8-2016

Investigating the Role of Testosterone Signaling at Androgen Receptors in Resiliency to Social Stress

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I am submitting herewith a dissertation written by Catherine Tucker Clinard entitled "Investigating the Role of Testosterone Signaling at Androgen Receptors in Resiliency to Social Stress." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Experimental Psychology.

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Investigating the Role of Testosterone Signaling at Androgen Receptors in Resiliency to Social Stress

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Catherine Tucker Clinard
August 2016

Dedication

To my husband

Jonathan Clinard

my mother and stepfather

Laurie Toth

Michael Toth

and my son

Cayden Clinard

Acknowledgements

As I reflect back on my educational journey there are multiple people who I owe a sincere thank you to. This accomplishment would not have been achieved without the love and support of the following individuals.

Dr. Matt Cooper has been the epitome of an excellent mentor. Dr. Cooper helped me transition from a naïve undergraduate to a confident graduate student. He challenged me most days, but encouraged me on the tough days. I appreciate the daily wisdom he shared, and for teaching me all the scientific techniques and skills I possess. He pushed me to be a more critical thinker, a better writer and a poised speaker. I leave graduate school knowing I am ready for my next professional endeavor because of Dr. Cooper. I also sincerely appreciate the mentorship that my committee members have shown to me. Drs. Todd Freeberg, Jim Hall, Theresa Lee and Rebecca Prosser have given their time over the past 5 years to attend my committee meetings and read my various papers and proposals. Collectively, their guidance and support has been instrumental in shaping me into the scientist and woman I am today. I would like to give an extra thanks to Dr. Prosser. As a wife, mother and a very successful scientist, she has been an admirable role model for me. I appreciate the extra support and encouragement she has shown me since I have become a mother.

I would like to thank a couple of key people from my undergraduate career that propelled me on the journey to graduate school. Dr. Brian Hock, an Assistant Professor of Psychology at Austin Peay State University, first introduced me to biological psychology. The idea that psychology was more than clinicians and counselors was new to me at this time, and learning about the biological underpinnings of mental illness was fascinating to me. Dr. Hock introduced me to the idea of graduate school in a biological psychology program where he explained I could conduct research to further investigate these interesting topics. Dr. Hock also helped me design my first research project, and mentored me throughout the project. I owe my first trip to Society for Neuroscience and my first publication to Dr. Hock. Despite attending a small university with limited research facilities, I was accepted into a PhD program because of the great mentorship of Dr. Hock. I would also like to acknowledge Sara Robson, the women's golf coach at Austin Peay State University. Coach was able to recognize that life meant more to me than just playing golf. She supported me in all of my academic endeavors, and taught me how to manage my time in a way that I could be successful both on the golf course and in the classroom. Because of my supportive coach I was able to excel in something other than athletics.

There is a team of students in the Cooper lab that I must also thank. My graduate student peers, Katie Morrison, Lauren Bader, Brooke Dulka and Alex Grizzell made research fun even when the results were not. They made the day-to-day grind of wet lab research much more enjoyable. I will forever cherish the friendships that have evolved from sharing a tiny office with each of you. There are so many exceptional undergraduate students who have passed through the Cooper lab during my time, many of whom have directly contributed to this dissertation. For the hours of animal testing, tissue slicing (oh, the slicing!), histology, dishes, behavioral scoring and more, I am eternally grateful. I have thoroughly enjoyed mentoring each of the undergraduates, and I am proud of the people they have become. But what these students don't know is they were

just as important in my life, as I was in theirs. I would like to especially recognize Samuel Adler and Abigail Barnes for their work in this dissertation. They were instrumental in getting this project across the finish line. Each of them worked many weekends for me so I could have a day off, and for that I am appreciative.

The administrative staff in the Psychology department has been critical in helping me navigate the administrative hoops that are part of graduate school. Connie Ogle, Jonathan Kelly and Mary Ellen Hunsberger have each played an important role in my success. Their warm, welcoming, and smiling faces will be missed.

Last but not least I owe the largest thanks to my family. My family has always been supportive of my academic and professional goals. My parents have provided endless love and encouragement, and provided the financial support to make my dreams possible. I am thankful for the strong influential women in my life; my mother who is my best friend and my Aunt Nancy who has loved me like her own child. My "adopted" parents in Knoxville, Nan and Bruce, have been critical in helping me adjust to life away from my immediate family. They have continuously welcomed Jon, Cayden and myself into their home, and loved us like we were apart of their family. A special thanks to Nan who voluntarily took care of Cayden every afternoon, so I could stay at school late in the evenings. My husband, Jon, has been on this journey with me since we were undergraduates and provided daily support along the way. He may not have always been able to understand the research or help me solve a scientific problem, but he made me believe that I could figure it out. He instilled confidence in me. Jon has been patient with me when I had to put work first, and understanding of the sudden demands of working in a lab with animals. I appreciate the sacrifices he has made in his own life, so I could attend graduate school. Finally, I hope my 1-year old son, Cayden, will one day read this and admire the work of his mother. Becoming a mother has been the ultimate transformation and it happened at the end of graduate school. Becoming a mother has further motivated me to finish this PhD, so I could begin a successful professional career providing for my family. With that said, Cayden has taught me there is so much more to life than just work. A bad day in the lab is suddenly not bad when you go home to a smiling little boy. I am thankful for all the joy he brings to my life, especially his "loves". To everyone in my family, whether mentioned here or not, I would like to say I love you, and that this is an accomplishment for us all because we were on this journey together.

Abstract

Social experience can alter how individuals cope with stressful events and contribute to individual differences in stress vulnerability. We have previously tested dominant and subordinate male Syrian hamsters (*Mesocricetus auratus*) in a conditioned defeat model and found that dominant individuals show reduced defeat-induced changes in behavior compared to subordinates. Dominant hamsters also show increased neural activation following social defeat stress in brain regions that regulate social behavior and coping with stress, including the medial amygdala (MeA). Because winning aggressive encounters generates a surge in plasma testosterone and androgen receptors are abundant in the MeA, we tested whether testosterone signaling at androgen receptors in the MeA contributes to the reduced effects of social defeat stress in dominant hamsters. Our overarching hypothesis was that dominant hamsters experience daily surges in plasma testosterone during the maintenance of their social status that increase the expression of androgen receptors in the MeA, which are necessary for their reduced conditioned defeat response compared to subordinates. We found that dominant hamsters experience a significant rise in plasma testosterone 15-min following an aggressive encounter compared to their pre-encounter baseline, whereas subordinates and control animals showed no change in testosterone. Furthermore, we investigated whether changes in androgen receptor and estrogen alpha-receptor immunoreactivity occur during the maintenance of dominance relationships. We paired male hamsters in daily agonistic encounters for 14 days to establish and maintain dominant/subordinate relationships. Dominant hamsters showed significantly more cells expressing androgen receptor immunoreactivity, but not estrogen alpha-receptor immunoreactivity, in the dorsal MeA

(dMeA) and ventral lateral septum (vLS), compared to subordinates and controls. Also, blockade of androgen receptors with flutamide during the maintenance of dominance relationships increased the conditioned defeat response in dominant animals compared to vehicle-treated counterparts. Flutamide-treated dominant animals also showed reduced androgen receptor immunoreactivity in the dMeA, but not the vLS, compared to vehicle-treated dominants. Altogether, these results suggest that the maintenance of dominant social status generates neural plasticity that is associated with an upregulation of androgen receptors in the dMeA, and that activation of androgen receptors is necessary for resistance to conditioned defeat.

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Abbreviations and Symbols

5-HT- serotonin

ACTH- adrenocorticotropic hormone

aMeA- anterior medial amygdala

BDNF- brain-derived neurotrophic factor

BNST- bed nucleus stria terminalis

CD- conditioned defeat

CRF- corticotropin-releasing factor

CRH- corticotropin-releasing hormone

CRH-R2- corticotropin-releasing hormone type-2 receptors

dMeA- dorsal medial amygdala

DRN- dorsal raphe nucleus

ES- escapable tail shocks

HPA- hypothalamic-pituitary-adrenal

IL- infralimbic (subregion of the ventral medial prefrontal cortex)

IS- inescapable tail shocks

LS- lateral septum

LSD- least significant difference

MeA– medial amygdala

MPOA- medial preoptic area

NAc- nucleus accumbens

NGS- normal goat serum

PBS- phosphate buffered saline

PBS-GT- phosphate buffered gelatin Triton

PTH2R- parathyroid hormone 2 receptor

PTSD- post-traumatic stress disorder

PVN- paraventricular nucleus of the hypothalamus

TIP39- tuberoinfundibular peptide of 39 residues

vLS- ventral lateral septum

vMeA- ventral medial amygdala

VMHL- ventromedial hypothalamus

vmPFC - ventromedial prefrontal cortex

VTA- ventral tegmental area

Chapter 1

General Introduction

The perception of aversive or threatening situations activates the hypothalamic-pituitary-adrenal (HPA) axis and initiates the neuroendocrine stress response, as well as activates extra-hypothalamic brain regions that regulate cognitive and emotional responses. Stressful situations disrupt physiological homeostasis and the purpose of the stress response is to restore homeostatic balance (Levine, 2005). However, stressors that are prolonged, intense, and exceed the body's ability to maintain homeostasis increase the risk for mood and anxiety disorders. For instance, stress is a contributing factor in the onset of psychopathologies such as major depression and post-traumatic stress disorder (PTSD) (Kendler et al., 1999; Vermetten and Bremner, 2002). However, exposure to stressful life events does not always lead to a stress-related psychological disorder. Interestingly, most stress-related psychiatric disorders are more prevalent in women than in men. For depression and PTSD, women are twice as likely as men to experience symptomatic episodes (Bangasser, 2013). Furthermore, although male soldiers often experience the same traumatic events during combat, only a small proportion will later develop PTSD. There is great interest in the underlying mechanisms controlling why some individuals are vulnerable to the development of stress-related mental illness, while others are resilient. Also, the National Institutes of Health have called for more research to investigate sex differences in susceptibility to stress-related disease. Moving forward, it is important to understand changes in neuroendocrine signaling that occur following stress and what protects some individuals from the negative consequences of prolonged and traumatic stress.

While translating pre-clinical findings to humans is an important goal, animal models must be utilized to investigate the cellular and molecular mechanisms underlying stress-related mental illness. Most animal models of stress utilize physical stressors such as foot-shock, forced swim or immobilization. While these stressors are useful, they don't represent the most common

type of stress experienced by humans. In westernized societies, psychosocial stress, including low social status and poor social support, is more commonly experienced than physical stress and thought to lead to poor psychological health (Mama et al., 2016). Because different stressors can activate different neural circuits and elicit unique behaviors, it is important for researchers to choose a stressor that best represents the human condition (Lopez et al., 1999; Vermetten and Bremner, 2002). For this reason, we use a social defeat model to investigate the neural mechanisms controlling stress-induced behavioral responses.

Social Defeat Stress

Social defeat is an ethologically relevant stressor that has been used to model stress-related behavior in a variety of species including rats, mice, hamsters, tree shrews, crayfish, and rainbow trout (Blanchard et al., 1995b; Fuchs and Flugge, 2002; Fujimoto et al., 2011; Huhman et al., 1990; Koolhaas et al., 1997; Larson et al., 2004; Trainor and Marler, 2001). Social defeat is a potent stressor that occurs when an animal is attacked and subjugated by an aggressive conspecific. Defeated individuals show a variety of behavioral changes, including decreased locomotor activity (Kramer et al., 1999), decreased self-grooming (Kramer et al., 1999), disturbed sleep patterns (Fuchs and Flugge, 2002) and increased social avoidance (Berton et al., 2006; Huhman et al., 1990; Kramer et al., 1999). In Syrian hamsters, a single social defeat causes a suite of responses in the loser, including a hormonal stress response that is characterized by elevated plasma adrenocorticotropic hormone, B-endorphin, cortisol and corticosterone (Huhman et al., 1990; Huhman et al., 1991). For these reasons, we elect to use a social defeat model in Syrian hamsters called conditioned defeat. In the conditioned defeat model, subjects receive an acute social defeat in the home cage of a larger, aggressive hamster and are then tested 24 hours later in their own home cage with a smaller, non-aggressive hamster. During testing, defeated

animals show a loss of species-typical territorial aggression and an increase in submissive and defensive behavior, such as flee, tail lift, paws raised, and tooth chatter, compared to non-defeated controls, and this defeat-induced change in future agonistic behavior is called the conditioned defeat response. Conditioned defeat has been shown to last at least 33 days without further social defeat experience, suggesting the response is robust and long-lasting (Huhman et al., 2003).

Syrian hamsters are an ideal species for a social defeat model because they are readily and reliably aggressive. Their aggression incorporates many ritualized behaviors, and as a result there is minimal wounding during agonistic encounters. Furthermore, agonistic behavior in Syrian hamsters is well characterized and easily quantified (Wommack and Delville, 2007). In the wild, hamsters are regarded as solitary and territorial animals (Nowak and Paradiso, 1983). This territoriality translates into the laboratory setting because when male and females are singly housed, residents will reliably attack intruders placed into their home cages. A resident-intruder paradigm simplifies social defeat in Syrian hamsters compared to other rodent species. For instance, in rat and mouse models researchers often have to manipulate housing conditions to either elicit aggression or reduce the degree and frequency of wounds (Meerlo et al., 1996). In the conditioned defeat model, an acute social defeat produces profound and lasting changes in behavior. This is unique compared to other rodent species in which chronic social defeat is necessary to elicit these behavioral changes (Berton et al., 2006). An acute model has the added benefit of allowing researchers to target the neural plasticity that controls the acquisition and consolidation of defeat-induced changes in behavior. Taken together, the reliably aggressive nature of the hamster, minimal wounding during defeat and potency of a single defeat makes this species ideal for studying the neural correlates of social stress.

The conditioned defeat model also allows for investigation of variability in the response to social stress. The degree of submissive and defensive behavior following social defeat can be variable between animals. We have previously shown male hamsters that have maintained dominant social status for 14 days show less submissive and defensive behavior following social defeat compared to subordinates (Morrison et al., 2012). Identifying the neurobiological mechanisms controlling resistance to the conditioned defeat response will improve our understanding of why some individuals develop stress-related mental illness after a traumatic event, while others do not. Several behavioral factors have been identified as important for human resiliency, including active coping (Steinhardt and Dolbier, 2008), cognitive flexibility (Dumont and Provost, 1999) and positive social support (Karatsoreos and McEwen, 2013), but the cellular and molecular mechanisms underlying resiliency remain unknown. Investigation into the biological basis of individual differences in stress resilience is a growing field of study, and there are several models for studying both individual differences in coping with stress and experience-dependent changes in stress vulnerability.

