast doubt on the view that the behavior of females is more variable than that of males, [73–77]. These findings do not confirm that concerns about sex differences in rats were unjustified. Rather, they provide a strong justification for using both sexes in basic research as a matter of routine, especially considering the fact that the prevalence of many human disorders shows clear sex differences too [78, 79]. Whatever the case, the National Institutes of Health (USA) and other funding agencies now expect the sex of the animals to be a variable that should be factored into the research design, analyses, and reporting of all experiments [80, 81].

Genetic background of the experimental animals is another biological determinant affecting the observed phenotype. In principle, laboratory rodents are divided into outbred stocks (heterogeneous populations) and inbred (isogenic) strains. It is interesting to note that the majority of rats used in biomedical research are regarded as outbred, whereas mice are mostly used as inbred strains. Also, there have been differences in standard practice that relate to specific scientific disciplines: e.g., in psychology and toxicology research, the use of outbred, heterogeneous animals has predominated.

There is no single or straightforward answer to the question of which genetic background should be used. Rather, the research question or hypothesis must inform the decision on which is the

most suitable species and strain. Recent studies, which provide some evidence that outbred stocks are not necessarily more phenotypically variable than inbred strains [82], have added a further complicating factor. Surely, inbred strains provide a stable system for studying the effects of single genes (in the case of genetic engineering) or endophenotypes, or other experimental manipulations. For generalization of the outcome (external validation), which is essential for successful translation into humans, it is suggested that the experiment should include more than one inbred strain in the design [83, 84], or use an outbred strain. If the panel of inbred strains is used for measuring any trait, the strain distribution can be revealed [85, 86]. However, knowing the typical characteristics of each strain is needed for determining possible ceiling or floor effects [87] which could confound the interpretation of the results.

1.4 Environmental and Procedural Factors

The list of variables that can affect the behavior of animals in an open field and other tests of exploratory behavior is not complete without considering the test situation itself: the characteristics of the techniques and apparatus, the testing environment, and the procedural details [88]. All such factors and details should be considered during the planning phase and taken into account when designing the experiments.

First of all, housing and husbandry conditions play a critical role [89]. For instance, single or group housing, open or individually ventilated cages, and nesting material all strongly influence the behavioral phenotype [90–92]. There is a great deal of evidence that the frequency and method of handling can also influence the behavior and underlying physiology of experimental animals [93–99]. There is even evidence that the sex of the experimenter can affect the stress response in mice [100], although the systematic follow-up studies to confirm that finding are lacking.

Historically, many experiments applied only a single test procedure to study individual animals. However, both the development of genetically modified mice and the increased attention to reducing unnecessary use of animals have led to the recommended use of test batteries for behavioral phenotyping [101]. Although reducing the number of animals used, this approach gives rise to the problem that animals' history of previous testing (including the order of tests) can affect the experimental outcome [102–106]. In general, if testing involves a battery of several tests, the series should start with the assessment of behavioral domains that are more influenced by repetitive exposures, handling, and other manipulations. In other words, the natural exploratory drive is studied first, using conventional approach-avoidance tests (e.g., open field, elevated plus (or zero) maze, light-dark box), followed by testing the domains that are thought to be less vulnerable to extensive

handling (e.g., motor abilities) or those that even require it (e.g., adaptation to human–animal interaction, which is needed for performing experiments that test cognitive performance).

Additional factors that can influence the behavior in OFT (and other behavioral experiments) and need to be accounted for at the stage of planning and design are outlined below. Importantly, all these details should be explicitly reported in publications arising from the work, because they are critical for objective appraisal of the work, especially when comparing the results with those from other studies.

As well as factors, such as the design and construction of the arena and its illumination, which are discussed in detail (below), several other factors can affect the behavior of animals in an open field:

- *Experimental procedures*, such as injections [107] and the choice of appropriate control animals. These could include treatment naïve animals, positive controls, and negative controls, which could comprise sham-lesioned or vehicle/saline-injected animals.
- *The presence of experimenter* [100, 108, 109]. It is strongly recommended that the experimenter should be as far away as possible from the animal during the experiment (behind the curtains or even in the neighboring room). Odors in the room (e.g., perfumes) should be avoided as much as possible, by the use of protective clothing, at least.
- *The circadian time of testing*. Mice and rats are nocturnal animals: because their activity is naturally higher during dark period, it would be preferable to test the animals during their active period by applying reversed or shifted light-dark cycle. Unfortunately, this is often not possible. In fact, testing either during dark or light period has shown that even if discrimination of strain differences can be better during the dark period, some measures are not affected at all, and spatial novelty is equally arousing regardless of circadian phase [110–113]. Nevertheless, circadian rhythms should be taken into account, and testing should be carried out approximately at the same time each day, or a randomized block design applied, whereby the time of testing is taken into account in the experimental design and statistical analysis of the results [114–116]. On the other hand, it is possible that the experimental intervention causes a phase shift in the animals' circadian rhythm, in which case, testing at the same time of day could produce a misleading snapshot of the behavioral phenotype [117]. Also, assessment of treatment-induced changes animals' spontaneous locomotor activity should not be based solely on its evaluation in the OFT and needs confirmation by monitoring the animals' activity in the home cage [118].

– *Test duration*. Longer testing may reveal behavioral patterns (habituation, sensitization), which can otherwise remain undetected [23, 119, 120].

1.5 Standardization and Reproducibility

How to deal with the long list of factors that may confound the outcome of any behavioral experiment? For many years, there has been considerable debate over the benefits versus the drawbacks of rigorous standardization of experimental procedures: in particular, how standardization might improve reproducibility [121–126]. However, a seminal study by Crabbe, Wahlsten and Dudek drew attention to the problem that extensive standardization of the equipment and methods rendered a high possibility for idiosyncratic findings [127]. This is because findings that emerge only under stringently controlled experimental conditions are unlikely to generalize to the animals' real world or be of translational significance. There may be many factors that underlie discrepant results, when comparing findings from studies within, or between laboratories, but there are several studies showing that the person carrying out the experiments may be a major source of variability [109]. On the other hand, that same study also showed the importance of detailed reporting of methods and scrutiny of the data analysis—in line with factors that the ARRIVE guidelines currently promote [128].

Scientific validity of preclinical research and animal experiments should be built on three pillars—construct validity, internal validity, and external validity [129]. Internal validity rests on items listed in the Essential 10 of ARRIVE guidelines (see below) and, in principle, ensures the quality of study conduct, its reliability, and consistency of the procedures. However, external validity should be achieved either by controlled and systematic heterogenization of study conditions (e.g., differential housing) by using a randomized block design or by replication of any findings in multi-laboratory studies [116, 123, 130].

To that end, the core facilities are major instruments for standardizing both the environment and the procedures for behavioral assessment [131–134]. In addition to validating and keeping a record of key test conditions, control experiments (to provide the reference baseline and to ensure that the data are of high quality) should be performed [133, 135]. Most importantly, staff working in the core facilities should be able and willing to support and advise researchers who may not have the necessary expertise with the methods they plan to use.

1.6 Resources for Rigor and Reproducibility

Unfortunately, several surveys have found a serious lack of rigor in respect of the quality of reporting of important details that can affect the results of the experiment, such as those discussed above [136, 137]. The ARRIVE guidelines were published in 2010 [138]

in order to help resolve this problem and improve the reporting of animal research. However, despite endorsement of the guidelines by hundreds of journals, ARRIVE did not have the expected beneficial effect on the quality of research publications [139].

As a further remedy, a revised version of the guidelines (ARRIVE 2.0) was published [140], together with extensive accompanying documentation, which explained and elaborated on the revisions [141]. The major change in ARRIVE 2.0 is the categorization of items that should be included in research reports into two sets: an "Essential 10" and a "Recommended." The Essential 10 comprises many items that are regarded as fundamental to the basics of the experimental design, whereas the Recommended list includes factors that might not be relevant for all types of experiments using animals. Conversely, it is notable that ARRIVE 2.0 has placed many of the items, discussed above, under the "Recommended" category, but it is important to note that this information should be regarded as mandatory when reporting behavioral research.

There are several other excellent resources that can help deal with all these variables when planning experiments using laboratory animals [142, 143], all of which similarly consider the minimum requirements for reporting the in vivo research [144]. Another valuable handbook, covering what is known about the genetic underpinnings of naturally occurring, non-pathological, individual differences in behavior, can be highly recommended for researchers who are interested in mice and their behavior [145] as can another in-depth book, which focuses on many practical aspects of mouse behavioral testing [58]. The legal and ethical aspects of animal experimentation have also been recently reviewed by [146]. Researchers might also find it helpful to consult the Experimental Design Assistant (EDA, available at https://www.nc3rs.org.uk/experimental-design-assistant-eda)—a novel tool bringing together machine-readable flow diagrams and computer-based logical reasoning to assist with the generation of robust and reproducible designs for animal experiments [147]. The contents of all these guidelines serve to emphasize how, even with a test as apparently simple as the OFT, the preparation of the procedure and interpretation of the findings requires full attention to every detail, including the full life history of the animals, which can affect the outcome of behavioral testing.

It should be recognized that the success of basic, preclinical studies and hope for translating the outcomes to clinical research is not possible if the challenges in the design and reporting of animal experiments are not addressed fully and satisfactorily. In this context, an excellent comprehensive review about a wide range of factors that could affect behavioral phenotyping, written in 2010, posed an apposite question in the title: "How many ways can mouse behavioural experiments go wrong?" [148].

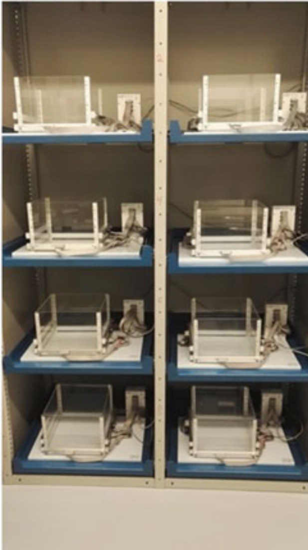
2 Materials

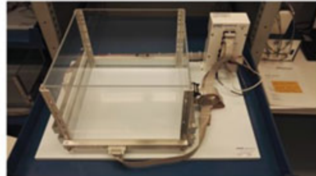
- *Recording Equipment and Setup* (see Fig. 2).

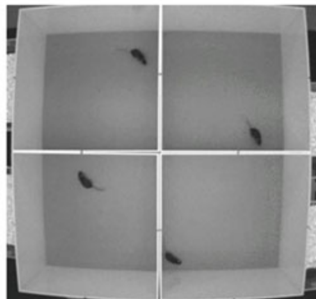
These days, tracking and recording of animals' movements is usually done either by infrared photocells or video-tracking systems. If infrared sensors are being used, they should be placed at a height that is appropriate for the size of the test animals. Infrared detectors are sometimes arranged in two levels, in which case the lower array of infrared sensors detects the ambulation and the second higher level records rearing events (vertical activity).

Video-tracking with a top-down camera requires a good contrast between the test subject and the background [149]. Infrared lights with IR-sensitive camera can be used for enhancing the detection. Additional equipment for refining commercially available systems is available for improving the analysis of video-tracking [150–152], and a number of open-source software platforms for tracking the animals are now available [153–156].

Both systems calculate and store the X–Y-coordinates of the center point (geometric center) of an animal, and, based on this information and calibration, its movement is calculated. On the basis of the shape, size, and mobility of the detected image, it may







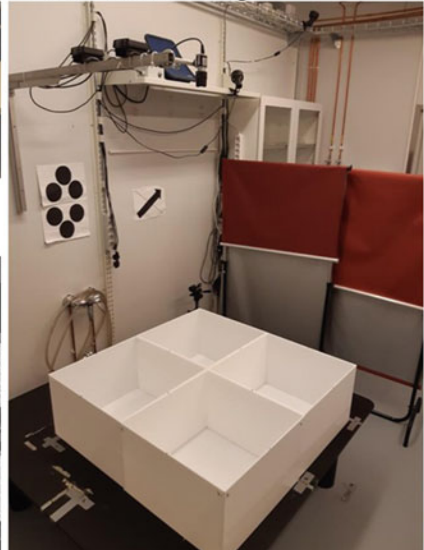


Fig. 2 Two configurations of open field setup at the Neuroscience Center, University of Helsinki. Eight arenas (30 × 30 cm floor) are surrounded by infrared beam arrays at two heights, for detecting horizontal (locomotor) and vertical (rearing) activity (Med Associates, St. Albans, VT)
Another setup consists of four custom-built open field arenas (50 × 50 cm floor) for use with a video-tracking system (EthoVision, Noldus Information Technology, Wageningen, the Netherlands)

be possible to count other behaviors, also: e.g., rearing behavior or freezing. In addition, the tracking systems may have a feature for manual scoring, meaning that some actions can be recorded during live-tracking or from a video file.

- *Physical Specifications of the OF.*

Larger arenas with a dark floor and low light intensity promote locomotion and exploration [93, 113, 157–160]. The early open field arenas were usually circular, but this is less common nowadays, probably because square or rectangular arenas are easier to construct. However, square arenas suffer the problem that animals will prefer the corners of the arena and are likely to show less exploratory activity in other regions of the arena. The walls should be high enough to prevent animals jumping out of the arena. The minimum floor area for mouse is 30×30 cm, and for rat 50×50 cm, but larger arenas are recommended as they will be more sensitive for detecting avoidance of the central region. For video-tracking, the recommended background (floor) is ideally of gray, nonreflective material, so that there is no light glare at floor level and both albino and pigmented animals can readily be detected.

- *Environment: Illumination, Ambient Noise.*

It is essential that the intensity of illumination is the same in all regions of the arena and that this is checked at floor level, where the animal will be. This can be difficult to achieve with square arenas, especially if they are constructed from shiny, reflective material (e.g., black Perspex). Also, square arenas are vulnerable to producing shadows in the corners, which will not only affect the behavior of the animals but can pose problem for accurate detection of the animals by video-tracking. The intensity of the illumination can be modified, empirically, according to the protocol (brighter light is generally aversive and can reduce exploratory activity), and so the light intensity at the floor level should be always reported.

Ambient noise at normal levels (<85 dB) has not been shown to influence the behavior critically [88, 161]. However, the environment for behavioral experiments should be protected against major interruptions (e.g., sudden noise bursts, vibration, or accidental disturbance by unrelated people).

3 Methods

Several step-by-step protocols for the OFT have been published [162, 163]. Despite that, the range of procedures in the literature shows that there is no universally accepted protocol. Our aim here is not to prescribe a preferred procedure: rather, we strongly emphasize the importance of attention to details of all the variables

discussed in the previous sections that can affect the animals' behavior in the OFT. We argue that every aspect of an animal's biology, life history, and environment will affect the results of the experiment. Therefore, a good knowledge and understanding of laboratory animal science, behavioral neuroscience, and experimental design are mandatory elements of training before starting with behavioral testing [164]. However, based on the hypothesis and study questions, standard operating procedures should be written and followed, with all the changes to those procedures documented fully and published transparently.

3.1 Procedure

- Prepare the animals and protocols—pay attention to identification, blinding, and randomization (the order of testing, counterbalancing the groups between arenas if more than one arena is used).
- If automated recording is used (video-tracking or infrared detection), then calibration and proper connection of hardware must be verified routinely.
- After taking the animals from the colony room to the test area, give them time to adapt (at least 30 min between transfer and start of test).
- Place the animal in the arena and check that the recording has started. Placement of the animal in either the corner or center of the arena can influence the resulting behavior. When placed in the center, some animals freeze before starting to move. One can measure the latency to move away from the center as "escape performance." When placed in the corner or close to the wall, the animals will most likely start moving along the wall (thigmotaxis), and it will be possible to measure the latency to enter the center (exposed) area.
- Monitor the animals and the system (remotely) throughout the experiment even if the recording is fully automated.
- After completing the trial, remove the animal from the arena and count the number of fecal boli. The latter variable may be indicative of emotionality (if the test duration was short, up to 10 min), but it can depend also on other factors (e.g., diet, time elapsed since the last eating).
- Clean the arena—not only different detergents have been used but also ethanol (5–70%) or water [165].
- At the end of the test, do not return the tested animal to the home cage (i.e., cage-mates) until all the animals from the same cage have been tested. It may be important to check for the order effects on behavior (possible differences between the first and last animal tested from the same cage). This can be achieved by incorporating each animal's position in the sequence of tests as a factor in the statistical analysis.

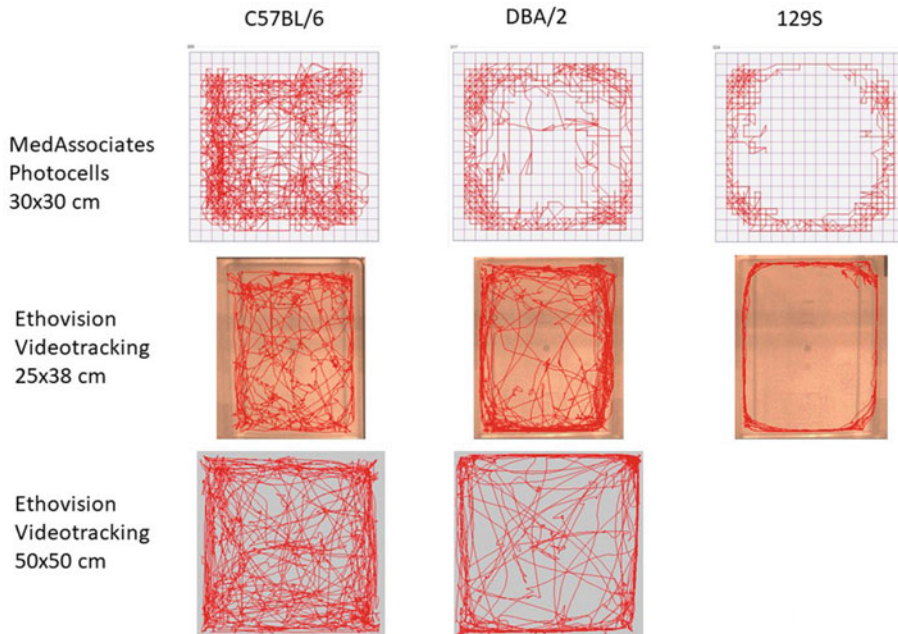


Fig. 3 The typical patterns of activity displayed by three commonly used inbred mouse strains, measured either by using infrared sensors or video-tracking, in arenas of different sizes. Note the difference between the strains in the density of the tracks in the center of the arena (C57BL/6 > DBA/2 > 129S2). The data are unpublished and derived from pilot experiments carried out at the University of Helsinki (e.g., a discussion on strain and equipment differences can be found in Ref. [160])

3.2 Results

These depend largely on the biological and environmental factors. Figure 3 shows representative tracks for different inbred mouse strains in the (square) open field. Proper use of positive and negative controls and strains with known behavioral characteristics will help to confirm that the results are reliable.

The following data are usually collected for analysis: total distance moved, proportion of distance and/or time in the center area (reference could be around 10–25% of total), number of rearings (distinguishing those that are supported by contact with the wall from those that are unsupported, in the center of the arena), speed of movement in periphery and center; time resting or not moving; and any ethological parameters presented previously.

Data analysis is usually done by Analysis of Variance between the groups (with treatment or genotype as a between subjects' factor, for instance). However, the results from the test can be split into time-bins (2–5 min, depending on the total duration) for analysis by repeated measures ANOVA to explore whether the influence of the factor(s) of interest (e.g., treatment or genotype) on animals' behavior(s) change with time in the arena. However, for a comprehensive interpretation of the data, it is important to keep in mind that the behaviors are not independent of each other,

and so factorial ANOVA should be used only when justified by a multivariate analysis, which should be carried out first (see, e.g., example [166]).

3.3 Further Factors to Consider in the Experimental Design and Data Analysis

- *Biological variables*: e.g., species, strain, sex, age (discussed above).
- *Environmental variables*: e.g., handling, housing and experimental conditions, testing history (discussed above).

To summarize, we fully agree with a recent viewpoint by Lisa Genzel: "In sum, behaviour is not a simple tool or measurement such as measuring the activity of individual neurons or a brain area. Instead, we should always keep in mind that behaviour is the outcome of many different factors. Thus, for one, we should be aware of these factors, keep track of them, and, ideally, control for them as best as we can." [164]. Moreover, the behavior of an animal in any test situation should be described in terms of exactly what it is without unjustified anthropomorphic interpretation [167, 168].

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Chapter 3

The Light–Dark Box Test in the Mouse

Solal Bloch and Catherine Belzung

Abstract

The light–dark box test is frequently used in rodents to assess anxiety-like behavior. The apparatus consists of a dark and a bright lit compartment. It relies on the aversion for brightly illuminated open spaces, more anxious animals being expected to prefer the more protected dark compartment. The origin, rationale, and methodological variations are further discussed in the chapter. We provide a detailed procedure addressing various environmental variables from housing conditions to data interpretations to successfully set up the test in any laboratory setting and provide representative results. The light–dark box is easy to use and implement. As observed with benzodiazepines and some antidepressants, it can be used for pharmacological studies to investigate potential anxiolytic or anxiogenic effects of drugs of interest.

Key words Light–dark box, Anxiety-like behavior, Mouse, Anxiolytic, Anxiogenic, Exploration

1 Background and History

Like other tests measuring anxiety behavior in laboratory rodents, such as the open field or the elevated plus maze, the light–dark box test (LDB) is based on the stressful exposure to a new place, in which the animal is given the choice to stay in a more protected and less stressful part (the dark box) and a brightly lit and more exposed environment (the light box). The subject can be placed in the light (which is more common) or the dark compartment at the beginning of the test. The latency to first transition as well as the number of transitions and the exploration of the light compartment are typically measured as a reflection of anxiety behavior, more anxious animals being supposed to prefer to stay in safer places, like the dark compartment.

Historically, the LDB was preceded by different emergence tests in rats and mice [1] using comparable measures, the first instances dating back as far as the 1930s from our knowledge of the literature [2, 3]. Likewise, the latency to exit was the main measure, and the start compartment could be an opened home cage or an unfamiliar start box [4, 5].

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The original idea of the LDB was to exploit the innate aversion of mice for unfamiliar environments (neophobia) and bright light in a test simple to implement and not requiring previous training [6, 7]. In this initial instance, the mice were placed in the light compartment of a box covered one-third in black paint, but the size, proportions, and durations have been modified in different ways over time [8]. In most instances, the box consists of two compartments of identical size (half/half) or of a dark compartment twice smaller than the light compartment (one-third/two-thirds), and mice are placed in the light compartment to start the test session. A study directly comparing both setups, manual and automatic tracking, as well as start sites shows that anxiety-like behaviors can be observed within all those variations [9]. However, albeit overall strain differences are maintained, there can be interactions between strains and behavioral measures following some modifications such as start site or luminosity, hence the need to adapt the setup carefully to the experiment.

2 Material and Setup

N.B: This is one example of a procedure we use in our laboratory. However, as mentioned above, the material (size/proportions of the box etc.) and procedure (length, start compartment...) can vary and be easily adapted according to one's own lab settings. However, the global workflow and measures remain the same. The LDB setup presented in Fig. 1 closely resembles previously used ones [10, 11].

This LDB consists of a light box (20 × 20 cm) brightly lit (e.g., 1000 lux) and a dark box with the same dimensions. The two compartments are linked by a 5.5-cm-wide circular hole, in which a tunnel can be easily adapted if need be. Dark boxes are covered with an opaque lid, and the lit box is covered by a transparent top (Fig. 1). It is preferable that both boxes are covered during testing, in order to limit interference with odors from the outside. Luminosity should be measured in the light compartment with a luxmeter before each behavioral session. In our setup the plastic has a clear off-white color because we use C57BL6/J animals: this allows a contrast between the black fur and the background, which is ideal for automated video tracking and distinguishing the animal. If using a strain with a light fur, you may, in turn, consider using dark plastic.

For behavioral analysis, we recommend a behavioral tracking software (such as EthoVision) in order to obtain reliable results more rapidly. To set up tracking analysis, a camera should be placed above the center of the light compartment to track the distance travelled, time spent, and transitions to this compartment. Video recording also allows other measures of stress, such as freezing, or

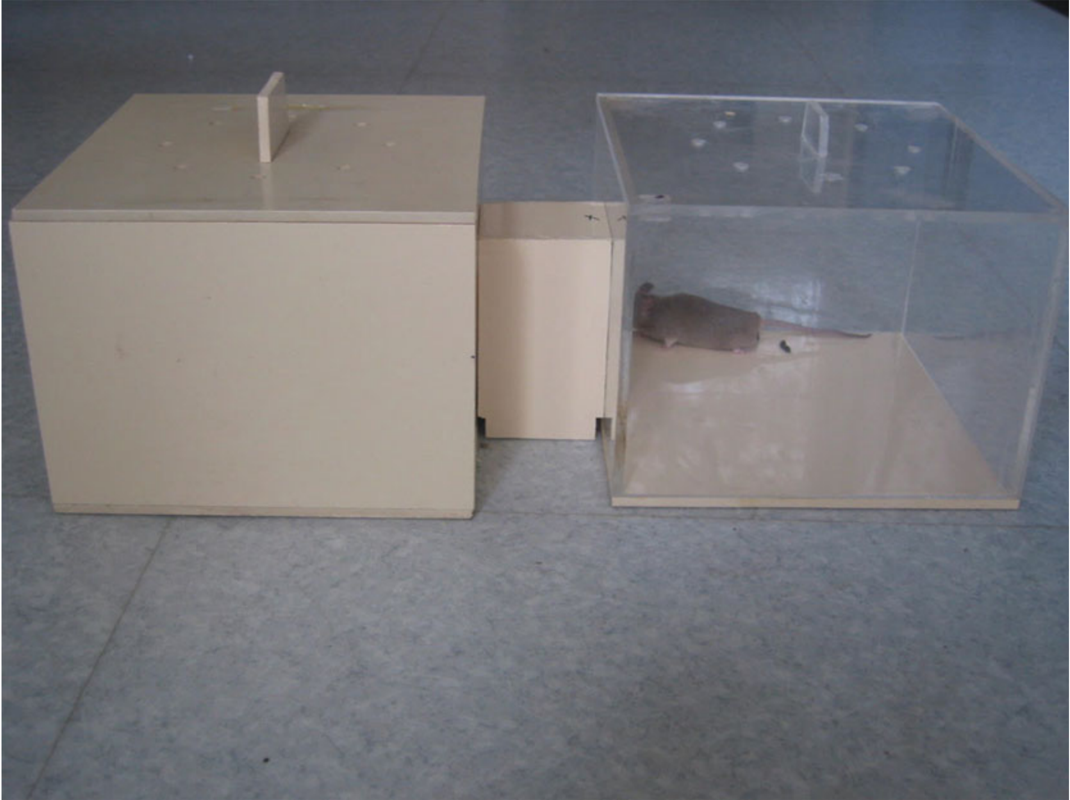

Fig. 1 An example of light–dark box. This is an apparatus with two compartments of equal size. Ideally, a translucid lid can be placed on top of the light compartment

exploration, such as rearing. In order to count a transition and the presence in the light compartment, the animal should have the four paws in the light compartment. In other words, head dips or elongations of the body and forepaws should not be computed in the time, distance, and transitions to the light compartment. Some authors also record the total distance including the dark compartment, thanks to a lid opaque to visible light but permeable to infrared [12, 13].

Alternatively, if video tracking is not available, manual tracking with three chronometers can be used to measure the main behavioral parameters of the test: the time in each compartment with the two first and the total time with the third. Transitions are also scored manually in this case following the same rules as with automated tracking.

3 Procedure

If the experimental protocol allows it, we recommend rearing the mice in social same-sex groups with physical enrichment (such as small tunnels, small igloos, nesting, and gnawing material such as

cardboard and paper), in reversed light cycle, in order to have basally unstressed animals. We prefer to perform behavioral tests during the dark cycle, as it is the normal activity period of rodents. The room in which behavioral tests are performed should be distinct and isolated from the housing facility in order to provide controlled conditions and not to disturb animals not tested. Mice are habituated to the behavioral room at least one hour before the start of the test with ambient light conditions of the behavioral room to minimize the stress induced by cage handling and abrupt changes in luminosity.

Before starting, the LDB floors and walls should be cleaned with 70% ethanol. This should be repeated between each mouse. If automated tracking is available, several LDB can be tracked at the same time by different cameras (2 arenas/camera); in this case the animals are hidden from one another.

One mouse is individually introduced in one of the far corners of the dark box (opposite to the hole, *see* **Note 1**). Although it is more common to place the mouse in the light box for start, we prefer to use the dark compartment as a start site to match the light cycle and to measure the emergence from a less stressful dark compartment to a more anxiogenic one (*see* **Note 2**). This choice is also justified by the strain C57BL6/J mice, typically exhibiting less anxiety behaviors than others, and by previous results obtained with this strain in a specific stress paradigm [12]. If automated tracking is available as in other tests, it is capital that every single mouse has the same start location for the test. Immediately, the lid is placed back on top of the dark compartment and the behavioral tracking starts (*see* **Note 3**). Since the latency is a key measure of the test, it is important that the behavioral tracking (either automated or manual) starts immediately after the animal is placed in the box and the lid back on top.

The behavior is monitored for 5 min, key measures being the latency to first entry, the number of crosses, time spent in each zone, and distance traveled in the light compartment. The animal is then returned to its home cage.

4 Anticipated Results

This test is based on (1) the aversion mice spontaneously exhibit for new environments into which they have been forced and on (2) their natural aversion for brightly lit spaces. The aversion for unknown places induces a motivation to escape from this situation: therefore, mice might display a tendency to move in the lit as well as in the dark box, in order to find a way to get out of the device. At the same time, mice try to avoid the brightly lit space and, therefore, might prefer the dark side of the apparatus. Because of the abovementioned (1) and (2), mice are exposed to an

unconditioned conflict between (1) entering the lit box to escape from the whole situation and (2) avoiding the lit box for which they display strong aversion. Anticipated results are, therefore, that mice display a strong preference for the dark box but might exhibit some entries in the lit box as well. Mice might, however, rapidly learn that the lit box does not offer the possibility to shield from the whole device: therefore, after 10–15 min, the incentive to enter in the lit box might decrease, which will translate in a decrease of the time spent in the lit box and in the number of transitions to the lit box with time. This is why the duration of the testing usually does not exceed 5 min. Another aspect relies on the intensity of the lighting: if the environment is very bright, this will decrease the time spent in the lit box as well as the number of transitions to it.

Any treatment that is decreasing anxiety-like behavior will increase the time spent in the lit box as well as the number of transitions. This has been observed with pharmacological treatments with anxiolytic drugs, such as benzodiazepines, benzodiazepines partial agonists, 5-HT_{1A} receptor agonists, 5-HT_{1B} receptor agonists, and 5-HT_3 receptor antagonists, but also with ethanol or some but not all treatments with antidepressants (paroxetine, imipramine, moclobemide) [8]. Conversely, anxiogenic treatment induces a decrease in the time spent in the lit box and in the number of transitions: this has been observed with benzodiazepine inverse agonists and after acute administration with some antidepressants, such as citalopram. The same is observed in mice with genetic or environmental manipulations aimed at inducing anxiolytic or anxiogenic effects: for example, physical enrichment can reduce anxiety behavior, while exposure to stress during development or adulthood might elicit the opposite pattern.

In the LDB, a huge interstrain variability has been observed. Usually, higher aversion for the lit box is observed in albino strains (Swiss mice, BALB/c mice, etc.), which is due to hypersensitivity to brightness in these strains in which light can induce ocular pain. This is mainly observed when light intensity is high. For example, in a study, authors compared C57BL/6 mice with albino C57BL/6 mice: entries in the lit box were identical between both strains at 0 and 500 lux but strongly decreased in the albino strain at 1000 lux [14].

When testing a population of mice, a normal distribution is usually observed. This is optimized when the number of mice is at least equal to 15–20 in each experimental group. This will then allow to perform statistical comparisons using ANOVAs, but nonparametric statistics are suited as well.

5 Experimental Variables and Troubleshooting

The experimental results can be impacted by several confounds, related either to the experimenters or to the experimental conditions. It is, therefore, crucial to ensure optimal conditions. Further, results should be interpreted with caution. This is detailed in the following section.

5.1 Experimental Conditions

5.1.1 Animals

As argued by the semiotic theories of Jakob von Uexküll as early as 1934 [15], each species is living in a specific "*Umwelt*" ("a self-centered world"), which depends upon its sensorial capacity and upon specific "*Merkwelt*" that determines which are the meaningful aspects of the environment that the animal will detect. Therefore, it is important to remind that mice mainly rely on olfaction: they have a very high odor sensitivity and detection, when compared to humans. It is, therefore, important that experimenters avoid heavy perfumes and aftershaves, as this can be meaningful to mice and change their behavior. In the same vein, as the experimental devices are usually cleaned with disinfectant solution, it is important to ensure that the odor of the solution is no more present at the time of testing, as this could also interfere with the behavior of the mice. Therefore, after cleaning the device with ethanol, it is necessary to wait 2–3 min before introducing the next animal. They are also able to detect ultrasounds ranging over 20–110 kHz. As frequency above 20 kHz are not audible by a human ear, experimenters have to be cautious about the utilization of devices emitting ultrasounds in animal facilities as this can interfere with ongoing experiments. Cell phone noise should also be avoided. Other aspects to consider is that mice are nocturnal species: it is, therefore, appropriate to perform experiments in inverted light–dark cycles in order to test the mice during the dark phase of the cycle. Their activity peak occurs just after the lights are off: at this moment, exploration is maximum, which is optimal for behavioral experiments. In case mice have not been reared in inverted light–dark conditions before their arrival at the animal facilities, it is to note that an adaptation period will be necessary. Mice might recover from the jet lag by 1 h/day: so, a period of at least 12 days of adaptation is necessary in case of a full shift of the light–dark conditions. Ultimately, mice are a social species, living in stable social groups. Therefore, social isolation and social instability can be stressful and might impact anxiety-like behavior in the light–dark box test.

An example of data obtained in female C57BL6/J in the LDB obtained after a 7-week-long protocol of social instability stress [12] is displayed, in which animals were subjected to cage changes twice a week. Controls remained in stable social conditions, while stressed animals were submitted to unstable social hierarchies, a

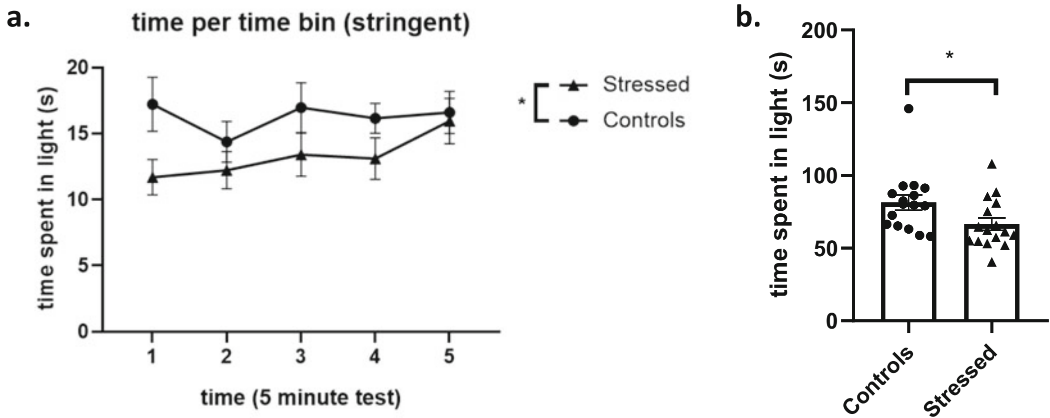

Fig. 2 (**a**) Time spent in light during the 5-min LDB test per minute following a chronic social instability stress. Stressed animals spend less time in the light than control animals, a difference that tends to disappear toward the end of the test. A two-way ANOVA yielded a significant group factor, with stressed animals spending more time in the light compartment ($p = 0.0346$). (**b**) Total time spent in the light compartment following a chronic social instability stress compared to controls. Control C57BL6/J spend significantly more time in the light compartment of the LDB (Mann–Whitney, $p = 0.017$). As normality was not respected all the datasets (D'Agostino–Pearson test), a nonparametric test was used (Mann–Whitney) instead of a t-test

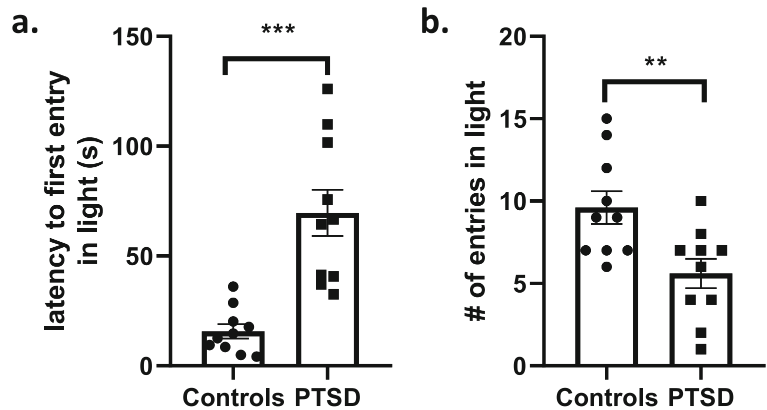

Fig. 3 (**a**) Latency to first entry in the light compartment 6 weeks after two 1.5-mA electric foot shocks. Shocked animals had a higher latency to enter the light compartment ($p = 0.0002$). (**b**) Number of entries (or transitions) to the light compartment. Shocked animals displayed less in the light compartment overall ($p = 0.0075$). All datasets passed respected normality (D'Agostino–Pearson test); therefore, an unpaired t-test was used for both (**a** and **b**)

single mouse being prevented from encountering the same cage-mate for five consecutive cage compositions (cages of 3–4 individuals, Fig. 2). Another example of data in male C57BL6/J from an independent experiment is displayed in Fig. 3. This study was done in order to further characterize a model of post-traumatic stress

disorder [16], in particular its long-term consequences. Interestingly, 6 weeks after the shocks, the latency to first exit was increased (Fig. 3a), while the number of transitions (Fig. 3b) was decreased in shocked mice. In addition to the information about the model, this highlights the fact that a brief but intense stressor can have a long-lasting impact on anxiety-like behaviors, hence the necessity to be careful about animal facility conditions (careful handling by experimented workers, quiet housing spaces, etc.).

The first point to mention is that anxiolytic or anxiogenic action can be lost by reason of floor or ceiling effects: therefore, the experimental conditions have to be set in order to avoid these confounds. Consequently, if an anxiolytic effect is expected, it is important to use high lighting conditions, as in this case the time spent in the lit box in the controls will be low, enabling to observe an increase after treatment. The opposite applies in case anxiogenic effects are expected. Another solution can be to use intermediate lighting conditions, as in this case both anxiolytic and anxiogenic effects can be observed.

In some protocols, long-term effects of experimental manipulations (chronic stress, chronic treatments, early-life manipulations, etc.) are assessed using a battery of behavioral tests. In this case, it is important to consider that the LDB is stressful for the mice. This can interfere with the results of other less stressful tests. Therefore, it is advised, when a battery of tests is done, to perform the non-stressful tests first in the schedule and the stressful tests at the end of the experimental schedule. Another aspect to consider is that, as the LDB partly relies on novelty, it is not relevant to repeat it in the same cohort of mice as in this case one component of the unconditioned conflict situation (the novelty) is lost. Indeed, some authors have, for example, shown that while the benzodiazepine diazepam elicited anxiolytic effects after the first confrontation with the LDB, this effect is completely lost in case testing is done the second time [17].

Finally, as behavior in this test can be modified by the time of the day, the surrounding noise, the composition of the social group within a given cage, and many other factors, a semi-random order of testing should be used. For example, we can imagine a theoretical experiment in which the experimenter is testing two doses of a pharmacological treatment in comparison to a control. Three groups of 15 mice will be set: a control group (denominated as Veh, for vehicle), a group with a low dose (denominated as Low), and a group with a high dose (termed as High) of the treatment. If animals are living in groups of 5, nine different cages will be used by the experimenter (cage 1 to cage 9). When doing the experimental schedule, the experimenter has to take into account the fact that the duration of the test is 5 min and the time of cleaning/ventilating the cleaned box is approximately 2 min. A semi-random schedule can be as follows: time 0, cage 1, Veh; time 7 min, cage 1, Low; time

14 min, cage 1, High; time 21 min, cage 1, Veh; time 28 min, cage 1, Low; time 35, cage 2, High; time 42, cage 2, Veh; etc. This enables that (a) in each cage there are one or two mice in each experimental group and (b) an equivalent number of mice in each treatment condition is tested at the different moments of the day.

5.2 Experimenter Interference

The experimenter's behavior can also interfere with the quality of the results. For example, an experimenter can handle mice in a stressful way or be tired: this might alter the quality of the results obtained. The experimenter has to observe the mouse without approaching the device, as movements of the experimenter can be detected by the mouse and interpreted in a fearful way. It is also very crucial to avoid movements over the LDB during testing, as this can be interpreted as related to the presence of a prey bird, for example. Indeed, rodents exhibit a defensive response (freezing or escape) to a looming disk, for example [18].

5.3 Interpretation of the Results

As in all experiments involving animal behavior, the data collected should be interpreted carefully. First, the results can be due to nonspecific effects. For example, if a mouse is blind (note that some strains such as C3H display retinal degeneration), it will not be able to distinguish the light and the dark box, and, therefore, that data collected cannot be interpreted in the frame of anxiety behavior. So, experimenters have to examine carefully that the treatments do not alter the visual aptitude of the animals. Another example of unspecific effects is related to the fact that a given treatment can also alter locomotion, thus interfering with the behavior of the animals. For example, anxiolytics, such as benzodiazepines, induce sedative effects at high doses: thus, a reduction of transitions could in fact be due not to anxiogenesis but to sedation. Similar effects (reduced locomotion) are observed when treatments eliciting ictus are administered: animals can display a postictal prostration. In this case, the reduction in the number of transitions is not to be interpreted as related to anxiogenesis. Therefore, it is important to always base the interpretation on both the time spent in the lit box and the number of transitions and not to only one variable.

6 Conclusion

The LDB is an excellent tool to assess anxiety behavior. However, we will highlight three limiting aspects as a conclusion. First, the LDB is not measuring anxiety but anxiety-like behavior. Indeed, anxiety is an emotion that also includes a physiological aspect (acceleration of heart rate, increase in blood pressure, dilatation of pupils, etc.), an expressive aspect (e.g., screaming), a cognitive aspect (increase of the memorization of the fearful moment in

episodic memory), and a subjective feeling. These aspects are not measured in the LDB that is only focusing on the behavioral component.

Second, anxiety can be divided in state and trait anxiety. While state anxiety corresponds to an emotion that is triggered by a threat, trait anxiety corresponds to a permanent trait of excessive worry that is present even if no threat is present in the environment [19]. The LDB, in which the animals are forced in a stressful environment, is measuring state and not trait anxiety behavior. This should be borne in mind. For example, if a given treatment has no effects in this test, it cannot be concluded that it has no effect on anxiety, as it is possible that it would have a specific effect on trait anxiety.

Finally, in this test the behavior of the mice is assessed at a specific moment termed as "test situation." It can be that the experimental manipulations done (here, introducing a mouse in the LDB) may reveal effects that would not be observed if the animal may be left undisturbed or, on the contrary, that the effects that were present in the animal when in the home cage are lost by the experimental manipulations.

7 Notes

1. It is capital to place the mouse in one of the corners opposite to the dark box entrance, head facing the corner. This is to a flight reaction due to handling toward the light compartment, which would be problematic for the interpretation of the results (in particular, the latency to first exit).
2. In the case of a modular LDB (two boxes, one for light, one for dark, possibly a tunnel), it is important that the dark and the light box are perfectly aligned so that the hole allowing the transition from one compartment to the other is fully opened. If the different components of the LDB are not carefully put together, there is a risk the transition port will be partially obstructed, which will make it difficult for the animal to transition and thus alter the results.
3. Placing the animal in the dark compartment and then closing the lid can disturb automatic tracking with software such as EthoVision. When this occurs, be careful to check live if the tracking has been altered and once the acquisition is done to correct the tracking manually.

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Chapter 4

The Olfactory Bulbectomized Rodent Remains a Valuable Preclinical Model of Depression and Antidepressant Activity

Michelle Roche and John P. Kelly

Abstract

Established over 40 years ago, the olfactory bulbectomized (OB) rodent model has been a widely used and validated preclinical model of depression and antidepressant activity. Due to the anatomical connections of the olfactory bulbs, their removal results in neuronal reorganization in limbic and cortical regions; alterations in neurotransmitter, endocrine, neurotrophin, and neuroimmune systems; and a constellation of behavioral changes, including hyperactivity, anhedonia, and cognitive impairment. Such changes are analogous to those observed in the depressed patients supporting the face validity of this model. Furthermore, similar to the clinical situation, antidepressants reverse neuronal and behavioral changes within in the model following chronic, but not acute, administration, confirming its predictive validity. Recent research has demonstrated that in addition to detecting classical monoaminergic antidepressants, antidepressant-like activity of non-monoaminergic pharmacological agents, such as ketamine, or non-pharmacological interventions, such as exercise and brain stimulation, can be detected in this model. The validity of this model in both mice and rats enables researchers to utilize new and evolving technologies to examine neurobiological underpinnings and treatment responses of relevance to depression using the OB model. This chapter describes in detail the protocol for the establishment of the OB model in rats and mice, examining antidepressant-like activity in the open field test.

Key words Animal model, Depression, Antidepressants, Bulbectomy, Open field test, Anhedonia, Cognitive impairment.

1 Introduction

The olfactory bulbectomized (OB) rodent has been examined as a preclinical model of depression and antidepressant potential for several decades. Numerous studies over the past 40 years have reported on the behavioral, neurotransmitter, neuroendocrine, and immune changes in the OB model, many of which resemble those reported in depressed patients [for reviews see [1–3]. Most notably OB rats exhibit a constellation of behavioral changes, including anhedonia [4–8], decreased social behavior [9–11], deficits in learning and memory [12–17], reduced sexual behavior

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Table 1
Distribution of behavioral tests used to assess antidepressant activity in the OB rat and mouse model

Behavioural parameters	No. of studies	%
Rat		
Open field test	105	81%
Forced swim test	30	23%
Sucrose/saccharin preference	19	15%
Hyperemotionality	16	12%
Elevated plus maze	15	12%
Passive avoidance test	7	5%
Social interaction test	7	5%
[a]Other	20	15%
Mouse		
Open field test	25	46%
Tail suspension test	12	22%
Forced swim test	10	19%
Splash test	10	19%
Novel object recognition test	9	17%
Passive avoidance test	8	15%
Sucrose/saccharin preference	7	13%
Y-maze	6	11%
Novel cage test	4	7%
Morris water maze	3	6%

Data analyzed from studies published from 1998 to 2021. Total of 130 rat and 54 mouse studies
[a]Other includes Morris water maze, novelty-suppressed feeding, T-maze, sexual behavior, splash test, object location, novel object recognition test, and muricidal behavior

[18, 19], and impaired reactivity to stressful environments [20–24] (*see* Table 1), all of which have been observed in the depressed patient. However, whether the underlying neurobiological mechanisms of this model resemble the neurobiological state of depressed patients is still a matter of debate.

The OB model has most widely used to examine antidepressant activity, demonstrating excellent predictive validity and making this a valuable tool for assessing the effects of novel antidepressant drugs. Although antidepressants have been demonstrated to reverse some, or all, of the behavioral alterations in the model, OB-related hyperactivity in the open field test is the most widely used test for assessing antidepressant activity in the model (Table 1). The first published findings examining antidepressant

activity in the OB rat was in the 1970s, where the model was used in assessing the potential of the atypical antidepressant mianserin using attenuation of OB-induced hyperactivity as a measure of efficacy [25]. However, it was not until the mid-1990s that the first published report of antidepressant-like effects on behavioral responding was reported in the OB mouse [26], and a further 10 years before reports of the OB mouse being used for antidepressant efficacy regularly appearing in the literature. Thus, there is a much longer history of the use of the rat model than the mouse, while at this point in time, both species are being used to a similar frequency.

Irrespective of species, a key feature of the OB model is that chronic (but not acute) administration with monoaminergic antidepressants is required to reverse changes in OB-related hyperactivity. This property of only detecting antidepressant effects following repeated administration has seen the OB model, playing a role in preclinical antidepressant drug evaluation, and means that there is less potential for acute drug effects to be detected, particularly if a period of drug "washout" is instituted prior to exposure to the behavioral tests. A number of currently marketed antidepressants have demonstrated efficacy in both rats and mouse (*see* Table 2). In more recent times, the model has been extended to examine non-pharmacological strategies, such as exercise [27–29], environmental enrichment [30], brain stimulation [31, 32], and (with the advent of the mouse model) genetic modification [33–36]. In addition, the potential to detect rapid-acting antidepressant effects has been examined, particularly in the case of ketamine [37–39].

This chapter provides detailed methodology on establishment of the OB model in rats and mice and assessing antidepressant-like activity using the open field test. We refer the reader to Volume I and II of the Neuromethods series on Mood and Anxiety Related Phenotypes in Mice [40, 41] for comprehensive protocols for assessing other behavioral changes, such as anhedonia, deficits in learning and memory, and neophobia/anxiety-like behavior. The notes and troubleshooting sections provide experimental variables and considerations when designing and analyzing OB rodent experiments.

2 Materials

All procedures should be carried out under appropriate ethical and animal welfare approval and comply with national and international regulations regarding the use of animals for research purposes (e.g., European Communities Council Directive 2010/63/EU). It is recommended that researchers conduct experiments and report results in line with the ARRIVE guidelines [42] (*see* **Note 4.9**).

Table 2
Pharmacological agents examined for antidepressant-like activity in the OB model in the open field test

Drug class	Drugs	Rat	Mouse
SSRI	Fluoxetine, paroxetine, citalopram/escitalopram, fluvoxamine Sertraline	Y Y	Y N
SNRI	Venlafaxine Duloxetine *Milnacipran*	Y N Y	Y N N
NRI	Reboxetine	Y	N
SARI	Trazodone, nefazodone	N	N
NaSSA	Mirtazapine, *mianserin*	Y	N
Melatonin agonist and 5-HT_{2C} antagonist	Agomelatine	Y	N
TCA	Amitriptyline Imipramine Desipramine, lofepramine	Y Y Y	Y Y N
NMDA/glutamatergic agent	*Ketamine,* *Tianeptine*	Y Y	Y N
MAOI	Moclobemide	Y	N
5-HT_{1A} agonist	Buspirone	Y	N

Data analyzed from studies published from 1998 to 2021. Total of 130 rat and 54 mouse studies

2.1 Animals and Housing

Animals may be housed under a variety of conditions (group or individually, open top or individually ventilated cages, with or without environmental enrichment), the choice of which will depend on experimental design and expected outcome (*see* **Note 4.2–4.4**). Animals are most commonly housed under standard housing conditions with food and water available ad libitum, constant temperature (20 ± 2 °C) and relative humidity (40–60%), and standard lighting (e.g., 12:12 h light–dark, stating when the lights are turned on). It is recommended that animals are allowed at least 7 days to acclimatize following any changes to environmental conditions prior to surgery or behavioral testing. In order to ensure sufficient power in studies, group size should be calculated in advance using statistical analysis (*see* **Note 4.5**).

2.2 OB and Sham Surgery: Equipment, Materials, and Setup

1. Appropriate surgical facilities and equipment are required. The surgical area should be cleaned and disinfected (e.g., 1–3% Milton disinfectant (Procter and Gamble, Ireland) or 70% alcohol). An aspirator pump (air or water, e.g., Vacu/Trol vacuum water aspirator, Spectrum Europe, Netherlands)

generates a constant pressure vacuum. Removal of the bulbs may be facilitated by attachment of a blunt 16G hypodermic needle or fine glass pipette to the vacuum hose on the aspirator (*see* **Note 4.1**). Alternatively, bulbs may be removed by mechanical compression or electrocauterization [43] or by photochemical means [44].

2. Additional surgical equipment required include stereotactic frame + non-rupture ear bars (Harvard Apparatus, UK), homoeothermic blanket with temperature controller or equivalent (CMA, Sweden or Harvard Apparatus, UK), high-speed micro drill with 2.1-mm burr (Fine Science Tools, Germany), hair clippers and surgical instruments (scalpel, retractors/bull dog clips, straight and curved forceps (Fine Science Tools, Germany)), and bead sterilizer. All equipment and surgical tools should be sterilized prior to, and between, surgeries.
3. Additional reagents required: 5% lidocaine cream (e.g. EMLA), Betadine or povidone (iodine) solution, sterile alcohol swabs, local anesthetic (e.g., norcaine), sterile cotton gauze/swabs/bubs, eye drops (e.g., fucithalmic, fusidic acid), hemostatic sponges (cut to 2 mm pieces; dental supply companies) and/or bone wax (Fine Science Tools, Germany), antibiotic powder (e.g., neomycin, Sigma, Ireland), sutures (3–0 Vicryl) [or equivalent (e.g., Histoacryl, Aesculap, Germany, or wound clips (7.5 mm Michel)], sterile injectable saline (0.89% NaCl), analgesics [e.g., the nonsteroidal anti-inflammatory agent, carprofen (1.25 mg/25 μl s.c., Rimadyl, Pfizer, UK)], hypodermic needles (25–30G), and sterile surgical gloves.
4. It is recommended that surgery be performed under inhalation anesthesia, such as isoflurane (IsoFlo (1–3% in O_2; 0.5 L/min), Abbott, Ireland), which allows for excellent control of anesthesia and rapid recovery following surgery. If choosing to use an injectable form of anesthesia, nonbarbiturates are recommended due to shorter recovery period and reduced complications associated with prolonged anesthesia. A combination of xylazine (80 mg/kg; Bayer, Germany) and ketamine (100 mg/kg; Aventis Pharma, Germany) has been widely used in the literature.

2.3 OB-Induced Hyperactivity and Assessing Antidepressant-Like Activity: Equipment, Materials, and Setup of Open Field Test

1. Open field arenas may be constructed of various materials (wood, metal, plastic), in numerous shapes (circular, square, rectangle) and sizes. Our studies have found that OB-induced hyperactivity is reliably detected when assessed in a large aversive arena [e.g., circular arena (75–90 cm diameter) with aluminum walls (60 cm high) and white floor] (*see* **Note 4.6**). Large arenas also allow for behaviors such as thigmotaxis (distance moved or time in outer perimeter) and anxiety-related

behaviors (time in the center zone) to be assessed. The arena should be cleaned with mild detergent or disinfectant (e.g., 1% Milton disinfectant solution or 10–50% alcohol), prior to and between testing of animals.

2. Various lighting conditions have been reported in the open field test; however, OB-induced hyperactivity is most consistently observed when testing occurs in brightly lit open field arena (Lux >100) (*see* **Note 4.6**). In general, the open field should be evenly illuminated, the light intensity recorded using a lux meter and reported in all publications.
3. To objectively assess behavior/locomotor activity, it is recommended to employ either photocell automated open field (e.g., Opto M3, Columbus, US) or video tracking software (e.g., EthoVision, Noldus, Netherlands, or open-source software (see https://edspace.american.edu/openbehavior/)) (*see* Fig. 2). Manual rating of open field activity involves determining the number of line crosses on the base of the arena (e.g., 10 × 10 cm squares painted on base of arena). It is advisable that behavior in the open field test be recorded to enable detailed behavioral assessments (e.g., latency to enter zone, time in zone/center, locomotor activity per time period, time/frequency of rearing, or grooming behavior) or reanalysis of locomotor activity to occur at a later date if required.
4. In evaluating antidepressant-like activity of chronically administered pharmacological agents in the OB model, it is recommended that sufficient time be given between administration of the last dose of the drug and testing in the open field to avoid acute short-term effects on locomotor activity. The length of time will depend on the half-life of the drug, but in general, a minimum of 12 h is recommended. In our experience, we would usually administer the drug in the afternoon and assess behavior in the following morning, a washout period of approximately 18 hr.

3 Methods

3.1 OB and Sham Surgery Procedure

1. Remove the animal from the cage and record body weight.
2. In a separate area to the surgical field, anesthetize the animal (e.g., induction 5% IsoFlo/oxygen 0.5 ml/min) and monitor the depth of anesthesia by toe and tail pinch. Once fully anesthetized, reduce the anesthetic delivery to maintenance level (e.g., 2% IsoFlo/oxygen 0.5 ml/min). Shave the superior surface of the head, removing all excess fur.
3. Transfer the animal to the surgical area, coat the ear bars in local anesthetic ointment (e.g., EMLA cream), and secure the

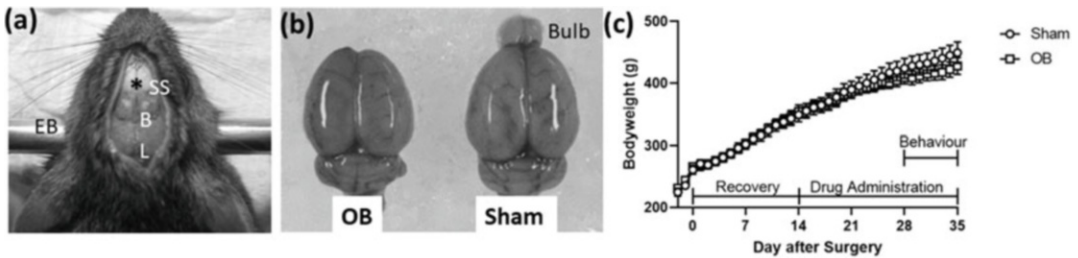

Fig. 1 (**a**) Landmarks on surface of skull used to determine location of burr hold for olfactory bulb removal (*) EB ear bars, L Lambda, B Bregma, SS sagittal sinus. (**b**) Verification of bulb removal in OB rats. (**c**) Body weight following OB or sham surgery. Data expressed as mean ± SEM. The rate of growth is almost identical in the first 3 weeks following surgery, with some blunting in growth rate evident in the OB group thereafter. Displayed on the graph is also the typically a 2-week recovery period, which allows for development of the OB syndrome. This is typically followed by drug treatment protocol (most commonly for at least 2 weeks), after which behavioral testing commences

head in the stereotactic frame using the ear bars and nose clamp/cone. Ensure that the head is horizontal to the stereotactic frame.

4. Place temperature probe into the animal's rectum and note the body temperature (~37 °C). Extra fabric/bubble wrap can be placed over the animal to prevent heat loss.
5. Apply eye drops (e.g., fucithalmic) to the animal's eyes to prevent corneal damage.
6. Administer analgesia (e.g., carprofen) to manage postoperative pain.
7. Put on sterile gloves.
8. With a sterile cotton bud, clean area to be incised using iodine solution followed by an alcohol swab.
9. Make a midline longitudinal incision (2–3 cm) with a sterile scalpel blade in the scalp over the junction of the frontal and nasal bone of the skull (midway between the eyes). Retract the skin, scrape back the periosteum, and dry the skull using a cotton swab (*see* Fig. 1a).
10. Apply local anesthetic (e.g., norcaine) to the area.
11. Identify the sagittal suture, bregma, and the dark diamond-shaped area along the sagittal suture directly between the two eyes (*see* Fig. 1a). Again, ensure the skull is parallel to the horizontal plane of the stereotactic apparatus, by adjusting the nose clamp/cone.
12. Mark the coordinates of the entry point on the surface of the skull. For mice, one hole (~2 mm diameter), 4 mm rostral to bregma (or 1 mm rostral to the sagittal suture) on the midline, is sufficient. For rats, mark two 2 mm holes directly above each bulb, 5 mm rostral to bregma (2 mm rostral to the sagittal

suture) and 2 mm lateral to midline (ML ± 1 mm) (*see* Fig. 1). Drill the burr hole(s) at the marked entry point(s), ensuring the drill is kept perpendicular to the skull surface, and pierce the dura with a fine hypodermic needle.

13. For sham surgery, stop any bleeding that may have resulted, dry the skull with a cotton swab, fill burr hole(s) with hemostatic sponge or bone wax, apply antibiotic power to the area, and close the skin with interrupted sutures or equivalent (proceed to point 16). Should damage to the olfactory bulbs during the procedure be suspected, it is advisable that the bulbs are removed.
14. For bulbectomy surgery, turn on air pressure or run water through the aspirator pump to create a vacuum (*see* **Note 4.1**). In order to remove the olfactory bulbs, pierce the bulbs with a sterile hypodermic needle and carefully aspirate the bulbs from the individual cavities with the aid of the blunt needle or glass pipette attached to the vacuum hose. Ensure that the needle is inserted vertically, and not at an angle, to avoid damage to the frontal cortex or olfactory tubercles. *Tip:* As the olfactory bulbs are positioned in a separate cavity, a hollow sound is heard when the bulbs have been successfully removed.
15. Fill the burr hole(s) with hemostatic sponge to stop any bleeding. Bone wax may be used to seal over the burr hole(s).
16. It is recommended that the needle and piping connected to the pump are cleaned immediately after removal of the bulbs to avoid clogging buildup of debris. This can be achieved by leaving the pump on and placing to needle into a beaker clean water to flush through the lines until cleared.
17. Apply antibiotic power (e.g., neomycin) to the wound, and close the skin using interrupted sutures or equivalent.
18. Remove the animal from the ear bars. If using inhalation anesthetic, turn off anesthetic but maintain oxygen delivery (0.5 ml/min) to aid recovery from surgery. Administer 1 ml of sterile saline (i.p.) to maintain hydration.
19. Transfer the animal to a clean warm cage on a heated recovery pad until recovery from anesthesia.
20. The duration of surgery time should be recorded for each animal and on average takes approximately 15 min.
21. As OB animals are rendered anosmic, it is advisable that food be placed in the base of the animals' home cage for the first 24 hr in order to encourage food consumption.
22. *Postoperative care:* Animal health and welfare, including body weight, should be carefully monitored in the 24–48 hr post-surgery (*see* **Note 4.1**). It is expected that OB animals might

exhibit a decrease in body weight (<20%) and possible loss of coat condition during this period, but adherence to the protocol will typically see animals gaining weight on a par with their sham-operated counterparts (*see* Fig. 1c). Animals should be checked for postoperative wound infection. Damage to the frontal cortex during surgery may result in animals displaying abnormal behavior (head tilt, rotational behavior, abnormal gait). Veterinary assistance should be sought for any animal that exhibits >20% loss of their presurgery body weight or show other adverse effects, which might require them to be excluded from the study. It is recommended that the body weight and general health status of animals be recorded throughout the study (*see* Fig. 1c) and animals routinely handled to reduce aggressiveness that may develop following OB surgery.

3.2 Anticipated Results

Following OB surgery, the olfactory bulbs should be completely removed, and the animal should be rendered anosmic. The most common means of verifying olfactory bulb removal is gross inspection following completion of the study (*see* Fig. 1b). Careful examination of olfactory cavity for incomplete removal of one or both bulbs or damage to the frontal cortex following OB surgery necessitates removal of the animal from analysis. Verification that neuronal damage to the frontal cortex was not induced following removal of the olfactory bulbs may also be confirmed using Nissl [45] or thionin [11] staining and histological examination. A criterion of removal of at least two thirds of the olfactory bulbs and lesioning of part of the olfactory nuclei has also been used as indications of successful bulbectomy. However, olfactory function can remain with relatively small bulb remnants [18], and therefore, should an investigator employ such criteria, it is important that anosmia is confirmed. Animals should be eliminated if damage to the olfactory bulb(s) is observed following sham surgery.

Although not routinely examined, anosmia may be confirmed following the removal of the olfactory bulbs by various means. Traditionally, animals which have been rendered anosmic are incapable of distinguishing between water and a bitter, scented solution [(0.1% amyl acetate +0.4% quinine) [11] or lithium chloride (0.12 M) [8]]. Sham animals learn to associate the smell with the bitter unpleasant taste and avoid the solution; animals have been rendered anosmic lick both solutions in order to differentiate between water and the unpleasant solution. Alternatively, anosmia may be determined by examining the latency to approach a novel aversive and/or pleasant odor [46] or find hidden food in the home cage [45].

3.3 OB-Induced Hyperactivity and Assessing Antidepressant-Like Activity

OB-induced hyperactivity (and other behavioral changes) is most commonly examined 14+ days following surgery, a time point at which surgery-induced neuronal reorganization is believed to be established, giving rise to the depressive-like behavioral phenotype. Animals may also be tested in the open field test prior to surgery to ensure baseline locomotor activity does not differ between animals allocated to sham or OB groups. The most common protocol for examining antidepressant activity in the OB model is the commencement of pharmacological treatment 14 days post-OB surgery (following the establishment of the hyperactivity response) and evaluation of efficacy after 14+ days of treatment (*see* **Note 4.8**). The open field test may also be conducted as part of a battery of behavioral tests to assess effects of OB and/or antidepressant-like activity in a variety of behavioral domains (*see* **Note 4.7** for considerations when designing such experiments).

3.3.1 Open Field Test Procedure

Prior to behavioral testing, it is recommended that animals are habituated to handling and the animals' home cage should not be changed within 24 hr of testing. These procedures act to minimize background stress that may impact on behavioral assessments.

1. As the open field test is used not only to determine locomotor activity but also behavioral responding of OB mice in a novel, stressful environment, animals do not be acclimatized to the testing room in advance.
2. Clean the open field arena thoroughly with mild detergent or disinfectant prior to testing.
3. Turn on the video camera, photocell, and/or video tracking software.
4. Place the animal in the open field. The position which investigators place the animal in the open field varies (center versus perimeter); however, positioning along the perimeter of a larger open field arena allows for the latency to enter and time in the center zone to be assessed, a useful measure of anxiety-related behavior. Animals are most commonly tested in the open field arena for up to 10 min.
5. At the end of the trial, the animal should be removed from the arena, placed back into their home cage and returned to the holding room. *Tip:* OB animals excrete more fecal boli in the open field test compared to sham-operated counterparts [47], and thus prior to cleaning the arena, it is recommended to record the number of fecal boli as an indirect measure of stress/anxiety in the model.
6. The arena should be thoroughly cleaned and dried between testing of animals.

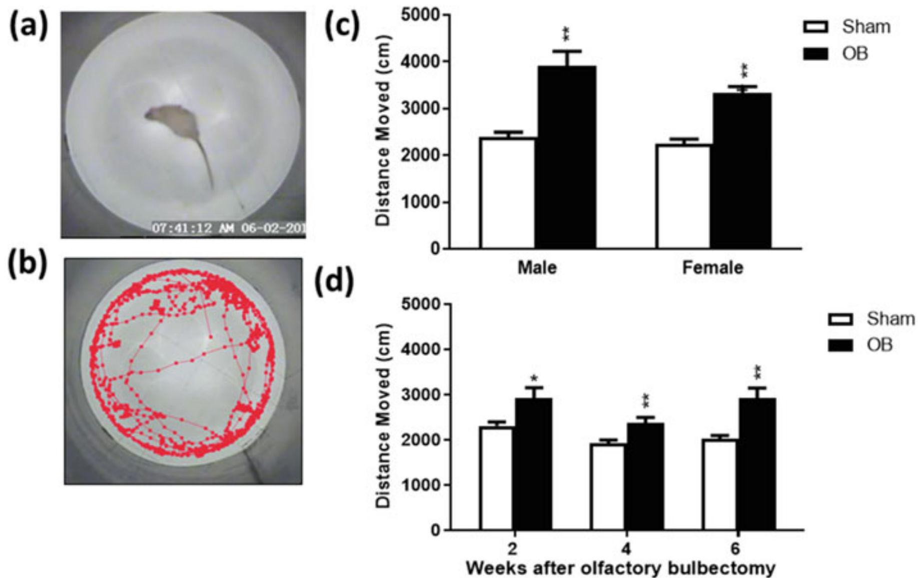

Fig. 2 (**a**) Diagram depicting open field test and (**b**) locomotor tracking using EthoVision software. (**c**) Distance moved in the open field of Sham and OB male and female rats 2 weeks post-surgery. (**d**) Distance moved of Sham and OB animals 2, 4, and 6 weeks post-surgery. $^{**}p < 0.01$ $^{*}p < 0.05$ vs. sham. Data is expressed as mean + SEM

3.3.2 Data Analysis and Anticipated Results

Data Collection The primary outcome of the open field test is change in locomotor activity (*see* Fig. 2). Locomotor activity may be assessed manually (number of line crosses) or using an automated system (beam breaks or video tracking); however, the latter will allow for analysis over discrete time periods and analysis of time in, and locomotor activity in, different areas of the arena (e.g., central zone). Furthermore, the use of automated systems eliminates much of the subjectivity associated with manual recording and often enables simultaneously recording of observed behaviors, such as freezing, rearing, and grooming, providing a full behavioral profile of the animal while in the test. Most studies report total locomotor activity (i.e., distance moved) over the entire duration of the trial; however, in cases where testing is >5 min, locomotor activity over discrete time periods may prove more informative. For example, discrete analysis over time may provide insight into the habituation profile of the animals to the novel open field arena.

Data Analysis All data should be tested for normality and homogeneity of variance prior to statistical analysis. If these conditions are met, then the effects of bulbectomy alone on behavioral responding (e.g., distance moved) are most commonly assessed using *t-test* (sham vs OB) or repeated measures ANOVA (sham vs OB effect over time). Two-way ANOVAs are routinely used to determine effects of drugs/treatments in the model (with the

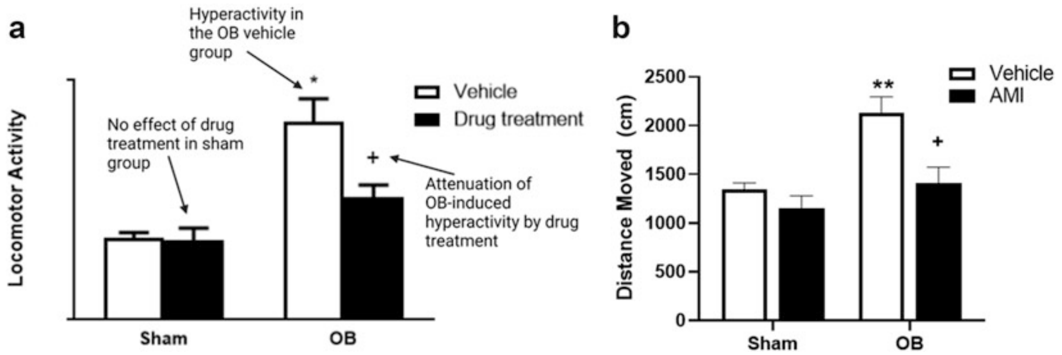

Fig. 3 (**a**) Features of the antidepressant response in open field test in the OB model. (**b**) Distance moved of Sham and OB animals chronically treated with amitriptyline (AMI) for 2 weeks. ** $p < 0.01$ vs. sham–vehicle. $^{+}p < 0.05$ vs OB–vehicle. Data is expressed as mean + SEM

factors of surgery and treatment). Should the data not be normally distributed (and not possible to transform), then nonparametric analysis should be employed.

Anticipated Results If animals are tested in the open field test prior to surgery, no difference in locomotor activity should be noted between groups assigned to sham or OB groups. OB animals exhibit enhanced locomotor activity (number of line crosses or distance moved) in the open field test (*see* Figs. 2 and 3). The antidepressant-like effect of pharmacological compounds may be determined by their ability to attenuate OB-related increases in locomotor activity in the open field, an effect observed by traditional antidepressants (e.g., tricyclics and SSRIs) following chronic, but not acute, administration (*see* Fig. 3). In addition to locomotor activity, studies frequently report effect of OB on latency to enter and time in the central zone, frequency and duration of rearing and grooming, and number of fecal boli. The inclusion of such data expands the behavioral profile of OB rats exposed to this novel stressful arena and provides further parameters by which to examine the effects of treatment regimens.

4 Notes

A number of experimental variables have been identified, which may impact the successful outcome of both the surgery and subsequent behavioral assessment in the OB model, and are presented as Notes below.

4.1 Surgical Procedure

While there are number of means of removing the olfactory bulbs, we have reported on the aspiration method and as such will discuss possible experimental variables pertaining to this method.

OB surgery is a relatively simple surgical procedure, with a high success rate even for those with relatively little surgical experience. However, as with any surgical procedure, the experience of the surgeon, aseptic technique, the ability to maintain core body temperature during surgery and appropriate postoperative care impact on the success of the surgery and the recovery of the animal. Consideration should also be paid to the appropriate coordinates for surgery, which may vary depending on the size/age/sex of the animals and the aspirator vacuum pressure, which if subject to variation may result in either an inability to remove the olfactory bulbs completely or damage to the frontal cortex. To reduce attrition within a study, it is recommended that all parameters are checked with 1–2 training animals prior to undertaking a full study. Where possible, surgery should be performed during the early in the light phase to allow for full postoperative recovery prior to the nocturnal dark phase, where rodents are naturally active and consume most food. OB animals will be rendered anosmic and possibly hypophagic, and as such it is recommended that food be placed within the home cage for 24–48 hr following surgery. These considerations aim to encourage feeding post-surgery, minimize body weight loss, and promote postoperative recovery.

4.2 Choice of Species: Rat vs. Mouse

The OB model has been established in a wide variety of species, including hamsters [22, 48], gerbils [49], prairie voles [50], gray mouse lemur [51], and musk shrew [52], but most extensively in rats and mice. [For a comparison of the effects of OB and chronic imipramine treatment in the rat, mouse, and gerbil, see [22]] Both rats and mice exhibit hyperactivity on exposure to the novel open field test, an effect reversed by chronic antidepressant administration, and thus the current protocol is applicable to both species. However, rats and mice differ in terms of their physiology and responses in different behavioral paradigms [53], and thus while OB-related behavioral changes are largely comparable between mice and rats, some notable differences have also been reported [54]. For example, OB mice, but not rats, exhibit reduced density of cholinergic neurons in the medium septum [55], and cholinesterase inhibition reverses OB-related memory impairments in mice but not rats [17, 54]. Furthermore, hippocampal BDNF levels are increased [56] and 5HT2 receptor levels remain unchanged [57] in OB mice, while BDNF levels are reduced [30] and 5HT2 receptors increased [58] in OB rats. Taken together, this suggests that the neurobiological changes following OB may differ between rats and mice. Thus, the choice of rats or mice for a particular study will depend on the experimental question. For example, the use of mice may be preferable if there is a requirement to use transgenic mouse lines/tools, optogenetics, costly pharmacological agents, employment of particular behavioral tests (e.g., tail suspension test), or ex vivo methodologies that have to date only been validated for mice

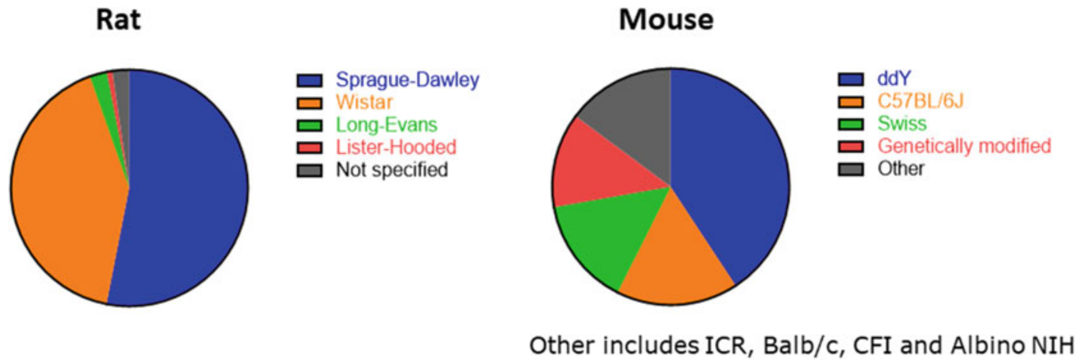

Fig. 4 Rat and mouse strains used in OB studies. Data compiled from 130 rat and 54 mouse studies examining antidepressant activity in OB model

(e.g., mass cytometry). Rats may be preferable if the aim is to examine effects on social or cognitive function (as rats perform better in such tasks) or if carrying out imaging techniques (e.g., fMRI, PET) as the larger rat brain offers between spatial resolution. Other factors that should be taken into consideration when choosing between rats and mice, which include the strain, sex, and age of the animal.

4.2.1 Strain

Sprague Dawley and Wistar rats are the most commonly used rat strains in OB studies, while C57Bl/6 and ddY are the most commonly used mouse strains (*see* Fig. 4). OB-induced hyperactivity has reliably been detected across multiple studies 14 days following surgery in these species and strains, effects reversed by chronic antidepressant treatment. However, strain-specific effects have been observed on behavioral responding in other paradigms. For example, OB in C57Bl/6 mice results in deficits in passive and active avoidance [24, 59], an effect not observed in OB DBA mice [59]. Thus, careful consideration should be paid to the strain of the rat/mouse, particularly if employing the use of transgenic animals.

4.2.2 Sex

The vast majority of studies using the OB model have been conducted in male rodents (*see* Fig. 5). However, there is an increasing awareness of the necessity to include females in preclinical research to greater correlate with the clinical scenario, a finding reflected in the funding policy of most state funded schemes (e.g., NIH: https://orwh.od.nih.gov/sex-gender/nih-policy-sex-biological-variable). This is highly relevant to extrapolation to the clinical situation, where females are twice as likely to experience depression and exhibit sex differences in underlying neurobiology and responses to antidepressant treatment. Although female rats and mice have been demonstrated to exhibit OB-induced hyperactivity, anhedonia, and cognitive deficits [55, 60–63], there have been a

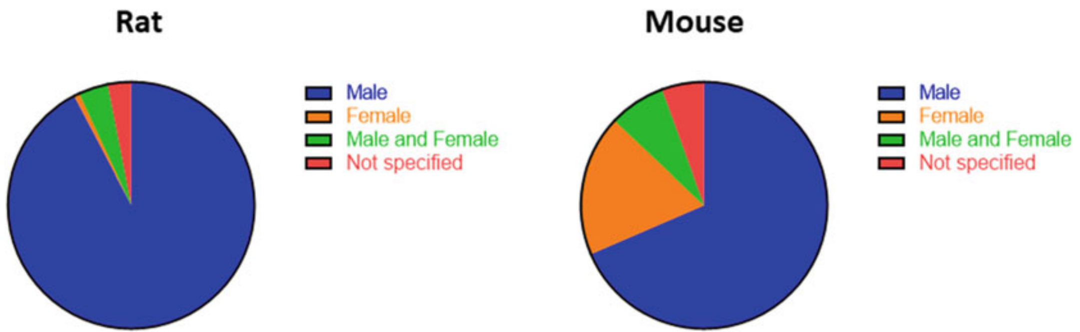

Fig. 5 Sex of animals used in OB rat and mouse studies. Data compiled from 130 rat and 54 mouse studies examining antidepressant activity in OB model

limited number of studies directly comparing effect of OB between male and female rodents. Data from our lab has demonstrated that OB male and female rats exhibit comparable increases in locomotor activity in the open field 2 weeks post-surgery (*see* Fig. 2a). This is in line with published research demonstrating no sex difference in OB-induced hyperactivity or defensive behavior; however, OB female rats exhibit greater decrease in sucrose preference compared to OB males and controls [6, 21]. Natural reward seeking behavior (for palatable food) is differentially altered in male and female OB rats, depending on the complexity of the task [64]. These data indicate that although OB-induced hyperactivity is comparable between males and females, sex differences may exist in relation to changes in hedonic/reward processing in the OB model. Thus, generalizing effects observed in one sex to the other is not appropriate, and where possible, studies should examine the impact of sex in the OB model and the response to antidepressant treatment, with sex recognized as a separate factor during analysis.

4.2.3 Age

The majority of studies using the OB model have been conducted in adult aged (2–4 months) rats (200–350 g) and mice (20–30 g body weight). Early OB studies indicated that adult OB rats (13 weeks) exhibit greater hyperemotional behavior when compared to younger (juvenile) counterparts (4 weeks) [65]. More recent studies examining OB in young adult (9 week) and aged (19 month) rats demonstrated that aged OB rats exhibit greater locomotor activity [66] and differential effects on 5HT system [67] compared to young adult counterparts. The authors propose that these data support that OB in aged rodents may provide a model of geriatric depression and a means of studying the differences in responses to antidepressants that act on 5HT in this population. OB has also been conducted in neonatal rats, resulting in hyperactivity and failure to habituate in the open field test during preadolescence and adulthood, respectively [68]. Furthermore, OB results in more profound cognitive impairment and interhemispheric EEG

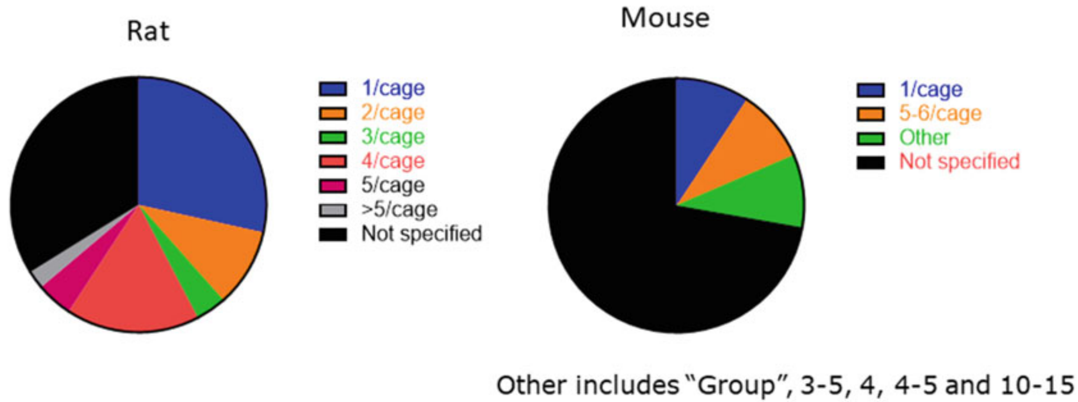

Fig. 6 Housing condition of animals used in OB rat and mouse studies. Data compiled from 130 rat and 54 mouse studies examining antidepressant activity in OB model

differences in the frontal cortex, 1 year following surgery [69]. Thus, when comparing across and between studies, it is important to be aware that the age of the animal may impact on the behavioral response observed in the model.

4.3 Housing Conditions

There is no consensus on whether OB animals should be group or singly housed; however, the majority of studies using the OB model have singly housed animals post-surgery (*see* Fig. 6). Two studies to date have directly compared housing conditions on OB-related behaviors in mice. Singly housed OB mice demonstrated enhanced emotional behavior as assessed, using the hole-board test, but did not differ in locomotor activity, when compared to group-housed counterparts [70]. In comparison in a separate study, singly-housed OB mice exhibited increased hyperactivity and rearing in the open field test, without altering novelty suppressed feeding or immobility in the forced swim test, when compared to group-housed counterparts [71]. Thus, housing configurations may impact on behavioral assessments in the OB model. In studies where animals are group-housed, there is a lack of detail on whether all sham, all OB, or equal amounts of both are housed together. Based on unpublished studies from our lab, we recommend if pair or group housing is to be employed, then equal numbers of sham and OB animals should be housed together. Research ethics and animal welfare recommend the provision of environmental enrichment to caged rodents, particularly when animals are singly housed. Environmental enrichment has been shown to increase the acute stress response of mice [72] and alter emotional behavior of mice in the open field test [73]. As regards the OB model, environmental enrichment normalizes OB-related hyperactivity of rats, but not mice, and normalizes passive avoidance deficits in OB mice, but not rats [30, 54]. Thus, depending on the experimental design

careful consideration should be paid to housing conditions (singly vs grouped; +/− environmental enrichment) of animals.

4.4 Handling

Although not consistently reported, most investigators employ a regime of regular handling of OB and sham animals prior to and post-surgery in order to reduce irritable and aggressive behavior that may occur [74]. However, repeatedly handling has been shown to induce, rather than reduce, acute stress in mice [72], although in naïve mice handling does not modify open field behavior [75]. Animals that remain irritable despite extensive handling may have a damage to the frontal cortex as a result of surgery and, shown to be the case, should be removed from all analysis. The time of day, means, and duration of handling may differ between laboratories; however, it is important that if employed the handling procedure remains consistent between studies.

4.5 Group Size Selection

In our review of the literature, the majority of rat studies use a group size that ranges from 6 to 12 (with the most common group size being 8), and in mouse studies ranging from 6 to 14 (with the most common group size being 10). Although the number of animals per group varies drastically between studies, based on sample size calculations in our laboratory, we would recommend 8–10 animals per group are required to detect OB-induced hyperactivity. It is increasingly becoming a requirement for a sample size calculation to be included in Animal Ethics applications to ensure that the study is sufficiently statistically powered. To help in reducing the extent of variation within a group, it should be ensured that all animals are of similar age and weight and have been housed and handled under similar conditions.

4.6 Open Field Dimensions, Shape, and Lighting

The design and aversiveness of open field test apparatus is particularly important for detecting OB-related hyperactivity [1, 76]. Hyperactivity of OB animals is most commonly assessed and robustly expressed in highly illuminated (100–320 lux) large (50–90 cm diameter) arenas (*see* Fig. 2). Large arenas also allow for anxiety-related behavior (time spent in and latency to enter center area) to be concurrently assessed. The open field arenas used are primarily either black or white and constructed of plastic to allow ease of cleaning. Testing is generally conducted without bedding in the arena. In order to increase the aversive nature of the test, several laboratories have included reflective walls into the area. Illumination is generally provided by light(s) positioned directly above the arena; however, care should be taken to ensure the arena is evenly illuminated, to avoid the natural tendency of the animal for darker areas.

4.7 Designing a Behavioral Testing Battery

In a review of the behavioral tests used for assessing antidepressant responses, we have found that 47% of rat studies and 42% of mouse studies use a single test (most commonly the open field test), while a third of rat and mouse studies have included an additional test, such as passive avoidance or the forced swim/tail suspension test. A number of studies have employed a battery of testing in OB animals to examine multiple behavioral alterations associated with depression (e.g., hyperactivity, anhedonia, impaired cognition and sociability) in the same batch of animals [55, 77–79]. There is no consensus as to the order or sequence of such a battery of testing nor have there been any studies directly examining different testing sequences. However, based on our own observations over the past 20 years, we recommend the following to be considered when designing a repeated battery schedule with the OB model. Animals will habituate to repeated testing, particularly if the test relies on behavioral responses to novelty, as is the case with the open field test. If OB-induced hyperactivity is the primary outcome of the study, then testing in the open field should be scheduled first. Researchers should consider the number of open field exposures within an experimental study and determine if separate batches of animals are required to assess effects over multiple time periods (e.g., time of onset studies). Recent studies have demonstrated sustained OB-related hyperactivity open field test on four separate occasions [80]. We have found that testing in the elevated plus maze does not alter locomotor activity in the open field test, and thus animals may be tested sequentially in these tests.

4.8 Assessing Antidepressant-Like Activity in the OB Model

The OB model is most commonly used to assess the antidepressant-like effects of chronically administered pharmacological treatments (*see* Fig. 3). Studies examining the time of onset of antidepressant activity have indicated a minimum of 14 days required for monoaminergic antidepressants (such as sertraline and paroxetine), to reverse the OB-induced hyperactivity [81, 82], and thus, it is the most commonly employed treatment schedule. In both OB rats and mice, the most commonly used comparator antidepressants to be used are the SSRI fluoxetine and the TCA imipramine, using the oral (gavage) or intraperitoneal routes as a bolus administration once or twice a day. In addition, the drugs have been formulated in the chow or the drinking water to reduce the regular handling that repeated drug administration requires. OB-induced hyperactivity may be attenuated with shorter duration of treatment (e.g., $5H_{T2C}$ agonist WAY after 5 days [58]) or combination drug treatment (e.g., ketanserin + citalopram after 7 days [83]). Thus, examining effects of treatment on OB-related hyperactivity over time may provide insight into potential early-onset antidepressant activity. Furthermore, a single administration of ketamine attenuates the OB-induced decrease in grooming in the splash test and increase in activity in the elevated plus maze without altering hyperactivity in

the open field test [37, 39]. Therefore, it is advisable that for a comprehensive evaluation of potential antidepressant-like effects in the OB model, responses in multiple behavioral tests representing the multiple facets of depression (e.g., anhedonia, cognitive impairment) are examined. When evaluating antidepressant-like activity of novel compounds in the OB model, it is advisable that effects are examined over multiple doses (dose response) and a reference antidepressant be included in the experimental design (e.g., see [80, 84–86]) (see Troubleshooting 4.4). The antidepressant-like effects of non-pharmacological treatments, such as exercise [27], sleep deprivation [87], and brain stimulation [31, 32, 88], have also been examined in the OB model.

4.9 Considerations for Experimental Design and Reporting

In order to improve the reproducibility of scientific research, transparent and accurate reporting is essential. This enables the methodological rigor of the studies to be scrutinized, assess how reliable the findings are, and repeat or build upon the work. Examination of over 200 studies investigating the antidepressant-like activity of pharmacological compounds in the OB model revealed that many lacked essential detail, such as strain, housing conditions, sex (*see* Figs. 4, 5 and 6), age, success rate of surgery, and inclusion/exclusion criteria, that would enable replication of the data. Embraced by the majority of leading international journals and endorsed by research funders, universities, and learned societies worldwide, it is recommended that researchers conform to the ARRIVE 2.0 guidelines [42] when designing, conducting, and reporting their research. Table 3 provides an overview of the Essential 10 ARRIVE 2.0 guidelines, which are the minimum that should be reported; however, we recommend that researchers consider employing the full Recommended ARRIVE 2.0 Set [42] where possible to ensure transparency and enhance reproducibility and robustness of finding using the OB model.

Increasingly, funders and the scientific community are embracing the move to Open Science, with open access a requirement on publications, resulting from most publicly funded research programs. This is a welcome progression in the field, resulting in research findings disseminated and readily accessible to the largest audience. In addition to publishing manuscripts and reports openly and to further increase the reliability of research, researchers may also consider the following when designing and reporting their data.

