Journal of Neuroendocrinology, 2004, Vol. 16, 767–775 doi:10.1111/j.1365-2826.2004.01233.x

Behavioural and Neuroendocrine Adaptations to Repeated Stress during Puberty in Male Golden Hamsters

J. C. Wommack,* A. Salinas,* R. H. Melloni Jr,† and Y. Delville*
*Psychology Department and Institute for Neuroscience, The University of Texas at Austin, Austin, TX, USA.
†Psychology Department and Program in Behavioral Neuroscience, Northeastern University, Boston, MA, USA.

Key words: plasticity, cortisol, conditioned fear, chronic stress, learned helplessness.

Abstract

In adult animals, the consequences of stress are often severe and long lasting. Repeated subjugation in adult male golden hamsters inhibits aggression and increases submissive and avoidant behaviours. By contrast, subjugation during puberty enhances offensive aggression. The goals of this study were to characterize behavioural and neuroendocrine responses of naïve and repeatedly subjugated juveniles to social defeat and to assess potential recovery from social stress. From the onset of puberty on postnatal day 28 (P28) to mid puberty (P42), animals were either socially subjugated or placed in a clean and empty cage for 20 min daily. The subjugated and control groups were further divided into subgroups and sacrificed under basal conditions or after social defeat on P28, P35 (early puberty), P45 (mid puberty) and P70 (early adulthood). On P35 and P45, repeatedly subjugated juveniles showed a complete inhibition of olfactory investigation (i.e. risk assessment) towards aggressive adults. Repeatedly subjugated animals also had lower postdefeat cortisol levels than controls on P45. Interestingly, basal cortisol levels increased gradually during puberty but did not differ between treatment groups at any point. Repeated subjugation was also associated with increased tyrosine hydroxylase immunoreactivity (ir-TH) within the extended medial amygdala. After a 4-week recovery period, none of these variables differed between subjugated and control groups. In an additional experiment, subjugated adults also had increased ir-TH in the medial extended amygdala, suggesting that these neurones are particularly responsive to social stress. In conclusion, puberty may be a developmental period characterized by behavioural and neuroendocrine plasticity in stress responsiveness. Furthermore, peri-pubertal changes in stress hormones may explain why juvenile hamsters are more resilient to social stress than adults.

In adult animals, the biobehavioural consequences of repeated social stress are often severe and long lasting. These effects include immunosuppression, increased anxiety, submissive behaviour and enhanced glucocorticoid synthesis (1). In adult male golden hamsters, repeated social subjugation causes a complete inhibition of offensive aggression, along with increased submission and avoidance, and enhanced plasma cortisol levels (2–6). These effects last for more than 1 month after subjugation (4) and may be sustained for the duration of the individual's life.

Interestingly, the consequences of repeated social stress during puberty are strikingly different. For example, subjugated juveniles shown enhanced aggression towards smaller and younger intruders (7, 8). Juveniles also differ from adults in that they do not show increased baseline plasma cortisol concentrations following repeated defeat (3, 9). These comparisons raise the possibility that behavioural and hormonal responses to social stress also undergo developmental changes during puberty. They also suggest developmental plasticity in behavioural and neuroendocrine stress responsiveness.

Repeated social stress profoundly affects areas in the brain (1). Recently, we reported that repeated subjugation during puberty causes a site-specific increase in the number of dopamine neurones within the posterior portion of the medial division of the bed nucleus of the stria terminalis (BST) and the posterodorsal part of the medial amygdala (MeA) (10). These areas are both highly responsive to stress, show stress-induced plasticity and are involved in anxiety behaviours (11–14). Consequently, it is likely that increased activity within these dopamine neurones correlates with the behavioural effects of social subjugation in hamsters.

The goal of this study was to characterize behavioural and neuroendocrine responses to social defeat in naïve (control) and experienced (subjugated) juvenile male hamsters. The

Correspondence to: Joel C. Wommack, Psychology Department, 1 University Station, A8000, The University of Texas at Austin, Austin, TX 78712, USA (e-mail: joelwommack@aol.com).

responses of individuals were compared between control and repeatedly subjugated animals on separate days across peripubertal development. To understand better the long-term effects of repeated subjugation, behavioural responses to social defeat were also observed after a 4-week recovery period. The behavioural responses included observations of defensive, submissive, avoidant and risk assessment behaviour. Neuroendocrine measures included assays of plasma levels of cortisol and labelling tyrosine hydroxylase immunoreactive (ir-TH) neurones in the BST and MeA.

Materials and methods

Animals and treatment

The animals (male golden hamsters) were bred in the laboratory from a colony that originated from Harlan Sprague Dawley (Indianapolis, IN, USA). Approximately 5 days after birth, all litters were culled to six pups including males and females. On postnatal day 25 (P25), all animals were weaned and individually housed in plexiglass cages ($20 \times 33 \times 13$ cm). Within 2 days of weaning, each animal was briefly (a few seconds) observed in the presence of an adult intruder. Individuals that immediately fled from the adult were considered to be inherently fearful (approximately one in 12) and were removed from the experiment. All animals received food and water *ad libitum* and were housed under a reversed 14 : 10 h light/dark cycle (lights off at 09.00 h).

Experimental design

On P28, animals were separated into two groups (control and subjugated). Social subjugation was performed according to a previously described protocol (7–10). Daily subjugation started on P28, which coincides with the onset of puberty (15), and ended on P42, near mid puberty. This period was approximately equivalent to the first half of puberty (16). Animals in the subjugated group were placed in the home cage of an aggressive adult for 20 min daily while controls were placed in a clean and empty cage for the same period. On the first day, either exposure to an aggressive adult or exposure to a novel cage comprises a stressor capable of increasing serum glucorticoids (9, 17). While juvenile hamsters habituate to daily exposure to a novel cage, social defeat is a stressor that causes an increase in cortisol, even after 14 consecutive days of exposure (9). As such, group comparisons were made between control animals exposed to a stressor versus the habituated and subjugated animals exposed to a social stressor to which they did not habituate. Subjugated juveniles were cycled through a group of aggressive adults ($n = 8$) for each subjugation day as previously described (7, 8). Before the experiment, adults were tested for offensive aggression by the resident–intruder paradigm (18). Smaller and younger intruders were placed in the home cage of adult hamsters. Adults that did not attack the intruder during screening were not used for in the experiment. During repeated subjugation, animals were observed daily while in the home cage of an adult to ensure they were chased and attacked. Daily subjugation and behavioural tests were performed during the second half of the dark phase.

At the onset of puberty, on P28, a group of animals that had not been assigned to either the control group or the subjugated group were sacrificed under basal conditions or immediately after 20 min in the home cage of an aggressive adult. These animals were tested on this day to establish a developmental trajectory. On P35 (early puberty), P45 (mid puberty) or P70 (early adulthood), both control and subjugated groups were further divided into subgroups: animals that were sacrificed under basal conditions ($n = 8$ per group) or after social defeat ($n = 9$–16 per group) (Table 1). These test dates were chosen to establish a developmental trajectory and to understand the time course of experimental effects. Each encounter was recorded with a Sony digital video camera for later review with iMovie software (Apple Computer Inc., Cupertino, CA, USA). The animals were sacrificed by rapid decapitation. Testes weights were recorded, trunk blood was collected and brains were extracted for each animal. As such, only a single blood sample was taken from each individual. This strategy was considered best for this experiment because multiple bleeds may have been a confounding stressor. In an additional experiment, adult male hamsters (born and raised in the laboratory) were separated into control and subjugated groups ($n = 6$ for each group) on P70.