Individual Variation in Stress Reactivity

Individual consistency in behavior that is both stable over time and across situations has been demonstrated in a wide variety of animals including rodents, birds, fish, insects and primates (Bell, 2007; Koolhaas, 2008; Sih et al., 2004). Many different terms have been used to classify these consistent types of behavior. For instance, behavioral syndromes are used to describe a suite of correlated behaviors, but terms such as temperament, personality and coping style are also interchangeably used. Across the literature it is well agreed that there are some behavior patterns that enable environmental control and are better suited to increase individual

fitness. Understanding the mechanisms underlying an individual's coping capacity and disease vulnerability is important for prevention and treatment of stress-related mental illness.

Proactive and Reactive Coping Styles

In stress research, there are two common coping styles that have been identified and studied for over 100 years. The first response was originally described as the fight-flight response, and is considered an active or proactive response (Cannon, 1915). Immobility and low levels of fight characterize the second response and is called the conservation-withdrawal response or a passive or reactive response (Engel and Schmale, 1972). In rodent models, animals are often categorized by their coping style and then tested to investigate population differences in response to multiple tasks. For instance, an animal's tendency to initiate aggressive behavior is predictive of the individual's reaction to other, non-social environmental challenges. Active or proactive rats are identified by increased levels of offensive aggression in a resident-intruder paradigm, and they will also actively bury a shock-probe during a defensive bury test and show high levels of swimming during a forced swim test. Conversely, passive or reactive rats show low levels of offensive aggression when faced with an intruder, avoid a shock-probe during a defensive bury test, and float during a forced swim test (Koolhaas et al., 2007). Several neurochemical and neuroendocrine differences exist between the two coping styles. Proactive animals show high sympathetic reactivity as measured by high epinephrine, norepinephrine, heart rate and blood pressure (Koolhaas et al., 2007). Reactive coping animals show a larger corticosterone response in response to a stressor, although there have been some inconsistencies across species (Overli et al., 2007; Veenema et al., 2003). Also, proactive rats show increased sensitivity of 5-HT1a and 5-HT1b autoreceptors compared to reactive rats, indicating that they have enhanced inhibitory control of the serotonin (5-HT) system (de Boer and Koolhaas, 2005).

Similarly, proactive and reactive coping styles have been investigated in mice. Mice have been bred to display a bimodal distribution in attack latencies in a resident-intruder test. The long-attack latency mice are less aggressive, and more vulnerable to the effects of chronic social defeat compared to the more aggressive, short-attack latency mice. In response to social defeat stress, long-attack latency mice show a longer lasting body weight loss, a greater increase in corticosterone and increased anxiety-like and depression-like behaviors compared to short-attack latency mice (Veenema et al., 2003). These mice also show differences in stress-related neuroendocrine measures and serotonin signaling. Consistent with their depressive-like behavior, the long-attack latency mice have a lower mineralocorticoid to glucocorticoid receptor ratio, which is characteristic of HPA axis dysregulation. In the forced swim test, the short attack latency mice show decreased concentrations of 5-HT in the frontal cortex, striatum, lateral septum, hippocampus, amygdala and brain stem compared to long-attack latency mice (Veenema et al., 2005). Similar to proactive rats, short attack latency mice have increasd 5-HT1a autoreceptor sensitivity (de Boer et al., 2009). Taken together, these data suggest that high aggression phenotypes often exhibit the neuroendocrine and neurochemical markers of a proactive coping style. Additionally, baseline differences in aggressive behavior are useful predictors of individual coping style.

Trait Anxiety in Rats

In another animal model of coping, male Wistar rats have been bred for baseline trait anxiety on the elevated plus maze. High anxiety rats spend significantly less time in the open arms, while low anxiety rats spend significantly more time in the open arms (Landgraf and Wigger, 2002). These rats have been useful in investigating population differences on a variety of neuroendocrine, neurochemical and behavioral measures. Interestingly, these rats have a

modality specific endocrine profile. The high anxiety rats show increased HPA axis activity following non-social stressors such as the elevated plus maze or open field test, but display no alteration following a social stressor compared to low anxiety rats (Landgraf and Wigger, 2002; Salome et al., 2004; Veenema and Neumann, 2007). Therefore, low anxiety rats appear to find social stimuli more stressful whereas high anxiety rats appear to find non-social stimuli more stressful. Low anxiety rats display more aggression than high anxiety rats when faced with an intruder, and accordingly show increased neural activation in brain regions associated with aggressive behavior (Veenema and Neumann, 2007; Veenema et al., 2007). During a fear-conditioning test, both types of rats acquire fear equally, but on subsequent extinction trials the high anxiety rats are slower to extinguish (Muigg et al., 2008). Similarly, following social defeat stress, the high anxiety rats show neural activity in brain regions associated with the production of fear and anxiety, such as the amygdala and hypothalamus, while the low anxiety rats show more activation in brain regions associated with stress resistance, such as the medial prefrontal cortex (Frank et al., 2006). Taken together, these findings parallel those from mice with short and long attack latencies. Overall, rats with low trait anxiety and high aggression display a proactive coping style, and rats with high trait anxiety and low aggression display a reactive coping style. This line of research was also among the first to indicate that a proactive coping style is associated with elevated stress-induced neural activity in the medial prefrontal cortex.

Individual Differences in Response to Chronic Social Defeat

There is a large body of research on susceptibility and resistance to chronic social defeat stress. Unlike the previous animal models that screen their animals on baseline measures of behavior, this model uses inbred C57BL/6 mice to investigate the molecular basis of latent phenotypic differences that only arise following social defeat (Krishnan et al., 2007). Mice are

subjected to daily bouts of social defeat, followed by continuous protected sensory contact with their CD1 aggressor mouse. Mice are exposed to a different CD1 aggressor mouse each day for 10 days and are then screened for social behavior. In the social interaction test mice are placed in a neutral arena that contains a mesh box housing a novel CD1 mouse, and the duration of time spent investigating and avoiding the mesh box is quantified. Susceptible mice show increased avoidance of the unfamiliar CD1 mouse compared to an empty mesh box, while unsusceptible mice are attracted to the CD1 mouse more than the empty mesh box. Approximately 30% of defeated mice fail to show social avoidance, and are deemed unsusceptible (Golden et al., 2011). Furthermore, this difference in defeat-induced social avoidance generalizes to other behavioral measures. Susceptible mice show decreased body weight and reduced sucrose preference, both consistent with increased depression-like behavior (Krishnan et al., 2007). Susceptible mice also show altered autonomic arousal and circadian amplitude of temperature fluctuations, and they display significant conditioned place preference to a low dose of cocaine. However, susceptible and unsusceptible mice do not differ on all types of stress-related behavior. Both susceptible and unsusceptible animals show increases in anxiety, by spending less time in the open arms of the elevated plus maze, and a sensitized corticosterone response to swim stress. Collectively, these data show that the development of defeat-induced social avoidance in susceptible mice is associated with depressive-like behaviors, but that chronic social defeat stress elicits anxiety-like behavior and altered corticosterone reactivity regardless of susceptibility.

The mesolimbic dopamine system is a critical neural circuit controlling susceptibility to chronic social defeat. Using a variety of techniques, Nestler and colleagues have proposed a model in which susceptible mice show heightened phasic firing of ventral tegmental area (VTA) dopaminergic neurons projecting to the nucleus accumbens (NAc), resulting in increased brain-

derived neurotrophic factor (BDNF) signaling in the NAc (Graham et al., 2009; Wang et al., 2013a). This heightened VTA-NAc BDNF signaling results in increased social avoidance, and may also cross-sensitize susceptible animals to drugs of abuse. Unsusceptible mice activate at least two mechanisms that diminish activity of the VTA- NAc circuit. First, they show resistance to the increased firing rate of VTA neurons by upregulating potassium channels in dopamine neurons, which functions to stabilize their excitability (Krishnan et al., 2007). Second, they display an induction of the transcription factor delta FosB in the NAc, increasing the expression of a number of genes involved in promoting resilience (Krishnan, 2014). Specifically, unsusceptible mice upregulate 159 genes in the NAc, compared to 91 upregulated genes in susceptible mice (Krishnan et al., 2007). Additionally, neural activity in the ventral medial prefrontal cortex (vmPFC) underlies individual differences in vulnerability to social defeat stress (Kumar et al., 2014). In mice that express a strong depressive-like phenotype following chronic social defeat stress (i.e. susceptible animals), optogenetic stimulation of the vmPFC exerts antidepressant-like effects (Covington et al., 2010). Interestingly, these findings suggest resilience is an active process, with its own cellular and molecular mechanisms, and is not simply the absence of susceptibility. These findings suggest that enhanced BDNF signaling within the mesolimbic dopamine circuit; and reduced activity of vmPFC neurons are critical neurobiological mechanisms promoting susceptibility to chronic social stress.

Experience-Dependent Resistance to Stress

Many lines of research indicate adverse experiences increase vulnerability to the negative effects of subsequent stress. Early life stressors, including prenatal stress and maternal deprivation, impoverished environmental housing and chronic subordination all increase susceptibility to the negative effects of future stressors. Interestingly, there are environmental

factors that can buffer an individual against the negative effects of stress, including environmental enrichment, exercise, stressor controllability, stress inoculation and dominant social status.

Environmental Enrichment

In rodent models, an enriched environment describes a housing environment that is more complex relative to standard laboratory housing conditions. Generally, enriched animals are kept in a larger cage, and in larger groups with the opportunity for more multifaceted social interaction. The enriched environment can be varied over time, but typically includes tunnels, plastic and wooden toys, running wheels, dietary treats and nesting material (van Praag et al., 2000). Results suggest that not any single element can account for the consequences of environmental enrichment, but instead an interaction of factors is essential. Environmental enrichment has been associated with improved learning and memory, as measured in a water-maze task (Leggio et al., 2005), novel object recognition test (Bruel-Jungerman et al., 2005), and radial arm maze (Leggio et al., 2005). Environmental enrichment has been shown to increase neurogenesis in the dentate gyrus of the hippocampus, increase dendritic growth, increase synapse formation and enhance gliogenesis, neurite branching and synapse formation in the cortex (van Praag et al., 2000). Taken together, these data suggest environmental enrichment increases neural plasticity in brain regions that modulate learning and memory processes.

Additionally, providing individuals with three weeks of environmental enrichment can confer resistance to future social defeat stress (Lehmann and Herkenham, 2011; Schloesser et al., 2010). In these studies, mice that are housed in an enriched environment show decreased anxiety and depressive-like behaviors, as measured by light/dark transition test, tail suspension test and forced swim test, following two weeks of social defeat stress. Specifically, mice that were

housed in an enriched environment showed a similar preference for the light and dark sides in the light/dark transition test and similar time spent immobile in both the tail suspension test and forced swim test as non-defeated controls. Furthermore, mice that were housed in an enriched environment show less defeat-induced social avoidance compared to non-enriched mice (Lehmann and Herkenham, 2011; Schloesser et al., 2010). Additionally, Lehmann and colleagues have identified the infralimbic subregion (IL) of the vmPFC as a critical structure mediating the effects of environmental enrichment (Lehmann and Herkenham, 2011). Specifically, mice housed in an enriched environment for three weeks display an increase in FosB/ΔFosB-positive cells in the IL following social defeat compared to non-enriched mice. Lesioning the IL prior to environmental enrichment eliminates resistance to social defeat, but lesioning the IL after enrichment but prior to social defeat does eliminate the resiliency conferred by enriched housing. Taken together, activation of the IL during environmental enrichment appears to be critical for the development of resilience.

Wheel Running

Exercise can prevent the development of stress-related mood disorders, and researchers have investigated the neural mechanisms underlying the protective effects of physical activity. A useful model for manipulating physical activity in rodents is to allow them voluntary access to running wheels. Voluntary wheel running avoids the confounding effects of forced exercise, and is actually rewarding for the animal. Fleshner and colleagues use a model in Sprague-Dawley rats to investigate the mechanisms by which voluntary wheel running modulates learned helplessness. Rats that are allowed six weeks of voluntary access to running wheels prior to exposure to a series of uncontrollable tail shocks are protected against the exaggerated fear and shuttle box escape deficits that are characteristic of learned helplessness (Greenwood and

Fleshner, 2008). More recently, voluntary wheel running has been shown to improve REM sleep following stress and attenuate stress-induced changes in diurnal rhythms, which can be characteristic of stress-related mood disorders (Thompson et al., 2016). Interestingly, voluntary wheel running does not dampen the stress response to uncontrollable stress because wheel run rats mount similar corticosterone responses compared to sedentary rats (Fleshner, 2000). Furthermore, c-Fos mapping studies indicate similar neural activation between wheel run and sedentary rats in stress-responsive brain regions such as the basolateral amygdala and central nucleus of the amygdala (Greenwood et al., 2005a). Therefore it seems plausible that voluntary wheel running produces adaptations in specific neural circuits, but does not globally modulate the physiological stress response.

Neurotrophic factors, serotonergic neurotransmission and noradrenergic neurotransmission have been investigated as factors modulating exercise-dependent resistance to stress. For example, voluntary wheel running decreases activation of norepinephrine-producing cells in the locus coeruleus during uncontrollable tail shock, which is believed to reduce norepinephrine inhibition within the dorsal raphe nucleus (DRN) (Greenwood et al., 2003). Increased neural activity was displayed in the mesolimbic dopamine pathway following voluntary wheel running, and changes in monoamine mRNA levels within this pathway were also detected, including increased tyrosine hydroxylase in the VTA and decreased D2 receptor mRNA in the NAc (Greenwood and Fleshner, 2011). Also, wheel running produces significantly increased BDNF mRNA in the dentate gyrus of the hippocampus and the basolateral amygdala compared to sedentary rats (Greenwood et al., 2009). The combination of these running-dependent changes may contribute to resistance to learned helplessness, but the role of 5-HT within the DRN is crucial. The DRN sends 5-HT efferent projections to many brain regions and

plays a key role in modulating depression, anxiety and fear responses. DRN 5-HT neurons are particularly sensitive to stressor controllability, and learned helplessness is tightly linked to the hyperactivation and sensitization of these neurons (Christianson et al., 2008; Maier and Watkins, 2005). Research suggests wheel running produces changes within the DRN that constrain the exaggerated 5-HT response characteristic of learned helplessness. For example, voluntary exercise results in increased 5-HT1a autoreceptor mRNA in the DRN, which constrains activation of DRN 5-HT neurons (Greenwood et al., 2005b). In summary, voluntary wheel running in rats has been a useful model for investigating experience-dependent neural plasticity underlying resistance to learned helplessness.

