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# Sex Differences In How Serotonin (5-HT) And Arginine Vasopressin (AVP) Mediate Aggression, Dominance, And Resistance To Social Stress In Syrian Hamsters

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# SEX DIFFERENCES IN HOW SEROTONIN (5-HT) AND ARGININE VASOPRESSIN (AVP) MEDIATE AGGRESSION, DOMINANCE, AND RESISTANCE TO SOCIAL STRESS IN SYRIAN HAMSTERS

by

JOSEPH I. TERRANOVA

Under the Direction of H. Elliott Albers, PhD

## ABSTRACT

There are profound sex differences in the incidence of many psychiatric disorders. While these disorders are frequently linked to social stress, little is known about sex differences in the underlying neural mechanisms. Individuals characterized by aggression and dominance are more resistant to social stress, whereas individuals characterized by docility and subordinace are more susceptible to social stress. The goal of this dissertation is to characterize sex differences in neural mechanisms that underlie aggression, dominance, and resistance to social stress. First, we explored sex differences in how serotonin (5-HT) and arginine vasopressin (AVP) regulate aggression and acquisition of dominance in Syrian hamsters. We tested the hypothesis that 5-HT in females and AVP in males facilitates aggression and acquisition of dominance. In females, 5-HT facilitates aggression and is associated with acquisition of dominance, whereas 5-HT reduces

aggression in males. AVP, on the other hand, facilitates aggression and is associated with acquisition of dominance in males, whereas AVP reduces aggression in females. Next, we explored the same 5-HT and AVP systems in the context of social stress. First, we paired hamsters in stable, 14-day dominant/subordinate relationships and subsequently measured activation of 5-HT and AVP-containing neurons after social defeat. We hypothesized that dominance is associated with activation of 5-HT-containing neurons in females and AVP-containing neurons in males after social defeat. Socially defeated dominant females had higher 5-HT neuron activation than subordinate or control females, whereas socially defeated dominant males had lower AVP neuron activation than control males. In another set of experiments, we pharmacologically manipulated the 5-HT and AVP systems and observed subsequent resistance to social stress. Systemic injection of the selective serotonin reuptake inhibitor, fluoxetine, in males increased social avoidance after social defeat stress whereas fluoxetine had not effect on social avoidance in females. Microinjection of AVP into the anterior hypothalamus (AH) of female after social defeat decreased social avoidance. Microinjection of a 5-HT1a receptor agonist into the AH of male after social defeat decreased social avoidance. Taken together, these data show that there are sex differences in how 5-HT and AVP regulate aggression, dominance, and resistance to social stress in Syrian hamsters.

INDEX WORDS: Gender differences, Agonistic, Subordinate, Social behavior, Fluoxetine, Coping style