Table 1. Sampling Days.

	Test days			
Subjects	P28	P35	P45	P70
Control[a]	Baseline[c] Postdefeat[d]	Baseline Postdefeat	Baseline Postdefeat	Baseline Postdefeat
Subjugated[b]		Baseline Postdefeat	Baseline Postdefeat	Baseline Postdefeat
	Early puberty[e]		Mid puberty[e]	Adulthood[e]

[a]Control animals were placed in a clean and empty cage for 20 min a day from postnatal day 28 (P28) to P42. [b]Subjugated animals were placed in the home cage of an aggressive adult for 20 min a day from P28 to P42. [c]Animals from both the control and subjugated groups were sacrificed under basal conditions. [d]Animals from control and subjugated groups were sacrificed after a 20 min in the home cage of an aggressive adult. [e]The postnatal days corresponding to early puberty, mid puberty and adulthood were determined by testosterone levels, testes weights and existing literature (15).

This period corresponds to early adulthood (15, 16). Experimental animals were subjugated (as previously described) daily between P70 and P84. On P85, both subjugated and control animals were sacrificed by rapid decapitation under basal conditions. For each individual, testes weights were recorded, trunk blood was collected, and brains were extracted.

Behavioural response to social defeat

During social conflict, golden hamsters display a number of stereotypical behaviours in response to an aggressor (19). These responses can be either defensive, submissive, avoidant or risk assessment. During 20-min exposures to unfamiliar aggressive adults, the responses for each individual in these behavioural categories were observed. (i) Defensive responses consisted of upright defences and were scored when the subjects stood upon their hind legs and orientated their face and forelimbs toward the resident to deflect attacks. (ii) Submissive responses consisted of tail-up and on-back submissive displays. Tail-ups and on-backs were scored as submissive responses because they did not involve active defence of attacks from the adults. During tail-ups, the hamsters maintained a lordosis combined with a raised tail while either moving slowly or standing still. By contrast, on-backs were scored when the juveniles remained motionless and supine (from seconds to minutes at a time) without using its forelimbs to defend from the attacks of the adults. (iii) Avoidant behaviour consisted of retreats. Retreats were scored when the juveniles retreated or fled from the adults. (iv) Risk assessment consisted of olfactory investigations and were recorded when the subjects actively approached and stretched their neck so that their snout made contact with the adults. This type of behaviour has previously been observed in defeated adult hamsters (6). Similar forms of risk assessment behaviour have also been observed in mice (20).

Additionally, we recorded the number of attacks received by individuals during the exposure to the adult. All behaviours were scored in terms of frequency (number/20-min period). For on-backs and olfactory investigations, durations (s/20-min period) were also recorded.

Cortisol and testosterone assays

Plasma cortisol levels were assayed in control and experimental animals in samples collected under basal conditions (baseline) or immediately following (postdefeat) social defeat on P28, P35, P45 or P70. Plasma testosterone levels were only assayed in samples collected under basal conditions. Subgroups of subjugated and control animals were sacrificed by rapid decapitation upon removal from their home cage or immediately following a 20-min period of social defeat ($n = 8$–16 per group). After decapitation trunk blood samples were collected and centrifuged at 5000 r.p.m. for 5 min. Plasma was saved at −20 °C. All cortisol assays were performed with Cortisol Correlate-EIA^{TM} kits (Assay Designs, Inc., Ann Arbor, MI, USA). Testosterone assays were performed with Testosterone Correlate-EIA^{TM} kits (Assay Designs). Samples were assayed in duplicate from 10-μl aliquots. Intraassay variability was 8.8% and interassay variability was 13.6%. For testosterone, intra-assay variability was 5.2%, and interassay variability was 10.0%. Plasma levels of testosterone

and cortisol were expressed as ng/ml. In addition, the percent increase over baseline was calculated to assess cortisol responses to social defeat. Each postdefeat cortisol level was divided by the average baseline concentration for respective group (subjugated or control) and postnatal day. For example, an individual cortisol level from a member of the control group defeated on P45 would be divided by the average baseline cortisol level of the P45 control group, then multiplied by 100.

Tyrosine hydroxylase immunocytochemistry

During sacrifice, brains were collected and fixed by overnight immersion in 10% acrolein in 0.1 M KPBS buffer (pH 7.2) at 4 °C and later saved in 20% sucrose/KPBS. The brains were then sectioned into 40-μm thick coronal sections with a freezing rotatory microtome and were stored in a cryoprotectant (21) at −20 °C until labelled by immunocytochemistry. Brains sections from animals sacrificed before subjugation were used for this procedure. Immunocytochemistry for TH was performed as previously described (10). Briefly, free-floating sections were pretreated in 1% sodium borohydrite (to remove residual aldehydes) followed by a preincubation in a solution containing 20% normal goat serum, 1% hydrogen peroxide and 0.3% Triton X-100 (respectively, to block nonspecific labelling, eliminate endogenous peroxidase activity, and permeabilize the tissue). Sections were then incubated in a mouse monoclonal antibody to TH (1 : 20 000; Sigma Chemical Co., St Louis, MO, USA), containing 2% normal goat serum and 0.3% Triton X-100 for 48 h at 4 °C. After washing, the sections were incubated in the secondary antibody (biotinylated goat antimouse IgG; 7.5 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by a tertiary incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Between incubations, sections were washed in 0.05 M TBS (pH 7.6). Finally, the sections were reacted with with diaminobenzidine (DAB, 0.5 mg/ml) and 0.05% hydrogen peroxide. This procedure is optimized for labelling of ir-TH neurones, dendrites and axons throughout the brain (10). We have previously labelled alternative sections by immunocytochemistry for dopamine β-hydroxylase, and found no cell bodies within the BST or MeA (10). Therefore, the neurones located within these nuclei are most likely dopaminergic, and not noradrenergic. Labelled sections were mounted on gel-coated slides, dehydrated in a series of alcohols, and coverslipped with permount. Later, TH-immunoreactivity (ir-TH) was observed with a Nikon Eclipse E600 microscope. Immunoreactive tyrosine hydroxylase neurones were counted with a camera lucida attachment.

The areas selected for cell counts were BST, MeA and the periventricular hypothalamic nucleus at the level of the optic chiasma (10, 22). These areas are interesting for a number or reasons. First, the BST and MeA are both involved in a number of social behaviours, including aggression (23–27). Second, these specific neurones are testosterone-dependent (28, 29) and may be sensitive to the physiological changes associated with puberty (30). These areas are also highly responsive to stress (11–14). The periventricular hypothalamic nucleus, an area rich in gonadal steroid receptors (31–33), was analysed as an additional control area to determine whether the changes in ir-TH were site-specific. Six to 12 cell counts were taken bilaterally from consecutive sections for each area analysed and each individual. Immunoreactive cells were counted only when their nucleus was clearly visible. The average number of ir-TH neurones per count and area was calculated for each individual.

