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Effect of social status on behavioral and neural response to stress

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Abstract

Individuals respond differently to traumatic stress. Social status, which plays a key role in how animals experience and interact with their social environment, may influence how individuals respond to stressors. In this study, we used a conditioned defeat model to investigate whether social status alters susceptibility to the behavioral and neural consequences of traumatic stress. Conditioned defeat is a model in Syrian hamsters in which an acute social defeat encounter results in a long term increase in submissive behavior and a loss of normal territorial aggression. To establish social status, we weight matched and paired Syrian hamsters in daily aggressive encounters for two weeks to create dominant/subordinate relationships. We also included controls which were exposed daily to a clean empty cage for the same 14 day period. Twenty-four hours after the final pairing or empty cage exposure, subjects were divided into defeat and no defeat groups. Individuals in the defeat group received three 5 minute social defeats at 5 minute intervals in the cage of a larger aggressive hamster. Individuals in the no defeat group were exposed to the empty cage of a larger aggressive hamster at the same time intervals. In experiment 1, subjects of both groups were tested for conditioned defeat with a non-aggressive intruder 24 hours after social defeat training. In experiment 2, brains were collected 65 minutes following social defeat training and immunohistochemistry was performed for c-Fos protein, a marker of neural activation. We quantified the number of c-Fos immunopositive cells in brain regions known to be involved in stress and aggression, including the ventral medial prefrontal cortex, medial amygdala, and lateral and ventromedial hypothalamus. We found that subordinate animals showed significantly more conditioned defeat behavior than did dominants or controls, and subordinates showed significantly less c-Fos immunoreactivity than did dominants in all these brain regions. These results suggest that decreased neural activity in these brain regions corresponds to an increased susceptibility to conditioned defeat. In sum, social status plays an important role in how animals respond to social stressors and this corresponds to activity in specific brain areas.

Introduction

Stress is an organism's subjective response to physical or psychological threat. This response is normally adaptive, but when a stressor is prolonged, severe, or uncontrollable there can be serious negative psychological and physiological consequences (Agid et al., 2000). Post-traumatic stress disorder (PTSD) is one well documented consequence of an acute but severe stress experience, such as that occurring during military combat (Sareen et al., 2007). Interestingly, only a fraction of individuals who experience this type of stressor will develop stress-related mental illnesses such as PTSD (Yehuda, 2004), indicating that individuals differ in their susceptibility to the negative consequences of traumatic stress. Understanding these individual differences is an important step towards better understanding these illnesses and developing more targeted treatments.

Previous experience is a critical factor affecting how individuals react to future stressors (Blanchard et al., 2001). Because social status is a key component in how animals interact with their social environment, differences in status may play an important role in how animals cope with stress. For example, dominant individuals tend to control social encounters with subordinates and this experience may help them to cope with future stressors (Chorpita & Barlow, 1998). Koolhaas and colleagues (2007) have suggested that dominant rodents exhibit a proactive coping style characterized by increased responsiveness with the environment, whereas subordinates exhibit a reactive coping style characterized by immobility and passivity.

In this study we tested the effect of social status on the behavioral and neural responses to a traumatic social stressor. We used a model with Syrian Hamsters established by Potegal and colleagues (1993) called conditioned defeat. Syrian hamsters are territorial animals that readily form dominant-subordinate relationships. In the conditioned defeat model, social defeat results in a loss of normal territorial aggression and an increase in submissive and defensive behavior in later non-aggressive social

encounters. These behavioral changes can last at least a month following the social defeat experience (Huhman et al., 2003), and in mice normal social behavior can be restored by treatment with antidepressant medications including fluoxetine (Berton & Nestler, 2006). These factors suggest that social conflict models like conditioned defeat may be particularly valuable as models for the study of stress related psychopathologies such as depression and PTSD (Huhman, 2006).

