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Dominance status alters restraint-induced neural activity in brain regions controlling stress vulnerability

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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. In Syrian hamsters, dominant and subordinate animals exhibit different behavioral and physiological responses to social defeat stress, with dominants showing stress resistance and subordinates showing stress vulnerability. We previously found that dominant and subordinate hamsters show different levels of defeat-induced neural activity in brain regions that modulate coping with stress, although the extent to which status-dependent differences in stress vulnerability generalize to non-social stressors is unknown. In this study, dominant, subordinate, and control male Syrian hamsters were exposed to acute physical restraint for 30 minutes and restraint-induced c-Fos immunoreactivity was quantified in select brain regions. Subordinate animals showed less restraint-induced c-Fos immunoreactivity in the infralimbic (IL), prelimbic (PL), and ventral medial amygdala (vMeA) compared to dominants, which is consistent with the status-dependent effects of social defeat stress. Subordinate animals did not show increased c-Fos immunoreactivity in the rostroventral dorsal raphe nucleus (rvDRN), which is in contrast to the effects of social defeat stress. These findings indicate that status-dependent changes in neural activity generalize from one stressor to another in a brain region-dependent manner. These findings further suggest that while some neural circuits may support a generalized form of stress resistance, others may provide resistance to specific stressors.

Keywords

Social dominance; Resilience; Coping; Anxiety; Medial amygdala; Dorsal raphe nucleus

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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 [1, 2]. 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 [3]. In the past decade, several animal models have been used to investigate the cellular and molecular mechanisms of stress resilience. This body of work indicates that stress resilience is characterized by active processes involving specific cellular and molecular mechanisms, rather than simply a lack of deleterious behavioral and physiological responses [4]. An improved understanding of the neurobiological mechanisms underlying stress resilience can lead to plausible targets for the treatment of a wide range of stress-related mental illnesses.

Several environmental factors that produce experience-dependent neuroplasticity and reduce the effects of subsequent stressful events have previously been identified. The opportunity to exert behavioral control over a stressor by terminating its occurrence blunts the behavioral and neurochemical consequences of an aversive event and prevents the heightened anxiety and impaired escape behavior characteristic of learned helplessness [5]. Rats exposed to uncontrollable tail shocks show elevated neural activity of serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN), which is essential for the development of learned helplessness [6]. Interestingly, animals that experience controllable tail shocks exhibit neural plasticity in the prelimbic cortex (PL) that inhibits DRN activity and prevents the development of learned helplessness when they are later faced with uncontrollable stress [7, 8]. Furthermore, prior experience with controllable shock prevents elevated 5-HT concentrations in the DRN after social defeat and prevents the impaired escape latencies and social interaction deficits associated with social defeat stress [9]. Together, these findings suggest that experience with a controllable stressor produces a generalized stress resistance to both uncontrollable tail shock and social defeat stress.

Environmental enrichment is another factor that modulates behavioral and physiological responses to stress and thereby promotes stress resistance. Exposure to an enriched housing environment prior to social defeat stress reduces defeat-induced social avoidance and increases FosB/ FosB immunoreactivity in the PL, infralimbic cortex (IL), anterior cingulate, and nucleus accumbens [10]. Interestingly, lesions of the IL disrupt the protective effects of environmental enrichment and decrease FosB/ FosB immunoreactivity in the PL, anterior cingulate, and nucleus accumbens. These findings suggest that the IL plays a key role in the cellular mechanisms by which environmental enrichment promotes stress resilience. However, not all forms of experience-dependent stress resistance depend on neural activity in the medial prefrontal cortex (vmPFC) 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 vmPFC [11]. All together, these findings suggest that multiple brain regions and neurochemical signals contribute to stress resilience.

Different types of resiliency training appear to generate varied forms of experience-dependent neuroplasticity and, perhaps, resistance to distinct stressors. We have used dominance relationships in Syrian hamsters to investigate the mechanisms by which social status reduces the behavioral and physiological effects of subsequent social defeat 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 [12]. A great deal of information indicates that the conditioned defeat response is an ethologically relevant form of conditioned fear. Several cellular and molecular mechanisms that regulate synaptic plasticity within the basolateral amygdala (BLA) are critical for the acquisition of conditioned defeat, including the activity of NMDA receptors, cyclic AMP response element binding protein, brain-derived neurotrophic factor, and activity-regulated cytoskeletal-associated protein [13–16]. In addition, neurotransmission in several cortical, limbic, and hindbrain regions modulate both the acquisition and expression of conditioned defeat, including the BLA, central amygdala (CeA), medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), lateral septum (LS), nucleus accumbens (NAcc), ventral hippocampus (vHP), vmPFC, and DRN [17–23]. 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 [24]. The maintenance of dominant social status also leads to elevated defeat-induced c-Fos immunoreactivity in the PL, IL, and ventral MeA (vMeA) [25]. On the other hand, vulnerability to the effects of social defeat stress in subordinate hamsters is associated with elevated c-Fos immunoreactivity in select subdivisions of the DRN [26]. These findings suggest that neural activity in certain limbic brain regions supports stress resistance in dominant hamsters, while neural activity in hindbrain regions such as the DRN promotes stress susceptibility in subordinates.

