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A NEURAL NETWORK UNDERLYING INDIVIDUAL DIFFERENCES IN EMOTION AND AGGRESSION IN MALE GOLDEN HAMSTERS

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Abstract—In rodents, aggressive behavior can be altered by experimental manipulations of emotional responsiveness. The goal of this study was to identify characteristics of emotional reactivity associated with individual differences in aggressive behavior and their integration within a common neural network. Male golden hamsters were first screened for offensive aggression. Then, the animals were trained through immediate reinforcement and tested for their adaptation to a delayed reward. Similar protocols have been used to test behaviors associated with frustration. At first, all hamsters showed increased frequency of bar pressing per reward during delayed reinforcement. However, Low-Aggression animals were able to adapt to the delay and showed a decreased rate of bar pressing per reward within 5 days. In contrast, High-Aggression animals maintained a high rate of bar pressing per reward. In addition, brains were collected after immediate reward training or delayed reward testing, and labeled for pCREB-immunoreactivity as a marker of trans-synaptic activity. In High-Aggression individuals, elevated density of cyclic AMP response element binding protein, phosphorylated (pCREB) immunostaining was found within the anterior hypothalamus, an area critical to the control of aggression. Delayed reinforcement was associated with enhanced pCREB immunostaining within the central amygdala, medial amygdala and preoptic area/hypothalamus continuum. Further analysis of the data also showed a positive correlation in labeling density between the lateral septum and the anterior hypothalamus, specifically in Low-Aggression animals exposed to delayed reward. Therefore, as High-Aggression individuals lack control of their emotional reactivity, they are also characterized by a de-synchronization between the inhibitory output of the septum and the aggression areas of the hypothalamus. Finally, our data also show that frustration is associated with an extensive activation of the preoptic area/hypothalamus continuum and amygdala.

Key words: impulsivity, learning, motivation, pCREB, catecholamines, limbic system.

Aggressive behavior can be studied as the response of an individual to a stimulus (Delville et al., 2003). This response can be either offensive or defensive (Blanchard and Blanchard, 1977). Offensive aggression is characterized by approach and initiation of attacks, while defensive aggression is performed in response to an attack. This behavior can be tested in a laboratory through a resident/intruder paradigm in which the intruder is smaller than the resident (Miczek, 1979). During these tests, animals may be recognized as more aggressive through their higher frequency and shorter latency of attacks and bites. However, these resident/intruder tests do not provide much information on behavioral traits capable of affecting the intensity of fights or an individual's chance of winning, such as emotional reactivity or impulsivity. In order to address these issues, it is necessary to develop new behavioral tests.

Golden hamsters constitute a convenient laboratory model for aggression studies, as they readily attack intruders placed in their home cage (Dieterlen, 1959; Grant et al., 1970). In addition, the neurobiology of aggression has been extensively studied in this species. In golden hamsters, offensive aggression is facilitated by vasopressin and inhibited by serotonin released within the anterior hypothalamus (Ferris et al., 1997, 1999). The anterior hypothalamus has reciprocal connections with several limbic areas that are activated during the consummation of offensive aggression (Delville et al., 2000). These areas include the ventrolateral hypothalamus, medial amygdala, medial division of the bed nucleus of the stria terminalis and the dorsolateral part of the midbrain central gray. This circuitry also includes other limbic areas that have been involved with emotional responsiveness such as the lateral septum and central amygdala (Taghzouti et al., 1985; Drugan et al., 1986; Hitchcock and Davis, 1986; Jellestad et al., 1986; LeDoux et al., 1988). These areas could participate in the control of aggressive behavior under specific emotional situations. For instance, as one element of the frustration theory (Amsel, 1992), discontinuation or reduction of reward (i.e. frustrative non-reward) enhances aggressive behavior (Gallup, 1965; Azrin et al., 1966; Thompson and Bloom, 1966; Davis and Donenfeld, 1967; Cherek and Pickens, 1970). Although little is known about the neural circuitry associated with frustration-based aggression, recent data showed that serotonin inhibits offensive aggression tested under various conditions including frustration (de Almeida and Miczek, 2002). This information suggests that frustration-based aggression may be considered as a subset of offensive aggression involving similar neurochemical control, although under a different emotional state. It is hypothesized that this enhanced aggression is modulated through areas associated with emotional responsiveness and integrated with the neural net-

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Abbreviations: DR, delayed reward; H-Agg, High-Aggression; IR, immediate reward; KPBS, potassium/phosphate-buffered, saline; L-Agg, Low-Aggression; pCREB, cyclic AMP response element binding protein, phosphorylated; TBS, Tris-buffered saline.

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work centered on the anterior hypothalamus and controlling offensive aggression.

During the present studies, we developed a conditioning procedure for the identification of emotional characteristics associated with aggression. Animals selected as either High-Aggression (H-Agg) or Low-Aggression (L-Agg) individuals under resident-intruder testing were compared for their adaptation to a change from an immediate reward to a delayed reward protocol. This change in reinforcement schedule produces frustrative emotional reactions similar to those observed with frustrative non-reward (Shanab et al., 1969; Moore and McHose, 1975; Spencer and Shanab, 1979; reviewed in Flaherty, 1982), but without any confound from extinction. We also compared neuronal activation between H-Agg and L-Agg animals killed directly following immediate reward training or just after delayed reward tests.

EXPERIMENTAL PROCEDURES

Animals and treatment

The present studies were performed with adult male golden hamsters (n=65) that were either purchased as adults (9–10 weeks old) from Harlan Sprague–Dawley (Indianapolis, IN, USA) or raised in the laboratory from our own breeding colony which is derived from animals originating from Harlan Sprague–Dawley. The animals were individually housed in Plexiglass cages (8×13×5 inches), in a reversed daylight cycle (14L-10D; lights on at 7:00 p.m.) and provided with food and water *ad libitum*. The hamsters were 11–12 weeks old at the onset of the experiments. The animals were housed according to NIH guidelines at the Animal Resource Center of the University of Texas at Austin, an AALAC accredited facility. All efforts were made to minimize the number of animals used and their distress.

Aggressive behavior

At the beginning of the study, all hamsters were tested for offensive aggression in the presence of the same intruder over 5 consecutive days (Fig. 1). The intruders were selected as younger and lighter (10–20%) male conspecifics. The resident–intruder tests consisted of daily 10-min periods carried out during the second half of the dark cycle. During this time, aggressive behavior was recorded as attack frequency, bite frequency, attack latency, bite latency, and contact time duration. Flank marking, a stereotyped form of scent marking in golden hamsters (Johnston, 1985), was also recorded. A wide range of individual differences was observed during these tests. On the first day of testing, some animals (n=24) were slow to attack (average: 418 s), and performed few attacks (average: 1) interspaced by long periods of rest or olfactory investigation. These animals, nevertheless, clearly dominated their intruders (which avoided contact with the residents and were submissive to them), and were considered as L-Agg individuals. Other animals (n=24) were initially fast to attack (average: 65 s), and performed several attacks (average: 13) intermitted by short periods of rest or olfactory investigation. These individuals also clearly dominated their intruders and were considered as H-Agg individuals. Animals (n=17) that were submissive or fearful of their intruders or neither clearly H-Agg nor L-Agg were taken out of the study. This selection of clearly different types of hamsters (L- vs. H-Agg) was necessary for the design of the study.