Social status may affect neural activation in brain regions related to stress and aggression during social defeat. c-Fos, which is the protein product of an immediate-early gene, has been used as a marker for neural activation (Fekete et al. 2009). In our study, stress-related regions of interest include the amygdala, ventral medial prefrontal cortex (vmPFC), and paraventricular nucleus of the hypothalamus (PVN). The amygdala is involved in the perception of threatening stimuli (Anderson, 2007) and uncertainty (Rosen & Donley, 2006). The vmPFC detects the controllability of stressors and inhibits stress related behavior such as learned helplessness (Amat et al., 2006). The PVN is well known for its role in initiating the neuroendocrine response to stress (Kiss et al. 1996). Other nuclei of the hypothalamus, including the lateral hypothalamus (LH) and lateral portions of the ventromedial hypothalamus (VMH-L), regulate aggressive behavior (Pan et al. 2010) and the communication of dominance status (Ferris et al. 1990; Ferris et al. 1987).

In Experiment 1 we tested the hypothesis that subordinate animals would show increased conditioned defeat behavior compared to dominants. In Experiment 2 we tested the hypothesis that subordinates would exhibit a different pattern of neural activation compared to dominants. We hypothesized that subordinates would show increased c-Fos expression in the PVN and amygdala which might be an indication of heightened stress and fear responses. In contrast we hypothesized that subordinates would show reduced c-Fos expression in the vmPFC, which may correlate with diminished resiliency to stressors. Furthermore, because subordinates have less previous experience as aggressors,

we hypothesized that they would show reduced c-Fos expression in aggression related nuclei of the hypothalamus compared to dominants.

Experimental Procedures

Subjects

Subjects were male Syrian hamsters (*Mesocricetus auratus*) acquired or bred from Charles River Laboratories (Wilmington, MA) stock. They weighed 140-160 g and were individually housed for one week prior to the study to allow for scent marking of territory. Older hamsters weighing 160-180 g were individually housed and used as resident aggressors for social defeat training. Younger hamsters weighing 90-110 g were housed in groups of four and used as non-aggressive intruders for conditioned defeat testing. All animals were housed in polycarbonate cages (12 cm x 27 cm x 16 cm) with corncob bedding, cotton nesting materials, and wire mesh tops. Animals were kept in a temperature controlled colony room (21±2 °C) and maintained on a 14:10 hour light:dark cycle with food and water available *ad libitum*. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee.

Experimental Design

Dominant-Subordinate Encounters

Subjects were weight-matched in pairs and randomly selected as either a resident or intruder. Pairs were exposed to each other in daily encounters for 14 days with all encounters occurring in the resident's home cage. The first encounter on day 1 was 10 minutes, while all subsequent encounters were 5 minutes. In pilot studies, we determined that a 10 minute encounter on day 1 facilitated the formation of a dominance relationship, and that 5 minute encounters were sufficient to maintain the dominance relationship while reducing the chance of wounding. One pair exhibited an inconsistent

dominance relationship and was removed from the study. Empty cage control animals were placed in clean empty cages for 10 minutes on day 1 and 5 minutes on days 2 through 14.

Social Defeat Training

Subjects were randomly assigned to receive social defeat or serve as no-defeat controls. One day following the final dominant-subordinate encounter or empty cage exposure, social defeat animals received three 5 minute defeats in the cage of larger resident aggressors, with 5 minute intervals between each defeat. A different resident aggressor was used during each 5 minute defeat. Dominants often fought back against the resident aggressor during the first defeat but eventually lost and did not fight back during subsequent defeats. To correct for potential variation in the amount of aggression subjects received, we defined social defeat as starting at the resident aggressor's first attack that was accompanied by submissive behavior in a subject. No animals were wounded such that they bled or required removal from the study. Two subjects failed to be defeated by the resident aggressors and were removed from the study. No defeat controls received three 5 minute exposures to a resident aggressor's empty cage, with 5 minute intervals between each exposure. They were exposed to a different resident aggressor's cage each time. Resident aggressor cages were used for the controls so that the defeat experience could be fully isolated from other stress potentiating cues such as scent.