Despite growing literature on the cellular and molecular mechanisms of stress resilience, there has been relatively little effort focused on better understanding the common neural correlates of coping with different types of stressors. The present study is focused on whether the cellular mechanisms controlling status-dependent differences in responses to social defeat stress generalize to acute physical restraint. Previous research indicates these stressors share some commonalities. Social defeat and physical restraint increase c-Fos expression within the lateral hypothalamus and the dorsal premammillary nucleus, which is thought to reflect activation of septo-hippocampal circuits that encode entrapment and restriction of environmental boundaries [27]. The goal of the present study is to determine whether the pattern of neural activation associated with vulnerability and resistance to social defeat stress in subordinate and dominant hamsters generalizes to restraint stress.

2. Methods

2.1 Animals

Male Syrian hamsters (*Mesocricetus auratus*) were obtained from our breeding colony derived from animals purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were 3–4 months of age and 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 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 Dominance encounters

Twenty-six animals were weight-matched and assigned to resident–intruder dyads. Resident or intruder status was randomly assigned and animals were exposed to daily dyadic encounters for two weeks. All encounters occurred in the resident's home cage and dominance status was found to be unrelated to residency status. Hamsters initially greet and sniff each other before one animal initiates aggression and the other responds with submissive and defensive behavior. Dominant animals are identified by their consistent display of aggressive behavior (e.g. chasing, attacking, biting, and displaying upright and side offensive postures), and subordinates are identified by their consistent display of submissive and defensive behavior (e.g. fleeing, avoiding partner, displaying upright and side defensive posture, tail-up, and stretch-attend postures). While mild aggressive postures are often observed during the first encounter, attacks from the dominant animal and a clear asymmetric pattern of agonistic behavior may not emerge for up to five days. We use 10-min encounters to encourage social investigation prior to the formation of a dominance relationship. After a dominance relationship is formed, we use 5-min encounters to maintain the relationship and prevent wounding of the subordinate. Using this model, we have found that dominance relationships remain stable for at least two weeks [24, 28]. Dyads that failed to establish a clear and stable dominance relationship by the fifth encounter were excluded from analysis (n = 4 dyads). To control for social status, a separate cohort of animals was individually housed and not exposed to daily dyadic encounters.

2.3 Restraint stress

Twenty-four hours after the final dominance encounter, hamsters were placed in ventilated Plexiglas restraint tubes to confine their movement. Dominants (n=13), subordinates (n=13), and social status controls (n=11) were exposed to restraint stress for 30 minutes. An additional cohort of animals (n=11) was not exposed to restraint stress or daily dominance encounters, and these animals were considered handled controls.

Because Syrian hamsters are burrowing animals with a high degree of flexibility, the effectiveness of a physical restraint stressor should be verified. Although some investigators have shown that acute physical restraint increases plasma cortisol in Syrian hamsters [29], others report that hamsters fall asleep in restraint tubes and use high intensity air puff to increase the stressful consequences of physical restraint [30, 31]. To confirm that 30 min of physical restraint was a potent stressor in our hands, hamsters without dominant or

subordinate status were anesthetized with isoflurane and decapitated immediately following exposure to acute restraint to assay plasma cortisol concentrations in trunk blood ($n_{\text{restraint}}=8$; $n_{\text{control}}=8$). Cortisol is the primary glucocorticoid in Syrian hamsters and levels were analyzed using an enzyme-linked immunoassay protocol following the manufacturer's instructions (Cayman Chemical). All samples were analyzed on the same plate to avoid inter-assay variability.

2.4 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.

A vibrating microtome was used to slice brains into 30 μm consecutive coronal sections and every third section was processed for c-Fos protein immunohistochemistry, which resulted in 60 μm between stained sections. To remove unbound reagents, sections were washed five times with PBS + 0.2% Triton before each incubation cycle. 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, followed by incubation for 24 h in rabbit anti-c-Fos polyclonal antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS + 0.2% Triton. Then, sections received a 60 min incubation in biotinylated secondary anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) with 0.5% GS and 0.2% Triton. Finally, sections were incubated for 60 min with avidin-biotin-complex reagent (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and 0.2% Triton. 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. Finally, sections were washed with PBS and then distilled water 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 sections from all animals were processed simultaneously for each brain region.

