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SOCIAL STATUS MODULATES RESTRAINT- INDUCED NEURAL ACTIVITY IN BRAIN REGIONS CONTROLLING STRESS VULNERABILITY

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SOCIAL STATUS MODULATES RESTRAINT- INDUCED NEURAL ACTIVITY IN BRAIN REGIONS CONTROLLING STRESS VULNERABILITY

SENIOR THESIS

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Abstract

Understanding the cellular mechanisms that control resistance and vulnerability to stress is an important step toward identifying novel targets for the prevention and treatment of stress-related mental illness. Dominant and subordinate animals have been shown to exhibit different behavioral and physiological responses to stress, with dominants often showing stress resistance and subordinates often showing stress vulnerability. We have previously found that dominant hamsters exhibit reduced social avoidance following social defeat stress compared to subordinate hamsters, although the extent to which stress resistance in dominants generalizes to non-social stressors is unknown. In this study, dominant, subordinate, and control male Syrian hamsters were exposed to acute restraint stress for 30 minutes. Brains were collected 60 minutes following restraint stress to quantify the number of c-Fos immunopositive cells in brain regions associated with stress-related behavior. Dominants and subordinates did not significantly differ in c-Fos immunoreactivity within subregions of the dorsal raphe nucleus or the paraventricular nucleus of the hypothalamus. However, compared to dominants and controls, subordinates displayed less restraint-induced c-Fos immunoreactivity in the infralimibic and prelimbic cortices. A similar trend was found in the ventral medial amygdala. These data are consistent with the reduced forebrain neural activity exhibited by subordinates following social defeat stress and suggest that subordinates exhibit a pattern of restraint-induced neural activity characteristic of stress vulnerability. However, dominant animals did not show restraint-induced changes in c-Fos immunoreactivity, suggesting that the cellular mechanisms controlling resistance to social defeat stress may not generalize to physical restraint.

Keywords

stress, dominance relationships, resilience, physical restraint, prefrontal cortex, medial amygdala, paraventricular nucleus, dorsal raphe nucleus, Syrian hamster

1. Introduction

A great deal of individual variation exists in vulnerability to the negative consequences of stressful life events. Although stress is a risk factor for a wide range of health problems, only a small portion of individuals exposed to stressful events develop stress-related psychopathology (Galea et al., 2005). Stress resilience refers to an individual's capacity to cope with adversity and avoid the negative behavioral and physiological consequences that would otherwise impair physical and psychological well-being (Luthar et al, 2006). In the past decade, several animal models have been used to investigate the neurobiological mechanisms of stress resilience. Overall, stress resilience is characterized by active processes that involve specific cellular and molecular mechanisms, rather than simply a lack of deleterious neural plasticity (Russo et al., 2012). Identifying neurobiological mechanisms controlling stress resilience is a key step toward developing novel therapeutic and preventative strategies for a range of stress-related mental illnesses.

Several animal models of stress resilience have focused on the mechanisms by which experience-dependent neuroplasticity reduces the effects of subsequent stressful events. Control over a stressful event is an experience known to generate behavioral immunization against future stress. In rodent models of learned helplessness, repeated exposure to uncontrollable tail shock has been shown to sensitize serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) (Amat et al., 2010). The heightened 5-HT

release from the DRN in response to subsequent stressors is essential for behavioral changes characteristic of learned helplessness (Amat et al., 2005). Interestingly, experience with controllable tail shocks prior to uncontrollable stress leads to neural plasticity in the prelimbic cortex (PL) that inhibits DRN activity and produces resistance to learned helplessness (Christianson et al., 2014). Furthermore, prior experience with controllable shock prevents elevated 5-HT concentrations in the DRN following subsequent social defeat stress and prevents the impaired escape latencies and social interaction deficits associated with social defeat stress (Amat et. al, 2010). These findings suggest that experience with a controllable stressor produces resistance to both inescapable tail shock and social defeat.

Other models of experience-dependent neuroplasticity and stress resilience have focused on the effects of environmental enrichment. Studies by Lehmann and colleagues have shown that an enriched housing environment prior to social defeat stress reduces defeat-induced social avoidance and increases neural activation in the PL, infralimbic cortex (IL), anterior cingulate cortex, amygdala, and nucleus accumbens—all brain regions involved in emotional regulation. Interestingly, lesions of the IL disrupted the protective effects of environmental enrichment and decreased Fos-B immunoreactivity in the nucleus accumbens and amygdala while increasing Fos-B immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN). These findings suggest that the IL plays a key role in the cellular mechanisms by which environmental enrichment promotes stress resilience (Lehmann & Herkenham, 2011). However, not all forms of experience-dependent stress resistance depend on neural activity in the medial prefrontal cortex (mPFC) or its PL and IL subregions. Voluntary exercise promotes resistance to the

exaggerated fear conditioning and impaired escape learning characteristic of learned helplessness, although exercise-dependent resistance is not lost following lesions of the mPFC (Greenwood et. al, 2013). All together, these findings suggest that multiple brain regions and neurochemical signals contribute to stress resilience.

Furthermore, separate types of resiliency training appear to generate different types of experience-dependent neuroplasticity and perhaps resistance to different stressors. We have used dominant-subordinate relationships in Syrian hamsters to investigate the mechanisms by which winning agonistic encounters reduces the behavioral and physiological effects of stress. In Syrian hamsters, acute social defeat leads to a conditioned defeat response, which is characterized by a decrease in normal territorial aggression and an increase in submissive and defensive behavior in future social interactions. Dominant hamsters exhibit a reduced conditioned defeat response, whereas subordinates exhibit an elevated conditioned defeat response compared to controls that do not have experience maintaining a dominance relationship (Morrison et al., 2012). The maintenance of dominant social status also leads to elevated defeat-induced c-Fos expression in the PL, IL, and ventral medial amygdala (vMeA; Morrison et al., 2014). On the other hand, vulnerability to the effects of social defeat stress in subordinate hamsters is associated with elevated c-Fos expression in select subregions of the dorsal raphe nucleus (DRN)(Gerhard et al., 2012). These findings suggest that neural activity in some brain regions promotes stress resistance in dominants, while neural activity in other brain regions promotes stress susceptibility in subordinates.

Despite a growing literature on the physiological underpinnings of stress resilience, there has been relatively little research directed at better understanding the

common neural correlates of coping across different types of stress. The present study aims to determine whether the cellular mechanisms controlling individual differences in responses to social defeat stress generalize to non-social stressors. Acute restraint, a form of physical stress, provides an interesting comparison to social stress. Previous studies have shown that both social defeat and restraint stress cause a significant increase in c-Fos expression within the PVN, lateral hypothalamic area, and the dorsal premammillary nucleus (Motta & Canteras, 2015). Overall, physical restraint and social defeat stress both activate neural circuits controlling entrapment and the restriction of environmental boundaries. The goal of this study is to determine whether the pattern of neural activation associated with vulnerability to social defeat stress in subordinate hamsters and resistance to social defeat stress in dominants generalizes to restraint stress.