Stressor Controllability

The degree of behavioral control an organism has over an aversive event potentially modulates the impact of that event. For instance, rats exposed to a series of inescapable tail shocks (IS) later fail to learn to escape from footshock in a shuttle box and show exaggerated fear conditioning. However, rats that are exposed to exactly equal amounts of escapable tail shocks (ES) do not show learned helplessness behavior in the shuttle box test (Maier et al., 1995). Maier and colleagues have extended their studies of stressor controllability to investigate the mechanisms by which exposure to controllable stress immunizes animals against the effects of subsequent uncontrollable stress. The vmPFC regulates the immunizing effect of controllable stress exposure, because it exerts inhibitory control over structures that mediate fear and stress-related processes, including the DRN (Jankowski and Sesack, 2004). Temporary inactivation of the vmPFC prior to ES leads to the development of learned helplessness as if the animal were exposed to IS (Amat et al., 2005). In addition, experience with ES one week prior to IS exposure buffers against the shuttle box escape deficit that typically occurs following IS. Once again

temporary inactivation of the vmPFC during the initial ES blocks the buffering effect of ES on later IS (Amat et al., 2006). Inactivation of the vmPFC during exposure to IS also blocks the buffering effects of prior ES, indicating the vmPFC is important for retrieving the memory of prior control (ES experience) (Amat et al., 2006). However, if the vmPFC was inactivated immediately after ES or during the shuttle box test there was no effect on learned helplessness behavior (Amat et al., 2006). Similarly, treatment of anisomycin, which is a protein synthesis inhibitor, into the vmPFC during ES prevented the buffering effect of ES on later exposure to IS. Taken together, these findings suggest vmPFC activation during experience with control is critical for resistance to learned helplessness. Work has additionally be done to investigate if activation of the vmPFC during IS is sufficient to produce ES-like effects on later IS exposure. Pharmacological activation of the vmPFC with the $GABA_a$ receptor antagonist, picrotoxin, while experiencing IS results in that uncontrollable stress producing resistance to learned helplessness following another experience with IS one week later (Amat et al., 2008). These results suggest that activation of the vmPFC during uncontrollable stress is sufficient to buffer against learned helplessness. Stressor controllability has been further investigated to see if its buffering effects extend beyond learned helplessness. Social defeat stress will produce shuttle box escape deficits similar to IS, and exposure to ES one week prior to social defeat stress prevents the social defeat induced shuttle box escape deficits (Amat et al., 2010). This suggests the experience of stressor controllability can buffer against the effects of both IS and social defeat.

Stress Inoculation

Stressful experiences increase susceptibility to the negative consequences of stress in the future. Interestingly, however, mild stressors have also been linked to the subsequent development of resilience. It is believed that stressful events that are not overwhelming, but

challenging enough to elicit emotional activation and cognitive processing may make future coping efforts more effective (Fergus and Zimmerman, 2005). The development of resilience through stress inoculation has been investigated in squirrel monkeys. In this model, monkeys are raised in groups of 3-4 mother-infant pairs, and stress inoculation occurs at 17 weeks of age. The stress inoculation protocol consists of 10 weekly 1-hour social separations, where each infant is individually housed, but can still see, hear and smell other monkeys. Separations increase plasma cortisol, and evoke distress calls and locomotor agitation, but this stress is considered mild and is not believed to overwhelm the capacity for coping with adversity (Coe et al., 1983). When the stress-inoculated monkeys are tested at 9 months of age in a novel environment stress test they show less anxiety, as inferred by decreased maternal clinging and increased object exploration, and decreased plasma cortisol compared to non-inoculated peers (Parker et al., 2004). These results suggest mild stressful early life experiences strengthen socioemotional and neuroendocrine resistance to subsequent stressors. More recently, Lyons and colleagues showed that stress inoculation is not restricted to critical or sensitive periods in development and protects adult monkeys against subsequent stress-induced anhedonia, as measured by sucrose preference (Lee et al., 2014). Additional work into the neural mechanisms underlying the buffering effect of stress inoculation has identified the vmPFC as a potential meditating structure. Stress-inoculated monkeys show increased vmPFC cortical volumes, as determined by high-resolution magnetic resonance imaging (Lyons et al., 2002). Prefrontal corticolimbic circuits are known to play a role in cognitive control of behavior, and increased vmPFC activation corresponds with diminished amygdala activity. Therefore, it is possible stress inoculation enhances prefrontal inhibition of amygdala activity in monkeys, and increases cognitive control of emotions (Lyons and Parker, 2007).

To investigate the cellular and molecular mechanisms that regulate the effects of stress inoculation in the future, Lyons and colleagues have recently extended their research from monkeys to mice. Stress-inoculated mice are exposed to an aggressor mouse for 15-min, every other day for 21 days, but a mesh screen separates the aggressor mouse (Brockhurst et al., 2015). The mesh screen provides a barrier between the animals to prevent physical contact, therefore the stressor is perceived as mild. The stress-inoculated mice perform better than non-inoculated mice in a range of behavioral and hormonal tests. The stress-inoculated mice show less immobility on a tail-suspension test, less freezing in an open field test, increased novel object recognition, and reduced corticosterone responses to repeated restraint stress compared to non-inoculated mice (Brockhurst et al., 2015). Taken together, models of stress inoculation are useful for investigating an experience-dependent learning-like process that resembles interventions designed to build resilience in humans.

Social Status

Social status has been shown to alter an individual's ability to cope with stress in a variety of species. Specifically, dominant individuals often display more effective coping strategies in the face of stress. In *Anolis carolinensis* lizards, dominant individuals display a proactive behavioral strategy during courtship, feeding and social encounters, while subordinates adopt a reactive strategy (Korzan et al., 2006; Ling et al., 2009). Dominant and subordinate lizards differ in neurochemical and neuroendocrine responses to restraint stress. For example, dominants mount an immediate corticosterone response to restraint, while subordinates have a delayed corticosterone response (Ling et al., 2009). In the visible burrow system (VBS), rats are housed in a semi-naturalistic mixed sex group for two weeks, in which dominance relationships form (Blanchard et al., 1995b). Social status acquired during this time alters how individuals

respond in an anxiety test. The dominant rats display more time investigating the open arms of an elevated plus maze compared to subordinates, indicating less anxiety (Davis et al., 2009). Chronic subordination in the VBS results in increased basal corticosterone compared to dominant and control rats. However, when challenged with restraint stress a proportion of subordinate rats fail to mount a normal neuroendocrine response (McKittrick et al., 1995). Stress-induced differences in 5-HT receptors are also noted between dominant and subordinate rats. For example, subordinates display a down-regulation of 5-HT1a receptors in the hippocampus and an up-regulation of 5-HT2 receptors in the parietal cortex compared to controls (McKittrick et al., 1995).

When housed in groups, primates form social hierarchies. Dominant long-tailed macaques have priority over access to resources such as food, water and primary resting sites, whereas subordinates receive more aggression, spend more time alone, and are groomed less often than dominants (Shively, 1998; Shively et al., 1997). Therefore, in this social system subordinates appear to be stressed relative to dominant peers. Social rank in primates is also associated with differences in neuroendocrine responses to stress and changes in brain regions controlling drug addiction. For instance, dominant male monkeys show a smaller hormonal stress response than subordinates following stressors such as capture and restraint (Honess and Marin, 2006). Furthermore, subordinate monkeys show reduced availability of dopamine D2 and D3 receptors in the caudate nucleus and putamen and increased vulnerability to cocaine addiction (Morgan et al., 2002; Nader et al., 2012). Interestingly, subordinates do not always show greater baseline cortisol levels compared to dominants. In long-tailed macaques (Shively et al., 1997), olive baboons (Sapolsky et al., 1997) and squirrel monkeys (Steklis et al., 1986) subordinate individuals show greater baseline cortisol levels than those measured in dominants, but in

marmosets (Saltzman et al., 1996), cotton top tamarins (Ginther et al., 2001) and rhesus macaques (Bercovitch and Clarke, 1995) subordinate individuals have similar or lower cortisol levels compared to dominants. The inconsistent relationship between social status and baseline cortisol levels is likely related to the perceived amount of stress and ability to cope. A meta-analysis supports the view that subordinates show a higher hormonal stress response only if they experience higher rates of stressors and fewer opportunities for coping compared to dominants (Abbott et al., 2003). Taken together, there are status-dependent differences in neuroendocrine and neurobiological factors that modulate susceptibility to stress and drug addiction in primates.

Recent work from our lab has shown that social status modulates the way hamsters initially respond to aggressive encounters and the long-term behavioral consequences of social defeat stress (Morrison et al., 2011). Hamsters quickly form dominance relationships and readily maintain their social status for up to two weeks. During social defeat, a dominant hamster will counter attack the resident aggressor, while subordinates will not, suggesting dominants use a more active coping strategy when faced with aggression than do subordinates. Dominant individuals also show a reduced conditioned defeat response compared to subordinates (Morrison et al., 2011). Investigations into the neurobiological mechanisms that underlie differences in stress-relate behavior between dominant and subordinate hamsters are ongoing. Subordinate hamsters show increased expression of 5-HT1a receptors in the medial amygdala (MeA), while dominants show reduced expression of 5-HT1a receptors in the paraventricular nucleus of the hypothalamus (PVN) (Morrison et al., 2011). Differences in defeat-induced neural activation between dominants and subordinates highlight a key role for several brain regions. Dominant individuals show increased defeat-induced neural activity in the vmPFC, lateral septum (LS) and MeA compared to subordinates (Morrison et al., 2012). Additionally,

pharmacological inactivation of the vmPFC during social defeat prevents the reduced conditioned defeat response characteristic of dominant animals (Morrison et al., 2013), indicating activation of the vmPFC during social defeat is necessary for the protective effects of dominant social status on the acquisition of conditioned defeat. Because dominant and subordinate hamsters self-select their social status, we investigated whether dominant animals needed to maintain their social status to acquire resistance to conditioned defeat. We found that 14 days of dominant social status is necessary for resistance to conditioned defeat, while one and seven days of dominant social status were insufficient to produce resistance to conditioned defeat (Morrison et al., 2014). In this same study, 14 days of dominant social status was also required for increased defeat-induced neural activation of the vmPFC and MeA (Morrison et al., 2014). Taken together, we have demonstrated that social-status associated resistance to conditioned defeat is experience-dependent, and this parallels the time course for defeat-induced neural activation in select brain regions. While there are several brain structures and neurochemical signals that modulate the experience-dependent plasticity underlying resistance to conditioned defeat, it appears activation of the vmPFC during social defeat is a necessary factor. However, a role for brain regions and neurochemical signals outside the vmPFC is less well understood.

Testosterone and Stress

The lifetime prevalence of anxiety and depressive disorders is twice as high in females as males (Holden, 2005), therefore gonadal hormones, including testosterone, likely influence sex differences in the risk for stress-related mental illness. Plasma testosterone levels fall during stress because cortisol suppresses plasma testosterone (Cumming et al., 1983; Doerr and Pirke, 1976). Furthermore, age-related decline in testosterone levels have been suggested as a causative factor in the more frequent incidence of depression in aged men (McIntyre et al., 2006; Shores et

al., 2004; Wainwright et al., 2011), and testosterone replacement therapy is mildly effective in improving depression symptoms in this population (Pope et al., 2003). Because of the positive correlation between testosterone and psychological well-being, researchers have investigated whether testosterone can promote resilience. Unfortunately, results obtained thus far are inconsistent. Lower testosterone levels were found in combat veterans with PTSD compared to controls (Mulchahey et al., 2001). However, other studies have not found such a testosterone difference in political refugees with PTSD (Bauer et al., 1994) or in patients with combat related PTSD (Karlovic et al., 2012; Lehrner et al., 2016; Spivak et al., 2003). Taken together, although testosterone reduces symptoms of depression, there is limited evidence that testosterone protects against PTSD.

Additional research has investigated whether circulating testosterone modulates anxiety levels. In humans, anxiety levels increase with declining levels of testosterone (Amore et al., 2009), and anxiety decreases with testosterone treatment (Wang et al., 1996). Common treatment for prostate cancer involves androgen receptor blockade, and concurrently patients report increased anxiety that is alleviated when treatment ends (Almeida et al., 2004). Also, women experiencing generalized anxiety disorder have lower salivary testosterone compared to female controls (Giltay et al., 2012). In rodents, testosterone also reduces anxiety levels. Male mice given testosterone spend more time in the open arms of the elevated plus maze than non-treated controls (Aikey et al., 2002). Female rats treated with testosterone have more entries into the center of the open field arena and open arms of the elevated plus maze (Frye and Lacey, 2001). Additionally, this anxiolytic effect of testosterone is mediated through activation of androgen receptors. Testosterone treatment reduced anxiety in wild type males, but had no effect on males with universally disabled androgen receptors caused by a spontaneous mutation (Zuloaga et al.,

2011). Additionally, in Syrian hamsters, testosterone replacement reduces submissive behavior during conditioned defeat testing compared to vehicle-treated, castrated controls, suggesting testosterone decreases social avoidance following social defeat stress (Solomon et al., 2009). Altogether, these results indicate testosterone feedback on androgen receptors diminshes anxiety-like behavior.

Testosterone also has the ability to modulate HPA-axis activity (Goel et al., 2014). In animals, castration and androgen replacement studies suggest that androgens inhibit stress-stimulated, but not basal, adrenocorticotropic hormone (ACTH) and cortisol concentrations (Handa et al., 1994; Papadopoulos and Wardlaw, 2000). Similarly, in men, CRH-induced cortisol concentrations were decreased during testosterone replacement compared to hypogonadal conditions (Rubinow et al., 2005). Although the mechanisms for how testosterone modulates the HPA-axis are unknown, it appears testosterone dampens PVN activity in response to stress. In males, stress-induced cFos expression in the PVN was inversely correlated with plasma testosterone, and gonadectomy led to prolonged PVN activation (Viau et al., 2003). Actions of testosterone on PVN function are likely indirect because this brain structure lacks androgen receptors (Bingham et al., 2006). Because prolonged HPA-axis activity can have deleterious effects on physiology and function, it is important to continue to investigate the mechanisms behind testosterone's dampening effect on HPA-axis activity. Taken together, testosterone has been shown to reduce depressive and anxiety-like behaviors, dampen HPA-axis activity, and increase psychological wellbeing.

Testosterone and Winning

In humans the experience of personal success, as well as a feeling of dominance in competitive situations, is associated with increased salivary and plasma testosterone

concentrations (Booth et al., 1989; Schaal et al., 1996; Suay et al., 1999). In human athletes, both judo competitors (Salvadora et al., 1999) and tennis players (Booth et al., 1989) experience a surge in plasma testosterone immediately after a victory. Moreover, testosterone surges are not dependent on a physical struggle during competition, as winners of chess tournaments also show elevations in plasma testosterone compared to losers (Mazur et al., 1992). A rise in plasma testosterone has been noted prior to competition in both tennis and chess players, suggesting the players anticipated the up coming contest (Booth et al., 1989; Mazur et al., 1992). This anticipatory rise in testosterone is believed to increase competitiveness and dominance behavior. Evidence of an anticipatory rise in testosterone in animal models is limited, and we found that dominant hamsters fail to exhibit an increase in plasma testosterone 10 min prior to an aggressive encounter (see Appendix A). In numerous other taxa, including primates, birds, rodents and reptiles, 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).