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# DEDICATION

*For Ignazio Terranova and John B. Terranova*

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# LIST OF ABBREVIATIONS

5-HT: Serotonin

5-HT-ir: Serotonin immunoreactivity

5-HT1aR: Serotonin 1a receptor

5-HT2aR: Serotonin 2a receptor

5-HT2cR: Serotonin 2c receptor

5-HT3R: Serotonin 3 receptor

8-OH-DPAT: 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol

ABS: Antibody solution

AH: Anterior hypothalamus

ANOVA: Analysis of variance

AVP: Arginine vasopressin

AVP-ir: Arginine vasopressin immunoreactivity

DA: Dopamine

DC: Defeated control hamster

diH2O: Deionized water

DAPI: 4',6-diamidino-2-phenylindole

DOM: 14-day established dominant hamster

DPAT: 8-OH-DPAT

DRN: Dorsal raphe nucleus

DRNa: Dorsal raphe nucleus anterior subregion

DRNp: Dorsal raphe nucleus posterior subregion

drDRNa: Dorsal rostral dorsal raphe nucleus subregion

Fos: c-Fos

Fos-ir: c-Fos immunoreactivity

GABA: γ-Aminobutyric acid

IP: Intraperitoneal

LAL: Long attack latency

LS: Lateral septum

MRN: Median raphe nucleus

mSON: Medial supraoptic nucleus

mRNA: Messenger ribonucleic acid

NAI: Non-aggressive intruder

NC: Nucleus circularis

NDC: Non-defeated control

NDS: Normal donkey serum

PBS: Phosphate buffered saline

PVN: Medial paraventricular nucleus

PTSD: Posttraumatic stress disorder

RA: Resident aggressor

SAL: Short attack latency

SUB: 14-day established subordinate hamster

SSRI: Selective serotonin reuptake inhibitor

V1aR: Vasopressin V1a receptor

vrDRNa: Ventral rostral dorsal raphe nucleus subregion

# 1 GENERAL INTRODUCTION

Anxiety and mood disorders are two of the most common psychiatric disorders in the United States, with a lifetime prevalence of 28.8% for anxiety disorders and 20.8% for mood disorders (Kessler et al., 2005). Of individuals diagnosed with anxiety and mood disorders, 5-10% are diagnosed with post-traumatic stress disorder (PTSD) and 11.7% are diagnosed with major depressive disorder (Merikangas et al., 2010; Horn et al., 2016). PTSD and major depressive disorder have serious consequences for afflicted individuals and their caregivers. Besides negative effects on mental health, both disorders are associated with precipitous decline in physical well-being (Roshanaei-Moghaddam et al., 2009; Pacella et al., 2013). Moreover, caregivers and family members are more likely to be stressed, depressed, and report lower overall quality of physical health (Sobieraj et al., 1998; Pinquart and Sörensen, 2003). Psychiatric disorders such as PTSD and major depressive disorder pose huge economic burdens, with can cost up to $97.3 billion dollars annually (Eaton et al., 2008; Ferry et al., 2015).

Given the frequency and severity of PTSD and major depressive disorder, much research has been dedicated to understanding the neural mechanisms of these psychiatric disorders and their treatments (de Mello et al., 2005; Brunoni et al., 2008; Rauch et al., 2012; Steckler and Risbrough, 2012; Bjorkholm and Monteggia, 2016). One area of interest is the neural mechanisms that underlie resistance to social stress. Broadly, stress is defined as challenges to the homeostasis of an individual that can induce many physiological changes (e.g. increased circulating glucocorticoids, adrenal enlargement, thymus reduction, etc.) and many behavioral changes (e.g. increased anxiety, increased depression, etc.) (Gold et al., 1988; Blanchard et al., 1993; Veenema et al., 2003; Chrousos, 2009). Social stress is one of the most common stressors experienced by humans and is an important risk factor for both PTSD and major depressive

disorder (Björkqvist, 2001; Almeida et al., 2002; Ozer et al., 2003). However, although social stress is a major risk factor for PTSD and major depressive disorder, only a subset of individuals who experience traumatic life events will go on to develop these psychiatric disorders (Merikangas et al., 2010; Cooper et al., 2015; Horn et al., 2016).

Why are some individuals susceptible to social stress (i.e. develop PTSD and major depressive disorder), whereas others are resistant to social stress? Even in rat models of PTSD, only a subset of individuals exhibit long-term maladaptive behavioral and physiological phenotypes (Cohen and Zohar, 2004; Cohen et al., 2004). Clues to variability in the neural mechanisms that underlie resistance social stress come from research on coping style, which is defined as the alternative response patterns to environmental challenges (Koolhaas et al., 1999; de Boer et al., 2017; Koolhaas et al., 2017). Two types of coping styles exhibited by rodents are proactive coping and reactive coping. Those that cope proactively actively engage stressful environmental stimuli, such that they are more aggressive, more dominant, engage in higher rates of defensive burying, and are less adaptable to day/night cycle reversal (Benus et al., 1987; Cooper et al., 2015; Gorka et al., 2016; de Boer et al., 2017). Conversely, those that cope reactively are docile, submissive, engage in low rates of defensive burying, and are more adaptable to day/night cycle reversal (Benus et al., 1987; Cooper et al., 2015; Gorka et al., 2016; de Boer et al., 2017). Interestingly, individuals that have traits associated with active coping strategies (e.g. aggression, dominance) are more resistant to social stress, whereas those with traits associated with reactive coping strategies (e.g. docility, submissiveness) are more susceptible to social stress (Veenema et al., 2003; Morrison et al., 2014a). The linkage between aggressiveness, dominance, and resistance to social stress suggests that they share at least some common neural mechanisms.