Experiment 1: Conditioned Defeat Testing

In Experiment 1, animals (N = 72) were tested for conditioned defeat 24 hours following social defeat training. Each subject was paired in their home cage with a non-aggressive intruder for 5 minutes. Non-aggressive intruders are younger, group-housed animals that display social and nonsocial behavior during testing. These encounters were digitally recorded and the behavior of subjects was quantified using Noldus Observer (Noldus Information Technology, Wageningen, Netherlands).

We quantified the total duration of 4 classes of behavior: (a) submissive and defensive (flight, avoid, tail up, upright and side defense, full submissive posture, stretch-attend, head flag); (b) aggressive (upright and side offense, chase, and attack including bite); (c) social (attend, approach, investigate, sniff, and nose touch); (d) nonsocial (locomotion, exploration, self-groom, nest build, and feed). These categories of behavior were adapted from Albers et al. (2002). The frequency of attacks, flees, and stretch-attend postures were also recorded. All behavioral scoring was performed by a researcher blind to the experimental conditions of the subject. Inter-rater reliability on the duration of submissive/defensive behavior was greater than 90%.

Experiment 2: Immunohistochemistry and Cell Counting

In Experiment 2, animals (N = 72) were anesthetized with a cocktail of 93% sodium pentobarbital and 7% isopropyl alcohol (Sleep Away, Webster Veterinary) 65 minutes after social defeat training. Animals were then transcardially perfused with 100ml of 0.1 M phosphate-buffered saline (PBS) followed by 100ml of 4% paraformaldehyde solution. Brains were removed and soaked in 4% paraformaldehyde for 24 hours, followed by 0.1 M PBS/30% sucrose solution for 48 hours, and were then stored in cryoprotectant.

Each brain was sliced into a series of consecutive 30 μm coronal sections on a vibrating microtome and stored in glass scintillation vials containing cryoprotectant. Tissue sections used in this study contained prefrontal cortex, hypothalamus, and amygdala. We performed immunohistochemistry on the free floating sections using a primary antiserum directed against the c-Fos protein (rabbit anti-c-Fos polyclonal antibody, 1:5000; Santa Cruz Biotechnology). All washes, rinses, and incubations were performed at room temperature in plastic well plates which were gently shaken on an orbital shaker throughout immunostaining. Sections were first rinsed with PBS-Triton and then incubated for 20 minutes with 0.3% hydrogen peroxide, then rinsed again with PBS-Triton. Sections were then incubated

overnight at room temperature in a PBS-Triton solution containing 1% normal donkey serum and the rabbit anti-c-Fos antibody. The next day sections were rinsed in PBS-Triton, and then incubated for 90 minutes in PBS-Triton containing 1% normal donkey serum and a biotinylated donkey anti-rabbit IgG polyclonal antibody (1:500, Vector Laboratories). Sections were then rinsed in PBS-Triton followed by a 90 minute incubation with an avidin-biotin complex reagent (Vectastain Elite ABC kit, Vector Laboratories). After rinsing with PBS-Triton, sections were placed in solution containing 3,3'-diaminobenzidene (DAB), hydrogen peroxide, and nickel ammonium sulfate for 10 minutes. The peroxide reaction was stopped with a series of 5 PBS rinses and five distilled water rinses. Sections were mounted onto microscope slides, air-dried, dehydrated with an ethanol series, cleared with citrisolv, and cover slipped using DPX mountant (Sigma-Aldrich, St. Louis, MO). The color product of c-Fos immunostaining was blue-black and localized to the cell nucleus.

Images of amygdala and PFC sections were captured at 20X magnification using an Olympus BX41 microscope, and hypothalamus images were captured at 40X magnification. The number of c-Fos immunopositive cells was determined in select brain regions using MCID Core image analysis software (InterFocus Imaging, Cambridge, England). The following brain regions were immunopositive and were quantified for cell count: dorsal medial amygdala (dMeA), ventral medial amygdala (vMeA), dorsal medial PFC (dmPFC), ventral medial PFC (vmPFC), lateral hypothalamus (LH), ventral medial hypothalamus (VMH-L), and paraventricular nucleus of the hypothalamus (PVN). For each region we quantified three sections per individual at select rostral-caudal locations. We defined immunopositive cells as those that showed staining 2X greater than background. The background was calculated by randomly sampling 10-20 points on each section where there was no staining and calculating the average densitometry value. A sample image of the LH shows the result of c-Fos immunostaining (Figure 1). Some animals had to be excluded from analysis because of poor tissue quality.