(a) Protocol registration: This enables researchers to demonstrate that the hypothesis, approach, and analysis were planned in advance and not shaped by data as they emerged; it enhances scientific rigor and protects the researcher against concerns about selective reporting of results. For example, the Open Science Framework (https://www.cos.io/products/osf)

Table 3
Overview of the ARRIVE Essential 10 guidelines

Characteristic	Elements
Study design	What groups are being compared, including control groups. The experimental unit (e.g., a single animal, litter, or cage of animals). [a]*Include an ethical statement: Name of the ethical review committee that has approved the use of animals, relevant license, or protocol numbers*
Sample size	Specify number of experimental units allocated to each group, total number in each experiment, and total number of animals used. Explain how the sample size was decided.
Inclusion and exclusion criteria	Describe criteria used for including or excluding animals. For each experimental group, report any data points not included in the analysis. For each analysis, report the exact value of n in each experimental group.
Randomization	State whether randomization was used to allocate experimental units and method used. Describe the strategy used to minimize potential confounders, such as the order of treatments and measurements or animal/cage location.
Blinding	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).
Outcome measures	Clearly define all outcome measures assessed (e.g., behavioral changes). For hypothesis-testing studies, specify the primary outcome measure, i.e., the outcome measure that was used to determine the sample size.
Statistical methods	Provide details of the statistical methods used for each analysis, including software used. Describe any methods used to assess whether the data met the assumptions of the statistical approach and what was done if the assumptions were not met.
Experimental animals	Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age, or developmental stage. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures. [a]*Provide details on housing and husbandry including any environmental enrichment* [a]*Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering, and distress* [a]*Report any expected or unexpected adverse events*
Experimental procedures	Describe the procedures in enough detail to allow others to replicate them, including: What was done, how it was done, and what was used. When and how often. Where (including detail of any acclimatization periods). Why (provide rationale for procedures).

(continued)

Table 3 (continued)

Characteristic	Elements
Results	For each experiment conducted, including independent replications, report: Summary/descriptive statistics for each experimental group, with a measure of variability where applicable. If applicable, the effect size with a confidence interval.

These constitute the minimum reporting requirement to ensure that reviewers and readers can assess the reliability of the findings presented [42]

[a]Included from the Recommend ARRIVE Set of guidelines

enables researchers to deposit PHISPS (Population; Hypothesis; Intervention; Statistical Analysis Plan; Primary Outcome Measure; Sample Size Calculation) protocols. In addition or alternatively, registered reports offered by an increasing number of journals undergo peer review, and if accepted, the journal commits to publishing the completed research, regardless of the results obtained. This latter option also enables negative data that may arise from a study to be published, which in turn further increases transparency, providing a more comprehensive profile of the model, reducing the possibility of repeating experiments that are likely to reveal negative data, and ultimately reducing the number of animals that may be used.

(b) Data sharing: Researchers and authors should also consider a data-sharing statement describing how others can access the data on which publications are based. Such data repositories enable others to replicate data analyses and independently test and verify results and enable data mining and combine data set to explore new research questions, which may in turn reduce the number of animals required. There are a number of repositories, where such data can be stored; however, all shared data should follow the FAIR guiding principles [89]; that is, data are findable, accessible (i.e., do not use outdated file types), interoperable (can be used on multiple platforms and with multiple software packages), and reusable (i.e., have adequate data descriptors).

5 Troubleshooting

5.1 Incomplete Removal of the Olfactory Bulbs or Damage to the Frontal Cortex During OB Surgery

Effects such as these may result from inappropriate vacuum pressure created by the aspirator. Placing a pressure gauge indicator on the pump will allow for the recording and maintenance of an appropriate vacuum pressure to aspirate the bulbs. Damage to the frontal cortex may also occur due to incorrect surgical coordinates. It is recommended that the OB surgery is carried out on a test

animal in order to confirm appropriate coordinates and vacuum pressure required for bulb removal prior to embarking on a large-scale study.

5.2 Irritability and Aggression Following OB

Irritability and aggression is most commonly observed in non-handled singly housed OB rats or damage to the frontal cortex during removal of the bulbs. As such, it is recommended that all sham and OB rats are habituated to handling prior to and following surgery. In comparison, group-housed OB mice are less aggressive than sham counterparts [11], although predatory aggression is increased 4 weeks following OB in mice [45]. OB may also enhance aggressive behavior in certain strains of mice. As such, handling prior to and following surgery may reduce aggressive behavior in such strains.

5.3 Inability to Detect OB-Induced Hyperactivity

This is most commonly attributed to the design of the testing apparatus or repeated testing in the open field. OB-induced hyperactivity is most pronounced when assessed in a large aversive (high illuminated) arena when compared to a small, low illuminated area (see experimental variables 3.5). Animals may become habituated to the open field test upon repeated testing, and thus if effects are to be examined over multiple time points, it may be necessary to use separate batches of animals.

5.4 Inability to Detect Reversal of OB-Induced Hyperactivity Following Treatment

Careful consideration should be given to dosing regimen (dose(s), frequency of administration, time-tested post-administration), species (rat vs mouse), and behavioral paradigm(s) employed. Certain treatment may demonstrate efficacy in one species/strain (e.g., efficacy of cholinergic drugs in OB mouse but not rat) or in different behavioral paradigms (e.g., effect of ketamine in splash test but not open field test). It is recommended that effects of compounds are examined over multiple doses and dose response curves established. The effects of novel compounds should be compared to those of a reference antidepressant (e.g., imipramine or citalopram) known to attenuate OB-induced hyperactivity (see experimental variables 3.6). Shorter or longer treatment regimens may be required when examining effects of novel compounds/treatments, particularly if not monoamine based.

6 Conclusion

In conclusion, the OB model is a robust, reproducible, and valid animal model of depression and antidepressant activity. Although initially employed exclusively in the rat for antidepressant efficacy, the last 20 years has seen its value in the mouse. In both species, a range of alterations have been identified, which have relevance to the human depressed condition, such as locomotor, cognitive, and

anhedonic changes that can be reversed by both pharmacological and non-pharmacological strategies. Although both species share certain behavioral hallmarks (such as a hyperactivity in a novel environment), there are differences that have been outlined that may impact on the selection of either the rat or mouse for investigation. This review has focused on this predictive validity of the model and, in particular, on the conditions pertaining to successful surgery and open field measurements. A greater emphasis in the future will be placed on examining rapidly acting antidepressant activity in the model, which will incorporate changes in central signaling and circuitry functioning. In addition, further development of a selection of behavioral assays that have a greater ethological relevance, while also attempting to model core symptoms of depression, will continue. This will enable a behavioral profile of a spectrum of OB-related alterations to be studied but will need to be decided upon with caution, in order to ensure that the same cohort of animals can be utilized in such a test battery. Such developments will enhance the value of the model in detecting promising compounds for further antidepressant evaluation.

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Chapter 5

Marble Burying in Mice

Jeffrey M. Witkin and Jodi L. Smith

Abstract

The discovery of new medicines requires large efforts on the part of medicinal chemists and their biology counterparts. Given the great numbers of compounds that require behavioral evaluation for the discovery of an optimized, new chemical entity for neurological or psychiatric disorders, rapid methods for screening compounds in small quantities are required. In addition to compound screening, marble burying can be used to assess the effects of nonpharmacological experimental changes (e.g., stress or dietary changes) and is a fast first method for comparing genotypic differences. In addition to the establishment of on-target activity of new compounds, the data can be used to make first approximations to potential therapeutic utility. Since 2005, there has been an exponential growth in the use of this assay. The procedure exemplified in the current protocol outlines testing of rotarod performance (2 min) followed by marble burying (30 min) in naïve, untrained mice. The marble-burying test is initiated by the placement of the mouse in a container with marbles resting atop sawdust bedding. The number of marbles buried (2/3 covered by sawdust) by the mouse after a fixed period is the primary dependent measure. The behavioral readouts of marble burying and rotarod performance help enable differentiation across some genetic lines and categorization of drug effects into pharmacological categories. The potential use of this assay system for the exploration of drugs that might be valuable in the treatment of anxiety and obsessive–compulsive disorder is discussed.

Key words Marble burying, Rotarod, Anxiety, Anxiolytic, Obsessive, compulsive behavior, OCD, SSRI

1 Background and Historical Significance

The discovery of new medicines requires large efforts on the part of medicinal chemists and their biology counterparts. Given the large numbers of compounds that require behavioral evaluation for the discovery of an optimized new chemical entity for neurological or psychiatric disorders, rapid methods for screening of compounds typically available in small quantities are required. Marble burying is a behavioral assay that fits this purpose. In addition to the establishment of on-target activity of new compounds, the data can be used to make first approximations to potential therapeutic utility. Since 2005, there has been an exponential growth in the use of this

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Table 1
Assay characteristics of the marble-burying test in mice

Assay characteristic	Value
Mouse appropriate	Reduced quantity of compound needed Ease of working with compounds difficult to dissolve or suspend
Transgenic mice	Wild-type and genetically modified mice can be compared
Target relevance	Genetic and pharmacological tests can establish an assay that detects the interaction of the compound with its specific protein target
Rapid throughput	Rapid testing – no training and brief test sessions
Minimal resources	Same mice are used for both rotarod and for marble burying
Rich biological readout	Both side effect and therapeutic potential can be assessed

assay. By April 1 of 2021, there were 588 research papers identified with the search in PubMed of marble burying. Prior to 2005, the number of publications per year was <10, whereas from 2011 to 2017, this value was >40/year, with 2018–2020 showing >70 publications/year.

Digging of many animals including mice is a natural behavior, the probability of which can be modulated by environmental context [1]. Thus, materials that are diggable, are typically dug by mice. The instinctive aspect to marble burying has several important advantages that include the lack of a training as a requirement for the establishment and maintenance of these behaviors and the ability to probe causal questions through evolutionary biology and modern neurobiological methods.

The value of marble burying for compound screening and behavioral studies in general is summarized in Table 1. The size of mice enables less compound to be required for testing, thus lowering the cost and burden on the medicinal chemistry teams. The concentration of compounds for in vivo testing is generally one tenth that needed for larger species like rats which provides the huge advantage of increasing the probability of compound dissolution for dosing. Genetically modified animals can be used for precise neurobiological inquiry. The use of combined genetic and pharmacological tools can enable rapid assays that detect the action of the compound at the protein target of relevance. For example, one could produce a decrease in marble burying with a selective agonist and block this with antagonists. Genetic deletion of the protein can be used to verify on-target activity of the new antagonists. Marble burying is a rapid throughput assay allowing for screening multiple compounds in vivo in a single day without the need for prior training. Finally, as with all in vivo assays, the

Table 2
Issues with the marble-burying test in mice

Assay issue	Implications
Behavior is not a steady state	Assay variability
Behavioral specificity	Secondary assays are needed
Pharmacological specificity	As a stand-alone assay, implications for predicting therapeutic potential is weak

biological output of a drug effect provides a first look into the potential side effects and therapeutic potential of the new chemical series. More will be said of these points later in the chapter.

The positive characteristics of the marble-burying assay come with some issues (Table 2), many of which are considerations that must be embraced by all in vivo model systems. The rapidity of the test comes with the issue that the behavior is not under firm regulatory control. As such, variability in behavior arises that needs to be controlled both environmentally and statistically. All behaviors have the issue of behavioral and pharmacological specificity, and marble burying is no exception. Thus, a compound that reduces marble burying can do so through a host of behavioral processes (e.g., motoric, sensory impairment, reduced obsessiveness, etc.). Parceling out the nature of the behavioral effect requires additional behavioral data for context. All drug effects also require evaluation for pharmacological specificity. Can the mechanism of the drug effect be precisely defined (e.g., interactions with receptor X) and can the therapeutic class of drug effect be assigned (e.g., anxiolytic)? More will be said on these points later in the chapter.

Therapeutic possibilities for compounds or manipulations that decrease marble burying with minimal ancillary effects are anxiety and obsessive–compulsive disorder (OCD). The basis for this possibility is based primarily, but not exclusively, on the pharmacological validation for selective serotonin reuptake inhibitors (SSRIs) [3] that remain the standard of care for both anxiety and OCD (data will be discussed below). Broekkamp et al. [4] provided the first report differentiating the effects of anxiolytics and antipsychotics in this assay.

SSRIs are the first-line therapy for the majority of patients with anxiety disorders [5] and depressive disorders [6]. Antidepressants including the SSRIs are also the first-line treatment for obsessive–compulsive disorder (OCD); other medicines are sometimes added since they are not always fully efficacious [7–9]. Methods for evaluating the antidepressant-like effects of SSRIs were described many years ago [10] and are widely used for their reliability and predictive validity [11]. In contrast, anxiolytic-like behavioral

effects of classical antidepressants and SSRIs are not generally detected by conventional anxiolytic drug screens [8, 12, 13]. Marble burying by mice is one of the few behavioral methods that has been used for reliably detecting the effects of SSRIs that may be anxiety related. Marble burying in mice has been used to model anxiety disorders including obsessive–compulsive disorders (OCD) due to the excessive nature of the behavior, ethological data [14–17], and the pharmacological effects of clinical standards [8, 15, 18–20]. Thus, drugs used in the treatment of generalized anxiety in humans (e.g., acute doses of benzodiazepines and chronic doses of SSRIs) as well as compounds used to treat OCD (e.g., SSRIs like fluoxetine) decrease burying.

Given the remarkable sparsity of animal models that are available for predicting pharmacological therapies for OCD, convergent data from multiple behavioral assays is generally utilized, and marble burying constitutes one such assay [20, 21].

2 Equipment, Materials, and Setup

2.1 Mice

Mice of any genotype can be used. We have primarily studied male, NIH Swiss mice (Harlan Sprague Dawley, Indianapolis, IN) weighing between 28 and 34 g. A number of receptor knockout mice on both a C57Bl/6 and a CD1 background have also been studied in our lab. The mice can be either housed in groups or in standard, individual mouse cages with continuous access to standard rodent chow and water. We have traditionally housed the mice under a 12-h light dark cycle (lights on: 06:00–18:00 h) with experiments conducted at about the same time daily to minimize any potential diurnal variation.

2.2 Test Environment

The following equipment has been utilized by our laboratory:

- Mouse home cages measuring 24 × 45 × 15 cm
- Sawdust bedding (Harlan Sani-Chips, Harlan-Teklad)
- A test room with lab bench space sufficient to house as many 17 × 28 × 12–cm high plastic tubs as will be used in one experimental run
- Rotarod or rotarods for mice (Ugo Basile model 7650)
- Mouse test cages: clear or opaque plastic tubs (17 × 28 × 12–cm high) containing sawdust shavings (Harlan Sani-Chips, Harlan-Teklad) tamped down by hand to about 5 mm high in the test cages
- Face mask for filtering airborne mouse particulate matter
- Disposable gloves
- Blue glass marbles (1.5 cm diameter)

- Marking pen for mouse identification by tail marking if necessary
- Plexiglas covers for the marble-burying cages to prevent escapes

2.3 Setup

Mice are initially brought into the housing vivarium for about 3 days to 1 week prior to setup in order for acclimation to occur. Mice can be housed individually or in group cages. Mice are given continuous access to standard rodent diet and water. We have housed our mice in 12 per cage (24 × 45 × 15–cm high) with sawdust bedding in a temperature-controlled vivarium with a 12-hr light–dark cycle (lights on: 0600 to 1800 h). On the day of the experiment, the mice are brought into the test room and are allowed to habituate to the testing environment for 60 min prior to dosing. The rotarod is set to rotate at 6 rpm (setting 2 on Ugo Basile model 7650). Marbles are removed from the test cages and are placed on a metal grate for sifting marbles from the sawdust bedding. We have utilized the mouse cage top for this purpose. If marble-burying cages are used on more than one occasion (which we have done), it is a good idea to wear a face mask to avoid breathing airborne particulates that arise in the sifting process. Then, tamp down the sawdust in the marble-free test cage using a gloved hand to a level of about 5 mm. On top of the sawdust, add 20 marbles to the center of each test cage without stacking marbles upon one another (patterns of marbles across the entire cage are also possible but setup takes more time and has not been shown to be essential) (Fig. 1).

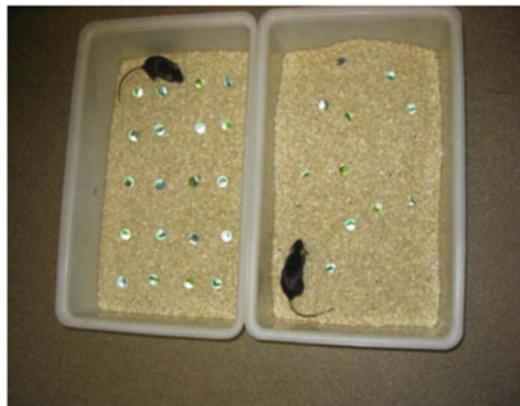

Fig. 1. Marble-burying apparatus and example data. The mouse on the right is a control animal and has buried several marbles. The mouse on the left received a complete hippocampal lesion and has not buried any. Covers for these chambers are not shown in this photograph. (The figure is from Deacon [1] with permission of the publisher)

2.4 Dependent Measures

The number of marbles buried and the number of rotarod failures are measured. The number of marbles buried in our lab has been accomplished by visual inspection by trained observers and was defined as a marble buried by 2/3 with the sawdust bedding.

Rotarod performance by our lab has been measured as a success (stays on rotarod for 2 min without falling twice) or a failure (falls off twice within the 2 min test period). Other measures are possible (e.g., latency to fall, or 0–4 falls, etc.).

3 Behavioral Procedure

3.1 Weighing and Administering Drugs or Non-pharmacological Manipulations

No prior training is necessary. However, if steady-state behaviors are desired, the mice could be given prior exposure to these two tests. Reports of prior training for marble burying have not be fully evaluated (however, see habituation data below). For the rotarod, mice could be trained to reach a criterion performance (e.g., able to stay on the rotarod for 2 min) prior to the test day.

After the setup procedure, weigh and mark the mice for identification if needed. After a mouse is marked, you can dose them with a test compound or provide a specific environmental impact (exposure to an odor or defeat stress, for example). Mice can be given test compound by any of many routes of administration (e.g., i.p., i.c.v., p.o., etc.). Dosing of compounds or exposure to a non-pharmacological stimulus should be done in groups of 5 every 3 min. This time allows for the testing of the first group of 5 mice on the rotarod prior to placing them into the marble-burying space. After dosing, the potential for fighting among mice could be minimized by placing them in individual holding cages instead of back into their home cages; however, lab space is required for this extra caging. After dosing, set a timer for the pretreatment interval (time from dosing to testing).

3.2 Testing

When the pretreatment time has elapsed, take 5 treated mice and place them on the rotarod for the 2 min test. If a mouse falls off, place it back on. If the same mouse falls again, that mouse can be placed into the marble-burying apparatus and the top placed on. Repeat this procedure with all groups of 5 mice. Handling of the mice and observation of the mice while in the experiment provide additional opportunities for detecting and noting any effects of the test compounds.

A timer is set so that the mice can be removed from the marble-burying apparatus after 30 min although other time periods could be established. Thus, the entire test procedure will last 32 min not including the weighing, dosing, and pretreatment times.

4 Data Analysis

Since the observation of marbles buried is scored by a trained observer, an observer blind to treatment is preferred. However, this is sometimes difficult to achieve in the lab setting. Therefore, it is reassuring that there is a high interobserver reliability for the detection of unburied marbles [3], a measure that is recommended by your lab as well. A single observer for one set of experiments is recommended for consistency. The use of positive controls and vehicle controls also provides a good assay calibration from one experiment and observer to the next. For dose–response data, the use of contemporaneous vehicle and positive controls creates a strong experimental design.

Analyze rotarod data using Fisher's exact probability test. If other measures of rotarod performance are used (e.g., latencies to fall), ANOVA should be utilized. Analyze marble-burying data using one-way ANOVA for a single compound. Analyze significant overall results by post-hoc Dunnett's test. Since the hypothesis is that an efficacious compound will impair rotarod performance and decrease marble burying, a one-tail test is justified if more conservative and conventional two-tail methods are not needed or desired. One could also establish a priori "statistical" values based upon historical data.

5 Anticipated Results

Our greatest experience with this assay system is with male NIH Swiss mice. Under the conditions described, the mean number of marbles buried by these mice in 30 min is 10.6 ± 1.4 marbles ($n = 48$). For the rotarod, the mean number of failures in the 2 min test period has historically been 8.5 + 1.6% ($n = 48$). Given variation from day to day, we have used contemporaneous controls rather than historical controls. Drug effects on marble burying as first reported by our group are shown in Table 3 where they are compared to nestlet shredding behavior and rotarod performances [3].

Note that the antidepressants studied produced decreases in marble burying at doses that did not impair rotarod performance with the exception of clomipramine. Anxiolytic compounds were either active at doses that reduced rotarod performance or impaired rotarod performance without impacting marble burying. The dopaminergic drugs shown in Table 3 were also distinct in their behavioral profile from the antidepressant agents. *d*-Amphetamine decreased marble burying without impacting motor performance but did stimulate locomotion [3]. The antipsychotic drugs, chlorpromazine and risperidone, decreased both rotarod and marble-burying behaviors. Note, too, differences in pharmacological

Table 3
Doses tested and minimal effective doses (MED) in a marble-burying test, nestlet-shredding test, and on rotarod performances of male, NIH Swiss mice[a]

	Marble burying		Nestlet shredding		Rotorod	
	Dose range	MED (mg/kg)	Dose range	MED (mg/kg)	Dose range	MED (mg/kg)
Monoamine uptake inhibitors						
Clomipramine	3–30	30	3–30	10	3–30	30
Citalopram	1–3	1	1–10	3	1–3	Inactive
Fluoxetine	3–30	10	1–10	10	3–30	Inactive
Venlafaxine	10–60	30	10–60	30	10–60	Inactive
Desipramine	3–30	30	3–30	10	3–30	Inactive
Nisoxetine	3–30	30	1–30	30	3–30	Inactive
Imipramine	5–30	15	3–30	3	5–30	Inactive
Anxiolytics						
Chlordiazepoxide	3–30	30	3–10	10	3–30	30
Buspirone	0.3–10	Inactive	0.3–3	Inactive	0.3–10	Inactive
Bretazenil	1–100	Inactive	3–30	10	1–100	Inactive
Pentobarbital	3–17	Inactive	5.6–17	10	3–17	5.6
Dopaminergics						
Amphetamine	0.1–10	1	1–10	1	0.1–10	Inactive
Risperidone	0.03–1	1	0.03–0.3	0.3	0.03–1	0.3
Chlorpromazine	1–10	10	1–10	10	1–10	10

The table is reproduced from Li et al. [3] with permission of the publisher
[a]Mean vehicle control values were 8.9 ± 0.8 marbles for marble burying, 0.91 ± 0.1 g for nestlet shredding, and 14.3 ± 2.4% for rotorod failures. Twelve mice were used for vehicle controls in marble burying, six were used for nestlet shredding and twenty–four were used for rotorod performance assessments. In some cases the MED is properly a least effective dose

profiles across the marble burying and nestlet shredding tests with these compounds.

An example of the use of marble burying in the process of drug discovery is shown in Figure 2. MGS-0039, an $mGlu_{2/3}$ receptor antagonist, was undergoing preclinical development for psychiatric disorders. Chaki and colleagues utilized marble burying as a potential predictor of anxiolytic activity and of in vivo target engagement. They demonstrated decreases in marble burying by MGS-0039. Importantly too, they showed that MGS-0039 was likely producing this effect in vivo by its actions as an antagonist of $mGlu_{2/3}$ receptors by preventing its effects by co-application of the specific mGlu2/3 receptor agonist LY354740 (Fig. 2). Thus, in quick

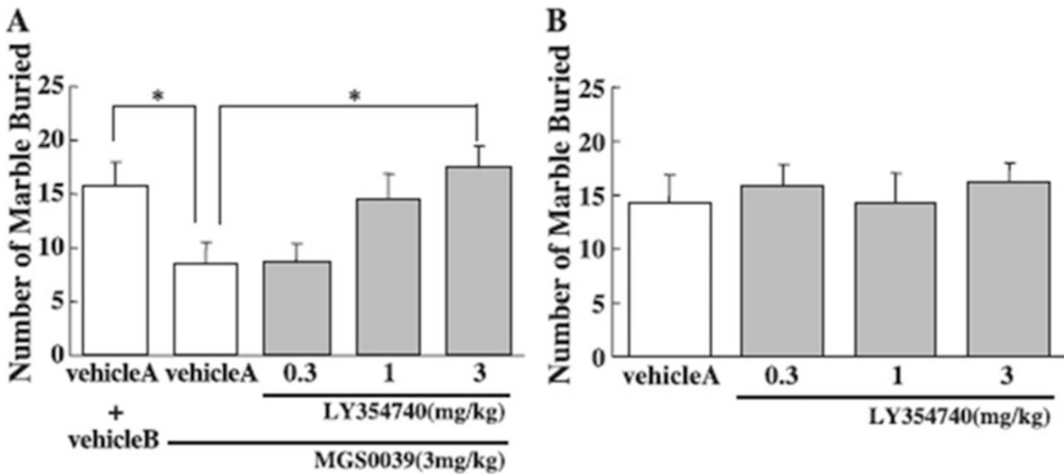

Fig. 2. (**a**) Effects of the mGlu2/3 receptor antagonist, MGS-0039, on marble burying of mice and blockade of MGS-0039-induced decrease in marble burying by the selective $mGlu_{2/3}$ receptor agonist LY354740. (**b**) The lack of effect of these doses of LY354740 on marble burying. Each bar represents the mean + SEM. * $p < 0.05$ compared to vehicle. (The figure is reprinted from Shimazaki et al. [41] with permission of the publisher)

order, the marble-burying test enabled the probing of two important biological questions surrounding this new $mGlu_{2/3}$ receptor antagonist.

6 Experimental Variables and Troubleshooting

6.1 Testing Time and Testing Environment

Time of day has not been explicitly explored as a factor that might modify either baseline behaviors or the impact of independent variables. In the absence of guiding data, it is suggested that time of day be held as constant as possible from day to day.

Given that variations in other conditions have not been specifically monitored for their potential impact on marble burying, holding these basic conditions as constant as possible is advised (e.g., room lighting, noise, etc.). The original report on nestlet shredding [3] utilized normal overhead fluorescent lighting during test conditions, whereas marble burying in this report used a dimly lit test room (~12 lux). Later studies (not published) have used higher-intensity lighting without changes in baseline behavior or effects of fluoxetine on both marble burying and nestlet shredding.

6.2 Training and Test Variations

The training parameters given above have been successfully implemented, but the full range of parametric variation that is acceptable or optimal has not been determined. A host of changes could be enabled for specific purposes. For example, variations in rotarod speed or rotarod pretraining could be implemented, or the amount of time in the marble-burying chamber might be reduced, etc. Other assays could be built into the test battery. The inverted

screen test [22], for example, has been used as a motor impairment test in place of the rotarod [23]. For such an assay battery, where the goal is rapidity and ease of testing, a series of consecutive behavioral/physiological assays could be established (e.g., pupillary reflexes, grip strength, a small-sized Irwin assessment battery, rotarod, and then marble burying).

In the work of Broekkamp et al. [4], grooming was used as the ancillary behavior against which to compare marble burying. Li et al. [3] used nontrained rotarod performance as the comparator behavior. Drug effects on behavior must be compared to their effects on other measures in order to interpret the behavioral specificity of drug action [2, 24, 25]. Although direct comparisons between methods have not been made, the use of grooming versus rotarod performance appears to detect overlapping yet distinct pharmacological effects. When drug effects on marble burying are compared to drug effects on grooming, the assay differentially detects anxiolytic agents. GABA-based anxiolytic compounds are not selective for marble burying when rotarod is the comparator behavior; SSRIs generally are. However, false positives also present themselves – *d*-amphetamine and imipramine also decrease marble burying at doses having no effect on rotarod performance. A locomotor stimulation assay was easily able to differentiate the effects of d-amphetamine from that of the SSRIs.

Additional dependent measures could be developed. For example, a camera atop each marble-burying chamber could be used to detect a variety of movements and movement patterns. With automated monitoring of behavior, one could ascertain the impact of the presence of marbles on burying behavior. One could also envision explorations of marble plus non-marble objects within the chamber and create phenotypic patterns for unique drug classes.

In addition, the assay system is open to evaluation of the effects of a range of independent variables such as dietary changes, stress manipulations, social interactions, etc.

The possibility of automation exists, though this aspect has not been reported. For example, photometric reading of the marble apparatus before and after marble burying might be possible and thereby eliminate the need for human observations for that purpose.

An illustration of the diversity of methods and experimental questions that can be applied with the marble-burying test is shown in Fig. 3. In this study, unpredictable chronic mild stress was applied to mice over a period of 3 weeks in BALB/c mice. A time course of marble burying was established by visual observations for each minute up to 5 min and then at each 5 min time point thereafter. Stress shifted the marble-burying curve to the left (Fig. 3, left). The AMPA receptor potentiator, LY392098, did not significantly alter marble burying. In contrast, chronic fluoxetine shifted the stress-induced curve for marble burying back in the

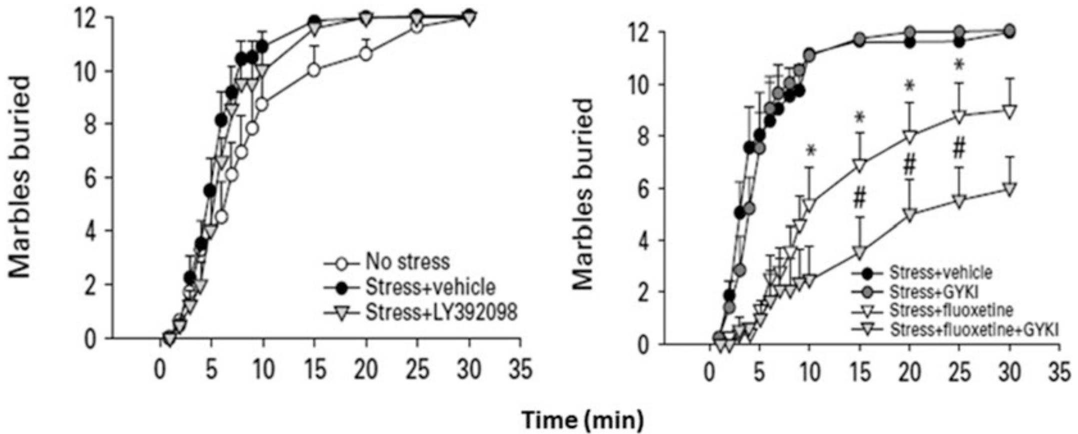

Fig. 3. Effects of unpredictable chronic mild stress and pharmacological manipulations on marble burying in mice. Each point represents the mean + SEM of 10 mice. * $p < 0.05$ compared to stress + vehicle control; # $p < 0.05$ compared to stress + fluoxetine group at each time point. (Data are from Farley et al. [42] with permission of the publisher)

direction of vehicle-control values (stress moderation), an effect further modulated by the AMPA receptor antagonist GYKI 52466 (Fig. 3, right).

6.3 Genotype Comparisons

The availability of transgenic and receptor-deficient mice provides a powerful tool alone and in combination with other tools (e.g., selective lesions or selective pharmacological agents) increases remarkably the ability of behavioral researchers to define neurobiological mechanisms of action. When conducting experiments with transgenic or receptor-deleted mice, a contemporaneous wild-type control mouse will have to be put through the same procedures. The use of appropriate counter-balancing practices for the experimental day is encouraged. Parametric variation might be needed for different strains. For example, a direct comparison of a drug effect in one transgenic mouse strain might require modifications in the testing conditions to enable the same baseline level (effect of drug vehicle).

Marble burying was used as an in vivo screening assay for the discovery of the $mGlu_2$ receptor potentiator THIIC. In a study with this compound, THIIC dose dependently decreased marble burying in $mGlu_2$+/+ mice (wild-type or WT mice). In contrast, no significant decreases were produced by THIIC in $mGlu_2$−/− mice (knockout or KO mice) demonstrating that the effects on marble burying were due to the interactions of THIIC with $mGlu_2$ receptors (Fig. 4). THIIC (30 mg/kg) did not impair rotarod performance in WT mice; chlordiazepoxide (30 mg/kg) decreased both marble burying and rotarod performance (Fig. 4).

In contrast to the anxiolytic chlordiazepoxide, KRM-II-81 and MP-III-80 are nonsedating. The later compounds are selective

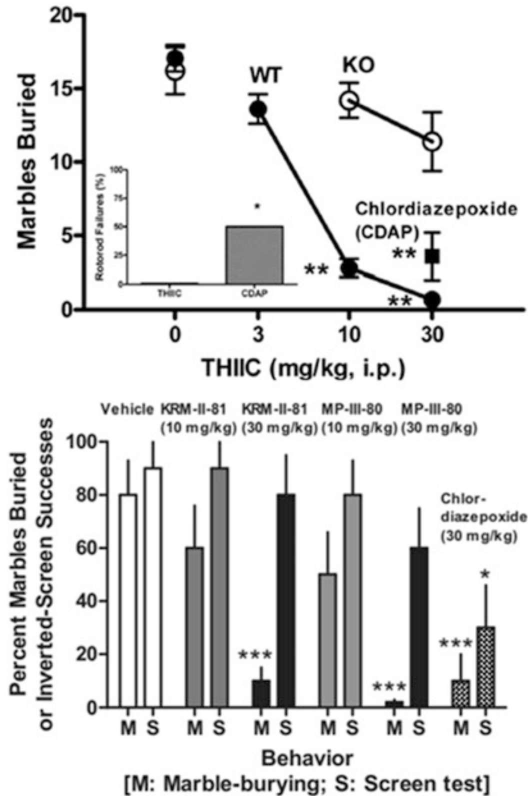

Fig. 4. Top. Effects of the $mGlu_2$ receptor potentiator THIIC on marble burying in wild-type (WT) and $mGlu_2$ receptor knockout (KO) mice compared to the effects of 30 mg/kg chlordiazepoxide in WT mice. Effects on rotarod performance of WT mice is also shown. **Bottom.** Effects of the alpha2/3-selective potentiators of $GABA_A$ receptors, KRM-II-81 and MP-III-80, compared to chlordiazepoxide on marble-burying behavior and inverted-screen performance. * $p < 0.05$, *** $p < 0.001$ compared to vehicle control values. (Data are shown as means ± SEM. THIIC data are reprinted from Fell et al. [43], and KRM-II-81 and MP-III-80 data are reprinted from Biggerstaff et al. [23] with permission from the publishers)

potentiators or GABAkines of alpha2/3-containing $GABA_A$ receptors and show anxiolytic-like activity in the Vogel conflict test [26, 27]. Both of these compounds decreased marble burying in C57Bl/6 mice without impairing inverted screen performance; chlordiazepoxide, which also increases Vogel conflict responding [26, 27], decreased both marble burying and inverted-screen performance of these mice (Fig. 4).

Studies in TARP- γ8-deficient mice (KO) show multiple measures of behavior that can be obtained to compare with the behavior of marble burying to enrich comparative findings (Fig. 5). TARP-γ8 is an auxiliary protein localized primarily in the cerebellum and relatively deficient in cortex [28]. Compared to intact mice (WT), TARP-γ8-deficient mice (KO) showed significantly less

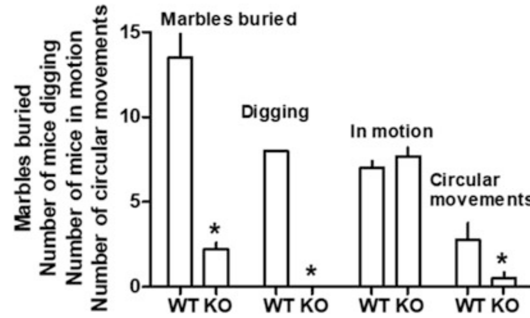

Fig. 5. Marble burying and ancillary behaviors of wild-type and TARP-gamma8-deficient mice. Each bar shows the mean + SEM of 8 male mice. * $p < 0.05$ compared to WT. (Data are extracted from Gleason et al. [44])

marble burying, digging behavior (by visual observation in the marble-burying chamber), and circular movements (measured by photocells on a circular track). In contrast to these differences in behavior, WT and KO mice did not differ significantly in their overall movement in the track (not shown) or the number of mice in motion (by visual observation in the marble-burying chamber) (Fig. 5).

6.4 Experimental Chambers

The size of the marble-burying chambers in all dimensions has not been precisely evaluated. The possibility of behavioral differences across dimensions is possible.

6.5 Statistical Power

We have successfully used 12 mice for a vehicle control group and eight mice/dose group. If the overall experiment uses more mice than allowed by test cage space, repeated iterations of the dosing and testing protocol can be utilized [3].

A dose–response curve for fluoxetine and a time course over 5 consecutive days for this compound are shown in Fig. 6. Note the dose-dependent decreases in marble burying at doses that also produce antidepressant-like effects in mice [3]. Mice given with drug vehicle for 5 consecutive days showed a relatively stable number of marbles buried thus demonstrating the perseverance of this behavior over time. The effects of fluoxetine over 5 days were also consistent with a decrease, but the statistical significance at this lower dose of 10 mg/kg was uncovered in only 3 out of the 5 days. These findings illustrate the lack of tolerance to the effects of fluoxetine. In addition, they point out the need for large effect sizes (e.g., at 30 mg/kg fluoxetine) and higher numbers of mice to achieve statistical separation than in assays in which variability is more controllable.

Given that relatively stable and persistent features of marble burying that occurs in the same mice over repeat testing also suggest the possibility of being able to use the same mice for multiple experiments can be a huge savings in costs.

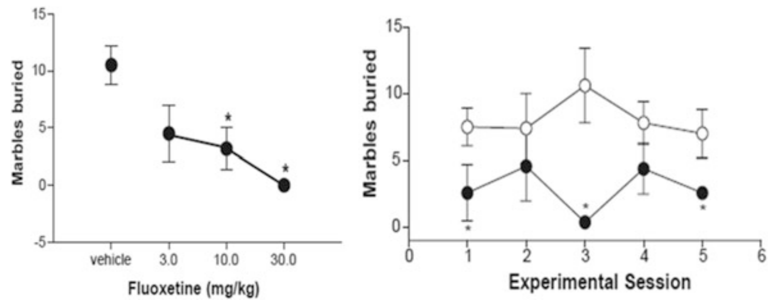

Fig 6. Comparison of effects of vehicle and fluoxetine on marble burying of male, NIH Swiss mice during a single 30 min experimental session (left panel) or over 5 consecutive days of testing of the same mice. Each point represents the mean ± SEM in 8 mice. (Data are reprinted from Li et al. [3] with permission of the publisher)

7 Concluding Remarks

The methods we describe of sequential rapid tests of motor function and marble burying has utility in the establishment of the in vivo profiles of novel potential therapeutic agents. The ability to utilize transgenic and receptor-deficient mice as well as other neurobiological methods (optogenetics, selective pharmacological tools, brain stimulation and lesions, etc.) increases dramatically the impact of such rapid behavioral assays in the fields of neurobiology and neuropsychopharmacology. It is, however, critical to remember that all in vivo assays are biological readouts and not disease models per se. Thus, the most powerful interpretation of data for decision-making comes with analysis of convergent sources of data in conjunction with human data if available.

In vivo modeling of OCD is not well advanced [9]. Adjunctive behaviors such as schedule-induced polydipsia [40] or high-rate behaviors such as chocolate-chip eating in rats have been used to explore the pharmacological basis of this disorder [20]. Other models have also been used where an SSRI has shown activity [29]. However, there has not been substantial pharmacological and behavioral validation of these models (e.g., test–retest reliability, is the drug effect behaviorally specific, false positives and false negatives, etc.). Marble burying was described many years ago by Broekkamp et al. [4] (see reviews [8, 18]); however, marble burying in this work was evaluated pharmacologically primarily for generalized anxiety disorder. Nestlet shredding was first described by Li et al. [3] as an excessive and non-habituating behavior like marble burying.

Marble burying has been an increasing-used behavioral test for OCD and OCD therapeutics. As of April 1, 2021, there were 67 literature citations in PubMed under the search marble burying

and OCD. The number of publications on this topic has increased over the years with ≤3/year as of 2009. From 2017 to 2020, that value has more than doubled with ≥7 publications/year.

As with the increasing use of marble burying in general and to investigate aspects of OCD, additional methods for studying OCD have been disclosed with rapid growth. A literature search on April 1, 2021, revealed a total of 344 publications under the search "OCD and animal model." Prior to 2007, there were <7 publications/year on this topic. From 2008 on, this number reached double digits with >20 papers published/year from 2011 to 2020. This chapter is not the place for discussion of the new work that has been done in this area. For the interested reader, it is enough to be said that models in addition to marble burying have been sought out. For example, genetically engineered mice have been used, including the 5-HT_{2C} receptor knockout mouse [30]. New conceptual models of OCD have also recently been presented [9]. Recent reviews and new thoughts on the topic should also be of value in guiding future model assessments and development [31–39].

A novel selective 5-HT_{2C} receptor agonist was tested for its possible efficacy as a therapy for OCD [21]. In this study, marble burying was one of the multiple assays employed. The compound decreased marble burying like that of the anti-OCD drug clomipramine, but unlike clomipramine, the 5-HT_{2C} receptor agonist did not impair rotarod performance (Fig. 7). Congruent with the marble-burying results, the compound also decreased the excessive

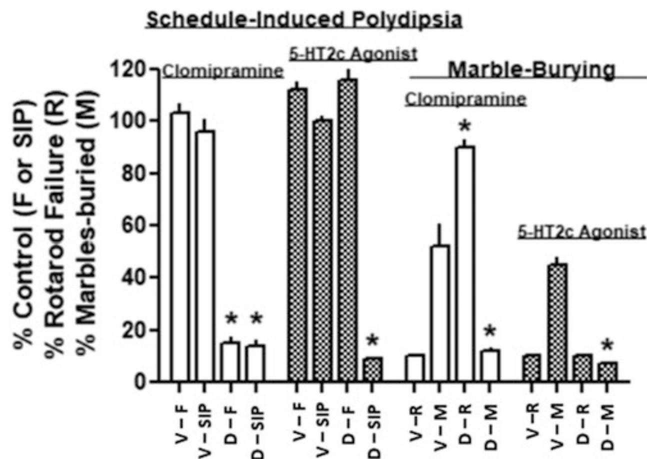

Fig. 7. Effects of the anti-OCD drug clomipramine in comparison with a selective 5-HT2C receptor agonist against marble burying (M), rotarod performance (R), food-maintained lever pressing (F), and schedule-induced polydipsia (SIP) [40]. Shown are data for vehicle controls (V) compared to the two drugs (D). Each bar represents the mean + SEM in 5–8 rats or 12 mice. * $p < 0.05$ compared to respective vehicle controls. (Data are extracted from Rodriguiz et al. [21])

and non-habituating behavior of schedule-induced polydipsia in rats [40]. As with the rotarod data, clomipramine, but not the 5-HT_{2C} receptor agonist, also decreased responding maintained by food delivery (Fig. 7). That 5-HT_{2C} receptor agonists might be of value for the treatment of OCD is given another degree of support from the OCD-like behaviors observed in mice in which the 5-HT_{2C} receptor has been deleted [30].

8 Notes

Although we have touched briefly on the topic of OCD, there is a broad literature that was not covered in this chapter. Another interesting model used to engender OCD-like behaviors is that of food deprivation-induced wheel running, where high and persistent rates of wheel running are engendered [45]. A few genetic models have also been introduced (c.f., [46, 47]). The animal models have been important also for generating data that has helped define neurobiological mechanisms associated with OCD (c.f., [48]). Importantly, data from the experiments with animal models have enabled and facilitated the discovery and development of new potential treatment options for OCD [49].

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Chapter 6

How the Sucrose Preference Succeeds or Fails as a Measurement of Anhedonia

Tatyana Strekalova

Abstract

The sucrose preference test is used in laboratory rodents as a reward-based test to indicate a decrease in the sensitivity to a reward (anhedonia), which is a key symptom of major depression. It is based on the animal's natural preference for sweets, with the assumption that this preference is in proportion to the pleasure that the animal experiences when it consumes sweetened solutions. As such, a reduction in the intake and preference of a sucrose-sweetened solution in rodents, typically compared to plain water over a fixed time frame, is considered as a manifestation of anhedonia. Despite the seemingly technical simplicity of the test, many laboratories experience serious difficulties in establishing this method in a sufficiently reproducible manner, especially in mice. Moreover, while a manifestation of anhedonia in the sucrose test parallels other depressive-like changes in rodents, the stratification of animals upon their hedonic responses as anhedonic and non-anhedonic is often problematic. Here, we address the most common technical, physical, and physiological phenomena that typically confound the evaluation of hedonic sensitivity with the sucrose test in laboratory mice and rats. The impact of these factors is often underestimated or neglected in routine laboratory work. When taken under control, however, the accuracy and sensitivity of the test is substantially increased and the abovementioned limitations with this method are resolved.

Key words Sucrose test, Reward sensitivity, Anhedonia, Drinking behavior, Interindividual variability, Depression models, Confounds, Mouse, Rat

1 Introduction

Several approaches have been applied to evaluate the ability of rodents to experience pleasure, including the sucrose intake/preference test, progressive ratio responding, intracranial self-stimulation, novel-object place conditioning, and conditioned place preference [1–6]. The sucrose intake/preference test measures the amount and the ratio of a sweet-tasting solution that the animal voluntarily ingests across a fixed period. It does not involve any learning, operant, or other laborious effortful behaviors [7, 8]. As such, this test is the only paradigm for a sensitivity to a

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reward that is based on spontaneous behavior. It is considered to reflect the animal's capacity to experience hedonic pleasure while not being substantially influenced by other behavioral biases.

Typically, a free choice two-bottle testing paradigm is used, where individually housed rats or mice are allowed to access one drinking bottle that contains sweetened solution and the other with plain water for 1–48 h [9–12]. Most commonly, sucrose solutions are used; the use of saccharin was proposed earlier to minimize a caloric component of the test [13], though current evidence demonstrates a negligible effect of this factor. Because of this, and the bitter taste of saccharin, the vast majority of researchers use sucrose, with solution concentrations 0.5–2% [3, 14]. The time of day that the test is initiated is typically close to the beginning of the animals' dark phase. Water deprivation for 1–20 h is employed with some protocol variants [1, 14, 15]. Preference for the sucrose solution can also be examined in automated systems, whereby licking frequency and duration are measured. The disadvantage of automated method is the as yet insufficient accuracy of these measurements, and the naturally large interindividual variability in the licking behavior of rodents [16]. The sucrose preference test can be carried out in a separate test cage. In this case, the animals are habituated to the testing conditions [17], and the amount of sweetened solution and plain water consumed across the testing period is measured by weight or volume. From these data, a sucrose preference is calculated as follows: preference = intake of sweet solution consumed/total intake of sweet solution + plain water.

As the sucrose preference test is a mechanistically undemanding experimental task, it can be deployed simultaneously in large cohorts of animals. Unlike the foregoing models that address reward-seeking behavior, it does not depend on learning, anxiety, and locomotion, which are frequently altered by various experimental manipulations. It is commonly used to study hedonic sensitivity in models of depression [18–21]. For these purposes, it was originally established in rats as an element of the chronic mild stress (CMS) paradigm [13]. With an increase of the use of mice in translational research, the use of the sucrose test was expanded to this species, with minor adjustments [2, 22, 23].

A number of protocols of the sucrose test have been developed over the decades, and, even with seemingly similar protocols, the outcomes vary greatly between different laboratories [3]. This is well illustrated by a comparison of 25 CMS rat studies, where similar CMS protocols and stress-induced induction of another hallmark of depressive-like behavior, such as helplessness, were performed in experimental rats (Table 1). These varieties in baseline and CMS sucrose preference values mirror the challenges with the standardization, reliability, and reproducibility of this method. While a manifestation of anhedonia in the sucrose test parallels other depressive-like changes in rodents, the stratification of

Table 1
Variable outcome from the sucrose test in CMS rat experiments

Rat strain	Sucrose concentration, %	Mean sucrose preference, %			Reference
		Stress group	Non-stressed control	Baseline	
Wistar	2	55	80	N.A.	[24]
Sprague-Dawley	1	60	85	N.A.	[25]
Wistar	1	60	80	N.A.	[26]
Sprague-Dawley	1	59	84	N.A.	[27]
Sprague-Dawley	1	55	80	80	[28]
Sprague-Dawley	1	80	92	N.A.	[29]
Sprague-Dawley	2	40	75-80	N.A.	[30]
Sprague-Dawley	2	65	95	N.A.	[31]
Wistar	1	71	85	86	[32]
Wistar	1	60	85	83	[33]
Sprague-Dawley	1	70	80	N.A.	[34]
Sprague-Dawley	1	50	75	N.A.	[35]
Sprague-Dawley	1	18	80	67	[36]
Wistar	1	40	70	65–70	[37]
Sprague-Dawley	1	40	80	N.A.	[38]
Wistar	1	86	93	93	[39]
Sprague-Dawley	1	68	83	83	[40]
Wistar	1	65	80	N.A.	[41]
Sprague-Dawley	1	62	70	70–71	[42]
Wistar	1	69	77	N.A.	[43]
Sprague-Dawley	1	35	75	N.A.	[44]
Wistar	1	45	80	80	[45]
Sprague-Dawley	1	80	78	70–75	[46]
Lister Hooded	0.9	82	75	80–82	[23]
Lister Hooded	1	75	88	88	[47]

The analysis of 25 CMS rat studies published between 1989 and 2019 (selection criteria: definition of anhedonia by a sucrose preference, occurrence of helplessness and molecular/physiological features of MDD) revealed great variability in mean sucrose preference values; e.g., Feng et al. [36] reported 18% as the mean sucrose preference in stressed rats and 80% as the mean sucrose preference in controls, whereas Shi et al. [39] reported the mean sucrose preference of 86% in the stressed group and the mean sucrose preference of 93% in control rats. Based on these observations, the need to consider the role of potential confounds and the demand to develop a more reproducible sucrose test protocol remain high

animals upon their hedonic responses as anhedonic and non-anhedonic is often problematic due to the limited accuracy of the test, especially in mice [1, 18, 21].

Indeed, drinking behavior during the sucrose test can be considerably influenced by many factors unrelated to the hedonic status of the animal, including general nonspecific effects of experimental chronic stress or "background noise" stressors on a consummatory behavior that particularly may imply effects of novelty, diet, and water deprivation, which are used in the CMS paradigm [3, 11, 12]. Stress might not only decrease but also increase liquid intake and sucrose intake and preference. This can result from stress-induced diabetes mellitus, a hypersecretion of corticotropin-releasing factor and vasopressin at hypothalamus and hypophysis manifesting as a hyper-compensatory "pro-hedonic" response to stress [2, 18, 48, 49]. Drinking behavior and sucrose ingestion in rodents can be compromised by bias related to social status or the sensitization to reward experiences caused by excessive intake of palatable solutions. The latter results in a "ceiling effect" in this test. Other factors that may compromise drinking behavior include alterations in the metabolic needs in calories and water due to change in body mass, strain differences, and inter-batch variability between cohorts of experimental animals [14, 17, 50]. Furthermore, a natural interindividual variability in circadian patterns of liquid intake, where individual laboratory rodents display maximum liquid intake at different periods of the day, may greatly confound the outcome of the sucrose test [3, 11, 12, 51].

Physical artifacts contributing to the difficulties with the sucrose test are rarely studied. Nevertheless, they can hamper measurements of sucrose intake and preference when not taken under control [2, 11, 12, 14]. The most common of these artifacts are the use of excessively leaking bottles and their erroneous handling, as well as a temperature imbalance between laboratory environment and drinking solutions, and the lack of proper control over potential contamination of material and equipment used scents, odors aversive to rodents. Below, we describe the sucrose test protocol that minimizes many physiological and physical confounds in mice and rats, thus increasing its accuracy to the extent that a stratification of experimental groups upon their individual hedonic responses becomes possible.

2 Materials

2.1 Bottles and Scale

To perform a two-bottle sucrose preference test on rats and mice, we used customized glass bottles of volume 150 mL, or 50 mL Falcon tubes with scent-free chemical rubber tops and glass drinking spouts (length of 6 mm; the thickness of glass is 1.5 mm, internal diameter of the tip is app. 2.5–3 mm). The rubber must

be flavor free as intense scent or resin can cause an aversive reaction in rodents and suppress drinking, sometimes entirely. The choice of glass drinking spouts is of preference over metal drinking spouts since metals can also exert aversive reactions in rodents due to the metallic taste/flavor and high thermal conductivity, whereas glass material is neutral in these aspects. The length of nickels is particularly critical for the mouse test. Spouts that are too long result in capillary effects that require an additional physical effort to suck a liquid. Furthermore, the optimal choice of the length and diameter of the spout is important for preventing bottles from leaking and should be tested prior to use.

To assess liquid intake, we weigh the bottles using scales with a resolution of 0.1 g. It is surprising how many labs use scales with a resolution of 0.5 or even 1.0 g, while mouse water intake during a 10-h preference test might be only 0.1–0.5 mL. The measurement of volumes of liquid drunk is normally a far less accurate method and is not recommended, especially when low volumes are consumed.

2.2 The Preparation of Sugar Solutions

To prepare sucrose solutions, we recommend using commercial sugar from food stores sold in paper packaging. Sugar easily absorbs flavor, in particular from plastic, which is beyond human but not rodent ability to sense. It is highly advisable, therefore, to make sure that sugar used for the sucrose test is not in contact with any plastic, e.g., during storage. In some laboratories, stock solutions are prepared to be diluted later. This is also not recommended because of the above-indicated scent contamination factor that is difficult to control. The concentration of sucrose solution can vary, dependent on the protocol, from 0.5% to 2.5% (*see below*), while the average optimal concentration is 1%. We prepare sucrose solution using tap water that the experimental animals normally consume, approximately 12 h prior the test. We fill the bottles and store them in the upside-down position in the laboratory room where the test takes place. This procedure greatly helps to avoid air bubbles in drinking spouts and to prevent leakage caused by a temperature difference between freshly used tap water and room temperature (*see* **Note 1**).

2.3 Controlled Laboratory Environment

Since the daily dynamics of drinking behavior of laboratory rodents is determined by the animals' active (dark) cycle, it is optimal to house them under a reverse 12 h:12 h light–dark cycle for studying liquid intake. Rats and mice display individual peaks in water intake during the dark period (*see* **Note 2**, Fig. 1; [11, 12]). A reversed lighting schedule is also important because of the need to swap the position of the bottles in a midway through the testing period, in order to avoid artifacts that may be caused by the individual preferences for one side of the cage when drinking [14]. It is also worth controlling the brightness of laboratory illumination which, if too high, can be a source of stress in rodents.

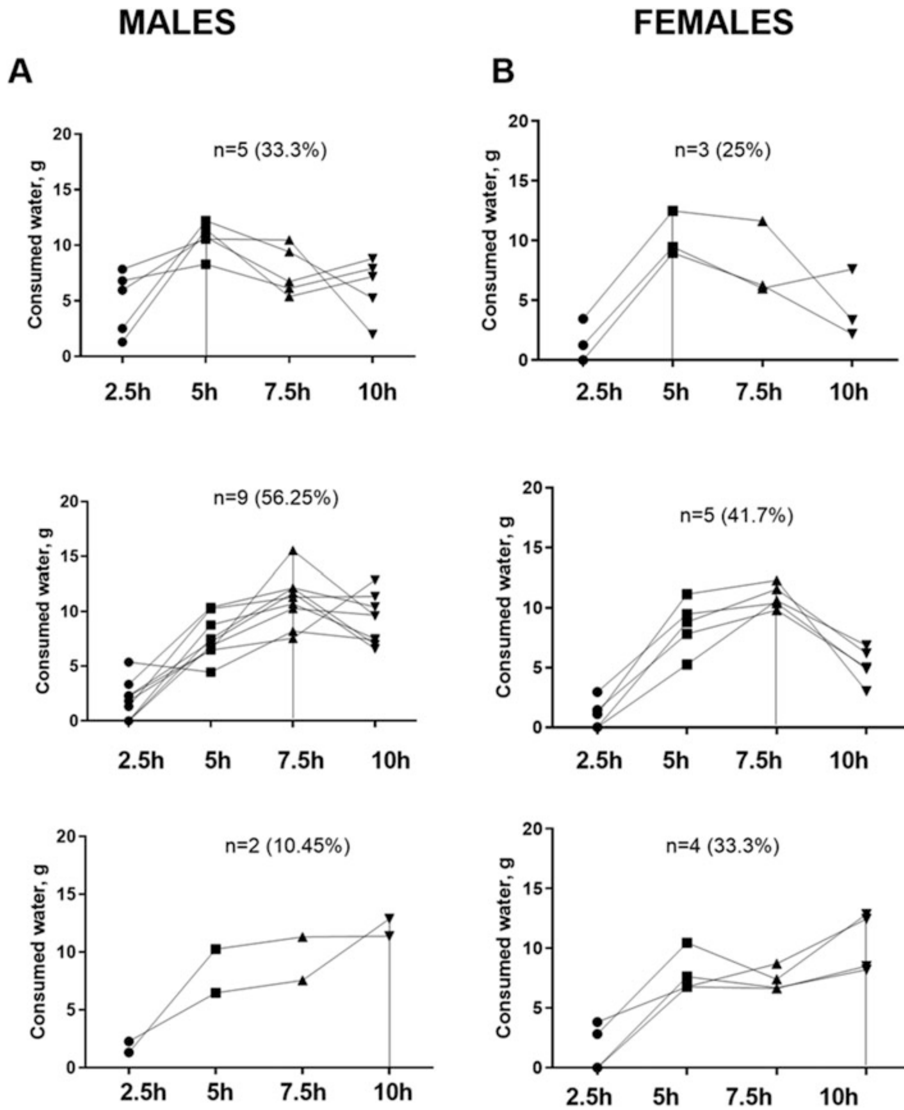

Fig. 1 Individual drinking patterns in male and female Wistar rats. (**a**) Males and (**b**) females showed peaks in water consumption after 2.5 h, 5 h, 7.5 h, or 10 h from the beginning of the test that coincide with the onset of dark (active) day cycle. Percentage of males and females from each rat group ($n = 16$ and $n = 12$, respectively) with individual drinking pattern is indicated. Notably, a substantial cohort of animals' peaks in drinking behavior is at 10 h, a time point that is unlikely covered during typical sucrose test protocols. This can be a source of artifacts that reduce the sensitivity of the sucrose test. Each line represents an animal and each dot the absolute water intake at that time point

Temperature control is a substantial element of the laboratory environment that defines the accuracy of the sucrose test. A maintenance of temperature at 22 ± 1 °C is optimal, and temperatures fluctuating by more than 3 °C can be considered suboptimal for the accuracy of the sucrose test. Increases in the air temperature in this range can result in the increased daily liquid intake up to 30% in

mice. Another important factor for drinking behavior is air humidity, which should be 55%. Lastly, excessive vibration and noise, even if imperceptible to humans, can greatly affect drinking behavior in rodents due to stress effects and must be avoided for the sake of reliable results.

2.4 Animals and Group Randomization

For the sucrose preference test, we use laboratory mice and rats obtained from licensed animal providers that are allowed to habituate to the laboratory conditions (housing, lighting) for at least 5 days. Because of a large interindividual and seasonal variability in drinking behavior and sucrose ingestion between cohorts of mice and rats, it is important to ensure that within one study all experimental animals are originated from the same cohort and tested simultaneously. As the test is carried out on single-housed animals, female mice and rats that are normally group housed have to be single housed 48 h prior; the duration of habituation can affect drinking behavior (*see* **Note 3**, Fig. 2). Although under normal conditions we have not observed any correlations between the sucrose test variables and body weight, the latter can play a role when various experimental challenges are employed. It is, therefore, highly recommended to balance experimental groups upon their weight.

3 Methods

With the above-described settings, when the bottles are prefilled and animals are individually housed under reversed light schedule, the experiment starts shortly before the onset of dark animals' light cycle (typically at around 9:00), with the removal of regular drinking bottles from cages, weighing the sucrose test bottles, and the placement of the sweetened and plain water bottles at two maximally distanced locations of each cage. We avoid any water and food deprivation prior to the test as this was shown to confound drinking behavior of rodents. The duration of the test varies between 5 and 48 h and the optimal duration is 8–10 h. To prevent possible effects of side preference in drinking behavior, the bottles are carefully swapped in a mid-time point of the test. The bottles are weighed immediately after the termination of the testing period.

3.1 Sucrose Test Parameters

Traditionally, the sucrose preference is calculated as a percentage of consumed sucrose solution from the total amount of liquid drunk. All absolute measures need to be considered while interpreting the results, to evaluate the possibilities of nonspecific changes in liquid intake, potential instrument error, and other artifacts. Since the physiological meaning of the sucrose preference test is the evaluation of preference to either solution above random choice (chance level of 50%), we have proposed an alternative formula for sucrose

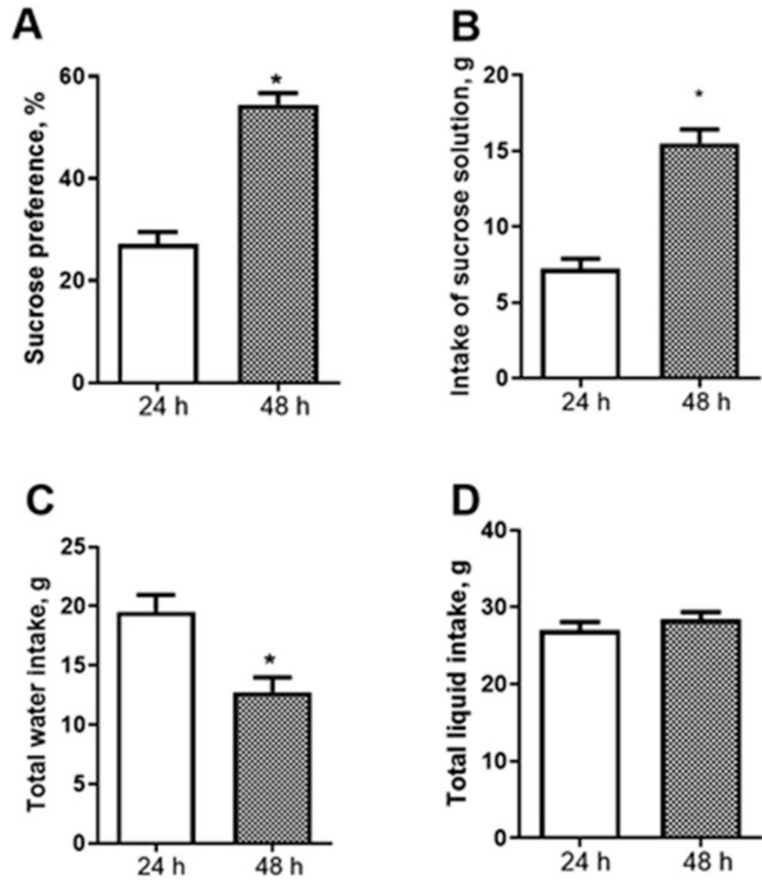

Fig. 2 Effects of habituation to single housing in the rat sucrose test. (**a**) Sucrose preference was lowered in comparison with a chance level of 50% at the time point of 24 h of single housing in new cages but not at 48 h. At the time point of 24 h, the (**b**) sucrose intake was significantly decreased, and (**c**) water intake was significantly increased in comparison with a 48-h time point. (**d**) Total liquid intake was not affected during a habituation of rats to new cages. Thus, habituation to new housing conditions in rats alone compromises sucrose intake/preference and needs to be of sufficient length to avoid confounds in the evaluation of these parameters. Bars are Mean $\pm$ SEMs, $*p < 0.05$, Wilcoxon. Each group had 13–15 young Wistar rats

preference that takes into account this factor and might be more accurate in mirroring hedonic sensitivity in the sucrose test:

$$\text{Sucrose Preference} = [\text{V (Sucrose solution)}/\text{V (Sucrose solution)} + \text{V (Water)} \times 100\%] - 50\%$$

A reduction of preference for highly palatable substances including sucrose is normalized by antidepressants, which validates this parameter as a measure of anhedonic behavior [3, 6, 22, 52]. Sucrose preference is less sensitive to various confounds than the absolute intake of sweetened solutions, especially in mice [1, 11, 12, 50]. The use of sucrose preference enables the

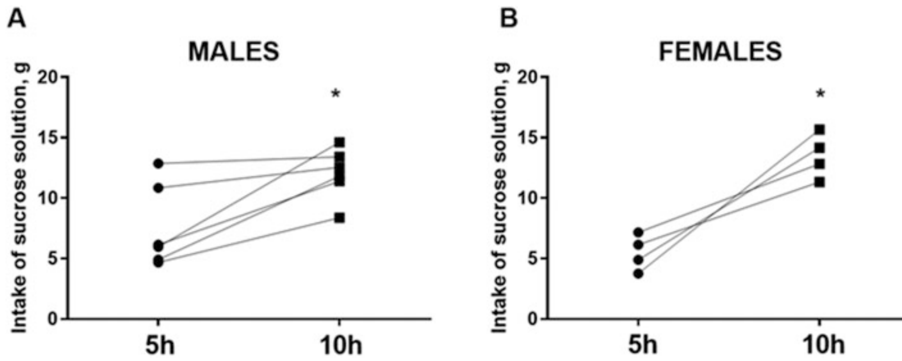

Fig. 3 Neophobic responses toward sucrose solution in Wistar rats naive for sucrose. The intake of a 1% sucrose solution was increasing in a course of a 10-h sucrose test in (**a**) male rats and (**b**) female rats. Each line represents an animal and each dot the absolute water intake at that time point. $*p < 0.05$, Wilcoxon

stratification of individual animals as anhedonic and non-anhedonic in the CMS [48, 53, 54] and as responders and nonresponders to standard antidepressant treatment [36, 39, 55, 56].

3.2 Single Sucrose Preference Assessment

Due to neophobic reactions toward sucrose taste in naive rodents—a phenomenon more typical in mice that may also occur in rats (*see* **Note 4**, Fig. 3), we run a presession of sucrose drinking 24 h prior the first test session. Therefore, animals are either allowed to drink 2.5% sucrose solution in a one-bottle paradigm for 2 h or receive access to a 1% sucrose solution/water in a two-bottle paradigm according to a regular protocol. Because of seasonal variations, sucrose intake and preference are likely to be higher in summer and lower in winter. Therefore, it is advisable to use the 2.5% sucrose method during winter and a regular protocol with 1% sucrose solution during summer. In summer months, neophobic responses and variability during the sucrose test can be low; thus, no taste familiarizing procedure may be required. Instead, data from the first standard sucrose test protocol can be used. A criterion of sufficient familiarization with a sucrose test that serves well in many studies is a combination of the mean sucrose preference of about 75% and absence/negligible percentage (<5%) of individual animals with sucrose preference <65%.

3.3 Repeated Sucrose Preference Assessment

Repeated sucrose test is frequently used with rodent studies addressing the effects of chronic manipulations and interventions, such as stress, drugs, diets, aging, and others. The main issue that needs to be taken into account with this type of experiment is the gradual increase in sensitization ("addiction") to the ingestion of sucrose, developed along repeated access of animals to the sucrose solution. This manifests as increases in sucrose intake, total liquid intake, and sucrose preference and results in the "ceiling effect" in sucrose preference (that approaches to 90% or higher values). Through this, the discriminative ability and sensitivity of the test is lost.

Careful planning of experimental design should be aimed to minimize the number and the duration of sucrose test sessions that can diminish sensitivity. A stepwise gradual decrease of the concentration of sucrose solution can be another option of maintaining optimal sucrose preference in controls.

4 Conclusions

While the sucrose test is one of the traditional animal tests used for decades in common laboratory practice, there remains a clear need for its refinement. In this chapter, we have summarized several frequently neglected factors that are critical to the robustness of the sucrose test as a measure of anhedonia. Furthermore, its optimized methodology can enable a stratification of experimental rodents upon their individual vulnerability/resilience to hedonic deficit and response to antidepressant treatment that is critical for more advanced translational research on depression, aging/neurodegeneration, neurotoxicity, and other medical conditions. One such example is the CMS mouse model, in which mice receive a chronic dosing with citalopram or celecoxib (Fig. 4). We hope that this chapter can refresh the view of the researcher on this classic though somewhat difficult to exploit test and encourage its broader use, to foster precision medicine.

5 Notes

1. A lack of a proper control over a temperature balance between laboratory facilities and drinking solutions can generate significant error in the evaluation of drinking behavior, typically, if freshly poured tap water is used to prepare drinking solutions *ex temporo* (of temperature about 17 °C). Because of the difference with laboratory room temperature (22 °C in average), the air pressure inside the water bottle gradually increases due to an increase of its temperature, resulting in leakage that can reach 0.5 g, i.e., amounts comparable to daily water intake in a mouse sucrose preference test [11, 14, 50].
2. The measurement of drinking activity can be corrupted by individual differences in animals' daily patterns of drinking behavior, as was shown in mice [2, 12]. Wistar rats that are often used in the sucrose test show similar interindividual variability in peaks with water consumption in a one-bottle 10-h test during four measurements spaced by 2.5 h (Fig. 2). These data highlight the importance of a prolonged testing of drinking behavior covering individual drinking peaks in the majority of experimental animals, not only in mice but also in rats, for higher accuracy of the sucrose test.

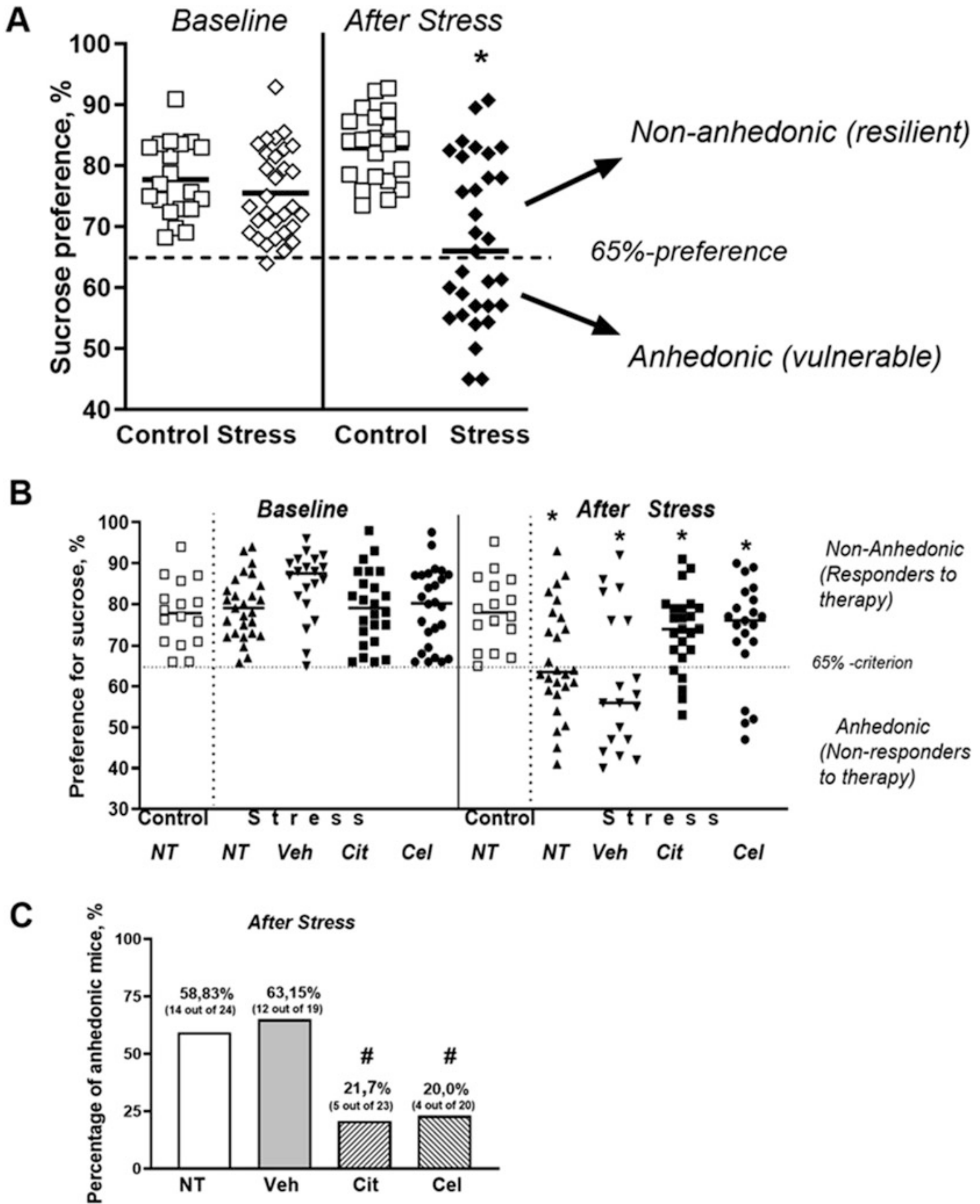

Fig. 4 A stratification of individual animals as resilient/vulnerable to stress-induced anhedonia and as drug responders/nonresponders in a CMS mouse paradigm. The refined sucrose test protocol in C57BL/6 mice enables the analysis of the interindividual differences in a reward sensitivity in the CMS model and a stratification of (**a**) anhedonic and non-anhedonic animals subjected to CMS upon anhedonia 65% threshold (that equals the lowest control value of sucrose preference), (**b**) responders and nonresponders to the antidepressant drug therapy among CMS mice treated with citalopram or celecoxib [2], and (**c**) a comparison of sizes of subgroups. *NT* not treated pharmacologically, *Veh* injections of saline, *Cit* injections of citalopram solution at the dose 15 mg/kg/day, *Cel* dosing with food pellets at the dose 30 mg/kg/day. *$p < 0.05$ vs. non-stressed control group (**a,** t-test; **b,** two-way ANOVA and Tukey's test); **#**$p < 0.05$ vs. respective stressed control group, Fisher test

3. The habitation of a novel housing environment can affect drinking behavior in both mice and rats. We have found that for group-housed male Wistar rats, the first 24 h after transition to single housing reduced sucrose preference to <50%. After 48 h, however, rats showed significant preference to a 1% sucrose solution (Fig. 3).
4. While rats were shown to display a limited neophobic reaction toward the taste of sucrose in comparison to mice [12, 23], the analysis of the dynamics of sucrose intake during the 8-h test revealed low sucrose intake and preference during the first hours of the test and high sucrose consumption at its end (Fig. 4). These data suggest that, even though this confound is regarded to be typical for mice rather than for rats, neophobic responses of rats toward sucrose solution need to be considered.

Acknowledgments

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Chapter 7

A Vogel Conflict Test Using Food Reinforcement in Mice

Jeffrey M. Witkin and Jodi L. Smith

Abstract

Control of operant behavior in mice represents a powerful technique for evaluating behavioral function, biobehavioral differences, and the impact of environmental and pharmacological stimuli. The present method provides a means for rapid behavioral assessments that contrasts with the many weeks or months required in most steady-state procedures. The use of mice in these tests has many advantages including the ability to evaluate transgenic animals. Effects of compounds on suppressed or punished responding are often used to predict anxiolytic efficacy in humans. In contrast to steady-state baselines that can take months to establish, the present method can deliver dose–response data within a few days. Mice are food deprived and placed in an experimental chamber with two nose poke holes. Every nose poke (FR1) produces a 20 mg food pellet. After 2 days of training, a drug or drug vehicle is administered, and the mice are exposed to a mixed FR1 (food), FR1 (food + shock) schedule in alternating, unsignaled periods of 4 and 10 min for three cycles. In the 10-min period, nose pokes produce both food plus brief electrification of the grid floor (0.5 mA for 100 ms); during the 4-min period, only food is delivered with each nose poke. Under these conditions, the introduction of shock can substantially decrease nose pokes during the 10-min punishment period without significantly affecting responding during the non-punishment periods. Results are shown that document the comparability of the drug effects in this procedure to that of other punishment procedures requiring protracted training. Importantly, the method has wide applicability in probing multiple aspects of murine behavior and behavioral differences such as acquisition and extinction of behavior, sensory differences, and memory.

Key words Vogel conflict test, Mouse, Anxiety, Anxiolytic, Transgenic mice, Operant, response.

1 Background and Historical Significance

In the era of modern neuroscience where genetic manipulation and targeted neurological and pharmacological manipulations are possible for investigation in live, behaving organisms, the potential for probing important neurobiological questions and their associated mechanisms requires the ability to accurately control and monitor behaviors. Operant behavior, or behavior controlled by its consequences [1], can be readily and precisely established, regulated, and monitored. The elegance of operant behavior methods for these purposes is undisputed but comes with the disadvantage of long-

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term behavioral studies that can require months to establish, maintain, and utilize these behaviors to probe neurobehavioral questions.

A method for rapidly establishing behavior and evaluating the impact of a host of interventions (behavioral, environmental, pharmacological, etc.) is presented here. The method simultaneously utilizes both suppressed and non-suppressed behavior to increase the diversity of the behavioral readouts. For example, responding suppressed by punishment is widely used as a method for detecting anxiolytic activity of compounds since increases in punished responding by drugs are often produced by drugs used in the management of generalized anxiety disorder and acute anxiety states [2, 3]. Although punished responding has been established in mice, there have been very few studies [4, 5]. The scarcity of such data is likely related to several factors. Food or water deprivation in mice must be very precisely controlled and monitored so as to preclude health problems and mortality. The training time required to establish stable baselines of responding under punishment procedures can be several months. Finally, faster procedures to evaluate anti-anxiety agents in mice exist and include the elevated plus maze and a host of other ethologically based methods such as open field, marble burying, and light/dark transit studies (see present volume).

Another method that has been used to some extent in mice is the Vogel conflict procedure that is a variant of the Geller–Seifter conflict procedure in which water-deprived animals are exposed to a drinking spout and, after a period of drinking, every lick produces an electric current that suppresses the rate of drinking. Drugs used in the treatment of anxiety prevent the shock-induced suppression [6, 7]. A drawback with the Vogel test is that it has not been studied in much detail with mice, and the difficulties in establishing a validated model have been acknowledged [8]. Methods for use of this procedure in mice has been communicated (see present volume). In addition, the traditional Vogel procedure may not be pharmacologically isomorphic with the food-based conflict methods that are more firmly under schedule control. For example, buspirone does not generally increase punished responding under the Geller conflict method but increases drinking in the Vogel conflict procures with greater proclivity [9]. False positives have also been reported. For example, isoproterenol increased Vogel conflict responding and water consumption but did not increase Geller conflict responding where food was the reinforcer [10].

The present procedure describes a Vogel-like method in mice utilizing food presentation and an arbitrary operant response (original report in [11]). We used the nose poke response for this purpose since it is readily emitted at a high basal rate so as to reduce the time for training. The nose poke response is also not the

consummatory response as is drinking in the Vogel procedure, and thus, we hoped to minimize the influence of appetite stimulants on this behavior.

2 Equipment, Materials, and Setup

2.1 Mice

Mice of any genotype can be used although there has been little comparative behavioral analysis with this procedure. We had originally studied male, NIH Swiss mice (Harlan Sprague Dawley, Indianapolis, IN) weighing between 28 and 34 g, as well as a number of transgenic mice bred on either a C57bl/6 or a CD1 background. The mice can be either housed in groups initially or in standard, individual mouse cages with continuous access to standard rodent chow and water. We have primarily used a 12-h light–dark cycle (lights on: 06:00–18:00 h), and experiments are conducted toward the dark cycle period, but other variations are not likely to impact results.

2.2 Test Environment

Experiments are conducted in mouse operant conditioning chambers (MED Associates, Inc., St. Albans, VT) (*see* Fig. 1). These chambers are equipped with two holes (13 mm diameter), 1 cm above a grid floor and spaced 9.7 cm apart. The holes could be illuminated with white light. A water bottle is located on the opposite wall. Nose pokes within the hole (>6 mm) are counted as responses and produce the audible click of a relay. Food pellets (20 mg, BioServe, Frenchtown, NJ) were delivered to a food trough (centered between the nose poke holes) coincident with an audible tone for 100 ms. The chambers are located within sound-attenuating cubicles supplied with ventilation and white noise to mask extraneous sounds. Scrambled electric shock could be delivered to the grid floor of the chamber by a constant current AC source (MED Associates). Experimental events and data were collected with MED-PC software (MED Associates).

2.3 Setup

Mice are acclimated to the vivarium for about 3 days to 1 week prior to experiments. Mice ideally will be individually housed in order to best control food intake in individual animals. However, creative variations with group housing are possible, the key being to control food intake in individual mice. During the acclimation period, mice are given continuous access to their standard rodent diet and water.

For 3 days before training begins, food pellets to be used as reinforcers are added to the food supply in order for the mice to become accustomed to these pellets. This can help prevent any novelty pellet avoidance. On the day prior to training, food restriction begins with each mouse being given ~3 g standard rodent chow plus two reinforcer pellets at about 16:00 h. Water remains continuously available.

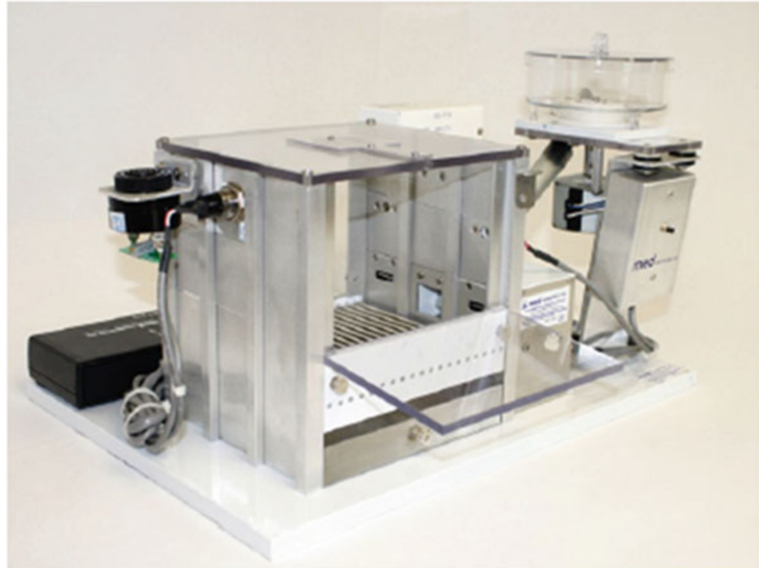

Fig. 1 Mouse operant conditioning chamber. The figure shows an opening on the front panel (center near grid floor) into which food pellets can be delivered by a pellet-dispensing device (behind front panel). On either side of the pellet opening would be the two nose poke holes (not shown in this photograph where retractable levers are pictured). A mechanical relay (not shown) is mounted behind the front panel to generate an audible click with each nose poke. Also not shown is a small sound speaker to deliver white masking noise into the chamber. In our work, a water bottle was at the back of the chamber with the spout accessible to the mouse. The entire chamber is housed within a sound-attenuating enclosure and operated and monitored by a remote computer. (Photograph courtesy of Steve Walsh, Med Associates, Inc.)

2.4 Dependent Measures

Rates of responding (responses/min) under each component of the mixed schedule are calculated from the number of responses and the elapsed time in each component. Dose–effect and/or time–effect curves for a compound are constructed from the data collected with drug vehicle and different drug doses and/or times post dosing.

3 Behavioral Procedure

Mice are brought into the experiment room with the test chambers and are allowed to acclimate in their home cages for approximately 60 min. During the 60 min acclimation period, five reinforcer pellets are placed within the food hopper of the operant chambers. The experimenter should also check that pellet delivery works properly and that there are no pellets on the chamber floor or stuck in the pellet delivery tube. After 60 min, the mice are weighed and placed into the operant test chambers.

3.1 Day 1: Training

Initial training occurs about 24 h after the removal of food from the home cage, but variation is likely tolerated. During training, mice remain within the operant chamber overnight with water available. During the evening experimental session beginning at about 1700 h, the mice are placed within the experimental chamber for the first time. After about 120 s, the left nose hole is illuminated, and every nose poke response within the right hole produces a single food pellet. The hole was illuminated as a discriminative stimulus associated with the experimental session being in operation. However, nose pokes within the illuminated hole have no scheduled consequences. A total of 200 food pellets can be delivered within this first session that terminates after that point or after 8 h, whichever occurs first. At ~09:00 the next morning (day 2), the mice are returned to their living cages. If the mice received at least 15 food pellets in the training evening, they are not fed. If the mice received less than 15 pellets, they are eliminated from the study.

3.2 Day 2: Further Training

At ~1500 h of day 2, the mice are returned to the test room, acclimated for about 60 min, and then dosed with physiological saline or relevant drug vehicle by the desired route of administration. The mice are then placed into the operant chambers without any reinforcer pellets having been placed into the food hoppers. Two minutes later, the left nose hole is illuminated, and as on day 1, every response in the right nose hole produces a single food pellet. This component ends after 4 min, or if there are no responses, the component terminates after a total of 15 min. Four minutes after termination of component 1, a 10-min period is introduced without any change in stimulus conditions; every response within the right nose hole continues to produce a food pellet during this period.

The 4- and 10-min periods then alternate for three cycles; the component durations are fixed with the exception of the first component as just described. The reason for the potential time extension in component 1 is to maximize the probability of occurrence of at least one reinforced response before the punishment component where shock will be introduced for the first time on experimental day 3. After the experimental session, the mice are returned to their home cages with ~3 g food. The mice that meet a response criterion of at least 18 responses on day 2 are then advanced to the day 3 schedule. Mice that do meet these criteria can either be eliminated from the study or run the next day as a third training session.

3.3 Day 3: Pharmacological Testing

The mice that have advanced to this stage are brought back into the test room at about 1500 h and acclimated to the test room for about 60 min. The mice are given a dose of a compound or given the compound vehicle, returned to their home cages for the appropriate pretreatment interval, and then placed within the

experimental chamber. The experimental session on this day is comparable to that which was used on training day 2 with the exception that electrification of the grid floor (0.5 mA for 100 ms) occurs in conjunction with food delivery during each of the three 10-min periods. Each mouse can be used either once for this purpose or brought back on subsequent experimental sessions for retraining and drug testing. It is recommended that pharmacological testing in the same mouse is done only after several nondrug sessions.

3.4 No Punishment Controls

A separate group of mice can be used to ascertain the levels of responding when electric shock is omitted on the day 3 test.

4 Data Analysis

Responses or rates of responding (responses/min) under each component of the mixed schedule can be calculated on days 2 and 3 from the number of responses and the elapsed time within each schedule component. Dose–effect curves for each compound are constructed from the data collected on day 3 and analyzed by one-way ANOVA for non-punished and punished responding separately. Given significant dose–effect changes, rates of responding during administration of drug vehicle are compared to the rates occurring during each drug dose by Dunnett's test (e.g., GraphPad Prism Software). At least 6 mice are studied for each drug dose and a total of 8 to 12 are evaluated for vehicle controls. Statistical probabilities of less than 0.05 were considered to be significant.

5 Anticipated Results

In NIH Swiss mice, our experience has seen good training results on day 1 with 33 ± 2.7 (mean ± SEM) and 100 ± 7.8 responses for left and right nose pokes observed, respectively. Performances on day 2 resulted in over 92.6% of mice advancing directly to the day 3 procedure by meeting the right nose poke response criterion on day 2. Introduction of shock on day 3 should result in the rate of unpunished responding being essentially unchanged, whereas the rate of responding in the shock component should be significantly reduced (by about 90% in NIH Swiss mice). Vehicle control responding has averaged 2.1 ± 0.2 and 0.2 ± 0.02 responses/min in components 1 and 2, respectively, with the range of punished responses being between 3 and 12 responses [11].

Classical anxiolytic agents that act upon $GABA_A$ receptors would all be anticipated to increase suppressed responding as exemplified by the effects of the benzodiazepine anxiolytic chlordiazepoxide (*see* Fig. 2). In contrast, other drugs are expected to

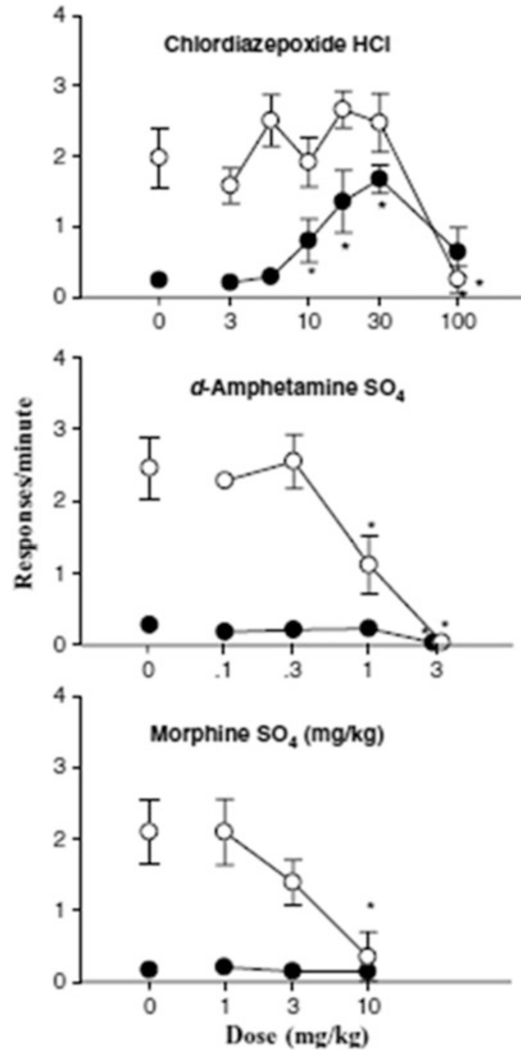

Fig. 2 Effects of the anxiolytic chlordiazepoxide, *d*-amphetamine, and morphine on punished and non-punished responding of mice. *Open circles*: non-punished responding. *Filled circles*: punished responding. Each drug dose represents the mean ± SEM of at least 6 mice; vehicle control values are from 8 to 12 mice. Significant differences ($p < 0.05$, Dunnett's test) from vehicle control values (points above 0) are represented by an *asterisk*. (This figure is reprinted from [22] with permission and is a rendering of data previously published [11])

decrease suppressed responding as exemplified by *d*-amphetamine and morphine (*see* Fig. 2). These compounds are also generally inactive in other more traditional punishment procedures [3]. The data with *d*-amphetamine show that drugs that can increase behavior per se do not necessarily increase punished responding. The data with morphine help to demonstrate that reduction of sensitivity to electric shock per se is not sufficient to increase punished responding. The original report on this Vogel

conflict method also reported that pentobarbital and bretazenil also increased punished responding, whereas buspirone and chlorpromazine were inactive.