There are sex differences in psychiatric disorders in which social stress is a risk factor, with women twice as likely than men to be diagnosed with PTSD or major depressive disorder (Kessler et al., 2005; Marcus et al., 2005; Cover et al., 2014). Nonetheless, current treatment strategies for these disorders are largely the same for both sexes with, at best, limited effectiveness (Sramek and Cutler, 2011). It seems likely that these disorders are so intractable because they stem from highly entrenched behavioral strategies for coping with social challenges that have evolved differently between the sexes. Therefore, to better address the questions regarding sex differences in the incidence and treatment of psychiatric disorders, we must investigate sex differences in the neurobiological mechanisms that underlie these disorders. This dissertation will explore the potential link between sex differences and the neural mechanisms that underlie aggression, dominance, and resistance to social stress using the Syrian hamster as a model species.

Syrian hamsters are an excellent model species to study the neurobiological mechanisms that underlie aggression, dominance, and resistance to social stress because they are quick to attack intruders, demonstrate stereotyped offensive and defensive behaviors, and quickly form stable dominance relationships (Payne and Swanson, 1970; Potegal et al., 1993; Olivier and Young, 2002; McCann and Huhman, 2012). Moreover, when paired with a same sex conspecific, both sexes engage in similar amounts of spontaneous aggression, unlike other rodent species where males are more aggressive than females (Payne and Swanson, 1970; Blanchard et al., 1988). Physical contact is not necessary for Syrian hamsters to maintain dominance status and thus, like in primates, social rank in hamsters is an ethologically relevant psychosocial stressor (Huhman et al., 1992; Meyer and Hamel, 2014). Hamsters that are socially defeated are no longer spontaneously aggressive and, instead, exhibit avoidance and submissive behaviors. This

behavioral response to social defeat stress is termed *conditioned defeat* (Potegal et al., 1993; McCann and Huhman, 2012). Critically, both female and male hamsters display the conditioned defeat phenotype after social defeat, which makes it possible to study resistance to social stress in both sexes (Huhman et al., 2003). Thus, Syrian hamsters are an excellent model species to study the neural mechanisms of aggression, dominance, and resistance to social stress.

Serotonin (5-HT) and arginine vasopressin (AVP) are critical regulators of aggression in rodents. Much of what is known about how 5-HT and AVP regulate aggression in rodents comes from data on males. Central administration of 8-OH-DPAT, a 5-HT1a receptor (5-HT1aR) agonist, decreases aggression in male hamsters, whereas microinjection of AVP into the AH of males increases aggression (Ferris et al., 1997; Ferris et al., 1999; Caldwell and Albers, 2004; Morrison and Melloni, 2014). Systemic administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, decreases aggression in male hamsters (Ferris et al., 1997). Microinjection of a AVP 1a receptor (V1aR) antagonist into the AH of male hamsters decreases aggression (Ferris and Potegal, 1988; Potegal and Ferris, 1989). Exposure of male hamsters to an aggressive encounter is associated with increased activation in AVP-containing neurons in the medial supraoptic nucleus and nucleus circularis compared to controls (Delville et al., 2000). In summary, 5-HT reduces aggression and AVP increases aggression in male hamsters.

There is considerably less data on how 5-HT and AVP control aggression in females. While there is no sex difference in aggressive behavior between same sex male and female pairings, in male-female pairings females tend to be more aggressive and dominant (Payne and Swanson, 1970). Microinjection of AVP into the AH of females reduces aggression, whereas microinjection of a V1a receptor antagonist increases aggression (Gutzler et al., 2010). Microinjection of 8-OH-DPAT into the 3$^{rd}$ ventricle of female hamsters failed to observe an

effect on aggression (Joppa et al., 1997). Data on the neural control of aggression in females are lacking and Chapter 2 of this dissertation will address this knowledge gap. Indeed, of the hundreds of studies that have been published demonstrating an inhibitory effect of 5-HT on aggression in males, our review of the literature found only a single study that investigated the role 5-HT and aggression in females (Joppa et al., 1997; Morrison and Melloni, 2014).