Data analysis

Parametric data (e.g. duration of behaviours, hormone levels, percent increases in hormone levels, average cell counts) were compared between groups over time by two-way analysis of variance (independent variables: treatment groups and age). In addition, group comparisons were performed for each day with the Student's t-test (two-tailed). Nonparametric data (behaviour frequencies) were compared separately with the Mann–Whitney test (two-tailed) for group comparisons, and with the Kruskal–Wallis test followed by the Mann–Whitney test for age comparisons (two-tailed).

Results

Behavioural responses to social defeat

Social subjugation during puberty did not alter defensive or submissive responses to social defeat. We observed neither age-related nor subjugation-induced changes in the frequency of upright defences, tail-up and on-back submissive displays (Fig. 1). Similarly, there was no statistically significant difference over time or between subjugated or control groups for the analysis of on-back durations displayed during social defeat. Subjugated animals appeared to have a slightly higher frequency of on back displays on P35 and longer duration of on-backs on P35 and P45, but these differences were not statistically significant.

Avoidance behaviour increased significantly during puberty, but this change was paralleled by increases in the number of attacks received. The frequency of retreats increased significantly from P28 to P70 in both subjugated and control hamsters [respectively, $H(3)14.7$, $P < 0.01$, Kruskal–Wallis; $H(3)15.5$, $P < 0.01$]. However, there was no statistically significant difference between these subjugated and control groups on any test day. In addition, the number of attacks received by the animals increased significantly for both subjugated and controls groups from P28 to P70 [respectively, $H(3)26.5$, $P < 0.001$; $H(3)19.9$, $P < 0.001$]. Similar to the number of retreats, the number of attacks received did not differ significantly between groups on any test day.

Risk assessment behaviour was completely inhibited in subjugated juveniles during the period of daily subjugation (Fig. 2). Compared to control animals, subjugated animals performed olfactory investigations less frequently (Mann–Whitney: $U = 8$, $U' = 92$, $P < 0.01$; $U = 16$, $U' = 74$, $P < 0.05$, respectively) and for a shorter duration [Student's t-test: $t(18) = 4.03$, $P < 0.001$; $t(18) = -2.43$, $P < 0.05$, respectively] when tested on P35 and P45. This effect was not long lasting. On P70, 4 weeks after the end of daily subjugation, no statistically significant difference in olfactory investigation frequency ($U = 73$, $U' = 107$, $P > 0.05$) or duration [$t(23) = -0.39$, $P > 0.1$] was observed between groups.

Cortisol

Plasma cortisol concentrations were analysed as baseline levels, postdefeat levels, and percent increase over baseline (Fig. 3). Baseline cortisol levels increased gradually during puberty from P28 to P70 in both groups [$F(3,58) = 8.1$, $P < 0.001$]. This developmental increase was not significantly affected by daily social subjugation [$F(1,58) = 0.6$, $P > 0.1$] nor was there a group–day interaction [$F(3,58) = 1.0$, $P > 0.1$]. Postdefeat cortisol levels also increased during puberty in both groups [$F(3,74) = 32.2$, $P < 0.001$]. Interestingly, repeated subjugation from P28 to P42 resulted in a statistically significant group difference for postdefeat cortisol levels [$F(1,74) = 7.4$, $P < 0.01$] and a statistically significant group–day interaction [$F(3,74) = 4.3$, $P < 0.01$]. On P45, subjugated animals showed postdefeat cortisol levels that were increased two-fold from baseline while control animals showed a six-fold increase from baseline. The group difference in postdefeat cortisol levels was statistically significant on this day [$t(18) = 3.25$, $P < 0.01$]. A group difference in postdefeat cortisol levels was not observed on any other test day. Percent increase over baseline also

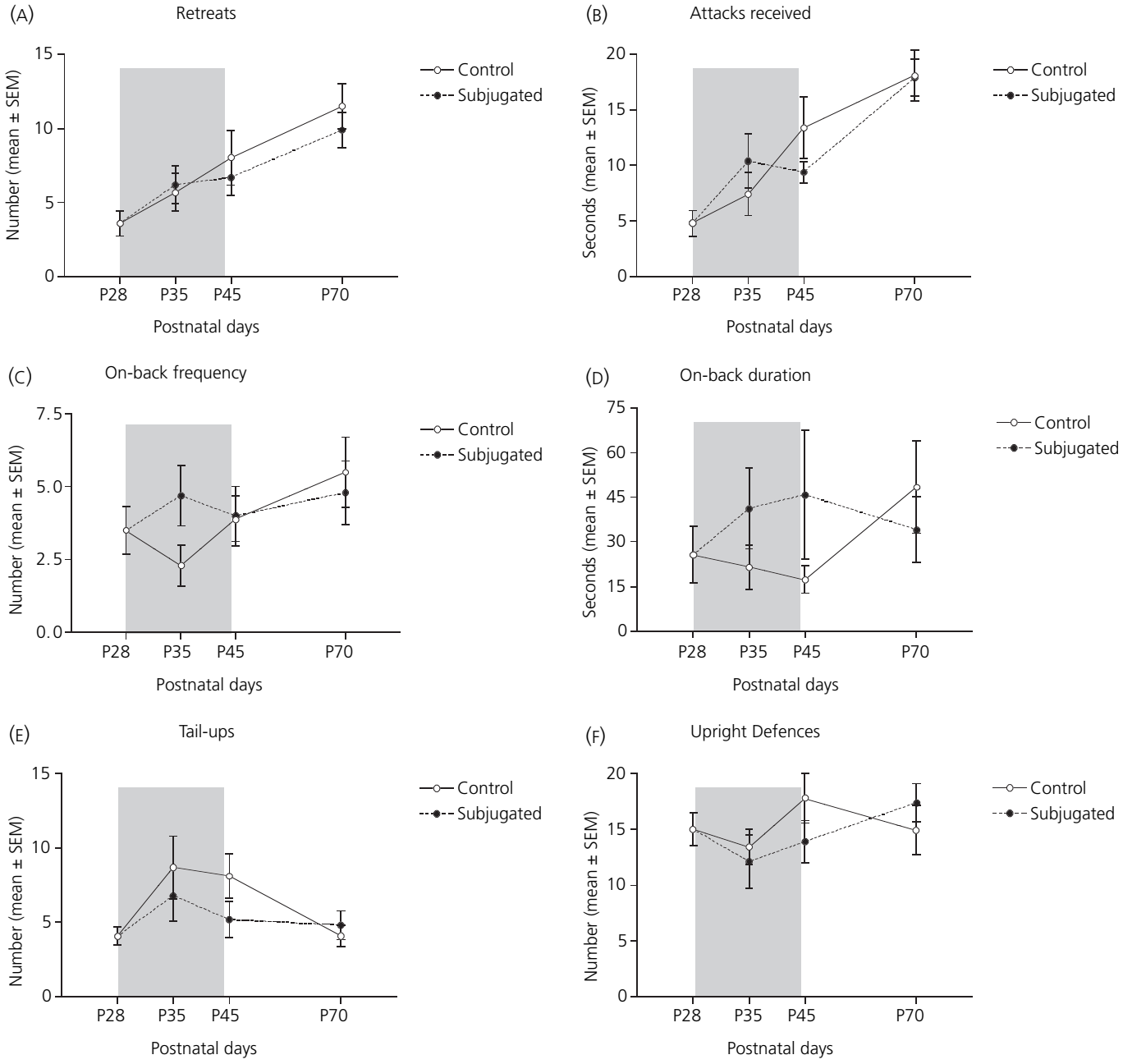

FIG. 1. Retreats (A), attacks received (B), number (C) and duration (D) of on-back submissive postures, tail-ups (E) and upright defences (F) as observed in subjugated and control animals during a 20-min exposure to an aggressive adult. The shaded area represents the period of daily subjugation.