2. Methods

2.1. Animals

Subjects (n=64) were male Syrian hamsters (*Mesocricetus auratus*) obtained from our breeding colony derived from animals purchased from Charles River Laboratories (Wilmington, MA, USA). Subjects (3-4 months of age) weighed 120-180 grams at the start of the study. All animals were individually housed in polycarbonate cages (12 cm×27 cm×16 cm) with corncob bedding, cotton nesting materials, and wire mesh tops. Food and water were available *ad libitum*. Animals were housed in a temperature-controlled colony room (21±2 °C) and kept on a 14:10-h light/dark cycle. All behavioral testing occurred during the first three hours of the animals' active period. Cages were not changed for at least one week prior to testing to allow individuals to scent mark their territory. Subjects were individually housed and handled (7-22 days) before dominant–

subordinate encounters in order to habituate them to the stress of human handling. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) and are in concordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Dominant–subordinate encounters

Animals were weight-matched in resident–intruder dyads and paired in 5-min daily social encounters for two weeks. Resident or intruder status was randomly assigned, and all encounters occurred in the resident's home cage. Dominant animals were identified by their consistent display of aggressive behavior (e.g. chasing, attacking, biting, and displaying upright and side offensive postures), and subordinates were identified by their consistent display of submissive/defensive behavior (e.g. fleeing, avoiding partner, displaying upright and side defensive posture, tail-up, and stretch-attend postures). Eight dyads did not form stable dominance relationships within five days and were excluded from the study. A separate cohort of social status control subjects was individually housed (7-22 days), not exposed to daily social encounters, and did not establish dominance relationships.

2.3. Restraint stress

Restraint stress occurred 24 hours after the final dominant-subordinate encounter. Hamsters were placed in ventilated Plexiglas restraint tubes to confine their movement for 30 min. Dominants (n=13), subordinates (n=13), and social status controls (n=11) were exposed to restraint stress. An additional group of singly-housed (7-22 days) animals (n=11) were treated as handled controls and were not exposed to restraint stress.

2.4. Enzyme-linked Immunoassay

To confirm that 30 min of physical restraint is a potent stressor for Syrian hamsters, animals were anesthetized with isofluorane and decapitated immediately following exposure to acute restraint for analysis of cortisol levels in trunk blood ($n_{restraint}$=8 ; $n_{control}$ =8). Plasma cortisol levels were analyzed using an enzyme-linked immunoassay protocol following the manufacturer's instructions (Cayman Chemical). Restraint-exposed animals indeed had significantly elevated cortisol levels compared to non-restraint controls (Appendix A). These preliminary results confirm that physical restraint initiates a neuroendocrine stress response in Syrian hamsters.

2.5 c-Fos immunohistochemistry

Sixty minutes following restraint stress, animals were anesthetized with isoflurane and transcardially perfused with 100 ml of 0.1 M phosphate buffered saline (PBS), followed by 100 ml of 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde for 24 h, transferred to 0.1 M PBS/30% sucrose solution for 48 h, and then stored in cryoprotectant solution until further use (all at 4 °C).

A vibrating microtome was used to slice brains into 30 μm consecutive coronal sections. Free-floating sections were stored in cryoprotectant (4 °C), and every third section was processed for c-Fos protein immunohistochemistry. To remove unbound reagents, sections were washed five times with PBS + 0.2% Triton before each incubation cycle (always at room temperature). Sections were incubated for 25 min in 0.3% hydrogen peroxide and methanol solution. Sections were then incubated with 0.5% goat serum (GS) in PBS + 0.2% Triton for 25 min before being incubated for 24 h in rabbit anti-c-Fos polyclonal antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS + 0.2% Triton. This was followed with five washes with PBS + 0.2% Triton,

and a 60 min incubation in biotinylated secondary anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) with 0.5% GS and 0.2% Triton. Sections were then incubated with avidin-biotin-complex reagent (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and 0.2% Triton for 1 h. The final product was visualized via a peroxidase reaction involving a 15-min incubation with 3,3′-diaminobenzidine (DAB tablet, Sigma–Aldrich, St. Louis, MO) and nickel dissolved in PBS. Sections were washed five times with PBS and 5 times with distilled H_2O prior to mounting on microscope slides. After air-drying, sections were dehydrated using a series of alcohols, cleared with Citrisolv and coverslipped using DPX mountant (Sigma–Aldrich, St. Louis, MO). Tissue from all animals was processed simultaneously for each brain region.

Images were taken at 10× magnification under an Olympus BX41 microscope. The number of c-Fos immunopositive cells was quantified in the PL, IL, dorsal MeA (dMeA), vMeA, and PVN. Because of heterogeneity within the DRN, it was divided into six subregions which include rostral dorsal DRN (rdDRN), rostral ventral DRN (rvDRN), rostral lateral DRN (rlDRN), caudal dorsal DRN (cdDRN), caudal ventral DRN (cvDRN), and caudal lateral DRN (clDRN). Background immunoreactivity was calculated for each image by quantifying optical density on regions of the tissue without c- Fos staining. Immunopositive cells were defined as those with nuclei stained 1.6-1.8 times darker than the specific background immunoreactivity for each image. MCID Core image analysis software (InterFocus Imaging, Cambridge, England) was used to automatically quantify the number of c-Fos immunopositive cells. C-Fos immunoreactivity was also manually quantified in a subset of sections, and software parameters were adjusted to reach 90% agreement with manual cell counts.

Photomicrographs were captured within defined boxes for each brain region, and c-Fos immunopositive cells were quantified from the images (Appendix B). For every brain region, three to six sections were quantified per individual. Sample sizes varied per brain region due to technical difficulties with tissue sectioning or poor staining quality.

2.6. Open Field Testing

Open field testing was conducted to measure anxiety-like behavior following restraint stress. Previous studies have demonstrated that social isolation in a brightly lit, novel, and unprotected arena is a stressful experience for rodents (File, 1980; Prut & Belzung, 2003). In rats and mice, increased time spent in the thigmotaxic zone versus the center zone of the arena is indicative of heightened levels of anxiety (Prut and Belzung, 2003). Although a common test of anxiety-like behavior in rats and mice (Hall, 1934; Christmas AJ, Maxwell, 1970), the open field is a less well-established measure of anxiety in hamsters.

Two preliminary studies were performed to optimize our open field testing for hamsters (Appendix C). First, animals were exposed to 30 min physical restraint and then placed in the open-field arena 10 min and 24 hrs later (Appendix D). These data indicated no significant difference in open field behavior between animals exposed to restraint and non-restraint controls. Second, non-restraint animals were tested in an open field arena under either high lux or low lux conditions. These data indicated that animals show greater locomotion in the low lux condition compared to the high lux condition (Appendix E).

Following these preliminary studies, animals were placed in open-field arena 24 hrs after 30 min of physical restraint stress and tested under low-lux conditions ($n_{restraint}$=6; $n_{control}$=11). The first 15 min of activity in the arena was recorded digitally and the total distance traveled (cm) and time spent in the thigmotaxic zone (sec) were later scored manually using EthoVision XT 9.0 video tracking software (Noldus Information Technology).