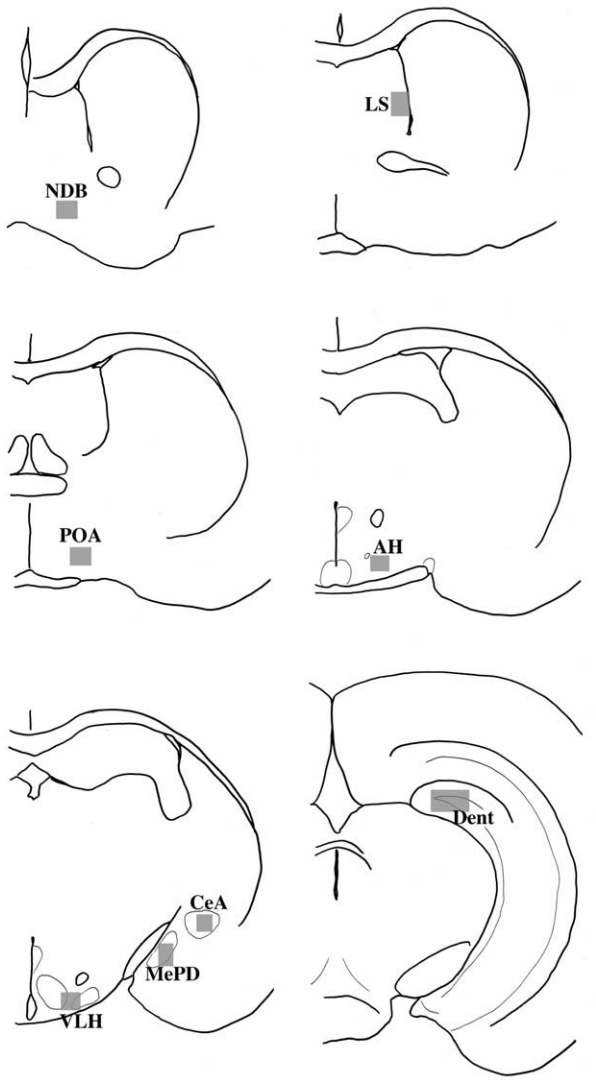

Fig. 1. Camera lucida drawings showing the location of the sites selected for quantification of pCREB immunoreactivity, including the anterior hypothalamus (AH), central amygdaloid nucleus (CeA), dentate gyrus (Dent), lateral septum (LS), medial preoptic area (MPA), medial amygdala (MeA), nucleus of the diagonal band (NDB), and ventrolateral hypothalamus (VLH).

Conditioning

The next week, the hamsters were trained for 5 consecutive days to bar press for food pellets under an immediate reward schedule of reinforcement (IR). The following week, the animals were tested in the conditioning chambers under a delayed reward schedule of reinforcement (DR) over 5 consecutive days. This paradigm was used to compare the emotional responses and adaptation capacity of H-Agg and L-Agg hamsters to a new environment, without the emotional baggage of agonistic interactions.

Conditioning chambers (Coulbourn Instruments, Allentown, PA, USA) were used to train and test the animals through a conditioning paradigm. The initial week was used for training (IR) and the second week for testing (DR). For each trial, each animal was placed in an operant chamber (12×12 inches) for a period of 10 min. Each chamber included two levers, two feeders and photocells to record movement. The actions of the animals in the chambers were recorded on a computer through a data acquisi-

tion software (Winlinc Version 3.001–00; Coulbourn Instruments). Only one feeder was programmed as active and readily provided the positive reinforcement/food pellet. The other feeder served as a decoy. Only one lever (correct lever) was programmed to deliver the reward, a 45-mg food pellet (Dustless Precision Pellets, product F0021; BioServ, Frenchtown, NJ, USA), while the second lever (dummy lever) was not reinforced. The levers were located on each side of the active feeder. These levers kept the same function throughout the study. During the training week, the hamsters were familiarized with these operant chambers and trained to press the correct lever to obtain food pellets. For every correct lever press, the hamsters would immediately receive one reward. These training trials were performed and recorded for 5 consecutive days, with each trial lasting for a period of 10 min. Beginning on the first day of training, the animals were food deprived at the beginning of the dark cycle as a motivational factor. The hamsters were placed in the conditioning chambers during the second half of the dark cycle. At the end of the week of training (IR5), hamsters (n=7 per group) were randomly chosen and killed by rapid decapitation immediately following their last exposure to the conditioning chamber. Brains were taken out and fixed for immunocytochemical labeling. The remaining animals were used for other purposes.

During the testing week (DR), the chambers were programmed to deliver the reward reinforcement 1 min after a lever press. As such, the hamsters were tested for their capacity to wait for their reward and learn to decrease their bar pressing frequency. These trials were repeated daily for 5 days, as during the training week. At the end of the testing week (DR5), animals (n=9 per group) were randomly chosen and killed by rapid decapitation immediately following testing. Brains were taken out and fixed for immunocytochemical labeling. The remaining animals were used for other purposes.

All data from the operant chambers was compiled as reward to pressing ratio (number of rewards/number of correct lever pressings×100), mistake ratio (number of dummy lever pressings/total number of lever pressing×100), and activity (recorded by photocells). The data were compared between groups during the testing week.

Immunocytochemistry

During the studies, brains were collected and prepared for immunocytochemical labeling. The brains were labeled with a monoclonal antibody for phospho-CREB (pCREB; Ser133). CREB is a transcription factor that binds to the cyclic AMP-responsive element and activates gene transcription in response to a variety of signals. CREB is activated (phosphorylated) at serine 133 through activation of the protein kinase A and/or calcium calmodulin-dependent protein kinase (Gonzalez and Montminy, 1989; Sheng et al., 1991). The antibody for pCREB used in the present study can, thus, be considered as a marker of trans-synaptic activity. The brains were fixed through a 6-h immersion in 10% acrolein in 0.1 M potassium/phosphate-buffered saline (pH 7.2) and later stored at 4 °C in 20% sucrose-KPBS. The brains were then sliced into 40-μm thick sections with a freezing rotary microtome and then stored in cryoprotectant (Watson et al., 1986) at −20 °C.

Immunocytochemical labeling for pCREB was performed as follows. Sections were first washed in 0.05 M Tris-buffered saline (pH 7.6) and were then pretreated with 1% sodium borohydride, followed by a pre-incubation solution containing of 20% normal goat serum, 0.3% Triton X-100 and 1% hydrogen peroxide. Sections were then incubated at 4 °C for 2 days in a mouse monoclonal antibody to phospho-CREB (1/8000; Cell Signaling Technology, Beverly, MA, USA), containing 2% normal goat serum and 0.3% Triton X-100. Following this incubation, tissue sections were then washed in 0.05 M TBS buffer (pH 7.6) and then incubated in the secondary antibody (7.5 mg/ml; biotinylated goat anti-mouse IgG; Vector Laboratories, Burlingame, CA, USA) for 45 min. After more washes, the sections were placed in a tertiary incubation (Vectastain ABC Elite kit; Vector Laboratories). Finally, the sections were stained and labeled with diaminobenzidine (0.5 mg/ml) in the presence of hydrogen peroxide (0.05%). All labeled sections were then mounted on gelatin-coated slides and coverslipped with permount.