The challenge hypothesis predicts 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 is also found in a variety of species, including fish, mammals, birds and invertebrates, and is characterized by an increased probability of winning an aggressive encounter following previous victories (Drummond and Canales, 1998; Oliveira et al., 2009; Oyegbile and Marler, 2005; Schuett et al., 1996). Testosterone modulates the winner effect in California mice because winning facilitates a surge in plasma testosterone and castration prevents the winner effect (Trainor and Marler, 2001). One hypothesis for the functional significance of a contest-related testosterone surge

proposes that transient increases in testosterone following an aggressive encounter promote neural plasticity that increases the probability of winning in the future (i.e. the 'winner-challenge hypothesis') (Oyegbile and Marler, 2005). In California mice, androgen receptors likely play a role in the winner-challenge effect because winning an aggressive encounter increases the expression of androgen receptors in brain regions associated with emotional reactivity and agonistic behavior, including the NAc, VTA and BNST (Fuxjager et al., 2010). Taken together, these results suggest there is a transient increase in plasma testosterone following a victory, and that this surge activates androgen receptors and generates neural plasticity that mediates winning ability in the future. Because dominant hamsters win agonistic encounters daily, my dissertation will investigate whether a winner-challenge effect can account for reduced conditioned defeat in dominant hamsters.

Medial Amygdala

The MeA is part of the social brain network, contains an abundance of androgen and estrogen receptors, and is an important node in the neural circuitry regulating many social behaviors including reproduction, aggression, territorial marking and maternal behavior (Newman, 1999). The MeA is located only two synapses away from the vomeronasal organ, a sensory epithelium that detects pheromonal signals (Dulac and Torello, 2003). Therefore, the MeA is situated at an early stage in sensory information processing, suggesting that it may function at a relatively high level in behavioral decision hierarchies. The MeA is a heterogeneous structure in which at least three subdivisions can be recognized. The anterior MeA (aMeA) is connected with structures implicated in defensive, agonistic and reproductive behaviors (Pardo-Bellver et al., 2012). The posterior dorsal subdivision (dMeA) contains the highest density of steroid receptors (Cooke, 2006) and is connected mainly with structures implicated in

reproductive behavior (Canteras et al., 1995). Finally, the posterior ventral subdivision (vMeA) is connected mainly with neural substrates involved with defensive behavior (Canteras, 2002). Altogether, the heterogeneity of the MeA's afferent and efferent projections give rise to this structure being important for the control of a broad range of species typical social behavior.

The Medial Amygdala's Role in Fear and Stress-Related Behavior

The MeA has both GABAergic and non-GABAergic projection neurons, making it difficult to decipher the mechanisms by which neurotransmission in the MeA modulates agonistic behavior and responses to aversive stimuli (Keshavarzi et al., 2014). In the case of aggression, MeA lesions both increase (Rosvold et al., 1954; Vochteloo and Koolhaas, 1987) and decrease aggression (Kemble et al., 1984; Takahashi and Gladstone, 1988; Wang et al., 2013b). Also, the posterior dMeA displays increased cFos expression during offensive aggression (Nelson and Trainor, 2007). Specifically, GABAergic neurons within this sub region promote aggressive behavior (Hong et al., 2014). Similar inconsistencies are found with the role of the MeA in modulating fear and stress-related behavior. Neural activity within the MeA has been shown to both promote fear-related behavior (Cousens et al., 2012; Muller and Fendt, 2006; Takahashi et al., 2007), and reduce the negative effects of stress (Tsuda et al., 2015). For example, the MeA shows increased activity when mice and rats are exposed to cat predator odor (Choi et al., 2005; Samuelsen and Meredith, 2009). Furthermore, lesions of the MeA reduce the neuroendocrine response to acute stress and fear-related behavior, including fear potentiated startle and conditioned freezing (Cousens et al., 2012; Muller and Fendt, 2006; Takahashi et al., 2007; Walker et al., 2005; Yoshida et al., 2014). In Syrian hamsters, pharmacological inactivation of the MeA reduces both acquisition and expression of conditioned defeat (Markham and Huhman, 2008). Additionally, injection of CRF into the MeA exerts anxiogenic effects

through activation of CRF type 1 receptors (Vicentini et al., 2014). Altogether, these data indicate the MeA promotes fear and stress-related behavior, but other findings suggest activation of the MeA reduces the negative effects of stress. In mice, activity of neuropeptide tuberoinfundibular peptide of 39 residues (TIP39) at its parathyroid hormone 2 receptor (PTH2R) in the MeA has been shown to reduce incubation of conditioned fear (Tsuda et al., 2015). Dominant hamsters show increased defeat-induced neural activation in the MeA compared to subordinates, while also displaying a reduced conditioned defeat response (Morrison et al., 2012). These findings suggest that a subpopulation of cells in the MeA can reduce stress-related behavior, including conditioned defeat. Altogether, the MeA is a neural substrate well positioned to integrate social context into stress, anxiety and fear-related behavior.

How Might the Medial Amygdala Modulate Resistance to Conditioned Defeat?

Neural activity in several brain regions is associated with resistance to stress, and much research has focused on the role of the vmPFC (Amat et al., 2006; Kumar et al., 2014; Lehmann and Herkenham, 2011; Morrison et al., 2013). Although activation of the vmPFC is necessary for resistance to conditioned defeat (Morrison et al., 2013), it is unlikely the only mechanism contributing to reduced conditioned defeat in dominant hamsters. The MeA modulates social behavior and responses to stress, but its role in stress resilience is poorly understood. Along with increased vmPFC activation, dominant hamsters show increased defeat-induced neural activity in the MeA compared to subordinates. Furthermore, we have shown resistance to conditioned defeat develops over a 14-day period of maintaining dominant status. Testosterone is a prime candidate for generating neural plasticity in the MeA to promote resistance to conditioned defeat because dominant hamsters repeatedly win encounters and may experience changes in testosterone during the maintenance of dominant status. My dissertation will investigate the

neuroendocrine mechanisms promoting experience-dependent plasticity within the MeA that underlie resistance to conditioned defeat in dominant hamsters.

Hypothesis: The overarching hypothesis is that dominant hamsters experience daily surges in plasma testosterone during the maintenance of their social status that increase the expression of androgen receptors in the MeA and lead to a reduced conditioned defeat response compared to subordinates.

Specific Aim 1: To determine whether winning an agonistic encounter increases plasma testosterone in male Syrian hamsters. (Chapter 2)

Specific Aim 2: To determine whether repeatedly winning agonistic encounters increases androgen receptor expression in select brain regions known to modulate agonistic behavior, including the MeA. (Chapter 2)

Specific Aim 3: To determine whether repeatedly blocking androgen receptors during daily agonistic encounters prevents resistance to conditioned defeat and the up-regulation of androgen receptors in the MeA in dominant hamsters. (Chapter 3)

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Appendix A

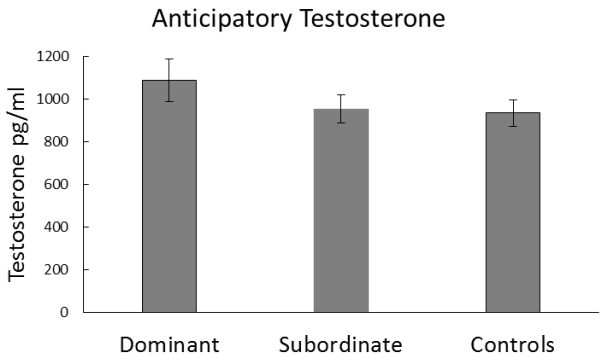

Figure 1.1 Amount (mean ± SE) of plasma testosterone 10 min prior to the 14th social encounter or control experience. Dominants did not significantly differ from subordinates or controls in plasma testosterone prior to the start of their final agonistic encounter, ($F_{(2,31)} = 1.12$, $p = .340$). n = 10 – 13 per group.

Chapter 2

Winning Agonistic Encounters Increases Testosterone and Androgen Receptor Expression in Syrian Hamsters

This chapter is adapted for dissertation formatting from the following publication:

C. T. Clinard, A. K. Barnes, S. G. Adler, & M. A. Cooper. *Hormones and Behavior*

(Under Review)

My primary contributions to this paper were in the following areas: designing the experiments, performing experimental procedures, collecting and analyzing data, interpreting data, and writing the manuscript.

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.

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 one or seven 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-HT1A 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 one-day and seven-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.

Materials and Methods

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 x 27 cm x 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

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 maintain their status as a resident and an intruder, and residency status does not predict who the winner will be. 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.

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 4400xg 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%.

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 H_2O 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.

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.

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.

Immunohistochemistry Quantification

Images were captured at 10X 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 quantifiy. 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 1.4-1.6X 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.

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.

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.

Experiment 3

Subjects (n = 24) were weight-watched 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.

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 3X3 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. All statistical tests were two-tailed, and the α level was set at $p \leq .05$.

Results

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 ($F_{(2,25)} = 4.807$, $p = .017$) (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 ($r(8) = .292$, $p = .412$). 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 ($t(8) = .438$, $p = .673$).

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 ($F_{(2,32)} = 6.16$, $p = .005$; Figure 3). The duration of submissive behavior displayed by subordinates on day 14 did not correlate with their plasma testosterone levels ($r(10) = -.400$, $p = .198$).

Following 14 days of aggressive encounters, dominant animals showed more androgen receptor positive cells in the dMeA ($F_{(2,35)} = 3.89$, $p = .03$) and vLS ($F_{(2,34)} = 3.948$, $p = .029$) compared to subordinates and controls (Figure 4a). There was a trend for dominant animals to have more androgen receptor immno-positive cells in the vMeA compared to subordinates and controls ($F_{(2,36)} = 2.8$, $p = .074$). There were no significant differences in androgen receptor immunoreactivity between dominants, subordinates and control animals in the VMHL ($F_{(2,33)} = 2.325$, $p = .114$) or MPOA ($F_{(2,31)} = 1.759$, $p = .189$). 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 ($F_{(2,34)} = .220$, $p = .804$) or vMeA ($F_{(2,34)} = .220$, $p = .803$) (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 (MeA: $t(12) = .111$, $p = .914$; dMeA: $t(12) = -.367$, $p = .720$; vLS: $t(11) = -.881$, $p = .397$).

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 ($t(20) = -1.113$, $p = .279$), vMeA ($t(20) = -.387$, $p = .703$) or vLS ($t(18) = -1.294$, $p = .212$) compared to subordinate animals (Figure 5).

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.

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., 2013b). 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., 2013b), 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 MeA 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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Appendix B

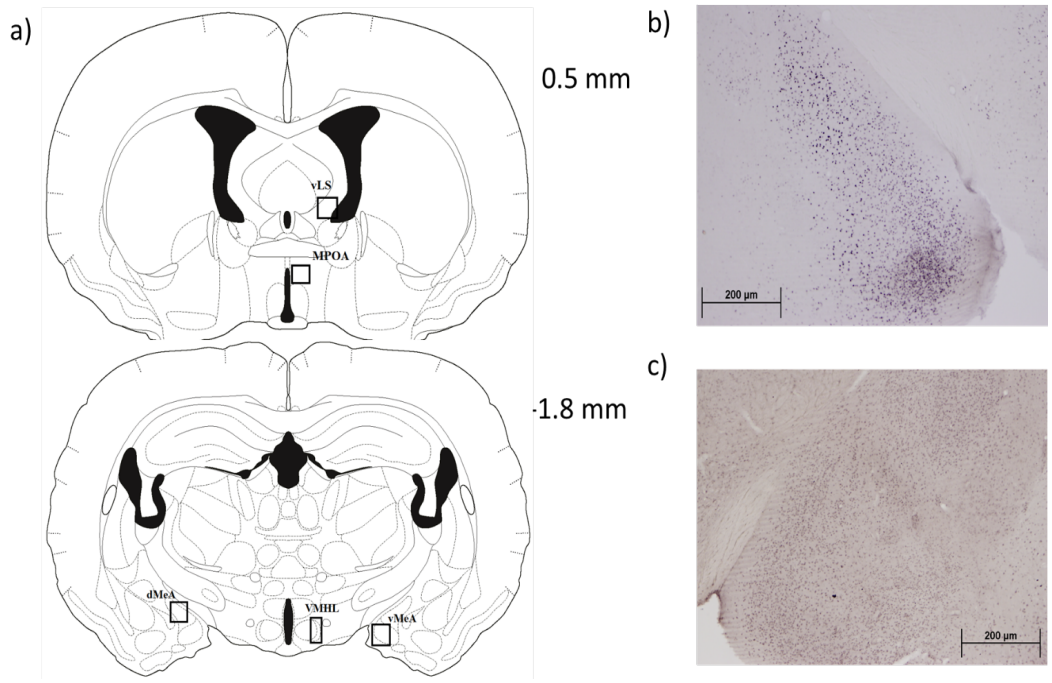


Figure 2.1 a) The diagrams indicate the location of brain regions selected for androgen receptor quantification. The diagrams were modified from the hamster atlas of Morin & Wood (2001) and values indicate the distance from bregma. The box sizes used for quantification were as follows (width x height): 325 µm × 650 µm (VMHL); 500 µm × 500 µm (vLS and MPOA); 870 µm × 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.

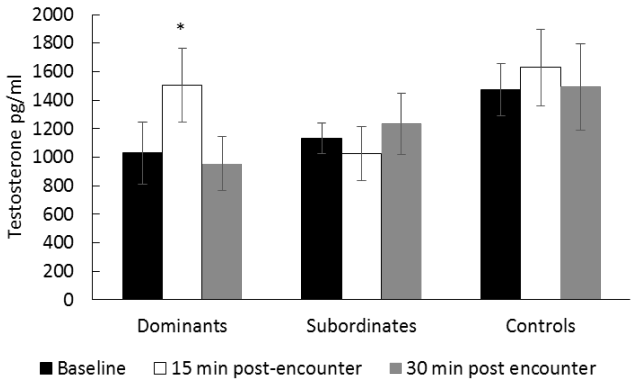


Figure 2.2 Amount (mean ± SE) of plasma testosterone at baseline,15 min, and 30 min following establishment of dominance relationships for dominants, subordinates and controls. We found a significant time x social status interaction, and an asterisk indicates a significant change from baseline ($P < 0.05$). n = 9 – 10 per group.