3.7. Statistical analysis

For immunohistochemical data, two separate two-way ANOVAs were performed. Social status (3 levels between-subjects factor) and brain region (11 levels within-subjects factor) were analyzed in a two-way repeated measures ANOVA, and one-way ANOVAs with LSD tests were used for *post-hoc* analysis. All restraint animals were pooled into a single group so that restraint (2 levels between-subjects factor) and brain regions (11 levels between-subjects factor) were analyzed in a two-way repeated measures ANOVA. Independent t-tests were used as *post-hoc* tests. Levene's test and Mauchy's test of sphericity were used to adjust for any violations in the homogeneity of variance assumption. For open field testing, independent t-tests were used to evaluate any significant mean differences between groups. All statistical tests were two-tailed, the alpha level was set at $p < 0.05$, and data are presented as mean ± S.E.

3. Results

3.1 Dominant-subordinate encounters

Dominant animals displayed stable levels of aggressive behavior during the two weeks of encounter, and subordinates maintained high levels of submissive and defensive behavior. As with our previous studies, dominance relationships remained stable once formed (i.e. dominants did not become subordinates and vice versa) (Morrison et. al, 2012).

3.2 c-Fos immunohistochemistry

To investigate the effects of restraint stress on c-Fos immunoreactivity, dominant, subordinate, and social status controls were pooled and compared to non-restraint controls. We tested for the effects of restraint stress within each brain region using a series of independent t-tests. Physical restraint produced a significant increase in c-Fos immunoreactivity in the IL ($t_{(35)}$=4.5, p= 0.000), PL ($t_{(35)}$=4.9, p= 0.000), vMeA ($t_{(38)}$=6.6, p= 0.000), dMeA ($t_{(37)}$=8.2, p= 0.000), and PVN ($t_{(27)}$=9.1, p= 0.000) (Figure 1). Restraint also increased c-Fos immunoreactivity within the rvDRN ($t_{(27)}$=3.4, p= 0.002), but not within other subregions of the DRN, including rdDRN, rlDRN, cdDRN, cvDRN, and clDRN (p> 0.05, Figure 2).

To investigate the effects of social status, we compared restraint-induced c-Fos immunoreactivity in dominants, subordinates, and social status controls. Repeated-measures ANOVA revealed a significant main effect of social status ($F_{(2)}$= 6.515, p=0.025), brain region ($F_{(10)}$=13.905, p=0.000), as well as an interaction of social status with brain region ($F_{(20)}$=2.512, p=0.002). One-way ANOVA and LSD *post-hoc* tests were used to further investigate the effect of social status within each brain region. There was a significant difference in c-Fos expression between groups in the IL ($F_{(2, 27)}$=6.44,

p=0.005). Specifically, subordinates had significantly fewer c-Fos immunopositive cells than both dominants and social status controls (*p*=0.018 and *p*=0.002, respectively, Figure 1a). There was also a significant difference in c-Fos expression between groups in the PL ($F_{(2, 27)}$=4.94, *p*=0.015). Specifically, subordinates had significantly fewer c-Fos immunopositive cells than both dominants and social status controls (*p*=0.009 and *p*=0.017, respectively, Figure 1b). There was also a notable trend towards an effect of social status on c-Fos expression within the vMeA ($F_{(2, 29)}$=2.554, *p*=0.095). Subordinates displayed a tendency towards having fewer c-Fos immunopositive cells than both dominants and social status controls within the vMeA (*p*=0.063 and *p*=0.065, respectively, Figure 1c). There was also a significant difference in c-Fos expression between groups in the clDRN ($F_{(2, 22)}$=4.392, *p*=0.025). Social status controls had significantly more c-Fos immunopositive cells than both dominants and subordinates (*p*=0.012 and *p*=0.033, respectively, Figure 2e). Lastly, there was a trend towards a between-group difference in c-Fos expression within the rvDRN ($F_{(2, 22)}$=2.914, *p*=0.075). Subordinates had more c-Fos immunopositive cells than dominants in the rvDRN (*p*=0.026, Figure 2b).

3.3. Open field testing

Restraint-induced anxiety in hamsters was assessed via analysis of locomotion and time spent in the thigmotaxic zone of the open field arena. There were no significant differences in total distance moved between controls and restraint animals ($p > 0.05$). Restraint-exposed animals spent significantly less time in the thigmotaxic zone than non-restraint controls in their first minute of exposure to the open field ($t_{(15)}$=3.25, *p*=0.0054,

Figure 3a). The avoidance of the thigmotaxic zone disappeared after several minutes of testing (Figure 3b-c).

4. Discussion

In this study, we characterized neural activation following restraint stress in dominant and subordinate hamsters. Dominant and subordinate animals did not significantly differ in c-Fos expression within the PVN, suggesting that dominance status does not alter restraint-induced activation of the HPA-axis. However, dominance status does modulate neural activation in brain regions that control stress-related behavior. Subordinate animals showed decreased neural activation in the IL and PL following restraint stress compared to dominants and social status controls. A similar trend was observed in the vMeA, although it did not reach statistical significance. Social status controls displayed significantly greater neural activation than both dominants and subordinates in the clDRN and than dominants in the rvDRN. These results suggest that there are social status-dependent differences in neural activation following restraint stress.

The IL inhibits the expression of fear memories through its projections to GABAergic neurons within the basolateral amygdala (BLA) (Cho et al., 2013). These projections serve to inhibit the amygdala and promote the extinction of conditioned fear (Sierra-Mercado et al., 2011). Also, neural activity in the IL is necessary for resistance to the effects of chronic social defeat in mice (Lehmann & Herkenham, 2011). The PL sends projections to lateral regions of the DRN, which function to inhibit serotonergic cells (Vertes, 2003). Activity of this PL-to-DRN neural circuit is necessary for resistance to learned helplessness in rats previously exposed to controllable stress (Baratta et al.,

2009). In Syrian hamsters, pharmacological blockade of the vmPFC, including the IL and PL, prevents resistance to the effects of acute social defeat in dominant hamsters (Morrison et al., 2013). The current findings are partly consistent with our previous studies on status-dependent changes in c-Fos immunoreactivity following social defeat stress. The reduced neural activity in the IL and PL shown by subordinate animals suggests that they may be less capable of coping with physical restraint. Whereas dominant animals show elevated neural activity in the IL and PL following social defeat stress (Morrison et al., 2014), this pattern of neural activity does not generalize to restraint stress.