The specificity of the antibody was tested through omission of the primary antibody and pre-incubation of the primary antibody with the pCREB control peptide (10 and 50 μg/ml; Cell Signaling Technology). In all cases, the sections remained devoid of immunocytochemical labeling.

Quantification of immunocytochemical labeling

The density of pCREB-immunoreactive nuclei was determined within selected neural sites using NIH Image software (v. 1.62, NIH, Bethesda, MD, USA) as previously described (Delville et al., 2000). The selected sites have reciprocal connections with the anterior hypothalamus, and are associated with the expression of offensive aggression (Delville et al., 2000). These areas included, the anterior hypothalamus (just ventrolateral to the nucleus circularis), lateral septum (at the junction of the intermediate and ventral portions near the lateral ventricle), medial preoptic area (at the junction of the medial preoptic nucleus, about 400 μm above the lateral edge of the optic chiasma), the posterodorsal part of the medial amygdaloid nucleus, the horizontal part of the nucleus of the diagonal band (at the level of the nucleus accumbens) and the ventrolateral hypothalamus (an area covering the ventrolateral part of the ventromedial hypothalamic nucleus and spreading leftward into the medial tuberal nucleus; Fig. 1). Other areas also included in the study, such as the dentate gyrus (in the dorsal part of the hippocampus, at the level of the posterior hypothalamus) and central amygdaloid nucleus, also have reciprocal connections with the anterior hypothalamus and were chosen as representative areas dealing with memory and emotion (Fig. 1). The sections containing these selected areas were observed (using a 20× objective) as digitized images captured with a Cohu CCD camera mounted on a Nikon microscope. Each image was then imported to a Macintosh computer through a frame grabber (LG3; Scion Corporation, Frederick, MD, USA). The density histograms for each area were adjusted (gain) to a standard value to normalize background levels. Cell counts were performed automatically using the Image software after standardized gray-level thresholding (Shipley et al., 1989). Each count was verified for clustered cells counted as a single unit. Counts were taken from standard samples (a square of 200×200 μm) placed over the areas of interest shown in the digitized images. The measures were repeated over adjacent sections within the area of interest. The measures (n=8 per area) were averaged for each area and for each individual animal. These individual averages were then compared between groups for each area sampled. The results were presented as a cell density (i.e. average number of cells per standard sample) in each group. A different procedure was followed in the dentate gyrus, as neurons are concentrated on a band and therefore difficult to count accurately through this method. In this case, pCREB-immunoreactive cells were counted on drawings made with a camera lucida. Cells were counted on each side (200 μm long) of a triangle pointed on the tip of the dentate gyrus. A total of eight measures were taken from adjacent sections. These measures were averaged for each individual and compared between groups.

Data analysis

Parametric behavioral data (latencies, durations, ratios) were analyzed through separate two-way ANOVAs (independent variables: H-Agg vs L-Agg groups and repeated daily observations) for each measure. Non-parametric behavioral data (number of attacks or bites) were analyzed through Wilcoxon tests (for re-

peated data) and Mann-Whitney tests (group comparisons). Neuroanatomical data were analyzed through separate two-ways ANOVAs (independent variables: groups and reward schedule) for each area.

Correlation coefficients were calculated between each area analyzed for each group independently. Each correlation coefficient was converted into a Z-value through the Fisher Z-transformation (Hays, 1994). These Z-values were compared between groups for each correlation between two areas using the following formula (Hays, 1994):

$$Z=\frac{Z_{ij1}-Z_{ij2}}{\sqrt{[[1/(n_1-3)]+[1/(n_2-3)]]}}$$

In this formula, Z_{ij} is the Fisher Z-transformed value for the correlation coefficient between areas 'i' and 'j', while n_1 and n_2 are numbers of animals in groups 1 and 2 respectively. These comparisons were used to identify significant changes ($P<0.05$) in correlations between two particular areas. Similar methods have been previously used to analyze fluorodeoxyglucose autoradiography into a form of functional imaging for Pavlovian conditioning paradigms (Jones and Gonzalez-Lima, 2001). Though these comparisons, areas showed significant increases or decreases in correlations. A significant increase in correlation between two areas and two conditions would be observed in the following situation. The individuals with the highest number of pCREB-immunoreactivity cells in one area would also become the individuals with the highest number of labeled cells in the other area. Such change in correlation would suggest a synchronization of the neural input causing the phosphorylation of CREB from one condition to the other. However, this change in correlation is not necessarily associated with increased labeling or similar increases in labeling. Alternatively, a significant decrease in correlation would suggest a de-synchronization of the input causing the phosphorylation of CREB.

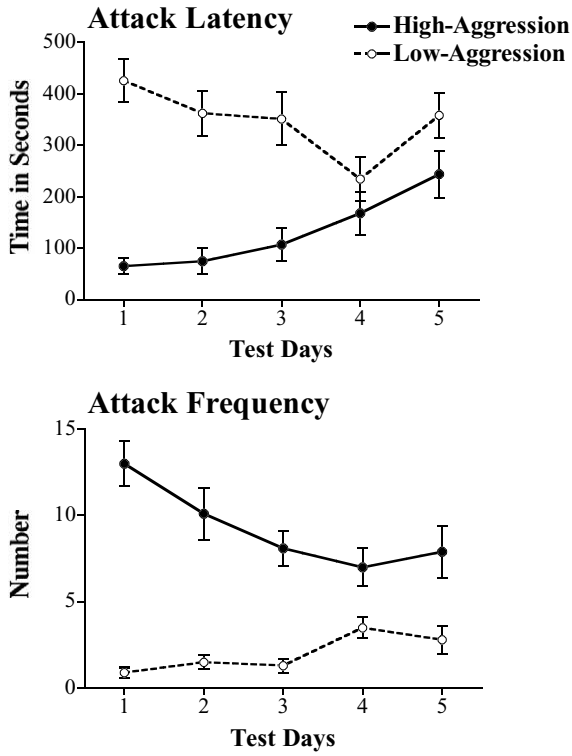

Fig. 2. Comparison of attack frequency and attack latency (mean±S.E.M.) between H- and L-Agg hamsters during repeated aggression tests over a 5-day period. Animals were tested for 10 min daily in the presence of the same smaller and younger intruders.