Neuroanatomical evidence provides further support that the hypothalamus, particularly the AH, is an important brain region for the control of aggression by 5-HT and AVP. 5-HT neurons from the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) densely project to the hypothalamus (Morin and Meyer-Bernstein, 1999; Delville et al., 2000). AVP neurons originating from hypothalamic nuclei such as the medial supra optic nucleus (mSON), nucleus circularis (NC), and paraventricular nucleus (PVN) project centrally (e.g. within the hypothalamus) in addition to projecting to the neurohypophysis (Mahoney et al., 1990; Ferris et al., 1992; Delville et al., 2000). Social isolation increases aggression in male hamsters and is associated with an increase in V1aR density in the AH and other hypothalamic brain regions (Albers et al., 2006). Testosterone in males appears to regulate aggression, at least in part, through the expression of the V1a receptor. Although testosterone fails to alter AVP immunoreactivity (AVP-ir) in male hamsters, testosterone is positively associated with V1aR expression (Albers et al., 1991; Johnson et al., 1995; Young et al., 2000).

In males, there is evidence for a relationship between 5-HT, dominance status, and resistance to social stress. Socially defeated male hamsters, regardless of dominance status, demonstrate elevated levels of c-Fos mRNA in the dorsal raphe nucleus compared to handled controls (Kollack-Walker et al., 1999). Central infusion of a 5-HT1aR agonist into the DRN of male hamsters, either prior to social defeat or prior to testing for conditioned defeat, reduces

submissive and defensive behaviors (Cooper et al., 2008). Conversely, central infusion of a 5-HT1aR antagonist into the DRN of male hamsters, either prior to social defeat or prior to testing for conditioned defeat increases submissive and defensive behaviors. (Cooper et al., 2008). 5-HT1a mRNA is increased in the DRN, a brain region containing many 5-HT-containing neurons, of dominant male hamsters in established dominance relationships compared to subordinates (Morin and Meyer-Bernstein, 1999; Cooper et al., 2009). 5-HT1aR stimulation initiates an inhibitory $G_i$ protein cascade that hyperpolarizes neurons (Polter and Li, 2010). Therefore, activation of DRN 5-HT neurons in males decreases resistance to social stress, whereas inhibition of these neurons increases resistance to social stress.

Several 5-HT receptor variants, besides 5-HT1aRs, play a role in modulating resistance to social stress. 5-HT2aRs and 5-HT2cRs mediate resistance to social stress in Syrian hamsters. Systemic injection of male hamsters with a 5-HT2aR agonist after social defeat and prior to testing for conditioned defeat decreases submissive behavior towards a non-aggressive intruder (NAI) stimulus animal (Harvey et al., 2012). Systemic injection of male hamsters with a 5-HT2cR antagonist prior to social defeat increases submissive behaviors towards a NAI during conditioned defeat testing (Harvey et al., 2012). The 5-HT3 receptor subtype is another 5-HT receptor that potentially regulates aggression, dominance, and resistance to social stress. 5-HT3a receptors are expressed throughout neural regions involved in the control of aggression and resistance to social stress (Carrillo et al., 2010). Systemic injection of a 5-HT3 receptor (5-HT3R) agonist increases aggression and systemic injection of a 5-HT3R antagonist decreases aggression in male hamsters (Ricci et al., 2004). Microinjection of a 5-HT3R into the AH of male hamsters decreases anxious behavior observed in an elevated plus maze task (Morrison et

al., 2015b). Thus, 5-HT, through several receptor subtypes regulates agonistic behaviors and how the response of an individual to social stressors.