The medial amygdala integrates olfactory information and hormonal signals and serves a key role in mediating social behavior. The vMeA has efferent projections to the ventral striatum, ventral tegmental area, hypothalamic defense system, hypothalamic reproductive system, and accessory olfactory bulbs (Pardo-Bellver et al., 2012). It thereby modulates motivated, defensive, and reproductive behavior. Similarly, the dMeA mediates reward, defensive, and reproductive behavior through its projections to the ventral striatum, ventral tegmental area, hypothalamic defense system, and the hypothalamic reproductive system (Pardo-Bellver et al., 2012). The vMeA and dMeA both project to the olfactory cortex, hippocampus, and several subregions of the amygdala and thereby control fear processing, anxiety, and emotional and contextual learning (Pardo-Bellver et al., 2012). While the vMeA and dMeA have many similar functions, the vMeA is known to play a greater role in sensory modulation, while the role that the dMeA plays is more pertinent to reproductive behavior (Pardo-Bellver et al., 2012). In this study, we found a trend for subordinates to show less neural activity than

dominants in the vMeA following restraint stress. This parallels previous findings demonstrating that subordinates had significantly fewer c-Fos immunopositive cells in the vMeA than dominants following social defeat (Morrison et al., 2012). These findings suggest that status-dependent differences in vMeA activity may not be limited to social stress and that the vMeA may play a role in how subordinates respond to physical stress.

The DRN is a heterogeneous structure with numerous subregions that send axonal projections to different areas of the brain (Hale & Lowry, 2011). Uncontrollable stress has been shown to increase the activity of neurons in the caudal portions of the DRN (Maier & Watkins, 2005; Grahn et al., 1999). On the other hand, social defeat stress leads to increased neural activation in rostral ventral portions of the DRN (Cooper et al., 2009). Susceptibility to the effects of social defeat is also associated with elevated DRN activity. We have previously shown that subordinate hamsters have significantly greater c-Fos expression in the rvDRN and cvDRN following social defeat, compared to dominants and social status controls (Gerhard et al., 2012). In the present study, social status controls displayed greater neural activation than dominants and subordinates in the clDRN and than dominants in the rvDRN. These findings suggest that the elevated c-Fos expression in subordinates that is associated with their susceptibility to social defeat stress does not generalize to physical stress. Furthermore, the decrease in restraint-induced c-Fos expression in both dominants and subordinates, compared to social status controls, is consistent with the possibility that daily agonistic encounters habituate the DRN to mild stress and thereby lead to reduced neural activity following acute restraint.

We found that acute restraint stress produced a significant increase in plasma cortisol levels in Syrian hamsters. Corticotrophin-releasing hormone (CRH) neurons in

the PVN initiate the neuroendocrine stress response, and the elevated c-Fos expression within the PVN of restraint-exposed animals indicates that physical restraint leads to robust activation of the HPA axis. However, we found no effect of social status on restraint-induced neural activity in the PVN. In contrast, subordinate rats display dysregulated HPA axis activity following chronic social stress. For example, subordinate male rats housed in a visible burrow system not only displayed higher basal corticosterone levels, but 40% of subordinates also had a blunted corticosterone response to restraint stress (Blanchard et al., 1994). The mild stress experienced by subordinate hamsters during the maintenance of their dominance relationship is likely not severe enough to alter HPA axis function.

Overall, the reduced neural activity in subordinates in brain regions regulating coping with stress suggests that they may be susceptible to the effects of restraint stress, which suggests that stress susceptibility in subordinates is domain-general. On the other hand, the finding that dominants do not show elevated neural activity in brain regions supporting coping— whereas they do following social defeat stress— suggests that stress resistance in dominants is domain-specific.

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6. Figures

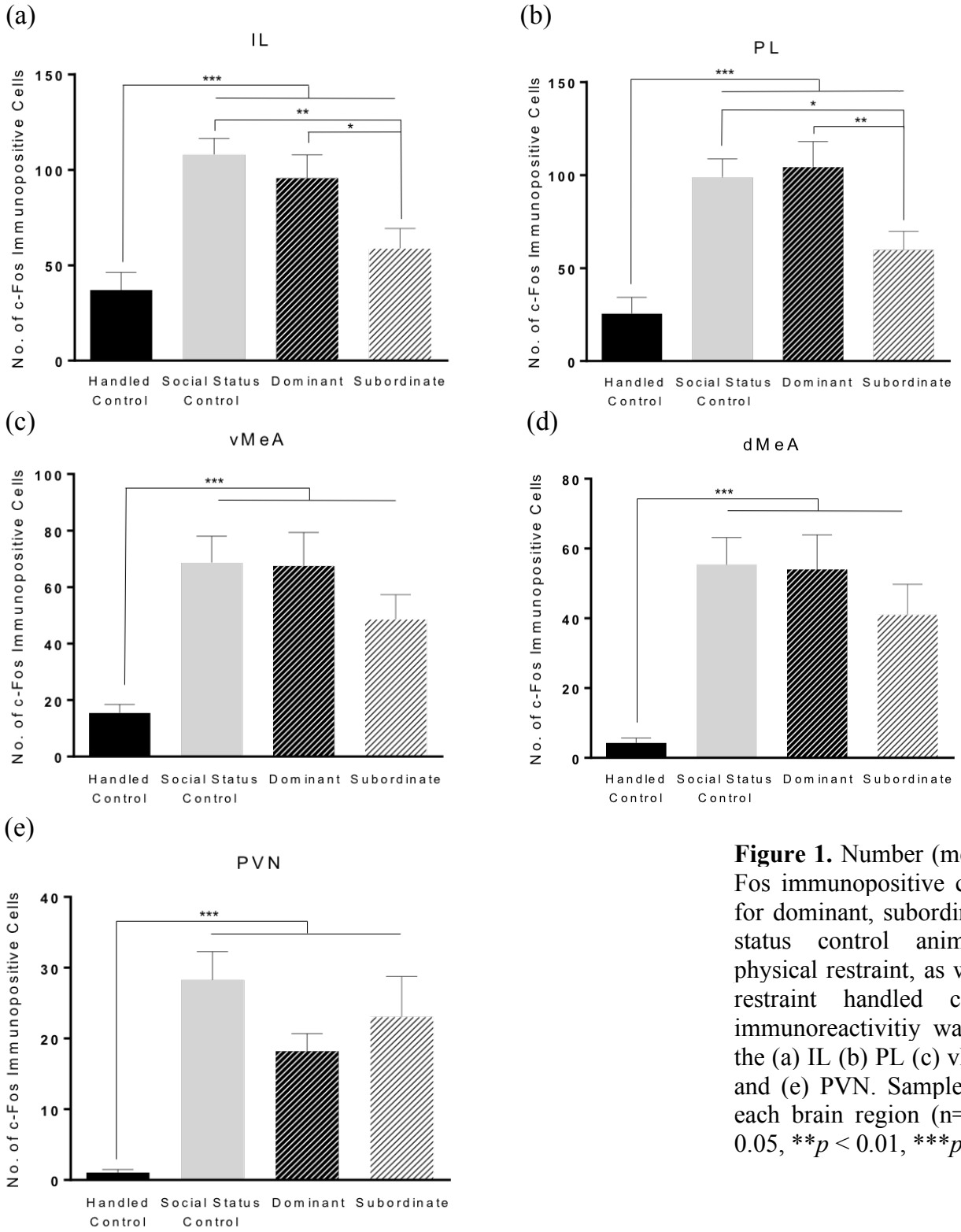


Figure 1. Number (mean ± SE) of c-Fos immunopositive cells are shown for dominant, subordinate, and social status control animals following physical restraint, as well as for non-restraint handled controls. c-Fos immunoreactivitiy was quantified in the (a) IL (b) PL (c) vMeA (d) dMeA and (e) PVN. Sample sizes differ in each brain region (n= 9- 13) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