RESULTS

Aggressive behavior

Two distinct patterns of changes were observed over the 5-day repeated aggression tests (Figs. 2 and 3). Animals rated as H-Agg on the first test became less aggressive and slower to attack. In comparison, animals rated as L-Agg became more aggressive and faster to attack by the fourth day of testing. Consequently, attack latencies were similar between H-Agg and L-Agg animals on the last day of testing. Analysis of attack latency data shows an overall significant effect of group [$F(1,176)=51.3$, $P<0.001$] and an significant interaction between group and repeated testing [$F(1,4,176)=5.4$, $P<0.001$]. The other recorded measures produced different outcomes. In H-Agg animals, attack frequency decreased gradually from the first testing day and stabilizing to an average of eight attacks per test within 3 days [$\chi^2(4)=18.8$, $P<0.001$, Friedman]. In L-Agg animals, attack frequency tended to increase from an average of one attack per test to three per test within 4 days [$\chi^2(4)=9.4$, $P=0.052$, Friedman]. However, attack frequency remained significantly higher in H-Agg animals throughout the 5-day testing period (days 1–3: $P<0.001$; day 4: $P<0.05$; day 5: $P<0.01$, Mann-Whitney). Bite frequency followed a similar pattern as H-Agg animals averaged between seven bites on the first test day, decreasing slightly to four bites on the last day [$\chi^2(4)=8.1$, $P<0.1$, Friedman]. In L-Agg animals, bites were rare in the first test days, but their frequency increased to one bite per test by the last day [$\chi^2(4)=15.6$, $P<0.01$, Friedman]. In H-Agg animals, attack frequency remained significantly higher from day 1 to day 4 (day 1: $P<0.001$; days 2–3: $P<0.01$; day 4: $P<0.05$) and still tended to be higher on day 5 ($P<0.1$). In contrast, contact times did not differ between H-Agg and L-Agg animals and remained at around 200 s across the 5 testing days.

Conditioning

During the training week (IR1-5), all animals learned to bar press for food when placed in the conditioning chambers. By the end of training (IR5), the animals pressed the correct lever approximately 12 times in the 10-min period. Often, the hamsters would return to their home cages with several food pellets stored in their cheek pouches. There was no difference between groups in the amount of bar pressing during the training week (IR1-5), although different observations were noted during the week of testing (DR1-5). On DR1, all animals pressed the correct lever three to four times more than they received food pellets (Fig. 4). H-Agg animals remained at the same reward to press ratio throughout the 5-day testing period. However, the reward to press ratio started increasing by the second day of testing in L-Agg hamsters. By DR5, the ratio returned to almost one press per pellet in these animals. These differences were statistically significant [group effect: $F(1,96)=14.1$, $P=0.001$; time effect: $F(4,96)=4.6$,

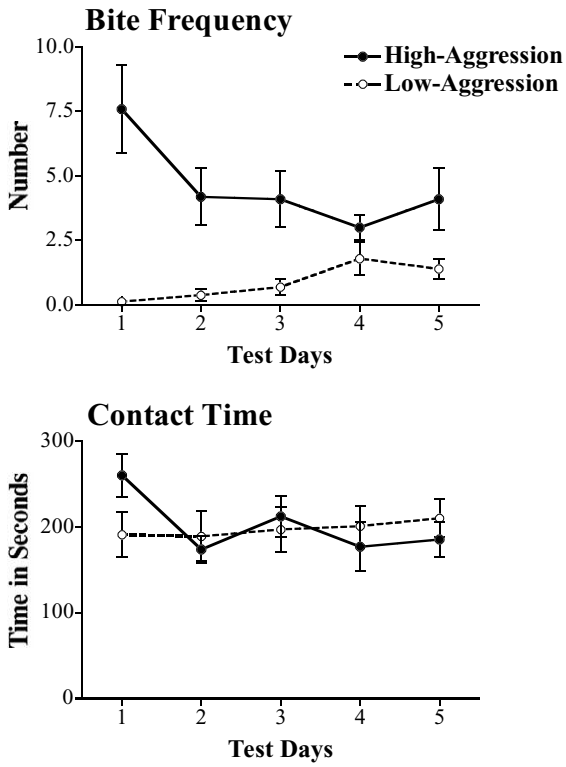

Fig. 3. Comparison of bite frequency and contact time (mean±S.E.M.) between H- and L-Agg hamsters during repeated aggression tests over a 5-day period. Animals were tested for 10 min daily in the presence of the same smaller and younger intruders.

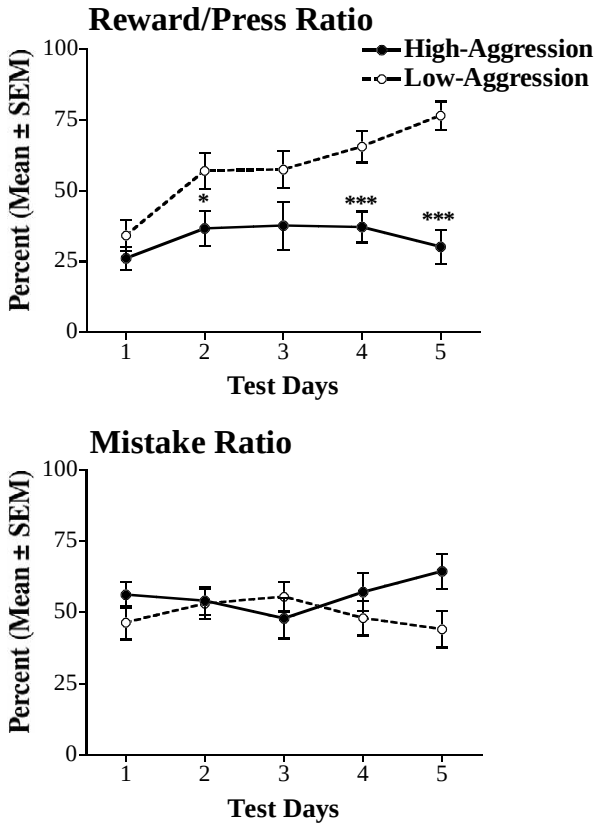

Fig. 4. Comparison of reward to press ratios (number of correct lever pressings divided by the number of rewards, multiplied by 100) and mistake ratios (number of dummy lever pressings divided by total number of lever pressings, multiplied by 100) between H- and L-Agg hamsters during repeated tests under DR over a 5-day period. During training, the conditioning chambers were programmed to deliver a food pellet immediately after each lever pressing (IR). During testing, the conditioning chambers were programmed to deliver a food pellet 1 min after each lever pressing (DR).

$P<0.01$; interaction group vs. time: $F(1,4,96)=2.9$, $P<0.05$].

Mistake ratios were calculated for all animals. These ratios remained constant (around 50%) throughout the testing week and similar between groups until the last day of testing. The overall differences over time and between groups were not statistically significant. Nevertheless, H-Agg hamsters had a slightly higher rate of mistake than L-Agg animals [64.4±6.2 vs 44.1±6.4; $t(45)=2.25$, $P<0.05$, Student's t-test] on that last day.