Images were captured at 10 $\times$ magnification on an Olympus BX51 microscope. We restricted c-Fos quantification to brain regions for which we previously found social status-dependent differences in c-Fos immunoreactivity after social defeat stress, including the PL, IL, dorsal MeA (dMeA), vMeA, and DRN [24–26]. Because of its heterogeneity, the DRN was divided into five subdivisions, which include rostral dorsal DRN (rdDRN), rostral ventral DRN (rvDRN), caudal dorsal DRN (cdDRN), caudal ventral DRN (cvDRN), and lateral DRN (lDRN) [32, 33]. We also include the paraventricular nucleus of the hypothalamus (PVN) to compare restraint-induced neural activity with our previous research on social defeat. Two-dimensional photomicrographs were captured within defined regions of interest that were tailored to the size of each brain region, and the number of c-Fos immunopositive cells was quantified in each image (Figure 1). We quantified 5–6 sections per individual in the PL, IL, dMeA, and vMeA, 4–6 sections per individual in the PVN, and 3–4 sections per individual in subregions of the DRN. We analyzed the average cell count per individual, and sample

sizes ($n = 8-10$) varied per brain region because individuals with poor tissue quality were excluded from analysis.

Immunopositive cells were defined as those with nuclei stained 1.6 times darker than the specific background immunoreactivity for each image. Background immunoreactivity was calculated by quantifying optical density on regions of the tissue without c-Fos staining. MCID Core image analysis software (InterFocus Imaging, Cambridge, England) was used to quantify the number of c-Fos immunopositive cells, although 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.

2.5 Defeat-induced c-Fos expression

Because we have previously shown that social defeat stress leads to status-dependent changes in c-Fos expression, we sought to compare restraint-induced c-Fos expression with our data previously published on defeat-induced c-Fos expression [24]. The details of this study are briefly presented below. Male hamsters were assigned to resident-intruder dyads and paired in 14 daily dominance encounters. Then, dominants, subordinates, and social status control animals that did not participate in daily dominance encounters received acute social defeat stress. Social defeat consisted of subjects being placed into the home cage of three separate trained aggressors for three 5-min aggressive encounters that occurred at 5-min inter-trial intervals. We also included a group of handled control animals that did not participate in dominance encounters and received three 5-min exposures to the empty cages of three trained aggressors. Sixty minutes after social defeat stress, animals were euthanized and brains removed for c-Fos immunohistochemistry. The immunohistochemistry and c-Fos quantification were performed as described above.

Because tissue samples for the defeated and restrained animals were processed at different times and by separate experimenters, we standardized stress-induced c-Fos immunoreactivity to the handled controls in each experiment. We calculated the relative number of c-Fos immunopositive cells for dominant, subordinate, and social status control animals as a proportion of the mean number of c-Fos-positive cells in the handled control group.

2.6 Behavioral testing

Before determining if restraint induces status-dependent changes in anxiety-like behavior, we first needed to verify whether restraint stress alters the behavior of Syrian hamsters in conventional assays of rodent anxiety. Therefore, our initial goal was to determine whether acute physical restraint would increase anxiety-like behavior in hamsters without social status. In these studies, hamsters received either 30 min physical restraint or brief human handling without restraint. Behavioral testing occurred 24 hours after restraint stress because preliminary studies revealed that hamsters spend the majority of their time self-grooming when tested either 10 or 60 min following restraint. Unfortunately, we failed to show that acute physical restraint altered anxiety-like behavior in Syrian hamsters and decided it was unnecessary to move forward with testing dominant and subordinate animals.

The first cohort of animals received restraint stress and then a social interaction test 24 hours later, which is similar to the conditioned defeat test we have previously used to investigate the effects of social defeat stress in Syrian hamsters [34]. A smaller, younger, non-aggressive hamster was placed for 5 minutes into the subject's home cage (n=12). We digitally recorded all testing sessions and quantified the behavior of subjects using Noldus Observer software (Noldus Information Technology). We quantified the total duration of the following categories of behavior: submissive/defensive (flee, avoid, upright and side defensive postures, tail-up, stretch-attend); aggressive (chase, attack, upright and side offensive postures); non-agonistic social (sniff, approach); and nonsocial (locomotion, grooming, nesting, feeding) [12]. We also recorded the frequency of attacks, flees, and avoids displayed by the subjects. A researcher blind to the experimental conditions completed behavioral quantification. To establish inter-rater reliability, we achieved 90% agreement on the duration of submissive, defensive, and aggressive behavior in a subset of videos.

A second cohort of restrained or control hamsters (n=6 per group) was exposed to a social avoidance test as previously described [35]. Briefly, subjects were tested in a novel arena (12 × 27 × 16 cm) with a caged, unfamiliar adult male hamster. Testing involved a 5-min session with an empty perforated box (7 × 14 × 7 cm) immediately followed by a 5-min session with a social target inside the perforated box. The perforated, plastic box allowed subjects to see, hear, and smell the stimulus animal, but not make physical contact. The testing arena was divided into 3 zones: interaction zone (subject's nose within 3 cm of target box), near zone (1/2 of arena containing target box), and far zone (1/2 of arena without target box). An observer blind to the testing conditions used Noldus Observer software to score the duration of time spent in each location, as well as several other types of behavior including flank mark, groom, avoid, flee, and stretch attend. We also calculated the interaction ratio, defined as the interaction zone time during the social target trial divided by the interaction zone time during the empty trial.