6 Experimental Variables and Troubleshooting

6.1 Time of Experimental Sessions

Although times of day were provided in the procedure sections, variation is likely without major impact. The first day of training that occurs overnight seems valuable for getting the most rapid training possible. Subsequent training sessions could likely be within variations of ±6 h without marked alterations in behavioral effect.

6.2 Food Deprivation Levels

The amount of food deprivation needed for acquisition and maintenance of the behaviors described here will vary depending upon a host of factors that include genotype (see below). The specific methods described above have worked well with several strains. It is critical that mice be carefully observed every day and that body weights be taken. It is recommended that during training days, food be placed on the floor of the cage for easy access.

6.3 Training Variations

The training parameters given above have been successfully implemented, but the full range of parametric variation that is tolerated and optimal have not been determined. A host of changes could be enabled for specific purposes as illustrated further below. For example, mice that do not meet criterion on one training day could be brought in for additional training at other times.

6.4 Test Variations

As with the training variables discussed above, testing variations are likewise possible. For example, mice could be used for drug testing on multiple occasions. Additional variations could include withholding of shock on the test day; this might enable increased sensitivity to drug effects; however, baseline changes over the course of the test session would also be anticipated. The procedure is also amenable to the detection of time course by appropriate drug pretreatments and to drug combination studies, etc. A host of other parameter changes can be implemented for the purpose of answering specific research questions – see below for some examples (*see* Subheading 6.5).

6.5 Genotype Comparisons

Different mouse genotypes have inherent differences in their food and water regulation and body weights. Hence, it is critical to know your mice from this vantage point to determine the parameters around the duration of food deprivation, the amount of food to deliver after the experimental session, and perhaps the number of days of training needed to reach criterion performances. When conducting experiments with transgenic or receptor-deleted mice,

a contemporaneous wild-type control mouse will have to be put through the same procedures. The use of appropriate counterbalancing practices for the experimental day should be used. Parametric variation might be needed for different strains. For example, the intensity of the punisher might need adjusting to have the same behavioral effects across strains.

The flexible use of this Vogel conflict method to evaluate the comparative behaviors of genetically modified mice is illustrated in the experimental literature. In a study aimed at understanding the role of MCH_1 receptors in foraging behavior for food, mice with constitutive deletion of MCH_1 receptors ($MCH_1-/-$) were compared to their wild-type controls ($MCH_1+/+$). In order to elucidate the potential difference in food foraging behaviors, multiple variations of the method were employed to probe specific aspects of the potential controlling variables of mouse foraging.

The acquisition of behavior was evaluated prior to the introduction of shock. In this study, the mice were given access to the operant chambers and were allowed to earn up to 100 food pellets for 7 experimental sessions. Under these conditions, large amounts of food were secured even within the first experimental session (*see* Fig. 3). In addition, discriminative control of behavior was also established rapidly (day 1) with more responses occurring on the right nose hole that produced food relative to the left nose hole for which nose pokes did not produce food. By day 7, the primary change in behaviors from day 1 was that significantly less responding occurred on the inactive left nose hole.

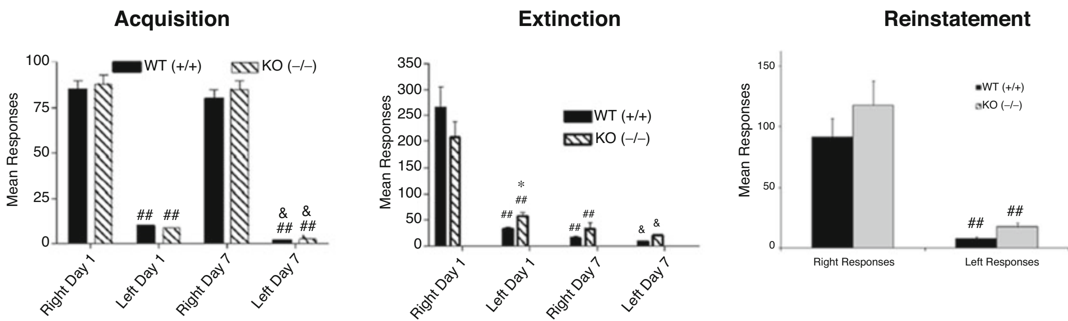

Fig. 3 Acquisition: MCH_1 −/− and MCH_1+/+ mice did not differ in their acquisition of food-maintained nose poke responding (right nose hole). Shown are the mean number responses on day 1 and day 7 of response acquisition on the right and left nose holes. Each bar represents the mean + SEM of 16–17 mice/group. ## $p < 0.01$ compared to right responses; $p < 0.05$ compared to left responses on day 1. **Extinction**: MCH_1 −/− and MCH_1 +/+ mice did not differ overall in the rate of extinction of food-maintained responding. Shown are the mean number of responses on day 1 and day 7 of response extinction on the right and left nose holes. Each bar represents the mean responses + SEM of 16–17 mice/group. * $p < 0.05$ KO vs WT; ## $p < 0.01$ compared to the right responses on day 1; $p < 0.05$ compared to left responses on day 1. **Reinstatement.** MCH_1R −/− and MCH_1+/+ mice did not differ in the responding in the presence of food-associated stimuli. Shown are the mean number of responses under conditions of food and food-paired stimulus reinstatement of extinguished responding. Each bar represents the mean responses + SEM of 16–17 mice/group. ## $p < 0.01$ compared to right responses. (Data are reproduced from [23] with permission of the publisher)

Extinction of behavior (no responses produced food) was then evaluated where it was observed that significant reductions in responding occurred from day 1 to day 7. As anticipated, increases in left nose poke responses occurred on day 1 (compared to the last day of acquisition) that were likely the result of extinction-induced foraging. Interestingly, the MCH1−/− mice showed more of this behavior on day 1 than their wild-type counterparts (*see* Fig. 3).

After extinction, food-paired stimuli were reinstated to evaluate whether food-related cues would differently reinstate responding in the MCH_1−/− mice. Although no genotypic differences were uncovered, responding was nicely reinstated within one experimental session. All of these methods have broader applicability. For example, reinstatement of behavior is widely used as a method for evaluating the ability of compounds to suppress drug or food craving [13, 23].

Additional pressures on food foraging were introduced in separate groups of mice. In the first study, mice could earn food under a progressive ratio schedule (no shock) in which the number of responses required to produce food increased after each food delivery. In this experiment, MCH_1−/− mice made significantly more responses and reached a higher level of response requirement (break point) than MCH_1+/+ mice (*see* Fig. 4). Quinine adulteration of the food pellets was used as another pressure on food-maintained behavior. After training with normal pellets, quinine-adulterated pellets were substituted in the next experimental session without great impact upon behavior of either genotype. During a day of free-feeding, responding maintained by the quinine pellets was reduced in both genotypes. However, if behavior was monitored 24 h after free-feeding, the MCH1−/− mice displayed significantly greater responses (*see* Fig. 4). The type of reinforcer has been shown to modify drug effects in mice previously [19], an effect that appears to be influenced by motivational variables as with the quinine experiments.

Another study was conducted in separate groups of mice to evaluate the impact of punishment of food foraging. In order to compare genotypes, the potential differential sensory sensitivity to the punisher (electric shock) was assessed. In this study, the MCH1−/− mice were more sensitive to shock reactivity than wild-type controls (*see* Fig. 4), a finding of interest in its own right. However, since this differential sensitivity existed, the punishment procedure was conducted in two ways, one in which the shock intensity was the same in each genotype and another separate study in which the shock intensity was based upon equivalent sensitivity values. When the shock intensity was 0.5 mA for both genotypes, suppression of responding by punishment was not significantly different across genotypes. In contrast, when the shock intensity was based upon equivalent sensitivity values (0.25 vs

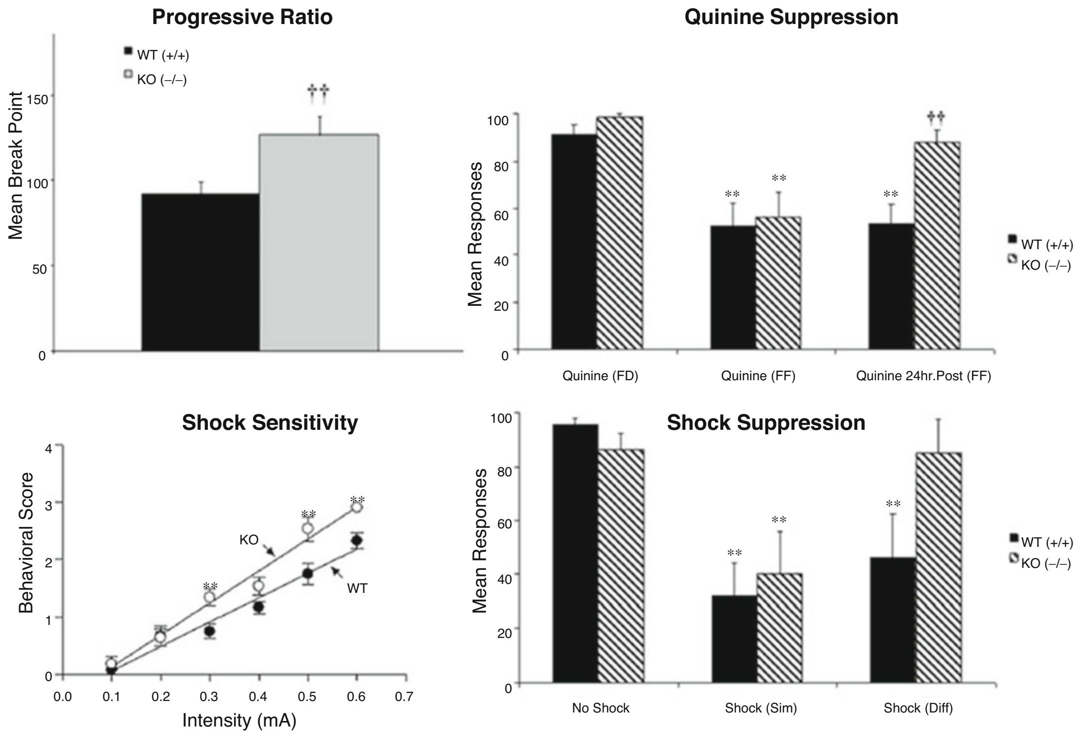

Fig. 4 Progressive ratio. MCH_1 −/− made more responses which had higher break points under a progressive ratio schedule of food delivery than MCH_1+/+mice. Each bar represents the mean responses + SEM of 16–17 mice/group. †† $p < 0.01$ compared to WT mice. **Quinine suppression**. MCH_1 −/− made more responses than MCH_1+/+ mice to produce quinine-adulterated food pellets 24 h after free access to food. Behavior of the mice was studied when they were food deprived prior to testing (FD), given free access to food prior to testing (FF), or when tested 24 h post access to free food (24 h post FF). Each bar represents the mean responses + SEM of 8–11 mice/group. ** $p < 0.01$ compared to FD condition. †† $p < 0.01$ compared to WT mouse under same conditions. **Shock sensitivity**. MCH_1 −/− mice were more sensitive (reactive) to electrical foot stimulation than MCH_1+/+ mice. Each point represents the mean responses + SEM of 8 mice/group derived from a rating scale of reactivity to electrical stimuli. ** $p < 0.01$ compared to WT. **Shock suppression**. MCH_1 −/− showed less suppression of responding than MCH_1 +/+ mice when evaluated under estimated equivalently detected shock conditions. Data are shown under no shock or response-produced shock at 0.5 mA intensity (similar across WT and KO; Sim) or 0.25 mA (KO) and 0.33 mA (different intensities between WT and KO; Diff). Each bar represents the mean responses + SEM of 8–10 mice/group. ** $p < 0.01$ compared to the no shock condition. (Data are reproduced from [23] with permission of the publisher)

0.33 mA in the knockout vs wild-type mice, respectively), significant suppression of responding in the MCH1−/− mice was not observed (*see* Fig. 4).

Another series of studies evaluated the role of $5\text{-}HT_7$ receptors in the regulation of affect in mice with constitutive deletion of $5\text{-}HT_7$ receptors. Prior to the introduction of punishment, baseline behaviors were compared where it was found that $5\text{-}HT_7$−/− mice had higher rates of responding in the periods of non-punishment (trend) and in the periods of the session during which punishment

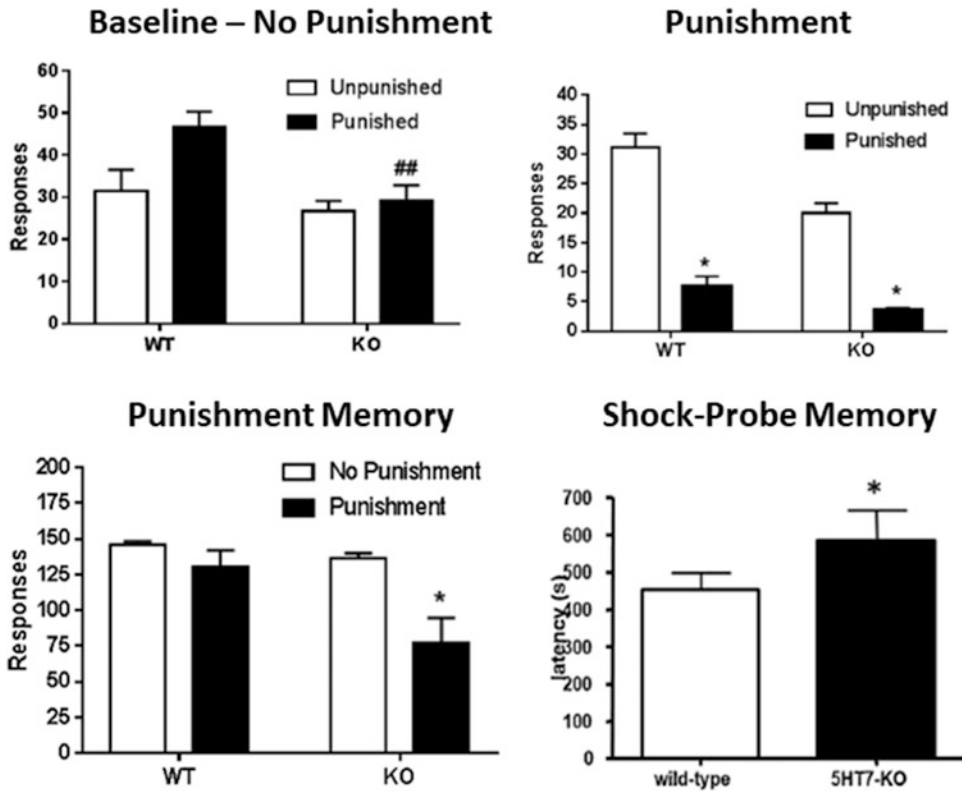

Fig. 5 Comparison of 5-HT_7 −/− (KO) and 5-HT_7 +/+ (WT) mice. **No punishment**. Right-hole responses of WT and KO mice prior to the introduction of punishment. Each bar represents the mean + S.E.M. for 13 mice per group. ##$p < 0.01$ WT vs corresponding KO data. **Punishment**. Unpunished and punished right-hole responses of WT and KO mice. Each bar represents the mean + S.E.M. for 13 mice per group. *$p < 0.05$ unpunished compared to punished responding within genotype. **Punishment memory**. Responses of WT and KO mice, sorted into "previous no punishment" and "previous punishment" periods. No shock was delivered on this day. Each bar represents the mean + S.E.M. for 13 mice per group. *$p < 0.05$ unpunished compared to punished responding within genotype. **Shock-probe memory**. Latencies of WT and KO mice to approach a shock probe that previously delivered shock but where no shock was given in the present test. Each bar represents the mean + S.E.M. for 8 mice per group. *$p < 0.05$ compared to WT group. (Data are from [24] with permission of the publisher)

would be introduced in the next phase of the study (*see* Fig. 5). Punishment decreased responding in the punishment component for both genotypes, but the rates of non-punished responding in the mice were also somewhat decreased (*see* Fig. 5). In order to investigate whether 5-HT_7 receptors regulate emotional memory, the punishment mice were evaluated the following day when shock was not delivered. Under these conditions, the behavior of the 5-HT_7−/− mice but not the 5-HT_7+/+ mice was significantly suppressed during the prior punishment periods (*see* Fig. 5). In concordance with this enhanced emotional memory, 5-HT_7−/− mice were also slower to approach a shock probe that had previously delivered electric shock (*see* Fig. 5).

6.6 Experimental Chambers

The experimental chambers used in the current procedure are relatively expensive and space consuming when considering the conduct of experiments in many mice. Given this fact, relatively few chambers can generally be made available for simultaneous mouse training or testing. These drawbacks are counterbalanced by advantages in the multiple and flexible uses of this equipment. Alternatives are possible, but are not commercially available to our knowledge. Thus, a stand-alone apparatus can be envisioned in which the minimal structure required for the conflict procedure is built.

6.7 Statistical Power

The number of mice required for the detection of a given level of drug effect has not been explicitly explored. The current use of this method has used six mice per dose group and 8–12 mice for vehicle control with good success in separating drug effects from that of drug vehicle.

6.8 Use of No-punishment Control Group

The data from this control group are used to determine the degree to which responding in the punishment group is reduced. Once these data are collected, routine dose–response studies could, if desired, be collected, using the punishment baseline without the use of the no-punishment control group. Since there is an ongoing non-punishment component built into the procedure, a concurrent no-punishment baseline is essentially always present. It is critical to remember that all behavior and behavioral effects of drugs must be interpreted by comparison to other behaviors [14–16].

6.9 Use of Food-Intake Controls

A host of ancillary behavioral assessments can be made against which to compare the effects of drugs on punished responding. Food intake is one that can provide information about the relationship of the drug effects on punished responding to food motivation.

7 Concluding Remarks

The ability to rapidly and accurately probe important questions of psychological and neurobiological import in mice is an absolute requirement in the age of transgenic mice. The methods described here have broad applicability to the assessment of the behavior of mice as demonstrated in this chapter by example from the experimental literature. In addition to the comparative study of genetic manipulations, other variables are equally assessable to experimental study that include prior or contemporaneous exposure to diverse environmental, pharmacological, and nutritional variables.

The Vogel conflict test using water drinking in mice is rarely seen in the experimental literature (see Vogel conflict drinking test, this volume) but is a highly used assay in rats (c.f., [17]). In a recent

report by Pisu and colleagues [18], 4 days were required for training and dose–effect analysis compared to the three reported here. Another study used mice to study drug effects with a total of 9 experimental sessions [19]. Very few studies using operant methods in mice have likewise been disclosed [12]. In contrast, methods using ethological methods (e.g., elevated plus maze, dark/light box, marble burying) have dominated the work with mice in the anxiolytic research area (c.f. see other chapters in this book series). The apparent greater promiscuity of the ethological methods for diverse pharmacological mechanisms along with the ease of testing is a likely contributor to their use in anxiolytic drug assessments. Although comparison of methods is not within the scope of the current discussion, the scientific literature suggests some of the drawbacks of such methods that include a lack of reproducibility of findings across labs that likely results from the use of behaviors that are less well controlled than those described here. Under the current method, drug effects on suppressed responding can be evaluated after only 2 days of training in mice.

Despite the fast turnaround for drug effects on operant behavior which involved minimal training, only 7.4% of NIH, Swiss mice tested by us [11] did not meet the training criteria on day 2. Moreover, vehicle control rates on test day 3 were generally comparable across many groups of mice over several years. The reproducibility of the drug effects was additionally verified in many experiments with chlordiazepoxide. Although the methods described permit rapid and reliable detection of drug effects on suppressed responding, no attempt was made to fully optimize the assay or to fine-tune it toward particular pharmacological activities. Parameters that may be worthy of manipulation could include addition of another training day, addition of discriminative stimuli associated with the non-punishment and punishment components, component durations, the inclusion of a schedule component under which responding has no scheduled consequences, and the use of intermittent schedules of food or shock delivery.

The possibility of detecting false positives in the mouse punishment procedure due to increases in food consumption per se was also a concern in the present assay (see [10] for a good example). The present procedure was designed to minimize the influence of effects of compounds on feeding on their effects on punished responding by having the operant response separate from the consummatory response. In addition, separate experiments have been conducted to evaluate the effects of the compounds studied on food consumption in mice showing that these behaviors can be dissociated [11].

Punishment procedures are not fully functional models of anxiety. Instead, as with most well-controlled behaviors, these methods provide stable baselines against which specific classes of pharmacological agents can produce increases in response rates. Although

capable of detecting the activity of a host of antianxiety agents used in clinical practice, others mechanisms are not always positive. For example, although selective serotonin-uptake inhibitors are widely used in the first-line treatment of some anxiety disorders, they are generally not reported to increase suppressed responding of this type but are active under other conditions in mice [20, 21].

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Chapter 8

Fear Conditioning in Laboratory Rodents

Iris Müller and Markus Fendt

Abstract

An animal's ability to avoid threat by learning about cues and places that predict the occurrence of a threat is pivotal for survival. This learning is a form of Pavlovian conditioning, called fear conditioning, and is seen across the animal kingdom. Importantly, it can be modelled in the laboratory setting by presenting the animal with a certain context or a discrete cue (e.g., tone or light stimulus) that are then paired with a threat (e.g., an electric stimulus). The learning is then assessed by measuring the acquired fear response to the respective context and/or cue. Variations of the parameters and the protocol allow the investigation of a multitude of physiological and pathological learning phenomena. In this chapter, we give a methodological overview of a standard fear conditioning setup, different protocols, behavioral read outs, and data analysis.

Key words Associative learning, Fear conditioning, Pavlovian conditioning, Classical conditioning, Freezing, Memory, Protocols

1 Background and Overview

In nature, animals regularly experience situations that threaten their well-being or even their life. For such situations, e.g., encounters with a predator, evolution has developed species-specific defensive behavior that helps animals to survive [1]. However, these evolutionary processes have also optimized the hunting behavior of predators [2]. Hence, it is obviously the best strategy to avoid encounters with predators and/or to be detected by predators [3]. To be able to do so, animals should "know" which environmental stimuli predict the appearance of predators. If these stimuli are predator-related, such as their odor or their vocalizations, they are often innately recognized by prey animals [4–6]. However, since nature is diverse and flexible, it is of advantage if places or other stimuli predicting predators can be learned. This process is called fear conditioning [7–10] or threat conditioning [11].

Fear conditioning is a type of Pavlovian learning in which the animal learns to associate a previously neutral environmental stimulus with an aversive event. These environmental stimuli can be

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either a discrete stimulus of any modality or the combination of several environmental stimuli that are present at a specific location, which is then also called "context". Of note, the stimulus "context" can include much more than just spatial stimuli [12], e.g., temporal components and internal factors (emotion, motivation, hormonal state, circadian rhythm, etc.).

Since fear conditioning is observed across the animal empire, it was and still is used by a plethora of studies investigating the behavioral, molecular, pharmacological, and neural characteristics of learning in different laboratory animals, such as rats and mice [7], rhesus monkeys [13], fruit flies [14], or nematodes [15]. Furthermore, fear conditioning is also used in human experiments, with the aim to better understand emotional learning processes not only in healthy subjects but also in patients with anxiety- and stress-related disorders [16, 17].

The present chapter will focus on methodological aspects of fear conditioning in laboratory rodents, i.e., mice and rats. In these species, fear conditioning is maybe the most used behavioral paradigm to investigate molecular and neural mechanisms of learning [7–10, 18]. Most of the studies measured the freezing response, i.e., the cessation of body movements except those necessary for breathing. Freezing behavior is a species-specific defensive response of rodents [19] but is also observed in other animals ranging from fruit flies [20] via tadpoles [21] to humans [22]. However, there are further defensive responses which can be measured across species, including startle potentiation [23, 24], escape behavior [25], heart rate variability [26, 27], skin conductance [28], or suppression of appetitive behaviors [29, 30]. In the present chapter, we will mainly focus on fear conditioning in mice, measured by freezing. However, we will also include sections dedicated to experiments in rats, as well as to other measures of fear, primarily startle potentiation.

2 Equipment, Materials, and Setup

Fear conditioning experiments are performed in setups consisting of at least one conditioning box, usually provided with a floor grid to administer electric stimuli (*see* Fig. 1). This box is placed into a sound-attenuating chamber, which is equipped with a loudspeaker and/or a light source to deliver tone or light stimuli, as well as with a ventilating fan. The presentation of these different stimuli is controlled by a computer with respective software. The behavior of the animals is typically recorded either by a camera, mounted on the ceiling of the chamber, or by infrared sensors, located in a frame around the boxes. The video recordings or the signals from the infrared sensors can be automatically analyzed with specified software (video tracking or infrared sensor tracking software). Another

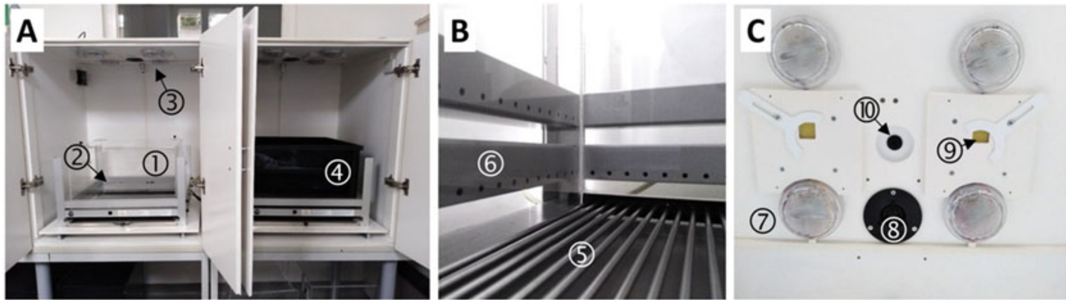

Fig. 1 Exemplary fear conditioning system. (**A**) Two of the system's chambers, equipped either with the conditioning box (①) or a box which can be used in retention tests (④). Details of the infrared sensor frame (②) and the floor grid are shown in (**B**); details of the chambers' ceiling (③) are shown in (**C**). (**b**) Floor grid with steel bars (⑤; 3 mm diameter, 10 mm distance between bars) and frames with infrared sensors (⑥; distance 14 mm). The lower frame is to detect horizontal movements (including freezing), the upper frame to detect vertical movements (e.g., rearing behavior). (**C**) The ceiling of the chamber is equipped with houselights (⑦), loudspeaker (⑧), and LED lights (⑨) for cue presentations, as well as with a camera for monitoring and/or video recording (⑩)

option is to manually analyze the video recordings with the help of an event recorder software. Of course, such a manual analysis can also be done by directly observing the animals; however, using a camera/monitor system is recommended, since then the experimental animals are not distracted by the experimenter.

Commercially available setups often consist of multiple conditioning boxes (e.g., 2, 4 or 8) in which several animals can be tested in parallel. In such setups, identical test sessions usually run simultaneously in the different boxes, i.e., that the manipulations of the animals (e.g., pharmacological treatments) have to be performed at least almost simultaneously. Dependent on the complexity of the manipulation and the number of available experimenters and/or additional setups necessary to perform the manipulation, only a small number of simultaneous tests can be run despite testing more animals in parallel, which would be theoretically possible.

2.1 The Conditioning Box

Most conditioning boxes are made from acrylic glass that is either transparent, unicolored, or with multicolored pattern. The layout is most often quadratic, with side lengths between 17 and 50 cm. Smaller boxes are intended for mice, while larger ones are used for rats. However, based on our experience, the larger boxes can also be used for mice. The height of the boxes is between 25 and 40 cm. Rats can easily jump out of such boxes and will most probably do so when they get aversive electric stimuli in the box. Therefore, it is highly recommended to use a lid that prevents this. Despite mice most likely can also jump out of boxes with these heights, we never observed this in our experiments.

As the name suggests, the conditioning boxes are used for fear conditioning sessions. During fear conditioning, the animals learn to associate the conditioning boxes with the unconditioned stimuli,

e.g., an electric stimulus (see below). This process is called contextual fear conditioning. Hence, the conditioning boxes are also used in the retention tests on conditioned contextual fear (see below).

2.2 Additional Box (es) for Retention Test(s)

In addition to the conditioning boxes, further boxes are used in the retention tests. These boxes can be totally different or similar to the one used for the conditioning. The more different the box should be, the more features of the box are changed. For clearly different boxes, the color of boxes' walls is usually changed, and the floor grid is replaced by a floor with a different structure (e.g., simply plain). Sometimes, also the layout of the box is changed, e.g., round, triangular, or octagonal layout are used. If a different layout is used, this layout should have the identical area with the conditioning boxes.

To create different or similar testing contexts, it is recommended to change further features of the context in addition to the boxes themselves. This includes olfactory stimuli (e.g., different solutions to clean the boxes such as soapy water, 70% ethanol solution, 1% acetate acid solution), different illumination conditions, and different intensities of the background noise. Sometimes, additional changes are done, e.g., the boxes are placed in other rooms and/or other experimenters perform the test.

Clearly different boxes are used to evaluate the animals' fear responses to discrete conditioned fear stimuli, e.g., a tone stimulus. Such retention tests for cued fear should be done in the absence of contextual fear, i.e., in the best scenario, the different boxes best should induce no fear responses at all.

Boxes similar to the conditioning boxes are typically used to test the specificity of conditioned contextual fear [31–33]. If contextual fear is very specific, the animals should express less contextual fear; the more features of the similar box are different from the conditioning box. If contextual fear is generalized, full contextual fear is also induced by similar or even different boxes.

2.3 The Unconditioned Stimulus (US)

Nearly all fear conditioning experiments use electric stimuli, administered via the floor grid, as unconditioned stimuli. Electric stimuli have the advantage that their parameters are well-defined and that they are easy to control and to administer. If the electric stimuli are administered to the feet of the animals, they are also called "foot shocks." However, in some experiments also "tail shocks" [34] or "back shocks" [35] were used. For such tail and back shocks, but rarely also for foot shocks [36], the electrodes are usually fixed, e.g., by tape, on the animal's tail, back, or foot.

Electric stimuli can differ in intensity and duration. Typically, intensities between 0.4 and 1.0 mA are used, with durations between 0.5 and 2 s. As for other stimuli used in fear conditioning experiments, also the intensity of the electric stimuli should be calibrated regularly to assure controlled conditions throughout.

Damage in the cables and/or connectors, as well as urinary calculus or other contaminants that may accumulate between the grids due to poor cleaning, can cause changes in the actual foot shock intensity. To prevent the latter, good cleaning of the grids and subsequent rinsing with demineralized water are recommended. Another reason why animals did not get the intended electric stimuli is that they just happened to be standing on grid bars with the same current polarity. To avoid this, most devices controlling the application of electric stimuli produce so-called scrambled stimuli, i.e., that the polarity of the grid bars switches in a certain frequency.

Due to such technical issues and/or accidentally not touching grids with different voltage poles, animals can miss some of the intended electric stimuli. Therefore, it is recommended to observe and/or analyze the animals' behavior during application of the electric stimuli. This can occur online via an observation monitor, offline by checking video recordings, or comparing the recorded locomotor activity or mean velocity during application of the electric stimuli with the activity in a control time window.

It is important to note that with a higher number of the electric stimuli, as well as with more intense and longer electric stimuli during fear conditioning, usually more fear behavior is expressed in the retention tests. However, of note, high intensities—and most likely also long durations—of electric stimuli should be used with caution, since they are associated with a less specific, i.e., generalized, fear memory [37]. Furthermore, high intensities can lead to unwanted behaviors, e.g., escape attempts. Usually, the same intensity of the electric stimulus for all test animals is used. However, in some studies the individual pain threshold, i.e., the minimal intensity to induce a pain response such as jumping or vocalizing, was first determined, and then, intensities relative to the individual threshold were used.

Several studies used aversive odors, e.g., predator odors, as an unconditioned stimulus in fear conditioning experiments. Cat odor was successfully used in some studies [38–40], while derived predator odor components, such as 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), were not effective ([41], but see [42]). To the best of our knowledge, other predator odor components such as 2-phenylethylamine [43] or 2-methyl-2-thiazoline [44] were not used so far as US in fear conditioning studies.

2.4 The Conditioned Stimulus (CS)

As mentioned above, during fear conditioning the animals will associate the conditioning box with the unconditioned stimuli, i.e., the conditioning box will become a conditioned fear stimulus for contextual fear. If during the fear conditioning session no discrete stimuli had been used to predict the unconditioned stimulus, the process is called foreground contextual fear conditioning. However, most often an additional discrete stimulus predicting the

unconditioned stimulus (forward paired) is used. In these cases, the contextual fear conditioning is in the "background" of the discrete stimulus, and the process is called background fear conditioning [45].

Of course, only stimuli that are perceptible to the respective animal species should be used. With this restriction, all sensory modalities can be used for discrete conditioned stimuli. Since the hearing curve of laboratory rodents is shifted to higher frequencies [46, 47], caution is recommended with acoustic stimuli of very low frequencies. The same is true for colors, as it is unclear whether and how well laboratory rodents can differentiate colors [48]. Further, it should be considered that transgenic lines and aged rodents might suffer from sensory deficits.

Importantly, the discrete conditioned stimulus should be well-defined in terms of timing (onset, duration, offset), intensity, and quality (sine tone, noise, wavelength, etc.). Controlling timing and intensity is technically challenging for some modalities (e.g., olfactory or tactile stimuli) but relatively easy for other modalities (e.g., acoustic or visual stimuli). Nevertheless, there are a number of studies successfully using olfactory fear conditioning [49] or tactile fear conditioning [50]. However, acoustic (e.g., a tone or white noise) or visual (e.g., light) stimuli are used in most studies as discrete conditioned stimuli. Usually, the stimuli have constant parameters, but also stimuli with modulations are used (e.g., flickering lights or sweeps).

The conditioned stimulus is usually described as "a previously neutral stimulus," i.e., it should not induce fear-related behavior. This can be checked by presenting the prospective conditioned stimulus during a habituation phase of the experiment. During presentations of this stimulus, the animal then should not express freezing behavior, for example. In our experience, the acoustic stimuli, especially those with high frequencies and intensity, rather than visual stimuli have the potential to induce freezing. Of note, certain stimuli can also inhibit freezing. Of course, such an ability cannot be tested during the habituation phase of an experiment, since the animals do not express freezing behavior during this part of the experiment. Instead, such stimuli should be tested in contextual fear conditioned animals during a retention test on contextual fear. Based on our experience, visual stimuli have a tendency to inhibit freezing behavior, especially in albino rats. If such freezing-inducing or freezing-inhibiting properties of discrete stimuli were detected, reducing the intensity might be a promising strategy to get rid of such unwanted effects.

The duration of the conditioned stimulus is also dependent on the used behavioral readout. If freezing is used, a duration of at least 10 s (better 20 or 30 s) is recommended, since freezing has a relatively slow dynamic. If the behavior of interest has a very fast dynamic, much shorter durations can be used. For example, a

100-ms tone was used as a conditioned fear stimulus in a study investigating the effects of attention on prepulse inhibition of the startle response [36].

3 Measurement of the Conditioned Fear Response

3.1 Freezing Behavior

Freezing is a cessation of all body movements except those which are necessary for breathing [19]. In fear conditioning experiments, it is the most used measure of conditioned fear, since it can be relatively easy quantified. The least technically demanding way to quantify freezing behavior is to directly observe the animals and "manually" score their behavior. However, it is better to use a camera/monitor system as this is less distracting to the animal. Scoring of freezing then can be done either by focal sampling [51], i.e., one particular animal is continuously observed, or by scan sampling [52], i.e., different animals are observed and the behavior of each individual animal is scored at regular intervals, e.g., every 10 s. Importantly, clearly defined criteria should be used in manual scoring, e.g., no movements longer than 1 s and all four paws on the floor. Recording of the freezing scores can be done in prepared tables or—if available—using a software for behavioral scoring. The scoring experimenter should be well trained and blind to experimental conditions. In addition, it is advisable to validate the scores (at least a part of them) by a second experimenter.

Commercially available fear conditioning setups most often use automated recording of freezing. For this purpose, a software that analyzes either the video recording or signals from infrared sensors is used. Rarely, signals from motion-sensitive platforms are used [53]. These automated analyses clearly have the advantage that they are not so time-consuming and less biased. However, very reliable automated analyses are dependent of either an optimal visual contrast between animals and background (floor of the setup) or a high density of the infrared sensors. First could be very challenging with similar colors of the animals and the background, multicolor animals, and reflecting floors (which is very likely with grids), while the latter is simply expensive.

Figure 2 shows freezing data of a small fear conditioning experiment in rats, analyzed by manual scoring (two trained observer), infrared sensors (TSE Fear Conditioning System; TSE Systems, Bad Homburg, Germany), and video tracking (EthoVision XT 11; Noldus, Wageningen, the Netherlands). Of note, the conditions for video tracking were suboptimal in this experiment, since white rats and a rather light floor with reflecting grids were used. Nevertheless, there was a very good correlation ($r^2 = 0.85$; Fig. 2a) between video-tracking analysis and observer-rated scoring. Freezing analysis by the infrared sensors is not dependent of visual

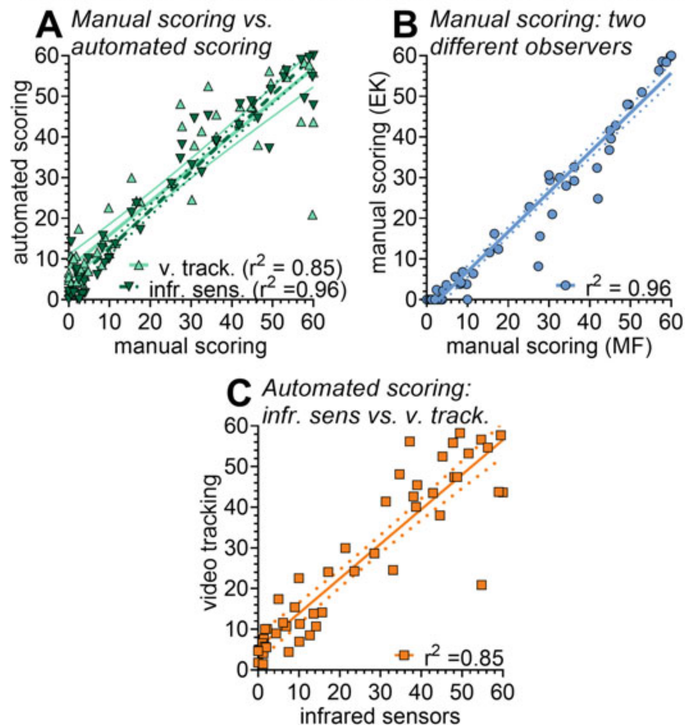

Fig. 2 Comparison of different methods to measure freezing behavior. Correlation analyses of freezing data measured or analyzed with different methods (video tracking, infrared sensor tracking, manual scoring). Data are from a fear conditioning experiment in rats (N = 6). Freezing was analyzed by infrared sensors (TSE fear conditioning system; freezing threshold: 1 s), by video tracking (Noldus EthoVision XL 11; sample rat: 5/s, averaging 5 samples, 1% change), or by focal observation (observer MF and EK). Abbreviations: *vs* versus, v. *track.* video tracking, *infr. sens* infrared sensor

contrasts and not disturbed by reflections, which is reflected in a better correlation with manual scoring ($r^2 = 0.96$; Fig. 2a). This correlation is very similar to the correlation of manual scoring by two different observers ($r^2 = 0.97$; Fig. 2b), while the two automated analyses correlated much less ($r^2 = 0.85$; Fig. 2c). Notably, there is usually a small "offset" with the automated freezing analyses, i.e., they generally detect a bit more (5–10%) freezing than human observers.

If automated recording of freezing is used, an optimization and validation of the parameters used in the freezing analysis software is necessary. For the detection of freezing, the different softwares use thresholds for inactivity and for the duration of this inactivity. For example, inactivity is defined by changes in activity below 1%, and this inactivity should last at least 1 s. These thresholds should be optimized in a way that the freezing values best correlate with manually scored freezing (cf. caption of Fig. 2).

3.2 Potentiation of the Startle Response

In rats, monkeys, and humans, conditioned fear can be also measured by utilizing the acoustic startle response. The startle response is elicited by sudden and intense stimuli and can be measured by a motion-sensitive platform (in rodents; [54]) or by electromyography (eyeblink response in monkeys and humans; [55]). During learning and/or testing conditioned fear, the test subjects are repeatedly exposed to startle stimuli, some of them in the presence, some of them in the absence of the conditioned stimulus. If the conditioned stimulus has gained negative valence, which should be the case after (but not before!) fear conditioning, the startle response of the subject should be potentiated in the presence of the conditioned stimulus [13, 23, 56].

Startle experiments can be performed with commercially available setups. These setups have specific software for controlling the delivery of the startle stimuli, the unconditioned and the conditioned stimuli, and for recording the startle magnitudes. The advantages of using the startle response as a behavioral readout is that this measure is used in many human studies, i.e., it is translational, and that the startle response is bivalent, i.e., potentiated by negative emotions of the subject and attenuated by positive emotions [57]. A disadvantage of the startle response is that its use in mouse fear conditioning experiments is very limited. For unknown reasons, most mouse lines (e.g., C57BL/6 mice) do not robustly express startle potentiation after fear conditioning (but see [58]). However, there are also mouse lines with robust fear-potentiated startle (e.g., DBA mice, [59]).

4 Procedure

In the following, general recommendation for fear conditioning experiments in male mice and rats are given. Unfortunately, fear conditioning is much more investigated in male mice, despite it is known for a long time that there are considerable sex differences in human fear learning and in anxiety disorders associated with fear learning [60]. Recently, however, more and more mouse studies are being conducted with both sexes and sex differences are regularly found (e.g., [61–64]). In addition to sex, the strains and even the substrains of mice (and rats) matter [65–68]. This also applies to transgenic mice that are often created in mouse strains with sensory deficits and/or behavioral impairments. Here, it is essential to consider the genetic background [69].

In general, fear conditioning parameters, like shock intensity and number of pairings, given below should be interpreted as examples and rough guidelines. The exact protocols have to be established and validated (see below) in each laboratory, since parameter features differ greatly between groups, as exemplary shown in the tables below. For instance, we observed specific fear

memory in a contextual fear conditioning task using two US with 1.2 mA intensity and 2 s duration [70], while others induced generalization with three US with 0.75 mA intensity and 1 s duration [71]. Such heterogeneity is likely caused by a combination of different conditions in the animal facilities, handling and genetic drift in the colony, as well as other modest differences in the protocol but could also simply be caused by different ways to measure the US intensities.

4.1 Basic Protocol

The majority of fear conditioning protocols contain a 1–2-min-long stimulus-free period in the beginning and the end of each session. The initial stimulus-free period is particularly important in contextual fear conditioning protocols, since it prevents the immediate shock deficit, a phenomenon that describes the inability to associate a context with foot shocks, if shocks are given immediately after placing the animal in a context, i.e., the conditioning box [72]. The contextual fear memory increases proportionally with the time between placement and first shock presentation [63, 72]. The stimulus-free period at the end of the session allows the animal to calm down after the shock experiences, which facilitates handling for the experimenter. Moreover, freezing during these intervals may be analyzed. The initial stimulus-free period can be used to determine baseline or unspecific freezing behavior, while the freezing behavior during the final stimulus-free period can be used to determine the short-term effects of the US administration.

As mentioned above, one can distinguish between contextual fear conditioning and cued fear conditioning. In foreground contextual fear conditioning, an association between the US and the conditioning context is learned, i.e., unsignaled electric stimuli are presented in an initially neutral context (Fig. 3a, d1). In cued fear conditioning, an association between the US and a cue is learned (Fig. 3b, d1). Hence, an initially neutral, discrete cue immediately precedes the US and thereby becomes the predictor of the US [9]. This protocol is called delayed fear conditioning, since the onset of the US is delayed to the CS onset (while the offsets are identical). As mentioned above, a US-context association is also learned during this procedure. This emerging background context fear memory is only marginally weaker than the foreground context fear memory [73].

The fear memory is tested in retention tests (also often called retrieval or recall). If short-term memory is of interest, these retention tests can be performed in the hours after the fear conditioning procedure. However, most often the long-term fear memory is of interest. Then, the retention tests are performed at least on the next day. If remote fear memory is of interest, retention tests can be performed several weeks [74, 75], months, or, in rats, even 1.5 years [76] after the fear conditioning procedure. Typically,

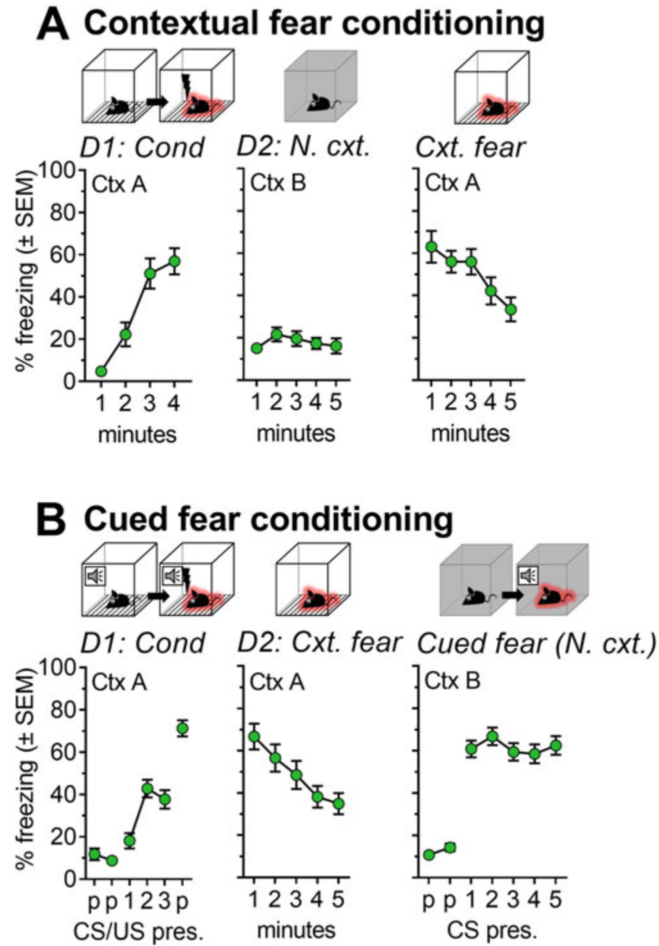

Fig. 3 Exemplary freezing data for the basic protocols. The sketches above the panels show the experimental procedure. The white box is the conditioning box (Ctx A), the black box a novel context (Ctx B). The flash symbolizes the electric stimulus (US), the loudspeaker a tone cue (CS). The color around the mouse symbolizes the emotional state of the mouse (green: no fear; red: fear). The experimental subjects were 8–10-week-old male C57BL/6 J mice (A: $n = 13$, B: $n = 20$). (**a**) Foreground contextual fear conditioning (3 US with 1 s duration and 0.5 mA intensity), (**b**) cued fear conditioning (3 US with 2 s duration and 0.6 mA intensity, CS was a 10 kHz tone with 30 s duration and 75 dB SPL intensity). Y-axis scale and units in the middle and right subpanels are the same as in left subpanels; shown are means (± SEM). Abbreviations: *Cond* conditioning, *CS* conditioned stimulus, *D* day, *Ctx* context, *p* pause (stimulus-free period), *N.* cxt., novel context; *pres* presentations, *SEM* standard error of the mean, *US* unconditioned stimulus

two retention tests are performed, separated by at least 2–3 h (Fig. 3, d2). After foreground contextual fear conditioning, the animals are put in a novel context, and freezing behavior is recorded (test duration: 3–5 min). The more specific contextual fear is, the

less freezing should be observed in this novel context. However, trait anxiety and sensitization by the conditioning procedure may also influence mice's freezing behavior in this novel context. In the second retention test, the animals are placed into the conditioning context, which should induce a robust fear response. After cued fear conditioning, the first retention test is typically performed in the conditioning context (test duration: 3–5 min) to evaluate background contextual fear. In the second retention test, the animals are placed into a novel context, which normally should not induce a fear response. After 1–2 min, the CS is presented, which then should induce a freezing response. It is recommended either to present the CS for 2–3 min [77] or to repeat CS presentations [78].

As these two protocols are standard, relatively easy to establish and usually produce robust results, they should be a starting point, when setting up fear conditioning in a laboratory. As simple as the principle is, as complex it can get given the multitude of variation possibilities of the different parameters involved. The two most important parameters are US intensity and number of US presentations. As mentioned, moderate US intensity and number lead to a specific fear memory and are therefore used to investigate general learning mechanisms. Stronger protocols are used to model the generalization of conditioned fear [37, 79], which is more pronounced and/or pathological in many anxiety disorders and post-traumatic stress disorder [80–82]. For example, in a cued conditioning protocol in mice, three CS/US pairings with a US intensity of 0.4 mA and a duration of 1 s lead to a specific fear memory, while ten pairings with an increased US intensity of 0.6 mA but equal US duration induce a generalization of conditioned fear, later tested in a retention test with presentations of a neutral cue that was never paired with the US [79]. In rats, seven CS/US pairings with a US intensity of 0.3 mA elicits fear to the CS only, while US intensities between 0.6 and 0.9 mA elicits fear to the CS and the conditioning context, and an US intensity of 1.2 mA elicits higher amounts of freezing even toward a novel context [37]. Variations in these two parameters of the US thus allow a finetuning on the memory specificity-generalization axis. In the following, we will assume moderate US settings and focus on CS variations, since these can result in qualitatively different memory phenomena. To do so, we distinguish three phases: before conditioning, conditioning (US presentation), and after conditioning. Examples of parameter settings for the particular protocols are given in the Tables below.

4.2 Some Exemplary Modifications of the Protocol

4.2.1 Modifications Before Fear Conditioning

Generally, pre-exposure of the animals to the conditioning context or to-be-learned cues has the potential to affect the learning and/or retention of conditioned fear. Depending on the amount of pre-exposure, two protocol modifications can be distinguished.

4.2.2 Familiarization

Before the actual conditioning sessions, habituation sessions (also called adaptation sessions) can be presented, in which the animals are familiarized with the fear conditioning chamber and the future fear cue [83] to reduce unspecific fear induced by novelty. If the purpose of the study is to investigate generalization of conditioned fear, one can present a cue similar, but not identical to the later fear CS. This cue will not be presented during the conditioning phase but during the retention to measure a transfer of fear memory to stimuli close to the fear cue [84]. Of note, only few cue presentations are given during these habituation session(s) (e.g., for neutral cues: two sessions á 6 cues [84] or four sessions á 4 cues [85], for the future fear CS: 1 session á 5 cues [83]), since an exaggerated number of pre-exposure to the future fear CS leads to latent inhibition (see next section). Likewise, the animals can be pre-exposed to the context in contextual fear conditioning session [86]. Examples of parameter settings are shown in Table 1.

Table 1
Exemplary studies with parameters for familiarization

Laxmi et al. (2003): Familiarization in male C57BL/6 J OlaHsd mice [79]	
Before	4 sessions (2/day): 6× 2.5 kHz, 85 dB, 10 s, 20 s ITI. 2-min initial and final pause, in shock context
Conditioning	Protocol A: 1 session, 3 pairings: CS+ (10 kHz, 85 dB SPL, 10 s), US (0.4 mA, 1 s), ITI (20 s) Protocol B: 1 session, 10 pairings: CS+ (10 kHz, 85 dB SPL, 10 s), US (0.6 mA, 1 s), ITI (20 s)
Result	Protocol A: Specific fear memory to 10 kHz tone Protocol B: Generalization to 2.5 kHz tone
Bergado-Acosta et al. (2008): Familiarization in male C57BL/6 mice [84]	
Before	2 sessions (on 1 day): 6× 2.5 kHz, 85 dB SPL, 10 s, 20 s ITI
Conditioning	1 session, 3 pairings: CS+ (10 kHz, 85 dB SPL,10 s), US: 0.4 mA (1 s), ITI (20 s)
Result	Specific fear memory to 10 kHz tone
Huff et al. (2005): Familiarization in male Sprague-Dawley rats [86]	
Before	1 session: 8-min shock context or neutral context
Conditioning	Protocol A: 1 session with 1 US (1.8 mA, 2 s), initial pause 30 s Protocol B: 1 session with 2 US (0.5 mA, ITI: 15 s), initial pause: 45 s (parameters varied depending on pharmacological treatment)
Result	Pre-exposure influences brain regions recruited during memory formation

Abbreviations: *CS* conditioned stimulus, *CS+* conditioned stimulus paired with US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *s* seconds, *SPL* sound pressure level, *kHz* kilohertz, *mA* milliampere

4.2.3 Latent Inhibition

Excessive pre-exposure to a cue inhibits fear learning when this cue is later paired with an US, a phenomenon called "latent inhibition" [87]. The number of pre-exposures is very different, ranging from relatively low numbers of pre-exposures (20 ([88]), 2× 20 pre-exposures [89]) to higher numbers (90 CS [90], 120 (*see* Fig. 4)). The massed pre-exposure of the prospective CS leads to learning that the cue is irrelevant, and the subjects later ignore this seemingly irrelevant cue during fear conditioning, thereby preventing its association with the US. Likewise, the context can be excessively pre-exposed, resulting in a similar impairment of contextual fear conditioning (e.g., daily 30-min exposures for 10 days [91]). Examples of parameter settings are shown in Table 2.

Since schizophrenia patients are impaired in filtering out irrelevant information from their environment [92], they are also impaired in latent inhibition [93, 94]. Animal models of schizophrenia thus employ latent inhibition paradigms to investigate this cognitive deficit of the disorder [95].

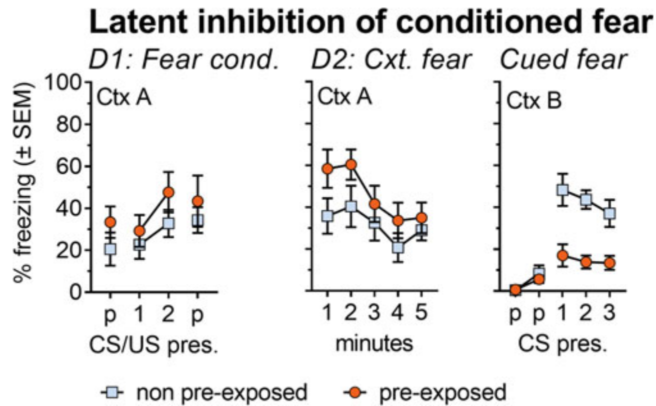

Fig. 4 Latent inhibition of conditioned fear. Experimental subjects were 8–10-week-old male C57BL/6 J mice (n = 9). There were three pre-exposures in context A (Ctx A) on three consecutive days with 40 CS presentations (CS: 10 kHz tone with 10 s duration and 75 dB SPL intensity) each day. Non-pre-exposed mice were also put into the boxes, but no tones were presented. Fear conditioning was then performed on the third day of pre-exposure, directly after the 40 CS presentations. Two pairings of the CS with electric stimuli (1 s duration, 0.6 mA intensity) were presented. One day later, retention tests on contextual (in Ctx A) and cued fear (in novel context B (Ctx B)) were performed. Note that the pre-exposed mice showed much less cued fear than the non-pre-exposed mice but more contextual fear. Y-axis scale and units in the middle and right subpanels are the same as in left subpanel; shown are means (± SEM). Abbreviations: *CS* conditioned stimulus, *cond* conditioning, *D* day, *Ctx* context, *p* pause (stimulus-free period), *pres* presentations, *SEM* standard error of the mean, *US* unconditioned stimulus

Table 2
Exemplary studies with parameters for latent inhibition

Lewis and Gould (2007): Latent inhibition (cued fear) in male C57BL/6 J mice [88]	
Before	1 session: 20 CS (white noise, 5 s, 85 dB SPL), ITI (15 s)
Conditioning	1 session: 2 CS+/US pairings, US (0.5 mA, 1 s), ITI (2.5 min)
Result	Decreased suppression ratio for water licking in pre-exposed mice
Mongeau et al. (2007): Latent inhibition (cued fear) in male C57BL/6 N mice [89]	
Before	1 session in shock context: 20× CS (9 kHz, 80 dB SPL, 10 s), random ITI; immediately followed by 1 session in retention context: 20× 9 kHz, 80 dB SPL (10 s), random ITI
Conditioning	Protocol A: 1 session, 2 CS+/US pairings. US (0.7 mA, 2 s), ITI (2 min) Protocol B: 1 session, 10 CS+/US pairings. US (0.4 mA, 1 s), ITI (1 min)
After	1 session: 18 min in shock context to extinguish background context fear
Result	Protocol A: Decreased suppression ratio for water licking in pre-exposed mice Protocol B: No difference in pre-exposed and not pre-exposed mice
Puga et al. (2007): Latent inhibition (cued fear) in male CBA/J mice [90]	
Before	1 h context habituation in shock context A 3 days in neutral context B: 90 CS (15 s), ITI (40 s)
Conditioning	2 days in context A: 4 CS+/US pairings. CS (1–2 kHz, 65 dB, 15 s), US (0.5 mA, 0.75 s), random ITI
After	2 sessions in neutral context B: 60 CS, 45 s ITI (15 s)
Result	Reduced freezing in latent inhibition group comparable to after extinction
Yamasaki et al. (2012): Latent inhibition (contextual fear) in male C57BL/6 mice [91]	
Before	10 sessions (1/day): 30-min context exposure
Conditioning	3 US (2 s, 1 mA), ITI (148 s)
Result	Reduced context fear memory compared to not pre-exposed group
Fendt (Fig. 4): Latent inhibition (cued fear) in male C57BL/6 mice	
Before	3 sessions (1/day): 40× CS (10 kHz, 10 s, 75 dB SPL)
Conditioning	Directly after last pre-exposure session: 2 CS+/US pairings. US (1 s, 0.6 mA)
Result	Less cued fear (freezing) in neutral context (and increased contextual fear) in pre-exposed mice

Abbreviations: *CS* conditioned stimulus, *CS+* conditioned stimulus paired with US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

4.2.4 Modifications During Fear Conditioning

Several qualitatively different learning phenomena can be investigated by varying the temporal contingency of CS and US during the conditioning phase.

Trace Fear Conditioning

In trace fear conditioning protocols, the US is presented in a relatively short and fixed time interval after the offset of the CS,

Table 3
Exemplary studies with parameters for trace fear conditioning

Holmes et al. (2002): Trace fear conditioning in male and female 129S6/SvEvTac and C57BL/6 J mice [96]	
Conditioning	2-min initial pause, 4 pairings: CS+ (800 Hz, 80 dB SPL, 30s), trace: 2.5 s, US (0.5 mA, 2 s), ITI (2 min) final pause: 2 min
Result	Freezing to cue and background context
Hwang et al. (2010) Trace fear conditioning in male C57BL/6 and DBA/2 mice [100]	
Before	1 session: 12-min shock context exposure
Conditioning	1 session, 6 pairings: CS+ (2 kHz, 80 dB, 20 s), trace: 18 s, US (0.5 mA, 2 s), ITI (60s)
Result	In neutral context: both strains freeze more to the CS compared to the unpaired control group, C57BL/6 mice freeze more than DBA/2 mice
Burman et al. (2014) Trace fear conditioning in male C57BL/6 J Crl mice [97]	
Conditioning	5-min initial pause, 10 pairings: CS+ (4 kHz, 67 dB SPL, 10s), trace: 20s, US (0.3 mA, 2 s), ITI: variable around 3.5 min
Result	Freezing to cue in neutral context comparable to delay fear conditioned and higher compared to unpaired group

Abbreviations: *CS+* conditioned stimulus paired with US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

e.g., after 2.5 s [96], 20 s [97], or 30 s [98]. Unlike for safety conditioning protocols, this gap is short enough to form an association between the CS and the US. Of note, the intertrial intervals between CS/US pairings should be much longer than usual, since intervals of 2.5–4.5 min, but not 1.5–3.5 min, lead to trace fear conditioning [97]. Several studies showed that trace fear conditioning is mediated by hippocampus plasticity [97, 99, 100]. Examples of parameter settings are shown in Table 3.

Delay Conditioning
As mention above, the standard cued fear conditioning protocol is delay conditioning, i.e., the CS co-terminates with the US. Examples of parameter settings are shown in Table 4.

Discriminative Conditioning
In this protocol, a second, non-reinforced cue is presented in addition to CS/US pairings. In this context, the term CS+ is commonly used for the CS that is reinforced with the US, while the unreinforced CS is called the CS−. Discriminative protocols allow the investigation of two different memory phenomena that are affected in PTSD patients, namely, increased fear generalization [82] and decreased fear inhibition [101]:

Table 4
Exemplary studies with parameters for delay fear conditioning

Phillips et al. (1992): Delay fear conditioning in male Sprague-Dawley rats [51]	
Before	Shock context exposure for 40 min
Conditioning	2 sessions (1/day): 2 pairings: CS+ (8 kHz, 80 dB, 20 s), US (0.3, 0.5, 1.0, 2.0 mA, 0.5 s) ITI (60–120 s)
Result	0.3 mA induce less freezing than 0.5 mA to CS and shock context
Balogh et al. (2002): Delay cued and contextual fear conditioning in male C57BL/6 and DBA/2 J mice [66]	
Before	Neutral context for 3 min (1 h before conditioning)
Conditioning	Protocol A: 1 session: 1 US (0.35 mA, 2 s) Protocol B: 1 session: Initial pause 2 min; 2 pairings: CS+ (clicker, 80 dB SPL, 30 s), US (0.7 mA, 2 s)
Result	Protocol A: C57BL/6 mice freeze more than DBA/2 J mice Protocol B: C57BL/6 mice freeze more than DBA/2 J mice and generalize more
Herry et al. (2003): Delay fear conditioning at different intensities in male C57BL/6 mice [113]	
Conditioning	1 session: 10, 4, or 2 pairings: CS+ (2.5 kHz, 80 dB, 30 s), US (0.9 mA, 1 s), ITI (60–180 s)
Result	In shock cxt: More freezing after 10 pairings than after 4 and 2 pairings; no difference between 4 and 2 pairings
Holmes et al. (2002): Delay fear conditioning in male and female C57BL/6 J mice [96]	
Conditioning	2-min initial pause, 4 pairings: CS+ (white noise, 80 dB SPL, 30s), US (0.5 mA, 2 s, co-termination), ITI (2 min), final pause: 2 min
Result	Freezing to shock context and in a neutral context to CS
Baldi et al. (2004): Delay fear conditioning in male Wistar rats [37]	
Conditioning	3-min initial pause. 7 pairings: CS+ (8 kHz, 75 dB SPL, 6 s), US (0, 0.15, 0.3, 0.6, 0.9 or 1.2 mA), ITI (30 s)
Result	No freezing after 0.15 mA, freezing to CS after 0.3 mA, freezing to CS and shock context after 0.6 mA, freezing to CS, shock context and neutral context (generalization!) after 0.9 and 1.2 mA
Burman et al. (2014): Delay fear conditioning in male C57BL/6 J Crl mice [97]	
Conditioning	5-min initial pause, 5 pairings: CS+ (4 kHz, 67 dB SPL, 10 s), US (0.3 mA, 2 s), ITI (variable around 3.5 min)
Result	More freezing to cue in neutral context compared to unpaired group
Huckleberry et al. (2016): Delay cued and contextual fear conditioning in male C57BL/6 J mice [71]	
Before	5 sessions (1/day): Handling for 2 min
Conditioning	Protocol A: 1 session with 3 pairings: CS+ (5 kHz, 85 dB SPL, 20 s), US (0.75 mA, 1 s) Protocol B: 1 session with 3 US (0.75 mA, 1 s) Protocol C: 1 session with 1 US (0.75 mA, 2 s)

(continued)

Table 4 (continued)

Result	Protocol A: Background context fear is decreased if scent and/or floor was changed Protocol B: Foreground context fear is stronger than background context fear, no generalization Protocol C: Foreground context fear is similar to background context fear (i.e., less than in protocol B), no generalization
Ramanathan et al. (2018): Delayed cued and contextual fear conditioning in male Long-Evans rats [151]	
Conditioning	Protocol A: Initial pause: 3 min, 5 pairings, CS+ (2 kHz, 80 dB SPL, 10 s), US (1 mA, 2 s), 70 s ITI Protocol B: Initial pause: 3 min, 5× US (1 mA, 2 s), 70 s ITI
Result	Protocol A: High freezing to CS in neutral context Protocol B: Freezing to the shock context, little freezing to neutral context
Glover et al. (2020): Delay fear conditioning in C57BL/6 J and 129S1/SvImJ mice [114]	
Conditioning	180 s initial pause; 3 pairings: CS+ (white noise, 75 dB, 30 s), US (0.6 mA, 2 s), ITI (60–90 s); final pause: 120 s
Result	In neutral context: More freezing compared to nonconditioned control; more freezing to CS+ compared to context; more CS+ freezing than nonconditioned control

Abbreviations: *CS+* conditioned stimulus paired with US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

- Generalization or specificity, respectively, of conditioned fear memory: Generalization of fear is the spillover of fear to cues that are similar to, but not identical with, the cue that was reinforced by the US during the conditioning procedure (reviewed in [102]). Generalization to non-reinforced cues (or the specificity for the fear cue or context) is assessed by comparing the fear response to both cues, i.e., CS+ and CS-. Fear specificity is better; the more the animals freeze to the CS+, the less they freeze to the CS−. The degree of similarity between cues can be varied depending on the research question. For the assessment of fear generalization, it is further possible to present this second cue only in the retention test, instead of the conditioning phase and the retention test.
- Fear inhibition/safety learning: Since the CS− was never followed by an aversive outcome, it may signal a period of safety and reduce fear (reviewed in [103, 104]). The CS− is therefore also called safety cue. If a safety memory and its interaction with the fear memory are the focus of interest, it is not enough to compare fear and safety cue responses, because little fear to the CS− can indicate both a lack of fear generalization and a safety association with the CS−. To investigate safety memory, a compound of CS+ and CS− should be presented in a retention test, and the response to this compound should be compared to the response to the CS+ alone. Because the response to the

compound CS+/CS− is the sum of fear and safety memories, this test is called summation test. A criterion of a successful summation test is that the fear response of the animals to the compound CS+/CS− is significantly less than to the CS+ alone [105]. An unfamiliar/novel CS− should not have this effect, since the animals did not have a chance to learn a safety association—a control test that is unfortunately rarely performed. Of note, some cues have the ability to reduce fear-like behavioral responses without any conditioning, a phenomenon that is called "external inhibition" [106]. While external inhibition is an interesting topic on its own, such cues are unsuited to serve as safety cues in fear inhibition paradigms, in which cues should be associated with an emotional valence solely through a learning process and not via physical properties of the cues. The summation test constitutes one of two tests for safety memory [105]. The other test is the so-called retardation of fear acquisition test, i.e., the acquisition of a CS− as a fear cue should be retarded compared with a cue, which has no meaning for the animal at the beginning of fear conditioning.

Of note, the compound CS+/CS− is usually first presented in the retention test [107]. However, sometimes the non-reinforced compound CS+/CS− is already presented during conditioning [83]. If latter is the case, compound-specific learning during this phase cannot be excluded, while this is not the case if the compound is first presented in the retention test.

During conditioning, the number of CS− may be equal [106, 108–111], higher [83], or even lower [112] than the number of CS+. In the optimal case, the two stimuli should be interchangeable, i.e., (a) if used as a CS+, both stimuli should induce the same amount of fear learning; (b) if used as a CS−, both stimuli should induce a similarly low amount of freezing; and (c) if presented as a compound, both possible combinations should result in similar fear inhibition. If these conditions are met, the cues should be used in a counterbalanced fashion. Examples of parameter settings are shown in Table 5.

Partial Reinforcement

In contrast to the discriminative learning protocol described in previous section, partial reinforcement regimens contain cues that are reinforced with a specific probability [113, 114]. Of note, different CSs could be associated with a different probability of reinforcement. Thus, such protocols are used to investigate the effects of uncertainty and ambiguity. Examples of parameter settings are shown in Table 6.

Table 5
Exemplary studies with parameters for discriminative fear and/or safety conditioning

Ostroff et al. (2010): Safety and fear conditioning in male Sprague-Dawley rats [152]	
Before	2 sessions (1/day): 30-min shock context exposure
Conditioning	Protocol A: 1 session with 5 pairings, CS+ (5 kHz, 80 dB SPL, 30 s), US (0.7 mA, 1 s), variable ITI around 5 min Protocol B: 1 session with 5 explicit unpairings of CS– (5 kHz, 80 dB SPL, 30 s) and US (0.7 mA, 1 s), 119 s and 180 s
Result	Protocol A: High freezing to CS, low freezing to context Protocol B: Freezing to context is suppressed during CS presentation
Sangha et al. (2013): Discriminative fear, safety, and reward conditioning in male Long-Evans rats [83]	
Before	(reward pre-conditioning), 5 CS each (continuous light: 28 V, 100 mA, 20 s and tone: 11 kHz, pulsing, 70 dB, 20s) in shock context
Conditioning	4 sessions (1/day): 4 pairings: CS+ (11 kHz, pulsing, 70 dB, 20s), US (0.4 mA, 0.5 s), 10 CS– (continuous light: 28 V, 100 mA, 20 s), 15 compound CS+/–, ITI (100–140 s), (reward conditioning continued)
Result	More freezing to CS+ than to CS- and CS+/– (discrimination and summation)
Likhtik et al. (2014): Discriminative fear conditioning in male 129Sv/Ev mice [108]	
Conditioning	3 sessions (1/day): 2–3-min initial pause, CS+/CS– counterbalanced; 5–6 pairings: CS + (8 kHz, 30 s), US (0.4 mA, 1 s), 5 CS–: CS– (white noise, 30 s), pseudo-random order; ITI (60–180 s)
Result	Individual differences in generalization and the ability to discriminate
Chen et al. (2016) Discriminative fear conditioning in male Sprague-Dawley rats [109]	
Conditioning	3 sessions (1/day): 70 s initial pause, a 1 kHz tone (5 s, 75 dB SPL) preceded each CS+ and CS–; 15 pairings, CS+ (flickering LED-light, 264 lux, 20 ms on/off, 15 s), US (1 mA, 0.5 s); CS– (white noise, 75 dB SPL), quasi-random order
Result	More freezing to CS+ than to CS– and shock context
Foilb et al. (2018): Discriminative fear conditioning in male and female Sprague-Dawley rats [111]	
Conditioning	1 session: a 1 kHz tone (75 dB, 5 s) preceded each CS+ and CS–, 15 pairings: CS+ (flashing white LED light, 264 lux, 200 ms on/off, 15 s), US (1.2 mA, 0.5 s), 15 CS– (white noise, 75 dB SPL, 15 s), quasi-random order, ITI (90 s)
Result	In shock context: Females generalize to CS–
Foilb et al. (2018): Discriminative fear and safety conditioning in male and female Sprague-Dawley rats [111]	
Conditioning	5 sessions (1/day in the afternoon): 1 kHz tone (75 dB, 5 s) preceded each CS+ and CS–; 15 pairings: CS+ (flashing white LED light, 264 lux, 200 ms on/off, 15 s), US (1.2 mA, 0.5 s), 15 CS– (white noise, 75 dB, 15 s), quasi-random order; ITI (90 s), 5 retention sessions along conditioning days starting 1 day after conditioning started
Result	On day 1 in shock context: both sexes freeze more to CS+ and CS+/– than to CS– (discrimination, but no summation) On day 5 in shock context: both sexes freeze more to CS+ than to CS+/– and to CS– (discrimination and summation)

(continued)

Table 5
(continued)

Meyer et al. (2019): Discriminative fear conditioning in male C57BL/6 mice [107]	
Conditioning	4 sessions (1/day): 2 pairings: CS+ (12.5 kHz, 80 dB SPL, 20s), US (0.5 mA, 1 s), 30 CS– (2.9 kHz, 80 dB SPL, 20s), daily variation of trial order
Result	More freezing to CS+ than to CS–
Takemoto et al. (2019): Discriminative fear conditioning and context fear in male B6 mice [153]	
Conditioning	Protocol A: 3 session (1/day): 4 pairings: CS+ (white noise, 80 dB SPL, 10 s), US (0.6 mA, 2 s), 4 CS– (12 kHz, 80 dB, 10 s), ITI (60–90 s) Protocol B: 3 session (1/day): 4 pairings: CS+ (12 kHz, 80 dB SPL, 10 s), US (0.6 mA, 2 s), 4 CS– (white noise, 80 dB SPL, 10s), ITI (60–90 s)
Result	Protocol A: Lower freezing to CS– than to shock context (safety) Protocol B: Similar freezing to CS– and shock context (no safety)
Zhang et al. (2019): Discriminative fear conditioning in male C57BL/6 J mice [154]	
Conditioning	3-min initial pause, 3 pairings: CS+ (3 kHz, 70 dB SPL, 30s), US (0.8 mA, 2 s); 3× CS– (white noise, 70 dB SPL, 30 s), pseudo-randomized order, 90 s ITI
Result	In a neutral context more freezing to CS+ than to CS–

Abbreviations: *CS* conditioned stimulus, *CS+* conditioned stimulus paired with US, *CS–* conditioned stimulus unpaired with US or CS+/US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *ms* milliseconds, *min* minutes, *s* seconds, *SPL* sound pressure level, *V* volt

Table 6
Exemplary studies with parameters for partial reinforcement

Herry et al. (2003): Partial fear conditioning and extinction in male C57BL/6 mice [113]	
Conditioning	1 session, 2 pairings: CS+ (2.5 kHz, 80 dB SPL, 30s), US (0.9 mA, 1 s), 2 CS+ alone (no US), intermingled, ITI (60–180 s)
After	3 sessions (1/day): In shock context, 4 CS alone
Result	Impaired extinction in the partial reinforcement group
Glover et al. (2020): Partial fear conditioning in C57BL/6 J &129S1/SvImJ mice [114]	
Conditioning	180 s initial pause, 3 pairings: CS+ (white noise, 75 dB SPL, 30 s), US (0.6 mA, 2 s), 3 CS+ alone (no US), intermingled, ITI (15–60 s), 120 s final pause
Result	Less freezing to CS+ compared to fully reinforced group in BL/6 J mice (but similar in S1 mice). Similar freezing to neutral context

Abbreviations: *CS+* conditioned stimulus paired with US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

Safety Conditioning Through Explicit Unpairing

Explicitly unpaired presentations of CS and US, e.g., more than 30 s apart [115, 116], are considered to induce another form of safety conditioning, since the CS signals the nonoccurrence of foot shocks [117, 118]. After such a conditioning procedure, animals

therefore show a reduction of freezing in the retention test, if the safety cue is presented in the conditioning context, i.e., the strength of safety memory is measured by the difference between freezing during exposure to the conditioning context alone and freezing during CS presentations. Two different types of protocols are used. Either the CS and US presentations are intermingled presented in a random order ([115, 116]; *see* Fig. 5) or they are presented separately from each other with, e.g., US presentations in the first

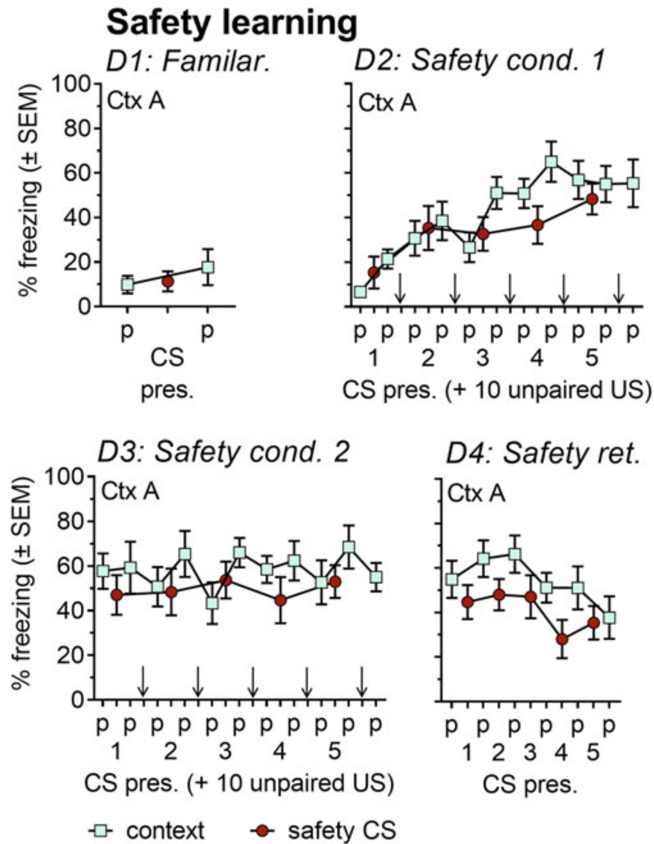

Fig. 5 Safety conditioning. Experimental subjects were 8–10-week-old male C57BL/6 J mice (n = 10). The CS was a 10 kHz tone with 30 s duration and 80 dB SPL intensity; the US had an intensity of 0.4 mA and a duration of 2 s. The protocol starts with a familiarization session (D1) in which one CS was presented. On the two following days (D2, D3), the US and the CS were presented five times daily, each in an explicitly unpaired manner, i.e., they were timely separated by 30–90 s. The aim of this conditioning procedure is that the US is associated with the conditioning context and the CS with the absence of the US (i.e., safety). In the safety retention test (D4), the ability of the CS to inhibit contextual fear was measured. Y-axis scale and units in the right subpanels are the same as in left subpanel; shown are means (± SEM). Abbreviations: *CS* conditioned stimulus, *Ctx* context, *D* day, *familiar* familiarization, *cond* conditioning, *p* pause (stimulus-free period), *pres* presentations, *ret* retrieval, *SEM* standard error of the mean, *US* unconditioned stimulus

Table 7
Exemplary studies with parameters for safety conditioning through explicit unpairing

Pollak et al. (2010): Safety conditioning in male C57BL/6 mice [117]	
Conditioning	3 sessions (1/day): 4 explicit unpairings: CS– (0.35 kHz, 72 dB, 20 s), 4 US (0.6 mA, 2 s), order of CS and US blocks was switched daily
Result	Less freezing to CS– compared to shock cxt
Kreutzmann et al. (2020): Safety conditioning in male and female C57BL/6 [115]	
Before	Shock context habituation for 3.5 min,1 CS (10 kHz, 85 dB SPL, 30 s)
Conditioning	2 sessions (1/day): 5 explicit unpairings: CS– (10 kHz, 85 dB SPL, 30 s), US (0.4 mA, 2 s), 30 s minimal interval between US and CS–
Result	Less freezing to CS– than to the shock context in males and females
Kreutzmann et al. (2020): Safety conditioning in male and female Sprague-Dawley rats [155, 156]	
Before	2 sessions (1/day): Shock context exposure and baseline startle measurement; 1 session: 20 startle alone and 10 CS (light, 1000 lx)/startle
Conditioning	2 sessions (1/day): 15 explicit unpairings: CS– (light, 1000 lx), US (0.6 mA, 0.5 s), variable ITI, 24 s minimal interval between US and CS–
Result	Reduced startle response following CS– compared to startle alone

Abbreviations: *CS–* conditioned stimulus explicitly unpaired with US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *lx* lux, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

half of the session and CS presentations in the second half [117]. If the second option is pursued, the order of the US and CS presentation should be switched on the next conditioning session to prevent timing effects. Examples of parameter settings are shown in Table 7.

Patients with post-traumatic stress disorder are impaired in safety learning [101]. Therefore, unravelling the underlying mechanisms will aid the understanding of pathological memory processes in this disorder. Moreover, since here the animals learn to reduce fear, safety learning protocols may bear considerable therapeutic potential for the treatment of other anxiety-related disorders.

Backward Conditioning

In the standard fear conditioning protocols, forward pairings of the CS and US are presented (see above), i.e., the CS predicts the occurrence of the US. In backward conditioning, this order is switched, i.e., the CS is presented immediately after the US. After such a backward conditioning protocol, the CS induces a relatively low amount of fear. Therefore, backward conditioning is also used to model "ambiguous fear" [119]. Others use backward conditioning as a model for safety conditioning since the CS later is able to inhibit fear behavior. Backward conditioning is also called "relief

Table 8
Exemplary studies with parameters for backward conditioning

Andreatta et al. (2012): Backward vs. forward conditioning in male Sprague-Dawley rats [157]	
Before	Context exposure and startle baseline
Conditioning	5-min initial pause, 15 CS-US pairings: CS+ (light, 5 s, 700 lux), US (0.8 mA, 0.5 s), different interstimulus intervals (ISI) between CS and US; ITI between CS-US pairings (90–210 s)
Result	ISI of 4.5 s (forward pairing) leads to increased startle response, ISI of −3 s (backward pairing) leads to decreased startle response
Goode et al. (2019): Backward conditioning in male Long-Evans rats [119]	
Conditioning	5-min initial pause. 12 US-CS pairings: US (1 mA, 2 s), CS+ (80 dB SPL, 2 kHz, 10 s), ITI (70 s)
Results	Less freezing compared to standard delayed fear conditioned group
Ressler et al. (2021): Backward conditioning and context extinction in male Long-Evans rats [158]	
Conditioning	Protocol A: Forward: 5-min initial pause, 12 CS-US pairings: CS+ (2 kHz, 80 dB, 10 s), US (1 mA, 2 s) ITI (58 s) Protocol B: Backward: 5-min initial pause, 12 US-CS pairings: US (1 mA, 2 s), CS+ (2 kHz, 80 dB, 10 s), ITI (58 s)
After	Shock context extinction: 2 sessions (30-min context exposure/day)
Result	Protocol A: No extinction of background context fear Protocol B: Extinction of background context fear

Abbreviations: *CS* conditioned stimulus, *CS+* conditioned stimulus paired with US, *ITI* intertrial interval, *ISI* interstimulus interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *lx* lux, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

conditioning" since the CS is presented during the relief from the US [120]. A relief CS is able to induce appetitive-like behaviors, such as approach behavior or attenuation of the startle response. Examples for parameter settings are shown in Table 8.

4.2.5 Modifications After Fear Conditioning

Extinction of Cued Fear Memory

In a classical protocol, extinction (or extinction training) is performed by increasing the number of CS presentations in the retention test, for example, the number of presentations is increased from 5 to 30. Often, the intertrial interval is shortened, e.g., only 5 or 10 s are used. Of course, all CSs are presented without reinforcement by the US. Thereby, a new memory is formed—the "CS-no US" memory. This extinction memory is tested in a further retention test, typically 1 day after the extinction. If the extinction was successful, reduced fear is observed in this retention test (also called "extinction retrieval"), as compared to the fear before extinction [121]. Since the early phase of extinction is equivalent to a fear retention test, the comparison between this phase and the extinction retention is an ideal readout for extinction success (Fig. 6).

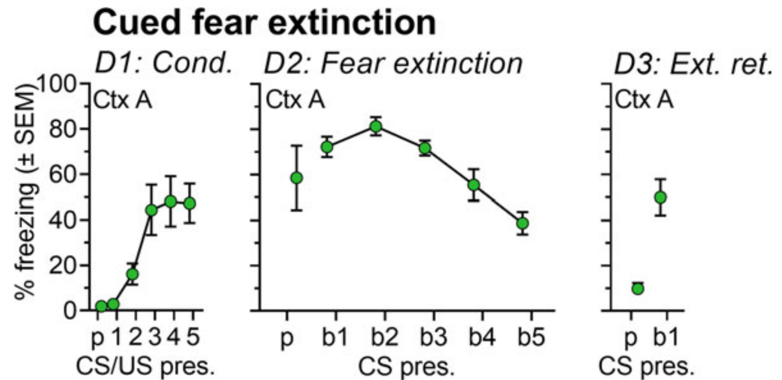

Fig. 6 Fear extinction. Experimental subjects were 10–11-week-old male C57BL/6 J mice (n = 6). The CS was white noise of 30 s duration and 80 dB SPL intensity. The US intensity was 0.4 mA and its duration was 2 s. On D1 of the protocol, mice were fear conditioned with 5 CS/US pairings. On the next day (D2), mice received 25 sole presentations of the CS (shown as blocks of 5 adjacent, averaged CS) without the US to extinguish the fear memory. On D3 extinction memory was retrieved by presenting 5 CS. Y-axis scale and units in the middle and right subpanels are the same as in left subpanel; shown are means (± SEM). Abbreviations: *b* block, *CS* conditioned stimulus, *Cond* conditioning, *D* day, *Ctx* context, *Ext. ret* extinction retention, *p* pause (stimulus-free period), *pres* presentations, *SEM* standard error of the mean, *US* unconditioned stimulus

Therefore, extinction training and extinction retention should be carried out in the same context, whether it is a novel or the conditioning context. If the conditioning context is used, the respective contextual fear memory is of course also affected by the extinction process. If a novel context is used, there might be second-order fear conditioning during the extinction, i.e., the fear induced by the CS is then also associated with the novel context [122]. These cue-context interactions can be both, subject of scientific interest and a confounding factor.

While a fear memory is formed after only few CS/US pairings, its extinction requires significantly more CS-alone presentations (15–30, or even more) within one session and/or over several sessions or days [123–129]. The exact parameters of the extinction protocol depend on the intensity of the fear conditioning protocol, but only 1 day or one session, respectively, of extinction are more commonly used than several days or sessions, and shorter intertrial intervals are more efficient than longer intertrial intervals [130].

Reinstatement, *renewal*, and *spontaneous recovery* (e.g., reviewed in 121): These three phenomena demonstrate that the original fear memory can return after extinction under certain experimental circumstances and thus provide evidence that extinction indeed is the formation of a novel CS-no US memory that

actively inhibits the former fear memory. *Reinstatement* describes the return of extinguished CS-fear, if the US is presented alone, without the CS, after extinction. *Renewal* refers to the return of extinguished CS-fear, if the CS is presented in a context different from the context in which extinction had occurred. *Renewal* illustrates the context dependency of an extinction memory. *Spontaneous recovery* is return to pre-extinction fear levels in a remote retention test (e.g., 10 days later [131]) without any other manipulation between the extinction and this retention test.

If the fear memory cannot be recovered by renewal, reinstatement, or spontaneous recovery protocols, this memory is considered to be not extinguished, but erased [132]. The particular case of fear memory erasure occurs, for example, if fear conditioning and extinction are carried out in juvenile animals [133] or if extinction was performed in a particular time window after a short retention test [132]. Fear extinction further has to be distinguished from forgetting, which is defined as a memory decay over time without any manipulation on the memory trace (CS, US, or context) between fear conditioning and fear retention [121].

Extinction is probably the most popular post-conditioning modification of fear memory due to its high translational relevance in the therapeutic setting. It is considered the laboratory equivalent to exposure therapy, a standard treatment approach for anxiety disorders in cognitive behavioral therapy, in which patients are repeatedly confronted with fear-associated stimuli without an aversive outcome [134]. Examples for parameter settings are shown in Table 9.

Extinction of Contextual Fear Memory

Similar to the extinction of cued fear memory, the extinction of contextual fear memory requires exposure(s) to the conditioning context without reinforcements by the US. Such exposures can include one or few long sessions or up to ten shorter sessions, typically with one session per day [135–137]. Examples of parameter settings are shown in Table 10.

Conditioned Inhibition

In a conditioned inhibition protocol, a second cue is introduced that either precedes the CS [112, 138] or is presented simultaneously with the CS [139]. This cue, called a conditioned inhibitor, predicts the non-reinforcement of the following CS. One or more conditioning sessions can be performed, in which the conditioned inhibitor is presented with the non-reinforced CS several times, but importantly also reinforced CS should be presented. In retention tests, the effects of the CS alone, the conditioned inhibitor, as well as the compound are tested. As explicit unpairing and backward conditioning, conditioned inhibition is regarded as a form of safety conditioning [140–142]. Examples of parameter settings are shown in Table 11.