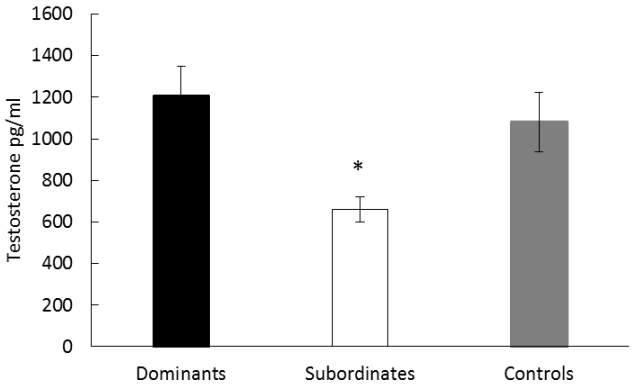
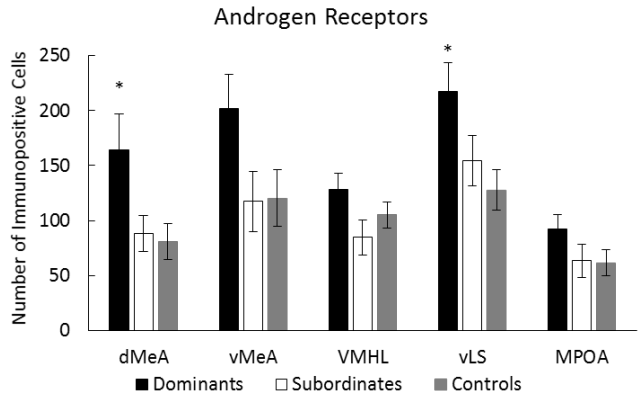


Figure 2.3 Amount (mean ± SE) of plasma testosterone following 14 days of social encounters. Asterisk indicates a significant difference compared to dominants and control animals ($P < 0.05$). n = 10 – 13 per group.

a)



b)

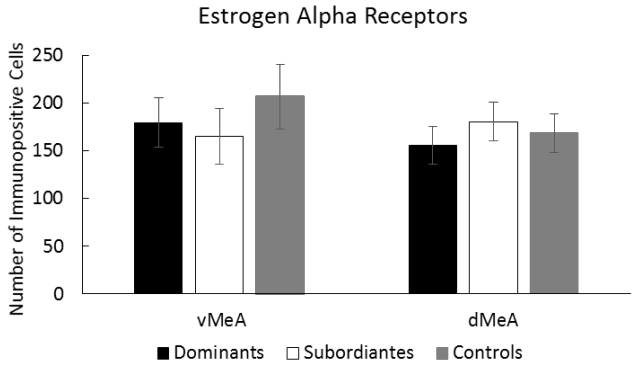


Figure 2.4 a) Number (mean ± SE) of androgen receptor immunopositive cells following 14 days of social encounters in the dMeA, vMeA, VMHL, vLS and MPOA. b) Number (mean ± SE) of estrogen alpha receptor immunopositive cells following 14 days of social encounters in the vMeA and dMeA. Asterisks indicate a significant difference compared to subordinate and control animals ($P < 0.05$). n = 10 – 15 per group.

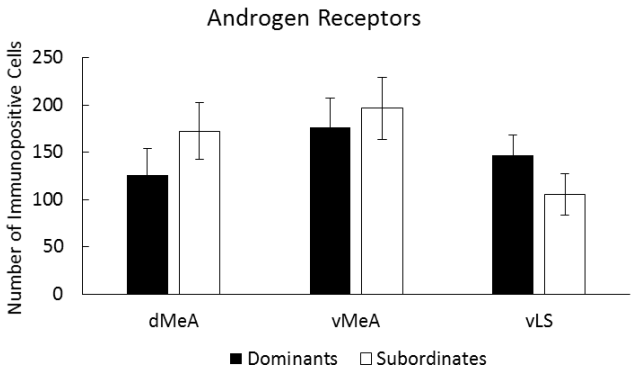


Figure 2.5 Number (mean ± SE) of androgen receptor immunopositive cells following a single social encounter in the dMeA, vMeA and vLS. n = 10 -11 per group.

Table 2.1 Subjects form stable dominance relationships

	Day 1	**Day 7**	**Day 14**
Subordinates – Total Submissive Behavior (mean ± SE)	87.6 s ± 30.49	176.8 s ± 17.02	196.7 s ± 18.31
Dominants – Total Aggressive Behavior (mean ± SE)	87.8 s ± 30.63	139.2 s ± 15.24	151 s ± 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.

Chapter 3

Blocking androgen receptors during maintenance of dominance relationships prevents resistance to conditioned defeat in hamsters

Abstract

In Syrian hamsters, dominant animals show a reduced effect of social defeat stress compared to subordinate animals, as indicated by a reduced conditioned defeat (CD) response. Furthermore, hamsters that win brief agonistic encounters show increased plasma testosterone, and those that win encounters for 14 days show increased expression of androgen receptors in stress-responsive brain regions, including the medial amygdala (MeA) and lateral septum. In this experiment, we investigated whether androgen receptor activity is necessary during the maintenance of dominance relationships for dominant hamsters to show resistance to CD and increased androgen receptor expression in the MeA and lateral septum. Male hamsters were weight matched and paired in 5-min aggressive encounters with the same individual for 14 days, during which time they established and maintained a dominance relationship. Injections of the androgen receptor antagonist flutamide (15 mg/kg, s.c.) or vehicle were administered daily prior to aggressive encounters. After maintaining stable dominance relationships for two weeks, each animal received acute social defeat stress in which they lost three consecutive 5-min encounters to a trained aggressor. The next day, animals received CD testing, which involved a 5-min social interaction test with a non-aggressive intruder. The following day brains were collected for androgen receptor immunohistochemistry. Dominant animals that received flutamide treatment for 14 days showed an increased CD response and significantly fewer androgen receptor immuno-positive cells in the dorsal MeA, but not lateral septum, compared to vehicle-treated dominants. In contrast, flutamide treatment did not alter the CD response or the number of androgen receptor immuno-positive cells in subordinate counterparts. These findings suggest that the upregulation of androgen receptors in the MeA is associated with dominant social status and

that activation of androgen receptors during the maintenance of dominance relationships is necessary for resistance to CD in dominant hamsters.

Introduction

Animal models often attempt to minimize genetic, physiological, and behavioral variability between individuals. While this approach is standard and necessary for understanding the biological basis of behavior, it can limit the study of individual differences. Humans show a great deal of variability in how they respond to stressful events, with some individuals coping successfully and others showing high risk for stress-related mental illness. In an attempt to identify novel targets for the treatment and prevention of stress-related disorders, there is growing emphasis on understanding the cellular mechanisms that control vulnerability and resistance to the negative consequences of stress.

To investigate the neurobiological mechanisms controlling vulnerability and resistance to the effects of stress, we use a social defeat model in Syrian hamsters called conditioned defeat (CD) (Huhman, 2006; Huhman et al., 2003). In the CD model, subjects receive an acute social defeat in the home cage of a larger, aggressive hamster and are tested 24 hrs later in their home cage with a smaller, non-aggressive hamster. At CD testing, defeated hamsters display a loss of species-typical territorial aggression and an increase in submissive and defensive behavior compared to non-defeated controls. Hamsters that have achieved dominant social status exhibit less submissive and defensive behavior during CD testing, compared to subordinates and controls, indicating experience winning aggressive encounters prior to social defeat stress is associated with resistance to CD (Morrison et al., 2012; Morrison et al., 2011). Resistance to CD in dominant animals is experience-dependent and requires animals to maintain dominant social status for 14 days. Likewise, defeat-induced neural activity changes with the maintenance of

dominance relationships. Dominant animals that have maintained their social status for 14 days show a reduced CD response and increased c-Fos expression in the ventromedial prefrontal cortex (vmPFC) and medial amygdala (MeA) compared to dominants that have maintained their status for one day (Morrison et al., 2014). These data highlight the importance of maintaining dominance relationships, and suggest that repeatedly winning is necessary for the development of CD resistance in dominant hamsters. Findings from hamsters are consistent with data from rats and mice indicating that aggressive behavior is associated with differences in how individuals cope with stress. Rats that show high offensive aggression in a resident-intruder paradigm also exhibit a proactive coping style, as indicated by burying a shock probe in a defensive burying test and displaying high amounts of swimming during a forced swim test (Koolhaas et al., 2007). Mice that display short attack latencies in a resident-intruder paradigm show reduced anxiety-like and depressive-like behavior following chronic social defeat stress compared to mice with long attack latencies (Veenema et al., 2003).

Neural activity in several brain regions is associated with stress resilience, and much research has focused on the role of the vmPFC (Amat et al., 2006; Kumar et al., 2014; Lehmann and Herkenham, 2011; Morrison et al., 2013). Other brain regions modulate stress-related behavior, such as the MeA, although their role in stress resilience is poorly understood. The MeA is part of the social brain network, and is an important node in the neural circuitry regulating many types of social behaviors including aggressive and defensive behavior (Newman, 1999). The MeA has a heterogeneous cell population throughout its various subregions, with both GABAergic and non-GABAergic projection neurons, making it difficult to decipher how changes in neural activity modulate agonistic behavior and responses to aversive stimuli (Keshavarzi et al., 2014). Neural activity in the MeA has been shown to promote fear-related

behavior (Cousens et al., 2012; Muller and Fendt, 2006; Takahashi et al., 2007), while activity of neuropeptide tuberoinfundibular peptide of 39 residues (TIP39) in the MeA reduces the incubation of conditioned fear (Tsuda et al., 2015). Mixed results are also found for the role of the MeA in aggression. Lesions of the MeA have both increased and decreased aggression (Kemble et al., 1984; Rosvold et al., 1954; Takahashi and Gladstone, 1988; Vochteloo and Koolhaas, 1987; Wang et al., 2013), while c-fos expression indicates elevated MeA neural activity during aggression (Delville et al., 2000; Hong et al., 2014; Nelson and Trainor, 2007; Newman, 1999).

The MeA also contains an abundance of androgen receptors, and testosterone signaling in the MeA is known to modulate aggressive as well as anxiety-like behavior (Wood and Newman, 1993). Testosterone treatment reduces anxiety-like behavior in wild-type 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 anxiolytic effect of androgens could, in part, be due to increased testosterone-signaling at androgen receptors within the MeA, because a partial knockout of androgen receptors in brain regions outside the amygdala and hypothalamus is not sufficient to alter anxiety-like behavior (Chen et al., 2016). Interestingly, castration with either testosterone or dihydrotestosterone replacement reduces the CD response in male hamsters, although it does not alter aggressive behavior in non-defeated control subjects (Solomon et al., 2009).

In a variety of species, winners of competitive interactions exhibit increased plasma testosterone compared to losers (Cavigelli and Pereira, 2000; Oyegbile and Marler, 2005; Smith et al., 2005; Yang and Wilczynski, 2002). The challenge hypothesis predicts 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 is also found in a variety of species, and is characterized by an increased probability of winning an aggressive encounter following previous victories (Oliveira et al., 2009; Oyegbile and Marler, 2005; Schuett et al., 1996). Testosterone modulates the winner effect in California mice because winning facilitates a surge in plasma testosterone and castration prevents the winner effect (Trainor and Marler, 2001). Based on these findings a winner-challenge effect was proposed, in which winning an aggressive encounter leads to a transient increase in testosterone that increases the probability of winning future encounters. In California mice, androgen receptors likely play a role in the winner-challenge effect because winning an aggressive encounter increases the expression of androgen receptors in brain regions associated with agonistic behavior (Fuxjager et al., 2010). Interestingly, we have shown a similar winner-challenge effect in dominant hamsters. We confirmed hamsters exhibit a transient rise in plasma testosterone after winning an aggressive encounter (Clinard et al., Under Review). Also, dominant hamsters exhibit increased androgen receptor immunoreactivity in the ventral lateral septum (vLS) and the dorsal medial amygdala (dMeA) after maintaining their dominance status for 14 days. The increase in androgen receptor immunoreactivity was not evident after winning a single encounter, which suggests that experience-dependent neural plasticity occurrs during the maintenance of dominance relationships.

This project is focused on whether changes in androgen receptor signaling contribute to the reduced CD response shown by dominant hamsters. The aim of this study was to determine whether androgen receptor activity during the maintenance of dominance relationships is necessary for the upregulation of androgen receptors in the dMeA and vLS, and for resistance to CD in dominant hamsters. We hypothesized that systemically blocking androgen receptors

during 14 daily aggressive encounters would prevent dominant animals from showing a reduced CD response and increased androgen receptor immunoreactivity in the vLS and dMeA compared to vehicle-treated dominant animals.

Materials and Methods

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 x 27 cm x 16 cm) with corncob bedding, cotton nesting materials, and wire mesh tops. Animals were reared with environmental enrichment, such as, plastic shelters and paper cups, although enrichment objects were removed prior to the start of the study. 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 that accompanies drug injections. 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.

Dominant-Subordinate Encounters

To allow animals to establish dominance relationships, animals within each cohort were weight-matched in resident-intruder dyads and paired in daily social encounters for 14 days. Subjects were randomly assigned as a resident or intruder, and all social encounters occurred in the resident's home cage. The encounters were 10 min in duration until a clear winner and loser were identified, 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 establish a dominance relationship after 5 days of encounters (3 out of 22 dyads) were excluded from the study. Control animals were not placed in daily agonistic encounters, but were handled for 14 days. 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).

Social defeat stress

Social defeat stress consisted of three consecutive 5 min aggressive encounters in the home cage of a larger aggressor with a 5 min rest in the subject's home cage between each defeat. To ensure each subject received similar amounts of aggression from the aggressor, timing of the first defeat did not begin until the first attack, which usually occurred within the first 60 s of the encounter. Defeats were digitally recorded and the behavior of the aggressor was quantified later using Noldus Observer. We quantified total number of attacks and total duration

of aggression displayed. Any animal with a wound extending beyond the epidermis and into the dermis layer was treated and removed from the study (n = 1).

Conditioned defeat testing

Conditioned defeat testing consisted of a 5 min social interaction test, during which a non-aggressive intruder was placed in the subject's home cage. Non-aggressive intruders were younger, group-housed animals that displayed social and nonsocial behavior, and at testing we excluded those intruders that displayed agonistic behavior. All testing sessions were digitally recorded and the behavior of the subject was quantified using Noldus Observer. We quantified the total duration of the following categories of behavior: submissive/defensive, aggressive, social, and non social. We also quantified the frequency of flees and attacks displayed by the subject. For all behavioral scoring, including dominance relationships, social defeats and conditioned defeat testing, the researcher was blind to the experimental conditions of the subject. Inter-rater reliability was established on a subset of videos by reaching 90% agreement on the duration of submissive/defensive and aggressive behavior.

Drugs

Flutamide (Sigma-Aldrich) was dissolved in DMSO and diluted with sesame oil to reach a final concentration of 5% DMSO (pH = 7.4). Sesame oil with 5% DMSO was used as a vehicle control at a similar pH. Flutamide is a competitive non-steroidal androgen antagonist, and was administered at 15 mg/kg on the basis of previous research (Nagypal and Wood, 2007). Once prepared, the drug was stored at 4° C overnight and brought to room temperature and vortexed daily before use.

Immunohistochemistry and Cell Quantification

Twenty-four hours after conditioned defeat testing, 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.

Sections were processed for androgen receptor immunohistochemistry according to a previously published protocol (Chen et al., 2014). Sections received 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 a 15 min incubation in 0.5% sodium borohydride in PBS-GT. Sections were then exposed to 10% normal goat serum (NGS) in PBS-GT for 1 h to block non-specific binding, and then incubated for 10-min in avidin block followed by 10-min in biotin block (avidin/biotin blocking kit, Vector: #SP-2001). Sections were then incubated for 24 h at 4 °C in 1% NGS in PBS-GT with an anti-androgen receptor antibody (rabbit monoclonal- Abcam: ab52615, 1:1000). 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 (Vector: BA-1000, 1:500). 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. After immunohistochemistry, all sections were washed five times with distilled H_2O 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 was processed simultaneously.