Statistical Analysis

Behavioral and immunohistochemical data were analyzed using 2-way analysis of variance (ANOVA) with social status and defeat condition as independent variables. In the cases of significant social status effects, an additional 1-way ANOVA was performed with a Tukey post hoc test. All statistical tests were two-tailed, and the α level was set at $p \leq 0.05$.

Results

Experiment 1: Social Status and Conditioned Defeat Behavior

Subordinate hamsters showed increased conditioned defeat behavior compared to dominants and controls (Figure 2). We found a significant interaction between social status and defeat condition such that among socially defeated animals, subordinates showed more submissive and defensive behavior than either dominants or controls ($F=3.179$, $P=.049$); post-hoc tests $P<.05$). Defeated subjects showed reduced aggressive behavior at testing compared to no defeat control, although social status did not alter aggression ($P=.093$). Social behavior differed significantly between social status groups ($F=6.130$, $P= .004$), although there was no interaction with defeat condition. Subordinate subjects showed significantly less social behavior than dominants ($P=.011$), while empty cage controls were intermediate. Similarly non-social behavior differed significantly between social status groups ($F=6.905$, $P=.002$), and there was no interaction with defeat condition. Subordinates showed significantly more non-social behavior than dominants ($P=.047$), while empty cage controls were intermediate.

Experiment 2: Social Status and Defeat-induced Neural Activation

Subordinate hamsters showed decreased c-Fos immunoreactivity in several key brain regions following social defeat (Figure 3). There was a significant effect of defeat in all brain regions ($P<.001$). In the vmPFC there was a significant effect of social status ($F=5.107$, $P=.009$). Specifically, subordinates had

significantly fewer c-Fos positive cells than dominants (P=.024), while empty cage controls were intermediate. In the LH there was a significant interaction of defeat and social status (F=4.278, P=.018). Among socially defeated animals, subordinates showed significantly fewer c-Fos positive cells in the LH cells than dominants (P=.023), and again empty cage controls were intermediate. There was also a significant interaction in the VMH-L (F=8.039, P=.001), and socially defeated subordinates showed significantly fewer c-Fos positive cells than corresponding dominants (P=.006) and controls (P=.012). The vMeA showed a similar interaction (F=3.786, P=.030) and defeated subordinates again had fewer c-Fos positive cells than corresponding dominants (P=.024) and controls (P=.049). There was no significant effect of social status on c-Fos immunoreactivity in the PVN, dMeA, or dmPFC.

Discussion

These results indicate that dominant and subordinate hamsters differ in their susceptibility to conditioned defeat and in their pattern of defeat-induced neural activation following social defeat. Subordinates showed significantly more submissive and defensive behavior than did dominants or controls when tested with a non-aggressive intruder. Aggressive behavior did not differ though, indicating that although social status may affect the severity of conditioned defeat it cannot eliminate it. Subordinates also showed significantly less neural activation than did dominants in the LH, vMeA, VMH-L, and vmPFC during social defeat. These results suggest that chronic subordination increases susceptibility to conditioned defeat and is associated with decreased neural activation in key brain regions that may modulate the development of conditioned defeat.

Some of these findings contradict our original hypotheses. We expected that empty cage animals would be intermediate to subordinate and dominant subjects in both their neural activation during social defeat and their later conditioned defeat behavior. Surprisingly though, controls were often statistically indistinguishable from dominants. The similarity between dominants and controls

raises two possibilities. Either the experience of living and establishing a territory in an empty cage is similar to gaining dominant social status, or dominant social status does not alter behavioral and neural responses to stress. The former explanation is more likely because the empty cage animals' aggressive and dominant like behavior can be reduced by individually housing them for less time. An additional control paradigm, in which controls experience a mixture of dominant and subordinate social encounters instead of an empty cage, could be investigated in future experiments.