changed significantly during puberty [$F(3,74) = 5.8$, $P < 0.01$]. Significant interactions for group and group × day were also observed [$F(1,74) = 11.4$, $P < 0.01$; $F(3,74) = 5.8$, $P < 0.01$, respectively]. On P28 and P35, subjugated and control animals each showed approximately a 250% increase from baseline following defeat. Throughout puberty, percent over baseline remained approximately 250% in subjugated animals. By contrast, control animals showed nearly a 700% increase on P45 followed by a 350% increase on P70. The group differences on P45 and P70 were statistically significant [$t(18) = 3.7$, $P < 0.01$; $t(24) = 4.8$, $P < 0.001$, respectively].

Testosterone and testes and body weights

During puberty, testosterone levels increased gradually in both control and subjugated hamsters [$F(3,58) = 21.5$, $P < 0.001$] (Fig. 4). However, subjugated juveniles had lower levels of plasma testosterone than controls on P45 [$t(14) = 3.6$, $P < 0.01$]. This effect did not last. On P70, plasma testosterone levels did not differ significantly between previously subjugated and control animals [$t(14) = 1.1$, $P > 0.1$]. In addition, testes and body weights were also recorded and compared between groups in this study. Testes grew from 0.51 ± 0.1 g on P28 to 4.0 ± 0.2 g and

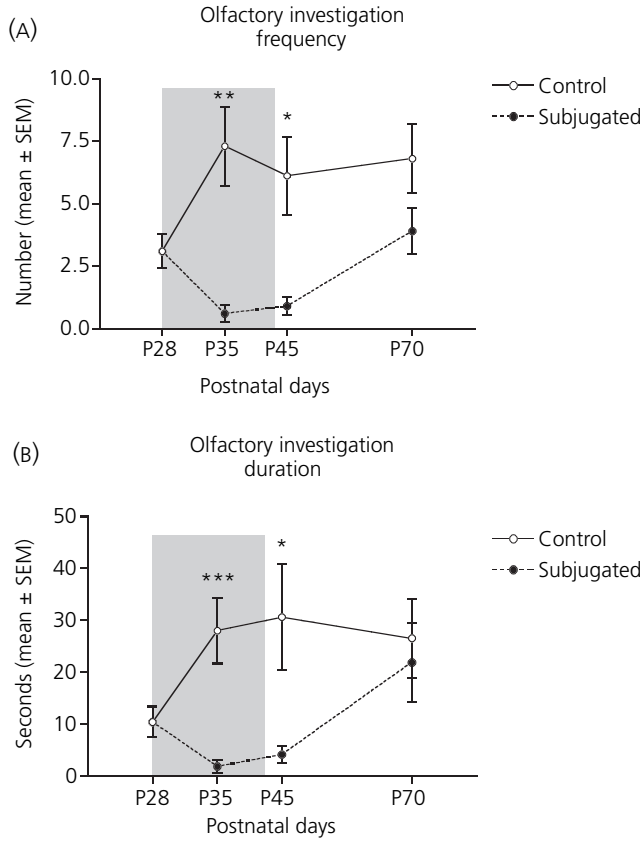

FIG. 2. Frequency (A) and duration (B) of olfactory investigations as observed in subjugated and control animals during a 20-min exposure to an aggressive adult. The shaded area represents the period of daily subjugation (Mann–Whitney or Student's t-test, two-tailed, *P < 0.05, **P < 0.01, ***P < 0.001).

3.8 ± 0.4 g (in control and subjugated animals, respectively) on P70. During that time, the animals grew from 49.0 ± 2.8 g on P28 to 109.5 ± 11.1 g and 104.2 ± 11.9 g (in control and subjugated animals, respectively) on P70. Neither measure was statistically different between groups at any point during the experiment.

Tyrosine hydroxylase

The analysis of ir-TH neurones showed increased number of labelled cells within specific areas in experimental animals starting 1 week after the onset of social subjugation (Fig. 5). In the BST, the number of ir-TH cells changed significantly during puberty [$F(3,40) = 8.2$, $P < 0.001$] decreasing from P28 to P70. The difference between groups was also statistically significant [$F(1, 40) = 30.5$, $P < 0.001$], so was the interaction between groups and age [$F(3,40) = 6.7$, $P < 0.001$]. Repeatedly subjugated animals had more ir-TH neurones than controls on P35 and P45 [$t(10) = 5.29$, $P < 0.001$; $t(10) = 3.39$, $P < 0.01$, respectively]. No group difference was observed on P70.

We also observed an increase in the number of ir-TH neurones within the MeA in subjugated animals during (P35) or just after (P45) the period of daily subjugation. The number of ir-TH neurones within this area decreased during

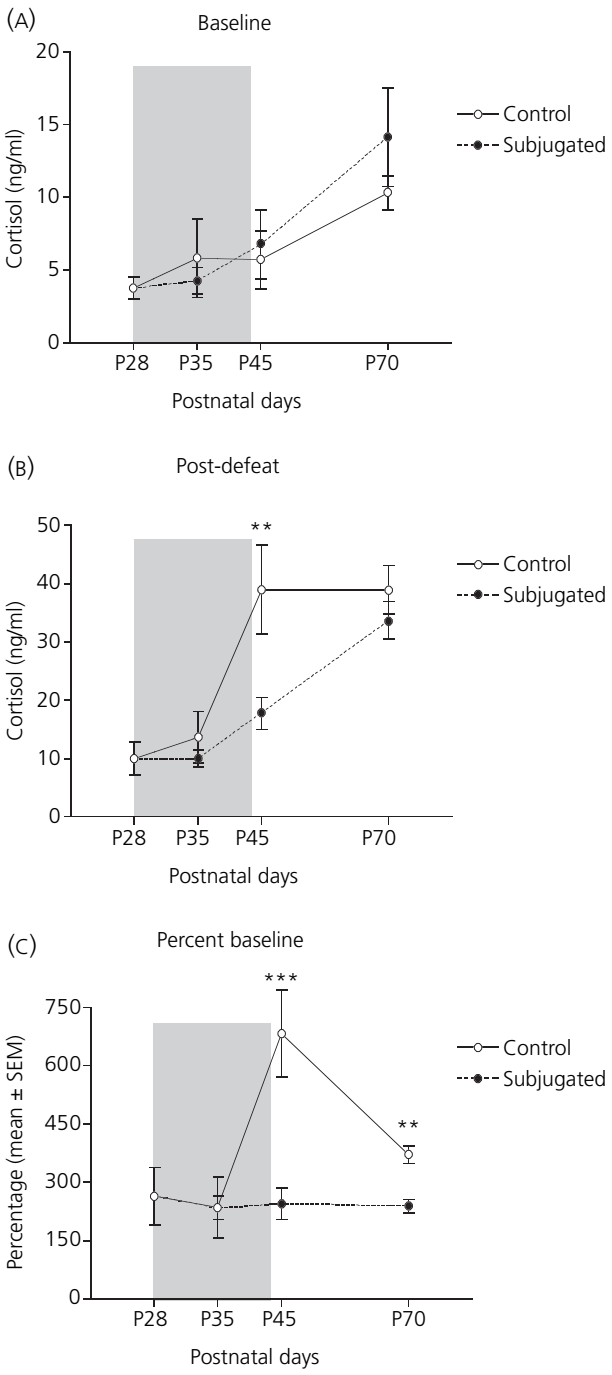

FIG. 3. Plasma cortisol concentrations assayed in subjugated and control groups under basal conditions (A) following defeat (B) and expressed as percent increase over baseline (C). The shaded area represents the period of daily subjugation (Student's t-test, two-tailed, *P < 0.05, **P < 0.01, ***P < 0.001).