Table 9
Exemplary studies with parameters for extinction of cued fear

Milad et al. (2002): Cued fear conditioning and extinction in male Sprague- Dawley rats [159]	
Conditioning	1 session: 5 CS alone followed immediately by 5 pairings: CS+ (4 kHz, 80 dB, 30 s), US (0.5 mA, 0.5 s), ITI (2–6 min)
After	1 h after conditioning: 20 CS
Result	Reduction of freezing during extinction and in extinction retention
Cain et al. (2003): Cued fear extinction with different ITI duration in male C57BL/6 mice [130]	
Before	10 min in neutral context, 1 h before conditioning
Conditioning	1 session: 2-min initial pause, 2 pairings: CS+ (white noise, 80 dB SPL, 2 min; US (0.4 mA, 2 s), ITI (2 min)
After	Protocol A: 20 CS in neutral context; different ITI (6 s) Protocol B: 20 CS in neutral context; different ITI (60 s) Protocol C: 20 CS in neutral context; different ITI (600 s)
Result	Protocol A: Reduction of freezing during extinction and in extinction retention Protocol B: Reduction of freezing during extinction but not in retention Protocol C: No reduction of freezing during extinction and in retention
Pfeiffer and Fendt (2006): Cued fear extinction in male Sprague-Dawley rats [126]	
Conditioning	1 session: 2 CS+ alone followed by 7 pairings: CS+ (4 kHz, 80 dB SPL, 30 s), US (0.5 mA, 1 s), ITI (120 s)
After	1 session: In shock context, 15 CS, ITI (120 s)
Result	Reduction of freezing during extinction and in extinction retention
Sangha et al. (2009): Cued fear extinction in male wild-type mice with C57BL/6 background [124]	
Before	2 sessions (2/day) in shock context with 6 CS– (2.5 kHz, 85 dB SPL, 10 s), ITI (20 s)
Conditioning	Protocol A: 2 sessions (1/day): 3 pairings: CS+ (10 kHz, 85 dB SPL, 10 s), US (0.2 mA, 1 s), ITI (10–30 s) Protocol B: 2 sessions (1/day): 3 pairings: CS+ (10 kHz, 85 dB SPL, 10 s), US (0.4 mA, 1 s), ITI (10–30 s)
After	6 sessions in neutral context (30 min apart) with 4 CS-, 4 CS+ presentations
Result	Protocol A: Reduction of freezing during extinction and in extinction retention, no generalization to CS– in wild-type control mice Protocol B: Reduction of freezing during extinction and in extinction retention, no generalization to CS– in wild-type control mice
Monfils et al. (2009): "Bounderies" of cued fear extinction in male Sprague-Dawley rats [132]	
Conditioning	1 session: Initial pause: 10 min; 3 pairings: CS+ (5 kHz, 80 dB SPL, 20 s), US (0.7 mA, 0.5 s), ITI (variable around 180 s)
After	Protocol A: Brief context exposure, 1-h pause, 19 CS in shock context, ITI (180 s) Protocol B: Brief context exposure, 1-h pause, 19 CS in shock context, ITI (180 s), 5 US 24 h later

(continued)

Table 9 (continued)

	Protocol C: Brief context exposure, 1-h pause, 19 CS in neutral context, ITI (180 s); extinction retention 24 h later in neutral context, extinction retention another 24 h later in shock context Protocol D: Brief context exposure, 1 h pause, 19 CS in shock context, ITI (180 s), 4 CS+ in shock context 24 h later, 4 CS+ in shock context another month later
Result	Protocol A: Reduction of freezing to CS+ during extinction and in retention Protocol B: **Reinstatement** of extinguished fear after US exposure Protocol C: **Renewal** of extinguished fear in shock context Protocol D: **Spontaneous recovery** of extinguished fear after 1 month
Fendt et al. (2010): Cued and background context fear extinction in male wild-type mice with C57BL/6 J background [122]	
Conditioning	60 s initial pause, 6 pairings: CS+ (8 kHz, 80 dB SPL, 30 s), US (0.6 mA, 2 s), ITI (90 s)
After	6 sessions (2/day each: One 3 min in shock context, 2 h later one in neutral context with 10 CS+)
Results	In wild-type control mice: Reduction of freezing in shock context from day 1 to day 3, in neutral context reduction of freezing to CS+ from day 1 to day 3
Çaliskan et al. (2016): Cued fear extinction in *male wild-type* mice with C57BL/6 background [125]	
Before	4 sessions (2/day): 6-min shock context exposure
Conditioning	1 session: 2-min initial pause, 3 pairings: CS+ (9 s, 10 kHz, 85 dB), US (0.4 mA, 1 s), ITI (20 s)
After	5 sessions in neutral context (1/day): 20 CS (10s), ITI (20 s)
Result	Reduction of freezing in neutral context over sessions in wild-type mice
Müller (Fig. 6): Cued fear extinction in male C57BL/6 mice	
Conditioning	1 session: 5 pairings: CS+ (white noise, 80 dB, 30 s), US (0.4 mA, 2 s)
After	25 CS+ in shock context
Result	Less freezing in extinction retention compared to beginning of extinction

Abbreviations: *CS* conditioned stimulus, *CS+* conditioned stimulus paired with US, *CS–* conditioned stimulus without US, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *h* hour, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

Second-Order Conditioning

A very similar protocol is used in second-order conditioning. Here, a second cue is repeatedly paired with an already well-conditioned CS. The second cue is then associated with the fear response induced by the CS and thereby becomes a fear CS by itself [143, 144]. Examples of parameter settings are shown in Table 12.

Table 10
Exemplary studies with parameters for extinction of contextual fear

Sangha et al. (2009): Contextual fear extinction in male wild-type mice with C57BL/6 background [124]	
Before	2 sessions (2/day): 10-min neutral context
Conditioning	3-min initial pause, 1 US (0.7 mA, 2 s)
After	6 sessions in shock context, 30 min apart
Results	Reduction of freezing over extinction and in extinction retention in wild-type control group
Çaliskan et al. (2016): Contextual fear extinction in male wild-type mice with C57BL/6 background [125]	
Conditioning	3 sessions (1/day): 1 US (0.4 mA, 1 s)
After	5 sessions (1/day): 10-min shock context
Result	Reduction of freezing from day 1 to day 5 in wild-type control group

Abbreviations: *US* unconditioned stimulus, *mA* milliampere, *min* minutes, *s* seconds

Table 11
Exemplary studies with parameters for conditioned inhibition

Vouimba et al. (2000): Conditioned inhibition in male C57BL/6 mice [112]	
Before	5 sessions (1/day): Handling and 10-min shock cxt
Conditioning	Phase 1: 2 sessions (1/day): 5 CS+/US pairings Phase 2: 5 sessions (1/day): 5 CS+/US pairings randomly mixed with 5 CI/CS+ pairings (no US), CS+ (1 kHz, 80 dB, 20 s), US (0.5 mA, 0.5 s), ITI (60–180 s), CI (light, 12 W, 20 s)
Result	In shock context less freezing to CS+ with the CI compared to without
Fendt (1998): Conditioned inhibition in male Sprague-Dawley rats [160]	
Conditioning	Phase 1: 5-min initial pause, 10 CS+/US pairings: CS+ (light, 3.7 s, 40 W), US (0.6 mA, 0.5 s), ITI (1.5–2.5 min) Phase 2: 5-min initial pause, 5 CS-US pairings randomly mixed with 15 CI/CS+ presentations, CS (white noise, 3.7 s, 70 dB SPL)
Result	Potentiated startle response during CS+ but not during CI/CS+ presentation

Abbreviations: *CI* conditioned inhibition, *CS* conditioned stimulus, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level, *W* watt

5 Data Analyses

In rodent experiments, freezing and startle response modulation are most commonly used as behavioral measures of conditioned fear. Time spent freezing is typically expressed as percentage of CS duration and averaged across all CS of the retention session. To measure contextual fear, the percent time spent freezing of the

Table 12
Exemplary studies with parameters for second-order conditioning

Gewirtz et al. (1997): Second-order conditioning in male Sprague-Dawley rats [143]	
Conditioning	5 sessions (last session intermingled in second-order conditioning sessions): First-order conditioning session: 16 CS+/US pairings, 4 CS alone, 3 sessions: second-order conditioning session: 10 (CS+/2nd CS pairings: CS+ (white noise, 70 dB, 6.7 s), US (1.2 mA, 0.5 s), 2nd CS (light, 8 W, 3.7 s), ITI (90–210 s)
Result	Potentiated startle during 2ndCS presentations

Abbreviations: *CS* conditioned stimulus, *2nd CS* second order conditioned stimulus, *ITI* intertrial interval, *US* unconditioned stimulus, *dB* decibel, *kHz* kilohertz, *mA* milliampere, *min* minutes, *s* seconds, *SPL* sound pressure level

session duration can be used. For extinction sessions, CSs are individually analyzed, since the decline in freezing and thus the acquisition of fear extinction is of interest. If an exaggerated number of CSs is presented and/or the curve appears very noisy, averaging, e.g., 2–5 consecutive CS and analyzing blocks of CS may help to streamline the curve. In the case of context extinction, the session can be broken down to, e.g., 1- or 2-min intervals to assess the acquisition of fear extinction. Another but less commonly used parameter is the amount of freezing episodes, expressed as the total number.

The startle response displays a certain degree of intra- and interindividual variability, so that many trials are needed and matching is recommended [106]. Matching means assigning mice or rats to experimental and control group according to a baseline startle response to assure that the group means do not differ from each other before the actual experiment has started. Retention tests always contain startle stimulus alone trials in addition to fear CS + startle stimulus trials. The difference in the mean responses to the "startle alone" and the "CS-startle" is called fear potentiation and is presented either as an absolute value or as the percent change to the "startle alone" magnitude [145].

Very often, it is recommended to not only analyze one single measure of conditioned fear, especially if setups and/or protocols are used that allow more fear responses than only one. For example, animals can switch their fear response from freezing to escape response [146–148]. Such escape response can also be automatically detected, e.g., by velocity threshold in the tracking software [149, 150]. Without the knowledge that escape responses are increased in an experiment, the observation that freezing is decreased in this experiment would be miss-interpreted as fear reduction. An increase in fear from before conditioning to the fear retention session (or the early phase of extinction in case CS-presentation is continued) indicates successful fear memory. Since fear conditioning in general produces robust and strong effects and rodents rather explore than fear non-aversive environments, it is common to report only the fear response to the

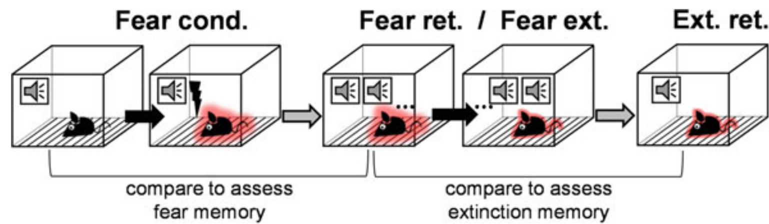

Fig. 7 Fear and extinction memory assessment. Successful fear conditioning is demonstrated by higher fear in the retention test than in the phases before US application. However, this comparison is usually omitted, once the protocol is established in a laboratory. Since an extinction memory often is less robust, it is recommended to compare the extinction retention with the fear retention test/ early extinction to demonstrate a decline in fear. Abbreviations: *cond* conditioning, *ret* retention, *ext/Ext* extinction

conditioned stimulus or context, after the protocol is established in a laboratory. This does not apply to safety learning paradigm. Here, the comparison with a fear-associated reference is mandatory. Fear extinction is less robust than fear conditioning, and freezing levels will most likely not return to baseline. To assess successful fear extinction, behavior in the extinction retention phase can be compared to the early phase of extinction, since this part is equivalent to a fear retention session (Fig. 7).

6 Notes

In fear conditioning, an association between a neutral cue or context and an aversive stimulus is learned through their co-presentation, so that the neutral cue/context becomes a conditioned cue/context capable of eliciting a fear response. Manipulations before, during, or after the conditioning procedure, as well as of the parameter settings themselves, allow the investigation of different aspects of healthy and pathological learning. One of the main readout parameters in rodents is freezing (immobility except for breathing), which can be measured automatically or manually. Both methods have potential pitfalls. Despite being more labor-intensive, manual scoring is also less objective. The latter can be overcome by proper study designs that allow blinding of groups or conditions, which are to be compared. When a software scores freezing, the thresholds should be set such that the automatic rating correlates high with the scoring of an experienced experimenter. This assures that what the software measures is freezing indeed. Both methods bear a certain degree of error, particularly when behavior is ambiguous.

If fear conditioning does not elicit freezing, possible reasons can be that the US was not applied (e.g., lose cables, other problems with the hard ware, dirty grid, or an unfortunate position of

the animal on the grid) and/or the CS and/or US was not perceived. Examining the setup and assuring that both stimuli are within the perceptible range of the species used is recommended. Since certain mutations might have unintended effects on the perceptive systems, wild types should be used to establish a protocol.

If conditioning induces freezing, the next step is to assure that it is indeed caused by the learning process and not by unspecific factors, like the conditioned cue or context being aversive per se. This possibility can be excluded, when freezing to this CS or context before conditioning is low or when unconditioned mice do not freeze. Further, it should be demonstrated that freezing is restricted to the conditioned cue/context. If protocols are too intense, animals will show a fear response to other (similar) cues or contexts as well, so that freezing to a neutral/novel cue or context should be assessed after conditioning as well (*see* Figs. 8 and 9).

6.1 Setting Up Contextual Fear Conditioning

Figure 8 presents a flowchart for setting up contextual fear conditioning. If mice or rats show high freezing levels to the conditioned context in the retention, it has to be demonstrated that these high freezing levels are indeed caused by a fear memory. This can be checked, for example, by comparing these freezing levels to those of animals that underwent the same conditioning paradigm except for US presentation. Likewise, the freezing levels can be compared to the freezing levels in the same context before conditioning. If these comparisons do not show differences, i.e., there is equally high freezing in nonconditioned animals or before conditioning, the context is likely aversive per se, which can strongly interfere with fear conditioning and/or can lead to a ceiling effect. In this case, the context and/or the experimental conditions should be adjusted, e.g., by decreasing intensity of the house light and downsizing the chamber. Further transport stress can be reduced by shortening the distance between animal facility and experimental room.

A further important question is whether the protocol induces a fear memory that is specific to the conditioning context or whether the memory is generalized to another novel/neutral context. Often, a reduction of US intensity is helpful to make the memory more specific. Similarly, high freezing levels to the conditioned and a novel/neutral context can also result, when contexts are too similar and the animals cannot discriminate between them. It is therefore recommended to make these two contexts as different as possible.

If no freezing at all is detected upon reexposure to the conditioning context, the reason is likely an issue with US delivery or with the detection of freezing behavior by the system. The first checkpoint is assuring that US intensity (e.g., 0.45–1 mA) and

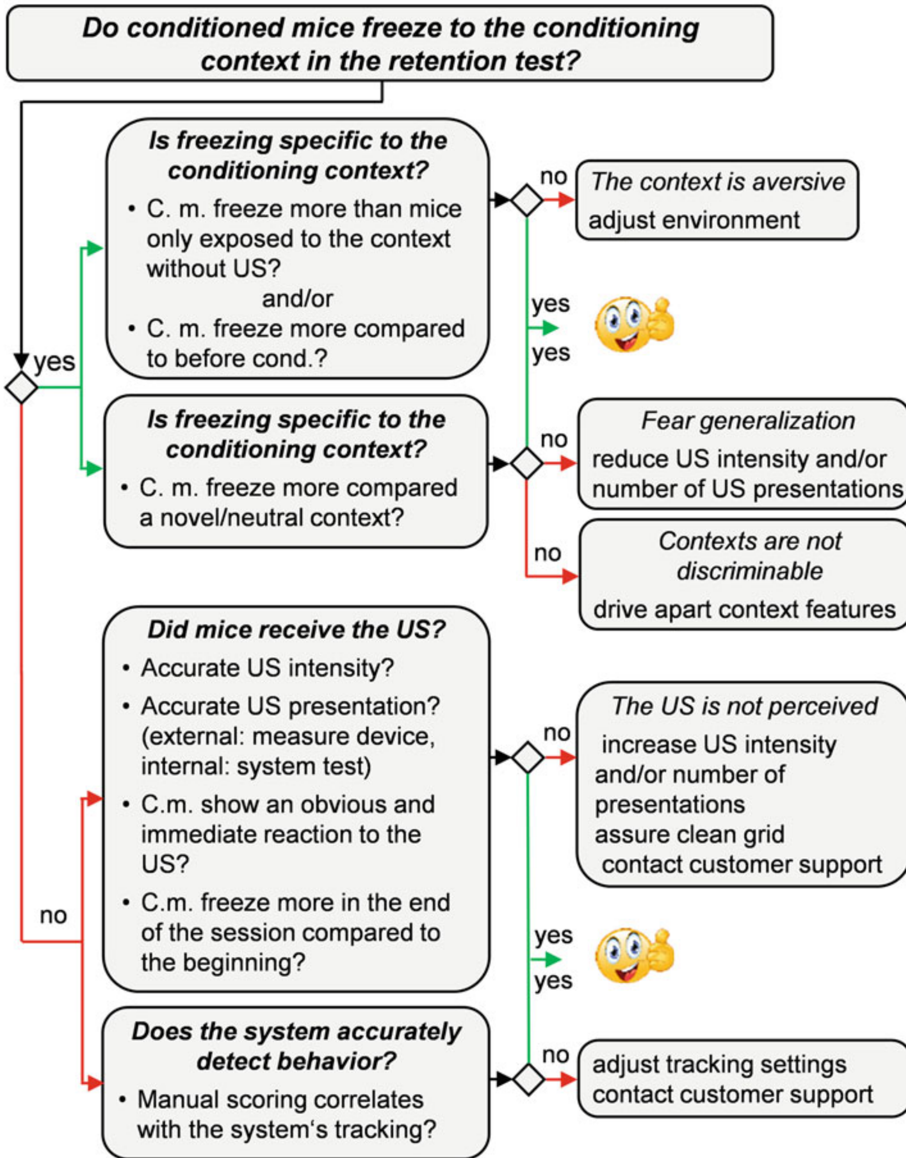

Fig. 8 Flowchart for setting up contextual fear conditioning. For details, see main text. (Smiley credits: DarkAthena on pixabay.com) Abbreviations: *C.m* conditioned mice, *cond* conditioning, *US* unconditioned stimulus

number of US presentations are in the range capable of inducing a fear memory and that the grid is clean. Further, animals should show an obvious and immediate reaction (jumps, twitches, startle) to the foot shock, and the system measures should indicate higher freezing in the final stimulus-free phase of the conditioning session compared to the initial stimulus-free phase before shock presentation. An external measure device (amperemeter) can be used to

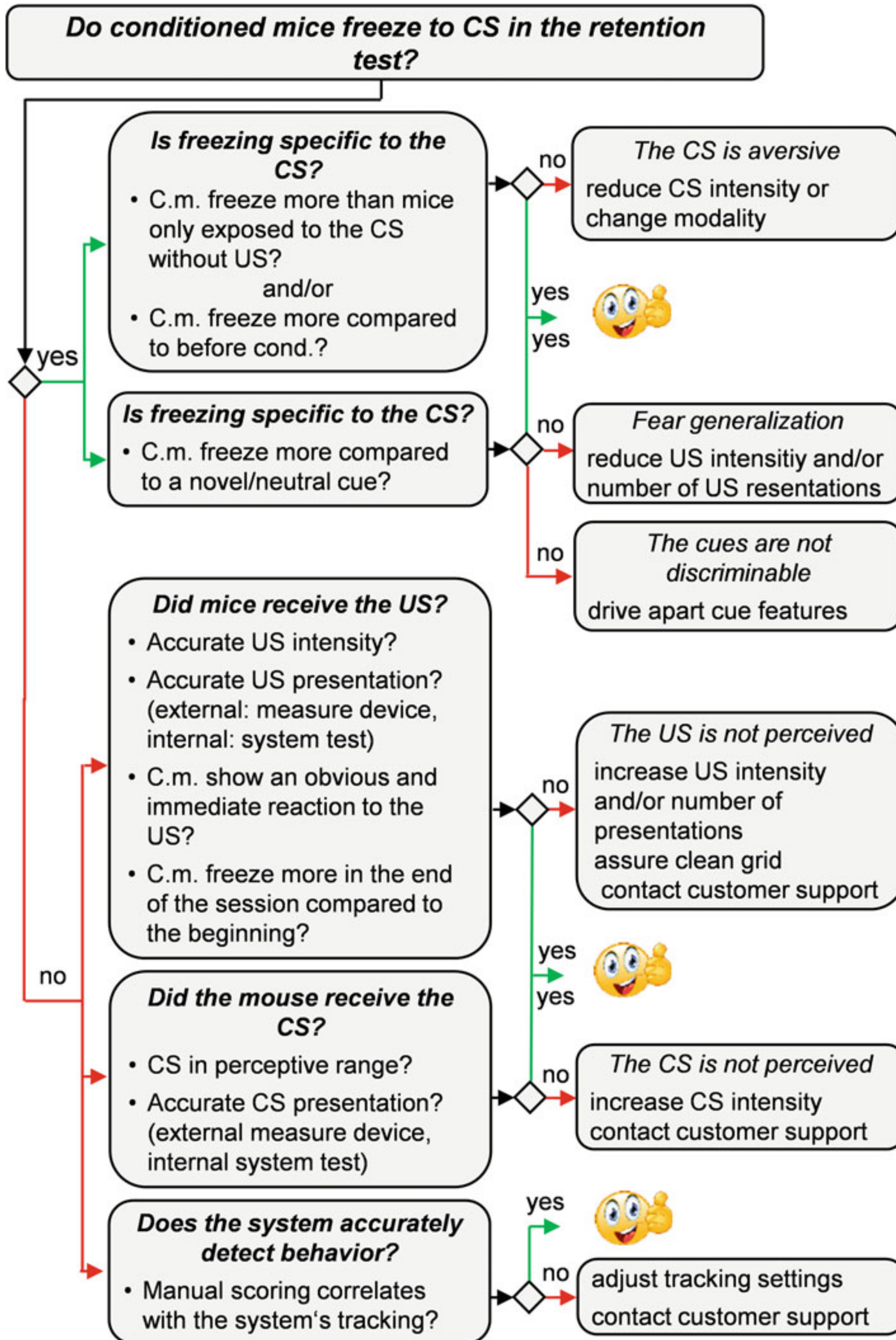

Fig. 9 Flowchart for setting up cued fear conditioning. For details, see main text. (Smiley credits: DarkAthena on pixabay.com) Abbreviations: *C.m* conditioned mice, *cond* conditioning, *CS* conditioned stimulus, *US* unconditioned stimulus

confirm that US intensity set in the program corresponds to the real US intensity delivered to the grid. We recommend to test this in an actual conditioning session with all boxes, in addition to the system test that most programs contain. To verify that accurate detection of freezing by the program, we recommend recording videos and correlating the system output with the manual scoring of an experienced experimenter (*see* Fig. 2).

6.2 Setting Up Cued Fear Conditioning

The approach for setting up cued fear conditioning is very similar (*see* Fig. 9). If mice or rats show high freezing levels to the CS in the retention test, it has to be demonstrated that this indeed is caused by a fear memory, e.g., by comparing these freezing levels to those of animals that underwent the same procedure (including CS presentation), except for US presentations. Likewise, the CS response during the retention session can be compared to the CS response before conditioning. If these comparisons show that the CS response is not increased by the conditioning procedure, the CS is likely aversive per se, leading to a ceiling effect. In this case, the CS can be adjusted by reducing intensity (loudness, brightness). In a next step, one would check if the protocol induces a specific fear memory or a fear memory that also generalized to novel or neutral cues. If this is observed, the US intensity should be adjusted. For an adequate test of fear specificity or generalization, two cues that are clearly different but which are both well perceivable for the animals should be used (e.g., 2.5 and 10 kHz tones).

If no freezing is detected upon CS presentations, the reasons can be problems with the US and/or the CS delivery, as well as a problem of the system with the detection of freezing behavior (see also above). The first checkpoint is assuring that US intensity (e.g., 0.45–1 mA) and number of US presentations are in the range capable of inducing a fear memory. Further, animals should show an obvious and immediate reaction (jumps, twitches, startle) to the US and the system measures should indicate higher freezing in the final stimulus-free phase of the conditioning session compared to the initial stimulus-free part before shock presentation. Similar to the US, also the CS has to be in a range detectable for the animal and of sufficient intensity. Frequencies between 2.5 and 10 kHz are commonly used. Since this range is also audible for most humans, a verification of CS delivery is simple. Accurate US and CS delivery can be tested using external devices (amperemeter; audiometer, luxmeter) during a fear conditioning session, in addition to an internal system test. To verify accurate detection of freezing by the program, we recommend correlating the system output with the manual scoring of an experienced experimenter (*see* Fig. 2).

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Chapter 9

Learned Helplessness in Rodents

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Abstract

Learned helplessness (LH) is a well-known phenomenon described in animals upon their exposure to uncontrollable and unpredictable aversive stimuli, usually inescapable shocks. It is often characterized by impairment in learning to escape or avoid controllable aversive stimuli in a subsequent behavioral test. In addition to that, previously stressed animals present a range of other behavioral and physiological changes, including cognitive deficits, decreased reward sensitivity (anhedonia), hypolocomotion, sleep and appetite changes, among others. Disturbances in different neurotransmitter systems in cortical and limbic brain regions as well as impaired neuroplasticity have been associated with the development of LH following exposure to an uncontrollable stressor. Since exposure to uncontrollable stress is a risk factor for developing psychiatric disorders and feelings of helplessness are part of the symptomatology in patients suffering from depression and posttraumatic stress disorder (PTSD), the LH paradigm has been used in animals to study the pathophysiology of such conditions and new treatment options. This chapter summarizes the most relevant discussions about the effects induced by inescapable shock that can be associated with LH development and depression neurobiology. Moreover, we present herein a protocol that can be used to test the effect of different pharmacological interventions and how this can be relevant to studying depression neurobiology.

Key words Learned helplessness, Uncontrollable stress, Depression, Rats, Animal model

1 Introduction

> We should never truly 'understand' this phenomenon, since the complete comprehension of life is beyond the limits of the human mind. But there are many degrees of 'elucidation'. (Hans Selye, 1950 [1])

Almost a century ago, Hans Selye first described that exposure to many different nocuous stimuli could trigger a set of physiological changes in the body, which he then called "general adaptation syndrome," and later stress [2]. Such changes were interpreted as an attempt of the organism to adapt to the new demands, which upon high intensity or prolonged periods could lead to exhaustion and eventually death [2]. Since then, a large number of studies have been developed with the aim to understand how the body and the

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brain react to stress and how it can be associated with disease development [3–5]. It is now a consensus that stress exposure, especially if uncontrollable and too intense or for prolonged periods, is a risk factor for the development of many diseases, ranging from cardiovascular problems to psychiatric disorders, such as depression, anxiety, and posttraumatic stress disorder (PTSD) [5, 6]. In this scenario, animal models have been important experimental tools to study how stress exposure can affect the brain and promote different physiological, behavioral, and emotional outcomes [7].

Animal models enable the investigation of brain-behavior relations, in a way that is not always possible in humans, with the aim to gain insight into the behavior and its underlying neurological, neurochemical, and molecular mechanisms [7]. The translational value of the findings from animal models to human conditions is, however, subject to important limitations, given the fact that they often provide a simplification of complex phenomena [8]. Upon evaluation of animal data, it is important, for example, to remember that many physiological, biochemical, and anatomical processes differ between species. Thus, the evaluation of animal data requires prior knowledge about the association between the pathogenesis in the animal model to that of the human disease and whether the behavioral measures are reliable enough to resemble those of the human situation [9]. This is further complicated by the lack of sufficient knowledge about the pathophysiology of many brain disorders. For example, it is difficult to ascertain if the behavior being modeled corresponds to a given symptom in a human condition. Moreover, some important emotional stressors in humans (e.g., shame, guilt, grief), which are core symptoms of some psychiatric disorders, cannot be assessed in animals.

In view of that, animal models to be considered as such must meet important validation criteria: face validity, based on the phenomenological similarity between the behavior in the model and the symptom in the human condition; etiology, when the causes of the human condition and the behavior in the animal are shared; construct, when physiopathological changes in the animal model reflect similar changes subsiding the symptomatology in the human condition; predictive (or pharmacological), when the behavior in the animal model is sensitive to drugs that are effective in treating the human condition and vice versa [10, 11]. These concepts have developed over the years to include other criteria [9, 12, 13], and it is currently argued that a model should be seen as a biological system reflecting an endophenotype/symptom-based model representing a distinct pathological process, related to a given symptom/set of symptoms, rather than a nosological entity [8, 12]. In general, animal models fulfil some, but not all, of the above-mentioned criteria and results obtained with them should always be interpreted in light of their limitations and strengths [9, 12, 13]. Herein, we

will consider the learned helplessness paradigm (LH) as an animal model to study the neurobiology of increased vulnerability to stress and depression, with perspectives of better understanding its physiopathological mechanisms and new options for interventions.

2 Learned Helplessness (LH)

2.1 Background and Historical Overview

While studying the effects of fear conditioning in later instrumental learning in dogs, Seligman and colleagues [14, 15] observed that dogs previously exposed to uncontrollable shocks in a shuttle box presented impaired ability to learn how to escape/avoid shocks in a subsequent behavioral test, when compared with non-stressed animals (not exposed to shocks in the pretest session). Following similar observations, Seligman and colleagues [14–16] assumed that during exposure to inescapable shocks, the animals would have learned that nothing they did would be efficient to interrupt or avoid the shock presentation, i.e., they learned to be "helpless." In other words, the animals learned that the outcomes were independent of their responses, thus resulting in an impaired escape or avoidance performance when exposed to escapable shocks afterwards. Such a revolutionary hypothesis was further investigated in a *triadic design*, which included three experimental groups: control (no shock, NS), escapable shocks (ES), and yoked inescapable shocks (IS, shock presentation is controlled by the behavior of the ES group). With this design, both the IS and the ES group received the same amount of shock, with controllability over shock presentation being the only difference between them. On the next day, all animals were tested in a different environment with escapable shocks. The results of such experiment demonstrated that only the IS group presented impaired escape performance in the test, while *ES and NS learned to escape equally well*, thus suggesting that the non-contingency between responding and shock presentation rather than the shock itself produced later passivity. In other words, it is the uncontrollability over the stressful situation rather than the stress of being shocked that triggered the behavioral deficits [14, 16, 17].

The observation that exposure to IS-, but not ES, induced behavioral deficits in animals boosted the development of several studies aimed at understanding how the different psychological variables (controllability, predictability, conflict) could affect stress responses, initially without any intention of associating such deficits with pathological human conditions [18].The cognitive theory about *learned helplessness* (LH), originally proposed by Seligman and Maier [16], faced important opposing interpretation from prominent behaviorists, who raised the alternative hypothesis that the failure to escape the shocks was rather a consequence of learned inactivity (i.e., the animals would have learned that the shock

termination would result from inactivity during the shock presentation) [18]. However, additional experiments developed by Maier [19] demonstrated this was likely not the case, since animals that could interrupt shock presentation by not moving during the pretest learned how to actively escape the shocks afterwards in the test session. Alternatively, Weiss and colleagues [20, 21] considered that a "motor activation deficit" would subside the escape failures as a result of depleted levels of monoamines in the brain of IS-exposed animals. Indeed, animals exposed to IS present reduced locomotor activity in a variety of tests, when compared to ES or NS [22]. However, since the initial experiments did not allow for the discrimination between reduced associability and decreased activity, the behavior of IS vs ES and NS animals was further investigated in different tests of associative learning, where the level of motor activity would not interfere with the behavioral outcome [22]. It was then shown that IS animals presented a slower learning rate and higher number of inaccurate responses in a Y-maze, where escape is accomplished by choosing the correct response from two available alternatives rather than by moving in a shuttle box [22, 23]. Similar deficits were observed in IS animals upon the acquisition of appetitive operant behavior and other associative and discrimination learning tasks, thus supporting the idea that escape performance impairments in IS animals would result from deficits in associative learning manifested during the test session [24–26]. Altogether, these studies pointed to a deficiency in attentional processes in IS animals, leading to delayed learning of escape/avoidance tasks during test [26]. Indeed, animals exposed to IS present a deficient learning curve during the test, thus resulting in increased latency and number of escape failures.

Another hypothesis proposed to explain the LH phenomenon was that it would result from the intense fear generated during IS presentation, which would promote decreased activity, impaired associative learning and delayed response to the escapable shocks, often resulting in more escape failures during the test. In this scenario, the possibility to control the shock during the pretest session (as in the ES group) would make so that the active avoidance would become a conditioned inhibitor of fear by signaling a shock-free interval of time ("safety signal"), thus decreasing fear during the test [18, 27]. Corroborating that view, Maier and colleagues demonstrated that animals experiencing control during a training session are immune to the IS-induced effects in subsequent shuttle box performance [28]. According to the authors, when the presence of control is identified by the medial prefrontal cortex, the overactivation of circuits associated with fear and exaggerated response to stress would be inhibited, thus decreasing the impact of the stressors. In this context, when control is not present, the disinhibition of circuits involved with the processing of fear and anxiety during the training would result in exaggerated responses to subsequent aversive situations [29].

Finally, reduced sensitivity to painful stimuli (hypoalgesia) dependent on the controllability of the aversive stimulus has also been suggested as an alternative explanation to the manifestation of behavioral deficits that characterize the LH [30, 31]. This hypothesis, however, has not been supported by other studies, which failed to identify differences in the pain threshold between IS-, ES-, and NS-exposed animals [32, 33]. Moreover, drugs that decrease pain sensitivity, such as analgesic opioids decrease instead of increasing LH development [34, 35].

A wide range of other behavioral changes were identified in IS animals, which made the discussion about the basis of LH even more complex. To briefly summarize, IS animals present hypolocomotion, reduced food and water intake, impaired performance in appetitive-motivated tasks, reduced preference for sweet tastes, reduced social interaction, and aggression, potentiated fear conditioning and impaired fear extinction, increased levels of stress hormones and anxious behavior, among others [11, 18, 36, 37]. Importantly, none of these behavioral changes occurred if the shocks were escapable, and they could last for weeks or months after the initial aversive situation, especially when the animal was reexposed to the aversive environment [38, 39]. Despite other important behavioral changes, the hallmark of the LH paradigm is the greater latency to escape or avoid the shocks and an increased number of failures during the escapable test session, in comparison to the non-stressed animal or to the animal that received escapable shocks in the training session [40].

Since the behavioral deficits observed in animals exposed to IS resemble some of the symptoms often described by depressed patients, the LH was soon associated with the feeling of helplessness that is observed in humans with depressive symptoms [14, 16, 41]. In line with that, a number of studies reported that the sense of lack of personal control over significant events in life was associated with the motivational and behavioral deficits that characterizes clinical depression [42, 43]. Disrupted emotional–motivational–cognitive states, of which the LH is a fundamental aspect, are actually considered the core/typical symptoms of depression [37]. Thus, the LH has been proposed as a major etiological process underlying vulnerability to and onset of major depression [37]. In this context, the study of the neurobiological basis of the LH contributes to the understanding of the mechanisms by which exposure to uncontrollable life events can increase vulnerability to the development of emotional and cognitive consequences associated with depression. Moreover, the manipulation of LH provides opportunities for identifying possible treatment options.

The phenomenon of learned helplessness also occurs in many species, such as dogs, rats, and mice [15, 44, 45], and has been translated even to non-mammals, such as zebra fish and flies

[46, 47]. Most of the work has been developed in rats, although more recently mice have been widely used as well, with the advantage of testing transgenic animals [37].

2.2 Neurobiological Mechanisms

The neurobiological mechanisms underlying the development of LH have been the subject of investigation for many decades and extensively reviewed elsewhere [18]. Maier's group has demonstrated in a series of works that IS exposure promotes sensitization of the serotonin neurons in the dorsal raphe nucleus (DRN), which is then overactived during the test, 24 h later. The DRN has projections to different brain regions involved in the modulation of fear and anxiety, such as the periaqueductal gray and the amygdala [48, 49]. The increased excitability of the DRN results in increased serotonin release in the projection areas during the test session, which causes inhibition of escape responses (in the periaqueductal gray) and increased anxiety (in the amygdala) [49, 50]. The animals would present increased escape failures in the test (LH behavior) due to the inhibition of fear/escape responses and increased anxiety (enhanced freezing). Such deficits would be more pronounced in IS-exposed animals, because exposure to ES would be identified by the medial prefrontal cortex (mPFC), which sends projections to GABAergic interneurons in the DRN, promoting its inhibition and protection against the stress effects during the pretest session [51].

The mPFC is also a target for the stress effects and shows reduced synaptogenesis and connectivity in IS-exposed animals [52, 53]. Similarly, the hippocampus, another brain region important to stress adaptation and memory, also shows loss of functional synapses [54] and impaired long-term potentiation (LTP) [55]. Altogether, a deficient mPFC-hippocampal connectivity might contribute to the impaired associative learning observed in IS-exposed animals [22, 53, 56]. Moreover, both the mPFC and the hippocampus are central brain regions in the negative feedback regulation of the HPA-axis activation during the stress [57]. Therefore, the impaired functionality of such brain regions can also contribute to an exacerbated endocrine response during subsequent stress exposure, which also plays a role in regulating behavioral adaptation to stress [58].

The molecular mechanisms subsiding the mPFC and hippocampus impaired functionality in the LH are not completely understood. The involvement of brain-derived neurotrophic factor (BDNF) and other neurotrophins, which are central to synaptic homeostasis and stress adaptation, has resulted in contradictory findings [59–62]. More recent evidence indicates that increased neuroinflammation can result from IS exposure and contribute to LH development [63, 64]. This is in line with the current hypothesis of impaired adaptation to stress and increased vulnerability to psychiatric disorders [65].

3 Validation

To be considered for translational use, a valid animal model has to fulfil specific validation criteria. The first attempt to propose criteria for external validation of animal models of affective disorders was published by McKinney and Bunney in 1963 [66]. Later on, Paul Willner (1984) [67] defined the three validation criteria, which an animal model of depression should fulfil to be considered as such: face, construct, and predictive validity. Most of the researchers working in the field of animal models of depression rely on this classification, although other authors have proposed changing some criteria in that list or including hierarchy between them [13]. Herein, we will consider Willner's classification to discuss the validation of LH as an animal model of depression.

3.1 Construct Validity

As much is still unknown about the neurobiology of depression, the construct validity for learned helplessness, as well as for other animal models of depression, becomes quite questionable. According to Willner [11], the construct validity for the learned helplessness model could be evaluated in three parameters: (1) animals subjected to uncontrollable aversive events become helpless; (2) similar states can be observed in humans exposed to uncontrollable events; and (3) helplessness would be the central symptom underlying depression in humans. These assumptions have been the subject of much controversy, but some studies have shown that animals subjected to different stressors developed the deficits that characterize helplessness [68] and that humans subjected to inescapable/uncontrollable stress (loud noise, unsolvable anagrams, frustration) presented performance deficits, which were more pronounced in depressed patients than in healthy controls [69]. It is also observed that high levels of helpless scores are good predictors of depression and suicide risk [70, 71]. Finally, there is strong evidence that exposure to uncontrollable aversive life events, early in life and/or in adulthood, is one of the major risk factors for depression, and it can trigger its development [72, 73].

It is currently accepted that major depression is a condition resulting from chronically altered emotional–motivational–cognitive processing of aversive and/or rewarding environmental events, in which genetic and environmental factors play a major etiological role. Even though helplessness is not considered a symptom, based on the current nosological classification, it is an emotional–cognitive state considered central in depression etiology [37]. Together, these data can provide some theoretical basis for the construct validity of the LH paradigm as a model of depression.

3.2 Face Validity

Face validity corresponds to the extent of similarity between the model and the disorder being modeled, including both pharmacological similarity and phenomenological identity [67]. Although many of the central symptoms of depression, such as depressed mood, guilt, or suicidal thoughts, cannot be modeled in animals, others can be translated from species to species and studied within specific settings. Animals exposed to IS present a series of behavioral changes that resemble important aspects of depression, such as the following: decreased sensitivity/responsivity to reward as an indicative of anhedonia [74, 75], a core symptom of depression [76]; impaired voluntary response initiation, as analogue to psychomotor retardation [77]; and changes in appetite and sleep patterns [78]. Neuroendocrine and immunological changes described in depressed patients have also been found in animals exposed to IS, e.g., altered HPA-axis responsivity and increased circulating levels of inflammatory cytokines [79–81]. No experimental paradigm is able to model depression in all its aspects, but rather specific aspects of this disorder. LH paradigm, in particular, seems to model aspects of depression related to increased vulnerability to uncontrollable aversive life situations and impaired stress coping [37].

3.3 Predictive Validity

Due to the limited knowledge of the etiology of depression and the difficulty in translating depression symptoms to animal behavior, predictive validity assumes an important value when choosing an animal model of depression. Predictive validity relies on the proper identification of effective interventions, without making errors of omission or commission and with corresponding sensitivity [13, 67]. A wide variety of antidepressants, including tricyclic antidepressants, selective serotonin-reuptake inhibitors, and MAO-inhibitors, attenuate the development of LH after chronic but not after acute administration, similar to what is observed in clinical settings [82]. Moreover, the fast-acting antidepressant drug ketamine is able to attenuate LH after a single intervention, as observed in humans [52, 83]. Non-pharmacological treatments with proven antidepressant effects in humans are also positively identified by the LH paradigm, such as electroconvulsive therapy and deep-brain stimulation [84, 85].

Finally, drugs that lack antidepressant activity, such as antipsychotics and benzodiazepines, are not effective in the LH, unless they interfere with the training session [58, 86, 87]. Altogether, this indicates that the profile of antidepressant effects in the LH reproduces many of the aspects associated with the antidepressant intervention in the clinical practice, thus indicating that the model has good predictability, and it is useful to detect new treatment targets, including fast-acting drugs and non-pharmacological approaches.

4 LH in Rats

The LH can be studied using a triadic design, in which animals exposed to non-shock (NS) or escapable shock (ES) are used as control groups for the yoked group (IS). This design is optimal when the aim is to discriminate the effects resulting from uncontrollability over an aversive situation from general stress effects [36]. However, individual differences in stress reactivity in the ES group during the pretest (PT) (good vs poor learners) can increase response variability during the test in both the ES and the yoked group, thus often requiring the exclusion of animals presenting extreme performances during the PT.

When the aim is to use the LH as an animal model of depression, the triadic design is not a requirement, since the goal is often to identify mechanisms associated with stress vulnerability and response to interventions. In this case, NS serves as control and differences between IS-exposed animals (helpless vs non-helpless) can be used to investigate stress vulnerability [45]. This poses an important difficulty, which is to differentiate helpless vs non-helpless animals (see below in the result section).

In our lab, we used the triadic design to validate the protocol and assess if it was sensitive enough to identify differences between NS, IS, and ES (*see* Figs. 1 and 2) [88]. To investigate depression neurobiology and treatment, we have used protocols including the NS and IS group, with different intervals between the PT and test (T) (*see* Figs. 3 and 4): 24 h or 6 days. When the interval is 24 h, the intervention (if chronic) has to be performed before the PT, which can potentially bias the results by interfering with the acquisition of LH. To avoid that problem, we established a protocol that allows us to perform interventions between the PT and T, with higher translational value as a model of depression. The protocol described herein allows for the evaluation of behavioral, physiological, neurochemical, and molecular changes associated with LH as an animal model of depression, and it provides opportunities for testing interventions (pharmacological and non-pharmacological).

4.1 Methods

4.1.1 Subjects

In our studies, we have used male Wistar rats with 7 and 8 weeks of age (250–300 g), at the start of the experiment. As the animals are bred in our university, we order them at least 1 week before the start of the experiment and allow them to acclimatize in the animal room next to our lab. The animals are housed in groups of four in propylene cages (41 × 33 × 16.6 cm) with wood bedding (sawdust) and no environmental enrichment and ad libitum access to tap water and food (commercial rodent cow, Nuvilab–Quimtia–Paraná, Brazil). The rats are kept in a controlled temperature room (24 ± 1 °C), under a 12 h light/dark cycle (lights on at 6 a.m.) and constant air renovation. The cages are cleaned 3 times/

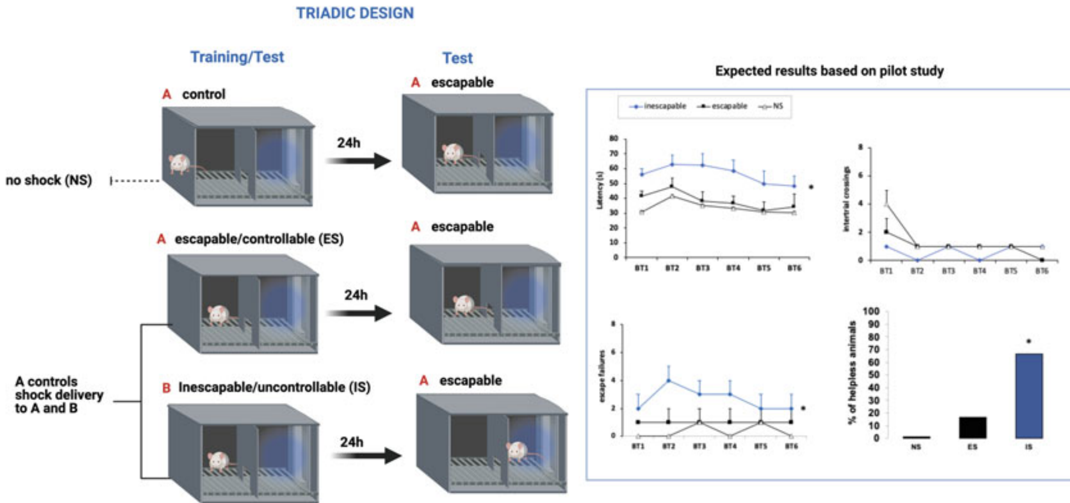


Fig. 1 Triadic design of the LH. Left panel: the protocol consists of a pretest (PT) and a test (T) session, with a 24-h interval. In the PT animals are exposed to inescapable footshocks (IS: 40 trials of 10s, 1.0 mA, scrambled interval of 30–90s), escapable footshocks (ES, 40 trials of 10 s, 0.8 mA, preceded by a 5 s tone), or non-shocks (NS, 30-min habituation in the shuttle box). In the test session, all animals receive 30 trials of escapable footshocks (30 trials of 10 s, 1.0–0.8 mA, preceded by a 5 s tone). Right panel: expected results from the protocol, based on a small sample (6–8 animals/group) in a pilot experiment using independent IS and ES groups. Results represent the mean ± SEM of blocks of five trials (BT) or percentage of helplessness. *indicates significant difference from NS group (ANOVA: time and condition as main factors). These results are published as part of a PhD theses (Joca, S. 2004. University of Sao Paulo-USP)

week. After the pretest section, they are kept isolated in propylene boxes (30 × 20 × 13 cm), and cages are cleaned just once a week. We observed that isolation improves the response to stress and the development of helplessness, making it easier to see the differences between the experimental groups. Many strains are used in LH testing, including outbred Wistar, Sprague-Dawley (SD), and Wistar Kyoto rats [89]. In our experiments, we used outbred Wistar rats which have been bred in our campus for several generations. More recently, we tested outbred Wistar Hannover rats, and they present a LH rate similar to Wistar rats, although their response to intervention is impaired (not published). The protocols described below have been approved by our local Animal Ethical Committee in different studies of our group, and it complies with the international laws and policies for the ethical use of animals under experimentation.

4.1.2 Procedure

Equipment

We use an automated apparatus connected to a shuttle box (or equivalent) and a computer. In our lab, the apparatus used is an EP 111 model from Insight Scientific Equipments (Brazil) connected to two shuttle boxes ("A" and "B"), which are connected to a computer and controlled by Esquiva software (Insight Scientific Equipments, Brazil).

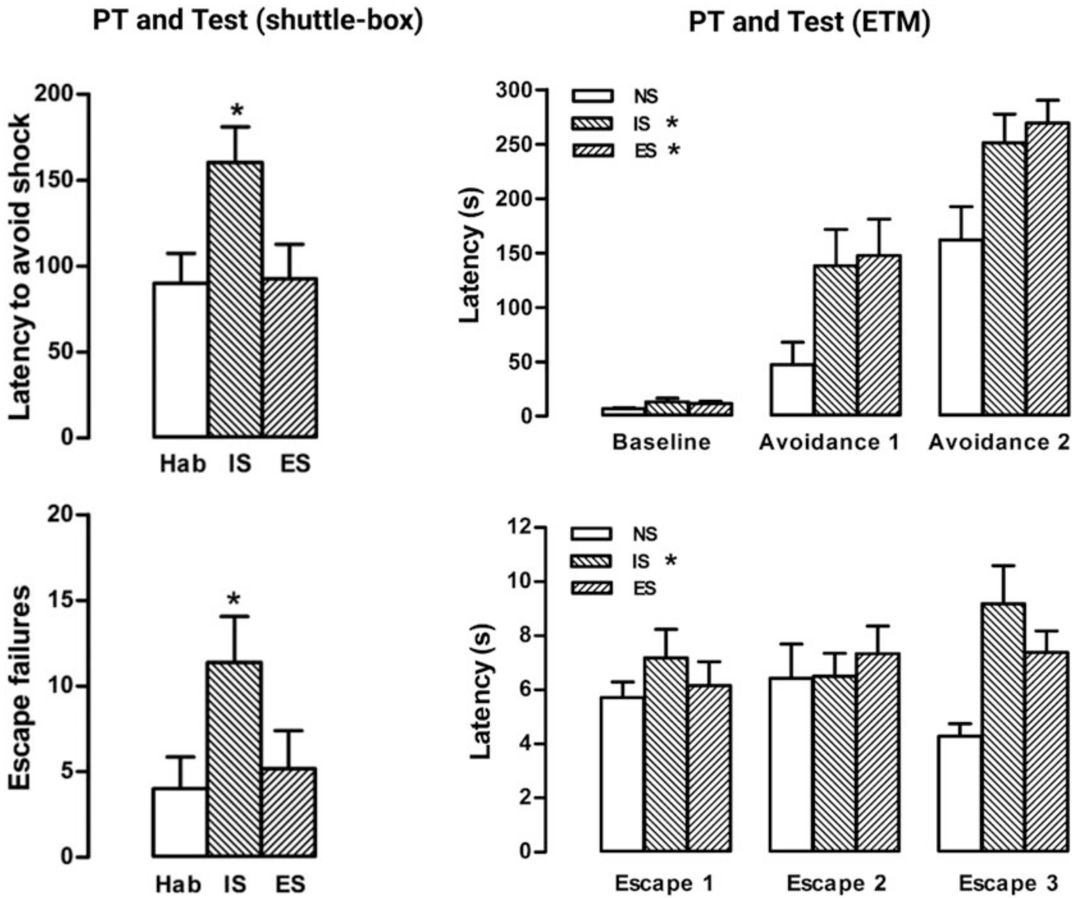


Fig. 2 Triadic design of LH. Animals were exposed to the pretest session (PT), as described in Fig. 1. Twenty-four hours later, their behavior was analyzed in the shuttle box (left panel), followed by the elevated T-maze (ETM, right panel). Animals exposed to IS present increased latency to escape/avoid the shocks and increased number of escape failures in the LH, as well as impaired inhibitory avoidance from the enclosed arms and facilitated escape from the open arms in the ETM, thus indicating increased anxiety and impaired fear responses, respectively [88]. Data represents mean ± SEM (n = 14–18/group). *indicates $p < 0.05$ vs NS group (habituate, hab group). Reprinted from Brain Research, 225(2): 590–595; De Paula Soares et al., Distinct behavioral consequences of stress models of depression in the elevated T-maze (2011) [88], with permission from Elsevier (License number: 5211590674199)

The shuttle boxes measure 30.7 cm × 33.3 cm × 56.2 cm and have two compartments of equal size separated by a wall with a central open door. The walls are made of transparent acrylic (5–10 mm), allowing visualization of the animal during the experiment, and the floor consists of stainless steel grids (4 mm in diameter and 13 mm of the distance between the grids), through which the footshocks are delivered. Moreover, the shuttle box "A" contains infrared sensors, which detect the rat's movement. In this setup, both shuttle boxes can deliver footshocks; however, only in cage "A" the rat could stop the footshocks by crossing to the

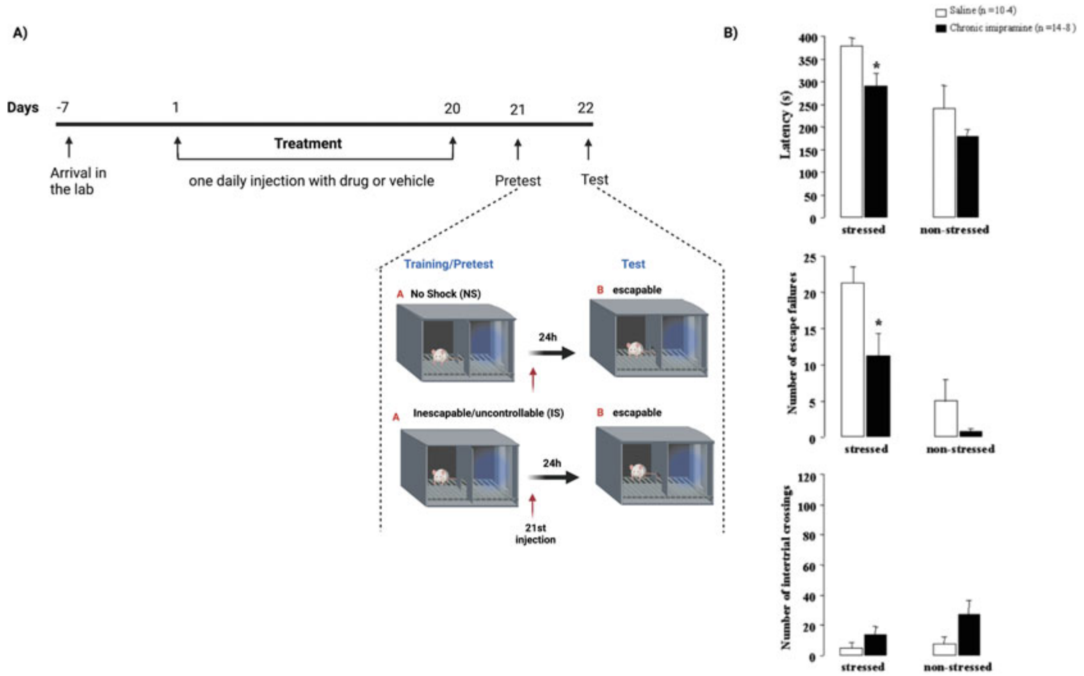


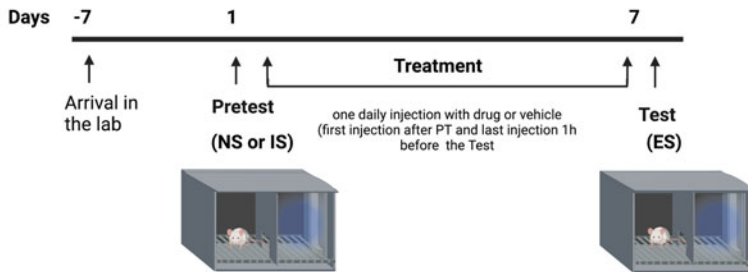
Fig. 3 Effect of chronic antidepressant treatment before the pretest (PT) in the LH model of depression. Animals were treated daily during 20 days with imipramine (i.p., 15 mg/kg, 1 mL/kg) or vehicle (1 mL/kg) exposed to the PT 24 h after the last injection. In the PT, animals received no shock (non-stressed, NS, 30 min of habituation in the shuttle box) or 40 inescapable footshocks (stressed, IS, 10s, 1.0 mA, random interval 30–90s). Immediately after the PT, all animals received a single injection of imipramine (i.p., 15 mg/kg, 1 mL/kg) and were tested 24 h later with escapable footshocks (ES, 30 shocks, 10s, sound-signaled by 5 s tone). Animals exposed to IS presented increased latency to escape/avoid the shocks and increased number of escape failures, which were attenuated by chronic antidepressant treatment. No significant effects of the drug were observed in the NS group. Data represents mean $\pm$ SEM ($n = 10$–14, IS; $n = 4$–8, NS). *indicates $p < 0.05$ vs NS group (2-way ANOVA, factors: condition and treatment). Reprinted from Brain Research, 978(1–2), Joca et al., Activation of postsynaptic 5-HT1A receptors in the dorsal hippocampus prevents learned helplessness development, PAGES 177–184(91), Copyright (2003), with permission from Elsevier (License number: 5212530070024)

opposite side of the chamber. Therefore, both cages are used for uncontrollable shocks, but only cage “A” is used for controllable footshocks.

Experimental Design

To ensure that the uncontrollable stress exposure is effective in inducing behavioral and molecular changes, we always include animals of both experimental groups, the stressed and non-stressed, on the same experimental day, and repeat the experiment to complete the required sample size in each group. We perform the pretest and test section from 8 a.m. to 5 p.m., but we carefully randomize the experimental groups throughout the day to minimize the effects of hormonal and neurochemical circadian changes.

A)



B)

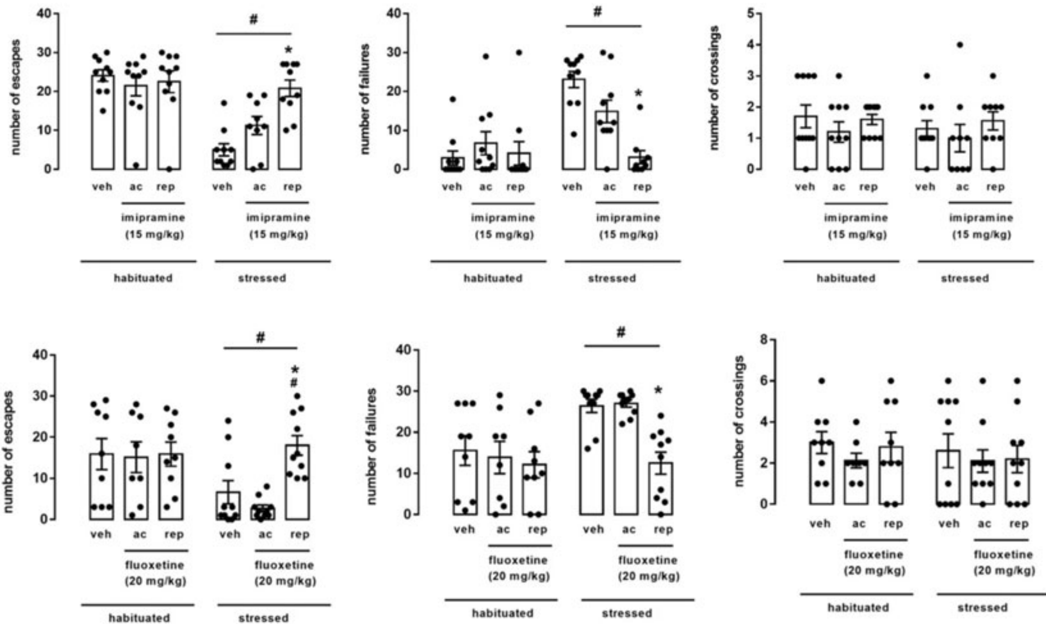


Fig. 4 Effect of chronic antidepressant treatment between the pretest (PT) and test (T) in the LH model of depression. (**a**) After 1 week of habituation, the animals were submitted to the PT, where they received no shock (NS, 30 min of habituation in the shuttle box) or 40 inescapable shocks (IS, 10s, 0.4 mA, random interval 30–90s). Immediately after the PT, all animals received a single injection (ac, acute treatment) of imipramine (i.p., 15 mg/kg, 1 mL/kg) or fluoxetine (i.p., 20 mg/kg, 1 mL/kg) or vehicle. The same treatment was repeated (rep, repeated treatment) for the next 6 days, with the last injection 1 h before the test, in which all animals received 30 sound-signaled (5 s) escapable footshocks (ES, 10s, 0.4.mA). (**b**) Animals exposed to IS presented increased latency to escape/avoid the shocks and increased number of escape failures, which were attenuated by chronic, but not acute, antidepressant treatment. No significant effects of the drug were observed in the NS group (habituate group). Data represents mean ± SEM. *indicates $p < 0.05$ vs NS group (2-way ANOVA, factors: condition and treatment). Reprinted by permission from Springer Nature: Springer, Molecular Neurobiology 58, 777–794 (Modulation of DNA Methylation and Gene Expression in Rodent Cortical Neuroplasticity Pathways Exerts Rapid Antidepressant-Like Effects, Salet et al) (94), COPYRIGHT (2020), License number: 5212600549186

On an experimental day (PT or test), the animals are brought to the laboratory and allowed to acclimatize in a waiting room, which is an annex to the experimental room, for at least 1 hr. before the start of the experiments (food and water are allowed during this time, but not during the experiment). Both the waiting room and the experimental rooms are dimly lit, with controlled temperature (24 ± 2) and constant air renovation. Background noise is provided by a small hood installed in the rooms to buffer possible incoming and outgoing sounds.

The animals are randomly assigned to the different conditions and individually brought to the experimental room, where the equipment is placed. After being placed in the shuttle box, a 2-min habituation period is allowed, so the animals can explore the environment. After that, the session is started and controlled by the computer. At the end of the training/pretest session, the animals are placed individually in their home cages and are kept in our local animal room (next to our lab) until the test. They are left undisturbed, except for cleaning, or receiving daily drug injections.

In the case of drug administration, the animals are weighted every day to provide accurate drug exposure. Treatments are randomly assigned to the different experimental conditions. On the test day, the animals are brought again to our lab and allowed to acclimatize in the waiting room for 1 h. After that period, each animal is brought individually to the experimental room to be tested in the shuttle box.

Pretest Section (PT)

The pretest consists of a 35–40-min session, where animals are exposed to either IS, ES, or NS (*triadic design*) or only NS or IS (*depression model*). We chose to use footshocks to induce LH instead of tail shocks, since the number of footshocks required is usually smaller [90]. Although we have used the triadic design, we currently use the dyadic design as a model of depression. Below we described the two protocols used by our group.

Triadic design (*see* Figs. 1 and 2): animals are placed individually in cage "A" and cage "B", and 40 footshocks, each one consisting of 10 s, 1.0 mA, given according to a variable time schedule with a mean interval of 60 s (range from 30 to 90 s) are delivered. The shocks are preceded by a 5 s tone (60 dB, 670 Hz) that initiates 5 s before shock delivery and lasts until its end. The animal placed in cage "A" can control shock delivery in cage A (ES group) and cage B (IS or yoked group) by crossing to the opposite side of the chamber. As a control group (non-stressed, NS), a pair of animals is individually placed in cage "A" and cage "B," but no shocks are delivered, and allowed to habituate for 30 min.

Depression model (*s*ee Figs. 3 and 4): a pair of animals are individually placed in cage "A" and cage "B". Since cage "B" reproduces the protocol applied in "A," the pair of animals belong to the same experimental group, which can be IS or NS. The IS

group is submitted to 40 inescapable footshocks, each one consisting of 10 s, given according to a variable time schedule with a mean interval of 60 s (range from 30 to 90 s). The intensity of the shock is usually 0.8 mA in the pretest for outbred Wistar rats [91–93], but more recently, we have had good results with lower intensities (0.4 mA in both PT and T) [94, 95], which have been used to reduce animal suffering. For the NS group (control), the animals are placed in the shuttle boxes for 30 min, but no shock is delivered. The session is carefully monitored by the experimenter to ascertain that the animals are receiving the shocks properly (see troubleshooting).

When PT is finished, the animals are gently removed, placed individually into an acrylic box (20 × 30 × 13 cm), and returned to their home cage in the animal room. The stainless steel grids are cleaned with a damp sponge, disinfectant, soap, or water and the walls with 70% alcohol, after each animal testing.

Test Section (T)

Twenty-four hours or 6 days later (depending on the duration of the intervention, see Figs. 3 and 4), the animals are submitted to the test section, which consists of exposure to escapable shocks. After the acclimation period in the waiting room, each animal is individually brought to the experimental room, which is dimly lit, temperature-controlled (24 ± 2), and equipped with a hood that produces background noise and guarantees constant renewal of the air. The rat is placed individually in the shuttle box "A" and allowed to habituate for 2 min. All animals are submitted to the same protocol, which consists of 30 escapable footshocks (0.4 mA), 10 s of duration each, given according to a random variable time schedule with a mean interval of 60 s (range from 30 to 90 s). Each footshock is preceded by a tone (60 dB, 670 Hz) of 5 s of duration.

The rats can avoid or interrupt the shock by crossing the door to the opposite side of the chamber during the tone or the shock presentation, respectively. If the animal crosses during the tone presentation, the program register the behavior as it as "avoidance," and if it crosses during the footshock presentation, it is registered as "escape". The absence of any of these responses is considered an "escape failure". The program also registers the "number of intertrial crossings" that can be used as a locomotor activity index [96]. All these behaviors are automatically registered by the equipment in connection with the interruption of the photobeans displaced in each side of the chamber. These variables are registered for each of the 30 trials, and a full report is provided for each animal at the end of the session.

At the end of each session (average duration of 30 min), the animals are carefully removed from the chamber and returned to their home cages or killed to have their brains removed for further molecular analysis. The cages are carefully cleaned before the start

of the new session: stainless steel grids are cleaned with a damp sponge, disinfectant, soap, or water and the walls with 70% alcohol.

It is important to highlight that the trans-situationality of LH is compromised in our protocol [50], since the animals are exposed to the same training cages (A and B are identical cages). Furthermore, exacerbation of contextual fear conditioning can play a role in the observed results. Nevertheless, our protocol has been effective in distinguishing between IS, ES and NS in other animal models [88], as well as positively identifying the effects of conventional and new antidepressant drugs [93, 94, 97, 98] (see below).

4.1.3 Results and Analysis

The behavioral parameters that best characterize learned helplessness are the increased latency to escape/avoid shocks and/or increased number of escape failures, often referred to as deficit pattern (DP) or failure pattern (FP), respectively [40, 45]. Since the FP is more specific in identifying LH when compared to DP, the number of escape failures is more often used to discriminate between helpless and non-helpless animals [40, 45].

To differentiate between "helpless" and "non-helpless" in our experiments, we use the FP with a cutoff of 10 escape failures out of 30 trials (animals with more than 10 escape failures are considered helpless) [91, 92]. With this approach, we observe LH in 50–60% of animals in the IS group and 10–30% in ES and NS animals (spontaneous helplessness) [91, 92]. The difference in the frequency of helpless animals between groups is analyzed with the Chi-square test. To avoid false-negative results of stress or intervention, we have also computed the latency to escape/avoid the shocks and the number of escape failures, either in blocks of five trials or in total. We perform parametric analysis of variance (two-way ANOVA or repeated measures) to compare differences in the latency; and nonparametric ANOVA (Kruskal-Wallis) to compare the number of escape failures and intertrial crossings, since these are noncontinuous variables. Due to the high variability in the IS group and small effect size, a sample size >12/group is often required to detect statistically significant differences between the groups.

4.1.4 Experimental Variables

The experimental variables that can interfere with the development of LH are very broad, including antidepressant treatment (monoaminergic, non-monoaminergic, and fast-acting), exposure to other stressors than the IS, as well as pharmacological and genetic manipulation of specific brain circuits.

The effects of antidepressants are observed after repeated antidepressant treatment (usually 7 days or more), unless the drug is a fast-acting antidepressant [82, 93, 98–101]. Prolonged treatment with antidepressants (>7 days) usually requires administration before PT, since the effects of IS tend to dissipate after 1 week [38, 96]. In our protocol, one daily injection with imipramine,

during 21 days, with the last injection immediately after the PT, was effective in attenuating the development of LH [91]. All animals were then tested 24 h after the PT (*see* Fig. 3). However, this protocol has the disadvantage of training the animals under the influence of antidepressant treatment. This can potentially skew the results by attenuating the impact of the emotional stress during the training rather than attenuating the emotional consequences of the training that can be observed later in the test.

To avoid problems with the interference of the treatment during the training, we have used an alternative protocol with a longer interval between PT and T (7 days), and the antidepressant is administered daily during this interval: first injection immediately after PT and last injection 1 h before the test [93, 97, 98]. The investigation of potential new fast-acting antidepressants is then performed by administering the drugs acutely, immediately after the PT, or 1 h before the test, using the same protocol [94, 97, 102, 103]. Non-pharmacological tools, such as deep brain stimulation, have also been described as effective antidepressant interventions in the LH [104, 105].

In the context of studying depression neurobiology, exposure to other stressors or circuit manipulations have also been used [51, 68, 91, 92, 106]. The effects induced by manipulation of specific brain regions and circuits can involve pharmacological and genetic tools, with the latter being more common in mice due to the availability of transgenic animals. Such studies have brought important understanding about the influence of stress controllability in determining vulnerability to its emotional consequences and depression-like behavior.

4.1.5 Troubleshooting and Limitations

Many technical considerations should be considered to provide good reliability when establishing the LH protocol, with good discrimination between IS and NS groups. One main problem is the high variability in the IS group in protocols using low-intensity shock and reduced number of trials [36, 45]. Although older studies have used high-intensity shocks (>1.0 mA), most studies have used the intensity ranging from 0.8 mA to 1 mA in the pretest [45, 62, 93, 107–109]. In our protocol, we were able to show that shocks of 0.4 mA effectively induced helplessness in Wistar rats [94, 98, 110]. In the test, the shock intensity is usually the same as in the PT or lower [92, 93, 107].

Besides the footshock intensity, it is important to be sure that the shocks are being appropriately perceived by the animals. The shock is only delivered if the animal touches at least two grids; if the rat is able to equilibrate in only one grid, it will not receive the footshocks, thus compromising the results in the test. Occasionally, the animal can hold in the door or in the wall, keeping itself footing in one grade during the experiment. The presence of fecal bolus between grids can also impair the current received by the animal.

Therefore, it is important to observe the rats during the tests, to make sure it presents behavioral responses compatible with a stressful situation.

The difficulty of the task that leads to interruption/avoidance of the shocks during the test can also be a problem for achieving good reliability and reproducibility of results. The tasks have to be easy enough to be learned by the NS group, but not too easy, so the learning impairments in the IS cannot be properly detected. When using rats, lever-press or wheel-turning paradigms with varying levels of difficulties are often used [45, 111]. Although shuttle boxes give better results for mice [44], we have successfully established our protocol using shuttle boxes with a fairly simple task (cross the door to the other side of the chamber). It should be noted, however, that our protocol faces a limitation related to training and testing in the same environment, which could skew the results by exacerbating aspects of contextual fear conditioning [112–114].

Differently from mice, which require a higher number of trials in the PT and T (>100) [44, 115, 116], in rats the number of trials is usually below 80. A new analysis of LH tests of both wild-type Sprague-Dawley and selectively bred animals (congenitally LH and non-LH rats) revealed, however, higher statistical power for detecting differences between helpless and non-helpless animals in trials 3 to 10 [117], thus suggesting that the number of trials in the test could be reduced to decrease animal suffering without compromising the detection of LH.

The rat strain used in the LH paradigm can also interfere with the vulnerability to the IS. Wieland et al. [89] demonstrated that Wistar Kyoto and SD rats from Charles River were more susceptible to LH training when compared to Brown Norway, Buffalo, Fischer 344, and Lewis rats. Although the low susceptibility can interfere in LH training, this is not a limitation, since by using an appropriate cutoff, it is possible to separate resilient and susceptible animals, allowing the study of neurobiological factors involved in resilience and susceptibility to stress [118–120].

A common criticism about LH is the lack of reproducibility [40, 121]. The lack of reproducibility intraindividually can occur when there are multiple tests, in which the animal improves or decreases its performance during the repeated testing [45]. Reproducibility can also be affected by data analysis with calculation of escape or avoidance failures instead of delayed responses [40, 45]. The results of the LH should, therefore, be discussed in light of the differences in the protocols used, the rat strain and the data analysis performed.

5 LH in Rats vs. LH in Mice

Although the LH paradigm is widely used in rodents, it is important to highlight that the protocol for rats and mice have considerable differences. The "helpless frequency," for example, is about 50% in rats and 70% in mice, when submitted to the LH paradigm, indicating that the shuttle boxes are more efficient in mice [44, 89, 122]. Moreover, the number and intensity of shock trials are also different. Rats are usually submitted to a protocol with two sections, pretest and test, characterized by uncontrollable and controllable shocks, respectively [45, 62, 107]; whereas mice are usually reexposed to shuttle box three or four times [44, 115]. The number and intensity of the shock can also vary between rats and mice, with the latter normally exposed to more than 100 trials [44, 115, 116], while rats are submitted to fewer trials of 0.8–1 mA [45, 62, 93, 107–109]. Previously published protocols for LH in mice have been published and provide detailed information of the experimental conditions and troubleshooting [123].

6 Conclusion

The LH is a depression model with high construct, face, and predictive validity in both rats and mice [11, 45], thus being an important experimental tool for the study of depression neurobiology and treatment. On the other hand, to establish a reliable protocol to induce LH, many aspects should be taken into account, such as the species (rat or mice) and the strain used, the number of shocks, and the difficulty of the task during the test. Nevertheless, regardless of such differences, the LH has been consistently demonstrated in a number of different paradigms, and it can be considered of high value to future translational research aimed at increasing the understanding of depression and its treatment [37].

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Chapter 10

Chronobiology of Mood States: Introducing Circadian Animal Models

Jorge Mendoza, Noëmi Billon, Guillaume Vanotti, and Viviane Pallage

Abstract

Mood disorders, such as depression and anxiety, are characterized by behavioral, physiological, and psychological alterations caused by a combination of genetic predispositions and environmental factors. Among the behavioral and physiological disturbances, changes in daily biological processes such as the sleep-wake cycle or hormonal rhythmic release are the most affected. People suffering of mood disorders (e.g., depression) show delays in the onset of sleep, awakenings during sleep, or sleepiness at daytime. Inversely, people experiencing circadian misalignment (e.g., jet lag, shift work) exhibit mood-related symptoms, emphasizing the reciprocal connection between affective and circadian disorders. Moreover, pharmacological and non-pharmacological treatments that improve daily rhythms have important benefit effects in emotional states, indicating that the circadian system is a good entrance for the successful treatment of mood disorders. Thereby, to understand the physiological mechanisms underlying mood alterations induced by disruptions of daily physiology, it is important to develop and use valid animal models that simulate human circadian activity.

Key words Circadian, Jet lag, Mood, Anxiety, Depression, Animal model

1 Introduction

1.1 The Circadian System

The circadian (circa = close to; *diem* = day) system controls the 24 h timing of behavior and physiology. The sleep-wake cycle, daily rhythms of temperature, and hormonal release are built by the circadian clock and genes underlying the clockwork of every cell in the body [1].

In mammals, the principal circadian clock is located in the brain. The anterior ventral hypothalamus harbors the suprachiasmatic nucleus (SCN), the timekeeper that orchestrates and synchronizes other peripheral clocks for the control of all body's circadian rhythms [2].

At the molecular level, each SCN cell contains a circadian machinery composed of several (clock) genes working into positive and negative feedback loops in a rhythmic manner [3]. At the core

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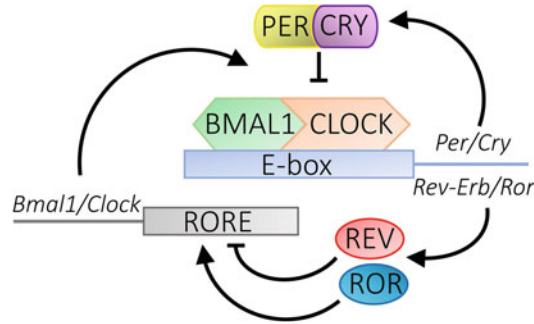

Fig. 1 Molecular mechanism of circadian clocks. The circadian activity of the SCN and other peripheral clocks depends on positive and negative feedback loops on the expression of genes and clock proteins. In a positive loop, the transcription factors BMAL1 and CLOCK form a dimer able to transcript the expression of the genes from the negative loop *Per* and *Cry* via the E-boxes in their promoters. PER and CRY protein products, similar to BMAL1 and CLOCK, form a dimer that enter the nucleus repressing their own transcription by an inhibitory action on BMAL1 and CLOCK. An additional loop is formed by the nuclear receptors *Rev-Erb* and *Ror*. These have principal and inhibitory and stimulatory rhythmic transcription activity on *Bmal1* gene expression, respectively, via the RORE-binding regions at the *Bmal1* promoter

of these loops, first, the transcription factors BMAL1 and CLOCK, once translated into proteins, form a dimer complex with the ability to get into the nucleus and bind the E-boxes contained in the promoters of *Period* (*Per1-3*) and *Cryptochrome* (*Cry1-2*) genes for their transcription. Then, PERIOD and CRY protein products form a second dimer in the cytoplasm that will enter into the nucleus to stop their own transcription by an inhibitory action on BMAL1/CLOCK [3, 4] (Fig. 1).

The SCN is anatomically divided into the subdomains called "core" and "shell" or ventrolateral and dorsomedial regions, respectively. The ventral part is characterized by vasoactive intestinal peptide cells (VIP), whereas the dorsal region contains mainly vasopressinergic neurons. A synchronized communication between SCN cells is necessary for the good functioning of the clock to keep synchrony among neurons and to transmit a strong rhythmic signal to the brain and body [5, 6]. To maintain this synchrony, SCN cells use VIP as a synchronizing molecule. Indeed, a lack of VIP or its receptor compromises circadian rhythms of electrical activity of SCN neurons and of behavioral and physiological cycles [7]. In addition to the coupling role, VIP is also relevant for the entrainment of the SCN to the light-dark (LD) cycle [8].

The solar time is transmitted to the SCN via a monosynaptic pathway from the retina to the ventral hypothalamus, the retinohypothalamic tract (RHT). In the retina, the ganglion cell layer harbors specific melanopsin producing photosensitive cells, known

as intrinsic photosensitive retinal ganglion cells (iPRGCs) [9]. Through the RHT, iPRGCs contact the VIP ventral part of the SCN permitting the entrainment of the clock to the LD cycle. Light at dusk and dawn induces the transcription of *Per* genes in SCN neurons which lead in the resetting of the clock in a time- and direction-dependent manner [10, 11]. Whereas light at the early night induces phase delays of the clock and circadian rhythms of behavior and physiology, at late night it leads in phase advances of the whole circadian system [12].

A proper entrainment to the solar cycle permits robustness and stability of the SCN clock leading in a functional and healthy circadian system. However, when alterations of the photic synchronization of the clock occur, rhythms of behavior and physiology are compromised. Daily rhythms of cortisol (CORT) and the pineal hormone melatonin (MEL), peaking at the beginning of the active phase and at night, respectively, are disturbed in current occupations or urban lifestyles (e.g., shift work, light exposure at night) leading in sleep and circadian disruptions. Importantly, metabolic, cognitive, and mood alterations are strongly correlated with perturbations of the circadian system [13, 14].

To understand the central and peripheral mechanisms underlying the link between circadian alterations and physiological disorders, it is necessary to generate valid and consistent animal models of circadian troubles (e.g., jet lag). This approach should allow a better comprehension of this link and the proposition of new treatments to alleviate the resulting pathologies.

In this chapter, we review how biological rhythms are affected in mood ailments (e.g., depression) and how the exposure to aberrant LD cycles (e.g., jet lag) might lead to affective-like disorders. Finally, we describe the experimental designs used in chronobiology to elucidate the underlying mechanisms that may conduct mood-related disturbances (Figs. 2 and 3).

1.2 Mood Disorders Accompanied by Circadian Rhythm Alterations: From Humans to Animal Models

1.2.1 Major Depression Disorder

Major depressive disorder (MDD) is characterized by low mood, a loss of pleasure (anhedonia) and motivation, cognitive and neurovegetative declines, and suicidal thoughts [15]. Importantly, in MDD, disruptions of daily rhythms of activity, eating, hormonal release (e.g., MEL), and body temperature are also evident [16].

Among the diverse diagnostic criteria for MDD, disturbances of the sleep-wake cycle are the most consistent symptoms. Often, MDD patients report a poor quality of sleep with difficulties to initiate and maintain sleep, frequent awakenings during the night, and recurrent insomnia or hypersomnia along the daytime [17]. Polysomnographic studies show changes in the sleep architecture from depressive individuals. Total sleep time is significantly reduced in MDD patients, and particularly REM (rapid eye movement) sleep shows a shorter latency and an increased duration [18–20], suggesting that REM sleep alterations are a good marker for MDD [21].

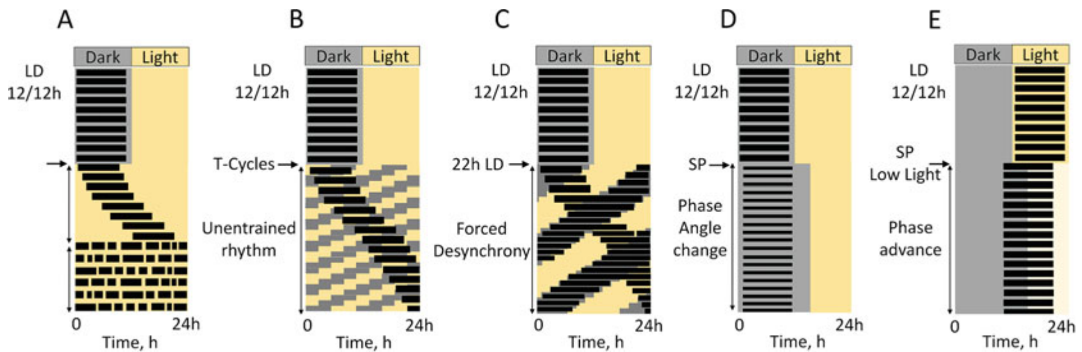

Fig. 2 Animal models of circadian disruptions. The light-dark cycle is the most important synchronizer for the circadian system. (**a**) Schematic single plotted actogram of a nocturnal rodent entrained to a 12h/12h light-dark (LD) cycle and then exposed to constant light conditions (LL). In this condition, animals show a lengthening of the circadian period of locomotor activity before becoming arrhythmic. (**b**) Schematic actogram of a nocturnal rodent entrained to a 12h/12h LD cycle and then kept in short photoperiods (LD 7:7) or T-cycles (T-7) [83]. Under these conditions, behavioral rhythms are not entrained, and these are expressed in a free running condition. (**c**) In a forced desynchrony model, animals are exposed to T-cycles of 22 h (LD 11:11) [84]. Under these conditions, behavioral rhythms desynchronize and show two independent activity rhythms; one entrained to the LD22h cycle and a second running freely. (**d**) Short photoperiods induce changes in the daily rhythms of locomotion in nocturnal rodents, mainly altering the phase angle of entrainment. (**e**) In the diurnal rodents *Arvicanthis ansorgei* and *niloticus*, short photoperiods induce changes in the entrained rhythms of locomotor activity. When SP is coupled with a reduced light intensity during the light phase, *Arvicanthis ansorgei* show a phase advance of the locomotor activity rhythm [45]. All schemes are single plotted (24 h). Black bars represent the animal activity. The grey-shaded area represents lights off (night) and the yellow-shaded area lights on (day)

In addition to the sleep architecture, the daily rhythm of locomotor activity is also disrupted in MDD patients. Indeed, MDD has been associated with a lower amplitude and a delayed locomotor activity rhythm, and these alterations are positively correlated with the severity of melancholic and depressive symptoms [22, 23]. Moreover, MDD patients who respond positively to antidepressant treatments such as light therapy or sleep deprivation show an increase in the amplitude and a phase advance of motor activity rhythms [24, 25].

At the hormonal level, the daily peak of MEL secretion at night shows a phase advance and a downregulated amplitude in MDD patients [26]. CORT release is significantly increased in depression. However, different to MEL, no evident changes in the phase of the CORT daily rhythms have been reported [27, 28]. Thus, a greater phase difference among the peaks of the two hormones is observed in MDD patients, suggesting that this might be used as a biomarker for the assessment of depressive states in humans [29].

In postmortem studies, it has been reported that the circadian expression of clock genes in the human brain is disrupted in relevant areas for the control of mood in depressive patients

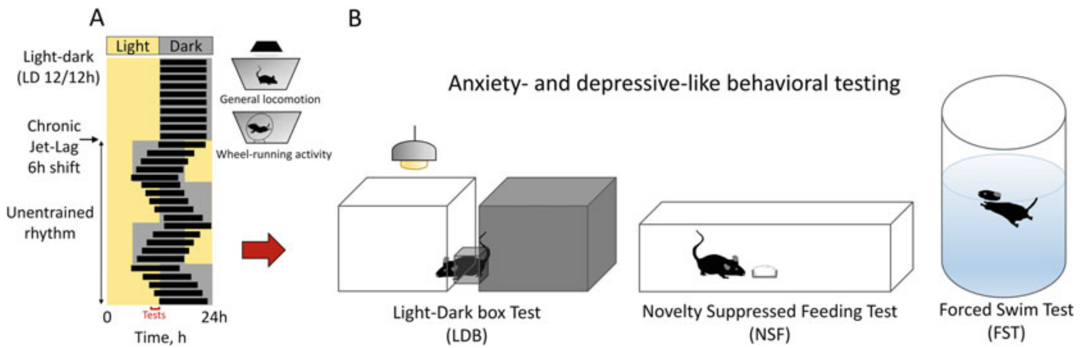

Fig. 3 Chronic jet lag model and mood-like behavioral testing. (**a**) Schematic actogram of the general locomotor activity (measured by infrared sensors) or wheel running activity of a nocturnal animal entrained to a 12h/12h light-dark (LD) cycle and then exposed to an experimental chronic jet-lag paradigm. In this, mice are subjected to a 6 h phase advance and delay in LD cycles every week during at least 6–8 weeks. Entrainment of locomotor activity rhythms to the phase advance is gradual and takes around 6–8 days to complete synchronization to the new LD cycle. This slow entrainment leads in transitory cycles, and animals cannot adjust their rhythm immediately to such shifts. Thereby, a stable synchronization is not established before the next LD cycle be advanced again. Yellow and grey background indicates light and dark phase, respectively. Black bars represent the animal activity. Top bars indicate the initial LD cycle. The red bracket indicates time of behavioral testing at the end of chronic jet lag exposure. (**b**) Anxiety- and depression-related behaviors are assessed over 6–8 weeks of chronic jet-lag exposure using a battery of tests: the light-dark box (LDB), novelty suppressed feeding (NFS), and forced swim (FST) test. Behavioral tests are performed in the last 2 h before lights off (ZT10-ZT12) and are separated for 1 week

[30]. These studies are relevant if we consider clock genes and circadian brain areas as important targets for classical and new treatments against depression.

Among animal models that lead in depressive-like behavior, the chronic mild stress (CMS) model is often used. In this model, animals are exposed to unpredictable stressor cues (e.g., physical immobility, food deprivation, LD cycle changes) twice per day for several days or weeks [31]. To note, when studying circadian rhythms, it is important to take into account certain considerations and to adapt the protocol accordingly, for example, to not include LD cycle changes as a stress stimulus and to expose animals to stressors at different times of day to avoid synchronizing effects [32].

CMS leads in behavioral and physiological alterations such as anxiety, depressive-like behavior, and hormonal changes (corticosterone levels increase). Additionally, antidepressant drugs (e.g., fluoxetine) reverse depressive-like behaviors induced by CMS indicating the good predictive validity of the model [31].

CMS-treated animals show reduced amplitude in daily rhythms of locomotor activity and temperature. Moreover, in mice exposed to an unpredictable CMS, the amplitude of the rhythms of clock gene expression in the SCN is reduced [32]. Along the same lines, a SCN-specific *Bmal1*-knockdown induces anxiety- and depressive-

like behavior in mice, indicating the main role of the SCN clock in the regulation of mood behavior and proposing it as an animal model of anxiety and depression [33]. Interestingly, the circadian activity of clock gene expression in the nucleus accumbens, a principal target for dopamine midbrain inputs, is amplified in CMS mice [32]. Thus, in affective behaviors the circadian activity of forebrain clocks is compromised.

1.2.2 Bipolar Disorder

Bipolar disorder (BD) is a psychiatric disease characterized by recurrent depressive and manic/hypomanic symptoms separated by a euthymic episode [34]. Alterations of daily rhythms of physiology have been reported in both depressive and manic episodes of BD [35]. For example, the daily rhythm of the release of the hormones MEL and CORT are phase-changed during the manic and depressive states, respectively. Furthermore, mood stabilizers (e.g., lithium or valproate) rescue daily rhythms of physiology (hormone release) and behavior (sleep-wake cycle) in BD patients [36].

At the molecular level, different studies have shown an important link between clock gene polymorphisms and BD [37]. Carriers of the G allele on Per2 rs2304672 present a greater risk to develop BD [38]. A single nucleotide polymorphism (SNP) on 3′-flanking region of the Clock gene (3111 T to C) is associated with sleep disruptions in BD patients [39]. Interestingly, in mice, a global mutation or a specific midbrain knockdown of the gene *Clock* lead in a mania phenotype (hyperactivity, decreased sleep) [40, 41]. Moreover, a lithium treatment rescues normal behavior in these mutants through the inhibition of the Glycogen Synthase Kinase 3 Beta (GSK3-β) which alters the stability of clock proteins and their nuclear translocation [42]. Altogether, these results point out the relevance of the circadian system in the pathophysiology of BD and highlight the use of clock mutant mice for the study of mood disorders.

1.2.3 Seasonal Affective Disorder

Winter depression or seasonal affective disorder (SAD) is characterized by the presence of depressive episodes associated to seasonal variations [43]. SAD occurs mainly during winter in countries of northern latitudes. Beyond mood changes, SAD-suffering people often experience sleep, appetite, and body weight changes, although some other behavioral or physiological changes (e.g., anxiety, drug intake) might appear. At the chronobiological level, there is an important phase delay of the daily rhythms of body temperature and locomotor activity in SAD patients [44].

The physiological mechanisms of SAD are not totally known, but as in MDD, the monoaminergic system can be compromised [45–48]. Importantly, monoaminergic activity (e.g., dopamine, serotonin release, or neural activity) shows strong circadian and seasonal rhythms in mammals including humans [49–53]. Indeed,

to understand the neural mechanisms involved in SAD, different models using both diurnal and nocturnal rodents have been proposed. For example, in diurnal rodents, such as the *Arvicanthis ansorgei* and *niloticus*, the exposure to short photoperiods conducts mood-altered behavior and brain dopaminergic modifications [45, 54]. In addition, mice exposed to 8–16 h LD cycles exhibit depressive and anxiety-like behaviors and decreased concentrations of central serotonin [55]. Thus, animals exposed to modified photoperiods might be a good model to study the pathophysiological mechanisms of SAD (Fig. 2).

1.3 Circadian Disruptions and Mood Disorders in Humans and Animal Models

1.3.1 Jet Lag

As mentioned before, the circadian system is principally synchronized to periodic environmental cues (e.g., LD cycle, feeding time) allowing certain flexibility of the system to adapt to a new environment. In humans, this is particularly true when we consider transmeridional travels. Indeed, the circadian system takes a few days to resynchronize progressively to the new phase of the environmental rhythm. During this period not all rhythmic functions are in phase with the new environment conducting to a state named jet lag [56].

In the context of transmeridional traveling, "lag" refers to a period of time during which biological clocks synchronize, by delaying or advancing their phase, to the new time zone. Many studies showed evidence that during this condition of circadian misalignment people suffer of insomnia, sleepiness, general malaise, or somatic symptoms such as headache and gastrointestinal discomforts [57]. These symptoms might vary according to the direction of the travel (East vs. West). In fact, people experience more intense jet-lag symptoms when flying from Eastern compared to flights from Western [58].

Circadian disruptions have negative impact on pre-existing psychiatric pathologies. Indeed, mood-affected people show exacerbated jet-lag symptoms when traveling [59]. Furthermore, jet-lag conditions might provoke manic or depressive episodes in BD patients. Interestingly, when flying from West to East (so generating a circadian phase advance), BD travelers are more likely to experience manic symptoms, while when flying from East to West they are more likely to experience depressive symptoms [60–62].