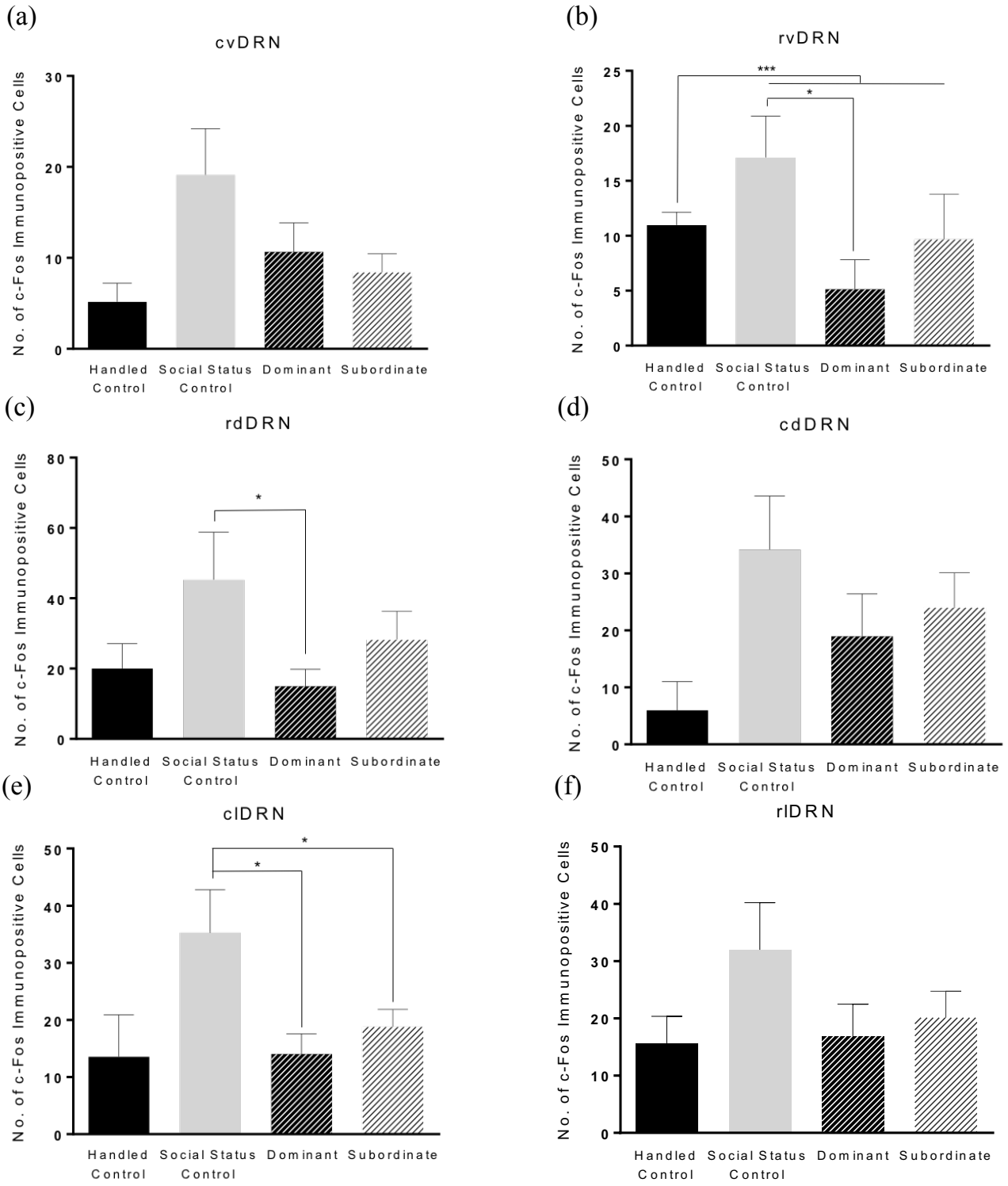


Figure 2. Number (mean ± SE) of c-Fos immunopositive cells are shown for dominant, subordinate, and social status control animals following physical restraint, as well as for non-restraint handled controls. c-Fos immunoreactivitiy was quantified in the (a) caudal ventral, (b) rostral ventral, (c) rostral dorsal, (d) caudal dorsal, (e) caudal lateral, and (f) rostral lateral DRN following restraint stress. Sample sizes differ in each brain region (n= 6-9). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

