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Winning agonistic encounters increases testosterone and androgen receptor expression in Syrian Hamsters

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Abstract

Winning aggressive disputes is one of several experiences that can alter responses to future stressful events. We have previously tested dominant and subordinate male Syrian hamsters in a conditioned defeat model and found that dominant individuals show less change in behavior following social defeat stress compared to subordinates and controls, indicating a reduced conditioned defeat response. Resistance to the effects of social defeat in dominants is experience-dependent and requires the maintenance of dominance relationships for 14 days. For this study we investigated whether winning aggressive interactions increases plasma testosterone and whether repeatedly winning increases androgen receptor expression. First, male hamsters were paired in daily 10-min aggressive encounters and blood samples were collected immediately before and 15-min and 30-min after the formation of dominance relationships. Dominants showed an increase in plasma testosterone at 15-min post-interaction compared to their pre-interaction baseline, whereas subordinates and controls showed no change in plasma testosterone. Secondly, we investigated whether 14 days of dominant social status increased androgen or estrogen alpha-receptor immunoreactivity in brain regions that regulate the conditioned defeat response. Dominants showed more androgen, but not estrogen alpha, receptor immuno-positive cells in the dorsal medial amygdala (dMeA) and ventral lateral septum (vLS) compared to subordinates and controls. Finally, we showed that one day of dominant social status was insufficient to increase androgen receptor immunoreactivity compared to subordinates. These results suggest that elevated testosterone signaling at androgen receptors in the dMeA and vLS might contribute to the reduced conditioned defeat response exhibited by dominant hamsters.

Keywords

Medial Amygdala; Estrogen Alpha Receptors; Androgen Receptors; Resiliency; Testosterone; Aggression

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1. Introduction

Stressful life events are a contributing factor in the onset of several mood and anxiety disorders, including post-traumatic stress disorder (PTSD) (Kuo et al., 2003; Vermetten and Bremner, 2002). However, exposure to stressful life events does not always lead to a stress-related psychiatric disorder. Identifying the cellular and molecular mechanisms controlling stress resilience is an essential step toward developing novel treatments for stress-related mental illness. While genetic factors likely contribute to individual differences in stress vulnerability, prior experience can improve an individual's ability to cope and modify how they respond to future stressors.

To investigate resiliency and vulnerability to the effects of stress, we use an ethologically relevant social defeat model (Blanchard et al., 1993). Specifically, we use conditioned defeat, a social stress model in Syrian hamsters in which a brief social defeat stress results in a loss of species-typical territorial aggression and an increase in submissive and defensive behavior when animals are later tested with a small, nonaggressive intruder (Huhman et al., 2003; Potegal et al., 1993). We have previously shown that pairs of Syrian hamsters with established dominance relationships respond differently to social defeat stress, such that dominant animals show a reduced conditioned defeat response compared to subordinate counterparts (Morrison et al., 2014; Morrison et al., 2013; Morrison et al., 2012; Morrison et al., 2011). However, hamsters must maintain social dominance for 14 days, and not for 1 or 7 days to exhibit resistance to conditioned defeat (Morrison et al., 2014). These findings suggest conditioned defeat resistance develops during the maintenance of dominance relationships. Additionally, we found that dominant hamsters show increased defeat-induced neural activation in several brain regions, including the medial amygdala (MeA), ventromedial prefrontal cortex (vmPFC) and ventral lateral septum (vLS) (Morrison et al., 2012). The elevated defeat-induced neural activation in the MeA and vmPFC of dominant animals also requires animals maintain social dominance for 14 days, and a similar trend is found in the vLS (Morrison et al., 2014). Altogether, these findings suggest the maintenance of dominant social status leads to neural plasticity in select brain regions that promotes resistance to social defeat stress.

Our findings are consistent with other animal models showing that specific environmental events can induce stress resistance. Stressor controllability (Maier, 2015), environmental enrichment (van Praag et al., 2000), brief maternal separation (Kinnally et al., 2010), and voluntary exercise (Greenwood and Fleshner, 2011) have each been shown to reduce the deleterious effects of subsequent stressors. Exposure to controllable stress induces neural plasticity within vmPFC neurons that enables these neurons to respond to subsequent uncontrollable stress and prevent the development of learned helplessness. These plastic changes include increased excitability of pyramidal neurons in layers 5 and 6 of the prelimbic cortex (Varela et al., 2012), and an upregulation of the ERK signaling pathway in the prelimbic cortex (Christianson et al., 2014). Also, lesions of the vmPFC block the ability of environmental enrichment from generating resistance to chronic social defeat stress (Lehmann and Herkenham, 2011). Likewise, young monkeys that are briefly separated from their mothers cope better with future stressors and have increased vmPFC cortical volumes (Lyons et al., 2002). However, it is not the case that all factors that produce stress resilience

do so via actions in the vmPFC. Voluntary wheel running in rats upregulates 5-HT_{1A} autoreceptors in the dorsal raphe nucleus, increases BDNF mRNA in the hippocampus and amygdala, and reduces the development of learned helplessness (Greenwood et al., 2003; Greenwood et al., 2009). However, vmPFC lesions do not reduce the ability of exercise to promote stress resistance (Greenwood et al., 2013). Altogether, stress resilience is not simply a passive response involving a failure to display the neuroendocrine, cellular and molecular changes characteristic of susceptible individuals, but instead it is an active process that involves distinct neural circuits and molecular mechanisms (Cooper et al., 2015; Russo et al., 2012).