In the light of modeling circadian disruptions in animals, phase changes of the LD cycle (being the strongest *zeitgeber* for mammals) mimic the human situation experienced when travelling from a time zone to another [63]. In laboratory conditions, animals are often exposed to an unshifted 24 h LD cycle usually with a 12 h of light and 12 h of darkness (or dim red light). Therefore, advancing or delaying the LD cycle in an acute (a single 6–8 h shift) or chronic manner (6–8 h shift every 3 or 4 days) has been traditionally used to induce phase shifts in circadian rhythms and reproduce symptoms of transmeridional travelling [64, 65]. However, when considering

animal models of jet lag, some methodological factors must be taken into account: (1) direction of the shift (advance vs. delay)/eastbound vs. westbound; (2) the phase (light or dark) to be shortened or lengthened (direct light effect)/flying night or day; (3) the intensity of shift (hours)/number of time zones.

1.3.2 Shift Work

If not everyone is flying every day changing from time zones, >20% of Europeans are night- or regularly shift workers and are therefore exposed to a chronic jet-lag condition (sixth European Working Conditions Survey 2017). Since the end of the twentieth century, there is growing evidence of adverse effects of shift working on human health [66].

Besides nightwork, shift work refers to many types of rotating timework schedules (morning-afternoon, morning-afternoon-night, morning-afternoon-night-off) and experienced by many occupations (e.g., physicians, nurses, truck drivers, pilots). Chronic shift work affects human health and leads in sleep, metabolic, cardiovascular, and mood disorders and cancer [66, 67].

Shift workers present sleep disruptions and a higher risk of developing depression [68]. Although the full mechanisms underlying the link between shift work and mood alterations are unknown, it has been proposed that circadian misalignment is in part one of the main causes [68, 69], although sleep deprivation may be also part of the causes in the development of pathological conditions (e.g., metabolic, cognitive, mood) [70]. Thus, nocturnal rodents exposed to an enforced daytime activity that bring sleep alterations can be a good model of human shift work [71].

1.3.3 Social Jet Lag

The use of alarm clocks to wake up in the morning may have an impact on sleep and mood. By using them, we interrupt our sleep at a possible suboptimal time regarding the sleep phase we are in, or shortening our sleep duration. When using an alarm clock during weekdays, a large part (70%) of the population experiences another biological shifted condition called social jet lag (SJL). SJL is a forced desynchrony between biological (internal) and social (external) time measured by the difference between mid-sleep times on free days and on workdays [72, 73].

Social refers to the social obligations we have in weekdays (e.g., work/school time schedules). Therefore, people will wake up earlier on weekdays and later during weekends guided by their biological clock, implying a misaligned condition between their internal clock and the one imposed by our social life, conducting to deleterious effects on health. SJL is associated with higher sleepiness and depressive symptoms, suggesting that SJL may promote mood disorders probably mediated by sleep debt [74, 75]. In BD patients the disruption of social rhythms and biological timing is associated with a higher risk to trigger manic episodes [76].

In adolescents, a higher level of SJL is associated with considerable anxious symptoms [77]. Moreover, the susceptibility to develop substance use- and risk-taking behaviors is higher in adolescents with a larger SJL [73, 78, 79]. Furthermore, a wide difference of sleep timing between week and weekend is correlated with decreased cortical and striatal reactivity to rewards, suggesting that mood-related symptoms in SJL-exposed subjects might be caused by alterations of the dopaminergic and reward system induced by the circadian misalignment [80].

2 An Animal Model of Circadian Disturbances to Evaluate Mood-Related Behavior

From many years ago, numerous lines of evidence indicate the significant relationship between alterations of circadian rhythms and mood disorders. At the experimental level, in humans and animals, the manipulation of the LD cycle leads to the desynchronization of the circadian system and changes in affective behaviors. In animal models, different LD cycle manipulations have been used. Among these, the exposure to constant light (LL) or darkness (DD) conditions [81, 82], the exposure to T-cycles (T-7) [83], to a forced desynchronization paradigm (T-cycles of 22 h; LD 11:11) [84], or to short photoperiods (SP) [45] (Fig. 2). In all of these, animals develop alterations of locomotor activity rhythms and behavioral manifestations of depression, then constituting important experimental models for the study of the etiology of mood disorders.

In the last paragraph of this chapter, we described the methodology of a chronic jet-lag model in mice that can be used to study mood-related behaviors induced by disruptions of locomotor activity rhythms, a major output of the circadian system.

2.1 Materials and Methods

2.1.1 Housing

Young (6–8 weeks old) male C57BL6/J mice are housed individually in polypropylene cages (29 × 22 × 14 cm) provided with bedding and one pressed white cotton Neslet (5 × 5 cm) in light-proof ventilated rooms (23 ± 1 °C) initially under a 12-12h light-dark cycle (LD; light, 300 lux; darkness, 5 lux red light). Under LD conditions, *zeitgeber* time (ZT) is defined relative to lights on (ZT0) and lights off (ZT12). Food and water are accessible ad libitum. Experiments are performed in accordance with the principles of international and local laboratory animal care.

2.1.2 Locomotion Recordings and Analyses

For locomotor activity recordings, cages are equipped with:

- An exercise wheel (10 cm diameter) permitting the record of the number wheel revolutions.
- Passive infrared motion captors above the cage permitting animal movement detection.

- Animals are implanted (under gaseous anesthesia; 2% isoflurane in O2/N2O (50:50)) with a telemetry transmitter in the peritoneal cavity (Mini-Mitter; VitalView 3000, Sunriver, OR, USA). Importantly, the advantage of the latter is that it also allows the continuous recording of core body temperature, another circadian rhythm affected in mood disorders.

Locomotion or wheel running activity signals are registered on an automated recording system (CAMS, Circadian Activity Monitoring System, Lyon, France) in 5–10 min bins. Then, data are plotted as actograms and analyzed using ClockLab software (Actimetrics, Evanston, IL, USA).

2.1.3 Jet-Lag Protocol

Chronic jet-lag animal model. We set up an animal model using mice exposed to a protocol of chronic weekly phase-shifted schedule of the LD cycle. After a baseline condition, animals are exposed to a 6 h phase advance of LD cycle. To this end, time of lights off is phase-advanced by 6 h, so the light period is shortened to 6 h. After 1 week under the new LD condition, the mice are exposed to a 6 h phase delay of LD cycle. On the day of the shift, time of lights on is delayed by 6 h. Animals are exposed to the 6 h phase-advance/delay paradigm during 6–8 weeks (Fig. 3). Control groups are exposed to a regular unshifted LD cycles.

2.1.4 Circadian Rhythms Analysis

Activity data are displayed as actograms and activity profiles (ClockLab software; Actimetrics, Evanston, IL, USA). Different activity parameters can be analyzed:

- The 24 h mean activity profiles to determine the amount and distribution (over the 24 h) of activity (e.g., movements, for general locomotion; number of turns, wheel running).
- The duration of nocturnal (*alpha*) and diurnal activity (*rho*) using the onset and offset of light.
- The rate of entrainment to a new LD cycle. This is defined as the number of days necessary for the animal to present activity onsets/offsets at fixed phases relative to lights off/on, respectively, and a duration of activity period similar to baseline values.
- The phase angle of entrainment of locomotor activity rhythms, defined as the time of activity onset relative to the time of lights on.
- The robustness of behavioral rhythms analyzing the amplitude of rhythm under each experimental condition using the fast Fourier transform, which estimates the relative power of 24 h period rhythms in comparison with other periodicities in the time series (ClockLab software, Actimetrics, Evanston, IL, USA).

The most common species used in behavioral neurosciences, including circadian biology, are nocturnal rodents (e.g., mice, rats). Therefore, circadian timing is a critical factor in behavioral analyses. To evaluate the effects of chronic jet-lag exposure in mood-related behavior, behavioral tests are performed in the last 2 h before lights off (ZT10-ZT12; Fig. 3). Furthermore, since each mouse is evaluated in different tests, the tests are separated from each other for 8 days to minimize interference, and no mouse is through the same test twice. The forced swim test is always considered a terminal behavioral evaluation (i.e., no other test is performed after mice are through forced swimming).

2.1.5 Light-Dark Box Test (LDB)

The light-dark box test (LDB) is widely used to evaluate anxiety-like behavior in mice. The apparatus consists of two polyvinylchloride chambers ($20 \times 20 \times 14$ cm) with a Plexiglas cover separating each other by a small tunnel ($5 \times 7 \times 10$ cm). One compartment is darkened and the other is transparent and lit by a compact fluorescent light bulb (~950 lumen) placed at a height of 25 cm (Fig. 3). Each mouse is placed in the center of the dark compartment, and then the door of the tunnel between the compartments is opened allowing the animal to explore freely either the light or dark side for 5 min. We recorded the time spent in the lit box and the number of lit box entries.

2.1.6 Novelty-Suppressed Feeding Test (NSF)

This test is used to evaluate aversion to novelty or hyponeophagia in rodents [85]. Animals are food deprived overnight before testing. Animals are removed from their home cage, and testing is performed in an open field ($60 \times 60 \times 40$ cm) as a new environment. During the test, the mouse has access to a novel highly palatable food, a 50% sweet condensed milk solution (Nestlé), placed in a small food cup (3 cm diameter) in the center of the open field (Fig. 3). The latency to the first contact to food or to eat is measured. Social food transmission is avoided by placing the mouse in another cage just after the test.

2.1.7 Forced Swim Test (FST)

The forced swim test (FST) is widely used to test behavioral despair and evaluate depression-like behavior. The mice are gently placed in a 2 L transparent cylinder (height 17.5 cm, diameter 12.5 cm) filled with water at 26 ± 2 °C to a height of 12 cm (Fig. 3). Water is changed between each subject. A mouse is considered as immobile when it floated in an upright position or making few movements to keep its head above the water level. The duration of immobility is measured during 6 min as the total immobility time and per periods of 2 min.

3 Conclusions

In spite of the tremendous progress of research in the neurobiology of depression and anxiety, there is still a need for a deeper understanding of the cellular, molecular, and brain networks underlying abnormal mood. Moreover, what are the environmental factors and how these trigger mood disorders are not totally known. From the examples discussed above, there is good evidence that a disruption of the circadian clock is one of these important factors of vulnerability that promote mood alterations. The proposition of a diverse array of useful animal models of circadian disruptions that can expand our understanding of mechanisms underlying depression is of enormous help. However, it is still necessary to develop new approaches or experimental models or to improve the known models that mimic better the human circadian conditions. This is possible if research realizes the strengths and limitations of each paradigm, for example, the limitation that we still have using nocturnal rodents rather than diurnal mammals that model better human physiology [86]. Improving animal models of circadian disruption will permit a better comprehension of the brain mechanisms of emotional states and the development of most sophisticated and effective next-generation treatments to fight against affective illness.

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Chapter 11

Telemetry in Rats and Mice: Methodological Considerations and Example Studies of Stress and Anxiety in Ground-Based Spaceflight Analogs

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Abstract

Telemetry can be a powerful tool for assessing physiological responses to emotional stimuli. It can provide continuously recorded data over the course of the stress response and can be incorporated into established behavioral paradigms and procedures in a relatively seamless fashion. Our lab has been using telemetry in conjunction with behavioral paradigms to study the relationship between anxiety and sleep in rat and mouse models for several years. This chapter discusses some of the current, commercially available telemetry systems, how telemetry compares to other recording methods, and a number of its advantages and disadvantages. A crucial requirement for implementing telemetry is successful surgery leading to fully recovered, healthy animals and ultimately the recording of the desired signals. We provide detailed surgical procedures for implanting telemetric devices in rodents. We also provide an example experimental protocol integrating telemetry with behavioral training and sleep recording in rats studied in the context of ground-based models of spaceflight stress and discuss solutions for problems that may be encountered in implementing telemetry in these and other studies.

Key words Anxiety, Cable recording, Fear, Individual differences, Sleep, Stress, Surgery, Telemetry

1 Background

A variety of training paradigms and behavioral assays have been developed to induce and measure emotional responses in rodents. These paradigms and assays have typically been used for examining overt behaviors during the experimental session. There can also be significant perturbations in stress- and emotion-related parameters that accompany these behaviors. Common examples include heart rate and blood pressure [1–4] and body temperature [3–5]. Arousal and sleep are also strongly impacted by stress and emotion, and there is a strong link between disturbed sleep and mood and anxiety disorders [6, 7].

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Our labs utilize a variety of stress paradigms as well as both behavioral methods and physiological recording to examine emotional responses in rats and mice. Concurrent, continuous measurement of neurobiological and physiological variables typically requires animals to be surgically implanted for recording via tethers or cables or via telemetry. Thus, incorporating recordings of physiological variables into studies of emotional behavior and stress may be seen as a daunting task because of the technical issues. Compared to non-implanted animals, there also may be additional environmental and experimental controls that have to be considered in order to obtain viable data, and the required data handling and analysis procedures can be very labor-intensive. The following is a discussion of some of the factors that need to be considered in using telemetry in rats and mice and procedures we have found valuable in using telemetry in conjunction with behavioral paradigms. We include an example protocol that utilizes telemetry in rats in current studies examining how ground-based models of inflight stress and space radiation impact sleep and fear-conditioned responses across systems.

2 Telemetry Basics

Telemetry has been used to record a number of variables in rats and mice including heart rate [8–11], blood pressure [8, 10, 12], the electroencephalogram (EEG) [13–16], electromyogram (EMG) [17, 18], movement [10, 11, 13], and body [9, 11] and brain [19] temperature. Based on acquired signals (e.g., EEG, EMG, and/or activity), telemetry has also been used to determine sleep–wake states in rats [20–26], mice [13–16, 18], and other rodents [27].

One of the main arguments in favor of the use of telemetry is the reduction of stress compared to conventional measurement techniques [12, 28]. This rationale for using telemetry is particularly relevant for studies which require the animal to be subjected to a stressful situation as part of an experimental protocol. For instance, in our work, we focus on the impact of aversive conditioning and stress on sleep. Telemetry allows the animals to be transferred from home cage to conditioning apparatus and back to the home cage very easily without being subjected to the potential extra stress of disconnecting and reconnecting recording cables for each manipulation. Animals also have relatively more freedom of movement within their home cages compared to animals recorded via cables. In addition, data generally can be collected continuously without the need of special animal care [28, 29].

Telemetry eliminates the need for restraint which can be a component of certain noninvasive recording procedures (e.g., tail–cuff manometry for monitoring blood pressure) and which

can be a source of experimental artifact and inter-animal variability [29]. The impact of restraint has been demonstrated for various measures including increases in body temperature, heart rate and blood pressure, plasma levels of epinephrine, norepinephrine, and corticosterone (for reviews see [12, 28]). Even brief restraint can produce significant alterations in subsequent behavior and physiological parameters. For instance, 5 minutes of manual restraint, such as that required for administering microinjections, can produce significant alterations in subsequent sleep that may not fully habituate across repeated sessions [30]. Changes in core body temperature can begin within 10 s of the onset of restraint and temperature can rise as much as 2 °C during the restraint period [31, 32].

The advent of commercially available systems has allowed the use of telemetry to become relatively widespread in rats and mice. A full discussion of the various types of telemetry systems is beyond the scope of this chapter. However, transmitters suitable for use in rats and mice are supplied by companies such as Data Sciences International (DSI, St. Paul, Minnesota) and Starr Life Sciences Corp (Oakmont, Pennsylvania). DSI offers a number of implantable telemetry transmitters appropriate for implantation in rats and larger animals. As of this writing, six models offer the capability of recording from one to four biopotential channels (PhysioTel® HD-S21, HD-S11-F0/F2, HD-S02, 4-ET, CTA-F40 and F50-EEE). All models are capable of recording temperature and activity and the HD-S21 and HD-S11 are capable of recording blood pressure. They range in volume and weight (e.g., HD-S02, 3.3 cc and 4.7 gm; 4ET, 8.8 cc and 12.8 gm) and in battery life. The 4ET is available in two frequencies that allow recording in pair housed animals that could be useful for examining social interactions in anxiety paradigms.

In principle, the implantation and usage of the transmitters are the same across species, as are many of the procedures described in this chapter. Mouse-specific applications are described in a previous chapter [33]. Current DSI telemetry models that include biopotential channels that are suitable for mice include the PhysioTel® ETA-F10, HD-X02, and HS-X11 models that vary in physical size, the number and types of signals that can be obtained, and the duration of battery life. For example, the ETA-F10 has a volume of 1.1 cc and weighs 1.6 gm, whereas the HD-X02 has a volume of 1.7 cc and weighs 2.2 gm. The ETA-F10 can record a biopotential (e.g., EEG or EMG), temperature, and provide a measure of relative activity. Battery life in the ETA-F10 is 2.0 months. The HD-X02 can record two biopotentials, temperature, and activity and has a battery life of 1.5 months. The HD-X11 is of similar size to the HDX02 and can also collect pressure information.

Starr Life Sciences offers transponders to record temperature, gross motor activity, and heart rate data via telemetry. An advantage

of this system is that the transponders are externally powered and thus do not require batteries. Without the requirement for battery power, the transponders can be smaller and lighter and do not require refurbishing to change the battery. The PDT 4000 HR E-Mitter® transponder allows recording of heart rate, core body temperature, and gross motor activity. It is 26 mm × 8 mm and weighs 2.2 gm. An even smaller transponder capable of these signals is the model G2 HR E-Mitter® which is 19.5 mm × 6.5 mm and weighs only 1.5 gm. These transponders are not capable of recording biopotentials; however, Kaha Sciences (a division of ADInstruments) provides inductive powered systems for both rats and mice that have transmitters with the capability of recording biopotentials. Additionally, both battery-free and battery-powered models capable of recording core body temperature [34] or temperature and activity [35, 36] are sometimes implanted in animals that are also recorded via cables for signals such as the EEG and EMG.

It should also be noted that external telemetric systems that do not require surgery and hybrid systems that pair implanted devices with external transmitters and/or external power sources are also commercially available. For real-time wireless recordings of brain activity with high sampling rates, paired sets with a wireless transmitter and a receiver unit are typically used. The transmitter may take the form of a miniaturized wireless headstage unit that includes a low-noise preamplifier, a wireless data transmitter, and a power module. This type of wireless neural recording systems is supplied by Multi Channel Systems (W2100 model, Baden-Württemberg, Germany) and Jaga Systems (JAGA-16 model, Sunnyvale, CA). These systems provide various wireless headstages with different numbers of data channels and can be particularly useful for real-time chronic neural recordings from freely moving animals. Recording time can vary with battery size, and signal range can reach up 30 m distance, depending on wireless communication protocols and antenna used.

For long-term recordings where batteries need to be routinely changed or recharged, we use a hybrid system. In this case, electrodes are permanently implanted within the brain, and the wireless transmitter and battery are housed in a backpack and is attached to a head mounted plug only during recording. For another type of untethered recordings, a headstage datalogger and a base downloader unit from Plexon Inc. (DataLogger model, Dallas, Texas) are used. A battery-powered headstage datalogger allows wideband continuous recording and consists of a low-noise data acquisition and storing unit, which has advantages in terms of size and battery life by removing the wireless transmitter. After recordings, stored data is downloaded at the base unit. In this system, an infrared link is applied to control the operation of the datalogger remotely.

3 Telemetry vs. Cable Recording

The major competing methodology for telemetric recording of physiological signals in rats and mice is the use of an attached cable. This approach has long been used for obtaining a variety of physiological and neural information through the surgical implantation of sensors or electrodes to record the parameter of interest. In many cases, a recording cable is attached via a plug permanently affixed to the research animal's skull, though the cable itself also may be permanently attached. The cable may be attached to the animal via a harness in certain applications. This is a valuable technique for collecting physiological information, though cables can restrict movement and potentially influence behavior in mice [37]. In addition to the simple effects that cable weight and stiffness could have on behavior are the more complex effects they may have on the amount of energy and force required to overcome inertia and to provide the twisting force necessary for the mouse to initiate and continue rotational movement. These may vary with the position of the animal in its cage and with the angle of the cable [37]. Relative freedom of movement can be increased by the use of a commutator that allows the cable to turn without twisting; however, proper implementation, especially in mice, can be challenging.

In a previous study in mice [37], we found that cables of increasing weight and decreasing flexibility produced relatively linear decreases in activity. Heavier, less flexible cables also produced increases in sleep, but alterations in sleep showed more variability across cables. Perhaps most importantly, variations in cables produced a differential impact on sleep across mouse strains. The relatively small physical size of mice suggests that they may be particularly sensitive to variations in cable weight and flexibility. When unrestrained, mice can be relatively faster-moving, with vertical movement such as climbing on the watering tube and cage bars, suggesting that that cables could limit their movement to a relatively greater degree than in larger animals. Cabling also could be a factor in studies of gene-altered animals, which may have motor or other deficits that affect their activity [38].

To determine how activity and sleep (non-rapid eye movement sleep (NREM) and rapid eye movement sleep (REM)) recorded via telemetry would compare to that recorded via cable, we compared results for BALB/cJ (C) and DBA/2J (D2) mice obtained using telemetry [13] with results obtained with a commercially (Plastics One Inc.) available commutator (SL6C) and lightweight cable (363-SL/6 cable, without vinyl and protective spring covering (gross weight, 4.5 g; resting weight, 1.5 g)). Approximate cable resting weights were determined by placing the free end of the cable on a scale placed at headstage height. Based on total distance traveled (TD PCRB; considered as a percentage change relative to

baseline travel distance exhibited prior to surgery), mice recorded via telemetry typically had greater reductions in activity than those recorded with a lightweight cable. However, mice in both strains recorded with telemetry showed less reduction in activity than the 60–80% reductions in TD PCRB in recordings obtained with medium (resting weight, 2.2 g) and heavy (resting weight, 3.0 g) cables. The head pedestal, required for attaching the cable, alone (total weight about 1.6 gram, including electrodes, plug and dental cement) significantly reduced activity in the more active C mice compared to their presurgery activity levels. By comparison, pre- and post-surgery activity did not significantly differ in less active D2 mice suggesting that activity in the C strain was more sensitive to the weight of the implant alone.

Total NREM and REM were quite similar over the total 24 h recording period in C and D2 mice with both recording techniques. Differences in total NREM across methods were 10 min for C mice and 18 min for D2 mice, and differences in total REM across methods were only 3–4 min in both strains. D2 mice also showed similar light and dark period values with both methods. Differences in REM duration between methods were no larger than 0.1 min in either strain. Compared to telemetry, NREM durations with cable recording were 0.9–1 min longer in C mice and 1.2–2.2 min shorter in D2 mice.

The lesson from this comparison is that any invasive method of recording in mice has the potential to impact behavior and neurobiological function. All things considered, lighter and less obtrusive methods are better. However, the characteristics that make inbred strains and gene-altered rodent lines good models may also interact differently with the properties of the recording methods. These factors have to be considered when determining which recording method to use.

4 Potential Disadvantages of Telemetry

The advantages of telemetry need to be carefully weighed with its disadvantages compared to other recording techniques. Compared to noninvasive recording, the use of telemetry in rats and mice typically requires surgical implantation of transmitters or transponders or, as indicated above, the use of a backpack that houses the electronics and power supply. Thus, personnel must be trained to perform the implant surgery as well as to monitor and take care of the animals during recovery. As indicated below, the recovery period can be quite long, and thus more expensive, as the animal must be maintained and cared for before useful data can be obtained.

Compared to simple cable recordings, surgical trauma for telemetry implants can be greater. In addition, most currently available transmitters are large relative to the body size of mice

and can have an impact on behavior, just as cables can. The equipment required for telemetry can be more costly, both in the initial startup and over time, as battery-powered transmitters must be refurbished periodically.

The extent to which the animal can maneuver (i.e., range of motion or extent of reach) is also an important factor that should be taken into consideration when considering the placement, either implanted or externally, of the transmitter. Implantation of the transmitter can be quite uncomfortable for the animal, and if proper surgical techniques are not followed, irritation of the incision sites may cause the animal to tamper with their sutures. This may prevent proper healing of the incision areas, possibly leading to infection, and must be properly monitored. Furthermore, if the animal is able to access the transmitter (implanted or external), they may also damage the integrity of the wire leads or the equipment itself, in which case recording signals will be lost and the animal generally must be removed from study.

Lastly, when considering implementing telemetry in a lab that does not have previous experience with other types of recording (e.g., via cables or noninvasive methods), it should be recognized that are a variety of currently commercially available telemetry systems. These can have a wide range of recording capacity, from one or two channels up to 32 channels, thus far, due to the limitation of wireless transmitter size and power consumption. These systems can require time consuming and complex procedures for data handling and analysis. They may also require specialized training (such as scoring sleep, conducting a Fourier analysis of the EEG, and analyzing heart rate variability or neural activity) before useful data can be obtained and/or interpreted.

5 Application of Telemetry to Behavioral Paradigms

The integration of telemetry recordings with behavioral paradigms can be relatively seamless with some limitations. One significant limitation is the restricted operating range of some current commercially available transmitters that are suitable for rats and mice. For example, RPC-1 receivers available from DSI have a maximum range of 16 in that is more appropriate for home cage recordings. This limitation can be circumvented by linking multiple receiver platforms. For example, multiple receivers could be combined into a single receiving system to extend the range of reception to cover a large area, such as an open field. Thus, it would be possible to integrate telemetric recordings into a variety of behavioral paradigms.

Telemetry can also easily be used to record in the period immediately after training or testing sessions when an animal has been returned to its home cage (*see* **Notes 1** and **2**). We have used

this approach to examine the post-stress period in a variety of paradigms including cued [39, 40] and contextual [41, 42] fear, controllable and uncontrollable footshock [43], and the open field [42]. It is also useful for paradigms in which stimuli can be presented in the home cage, e.g., fear-conditioned auditory cues [39, 40] and novel objects [44].

6 Implantation Surgery and Recovery

The primary requirement for implementing telemetry in rats and mice is successful surgery leading to fully recovered, healthy animals and ultimately the recording of the desired signals. A major concern for surgery in mice is the size of currently available telemetry transmitters (*see* **Note 3**). Surgery trauma is greater for transmitters placed intraperitoneally, and longer recovery periods may be needed after surgery for certain types of implants. Thus, other than the type of data one desires to collect and the appropriate transmitter to use, one of the most significant considerations is the size of the subject. DSI suggests minimal animal weights of 19 gm for implantation of the HD-X11 and HD-X02 a minimal animal weight of 17 gm for the ETA-F10. These weights are good starting points; however, the success rates of implants also can vary with mouse strain and with surgical skill. Some strains (e.g., C57BL/6) are more robust, and an excellent surgeon and excellent post-surgery care can provide good results with smaller animals, whereas less robust strains may require larger sizes for success.

Proper surgical technique, post-surgery care, and adequate recovery time are crucial for obtaining the best results in mice implanted for recording via telemetry. Following is a detailed protocol for subcutaneous implantation of a telemetry transmitter (DSI ETA-F10 or similar) in rats using procedures we have utilized and refined for over 20 years. Implanting the transmitter subcutaneously on the back in mice is also possible [45, 46], though in our experience this type of implant is more prone to problems in long-term studies that are carried out over the course of several weeks or months. Over time, the skin, which typically is thin in mice, above the transmitter can degrade though this may not be a significant concern for short-term experiments.

We use isoflurane as the surgical anesthetic, and we also cover the cranial implant with dental acrylic which results in an exposed mound. Others may prefer to use injectable anesthetics and to close the skin over the cranial mound for either aesthetic reasons or to lessen the potential for infection at the incision site. There may also be variations in acceptable anesthesia, analgesia, antibiotic regimens, or other procedures across institutions. However, we have had minimal problems with infection at the incision sites. We also often pair telemetry recordings with intracranial injections (either

intracerebroventricular (ICV) or locally into specific brain regions) which necessitates a cranial mound to support the implanted injection cannula(e). Similar procedures can be used to pair optogenetics with telemetry [47]. A description of the integration of the surgical procedure for implanting cannulae with that required for the telemetry implant is included below though others have performed the implantation of an ICV cannula as a separate surgical procedure [15].

The procedures described in this section should be easily used or adapted across species by researchers with experience in animal surgery. A specific description of surgery in mice is provided in a prior chapter on telemetry [33]. Either version should be a good starting point for researchers with minimal experience who are encouraged to obtain training in basic surgical skills. Note that the surgery requires two people: the surgeon who conducts the surgery and aseptic procedures and an assistant who monitors the animal and performs tasks such as mixing dental cement and providing supplies. Table 1 provides a list of the instruments and supplies required to complete the procedures described below.

Table 1
Instruments and supplies used for implanting a telemetry transmitter (ETA-F10 or similar)

General instruments and supplies	Scale, cautery, UV light, water blanket, surgical drill, #9 drill bit, #14 drill bit, 10 mL syringe, 1 mL syringes, 3M ESPE Scotchbond Dual Cure Dental Adhesive Liquid System, dental acrylic, tape, sutures, scalpel blade (#10 or similar), sterile saline
For surgeon	Lab coat, surgical gloves, mask, scrub, sterile towels
Surgical area	Electric clippers, Betadine-soaked gauze, 70% alcohol-soaked gauze
Drugs	Antibiotic ointment, Puralube® ocular ointment, gentamycin (0.005 mg/gWT), potassium penicillin (100 IU/gWT), ibuprofen (30 mg/kg)
Anesthesia system	Vaporizer, induction chamber, isoflurane, oxygen, tubing, scavenger
Autoclave pack	Drapes, 2 × 2 gauze, cotton swabs, stainless steel tube (trocar), needle point forceps, curved point forceps, toothed forceps (large and small), microdissection scissors, dull scissors, scalpel handle, needle driver (holder)
Cold sterilized items	Screw driver, scissors, anchor screws
Surgical board	Covered with 4 × 4 gauze
Forms	Anesthesia monitor log, animal health action, and immediate recovery/pain forms
Extras for cannula implants	18-gauge needles, gel foam, dexamethasone (0.4 mg), dental pick (in the autoclave pack), stereotaxic apparatus, cannulae and dummy cannulae (in cold sterile)

6.1 Preoperative Procedures

Preoperatively, the rat should be prepared for surgery by providing it with analgesic. For each rat, we make available ad lib access to ibuprofen (30 mg/kg; 4.75 mL children's Motrin® (or similar) in 500 mL water) in its water bottle for 24–48 h prior to surgery and continuing for at least 72 h post-surgery. Antibiotics (gentamicin 5–8 mg/kg IM and procaine penicillin 100,000 IU/kg (0.2 mL total dosage)) are given subcutaneously preoperatively and may be used additionally if infection occurs following surgery. If intracranial cannulae are to be implanted, dexamethasone (0.5–2 mg (0.2 mL total dosage)) may be administered preoperatively to reduce brain swelling.

For the surgical implantation of electrodes and transmitter, the rat is initially anesthetized with isoflurane (5% induction, 2% maintenance (note: the requirement for maintaining anesthesia may vary depending on how the animal responds)) in an induction chamber. In our case, this is a plastic rectangular chamber (30 cm × 19 cm × 13 cm). Following attainment of surgical anesthesia, eye lubricant (Puralube® sterile ocular ointment) is then applied to the eyes.

6.2 Operative Procedures

After it reaches a surgical plane of anesthesia, the animal is placed on a surgical board in the prone position, and the head is cleaned using gauze soaked in Betadine® (10% povidone–iodine) followed by cleaning using gauze soaked in 70% alcohol. Afterward, an incision approximately 1.5 cm in length is made using microdissection scissors (or scalpel blade). A scalpel blade is used to scrape away the membrane of connective tissue covering the skull. A cautery may be used to stop excessive bleeding. A drop of phosphoric acid is placed on the skull, and a cotton swab is used to "scrub" the skull (avoiding the skin) to ensure all connective tissue has been removed. The skull is then rinsed three times with saline. During surgery, operative tissues are kept moist with applications of saline warmed to approximately normal body temperature 37 °C (98 °F).

An incision is made in the skin (approximately 2.0 cm in length) about 1 cm behind the scapulae using a scalpel blade, and blunt dissection is used to separate the skin from the underlying muscle. The transmitter is placed subcutaneously, and the wires are superficially routed thru to the skull with the aid of a small stainless steel tube (this must be large enough such that the wires from the transmitter can easily slide through it (no smaller than 3 mm in diameter is recommended)) which is used as a trocar to create a subcutaneous path from the head incision to the back incision. The wires are routed thru the tube and it is removed. The transmitter is then sutured to the back skin, and the surgical site closed. This is done using simple single sutures. Once closed, antibiotic ointment is placed on the incision along with sterile gauze.

Four holes are then drilled in the skull. Two holes (contralateral placement with one anterior and one posterior to lambda) are made with a #14 drill bit for placing anchor screws (0–80 × 3–32). Two additional holes (one in the left anterior quadrant and one in the right posterior quadrant around bregma (A:2.0, L:2.0; P:4.0, L:4.0) are made with a #9 drill bit for the lead wires from the transmitter. The anchor screws are inserted and left raised off the skull. These screws are placed to aid in permanently affixing the implant to the skull. The wires are cut to length (use dull scissors as the wire ruins surgical scissors), and the insulation is removed approximately 1.0 mm from the cut end. The wire tips are each bent into a "Z" shape and then placed into the hole such that the "Z" is completely under the skull. The wires are secured to the skull using 3M dual cure dental glue (activated by UV light (Patterson Brand, Model TCL490 Plus, visible light curing unit)). Dental acrylic is placed over the wires and exposed skull to finish the head cap.

Cannula(e) for the local or ICV administration of drugs or other compounds can be placed during the same surgical procedure with modifications to account for stereotaxic placement of the cannulae. For ICV microinjections, a 1-mm hole is drilled 1.4 mm lateral (L) and 0.8 mm posterior to the Bregma. The dura is then pierced with a dental pick. Afterward, the tip of a 26-gauge stainless steel infusion cannula (e.g., P1 Technologies, part number 315G with custom length for use in mice) is stereotaxically placed 3.8 mm below the skull surface in the lateral ventricle. Gelfoam is carefully inserted around the cannula to prevent dental cement from touching the brain. The cannula is then secured to the skull with dental cement, and a stylus is inserted to maintain patency. Simple variations of this procedure can be used to place cannulae for local delivery of drugs in specific brain regions. Note also that the position of the EEG and anchor screws may need to be moved to accommodate certain cannula placements.

6.3 Postoperative Period

Once an animal is taken off anesthesia, it is returned to its home cage and kept warm by an under cage (water circulating) heating pad. We place the animal on a clean paper towel to prevent the incision from coming in contact with the bedding material and to ensure that the animal does not inspire the bedding as they are recovering. Initially, and every 15 min for at least 2 h postoperatively, a record is made of the animal's respiration, coloring, returning reflexes, and an assessment of the animal's pain. The time it took the animal to wake up and ease of recovery is also recorded.

In addition, we have found that allowing the animal access to highly palatable food post-surgery aids in weight maintenance during the recovery period.

6.4 Post-surgery Recovery Period

An adequate post-surgery recovery period is essential for obtaining data that are not potentially affected by the recovery process. At present, we allow a minimum of 21 days for the rats to recover from an intraperitoneal implant prior to beginning baseline sleep studies and typically 28 days prior to beginning any experimental manipulation. Others report periods ranging from 28 days [14] up to 8 weeks [48] prior to beginning experiments. These may seem like long recovery periods, but we have found that the longer recovery periods provide more consistent data across animals. Fully recovered and stabilized animals are important to insure that stressful manipulations or behavioral tests used to induce or assess emotional state are not interacting with the recovery process.

Studies using telemetry in rats report a large range of recovery times allowed before experiments are started, and the time required for post-surgery stabilization may vary with the parameter of interest and surgical site. The best course of action is to empirically verify stabilization prior to beginning studies. For instance, rats with subcutaneous transmitter showed stabilized NREM and total sleep amounts within 2–3 days (compared to sleep at 15 days post-surgery), whereas REM amounts may require 7 or more days [21].

7 Sample Protocol for Examining Fear Conditioning in Inflight Stress Models

7.1 Background

Astronauts on the long duration of the proposed mission to Mars will face several physical and psychological challenges. They will constantly be exposed to microgravity which has well-documented effects on the skeletal, microbiome, gut mucosa, sensorimotor, and ocular systems (e.g., [49–55]). There also are significant effects on the brain, particularly in cerebellar, sensorimotor, and vestibular brain regions (Reviewed in [56]) and recent studies observed structural changes in the brain (rotation of the cerebral aqueduct, changes in ventricular volume, and narrowing of CSF spaces at the vertex) after spaceflight in astronauts, predominantly after long duration stays on the International Space Station [57]. Data from ground-based analogs for team cohesion and astronaut psychological status also suggests that some astronauts will have problems with sleep, interpersonal interactions, stress, and issues related to isolation (Mars 500, Antarctic confinement studies). They will also have a predicted exposure to ~13 cGy/Yr space radiation (SR) that can impair executive functions [58–61] and produce physical changes in the brain including marked and persistent gliosis [62–64].

In this project, we are using established *ground-based* models of microgravity (hindlimb unloading [49, 50]), social isolation [65], and exposure to SR [58, 66] in combination with telemetry to record real-time indices of how inflight stress and SR impact

physiological state and the stress system and how that impact is altered over time under continuing stress. These indices include *sleep*, which is a sensitive marker of stress that also has a functional role in mediating stress outcomes, and core body temperature which will enable assessing *stress-induced hyperthermia* (SIH), which responds in parallel with increased corticosterone and HPA activation [67]. We are assessing the effects of these stressors and SR on *fear learning and extinction* to assess how chronic inflight stressors may impact the ability of astronauts to respond to additional acute stress. The effects of fear and extinction learning on behavior, sleep, and physiological stress are being used to determine how continued inflight stress and SR alter the ability to cope with additional acute stress and its effects on physiological state. We are also using multiple measures of fear memory because although freezing, the standard behavioral measure of fear memory in rodents, is increased during extinction in SR exposed mice [68], freezing and its extinction are not predictive of fear-conditioned changes in sleep, EEG parameters, or SIH [69].

7.2 Subjects

Our subjects in these studies are outbred Wistar rats which have demonstrated individual differences in stress responsivity (vulnerable (Vul) and resilient (Res)) that appear predictable by plasma levels of BDNF [70]. Vul rats show significant reductions in REM after stress and fearful memories, whereas Res rats maintain REM amounts or show increases in REM sleep after the same experiences; these changes in sleep occur despite virtually identical freezing and SIH responses [69–73].

7.3 Experimental Procedure

The paradigm we use for the fear conditioning component of these studies is a variant of the inescapable footshock training paradigm we have used for years in rats and mice:

1. The rats are implanted for recording via telemetry and allowed to completely recover as described above. When the animals are not on study, the transmitter is inactivated to preserve battery life. The recording room is kept on a 12:12 light/dark cycle, and ambient temperature is maintained at 24.50 ± 0.5 C. Cages are changed 2 days prior to recording onset and then timed such that they do not impact subsequent phases of the study. Two days are allowed to provide sufficient time for the rats to habituate at each cage change.
2. A minimum of 2 days of uninterrupted, baseline sleep are recorded for each rat (*see* **Note 4**). For recording, the rats are housed in individual cages with food and water available ad libitum. Individual home cages are placed on a DSI telemetry receiver (RPC-1), and the transmitter is activated with a magnetic switch. Data acquisition is conducted using a configuration file containing pre-entered information regarding the

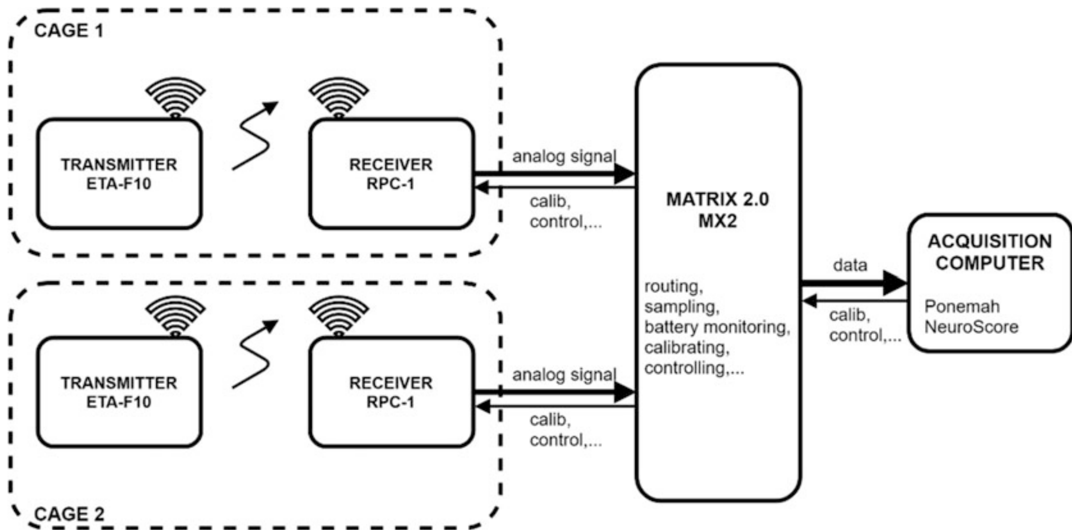

Fig. 1 Diagram showing major components of DSI telemetry system and their functions. The transmitters (ETA-F10 or similar) are implanted in the animals and relay signals to the receivers

transmitter, its calibration, and type of signals to be collected. Setting up the configuration is carried out using instructions in the DSI system manual. We currently use DSI Ponemah software with Neuroscore Core and Sleep modules. A diagram of system basics are provided in Fig. 1.

3. After baseline recording is completed, the rats are trained inescapable footshock using a custom made acrylic shock chamber with a grid floor for presenting shock (Coulbourn Instruments, Model E10-18RF). We use custom made chambers to allow greater headspace space for larger animals used in our NASA studies and for animals with various implants. Training takes place in a room separate from the colony/recording room. The rats are gently transported in their home cages to the training room and placed in the shock chamber and allowed to freely explore for 5 min after which they are presented with 20 footshocks (0.8 mA, 0.5 s duration) at 1.0 min intervals. Five min after the last shock, the rats are returned to their home cages. Training is conducted during the same h if multiple sessions are conducted across days. The chamber is thoroughly cleaned with diluted alcohol prior to each conditioning session.

4. Training parameters are controlled by computer. We use Coulbourn Graphic State software running on a personal computer to control the administration and timing of footshock. Footshock is produced via Coulbourn Precision Regulated Animal Shockers (Model E13–14) and administered via grid floors in the shock chambers.

5. Following training, the rats are returned to the colony/recording room and are monitored for the subsequent 20 h. For experimental paradigm changes or studies in new strains, recording is continued for an additional 24 h to follow the time course of the impact of the stress and to determine whether baseline sleep is re-established on the post-training day.
6. Six to 7 days following training, the rats are returned to the shock chamber and allowed to freely explore for the same amount of time as required for the training sessions but receive no shock. The animals are videotaped during this time for subsequent scoring of behavioral freezing. These tests take place at the same circadian time as the shock training.
7. Following exposure to context, the rats are recorded for 20 h on the day of exposure and for an additional 24 h, if needed.

7.4 Data Collected

1. *Freezing*: Freezing is scored in 5-s intervals over the course of the initial 5-min period prior to shock training and the context re-exposure session. From these data, the percentage time spent in freezing is calculated (FT%: freezing time/observed time $\times$ 100) for each animal for each observation period.
2. *Telemetry Data*: Telemetric data include the EEG (and EMG with the appropriate transmitter model), a relative measure of activity and core body temperature. These signals can be used to provide relevant information on their own. For example, the EEG can be subjected to a Fourier analyses, and the temperature measure can be used to assess stress-induced hyperthermia. With appropriate training, they can also be used to derive basic sleep parameters such as total sleep time, number of NREM and REM episodes, number of arousals, and duration of NREM and REM episodes. Example recordings of telemetrically recorded EEG, activity, and temperature and examples of active and quiet wakefulness, NREM (slow wave sleep), and REM (paradoxical sleep) are provided in Fig. 2.

8 Notes

This section provides discussion of issues that can be concerns in telemetric recording and some of the ways that we have dealt with them in designing and interpreting our experiments.

1. *Husbandry and Housing*

   Simple husbandry requirements such as routine changes of an animal's home cage can be stressful. In fact, transferring an animal to a clean cage and the introduction of novel objects (even those used for environmental enrichment) into the cage

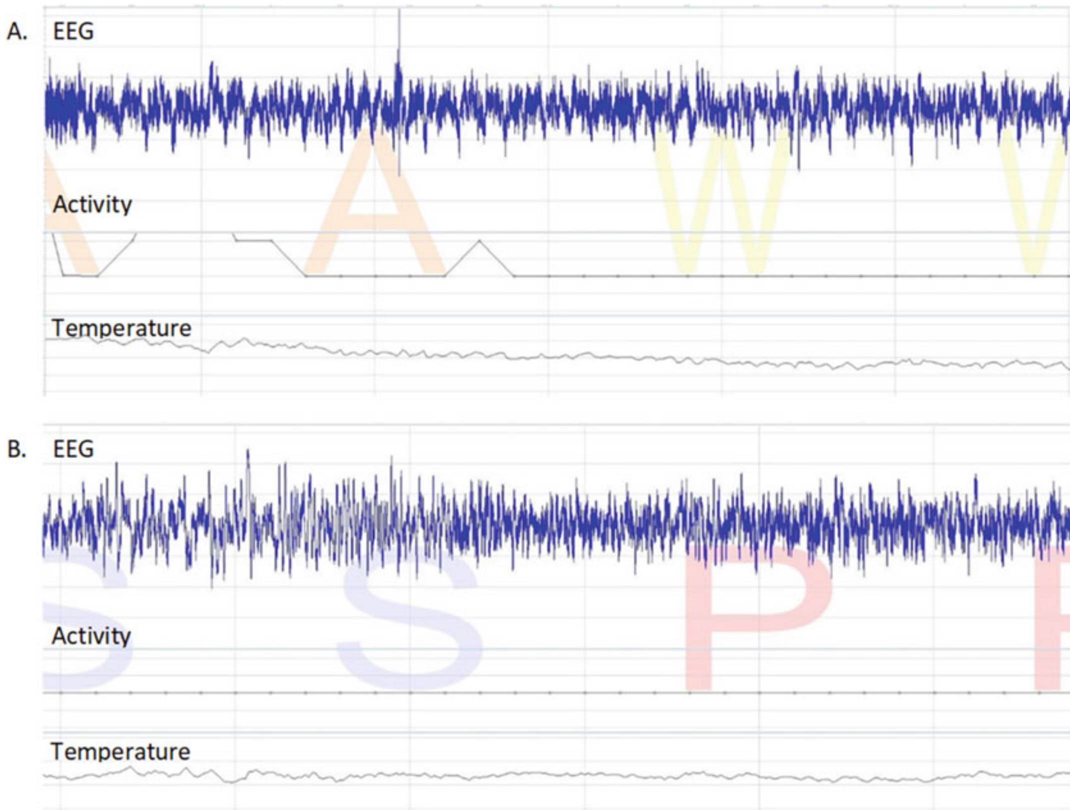

Fig. 2 Example sleep scoring in the DSI Neuroscore program showing (**a**) wakefulness and (**b**) transition from slow wave to paradoxical sleep recorded via telemetry. EEG and activity signals allow determining active waking (A), quiet waking (W), slow wave sleep (S), and paradoxical sleep (P). Also shown is continual recording of core body temperature

can be used as mild stressors for experimental purposes and can produce significant alterations in activity [44, 74], heart rate and blood pressure [75], and sleep and wakefulness [44, 74, 76–79]. Animals also do not appear to habituate to these procedures [75, 80], and responses can vary across inbred and outbred strains of rats and mice [44, 74] and in gene-altered mice [77–79]. Thus, it is very important that the timing of behavioral procedures and recording session take the amount of time since the last cage change into account.

2. *Effects on Activity*

As discussed above, mice intraperitoneally implanted with transmitters may show significantly decreased home cage activity compared to pre-surgical levels [13]. Intraperitoneally implanted transmitters have also been reported to decrease running wheel activity [9, 10]. This suggests that while current telemetry recording devices may allow a greater freedom of movement for certain activities, the overall level may still be

lower than before implantation. The reduction in activity should be carefully considered when using implanted animals in behavioral tests as many tests of emotion have a significant motor component [38, 81–83]. Unfortunately, the amount of impact on activity can vary with mouse strain [13] adding a potential confound that must be considered. To our knowledge, whether or not telemetry significantly impacts activity in larger animals has not been fully assessed.

3. *Survival Rates*

   Both rats and mice, of appropriate size, are highly tolerant of implant surgery. In earlier work in mice, we had a mortality rate of 9.3% between 3 and 9 days after surgery [13]. These rates are similar to the survival rate of over 90% reported for telemetric implants used for recording blood pressure and heart rate in B6 mice [10]. With good surgical technique and postsurgical care, it is possible to increase the number of viable implants, and our current mortality rate in mice is around 7%. Surgery-related morbidity and mortality in rats are negligible and are more apt to be due to complications with surgical recovery (e.g., infection, head tilt development, difficulty eating/drinking, impaired motor function).

   For intraperitoneal implants, proper placement and securement of the transmitter are crucial to prevent ileus and potential problems with bladder and diaphragm function. This is a consideration for both rats and mice. Interestingly, D2 mice had higher mortality (16.7%) that could be associated with the strain's naturally lower activity, which could possibly lead to more chances of having secondary abdominal complications [13].

4. *Signal Quality*

   Sources for decreased signal quality can come from a failing battery, degradation in the interface between the animal and the transmitter, signal dropout when the animal moves outside the range of the receiver, or environmental factors.

   Problems with the battery and implant are best prevented rather than remedied. For the battery, turn off the transmitter when it is not in use and keep accurate notes as to how long it has been in use. Current telemetry systems provide battery life information that assist with this task. By keeping track of the projected life of the battery, one can obtain a reasonable estimate of whether a battery will last through a study. Using these simple steps, we routinely implant transmitters in multiple animals before having them refurbished. With respect to the animal interface, repair may be possible in certain cases. However, firmly affixing the lead wires as described in the surgical section should provide adequate signal quality for the life of most studies.

One problem that we have encountered that may be unique to our space analog studies is an apparent shortening of battery life produced by exposure to simulated SR. Radiation is known to have the potential to reduce lithium–ion battery life [84, 85], and this reduction can be a critical factor in studies that require long-term recording. We solved the problem by implanting animals only after SR exposure but at the expense of loss of within subject baseline recordings. This also required the implementation of laborious behavioral assessment of sleep in pre-irradiated rats. The use of telemetry systems that use inductive power transfer and/or rechargeable batteries also may be a solution, but this would have to be verified. It may also be possible to shield the transmitters for the effects of radiation, but this, too, will need to be verified.

If signal dropout occurs, the most likely source is that the animal cage or test area is too large and allows the animal to move outside the range of the receiver. Environmental sources of signal interference may include "cross talk" between two or more receivers and their respective transmitters or electromagnetic interference. Detailed instructions for testing for and dealing with these problems should be included in system manuals. Solutions may be as simple as using a smaller cage for signal dropout or moving receivers farther apart for cross talk. They may also be more complicated such as shielding the receivers and cage or making other changes to protect the system from electromagnetic interference. Fortunately, these types of problems can typically be solved permanently unless there is a subsequent change in the recording environment. Our lab has two telemetry systems currently running (one in a fully shielded room and one in an unshielded room) that have provided noise free recordings for several years.

9 Conclusion

Telemetry can provide physiological recordings to complement data obtained in behavioral tests. It can provide continuously recorded data over the course of the stress response. It also has the advantage in that telemetered animals can be tested using established behavioral paradigms and procedures in a relatively seamless fashion. Drawbacks include the potential need for lengthy surgery recovery times and the fact that the implanted transmitter may significantly alter activity levels. However, with proper consideration of these factors, telemetry can be a valuable tool in the use of rodent models in research on mood and anxiety disorders.

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Chapter 12

Utility and Implementation of Oral Corticosteroid Exposure in Behavioral Neuroscience Research with Rodents

Michelle K. Sequeira, Jane R. Taylor, and Shannon L. Gourley

Abstract

Multiple biological factors are implicated in the neurobiology of depression. Historically, many functions were identified based on the characterization of antidepressant action in naïve rodents, rather than in models that recapitulate behavioral features of depression. In order to address this issue, Gourley and Taylor developed protocols in rats and mice in which organisms were exposed chronically to the stress-associated adrenal hormone, corticosterone (CORT), in the drinking water. Chronic CORT exposure results in anhedonic- and helplessness-like behaviors, as well as alterations in reward-related decision-making, that are persistent yet reversible by chronic antidepressant treatment. The present authors provide an updated guide for implementation and comment on the utility of this procedure. Chronic CORT exposure provides an alternative or complementary method to chronic mild stress or other models for studying aspects of depression-like phenomena. Such an alternative is valuable because these models can be difficult to implement consistently across laboratories or even cohorts of animals within the same laboratory or in particularly resilient mouse strains like the common C57BL/6 mouse. By contrast, the CORT exposure procedure described here is easily replicable, and behavioral and neural phenotypes persist well beyond the CORT exposure period, thereby modeling persistent depressive-like states in humans.

Key words Stress, Corticosterone, Dendritic spines, Neurotrophin, Rodent, Glucocorticoid

1 Introduction

Depression is a debilitating and chronic illness characterized by feelings of helplessness, anhedonia, and loss of motivation to perform even everyday tasks. Although the relationship between stress and major depressive disorder (MDD) is incompletely understood, stress-induced cortisol release from the adrenal glands and subsequent activation of glucocorticoid receptors in the brain [1] likely plays a crucial role, as stressful life events are potent factors that trigger, induce, or exacerbate major depressive episodes [2]. While no stress-induced depression-like behavioral phenotype in rodents can fully recapitulate the human condition, a procedure that produces a *persistent*, *varied* behavioral sequela (including

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helplessness, anhedonia, and loss of sensitivity to reward) and that is sensitive to chronic antidepressant treatment would be a major advance for understanding the neurobiology of depression [3, 4]. This unit presents a procedure for studying depression-related behaviors based on oral exposure to the stress-associated hormone corticosterone (CORT) in its hemisuccinate form in rodents (also see [5–7]). The use of CORT hemisuccinate for oral administration provides an alternative to dissolving CORT in small amounts of ethanol, which requires treating control animals with ethanol (e.g., [8, 9]). Either of these methods, however, allows for the identification of how CORT impacts neurobiology and behavior.

Historically, the dilution of CORT in rodents' drinking water was also used to administer CORT to nursing pups via the lactating dam [10, 11] and to evaluate the reinforcing properties of the hormone [12] and its contribution to vulnerability to drugs of abuse [13]. The CORT exposure protocol described here is a chronic exposure method that has been optimized for use in modeling a persistent depressive-like state in rodents. It has the advantage of allowing for multiple behavioral tests after CORT washout in the same animals, if one wishes. It is easily replicable between and within laboratories, and depression-like behaviors do not require CORT to be on-board to manifest. This advantage is valuable because some effects of acute CORT could confound responding in a given task (e.g., anxiogenic responses to novelty soon after animals consumed CORT have been previously reported [14]). Optimized dosing protocols for mice and rats are provided here.

2 Materials

- Corticosterone hemisuccinate (4-pregnen-11β 21-DIOL-3 20-DIONE 21-hemisuccinate) (Steraloids, Inc., Newport, RI).
- Mice or rats (e.g., from Charles River Laboratories, Jackson Labs).
- Standard laboratory housing, diet, and light cycle regulation.
- 10 N NaOH and HCl for adjusting pH of solutions.
- pH meter.
- Disposable Pasteur pipets (for pHing solutions, e.g., Fisher Scientific) stir plate, and magnetic stir bar.
- Standard laboratory rodent water bottles metric balance.
- or 2-liter clean glass bottles with lids for solution storage.
- Tap water.

3 Methods

Note: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or conform to governmental regulations regarding the care and use of laboratory animals.

3.1 Oral Corticosterone Exposure in Rats and Mice

CORT hemisuccinate is dissolved in tap water in a concentration of 25 μg/mL for mice and 50 μg/mL for rats (free-base) and administered chronically with a weaning period (rather than an abrupt withdrawal from CORT) at the end of the exposure period (*see* **step 5** below). Behavioral effects of CORT are expected to be persistent and long-lasting. This procedure provides a simple, convenient, and easily replicable alternative to stress procedures for studying aspects of depression in rodents. The authors' initial studies largely used mature male animals, but the procedure was recently applied to adolescent mice, as well as female mice [15, 16], and concentrations up to 100 μg/mL can have depressive-like consequences [6].

1. Weigh out the appropriate amount of CORT hemisuccinate, depending on the desired concentration and volume, correcting for the added molecular weight of the salt by multiplying the desired amount by a factor of 1.289. In other words, for a desired concentration of 25 μg/mL, dissolve 32.22 mg CORT hemisuccinate into 1 L of tap water. The molecular weight of CORT is 346.46; the molecular weight of CORT hemisuccinate is 446.53. Concentrations in this chapter are expressed as free-base values.
2. Add CORT to tap water in a clean glass storage container. To facilitate dilution, one can increase the pH to 12–13 with 10 N NaOH using a sterile disposable Pasteur pipette. Stir at room temperature or at 4 °C until dissolved (3–7 h). Stirring at 4 °C is preferable because it will slow decay. *See* Subheading Reagents and Solutions for recommended CORT (solid and in aqueous solution) storage conditions and shelf-life.
3. Following dilution, neutralize the pH to 7.0–7.4 with 10 N HCl. Add HCl slowly, as the pH will rise and fall dramatically because the solution is not buffered.
4. Once the CORT solution is prepared, present animals with the CORT *in place* of normal drinking water for 14 days so CORT provides the only source of hydration throughout the exposure period. To generate an average dose of CORT, weigh bottles daily, and divide consumption values by the total body weight of the animals in the cage. In the authors' experience, adult male C57BL/6 mice typically consume 4- to 6-mL dissolved CORT per day, while adult male Sprague–Dawley rats typically consume 30- to 60-mL dissolved CORT per day. Over the

course of this 2-week period, the average CORT doses the authors have observed range from 5 to 7 mg/kg/day for mice consuming a concentration of 25 μg/mL; values are comparable for the rat consuming 50 μg/mL, as rats consume less fluid per gram of body weight than mice.

5. Wean as follows: 3 days with 50% of the original CORT concentration and 3 days with 25%, to allow for recovery of endogenous CORT secretion, finally followed by a return to regular drinking water. **The entire exposure period is 20 days.**
6. Monitor body weights periodically during and after the CORT exposure period to confirm weight loss is not excessive or injurious to the animal. Watch for increased conspecific-directed aggression during CORT exposure, and separate animals when appropriate to prevent injury.
7. For those interested in the long-term consequences of excess CORT exposure, allow a wash-out period (in which animals are given free access to food and regular drinking water) after the cessation of CORT drinking. For instance, some biochemical effects of CORT exposure in the dorsal hippocampus evolve only following a wash-out period [7].

Reagents and Solutions CORT solutions should be changed within 72 h of dilution, as CORT will begin to degrade once in solution. Dissolved CORT should be stored at 4 °C, and stirring at 4 °C will also slow degradation. In solid form, CORT hemisuccinate is stable and can be used until the manufacturer's expiration date. Solid CORT can be stored at room temperature or at 4 °C unless otherwise advised by the manufacturer.

4 Notes

4.1 Time Considerations

Experimenters should ideally prepare CORT every 3 days. Further, the exposure period is substantial—20 days—and a wash-out period after CORT exposure is recommended to model the chronic depressive-like state (*see* Subheading 4.2), potentially resulting in the addition of several weeks to the experiment duration. Pilot experiments will be required to determine an appropriate timeline for each experiment, depending on the experimental aims. One additional factor to consider in behavioral testing is the possibility of testing animals before and after CORT exposure, allowing for within-subjects comparisons, which may be statistically advantageous and potentially minimize the number of animals used for a given project. This approach would add several days to the experimental timeline.

4.2 Critical Parameters

CORT Dose Sensitivity Despite evidence of morphological and neurochemical alterations in the hippocampus after high concentrations of oral CORT in rats (400 μg/mL, [8, 9]), the highest CORT concentrations used in our initial unpublished behavioral studies (300 and 400 μg/mL) were ineffective in producing a persistent depressive-like phenotype in mice, as measured by sucrose consumption, mobility in the forced swim test, and instrumental assays of sensitivity to reward (Gourley and Taylor, not published). We did not pursue these negative findings because the health of the mice also suffered, sometimes lethally, with these CORT concentrations. By contrast, behavioral and biochemical findings after "low-dose" CORT (25–100 μg/mL) are robust and replicable without excessive threat to animal health, particularly at the lower 25 μg/mL concentration. In adult, female C57/BL6 mice, a 35 μg/mL concentration may be even more optimal for inducing instrumental decision-making deficits and anxiety-like behavior ([17]; our unpublished data). In Sprague–Dawley rats, we find that 50 and 100 μg/mL exert comparable, if not indistinguishable, effects on behavioral responding; concentrations >100 μg/mL have not been tested by us.

The Use of a Wash-Out Period After CORT The investigations that the authors have pursued for nearly two decades relate to the long-lasting and persistent effects of prior CORT excess on behavioral and biochemical measures. With few exceptions, it is not known if animals tested immediately after CORT exposure will resemble those given a wash-out period. Certainly, some immediate and long-term biochemical consequences are similar: For example, levels of tropomyosin receptor kinase B (trkB), the receptor for brain-derived neurotrophic factor (BDNF), are decreased in the dentate gyrus of the hippocampus immediately after CORT exposure; meanwhile, phosphorylation of the receptor is attenuated 2 weeks and 1 month after exposure [7]; both outcomes would be expected to dampen trkB-mediated intracellular signaling. In another example, excitatory neurons in frontal cortical regions undergo dendritic spine loss upon CORT exposure; however, the timeline of recovery is region-dependent [18]. These and other data indicate that some consequences of prior CORT exposure are temporally dynamic, changing depending on temporal proximity to the timing of CORT administration. Further elucidating the mechanisms by which chronic stress hormone exposure and wash-out influences the development, expression, and progression of depressive-like symptomology in rodents may provide additional insight into stress-associated psychopathologies in humans and new avenues for antidepressant drug development.

4.3 Troubleshooting

At higher concentrations, CORT may not dissolve within the recommended time window. Stirring longer (e.g., overnight at 4 °C) does not obviously harm the compound; all concentrations discussed here would be expected to dissolve within 24 h. After dissolving CORT, the authors have not experienced it coming out of solution again even if the pH drops below the desired range. If CORT "falls out," the authors recommend discarding the solution and starting over.

Increased conspecific aggression may be observed while animals, particularly C57BL/6 mice, are consuming CORT. In this instance, animals should be rehoused separately, as increased fighting may result in death. Over-grooming may also occur, such that the animals remove patches of fur. Fur should regrow when the CORT consumption period ends. If the skin is broken, veterinary care should be sought.

The authors have not experienced a situation in which the animals refuse to drink CORT, but it is possible that certain strains of mice or rats might find the compound aversive. Adding 2% w/v saccharin increases the palatability of other compounds, such as chemical antidepressants [7, 19]; adding saccharin to CORT would not be expected to alter its pharmacological effects.

5 Commentary/Conclusion

This unit instructs the user on how to use exogenous orally available CORT (translating to 5–7 mg/kg per day *p.o.*) to induce a persistent depression-like state that is sensitive to antidepressant treatment in rodents. Prior CORT exposure has several behavioral consequences, including increased immobility in the forced swim test and decreased sucrose consumption in a model of anhedonia [6], as well as depressed instrumental (i.e., reward-seeking) behavior and decision-making strategies that are biased toward habitual behavior at the expense of goal-directed action [15, 20–22]. Exploration of an elevated plus maze, baseline daytime endogenous CORT, and adrenal gland weights are unchanged 2 weeks after exposure, thus providing a depressive-like behavioral phenotype without obvious anxiety-like behavioral or physiological confounds [6]. The authors have also previously confirmed that basic gustatory discrimination processes—as measured by quinine/water discrimination—are intact, indicating the effects of prior CORT on sucrose consumption are unlikely to be due to nonspecific effects on sensory discrimination skills [6].

5.1 CORT Is Sufficient to Alter Molecular Targets Implicated in Depression and Antidepressant Efficacy and Modify Neuron Structure

The neurotrophic hypothesis of depression and antidepressant efficacy [23] was based in part on observations that stress, and stress-associated hormones decrease activity of hippocampal cAMP-response element-binding protein (CREB), a transcriptional regulator of the growth factor, BDNF, or BDNF itself, while antidepressants produce opposite effects [24–28]. In one report addressing the long-lasting consequences of stress exposure on neurotrophin-regulated signaling targets, Laifenfeld et al. [29] reported decreased hippocampal CREB phosphorylation 4 months after chronic environmental stress. Similarly, we have demonstrated that CORT, a major stress substrate, is itself sufficient to persistently decrease hippocampal CREB phosphorylation up to 1 month after CORT exposure, and subsequent treatment with chronic amitriptyline or fluoxetine can normalize levels [7]. Additionally, CORT exposure regulates phosphorylated extracellular signal-regulated kinase 42/44 (ERK 42/44) a downstream substrate of trkB signaling [7]. Recently, these findings culminated in reports revealing that chronic exposure to CORT in the drinking water causes a shift in the balance of full-length/truncated isoforms of trkB (the BDNF receptor), in corticolimbic regions, and a trkB agonist mitigated CORT-induced behavioral deficits [15, 16]—one example of the translational utility of the CORT exposure procedure described here.

It is widely appreciated that stress remodels excitatory neurons throughout the frontal cortical regions of the brain that control mood and decision-making behavior [30, 31], as well as altering BDNF-trkB expression and activity in these regions [32]. Because of the complexity of the stress response, though, it can be difficult to pinpoint mechanisms. Using CORT in the drinking water allows researchers to isolate CORT-related phenomena and, potentially, their mechanisms and to develop novel strategies for recovery. In one particularly exciting report, chronic exposure to CORT caused clustered dendritic spine loss and behavioral deficits in mice, which were rescued by treatment with subanesthetic doses of ketamine. Notably, the long-term behavioral effects of ketamine *required* this spinogenesis [33]. In another report, stimulation of a cytoskeletal regulatory factor, Abl2 kinase, caused the recovery of structural alterations in hippocampal neurons, which otherwise atrophied with CORT exposure, and resulted in concomitant antidepressant-like consequences [34]. These and many other recent discoveries add to mounting evidence that dendritic spine plasticity and their regulation may be an important target in effectively treating MDD.

In summary, the CORT exposure protocol outlined here is a powerful and reproducible method to model multiple dimensions of MDD, as well as understand the manner in which antidepressants work in the brain and identify new compounds with therapeutic potential. We have also utilized this protocol to determine

effects of excess CORT on decision-making behavior, including during vulnerable adolescent periods [15, 20], explore individual differences in CORT vulnerability [16], identify neuron populations that are more or less resilient to corticosteroid excess [18, 20, 35], and reveal CORT-sensitive gene expression patterns in specific regions of the brain [36]. Our hope is that other investigators will also find this tool fruitful in advancing new knowledge regarding the brain and behavior.

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Chapter 13

Animal Models for Mania

Kirsten Schmerder and Nadja Freund

Abstract

Manic episodes are assigned to the classification of affective disorders and oftentimes occur as a mood episode in the course of bipolar disorder (BD). Manic symptoms include elevated energy levels, hyperactivity, reduced need for sleep, and increased impulsivity as well as risk-taking. Unfortunately, efficacy of treatment options is limited, and further research is desperately needed to improve quality of life of those suffering. Research in animal models is a critical tool in revealing mechanisms underlying the pathophysiology of bipolar mania and supports the assessment of new potential treatment targets. This chapter aims to give an overview of the current status and availability of animal models in BD research with a strong focus on modeling the manic pole. Different approaches are used by researchers in order to develop models of mania, and, thus, pharmacological, environmental, as well as genetic animal models are presented in this chapter. Some of these models display both mania- and depressive-like behavior within one animal, giving rise to the potential of investigating mechanisms behind the mood switch in BD. For each animal model, the three axes of validity are discussed, and strengths as well as limitations are described. Furthermore, detailed protocols for two animal models covering manipulation of the photoperiod and stereotactic injection of a lentiviral construct are provided at the end of this chapter.

Key words Mania, Bipolar disorder, Animal model, Mood, Depression

1 Introduction

Manic episodes are classified as an affective disorder according to the ICD-10 (F30). Patients with acute manic episodes experience elevated hedonia, hypersexuality, decreased need for sleep, heightened aggression, and increased goal-directed, risk-taking and impulsive behavior [1–5]. Moreover, manic episodes can be accompanied by psychotic states [6] and can be further categorized in euphoric and dysphoric mania. Hypomanic individuals experience the same symptoms even though the symptomatology is not as pronounced and long-lasting. Manic episodes can be present in schizoaffective disorders, can be caused by general medical conditions, or are precipitated side effects of neurological medication. In most cases, however, episodes of mania or hypomania occur as one

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pole next to the depressive pole in the course of BD [7]. Bipolar disorder (BD) is characterized by recurrent episodes of depression and mania or hypomania with intervening phases of euthymia [8]. BD affects more than 1% of the world's population, regardless of nationality, ethnic origin, and socio-economic status [9]. Yet, despite this widespread prevalence and severe symptomatology, the underlying pathophysiology of BD is still not fully understood, and already available treatment options are not able to alleviate symptoms in all patients [8]. Therefore, the need for appropriate animal models to further investigate physiological processes and risk factors as well as discover potential treatments for BD is huge. Ideally, to achieve a satisfying translationality, adequate animal models require high levels of face, predictive, and construct validity [10]. Face validity refers to the degree to which the animal model recapitulates the phenotypes (biochemical, neuroanatomical, and behavioral) seen in humans suffering from the disease. Predictive validity is the extent to which the model responds to a treatment that is efficacious in patients and construct validity reflects the degree to which the model measures which it claims to measure. High construct validity can be achieved by either manipulating the genetic phenotype and therefore interfering with specific signaling pathways or altering the activity levels of key proteins that are known to be involved in the pathophysiology of BD, exposing the animal model to environmental circumstances that are hypothesized to be risk factors in the etiology of BD or a combination of these methods. Since neither the pathophysiology nor the etiology of BD has been completely uncovered yet, the level of construct validity is often the limiting factor in establishing adequate animal models [11]. However, in the last decades, major progress has been made creating animal models for BD (for reviews see [12–15]), which helped to begin unraveling the fundamental underlying neurobiology. The greatest challenge in establishing these models is to recapitulate the cyclical nature of the disease [16]. Thus, the depressive and manic poles are typically modeled in separate animal models [17]. Importantly, models for the depressive pole of the disease are more abundant since most of them are also used in the context of unipolar depression research [16, 18] and, therefore, especially the small number of validated animal models for the manic pole of BD is contributing to the rate-limiting factors in advancing BD-related research. This book chapter aims to give an overview of the currently established animal models for mania.

2 Animal Models of Bipolar Mania

There are four main mechanisms to model mania in animals: by pharmacological treatment, via manipulation of the environment, through a genetic approach, and by harnessing the intrinsic mania-

like behavior of specific rodent strains. Each animal model and every approach has different strengths and weaknesses regarding the three different axes of validity.

2.1 Pharmacological Animal Models of Mania

The initial animal models of human mania were based on drug-induced mania-like behavior, mostly via pharmacological modulation of dopaminergic activity. Administering psychostimulants was long thought to be the gold standard and is still frequently used to this day in order to induce a mania-like status in rodents and evaluate potential novel treatments [12, 19–26]. The mania-like behavior is expressed as an increase of locomotor activity (i.e., hyperactivity) which can be easily assessed in rodents. Since BD patients oftentimes experience increased motor agitation during manic episodes, psychostimulants can produce mania-like symptoms in healthy subjects and even trigger or worsen manic episodes in patients, and the level of face validity of these animal models seems to be high [27, 28]. An overview of the pharmacological animal models discussed in this chapter and their triaxial validity can be found in Table 1.

2.1.1 Amphetamine-Induced Hyperactivity

The most prominently used drug in the context of pharmacological animal models of mania is amphetamine. Acute doses of amphetamine (3 mg/kg dexamphetamine or 1 mg/kg methamphetamine, respectively) induce locomotor hyperactivity in rodents, which can be attenuated with either acute lithium treatment in a dose-dependent manner (150 mg/kg and 300 mg/kg lithium [24]) or acute microinjections of valproate (300 μg [29]). A more recent study demonstrated that chronic treatment with amphetamine (five repetitions of 7 days of amphetamine injections (2 mg/kg) followed by a 7-day wash-out period to mimic between-episode intervals) results in cognitive deficits (i.e., impaired habituation memory) and in increased levels of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex and amygdala [30]. Altered BDNF levels are also known to be present in BD patients [31, 32]. Therefore, prolonged application of amphetamine might model the neuroprogression and cognitive aggravation seen in BD.

Despite the seemingly high levels of face validity (hyperactivity, cognitive impairment), predictive validity (attenuative effect of lithium and valproate), and construct validity (hyperdopaminergia), amphetamine-induced hyperactivity as a model for bipolar mania holds several limitations. Firstly, Gould and colleagues demonstrated that the attenuative effect of acute lithium treatment on amphetamine-induced hyperactivity is mice strain specific [25]. Even if the genetic-dependent impact (or lack of, respectively) of lithium might yield interesting insights into the effect of lithium (responders vs nonresponders), it is still an important factor that needs to be considered. The predictive validity of this animal model

Table 1
Pharmacological models of mania

Model	Validity			Mania-related behavior	Limitations	References
	Face	Predictive	Construct			
Amphetamine-induced hyperactivity	•	•	+	Hyperactivity	AMP-induced alterations in behavior differ from that seen in healthy subjects, chronic treatment not efficacious, limited facets of mania	[24, 29, 30]
Amphetamine + chlordiazepoxide	•	-	•	Hyperactivity, stereotypy	Huge number of control groups required, limited facets of mania, chlordiazepoxide potentiates locomotor activity-suppressant effects of treatments	[38, 39];
Quinpirole	•	•	•	Hyperactivity	Limited facets of mania, treatment not approved for mania efficacious	[46]
Ouabain	•	•	+	Hyperactivity	Limited facets of mania, novel and anxiogenic environment required to see ouabain-induced hyperactivity, controversial effects of lithium treatment	[48–52, 56]

Green plus sign indicates good validity; yellow dot indicates moderate validity; red minus sign indicates poor validity
AMP amphetamine

is further diminished by the fact that the effect of lithium is confounded by a decreased activity in vehicle alone-treated mice [24] and only acute but not chronic lithium (pre)treatment (14 days prior to testing) is able to reverse behavioral effects of amphetamine [21]. A recent study once more challenged the predictive validity of amphetamine-induced hyperactivity as it indicates no efficacy of

chronic treatment with lithium (5 days of 0.2% lithium succeeded by 10 days of 0.4% lithium in powdered rodent chow) followed by acute administration of amphetamine (single dose; 1 mg/kg). Here, lithium was not able to hinder psychostimulant-induced hyperactivity [33]. The lack of effectiveness of chronic treatment in animal models is an especially crucial aspect to acknowledge since a chronic treatment with lithium is required to achieve symptom relief in humans [22, 34]. Secondly, amphetamine-induced alterations of behavior and, importantly, the effect of lithium on these behavioral alterations in healthy subjects are different to those seen in rodents [35]. Lithium does not weaken the effect of amphetamine in humans not suffering from BD. However, it is noteworthy that subjects were treated with lithium for 7 days prior to amphetamine administration which does not resemble an acute treatment but might be considered a sub-chronic period. Thirdly, as aforementioned, human mania is characterized by a broad spectrum of symptoms of which a set of symptoms needs to be experienced by the patient to meet diagnostic criteria for bipolar mania. Yet, amphetamine-induced behavioral changes mostly only comprise locomotor hyperactivity. Increased motor agitation is only one facet of the disease, and not all patients suffer from elevated need for movement. Lastly, amphetamine administration is not exclusively used and interpreted as a model for mania but is also utilized in schizophrenia and drug abuse research [36, 37].

2.1.2 Amphetamine + Chlordiazepoxide

In an attempt to model more facets of human mania, another animal model was established that uses amphetamine (1.25–5 mg/kg) in combination with the benzodiazepine derivative chlordiazepoxide (25 mg/kg or 6.25 mg/kg chlordiazepoxide hydrochloride, respectively [38, 39]. Chlordiazepoxide alone acts as a sedative and anxiolytic drug. Combined with amphetamine, applications in rodents result in the commonly seen amphetamine-induced elevated locomotor activity as well as in increased amounts of head dips. Acute pretreatment with lithium (3 mEq/kg or 10 mL/kg, respectively) is able to reverse both behavioral abnormalities [19, 40]. Several additional studies tested the efficacy of other drugs in decreasing the induced hyperactivity in this animal model. Next to lithium, acute administration of retigabine (10 mL/kg), lamotrigine (10 mL/kg), and levetiracetam (dose-dependent with 17 and 54 mg/kg) was also efficacious in reversing the manipulation effects [40–42]. One of the major limitations of this model of mania is the number of control groups that is required for each study (vehicle alone, amphetamine alone, chlordiazepoxide alone, amphetamine + chlordiazepoxide, amphetamine + chlordiazepoxide + each treatment, amphetamine + each treatment, chlordiazepoxide + each treatment). In fact, a complete control comparison was conducted and revealed that chlordiazepoxide

alone often potentiated the locomotor activity-suppressant effects of treatments [39]. Therefore, it is unclear whether data from former studies simply represent additive drug effects. This fact complicates the assessment of potential novel anti-manic agents and the interpretation of obtained data. Another factor diminishing the predictive validity is the efficacy of lamotrigine and levetiracetam in studies assessing treatment effects since both drugs seem to have limited success managing acute manic episodes in patients [43–45]. Similar to the amphetamine only model, acute doses of both amphetamine and chlordiazepoxide and the treatment drugs were examined separately, further limiting the predictive validity of this model of human mania.

2.1.3 Quinpirole (D2 Receptor Agonist)

Apart from amphetamine, the manipulation of the dopaminergic system in animal models of mania can also be achieved by dopamine receptor agonists. The dopamine D2 and D3 specific agonist quinpirole was utilized in this context. Increased dopaminergic signaling via acute administration of quinpirole (0.5 mg/kg) results in hyperactivity, which was attenuated by chronic treatment (added to food chow) with valproate (1.2 g/kg daily), carbamazepine (0.8 and 1.2 g/kg daily), and topiramate (30 mg/kg daily [46]. However, topiramate was more effective than valproate and carbamazepine in attenuating the quinpirole-induced effect even though topiramate is not approved for treatment of mania and might in fact induce manic episodes in BD patients [47].

2.1.4 Ouabain

Not only pharmacological manipulation of the dopaminergic system but also manipulation of the energy metabolism is utilized to model human mania in animals. In this context, the adenosine triphosphate inhibitor ouabain has been evaluated. Acute i.c.v. application (5 μL 10^{-5} M) produces hyperactivity in rats which is attenuated by acute haloperidol (21 mg/kg) and sub chronic lithium (1.994 g/kg in rodent chow for 7 days) treatment [48–50]. Interestingly, one study demonstrated that a single i.c.v. infusion of ouabain (5 μL $10^{-2}/10^{-3}$ M) is sufficient to induce a lasting effect on activity levels for up to 7 days [51]. Next to the behavioral assessment of the ouabain animal model, levels of oxidative stress were analyzed. Several studies found elevated oxidative stress factors, whereas anti-inflammatory agents as well as BDNF levels were decreased [51, 52]. Chronic lithium (47.5 mg/kg twice a day for 7 or 13 days) and valproate (200 mg/kg twice a day for 7 or 13 days) improve most but not all measures [52, 53]. The effect of ouabain on oxidative stress highlights the high construct validity of this animal model since it is known that BD is associated with higher degrees of oxidative stress and decreased BDNF levels [54, 55]. However, as all animal models for human mania, the overall validity is challenged by several

parameters. The effect of haloperidol on the ouabain-induced hyperactivity is confounded by the reduction of activity levels in haloperidol only experiments [50]. Furthermore, it is important to note that ouabain induces hypoactivity in dark, small arena setups contrary to the hyperactivity seen in the open field test. Here, pretreatment with lithium (daily 2.3 mEq/kg for 7 days) increased locomotor activity. However, lithium's effect on reducing activity might have been masked by already low baseline locomotor activity levels due to habituation to the environment prior to testing [56]. A novel and anxiogenic environment seems to be required to see an ouabain-induced increase in activity [57].

2.2 Environmental Animal Models of Mania

Even though the pathophysiology and etiology of BD is still not fully deciphered, the notion that the disease is a result of an interplay of several genetic predispositions and environmental factors is well established [58, 59]. With regard to environmental factors, stressful events such as unpleasant experiences or unstable sleep patterns seem to provoke mood episodes in BD patients. Circadian rhythm disturbances have been shown to trigger or exacerbate mood symptoms in humans [60, 61]. For example, it is known that disturbances in the wake–sleep cycle of BD patients increase the risk of mood episodes or worsens them [62–65]. Several treatment or therapy options, namely, sleep deprivation, interpersonal social rhythm therapy (IPSRT), and bright light therapy (BLT), intervene in the circadian rhythm of patients to achieve symptom relief. Sleep deprivation has been clinically used for a long time to achieve prompt anti-depressant effects in suicidal and depressed patients [66, 67]. IPSRT emphasizes the importance of daily routines including wake and sleep times and is designed to stabilize disrupted circadian rhythms [68]. Also effectively used and well established in the therapy of patients suffering from seasonal affective disorder or major depressive disorder is the BLT [69–71] in which patients are exposed to bright light (10000 lux) for a certain time. In stark contrast, exposure to bright light during the BLT might even trigger a switch to manic or hypomanic episodes in BD patients [72]. Given all of this data suggesting a critical involvement of the circadian system in the pathophysiology of BD, several genetic mutants targeting the circadian clock (*see* Chap. 2c) and animal models exposed to a manipulated sleep rhythm have been developed. The environmental animal models and their validity discussed here are summarized in Table 2**.**

2.2.1 Sleep Deprivation

With first studies already being conducted in 1979, rodent sleep deprivation has a long history of being utilized in research of human mania [73–75]. The canonical paradigm typically involves 72 h of sleep deprivation. This is ensured by placing the rodents on a small platform that is surrounded by water. As soon as the rodent falls asleep, the associated relaxation of muscles results in the animal

Table 2
Environmental models of mania

Model	Validity			Mania-related behavior	Limitations	References
	Face	Predictive	Construct			
Sleep deprivation	+	+	•	Hyperactivity, insomnia, aggression, hypersexuality, increased stereotypy	Various stressors might contribute to behavioral phenotype	[73–76]
Circadian rhythm disruptions	•	?	+	Less anxiety and depressive-like behavior	Studies show contradictory results, predictive validity not assessed	[94, 97]
Resident intruder paradigm	•	+	•	Aggression	RI paradigms usually used for depression models, paradigm sensitive to anxiolytic and anxiogenic drugs – possibly anxiety-related phenotype	[116, 123]

Green plus sign indicates good validity; yellow dot indicates moderate validity; black question mark indicates no available data on validity
RI resident–intruder

falling in the water and thereby waking up the rodent. Sleep-deprived animals display a variety of mania-like behavior that ranges from hyperactivity, insomnia, and aggression to hypersexuality and increased stereotypy. This behavior peaks 30–40 min after the end of the sleep deprivation paradigm [73–75] and can be attenuated by lithium treatment during testing (added to chow; 0.7–0.1 mEq/L serum levels) or haloperidol administration (0.2 mg/kg) in the last hours of sleep deprivation [76]. A more recent study revealed individual differences in response to this circadian rhythm disruption: mice with a delayed normalization of sleeping behavior after being deprived are more susceptible to quinpirole-induced hyperactivity 10 days after the sleep deprivation [77] which suggests a somewhat lasting effect of this short-term manipulation on mania-like behavior. Another study investigated the effect of sleep deprivation on protein kinase C (PKC) activity levels [78]. PKC is an enzyme implicated in neurotransmitter release; it regulates the neuronal excitability and is involved in long-term alterations of gene expression and neuronal plasticity [79, 80]. It is known that BD patients exhibit elevated PKC activity especially during manic phases [81] and that treatment with the mood stabilizers lithium or

valproate inhibits the PKC [82, 83]. Moreover, increased PKC activity has been associated with locomotor hyperactivity, risk-taking behavior, and increased hedonia [78]. Interestingly, animals that were formerly sleep-deprived for 4 h display increased PKC-mediated phosphorylation of AMPA receptors in the frontal cortex. This overactivity can be reversed by chronic lithium treatment (1.2 g/kg and 2.4 g/kg added to chow [78]. Overall, the sleep deprivation animal model for mania holds high face validity since it displays several facets of mania-like behavior beyond hyperactivity. Likewise, the predictive validity is adequate given the efficacy of both chronic lithium and valproate. Altered PKC signaling as well as the fact that acute circadian rhythm and sleep pattern disruption can precipitate mania in humans highlights the construct validity of this animal model. However, sleep deprivation in healthy subjects does not trigger full-blown episodes of mania, and considering that behavioral and signaling pathway changes in these studies have been seen in sleep-deprived but otherwise not manipulated healthy animals, the effectiveness of the treatments might only represent symptomatic treatment rather than specific treatment of mania. Furthermore, sleep deprivation is not the only stressor animals are exposed to in this paradigm. Due to the experimental design, animals experience prolonged isolation, immobilization, and water as an anxiogenic factor. However, it is noteworthy that one study examined differences regarding mania-like behavior between a sleep-deprived and a stress control group. This study indicates that sleep loss indeed seems to be the key for developing mania-like behavior [84].

2.2.2 Circadian Rhythm Disruptions

Besides total sleep deprivation, there have been attempts to model human mania in rodents by manipulating their photoperiod exposure. It is known that the visual perception of (natural) light phase shifts circadian rhythms [85, 86] by retinal inputs to the master pacemaker suprachiasmatic nucleus. Therefore, it is possible to interfere with the circadian clock of animal models by alternating light exposure duration. Several studies are investigating behavioral changes in animals exposed to extended resting phases [87–92] demonstrating a depressive-like phenotype similar to MDD or SAD in humans. Only few have examined the effect of extended activity phases in animal models [93–97] mimicking the decreased amount of sleep that, for example, BD patients experience during manic phases. In this paradigm, rodents are exposed to a dark/light cycle that deviates from the normal 12/12 h rhythm and is shifted toward a longer dark period (16 h dark/8 h light or 19 h dark/5 h light). Three weeks of adaption to the new light cycle are sufficient to impact rodents' behavior [95, 97]. Most of the studies analyzed the behavioral phenotype of these animals in the elevated plus maze and forced swim test. Experiencing long activity phases (i.e.,

exposure to shortened photoperiods) seems to induce mania-like behavior in both mice and rats that goes along with a neurotransmitter switch from somatostatin to dopamine, favoring dopamine during shortened photoperiods [94, 97]. These findings underline the construct validity as they support the dopamine hypothesis that postulates that depressive and manic episodes in bipolar disorder are due to hypo- and hyperdopaminergic signaling, respectively [98]. However, other studies have not seen any behavioral differences in animals exposed to extended activity phases or have even demonstrated depressive-like behavior in the elevated plus maze [93, 95]. This limited reproducibility of behavioral phenotypes, and contradictory results of these studies might be reflective of not standardized experimental design (light exposure duration: 16 h dark/8 light vs 19 h dark/5 h light, behavioral assessment during the animal's dark vs light phase, single- vs group-housing) since several studies have demonstrated the urgent need for standardization of behavioral testing protocols to enable precise interlaboratory comparison of behavioral data [99–101] with special regard to single vs group housing, which seems to have a huge impact on anxiety-related behavior because of stress induced by social isolation [102]. However, whether differences in the experimental setup explain the inconsistent outcomes is unclear and therefore clearly represents a limitation of this animal model. The predictive validity is still to be determined since, to the best of our knowledge, no pharmacological studies have been conducted yet. Nonetheless, altered circadian rhythms and disrupted circadian clocks are known to be implicated in bipolar disorder [103–106], and mood episodes can feature seasonality, [107] as well as distinct shifted circadian rhythms during (hypo-) manic versus depressive phases [108]. Therefore, given the major implications of the circadian clock in bipolar disorder and the demonstrated hyperdopaminergic activity in rodents exposed to extended activity phases [94, 97], this animal model holds high construct validity. Moreover, lithium is known to lengthen circadian periods [109–111]. Thus, further validating this animal model by assessing the effectiveness of mood stabilizers on photoperiod manipulation-induced behavioral abnormalities would be of great interest. The greatest limitation of this animal model is the questionable translationality regarding nocturnal animals used as a model in these studies versus diurnal humans suffering from bipolar disorder. Most of the abovementioned studies worked with the inbred mouse strain C57BL/6. However, it is controversial whether Bl6 mice are able to synthesize a detectable amount of melatonin, one of the most important hormones acting on the sleep–wake cycle in humans [112]. It has also been shown that melatonin levels are elevated during daytime in BD patients that experience an acute manic episode [108]. It is known that the synthesis of melatonin in

Bl6 mice is impacted due to a truncated enzyme in the synthesis pathway. Nonetheless, the synthesis in the pineal gland generally seems to be functional and mice express both melatonin receptors [113]. Still, the face validity of this animal model is diminished since the melatonin levels of Bl6 mice cannot be analyzed accurately. Otsuka et al. compared the behavioral phenotype of Bl6, CBA/N (melatonin-proficient) and ICR (melatonin-deficient) mice after exposing them to extended activity phases [95]. The behavioral effects of altered photoperiod lengths were Bl6 specific and neither seen in the melatonin-proficient nor the melatonin-deficient mouse strain. The authors therefore argued that the effect was melatonin pathway-independent. However, it is important to note that Otsuka and colleagues have seen depression-like behavior in mice exposed to extended activity phases contrary to the mania-like behavioral phenotype that was intended to be induced by extended activity phases. As humans are diurnal, studies have also proposed that utilizing nocturnal rodents in circadian rhythm-related research might be ill-suited. Challet reviewed the conformities and differences in the circadian system of diurnal and nocturnal rodents [114]. A lot of SCN-related processes seem to be similar such as the melatonin secretion (peaks during the night in both diurnal and nocturnal animals) and the clock genes expression. He concluded that arousal-dependent factors modulate opposite effects, but the synchronizing effects of light are the same in both nocturnal and diurnal rodents. Since this animal model focuses exclusively on the alteration of photoperiod lengths, the manipulation is solely arousal-independent and therefore probably has the same effects on diurnal animals. Nevertheless, several studies have worked with diurnal animals. For example, Goda and colleagues compared neurotransmitter levels of serotonin (5HT) and dopamine in nocturnal and diurnal rodents exposed to both extended and shortened activity phases [96]. The authors found increased 5HT levels in the dorsal raphe nucleus and the amygdala after exposure to long day conditions (16 h light/8 h dark) in mice and diurnal chipmunks, suggesting the 5HT system responses to photoperiod lengths to be similar. However, the dopaminergic system revealed different, partly opposite responses: extended photoperiods lead to an increase of dopamine in the hypothalamus of mice but to a decrease of dopamine in the hypothalamus of chipmunks. As the dopaminergic system is broadly involved in mood and behavioral functions, these findings need to be considered with regard to translational research. For the detailed protocol of this animal model we use in our lab see Chapter 4a.