Photomicrographs were captured at 10X magnification using an Olympus BX41 microscope. The number of androgen receptor immuno-positive cells was quantified 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), and ventral lateral septum (vLS). These brain regions were selected for quantification because they showed status-dependent changes in androgen receptor immunoreactivity in our previous study (Clinard et al., Under Review). 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 1.6X 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. The box sizes used for quantification were as follows (width x height): 500 μm x 500 μm (vLS), and 870 μm x 660 μm (dMeA and vMeA). For each brain region we quantified three to six sections per individual along a rostral-caudal axis. MCID software settings were calibrated to yield cell counts that were reliable with human counting.

Experimental Design

Subjects (n = 44) were weight-matched and assigned to resident-intruder dyads for dominant-subordinate encounters (Days 1-14). All dyads were randomly assigned to receive 14 days of vehicle or flutamide (15 mg/kg, s.c.) injections 1 hr prior to each daily aggressive encounter. Both animals within a dyad received identical drug treatments. To investigate the effect of flutamide on the CD response in hamsters without social status, a separate cohort (n =

22) of control animals received 14 daily injections of vehicle or flutamide (15 mg/kg, s.c.), but was not exposed to daily aggressive encounters. On day 15, all subjects received social defeat stress. On day 16, all subjects received CD testing, and on day 17 animals were euthanized for brain collection.

Data Analysis

We performed two-way ANOVAs to investigate an interaction between social status (3 levels) and drug treatment (2 levels) on behavior at CD testing, and androgen receptor immunoreactivity in each brain region. For both CD behavior and androgen receptor immunoreactivity, we performed planned comparisons to test for differences between vehicle-treated dominant animals and subordinate animals and between vehicle-treated and flutamide-treated dominant animals (t tests). All statistical tests were two-tailed, and the *a* level was set at $p \leq .05$.

Results

Agonistic behavior during the maintenance of dominance relationships

On average dominance relationships were decided on day 1.27 (SE = 0.04). After dominance relationships were established, all dyads remained stable during the 14 days of agonistic encounters (Table 1). The duration of aggressive behavior during the maintenance of dominance relationships did not differ between vehicle-treated dominant animals and flutamide-treated dominant animals ($F_{(1,15)} = .85$, $p = .371$). Similarly, the duration of submissive behavior during the maintenance of dominance relationships did not differ between vehicle-treated subordinate animals and flutamide-treated subordinate animals ($F_{(1,16)} = 1.33$, $p = .267$). Additionally, there was no main effect of time on the duration of aggressive or submissive

behavior ($F_{(1,15)} = 8.57$, $p = .369$; $F_{(1,16)} = 1.55$, $p = .231$, respectively). Regardless of drug treatment, there were no differences in the amount of agonistic behavior between days 6 and 14.

Intensity of social defeat stress

To test whether social status or flutamide treatment altered the intensity of social defeat, we quantified the duration of aggressive behavior displayed by the resident aggressors during social defeat (Table 2). There was not a significant drug x social status interaction in the amount of aggression displayed by the resident aggressor ($F_{(5,52)} = .23$, $p = .796$). There were no main effects of flutamide treatment or social status on the amount of aggression displayed by the resident aggressor ($F_{(5,52)} = .83$, $p = .367$; $F_{(5,52)} = 2.84$, $p = .067$, respectively). There was also no significant drug x social status interaction in the number of attacks initiated by the resident aggressors ($F_{(5,52)} = 2.11$, $p = .131$). There were no main effects of flutamide treatment or social status on the number of attacks initiated by the resident aggressors ($F_{(5,52)} = .47$, $p = .498$; $F_{(5,52)} = 1.51$, $p = .230$, respectively). Altogether, these data indicate that subjects did not significantly differ in the amount of aggression received during social defeat stress.

Effects of flutamide and social status on conditioned defeat behavior

Flutamide treatment increased the amount of submissive behavior displayed by dominant animals, but not by subordinate animals (Fig. 1a). We found a significant drug treatment x social status interaction for the amount of submissive behavior displayed ($F_{(5,50)} = 4.12$, $p = .02$). Importantly, dominant animals treated with flutamide displayed a greater duration of submissive behavior compared to vehicle-treated dominant animals ($t(15) = 4.16$, $p < .01$). In a planned comparison, dominant animals treated with vehicle show a lower duration of submissive behavior compared to both subordinate and control animals treated with vehicle, respectively ($t(13) = -4.01$, $p < .01$; $t(15) = -2.18$, $p = .045$).

Flutamide treatment did not alter the amount of aggressive behavior, social behavior or non social behavior displayed during conditioned defeat testing (Fig. 1b,c,d). There was a main effect of social status on the amount of aggressive behavior displayed ($F_{(5,50)} = 3.84$, $p = .03$). Regardless of drug treatment, dominant animals displayed more aggressive behavior during conditioned defeat testing compared to their subordinate counterparts.

Effects of flutamide and social status on androgen receptors

Flutamide treatment reduced the number of androgen receptor positive cells in the dMeA in dominant, but not subordinate, animals (Fig. 2a). There was a significant drug treatment x social status interaction for the number of androgen receptors in the dMeA ($F_{(3,30)} = 15.78$, $p < .01$). Importantly, dominant animals treated with flutamide showed fewer androgen receptor positive cells in the dMeA compared to vehicle-treated dominant animals ($t(15) = 4.2$, $p < .01$). In a planned comparison, dominant animals treated with vehicle showed more androgen receptor positive cells in the dMeA compared to vehicle-treated subordinate animals treated with vehicle ($t(13) = 4.65$, $p < .01$).

Flutamide treatment produced a similar change in androgen receptor expression in the vMeA, although the differences were not as pronounced as in the dMeA (Fig. 2b). There was a trend for a main effect of social status on the number of androgen receptor positive cells in the vMeA ($F_{(3,29)} = 3.95$, $p = .056$), while the drug treatment x social status interaction was not statistically significant ($F_{(3,29)} = 1.63$, $p = .211$). A planned comparison indicated dominant animals treated with flutamide showed fewer androgen receptor positive cells in the vMeA compared to vehicle-treated dominant animals $t(15) = 2.17$, $p = .046$. Additionally, vehicle-treated dominant animals have a greater number of androgen receptor immunopositive cells in the vMeA than do vehicle-treated subordinate animals $t(12) = 2.42$, $p = .032$.

Flutamide treatment did not alter the number of androgen receptor positive cells in the vLS of dominant or subordinate animals (Fig. 2c). There was a main effect of social status on the number of androgen receptor positive cells in the vLS ($F_{(3,27)} = 4.11$, $p = .05$). Regardless of drug treatment, dominant animals had more androgen receptor positive cells in the vLS compared to their subordinate counterparts. A planned comparison indicated vehicle-treated dominant animals have more androgen receptor positive cells in the vLS compared to vehicle-treated subordinate animals $t(10) = 1.81$, $p = 0.042$.

Discussion

We found that vehicle-treated dominant hamsters exhibit a reduced CD response and have more androgen receptor positive cells in the dMeA, vMeA and vLS, compared to vehicle-treated subordinates and controls, and these results are consistent with our previous findings (Clinard et al., Under Review). These data suggest that winning daily aggressive encounters for two weeks produces testosterone-dependent plasticity in brain regions known to modulate the CD response. To further investigate the link between increased androgen receptor expression and resistance to CD, we chronically blocked androgen receptors during the maintenance of dominance relationships. We found that flutamide treatment during the maintenance of dominance relationships prevented resistance to CD in dominant hamsters. Also, chronic flutamide treatment in dominant hamsters prevented an increase of androgen receptors in the dMeA and vMeA, although flutamide did not alter androgen receptor expression in the vLS. Together, these results suggest that the maintenance of dominance relationships leads to an upregulation of androgen receptors in the MeA and that androgen receptor activation during this time is necessary for resistance to social defeat stress in dominant hamsters.

Although flutamide was administered daily during the maintenance of dominance relationships, it did not alter agonistic behavior during daily aggressive encounters. Dominant animals displayed high levels of aggressive behavior regardless of drug treatment, and subsequently subordinate animals displayed high levels of submissive behavior regardless of drug treatment. The amount of aggressive and submissive behavior displayed by dominants and subordinates did not differ between days 6 and 14. Previously, agonistic behavior in hamster dominance relationships was found to be stable or even decrease over time (Ferris et al., 1987; Johnston, 1975; Morrison et al., 2011). The finding that aggressive behavior fails to decrease in dominants in our studies could be a consequence of our experimental design. The brief daily encounters in a resident animal's home cage likely encourage territorial aggression, and the small cage dimensions prevent the subordinate animal's escape. Importantly, persistent aggression from dominants leads to a reduction in plasma testosterone in subordinate hamsters, which is characteristic of chronic stress (Clinard et al., Under Review; Huhman et al., 1990).

Flutamide treatment did not alter the intensity of the social defeat, as indicated by the amount of aggression received. Therefore, the effect of flutamide on CD behavior in dominant animals is not accounted for by the nature of the stressor, and is instead an effect of drug treatment. Flutamide is a competitive androgen receptor antagonist, which has the ability to block testosterone's action at either genomic or non-genomic receptors (Farla et al., 2005; Gorczynska and Handelsman, 1995). We selected a dose of 15 mg/kg because it has been shown to block the classic genomic actions of testosterone (Nagypal and Wood, 2007). We expect that testosterone is acting on androgen receptors to alter genomic activity in dominant animals because they have lasting behavioral and neural changes. Furthermore, dominant hamsters display increased c-Fos expression following social defeat in the MeA (Morrison et al., 2014),

the same brain region we also see an increase in androgen receptors. Because the induction of Fos protein requires transcriptional activation, it has often been used a marker for genomic effects (Nagypal and Wood, 2007). Although we hypothesize testosterone is acting through genomic mechanisms to upregulate androgen receptors during the 14 days of dominance relationships, it is possible activation of non-genomic androgen receptors during CD testing leads to less submissive and defensive behavior. The mechanisms by which increased androgen receptors in dominant animals contribute to CD resistance still need to be explored. A role for the aromatization of testosterone into estrogen seems unlikely because dominant animals show an increase in androgen receptors but not estrogen receptors in the MeA (Clinard et al., Under Review).

We have shown that activation of androgen receptors during the maintenance of dominance relationships is necessary for the upregulation of androgen receptors in the dMeA and vMeA in dominant hamsters. In Syrian hamsters, winning aggressive encounters produces a surge in plasma testosterone that lasts approximately 15 min, and is associated with lasting changes in androgen receptor expression (Clinard et al., Under Review). In California mice, the increased likelihood of winning a future encounter after winning a previous encounter (i.e. the winner effect) cannot be mediated by plasma testosterone levels alone because testosterone levels quickly return to baseline after winning, and therefore are not still high when the animal is placed in an aggressive encounter the following day (Fuxjager et al., 2010). Instead the winner effect is hypothesized to result from an upregulation of androgen receptors in brain regions such as the bed nucleus of the stria terminalis (BNST) and nucleus accumbens. In our model, dominant hamsters win encounters against the same opponent for 14 days and experience some of the neuroendocrine changes characteristic of the winner effect. Our findings suggest that the

surge in plasma testosterone experienced by dominant hamsters leads to an upregulation of androgen receptors in several brain regions, including the dMeA, vMeA and vLS. Because flutamide specifically blocked the upregulation of androgen receptors in the dMeA and vMeA, it suggests that androgen receptor signaling in the MeA is critical for reduced CD in dominant hamsters.

In addition to blocking androgen receptors, flutamide can alter testosterone production in the testes (Ayub and Levell, 1987; Clos et al., 1988). If testosterone production were increased following flutamide treatment then it would increase testosterone in all animals receiving flutamide, including dominants, subordinates and control animals. Flutamide treatment did not alter behavior at CD testing or androgen receptor expression in any brain region for subordinate and control animals compared to vehicle counterparts. This suggests that a compensatory change in testosterone production does not account for the current findings. The lack of effect of flutamide in subordinates and controls also suggests that flutamide is blocking the actions of a testosterone surge that only occurs in dominants.

The MeA is a heterogeneous structure in which at least three subregions can be clearly recognized (anterior MeA, posterior vMeA and posterior dMeA). The neuroanatomical heterogeneity gives rise to the many different types of behavior this brain region modulates. For instance, the MeA has a wide range of efferent and afferent connections with other structures in the social brain network, and together are implicated in olfactory processing, defensive, agonistic and reproductive behaviors (Newman, 1999). Here we investigate androgen receptors within the posterior MeA, and we include both the dorsal and ventral subregions in our analysis. The posterior vMeA has strong efferent connections to the medial BNST, and is well known for modulating defensive behaviors, including response to predator odor (Choi et al., 2005; Pardo-

Bellver et al., 2012). Previously, we showed dominant hamsters had more cFos expression in their vMeA, but not dMeA, following social defeat stress compared to subordinate and control animals, suggesting a role of the vMeA in modulating responses to social stress (Morrison et al., 2014; Morrison et al., 2012). The posterior dMeA has the largest population of steroid receptors, and its densest projections are to brain regions that control reproductive behaviors, although axonal projections to defensive-related nuclei also exist (Pardo-Bellver et al., 2012). Also, the dMeA shows increased neural activation during offensive aggression (Hong et al., 2014). All subregions of the MeA have moderate projections to the central and basolateral amygdala, which may modulate fear learning (Pardo-Bellver et al., 2012). For example, the MeA can modulate olfactory fear conditioning likely via its connections to the central and basolateral amygdala (Cousens et al., 2012; Takahashi et al., 2007). Similarly, pharmacological inactivation of the MeA reduces both the acquisition and expression of CD, further suggesting a role for the MeA in modulating the CD response (Markham and Huhman, 2008). Additionally, corticotropin-releasing factor (CRF) in the MeA exerts anxiogenic effects through activation of CRF type 1 receptors, suggesting a role for the MeA in the stress-related behavior (Vicentini et al., 2014). Taken together, the MeA is a neural substrate well positioned to integrate social context into stress, anxiety, and fear-related behavior.

In the present study, we have shown that vehicle-treated dominant animals have more androgen receptor positive cells in both the vMeA and dMeA compared to vehicle-treated subordinates. Previously we found dominants have more androgen receptor positive cells in the dMeA compared to subordinates, but only a trend in the vMeA. Flutamide prevented an upregulation of androgen receptors in the dMeA of dominant animals, and planned comparisons indicate a similar change in the vMeA. It is possible the effect of flutamide is stronger in the

dMeA because of the greater population of androgen receptors in this subregion (Wood et al., 1992). While androgen receptors in both the dMeA and vMeA may play a role in modulating resistance to CD in dominant animals, at this time the role of androgen receptors in the dMeA appears more substantial. Dominant animals also showed more androgen receptor positive cells in the vLS, compared to subordinates and controls. Thus, upregulation of androgen receptors within this brain region may be a result of repeatedly displaying aggressive behavior or winning. Because there was no effect of flutamide on androgen receptors within the vLS, it is unlikely the vLS is a key brain region modulating status-dependent differences in CD.