In numerous species, winners of competitive interactions and social challenges exhibit increased plasma testosterone compared to losers (Cavigelli and Pereira, 2000; Oyegbile and Marler, 2005; Smith et al., 2005; Yang and Wilczynski, 2002). It is possible that changes in testosterone signaling modulate the development of conditioned defeat resistance, because dominant individuals gain resistance after repeatedly winning aggressive social encounters. The link between fluctuating levels of testosterone and aggression has been described in the challenge hypothesis, which states that testosterone levels rise and facilitate aggression during social challenges that occur in a reproductive context such as territory formation, dominance disputes, and mate guarding (Wingfield et al., 1990). The winner effect, found in a range of species including mammals (Oyegbile and Marler, 2005), fish (Oliveira et al., 2009), and reptiles (Schuett et al., 1996), is characterized by an increased probability of winning an aggressive encounter following previous victories. In California mice, castration prevents the winner effect, and winning multiple agonistic encounters creates a post-victory surge in plasma testosterone (Trainor and Marler, 2001). These findings suggest a winner-challenge effect in which winning an aggressive encounter leads to a transient increase in testosterone that increases the probability of winning future encounters. Furthermore, the winner-challenge effect appears to be mediated by androgen receptors because winning an aggressive encounter increases the expression of androgen receptors in brain regions associated with agonistic behavior, including the bed nucleus of stria terminalis, nucleus accumbens and ventral tegmental area (Fuxjager et al., 2010). Additionally, testosterone activity at androgen receptors has also been implicated in reduced anxiety-like behavior. Testosterone treatment reduces anxiety-like behavior in rats and mice, but has no effect on the animals with a testicular feminization mutation that disables androgen receptors (Zuloaga et al., 2008; Zuloaga et al., 2011).

The MeA is part of a social brain network, where an abundance of androgen receptors are located (Wood and Newman, 1993). The MeA is an important node in the neural circuitry regulating many social behaviors including reproduction, aggression, territorial marking and maternal behavior (Newman, 1999). Neurons in the MeA are also activated by aversive stimuli and emotional events, including conditioned fear (Milanovic et al., 1998). Lesions of the MeA reduce the neuroendocrine response to acute stress and several fear-related behaviors, including predator odor-evoked freezing, fear potentiated startle and conditioned fear memory (Cousens et al., 2012; Muller and Fendt, 2006; Takahashi et al., 2007; Trogrlic et al., 2011; Walker et al., 2005; Yoshida et al., 2014). Furthermore, in Syrian hamsters it has been shown that pharmacological inactivation of the MeA during either social defeat stress or behavioral testing reduces the acquisition and expression of the conditioned defeat

response, respectively (Markham and Huhman, 2008). While these findings suggest that neural activity in the MeA promotes stress-related and fear-related behavior, others research indicates that activity of the MeA neurons reduces the effects of stress. Activity of neuropeptide tuberoinfundibular peptide of 39 residues (TIP39) at its receptor, parathyroid hormone 2 receptor (PTH2R), in the MeA has been shown to reduce incubation of conditioned fear (Tsuda et al., 2015). Also, we showed that dominant hamsters that maintain their social status for 14 days exhibit increased c-Fos immunoreactivity in the vMeA following social defeat stress, compared to 1-day and 7-day dominants (Morrison et al., 2014). Some of the inconsistency in the contribution of the MeA to stress-related behavior is likely related to the heterogeneity of GABAergic and non-GABAergic projection neurons in the MeA (Keshavarzi et al., 2014).

In this study, our goal is to identify neuroendocrine mechanisms that control a reduced conditioned defeat response in dominant hamsters. The overarching hypothesis for these experiments is that dominant hamsters experience daily surges in plasma testosterone during the maintenance of their social status that increases the expression of androgen receptors in the MeA. In experiment 1, we tested the predication that dominant animals experience a rise in plasma testosterone following an aggressive encounter but that subordinates do not. In experiment 2, we tested the prediction that 14 days of dominant, but not subordinate, social status increases androgen receptor expression in select brain regions, including the MeA. Finally, in experiment 3, we tested the prediction that dominant animals would exhibit increased androgen receptor expression following a single winning encounter compared to subordinates.