Behavioral activity recorded by the photocells located inside the chambers did not differ between groups or across days. A separate recording was also made for the first minute of testing on DR1-5 and also failed to show any statistically significant difference.

pCREB Immunocytochemistry

The density of pCREB-immunoreactive cells was quantified for each group of animals (H-Agg vs. L-Agg) and between IR5 and DR5. Different patterns of labeling were observed in specific areas (Figs. 5–7). Within the anterior hypothalamus, more pCREB-immunoreactive cells were counted in H-Agg than in L-Agg animals, regardless of reward schedule [$F(1,24)=13.9$, $P=0.001$]. A different pattern of differences was observed in all other areas analyzed. In many of those areas, more PCREB-immunoreactive cells were counted in DR5 than IR5 animals, regardless of aggressive behavior. These other areas included the ventrolateral hypothalamus [$F(1,26)=21.7$, $P<0.001$], central amygdala [$F(1,26)=9.2$, $P<0.01$], medial amygdala [$F(1,26)=6.8$, $P<0.05$], preoptic area [$F(1,26)=14.3$, $P<0.001$], and nucleus of the diagonal band [$F(1,21)=5.5$, $P<0.05$]. No statistically significant interaction was observed between the independent variables (groups and reward schedule) in any of the areas analyzed in this study. No statistically significant differences were observed between groups and reward schedule in the lateral septum, and dentate gyrus.

Correlations were performed between areas within each group and reward schedule (Table 1). Only one statistically significant correlation was found in Agg animals killed on IR5 (central amygdala–ventrolateral hypothalamus: $r=0.92$, $P<0.01$). A larger number of statistically significant correlations was found in H-Agg animals on DR5 (central amygdala–preoptic area: $r=0.82$, $P<0.01$; nucleus of the diagonal band–preoptic area: $r=0.81$, $P<0.05$; anterior hypothalamus–ventrolateral hypothalamus: $r=0.73$, $P<0.05$; anterior hypothalamus–medial amygdala: $r=0.75$, $P<0.05$; dentate gyrus–nucleus of the diagonal band: $r=-0.91$, $P<0.001$). Several statistically

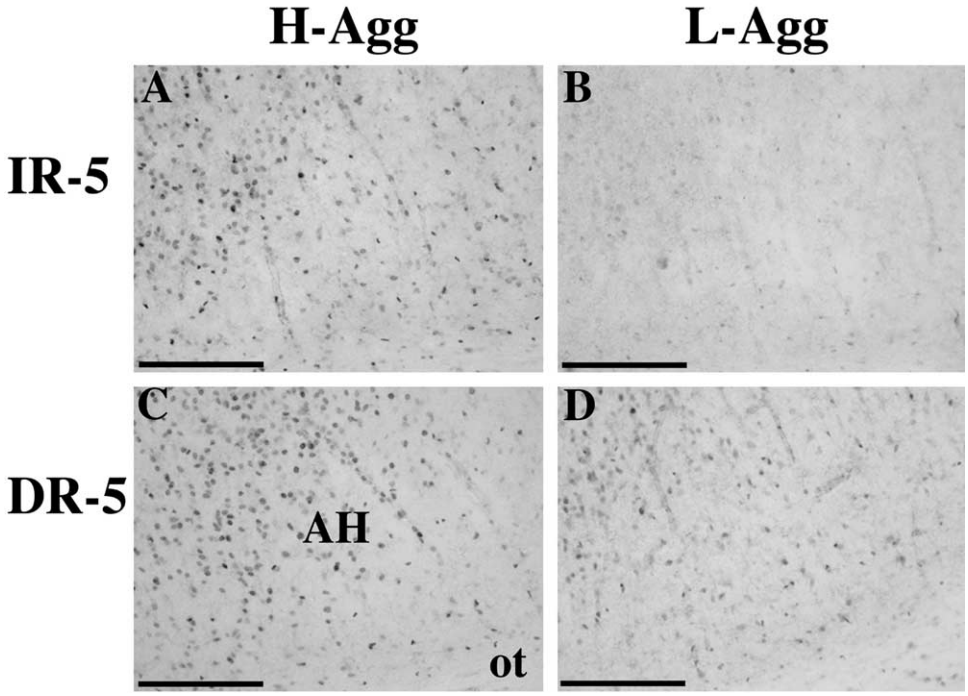

Fig. 5. Photomicrographs showing pCREB immunoreactive nuclei within the anterior hypothalamus (AH) in H-Agg (A, C) and L-Agg (B, D) hamsters killed immediately after a 10-min period in conditioning chamber on the fifth day of IR (IR5; A, B) or on the fifth day of DR (DR5; C, D). ot, optic chiasma. Scale bar=200 μm.

significant and positive correlations were found in L-Agg animals killed on IR5. These correlations were found between the following areas: nucleus of the diagonal band, preoptic area, anterior hypothalamus, ventrolateral hypothalamus, and medial amygdala. Finally, significant correlations were also found in L-Agg animals killed on DR5.

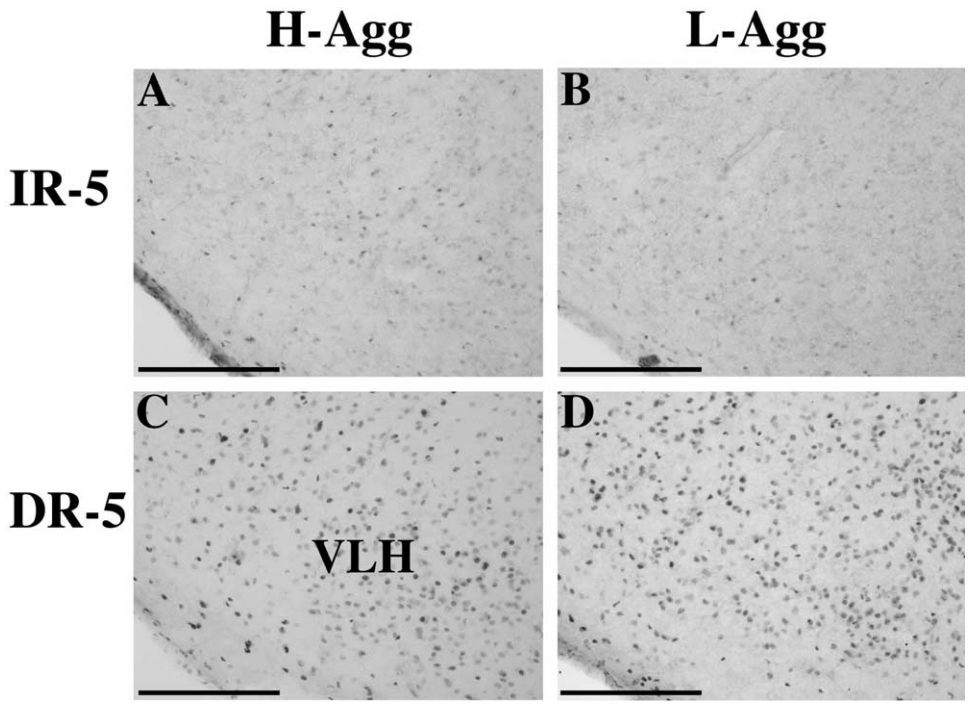

Fig. 6. Photomicrographs showing pCREB immunoreactive nuclei within the ventrolateral hypothalamus (VLH) in H-Agg (A, C) and L-Agg (B, D) hamsters killed immediately after a 10-min period in conditioning chamber on the fifth day of IR (IR5; A, B) or on the fifth day of DR (DR5; C, D). Scale bar=200 μm.