The present study suggests that the activation of androgen receptors during the maintenance of dominance relationships is necessary for resistance to CD in dominant hamsters. More broadly, we have identified testosterone as a neuroendocrine signal promoting experience-dependent neural plasticity at androgen receptors in the MeA, and that it is associated with resistance to social stress. Because there are limited available interventions to promote stress resilience, androgen receptors are a worthwhile target for future research. Overall, experiences that promote winning and/or personal success may facilitate the neuroendocrine changes in the brain that protect against stress-related mental illness.

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Appendix C

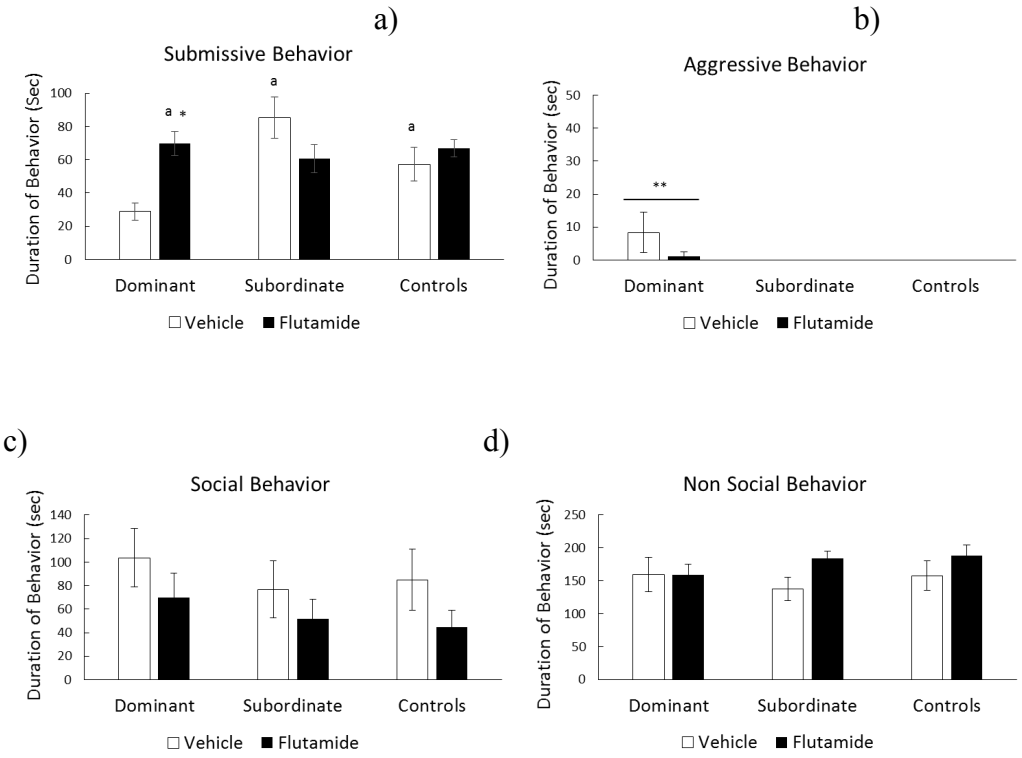

Figure 3.1 Durations (mean ± SE) of a) submissive behavior, b) aggressive behavior, c) non-agonistic social behavior, and d) non social behavior are shown for a 5-min conditioned defeat test. Animals received either flutamide (15 mg/kg) or vehicle injections during the 14 days of maintaining their dominance relationship. All animals received social defeat 24 hrs prior to conditioned defeat testing. *Indicates significant drug treatment x social status interaction; [a] indicates significant difference compared to vehicle-treated dominant animals ($p < .05$), n = 7-10.

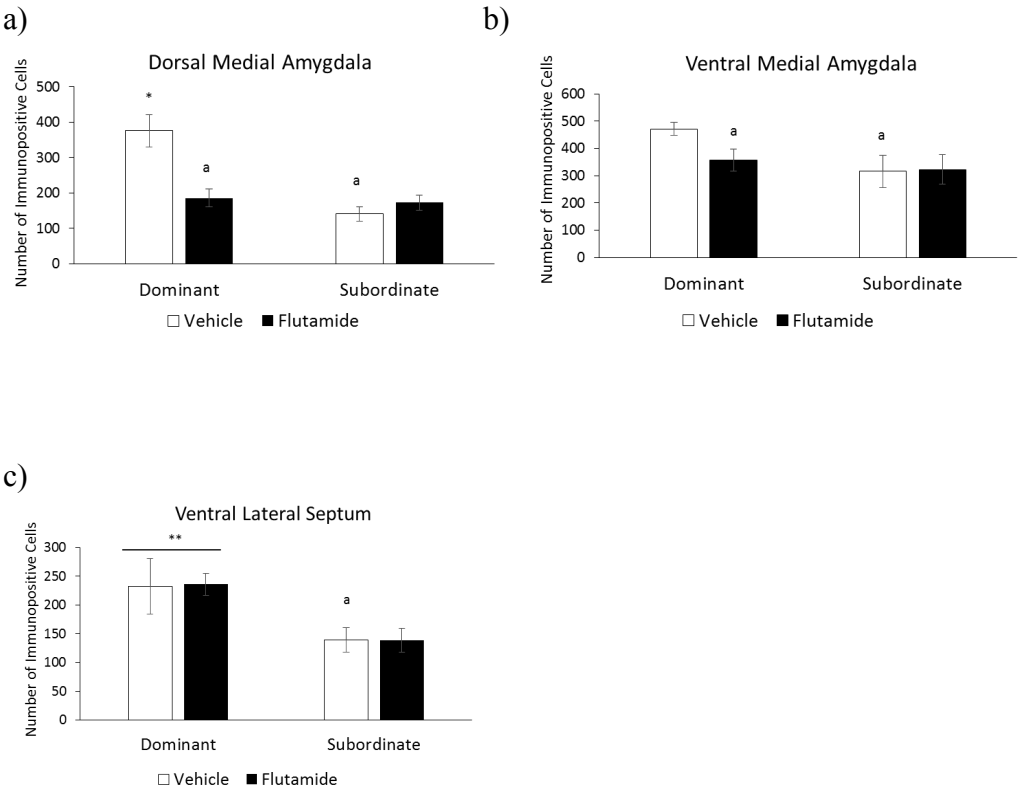

Figure 3.2 Number (mean ± SE) of androgen receptor positive cells in the a) dorsal medial amygdala b) ventral medial amygdala and c) ventral lateral septum. Animals received either flutamide (15 mg/kg) or vehicle injections during the 14 days of maintaining their dominance relationship. *Indicates significant drug treatment x social status interaction; **Indicates main effect of social status; [a] indicates significant difference compared to vehicle-treated dominant animals ($p < .05$), n = 6-10.

Table 3.1 Agonistic behavior during the maintenance of dominance relationships.

Social status*	Drug treatment	Submissive behavior in seconds (mean ± SE)		Aggressive behavior in seconds (mean ± SE)	
		Day 6	Day 14	Day 6	Day 14
Subordinate	Vehicle	121.5 ± 27.3	141.5 ± 24.9	___	___
Subordinate	Flutamide	137.8 ± 20.6	180.7 ± 13.7	___	___
Dominant	Vehicle	___	___	90.0 ± 19.3	109.0 ± 22.9
Dominant	Flutamide	___	___	109.1 ± 17.1	125.9 ± 15.3

**N = 8 – 10 per group*

Table 3.2 Aggression received during social defeat.

Social status*	Drug treatment	Aggression received in seconds (mean ± SE)	Number of attacks received (mean ± SE)
Subordinate	Vehicle	119.1 ± 18.4	5.1 ± 1.0
Subordinate	Flutamide	118.6 ± 17.0	3.6 ± 0.6
Dominant	Vehicle	124.2 ± 23.0	4.9 ± 0.8
Dominant	Flutamide	149.6 ± 22.1	6.6 ± 1.1
Control	Vehicle	154.3 ± 20.2	4.0 ± 0.7
Control	Flutamide	172.1 ± 10.5	5.2 ± 0.5

**N = 8 – 11 per group*

Chapter 4

General Conclusions

Summary of Findings

The aim of my dissertation was to investigate the neuroendocrine mechanisms promoting experience-dependent plasticity within the medial amygdala (MeA) that underlie resistance to conditioned defeat in dominant hamsters. Dominant hamsters respond to social defeat stress with reduced submissive and defensive behavior at testing compared to subordinates and controls, and thus have a reduced conditioned defeat response. Importantly, this resistance to conditioned defeat requires 14 days of dominant social status, and not one or seven days (Morrison et al., 2014). Previously, we showed that dominant animals exhibit greater defeat-induced neural activation within the MeA, compared to subordinates (Morrison et al., 2014). Here, we hypothesized that dominant hamsters experience daily surges in plasma testosterone during the maintenance of their social status that increase the expression of androgen receptors in the MeA and lead to a reduced conditioned defeat response compared to subordinates. The experiments presented here provide support for this hypothesis. Dominant individuals exhibited a significant rise in plasma testosterone 15-min following an aggressive encounter compared to their baseline testosterone level, whereas subordinates and control animals showed no change in testosterone (Chapter 2). Furthermore, we investigated whether changes in androgen receptor and estrogen alpha-receptor expression occur during the maintenance of dominance relationships. After maintaining dominance relationships for 14 days, dominant animals showed significantly more cells expressing androgen receptor, but not estrogen alpha-receptor, immunoreactivity in the dorsal MeA (dMeA), ventral MeA (vMeA) and ventral lateral septum (vLS), compared to subordinates and controls (Chapters 2 and 3). Additionally, one day of dominant social status was not sufficient to increase androgen receptor immunoreactivity in the dMeA, vMeA or vLS (Chapter 2). Pharmacological blockade of androgen receptors with flutamide during the

maintenance of dominant social status prevented resistance to conditioned defeat in dominant animals compared to vehicle-treated dominants (Chapter 3). Flutamide treatment during the maintenance of dominant social status also prevented the increase in androgen receptor immunoreactivity in the dMeA and vMeA, but not in the vLS, compared to vehicle-treated dominants (Chapter 3). While we were unable to selectively block androgen receptors in the MeA, their role in resistance to conditioned defeat remains an important focus for future studies. Altogether, these results suggest that the maintenance of dominant social status leads to an upregulation of androgen receptors in the MeA, and that the activation of androgen receptors during the maintenance of dominance relationships is necessary for dominant hamsters to show resistance to conditioned defeat.

Control Groups

Understanding neuroendocrine differences and their contribution to stress resistance requires establishing the appropriate control groups. Our control animals serve an important function, because our interpretation of stress vulnerability depends on the response of control animals. If control animals are similar to subordinates and show high levels of stress reactivity then we conclude dominant animals are resistant. Conversely, if control animals are similar to dominants and show low levels of stress reactivity then we conclude subordinates are susceptible. For instance, we showed dominant animals have more androgen receptor positive cells in several brain regions compared to subordinates and controls, suggesting the upregulation of androgen receptors is associated with resistance to conditioned defeat. On the other hand, after maintaining subordinate social status for 14 days subordinates showed lower plasma testosterone levels compared to dominants and controls, suggesting 14 days of losing encounters leads to stress-induced decreases in plasma testosterone.

In each of the experiments presented here different types of control groups were used. In Chapter 2, control animals were singly housed at the same time as corresponding experimental animals and brought into the lab for blood collection along with experimental animals. These control animals received no experimental manipulation other than human handling. We elected not to repeatedly expose our control animals to empty cages during the maintenance of dominance relationships because we previously found that if control animals were exposed to a novel, clean cage everyday for two weeks then they would behave more like dominant hamsters (Morrison et al., 2012). Furthermore, because we were measuring plasma testosterone and androgen receptors, we were especially concerned about how repeated exposures to a novel, empty cage might alter plasma testosterone in our control groups. When establishing a territory in a new environment, hamsters typically display a form of scent marking called flank-marking (Ferris et al., 1987). In hamsters, flank-marking behavior is sensitive to testosterone manipulation (Albers et al., 1988; Vandenbergh, 1971), and therefore we did not want to increase the occurrence of flank-marking in our control animals. For these reasons, while the experimental animals are exposed to two weeks of social encounters, we chose not to expose control animals to a novel environment each day.

In the final experiment of Chapter 2, we investigated whether one day of winning would increase androgen receptor expression. Our main question of interest was whether dominant and subordinate animals entered the experiment with differences in androgen receptor expression. Control animals were not needed to address this question. Additionally, we believed we would be able to compare the number of androgen receptor immuno-positive cells in our one-day dominant and subordinate animals to the number of androgen receptor positive-cells in our 14-day controls, because experimentally one-day and 14-day controls are similar. We found no

significant differences between one-day dominants, one-day subordinates and 14-day controls in the number of androgen receptor immuno-positive cells in the dMeA, vMeA or vLS ($p > .05$ for all tests). These results indicate androgen receptor expression after one social encounter in both dominants and subordinates is similar to control levels. We recognize it would have been ideal to run an additional set of controls because immunohistochemistry on these subjects was run at a different time. Moving forward, the most thorough approach would be to test one-day control animals and perform immunohistochemistry for androgen receptors alongside stored tissue from one-day dominant and subordinate animals.

In Chapter 3, we ran two sets of controls. First, because we administered flutamide chronically, it was necessary to control for the effects of the repeated injection process and the solution used to dissolve the drug. A vehicle control group received s.c. injections similar to the flutamide-treated animals, but instead of receiving flutamide the solution only consisted of 5% DMSO and sesame oil. The vehicle-treated animals were placed in daily social encounters for 14 days, followed by social defeat stress and conditioned defeat testing similar to flutamide-treated animals. Both dominant and subordinate animals in a dyad received the same drug treatment, and our main comparison of interest was whether vehicle-treated dominant animals differed from flutamide-treated dominant animals in conditioned defeat behavior and the number of androgen receptor immuno-positive cells. Additionally, we recognized flutamide treatment could alter conditioned defeat behavior or androgen-receptor immunoreactivity independent of social status. Therefore, we ran an additional control group that received either vehicle or flutamide for 14 days, but were not exposed to daily agonistic encounters. Therefore, they did not attain dominant or subordinate status, but were still exposed to social defeat stress. In an effort to reduce the number of animals, we did not run non-defeated control groups for this experiment. Previously,

our lab has investigated the effects of social status in non-defeated subjects and found little effect on agonistic behavior and c-Fos immunoreactivity (Morrison et al., 2012).