A third cohort of restrained or control hamsters (n=6 per group) was placed in a randomly assigned corner of an open field arena (100 cm²) under low-light conditions (approximately 15 lux) for 20 minutes. Preliminary open field tests revealed that higher light conditions greatly diminished ambulation in both restrained and non-restrained hamsters. We quantified the duration of time spent in four zones: thigmotaxis zone (5.5 cm from wall), extended perimeter zone (11 cm from wall), center zone, and corners (11 cm²). We also recorded the total distance traveled, mean velocity, latency to leave thigmotaxis zone, and latency to enter center zone.

A fourth cohort of restrained or control hamsters (n=9 and 4, respectively) was exposed to a light/dark transition test arena for 5 minutes. The arena consisted of one enclosed and one opened compartment with opaque and transparent plastic walls, respectively. A small opening (8 × 6 cm) allowed free passage between the equally sized compartments (60 × 45 × 40 cm). A preliminary study revealed that placing hamsters in the light compartment (50 lux) at the start of testing greatly diminished ambulation and resulted in little movement into the dark compartment and >70% time spent in the light compartment. Consequently, the hamsters reported here were placed inside the dark compartment. We quantified the following behavior in the light compartment only: latency to enter light compartment, time

spend in light compartment, mean velocity, and total distance traveled, which was normalized to total time spent in the light compartment. Noldus Ethovision software (Noldus Information Technology) was used to quantify behavior in both the open field and light/dark transition tests.

2.7 Statistical Analysis

To analyze the effects of restraint stress on c-Fos expression, we performed a two-way repeated measures ANOVA in which social status (4 levels) and brain region (10 levels) were factors. Because the two-way interaction was significant, we followed this analysis with separate one-way ANOVAs in each brain region and used Fisher's LSD tests for post-hoc analysis. To compare the effects of restraint and social defeat stress, we performed separate two-way ANOVAs for each brain region with stressor (2 levels) and social status (3 levels) as factors. We were specifically interested in two-way interactions showing that the effects of social status depend on stressor type and performed pairwise comparisons only when this interaction was significant. Independent t-tests were used to compare restrained and non-restrained animals in each behavioral test and for plasma cortisol levels. All statistical tests were two-tailed, the alpha level was set at $p < 0.05$, and data were reported as mean $\pm$ SEM.

3. Results

3.1 Physical restraint and plasma cortisol

Hamsters exposed to 30 min physical restraint had a plasma cortisol concentration of 3169.2 pg/mL ($\pm$ 729.3) compared to 475.2 pg/mL ($\pm$ 122.7) for non-restrained animals ($t(14) = 3.64$, $p = 0.0001$). These findings confirm that acute physical restraint is a potent stressor for Syrian hamsters. Next, we tested whether physical restraint leads to status-dependent changes in c-Fos expression as previously observed for social defeat stress [24, 26].

3.2 Physical restraint and status-dependent c-Fos immunoreactivity

Dominance relationships were established on day 2.29 (SD = 0.91, $n = 13$ dyads) and the direction of aggressive and submissive behavior remained stable thereafter. The effects of physical restraint on c-Fos immunoreactivity were found to be brain region dependent. We found a significant interaction of stress condition and brain region ($F_{(3, 31)} = 8.19$, $p = 0.0003$), and followed this analysis with one-way ANOVAs for each brain region. There were significant effects of stress condition in the IL ($F_{(3, 38)} = 12.69$, $p = 0.0002$), PL ($F_{(3, 38)} = 13.65$, $p = 0.0001$), dMeA ($F_{(3, 37)} = 8.89$, $p = 0.0003$), vMeA ($F_{(3, 37)} = 9.64$, $p = 0.0003$), and PVN ($F_{(3, 37)} = 11.53$, $p = 0.0002$). In the IL, PL, dMeA, vMeA, and PVN, handled controls exhibited fewer c-Fos immuno-positive cells compared to each of the other groups that received restraint stress ($p < 0.05$; Figure 2a). Also, in the IL, subordinate animals showed fewer c-Fos-positive cells compared to both dominants and social status controls ($p < .05$). In the PL, subordinate animals showed fewer c-Fos-positive cells compared to dominants only ($p < .05$). In the vMeA, subordinates showed fewer c-Fos-positive cells compared to both dominants and social status controls ($p < .05$).