2. Materials and Methods

2.1 Subjects

Subjects were male Syrian hamsters (*Mesocricetus auratus*) obtained from our breeding colony that was originally derived from male and female hamsters from Charles River Laboratories (Wilmington, MA). Subjects were 3–4 months old (120–180 g) at the start of the study and were individually housed one week prior to the start of the study. All animals were 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*. Cages were not changed for one week prior to dominant-subordinate encounters to allow individuals to scent mark their territory. Subjects were handled daily for one week prior to dominant-subordinate encounters to habituate them to the stress of human handling. Animals were housed in a temperature controlled colony room (21 ± 2 °C) and kept on a 14:10 hr light:dark cycle to facilitate testes development and aggressive behavior. All behavioral protocols were performed during the first three hours of the dark phase of their light:dark cycle. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals

2.2 Dominant-Subordinate Encounters

To allow animals to establish social status, subjects within each cohort were weight-matched in resident-intruder dyads and paired in daily social encounters for up to 14 days. Subjects were randomly assigned as a resident or intruder, and all social encounters occurred in the resident's home cage. Residents and intruders maintained their residency status throughout the experiment, and residency status did not predict which animal became dominant. The encounters were 10 min in duration until a stable dominance relationship was formed, and all subsequent encounters were 5 min. We have previously determined 10 min encounters help facilitate the formation of a dominance relationship, and that 5 min encounters on subsequent days maintain the dominance relationship and reduce the chance of wounding. Pairs that did not form a stable dominance relationship after 5 days of encounters (approximately 25% across all three experiments) were excluded from the study. Control subjects were individually housed at the same time as experimental animals and were handled daily for 14 days instead of being paired. We digitally recorded daily aggressive interactions and quantified the behavior of subjects using Noldus Observer software (Noldus Information Technology). In a subset of videos, we quantified the total duration of the following categories of behavior: submissive/defensive (flee, avoid, upright and side defensive postures, tail-up, stretch-attend, head flag); aggressive (chase, attack including bite, upright and side offensive postures); nonagonistic social (sniff, approach); and nonsocial (locomotion, grooming, nesting, feeding). A researcher blind to the experimental conditions of the subject performed all behavioral scoring. Inter-rater reliability was established in a subset of videos by reaching 90% agreement on the duration of submissive/defensive and aggressive behavior.

2.3 Blood Collection and Enzyme Immunoassay

Retro-orbital bleeds were conducted under 4% isoflurane anesthesia prior to and 15-min after aggressive encounters. Trunk blood was collected under 4% isoflurane anesthesia 30-min after aggressive encounters. Blood was collected in rapid sequence for dominants and subordinates in a dyad, which resulted in a difference of approximately 2 minutes. Blood was centrifuged at 4400×g for 15-min, and then the plasma layer pipetted off and stored at -80°C until assayed. Blood was assayed using a commercial testosterone EIA kit (Cayman Chemical, # 582701). Samples were treated with the plasma extraction protocol recommended by Cayman and were run in duplicates with 50µl per well. Inter-assay reliability between plates was 7.6%, while intra-assay reliability within a single plate was found to be 8.6%.

2.4 Immunohistochemistry

Forty-five minutes after the last aggressive encounter, animals were anesthetized with isoflurane and 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 then were stored in cryoprotectant, all at 4°C. A consecutive series of 30 µm coronal sections were sliced on a vibrating microtome, collected into twelve vials, and stored as free floating sections in cryoprotectant at 4°C. The collected sections were

processed for either androgen receptor or estrogen alpha receptor immunohistochemistry. After immunohistochemistry, all sections were washed five times with distilled H₂O prior to being mounted onto glass microscope slides. After air-drying, sections were dehydrated using a series of alcohols, cleared with citrisolv and coverslipped using DPX mountant (Sigma-Aldrich). All tissue for each brain region and receptor type was processed simultaneously.

2.4.1 Androgen Receptor Immunohistochemistry—Sections were processed for androgen receptor immunohistochemistry according to a previously published protocol (Chen et al., 2014). Sections were rinsed in three 10 min washes in a phosphate-buffered gelatin Triton solution (PBS-GT; 0.1% gelatin, 0.3% Triton X-100, in PBS, pH 7.4), followed by 0.5% sodium borohydride in PBS-GT for 15 min. Sections were then incubated in 10% normal goat serum (NGS) in PBS-GT for 1 h to block non-specific binding and then incubated 10-min in avidin block followed by 10-min in biotin block (avidin/biotin blocking kit, Vector: #SP-2001). Sections were then incubated 24 h at 4 °C in 1% NGS in PBS-GT with an anti-androgen receptor antibody at 1:1000 concentration (rabbit monoclonal-Abcam: ab52615). Following incubation in the primary antibody, the sections were rinsed in PBS-GT, and incubated 1 h in 1% NGS in PBS-GT with biotinylated goat anti-rabbit antibody at 1:500 concentration (Vector: BA-1000). Brain sections were then incubated 1 h in PBS-GT with an avidin–biotin complex (ABC Kit, Vector Laboratories: PK6100), and the peroxidase reaction was visualized using a 10 min incubation in 3,3'-diaminobenzidine (DAB tablet, Sigma: D5905) and nickel dissolved in PBS.