2.2.3 Resident–Intruder Paradigm

Human mania is often accompanied by increased aggressive behavior as well as agitation, and stress is frequently associated with changes in mood state in BD patients. Therefore, paradigms to induce this behavior in rodents have been established. Utilized

paradigms are variants of the resident intruder paradigm. During this task, a group-housed intruder is introduced into the home cage of an individually housed resident rodent [115]. Aggressive behavior (biting, tail rattling, threatening postures) committed by the resident and defensive acts of the intruder are quantified. Treatment with chronic lithium is able to alleviate aggressive attacks (for review see [116]) without affecting the amount of nonaggressive interactions [117]. Exposure to acute stressors such as foot shocks or prolonged isolation is able to increase the aggressive phenotype of the resident [118, 119] which then in turn can be attenuated by chronic lithium administration (20 mEq/L presented in drinking bottle [120]). However, it should be noted that lithium treatment decreases shock responses in the jump–flinch test [121], which might confound the effect of lithium on reducing shock-induced aggression in the resident intruder task. Nevertheless, mood stabilizers are efficient in reducing aggressive behavior in the resident rodent, and, in contrast, chronic application of antidepressants is able to augment aggressive behavior [122]; therefore this paradigm might represent a model for human mania and antidepressant-induced manic episodes in BD patients with good predictive validity. A validity limiting factor is the canonical employment of this task as a model for depression due to its social defeat characteristics.

Inspired by the resident intruder task, another paradigm was postulated as an animal model for human depression and mania. This test focuses on dominant–submissive behavior of rodents and is based on the reduced locomotor activity and increased submissive behavior of depressed humans (or subordinate animals) versus elevated locomotor activity and dominant behavior that manic patients (or dominant animals) typically display [123]. In this paradigm, two rodents are competing for food by being placed in opposite chambers which are connected via a narrow tunnel that allows only one animal at a time to have access to the feeder. Over a period of 2 weeks with daily 5 min trials, approximately 50% of the animal pairs develop a submissive–dominant relationship. Submissive rats treated with antidepressants (imipramine, desipramine or fluoxetine (all administrated as 10 mg/kg for 21 days) become more assertive and gain significantly more access to the food source over a time course of 2–4 weeks. Conversely, dominant animals that are treated with mood stabilizers (lithium (100 mg/kg), carbamazepine (20 mg/kg), or valproate (30 mg/kg)) become less aggressive and lose their dominance over the feeder [123, 124]. A strong indicator for the predictive validity of this animal model is the efficacy of lithium, carbamazepine, and valproate that all exert their effect only after 2–3 weeks of treatment as well as the time course of the antidepressant treatments. Both suggests a similar timeline of treatment effects compared to humans since only chronic treatment with mood stabilizers and often also

antidepressants alleviates symptoms in BD and major depressive disorder patients, respectively [22, 34, 125]. However, it is important to consider that resident intruder paradigms are also generally sensitive to anxiolytic and anxiogenic drugs which might indicate behavioral phenotypes that are due to anxiety-like rather than depression- and mania-like status [126]. Given that only half of the animal pairs develop a submissive–dominant relationship is a further validity diminishing aspect.

2.3 Genetic Animal Models of Mania

The major progress that has been made in molecular biology and the new tools that arose as a consequence during the last few decades provide novel methods to generate animal models. Targeted mutations allow manipulation of genes and alteration of protein functions that are specifically implicated in the pathophysiology of BD. Therefore, transgenic animal models often represent a reverse translation approach. However, it is crucial to acknowledge that knockouts or knockdowns of a single gene in animals are not able to represent a full resemblance of the pathogenesis of the human disorder since it is highly unlikely that BD is caused by a distinct gene mutation. Table 3 provides an overview of the genetic animal models listed in this chapter.

2.3.1 ClockΔ19 Mice

As mentioned before, circadian rhythm disruptions seem to have major implications in the pathophysiology of BD [103–106]. Based on that notion, several transgenic mouse models have been established that target genes and proteins that are involved in the circadian clock. One of the most researched and characterized animal model is the ClockΔ19 mouse. This mouse carries a single-base mutation in the *Clock* gene exon 19 that causes no CLOCK-mediated transcription which in turn then results in a severe disruption of circadian gene expression [127]. In humans, a polymorphism in the 3′ flanking region of the *Clock* gene has been associated with more frequent manic episodes as well as insomnia and a reduced need for sleep in BD patients [128]. All in all, this animal model holds high face, predictive, and construct validity. ClockΔ19 mice display decreased anxiety- and depression-like behavior, increased impulsivity and reward-seeking, locomotor hyperactivity as well as impaired decision-making and sensorimotor gating [129–133]. Furthermore, this model exhibits disrupted circadian rhythms and sleep [134]. Interestingly, anxiety-related and depression-like behavior naturally varies across the circadian day with a reduction in this behavior during the day that fluctuates back to wild-type levels during the night [135] which could be interpreted as attenuated rapid cycling between mania-like behavior and euthymia. Chronic lithium treatment (600 mg/L in drinking water for 10 days) is effective in normalizing anxiety- and depression-related behavior [136]. The construct validity is supported by the *Clock* polymorphism detected in humans suffering

Table 3
Genetic models of mania

Model	Validity			Mania-related behavior	Limitations	References
	Face	Predictive	Construct			
ClockΔ19 mice	•	+	+	Decreased anxiety- and depression-like behavior, increased impulsivity and reward-seeking, locomotor hyperactivity, as well as impaired decision-making and sensorimotor gating	Model might emulate addiction-related phenotype	[129–133]
Glycogen synthetase kinase 3β overexpressing mice	+	?	•	Increased locomotor activity, reduced depression-like behavior, impaired habituation	Another mouse model with increased GSK-3β expression (P- GSK-3β-KI) shows increased anxiety- and depression-related behavior	[145, 148]
Dopamine transporter knockdown mice	•	+	•	Hyperactivity in novel environments, increased goal-directed behavior and risk-taking behavior, impaired decision-making	Also utilized as models in ADHD and schizophrenia research	[151–154]
SHANK3 overexpressing mice	+	•	+	Hyperactivity, increased sensitivity to AMP, impaired sensorimotor gating, reduced depression-like, altered circadian locomotor activity	Chronic lithium has no effect	[165]
Ankyrin G 3-disrupted mice	•	+	+	Decreased anxiety-related behavior, increased locomotor activity during the light phase, increased reward-seeking behavior	No behavioral alterations at baseline (without additional stressor)	[172]
Glutamate–cysteine ligase modifier unit KO mice	+	?	+	Heightened sensitivity to AMP, impaired social behavior and sensorimotor gating, stress induces reduction in depression- and anxiety-related behavior, increased risk-taking behavior	Model has also been used for schizophrenia and Alzheimer's disease	[177]
Myshkin mutant	+	+	+			

				Hyperactivity, sensitivity to repeated amphetamine applications, increased novel object exploration, reduced anxiety, impaired sensorimotor gating, disrupted sleep pattern, altered circadian behavioral rhythms		[182, 184, 187]
GluR6 knockout mice	+	•	•	Hyperactivity, elevated sensitivity to AMP, increased aggression and risk-taking, reduced anxiety-like behavior	Null mutation does not emulate reduced GluR6 mRNA expression in patients, only lithium assessed for predictive validity	[201]
Forebrain-specific Plcg1 knockout mice	+	+	+	Hyperactivity, hyperhedonia, reduced anxiety- and depression-like behavior, cognitive deficits	Not all abnormal behavior is reversed by treatment	[203]
ERK1 knockout mice	+	•	+	Hyperactivity enhanced response to AMP, increased risk-taking, impulsivity and hedonia, reduced depression-like behavior	Efficacious agents alone reduce activity	[206]
BDNF-haploinsufficient mice	•	?	+	Hyperactivity, emphasized sensitivity to AMP, enhanced risk-taking, aggression, and hyperphagia	Patients might experience hypo- or hyperphagia, lacks specificity as a model for mania	[208, 209]
Bcl-2 heterozygous knockout mice	-	•	•	Increased risk-taking, increased locomotor response to AMP	Partially anxiety-like phenotype, poor efficacy of lithium	[214, 215]

Green plus sign indicates good validity; yellow dot indicates moderate validity; red minus sign indicates poor validity; black question mark indicates no available data on validity
GSK-3β glycogen synthetase kinase 3β, *ADHD* attention deficit hyperactivity disorder, *AMP* Amphetamine

from BD, as well as by the reduced circadian amplitude and delayed circadian phase [137] and the hyperdopaminergic signaling that stems from an enhanced dopamine release from ventral tegmental area neurons [130] that ClockΔ19 mice display. Nevertheless, a few factors are limiting the overall validity of this animal model. A cross species paradigm, the behavioral pattern monitor, was established to assess organization, level, and patterns of locomotor activity as well as goal-directed behavior in humans. Manic patients display hyperactivity with an elevated exploratory drive and increased linear, direct movements in this paradigm which is reflected in the so-called spatial d value. The spatial d value ranges from 1 to 2, whereas 1 represents a direct, linear path and 2 represents small, highly nonlinear movements. Since manic patients have expanded goal-directed behavior, their spatial d value is rather low [2]. In contrast, ClockΔ19 mutant mice tend to perform smaller, repetitive movements and have therefore a higher spatial d value compared to patients during a manic episode [138]. Furthermore, the circadian rhythm disruptions in these mutant mice are more severe than the one seen in humans suffering from manic episodes, since ClockΔ19 mice are not able to maintain behavioral rhythms under constant conditions which is not the case to this extent in BD patients [10]. Authors have also proposed that this model might emulate an addiction-related phenotype in addition to the mania-like status because of the hyperhedonic behavior seen in various reward-sensitivity, motivation, and self-administration paradigms [130–132]. Finally, next to the ClockΔ19 mice, transgenic mice have been developed with knockdown of *Clock* specifically within the ventral tegmental area. Surprisingly, these mice display a behavioral phenotype that is reminiscent of mixed states in BD since mice also exhibit increased depression-like behavior next to mania-like hyperactivity and elevated exploratory drive [139]. These findings complicate the interpretation of CLOCK's exact involvement in the behavior seen in ClockΔ19 mice.

2.3.2 Glycogen Synthetase Kinase 3β Overexpressing Mice

Polymorphisms in the gene coding for the glycogen synthase kinase 3 beta (GSK-3β) have been associated with BD and the degree of lithium response in patients [140]. Thus, transgenic mice overexpressing GSK-3β have been postulated as a model for human mania. GSK-3β is implicated in a variety of cellular functions such as gene transcription, neurogenesis, and apoptosis [141]. In this context, GSK-3β is also able to intervene in the circadian rhythm by phosphorylation of core circadian clock proteins [111]. Likewise, the transcription factor β-catenin is phosphorylated for ubiquitin-dependent degradation by the GSK-3β [142] which results in a reduced protein expression. Studies have revealed a significant reduction in β-catenin expression in postmortem brains of humans suffering from BD [143] supporting the construct validity of this

animal model. Furthermore, chronic lithium treatment leads to an inhibition of GSK-3β and therefore to an increase of gene expression due to higher levels of undegraded β-catenin and a modulation of the circadian rhythm via lengthening of circadian periods [109, 110, 144]. GSK-3β-OX mice display a mostly mania-like phenotype with increased locomotor activity, reduced depression-like behavior in the forced swim test, and an impaired habituation in the open field test [145]. However, an aspect that might stand in contrast to the phenotype of BD patients experiencing an acute manic episode is the increased acoustic startle response seen in GSK-3β OX mice [145] compared to the reduced response seen in humans [146]. Also contrary to the phenotype seen in individuals suffering from BD is the corticosterone response to acute stress which is similar to that of wild-type mice, while BD patients exhibit a blunted response [147]. Another mouse model with increased GSK-3β expression (viz., P- GSK-3β-KI) features increased anxiety-related and depression-like behavior in addition to hyperactivity and increased amphetamine sensitivity [148] which limits the construct validity together with reduced protein GSK-3β expression that has been demonstrated in postmortem brains of humans suffering from BD [143] opposingly to the overexpressed GSK-3β in this animal model. The predictive validity of GSK-3β OX mice as a model for human mania is still to be determined as no pharmacological studies have been conducted yet. However, it is important to consider that the effect of lithium and valproate on GSK-3β has already been confirmed in the mouse brain [149].

2.3.3 Dopamine Transporter Knockdown Mice

The so-called dopamine hypothesis states that mood episodes of BD are due to abnormal dopaminergic signaling and was postulated by Berk and colleagues in 2007 [98]. Indeed, studies in humans have revealed *DAT* gene polymorphisms as well as reduced DAT protein expression in humans suffering from BD [10, 150]. Based on these findings, animal models have been established that exhibit approximately 10% of DAT function compared to wild-type mice [151]. The modified mice display various mania-like behavior such as hyperactivity in novel environments, increased goal-directed behavior and risk-taking behavior, and impaired decision-making [151–154]. Chronic valproate administration (15 g/kg chow for 4 weeks) is able to reduce locomotor activity [133]. Some behavior however is contrary to that seen in BD patients. For example, even though DAT knockdown (DAT-KD) mice show increased goal-directed behavior, their spatial d value is greater compared to the value of manic patients [138, 153] indicating less direct, linear movements. DAT-KD mice also lack impaired sensorimotor gating during a prepulse inhibition task like it is observed in individuals suffering from BD [155]. Interestingly, a complete knockout of the *DAT* gene

(DAT-KO mice) induces impaired sensorimotor gating and hyperactivity in mice [156]. The predictive validity of DAT-KO mice is supported by the efficacy of the atypical antipsychotics clozapine and quetiapine which reverse the effect of lost DAT function in the prepulse inhibition task [157]. Summed up, DAT-deficient mice resemble several mania-like behavioral features but also exhibit face validity diminishing behavior. The construct validity is given by the altered dopaminergic neurotransmission. However, it is important to note that humans affected by BD express approximately 80% of healthy subjects' DAT levels, whereas DAT-KD mice only express 10% of wild-type DAT levels [158]. Furthermore, DAT-manipulated rodents are not specifically utilized as models for mania but have also been used in ADHD and schizophrenia research [159, 160] since abnormal dopaminergic signaling has also been implicated in these disorders.

2.3.4 SHANK3 Overexpressing Mice

The SHANK3 protein is a scaffolding protein that is crucial for synapse formation, maintenance, and modulation of the excitation/inhibition (E/I) balance which is located in the postsynaptic density of mostly excitatory synapses [161, 162]. Human studies have proposed an implication of altered SHANK3 function in the etiology and pathogenesis of BD [10, 163], but SHANK3 gene variants are also often associated with autism spectrum disorders [164]. Mice that express 150% SHANK3 (SHANK3-OX) display various mania-like behavior including hyperactivity, increased sensitivity to the locomotor-stimulating effect of amphetamine, impaired sensorimotor gating, reduced depression-like behavior in the tail suspension test, and altered circadian locomotor activity [165]. The abnormal locomotor activity, increased sensitivity to amphetamine, and sensorimotor deficits are attenuated by acute valproate treatment (200 mg/kg [165]). On the molecular level, it has been demonstrated that overexpressing SHANK3 promotes a shift toward excitation regarding the E/I ratio in the hippocampus [162, 165]. This might support the construct validity considering altered GABAergic signaling is suggested to be involved in the pathophysiology of BD [166, 167]. However, a more recent meta-analysis of proton magnetic resonance spectroscopy data revealed no significant differences in GABA levels in BD patients compared to controls [168]. Surprisingly, chronic lithium (presented in food resulting in serum levels of 0,091 mmol/L) has no effect on any of the mania-like behavior. This arguably improves predictive validity since a subset of BD patients fails to respond to lithium. Similar to the former animal models, an aspect possibly limiting the face validity is the elevated acoustic startle response that these SHANK3-OX mice exhibit.

2.3.5 Ankyrin G 3-Disrupted Mice

Another scaffolding protein that has been identified as an associated gene for BD in a meta-analysis of genome-wide association studies is Ankyrin G [169, 170]. This protein plays a role in the formation and maintenance of the axon initial segment and is therefore essential for action potential propagation [171]. Inspired by the results of the meta-analysis of the genome-wide association study, transgenic animals with a specific knockdown of Ankyrin G 3 (ANK3) in the dentate gyrus of the hippocampus have been developed (ANK3-DG-KD). These mice exhibit a decreased anxiety-related behavioral phenotype in the novelty-suppressed feeding task, the elevated plus maze, and the dark/light box as well as increased locomotor activity during the light and therefore rather inactive phase [172]. This data might indicate a disruption of the circadian rhythm in these animals. Behavioral abnormalities can be reversed with chronic lithium administration (85 mg/kg daily for 14 days). Next to the specific ANK3 knockdown in the dentate gyrus, a heterozygote animal model has been established that lacks the ANK3 1b isoform [172]. These mice have similar behavioral features compared to the ANK3-DG-KD mice. Additionally, they show increased reward-seeking behavior that supports the mania-like phenotype. Exposure to isolation leads to increased depression-like behavior in the heterozygote animals which manifests as increased latency to enter the open arms in the elevated plus maze and the light side of the dark/light chamber as well as in the immobility time in the forced swim test. Stress also normalized the elevated sucrose preference [172] suggesting some sort of stress-induced switch of mood episodes from mania-like to depression-like in the heterozygote ANK3 mice. However, it is important to mention that the face validity is diminished by the fact that both models display no behavioral alterations in the open field task, forced swim test, acoustic startle response, or prepulse inhibition without additional stressors (i.e., prolonged social isolation prior to testing). Still, the construct validity is great as the candidate gene was identified in a human GWAS meta-analysis.

2.3.6 Glutamate–Cysteine Ligase Modifier Unit KO Mice

Elevated oxidative stress levels have been described in brains of BD patients [54, 55], and postmortem studies have also revealed reduced glutathione (GSH) in the prefrontal cortex of humans suffering from BD [173, 174]. GSH is one of the major antioxidants synthesized in the cell and therefore protects against reactive oxygen and reactive nitrogen species [175]. Glutamate–cysteine ligase modifier unit (GCLM) knockout mice have approximately only 20–30% of wild-type GSH levels [176]. These mice are more sensitive to amphetamine-induced locomotor activity and show impaired social behavior and sensorimotor gating. After exposure to mild stress, GCLM-KO mice develop a reduced depression- and anxiety-related phenotype and exhibit increased risk-taking behavior [177]. Interestingly, a transient depletion of GSH leads to a

different behavioral phenotype that is more evident as impaired performance in cognitive tasks such as object and spatial working memory [178]. Unfortunately, predictive validity of these animal models has not been assessed yet. Furthermore, transient depletion of glutathione has also been used as a model for schizophrenia and Alzheimer's disease and therefore lacks specificity for bipolar mania [178, 179]. Nevertheless, the construct validity of this animal model is supported by the disrupted redox signaling and by the hyperdopaminergic state indicated by the increased sensitivity to amphetamine [177].

2.3.7 *Myshkin* Mutant

By backcrossing *Myshkin*-mutant mice with a C57BL/6NCr strain, a new animal model for bipolar mania has been developed. *Myshkin*-mutant mice were originally established as a model for epilepsy because their *Myshkin* allele contains a mutation in the coding region for the Na^+, K^+-ATPase α3 isoform that leads to its loss of function and consequentially promotes seizure-susceptibility [180, 181]. By crossing them with seizure-resistant C57BL/6NCr mice, mutant mice have been produced that display a range of mania-like behavior without being affected by excessive neuronal excitability. These mice show hyperactivity, magnified locomotor sensitivity to repeated amphetamine applications, increased novel object exploration, as well as reduced anxiety and impaired sensorimotor gating [182]. They also exhibit a disrupted sleep pattern and altered circadian behavioral rhythms that seem to be independent of deficits in molecular clock function [183]. Abnormal activity levels and anxiety-related behavior is normalized with chronic lithium (0.4% in chow for 28 days) and valproate (150 mg/kg daily for 28 days) treatment suggesting high predictive validity [184]. Intriguingly, next to pharmacological treatment, adjunct therapies such as prolonged exercise or chronic melatonin supplementation that are approved for treatment of BD patients [185, 186] are also effective in attenuating mania-like behavior and impaired sleep in the *Myshkin*-mutant mice [187]. Supporting of the construct validity of *Myshkin*-mutant mice, polymorphisms in the *ATP1A3* gene that encodes the Na^+, K^+-ATPase α3 isoform have been associated with BD [188]. Expression of the α2 isoform has also been reported to be decreased in specific regions of postmortem brains of individuals suffering from BD [189, 190]. Loss of Na^+, K^+-ATPase α3 function also seems to lead to impairments in the ERK signaling pathway [191]. As several regulators upstream of ERK have been identified to be involved in the pathophysiology of BD [192, 193], this once again underlines great construct validity of this animal model.

2.3.8 GluR6 Knockout Mice

The chromosome 6q has been linked to BD in several genome-wide studies during the last two decades [194–196]. GluR6 knockout mice hold great construct validity since the *glutamate 6 receptor (GluR6)* gene is located on the chromosome 6q21 [197]. Moreover, abnormal glutamatergic signaling and significantly reduced GluR6 mRNA levels in postmortem BD brains have been reported [198–200]. Indeed, mice lacking the *GluR6* gene display various mania-related behavior such as hyperactivity, elevated locomotor sensitivity to amphetamine, increased aggression and risk-taking, as well as reduced anxiety-like behavior [201]. Supporting the predictive validity, chronic lithium treatment (2.4 g/kg in chow for 4 weeks) is able to reverse this behavior. However, since lithium fails to effectively treat all BD patients, other approved treatments should be assessed to further validate this animal model. Providing another aspect limiting validity, BD patients may have reduced GluR6 mRNA levels but no complete null mutation like GluR6 knockout mice.

2.3.9 Forebrain-Specific Plcg1 Knockout Mice

Several components of the extracellular signal-regulated (ERK)/ mitogen-activated protein kinases (MAPK) signaling pathway have proposed to be altered in BD patients. This pathway mediates neuronal survival, growth, and plasticity but also apoptosis [202]. Based on this data, various transgenic mice have been suggested as an animal model for human mania. One of these models is the forebrain-specific *Plcg1* knockout mouse. A complete knockout of phospholipase Cγ1 leads to high lethality already at the embryonic state. Therefore, to enable behavioral assessment, the *Plcg1* is only ablated in the forebrain in this model. The *Plcg1* is activated by BDNF binding to its TrkB receptor and once activated hydrolyzes membrane-bound Pip_2 to generate IP_3. Plcg1 knockout mice exhibit diverse mania-like behavior including locomotor hyperactivity in the open field, increased hedonia, as well as reduced anxiety-like behavior in the elevated plus maze and reduced depression-like behavior in the forced swim test [203]. Interestingly, Plcg1-KO mice also display cognitive deficits in form of impaired learning and memory and conditioning paradigms. Most of the mania-related behavior can be reversed by lithium (300 mg/L in drinking water for 14 days or acute injection of 100 mg/kg) and acute valproate treatment (200 mg/kg [203]). Therefore, face and predictive validity of this animal model is great. However, similar to what is seen in most animal models of mania, the acoustic startle response is increased, possibly diminishing validity. Still, polymorphisms in the *Plcg1* gene have been linked to BD by several studies, highlighting the construct validity of this animal model [204, 205].

2.3.10 ERK1 Knockout Mice

Another animal model that is based on the involvement of the ERK/MAPK signaling cascade in the pathophysiology of BD is the ERK1 knockout mouse. ERK1 is a serine/threonine kinase that activates transcription factors as well as downstream protein kinases. In fact, ERK1 KO mice display a mania-like behavioral phenotype. They show locomotor hyperactivity (during the open field task and in a home-cage setting), enhanced responses to psychostimulants, increased risk-taking, impulsivity, and hedonia, as well as reduced depression-like behavior in the forced swim test [206]. Regarding the predictive validity of this animal model, lithium (2.4 g/kg given in chow) is not efficient to attenuate abnormal locomotor activity, but acute olanzapine (2 mg/kg) and chronic valproate (24 g/kg given in chow) treatment normalize activity levels and response sensitivity to amphetamine. However, this effect might be confounded by the reduction of activity levels induced by these drugs alone [206]. Still, this animal model might provide a tool to investigate differences between lithium responders vs nonresponders.

2.3.11 BDNF-Haploinsufficient Mice

As BDNF activates the ERK/MAPK signaling pathway, the neurotrophic factor is an essential component of this signaling cascade. BDNF-haploinsufficient mice express approximately 50% of wild-type BDNF protein throughout the brain [207]. Reduced BNDF levels seem to have an impact on the behavioral phenotype of these mice which is reflected in increased locomotor activity, emphasized sensitivity to psychostimulants, as well as enhanced risk-taking, aggression, and hyperphagia [208, 209]. However, it is noteworthy that BD patients may experience hyper- or hypophagia [210]. Neither of these two animal studies assessed the efficacy of treatment agents approved for mania. Therefore, the predictive validity of this animal model has not been determined. Still, BDNF has been suggested to be implicated in the pathophysiology of BD by several studies [31, 32], providing an aspect that supports the construct validity. A recent study has used the BDNF-haploinsufficient mice as a model for schizophrenia, diminishing the specificity for bipolar mania [207].

2.3.12 Bcl-2 Heterozygous Knockout Mice

The B-cell lymphoma 2 (Bcl-2) family consists of regulatory proteins in the ERK/MAPK signaling pathway that play a key role in cell regulation and neurogenesis by inhibiting apoptosis [211, 212]. There are several animal models targeting the Bcl-2 family for manipulations. For example, mice with elevated Bcl-2 levels show decreased anxiety-related behavior [213]. Contrary, Bcl-2 heterozygous knockout mice with therefore decreased Bcl-2 levels exhibit enhanced anxiety-related behavior [214]. Increased risk-taking and increased locomotor response to amphetamine administration (1 mg/kg) emulate the manic behavioral phenotype seen in bipolar patients [215]. Enhanced sensitivity to

amphetamine is attenuated by daily pretreatment with lithium (100 mg/kg). To the best of our knowledge, further studies assessing the efficacy of mood stabilizers in Bcl-2 heterozygous knockout mice have not been conducted. Some research suggests the upregulation of Bcl-2 by inhibition of protein kinase C as one of lithium's and valproate's mechanisms of action (for reviews see [82, 216]). However, a recent meta-analysis revealed that most studies were not able to detect a significant effect of lithium on Bcl-2 levels [217]. Therefore, proposing high construct validity based on this aspect is questionable. The somewhat mixed behavioral phenotype that Bcl-2 heterozygous knockout mice display and controversial effect of lithium on Bcl-2 indicates an overall rather poor validity of this animal model.

2.4 Strains with Intrinsic Mania-Like Behavior

Another strategy to model mania-like behavior in rodents independently from genetic manipulation is identifying strains whose intrinsic behavior imitates behavioral phenotypes seen in patients from BD. Mostly, two mice strains have been investigated in this context: Black Swiss mice and the Madison mice. Even though it might be possible to explore underlying mechanisms of behavioral abnormalities without any intervention in these animal models, it is important to consider that these rodents are not "sick" but rather display their physiological baseline behavior. Therefore, the translationality might be problematic. An overview of the validities and phenotypes of mania-like strains included in this chapter can be found in Table 4.

Table 4
Strains with intrinsic mania-like behavior

	Validity					
Model	**Face**	**Predictive**	**Construct**	**Mania-related behavior**	**Limitations**	**References**
Black Swiss mice	+	+	-	Hyperactivity, heightened sensitivity to AMP, increased aggressive behavior, risk-taking and hedonia, decreased depression- and anxiety-like behavior	Not all behavioral alterations are reversed by treatment with mood stabilizers	[23, 218]
Madison mice	+	•	+	Increased in-cage locomotor activity, heightened sex drive, decreased despair-like behavior, altered circadian behavior	Limited efficacy of treatment agents	[223, 224, 226]

Green plus sign indicates good validity; yellow dot indicates moderate validity; red minus sign indicates poor validity
AMP amphetamine

2.4.1 Black Swiss Mice

By crossing Swiss outbred and C57BL6/JN inbred, Black Swiss mice have been developed. This mouse strain models a range of mania-like behavior including locomotor hyperactivity, heightened sensitivity to amphetamine; increased aggressive behavior, risk-taking, and hedonia; as well as decreased depression-like and anxiety-related behavior [218]. The validity of this model is further supported by the efficacy of lithium (200 mg/kg twice a day throughout testing) and valproate (100 and 200 mg/kg twice a day throughout testing) in normalizing reward-seeking behavior, aggression, and locomotor response to psychostimulant application. Additionally, valproate (100 and 200 mg/kg twice a day throughout testing) attenuates the depression-like phenotype. Overall locomotor activity and risk-taking behavior are affected by neither of these mood stabilizers. [218, 219]. Asenapine, an atypical antipsychotic, which is also approved for bipolar mania [220], is more efficient in normalizing various mania-like behavior in the Black Swiss mice compared to lithium and valproate (asenapine: 0.3 mg/kg twice daily for 3 days [221]). Regarding the construct validity of Black Swiss mice, β-catenin protein levels are reduced in the hippocampus, whereas BDNF brain levels are not altered [23] which stands in stark contrast to reduced BDNF protein levels in the hippocampus of BD postmortem brains [222] and therefore diminished the construct validity of this animal model. Generally, a factor suggesting overall high validity is the increased genetic heterogeneity as it is physiologically the case in outbred mouse strains, because high genetic heterogeneity might be more representative of the interplay of various factors (biological, genetic, and environmental) that leads to the development of BD compared to single-gene mutations induced in genetic animal models of mania. Taken together, Black Swiss mice hold a high face and predictive validity whereas the construct validity needs more investigation.

2.4.2 Madison Mice

Another mouse strain with a naturally occurring set of behavioral characteristics that mimic manic episodes in BD patients is the Madison strain. These inbred mice display increased in-cage locomotor activity, heightened sex drive, and decreased despair-like behavior in the forced swim test. Furthermore, they show altered circadian behavior with reduced resting phases [223, 224]. Treatment with the atypical antipsychotic olanzapine (1 mg/kg daily) normalizes sleep patterns and hyperactivity, while lithium (0.2–0.4% chow throughout experiments) only attenuates the locomotor activity [223]. Further assessment of pharmacological agents such as valproate might be of interest to expand the predictive validity of this model. Two studies established the construct validity of this mouse strain as an animal model for mania by identifying gene variants and altered gene expression in the Madison mouse that have been implicated in the pathophysiology of BD [225, 226].

3 Animal Models of BD Displaying a Switch in Behavioral Phenotypes

Investigating and exploring the molecular mechanisms underlying the mood switches that are characteristic for bipolar disorder remains one of the most critical steps in determining the pathophysiology. As mentioned before, modeling both the depressive and the manic pole within one animal is the most challenging part developing animal models for BD. Some of the animal models noted above display such a switch in their behavioral phenotype to a certain extent. For example, behavioral aspects of the ClockΔ19 mutant mice vary in a circadian manner or manipulation of the photoperiod exposure and therefore shortening or lengthening the activity phase results in depressive-like and mania-like behavior, respectively. However, only few models exist that emulate various facets of the switch in mood episodes seen in BD patients within the same animal. Table 5 provides a summary of the phenotypes and validity of these animal models.

3.1 Amphetamine Sensitization

There have been several approaches to model BD by administration and withdrawal from psychostimulants since it has long been hypothesized that sensitization of the dopaminergic system upholds both the manic and the depressive episodes in BD patients [98]. Already in 1978 it has been demonstrated that abuse and subsequent withdrawal from amphetamine produces manic and depressive states that are reminiscent of bipolar mood episodes in humans [227]. Several studies in animals have used protocols of administration and withdrawal of amphetamine to induce mania-like or depression-like behavior, respectively, in rodents. However, only two studies focused on applying a protocol that produces both the mania- and the depressive-related behavioral phenotype within one animal [228, 229]. This sensitization protocol consists of daily injections of amphetamine (1.8 mg/kg) on 5 consecutive days and a subsequent withdrawal period of up to 31 days. Withdrawal from amphetamine results in depressive-like behavior that is represented by decreased sucrose preference and deficits in motor memory, specifically in spontaneous improvement. BD patients often suffer from psychomotor impairments, and individuals suffering from depression show no spontaneous improvement [230–232]. Interestingly, the functional outcome of mice has also been assessed since bipolar disorder is oftentimes accompanied by poor functional outcome. Here, nest-building typifies as an ethologically relevant activity of daily living, and in fact, nest-building is impaired in amphetamine-sensitized mice [229]. During the withdrawal period, amphetamine-sensitized mice challenged with either a low dose of amphetamine or a restraint stressor exhibit locomotor hyperactivity, whereas the baseline locomotor activity of amphetamine-sensitized mice is not altered [229]. Therefore,

Table 5
Animal models of bipolar disorder displaying a switch in behavioral phenotypes

Model	Validity			BD-related behavior	Limitations	References
	Face	Predictive	Construct			
Amphetamine sensitization	+	•	+	Depression-related: decreased sucrose preference, deficits in motor memory, impaired nest-building Mania-related: locomotor hyperactivity after AMP challenge or stressor	Limited facets of mania, treatment efficacy only assessed for mania-like behavior	[228, 229]
D-box-binding protein knockout mice	•	•	+	Depression-related: abnormal circadian rhythms, disrupted sleep homeostasis, hypoactivity, blunted response to AMP Mania-related: hyperactivity, increased drug-taking after exposure to chronic stress or acute sleep deprivation	Efficacy of lithium not assessed, chronic stress might lead to anxiety-related behavior	[236]
Inducible cortical overexpression of dopamine D1 receptor and termination thereof	+	?	+	Depression-related: increased learned helplessness (LH), reduced activity, decreased hedonia, increased anxiety-like behavior after termination of overexpression Mania-related: emphasized sexual drive, increased drug conditioning and consumption, elevated impulsivity, hyperhedonia, reduced anxiety-related behavior during overexpression	Predictive validity has not been determined	[240–242]

Green plus sign indicates good validity; yellow dot indicates moderate validity; black question mark indicates no available data on validity
AMP amphetamine

mice display various bipolar disorder-relevant behaviors that are dependent on the environmental challenge they are exposed to with euthymic-like baseline locomotor activity after sensitization, depression-like behavior during the withdrawal period, and mania-related behavior after exposure to the stressor. The predictive validity of this animal model has been tested with lithium (100 mg/kg) and quetiapine (1 and 3.2 mg/kg). Lithium decreases locomotor hyperactivity in both amphetamine-sensitized and vehicle-treated mice to a similar extent after an amphetamine challenge. Quetiapine's effect, however, is more pronounced in amphetamine-sensitized mice compared to the vehicle control group [229]. The behavioral phenotype of this animal model provides not only validity but also the alterations on molecular and neuronal circuit level. Amphetamine-sensitized mice display an increase in neural circuit activation in several brain regions as it has been assessed by Arc mRNA measurements. Correlation analyses of Arc mRNA levels revealed decreased functional connectivity between parts of the frontal cortex and the striatum, whereas connectivity between the amygdala and hippocampus seems to be increased in amphetamine-sensitized animals [229]. Both alterations in neural circuit activation and functional connectivity have been proposed to be implicated in the pathophysiology of BD, highlighting the level of construct validity [233]. Additionally, histone modifications are altered in BD patients [234]. In fact, a histone implicated in stress responses exhibits higher degrees of methylation in the hippocampus of sensitized mice compared to the vehicle-treated group. Summed up, the amphetamine sensitization protocol seems to provide an animal model with high face and construct validity. Nonetheless, similar to other psychostimulant paradigms, animals display locomotor hyperactivity as the only facet of mania. Furthermore, the predictive validity needs more investigation regarding the efficacy on depression-like behavior.

3.2 D-Box-Binding Protein Knockout Mice

Similar to the amphetamine sensitization model, the circadian gene D-box-binding protein knockout (Dbp KO) mice exhibit a switch in their behavioral phenotype after exposure to stressors. The Dbp is a basic leucine zipper transcription factor which is regulated by the CLOCK protein and therefore a component of the circadian clock [235]. As expected, Dbp KO mice exhibit abnormal circadian rhythms and disrupted sleep homeostasis. Moreover, mutant mice are hypoactive and show a blunted response and stereotypy following amphetamine administration (10 mg/kg methamphetamine). This somewhat depressive-like behavior switches to a mania-like phenotype (i.e., hyperactivity and increased drug-taking) after exposure to chronic stress or acute sleep deprivation [236]. Treatment with valproate (200 mg/kg) prior to sleep deprivation prevents the switch to mania-related behavior. The efficacy of lithium has not been assessed in the study but might be helpful in further

validating predictability. As noted above, the circadian clock of BD patients is altered, and several circadian clock genes seem to be heavily implicated in the pathophysiology. Therefore, manipulation of a circadian clock component holds high validity. Several other aspects are further supporting the construct validity of this animal model. The *Dbp* gene, proposed as one of the highest-ranking candidate genes related to BD [236], seems to be affected in individuals suffering from BD, and its expression is influenced by lithium [237]. A potential limiting factor is the exposure to long-term stressor which is required to induce mania-like behavior in this animal model. Whether this also produces anxiety- and depression-related behavioral characteristics has not been determined yet. However, it is important to consider that stressful experiences can trigger mood episodes in BD patients.

3.3 Inducible Cortical Overexpression of Dopamine D1 Receptor and Termination Thereof

Another animal model based on the dopamine hypothesis has been established by Freund and colleagues. As aforementioned, the hypothesis states that mood episodes of BD are due to hyper- and hypo-dopaminergic transmission [98]. During a manic state, excessive dopamine levels activate endogenous, homeostatic mechanisms that lead to a secondary downregulation of key components of the dopaminergic system in the pre- and post-synapse including dopamine receptors. This negative feedback loop then results in a reduced dopaminergic signaling, and the depressive episode is initiated. Subsequently, a secondary upregulation of dopaminergic system elements takes place, and another manic episode begins [98]. Former studies revealed the potential impact of the dopamine D1 receptor (D1R) in the pathophysiology of BD. Positron emission tomography and single-photon emission computed tomography demonstrated elevated D1R levels and decreased binding capacity in humans [238, 239]. Inspired by these observations, an animal model was developed that mimics the cyclical nature of BD. Through the introduction of an inducible lentiviral system to the medial prefrontal cortex, an overexpression of the D1R and a termination thereof can be accomplished. Indeed, overexpression results in emphasized sexual drive, increased drug conditioning and consumption, elevated impulsivity in a delay discounting task, and hyperhedonia in the two-bottle preference test. Anxiety-related behavior in the elevated plus maze is reduced [240]. Therefore, mania-like behavior can be produced by overexpressing the D1R in the medial prefrontal cortex of rodents. Intriguingly, termination of the D1R overexpression induces depressive-like behavior which is reflected in the triadic paradigm of learned helplessness, a reduction in activity, and a decreased hedonia in the sucrose preference test [241]. Additionally, animals display elevated anxiety-related behavior in the marble-burying test after termination of the D1R overexpression which is positively correlated with increased interleukin-6 levels in the hippocampus [242]. The latter supports

the construct validity of this animal model since it has been suggested that levels of the pro-inflammatory cytokine interleukin-6 are elevated in the blood serum of BD patients [243]. Another study, however, only found higher degrees of interleukin-6 in BD patients during a manic or euthymic episode [244]. Even though the data regarding interleukin-6 is not consistent, a general pro-inflammatory state is well established to be part of the pathophysiology in BD [245]. Another aspect regarding the construct validity is the expression of the dopamine D2 receptor (D2R) in the nucleus accumbens. While bipolar patients with psychotic symptoms show elevated levels of D2R in the nucleus accumbens [246], rodents exhibit a reduced levels of D2R in this brain region during viral overexpression [240]. In spite of this discrepancy, it is very interesting and worth of consideration that the availability of D2R and D3R seems to be lower in impulsive individuals [247, 248]. Indeed, animals exhibit increased impulsivity-related behavior during the D1R overexpression [240], and impulsivity is considered a trait characteristic of bipolar patients [249]. Overall, this animal model has great face validity showing both mania- and depressive-like behavior within one animal independently of exposure to external stressors and holds high construct validity considering the dopamine hypothesis as well as the increased inflammation seen during the depressive-like state. To further validate the model, the efficacy of mood-stabilizing agents approved for BD needs to be assessed. For a detailed protocol of this animal model *see* Chap. 4b.

4 Protocols

4.1 Circadian Rhythm Disruptions

4.1.1 Animals and Housing

Adult, male C57Bl/6J mice (P60) are either group-housed or housed individually depending on behavioral test requirements (e.g., food restriction for weight control and motivation) under standard housing conditions (22 ± 2 °C, 45 ± 5% humidity). The control group is housed in a 12/12 h dark/light cycle, whereas the experimental group is adapted to a 19/5 h dark/light cycle for 3 weeks prior to the behavioral testing. Previous studies have shown that a switch of the dark/light cycle for 3 weeks beforehand is sufficient to impact mice behavior [95, 97]. Switching to the new dark/light cycle happens abruptly by simply extending the dark phase to 19 hours on day 1 of the adaption.

4.1.2 Equipment

– Scantainer, completely opaque and lined with white LED lights connected to a clock timer or housing of the two groups of mice in different rooms to ensure correct photoperiod manipulation. If a clock timer is used, make sure it is plugged into the emergency power outlet in case of power outage.

– Darkened room illuminated with red LEDs to allow supervision of animals during behavioral tasks.
– Camera that can be adjusted to dim light conditions for recording animals during the behavioral tasks.

4.1.3 Time Considerations

Animals need to be habituated to the altered dark/light cycle for 3 weeks before any behavioral testing can be performed. During habituation, animals only need to be provided with food, water, fresh bedding, and daily visual inspection. Therefore, time expenditure is minimal.

4.1.4 Troubleshooting and Critical Parameters

1. **Testing conditions:** Since this animal model is based on the disruption of the circadian clock, behavioral testing has to be conducted in the dark, during the mice' active phase in order to prevent any further disturbance of the circadian rhythm. Do not start behavioral testing earlier than 1 hour after the lights go off.
2. **Counteracting stress:** Manipulation of the photoperiod exposure can be interpreted as a chronic mild stressor. It is known that housing conditions can counteract stress-related behavior (i.e., anxiety-relevant behavior and impaired learning [102, 250–255]. Effects of altered photoperiod duration might therefore be dampened in group-housed animals and/or animals provided with an enriched environment. To avoid stress artifacts in the mice' behavior, do not change cages or bedding shortly before behavioral testing starts.
3. **Reward habituation:** If behavioral tasks that involve a reward of any kind are conducted (e.g., learning paradigms or impulsivity tasks), it might be beneficial to habituate the animals to the reward a few days before testing starts. This prevents initial shyness around the new food.
4. **Choice of mouse strain and age:** As the sleeping hormone, melatonin is a substantial component of the sleep–wake cycle in humans, and levels have been found to be affected in some psychiatric disorders. For example, daytime levels of melatonin are elevated in acute manic patients [108]. Therefore, assessment of melatonin levels during the experiment or after the animal is sacrificed might be of interest. However, not all laboratory mice synthesize detectable amounts of melatonin in the serum or produce the hormone in a rhythmic manner. For example, it is known that melatonin synthesis in C57Bl/6J mice is impacted due to a truncated enzyme in the synthesis pathway. Nonetheless, the general synthesis on the pineal gland seems to be functional and mice express both melatonin receptors [113]. Still, the literature is divided by disagreement whether melatonin levels of C57Bl/6J mice or mouse strains

with a C57Bl/6J genetic background are analyzable [112]. This has to be taken into consideration when planning the experiment and choosing a suitable mouse strain. Furthermore, circadian rhythms change with age in both humans and mice [256, 257]. Consequently, mice should be age-matched throughout the experiment.

4.2 Inducible Cortical Overexpression of Dopamine D1 Receptor and Termination Thereof

4.2.1 Animals and Housing

Adult, male rats weighing 350–375 g are group-housed under standard conditions (22 ± 2 °C, 45 ± 5% humidity and reversed 12/12 h light/dark cycle – lights on 7 pm–7 am) with food and water access ad libitum. Our lab uses Sprague–Dawley rats but any wild-type rat strain is suitable. However, take potential intrinsic differences in baseline behavior between rat strains into consideration when planning your experiments. Behavioral testing is preferentially conducted during the rat's active and therefore dark phase under red fluorescent light allowing the experimenter to observe the animal.

4.2.2 Equipment and Materials

- Appropriate medication for (local) anesthesia and analgesia approved by your Animal Welfare Agency as well as syringes and cannulas for subcutaneous and intraperitoneal injections. The step-by-step protocol below states medication and dosage used in our lab.
- Hamilton syringe (Gastight™ syringe, #1701) with attachment (Hamilton, #65461-02) or custom-pulled glass pipette (tip diameter ~10 μm) with tubing connected to a regular syringe for pressure injection of the viral vector.
- Tetracycline-on inducible lentiviral vector containing the rat dopamine D1 receptor (or a fluorescent protein for the control group) and a CamKIIα promoter to enable specific expression in glutamatergic neurons (ca. 10^9 transducing units per mL [241]).
- Isoflurane vaporizer (Anesthesia Unit, Univentor 410 or equivalent) and connected anesthesia nose mask to ensure steady anesthesia while stereotactic surgery
- Induction chamber (plexiglass, 19.5 × 10.5 × 9.5 cm).
- Stereotactic frame with attached micromanipulator (RWD, #E03135-002 or equivalent) providing a steady fixation of the animal's head and allowing exact determination of stereotactic injection coordinates.
- Sterile surgery equipment (small scissor/electric shaver, forceps, scalpel, bulldog clamps, cotton swabs, dental's drill (SMT, K-38 or equivalent)), antiseptic solution of preference as well as suture material and paper towels.

- Syringe pump (in case you are using a syringe for pressure injections; kdScientific, LEGATO® 131 or equivalent).
- Doxycycline to induce virus expression.

4.2.3 Surgical Procedure

- Ensure sufficient intrasurgical analgesia by subcutaneous injection of buprenorphine (0.04 mg/kg) 20 minutes prior to anesthetic induction.
- Load Hamilton syringe with 2 μL virus (1 μL for each injection site).
- Induce initial anesthesia by placing the animal in an induction chamber filled with 4–5% isoflurane oxygen mixture until animal is deeply anesthetized which is indicated by nonexistent reflexes (eye blinking and toe pinch) and deeper breathing.
- Place animal in the stereotactic frame, cover the animal's nostrils with an anesthesia nose mask connected to the isoflurane vaporizer, and set isoflurane to 1.5–3% to ensure steady anesthesia during the surgical procedure. Adjust isoflurane concentration according to the animal's physiological conditions throughout the entire surgery.
- Secure animal's head in the stereotactic frame using ear bars, and apply eye gel to prevent dehydration of the eyes. Make sure the head alignment is straight and the fixation is steady.
- Shave surgical site with either a small scissor or electric shaver. Disinfect the scalp and ensure local anesthesia by applying 2% lidocaine hydrochloride with a cotton swab.
- Once again check for the animal's anesthetic state by verifying a nonexistent toe pinch reflex and adjust the isoflurane concentration accordingly if needed.
- Expose the cranium by making a sagittal incision (1–1.5 cm) with a scalpel starting at the most rostral point right at the edge of the nose mask.
- Again, apply the local anesthetic lidocaine hydrochloride on the wound margin, and expose the cranium using bulldog clamps on both sides.
- Check the straight and steady alignment of the animals head in the stereotactic frame once more (ears on the same horizontal level and bregma and lambda on the same horizontal level). Adjust stereotactic fixation if necessary.
- Clean the skull with a cotton swab soaked in Ringer solution/ 70% ethanol.
- Determine injection sites (medial prefrontal cortex) on both hemispheres with the help of the micromanipulator and bregma as the point of origin (AP +2.7 mm; ML ±0.4 mm), and perform the craniotomies (approx. 1 mm in diameter) using a

dental's drill. If bleeding occurs, stop it with cotton swabs or hemogelatin sponges when necessary. Make sure craniotomies do not get clogged by coagulated blood.

- Place Hamilton syringe above a craniotomy, and lower it until it is placed right above the dura mater. Reset the micromanipulator. Now lower the syringe to DV −2.8 mm, and hold it in place for 5 min to allow tissue to recover from mechanical irritation.
- Start the pressure injection of a total volume of 1 μL virus with a flow of 0.1 μL/min. After the injection, hold the syringe in place for another 5 min to allow virus spread and recovery of the tissue before withdrawal.
- Repeat **steps 13** and **14** for the second injection on the other hemisphere.
- Carefully suture the wound with single button sutures and disinfect wound area.
- Remove animal from stereotactic frame, turn off isoflurane vaporizer, and ensure analgesia by subcutaneous injection of meloxicam (1 mg/kg).
- Return animal into its home cage, and keep it single housed for at least 1 h after the end of surgery or until animal returned to freely moving in its cage in order to avoid adverse interactions of cage mates. Use a heating lamp or heating plate, or wrap the animal in paper towels to prevent hypothermia until animal recovered from anesthesia.
- Provide post-surgery analgesia by administering meloxicam (p.o. 0.5 mg/kg) for 3 days.

4.2.4 Doxycycline Treatment and Termination

The expression of the tetracycline-on viral vector is induced by administering doxycycline (0.5 g/L) in drinking water for 7 days. For testing animals during the "on phase" (i.e., during overexpression of the dopamine D1 receptor or control fluorophore, respectively), animals are continuously treated with doxycycline until behavioral testing is completed or animal is sacrificed. For testing animals during the "off phase" (i.e., after the termination of the dopamine D1 receptor or control fluorophore overexpression), the doxycycline treatment is terminated after 7 days. 5 additional days after withdrawal and before behavioral testing starts ensure complete termination of the overexpression [241]. Animals do not need additional recovery time after surgery before doxycycline treatment is initiated.

4.2.5 Troubleshooting and Critical Parameters

1. **Assistance:** Concentrating on the surgical procedure and keeping an eye on the animal's breathing rate and anesthetic stability might seem like juggling tasks especially for unexperienced experimenter. Therefore, a second experimenter to assist as a helping hand is recommended.

2. **Anesthesia:** Experience has shown the tolerance of rodents to isoflurane anesthesia seems to vary between different strains and individual animals. This has to be considered when deciding on isoflurane dosage, and the animal undergoing surgery has to be monitored constantly while anesthesia is adjusted according to the animal's condition. Too low isoflurane concentration is usually indicated by a high breathing rate and toe pinch reflex. Contrary, too high isoflurane concentration is generally indicated by gasping respiration. The phase of anesthesia induction with 4–5% isoflurane has to be kept short to prevent overdosage.
3. **Hypothermia:** Narcotized rodents are prone to hypothermia due to impaired thermoregulatory mechanisms, and general anesthesia can cause rapid decrease in body temperature especially during long surgery and amid recovery time from anesthesia. A heating plate integrated in the stereotactic frame, a heating lamp, or paper towels to cover the cold surface or wrap the animal in might provide remedy. A recent study demonstrated pre-warming of animals prior to anesthesia induction with isoflurane can delay the onset of hypothermia [258]. However, make sure the heating plate is set to an appropriate temperature and the heating lamp is not placed too close to the animal to prevent overheating.
4. **Eye dehydration:** Keeping the eyes hydrated during surgery is important to avoid damage to the cornea. Make sure to reapply eye gel regularly. Using tinted instead of translucent eye gel is beneficial especially while working with bright lights at the surgical setup. The eye blink reflex will return soon during the recovery from anesthesia. However, depending on the form of anesthesia, the recovery will take some time. Therefore, reapplying eye gel at the end of surgery is recommended.
5. **Fixation and alignment in the stereotaxic frame:** When using a stereotaxic frame that has ear bars incorporated to fixate the animal's head, be careful adjusting the bars to avoid damage to the tympanic membrane. Nonetheless, a steady fixation is crucial to prevent any harm caused by yielding of the head in response to the pressure that is applied while performing craniotomies. Repeated small readjustments until the animal's head is completely secured are recommended. Straight alignment of the head must be accomplished by correcting the ear bars and vertical adjustments of the mouthpiece. Some stereotaxic frames provide a supplementary alignment tool to help position bregma and lambda on the same horizontal plane. A cannula fixed in the stereotaxic holder can also be used to check for an even gap between cannula and skull at all coordinates on an imaginary line between bregma and lambda.

6. **Bleeding:** While performing the craniotomies and the pressure injections of the viral vector, irritation of the tissue or minor damage of small blood vessels beneath the skull can cause bleeding. In most cases, bleeding stops on its own or can be easily arrested with cotton swabs. If that is not the case, hemogelatin sponges are a great tool to help arrest the bleeding. Make sure the bleeding has stopped and the craniotomies are not blocked by coagulated blood before lowering the Hamilton syringe or custom-pulled glass pipette into the brain to prevent clogging of the injection tool. Do not forcefully pressure inject if you notice any resistance.
7. **Placement verification:** After the behavioral testing has been conducted and animals have been sacrificed, the correct placement of the virus has to be verified. Therefore, usage of a viral construct that is tagged with either a fluorophore or a horseradish-peroxidase, which is expressed regardless of doxycycline treatment, is recommended.

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Chapter 14

Social Behavior Testing in Mice: Social Interest, Recognition, and Aggression

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Abstract

Humans spend the majority of our time interacting with others, and thus social skills are essential. Therefore, advances in our understanding of the neurobiological underpinnings of social behavior are of critical importance. Social behavior is a broad, complex category involving many different neural circuits, skills, and motivations. In this chapter, we describe assays of three aspects of social behavior well suited to testing in mice in the laboratory: social interest, social recognition, and aggression (in the context of a free social interaction). We also discuss general recommendations for social behavior testing and compare different scoring and data analysis options. This chapter intends to be a useful resource for researchers of varying experience levels and to provide protocols for three basic tests of social behavior in mice.

Key words Social behavior, Social investigation, Social interest, Social recognition, Aggression, Free social interaction, Behavioral analysis

1 Introduction

1.1 Why Study Social Behavior?

As a social species, we spend most of our lives interacting with others. Therefore, we benefit from advances in our understanding of the neurobiological underpinnings of social behavior. Many cognitive behaviors are related to, or dependent on, social interactions, and the majority of human learning is either social or takes place in a social context [1]. While clearly conveying several benefits (e.g., better defense of resources and against predators, improved thermoregulation, additional caregivers for offspring), social living also comes at a cost, including increased competition for resources, social cognitive demands, and increased stress associated with dominance hierarchy establishment and maintenance. Social behaviors are increasingly studied in mice as a controlled means to understand the etiology of human mood and anxiety disorders, as well as disorders related to social impairments, such as autism spectrum disorder, schizophrenia, and Williams syndrome [2].

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Mice are social animals that live in colonies and exhibit complex relationships and behaviors, such as sex-specific interactions, aggression, and dominance hierarchies [3]. Mice can recognize conspecifics through a variety of sensory modalities, including olfaction, vision, and touch. Social behaviors can be studied across the life span of the mouse, from ultrasonic vocalizations in neonates to a broad range of social behaviors in adults (e.g., social interest, social recognition, aggression and dominance, mating, maternal behavior [4–14]). Thus, "social behavior" is a very broad category that encompasses many different motivations, skills, and neural circuits. In this chapter, we focus on three aspects of social behavior than can be easily tested in laboratory mice: social interest, social recognition, and aggression in the context of a free social interaction ("resident–intruder test of free social interaction"; free social interaction indicates that neither mouse is confined or tethered but are allowed to interact freely).

1.2 Social Interest

Social interest is one of the most widely tested types of behavior across mouse models of ASD and represents one of the most fundamental aspects of social behavior. At the most basic level, measurement of social interest involves the observation of a subject mouse and a stimulus mouse interacting, which takes advantage of the natural tendency of a mouse to approach and interact with an unfamiliar conspecific through following, sniffing, allogrooming, and other such behaviors [3, 15]. To minimize confounds and keep focus on the social interest aspect of behavior, stimulus mice are usually wild-type controls of the same sex (minimizes mating-motivated behaviors), tested in a neutral location (minimizes territorial aggression), and typically tethered or confined in a cage or tube (dissociates social interaction initiated by the subject mouse from that initiated by the stimulus mouse).

Mice with altered social interest may spend less time engaged with the stimulus mouse, and this can be interpreted as a deficit in sociability. As a measure of innate behavior, social approach is highly reproducible within mouse strains [16], while strain-specific changes in social behavior can be detected [17, 18]. Genetic mutations that alter neural development have been shown to influence social approach (e.g., [8, 10, 19–27]), as have pharmacological agents (e.g., [6, 28–33]) and environmental modifiers (e.g., [34, 35]).

There are two main ways in which social interest is analyzed in animal models. The first is by simply measuring the amount of time a test mouse spends interacting with a novel conspecific in a neutral environment (i.e., not the territory of either the test or stimulus mouse). The second employs the three-chamber social approach test, which gives an experimental animal a choice between interacting with a confined stimulus mouse and an inanimate object [12, 32, 36]. Details of the latter protocol are described below (Subheading 3).

1.3 Social Recognition

Social recognition is the basis for social living and all social behaviors [37]. In order to interact appropriately and form hierarchies and other social relationships, one must be able to recognize and distinguish between conspecifics and adjust one's behavior based on one's previous experience with that individual [38–40]. Social recognition, in its simplest form, is the ability to distinguish between a novel conspecific and one that has been encountered before ("familiarity recognition"). It can be classified into different levels, which can range from true individual recognition to simple species recognition, and varying degrees are essential for determining if other individuals are related and/or familiar, potential mates (i.e., adults of the opposite sex), and/or sexually receptive (e.g., females in behavioral estrus). It can also allow one to distinguish between the animal from which one can take resources (lower hierarchical status) and the animal to which one should relinquish resources (higher status), those that are healthy and those that are not, and mates or group members and strangers or intruders (reviewed in [38]).

The majority of laboratory research has focused on familiarity recognition [38]. Social recognition can be tested in several ways using mouse models. Three-chamber social novelty follows from the three-chamber social approach test, with a novel social stimulus being placed into the tube that previously was empty or held a nonsocial object; the test animal should show a preference for the novel social chamber, tube, or stimulus (*see* Subheading 3.2.3). Alternatively, social recognition can be assessed using habituation (with or without dishabituation; *see* Subheading 4). This takes advantage of the fact that mice will spend decreasing amounts of time investigating a conspecific with repeated exposures (habituation). In some cases, this is followed by a dishabituation trial in which a novel stimulus is introduced, and the test animal should show an increase in investigation. This second step is particularly important to ascertain whether the experimental animal is habituating to the testing experience per se, or to the stimulus animal specifically.

1.4 Social Interaction and Aggression

In animals that share territory, social recognition often governs the nature of agonistic interactions with conspecifics. Aggression is typically high when a new social group is initially formed, but mice, especially females, will usually settle into a hierarchy within a few days [41, 42]. Dominance hierarchies are established through agonistic encounters that can include preliminary "threat" behavior (e.g., tail rattle), chasing, the attacks themselves, defensive behavior, and other less overtly aggressive behaviors (e.g., aggressive grooming, pinning [43, 44]). Once established, maintenance of these dominance hierarchies tends to consist of threats and aggression in high-ranking animals and submissive, defensive, and evasive behaviors by lower-ranked individuals, resulting in decreased risk of injury and energy costs associated with fighting [44–46].

The majority of aggression research has used male subjects [47], as it has long been claimed that females are not agonistic and do not fight outside of so-called maternal or postpartum aggression. This maternal aggression occurs when females are protecting their offspring and is qualitatively different from the territorial aggression typically studied in males, being less ritualized and more violent (e.g., [44, 47–49]). While females will show agonistic behavior even when not defending their litters (e.g., [50–53]), females of most laboratory rodent species do, in general, fail to attack and demonstrate the overt aggression seen in males and in wild-caught female mice, likely because female laboratory mice appear to have had most of their overt intrasexual aggression bred out to facilitate breeding [52]. Instead of attacks, they frequently perform other agonistic behaviors aimed at establishing a dominance hierarchy, such as following or chasing, pinning down, or aggressively grooming the intruder [43, 54–56]). Thus, as has long been recommended (e.g., [44]), the study of agonistic behavior in female laboratory animals requires the use of more sensitive and comprehensive ethological analyses which include both overt and more subtle forms of agonistic behavior and the contexts in which they occur.

One of the most common tasks used to assess aggression is the resident–intruder test of free social interaction, in which an "intruder" animal is introduced into the home cage of a "resident" conspecific and their interactions are observed and scored (*see* Subheading 5).

2 General Notes on Social Behavior Testing

Social behavior involves numerous cognitive and neurobiological elements and is particularly sensitive to a variety of factors that require consideration. Some of these are outlined below. The protocols included in this chapter describe procedures for testing social approach, social recognition, and the resident–intruder test of free social interaction that we have found effective across different locations and experimenters [20, 26, 31, 33, 54, 55, 57–62]. However, there are numerous variations of these paradigms that have also been successfully employed, and the reader is encouraged to investigate the range of methods and testing parameters used across various laboratories.

2.1 Behavior Room and Personnel

Rooms used for testing social behavior should be located in a quiet area with minimal traffic and should not be used to test or hold any other animal species, as the odors of rats, ferrets, and other experimental animals may elicit a fear response that can lead to altered social behavior [63, 64]. If possible, it is preferable to have a separate area (or additional room) in which to hold subject mice before and after testing to limit exposure to distracting odors. Mice

should be moved to this holding room or area at least 1 h before testing to eliminate effects of stress associated with transportation and to habituate to the testing area.

Apparati should be placed in consistent locations, with even lighting, far from drafts or air flow, and such that mice have limited perception of personnel moving around the room. The individual(s) conducting experiments should ideally be consistent for the length of a study. Additionally, it is essential that strong perfumes, colognes, and similar scented products be avoided during testing, as olfaction is the primary sense involved in mouse social behavior. Similarly, apparati should be thoroughly cleaned with a disinfectant and cleaner (e.g., Quatricide PV, Pharmacal, Naugatuck, CT, USA) between animals to prevent scent trails or other cues from passing to subsequent subjects.

In all cases, experimenters should be blind to "group" (i.e., sex, genotype, pharmacological or other treatment, or any combination thereof) whenever possible. This is true for both the physical running of the experiments and any scoring done from video afterward.

2.2 Lighting Conditions and Husbandry

Lighting conditions can have significant impact on social behaviors. We have found that dim white light is necessary when testing social approach but that red light is preferable for social recognition and resident–intruder test of free social interaction, especially as bright lighting can increase anxiety in mice. However, it is most important that lighting is even across the apparatus and consistent across trials within an experiment.

It is also important to consider the timing of testing relative to the light/dark cycle. While there has been no observed effect of testing in the light vs. dark phase of the circadian cycle for some social behaviors (e.g., social approach [65]), mice are nocturnal, and thus it is generally preferable to test mice during their active (dark) phase. This can be easily accomplished by housing mice on a reversed 12:12 h light/dark cycle and using red lights, which cannot be detected by mice, to allow personnel to perform routine maintenance and husbandry and to carry out experiments. To avoid circadian rhythm effects, it is advisable that behavioral testing take place during the same time of day for a given experiment.

Additionally, one should consider the potentially disruptive effects of cage changes on behavior, and when possible, avoid testing mice on the day of or after placement into new, clean cages. Unless required for experimental manipulations, all mice should be group-housed throughout testing, on a 12-h reversed light/dark cycle, with ad libitum access to food and water. One exception is that experimental mice should be single-housed for at least 7 days prior to being tested in the resident–intruder test of free social interaction (*see* Subheading 5).

2.3 Characteristics of Subject Mice

Ideally, experimental mice should be naïve (i.e., no previous behavioral testing experience). However, given how valuable mice can be, due to rare genotypes, intensive treatment protocols, or other manipulations, and in the interest of reducing the number of mice used for ethical purposes, mice often undergo several tests in a battery. If mice are to be tested in this manner, it is important to consider the timing and order of these assays, especially if more than one test of the same modality is included. Tests should be spaced out by at least 3 days, with at least a week between tests of similar behaviors (e.g., two social or two anxiety tests) to minimize carryover effects. We also recommend limiting the total number of tests to six to eight. Extra care must also be taken in the selection of stimulus mice if more than one social test is included in the battery (i.e., stimuli for each test should be from different breeding cages from each other and the test mice).

Other considerations for selecting experimental mice concern age, reproductive status, and sex. To avoid increasing variability, test mice should be of similar ages (e.g., 2–6 months of age for young adults) and virgin unless necessary for experimental manipulations (as mating experience can change neuroanatomy, neurochemistry, and behavior). It is also essential to test mice of both sexes. In addition to being required by many funding agencies (e.g., NIH), combining sexes, especially if sex ratios are different between groups, can also increase variability and mask effects of group due to sexual dimorphism. We thus strongly recommend testing both male and female subjects and analyzing them separately and/or including sex as an independent variable in all analyses. Unfortunately, many studies still do not include both sexes or analyze sexes separately and in some cases even fail to identify the sexes or sex ratio being tested.

Finally, before testing social behavior in a new mouse line, these animals should first be screened for deficits in locomotor (e.g., open field test) and sensory function, especially in the domain of olfaction (e.g., chocolate chip test, olfactory habituation/dishabituation). Any deficits in these areas represent a potential confound that needs to be taken into account in the design of experiments for the mouse line under consideration.

2.4 Characteristics of Stimulus Mice

Stimulus mice can have a significant effect on the type and nature of social behavior, and thus the characteristics of stimulus mice need to be considered. In all three assays described below (Subheadings 3, 4 and 5), stimuli should be sex-matched, wild-type, virgin mice from different breeding cages than the test mice, and group-housed throughout testing under standard housing conditions. Often these mice are from the same strain or background strain as the subject mice.

Age is also an important consideration. When testing adult mice on the social approach test, we have found that, at least in mice on the C57BL/6J background, stimulus mice that are

approximately postnatal day 60 (P60) tend to elicit stronger responses in adult test subjects than older animals, regardless of the test mouse's age. For social recognition, we typically use juvenile stimuli (P21–28), as we have found this increases the interest of the test mice. For the resident–intruder test of free social interaction, adult mice that do not show extreme deviation from test mice in weight or age are preferable, as these factors can alter agonistic behavior (e.g., [42, 48]). Often group-housed, gonadectomized, and/or olfactory-bulbectomized conspecifics, which show little or no aggression, are used as intruders [48, 66, 67]. This makes for more consistent intruders and thus better assessment of the aggression level of the experimental animals, although group-housed gonadally intact mice can also be used, especially if from a less aggressive strain (e.g., BALB/c).

3 Three-Chamber Social Approach and Social Novelty Paradigm

This paradigm measures social interest, as indicated by a mouse's preference to spend time with a conspecific over an object. It can also be used to test if a mouse prefers a novel mouse over a familiar one, which is suggestive of social recognition.

3.1 Setup and Organization

3.1.1 Animals

For assaying social approach behavior in adult mice, test subjects should be at least 8 weeks old. It is also possible to test juvenile mice; we have successfully tested mice as young as P21 [68]. If test mice are younger than 8 weeks of age, stimulus mice should be approximately age-matched to the test subjects. A large number of inbred and outbred strains have been tested in social approach behavior, and strain-specific behavior differences are common [9, 18, 69]; therefore, care should be taken when using transgenic animals to maintain consistency in genetic background.

3.1.2 Apparati

A social approach apparatus can be purchased from a number of retailers or can be easily constructed in-house. As an example, one may use opaque acrylic sheets (e.g., US Plastics, Lima, OH, USA) bonded using acrylic solvent (e.g., GE Polymershapes, Huntsville, NC, USA) to form an open top box with three chambers, as shown in Fig. 1a. A commonly used box size is 60-cm (24″)-long × 30-cm (12″)-wide × 30-cm-(12″)-high, divided into three chambers 20-cm (8″)-long × 30-cm-(12″)-wide × 30-cm (12″)-high by acrylic sheets with 7.5-cm (3″) × 7.5-cm (3″) square openings cut into them to allow test mice to move between chambers (*see* Fig. 1a). The floor of the apparatus should contrast with the coat color of the mouse; in experiments using strains with dark or black coats, a white floor is recommended. If lines with a range of coat colors will be tested, a red or gray floor may provide the best overall contrast.

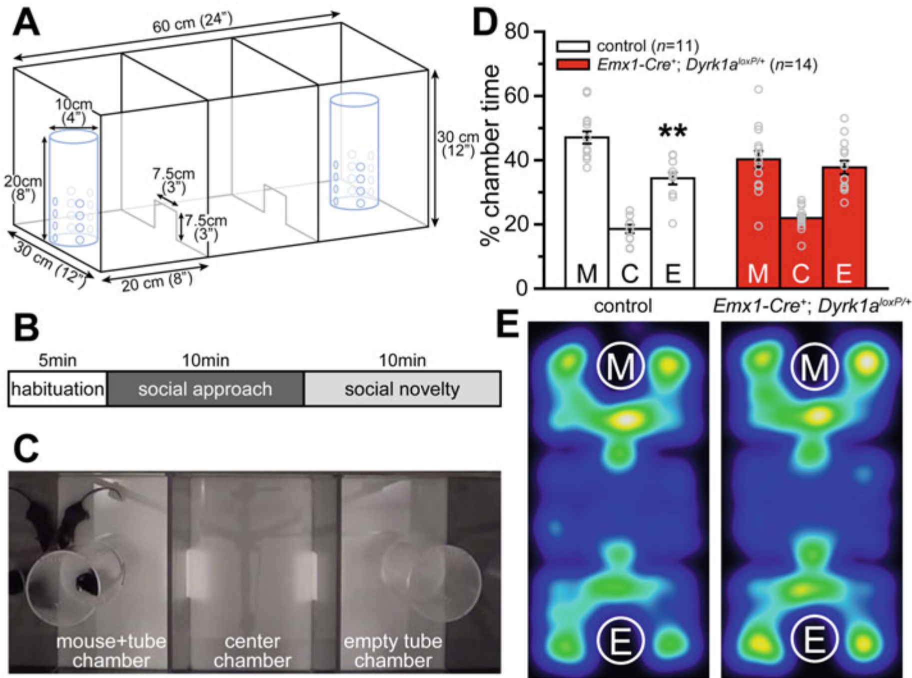

Fig. 1 Three-chamber social approach. (**a**) Three-chamber apparatus and acrylic tube enclosures for stimuli, including dimensions. (**b**) Timeline of the three-chamber social approach and social novelty procedure. (**c**) Top-down view of social approach phase of testing, with test mouse in the mouse+tube chamber sniffing the social stimulus. (**d–e**) Example data in male mice from [33] showing a significant preference for the social chamber in control mice but no significant preference in mice with a conditional heterozygous deletion of *Dyrk1a* (*Emx1-Cre*$^{+}$; *Dyrk1a*$^{loxP/+}$). Data scored and heatmaps generated using EthoVision (Noldus). (**d**) Quantification of chamber time. (**e**) Average heatmaps of test mouse location for control (left) and *Emx1-Cre*$^{+}$; *Dyrk1a*$^{loxP/+}$ (right) mice. M, mouse+tube chamber or tube containing stimulus mouse; C, center chamber; E, empty tube chamber or empty tube. ** $p < 0.01$, paired *t*-test mouse+tube chamber vs. empty tube chamber

During social approach and social novelty testing, the left and right chambers hold enclosures, while the center chamber is empty (no enclosure) and serves as a passage between the left and right chambers. The enclosures may be wire cup "cages" purchased commercially or tubes built from clear acrylic cylinders measuring 20 cm (8″) high × 10 cm (4″) in diameter (8″ × 4″; US Plastics, Lima, OH, USA) drilled with holes to allow for olfactory, visual, and tactile investigation of the stimulus mouse by the subject mouse (*see* Fig. 1a). Using acrylic tubes has the advantage of reducing climbing up and onto the enclosures. The enclosures should be large enough for an adult mouse to move about comfortably and heavy enough to prevent a stimulus mouse from moving them during normal exploratory behavior, and the holes should not be so large as to allow stimulus mice to escape (this is particularly important if using juvenile stimuli). In the social

approach phase, one of the enclosures holds the social stimulus mouse; the enclosure in the other chamber remains empty or may contain a nonsocial object as a control. In the social novelty phase, one enclosure contains the same ("familiar") mouse used in the social approach phase, while the previously empty (or nonsocial object) enclosure holds a novel stimulus mouse.

3.1.3 Side Preference Pretest

When starting to use a new apparatus or testing room, it is necessary to observe the behavior of a cohort of control mice in an empty behavior apparatus (no stimulus mice or enclosures) before beginning any testing, to ensure that no side preference within the three-chamber apparatus is observed. It is also important to alter the orientation of the apparatus relative to the testing room and record movement of the mice to verify that subject mice do not display positional preference. If subject mice display differential exploration of the left and right chambers during habituation, then a new testing room should be found.

3.2 Procedure

3.2.1 Preparation, Acclimation, and Habituation

Identifying the test mice by marking the tail either the day before or the day of testing (when mice are brought into the testing area to acclimate, at least 1 h before the start of testing) helps to minimize stress associated with scruffing mice to check ear tags, tattoos, or other identification. To begin testing, mice should undergo a 5-min habituation to the behavior apparatus by being placed into a clean, empty three-chamber social approach box (without enclosures present) and allowed to explore the box (*see* Fig. 1b). Recording this habituation will allow for analysis of initial chamber preferences, especially if abnormal results are found in control mice.

3.2.2 Social Approach

After the 5-min habituation to the social approach apparatus, test mice should be removed and placed into a clean holding cage while the two enclosures are placed into the left and right chambers, and a stimulus mouse is placed into one of these (designated as the "mouse+tube chamber," as opposed to the "empty tube chamber"); this should be done as rapidly as possible and is unlikely to add significant time to the assay. The location of the stimulus mouse should be counterbalanced within groups across trials, such that each group (i.e., sex, genotype, pharmacological or other treatment, or any combination thereof) has approximately half of the stimulus mice on the left and half on the right. The test mouse should then be returned into the center chamber and videotaped exploring for 10 min (*see* Fig. 1b, c). Following this, either proceed to the social novelty phase below (*see* Fig. 1b) or return the test and stimulus mice to their respective home cages. If not performing social novelty, clean the enclosures and social approach box thoroughly with a cleaner and disinfectant to remove odors (e.g., Quatricide PV, Pharmacal, Naugatuck, CT, USA), and allow the apparatus to dry prior to testing the next mouse.

3.2.3 Social Novelty

Optionally, immediately following the social approach assay, it is possible to include the social novelty test (*see* Fig. 1b). This involves briefly removing the test mice into their holding cage and adding a second, novel stimulus mouse into the enclosure that was previously empty (or contained a nonsocial control object) while leaving the original stimulus mouse (now "familiar") undisturbed; this novel mouse should be from a different breeding and home cage than both the familiar stimulus mouse and the experimental animal. While this test can be suggestive of social or familiarity recognition, it is frequently less reliable than the social approach test in control mice of both sexes (who do not always show a significant preference for the novel stimulus (e.g., [20, 61]), and there is some data suggesting that it may not necessarily indicate recognition of the individual stimulus mice but rather the presence of a mouse where no mouse was previously [16]. Thus, the social habituation/dishabituation assay described below (Subheading 4) may be a better, more reliable way to determine if social recognition is affected by experimental manipulations.

3.3 Video Acquisition, Outcome Measures, and Statistical Analysis

3.3.1 Video Acquisition

While live scoring is possible, it is preferable to take video recordings of trials with a digital camcorder (e.g., Sony Handycam) held directly above the apparatus using a tripod or ceiling mount. Consistent positioning of the camera is essential – ideally, the social approach box(es) and camera would be fixed in place. As this is not always possible, care must be taken to place the box(es) and camera in the same location to keep lighting and external cues (e.g., door location, items on walls, overhead lights, etc.) consistent across trials.

3.3.2 Outcome Measures

There are two main aspects of social interest that can be assessed using the social approach and social novelty aspects of the protocol. The first, which is simplest and can be easily automated, is measuring chamber time (*see* Fig. 1d–e). This is used as a proxy for social interest and was the initial manner in which the assay was scored. The second outcome that can be assessed is sniffing time, which is usually operationally defined as the nose of the test mouse being within 1–2 cm of the openings in each enclosure. These measures can be acquired in several different ways, ranging from manual scoring with a stopwatch to sophisticated deep learning software (*see* Subheading 6 for a discussion of these options). As a control measure, locomotion should also be analyzed if possible (i.e., if using tracking or deep learning software; number of crossings between chambers can be used as a proxy if manually recording behavior).

3.3.3 Statistical Analysis

The dichotomous social approach variable (the presence or absence of a social preference) is determined by comparing the time spent with the social stimulus to that spent with the empty tube (whether

assessed by chamber time or sniffing). These data can be analyzed using two-way mixed-model analyses of variance (ANOVAs), with group as the between-subjects variable and chamber or stimulus type as the within-subjects factor. Post hoc comparisons (e.g., Tukey, Sidak) can be employed if the ANOVA shows a significant group x stimulus interaction. Additionally, paired-sample *t*-tests can be used as planned comparisons to determine the presence or absence of a social preference within each group separately. Importantly, one cannot perform direct comparisons across groups using this method [70], as it does not produce a measure of the strength of the preference but simply indicates whether or not it exists. However, it is informative to know which groups show a preference and which do not, and this was the analysis for which the test was designed [71].

To allow for direct comparisons across groups, a preference index can be used to determine the magnitude of difference in time spent between social and nonsocial chambers (i.e., the strength of the preference), distinct from the presence or absence of a social preference. This preference index can be calculated as:

$$\frac{(\text{mouse} + \text{tube time}) - (\text{empty tube time})}{(\text{mouse} + \text{tube time}) + (\text{empty tube time})}$$

or

$$\frac{(\text{mouse} + \text{tube time})}{(\text{mouse} + \text{tube time}) + (\text{empty tube time})}$$

where "time" indicates either (a) time spent sniffing a given tube or (b) time spent with the majority of the body inside a given chamber. Independent-samples *t*-tests (two groups) or one-way between-subjects ANOVAs (more than two groups) should be used to analyze the effects of group on the preference index, with post hoc analyses if a significant main effect of group is found in the ANOVA. Similar analyses can be used for other measured variables (e.g., distance traveled or locomotion).

3.4 Tips and Troubleshooting

3.4.1 Stress Effects on Social Approach Behavior

Social approach behavior is sensitive to environmental stressors, such as aversive sounds, odors, or handling, that can occur in a laboratory testing environment. Mice that are stressed or fearful may be observed freezing, hiding, or darting during a social approach trial, creating a large number of chamber crossings or unusually long chamber stays. For example, we have found that most mice on the C57BL/6J background spend approximately 20% of their time in the center chamber; if a mouse or a group spends substantially more time in the center, it may indicate increased anxiety or locomotor deficits and typically requires further investigation to determine the cause. If a cohort of control mice exhibits abnormal social approach behavior, then it is

necessary to examine whether the mice were exposed to an uncontrolled environmental stressor and, if so, use data collected from another cohort of mice once that variable is removed.

3.4.2 Acclimation of Anxious Mice

In strains that are more anxious, it may be beneficial to further acclimate the mice to the behavior room, apparati, and handling for several days prior to the start of experiments, as previously described [65]. Mice may be moved to the behavior room in their home cages around the time of day that the experiment will be taking place. Mice should be acclimated to handing to a point where they can be removed from the home cage with minimal stress. It is generally sufficient for mice to be handled by personnel for approximately 5–10 min a day for several days prior to testing. If mice will be identified by viewing ear tags or tattoos, mice should also be acclimated to this aspect of handling. If injection of drug or vehicle will be part of the experiment, mice should be acclimated to the restraint necessary to humanely administer an injection.

Mice can also be acclimated to the behavior apparatus before testing. This may be accomplished at the same time that acclimation to handling is taking place. Test mice should be placed into the clean three-chamber social approach box (without enclosures) and allowed to explore the chambers for 5–10 min.

3.4.3 Environmentally Induced Variability

Variability may be reduced through controlling the environment of the mouse being tested. Mice can be acclimated to the behavior apparatus and handling as described above, noting that time spent acclimating may change between mouse strains. Subject mice should not be housed individually unless required for or used as experimental manipulations, as isolation housing leads to various behavioral effects including increased aggression and anxiety that may alter baseline social behavior. Levels of home cage enrichment should be kept consistent among cohorts of mice for a given experiment. Inconsistent handling between personnel can be a source of uncontrolled variability; it is advisable to have one person carry out all mouse handling for a given experiment to remove this variable. While it is possible to successfully carry out trials using intraperitoneal or subcutaneous injected drugs, we have noted that if the latency between injection and social approach testing is less than 15 min, wild-type C57BL/6 animals injected with control vehicle (saline) will sometimes show altered patterns of behavior in the apparatus, with increased chamber crossings or freezing being the most notable effect.

3.4.4 Counterbalancing and Simultaneous Testing of Multiple Animals

It is essential to counterbalance the side on which the stimulus mouse is placed in order to avoid any previously undetected side biases (*see* Subheading 3.1.3). One should also take note if the time spent in the mouse+tube chamber vs. the empty tube chamber during the social approach phase varies significantly for the control

subject mouse population depending on the side on which the mouse is placed, as this suggests a side bias and may require the use of a different room or location. This requires planning and good record-keeping, especially if mice for a single experiment are being tested in multiple cohorts. Counterbalancing should be planned such that approximately half of the mice tested on a given day *within* a given group (i.e., sex, genotype, pharmacological or other treatment, or any combination thereof) have the stimulus mouse in the left chamber and half in the right. Additionally, approximately half of all mice in a given group within an experiment (across cohorts/testing days) should have stimuli placed on each side. In order to allow for the experiment to be performed blind, this organization can be done by a different individual that the one physically performing the test. Alternatively, if sufficient numbers of mice are used, one is usually unable to remember which mice are of which group without effort (which should be avoided), particularly if planning is done in advance. Similarly, coding vehicle and drug doses for treatments can also assist in blind testing.

While it is possible to test multiple animals simultaneously (i.e., in several apparati, one mouse per box), the location of the boxes and the stimulus mice must be taken into consideration. It would be preferable to place each box under its own camera with as much distance between boxes as possible, but if boxes must be adjacent (e.g., under a single camera), it is important to keep stimuli on the same side in these boxes so as to avoid the confound of scent transfer from the other box(es). This adds an additional level of complexity to the counterbalancing and thus requires further advanced planning of stimuli locations.

4 Social Recognition

This paradigm requires the mice to remember and indicate recognition of a familiar conspecific by decreasing social investigation across four presentations of the same mouse (habituation). An increase in investigation during the presentation of a novel mouse (often to a level similar to that observed in the first habituation trial) indicates dishabituation and is an important control for general habituation to the testing situation. Additionally, this task included a built-in measure of social interest, which is observed in the first habituation trial.

4.1 Setup and Organization

4.1.1 Room Setup and Lighting

Social recognition testing should take place in a quiet room with even red lighting across the testing cage and should be recorded from above using a camera with low-light capabilities.

4.1.2 Apparati

Mice are tested in clean cages identical to their home cages, which should be covered with clear lids to allow for videotaping. Stimulus mice should be contained within clear acrylic tubes [7.25 cm (3″) in diameter, 12.5-cm (5″)-tall] with holes in the bottom (3 offset rows of 12 4-mm (0.16″) diameter holes).

4.2 Procedure

4.2.1 Preparation and Acclimation

Two hours prior to the start of testing, mice should be individually placed into clean cages of the same type as their home cages containing a clear acrylic tube [72]. This will allow the formation of a territory without requiring long-term isolation housing and will acclimate the test mice to the stimulus mouse enclosure.

4.2.2 Habituation and Dishabituation Trials

To commence testing, place the juvenile (P21–P28), same-sex stimulus mouse into the enclosure, and allow the test mouse to investigate for 5 min. Remove the stimulus mouse (but not the enclosure), and leave the cage undisturbed for a 10 min inter-trial interval (ITI). Repeat these two steps (trial and ITI) for a total of four exposures to the same stimulus mouse, and then introduce a novel juvenile stimulus for the final (dishabituation) trial (*see* Fig. 2a).

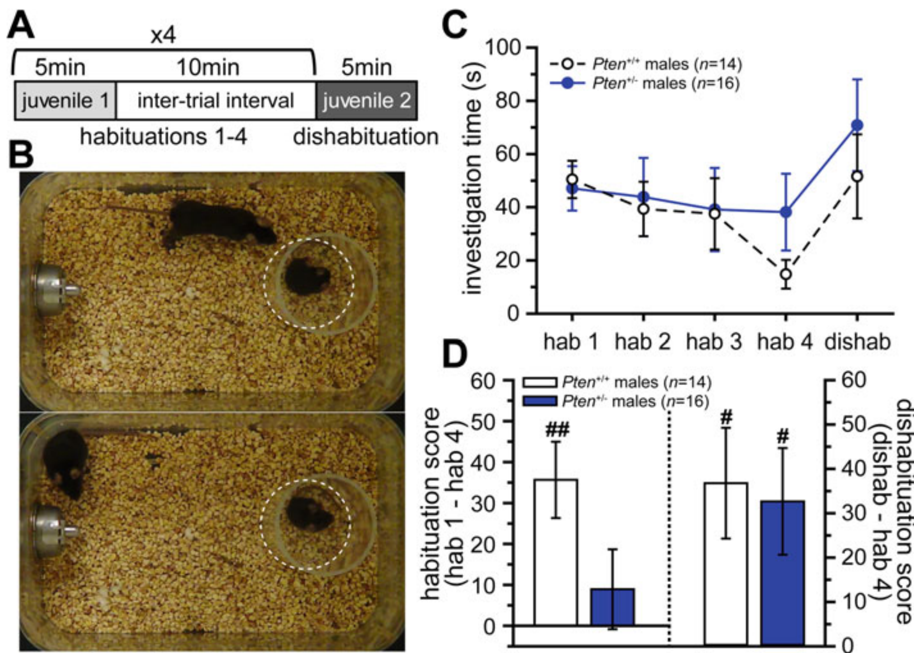

Fig. 2 Social recognition. (**a**) Timeline of the social recognition test. (**b**) Top-down view of social recognition testing, showing the test mouse investigating (top panel) and not investigating (bottom panel) the stimulus mouse. Dashed circle indicates bottom of the tube. (**c–d**) Example data in male mice from [20] showing significant habituation in control mice but no habituation in mice with germline haploinsufficiency for *Pten* (*Pten*$^{+/-}$). Dishabituation was normal in both groups. Hab, habituation trial; dishab, dishabituation trial. # $p < 0.05$, ## $p < 0.01$, one-sample *t*-test vs. 0

4.3 Outcome Measures and Statistical Analysis

4.3.1 Outcome Measures

Social investigation is the primary measure for the social recognition test and simply involves recording the amount of time the test mouse spends sniffing the social stimulus during each trial (*see* Fig. 2b–d). This can be done in a number of ways, from manual scoring with a stopwatch to sophisticated deep learning software (discussed in Subheading 6).

The duration of investigation in the first habituation trial provides a measure of initial social interest; if this is less than 10 s, the mouse does not show sufficient interest in the social stimulus and should be removed from the analysis. In addition to investigation time per trial (Fig. 2c), two other measures can be calculated (Fig. 2d): a habituation score [(time spent sniffing during habituation trial 1) – (time spent sniffing during habituation trial 4)] and a dishabituation score [(time spent sniffing during the dishabituation trial) – (time spent sniffing during habituation trial 4)]. The former indicates that the test mouse habituated to the social stimulus over the four exposures and the latter that the test mouse was able to identify the new social stimulus as "unfamiliar" or different from the habituation stimulus (*see* Fig. 2d).

4.3.2 Statistical Analysis

Social investigation should be analyzed using two-way mixed-model ANOVAs, with group (i.e., sex, genotype, pharmacological or other treatment, or any combination thereof) as the between-subjects variable and trial as the within-subjects factor. Post hoc analyses can be performed if there is a significant group x trial interaction. Planned comparisons can also be used to compare groups for each trial and for habituation and dishabituation scores (two groups, independent sample *t*-tests; more than two groups, one-way ANOVAs, with post hoc tests if significant effect of group).

To determine if a group showed significant habituation or dishabituation, the scores can be compared to zero using one-sample *t*-tests.

4.4 Tips and Troubleshooting

As in the social approach test above, it is important to minimize stress (Subheading 3.4.1), acclimate mice to handling and procedures if they show high anxiety in response to the test (Subheading 3.4.2), and reduce environmental variability (Subheading 3.4.3).

4.4.1 Lineage and Strain of Stimulus Mice

As mentioned in the general notes above, it is essential that stimulus mice and test mice be from different breeding cages to avoid a difference in baseline familiarity due to being siblings. Using inbred stimulus mice of the same background strain as the test mice can be convenient, especially if they are breeding in-house and given the relatively small target age range. However, if control mice fail to show significant habituation and dishabituation, using mice from an outbred strain can lead to improved recognition and discrimination. As with all stimulus mice, they should be group-housed.

4.4.2 Simultaneous Testing of Multiple Animals

If multiple mice are being tested simultaneously, cages should have dividers between them to avoid test mice being distracted by animals in adjacent cages. These dividers can be easily constructed by cutting the front and back covers from opaque plastic binders. One advantage of simultaneous testing, especially of an even number of mice, is that it allows for the novel mouse used during dishabituation to have received the same number of experiences in the tube as would the familiar mouse in a fifth exposure. This can be accomplished by switching the stimuli from cages 1 and 2, 3, and 4, etc. for the final dishabituation trial, although care must be taken to ensure that the novel and familiar stimuli are from different parent and holding cages.