We also hypothesized that subordinates would show increased c-Fos immunoreactivity in certain stress related brain areas, specifically the PVN and amygdala. In the PVN there was no effect of social status on c-Fos immunoreactivity. Activation of the PVN is correlated with activation of the HPA-axis (Cook, 2004) and research in primates has shown that subordinates show increased HPA-axis activity (Sapolsky et al., 1997). Our results suggest that all subject groups have similar PVN activation during social defeat. Thus susceptibility to conditioned defeat may be independent of activation of the PVN. Future research is required to test whether subordinates and dominants in fact have a similar neuroendocrine response to social defeat.

The basolateral nucleus of the amygdala (BLA) is a key brain region controlling the development of conditioned defeat (Jasnow et al., 2004). Recently, pharmacological inactivation of the MeA was shown to impair the acquisition of conditioned defeat, suggesting that this region modulates conditioned defeat development via its projections to the BLA (Markham & Huhman, 2008; Walker et al., 2005). We found that subordinates showed less defeat-induced c-Fos immunoreactivity compared to dominants and controls. This result was surprising because reduced activation of the MeA is associated with less conditioned defeat. This seeming paradox highlights one of the key limitations of c-Fos as a marker of neural activation. c-Fos only indicates that a cell has been activated, but does not indicate the phenotype of the cell. It may be that inhibitory GABA cells have increased activity in dominants and

controls which would better explain the behavioral results. This possibility is supported by existing literature which shows that GABA cells in the amygdala can be activated during stress (Cook, 2004) and can even block the formation of conditioned defeat (Jasnow & Huhman, 2001). Future experiments will be needed to determine what kinds of cells in the MeA are activated during social defeat.

Amat and colleagues (2006) have shown that having the experience of control over stressors activates the vmPFC and reduces the behavioral and physiological reaction to future uncontrollable stressors. These findings are consistent with our results and suggest that reduced activation of the vmPFC during social defeat may contribute to the increased conditioned defeat seen in subordinates. Subordinates also had reduced neural activation in the LH and VMH-L compared to dominants. These adjacent brain areas are involved in a host of functions including aggression, so perhaps low activation of the LH and VMH-L is related to the observation that subordinates did not fight back during social defeat training. It is important to note though that dominant and subordinate no defeat controls displayed equivalent amounts of aggression during testing, indicating that subordinate status alone does not induce a loss of normal territorial aggression.

The study of individual differences and how past experience mediates resiliency and likewise susceptibility to stress is an important avenue of research. Our results show that subordinate animals are more susceptible to conditioned defeat compared to dominants and controls. This indicates that social status has a significant effect on how animals respond to stress. Animal models such as this one may not be directly applicable to humans but allow for a much more detailed and intensive analysis than is possible with human subjects. We believe that experiments such as ours will continue to clarify the role played by relevant brain areas and shed light on what directions new medical and therapeutic treatments should take to help protect individuals from trauma-induced mental illness.

Figure captions

Figure 1 Representative photomicrograph of a hamster coronal brain section used for immunohistochemistry analysis showing box size for analysis of lateral hypothalamus c-Fos immunoreactivity.

Figure 2 Durations (mean ± SE) of submissive/defensive, aggressive, social, and nonsocial behaviors are shown during a 5 minute conditioned defeat test with a non-aggressive intruder. Some dominant (N=11), subordinate (N=11), and empty cage controls (N=11) received three 5 minute social defeats 24 hours prior to testing. Other dominant (N=12), subordinate (N=13), and empty cage controls (N=10) were exposed to an empty cage. A single asterisk (*) indicates an effect of social status compared to dominants ($P<.05$) and a second asterisk indicates an effect of social status compared to empty cage controls ($P<.05$). A bar indicates that social status is the main effect, with no interaction between defeat condition and social status ($p<.05$).