2.4.2 Estrogen Alpha Receptor Immunohistochemistry—Sections were processed for estrogen alpha receptor immunohistochemistry according to a previously published protocol (Trainor et al., 2007). Sections were washed three times in PBS before each incubation, which were conducted at room temperature unless otherwise stated. Sections were incubated for 10 min in 1% sodium borohydride in PBS, followed by a 20 min incubation in 20% NGS with 0.3% hydrogen peroxide in PBS. Sections were incubated at 4°C on the shaker in rabbit anti-estrogen alpha receptor antibody (EMD Millipore: 06-935) at a final dilution of 1:25,000 in PBS + 0.2% Triton with 1% NGS. Sections were then incubated for 60 min in biotinylated goat anti-rabbit (Vector Laboratories: BA-1000) at a final dilution of 1:200 in PBS-Triton. Sections were incubated in avidin-biotin-complex (ABC Kit, Vector Laboratories: PK6100) for 60 min, and the peroxidase reaction was visualized using a 5 min incubation in 3,3'-diaminobenzidine (DAB tablet, Sigma: D5905) and nickel dissolved in PBS.

2.4.3 Immunohistochemistry Quantification—Images were captured at 10× magnification using an Olympus BX41 microscope. The number of androgen receptor and estrogen alpha receptor immuno-positive cells were determined in select brain regions using MCID Core image analysis software (InterFocus Imaging). We quantified the number of androgen receptor immuno-positive cells in the following brain regions: dorsal medial amygdala (dMeA), ventral medial amygdala (vMeA), ventromedial hypothalamus (VMHL), ventral lateral septum (vLS) and medial preoptic area (MPOA) (Figure 1). These brain regions were selected for quantification because they exhibited strong androgen receptor

immunoreactivity and showed status-dependent differences in defeat-induced c-Fos immunoreactivity in previous studies (Morrison et al., 2014). Androgen receptor immunoreactivity was not quantified in the vmPFC because staining was not visible in this region. Additionally, androgen receptor immunoreactivity was not quantified in the NAc or BNST because staining was too faint to quantify. We quantified the number of estrogen alpha receptor immuno-positive cells in the dMeA and vMeA. For each brain region, we recorded background immunoreactivity in unstained regions of each image. We then defined immuno-positive cells as those that showed staining 1.4–1.6× darker than the specific background immunoreactivity calculated for each image. Cell counts were limited to the area within defined boxes that were tailored to the size of each brain region. For each brain region we quantified three to six sections per individual along a rostral-caudal axis.

2.5 Experiment 1

Subjects (n = 32) were weight-matched and assigned into resident-intruder dyads and blood was collected via retro-orbital bleed 10 min prior to aggressive encounters. Then, subjects were placed in daily 10 min aggressive encounters until the formation of a dominance relationship. Winner and losers were identified by direction of agonistic behavior within each dyad. Fifteen minutes after establishment of dominance relationships, blood was collected from both animals via retro-orbital bleed for testosterone assay. Thirty minutes after the establishment of a dominance relationship, animals were euthanized and trunk blood was collected.

2.6 Experiment 2

Subjects (n = 62) were weight-matched and assigned into resident-intruder dyads for dominant-subordinate encounters for 14 consecutive days. Trunk blood and brains were collected 45 min after the 14th aggressive encounter for testosterone assay and androgen and estrogen alpha-receptor immunohistochemistry.

2.7 Experiment 3

Subjects (n = 24) were weight-matched and assigned into resident-intruder dyads and placed in daily 10 min aggressive encounters until the establishment of a dominance relationship. Winner and losers were identified by direction of agonistic behavior within each dyad. Brains were collected for androgen receptor immunohistochemistry forty-five minutes after the first day which dominance relationships were established.

2.8 Data Analysis

Plasma testosterone and immunohistochemical data were analyzed using t-tests, or one-way ANOVA's followed by Fisher's protected least significant difference (LSD) post hoc test. The time course of plasma testosterone levels was analyzed using a 3×3 repeated measures ANOVA with a quadratic function. Pearson correlation coefficient was used to measure the strength of the linear relationship between plasma testosterone and submissive or aggressive behavior. Effects size estimations were calculated for all comparisons (partial η^2 for ANOVA and Cohen's d for t-tests). All statistical tests were two-tailed, and the α level was set at $p < .05$.