Stress- and status-dependent changes in c-Fos expression in the DRN were different from the patterns observed in the forebrain (Figure 2b). We found a significant effect of stress condition in each DRN subregion, including the rdDRN ($F_{(3, 34)} = 7.50, p = 0.001$), rvDRN ($F_{(3, 34)} = 5.14, p = 0.005$), cdDRN ($F_{(3, 34)} = 8.37, p = 0.0004$), cvDRN ($F_{(3, 34)} = 14.31, p = 0.0002$), and lDRN ($F_{(3, 34)} = 11.53, p = 0.0003$). In each DRN subregion, social status controls showed a greater number of c-Fos-positive cells compared to all other groups ($p < .05$). These findings suggest that repeated agonistic encounters decreased restraint-induced c-Fos expression in both dominants and subordinates. It is noteworthy that physical restraint failed to increase c-Fos immunoreactivity in dominant hamsters compared to non-restrained handled controls, whereas subordinates showed elevated c-Fos immunoreactivity in several DRN subdivisions. Subordinates showed increased c-Fos immunoreactivity in the rdDRN compared to dominants ($p < .05$) and increased c-Fos immunoreactivity in the cdDRN compared to handled controls ($p < .05$).

3.3 Comparing restraint-induced and defeat-induced c-Fos expression

We used our previously published data on social defeat to compare status-dependent differences in restraint-induced and defeat-induced c-Fos immunoreactivity [24, 26]. We found that social defeat and physical restraint produced a similar pattern of status-dependent c-Fos immunoreactivity in limbic brain regions (Figure 3a). The stress $\times$ social status interactions were not statistically significant in the IL ($F_{(2, 57)} = 0.10, p = 0.90$), PL ($F_{(2, 57)} = 0.81, p = 0.45$), dMeA ($F_{(2, 52)} = .30, p = 0.74$), vMeA ($F_{(2, 52)} = 0.18, p = 0.84$), and PVN ($F_{(2, 51)} = 0.11, p = 0.89$). Significant main effects of social status were found in the IL ($F_{(2, 57)} = 8.07, p = 0.001$), PL ($F_{(2, 57)} = 4.22, p = 0.02$), dMeA ($F_{(2, 52)} = 3.40, p = 0.04$), and vMeA ($F_{(2, 52)} = 7.68, p = 0.001$). These findings indicate that for both restraint and social defeat stress, dominant animals exhibit increased c-Fos immunoreactivity compared to their subordinate counterparts ($p < .05$). There was also a significant main effect of stress condition in several brain regions. Physical restraint produced increased c-Fos immunoreactivity compared to social defeat in the PL ($F_{(1, 57)} = 3.96, p = 0.05$), dMeA ($F_{(1, 52)} = 16.47, p = 0.0002$), and PVN ($F_{(1, 51)} = 32.94, p = 0.0001$). However, in the vMeA, social defeat led to increased c-Fos immunoreactivity compared to restraint ($F_{(1, 52)} = 7.06, p = 0.01$).

In contrast to limbic forebrain regions, physical restraint and social defeat produced a different pattern of status-dependent changes in c-Fos immunoreactivity in the DRN (Figure 3b). Stress $\times$ social status interactions were found in the rdDRN ($F_{(2, 54)} = 7.97, p = 0.001$), rvDRN ($F_{(2, 54)} = 3.79, p = 0.029$), cdDRN ($F_{(2, 47)} = 3.55, p = 0.037$), cvDRN ($F_{(2, 50)} = 9.94, p = 0.0003$), and lDRN ($F_{(2, 54)} = 7.59, p = 0.001$). A primary difference between these types of stressors was that physical restraint produced increased c-Fos immunoreactivity compared to social defeat for social status controls, but less so for dominant and subordinate animals. This finding was indicated by significant pairwise comparisons for social status controls in the rdDRN ($F_{(1, 54)} = 19.35, p = 0.0001$), cdDRN ($F_{(1, 47)} = 33.72, p = 0.0001$), cvDRN ($F_{(1, 50)} = 41.24, p = 0.0001$), and lDRN ($F_{(1, 54)} = 22.95, p = 0.0001$). Interestingly, the difference between physical restraint and social defeat was less pronounced in the cdDRN, such that physical restraint produced greater c-Fos immunoreactivity compared to social defeat in both dominants ($F_{(1, 47)} = 5.19, p = 0.027$) and subordinates ($F_{(1, 47)} = 7.74,$

$p = 0.008$). Finally, the rvDRN was unique insofar as subordinates exhibit greater c-Fos immunoreactivity to social defeat compared to physical stress, while dominants and social status controls do not ($F_{(1, 54)} = 4.20$, $p = 0.045$).

3.4 Changes in anxiety-like behavior following physical restraint

Our next goal was to determine whether status-dependent differences in c-Fos expression following physical restraint correspond to restraint-induced changes in anxiety-like behavior. However, this goal first required us to test whether physical restraint would lead to changes in anxiety-like behavior irrespective of dominance status. We used a variety of behavioral tests, in part, because few anxiety tests have been validated for Syrian hamsters [36].