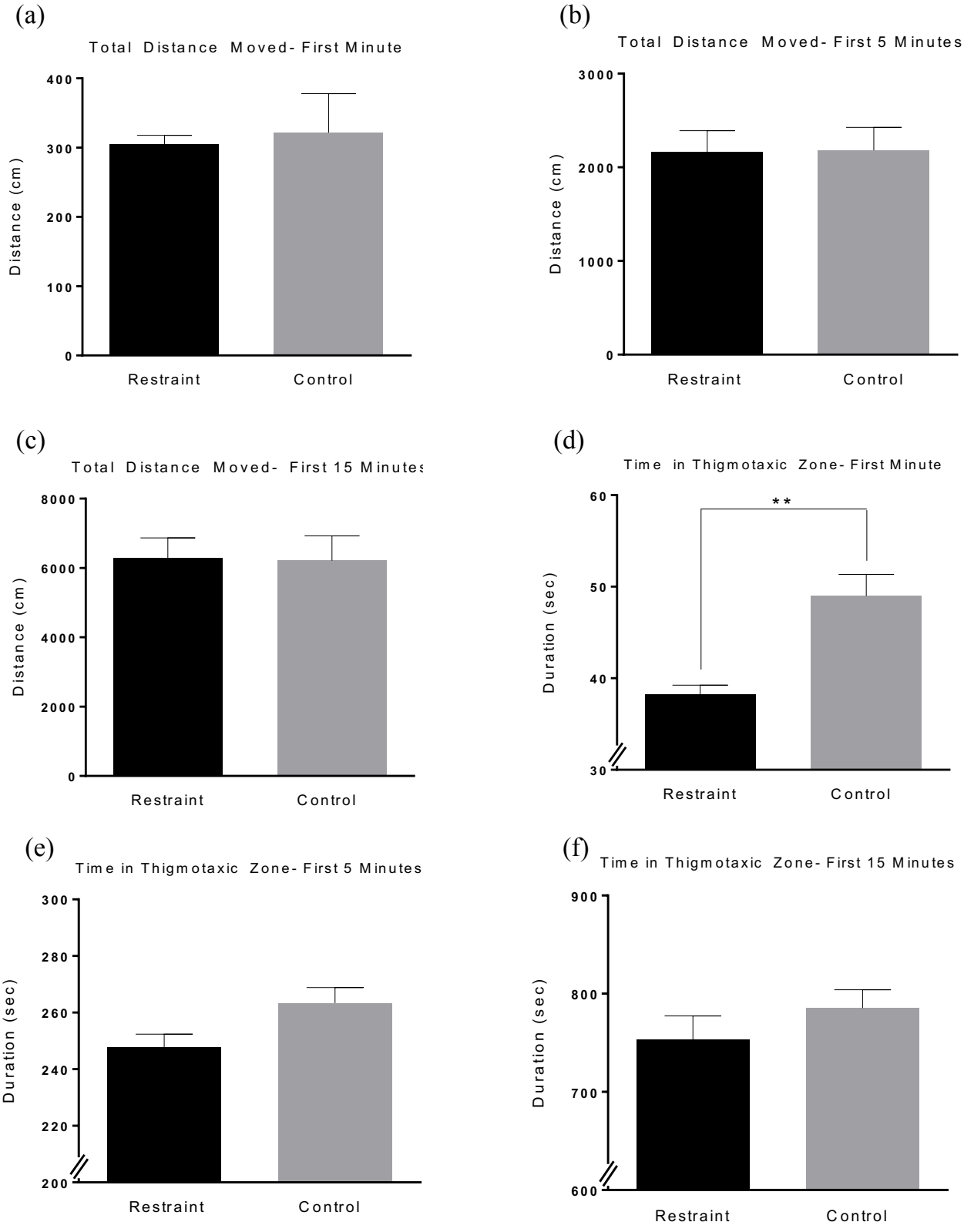
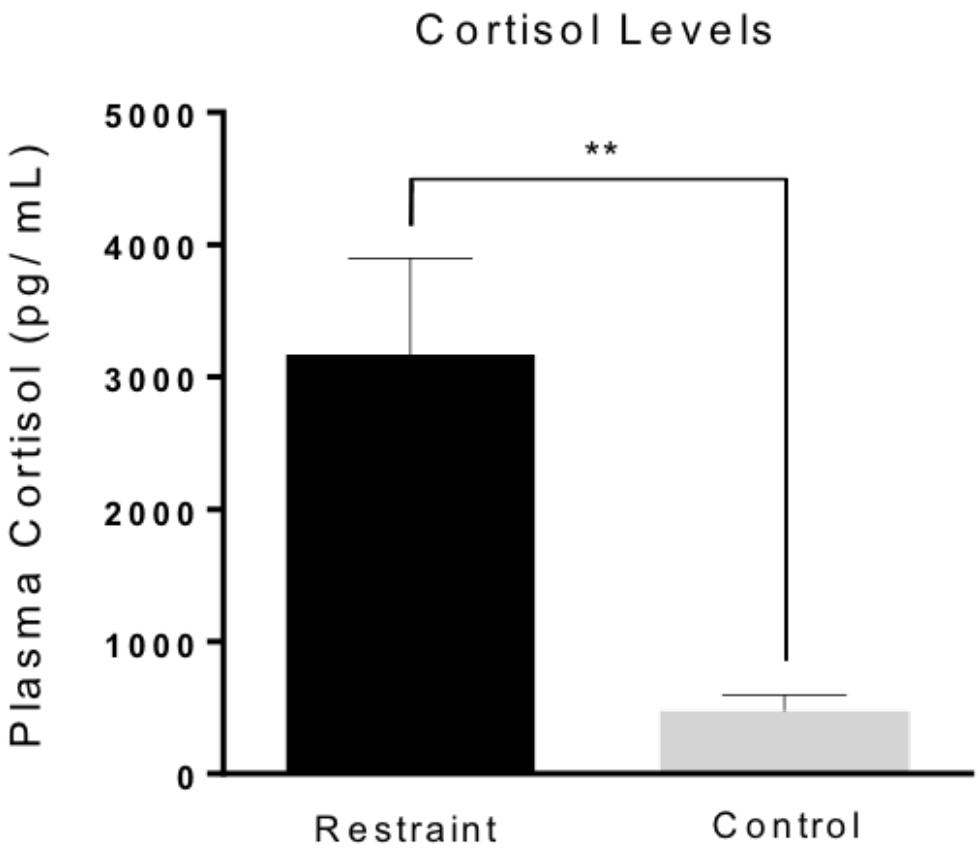


Figure 3. Figures a-c show the total distance (cm) moved in the open field by control and restraint animals 24 hours after physical stress exposure, as broken down into (a) the first minute (b) the first five minutes and (c) the first fifteen minutes of exposure to the open field arena. There were no significant differences in the total distance moved between controls and

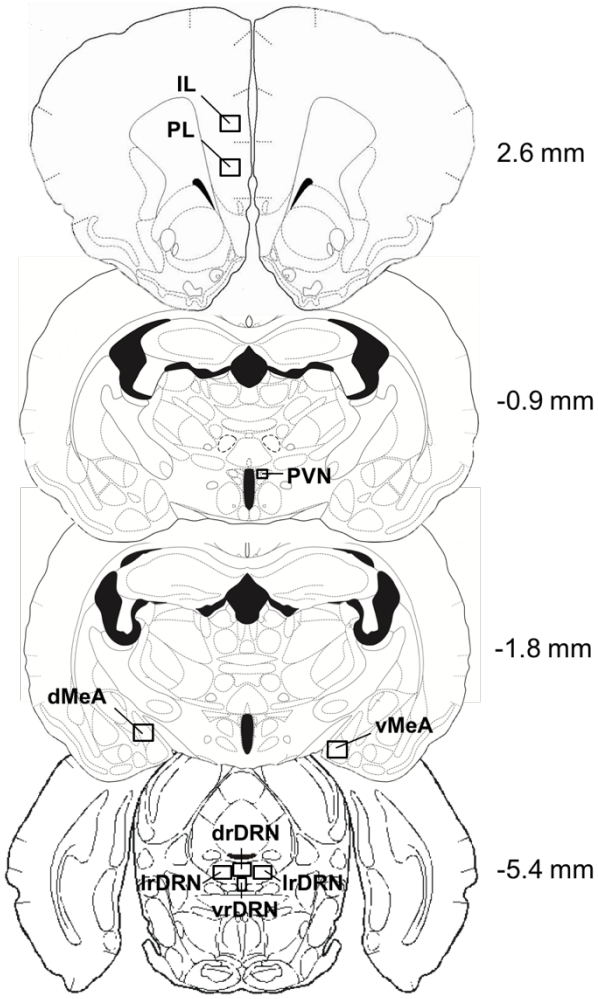
restraint animals. Figures d-f show the total time (sec) spent in the thigmotaxic zone of the open field arena by control and restraint animals 24 hours after physical stress exposure, as broken down into (d) the first minute (e) the first five minutes and (f) the first fifteen minutes of exposure to the open field arena. Controls spent significantly more time in the thigmotaxic zone than restraint animals in the first minute of exposure to the open field ($t_{(15)}$=3.25, p=0.0054), but this difference gradually disappeared as the test continued. ** indicates p <0.01.