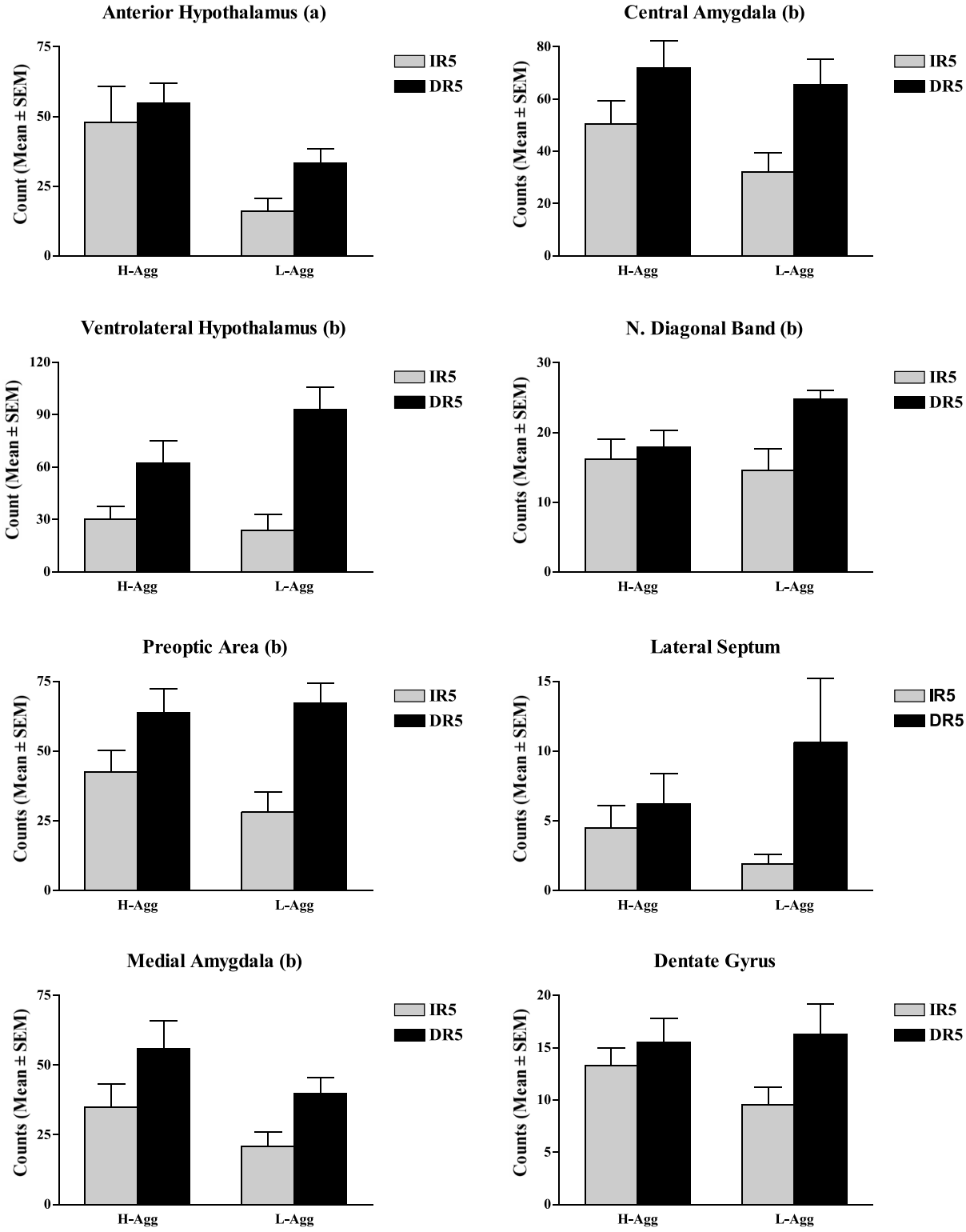

Fig. 7. Comparison of the number of pCREB immunoreactive nuclei within the anterior hypothalamus, preoptic area, ventrolateral hypothalamus, nucleus of the diagonal band, medial amygdaloid nucleus, central amygdaloid nucleus, lateral septum and dentate gyrus between H-Agg and L-Agg hamsters killed immediately after a 10-min period in conditioning chambers on the fifth day of IR (IR5) or on the fifth day of DR (DR5). (a) Statistically significant effect between H-Agg and L-Agg (at least $P<0.05$, ANOVA). (b) Statistically significant effect between IR5 and DR5 (at least $P<0.05$, ANOVA).

Table 1. Correlations of pCREB-immunoreactivity[a]

	Dent	LS	NDB	MPA	AH	VLH	MeA
L-Agg, IR5							
CeA	0.012	0.111	0.694	0.559	0.623	0.670	0.534
MeA	−0.113	−0.027	**0.893**	**0.876**	**0.868**	**0.891**	
VLH	0.073	0.265	**0.870**	**0.967**	**0.770**		
AH	−0.175	−0.355	**0.930**	0.699			
MPA	0.232	0.263	**0.901**				
NDB	0.013	−0.384					
LS	0.211						
N=6–8 per group							
L-Agg, DR5							
CeA	**0.789**	**0.961**	−0.446	**0.782**	0.748	**0.800**	0.496
MeA	−0.013	0.269	**−0.857**	0.736	0.286	0.228	
VLH	0.728	**0.857**	−0.036	0.692	0.667		
AH	0.685	**0.815**	−0.405	0.561			
MPA	0.454	0.700	−0.289				
NDB	−0.003	−0.223					
LS	**0.880**						
N=6–7 per group							
H-Agg, IR5							
CeA	−0.377	−0.028	0.398	0.419	0.407	**0.921**	0.440
MeA	0.333	0.009	−0.027	0.240	0.778	0.480	
VLH	−0.203	0.281	0.597	0.740	0.191		
AH	0.104	−0.599	−0.192	−0.269			
MPA	0.054	0.658	0.717				
NDB	0.326	0.788					
LS	0.559						
N=5–7 per group							
H-Agg, DR5							
CeA	−0.085	0.072	0.681	**0.820**	0.048	0.328	0.532
MeA	0.290	−0.311	0.298	0.542	**0.754**	0.541	
VLH	−0.431	−0.349	0.631	−0.693	**0.729**		
AH	−0.042	−0.251	0.295	0.319			
MPA	−0.469	−0.187	**0.809**				
NDB	**−0.908**	−0.139					
LS	−0.022						
N=7–9 per group							

[a] Correlations of pCREB-immunoreactive cell densities within various brain areas in each separate group. H-Agg vs L-Agg animals, animals killed on the fifth day of IR5 vs DR5.
Statistically significant correlations ($P<0.05$) are printed in **bold.**

Table 2. Comparisons of pCREB-immunoreactivity[a]