We found that acute physical restraint did not significantly alter anxiety-like behavior in a social interaction test, social avoidance test, open field test, or light/dark transition test (Table 1). In the social interaction test, one restrained animal briefly showed submissive behavior toward the intruder, and several restrained animals displayed aggressive behavior. These data are similar to agonistic behavior displayed by non-stressed hamsters in a resident-intruder paradigm, and indicate that acute restraint stress does not alter subsequent agonistic behavior [16, 19, 34, 37]. In the other behavioral tests, restrained animals tended to differ from non-restrained controls on some measures, but not always in a direction that would be considered anxiety-like. Restrained animals showed a trend for increased time spent investigating a social target in a social avoidance test ($t_{(10)} = 1.82$, $p = 0.09$), increased center time during the first 5 min of an open field test ($t_{(15)} = 1.87$, $p = 0.08$), and decreased time in the light zone in a light/dark transition test ($t_{(11)} = 1.76$, $p = 0.10$). However, these findings were not corrected for multiple comparisons and should be treated with caution. Because we did not find robust changes in anxiety-like behavior following restraint stress, we did not pursue status-dependent differences.

4. Discussion

This study characterized restraint-induced neural activity in dominant and subordinate hamsters in select brain regions. Our initial observations indicated that the amount of c-Fos immunoreactivity following physical restraint differed from similar data using social defeat stress, but only in specific brain regions. We used our previously published data to compare the effects of dominance status on restraint-induced and defeat-induced c-Fos immunoreactivity. To enable a quantitative comparison we corrected for c-Fos immunoreactivity in non-stressed controls in each experiment. We found that subordinate animals showed reduced c-Fos immunoreactivity compared to dominants in the IL, PL, and vMeA following physical restraint. These findings are consistent with the pattern of neural activity found in subordinate animals following social defeat stress [25]. In contrast, social defeat leads to status-dependent changes in c-Fos expression in the rvDRN and cvDRN [26], but similar patterns of c-Fos expression were not found following physical restraint. Overall, these findings suggest that status-dependent differences in stress-induced neural activity in the IL, PL, and MeA generalize from social defeat to restraint stress, whereas status-dependent differences in neural activity in the DRN are sensitive to the type of stressor. In this study, we were not able to show that acute physical restraint increased anxiety-like

behavior in Syrian hamsters. Although our ultimate goal was to link status-dependent differences in c-Fos expression with status-dependent differences in anxiety-like behavior, we did not move forward to test restraint-induced anxiety in dominant and subordinate hamsters. While hamsters show dramatic changes in agonistic behavior following social defeat stress, other tests of anxiety-like behavior can be difficult to apply to Syrian hamsters. The light/dark transition test was the only test in which restrained hamsters showed a tendency toward the type of anxiety-like behavior expected in rats and mice. While it might be worth using a longer duration of physical restraint in future studies, we selected 30 min restraint in this study to match the duration of social defeat in previous studies and thereby facilitate the comparison of c-Fos immunoreactivity.

We found that acute physical restraint increased plasma cortisol levels in Syrian hamsters, irrespective of dominance status. Corticotrophin-releasing factor (CRF) neurons in the PVN initiate the neuroendocrine stress response, and the elevated restraint-induced c-Fos immunoreactivity in the PVN found here is consistent with physical restraint producing greater PVN c-Fos expression compared to social defeat stress [27]. We found no effect of social status on restraint-induced c-Fos expression in the PVN, which is similar to our previous findings using social defeat stress [24]. Likewise, we recently found that dominant and subordinate hamsters do not significantly differ in plasma cortisol concentrations immediately or 60 min following social defeat stress [38]. Furthermore, pharmacological blockade of glucocorticoid synthesis during social defeat stress does not alter the acquisition of the conditioned defeat response in hamsters [39]. Although exposure to controllable stress can reduce the subsequent development of learned helplessness, hypothalamic pituitary adrenal (HPA) axis activity is not modulated by control over the stressor [40]. Also, although chronic social defeat stress produces individual differences in social avoidance and depression-like behavior, both susceptible and unsusceptible mice show elevated serum corticosterone following an acute swim stress [41]. These findings suggest that individual differences in stress resistance and susceptibility are not always associated with changes in HPA axis activity.

The PL and IL provide top-down inhibitory control over several structures in the limbic system. Dominant hamsters exhibit elevated defeat-induced c-Fos expression in the PL and IL after they have maintained their social status for 14 days [25]. Moreover, pharmacological inhibition of the vmPFC with a muscimol injection prior to social defeat stress reinstates social avoidance in dominant hamsters [42]. These findings are consistent with reports showing that rats bred for a high anxiety phenotype exhibit reduced c-Fos expression in the mPFC during stressful situations compared to low anxiety rats [43–45]. Neural activity in the PL also reduces the neuroendocrine stress response [46, 47], and prevents the development of learned helplessness [48, 49]. Likewise, neural activity in the IL promotes the extinction of conditioned fear [50, 51]. Interestingly, neurotransmission in both the PL [52] and IL [10] supports resistance to the effects of chronic social defeat stress. In this study, we found that subordinates show a decrease in restraint-induced c-Fos immunoreactivity in the PL and IL, suggesting that they have less top-down inhibitory control over limbic structures, which may impair their ability to cope with stressful events. Overall, neural activity in both the PL and IL is capable of enhancing the ability to cope with stress, although likely via separate efferent projections.