puberty from P28 to P70 [$F(3,40) = 45.4$, $P < 0.001$]. A statistically significant group difference [$F(1,40) = 24.1$, $P < 0.001$] and a group–age interaction [$F(3,40) = 7.6$, $P < 0.001$] were also observed in this area. On P35 and P45, subjugated animals had higher numbers of ir-TH neurones [$t(10) = -3.37$, $P < 0.01$; $t(10) = -5.76$, $P < 0.001$, respectively]. However, subjugated and control animals had similar numbers of ir-TH neurones by P70.

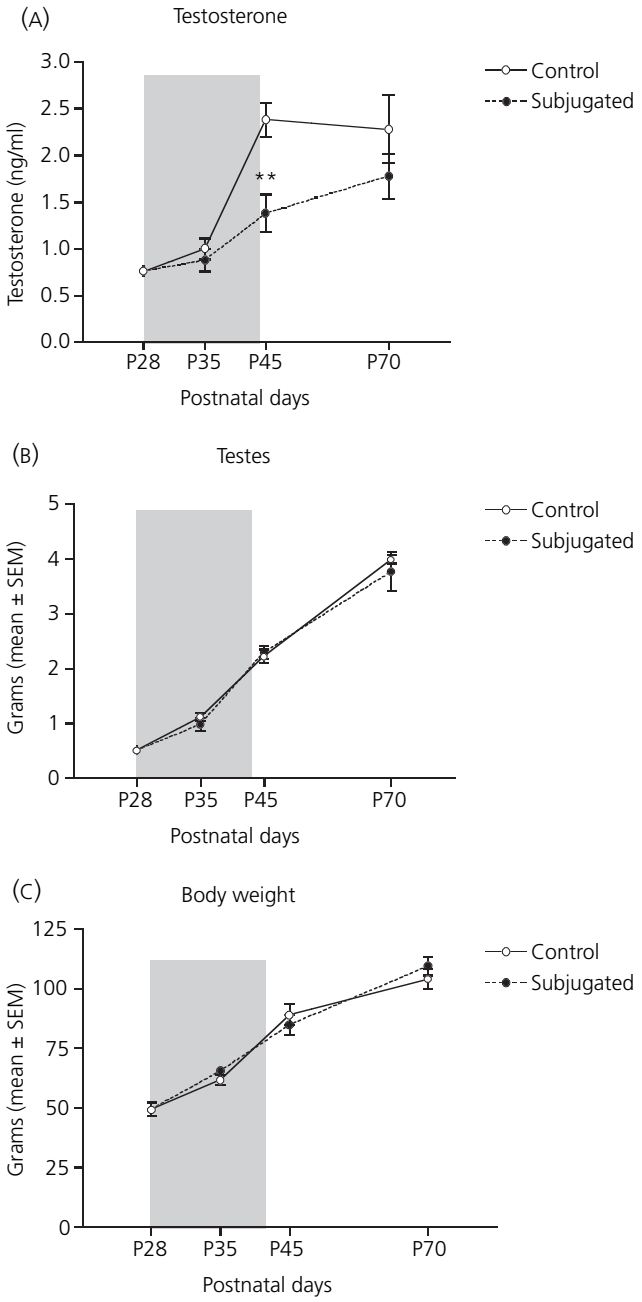

FIG. 4. Plasma testosterone concentrations (A), testes weights (B) and body weights (C). Testosterone was assayed in samples collected under basal conditions. Shaded area represents the period of daily subjugation. The shaded area represents the period of daily subjugation (Student's t-test, two-tailed, $^{**}P < 0.01$).

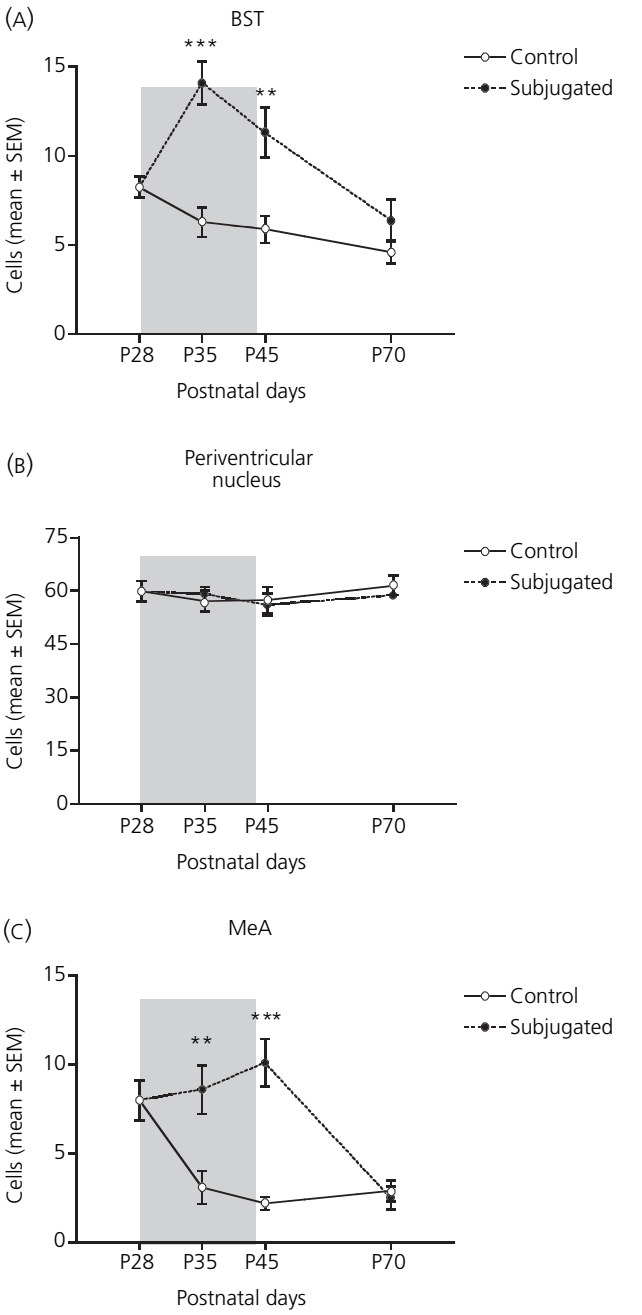

FIG. 5. Comparison of tyrosine hydroxylase immunoreactivity (ir-TH) between subjugated and control groups. Cell counts are expressed as average cell number per count per area. The shaded area represents the period of daily subjugation. BST, Posterior part of the medial division of the BST; MeA: posterodorsal part of the medial amydaloid nucleus (Student's t-test, two-tailed, $^{**}P < 0.01$, $^{***}P < 0.001$).