	Dent	LS	NDB	MPA	AH	VLH	MeA
L-Agg, IR5 vs DR5							
CeA	1.57	**2.74**	−1.64	0.63	0.36	0.43	−0.08
MeA	0.15	0.45	**−3.33**	−0.62	−1.68	−1.78	
VLH	1.27	1.50	−1.68	−1.75	−0.32		
AH	1.51	**2.25**	**−2.56**	−0.34			
MPA	0.38	0.89	**−2.17**				
NDB	−0.02	0.22					
LS	1.73						
H-Agg, IR5 vs DR5							
CeA	0.46	0.15	0.39	0.97	−0.46	−1.86	0.18
MeA	−0.07	−0.49	0.38	0.50	−0.09	0.12	
VLH	−0.38	−0.97	0.06	−0.14	0.88		
AH	−0.17	0.52	0.44	0.72			
MPA	−0.79	−1.38	0.26				
NDB	**−2.22**	−1.44					
LS	−1.01						
IR5, H- vs L-Agg							
CeA	−0.61	−0.21	−0.48	−0.25	−0.36	1.16	−0.18
MeA	0.68	0.05	−1.60	−1.52	−0.57	−1.34	
VLH	−0.41	0.03	−0.71	−1.48	−0.99		
AH	0.33	−0.38	−1.60	−1.36			
MPA	−0.25	0.71	−0.63				
NDB	0.36	1.61					
LS	0.62						
DR5, H- vs L-Agg							
CeA	1.71	**2.79**	−1.62	−0.16	1.37	1.13	−0.07
MeA	−0.46	0.89	**−2.08**	0.49	−1.03	−0.56	
VLH	**2.06**	**2.45**	−1.02	0.00	−0.18		
AH	1.31	**2.08**	−0.96	0.45			
MPA	1.54	1.63	−1.94				
NDB	**2.07**	−0.12					
LS	**2.17**						

[a] Comparison of correlations of pCREB cell densities between groups (H- vs L-Agg, IR5 vs DR5).
Number represented originate from calculated Z values as explained in the Experimental Procedures.
Statistically significant values ($P<0.05$) are printed in **bold.**

These correlations involved the lateral septum, dentate gyrus, central amygdala, nucleus of the diagonal band, ventrolateral hypothalamus, preoptic area, medial amygdala and anterior hypothalamus. In particular, significant correlations were found between the lateral septum and the following areas: dentate gyrus ($r=0.88$, $P<001$), central amygdala ($r=0.96$, $P<0.001$), ventrolateral hypothalamus ($r=0.86$, $P<0.05$), and anterior hypothalamus ($r=0.82$, $P<0.05$).

Comparisons of correlation coefficients were performed between groups and reward schedules for each pair of area analyzed (Table 2). In H-Agg animals, significant changes in correlation coefficients were found from IR5 to DR5 only between the nucleus of the diagonal band and dentate gyrus ($Z=-2.22$, $P<0.05$). In L-Agg animals, significant increases in correlations coefficients were found from IR5 to DR5 between the lateral septum and the anterior hypothalamus ($Z=2.25$, $P<0.05$), as well as between the lateral septum and the central amygdala ($Z=2.74$, $P<0.01$). In addition, significant decreases in correlation coefficients were found between the nucleus of the diagonal band and the following areas: anterior hypothalamus ($Z=-2.56$, $P<0.05$), preoptic area ($Z=-2.17$, $P<0.05$), and medial amygdala ($Z=-3.32$, $P<0.001$). No significant change in correlation coefficient was found between H-Agg and L-Agg animals on IR5. However, a number of significant changes in correlation coefficients were found from H-Agg to L-Agg animals on DR5. In particular, significant increases in correlation coefficients were found between the lateral septum and the following areas: anterior hypothalamus ($Z=2.08$, $P<0.05$), dentate gyrus ($Z=2.17$, $P<0.05$), ventrolateral hypothalamus ($Z=2.45$, $P<0.05$) and central amygdala ($Z=2.79$, $P<0.01$). Significant increases were also found between the dentate gyrus and the following areas: lateral septum ($Z=2.17$, $P<0.05$), nucleus of the diagonal band ($Z=2.07$, $P<0.05$), and ventrolateral hypothalamus ($Z=2.06$, $P<0.05$). Finally, a significant decrease in correlation was found between the

nucleus of the diagonal band and the medial amygdala ($Z=-2.08$, $P<0.05$).

DISCUSSION

During the present study, it was possible to clearly identify different behavioral characteristics associated with aggressive behavior. Animals classified as H-Agg individuals were consistently more likely to attack and bite their intruders. Conversely, animals classified as L-Agg individuals were consistently less likely to attack and bite their intruders. These animals also differed in response to shifts in reward presented in the conditioning chambers. L-Agg individuals gradually reduced their ratio of lever pressing activity in response to a change from immediate to DR. This adaptation took place within 5 days. In contrast, H-Agg individuals did not decrease their level pressing activity in response to the change in reinforcement schedule. However, these data do not mean that H-Agg individuals are incapable of learning or adapting their behavior. H-Agg hamsters were capable of decreasing their aggression during repeated exposure to the same intruders. This observation is consistent with previous reports on decreased aggression in hamsters (Ferris et al., 1987) and rats (de Almeida and Miczek, 2002). L-Agg animals also appeared capable of changing their aggressive behavior during repeated exposure to the same intruder. In this case, they became slightly more aggressive. This slight increase in aggression may reflect a habituation to the testing procedure, but may not necessarily be long lasting. Nevertheless, these observations show that both types of animals were well capable of adapting their behavior to a specific social situation. The difference between both types of testing involves predictability and emotional control. The change in reinforcement schedule includes a frustrative component that is not present during aggression testing. Similar or related experimental designs have been used to test behavioral responses to frustrative non-reward situations and have been associated with enhanced aggression (Flaherty, 1982; Amsel, 1992). Such experimental design clearly shows a relationship between emotional responsiveness and aggression. As such, the present data suggest that differences in aggression are associated with a difference in the control of emotional reactivity in golden hamsters. Individuals most capable of controlling their emotional responses would quickly establish dominant/subordinate relationships without a need for excessive aggression. It could be argued that this capacity is advantageous to these animals, as they would be able to invest more time in other activities such as mating or food collection. These animals may also be less stressed while remaining at the top of a social hierarchy.

The analysis of pCREB immunolabeling supports this conclusion and extends it to a level of functional neuroanatomy. Two main patterns of differences were found within the neural circuitry connected to the anterior hypothalamus and associated with the control of aggressive behavior in hamsters. In one area, the anterior hypothalamus, more immunoreactive cells were counted in H-Agg animals regardless of reinforcement schedule. This area is central to the control of aggression in golden hamsters (Ferris and Potegal, 1988; Ferris et al., 1997, 1999). This observation suggests that the neural input responsible for the phosphorylation of CREB within the anterior hypothalamus is more active in H-Agg animals. In other areas, more immunoreactive cells were counted in animals killed under a DR, regardless of aggression. These areas, the preoptic area, nucleus of the diagonal band, ventrolateral hypothalamus, medial amygdala, and central amygdala have reciprocal connections with the anterior hypothalamus (Delville et al., 2000). The ventrolateral hypothalamus and medial amygdala have been associated with the control of offensive aggression in hamsters (Delville et al., 1996, 2000; Potegal et al., 1996). These data show that the change in reinforcement schedule is associated with an activation of the neural input responsible for the phosphorylation of CREB within these areas. As such, the present data show that frustrative non-reward and its emotional component are associated with marked increase in synaptic activity within the neural network controlling aggression in hamsters. This finding may explain the intense aggression previously reported under such experimental design (Azrin et al., 1966; Cherek and Pickens, 1970; Davis and Donenfeld, 1967; Gallup, 1965; Thompson and Bloom, 1966).