The MeA integrates olfactory information and hormonal signals and serves a key role in mediating aggressive, defensive, and reproductive behavior. The posterior dMeA contains a high density of steroid hormone receptors and has been implicated in the control of reproductive behavior [53, 54]. On the other hand, the posterior vMeA is more closely associated with the regulation of defensive behavior [55, 56]. We have shown that subordinate hamsters exhibit reduced c-Fos immunoreactivity in the vMeA following social defeat stress compared to both dominants and social status controls [24]. These findings are consistent with the attenuated restraint-induced c-Fos expression found here. Although the MeA is a component of the neural circuit regulating defeat-induced social avoidance in hamsters, the mechanisms by which reduced neural activity in the MeA might promote stress susceptibility in subordinates remains unclear. Rats bred for high anxiety exhibit increased c-Fos expression in the dMeA and vMeA following social defeat stress [43]. Also, pharmacological inhibition of the MeA reduces defeat-induced social avoidance in Syrian hamsters [20]. On the other hand, neural activity in a select population of MeA neurons may reduce fear and anxiety. Increased activity of neuropeptide tuberoinfundibular peptide of 39 residues (TIP39) in the MeA reduces the incubation of conditioned fear [57]. Overall, our findings suggest that status-dependent neural activity in the MeA generalizes from social defeat to restraint stress.

The DRN is a heterogeneous structure with numerous subdivisions that send efferent projections to separate areas of the brain [58]. Social status interacts with both physical restraint and social defeat to produce a complex effect on DRN neural activity. We found that control animals show greater restraint-induced c-Fos immunoreactivity throughout the DRN compared to dominants and subordinates, which suggests that repeated dominance encounters desensitize DRN neurons to subsequent restraint stress. However, repeated dominance encounters may not desensitize DRN neurons to social defeat because subordinates, and not social status controls, show increased c-Fos immunoreactivity [26]. Here, we confirmed this difference by showing that social status controls exhibit greater c-Fos immunoreactivity following restraint compared to social defeat. Likewise, acute social defeat in both rats and hamsters produces robust c-Fos expression in the DRN with modest habituation after repeated social defeat [59, 60]. We found that all subjects, regardless of social status, showed increased c-Fos immunoreactivity in the cdDRN following restraint compared to social defeat. The cdDRN sends efferent projections to the hippocampus, thalamus, and the ependymal lining of the ventricular system [33]. Also, activation of CRF₂ receptors specifically increases c-Fos immunoreactivity within 5-HT neurons in the cdDRN [61, 62]. Subordinate hamsters showed increased c-Fos immunoreactivity specifically in the rdDRN following restraint stress compared to dominants. The rdDRN modulates anxiety-related changes in physiology and behavior, and sends efferent projections to limbic structures including the CeA, BLA, BNST, NAcc, and dorsal hypothalamus [33]. This suggests that subordinate social status specifically sensitizes the rdDRN to subsequent restraint stress. This is consistent with previous findings showing that Syrian hamsters that lose an aggressive encounter exhibit reduced mRNA for the 5-HT_{1A} autoreceptor in the DRN [63].

Hamsters that lose an aggressive encounter also have elevated c-Fos immunoreactivity in the rvDRN compared to winners and handled controls [63]. Recently, it was shown that losing

an aggressive encounter leads to elevated c-Fos expression in the rvDRN of male hamsters, but not females [37]. Similarly, hamsters with subordinate social status exhibit increased defeat-induced c-Fos immunoreactivity in the rvDRN compared to their dominant counterparts [26]. In contrast to social defeat stress, here we show that physical restraint did not increase c-Fos expression in the rvDRN in either subordinates or dominants. The rvDRN sends efferent projections to sensorimotor cortex, ventrolateral orbital frontal cortex, and dorsal striatum (DS), and is implicated in serotonergic modulation of motor and cognitive function [33]. One interesting possibility is that the motor requirements of social defeat may activate the rvDRN in subordinate animals to a greater degree than physical restraint. The learned helplessness and conditioned defeat models are similar insofar as acute traumatic events sensitize the 5-HT system and increase anxiety-like behavior, however these models differ in the role of specific DRN subregions [64]. In the learned helplessness model, uncontrollable tail shock sensitizes the caudal DRN, and serotonergic projections from the caudal DRN to the DS and BLA mediate escape deficits and anxiety-like behavior, respectively [65–68]. In the conditioned defeat model, acute social defeat activates the rostral DRN and serotonergic projects to the BLA modulate the changes in agonistic behavior characteristic of the conditioned defeat response [16, 34, 63, 69]. Interestingly, subordinate social status appears to sensitize several DRN subregions such that subsequent social defeat stress leads to elevated neural activity in the rvDRN whereas subsequent restraint stress increases neural activity in the cdDRN.