7. Appendix

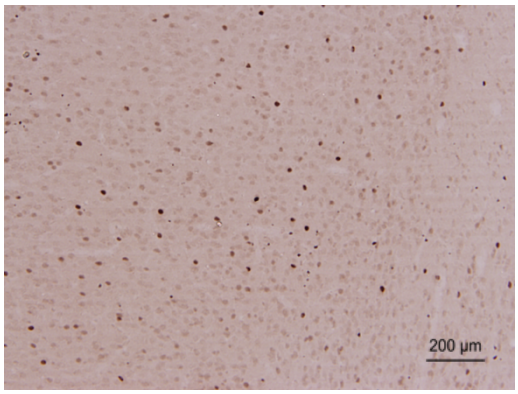


Appendix A. Hamsters exposed to 30 min restraint stress (n=8) show significantly higher plasma cortisol levels compared to non-restraint controls (n=8) ($t_{(14)}$=3.64, p= 0.003). ** indicates p <0.01.

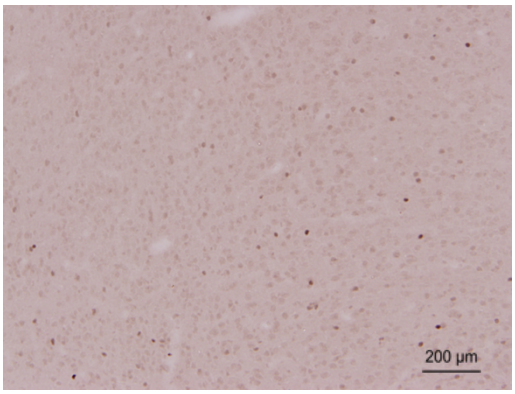
(a)



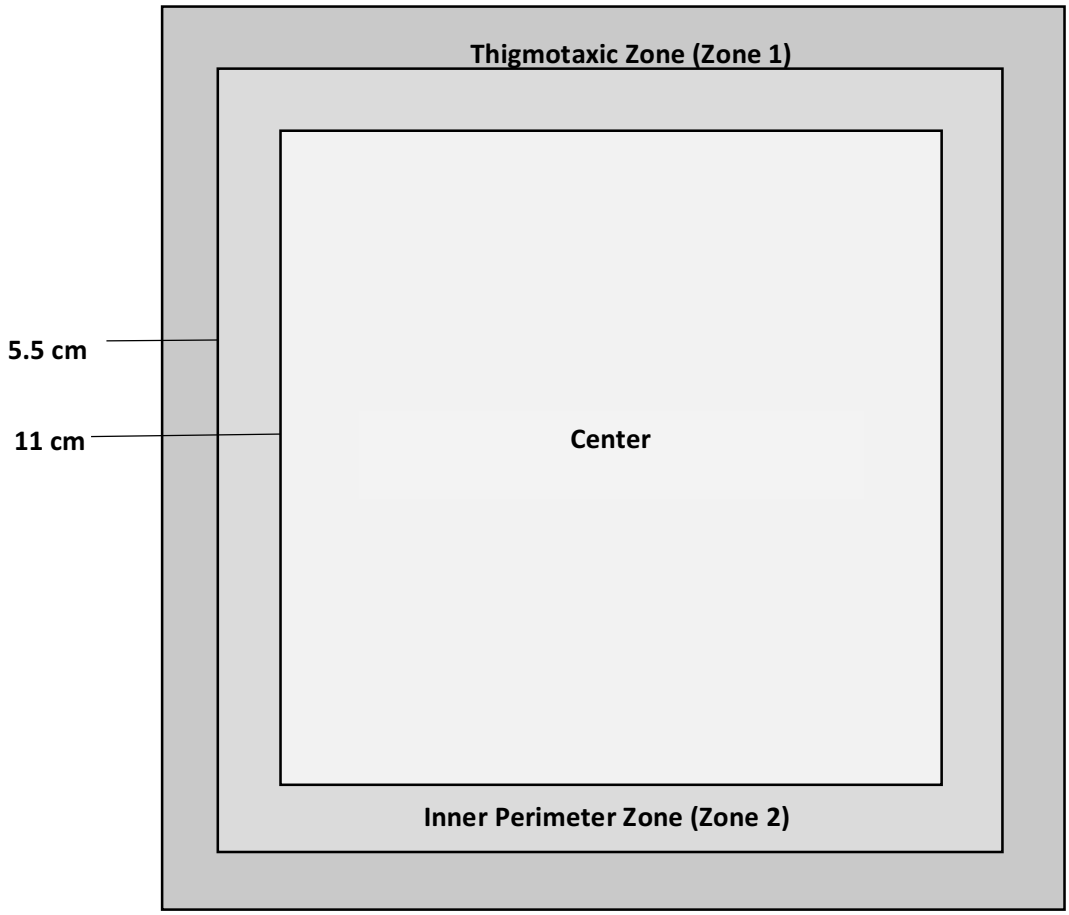
(b)



(c)