Within the periventricular hypothalamic nucleus, ir-TH did not change during puberty nor was affected by repeated subjugation.

Adult subjugation

Subjugated adults showed two-fold increases in ir-TH neurones within the BST [$t(10) = 3.8$, $P < 0.01$] and in the medial amygdala [$t(10) = 4.1$, $P < 0.01$] (Fig. 6). Importantly, subjugation during adulthood, did not affect body weights or testes weights.

Discussion

The current study reports several key findings on the behavioural and neuroendocrine adaptations to repeated social stress during puberty. First, repeated subjugation during puberty did not affect defensive, submissive or

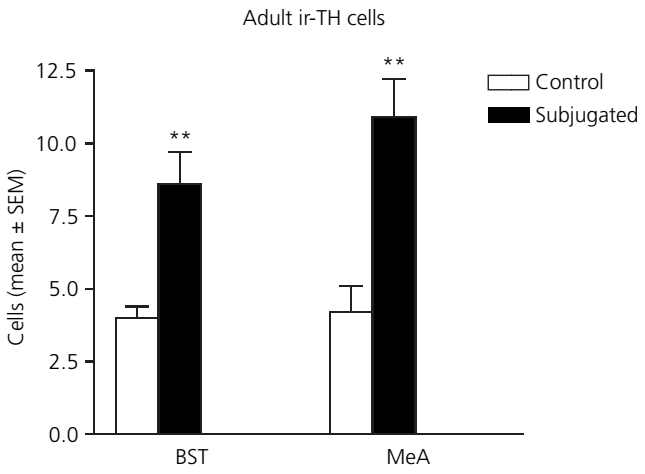

FIG. 6. Comparison of tyrosine hyrdoxylase immunoreactivity (ir-TH) between subjugated adults and controls. Cell counts expressed as average cell number per count per area. The shaded area represents the period of daily subjugation (Student's t-test, two-tailed, **$P < 0.01$).

avoidance behaviour; however, risk assessment behaviour (olfactory investigation) was completely inhibited in subjugated juveniles. Second, both baseline and postdefeat cortisol levels increased during puberty in control animals, with postdefeat levels peaking at approximately P45, suggesting a change in the activity of the hypothalamic–pituitary–adrenal (HPA) axis at this time. Daily subjugation did not alter the pubertal increase in basal cortisol but did result in blunted postdefeat levels on P45. Subjugated animals also had lower testosterone levels on this day. Next, repeated social subjugation was associated with enhanced ir-TH within the BST and MeA. Finally, neither the behavioural nor neuroendocrine effects of repeated social subjugation during puberty were long lasting. Four weeks after the end of the period of repeated social subjugation, the group differences in risk assessment behaviours, hormone levels and ir-TH were no longer statistically significant. These data are important for two main reasons. First, they contrast with previous studies of social stress in adult rodents, including hamsters (1, 4), and reinforce the hypothesis of differential stress responsiveness across puberty (16). Second, they show that puberty is a critical period for behavioural and neuroendocrine plasticity. Pubertal plasticity in the physiological mechanisms responding to and/or controlling stress responsiveness allows juveniles to overcome the consequences of social defeat, whereas adults remain socially subordinate after a similar experience.

As we have proposed previously, the resiliency that juveniles are capable of showing toward social stress may be ecologically relevant (9). In areas of high population density, the species would benefit if younger generations were not as susceptible to the negative effects of social stress. The lack of negative consequence from social defeat could help to ensure that juveniles are capable of becoming dominant upon reaching adulthood. This idea is supported by the current study in which subjugated juveniles showed no long-term behavioural inhibitions or neuroendocrine adaptations.

Daily subjugation did not produce any clear difference in defensive or submissive responses to social defeat, nor did these behaviours appear to undergo developmental transitions during puberty. Repeatedly subjugated animals showed a higher frequency and duration of on-back submissive responses during the defeat tests on P35 and P45. However, these differences were not statistically significant, possibly due to a high variability in subjugated animals. Previously reported individual differences in on-back responses may account for this high variability (9). By contrast, social subjugation during adulthood leads to a significant increase in on-back submissive responses in hamsters (4, 6). These observations suggest a difference in behavioural responses to social stress during peripubertal development.

In addition, social subjugation did not affect avoidance behaviours (i.e. retreats), although they did increase during puberty. Interestingly, this developmental increase corresponded with an increased number of attacks received from the adults. As such, this developmental trend may not solely be attributable to the juveniles, but rather to increased responsiveness of the adult residents towards larger juvenile intruders.

During the period of daily subjugation, repeatedly subjugated individuals did not engage in risk assessment (i.e. olfactory investigation) during the defeat tests. These findings are consistent with reports of decreased olfactory investigation in repeatedly subjugated adult hamsters (4, 6). Similarly, defeated adult hamsters also show a learned avoidance to the odour of a familiar winner (34). Importantly, subjugated animals in this study were not paired with familiar conspecifics during daily subjugation or on tests days to avoid a confounding effect related to this previous report. This decreased risk assessment behaviour in socially subjugated hamsters may be comparable to learned helplessness in rats. Learned helpless rats fail to escape from electrical shocks after receiving inescapable and unpredictable shocks (35). Similar to learned helpless rats, subjugated hamsters show a poststress behavioural depression when faced with an aversive stimulus. Interestingly, on P70, 4 weeks after the period of daily subjugation, previously subjugated hamsters once again engaged in normal levels of olfactory investigation. By contrast, the behavioural effects of social defeat last more than 1 month in adult hamsters (4), and similar long-term effects have been reported in fear conditioning studies in adult rats (36, 37). Therefore, the current findings show that the consequences of repeated social stress and the capacity to recover from this type of experience changes during peripubertal development.

Social subjugation during puberty also decreased adrenocortical responsiveness to further subjugation without affecting baseline cortisol levels. The lack of effect on basal cortisol levels is consistent with a recent report in golden hamsters (9). This observation contrasts with previous reports in adult hamsters and rats (1, 2). On P45, control animals had higher postdefeat cortisol levels that subjugated animals. Controls also showed significantly higher increases from baseline than subjugated animals on P45 and P70. This decreased responsiveness following defeat in the repeatedly subjugated juveniles possibly suggests a partial habituation to social stress. However, it remains unclear whether this effect is due to a stimulus-specific adaptation or a general decrease in stress responsiveness. The latter explanation appears unlikely because adult male rats exposed to repeated social stress

show facilitated HPA responsiveness when later exposed to a restraint stressor (38). Future studies will be required to fully address this question.