3. Results

3.1 Experiment 1

On average, dominance relationships were decided on day 1.9 ($SE = 0.28$), and three pairs were excluded because they did not form a stable dominance relationship after five days of aggressive encounters. Fifteen minutes after an aggressive encounter, dominant animals showed an increase in plasma testosterone compared to their baseline, whereas subordinates and controls did not ($F_{(2,25)} = 4.81$, $p = .017$, $\eta^2 = .28$) (Fig. 2). Dominant animals showed a 64.1% ($SE = 20.8$) increase in plasma testosterone 15 min after the aggressive encounter, whereas subordinates showed an 8.6% ($SE = 17.9$) decrease and controls showed a 10.7% ($SE = 15.5$) increase. Baseline plasma testosterone levels were not significantly different in dominant, subordinate, and control subjects, and plasma testosterone in dominant animals returned to baseline 30-min following the aggressive encounter.

The duration of aggressive behavior displayed by dominant animals on the day dominance relationships were established did not correlate with their peak plasma testosterone levels ($r(8) = .29$, $p = .412$). Dominance status was not related to whether animals were residents or intruders during the daily aggressive encounters. Five dominant animals were residents during the daily aggressive encounters whereas five dominant animals were intruders. Dominant residents showed a 73.7% ($SE = 34.75$) increase in plasma testosterone 15 min after the aggressive encounter and dominant intruders showed a 54.6% ($SE = 26.25$) increase, and these changes in testosterone were not significantly different from one another ($t(8) = .44$, $p = .673$, Cohen's $d = .28$).

3.2 Experiment 2

On average, dominance relationships were decided on day 1.9 ($SE = 0.15$), and nine pairs were excluded because they did not form a stable dominance relationship. Some animals were also excluded from analysis due to vibratome attrition or if cell quantification was impossible because of folds or tears in the tissue.

After dominant-subordinate pairs were established, animals maintained a stable relationship (Table 1). Dominant animals maintain high rates of aggressive behavior throughout the 14 days of encounters. After maintaining their social status for 14 days subordinate animals have lower plasma testosterone levels compared to dominants and controls ($F_{(2,32)} = 6.16$, $p = .005$, $\eta^2 = .28$; Figure 3). The duration of submissive behavior displayed by subordinates on day 14 did not correlate with their plasma testosterone levels ($r(10) = -.40$, $p = .198$).

Following 14 days of aggressive encounters, dominant animals showed more androgen receptor positive cells in the dMeA ($F_{(2,35)} = 3.89$, $p = .03$, $\eta^2 = .18$) and vLS ($F_{(2,34)} = 3.95$, $p = .029$, $\eta^2 = .19$) compared to subordinates and controls (Figure 4a). There was a trend for dominant animals to have more androgen receptor immunopositive cells in the vMeA compared to subordinates and controls ($F_{(2,36)} = 2.8$, $p = .074$, $\eta^2 = .14$). There were no significant differences in androgen receptor immunoreactivity between dominants, subordinates and control animals in the VMHL ($F_{(2,33)} = 2.33$, $p = .114$, $\eta^2 = .12$) or MPOA ($F_{(2,31)} = 1.76$, $p = .189$, $\eta^2 = .1$). After maintaining social status for 14 days, dominants, subordinates and controls animals did not show a difference in the number of estrogen

alpha-receptor positive cells in the dMeA ($F_{(2,34)} = .22$, $p = .804$, $\eta^2 = .01$) or vMeA ($F_{(2,34)} = .22$, $p = .803$, $\eta^2 = .01$) (Figure 4b). Dominance status was not related to residency status during the daily aggressive encounters, and seven dominants were residents and nine dominants were intruders. Dominant residents showed 206.4 (SE = 49.8), 150.9 (SE = 44.36), and 192.7 (SE = 25.95) androgen receptor immuno-positive cells in the vMeA, dMeA and vLS, respectively. Similarly, dominant intruders showed 198.9 (SE = 45.47), 174.8 (SE = 45.6) and 239.0 (SE = 43.2) androgen receptor immuno-positive cells in the vMeA, dMeA and vLS, respectively. In each of these brain regions the difference between dominant residents and dominant intruders was not statistically significant (vMeA: $t(12) = .11$, $p = .914$, Cohen's $d = .06$; dMeA: $t(12) = -.37$, $p = .720$, Cohen's $d = -.20$; vLS: $t(11) = -.88$, $p = .397$, Cohen's $d = -.50$).

3.3 Experiment 3

On average, dominance relationships were decided on day 1.3 (SE = 0.1), and one pair was excluded because they did not form a stable dominance relationship. After the first day in which dominance relationships were established, dominant animals did not show a significant difference in the number of androgen receptor positive cells in the dMeA ($t(20) = -1.11$, $p = .279$, Cohen's $d = -.47$), vMeA ($t(20) = -.39$, $p = .703$, Cohen's $d = -.17$) or vLS ($t(18) = -1.29$, $p = .212$, Cohen's $d = -.58$) compared to subordinate animals (Figure 5).