Individual Differences

One limitation of our dominant/subordinate model is animals self-select their social status. Therefore, it is difficult to know whether differences between dominants and subordinates are attributable to their acquired status or whether they reflect pre-existing traits. Hamsters are group-housed prior to experimental manipulation, during which time they form dominance relationships with other individuals in their cage. Thus, prior social experiences could influence the establishment of dominance relationships and subsequent differences in brain and behavior. Our lab has acknowledged this possibility and tried to address this concern. For example, we have previously shown that maintaining social status for one or seven days is not sufficient to produce resistance to conditioned defeat in dominant animals, suggesting resistance develops during the long-term maintenance of dominance relationships (Morrison et al., 2014). In Chapter 2, there were no differences in plasma testosterone prior to the beginning of the 1st social encounter, suggesting individual differences in plasma testosterone do not predict winners or losers. Additionally, we found one day of dominant social status is not sufficient to increase the number of androgen receptor immunopositive cells in the dMeA, vMeA or vLS, suggesting that repeatedly winning encounters is required to achieve differences in androgen receptor expression between dominants and subordinates (Chapter 2). Taken together, our findings indicate that differences between dominant and subordinate animals in resistance to conditioned defeat and number of androgen receptor immunopositive cells develop during the long-term maintenance of dominance relationships. Furthermore, while it is possible there are pre-existing differences that

contribute to which animal becomes the dominant in a dyad, basal plasma testosterone levels and androgen receptor density are not predictors.

Effects of Social Status on Testosterone

The findings in Chapter 2 suggest that winning an agonistic encounter increases plasma testosterone in Syrian hamsters. This is consistent with literature from other taxonomic groups, including primates, birds, reptiles and other rodents; suggesting winners of competitive interactions and social challenges exhibit increased plasma testosterone (Cavigelli and Pereira, 2000; Oyegbile and Marler, 2005; Smith et al., 2005; Yang and Wilczynski, 2002). This testosterone surge was apparent after winning one social encounter. While we did not measure plasma testosterone after all 14 social encounters, our working hypothesis postulates that because dominant animals win dyadic contests for 14 days they likely experience a similar testosterone surge following each social encounter. We propose dominant hamsters experience a transient surge in testosterone following each aggressive encounter and that these rapid increases in testosterone increase the probability of winning in the future (i.e. 'the winner-challenge hypothesis). Furthermore, we are the first to provide evidence for an additional functional significance of a contest-related testosterone surge. Our findings suggest that brief increases in testosterone following victories may promote neural plasticity that enables resistance to social stress in dominant hamsters. Interestingly, on the 14^{th} social encounter dominant hamsters did not show increased plasma testosterone compared to controls. The failure of dominant hamsters to show a rise in plasma testosterone could be related to the time point for blood collection in this experiment. Blood was collected 45-min post-encounter, and it is likely we missed the surge because our subsequent experiment showed a rise in testosterone 15-min after the dyadic encounter. 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, basal plasma testosterone was reduced in subordinate hamsters following 14 days of losing. This is consistent with other data suggesting subordinate social status is a chronic stressor. After living in the visible burrow system for 14 days, subordinate rats display increased basal plasma corticosterone, lower testes weight and lower plasma testosterone compared to dominants and controls (Blanchard et al., 1995). In the future, it would be interesting to investigate whether basal plasma cortisol levels negatively correlate with plasma testosterone levels in subordinate hamsters. Altogether, the maintenance of dominant social status in hamsters produces transient increases in testosterone, which alters androgen receptor expression, and together contributes to the differences in the conditioned defeat response between dominants and subordinates.

Effects of Social Status on Androgen Receptors

The results presented here suggest that transient increases in testosterone increase the number of androgen receptors in brain structures that is necessary promote resistance to conditioned defeat in dominant hamsters. In both chapters 2 and 3, dominant animals showed more androgen receptor immunopositive cells in the dMeA, vMeA and vLS compared to subordinates, controls and flutamide-treated dominants. While the surge in testosterone is transient, the change in androgen receptors is not. The upregulation of androgen receptors takes more than one winning experience, and is stable for at least three days after the last winning experience. In chapter 3, brains were collected three days after the final winning experience, after both social defeat and conditioned defeat testing. This suggests an upregulation of androgen receptors is maintained well past the last testosterone surge and is not obscured by social defeat stress or conditioned defeat testing. In chapter 3, we showed androgen receptor activation during

the maintenance of dominance relationships is necessary for dominant animals to show resistance to conditioned defeat. In addition, blocking androgen receptors during the maintenance dominance relationships prevents the upregulation of androgen receptors in the dMeA and vMeA, but not in the vLS, of dominant hamsters. Future studies should address whether increased androgen receptor expression in the MeA during social defeat stress or conditioned defeat testing reduces the acquisition or expression of conditioned defeat, respectively. Altogether, the maintenance of dominance relationships alters androgen receptor expression in key brain regions, such as the MeA, which contributes to status-dependent differences in the conditioned defeat response.

Estrogen Receptors

Testosterone can be aromatized into estradiol and act on either estrogen receptor alpha or beta. Therefore, we investigated whether the testosterone surges occurring during the maintenance of dominant social status altered the expression of estrogen receptors (chapter 2). Using alternate sections from the same animals used for androgen receptor immunoreactivity, we performed immunohistochemistry for estrogen receptor alpha. We focused on estrogen receptor alpha, instead of estrogen receptor beta, because of its role in aggression. Stimulation of estrogen receptor beta has consistently shown anxiolytic effects (Hughes et al., 2008; Lund et al., 2005; Walf et al., 2008), whereas estrogen receptor alpha is implicated in the regulation of male sexual and aggressive behavior (Paredes, 2003; Sano et al., 2013; Shimura et al., 1994). We found that dominant and subordinate hamsters did not significantly differ in estrogen receptor alpha expression in the MeA, suggesting that the maintenance of dominance relationships in male hamsters is associated with long-term changes in androgen receptor, but not estrogen receptor alpha, signaling in the MeA.

The Lateral Septum

The LS has been implicated in the regulation of emotion, social behavior and the HPA-axis (Herman and Cullinan, 1997; Sheehan et al., 2004). Additionally, the LS has been shown to modulate aggressive behavior (Albert and Chew, 1980; Albert and Richmond, 1976; Sodetz and Bunnell, 1970). Pharmacological inactivation of the LS has been shown to increase aggression in non-defeated hamsters and reduce the conditioned defeat response in defeated hamsters (McDonald et al., 2012). We had previously shown the 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 on defeat-induced neural activity are hard to rectify with lesion studies, the LS contains a heterogeneous population of cells. For instance, the LS sends GABAergic projections to a variety of limbic, hypothalamic and midbrain structures and also contains GABAergic interneurons that can inhibit the projection neurons (Risold and Swanson, 1997a, b). Here, we investigated the role of androgen receptors in the vLS in the development of resistance to conditioned defeat. Because the LS contains a high density of androgen receptors (Roselli et al., 1989) we hypothesized that elevated androgen receptor expression in the vLS would contribute to conditioned defeat resistance in dominant hamsters. We found two weeks of dominant social status increased the number of androgen receptor immunopositive cells in the vLS (chapters 2 and 3), suggesting repeatedly displaying aggressive behavior, and winning social encounters increases androgen receptors in this brain region. However, blocking androgen receptors with flutamide did not prevent the upregulation of androgen receptors in the vLS. These results suggest the upregulation of androgen receptors in the vLS may contribute to the establishment and maintenance of dominant social status, but it

likely does not modulate resistance to conditioned defeat. Nonetheless, there are status-dependent differences in androgen receptors in the vLS, and future investigations could help delineate its functional significance.

Medial Amygdala

The MeA is a heterogeneous structure that contains multiple subregions, and has projections to brain structures that are implicated in a variety of social behaviors. For instance, the MeA has connections with other structures in the social brain network, including the bed nucleus stria terminalis (BNST) and central and basolateral amygdala, and together are implicated in olfactory processing, defensive, agonistic and reproductive behaviors (Newman, 1999). The role of the MeA in coping with stress is less well known, but it is a neural substrate well positioned to integrate social context into stress, anxiety and fear-related behavior. Previously, we showed dominant hamsters had increased c-Fos expression in the vMeA, compared to subordinates and controls following social defeat stress, and a similar trend was found in the dMeA (Morrison et al., 2012). Here, we further investigated the neuroendocrine signaling within the MeA that changes during the maintenance of dominance relationships and contributes to resistance to conditioned defeat. We found two weeks of dominant social status increased androgen receptor expression in both the dMeA (chapters 2 and 3) and vMeA (chapter 3) compared to subordinates and controls. Blocking androgen receptors with flutamide during the maintenance of dominance relationships prevented resistance to conditioned defeat in dominant hamsters and prevented an upregulation of androgen receptors in the dMeA and vMeA. It is possible the difference between the cFos study, which emphasizes a large change in vMeA neural activity, and the flutamide study, which emphasizes a large change in dMeA androgen receptor expression, is related to the greater population of androgen receptors within the dMeA

(Wood et al., 1992). Therefore, both subregions of the MeA may contribute to reduced conditioned defeat in dominant hamsters, but the effect of flutamide may have been more apparent in the dMeA. While we did not directly test the causality between androgen receptor signaling within the MeA and a reduced conditioned defeat response, our results suggest an upregulation of MeA androgen receptors is linked to resistance to conditioned defeat in dominant hamsters. Future studies should investigate the mechanisms by which elevated androgen receptors in the MeA during social defeat stress and conditioned defat testing modulate the acquisition and expression of conditioned defeat in dominant hamsters.

Future Directions

One limitation of the current working model is the inability to know whether androgen receptor activation is only critical during the maintenance of dominance relationships or whether the upregulation of androgen receptors during social defeat stress and conditioned defeat testing is also critical. Here, flutamide was administered during two weeks of daily dyadic encounters, but not prior to social defeat stress or conditioned defeat testing. In the future, it would be important to block androgen receptors at both of these time points, and investigate whether signaling at androgen receptors during social defeat or conditioned defeat testing is necessary for dominant animals to show reduced conditioned defeat. We hypothesize the upregulation of androgen receptors in dominant animals is especially critical during conditioned defeat testing, and that elevated signaling at non-genomic androgen receptors might reduce the expression of the conditioned defeat response. Additionally, when blocking androgen receptors at these time points, we could address another limitation. In the work presented here, flutamide was administered peripherally and therefore blocked androgen receptors throughout the brain and body. Our work identifies the MeA as a brain structure where androgen receptors might

modulate the conditioned defeat response, but we did not selectively target this structure. Future studies could microinject flutamide into the MeA, to investigate whether androgen receptor signaling within the MeA reduces the acquisition and/or expression of conditioned defeat. The feasibility of daily microinjections during the maintenance of dominance relationships is low, but one-time microinjections of flutamide at either social defeat stress or conditioned defeat testing would address both limitations of neuroanatomical specificity and critical time points for receptor upregulation.

Another limitation to our working model is flutamide has the ability to block testosterone's action at either genomic or non-genomic receptors, and we cannot be certain which is occurring (Farla et al., 2005; Gorczynska and Handelsman, 1995). We expect that testosterone is acting on genomic androgen receptors to alter gene expression during the maintenance of dominance relationships because dominant animals have lasting behavioral and neural changes. Furthermore, dominant hamsters display increased c-Fos expression following social defeat in the MeA, the same brain region we also see an increase in androgen receptors. Although cFos has previously been used as a marker for genomic effects (Nagypal and Wood, 2007), activation of non-genomic receptors could also lead to changes in Fos activation. Further research would be needed to confirm if activation of genomic androgen receptors, as opposed to non-genomic receptors, during the maintenance of dominance relationships is necessary for resistance to conditioned defeat in dominant animals.

It would be interesting to investigate if a change in activation of androgen receptors during the maintenance of dominance relationships modulates activity in the broader neural circuit that we believe is important for resistance to conditioned defeat in dominant animals. One way to address this would be to administer flutamide during the 14 daily dyadic encounters, and

investigate whether flutamide treatment alters the pattern of defeat-induced neural activity that we have previously seen in dominant animals. Specifically, would flutamide treatment prevent the increase in defeat-induced c-Fos expression in dominant animals in brain regions outside the MeA, including the vmPFC, compared to vehicle-treated dominants? Another important goal for the future is to identify the phenotype of the cells expressing androgen receptors (glutamatergic, GABAergic, projection neuron, or interneuron). Our working hypothesis is that repeated and transient surges in testosterone, during the maintenance of dominance relationships, are acting through genomic androgen receptors in the MeA to induce neural plasticity leading to resistance to conditioned defeat in dominant hamsters. Future studies should address whether the upregulation of androgen receptors themselves are the form of neural plasticity that reduces the conditioned defeat response. Altogether, these studies would address how activation of androgen receptors during the maintenance of dominance relationships promotes resistance to conditioned defeat in dominant hamsters.

Conclusions

In conclusion, the dominant and subordinate paradigm in Syrian hamsters is a useful model for investigating the cellular and molecular mechanisms underlying experience-dependent resistance to stress. While much previous literature has identified the importance of neural plasticity within the vmPFC for experience-dependent resistance to stress, we propose androgen-dependent plasticity in other brain regions, including the MeA, as an important mechanism contributing to experience-dependent resistance to stress. Additionally, we confirmed a winner-challenge effect, in which dominant hamsters experience a surge in plasma testosterone and an increase androgen receptor expression after winning. Along with increasing the probability of

winning in the future, we propose a surge in plasma testosterone and increase in androgen receptor expression contributes to resistance to social stress in the future.

Overall, research into the neuroendocrine mechanisms underlying status-dependent differences in responses to social defeat should provide novel targets for the prevention and treatment of stress-related mental illness. Here, we have identified increased testosterone signaling at androgen receptors as an important factor promoting stress resilience. Because there are limited available interventions to promote stress resilience, future investigations into the mechanisms underlying testosterone's action on MeA activity could be beneficial. In addition, non-pharmacological treatments that promote winning and/or personal success may facilitate the neuroendocrine changes in the brain that protect against the development of stress-related psychopathology.

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Vita

Catherine Tucker Clinard was born to parents Mark Tucker and Laurie Toth in Princeton, NJ in July 1989. Catherine attended elementary, middle and high school in Ooltewah, TN. Catherine began her education at Wallace A. Smith elementary school, followed by Hunter Middle School and graduating with honors from Ooltewah High School in 2007. She attended college at Austin Peay State University, where she played collegiate Division 1 women's golf and received a degree in Psychology (B.S.) with a minor in health. Additionally, it was due to experiences at Austin Peay where Catherine realized her desire to pursue graduation education. After gaining biopsychology research experience under the direction of Dr. Brian Hock at Austin Peay, Catherine was admitted to the University of Tennessee to study in the behavioral neuroscience lab of Dr. Matthew Cooper in August 2011. She received a Ph.D. from UT in Biological Psychology in August 2016. Following graduation, Catherine accepted a position as assistant professor of psychology at Dalton State College in Georgia.