Analysis of baseline plasma cortisol concentrations produced an unexpected outcome. Plasma levels of cortisol both before and after stress were up to three-fold lower on P28 compared to P70. Interestingly, pubertal increases in cortisol have also been reported in human adolescents (39–41). The observed increases in stress hormones suggest that puberty is not limited to an activation of the hypothalamic–pituitary–gonadal axis, but also includes an activation of the HPA axis. Although the testosterone levels raised most dramatically around P45, so did cortisol levels. The present data also show enhanced cortisol response to stress on P45 in control animals compared to the other days. Obviously, the HPA axis changes around mid-puberty. The cause of this enhanced responsiveness is still unclear; however, it is important to note that a similar observation has been recently reported in rats (42). In rats, mid-puberty is also associated with enhanced responsiveness of the HPA axis to stress compared to adulthood (42).

Previous reports have stated that repeated social subjugation during puberty causes site-specific increases in ir-TH within the BST and the MeA, which were also confirmed in this study (10). Repeatedly subjugated animals showed an increased number of ir-TH neurones within the BST and MeA on P35 and P45. From our perspective, the effect was caused by an increase in ir-TH neurones in the BST from prepubertal levels, while at the same time being associated with a maintenance of previous levels in the MeA. These changes are likely to reflect increased TH expression and neuronal activity in stressed individuals. Chronic stress is known to increase TH mRNA expression (43–45) and enhance dopamine release in the brain (46). However, in the present study, the stress-induced changes in ir-TH were site-specific. Within the periventricular hypothalamic nucleus, an area rich in gonadal steroid receptors, ir-TH was not affected by repeated subjugation or puberty (10, 31–33). The enhancement of ir-TH within the BST and MeA in subjugated animals is reversible. By P70, control and subjugated animals had equal numbers of ir-TH neurones within both areas. In addition, the observation of enhanced ir-TH within the same areas in subjugated adult shows that this effect is not age-specific, but we have yet to determine whether ir-TH in subjugated adults and juveniles undergoes similar decreases during the recovery period. Nevertheless, our data show that increased ir-TH within the BST and MeA is a reliable neurobiological marker of repeated exposure to social stress. This possibility is supported by previous observations. The BST and MeA show enhanced neuronal activity after both acute and chronic exposure to social stress in hamsters and rats (11, 12) and undergo neurobiological changes following fear conditioning (14). In addition, acute restraint stress enhances neuronal remodelling within the MeA in association with anxiety-induced behaviours (13).

Our data support an association between the MeA and stress and indicate a role for ir-TH neurones within the BST and MeA in the expression of anxiety-induced behaviours. We originally hypothesized that increased ir-TH in the BST and MeA of subjugated juvenile hamsters is associated with the enhanced aggression specifically observed in these animals. In view of the present data, this hypothesis has to be rejected. Immunoreactive TH decreases over time in control animals and, as such, the transient increase in ir-TH within the BST and MeA of subjugated animals does not correspond to a developmental acceleration. Additionally, ir-TH was similar between groups in early adulthood, although subjugated animals were more aggressive than controls at that time (8). Together, these data fail to support the hypothesis that ir-TH within the medial and extended amygdala is related to group differences in aggressive behaviour. Instead, the changes in ir-TH expression within the BST and MeA in subjugated animals was perfectly correlated with the inhibition of risk assessment behaviour. Accordingly, ir-TH could be critical for the expression of fear conditioning and learned helplessness in repeatedly subjugated hamsters.

Social subjugation caused a transient decrease in plasma testosterone levels, while body and testes weights did not differ between subjugated and control animals. Testosterone levels increased from P28 to P70 in subjugated and control animals. However, repeatedly subjugated juveniles showed lower testosterone levels than controls on P45, but this difference did not last until P70. Similar decreases in plasma testosterone have been reported in adult males exposed to stress (1, 2).

In summary, repeatedly subjugated juveniles show a complete inhibition of olfactory investigation during further encounters with aggressive adults. This learned avoidance coincides with a blunted cortisol response to social defeat and increased ir-TH within the BST and MeA. In addition, these behavioural and neuroendocrine adaptations to social stress during puberty are transient, and suggest that puberty is a critical period of behavioural and neuroendocrine plasticity. These observations contrast from the effects of repeated social stress in adults (4) and support the hypothesis that stress responsiveness is not fully developed until after puberty. Our current data suggest pubertal changes in physiology may enable juveniles to more readily overcome the negative consequences of defeat. Furthermore, the ongoing development of the HPA axis during puberty is likely to be a contributing factor to the differing consequences of social stress during adulthood versus puberty. The issue of pubertal plasticity has broad implications and will be addressed in futures studies.

Acknowledgements

We are grateful to Catalina Cervantes, Tracey David, Jennifer Gench and Kereshmeh Taravosh-Lahn for their expert technical assistance. This study was supported by grant ES10385 from the National Institutes of Health to Y.D. J.C.W. was supported a predoctoral fellowship from the Hutchinson Endowment of The University of Texas.

Accepted 26 July 2004

References

1 Blanchard DC, McKittrick CR, Hardy MP, Blanchard RJ. Effects of social stress on hormones, brain, and behavior. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, eds. *Hormones, Brain, and Behavior*. Boston: Academic Press, 2002: 735–772.

2 Huhman KL, Moore TO, Ferris CF, Mougey EH, Meyerhoff JL. Acute and repeated exposure to social conflict in male golden hamsters: increases in POMC-peptides and cortisol and decreases in plasma testosterone. *Physiol Behav* 1991; **25:** 206–216.

3 Huhman KL, Moore TO, Mougey EH, Meyerhoff J. Hormonal responses to fighting in hamsters: separation of physical and psychological causes. *Physiol Behav* 1992; **51:** 1083–1086.

4 Huhman KL, Solomon MB, Janicki M, Harmon AC, Lin SM, Israel JE, Jasnow AM. Conditioned defeat in male and female syrian hamsters. *Horm Behav* 2003; **44:** 293–299.

5 Jasnow AM, Banks MC, Owens EC, Huhman KL. Differential effects of two corticotropin-releasing factor antagonists on conditioned defeat in male Syrian hamsters (*Mesocricetus auratus*) *Brain Res* 1999; **846:** 122–128.

6 Potegal M, Huhman KL, Meyerhoff JL. Conditioned defeat in the syrian hamster (*Mesocricetus auratus*). *Behav Neural Biol* 1993; **60:** 93–102.

7 Delville Y, Melloni RH Jr, Ferris CF. Behavioral and neurobiological consequences of social subjugation during puberty in golden hamsters. *J Neurosci* 1998; **18:** 2667–2672.

8 Wommack JC, Taravosh-Lahn K, David JT, Delville Y. Repeated exposure to social stress during puberty alters the development of agonistic behavior in male golden hamsters. *Horm Behav* 2003; **43:** 229–236.

9 Wommack JC, Delville Y. Repeated social stress and the development of agonistic behavior: individual differences in coping responses in male golden hamsters. *Phyiol Behav* 2003; **80:** 303–308.

10 Wommack JC, Delville Y. Chronic social stress during puberty enhances tyrosine hydroxylase immunoreactivity within the limbic system in golden hamsters. *Brain Res* 2002; **933:** 139–143.

11 Kollack-Walker S, Watson DC, Akil H. Differential expression of c-fos mRNA within neurocircuits of male hamsters exposed to acute or chronic defeat. *J Neuroendocrinol* 1999; **11:** 547–559.