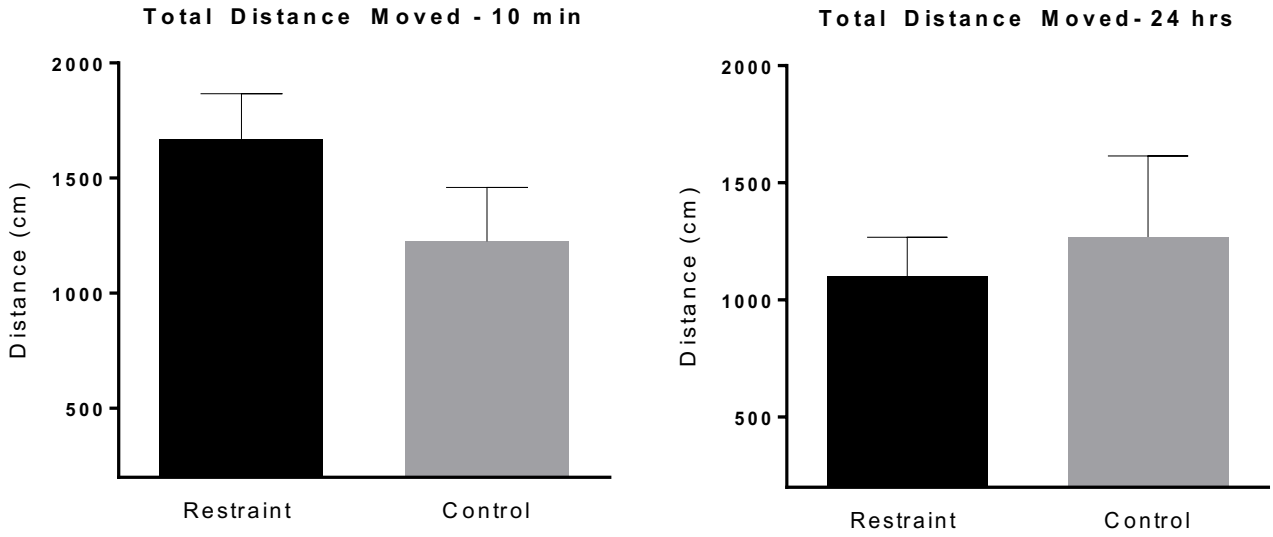


Appendix B. (a) The stereotaxic atlas images indicate the location of brain regions selected for c-Fos quantification (Morin and Wood, 2001). Although some boxed regions are indicated unilaterally within the respective brain region, images were collected from both hemispheres and averaged for analysis. Values on the right signify distance from bregma point on the skull. The box sizes used for quantification are as follows (width×height): 870 µm×660 µm (PL, IL, dMeA, vMeA); 439 µm×330 µm (PVN); x 500 µm×300 µm (lcDRN, lrDRN); x 500µm×300 µm (dcDRN, drDRN); 250 µm× 300 µm (vcDRN, vrDRN). (b) Representative photomicrograph of the IL from a dominant animal used in c-Fos quantification. (c) Representative photomicrographs of the IL from a subordinate animal used in c-Fos quantification. Brown dots indicate c-Fos immunopositive nuclei.

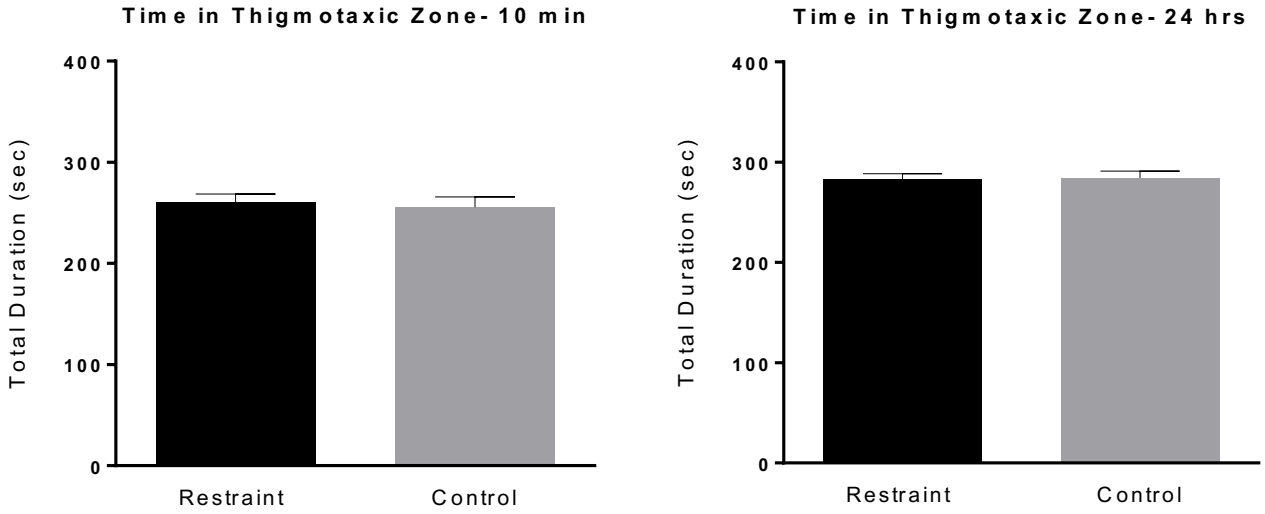


Appendix C. Diagram of the open field arena. The thigmotaxic zone was considered to be the region within 5.5cm of the arena walls.

(a)

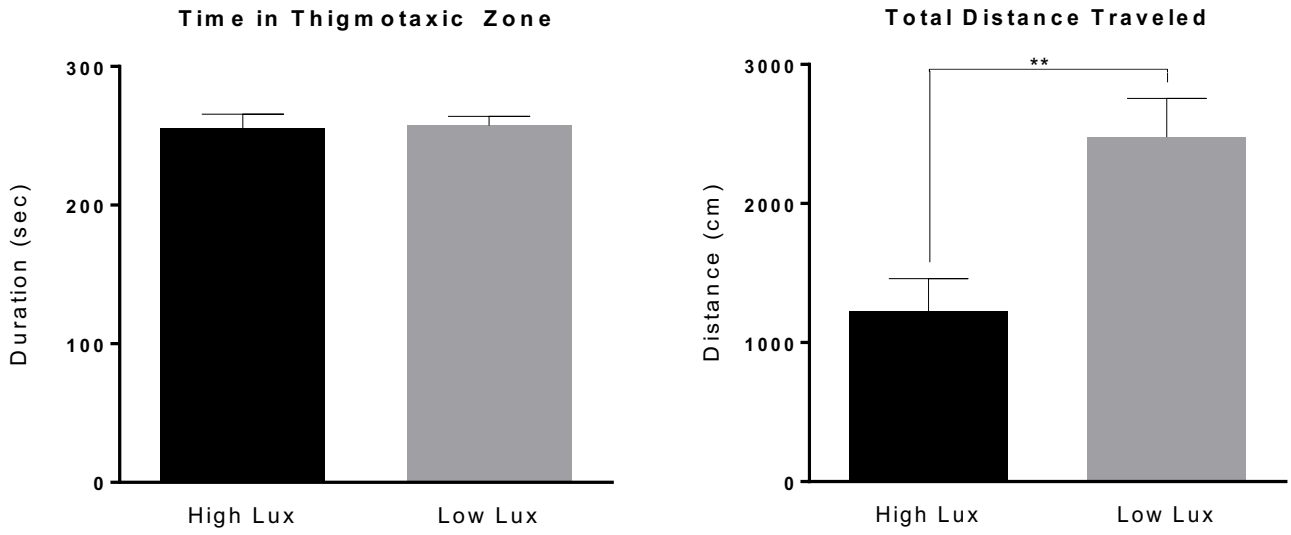


(b)



Appendix D. In optimizing the open field protocol for hamsters, restraint-exposed (n=6) and non-restraint (n=5) animals were first placed in the open-field arena (80 cm x 80 cm x 40 cm) ten minutes following restraint, and again 24 hours post-restraint under high lux conditions. High lux conditions were defined as testing with the overhead lights on. Animals spent 5 min in the arena and total distance traveled (cm) and time spent in the thigmotaxic zones (sec) were later scored via Noldus Ethovision. There were no significant differences between restraint and non-restraint animals in (a) total distance moved or (b) time spent in the thigmotaxic zone. Some animals showed higher rates of self-grooming and inconsistent ambulation following restraint stress and, therefore, all future testing occurred 24 hrs after restraint stress.

(a) (b)



Appendix E. In a second pilot experiment, non-restraint animals were exposed to the open field arena for 5 min under high lux (n=5) and low lux (n=7) conditions. Low lux conditions were defined as testing with lamp (25 watt bulb) pointed toward the floor. Animals in the low lux condition traveled a greater distance compared to those in the high lux condition ($t_{(10)}$= 3.230, p= 0.009). Future testing therefore occurred under low lux conditions.