5 Resident–Intruder Test of Free Social Interaction

This assay analyzes the aggressive and nonaggressive social behavior, as well as nonsocial behavior, that a resident mouse exhibits in response to the presence of a same-sex intruder in its home cage.

5.1 Setup and Organization

5.1.1 Animals

At least 7 days prior to testing, experimental mice should be separated and housed individually, with no cage changes but under otherwise normal conditions. This isolation allows test mice to establish a territory and increases the likelihood of attack (e.g., [42, 48, 73]). This is particularly important for less aggressive strains, including C57BL/6 mice [52, 74, 75]. Intruder mice should be housed in groups throughout the experiment.

5.1.2 Apparati

Testing takes place in the resident mouse's home cage, which should have a clear lid to allow for videotaping from above. Intruders with dark coats should be identified with nontoxic white paint (e.g., 3503 – white water-soluble ink, Speedball, Statesville, NC, USA) to assist in identification during behavioral scoring; light-coated intruders can be marked using a black Sharpie. Repeated use of the same intruders is possible, although it should be minimized, and there should be at least 1 h between uses, as repeated experience as an intruder can lead to increased aggression in response to investigation or agonistic behavior by the resident.

5.2 Procedure

During the active (dark) phase of the light/dark cycle, a marked intruder should be placed into the resident's home cage. Mice are then left undisturbed to be videotaped freely interacting for 15 min. The interactions should be monitored for excessive fighting, especially in more aggressive strains, and both resident and intruder should be checked for injuries following the test. However, both excessive fighting and injuries are uncommon during a brief 15 min interaction.

5.3 Outcome Measures and Statistical Analysis

5.3.1 Outcome Measures

The simplest measures for the resident–intruder test of free social interaction are the latency to the resident's first attack or the number of attacks made by the resident mouse (Fig. 3a). This can be done with a stopwatch or tally counter, but other, more advanced software can provide a richer analysis of the interaction (*see* Subheading 6). This is particularly important if test mice are female. Both sexes show agonistic behavior (e.g., [42, 54, 55]), but overt aggression, including attacks and aggressive postures, are almost exclusively performed by males, especially in inbred strains like C57BL/6 [52]. Thus, more detailed ethological analyses are required to assess agonistic behavior in females.

5.3.2 Ethological Behavioral Analysis

In addition to overt attacks or fighting, other agonistic, non-agonistic, and nonsocial behaviors are being increasingly included in the analysis of the resident–intruder paradigm (e.g., [54, 55, 58, 76–82]. We score for 23 behaviors (some examples in Fig. 3) based on the ethogram by Grant and Mackintosh [43, 54, 55, 58], as described in Table 1 (modified from [58]). This scoring should be done blind to group (i.e., sex, genotype, pharmacological or other treatment, or any combination thereof) and does require training and practice to accurately document behaviors. Manual scoring can use BORIS [83], The Observer video analysis software (Noldus Information Technology, Wageningen, the Netherlands), or a similar program; some deep learning programs may also be able to perform this scoring (*see* Subheading 6). Behavioral analysis focuses on the resident (experimental) mouse, and behavior of the intruder is collected only in relation to the behavior of the resident and in the reciprocal pairs of behaviors (i.e., resident- or intruder-initiated fighting, attacks delivered or received, dominant or submissive behavior, chasing or avoiding the intruder, aggressive postures, and reciprocal attacks). Typically, dominant behavior on the part of one mouse will be met by submissive behavior on the part of the other.

To gain an overall impression of the animals' behavior, individual behaviors can also be combined to form ten categories of individual behaviors (*see* Table 1), thus providing insight into whether mice of different groups show phenotypic alterations in general categories of behavior or only specific individual behaviors (*see* Fig. 3b). This is particularly important for agonistic behaviors, as the total agonistic category includes types of aggression-related behavior with divergent functional implications (e.g., *dominance behaviors intended to establish a dominance hierarchy* vs. *overt attacks aimed at establishing an exclusive territory;* [44, 84]). Total agonistic behavior also includes agonistic behaviors performed by the resident, the intruder, or both (in cases in which the aggressor cannot be identified). To determine which animal of the pair is dominant, a "dominance score" can be calculated (agonistic behavior delivered–agonistic behavior received; *see* Table 1

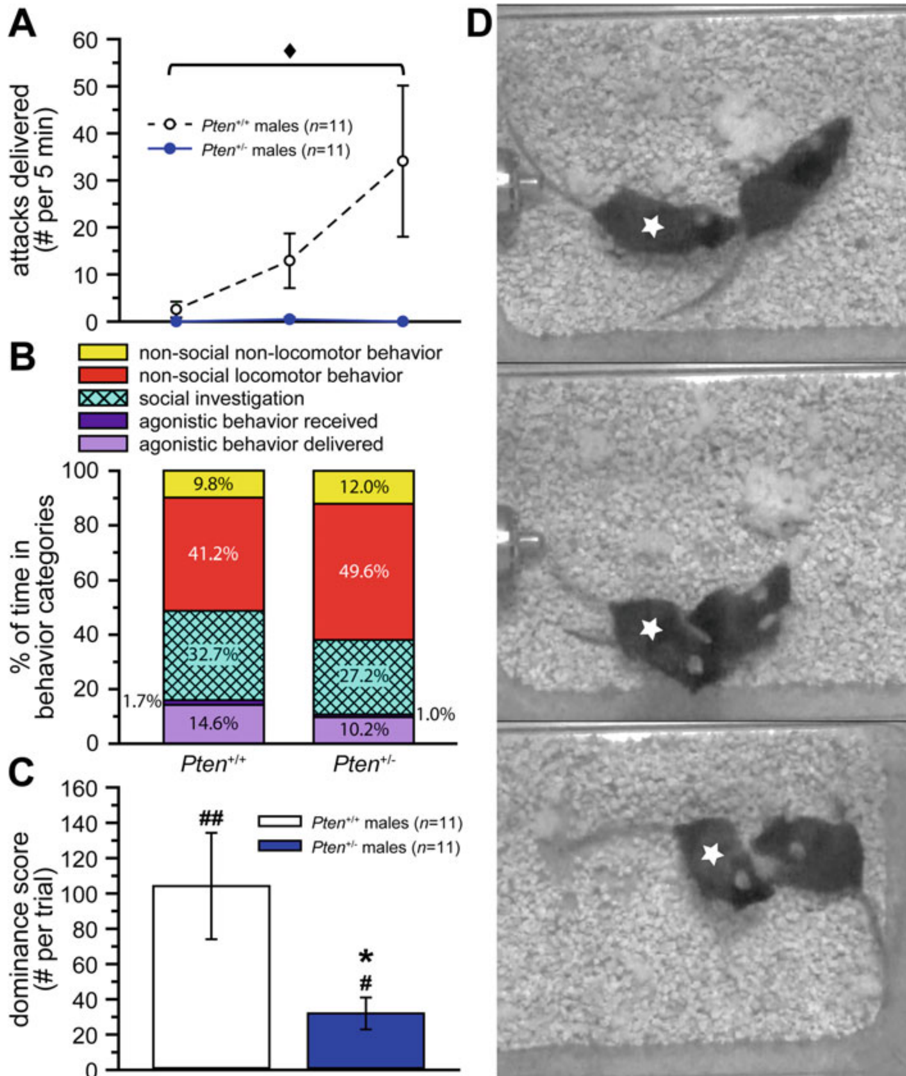

Fig. 3 Resident–intruder test of free social interaction. (**a–c**) Example data in male mice from [58] showing decreased agonistic behavior and increased nonsocial locomotor behavior in mice with germline haploinsufficiency for *Pten* (*Pten*$^{+/-}$). (**a**) Number of attacks delivered by the resident in 5 min bins. (**b**) Breakdown of behaviors performed during the test by category. (**c**) Dominance score (agonistic behavior delivered–agonistic behavior received by the intruder) across the 15 min trial. (**d**) Top-down view of social interaction, showing anogenital investigation (top panel), dominant behavior (specifically, aggressive allogrooming, middle panel), and aggressive postures (bottom panel). Resident mice are identified by star. * $p < 0.05$, genotype comparison; # $p < 0.05$, ## $p < 0.01$, one-sample *t*-test vs. 0; ◆ $p < 0.05$, significant effect of time in *Pten*$^{+/+}$ mice

and Fig. 3c), with a high positive score indicating that the resident mouse was dominant (delivering the majority of the agonistic behavior and receiving very little), while a negative score indicates a submissive resident.

Table 1
Descriptions of scored behaviors, composite behaviors, and categories

Behavior	Description
Scored behaviors	
Mutual agonistic behaviors	
Aggressive postures	Physical attacks which include box/wrestle, offensive and defensive postures, lateral sideway threats, and tail rattle
Reciprocal attacks	Physical attacks with a locked fight including tumbling, kick-away, and counterattack where the attacker cannot be identified
Agonistic behaviors delivered by resident	
Resident-initiated fighting	Multiple consecutive physical attacks, including dorsal/ventral bites, kick-away, tumbling, and counterattack, initiated by the resident
Attacks delivered	Count of, and latency to, individual physical attacks by the resident, including bites to dorsal/ventral regions
Dominant behavior	The resident mouse is in control; includes pinning of the intruder, aggressive grooming, crawling over or on top, pushing under, and mounting attempt; reciprocal to submissive behavior
Chasing the intruder	The resident mouse actively follows or pursues and chases the intruder; reciprocal to avoidance of the intruder
Agonistic behaviors delivered by intruder	
Intruder-initiated fighting	Multiple consecutive physical attacks, including dorsal/ventral bites, kick-away, tumbling, and counterattack, initiated by the intruder
Attacks received	Count of, and latency to, individual physical attacks by the intruder against the resident, including bites to dorsal/ventral regions
Submissive behavior	The intruder is in control; includes supine posture (ventral side exposed), prolonged crouch, and any other behavior by the resident in which the intruder is dominant (e.g., the intruder pins, aggressively grooms, etc., the resident); reciprocal to dominant behavior
Avoidance of the intruder	The resident withdraws and runs away from the intruder while the intruder is chasing; reciprocal to chasing the intruder
Defensive upright posturing	Species-typical defensive behavior; upright with the head tucked and the arms ready to push away
Non-agonistic social behaviors	
Social inactivity	Includes sit/lie/sleep together
Oronasal invest.	Active sniffing of the intruder's oronasal area
Body invest.	Active sniffing of the intruder's body, excluding the oronasal or anogenital regions
Anogenital invest.	Active sniffing of the intruder's anogenital area

(continued)

Table 1
(continued)

Behavior	Description
Stretched approaches	Risk assessment behavior; back feet do not move and front feet approach the intruder. Only number and latency were measured
Approaching and/or attending to the intruder	Often from across the cage; the resident's attention is focused on the intruder, head tilted toward the intruder, and movements toward the intruder; this becomes "chasing the intruder" once along the tail or sniff if within 1.5 cm of the intruder
Nonsocial behaviors	
Horizontal expl.	Movement around the cage; includes active sniffing of air, ground.
Vertical expl.	Movement to investigate upward, both front feet off the ground; includes sniffing, wall leans, and less than three lid chews
Digging	Rapid stereotypical movement of forepaws in the bedding
Solitary inactivity	No movement; includes sit, lie down, and sleep
Self-grooming	Rapid movement of forepaws over facial area and along body
Abnormal stereotypies	"Strange" behaviors, including spinturns, repeated jumps/lid chews/ head shakes (more than three)
Categories and composite behaviors	
Total activity	All active behaviors, both social and nonsocial. Excluded from this group are solitary and social inactivity, and self-grooming
Total social behav.	Any behavior involving both the resident and the intruder: Aggressive postures, reciprocal attacks, resident-initiated fighting, attacks delivered, dominant behavior, chasing the intruder, intruder-initiated fighting, attacks received, avoidance of the intruder, submissive behavior, defensive upright posturing, social inactivity, oronasal investigation, body investigation, anogenital investigation, stretched approaches, and approaching and/or attending to the intruder
Total agonistic behav.	This composite behavior represents the overall levels of agonism present in the resident–intruder interactions and does not indicate the direction of the agonistic behavior (i.e., whether agonistic behavior is directed toward the resident or toward the intruder). Includes aggressive postures, reciprocal attacks, resident-initiated fighting, attacks delivered, dominant behavior, chasing the intruder, intruder-initiated fighting, attacks received, avoidance of the intruder, submissive behavior, and defensive upright posturing
Agonistic behav. delivered	Resident-initiated fighting, attacks delivered, dominant behavior, and chasing the intruder
Agonistic behav. received	Intruder-initiated fighting, attacks received, avoidance of the intruder, submissive behavior, and defensive upright posturing

(continued)

Table 1 (continued)

Behavior	Description
Dominance score	Total agonistic behavior delivered minus total agonistic behavior received (both duration and number of bouts). A negative score indicates that the resident was the submissive animal in the pair, while a positive score signifies that the resident was the dominant animal
Social investigation	Oronasal investigation, body investigation, anogenital investigation, stretched approaches, and approaching and/or attending to the intruder
Nonsocial behav.	Any behavior involving only the resident: Horizontal exploration, vertical exploration, digging, solitary inactivity, self-grooming, and abnormal stereotypies
Non-soc. loc. behav.	Horizontal exploration, vertical exploration, and digging
Non-soc. non-loc. behav.	Solitary inactivity and self-grooming

Modified from Ref. [58]

Invest. investigation, *expl.* exploration, *behav.* behaviors, *soc.* social, *loc.* locomotor

5.3.3 Statistical Analysis

Once scored, behavior can be analyzed both across the whole 15-min test and in bins (e.g., of 5 min; *see* Fig. 3a), and one can look at the number of bouts, duration, and latency of each behavior (with a latency of 900 s assigned if a behavior is not performed by a given animal). For whole-trial data, an independent-samples *t*-test (two groups) or one-way ANOVA with post hoc tests if a significant effect of group is found (more than two groups) can compare groups for each behavioral measure. One-sample *t*-tests within each group will determine if the dominance score is significantly different from zero.

For binned analyses, a two-way mixed-model ANOVA, with group as the between-subjects factor, time as the within-subjects variable, and post hoc tests carried out in the case of a significant group x time interaction, should be used. Planned comparisons (as for the whole-trial data above) can also be used to compare behavior between groups for each bin.

5.4 Tips and Troubleshooting

As in both the social approach and social recognition tests above, minimizing stress (Subheading 3.4.1), acclimating mice to handling and procedures if they show high anxiety in response to the test (Subheading 3.4.2), and reducing environmental variability (Subheading 3.4.3) remain important.

The type and severity of these aggressive and agonistic interactions can be affected by a number of factors. Older and/or larger mice tend to be dominant [42], and, in what is known as the prior residency effect (Archer, 1988), an animal is more likely to win agonistic encounters and/or become dominant if tested in its home

cage against an intruder [42]. Mice will also respond to the behavior of their opponents [48]. The likelihood and intensity of aggression can also be increased by isolation, pain, or housing with the opposite sex [44, 48, 85]. Additionally, more aggressive males may be more anxious than less aggressive males [74, 86], and benzodiazepines can reduce both anxiety and aggression in these animals [52].

6 Scoring Social Behavior

There are several ways in which the assays described above can be measured. These all have their own advantages and disadvantages (*see* Table 2 for a comparison).

6.1 Manual Scoring with Stopwatch and/or Tally Counter

The most basic method involves simply using stopwatches or tally counters to measure the duration (stopwatch) or frequency (tally counter) of specific behaviors. While effective, manual scoring of this nature is labor-intensive; additionally, there is no record beyond the total time or count, and in order to look at different intervals, or to verify accuracy, one would need to completely rescore each trial. It also limits the number of variables that can be simultaneously scored, leading to the need for multiple viewings of each trial, and duration measures require either scoring in real time or calculating based on the factor by which the trial has been slowed.

6.2 Manual Scoring with Software

A slightly more involved option is to use software to record the manual scoring. One open-source program that lends itself well to this is BORIS [83], which allows one to import videos into the software and code for a variety of behaviors by assigning them a letter. In this type of program (The Observer from Noldus operates in a similar manner), behaviors are defined as “point” events (not associated with durations but single isolated events, e.g., a bite or a jump) or “state” events (an ongoing activity that measures both number of instances and duration of the behavior, e.g., chamber or sniffing time). As one sets up a defined list (or “ethogram”) of behaviors and the letters associated with them, this allows for scoring numerous behaviors concurrently. Thus, it is possible to score at a basic level (e.g., just chamber and/or sniffing time in social approach, social investigation in social recognition, or attack number, and/or latency in the resident–intruder test of free social interaction) or to include a variety of other, potentially interesting variables, as well as defining which behaviors can occur simultaneously (e.g., center chamber and inactivity in social approach) and which are mutually exclusive (e.g., mouse+tube chamber and center chamber in social approach). While practice is required to gain sufficient proficiency in the coding to be accurate, most programs

Table 2
Comparison of different methods and software for scoring social behavior

Method	Sec.	Example	Capabilities						Other advantages/disadvantages
			Auto.	Bin trials	Distance/ Locom.	Heat maps	Complex behav.[1]	Custom behav. (unltd. #)	
Manual scoring	6.1	n/a	No	No	No	No	Yes	With repetition	Low-cost Labor-intensive No way to verify accuracy
Manual scoring with software	6.2	BORIS [83] Observer (Noldus)	Semi	Yes	No	No	Yes	Yes	Open-source options (otherwise expensive license) Labor-intensive
Auto. Tracking software	6.3	EthoVision (Noldus)	Yes	Yes	Yes	Yes	No	No	Open-source options (otherwise expensive license) Low labor Cannot score sniffing except by proximity proxy Possibly unreliable head/tail tracking
Deep learning software	6.4	DeepLab Cut [88, 89]	Yes	Yes	Yes	Yes	Yes	Yes	Open-source options Low ongoing labor Initial setup: Somewhat labor-intensive, requires training data "Black box" analysis requires (at least initial) verification of accuracy

Sec. section, *Auto.* automated, *Locom.* locomotion, *Behav.* behavior(s), *Unltd.* #, unlimited number of custom behavior variables. [1]Complex behaviors such as nuanced social interaction, sniffing, etc.

(including BORIS and The Observer) will allow the speed of the trial to be adjusted, and it is possible to verify coding accuracy using the video and the scored trial. While neither manual option gives a direct measure of locomotion, recording the frequency of chamber crossings can provide a proxy for activity levels in the three-chamber assay.

6.3 Automated Tracking Software

More automated software is also available, especially for measuring variables like social approach chamber time and locomotion. A number of these are open-source, with varying capabilities (see [87] for a review). A commonly used commercial software is Etho-Vision (Noldus). This program can acquire videos and control trials, if the proper add-ons are included, but can also be used to score previously recorded videos. Automated tracking programs of this nature usually have the capability to track the head or nose, center of the body, and tail "points." In addition to decreasing the time and labor involved in extracting the data, these automated tracking programs are able to provide data not available from manual scoring, including distance, velocity, trajectory, and head orientation, as well as producing average heatmaps (*see* Fig. 1e) for a specific group (i.e., genotype, sex, treatment, or any combination thereof). A proxy of sniffing can also be measured by analyzing when the mouse's "nose point" is in a defined area around the enclosure, although care must be taken to ensure that the nose is properly identified (in some cases, the nose and tail points will "flip" during tracking).

6.4 Deep Learning Software

The ideal software would combine aspects of both the manual scoring software (Subheading 6.2) and the automated tracking (Subheading 6.3) to provide accurate quantification of social and nonsocial behavior without labor-intensive, time-consuming manual scoring. Recent advances in so-called deep learning (machine learning using deep-type artificial neural networks) are making this a possibility. One stand-out, open-source example that is being increasingly used to extract large amounts of valuable data from behavior videos is DeepLabCut [88, 89]. This software is able to track multiple body parts of the test animal and use this data to determine the "pose" (geometric configuration of body points) of the animal without requiring any markers or other invasive identification methods [88]. While it does require manual labeling to train the network (approximately 100–200 frames), following training DeepLabCut is able to use transfer learning to obtain accuracy comparable to human manual scoring and is even able to tolerate challenging videos with inconsistent or uneven lighting, distortions due to camera angle, shadows, and similar complications [88]. Furthermore, there is evidence that this software is capable of tracking multiple mice within a single video simultaneously, even when trained with a single animal, an essential requirement for social behavior scoring [88]. While occlusions

(such as one mouse blocking the view of the other) and dynamic visual environments remain difficult for automated scoring, refinements to the software improve its ability to cope with these challenges [89]. Furthermore, the recent versions use Python to minimize the amount of programming knowledge necessary for new users [89].

While there are clearly many advantages to this type of analysis tool, there are some limitations. Firstly, a significant initial investment of time is required, as are the initial "training" frames, which will need to be manually labeled. Additionally, as it can be something of a "black box" in terms of how the analysis is performed, manual verification, at least in the first few trials, is essential to ensure accuracy.

7 Summary and Conclusions

The category of social behavior involves many aspects, from a simple question of whether one prefers to interact with or avoid a social stimulus to more detailed examinations of the nature of social recognition, aggression, and interaction. While we do not claim that the tests described here cover all types of social behavior (e.g., they do not touch on intersexual behavior like mating, maternal behavior, or social learning), we hope the three assays described here provide a good battery to address some of these behaviors and give a general overview of whether social deficits are present in the mice being tested.

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Chapter 15

Rodent Models for Studying the Impact of Variation in Early Life Mother–Infant Interactions on Mood and Anxiety

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Abstract

The early life origins of risk for mood disorders and anxiety are supported by longitudinal studies in humans, particularly those focused on the experience of childhood adversity. Animal models have further supported the association between stress exposure during infancy and neurodevelopmental and physiological processes that shape behavioral indices of depression and anxiety. In rodents, these models have focused on disruption to mother–infant interactions that occur either naturally or through manipulation of the quantity and/or quality of maternal care. Though these models are challenging to implement, they serve as essential tools for establishing or characterizing the neurodevelopmental trajectories that lead to increased risk of psychopathology. In this chapter, we will describe methods that can be used to quantify maternal behavior in rats and mice, with particular emphasis on the use of newly developed video recording and machine learning approaches. We will also describe the premise and protocol for an established methodology to effectively manipulate mother–infant interactions within experimental designs that are exploring neurobiological and behavioral outcomes in offspring. This limited bedding and nesting material (LBN) manipulation is used broadly to study the impact of early life stress in rodents and when combined with high-resolution behavioral quantification approaches can yield novel hypotheses regarding the molecular, cellular, physiological, and behavioral mechanisms that contribute to altered brain function across the life span.

Key words Maternal behavior, Home-cage, Rodent, Machine learning, Limited bedding

1 Introduction

The experience of adverse early life experiences is associated with a broad range of physical and psychiatric health outcomes [1]. In particular, risk of mood disorders and anxiety are associated with disruptions to the quality and quantity of parental interactions during infancy and childhood, occurring in cases of abuse and neglect [2]. Though advances in human neuroimaging and molecular analyses have contributed to a deeper understanding of the potential pathways linking the experience of adversity and adult mental health outcomes, animal models continue to serve as a

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critical methodological tool for exploring these pathways [3]. The ability to manipulate and characterize the early environments of mice and rats, combined with the high fecundity and relatively short life span of these species, has contributed to an extensive literature exploring the molecular, neurobiological and physiological effects of early life adversity and the link between these effects and behavioral indices of depression- and anxiety-like behavior. This literature has integrated classic approaches, such as neonatal handling and maternal separation (MS), as well as relatively more recent adaptations of these approaches (e.g., maternal separation combined with maternal stress; see [4, 5]) and new approaches (e.g., limited bedding and nesting material (LBN) [6]). Here we will focus on the LBN model and more generally on methodologies for characterizing maternal behavior (see [7] for a discussion of handling and maternal separation).

The LBN manipulation is founded on the premise that altering the maternal environment by reducing nesting material resources induces maternal stress that in turn alters dam–pup interactions. This premise has led to interpretation of LBN as an early life stress manipulation comparable to various forms of early life stress observed in humans, such as abuse/neglect or institutional rearing [6]. The LBN procedure involves providing very little or no bedding material in the home-cage after pups are born which induces robust changes in dam–pup interactions. Some LBN studies have found no differences in global measures of maternal behaviors summarized from observations but have revealed substantial differences in patterns of maternal behavior [8]. Somatosensory cues scaffold central nervous system development and are transmitted to pups through maternal behavior, so changes in predictability or patterns of maternal behavior can have considerable effects on the developing pup brain during sensitive periods of central nervous system development that endure through to adulthood. While changes in pup-directed maternal behavior is certainly a primary contributor to pup outcomes, other factors associated with LBN may also influence pup development, including changes in pup nutritional intake and metabolism, nest temperature, pup huddling behavior, and altered olfactory cues due to the inadequate nest [9, 10]. LBN can be applied during postnatal periods specific to development of particular brain regions or applied more generally during the first 2 postnatal weeks. This increasingly popular early life stress paradigm approach is flexible and adaptable to different environments, relatively easy to implement with limited need for experimenter intrusion, and has translational potential to inform mechanisms underlying the relationship of early adversity and health outcomes in humans [9].

Changes in early life dam–pup interactions induced by LBN can lead to both immediate and enduring changes to pup stress physiology and behavior. The impact of LBN on hypothalamic–

pituitary–adrenal (HPA) axis-related outcomes depends on the timing of LBN exposure during development, specific LBN variation used, species and strain, age, and sex of offspring. For example, studies have found elevated, reduced, or no change in basal plasma corticosterone in LBN offspring immediately following LBN and in adulthood in rats and mice [6, 11–14]. Adrenal hypertrophy has also been reported for rat and mouse pups subject to LBN [6, 15]. Altered levels corticotrophin-releasing factor (CRF) in the paraventricular nucleus (PVN) and hippocampus have also been reported for both rats and mice [16–18].

Cognitive and emotional outcomes in adult rodents exposed developmentally to LBN offspring are also observed. Studies report increased anxiety-like behavior during the open field test [16, 19], reduced spatial memory during Morris water maze [6, 15] and novel object recognition test [6, 15, 20]), earlier onset of the development of contextual fear conditioning in mice [11], and increased depressive-like behaviors during the forced swim test [21] following LBN, although these deficits are not always observed.

Studies of the neurobiological consequences of LBN have focused primarily on the hippocampus and amygdala. In the hippocampus, LBN leads to dendritic atrophy [15], increased CRF expression, reduction in plasticity and synaptic density, and increased cellular proliferation and volume reduction in the dentate gyrus [16, 22]. LBN can perturb amygdala connectivity, including increasing spine density and altering connectivity with prefrontal cortex [23, 24]. In addition, LBN exposure during development has been found to enhance amygdala activity, particularly following stressful experiences such as novel context exposure or a forced swim test [18, 25].

Though the LBN approach to shifting offspring neurodevelopmental and behavioral trajectories provides a robust strategy for studying depressive- and anxiety-like behavior in rodents, a critical element of this approach is the quality, quantity, and patterns of maternal behavior experienced by pups within both the LBN and control conditions. Natural variations in maternal behavior have been documented in laboratory mice and rats, and there may be variation in how individual dams respond to postnatal manipulations [26, 27]. There are considerable species, between-strain and within-strain differences in the frequency of the various aspects of maternal behavior. In addition, studies in mice and rats highlight the enduring impact of variation in the quality and quantity of mother–infant interactions on a broad range of neurobiological and physiological systems with consequences for depressive- and anxiety-like behavior [28, 29].

Assessment of variations in maternal behavior in rodents, either within the context of a manipulation of the early rearing environment or to gauge naturally occurring variations is challenging but if

successful can yield exciting new insights into the pathways linking early life experience to molecular, neurobiological, and behavioral outcomes relevant to studies on mood and anxiety. Though there are other strategies for assessing maternal behavior in rodents, such as pup retrieval tests or assessment of maternal aggression [30–33], these approaches do not characterize the day-to-day experiences of offspring that may shape neurodevelopment. Thus, methodological approaches involving home-cage maternal observations and assessment of individual differences in maternal care toward offspring are essential in studies examining the direct impact of maternal care on offspring development as well as the moderating effect of these experiences on the influences of environment, genotype, and strain. In the subsequent sections, we will provide a detailed description of this methodology, outline strategies for analyzing maternal data, and discuss tips for troubleshooting this protocol.

2 Materials

2.1 Animals

Both inbred and outbred strains of laboratory mice and rats are suitable for measuring home-cage maternal behavior. However, as mentioned in the previous section, strains vary considerably in the frequency of mother–pup interactions, particularly in licking/grooming (LG) behavior. For example, Balb/c and 129Sv mice engage in significantly reduced levels of LG compared to B6 and Swiss. Similarly, Long–Evans rats display more maternal behaviors including more pup contact, better maintenance of pups in a central nest, more active nursing postures [34], and more licking and grooming of pups compared to other rat strains [35]. If the goal of the study is to assess offspring who have received low vs. high levels of LG in a within-strain model, then selecting a strain with a high average level of LG (i.e., B6 mice or Long–Evans rats) would be recommended. Alternatively, a cross-fostering design could be used between strains that exhibit low (i.e., Balb/c mice, Fischer 344 rats) vs. high (i.e., B6 mice, Long–Evans rats) levels of LG [36, 37]. To generate litters for these studies, female and male rodents can be successfully mated at 8 weeks of age with mating success declining after 32 weeks. Within a cohort of mating females, it is best to use animals that are similar in age and reproductive experience. When breeding in-house, females from the same lineage should be equally distributed between conditions since maternal care phenotypes can be passed across generations and may interact with treatment conditions [38]. For example, lineage was found to be an important contributor to several maternal behaviors including nest attendance, time spent nursing, and food-hoarding behavior among dams exposed to LBN and control conditions [39].

2.2 Animal Facility

The maternal behavior and rearing experiences of laboratory animals can be easily disturbed by the routine traffic and maintenance activities that occur in all animal facilities. Ideally, these studies should be conducted in smaller animal housing rooms separate from large vivarium housing. Though investigators often do not control the light cycle within animal housing rooms, studies should avoid transferring mice to and from rooms that differ in this cycle. For example, if offspring are going to be tested on measures that require reverse lighting (i.e., dark during the day, lights on at night), it would be preferable that they are reared under these same lighting conditions. There has been no systematic study of whether maternal data collected exclusively under light vs. dark conditions are better predictors of the overall pattern of maternal care and both strategies have been successful approaches in studying mother–pup interactions in rodents [40–42]. If observations are being conducted during the dark phase of the cycle, lamps with red light bulbs can be arranged within the observation area to provide suitable illumination. However, the type of light source should be carefully considered to avoid white light exposure and ultrasonic frequency noise generated by the light source.

Additional animal facility considerations are necessary for video recording of mother–pup interactions. Red lighting or infrared lighting allow for recording during the dark phase of the light cycle and lighting setups should be tested to ensure glare from light bulbs do not interfere with video observations. To avoid this issue, LED strip lights can be applied under shelving units and are typically bright enough to illuminate the entire cage. Camera angle and positioning on the nest are essential for collecting good-quality video. Many dams construct nests away from noise and disturbances (e.g., end of the cage close to the wall), and cameras should be set up with a clear view of the nest. In addition, disturbing cages should be avoided prior to and during recording. If adjustments to the cage need to be made, such as replacing the cage top with Plexiglass in order to record from above, the dam and pups should be permitted to habituate for 15 min to the new setup before data collection commences.

A wide range of cameras are appropriate for recording home-cage maternal behavior including traditional camcorders (with or without infrared lenses), GoPros, and higher-end webcams, although cameras with low-light setting or infrared/night vision capabilities are best for recording in the dark (*see* Fig. 1b). In an ideal setup, each cage is equipped with its own camera to avoid the need to move cameras or cages each day (*see* Fig. 1a). Furthermore, beginning recording remotely, for example, through an app that controls the camera via WiFi or Bluetooth, or through scheduled automatic recording, reduces the potential influence of experimenter presence on maternal behavior. Headless Raspberry Pi computers connected to NoIR infrared cameras can be

a

b

Fig. 1 Home-cage recording setup. (**a**) Example setup of a headless Raspberry Pi computer with NoIR infrared camera set up to record home-cage behavior. Raspberry Pis can be accessed remotely to program recordings. (**b**) Camera view during dark phase under infrared light of a lactating Long–Evans dam and pups

programmed to record videos during specific times of the day, automatically upload the videos to cloud storage, clear space for the next recording, and be controlled remotely by connecting via VPN or SSH [43, 44]. With one computer/camera per cage, all

cages can be recorded simultaneously, thus removing the need for repeated recording setup by the experimenter, eliminating time of day variability in behavior, and allowing for data collection during any period in the 24-h cycle without experimenter supervision. These and similar "mini computers" are becoming more prevalent in behavioral research and produce high-definition video quality at a fraction of the price of traditional camcorders. However, several computer/cameras transferring videos to cloud storage requires a high volume of data transfer, so it is advisable to consult with your institution's IT department to avoid issues with network data transfer limits or Wi-Fi connection. A final consideration when configuring recording setup and lighting is wire management and ensuring proper use of electrical outlets and surge protectors.

2.3 Housing and Husbandry

The observation of home-cage maternal behavior will require that rodents are housed in Plexiglas or equivalent cages that permit a clear view of the dam and litter. Though the standard cages that are typically used for housing mice and rats will be sufficient, larger cages permit the observation of more dynamic interactions between the dam and pups. The bedding material is also an important consideration. Corncob bedding does not provide sufficient nesting material for lactating females, and if using this type of bedding, supplemental bedding such as nestlets or cellulose bedding will need to be provided. However, these materials allow dams to build very elaborate nests which will prevent reliable observations of mother–pup interactions. An alternative is to use pine shavings as the bedding material. Mice and rats can build nests from this bedding yet still be viewed from the exterior of the cage, making nestlets unnecessary.

2.4 Mating

It is recommended that if rodents are being used from a commercial breeder, a 2-week period of acclimatization to the animal facility should be implemented to promote reduced stress at the time of mating. Multiple females (two to three) can be housed together with one male to promote the breeding of multiple litters. Males can be removed after a 2-week period to avoid any contact with postnatal pups which will reduce the risk of infanticide and prevent the male from influencing the postnatal development of offspring. Alternatively, dams placed with males can be checked daily for vaginal plugs and can be separated from males when the presence of a plug is confirmed.

2.5 Postpartum Monitoring and Husbandry

Mated females should be routinely monitored and singly housed a few days prior to parturition. Though it is often difficult to ascertain when a female will give birth (particularly if the litter is small), the weight gain of pregnant females will be evident 2 weeks after mating. Once singly housed, females should be monitored daily to

establish the date of birth of the litter. The process of parturition may take over an hour to complete, particularly if the litter is large, and it is best to leave the female undisturbed until this process is completed. After birth, the dam will retrieve her pups to the nest and clean them to remove remnants of the placenta. Once this process is complete, pups can be weighed and counted and placed with the dam into a clean cage, mixing in some of the soiled bedding material from the old cage to provide olfactory continuity and reduce rehousing stress. During the postnatal observations of maternal care, cage cleaning is very disruptive. Cleaning the cage on the day of birth (day 0) will allow for undisturbed assessment of home-cage behavior from postnatal days 1–6, and cages should not be cleaned until after observations are completed on postnatal day 6. We recommend cleaning the cage every 7 days thereafter until weaning. Cleaned cages can then be positioned on the racks in the animal housing room to permit viewing of activity within the cage. Observations of maternal behavior can then commence the following day on postnatal day 1. It is important to communicate with animal care facility staff regarding the husbandry arrangements for dams and litters to avoid unanticipated disruption to the periods of observation/recording (*see* **Notes 5.1** and **5.2**).

2.6 Implementing the LBN Manipulation

Though LBN was originally developed in rats and applied during postnatal (PN) days 2–9, it is now widely used with mice and rats, and several variations of LBN have emerged [6, 9]. All dams are maintained in cages with standard levels of bedding until birth or the onset of the LBN period. On the first day of LBN, dams are randomly assigned to LBN or control condition, and all dams and litters undergo a cage change. The most common variation involves insertion of plastic-coated mesh approximately 2.5 cm above the floor of the home-cage with a thin layer of bedding underneath the mesh that is inaccessible to the dam with limited potential to absorb urine and feces. LBN dams are given very little nesting material, usually a half nestlet or paper towel, to construct a nest. Control dams receive normal amounts of bedding according to standard operating procedures for the lab, which may vary from institution to institution but has been reported to be as much as three inches of bedding. The timing of the LBN manipulation also varies between labs and studies but often lasts four to 12 days between PN2 and PN14 during which the home-cage is left undistributed. Other versions of LBN, more frequently used with rats, include eliminating the wire mesh (commonly known as the "scarcity model" [45]) or brief, intermittent exposures to LBN [46]. LBN has also been used in combination with other manipulations of maternal care, including maternal separation [47].

3 Methods

The acquisition of behavioral data from the home-cage can be achieved either through video recordings or by observers who document the behavior in the home-cage from within the animal housing room. In both cases, raters/observers will need to be trained to a high degree of inter-rater reliability (see **Notes 5.4** and **5.5**). The description of maternal behaviors that can be observed when coding rats and mice is provided in Table 1 and includes nursing postures, licking/grooming pups, nest-building, self-grooming, eating, and drinking. These behaviors are not mutually exclusive and can occur in a variety of combinations. There may be a wide variety of nursing postures that can be observed, and a more detailed account of these postures has previously been described [40]. However, for most studies, the behaviors outlined in Table 1 will capture the features of mother–pup interactions that are predictive of long-term outcomes in offspring. Each individual behavior should be assigned a unique alphanumeric identifier (e.g., nursing = N, nest-building = B), and raters/observers should be provided with a detailed legend outlining these identifiers. The schedule of observations during the day relative to the light cycle should be consistent across days and for each litter. Furthermore, it is important to avoid conducting observations within an hour before or after the light–dark transition in room lighting. It is recommended that a minimum of 4 h of observations (e.g., 11am, 12pm, 3pm, 4pm) be conducted each day for each litter. Across the postpartum period, there is a significant decline in maternal behavior (particularly LG [26, 27]), and it is generally accepted that the critical window for many long-term developmental effects will require assessment of maternal care from the time of birth for at least 6 consecutive days.

3.1 Live Observation of Maternal Behavior

When conducting live observations of maternal behavior, the data collection strategy can generally follow a time-sampling method, where behaviors are coded at evenly spaced time intervals within an observation session, or focal observations, where coding reflects a continuous observation of the dam behavior including transitions from one behavior to the next. The time-sampling approach takes a "snapshot" of behavior and is an effective methodology for generating overall frequencies of a behavior. This method is particularly recommended when there is a high volume of litters to observe within a single observation session. The focal observation method provides information on the occurrence and temporal patterning of behavior but is more suited to live observation setups where there are few litters to observe (*see* Fig. 2). For a detailed description of implementing live observation protocols for data collection, see [7].

Table 1
Description of home-cage behaviors in rats and mice

Behavior	Description	Code
Nursing (crouch)	Dam is positioned over the pups to permit sucking or thermoregulation with a low to moderate arch in her back	N
Arched nursing	Dam is positioned over the pups with a high arch in her back to permit sucking and pup movement	A
Passive nursing	Dam is lying on her side with her ventrum exposed to the sucking pups	P
Licking/ grooming	Dam is licking pups (anogenital or body region)	G
Self-grooming	Dam is licking herself (often occurs during bouts of pup licking)	S
Nest-building	Dam is picking up pieces of bedding and retrieving these to the nest or moving bedding in the nest with her snout	B
Eating	Dam is eating	E
Drinking	Dam is drinking	D
Contact with pups	Dam is in contact with the pups but not in a posture that promotes sucking (i.e., sitting next to pups)	C
No contact with pups	Dam is off the nest and not in contact with any pups (and not engaging in any of the other behaviors noted above)	X
Pup retrieval	Dam picks up pup with her mouth and carries it to the nest	R
Tail chasing[a]	Dam chases her tail or carries her tail in her mouth	T
Tough handling[a]	The dam drags by limb, drops, steps on, or kicks a pup	H

[a]These behaviors can be scored when using LBN

3.2 Manual Scoring of Maternal Behavior from Video Recordings

Video recording the home-cage allows for continuous focal observations on a more flexible schedule. Video recordings afford the option of stopping, pausing, rewinding, or playing video in slow motion which allows for scoring of more behaviors with improved accuracy. Video recordings can be scored manually or may be scored using free event-logging software such as CowLog or Behavioral Observational Research Interactive Software (BORIS) or paid software such as The Observer XT or EthoVision (Noldus [48, 49]). These programs speed coding with programmable hotkeys for experimenter-defined "point event" (behavior with no duration) or "state event" (behaviors coded with stop and start times) behaviors and have built-in options for plotting and exporting data for statistical analysis. A major drawback to the use of video recordings is the inability to change perspective for a better view of the dam and pup which made lead to missing data, for example, when the dam or nesting materials block the view of the pups from the camera.

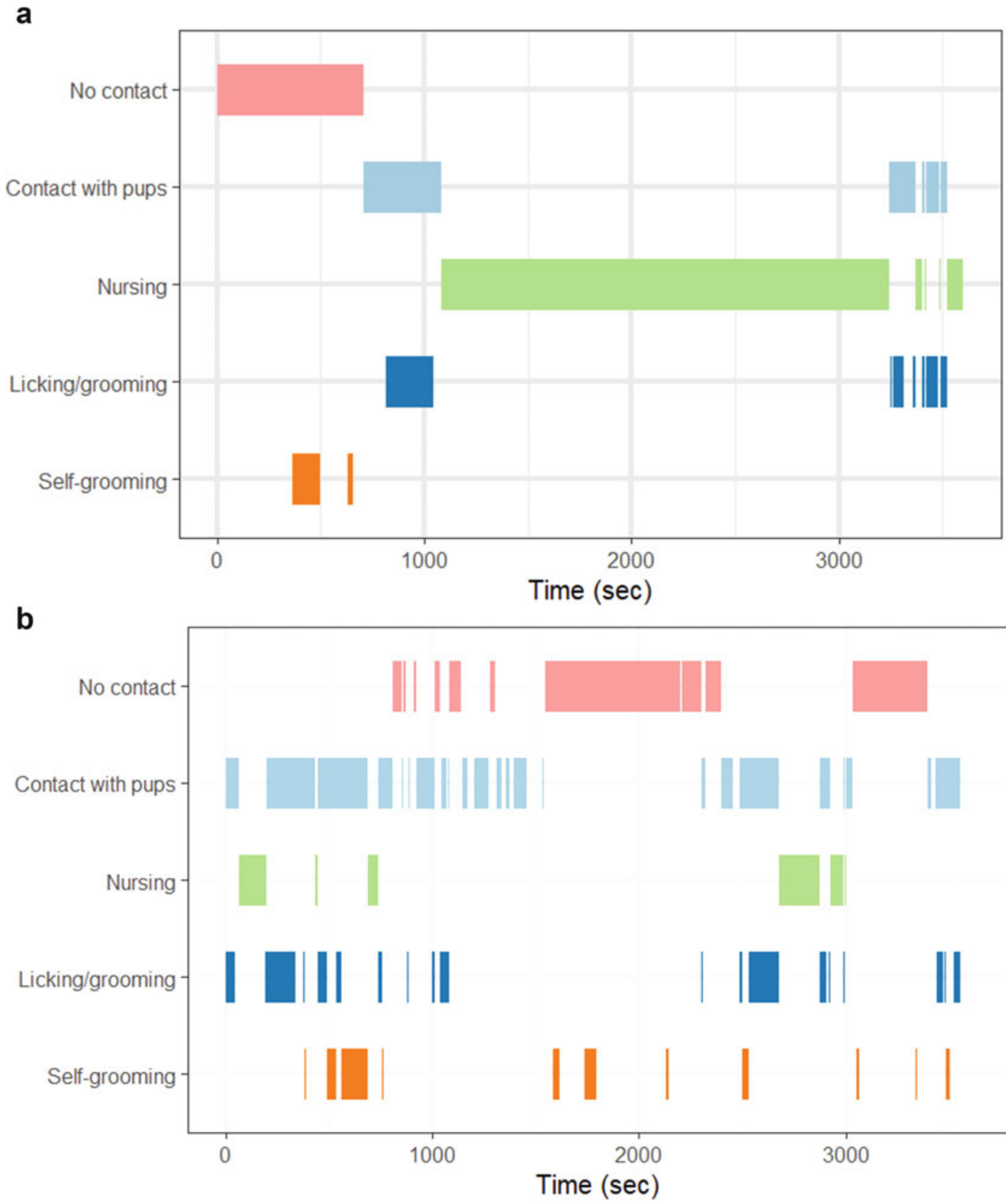


Fig. 2 Gantt plots of maternal behaviors scored from a 1-h home-cage video recording. (**a**) A Gantt plot from a focal observation of a dam exhibiting predictable behavior including a long nursing bout and maintaining pup contact for the majority of the observation period. (**b**) A Gantt plot from a dam demonstrating frequent behavioral transitions including short nursing bouts and initializing and terminating pup contact several times throughout the observation period

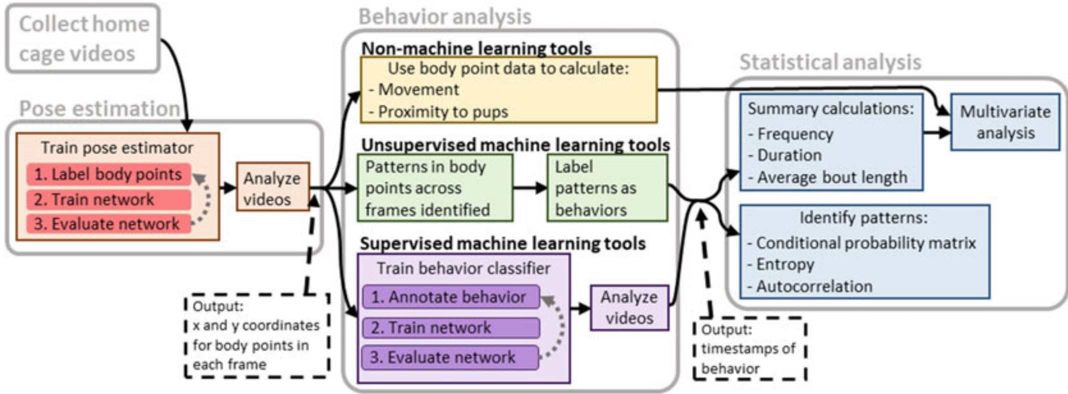
3.3 Observing Maternal Behavior During LBN

Maternal behavior during LBN is evaluated either through live observations or home-cage video recordings. The hallmark of the LBN procedure is fragmented patterns of maternal care, rather than changes to the quantity of any specific maternal behavior. For example, nest attendance bouts are shorter, although the total time LBN dams spend on the nest may be the same as control dams [9]. Long focal observations are better suited to capture this effect on maternal care. LBN also leads to an increase in rough handling of pups (e.g., dragging by limbs, dropping, kicking, stepping on pups) and tail chasing/carrying, so these behaviors should be scored as well (8; Table 1, *see* **Note 5.2**).

3.4 Machine Learning Approach to Video Processing of Recorded Maternal Behavior

Recent advances in machine learning tools for computer vision and automated classifiers hold promise of improving how rodent maternal behavior is measured. Generally, the process of using machine learning for behavior coding involves first tracking body points in a video frame by frame and reducing the video data to a list of location coordinates of each body point in the frame (e.g., in a csv file (*see* Fig. 3a)). The second step of the process is to identify and extract markers of behavior from those coordinates using machine learning or non-machine learning tools. Several open-source deep learning tools for pose estimation can be applied to dam–pup recordings including SLEAP and DeepLabCut [50–52]. With these tools, users can define body parts and label frames from videos to train the network (*see* Fig. 3b). Once the network is performing well, it can be applied to any video to obtain pose estimations. These methods allow for flexibility in lighting, context, camera angle, and have multi-animal versions enabling tracking of more than one individual [50, 53]. This innovation addresses some of the limitations that have prevented proprietary software from being used to code maternal behavior.

Once pose estimation is complete, the body coordinate data can be used to infer behavior. Some information that can be easily extracted from coordinates, like proximity to pups or overall movement, using R or another programming language without the use of machine learning tools. For more complex analyses, machine learning tools can be applied such as Motion Sequencing (MoSeq) or B-SOiD [54, 55]. Unsupervised machine learning tools extract spatiotemporal patterns or features from the body coordinate data with no instruction as to what to look for. These clusters of body coordinate patterns across frames (i.e., types of behavior) can then be examined by the user to determine what they are mapping onto preexisting ethograms [55]. Behavior extraction using unsupervised machine learning tools eliminates user bias in what behaviors are scored, allowing for detection of behavior that may otherwise be missed. However, the translation of the machine-defined clusters of body coordinates to meaningful behavior may not be straightforward. Once the network is generated using these



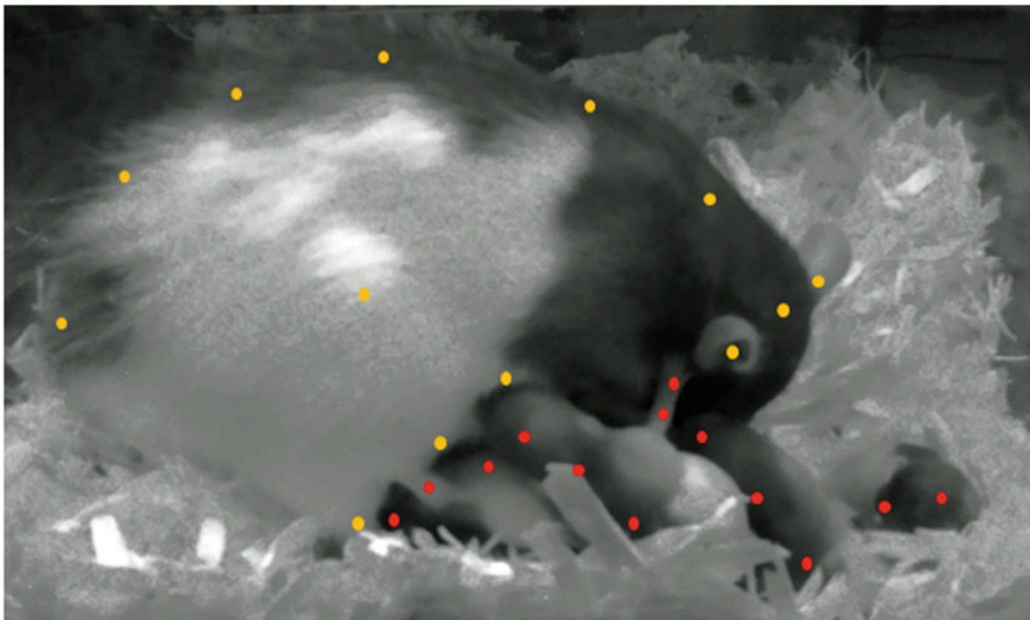


Fig. 3 Machine learning video processing overview. (**a**) Workflow for using machine learning tools to code maternal behavior from video recordings. First, a pose estimator must be trained to track body coordinates on the dam frame by frame in the videos. The coordinate data is then recorded in a csv file which can be exported for use in behavioral analysis. Non-machine learning tools and machine learning tools can be used to analyze body coordinate data. With unsupervised machine learning tools, patterns from the body coordinate data are identified and can then be labeled as specific behaviors by the user. With supervised machine learning tools, the user can train a behavior classifier to automatically identify user-defined behaviors by first training a network by providing annotated behavior and corresponding body coordinate data. After behavior identification, statistical analysis is performed on frequency, duration, bout length, or patterns of maternal behavior. (**b**) Example of a labeled frame used to train pose estimation networks. Dam body points are shown in yellow and pup body points are shown in red

unsupervised methods, the same network can be applied to infinite videos to analyze those behaviors. However, the output of the network depends on the videos it is trained on, so generating a network from different videos may lead to different behavioral output.

Unlike unsupervised methods, supervised machine learning tools for behavioral classification allow the user to define which behaviors are scored and provide examples of those behaviors. Supervised behavioral classifiers currently available include Simple Behavioral Analysis (SimBA), Janelia Automatic Animal Behavior Annotator (JABA), and DLCAnalyzer [56–58]. SiMBA allows the user to input pose estimation (e.g., from DeepLabCut) and accompanying behavioral annotations for a training set of videos [57]. During training, the network learns to identify the defined behaviors based on the pose estimate data. Once trained, the network can be applied to body coordinate data from any video, provided that the body parts tracked are the same as those tracked in the training set. Because behaviors are user-defined and the network is trained on manually scored data with supervised machine learning methods, this method may resemble manual coding. However, a high degree of user input makes this method more susceptible to user bias, and it will only produce information for specified behaviors.

While many new tools for automating behavioral coding are being developed, there are several challenges in implementing the tools currently available for scoring dam–pup interactions in rats and mice. First, these tools have been primarily used on video recordings from directly above or below the cage. For home-cage maternal behavior, this approach would require specialized housing when the top of the cage is occluded by food and water, while manual scoring can be conducted in standard cage setups where wire tops, food, and water block the top-down view. Second, multi-animal tracking and behavioral classification may not be able to track individual pup identity, especially in the case where pups become occluded and reemerge later in the video. This issue is a focus of tracking program developers and may be solved with future releases of tracking software. Third, effort required to generate a pose estimation network and develop a behavioral classifier is not trivial and may exceed the effort of manually coding videos. However, the use of machine learning tools for pose estimation and automated classifying of maternal behavior allows for improved standardization, better temporal resolution, and more efficient data-processing compared to other methods.

4 Data Analysis

The observation and coding protocols outlined in the previous section result in numerous individual data points that can be used in multiple ways depending on the overall experimental design of the study. There are multiple statistical approaches to using this data, and the choice of approach will depend on the independent or

dependent variables associated with maternal behavior. For a detailed description of calculating maternal behavior frequencies and behavioral bout length, see [7].

4.1 Identifying Patterns of Maternal Behavior

Predictability and patterns of maternal behavior can be detected with data from focal observations (e.g., *see* Fig. 2). A conditional probability matrix can be used to assess predictability of maternal behavior transitions, where the high or low probabilities suggest structured patterns and mid-range probabilities indicate random behavioral sequences. The entire transition matrix can also be reduced to one entropy score by calculating the entropy for each row's probability distribution and combining to calculate a final entropy score [8]. Similarly, maternal behavioral contingency can be calculated by scoring the primary behavior observed in time bins (e.g., every 10 s) appropriate to the length of the observational period using time series analysis. Autocorrelations can then be calculated by comparing the sequence of behaviors to the lag version of scored behaviors to determine the degree of stability in maternal behavior across the observational period.

4.2 Using Multilevel Modeling in Studies of Maternal Behavior

Multilevel (hierarchical or random effects) modeling is now well established as a standard technique appropriate for analyses of the type of data generated from observation or coding of maternal behavior [59, 60]. By accounting for repeated observations within a single group (i.e., multiple observations of a single litter), multilevel modeling allows researchers to retain the bulk of their data in raw unaggregated form, rather than using average levels, thus gaining statistical power and permitting more refined experimental questions. To use this approach, the variables to be included would be (1) observed behavior, (2) observation type (e.g., postnatal day, time), and (3) litter ID. The data are then analyzed as with GLM or regular regression but using a multilevel error structure. These techniques are easily applied in most current statistical packages [59, 60].

5 Notes

5.1 Low Levels of Maternal Behavior with Minimal Variation

Though this may be due to specific characteristics of the strain or genotype being used, a common cause of reduced maternal behavior in rodents is stressors within the physical or social environment. Fluctuations in temperature, humidity, and noise can disturb patterns of mother–pup interactions. Collaborating with animal care staff is essential to maintain appropriate conditions for assessing home-cage behaviors, and observations should be conducted when there are few if any ongoing activities in the housing room. Schedule observations during the day so as to avoid times when routine cage maintenance/cleaning are being conducted in the room.

5.2 Outliers in Maternal and Offspring Behavior

It is not uncommon for there to be dams or offspring that exhibit extremes of behavior. In some cases, there are predictors of when this will occur. Within a very small litter (i.e., fewer than four pups) or very large litter (i.e., greater than 14 pups), sizes can influence the pattern of maternal behavior and average pup weight and affect the experience of individual pups within the litters. One approach to address this issue is to cull litters to a standard size (i.e., eight pups) and to not use very small litters. However, litter size and average pup weight can also be used as covariates in data analyses and may yield interesting interactions between life history variables and outcome measures. In general, it is best to record as much information as possible on each litter, particularly any adverse experiences that may alter the data.

A subset of dams, particularly rat dams, exposed to the LBN procedure may use food pellets as supplemental nesting material. Dams that food-hoard during the first days of the LBN procedure are very likely to repeat this behavior throughout the manipulation period if food is still available to them, even if hoarded food pieces are removed. Food-hoarding can be addressed by removing food pieces from the bottom of the cage and limiting the amount of food available to the dam daily. Although food pellets are certainly qualitatively different than standard bedding, this behavior increases the ability of the dam to construct nest walls which compromises the intended effect of LBN and presents a possible confound in the environmental manipulation. A plan for addressing this behavior should be decided on prior to the start of the experiment. For example, access to excess food can be limited for these dams, food-hoarding behaviors can be included as covariates in statistical analyses, or dams showing this behavior can be excluded from analyses altogether.

5.3 High Levels of Mortality

There are often periods of time during the course of the year where breeding success is very low and the mortality of litters is very high. Reproduction in rodents is highly dependent on olfactory cues, and any change in the olfactory environment may lead to a reduction in the number of litters produced that survive to weaning age. If the source of the reduced reproductive success can't be identified, it may be recommended to use a different mouse or rat strain, as there are likely to be strain difference in resilience to these environmental fluctuations. Rats are generally more resilient to these influences on breeding; however strain, age, and previous reproductive experience can all affect breeding success.

5.4 Avoiding Observer Effects

Variability in behavioral data can often be linked to the particular characteristics of individual experimenters/observers. In addition to appropriate training to obtain an inter-rater reliability greater than 90%, observers should be provided with detailed instructions regarding the importance of not disturbing the litters while

conducting the observations. Cell phones, loud noises, lighted computer screens, lighted smartwatches, and heavy perfumes/ aftershaves are definitely to be avoided. Observations based on video recordings help to ameliorate these effects and increase the reproducibility of findings.

5.5 Making Fine-Tuned Behavioral Distinctions

Training observers is a difficult task. In addition to using video recordings to generate inter-rater reliability, accompanying observers during sessions and providing feedback will improve the quality of the maternal data for live scoring. This will be particularly important for difficult behavioral categories, such as the distinction between contact and nursing or between self-grooming and pup-grooming. Group discussions and training sessions can help cultivate a team of observers that can reliably assess maternal behavior.

6 Concluding Remarks

Across species, the quality of the early life environment can shape development leading to increased risk or resilience to later-life psychiatric outcomes. Measures of variation in maternal behavior have proven to be valuable tools in understanding the mechanisms of these developmental effects, and the successful use of mice and rats as models for these studies is dependent on the appropriate assessment of home-cage maternal behavior. This assessment can be conducted under standard rodent housing conditions when using manipulations such as LBN. Maternal behavior is dynamic, and temporally sensitive and detailed observation protocols are required to assess maternal behavior to generate robust and reproducible data. However, if there is careful attention to the parameters that impact this assessment, the data generated can provide meaningful insights into the origins of neurodevelopmental and behavioral trajectories.

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Chapter 16

Principles of Recording Rodent Ultrasonic Vocalizations

Stefan M. Brudzynski and Sylvie Granon

Abstract

The chapter presents a general method of recording ultrasonic vocalizations from rats and mice. There is a growing interest in recording and analyzing ultrasonic vocalizations emitted by rodents. These calls are frequently produced by animals and serve for emotional expression in intraspecies communication. Thus, vocalization opens a window into the emotional life of animals and allows scientists to study the emotional brain both in physiological and pathological situations. Rats and mice are used as animal models of neurodegenerative, developmental, or psychiatric disorders. In all these neuropathological conditions, social, and accompanying emotional disturbances are frequently associated with changes in vocal communication and in structure of emitted calls. This chapter will explain why rodents emit vocalizations and what their functions are, as well as why recording of ultrasonic calls in rats and mice present an important research tool. The chapter focuses on details of laboratory and behavioral methodology of recording ultrasonic calls, which differs from recording of audible sounds, as well as provides guidance as to the analysis of these calls. The procedure of recording ultrasonic vocalizations, further processing, and digitizing the signals is described, together with detailed setting of parameters controlling data acquisition using the digital signal processing technology.

Key words Ultrasonic calls, Expression of emotion, Social behavior, Recording, Digital signal processing, Ultrasonic microphone, Spectrogram, Power spectrum, Rats, Mice

1 Why Rodents Vocalize?

Ultrasonic vocalization evolved in rodents initially from mother–infant vocal communication and was further retained in adulthood [1]. Vocal communication is particularly useful because it is not dependent on the daylight, can reach the recipients from a considerable distance, and does not leave any trace in the environment. For protection, rodents developed communication in the ultrasonic range of frequencies, even though they retained the ability to emit audible sounds (squeals).

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There is a great diversity within rodent social systems and associated vocal communication [2] that is only represented in a limited way in laboratory animals used to model social behavior. For example, some mice, such as grasshopper mice (*Onychomys* sp.) with a predatory lifestyle, can produce both ultrasonic and audible sounds, for short-range and long-range communication, respectively, depending on behavioral contexts [3]. As in other mammals, the audible calls are used to warn conspecifics, to distract predators, or even to attract other predators and create competition [4]. It is interesting to note, however, that audible vocalizations below 18 kHz were recorded in mice when animals were subjected to restraint stress [5], reflecting the level of anxiety. Although recording audible calls is outside the scope of this chapter, it is important to note that both rats and mice can emit audible calls, when the environment favors them. This might not be observed in the laboratory because the laboratory environments are modified or have impoverished social conditions that may alter vocal communication [6].

The selection of high sound frequencies in rats and mice was dictated by predatory pressure of large number of birds of pray, which are the most dangerous predators for rodents [7]. Birds of pray can move very fast and/or fly silently as owls, making rodents defenseless [8], however, birds, unlike large mammals, cannot hear ultrasounds. The ability to emit ultrasounds most probably appeared in evolution as exaptation, i.e., rodents have taken advantage of high sound frequencies that appeared spontaneously by forcing air through narrowed vocal folds, for instance, in the cold [8, 9].

Communication with ultrasonic vocalizations takes place for relatively short distance [10] and evolved as a social phenomenon, i.e., for communication mainly within a colony of animals. Variety of calls and their complexity is dependent on the size and organization of social groups [11]. Thus, emission of ultrasonic vocalizations is socially adaptive and remains an important regulator of rodent social behavior. Over many decades of studies of ultrasonic calls, it was consistently observed that these calls are emitted in biologically important situations, such as mating, raising young, establishing dominance ranks, social interaction with unfamiliar conspecifics, defending against predators, etc.

Moreover, emission of ultrasonic vocalizations requires social reciprocity because the presence of a conspecific without direct interaction is not always sufficient for triggering sustained calling [12]. Vocal communication facilitated social interactions, increased security of the social group, and increased survival. It is beneficial to briefly review biological functions ultrasonic vocalization plays in rodents' lives.

2 Biological Functions of Vocalizations

There are many functions ultrasonic vocalizations play in rodents' life, and they were recently fully reviewed elsewhere [8, 13, 14]. The most primary functions of vocalization are originating from mother–infant interactions. Infant calls (isolation calls) serve self-preservation function, calling for help while outside the nest (locating function) promoting retrieval and warming and securing continuous maternal help (protective function). Many papers report the use of maternal behavior and emission of pup ultrasonic vocalizations as a noninvasive readout of healthy or pathological oxytocin-related processes (reviewed in [15]). More recently, the naturally rewarding to mother, behavior of pup retrieval in mice was used to study how maternal behavior is organized and maintained. It was shown that, although relying on innate behavior, dams learned to use a novel pup-associated sound to guide more efficient search and approach, showing the critical role of pup calls not only in the innate process of establishing mother–pup bond but also in shaping of a learned strategic search behavior [16].

At older age, young rodents emit ultrasonic vocalizations during juvenile play behavior, and these calls coordinate and facilitate play (ludic function). Juvenile rats, for instance, are known from rough-and-tumble play, during which they emit large numbers of positive 50-kHz vocalizations [17, 18]. The vocalizations were postulated to serve as play signals and to maintain playful mood in rats [19, 20]. Play behaviour was less reported in mice. However, play was shown in grasshopper mice and other mice strains [21, 22] with similar dynamics than that observed in rats [23], but vocalizations were not recorded. Whether mice emit little or no vocalization during social play remains yet an unexplored area.

In juvenile and adult rats, emission of 50-kHz ultrasonic calls serves important affiliative function that facilitates close interactions and promotes direct contacts among individuals and staying in close groups (affiliating and socio-coordinating functions, [8]). The affiliative vocalizations may also play social buffering function, as it was postulated for rats [8]. In mice, emission of ultrasonic calls was mostly observed in interaction between males and females, and these calls influenced behavior of the recipients [24]. In the male–female interactions, emission of ultrasonic calls facilitated female approach both in rats and mice [25, 26]. This is not an automatic behavior, and some studies have not replicated this response in either of these species [24, 27]. This response is sensitive to stress, frequent repetition of stimuli, and other factors.

Finally, it has been shown in rats that ultrasonic communication played also cooperative function. During longer training, two rats learned simultaneously touch a nose hole to receive reward. Trained rats could not achieve this goal, when their vocal communication

was blocked [28]. Although more limited amount of data exists in adult mice, male–male interactions trigger a large number of ultrasonic calls, at high frequency (>65–70 kHz), with a varied repertoire [29, 30], providing evidence that mice are motivated by social interaction [12, 29, 31, 32].

Ultrasonic vocalizations play an important regulatory role in reproduction (mating function). These calls contribute to selection of partners, soliciting sexual contact, and initiating copulation. For instance, female mice and rats will approach vocalizing males more frequently than devocalized males or approach male's vocalization playback [25, 33]. Also, female rats and mice produced higher number of vocalizations to intact males than to castrated males [34, 35], and female mice preference for their mating partners was influenced by their ultrasonic calling [36]. Nicolakis and collaborators [37] showed that mice displayed distinct ultrasonic calls associated with mating depending on the genetic proximity of their partner. Unrelated pairs emitted longer and more complex vocalizations compared to related pairs during direct interactions, and this was associated with greater reproductive success compared to related pairs. This finding suggests that mice can modulate the emission of courtship ultrasonic calls and that these calls can serve as markers of reproductive success. In the same vein, it was shown in adult mice that sexual priming (i.e., prior exposure to a female) promoted emission of ultrasonic calls, increased call repertoire, and changed acoustic parameters of calls (e.g., frequency) [38].

Also, emission of 50-kHz calls by male rats makes females more receptive than devocalized animals [39, 40]. Finally, male rats emit long aversive 22-kHz calls after copulation that are termed post-ejaculatory calls. These calls paly a social detachment function and were suggested to keep the females away right after successful copulation [41].

Taken together, the data obtained from rats and mice related to vocal communication during courtship and mating indicate that ultrasonic vocalizations modulate mating and, reciprocally, mating alters pattern and features of calling. In a well-documented review, Asaba and collaborators described how ultrasonic and olfactory information are integrated and processed in the brain networks to eventually regulate mating and reproduction [42].

Another important vocalization function is in agonistic (combative) behavior. Agonistic function of vocalizations serves establishment of dominance hierarchies, aggressive/defensive behavior, and play role during defending territory. In these behaviors, vocalization plays many roles. For instance, in aggressive encounters in rats, 50-kHz calls will be emitted mostly by the approaching or offensive animal, while 22-kHz calls will be emitted by the defensive or defeated rat (appeasement function) [43]. Although, there are no clear studies on territorial defense in the rat (excluding the intruder paradigm), several types of vocalizations were identified in

territorial defense in mice [44]. In mice, however, it is frequently observed that ultrasonic vocalizations are absent during aggressive behavior. This was particularly the case in mouse strains showing aggressive male–male interactions, such as CD-1 or 129SV, or in strains with high anxiety levels (e.g., DBA2) [31].

One of the best-known and studied function of vocalizations is their antipredator alarming function in rats in a form of long 22-kHz calls [45, 46]. Mice, on the other hand, do not show such a vocal alarm system [47]. As it was experimentally shown, rats also emit 22-kHz calls in any dangerous situation, including unpleasant physical stimuli, sudden unpredictable loud noise, etc. Thus, these calls have a general warning-alarmin function in face of any danger. Similar 22-kHz vocalizations were also reported to have frustration–expression function. As it was clearly demonstrated in an experiment in the laboratory, these calls were emitted when a rat could detect and smell receptive female but had no access to her through a barrier [48].

The described functions of vocalization are not the complete list, and ultrasonic calls play several other functions as phatic communication function (bonding function), investigative function, alimentary function, food provisioning function, security function, and others (for full review, see [8]. What is common to all these functions is an emotional nature of this communication.

3 Vocalizations as Emotional Expressions

All the functions of ultrasonic vocalizations, which were listed in the previous subsection, are associated with highly emotional situations. This is not surprising because emission of ultrasonic vocalizations is costly, and animals emit them only in biologically important situations. Based on large number of investigations, it has been concluded that all emissions of ultrasonic vocalizations in rodents represent emotional expression, and, therefore, all ultrasonic vocalizations are initiated by brain mechanisms of emotional arousal (for review, see [8, 14]).

Emotional arousal is a central process carried out by ascending tegmental neuronal systems that influence activity of most of the brain. Emotional arousal is distinct from cognitive arousal that is aimed at neocortex. The emotional arousal is initiated by two parallel ascending systems, the mesolimbic cholinergic and mesolimbic dopaminergic systems that target mostly the limbic regions of the brain [49]. The mesolimbic cholinergic system is responsible for the initiation of aversive arousal (akin to anxiety) and the mesolimbic dopaminergic system for the initiation of appetitive arousal (akin to joy). This dichotomy in emotional arousal is needed because, depending on the conditions, positive or negative situations require different preparation of the organism and different responses.

Interestingly, it was observed in studies on rats that initiation of appetitive or aversive emotional arousal is associated with emission of valence-specific ultrasonic vocalizations. In rats, aversive situations are accompanied by emission of 22-kHz type of calls, while 50-kHz type calls are emitted in appetitive situations. In mice, vocalizations produced in aversive or appetitive situation also differ, mostly in sound frequency, with an average frequency of calls about 52 kHz in aversive situation and about 73 kHz in appetitive situation [50]; however, no specific type of calls was ascribed to express these opposite emotional states.

Ultrasonic vocalizations are usually emitted in large numbers and often in series of calls. As shown in rats, ultrasonic vocalizations are directed to conspecifics [45, 51] and are not emitted toward other species or predators. The main biological role of emitting vocalizations is not to serve for sending specific information in a "lexical" sense, but vocalizations are emotional means used to influence behavior of other members of the social group [52].

In mice, rats, and other mammals, emotional behavior in social contexts is complex. Social contexts are triggering a large range of emotional states, and these states further modulate social behaviors. It was particularly interesting, as described below, that specific acoustic features such as vocalization frequencies are markers of emotional states and are modulated by social contexts.

4 What Does Emission of Ultrasonic Calls Indicate?

Emission of ultrasonic vocalizations, particularly its specific call types, informs us about animal emotional state and about its valence. The valence is signaled by emission of state-specific calls or state-specific frequency ranges, and these signals are addressed to other conspecifics. The main factor for discrimination of calls by the recipients is sound frequency of the vocalizations and to a lesser degree the duration of individual calls [53]. In rats, emission of long 22-kHz calls is associated with negative, aversive interactions and situations and dangerous stimuli, while emission of short 50-kHz calls is associated with positive, appetitive interactions and situations and social stimuli. Mice produce several different types of ultrasonic vocalizations that do not exactly match those of rats [13], although some frequency-based types were associated with positive or negative emotional states [50]. Thus, emission of 40-kHz calls in adult mice is associated with negative states, while emission of 65 kHz and above is associated with positive social interactions.

Emission of appetitive 50-kHz calls in rats is also associated with increased general activity and locomotor activity, investigation, and approach [54, 55], while 22-kHz calls are associated

with behavioral inhibition, decreased locomotor activity, or freezing and escape [45]. In general, rats have well-structured vocalizations, while mice seem to have variable vocalizations [13].

Not only the type of emitted calls is important but also the number of emitted vocalizations per time unit. The larger is the number of emitted calls, the higher is the emotional arousal and bigger urgency of the response. Other acoustic parameters are also important, and animals may prolong or shorten duration of individual calls or modulate the sound frequency, depending on the biological situation and their internal state.

5 Physical Characteristics of Animal Ultrasounds

The ultrasounds have different characteristics of propagation than audible sounds. Firstly, ultrasound emitted by animals has features of a beam, and this creates directionality of emission. The width of the beam is difficult to calculate because it is dependent on the sound frequency, i.e., the higher the sound frequency, the narrower the beam, and is also dependent on the diameter of the mouth opening and the distance from the animal's mouth. The narrower the mouth opening, the broader is the diameter of the beam, while fully open mouth narrows the beam [56]. Assuming an external mouth opening of a rat as approximately 10 mm and measurement from a certain short distance, the half-width will be of about 50° for 20 kHz and 20° for 70 kHz [10]. In some animals as bats, the width of the ultrasonic beam is regulated during flight and locating pray by changing the mouth opening [56]; however this phenomenon was not observed in rodents.

Secondly, ultrasonic vocalizations are quickly attenuated by atmospheric absorption and air humidity, and the higher sound frequency, the greater the absorption, as it is also for audible sounds [57, 58]. Moreover, absorption is dependent on the temperature, and the higher the air temperature, the bigger sound absorption [58]. In addition to that, ultrasounds are easily scattered by objects in the environments (leaves, branches, rocks, etc.), and the scattering is greater when the size of these objects is larger than the sound wavelength (usually about 1 cm). From these reasons, emissions of ultrasonic calls in rodents are used for a short-distance communication [10]. For the molecular composition of our atmosphere and low altitude, the speed of propagation of ultrasounds is only dependent on temperature of air (increasing approx. 0.6 m/s per 1 °C). Although ultrasounds do not reach far, propagation of ultrasonic vocalization in underground tunnels can travel further than in an open area [10].

Ultrasonic vocalizations are easily reflected from smooth surfaces, such as walls of plastic cages. Most of these multiple reflections are not recorded, but some may be picked up as echoes.

Echoes are very difficult to notice, and usually in ultrasonic vocalization research, these echoes are neglected since they are constant and omnipresent. Their effect on recorded calls in small cages is minimal and causes negligible extension of call durations. The echoes are only important in research requiring sound source localization by animals, particularly for longer distance. In these instances, smooth walls and other polished surfaces should be avoided. This could be done by appropriate sound-reducing paints, acoustic foams, or other sound-absorbing materials (e.g., mineral wool). For further details, *see* **Note 1**.

6 Recording Procedure of Ultrasonic Vocalizations

Most ultrasonic signals originating from animals are generated with sound frequencies between 20 and 100 kHz. Ultrasonic signals emitted by rodents are within this range of frequencies and can be properly recorded and analyzed using digital signal processors, i.e., set of techniques and algorithms, aided by computers, used to manipulate signals, after they have been acquired and converted into a digital form. Technical developments in mid-1980s, including high-speed dedicated hardware processors and computer technology, opened a new era of recording and processing ultrasonic signals in real time [59]. There are complete systems for recording and analyzing ultrasounds available commercially with hardware and software that can be connected to personal computers.

Rodent vocalizations are sound waves propagated in air with varying sound frequencies, amplitudes, and characteristic duration of single calls. The first step is recording of these signals (pressure waves) by an ultrasonic microphone that produces a continuous analog voltage waveform, characteristic for the recorded sound wave. This waveform is amplified, filtered, and digitized. Filtering minimizes noise and aliasing distortions, and digitization is done by an analog-to-digital converter board (ADC) in the computer. The ADC coverts the analog voltage signal to a binary number at successive discrete time intervals. Then, the signal is passed to many algorithms that process it in time and frequency domains (see [60]). This acquisition process is illustrated in Fig. 1.

The original signal from the microphone may be presented as a waveform image, i.e., signal's amplitude (in mV on the y-axis) over time (in seconds on the x-axis). These images present the dynamics of calls, i.e., amplitude (loudness) of the vocalization components, as well as the start and end of the vocalization. Waveforms, however, cannot inform us about sound frequency, its modulation, or harmonics.

After digitization, the signal can be also used for graphical representation of the recorded sound, usually as changes of frequency domain in time domain. In broad terms, any visual image of

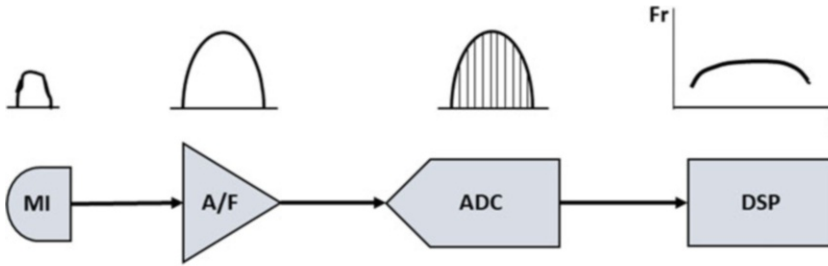

Fig. 1 The diagram is illustrating the process of recording, amplifying, filtering, and digitizing the ultrasonic vocalizations. The upper simplified graphs show how the signal is changing. The digitized signals can be visualized on the computer's monitor. Abbreviations: *MI* microphone recording the original signal, *A/F* amplification and filtering, *ADC* analog-to-digital-converter digitizing the signal, *DSP* computer digital signal processing, allowing visualization of the digitized signal in in time (t) and frequency (Fr) domains

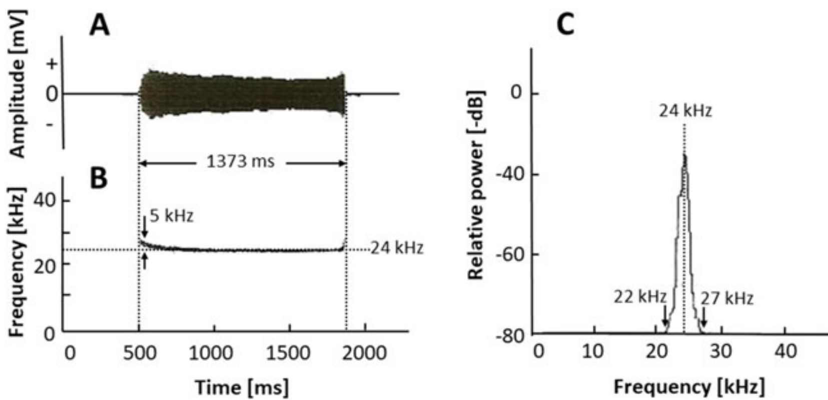

Fig. 2 Waveform and spectrograms of an exemplary single 22-kHz vocalization of a rat. (**a**) a waveform of the call is shown with amplitude measured in [mV] over time [ms]. It does not inform about the sound frequency. (**b**) Spectrogram of a single 22-kHz vocalization (the same as in (**a**) visualized as changes of sound frequency [kHz] over time [ms]. This call lasts 1373 ms, and this can be read from both waveform and spectrogram on the time scale. (**c**) Power spectrum of the same signal is shown, and it illustrates sound frequencies [kHz] with their power expressed in negative decibels [-dB] in comparison to the maximal signal (0 dB). The dominant frequency (the highest peak) shows the peak frequency for the entire call (24 kHz for this call). The peak frequency is also marked in (**b**). In addition to that, the power spectrum provides information about the minimal and maximal frequencies within this call. They are marked on the power spectrum with small arrows (22 kHz and 27 kHz, respectively). The difference between these frequencies, i.e., 27 kHz–22 kHz = 5 kHz is the bandwidth of the call. The bandwidth of 5 kHz is also marked on the spectrogram in (**b**) between small arrows

ultrasonic (or other) sound is termed sonogram or sonographic image. The sonographic image may be presented, as it mostly is, as changes of sound frequency in time or distribution of energy at different frequencies over time. In narrower sense, spectrum of sound frequencies and their changes over time are called spectrograms, while representation of relative power at different sound frequencies is termed power spectrum. Examples of a waveform, spectrogram, and power spectrum are shown in Fig. 2.

Using spectrograms, several parameters of ultrasonic vocalizations may be calculated. The most important are duration of signals and their sound frequency. Spectrogram will serve to measure duration of single calls, time between calls (inter-call interval), and the number of calls per time unit. Duration of calls may be read from waveform or spectrogram (Fig. 2a, b). Further analysis of spectrograms and power spectra will provide peak frequency (i.e., frequency of the call component with the highest power, Fig. 2c), minimal and maximal frequencies within single vocalization, frequency bandwidth (i.e., difference between minimal and maximal frequency within a call; see legend to Fig. 2), and fundamental and harmonic frequencies, if overtones are present. The exemplary spectrogram in Fig. 2 has no detectable harmonics. The spectrogram informs also about frequency modulation, i.e., changes in sound frequency within a single call over its duration. Calculation of these parameters is done by several algorithms and processing functions of the digitized signals. It may be done semi-manually by operation of cursors placed in critical points of the spectrogram and reading the values at or between the cursors, or it may be calculated automatically by dedicated programs.

7 Technical Arrangement of Recording

The first element of the recording setup is the microphone (Fig. 1, MI). They are polarized condenser microphones and should have relatively flat response up to not less than 100 kHz. Higher-quality externally polarized microphones have flat response up to 150 kHz or higher. Microphones are characterized by their functional range, i.e., range of sound frequencies that can be reliably recorded. Flat response means that the microphone will have similar frequency response over its functional range. The range can be from 10 to 100 kHz or as broad as 5–250 kHz for some types of expensive microphones. Other types of non-ultrasonic microphones cannot reach high sound frequencies. Ultrasonic microphones should be handled with care because of their delicate construction and sensitivity to moisture. Microphones are usually supplied with the whole systems for recording and analyzing ultrasonic vocalizations.

Placement of a microphone for recording is important to maximize catching all calls emitted by animal and to minimize reflections and echoes. The microphone usually comes with its own stand or mounting clamp and should be located 20–40 cm from the animal. If the animal is not freely moving, the microphone should be aimed at the front of the animal's head. Like ultrasounds themselves, microphones also have their own directionality, and the distance from the animal to microphone and orientation of animal's head will influence the loudness and quality of recorded vocalizations. If an animal, like a rat, turns back to the axis of the

microphone, all emitted calls may be reflected, muffled, or missed. Thus, the microphones are usually placed above the cage to cover the entire area where the animal may be moving. Recoding cages are small to ensure that from any location in the cage, the distance between the animal and the microphone will be comparable.

It is recommended to record ultrasonic calls in a sound-attenuated chamber, in which the recording cage is placed, or by sound-proofing the recording cage itself. This eliminates reflections from distant walls. The recording cage should not be too small (*see* **Note 2**). If animal is very active and creates many accidental sounds, preamplifier filters are recommended to reduce interfering noises below 10 kHz and above 100 kHz or 150 kHz. Regular, granular bedding is usually removed from the recoding cage and replaced with absorbent paper (as paper towel) or paper-based bedding. This reduces movement-related noises. Recording animal's calls in a plastic cage without any lining or bedding creates another danger of creating high-frequency short-duration screeching noises coming from animal's claws moving against the plastic floor.

Before the beginning of the experiment, it is recommended to record background noise that will show any other sources of interfering ultrasonic sounds in the laboratory room. These sounds can originate from working nearby engines, as compressor of a refrigerator, freezer, operating computers, and/or fluorescent lighting. All these devices can produce constant ultrasounds between 20 and 40 kHz of different amplitude that may interfere with recording of ultrasonic vocalizations. Ultrasounds can also be generated by researchers themselves, for instance, by opening and closing the doors, using jingling keys, etc., that generate sometimes very strong ultrasonic noise. Although, human voice is beyond the detection of the system, some elements of loud speech may be detected by the microphone and by the rat. Total elimination of all noises is not possible because movements of the animal itself can generate some ultrasonic sounds that come from scratching, grooming, teeth chattering, rubbing against walls, sneezing, etc. All these accidental noises should be eliminated from the recording usually by high- and low-band-pass filtering but sometimes by visual inspection of the recordings in offline scrolled spectrographic images.

The next step in recording is selection of the ADC board with appropriate resolution and sampling rate capabilities (Fig. 1, ADC). Computers operate in a binary code, and the term "bits" refer to the digits of that code. The bit count and sampling rate (in kHz) will determine the resolution and frequency response. Increase in ADC bits increases the dynamic range and resolution, while sampling rate of the convertor will determine the maximum frequency that can be measured. From a theoretical point of view, the highest frequency that can be measured is one-half of the sampling rate

[59]. Thus, for ultrasonic call's maximum frequency of 100 kHz, the minimum sampling rate should be 200 kHz, and for ultrasonic frequency of 150 kHz, 300 kHz sampling rate is required. In practice, higher sampling rates are selected, and an optimum ADC with 16 bits/500-kHz sampling rate for most rodent vocalizations is recommended [60]. However, high sampling rate will generate larger files and will require larger computer memory storage and longer time to analyze the recordings.

The computer performing the DSP is the heart and the central component of the acquisition of vocalizations and their analysis (Fig. 1, DSP). While data acquisition from the animal occurs in real time, the analysis of signals is usually performed on stored data offline after the acquisition. The dedicated data acquisition hardware should be connected to the computer directly by USB port. This guarantees high-speed performance without cumbersome installation of the acquisition hardware within the computer. The most important, critical, and difficult point of this process is proper setting of all parameters controlling the acquisition process. These parameters are presented and described in the next subsection.

8 Parameters Controlling Data Acquisition Process

8.1 Gain Parameter

The data acquisition system has gain control of the input to maximize the dynamic range, i.e., the ratio of the smallest to the largest signal that can be measured. It is important not to use too high amplification. If the recorded signals have too high amplitude, the signals will be clipped at the top and this will introduce distortion that will create false frequency harmonics [60]. It is frequently observed in published papers that pure ultrasonic vocalizations, which should not show harmonics, have multiple artifactual overtones. The gain can be manipulated directly or also decreased at the level of preamplifier. It is often beneficial to experiment with a known signal to set appropriate gain that gives clear signal.

8.2 Sampling Rate and Buffer Size Parameters

As mentioned before, the acquisition or sampling rate must be selected, which will determine the number of data points acquired per second. This rate should be at least twice, or more, the maximal frequency expected in the recording vocalizations (e.g., 200–500 kHz). In addition to that, to ensure gap-free recording, buffer size parameter need to be determined. At low buffer size, the number of gap errors increases, while larger buffers reduce the error but creates some increased latency of the system response. In general, in real-time processing of signals, the buffer should be smaller, while analyzing pre-recorded data, the buffer size should be increased. Often, overlapping buffers may be used to increase the resolution in time and frequency domains. Buffer size is related to the binary configuration of the hardware memory, and maybe,

for instance, 256, 512, or 1024 points, which, at the same time, is indicating the delay of the system's response by 0.256 s, 0.512 s, and 1.024 s, respectively.

8.3 Digital Input Filters Parameters

The filters are usually needed to eliminate unwanted or disturbing frequencies that are lower or higher than the recorded signal. A low-pass filter will eliminate high frequencies, which are above the signal frequency. The high-pass filter eliminates frequencies below the signals. This filter may be used to eliminate 60-Hz frequency originating from electric power lines and harmonic they produce, which may interfere with the recording. Combination of these two filters together will give band-pass filter with elimination of unwanted frequencies below and above the signal. The band-pass filter, if used, should be adjusted to the type of vocalization emitted by animals. For example, infant rats and mice may emit calls in a very broad range of frequencies encroaching even the audible sounds, so the high-pass filter could potentially cut off parts of these calls.

8.4 Recoding Mode Parameter

The mode informs the acquisition system whether recording is continuous without stops, or as triggered events selected for a specified parameters or time. The amplitude trigger should be set above the floor noise level and trigger holding time should be wide enough to prevent triggering by accidental very short noise spikes. The recording time should be also determined by trigger window time, i.e., the duration of data recording for a triggered event. Triggers are usually used for very long recordings, e.g., overnight, to save computer memory. For short laboratory recordings, triggering is not needed, particularly, when the researchers need to know the real-time gaps between infrequent calling events.

8.5 Fast Fourier Transform (FFT) Parameters

One of the principal algorithms in digital signal processing is fast Fourier transform (FFT) that converts the signal from its original time domain into representation in frequency domain. The FFT calculations are needed for creation of spectrograms. The parameters controlling the FFT calculations must be set up before the operation of the system. The principal parameter is the length of FFT analysis that determines the number of data points used for FFT calculation. This parameter is a multiple of two, and usually selected as 512 or 1024 points. The length of the FFT will determine the frequency resolution, i.e., the smallest frequency difference detected and visualized on the spectrogram, and the delay time needed to collect and calculate the data points of a given length. Thus, for the sampling rate at 200 kHz and 512 points, the frequency resolution is 390.6 Hz and latency time of processing is 5.1 ms, while for 1024 points, the resolution is 195.3 Hz, but the latency is 10.2 ms [60]. Thus, increase in the FFT size (length) will improve frequency resolution, but processing time will be longer.

8.6 Functions Used to Enhance FFT Calculations

FFT analysis has some inherent problem of discontinuities at each end of the FFT window, which is often called spectrum leaking. To correct or reduce this problem, the raw data are multiplied by a certain function that minimizes the problem. There are many types of such FFT window functions, but the most frequently used and suitable for recording ultrasonic vocalizations are Hanning window (more properly "Hann window," from the name of Julius von Hann, 1839–1921) or Hamming window (from the name of Richard Hamming, 1915–1998). The Hann ("Hanning") window gives better results than Hamming one and removes the discontinuities entirely giving better frequency resolution. It is recommended for analyses of ultrasonic vocalizations. For further details, see [60].