4. Discussion

We have shown that in male Syrian hamsters plasma testosterone increases 15-min after winning a single agonistic encounter and returns to pre-encounter baseline after 30-min. Also, dominant animals that repeatedly win agonistic encounters for 14 days show increased androgen receptor expression in the dMeA and vLS. These experience-dependent changes in plasma testosterone and androgen receptor expression are mediated by the establishment and maintenance of dominance relationships and not by residency status in a resident-intruder paradigm. Together, these results suggest dominant animals experience daily, transient surges in testosterone during the maintenance of their dominance relationship, which may lead to increased androgen receptor expression in brain regions that are known to regulate social behavior and responses to stress.

Hamsters in our model self-select into dominant/subordinate roles, thus it is possible plasma testosterone levels prior to dyadic encounters could predict winners and losers. However, we show here that animals did not differ in plasma testosterone at pre-interaction baseline, suggesting individual differences in plasma testosterone prior to aggressive encounters do not predict future social status. The post-victory surge in plasma testosterone peaked 15-min after the encounter. This testosterone surge is quicker than in guinea pigs and California mice, where 45-min is the ideal interval to capture a post-victory rise in testosterone (Marler et al., 2005; Sachser and Pröve, 1984). However, our findings are consistent with Siberian hamsters, where plasma testosterone increases immediately after winning an aggressive encounter (Scotti et al., 2009). Despite plasma testosterone peaking more quickly in Syrian hamsters than in California mice and guinea pigs, all three of these rodent species show a testosterone surge within a brief, 15-min time window. In Experiment 2, we collected blood

and brains 45-min following the 14th aggressive encounter to be consistent with previous research on post-victory changes in androgen receptor expression (Fuxjager et al., 2010). The failure of dominant hamsters to show a rise in plasma testosterone at 45-min compared to controls could be related to the time point for blood collection. Although, we expect post-victory surges in plasma testosterone to continue during the maintenance of dominance relationships, the present data cannot address habituation in post-victory testosterone surges. Additionally, we expect that rapid and transient surges in testosterone increase the expression of androgen receptors, which is unlike the decrease in androgen receptor expression observed in the medial preoptic nucleus following chronic testosterone treatment (Tetel et al., 2004).

We showed 14 days of subordinate status decreases plasma testosterone compared to dominant and control animals. The reduction in plasma testosterone in subordinates is consistent with previous data in Syrian hamsters, where nine encounters with a dominant opponent suppresses plasma testosterone in subordinates (Huhman et al., 1991). Similarly, in many other species, including rats, tree shrews, primates, and humans, chronic stress has been shown to reduce plasma testosterone (Fischer et al., 1985; Kreuz et al., 1972; Razzoli et al., 2006; Rose et al., 1971; Tamashiro et al., 2004). Thus, in our model the maintenance of subordinate status for 14 days produces changes in basal plasma testosterone similar to chronic stress. Interestingly, plasma testosterone levels in our hamsters did not correlate with the amount of submissive or aggressive behavior displayed by either subordinates or dominants during their 14th dyadic encounter. This suggests that the outcome of the aggressive encounter is more strongly associated with changes in plasma testosterone than the intensity of the aggressive encounter.

The MeA plays a critical role in the regulation of agonistic behavior (Cheng et al., 2008; Rosvall et al., 2012; Wang et al., 2013). Although lesion studies have supported this view, the direction of influence is not clear. MeA lesions decrease aggression in some studies (Kemble et al., 1984; Takahashi and Gladstone, 1988; Wang et al., 2013), while they increase aggression in others (Rosvold et al., 1954). We have previously shown dominant hamsters exhibit more c-Fos expression in the vMeA following social defeat, and a reduced conditioned defeat response the following day compared to subordinates (Morrison et al., 2014). These findings suggest neural activity in the MeA during social defeat stress contributes to resistance to conditioned defeat. However, pharmacological inactivation of the entire MeA with muscimol injection prior to social defeat reduces the conditioned defeat response (Markham and Huhman, 2008), suggesting activity of MeA neurons increases conditioned defeat behavior. Importantly, c-Fos expression in a subset of MeA neurons could reflect critical neural activity underlying resistance to conditioned defeat that is obscured by pharmacological inactivation of the entire MeA. Future studies should phenotype the c-Fos-positive and androgen receptor-positive cells in the vMeA of dominant hamsters. The MeA contains heterogeneous neuronal subpopulations and its role in modulating aggressive behavior could be specific to a distinct cell type or subregion (Hong et al., 2014). For instance, c-Fos expression studies have shown that the posterior dorsal subdivision of the MeA is activated during offensive aggression (Nelson and Trainor, 2007; Newman, 1999; Veening et al., 2005), and that selective activation of a GABAergic subpopulation within this subregion promotes aggressive behavior (Hong et al., 2014).

Overall, a better understanding of the heterogeneity within the MeA will be needed to delineate the mechanisms by which MeA activity regulates agonistic behavior and resistance to conditioned defeat.