Interestingly, there was no statistically significant interaction between the two independent variables (aggression and reinforcement schedule) within any of the areas analyzed in this study. Indeed, we did not find any statistically significant changes in labeling within a specific area in association with the modulation of lever pressing by L-Agg individuals. Such an area would have been involved with this type of learning. However, this does not mean that no such area exists. The analysis of correlations between areas points to specific relationships in association with adaptation to a DR. L-Agg animals killed under a DR had a unique and positive correlation between the anterior hypothalamus, central amygdala, and the lateral septum. These positive correlations did not appear under IR or in H-Agg individuals. The comparison of correlation coefficients is statistically significant in L-Agg animals between immediate and DR, as well as in DR between H-Agg and L-Agg animals. These data suggest that the neural input responsible for the increase in pCREB immunostaining within the anterior hypothalamus and central amygdala may be synchronized with the input to the lateral septum in these L-Agg animals, specifically as they were adapted to DR. This conclusion is consistent with previous observations using fluorodeoxyglucose autoradiography into a form of functional imaging (Nair and Gonzalez-Lima, 1999). In rats, the postnatal development of behavioral extinction (a non-reward situation) is associated with the establishment of a functional network correlating neural activity within the lateral septum with the hippocampus and ventral tegmental area (Nair and Gonzalez-Lima, 1999).

As CREB can be phosphorylated at serine 133 through the protein kinase A and calcium calmodulin protein kinase pathways, several types of neurotransmitter receptors

could be involved in this activation. However, one class of neurotransmitters has been associated with emotional and aggressive responses. Stress and aggression have been associated with increased release of dopamine and/or serotonin within various parts of the brain (Jordan et al., 1994; Tidey and Miczek, 1996; van Erp and Miczek, 2000; Summers, 2001). During encounters between individuals social dominance appears to be determined by rapid changes in catecholamine release in the brain in lizards (Summers et al., 2003). A number of catecholamine and serotonin receptors are linked to an adenylyl cyclase and activation of protein kinase A (Greengard, 2001; Svenningsson et al., 2002). Based on the present data, it could be hypothesized that the lack of control of emotional reactivity in H-Agg animals is caused by a de-synchronization of the dopamine and serotonin input to the hypothalamus–amygdala and lateral septum.

This hypothesis is supported by previous observations. In rats, non-reward effects are enhanced by neurochemical (6-OHDA) lesions of the dopamine innervation of the lateral septum (Taghzouti et al., 1985). It is possible that such lesions would also affect aggressive behavior in hamsters. Indeed, the lateral septum has been involved in the control of aggression in a number of species, including hamsters (Potegal et al., 1981a,b; Albert and Walsh, 1984). Aggressive behavior is enhanced by lesions and inhibited by electrical stimulation of the lateral septum in golden hamsters (Potegal et al., 1981a,b). The lateral septum has extensive reciprocal connections with the hypothalamus in hamsters and rats (Meibach and Siegel, 1977; Swanson and Cowan, 1979; Ferris et al., 1990; Risold and Swanson, 1996; Delville et al., 2000). In rats, the lateral septum contains an elevated density of GABA neurons (Jakab and Leranth, 1990; Risold and Swanson, 1996). These neurons integrate inputs from the hippocampus with other parts of the brain, including catecholaminergic cell populations (Jakab and Leranth, 1990, 1991, 1993; Jakab et al., 1991; Risold and Swanson, 1996) and are suspected to provide an inhibitory input to the hypothalamus (Cirino and Renaud, 1985; Saphier and Feldman, 1987; Jakab and Leranth, 1995; Risold and Swanson, 1996; Herman et al., 2002). As such, it is likely that the correlated activation of pCREB immunolabeling within the lateral septum of L-Agg animals reflects the activation of an inhibitory pathway to the hypothalamus.

In this study, DR caused an increase in the density pCREB immunostaining within the central amygdala. This area is critical to the expression of emotions in rodents (Pitkanen et al., 1997). Lesions of the central amygdala block emotional reactions (Hitchcock and Davis, 1986; Jellestad et al., 1986). As such, the increased pCREB immunolabeling within this area supports its role in emotional reactions. However, it is also important to note that a positive correlation was also found between the lateral septum and central amygdala in L-Agg animals adapted to DR. These correlations were not found on other animals under other conditions. Although there is no direct pathway between these two areas, the present data suggest they are integrated within an extended neural network modulating emotional reactivity.

Our data show that DR was associated with enhanced density of pCREB immunolabeling within a number of areas connected to the anterior hypothalamus. The density of pCREB immunolabeling was positively (although not always statistically significantly) correlated among most of these areas, with the exception of the nucleus of the diagonal band. Although DR was associated with increased pCREB immunolabeling in this area, the correlations with the hypothalamus and amygdala were negative particularly in L-Agg individuals adapted to DR. The change in correlation was significant between the nucleus of the diagonal band and medial amygdala. This observation suggests that the neural mechanisms responsible for these increases in the density of pCREB immunolabeling differ between the nucleus of the diagonal band and medial amygdala. It is also possible that the nucleus of the diagonal band plays a role in the adaptation to DR. This possibility is supported by previous data showing a role for this area in the control of emotion and rewarded behaviors. Neural activity within the nucleus of the diagonal band is associated with self-stimulation (Rompre and Shizgal, 1986; Konkle et al., 1999). Lesions of the area are capable of altering the acquisition of rewarded tasks (Roman et al., 1993). In addition, neural activity within the area is also decreased in association with inhibition of fear responses through Pavlovian conditioning (McIntosh and Gonzalez-Lima, 1994; Jones and Gonzalez-Lima, 2001).

In conclusion, the present data show an association between individual differences in aggressive behavior and emotional adaptation. The most aggressive individuals are not capable of adapting to a frustrative context. This lack of adaptation is associated with a putative de-synchronization of the activation of the anterior hypothalamus and central amygdala with the lateral septum. These areas are incorporated within a neural network centered on the anterior hypothalamus. The lateral septum provides an inhibitory input to the anterior hypothalamus and ultimately to the central amygdala. Considering the role of these areas in aggression and emotional reactivity, it is likely that the behavioral differences are related to a dysfunction of the activation of the lateral septum under the test conditions. One possible mechanism would be an abnormal activation of catecholamine or serotonin receptors within the lateral septum. This possibility will be tested in future studies.

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