Another function, called FFT overlap, is used to enhance spectrogram performance in the time domain. The overlap function visualizes better gradual shifts of frequencies within the FFT processing block of time and improves time resolution. Overlap function (expressed in %) causes that data acquisition frames are overlapped during analysis. Thus, 50% overlap for the FFT transform size of 1024 points would cause that every 512 points, a new FFT calculation will start out of 1024 previous points. This will increase time resolution but decrease frequency resolution. A 100% overlap is impractical, so 30–50% overlaps are recommended.

9 Behavioral Conditions of Recordings

There is a multitude of behavioral arrangements and experimental designs with recording of ultrasonic vocalizations. Each of these situations may have its own benefits and disadvantages, but there are some common rules that should be maintained during recordings. The first and important rule is to record ultrasonic calls emitted by single animal that is recorded individually. This is done in most pharmacological studies or in recording responses to some stimuli. In these situations, the recording cage with animal is moved to another room, what eliminates possibility that recorded animals can hear other animals from their colony, as well as to leave them without interference form researchers. Emission of calls could be influenced by any accidental stimuli, acoustic, visual, or olfactory ones, coming from other animals, from environment, or from researchers. Also, the general background noise in the recording room should not exceed 40 dB, because any acoustic noise above this level may influence calling behavior. The general background noise usually comes from such sources as ventilation system of the room/building, fans of heating/cooling systems, and often from sources outside of the building. If combination of these noises is too high, soundproofing would be needed.

In many behavioral interaction situations, there is a need for recording from two animals simultaneously [29, 54, 61], because reciprocal social interactions promote emission of ultrasonic calls [12]. Social interactions between adult animals represent a large repertoire [62], and it was shown that distinct patterns of vocalization are associated with specific social actions [29, 63]. Thus, many interactions must be studied in animal pairs. For example, social dominance in mice is one of the main modulators of emission of ultrasonic calls. Social situations that promote dominance increase call emission [29], and dominant animals are more likely to emit different vocal signals than mice avoiding social interactions [63, 64]. Theoretically, it is not possible to distinguish similar calls emitted by individual animals recorded together, although there are sometimes some imperfect methods of recording from many microphones, and based on the amplitude of the signal, to estimate the vocalizing animal in this way. At present, the best solution, however, is treating the recording as originating jointly from a pair of animals. Recording from larger groups is discouraged. In rare cases, the distinction between calls emitted by two animals recorded in a common cage is possible, if these two individuals are vocalizing with dissimilar calls (or dissimilar frequencies, or different call types). However, even in this situation, there is no absolute guarantee of proper identification of the caller.

The second rule is that the vocalizing animals should not be disturbed unless this is a necessary part of the experimental design. If during recording of a call, animal is physically pushed (or by a researcher or companion animal), the structure of the emitted vocalization is altered and presents an artifactual recording with distorted frequency or its modulation and usually shorter duration (Fig. 3b). Such events should be considered in the analysis. The disturbance may come from the animal itself, and the most frequent one is scratching itself or sneezing while vocalizing. It is interesting that animals, particularly rats emitting their long alarm calls, will not stop vocalizing white scratching themselves and will continue the call. This rarely happens, but it produces artifactual calls with rapidly fluctuating frequency, which does not represent any natural call.

Finally, it is worth mentioning that experimental animal should be healthy and not suffering from any chronic condition as pain (unless this is part of the experimental protocol). Different health conditions may affect calling in different way (*see* **Note 3**).

10 Basic Characteristics of Rat Ultrasonic Vocalizations

Rats' ultrasonic vocalizations are usually divided into three major groups: (1) infant calls, (2) a group of 22-kHz calls (aversive), and (3) a group of 50-kHz calls (appetitive). The division of

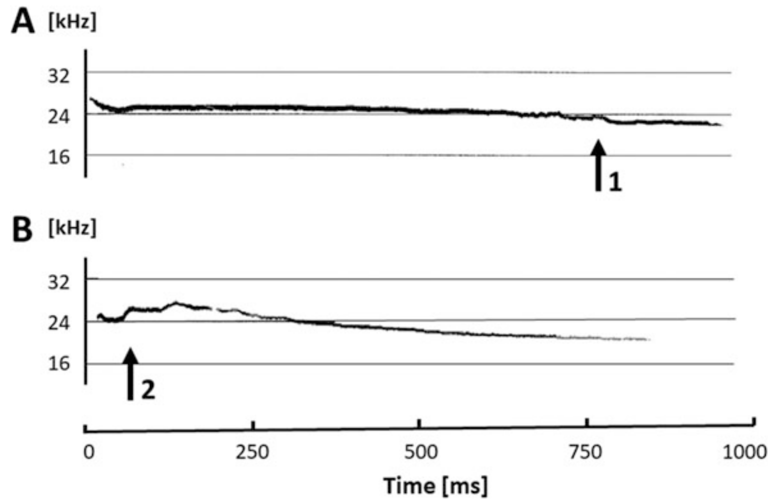

Fig. 3 Artifacts caused by external stimuli disturbing emission of vocalization. (**a**) During emission of a typical 22-kHz call, the experimenter suddenly extended his hand toward the rat's snout and stopped it at about 20 cm from the rat at the time marked with an arrow numbered 1. The rat continued its call; however, one may notice that the sound frequency was somewhat lowered after the stimulus. The peak frequency of the call is 24.5 kHz, but the part of the call after the hand approach is 23.2 kHz, so lowered by 1 kHz. The duration of this call is 942 ms and its overall bandwidth is 4.4 kHz. (**b**) The same rat was emitting another 22-kHz call, but this time, the experimenter extended his hand toward the rat and pushed it gently on the flank of the body at the point marked with an arrow numbered 2. This tactile stimulus caused artifactual deformation of the vocalization. The frequency was no longer maintained flat and showed artifactual frequency modulation, as well as the duration of the call was shortened, and its power decreased. The overall peak frequency of this call was 23.8 kHz; however, the tactile stimulus disturbed the call. The first half of the call has peak frequency of 25 kHz, while the second half of the call has 22.7 kHz. Thus, the touch of the rat during emission of the call caused forced fluctuation of approximately 2.3 kHz. The duration of this call is 857 ms and its overall bandwidth is 7.4 kHz

vocalizations into these groups is mostly based on the function these vocalizations play in rats' life and to some degree on sonographic appearance.

The infant calls, also termed isolation calls or distress calls, are vocalizations directed to pups' mother and express negative infantile emotional state aimed at obtaining maternal help. They are characterized by large acoustic variability, mostly as to the sound frequency range and frequency modulation. Infant calls do not have specific subtypes of vocalizations and have been classified based on frequently observed spectrographic images and divided to 10–12 most typical sonographic appearances [65, 66]. Infant calls change during rat development from simpler to more complex, with

deeper frequency modulation and longer duration [65]. Infant isolation calls have the largest frequency changes within some vocalizations as compared to adult repertoire.

The group of 22-kHz vocalizations includes calls of peak sound frequency 20–30 kHz and has two subtypes. The long calls have minimal frequency modulation (3–4 kHz) and very long duration from approximately over 300 ms to over 3000 ms. One such call is shown in Fig. 3a reaching close to 1000 ms in duration. The other subtype represents short 22–kHz calls that have similar characteristics, but they usually last from 50–300 ms, or longer.

The group of 50-kHz vocalizations has several call subtypes depending on frequency modulation and its pattern [67]. The sound frequency is often changing between 45 and 80 kHz. Some patterns of 50-kHz vocalization can frequently be joined in more complex calls with acoustic components around 50 kHz. Two main subtypes are “flat” calls (i.e., without frequency modulation, or with minimal modulation, Fig. 4a) and the frequency-modulated vocalizations. The frequency-modulated calls may have several elements. The most frequent is, so-called “step” call, i.e., abrupt jump of sound frequency up or down without any gap between jumps (Fig. 4b). These jumps were explained as hyperphonation, similar to those found in some pathological cries of human infants [66] as an instantaneous frequency changes with no break in time. Finally, the other form of frequency-modulated calls are “trills” (Fig. 4c).

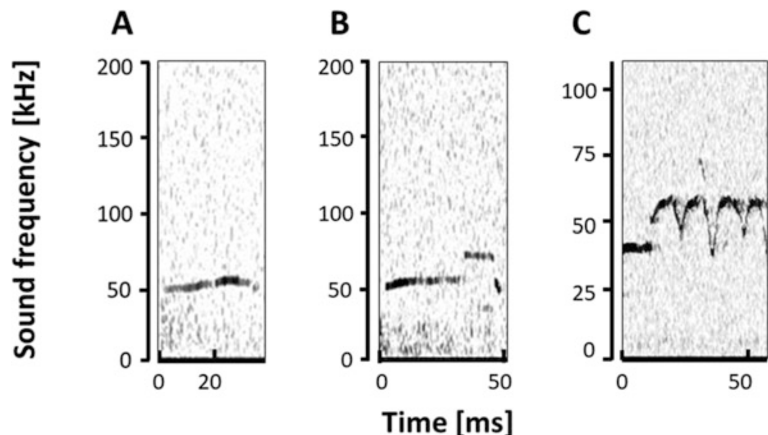

Fig. 4 Spectrograms of selected subtypes of 50-kHz vocalizations emitted by a rat. (**a**) A call classified as flat type with peak frequency of 50.2 kHz and duration of 35.3 ms. (**b**) A call classified as step call (up and down) with the peak frequency of the lower component of 50 kHz and the higher component of 72.5 kHz and call duration of 45 ms. (**c**) A call classified as flat-trill combination with the peak frequency of the flat component of 44. 5 kHz and the peak frequency of the trill component of 60 kHz and call duration of approx. 55 ms (the very beginning of the call was cut off). The frequency scale in (**c**) is narrower (0–100 kHz)

Trills are quickly occurring, rhythmic fluctuations of sound frequency like sine wave or sine half-waves (Fig. 4c). They may appear as independent calls or could be joined with step calls or flat calls. This produces several combinations of vocalization patterns within 50-kHz group [67, 68]. More careful analyses of 50-kHz vocalizations have identified up to 14 subtypes of these calls [68]. This classification includes also calls that have steadily rising or falling frequency, called "ramp" calls, and multistep or composite calls, i.e., calls representing combinations of mentioned elements. Usually, calls of the same type or subtype are not identical, and some variability exists (*see* **Note 4**).

11 Basic Characteristics of Mouse Ultrasonic Vocalizations

Mouse ultrasonic calls present more types or patterns of ultrasonic calls than rat vocalizations, and the calls also differ among the strains [69] to a greater degree than among rat strains. Terminology of call patterns is based on spectrographic temporal–structural shape of the calls, e.g., frequency steps, calls with harmonics, composite or complex calls, chevron-shaped calls, flat calls, and downward or upward ramps [30, 69, 70]. In general, as compared to rats, adult mouse ultrasonic calls have larger variability of sound frequency within most vocalizations (Fig. 5) and low fraction of flat calls (3–6%, [71]). Although mouse ultrasonic vocalizations may contain similar basic patterns observed in rats, as flat call, frequency steps, short trill-like frequency modulations called complex calls [69, 70], they

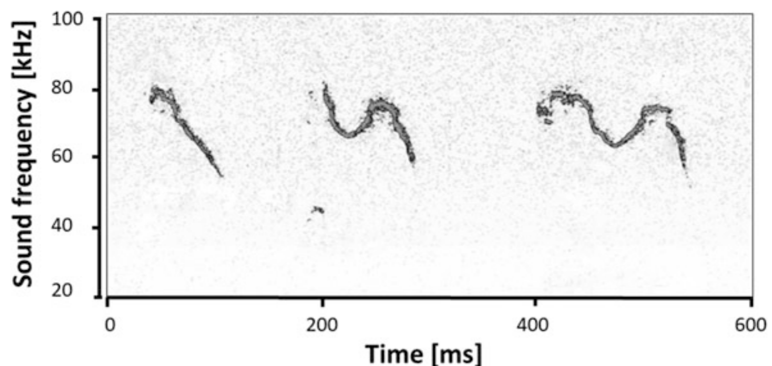

Fig. 5 Spectrogram of selected forms of vocalizations emitted by a dyad of adult male mice during reciprocal social interaction. All three vocalizations are probably emitted by the same animal in a series. The ultrasonic call types are (from left to right), downward call (duration 70 ms), small step-up with wavy shape (99 ms), and complex call (141 ms). The range of frequencies are similar for all three calls, with maximum frequencies between 80 and 82 kHz and minimum frequencies of 54–58 kHz (not counting the small step) and bandwidth of 24–27 kHz. Peak frequency of the calls is from 74 to 77 kHz

demonstrate large diversity of other types of calls that have not been unequivocally assigned to specific patterns of behavior. Usually, there are 8–12 types of mouse calls distinguished [30, 69–72]. Recent studies using four feature selection algorithms have confirmed nine distinct categories of calls termed short, chevron, complex, flat, downward, upward, two components, frequency steps, and composite call categories (e.g., *see* Fig. 5; [30]; *see* **Note 4**).

Despite the variability of vocalization types not characterized behaviorally, mice can express valence of their emotional arousal, so, for instance, mice emit calls of different sounds frequency in stressful or other emotional situations. In stressed mice, ultrasonic vocalizations may be emitted by adult animals even if they are alone [5, 29, 73, 74]. In general, calls are emitted at much lower frequencies in aversive situations (45–50 kHz) than in social, appetitive situations (approximately 70 kHz and above), indicating that call frequencies are reliable markers of emotional states [50].

12 Concluding Remarks

In rats and mice, ultrasonic vocalizations are triggered by emotional and social situations. It is therefore crucial to establish appropriate experimental conditions for recording of ultrasonic calls, which are noninvasive markers of emotional states. In adult or adolescent animals, in addition to social contexts, danger and novelty of the environment are important factors for triggering and recording ultrasonic calls. Careful setting of parameters controlling data acquisition by the digital signal processing technology is critically important in revealing acoustic details of vocal communication. Important factors in analyzes of vocalizations, in addition to the number of emitted calls, are call frequency, as the major index of the emotional state, patterns of frequency modulation, and call duration. A major limitation of recordings in social environment is the identity of the vocalizing animal in a dyad or a group of recorded individuals.

13 Notes

1. In general, it is important to remember in recording ultrasonic signals that these vocalizations are subjected to reflection from different objects. There might be numerous objects and walls around. The structure of the objects is less important, but the distance of these objects from the animal is essential. This distance will determine how quickly the reflected sound will return to the animal. The reflected sound that returns to the rat's ear within about a couple of hundreds of milliseconds will not be noticed and not perceived as a separate sound. Thus, all

reflections or echoes from the walls of a small cage have no significance for animal's behavior.

2. The size of the recording cage should not be too small. In very small and insulated cages, care should be taken not to overheat the cage's interior. Overheated cage may change animal's behavior. So, monitoring temperature inside the cage is recommended. Recording from a regular polycarbonate cage used for housing animals is appropriate. The recording cage should not be larger than that but may be reduced up to about 50% of its original size for short time recording (e.g., 10 min).
3. There are pathological conditions that will influence the parameters of vocalizations, as well as they may potentiate emission of vocalizations or decrease emission and stop calling all together. For instance, animals may stop calling during the continuing epileptic afterdischarges. Without EEG recording, the infrequent afterdischarges might not be observable in behavior. In general, sharp pain will induce immediate audible squeak without ultrasonic vocalizations. However, chronic pain may potentiate emission of aversive vocalizations after some time.
4. It is important to emphasize that all naturally emitted ultrasonic vocalizations are not perfect and we should expect some variability in recording. For classification of calls, the most important are the basic acoustic features as range of sound frequencies and durations, as well as overall sonographic structure of calls. This variability comes from two sources: firstly, the imperfect reproduction of successive calls of the same type by the vocalizing animal and, secondly, from an interindividual variability among individual animal. This variability is much larger in mice than in rats.

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Chapter 17

Development of a Selectively-Bred Mouse Model of Dominance and Submissiveness: Technical Considerations

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Abstract

Social interactions play an important role in the shaping of individual personalities and development of behavioral and physiological disturbances. Animal models represent a valuable tool in the study of the molecular and biochemical basis of social behaviors. Dominant–submissive relationship (DSR)-based models have been developed in both mice and rats for the purposes of studying the molecular basis of social behavior and psychotropic agent screening. These models have been established on the basis of the food competition paradigm. Whereas DSR models have been proven to be valid for drug testing, they have also been associated with different challenges, including low efficiency, experimental reproducibility, and testing time duration. To overcome these challenges, we employed the selective breeding approach, which has allowed us to develop mouse populations with strong characteristics of dominance and submissiveness. This model allows a dramatic increase in test efficiency, stability, and reproducibility as well as a substantial decrease in experimental duration. The selectively-bred dominant (Dom) and submissive (Sub) mice exhibit distinct differences in (a) stress-coping abilities, (b) responses to psychotropic agents, (c) inflammatory profiles, (d) gut microbiome profile, and (e) life span. Herein, we describe in detail the process of selective breeding as well as the behavioral, biochemical, and physiological characteristics of the Dom and Sub mice. We also discuss the different research directions that can be pursued by employing this model.

Key words Dominance, Submissiveness, Stress resilience, Selective breeding, Social interactions, Dominant–submissive relationship test, Antidepressants, Psychotropic agents, Depression, Anxiety

1 Background

During our everyday lives in a socially constructed world, we are constantly involved in and influenced by intricate and complex social interactions, which can be defined as situations in which two or more individuals communicate with each other or accomplish tasks together. Social relationships involve all forms of communication, including cooperation, competition, imitation,

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assistance, play, negotiation, and bluffing [1–7]. As the basic elements of societal structures, social interactions are critical for the shaping of personality, often determining the hierarchical rank of an individual [8–11]. In most animal societies, hierarchical differentiations between individuals are the result of competition, the societal position distinction of a higher-ranking individual effectively codifying their ability to realize their interests at the expense of their lower-ranking counterparts [9, 12–14]. Thus, hierarchical relationships result in conditions under which one member of a given pair achieves dominant status, while its counterpart is relegated to submissiveness.

Many studies have linked social rank with different behavioral and physiological deviations. Thus, dominant status has been shown to correlate with a suite of behavioral and physiological parameters, including lower stress hormone levels [15], altered serotonergic neurotransmission [16], enhanced metabolic profile [17], and higher growth rates [18]. In contrast, submissiveness can be accompanied by emotional instability [19], chronic hypothalamic–pituitary–adrenal axis (HPA) dysregulation [20], decreased innate immunity, decreased resistance to implantation [21], as well as an increased risk of development of diabetes [22, 23] and cancer [24]. In addition to this, the hierarchical rank of an individual influences many of their physiological parameters, including weight gain [25], fertility [26, 27], and offspring viability [28, 29].

Behavioral testing in animals is crucial in neuroscience research. In particular, animal models of human diseases or behavioral symptoms, such as anxiety and depression, should ideally be tested in situations where long-standing or permanent stable features of such behavior are manifested. This is especially relevant for the research of social and hierarchical interactions. Different models have been developed in rodents to study social relationships [30, 31]. Initially, these tests were developed to study distinct behavioral components [32, 31] and the ability of psychotropic agents to influence individual behavior in a socially-predefined environment [30, 33, 34]. The investigation of social interactions in rodents may rely on competition for food [35], water [36], territory [37], and other resources [36] or on the observation of the interactions of the subject and stimulus animals.

Our research group specializes in the study of the molecular and neuropharmacological aspects of social behavior by employing the dominant–submissive relationship (DSR) food competition paradigm, which was developed several decades ago for antidepressant and antimanic drug testing [38–41]. DSR-based models have been established in both mice [42, 35] and rats [38, 35]. Whereas DSR models have been proven to be valid for drug testing, they have also been associated with different challenges, including low efficiency, experimental reproducibility, and test time duration.

To overcome the abovementioned challenges, we have developed a population of animals exhibiting stable and strong behavioral characteristics of social dominance and social submissiveness. These animal populations have been derived using the selective breeding approach and food competition DSR paradigm. Herein we describe the procedures comprising the selective breeding process, behavioral, biochemical, and physiological characteristics of the Dom and Sub mice, as well as the different research directions that can be pursued by employing this model.

2 Historical Overview of DSR Testing

The DSR test is a modification of the tube test and was initially developed by Ewa Malatynska in rats [38, 39, 41, 43] and later on in mice [44]. The DSR paradigm relies on repeated competition for sweetened milk between fixed pairs of food-deprived animals. It has been found that after repeated interactions, some pairs develop relatively strong DSRs [40, 41]. The development of a DSR by a pair is subject to the satisfaction of the following criteria (also depicted in Table 1): (a) the time that the dominant animal spends at the feeder with sweetened milk must be significantly higher (using a *t*-test) than that of its submissive counterpart; (b) the average time that the dominant animal spends at the feeder must be at least 40% higher than that of its submissive counterpart; and (c) there is absence of "reversal of dominance," defined as occasions when the submissive animal outscores its counterpart during the 2nd week of testing. Only if a pair satisfies all of the abovementioned criteria is it defined as a pair that has developed a DSR. Approximately 25% of animals develop relatively stable DSRs which fulfill the above conditions, with all other pairs being defined as having not developed stable DSRs and are thus designated as "neutral." The major difference that distinguishes the DSR test

Table 1
Criteria required for the selection of animal pairs which have developed DSRs

DSR pair selection criteria	Values of properties required for the selection of dominant–submissive pairs
Statistical significance	$P < 0.05$
Satisfactory percentage of difference of the higher scoring animal	>40%
Absence of "reversal of dominance" (occasions when the submissive animal outscores its partner during the 2nd week of testing)	No cases of "reversal of dominance"

The selection criteria are based on the time each animal spends at the feeder of the DSR apparatus. The significance of the results is determined by an unpaired *t*-test

from the other social interaction paradigms, such as the tube and resident–intruder tests, is that the DSR test does not necessarily rely on aggressive behavior and represents the development of social interactions within the defined pair during a 2-week interval [45].

The DSR paradigm possesses a unique assessment advantage in terms of the time spent at the milk feeder serving as a quantifiable endpoint of social dominance within each rodent pair. Using this experimental design, it has been shown that submissive behavior is sensitive to and is selectively reduced by antidepressants [39] and that dominant behavior is sensitive to a range of drugs that are used to treat mania in humans [40].

3 Selective Breeding Protocol

3.1 Rationale for the Selection of the Sabra Mouse Strain

Selective breeding experiments were initiated in our laboratory in August of 2007, starting with the selection of the background strain. The selection of the background strain is critical for the successful performance of the selective breeding procedure, particularly in the case of the enrichment of phenotypic characteristics. The primary criteria for the background strain selection are the specific research goals and the strain properties required for the experiment [46]. We have identified the Sabra outbred strain [47] as a suitable basis for the development of a mouse model with strong dominant and submissive traits. This strain was established more than 45 years ago at the Hebrew University, Israel. Since 1993, the Sabra strain has been maintained at the Harlan Laboratories (currently Envigo, Ltd.) and has been used for neuropharmacological studies by several research groups in Israel [48–53]. While the origin of this strain is unknown, unconfirmed evidence indicates that the Sabra strain is derived from the ICR strain.

The rationale behind the selection of the Sabra mouse strain for the development of a model with enriched dominant and submissive characteristics was as follows: (a) the strain has an outbred origin, which is an important criterion for successful selective breeding, (b) Sabra mice easily develop DSRs, (c) although the Sabra strain is larger and heavier than the ICR, Balb/c, and C57BL/6J strains, comparison of their physiological, biomolecular, and behavioral characteristics has revealed no dramatic differences between these strains in terms of these properties [54]. A more detailed comparison of the Sabra strain with other mouse strains is given elsewhere [54]. It was thus concluded that the Sabra strain is suitable for DSR-based selective breeding for the behavioral traits of interest.

3.2 DSR Procedure for Selective Breeding

For initiation of the breeding procedure, groups of 10-week-old Sabra mice of both sexes were housed according to sex, weight, and age in groups of five per home cage. DSR testing was performed for

each predefined pair for 2 weeks, 5 consecutive days per week. During each 16-h period preceding the testing, the mice were fasted with water available *ad libitum*. Two-day breaks were applied after each 5-day testing period, during which the mice had free access to food until the night before the beginning of the next 5-day testing period.

Pairs of mice of the same sex and of relatively similar weight (42 ± 3 g) from different home cages were tested daily with the DSR apparatus (*see* Fig. 1). The animals were placed in the experimental room 30 min prior to the beginning of the testing for habituation. The experiment was conducted in single 5-min sessions on each day of the 5-day testing period at the same time of day (between 9:00 and 11:00 a.m. local time). During these 5-min sessions, milk drinking scores were recorded manually. After 2 weeks of testing, the pairs that developed DSRs were selected based on the defined criteria given in Table 1 and as described in the previous section. It is important to specify that only the second week scores were taken into consideration with the first week was used to acclimatize the mice to the testing environment. Both male or female pairs that satisfied the prior selection criteria were defined as pairs that developed DSRs, were designated as the parental (P) generation, and bred according to their behavioral traits. Thus, Dom females were home-caged with Dom males, and Sub females were caged with Sub males. Usually, two females were caged with one male (*see* Fig. 2). One week after mating, the males were removed, and the females were housed in individual cages until pup delivery. At 21–25 days of age, the offspring (generation F1) were weaned from their mothers, weighed, ear clipped for identification, and housed in groups of five by sex and parental phenotype until 10 weeks of age. At this point, offspring of the Dom mice were paired with offspring of the Sub mice for 2 weeks of DSR testing. The individuals which satisfied the above-described criteria for a stable DSR were further selected for the breeding protocol. In each subsequent generation, the number of animal pairs subjected to the DSR test was dependent on the number of offspring. The derivative generations that followed were designated as F2, F3, F4, F5, etc. The percentage of mice that developed DSRs in each generation during the selective breeding is demonstrated in Fig. 3. Behavioral traits of selectively-bred Dom and Sub mice become pronounced with the progression of selective breeding, with more than 95% of pairs forming stable and strong DSRs by generation 10. Furthermore, the formation of DSRs was observed from the first days of the experiment, allowing us to reduce the DSR selection procedure to only 4 days starting from generation 30 (Table 2).

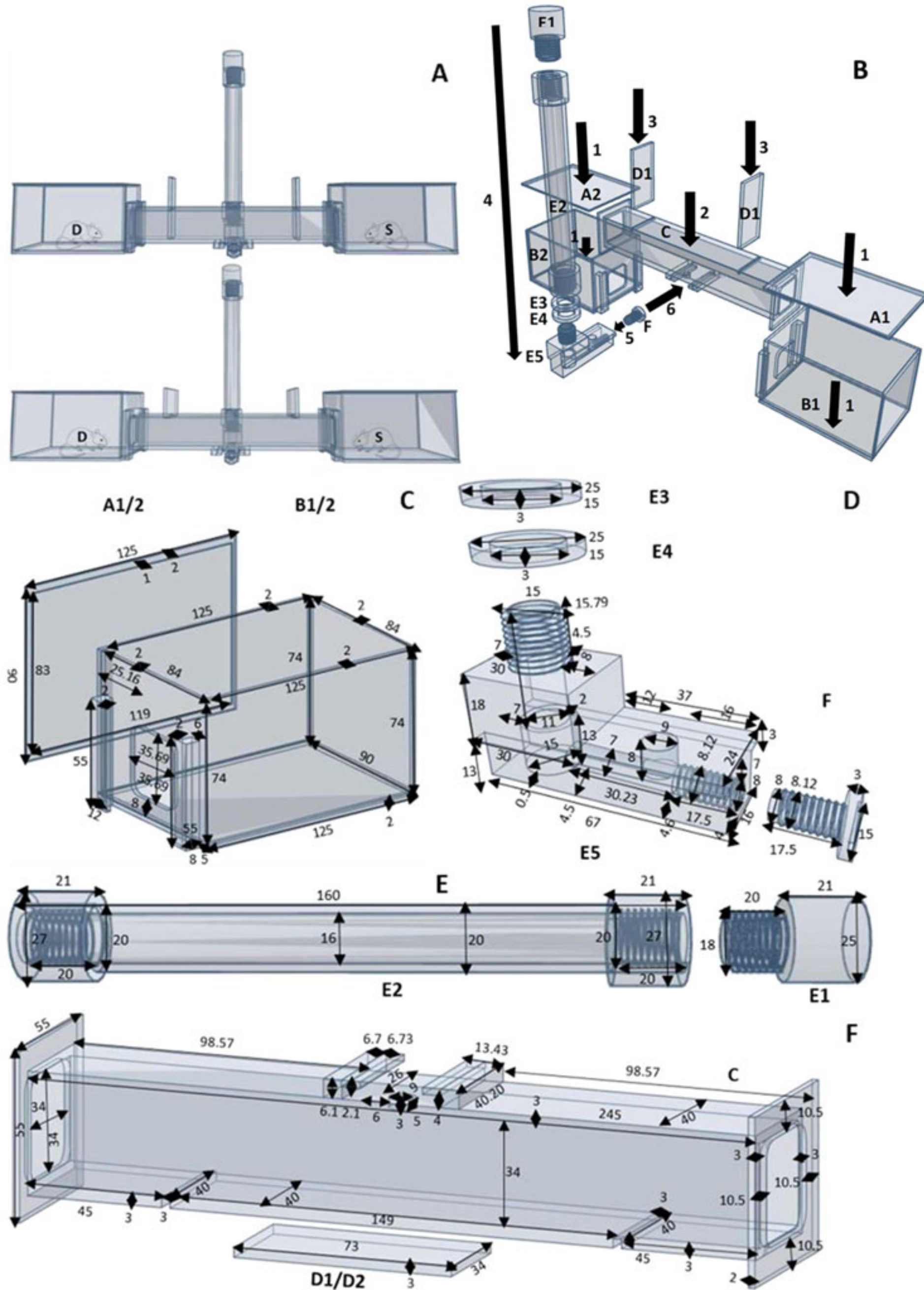

Fig. 1 Diagram of the components, assembly procedure, and structure of the DSR apparatus. (**a**) DSR apparatus and positioning of mice at test initiation. (**b**) Components and assembly order of the DSR apparatus. (**c–f**) Components of the DSR apparatus showing their component dimensions (mm). (**c**) Dimensions of components of C1, C2, C3, and C4 of the cages. (**d**) Dimensions of the components D1, D2, D3, and D4 of the feeder. (**e**) Dimensions of the components of E1 and E2 of the feeder. (**f**) Dimensions of the components F1, F2, and F3 of the tunnel. The diagrams were produced with Tinkercad

10 weeks old

DSR test

Unformed DSR pairs

Formed DSR pairs
(Generation P (♂ or ♀))

Mating

F1

DSR test at 10 weeks, selection and mating

DSR test at 10 weeks, selection and mating

F2

F3, F4, F5 and etc

Fig. 2 Summary of the selective breeding procedure. A complete description of the breeding procedure is given in Subheading 3.2

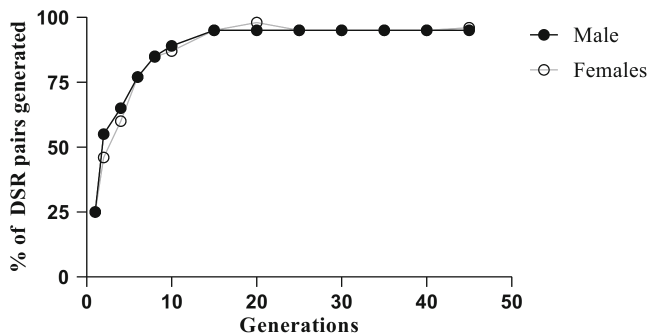

Fig. 3 Formation of DSR pairs throughout the generations. The selective breeding procedure increased Dom–Sub pair formation, reaching a success rate of over 95% from generation 10 in both males and females

Table 2
DSR test performance criteria for outbred and selectively bred Dom and Sub mice

Criteria	Outbred mice	Selectively-bred Dom and Sub mice
Test duration	14 days	4 days
DSR pair formation rate	25–30%	>95
Endpoint measurement	Time spent at feeder	Time spent at feeder
Measurement time	5 min	5 min
Device	DSR apparatus	DSR apparatus

Table 3
Assessment of dominant and submissive phenotypes in various behavioral tests

	Behavioral outcomes		
Behavioral tests	Dom	Sub	References
DSR test	Greater drinking time	Lower drinking time	[45, 58]
Forced swim test	Greater mobility	Lower mobility	[58]
Resident–intruder test	Social win	Social defeat	[58]
Three-chamber test	Higher sociability	Lower sociability	[71]
Chronic mild stress test	Resilience to stress	Susceptibility to stress	[71]

3.3 Confirmation of the Behavioral Phenotypes of the Selectively-Bred Mice

One of the most important questions that arise during enrichment of traits of interest through the selective breeding approach is the confirmation of the developed behavioral phenotype in different behavioral paradigms. Thus, after the successful development of the Dom and Sub mouse populations, we asked the question of whether this selection reflects the social attitudes of the generated population and not of other parameters such as the preference for sweetened milk, extreme aggression, or other physiological deviations.

To answer this question, we performed a series of experiments that included DSR testing at different age points as well as the confirmation of behavioral phenotypes of selectively-bred Dom and Sub mice by the means of the resident–intruder, forced swim, and three-chamber tests (Table 3). These tests are commonly used to assess the different components of social behavior and/or stress-coping abilities [55, 56].

3.3.1 Confirmation of Dom–Sub Phenotypes with the Resident–Intruder Test (RIT)

Rationale The DSR test is designed to assess the social interactions of animals in an environment with relatively mild stressogenic conditions, wherein a feeder with palatable food is located outside of the starting territory of each animal. Hence, it is important to

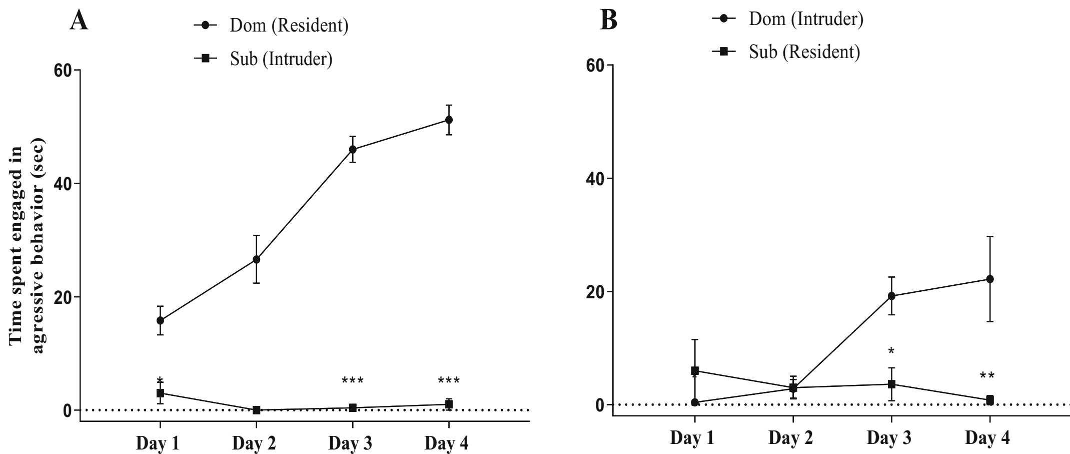

Fig. 4 Assessment of dominance and submissiveness with the RIT. Data indicate mean (±SE) aggressive behavior time (s) of generation F-37 males. (**a**) Sub intruders did not challenge Dom residents. (**b**) In contrast, Dom intruders exerted supremacy over Sub residents. Two-way ANOVA with a Dunnett's multiple comparison test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

verify the Dom-Sub phenotypes observed in the DSR test with the RIT, where territory serves as a critical component of the test [57].

Experimental Design The detailed design of the RIT is described elsewhere [58]. Engagement of the mice in aggressive behavior (biting, scratching, or chasing after the cagemate) during the test was recorded. Attack initiation toward the counterpart or a fighting response to attacks was defined as Dom (aggressive) behavior. Each animal pair (Dom resident–Sub intruder or Sub resident–Dom intruder) was fixed and maintained through the entire experiment.

Outcomes The results of the experiment were clear and straightforward (*see* Fig. 4). The Dom residents demonstrated obvious belligerent behavior toward their Sub intruders. Likewise, the Dom intruders expressed ascendency over Sub residents from the third day of the experiment [58].

3.3.2 Influence of Social Interactions on the Behavior of Dominant and Submissive Mice

Rationale Environmental social factors may trigger and influence the establishment of social status and personality traits in humans [59, 60]. In many cases, subordinate or submissive behaviors result from social triggers, particularly the existence of a stronger Dom partner [61–63]. To examine the influence of social triggers, we explored the behavior of Sub mice in the absence of Dom partners and in the presence of other Sub counterparts.

Experimental design Two types of experiments with different experimental paradigms of the DSR test were performed. In the first paradigm, the DSR test was performed for 3 weeks (*see* Fig. 5). During the first week, animals were subjected to standard DSR testing, where Dom mice were paired against Sub counterparts.

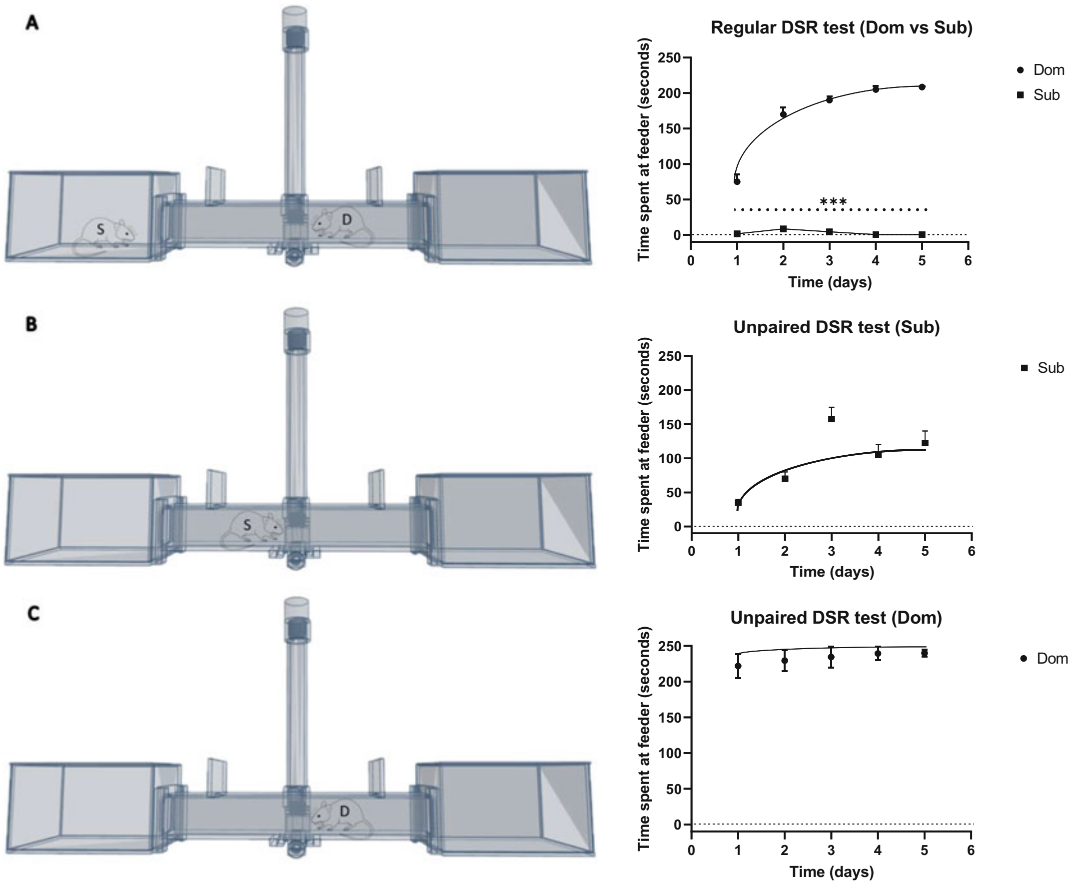

Fig. 5 Dependence of submissive behavior on social interactions. Mean (±SE) feeder time (s) in DSR test of generation F-34 male mice. (**a**) Dom and Sub mice established stable DSRs according to the regular protocol. (**b**) When each animal was present in the DSR apparatus alone, drinking scores of the Sub mice were significantly increased, whereas (**c**) drinking times of the Dom mice did not change. When the mice were again paired (Dom vs. Sub) in the third week of testing, drinking times returned to the pattern previously established in week 1 (**a**). Two-way ANOVA with Bonferroni test: ***, $p < 0.001$. Abbreviations: S or Sub, submissive; D or Dom, dominant

During the second week of testing, the mice (either Dom or Sub) were tested daily in the DSR apparatus alone, thus being allowed to access the feeder with sweetened milk without any interference or competition. During the third week, the mice were returned to testing with the standard DSR procedure using the same counterparts as in the first week of testing [58]. In the second experimental setup, Sub males were paired with Sub males from different cages and were subjected to DSR testing for 10 days [58].

Outcomes The results of the 3-week DSR test showed that both the Dom and Sub animals behaved in an expected way during the first week of testing (Dom vs. Sub): the drinking score of the Dom mice was significantly higher than that of their Sub counterparts.

During the second week of testing, where the mice were allowed to stay in the DSR apparatus without social contact (Sub only or Dom only), the drinking time of the Sub animals significantly increased and the behavior of the Dom mice remaining unaffected by the absence of their Sub counterparts. When the animals were again paired in the third week of testing (Dom vs. Sub), their drinking times returned to the patterns that were established in the first week of testing. Thus, this experiment clearly demonstrates that the behavior of Sub mice is dependent on environmental social triggers.

The experimental setup where the Sub mice were paired against each other (Sub vs. Sub) did not result in the development of strong and stable DSRs. Despite the fact that Sub mice spent more time at the feeder when paired with other Sub mice, the interactions between the pairs of Sub animals in the DSR test were not remotely comparable in terms of proportions to those observed between Dom and Sub mice. This experiment further demonstrates the inherited nature of submissiveness in selectively bred Sub mice.

3.3.3 Monitoring Behavior of Dom and Sub Mice with the Forced Swim Test (FST)

Rationale The FST is primarily used for the screening of antidepressants and for the assessment of depressive-like behaviors [64–66]. Immobility is considered an expression of depressive-like, situation-defeated behavior. In addition, FST is often used to assess stress-coping abilities in rodents [55]. Submissiveness is considered to be an element of depressive behavior which may also be accompanied by reduced stress-coping abilities [67, 68]. Thus, we assessed the performance of Dom and Sub mice in the FST.

Experimental Design Mice were individually tested in a glass cylinder (30 cm in height and 10 cm in diameter) filled with 25 ± 2 °C water up to the 25-cm high mark for 6 [69] or 9 [58] minutes. Immobility time of each mouse was manually recorded. A mouse was considered to be "immobile" if it displayed no activity except for what was required to keep its head above the water. Only immobility times for the final portion of the test period (last 4 min for the 6-min test, last 6 min for the 9-min test) were used for analysis.

Outcomes Independent of experimental settings [58, 69], the immobility time of Sub animals in the FST was significantly higher than that of their Dom counterparts (*see* Fig. 6). Thus, this test further confirmed the behavioral characteristics of the selectively bred Dom and Sub mice.

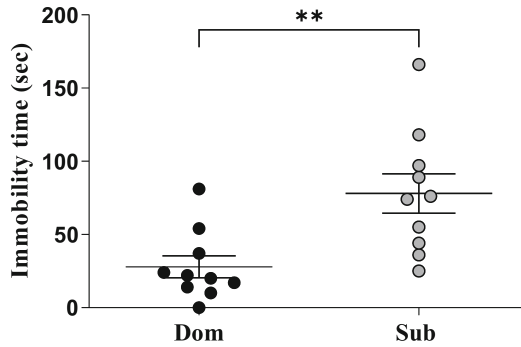

Fig. 6 Confirmation of Dom and Sub phenotypes with the FST. Two-month-old Dom and Sub male mice were subjected to FST for 6 minutes. Data represent mean (±SE) immobility time (s) of the final 4 min of testing for generation F-41 male mice. Immobility time of the Sub mice was significantly higher than that of the Dom mice (Student's *t*-test: **, $p < 0.01$)

3.3.4 Confirmation of the Behavioral Characteristics of Dom and Sub Mice Using the Three-Chamber Test (TCT)

Rationale Rodents generally choose to spend more time among cagemate cohort familiars. In this regard, the TCT is used to assess social behaviors and to identify individuals with deficits in sociability. For example, this test has helped to quantify deficits in the social behavior of transgenic animals exhibiting autistic-like traits and to evaluate the effects of novel chemical substances on social behavior [70]. In contrast with DSR and RIT, in which the major components of the experimental design are competition for food resources and territory, respectively, the endpoint of TCT relies on noncompetitive social interactions.

Experimental Design We followed the standard experimental design as established by Gross and Pinhasov [71]. The dwell time duration in each chamber and the total number of chamber entries was measured.

Outcomes The results of the TCT showed that the Sub mice spent a significantly lower amount of time in the chamber with the unfamiliar mouse and in the region of the cage where the stranger mouse was held than did the Dom mice (*see* Fig. 7) [71]. Thus, as was expected, the Sub animals showed a significantly lower social preference in comparison with the Dom animals. Hence, this test further confirmed the pattern of social behavioral properties of the selectively bred Dom and Sub mice.

3.3.5 Assessment of the Alterations in Social Defeat-Induced Hierarchical Status

Occasionally, strong environmental triggers lead to a change in social status that can cause mood and personality disorders [67, 68, 72–75]. Using the DSR model, we studied the effects of social status change on the behavior of Dom mice. Thus, during the repeated interactions of fixed pairs of Dom males in the DSR test (Dom vs. Dom), most of these mice maintained their dominance. However, some of these animals acquired a strong subordinate

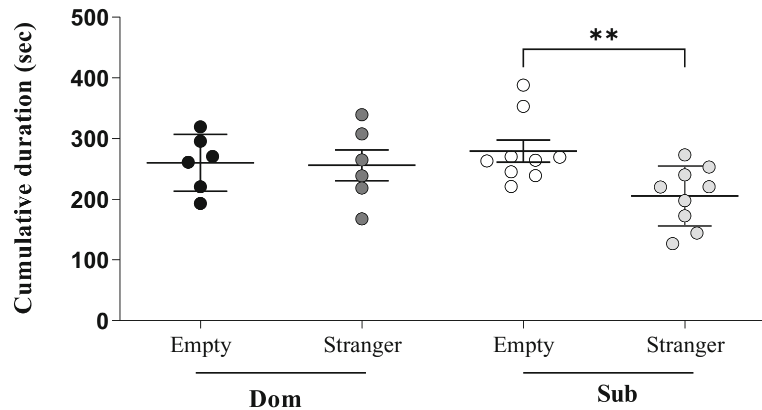

Fig. 7 Assessment of Dom and Sub mouse behavior with the TCT. Sub mice were spent greater amounts of time than Dom mice in the empty chamber, which did not contain the stranger mouse and represent asocial behavior (Student's *t*-test: **, $p < 0.01$)

phenotype despite their Dom genetic background, which we defined as "subdued dominance" (dSUB) [58]. When these dSUB males were matched against Sub males in the DSR test, they did not recover their Dom behavior. The dominance of dSUB mice was only restored by lithium chloride, a well-known potent mood stabilizer [58]. Notably, lithium chloride did not affect the behaviors of the Sub animals [58]. Therefore, it appears that the mechanisms responsible for the submissive behavior of the dSUB animals are different than those governing the behavior of selectively-bred Sub animals. The described modifications of the DSR test (Dom vs. dSUB) can be used to develop and test drugs with possible antimanic- or antidepressant-like effects. In humans, changes in socioeconomic status lead to an increase in psychiatric disturbances [67, 68, 74, 76], thus making DSR-based models a valuable tool in the study of such phenomena.

4 Dom and Sub Mice as a Valuable Basis for Prospective Research Directions

The characterization of the physiological features of mice with enriched dominant and submissive behavioral phenotypes has revealed distinct and substantial differences in terms of their stress-coping abilities, metabolic features, aging processes, inflammatory response, and other physiological features. Thus, by using this model, we have the potential to explore different pathological conditions that may be linked to or influenced by social behavior. The main research directions for which this model can be used are shown in Fig. 8 and are discussed below.

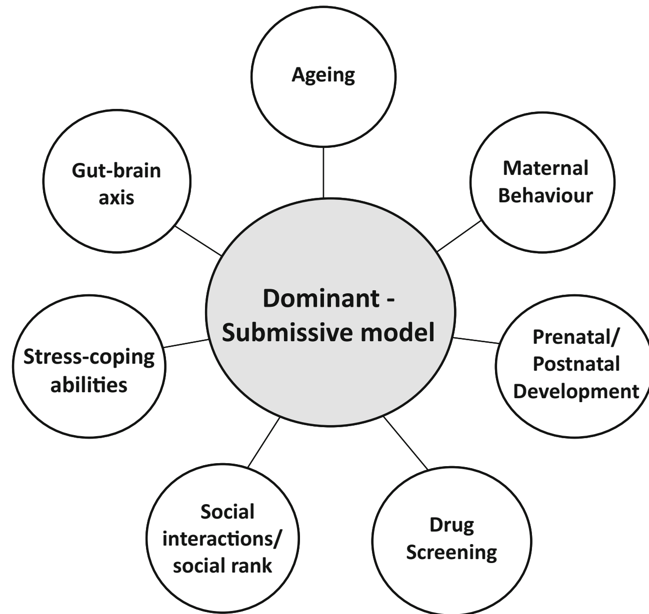

Fig. 8 Potential applications of the dominant–submissive mouse model

4.1 Assessment of Stress-Coping Abilities

Appropriate stress-coping abilities are critical for constructive response to environmental stimuli, and a proper stress response is essential for the maintenance of homeostasis [77–80]. We have found that Sub mice that were exposed to chronic mild stress (CMS) for 5 weeks exhibited dramatic reductions in their sucrose preference in comparison with Dom mice that successfully resisted various stressful triggers [71]. It has been documented that failure in homeostasis leads to chronic inflammation and to the accumulation of allostatic load [81, 82]. The prior observations are supported by our findings which demonstrate that innately stress-vulnerable Sub mice exhibit an aging-dependent increase in the levels of proinflammatory cytokines, with metabolic changes being accompanied by splenomegaly and a shortened life span [83]. These results are supported by other studies, which demonstrate the impact of stress on the development of chronic inflammation [84, 85]. Thus, this model may be valuable for the investigation of the link between impaired stress-coping abilities, the development of chronic inflammation, and accelerated aging [83].

Impaired stress-coping abilities are often associated with a propensity to manifest developmental abnormalities [86–89]. Healthy prenatal programming may be hampered by prenatal stress, which may alter the development of the HPA axis and increase the risk of subsequent behavioral disorders [90–95].

We have shown that the adult offspring of prenatal restraint stress (PRS)-exposed Sub and Dom dams demonstrate contrasting stress-induced stimuli-coping abilities. Sub offspring born to PRS pregnancies developed depressive-like and anxiety-like behaviors, which were not observed to occur in their Dom counterparts. Furthermore, PRS exposure has been shown to markedly facilitate glucocorticoid receptor recruitment to the hippocampi of Dom mice, which may be responsible for their resilience to stressful challenges [96, 97].

4.2 Response to Psychotropic Agents

The investigation of the effects of psychotropic agents, including those of antidepressants, mood stabilizers, and psychostimulants is one of the major research areas of psychiatry [98, 99]. It is known that the effects of psychotropic agents are highly diverse within the human population; however, no clear mechanism to explain these variabilities in response has yet emerged [100–102]. This diversity includes variable efficacy, time of onset, as well as side and paradoxical effects [98, 103–106]. As DSRs depend upon the functionality of the neural circuits, which involve monoaminergic neurotransmission [69], the behavioral profiles of selectively bred Dom and Sub mice have been proposed to mimic the hyperthymic-like and depressive-like temperaments observed in patients with affective disorders [69]. Indeed, our studies have demonstrated that Dom and Sub mice differentially respond to antidepressants [58, 69], mood stabilizers [58], and anxiolytic compounds [58] (Table 4). For example, paroxetine, a well-known selective serotonin reuptake inhibitor (SSRI), has been shown to dose-dependently reduce the immobility time of Sub mice in the FST, which was interpreted to represent a reduction in their depressive-like phenotype. In contrast, the same doses of paroxetine have been shown to cause a paradoxical effect in Dom mice comparable to the effect of antidepressants on individuals with manic behavior [107–109].

Diazepam, a benzodiazepine $GABA_A$ receptor agonist, has been shown to exert anxiolytic effects in Sub animals, reflected by reduced anxiety-like behavior in the elevated plus maze test (EPM) and sedated behavior in the open field test (OF). In Dom animals, diazepam causes anxiogenic effects in the EPM yet produces an increase in OF activity. In addition to the above features, diazepam significantly strengthened the dominance of animals in the DSR paradigm relative to their vehicle-treated Dom counterparts [58]. One explanation for these effects posits that the enriched dominant phenotype of Dom mice reflects the behavioral characteristics of a portion of the human population with a hyperthymic temperament and enhanced genetic predisposition to the paradoxical reaction to benzodiazepines [110, 111].

Table 4
Differences in response to various psychotropic agents in Dom and Sub mice

Psychotropic agent	Dom	Sub	References
Paroxetine	Paradoxical effect	Reduction of depressive-like behavior	[58]
Diazepam	Anxiogenic effect Increased dominance	Anxiolytic effect Reduction of anxiety-like behavior	
THC (low dose)	No influence on behavior in the CPP test	Reduction of depressive-like behavior	[113]
THC (high dose)	Development of depressive-like behavior	Drug aversion	
Cocaine	Attraction to cocaine in the CPP test No influence of CMS on attraction to cocaine	Aversion to cocaine in the CPP test Increase in cocaine attraction after CMS	[112]

Abbreviations: *THC* Δ-9-tetrahydrocannabinol, *CMS* chronic mild stress test, *CPP test* conditioned place preference test

Our psychostimulant studies also indicate that dissimilarities in stress-coping abilities of Dom and Sub mice result in different addictive responses [112, 113]. Thus, Dom and Sub mice react differently to high and low doses of Δ-9-tetrahydrocannabinol (THC). Following short-term repeated treatments with low (1.5 mg/kg) and high (15 mg/kg) THC doses, Sub mice demonstrated significant place aversion in the conditioned place preference (CPP) test at the high dose, whereas Dom mice displayed either no place preference or aversion. After 6 weeks of treatment, Dom and Sub mice presented different and dose-dependent behavioral patterns in the FST [113]. The low dose of THC reduced immobility in both Dom and Sub groups, whereas the high dose significantly increased depressive-like behavior in Dom mice only. It is thus possible to conclude that behavioral dominance and stress vulnerability differences are involved in differences in cannabis response among users [113].

Cocaine studies revealed that Sub mice display a drug aversion in the CPP paradigm test, whereas Dom mice display drug attraction. However, following a 4-week regimen of CMS, the Sub mice displayed a dramatic increase in cocaine attraction, yet no changes occurring in the drug attraction of Dom mice [112]. It can thus be concluded that psychotropic agents differentially affect individuals with different stress-coping abilities. Understanding the molecular mechanisms responsible for the distinct responses of Dom and Sub mice to pharmacological agents has the potential to aid the design of better clinical strategies in the treatment of substance addictions.

4.3 The Gut–Brain Axis and Its Role in Innate Stress Responsiveness

The gut microbiota is increasingly recognized as one of the factors shaping the brain and its functions, its effect being mediated by the gut–brain axis through bidirectional cross talk which involves neurological (via the vagus nerve), metabolic, nutritional, endocrine, and immunological aspects [114–117]. Since the alimentary tract is a large interface with the environment and hosts an immensely diverse microbial ecosystem, it follows that alterations of the microbiome profile and its resultant metabolic cross talk would have profound effects on a global level for any organism.

We have recently discovered that Dom and Sub mice possess different gut microbiota compositions, which correlate with their behavioral features. Specifically, we have found that Sub mice have a reduced α-diversity of the microbiome [118], similar to individuals suffering from depression and anxiety [119–122]. Remarkably, fecal microbiota transplantation (FMT) of Sub mice into germ-free (GF) Swiss Webster mice resulted in significant behavioral changes, including social skills impairment and depressive-like symptoms, which are typically observed in our Sub mice [118]. FMT-induced physiological changes in GF mice included reduction of epididymal white adipose tissue (eWAT) mass and reduction in adipocyte volume, as well as alterations of the inflammatory profile [118]. Thus, the Dom–Sub model can be used to study the gut–brain influence in individuals with opposite social phenotypes, allowing for identification of links between inherited stress vulnerability, gut microbiome composition, behavioral patterns, and metabolic and inflammatory characteristics.

5 Notes (Recommendations, Guidance, and Troubleshooting)

This section contains our remarks and suggestions for overcoming difficulties and pitfalls that may arise during the process of developing of mice with strong features of dominance and submissiveness using DSR-based selective breeding. For interested researchers, breeding pairs of Dom and Sub mice can be provided upon request to establish their own mouse colonies.

1. An important condition for the successful development of mice populations with strong features of dominance and submissiveness is the selection of an appropriate mouse strain. Our strategy was based on employment of an outbred strain; however, wild-type animals also may be considered. Obviously, inbred strains are not appropriate for this type of behavioral selection. We also recommend using mouse strains that have been previously used in neurobiological research and/or drug screening in the field of neuropsychopharmacology.

2. Based on the above stated stipulations, we recommend that animals are bred on the basis of their dominant and submissive phenotypes for at least five generations before the selection of the colony that will be utilized for the inbreeding procedure. Phenotypes of every generation should be verified with the DSR test and approximately at generation five with other behavioral tests such as RIT and TCT.
3. To avoid genetic complications, mating between siblings should be avoided.
4. Additional features to consider for the performance of the DSR procedure and its preparatory procedures.
   A. DSR pairs should be selected with respect to weight and age similarity, the acceptable age difference being no more than 5 days.
   B. It is recommended to perform male and female DSR tests during different weeks. If this is not possible, it is recommended to use different DSR devices and behavioral rooms for each of these tests. The prior precautions are due to the strong effects of female odors on male behavior which are difficult to remove from the test apparatus and may affect DSR pair formation.
5. We recommend dyeing the coats of the tested individuals with special dyes that do not harm the health of the animals and remain on their coats for at least one month. Our laboratory group sources animal dyes from Stoelting, USA.
6. DSR behavioral testing should not be performed before 8 weeks of age. Although mice reach sexual maturity as early as 35 days postnatal, physiological development and weight gain continue up to approximately 3 months of age [123]. Use of developmentally-stable adults at the beginning of DSR pairing will ensure more consistent DSR pair establishment.

6 Conclusions

Social interactions play an important role in shaping the personality of individuals and in development of behavioral and metabolic features. The biological nature of social interactions is complex and involves different mechanisms which require valid biological models. We have shown here that selective breeding for traits of interest may partially answer these needs by improved segregation and stabilization of those traits.

The DSR-based selective breeding approach has allowed us to produce mouse populations with stable and strong dominant and submissive phenotypes across generations. The implementation of this approach resulted in a dramatic increase in phenotype stability

and reproducibility of behavioral testing, substantial reduction in experimental time, as well as a high rate of DSR pair formation. Behavioral feature enrichment can allow us a better understanding of submissive and dominant traits as basic elements of the hierarchical spectrum, their genetic and epigenetic nature, and the associated gene-environment interactions. In particular, the distinct inherited stress vulnerabilities of Dom and Sub mice allow us to research the influence of stress and stress-coping abilities under various environmental conditions including prenatal and postnatal stages, aging, pharmacological interventions, and presentation of various pathologies.

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Chapter 18

Prediction of Susceptibility/Resilience Toward Animal Models of Post-traumatic Stress Disorder (PTSD)

Maria Morena, Giulia Federica Mancini, and Patrizia Campolongo

Abstract

In the western countries, the lifetime prevalence of experiencing high stressful events is estimated to be about 75–80%; however, only a small proportion of those individuals (about 10%) exposed to stress ultimately develops PTSD. This represents a critical aspect that must be considered when modeling PTSD-like symptomatology in animal models. The vulnerability toward the development of PTSD may be conferred by exposure to risk factors and individual predisposition, and both factors can be incorporated in animal models.

Here we describe an animal model for PTSD-like symptomatology that combines to the traumatic experience (footshock), exposure to risk factors (i.e., social isolation), and behavioral profiling to enable the differentiation between susceptible and resilient phenotypes to the development of long-lasting trauma-induced cognitive and emotional alterations. Notably, this model allows an early post-trauma identification of a reliable predictor (i.e., exploratory activity in a novel environment) of susceptibility and resilience. The present animal model provides a relevant behavioral tool to understand the neurobiology of PTSD and, most importantly, the neurobiology of resilience and susceptibility toward the development of the psychiatric disorder. Understanding the mechanisms of resilience and susceptibility will unravel new targets for prophylactic and/or therapeutic interventions aiming at promoting resilience or reduce vulnerability.

Key words PTSD, Traumatic memory, Stress, Rats, Psychiatric disorder, Experimental models, Behavioral profiling

1 Introduction

Exposure to stress activates neuronal and endocrine responses that promote effective physiological and psychological changes to cope with it [1]. However, when stress is excessive, these mechanisms can become dysfunctional leading to the development of trauma-related disorders, such as post-traumatic stress disorder (PTSD) [2, 3]. PTSD is classified as a chronic psychiatric disease with high prevalence and comorbidity with other disorders (e.g., depression, anxiety, and drug addiction) [4]. According to the last edition of the Diagnostic and Statistical Manual of Mental Disorders

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(DSM-5), PTSD is classified as a trauma-related disorder and might develop in subjects after direct or indirect exposure to a traumatic event (e.g., witnessing the trauma, learning that a close person was exposed to a trauma, exposure to aversive details of the trauma) [5]. Overall, PTSD patients manifest a constellation of symptoms including hyperarousal, hypervigilance, increased anxiety, altered sociability, and cognitive alterations (e.g., over-consolidation of the traumatic memory, fear generalization, and impaired extinction of the trauma) [6]. It is estimated that even if approximately one third of the world population will experience a trauma once throughout their lifetime [7], only a small subset of them (vulnerable individuals) ultimately develops this disorder [4], with women twice as likely to develop the pathology as compared to men [4, 8]. Although several studies on patients and animal models have focused on the pathophysiology of this disorder [6, 9], it remains a critical question that still needs to be clarified: why do some individuals develop PTSD after the exposure of a traumatic event while others do not? Preclinical models resembling the hallmark symptoms observed in PTSD patients represent a useful tool to gain insight into understanding the etiopathogenesis of this disorder and for the development of effective therapeutic strategies. Generally, animal models of psychiatric disorders refer to three criteria: "face validity" consisting in observable behavioral outcomes, "construct validity" demonstrating the same underlying neurobiological mechanisms seen in human subjects, and "predictive validity" providing a similar response to treatment as observed in humans [10–12]. However, this is extremely difficult when modeling PTSD and in general for most psychiatric disorders, as the neural basis are not clearly understood, and there is no gold standard pharmacological treatment for PTSD [13], which complicates the achievement of the construct and predictive validity criteria. Although the etiology and symptom manifestation of PTSD are notably complex due to its high interindividual variability, numerous preclinical models have been developed and used for their ability to mimic some of the core symptoms observed in PTSD patients. These paradigms are based on the exposure to stressors of different type, intensity, duration, and frequency [9, 12, 14–20]. For example, they may include physical, psychological, and/or pharmacological stressors, such as footshock exposure [21–26], single prolonged stress [27–33], underwater trauma [34–37], restraint stress/immobilization [38–43], stress-enhanced fear learning (SEFL) [44–47], social defeat stress [48, 49], and predator/predator odor exposure [36, 50–53]. However, the majority of these animal models lacks of construct and predictive validity [54] and presents important shortcomings which, often, limit their translatability to the human pathology. An important aspect, often underestimated in animal models, is the chronicity of the pathology. PTSD is not defined by the immediate response to

the trauma, which often expresses a physiologic rather than a pathologic reaction, but consists of a plethora of long-lasting neurobiological, physiological, and behavioral alterations, which should persist in humans for at least 1 month after trauma, according to the diagnostic criteria of DSM-5 [5].

Moreover, there are critical aspects that deserve more attention and need to be taken into consideration when modeling such a complex human disorder, such as the individual predisposition, sex differences, and the contribution of risk factors [15]. All of these determine the resilience or susceptibility to develop the disorder, given that, as mentioned above, only a subset of individuals experiencing a traumatic event develops PTSD.

1.1 Animal Models of Resilience and Susceptibility to Develop PTSD-Like Symptoms

The development of an animal model able to determine the factors leading to the susceptibility or resilience toward this disorder is crucial for understanding the neurobiological underpinnings of the individual variability in the development of PTSD. In this regard, some studies found validated classification criteria able to identify animals susceptible and resilient to develop PTSD-like symptomology among trauma-exposed rats [36, 37]. Cohen and colleagues classified rats according to their performance in two consecutive behavioral tasks (i.e., enhanced fearfulness in the elevated plus maze and exaggerated mean startle amplitude in the acoustic startle response task) evaluated in two different PTSD-like animal models, such as the predator exposure and underwater trauma [36]. They reported that, while in the acute post-traumatic phase 90% of rats presented maladaptive responses to stress, this percentage dropped to 25% 1 week after trauma and remained stable up to 30 days post-trauma [36]. In another study, rats were classified as affected or unaffected based on their position in the upper or lower 20th percentiles of the control group distribution of anxious-like behavior in the water zero maze and anhedonic-like behavior in the continuous saccharine preference test, shown 1 month after an underwater trauma stress [37]. Interestingly, several preclinical models using different types of trauma exposure found a specific variable which is able to predict which animal will be susceptible or resilient toward the development of PTSD-like phenotype after experiencing a traumatic event [45, 55–57]. However, some of these animal models capture only cognitive or emotional features of PTSD [55] or do not test for the persistence of the cognitive and emotional alterations in the long term (i.e., testing up to 13 days after trauma for the cognitive and emotional alterations and up to about 3 months after trauma for alcohol drinking behavior only) [45]. For instance, the study by Jeong and colleagues found that mice with enhanced freezing behavior during the intertrial interval (i.e., interval between two consecutive tone presentations) of a SEFL paradigm showed alterations in learning and memory functions, such as increased freezing to

novel cues, increased generalization indices, and extinction resistance after trauma exposure which were tested, however, relatively shortly (i.e., 14 days) after trauma [56]. Whereas in the study by Olson et al. mice were subjected to the traumatic experience with reminders of stress (TERS) model in which animals were exposed to a single shock (2 mA, 10 s) followed by exposure to six contextual reminders of the shock over a 25-day period. Mice were classified in resilient and susceptible based on the differences in the acoustic startle responses obtained before and after the TERS procedure and found that susceptible mice exhibited emotional alterations, such as social withdrawal and increased aggression, up to 1 month after the last reminder of stress session [55]. PTSD is a chronic psychiatric disease characterized by both cognitive and emotional alterations, considering both features in animal models is therefore important. A recent study in mice discriminated between susceptible and resilient subjects based on the animals' post-trauma arousal reactivity. Specifically, mice were subjected to a trauma (i.e., 24h-restrain stress) and assigned to susceptible and resilient groups based on their acoustic startle reactivity measured 15 and 29 days post-trauma; this categorization allowed authors to observe both emotional and cognitive alterations up to 75 days after trauma [43].

Furthermore, besides the interindividual variability, exposure to risk factors also plays a central role in determining the occurrence of the disorder following exposure to a trauma. Early life adversities or preexisting conditions, such as substance abuse and social isolation, in proximity of a stress experience, for instance, have been reported to increase the susceptibility to develop stress-related disorders [30, 58–61]. Thus, risk factors represent essential components to be included as part of the animal models for PTSD, being highly reflective of the human condition.

Based on these premises, in our proposed rat model for PTSD, we combined the incorporation of risk factors (i.e., social isolation) with behavioral profiling to allow the discrimination between subjects exposed to the trauma who developed PTSD-like symptomatology (susceptible) and subjects exposed to the trauma who became resilient to subsequent PTSD-like symptom development. Importantly, our rat model also resembles both cognitive and emotional aspects of the PTSD-symptomatology and the chronicity of it [25, 26] and allows the identification of a variable which can be identified early after trauma to predict the susceptibility or the resilience to subsequent development of the disorder [26]. Specifically, we have identified the explorative activity of a novel environment early after trauma as a robust predictive variable of susceptibility (i.e., low explorative activity) or resilience (i.e., high explorative activity) to subsequently develop PTSD-like symptomatology.

2 Materials

- **Animals**. Adult (from 60- to 90+-day-old) male or female rats (*Rattus norvegicus*) of the selected rat strain, kept in an air-conditioned colony room (temperature: 21 ± 1 °C; 12h/12h light/dark cycle) with pellet food and water available ad libitum.
- **Experimental rooms**. Sound-attenuated and dimly illuminated experimental room, connected by a door to an adjacent room.
- **Lamps**. One or more lamps with (i) red light bulbs to provide illumination (~2 lux) to the experimental room and apparatus on the social interaction testing days and (ii) white light bulbs to provide illumination (~2 lux) to the experimental room and apparatuses all the other days. Please, *see* **Note 1** for additional information.
- **Experimental apparatuses**:
  - *Fear chambers*. One or more experimental apparatuses consisting of custom-made metal trough-shaped alley (60-cm-long, 15-cm-deep, 20-cm-wide at the top and 6.4-cm-wide at the bottom) connected to a shock generator [25]. Alternatively to the custom-made metal chambers, classic fear-conditioning chambers may be used (30 × 24 × 21 cm; MED-Associates): the classic fear-conditioning chambers are constructed of aluminum side walls and Plexiglas ceiling and front door, and are situated in sound-attenuating cabinets. The floor of each chamber consists of stainless steel rods (4 mm in diameter) spaced 1.5 cm apart. Rods are wired to a shock source for the delivery of footshocks.
  - *Open field arena*. One or more testing arenas in opaque Plexiglas or any other nonabsorbent opaque material (80 × 80 × 60 cm).
  - *Social interaction arena*. One or more testing arenas made of transparent Plexiglas (40 × 40 × 60 cm) with the floor covered in clean sawdust to a maximum height of ~2 cm.
- **Computer** that will run a software and **interface** with the fear chambers for stimulus delivery.
- **Automated video-tracking** system (e.g., Smart Panlab; Observer XT, Noldus EthoVision XT; ANY-maze).
- **Infrared video camera** on a stand and/or fixed on the ceiling to record all the experimental sessions.
- **Voltmeter** to check the shock intensity.
- **Lux meter** to check the light intensity.
- **Weight scale**.
- **Stopwatch or timer** to time experimental sessions and for manual scoring of behavior.

- **Black marker and paper labels** for subject identification.
- **Cleaning solution (e.g., 70% ethanol solution) and paper towels** for wiping out the experimental apparatuses and eliminate olfactory cues in between each behavioral session.

3 Methods

3.1 Experimental Design

All experiments should be performed during the light phase of the cycle (between 10:00 am and 16:00 pm). The experimental protocol consists of different spaced behavioral sessions run across a comprehensive period of 24 days, during which socially isolated rats are exposed to a footshock trauma and screened, 5 days later, for their exploratory activity in a novel arena. Subsequently, the animals are re-exposed to the trauma context for four spaced sessions (at post-trauma days 7, 10, 13, and 16) to evaluate their fear memory retention/extinction and, lastly, pairs of rats belonging to the same experimental group are tested in a social interaction test (day 19) (*see* Fig. 1).

Handling (Day -4; Day -3; Day -2) and Social Isolation (from Day -3). To decrease the stress associated to the experimental procedure, all rats should be habituated to the experimenter manipulation and to the transfer from the housing room to the experimental room (*see* **Note 2** for additional information). Thus, all rats are transferred on wheeled racks in the room adjacent to the experimental room, under dim light and sound-attenuated conditions, and gently handled for 1–2 min each, for 3 consecutive days (day -4, day -3, day -2 before the trauma exposure). Rats should be left undisturbed in the room for at least 30 min before and after the handling procedure. At the end of the handling procedure on day -3, all rats are put back into individual cages for social isolation. Rats remain singly housed until the end of the behavioral testing (day 19) for a total of 23 days. Social isolation is a common risk factor for the development of PTSD, and it is necessarily required to develop enduring signs of emotional distress upon exposure to a traumatic event in our model [25]. Please, *see* **Note 3** for additional information.

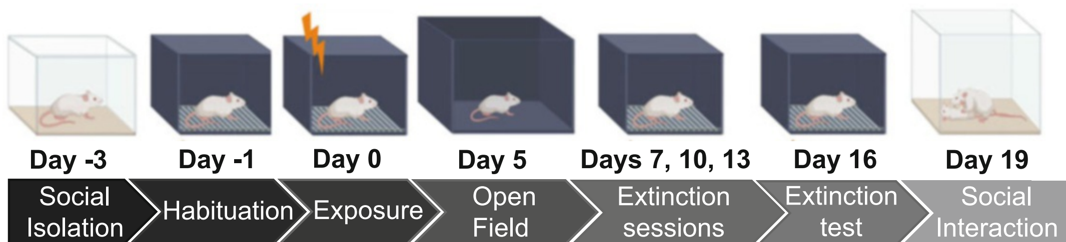

Fig. 1 Experimental timeline of the protocol illustrating all behavioral sessions

Habituation to the Fear Chamber (Day -1). Rats are transferred on a wheeled rack to the room adjacent to the experimental room 2 h before the beginning of the procedure, under dim light conditions, and remain undisturbed in the room for an additional 2 h after the end of the session. Rats are taken from their own home cage, moved to the adjacent experimental room (light ~2 lux) and placed in the fear chambers for 5 min to allow familiarization with the apparatus. Before and after each habituation session, fecal boli are removed from the fear chambers, which are, then, cleaned with 70% ethanol solution.

Trauma Exposure Session (Day 0). During this behavioral session, rats are exposed to the traumatic experience (footshock). We have previously shown that this footshock exposure model coupled with social isolation induces long-lasting (up to 8 weeks after trauma) enhanced traumatic memory recall and social impairments [25]. Please, *see* **Note 3** for additional information.

1. Before starting with the experimental procedure, turn on all the necessary equipment (computer, shock generator, lamps – ~2 lux).
2. Set up the software/equipment for the shock delivery protocol.
3. Calibrate the shock intensity (0.8 mA, 2 s) (*see* **Note 4**).
4. Clean all chambers thoroughly with 70% ethanol solution.
5. Transfer rats from the vivarium to the room adjacent to the experimental room, and leave them undisturbed for at least 2 h before the beginning of the experimental sessions.
6. Turn on the video cameras and the computer programs.
7. Move each subject to the experimental room, and place it into the fear chamber for a total duration of 6 min.
8. Two minutes after placement in the chamber, present five footshocks (0.8 mA, 2 s), randomly, with the last one always given at the end of the fifth minute. After the last footshock, keep rats in the apparatus for 1 min to facilitate the association of the context to the aversive stimuli. It is important to randomize the timing of footshock delivery to avoid any temporal conditioning which it has been shown to occur in fear-conditioning procedures when delivering the aversive stimuli at same time intervals [62].
9. Remove the animals at the end of the session and place them back to their home cages in the adjacent room.
10. Remove fecal boli, clean the chamber with 70% ethanol solution thoroughly, and reset all computer programs.
11. Test the next animal/s and repeat steps from **step** 7 to **10**.

12. At the end of the experimental sessions, leave the rats undisturbed in the room adjacent to the experimental room for 2 h before bringing them back to the vivarium.

Open Field Test (Day 5). This experimental session allows the screening of susceptible and resilient populations of rats to the subsequent development of PTSD-like symptomatology:

1. Before starting with the experimental procedure, turn on all the necessary equipment (computer, white light lamps – ~2 lux in the open field arena).
2. Set up the software for the animal tracking and the behavioral scoring.
3. Clean all arenas thoroughly with 70% ethanol solution.
4. Transfer rats from the vivarium to the room adjacent to the experimental room, and leave them undisturbed for at least 2 h before the beginning of the experimental sessions.
5. Turn on the video cameras and the computer programs.
6. Move each subject to the experimental room, and place it at the center of the open field arena, facing away from the experimenter, for 15 min.
7. Remove the animal from the arena at the end of the session, and place it back to its home cage in the adjacent room.
8. Remove fecal boli, clean the arena with 70% ethanol solution thoroughly, and reset all computer programs.
9. Test the next animals and repeat steps from **step 6** to **8**.
10. At the end of the experimental sessions, leave the rats undisturbed in the room adjacent to the experimental room for 2 h before bringing them back to the vivarium.

Extinction Sessions (Day 7, Day 10, Day 13) and Extinction Test Session (Day 16). These four sessions are carried out identically. During the three extinction sessions (days 7, 10, and 13), rats are tested for traumatic memory consolidation and recall (evaluated mostly on day 7) and trained to extinguish the aversive context–footshock association, as they are repeatedly exposed to the fear context without receiving the aversive stimuli (days 7, 10, and 13). During the extinction test session (day 16), rats are tested for recall of fear memory extinction.

1. Before starting with the experimental procedure, turn on all the necessary equipment (computer, lamps).
2. Set up the software for the animal tracking and the behavioral scoring.
3. Clean all chambers thoroughly with 70% ethanol solution.

4. Transfer rats from the vivarium to the room adjacent to the experimental room, and leave them undisturbed for at least 2 h before the beginning of the experimental session.
5. Turn on the video cameras.
6. Move each subject to the adjacent experimental room, and place it into the fear chamber for a total duration of 10 min.
7. Remove the animal at the end of the session, and place it back to its home cage in the adjacent room.
8. Remove fecal boli, clean the chamber with 70% ethanol solution thoroughly, and reset all computer programs.
9. Test the next animal/s and repeat steps from **step 6** to **8**.
10. At the end of the experimental sessions, leave the rats undisturbed in the room adjacent to the experimental room for 2 h before bringing them back to the vivarium.
11. Repeat **steps** from **1** to **10** for all the extinction sessions run on days 7, 10, and 13 and for the extinction test on day 16.

Social Interaction Test (Day 19). During this experimental session, a pair of rats is placed in the experimental arena for the evaluation of social impairments.

Pairs for the social interaction test should be decided the day before the experiment, according to the following criteria: (1) belonging to the same sex and experimental condition; (2) unfamiliarity, i.e., the two rats of each pair must never have been housed in the same cage, before social isolation; and (3) least weight difference (*see* **Note 4**). Thus, the day before the test, animals are weighed, and after deciding the couples, one rat of each pair is marked with a black nontoxic marker to allow the operator to discriminate between the two rats belonging to the same pair during the social encounter.

On the experimental day:

1. Before starting with the experimental procedure, turn on all the necessary equipment (computer, red light lamps – ~2 lux in the experimental arena).
2. Set up the software for the animal tracking and the behavioral scoring.
3. Clean all the arena/s thoroughly with 70% ethanol solution, and cover the floor in clean sawdust to a maximum height of ~2 cm (*see* **Note 5**).
4. Transfer rats from the vivarium to the room adjacent to the experimental room, and leave them undisturbed for at least 2 h before the beginning of the experimental session.
5. Turn on the video cameras and the computer programs.

6. Move a pair of rats to the experimental room and place it in the arena for 10 min.
7. Remove the animals form the arena at the end of the session, and place them back to their home cages in the adjacent room.
8. Remove fecal boli, blend the sawdust, clean the arena's walls with 70% ethanol solution, and reset all computer programs.
9. Test the next animals and repeat steps from **steps 6** to **8**.
10. At the end of the experimental sessions, leave the rats undisturbed in the room adjacent to the experimental room for 2 h before bringing them back to the vivarium.

3.2 Behavioral Scoring

Behavioral scoring can be performed manually by well-trained operators, who need to be blind to the experimental conditions or by automated systems (*see* **Note 6**). When scoring is performed manually by one or multiple observers, a critical point to reduce bias and variability of the data obtained is to calculate intra-rater and inter-rater reliability. Ideally, manual scoring should be performed by at least two observers with high intra- and inter-observer reliability and then averaged to generate a single human score. When using scoring automated systems, instead, it is critical to ensure a proper calibration and standardization of the video-tracking system before each session (e.g., camera calibration, setting of lightning conditions; *see* **Note 4**). It is also advisable to make sure inter-rater reliability is very high between well-trained human observers and the automated scoring system.

Intra- and inter-rater variability for continuous measures can be calculated by using correlation and linear fit analyses. A perfect reliability analyses would generate a correlation of 1.0, slope of 1.0, and y-intercept of 0 (i.e., $y = 1\ x + 0$).

Trauma Exposure, Extinction, and Extinction Test Sessions. To index the acquisition, recall, extinction, and extinction recall of the traumatic memory, this protocol uses freezing behavior. Freezing behavior was defined as the absence of all movements, aside from those required for respiration (without regard to posture) [63–66]. Freezing represents a prominent passive fear/defense reaction in rodents [67] that mice and rats develop after a context and/or cue pairing with an aversive stimulus. Freezing can be scored manually by well-trained operators, who need to be blind to the experimental conditions or by automated systems (i.e., VideoFreeze, Med Associates).

Freezing can be measured continuously with a stopwatch as a percent time spent freezing for a given test period [e.g., (time spent freezing, sec/total session time, sec) × 100] or by instantaneous time sampling every 3–10 s of a freezing bout, defined as the animal

presenting freezing behavior for 1 (or more) sec, [e.g., (number of freezing bouts observed every predetermined time interval/total number of observations made in the entire session) $\times$ 100] [64, 66].

Open Field Test. Out of all the different behaviours shown by trauma-exposed rats that were analyzed to determine a potential predictive variable for the development of PTSD-like symptomatology, the rats' exploratory activity of the open field arena was identified as the best fitting and consistent predictor of several PTSD-like behavioral outcomes shown by rats long after trauma [26]. Rats' exploratory activity can be determined as the total distance traveled in the arena (cm), scored automatically through a video-tracking system (e.g., Smart Panlab; Noldus EthoVision XT; ANY-maze). Alternatively, in the absence of an automated scoring system, the total distance traveled can be scored manually as the number of crossings, with all four paws, through ideal (or real) grid lines dividing the floor of the arena in squared sections. Each of these identical squared sections forming the grid should be sized to perfectly enclose the entire body of one rat. The grid lines can be actually drawn on the floor of the apparatus (i.e., real lines) or, ideally, might be drawn on a transparent paper and placed on the monitor at the time of scoring (i.e., ideal lines).

It is important to clarify that both the total distance traveled and the number of crossing events besides being indicators of exploratory behavior are dependent on the motor activity; however, we excluded that the pure motor activity predicted the vulnerability to develop PTSD-like symptomatology, as the performance of trauma-exposed rats on a rotarod test (a test widely used to assess general motor activity and coordination in rodents) did not correlate with PTSD-related behavioral outcomes [26].

Social Interaction Test. The total number and/or total duration of the below listed social and nonsocial behaviors can be scored in this test.

Nonsocial behaviors:

1. Wall rearing, defined as the rat standing on its hind limbs against the wall.
2. Rearing, defined as the rat standing on its hind limbs not contacting the wall.
3. Crossing, defined as the rat crossing with all four paws, through ideal (or real) grid lines dividing the floor of the arena in identical squared sections.

Social behaviors:

1. Following, defined as one rat following the direction of the other.
2. Sniffing, defined as one rat sniffing the other in any part of the body.
3. Pouncing, defined as one rat nosing or rubbing the nape of the neck of the other, it is intended as an index of play solicitation.
4. Pinning, defined as the rotation of one rat to its dorsal surface after receiving a pounce from the other, it is intended as the response to play solicitation.
5. Boxing/wrestling, defined as both rats standing on the hind legs in front of each other moving the forepaws.
6. Crawling over and under, defined as one rat passing over or under the other.

The total social interaction time is obtained by summing together all the discrete durations of each social behavior in seconds.

Social behaviors can be scored manually by well-trained observers or through automated scoring systems. Manual scoring of social interaction behavior can be time-consuming and requires very well-trained observers; however, the usage of automated scoring systems is also challenging as the system needs to be programmed and calibrated appropriately for the task, to distinguish between two identical animals and different body parts. Moreover, most of the times, automated scoring systems for social interaction behaviors require a human intervention to label the data appropriately (e.g., to restore lose point position and animal identities).

An important consideration to be taken into account when scoring social interaction is that the behaviors of the two interacting animals are dependent upon each other; thus, in most cases the total score of the pair, as supposed to the individual score of each rat forming the pair, is used for statistical analyses.

3.3 Data Analyses: Screening of Susceptible and Resilient Rats

In the present protocol, the rats' exploratory activity in the open field test has been identified as the best predictor of vulnerability or resilience to develop PTSD-like cognitive and emotional symptoms in trauma-exposed rats, in terms of over-consolidation and excessive recall of the traumatic memory (assessed as the freezing at day 7), impaired extinction (assessed as the freezing at day 16), and reduced social interaction (assessed as the total social interaction time at day 19). This is based on our previously reported findings demonstrating strong negative correlations between the total distance traveled in the open field arena and the percentage of total freezing shown at day 7 and 16, while the total distance traveled was positively correlated with the social interaction time (*see* Table 1) [26].

Table 1
Summary table showing the relation between rats' post-trauma exploratory activity in an open field arena and subsequent indices of traumatic memory recall, extinction, and social interaction

Phenotype	Distance traveled in the open field	Freezing at day 7 (traumatic memory recall)	Freezing at day 16 (extinction recall)	Social interaction time
Susceptible	Short	High	High	Low
Resilient	Long	Low	Low	High

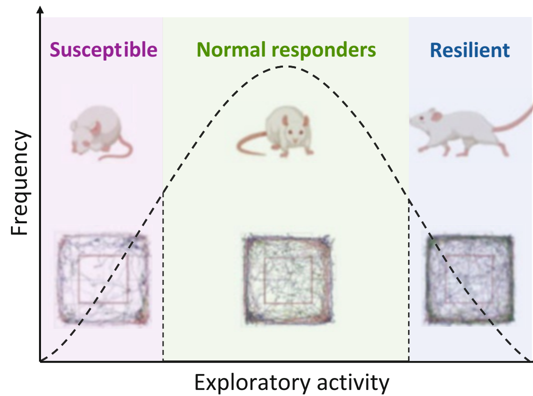

Fig. 2 Representative image indicating the screening procedure between the susceptible and resilient phenotypes. In an ideal Gaussian distribution of the exploratory activity in a novel environment of trauma-exposed rats, animals presenting values falling below the 25th percentile become susceptible, while rats presenting values distributed above the 75th percentile become resilient to subsequent development of PTSD-like behavioral alterations

Therefore, susceptible and resilient rats are identified according to the extremes (25th or 75th percentile) of the exploratory activity distribution. Rats scored below the 25th percentile are classified as susceptible, while rats positioned above the 75th percentile are considered resilient. Rats presenting exploratory activity values falling between the 25th and the 75th percentile are considered normal responders (*see* Fig. 2).

Once identified the three different phenotypes, rats can be assigned to the desired experimental groups, and statistical analyses can be performed as appropriate, depending on the experimental design.

Lastly, there are two important aspects that the experimenter needs to take into account when calculating sample size to ensure statistical power of the study: (i) considering the methodology of the screening used to select the different phenotypes, rats falling into the susceptible and resilient classification criteria will represent a small portion of the initial population (distributed below the 25th

or above the 75th percentiles), and (ii) for the social interaction test the sample size corresponds to the number of pairs (not the number of individual rats), as, in most cases, the two rats of the pair are scored together as a single experimental unit.

Taken together, the present experimental protocol, besides being versatile and very reliable [24–26], presents a number of advantages, as it allows to:

1. Model in animals both long-lasting cognitive and social alterations observed in PTSD. Excessive recall of the traumatic memory and impaired social interaction can be observed up to 56–58 days after trauma exposure in the above described model [25].
2. Identify a predictive variable (e.g., exploration of a novel environment after trauma) for early identification of susceptible or resilient phenotypes for the development of a long-lasting PTSD-like cognitive and emotional symptomatology.
3. Mimic behavioral exposure therapy in humans through the spaced re-exposure sessions run at days 7, 10, 13, and 16 after trauma.

4 Notes

1. To carry out behavioral experiments which do not rely on the light/dark conflict such as the elevated plus maze, the light dark box, and the step through inhibitory avoidance, it is important to provide uniform illumination to the experimental arenas and avoid the formation of shadows. Shaded areas might be perceived as safe spots where the animals would rest and hide from potential predators without exploring the testing arena or interacting with the conspecific.
2. To decrease the stress associated with the human manipulation and contact and to avoid extra olfactory stimuli which would affect (and increase the variability of) the obtained results, (i) the experimenter/s should be the same from the beginning to the end of the protocol; (ii) it is advisable for the experimenter/s to use a dedicated (and the same) lab coat throughout the protocol and to not wear perfume with intense scent; and (iii) routine cage, bedding, and water bottle changing procedures should be avoided in proximity of or the same experimental days.
3. To reproduce the chronicity of the PTSD-like symptomatology, it is of utmost importance to pair the trauma exposure with social isolation. We have previously shown that the exposure to a series of inescapable footshocks is able to induce in rats

a long-lasting memory trace for the traumatic event (up to 56 days after trauma), accompanied by enduring changes in social behavior (up to 58 days after trauma), only if paired with social isolation. When animals are pair housed, the social buffering operated is able to markedly attenuate the emotional dysfunction observed in the footshock-exposed animals, while keeping the trauma-related memory unaltered. Thus, the social isolation could be considered a precipitating factor leading to an enhanced susceptibility to adverse emotional outcomes after trauma exposure [25].

4. To minimize errors and variability within experimental groups, it is necessary to reduce weight differences among tested animals and to properly calibrate the experimental apparatuses before each experiment. Variations in footshock intensity, light conditions, etc. between apparatuses add additional variables that make it difficult to interpret the data.
5. When testing the animals' social interaction, it is advisable to fill the arena with approximately 2 cm of clean bedding since too much bedding would stimulate burrowing behavior and thus decrease social interaction.
6. When using automated scoring systems, it is recommended to video-record the rats throughout the experiment with a separate camera as a backup. In case the automated scoring fails, or the program crashes, the video files can be used to manually score the behaviors at a later time.

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