Androgen receptors are abundant in the MeA (Wood and Newman, 1993), and it is well established that they regulate reproduction, aggression, and processing of chemosensory information, but less is known about their role in stress-related behavior (Blake and Meredith, 2011). We have shown the maintenance of dominant social status for two weeks leads to an up-regulation of androgen receptors in the dMeA with a similar trend found in vMeA. Male rats with a testicular feminization mutation that globally renders androgen receptors dysfunctional exhibit increased anxiety-related behavior and a greater stress-induced corticosterone response (Zuloaga et al., 2011). If androgen receptors regulate responses to social stress, one possibility is that an up-regulation of androgen receptors during social defeat leads to increased neural activity within a subpopulation of MeA neurons that, in turn, reduces the conditioned defeat response. Future research will be needed to link androgen receptor expression within the MeA to a reduction in conditioned defeat in dominant hamsters. We have found Syrian hamsters exhibit an increase in androgen receptor expression in the MeA and vLS after winning 14 encounters, while California mice show elevated androgen receptor expression in the mesolimbic dopamine system after winning three encounters (Fuxjager et al., 2010). While this might be a species difference, it is possible that more than three winning experiences are required to up-regulate androgen receptors in brain regions outside the mesolimbic system.

Because testosterone can be aromatized into estradiol, the maintenance of dominant social status might alter the expression of estrogen receptors. The estrogen receptor alpha and estrogen receptor beta subtypes are widely distributed in the brain and have distinct effects on sexual behavior, aggression and anxiety. Stimulation of the estrogen beta-receptor has consistently shown anxiolytic effects (Hughes et al., 2008; Lund et al., 2005; Walf et al., 2008). Estrogen alpha-receptors are found in abundance in the MeA, MPOA, and VMHL, brain regions involved in the regulation of male sexual and aggressive behavior (Newman, 1999; Paredes, 2003; Sano et al., 2013; Shimura et al., 1994). We focused on estrogen alpha-receptors because of their role in aggression and found that dominant and subordinate hamsters did not significantly differ in estrogen alpha-receptor expression in the MeA. These findings are consistent with research showing that knockdown of estrogen receptor alpha in the MeA of mice has no effect on aggressive behavior (Sano et al., 2013). Overall, our results suggest the maintenance of dominance relationships in male hamsters is associated with changes in androgen receptor, but not estrogen-alpha receptor, signaling in the MeA.

The LS has been implicated in the regulation of emotion, social behavior, and the hypothalamic-pituitary-adrenal axis (Herman and Cullinan, 1997; Sheehan et al., 2004). Lesions of the LS produce septal rage, which is characterized by unusually high levels of inappropriate aggression (Albert and Chew, 1980; Albert and Richmond, 1976; Sodetz and Bunnell, 1970). Indeed, pharmacological inactivation of the LS has been shown to increase aggression in non-defeat hamsters and reduce the conditioned defeat response in defeated hamsters (McDonald et al., 2012). We have previously showed that maintenance of dominant social status for two weeks increases defeat-induced c-Fos expression in the vLS

compared to subordinates and controls, and these findings indicate that neural activity in the vLS is associated with resistance to the conditioned defeat response (Morrison et al., 2012). While our results are hard to rectify with the lesion studies, the heterogeneity of cell types within the LS is likely part of the explanation. The LS sends GABAergic projections to a variety of limbic, hypothalamic, and midbrain regions and also contains GABAergic interneurons that can inhibit the projection neurons (Risold and Swanson, 1997a, b). The LS also contains a high density of androgen receptors (Roselli et al., 1989). Systemic dihydrotestosterone treatment has been shown to increase the density of corticotropin-releasing hormone type-2 receptors (CRH-R2) in the LS (Weiser et al., 2008). Because CRH-R2 activity is known to modulate anxiety-like behavior (Bale et al., 2000; Kishimoto et al., 2000), these findings might provide a mechanism by which LS activity leads to the inhibition of stress-related and fear-related behavior (Thomas, 1988). Overall, our findings suggest that an up-regulation of androgen receptors in the vLS in dominant hamsters might contribute to changes in aggression and anxiety-like behavior that reduces the expression of the conditioned defeat response.

There might be several behavioral consequences of status-dependent changes in androgen receptor expression in the MeA and vLS. First, an up-regulation of androgen receptors might facilitate aggressive behavior and increase motivation to fight. We have shown that dominant hamsters are more likely to attack and fight back against larger, resident animals during social defeat encounters (Morrison et al., 2013). However, the effect on aggression may be limited because we have shown dominant hamsters are not significantly more aggressive toward novel intruders than are subordinates (Morrison et al., 2012). Second, it is possible an increase in androgen receptor expression increases mate preference and/or increases the probability of copulation for dominant animals. Sexual behavior and dominance status are closely associated in males, with dominant animals showing increased sexual behavior in a variety of species (Blanchard and Blanchard, 1989; D'Amato, 1988; Dewsbury, 1988; Perret, 1992; Perret, 1977). Interestingly, female Syrian hamsters prefer dominant males over subordinates in a mate choice test (Brown et al., 1988). This female preference is important for males because there is a first male advantage in siring offspring (Huck et al., 1985). Finally, it is possible the increase in androgen receptor immunoreactivity within the MeA and vLS could decrease defeat-induced changes in behavior. This is consistent with our previous data showing dominant hamsters have a reduced conditioned defeat response compared to subordinates (Morrison et al., 2014; Morrison et al., 2012). While these possibilities are not mutually exclusive, future studies would need to manipulate androgen receptors within select brain regions to address a causal link.