Figure 3 Number (mean ± SE) of c-Fos immunoreactive cells measured in each brain region 65 minutes following social defeat or no defeat empty cage exposure. Sample sizes range from (N=12) to (N=9). Sample sizes differ in each group because of variation in tissue quality. A single asterisk (*) indicates an effect of social status compared to dominants ($P<.05$) and a second asterisk indicates an effect of social status compared to empty cage controls ($P<.05$). A bar indicates that social status is the main effect, with no interaction between defeat condition and social status ($p<.05$).

Figure 1

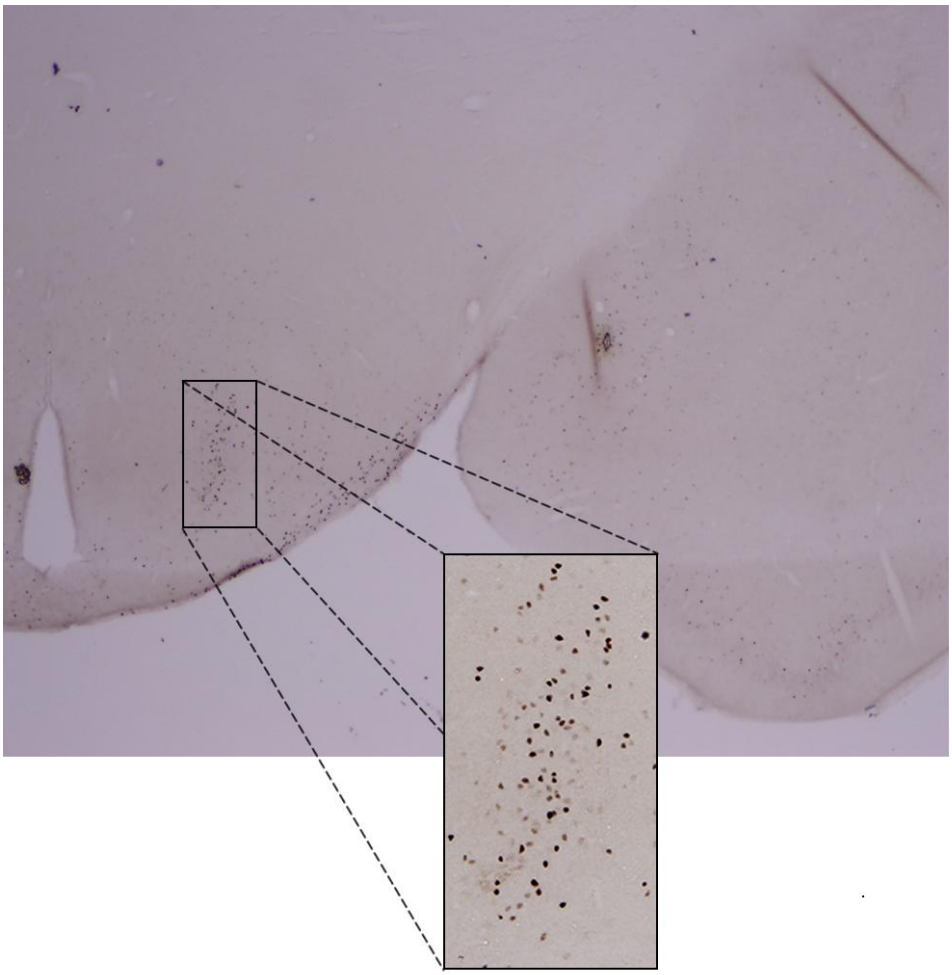


Figure 2

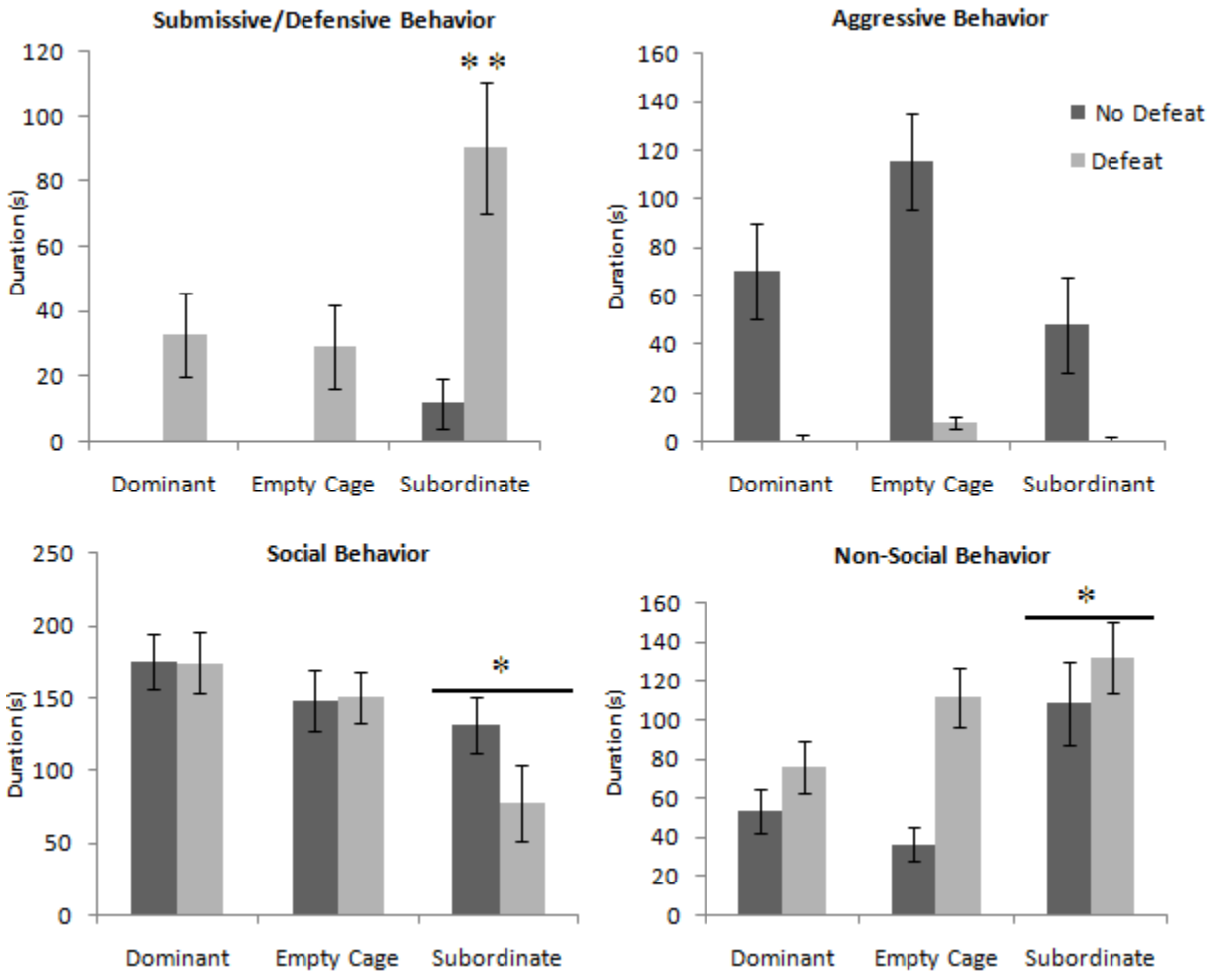
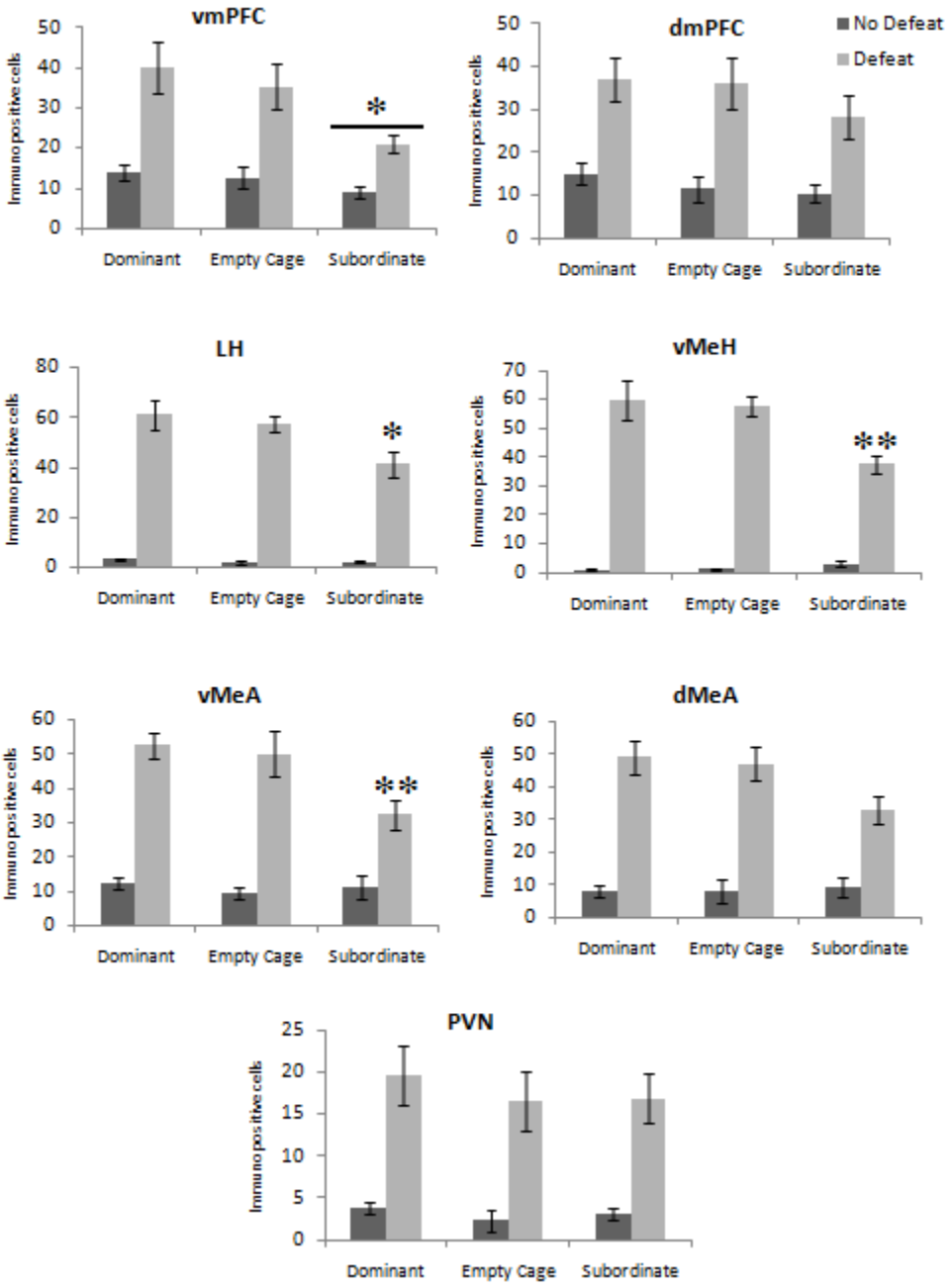

Submissive/Defensive Behavior
Duration (s)
120
100
80
60
40
20
0
* *
Dominant
Empty Cage
Subordinate
Aggressive Behavior
Duration (s)
160
140
120
100
80
60
40
20
0
No Defeat
Defeat
Dominant
Empty Cage
Subordinant
Social Behavior
Duration (s)
250
200
150
100
50
0
*
Dominant
Empty Cage
Subordinate
Non-Social Behavior
Duration (s)
160
140
120
100
80
60
40
20
0
*
Dominant
Empty Cage
Subordinate

Figure 3


vmPFC
dmPFC
No Defeat
Defeat
LH
vMeH
vMeA
dMeA
PVN
Immunopositive cells
Dominant
Empty Cage
Subordinate
*
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