12 Martinez M, Phillips PJ, Herbert J. Adaptation in patterns of c-fos expression in the brain associated with exposure to either single or repeated social stress in male rats. *Eur J Neurosci* 1998; **10:** 20–33.

13 Pawlak R, Magarinos AM, Melchor J, McEwen BS, Strickland S. Tissue plasminogen activator in the amygdala is critical for stress-induced anxiety-like behavior. *Nature Neurosci* 2003; **6:** 168–174.

14 Roozendaal B, Van Der Zee EA, Hensbroek RA, Maat H, Luiten PGM, Koolhaas JM, Bohus B. Muscarinic acetylcholine receptor immunoreactivity in the amygdala-II. Fear-induced plasticity. *Neuroscience* 1997; **76:** 75–83.

15 Vomachka AJ, Greenwald GS. The development of gonadotropin and steroid hormone patterns in male and female hamsters from birth to puberty. *Endocrinology* 1979; **105:** 960–966.

16 Delville Y, David JT, Taravosh-Lahn K, Wommack JC. Stress and the development of agonistic behavior in golden hamsters. *Horm Behav* 2003; **44:** 263–270.

17 Weinberg J, Wong R. Adrenocortical responsiveness to novelty in the hamster. *Phsyiol Behav* 1986; **37:** 669–672.

18 Miczek KA. A new test for aggression in rats without aversive stimulation. differential effects of d-amphetamine and cocaine. *Psychopharmacology* 1979; **60:** 137–146.

19 Albers HE, Huhman KL, Meisel RL. Hormonal basis of social conflict and communication. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, eds. *Hormones, Brain, and Behavior*. Boston: Academic Press, 2002: 393–433.

20 Blanchard DC, Griebel G, Blanchard RJ. Mouse defensive behaviors. pharmacology and behavioral assays for anxiety and panic. *Neurosci Biobehav Rev* 2001; **25:** 205–218.

21 Watson RE Jr, Weigand SJ, Clough JA, Hoffman GE. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 1986; **7:** 155–159.

22 Morin LP, Wood RI. *A Stereotaxic Atlas of the Golden Hamster Brain*. San Diego, CA: Academic Press, 2001.

23 Bunnell BN, Sodetz FJ Jr, Salloway DI. Amygdaloid lesions and social behavior in the golden hamsters. *Physiol Behav* 1970; **5:** 153–161.

24 Delville Y, De Vries GJ, Ferris CF. Neural connections of the anterior hypothalamus and agonistic behavior in golden hamsters. *Brain Behav Evol* 2000; **55:** 53–76.

25 Lehman MN, Winans SS, Powers JB. Medial nucleus of the amygdala mediates chemosensory control of male hamster sexual behavior. *Science* 1980; **210:** 557–560.

26 Lehman MN, Powers JB, Winans SS. Stria terminalis lesions alter the temporal pattern of copulatory behavior in the male golden hamster. *Behav Brain Res* 1983; **8:** 109–128.

27 Potegal M, Herbert M, DeCoster M, Meyerhoff JL. Brief, high-frequency stimulation of the corticomedial amygdala induces a delayed and prolonged increase of aggressiveness in male Syrian golden hamters. *Behav Neurosci* 1996; **110:** 401–412.

28 Asmus SE, Kincaid AE, Newman SW. A species specific population of tyrosine hydroxylase immunoreactive neurons in the medial amygdaloid nucleus of the Syrian hamster. *Brain Res* 1992; **575:** 199–207.

29 Asmus SE, Newman SW. Tyrosine hydroxylase neurons in the male hamster chemosensory pathway contain androgen receptors and are influenced by gonadal hormones. *J Comp Neurol* 1993; **331:** 445–457.

30 Romeo RD, Sisk CL. Pubertal and season plasticity in the amygdala. *Brain Res* 2001; **889:** 71–77.

31 Li HY, Blaustein JD, De Vries GJ, Wade GN. Estrogen-receptor immunoreactivity in hamster brain: preoptic area, hypothalamus, amygdala. *Brain Res* 1993; **631:** 304–312.

32 Wood RI, Brabec RK, Swann JM, Winans-Newman S. Androgen and estrogen concentrating neurons in chemosensory pathways of the male Syrian hamster brain. *Brain Res* 1992; **596:** 89–98.

33 Wood RI, Winans-Newman S. Androgen receptor immunoreactivity in the male and female hamster brain. *J Neurobiol* 1998; **39:** 359–370.

34 Lai WS, Johnston RE. Individual recognition after fighting by male golden hamters: a new method. *Physiol Behav* 2002; **76:** 225–239.

35 Seligman ME, Beagley G. Learned helplessness in the rat. *J Comp Physiol Psychol* 1975; **88:** 534–541.

36 Kim JJ, Fanselow MS. Modality-specific retrograde amnesia of fear. *Science* 1992; **256:** 675–677.

37 Van Dijken HH, Mos J, van der Heyden JA, Tilders FJ. Characterization of stress-induced long-term behavioural changes in rats: evidence in favor of anxiety. *Physiol Behav* 1992; **52:** 945–951.

38 Bhatnagar S, Vining C. Facilitation of hypothalamic-pituitary-adrenal responses to novel stress following repeated social stress using the resident/intruder paradigm. *Horm Behav* 2003; **43:** 158–165.

39 Jonetz-Mentzel L, Wiedeman G. Establishment of reference ranges for cortisol in neonates, infants, children, and adolescents. *Eur J Clin Chem Clin Biochem* 1993; **8:** 525–529.

40 Kiess W, Meidert A, Dressendorfer RA, Schriever K, Kessler U, Konig A, Schwarz HP, Strausburger CJ. Salivary cortisol levels throughout childhood and adolescence. relation with age, pubertal stage, and weight. *Pediatr Res* 1995; **37:** 502–506.

41 Elmlinger MW, Kuhnel W, Ranke MB. Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E_2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children, and young adults. *Clin Chem Lab Med* 2002; **40:** 1151–1160.

42 Gomez F, Houshyar H, Dallman MF. Marked regulatory shifts in gonadal, adrenal, and metabolic system responses to repeated restraint stress occur within a 3-week period in pubertal male rats. *Endocrinology* 2002; **143:** 2825–2862.

43 Angulo JA, Printz D, Ledoux M, McEwen BS. Isolation stress increases tyrosine hydroxylase mRNA in the locus coeruleus and midbrain and decreases proenkephalin mRNA in the striatum and nucleus accumbens. *Brain Res Mol Brain Res* 1991; **11:** 301–308.

44 Ortiz J, Fitzgerald LW, Lane S, Terwilliger R, Nestler E. Biochemical adaptations in the mesolimbic dopamine system in response to repeated stress. *Neuropsychopharmacology* 1996; **14:** 443–452.

45 Serova L, Sabban EL, Zangen A, Overstreet DH, Yadid G. Altered gene expression for catecholamine biosynthetic enzymes and stress response in rat genetic model of depression. *Brain Res Mol Brain Res* 1998; **63:** 133–138.

46 Jordan S, Kramer GL, Zukas PK, Petty F. Previous stress increases in vivo biogenic. amine response to swim test. *Neurochem Res* 1994; **631:** 1521–1525.