Conclusions

The present study indicates that winning aggressive encounters increases plasma testosterone, and that repeatedly winning increases androgen receptor expression in the MeA and vLS. Because dominant hamsters exhibit a reduced conditioned defeat response compared to subordinates (Morrison et al., 2012), we propose repeated and transient increases in testosterone signaling at androgen receptors in the MeA and vLS as a possible mechanism promoting resistance to conditioned defeat in dominant hamsters. Overall, research into the neuroendocrine mechanisms that underlie status-dependent changes in

responses to social defeat should provide novel targets for the prevention and treatment of stress-related psychopathology.

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Abbreviations

CRH-R2	corticotropin-releasing hormone type-2 receptors
dMeA	dorsal medial amygdala
LS	lateral septum
LSD	least significant difference
MeA	medial amygdala
MPOA	medial preoptic area
NGS	normal goat serum
PBS	phosphate buffered saline
PBS-GT	phosphate buffered gelatin Triton
PTH2R	parathyroid hormone 2 receptor
PTSD	post-traumatic stress disorder
TIP39	tuberoinfundibular peptide of 39 residues
vLS	ventral lateral septum
vMeA	ventral medial amygdala
VMHL	ventromedial hypothalamus
vmPFC	ventromedial prefrontal cortex

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Highlights

- Dominant hamsters showed a rise in plasma testosterone 15-min after winning.
- Dominants have increased androgen receptor expression compared to subordinates.
- This increase in androgen receptor expression requires repeatedly winning social encounters.

Figure 1.

a) The diagrams indicate the location of brain regions selected for androgen receptor quantification. The diagrams were modified from the hamster atlas of Morin & Wood (2001) and values indicate the distance from bregma. The box sizes used for quantification were as follows (width $\times$ height): 325 $\mu\text{m} \times$ 650 μm (VMHL); 500 $\mu\text{m} \times$ 500 μm (vLS and MPOA); 870 $\mu\text{m} \times$ 660 μm (dMeA and vMeA). Estrogen alpha receptor immunoreactivity was also quantified in the dMeA and vMeA. b) Representative photomicrograph of the medial amygdala from a dominant hamster used for androgen receptor quantification. c) Representative photomicrograph of the medial amygdala from a dominant hamster used for estrogen alpha receptor quantification.

Figure 2.

Amount (mean $\pm$ SE) of plasma testosterone at baseline, 15 min, and 30 min following establishment of dominance relationships for dominants, subordinates and controls. We found a significant time $\times$ social status interaction, and an asterisk indicates a significant change from baseline ($P < 0.05$). $n = 9-10$ per group.

Figure 3.

Amount (mean $\pm$ SE) of plasma testosterone following 14 days of social encounters.

Asterisk indicates a significant difference compared to dominants and control animals ($P < 0.05$). $n = 10-13$ per group.

Figure 4.

a) Number (mean $\pm$ SE) of androgen receptor immunopositive cells following 14 days of social encounters in the dMeA, vMeA, VMHL, vLS and MPOA. b) Number (mean $\pm$ SE) of estrogen alpha receptor immunopositive cells following 14 days of social encounters in the vMeA and dMeA. Asterisks indicate a significant difference compared to subordinate and control animals ($P < 0.05$). $n = 10-15$ per group.

Figure 5. Number (mean $\pm$ SE) of androgen receptor immunopositive cells following a single social encounter in the dMeA, vMeA and vLS. $n = 10-11$ per group.

Table 1

Subjects form stable dominance relationships

	Day 1	Day 7	Day 14
Subordinates – Total Submissive Behavior (mean $\pm$ SE)	87.6 s $\pm$ 30.49	176.8 s $\pm$ 17.02	196.7 s $\pm$ 18.31
Dominants – Total Aggressive Behavior (mean $\pm$ SE)	87.8 s $\pm$ 30.63	139.2 s $\pm$ 15.24	151 s $\pm$ 18.84

Subjects were weight-matched in resident-intruder dyads and paired daily in social encounters for 14 days. Day 1 encounters were 600 s in duration, while days 7 and 14 encounters were 300 s in duration. Dominants continuously displayed high rates of aggression throughout all 14 days, while subordinates maintained high rates of submissive behavior. n = 16 per group.