Other models of individual differences in stress susceptibility have noted that resilient animals display reduced effects of stress on some, but not all, behavioral and physiological measures [41, 70]. Also, the resistance that develops to one type of stressor may generalize to another stressor when there is sufficient overlap in the neurocircuitry [9]. Our findings suggest that the ability of a stress resistant phenotype to generalize from one stressor to another may dependent on the brain regions underlying the phenotype, such that the vmPFC and MeA may respond similarly to different stressors while the DRN may not. The cellular mechanisms in the vmPFC and MeA that support status-dependent differences in stress susceptibility may generalize from social defeat to physical restraint, but those in the DRN may be stressor specific. Overall, these findings should improve understanding of the neural circuits that regulate stress resistance and the ability of resilience to generalize from one context to another.

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Highlights

- Social status modulates restraint-induced c-Fos immunoreactivity
- Status-dependent differences in vmPFC neural activity generalize across stressors
- Status-dependent DRN neural activity depends on the type of stressor

Figure 1.

A) Stereotaxic atlas images indicate the location of brain regions selected for c-Fos quantification [71]. Although some boxes are indicated unilaterally for bilateral brain regions, images were collected from both hemispheres when possible. Values on the right side indicate distance from bregma. Dimensions of the photomicrographs used for quantification were as follows: $870 \times 660 \mu\text{m}$ (PL, IL, dMeA, vMeA); $440 \times 330 \mu\text{m}$ (PVN); $500 \times 300 \mu\text{m}$ (rdDRN, cdDRN, IDRN); $250 \times 300 \mu\text{m}$ (rvDRN, cvDRN). B) Representative photomicrographs at 4 $\times$ magnification for each of the regions of interest. Black nuclear staining represent c-Fos positive cells and scale bars are 500 μm .

Figure 2.

The number (mean ± SEM) of c-Fos immunopositive cells were quantified in (a) the IL, PL, dMeA, vMeA, and PVN, as well as in (b) several subdivisions of the DRN. Dominant and subordinate animals were exposed to daily dyadic encounters for two weeks during which time they established a dominance relationship. Then, dominant, subordinate, and social status control animals received 30 min physical restraint, whereas handled controls did not establish dominance status and were unrestrained. Sample sizes range 8–10 per group. * indicates a significant difference compared to all other groups in that brain region ($p < 0.05$). ^a indicates a significant difference compared to dominants in that brain region ($p < 0.05$). ^b indicates a significant difference compared to dominants and social status controls in that brain region ($p < 0.05$). ^c indicates a significant difference compared to handled controls in that brain region ($p < 0.05$).

Figure 3.

The relative number (mean $\pm$ SEM) of c-Fos immunopositive cells was calculated as a proportion of non-stressed handled controls in each brain region. c-Fos immunoreactivity was quantified in (a) the IL, PL, dMeA, vMeA, and PVN, as well as in (b) several subdivisions of the DRN. Social status controls (SC), dominants (D), and subordinates (S) were exposed to either 30 min physical restraint or three 5-min social defeat episodes at 5-min intervals. Handled controls for the restraint study were transported to the testing room and gently handled by experimenters, whereas handled controls for the social defeat study were exposed to the empty cages of three unfamiliar animals. The c-Fos data for socially defeated animals were previously reported (Morrison et al., 2012; Cooper et al., 2015). We specifically tested for restraint $\times$ social defeat interactions, and * indicates a significant pairwise comparison when the overall interaction was significant ($p < 0.05$).

Table 1

Restraint-induced changes in behavior

Behavioral Tests		Behavioral Measures, Mean (SEM)		
Social Interaction Test	Aggression (s)	Submission (s)	Social (s)	Nonsocial (s)
Restrained	49.5 (23.6)	0.7 (0.7)	55.1 (15.5)	196.2 (30.5)
Social Avoidance Test	Inter. Zone Time (s)	Far Zone Time (s)	Flank Marking (#)	Inter. Ratio
Restrained	162.3 (23.0) [†]	78.3 (20.6)	3.5 (1.3)	1.9 (0.2)
Non-restrained	117.6 (8.8)	90.7 (10.0)	3.0 (1.0)	1.6 (0.1)
Open Field Test	Thig. Zone Time (s)	Center Time (20 min) (s)	Center Time (1 st 5 min) (s)	Total Dist. Traveled (cm)
Restrained	753.2 (24.1)	62.0 (16.6)	15.4 (4.1) [†]	627.2 (5.9)
Non-restrained	785.4 (18.6)	33.0 (10.1)	8.0 (1.9)	620.3 (7.2)
Light/Dark Transition	Light Zone Time (s)	Light Zone Latency (s)	Light Zone Entries (#)	Dist.(cm)/Light Zone Time(s)
Restrained	79.9 (13.9) [†]	110.3 (18.5)	4.6 (0.7)	6.6 (0.7)
Non-restrained	120.7 (13.9)	77.3 (37.5)	6.0 (0.9)	7.6 (0.9)

[†] indicates $p < .10$ (t-tests). Inter: Interaction, Thig: Thigmotaxis, Dist: Distance.