There is a link between dominance status and AVP in male Syrian hamsters. Dominant males have an increased number of AVP cells in the NC compared to subordinates (Ferris et al., 1989). Dominant males that are paired daily with a subordinate partner have increased V1aR density in the hypothalamus compared to subordinates (Cooper et al., 2005). In contrast to males, there is little published data in females on how 5-HT and AVP regulate dominance status and resistance to social stress. In female macaques, there is a positive correlation between aggression, dominance, and levels of 5-Hydroxyindoleacetic acid, a metabolite of 5-HT, in the cerebral spinal fluid (Westergaard et al., 1999). Socially defeated female and male California mice both demonstrate changes in AVP cell expression after social defeat. AVP immunoreactivity (AVP-ir) is reduced in the PVN of both sexes and, in females, there is an increase AVP-ir/fos-ir when exposed to an NAI (Steinman et al., 2015; Steinman and Trainor, 2017). How 5-HT and AVP mediate dominance and resistance to social stress in females is not fully understood.

The goal of this dissertation is to better understand sex differences in the neural mechanisms that underlie aggression, dominance, and resistance to social stress. To address this goal, I will first investigate the neural mechanisms that control aggression and acquisition of dominance in both female and male hamsters. Chapters 3 and 4 expand upon the findings in Chapter 2 and apply these mechanisms to questions about social stress. In Chapter 3, I will investigate sex differences in how established dominant or subordinate status in hamsters affects activation of 5-HT and AVP-containing neurons in response to social defeat stress. In Chapter 4, I will investigate sex differences in the neural mechanisms that mediate resistance to social stress using a behavioral pharmacology approach to manipulate neural systems that regulate

aggression. Specifically, I will explore how 5-HT and AVP systems, which are known to control aggression, mediate resistance to social stress. Together, these data will provide valuable insights about sex differences in the neural control of resistance to social stress. Furthermore, these data will provide a better comprehension of sex differences in neural systems that are implicated in the pathogenesis and treatment of psychiatric disorders.

# 2 SEROTONIN AND ARGININE-VASOPRESSIN MEDIATE SEX DIFFERENCES IN THE REGULATION OF DOMINANCE AND AGGRESSION BY THE SOCIAL BRAIN

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## 2.1 Abstract

There are profound sex differences in the incidence of many psychiatric disorders. Although these disorders are frequently linked to social stress and to deficits in social engagement little is known about sex differences in the neural mechanisms that underlie these phenomena. Phenotypes characterized by dominance, competitive aggression, and active coping strategies appear to be more resilient to psychiatric disorders such as posttraumatic stress disorder (PTSD) compared to those characterized by subordinate status and the lack of aggressiveness. Here, we report that serotonin (5-HT) and arginine-vasopressin (AVP) act in opposite ways in the hypothalamus to regulate dominance and aggression in females and males. Hypothalamic injection of a 5-HT1a agonist stimulated aggression in female hamsters and inhibited aggression in males whereas injection of AVP inhibited aggression in females and stimulated aggression in males. Striking sex differences were also identified in the neural mechanisms regulating dominance. Acquisition of dominance was associated with activation of 5-HT neurons within the dorsal raphe in females and activation of hypothalamic AVP neurons in

males. These data strongly indicate that there are fundamental sex differences in the neural regulation of dominance and aggression. Further, because systemically administered fluoxetine increased aggression in females and substantially reduced aggression in males there may be substantial gender differences in the clinical efficacy of commonly prescribed 5-HT-active drugs such as selective 5-HT reuptake inhibitors. These data suggest that the treatment of psychiatric disorders such as PTSD may be more effective with the use of 5-HT-targeted drugs in females and AVP-targeted drugs in males.

## 2.2 Introduction

Prominent sex differences occur in the incidence, development, and clinical course of many psychiatric disorders. Women, for example, have higher rates of depression and anxiety disorders such as posttraumatic stress disorder (PTSD), while men more frequently suffer from autism and attention deficit disorders (Kessler et al., 1994; Kessler et al., 1995; Ramtekkar et al., 2010; Werling and Geschwind, 2013). Because little is known about sex differences in the efficacy of treatments for these disorders, current treatment strategies are largely the same for both sexes. The development of effective treatments for both women and men can proceed with a clear understanding of sex differences in the mechanisms and etiology of psychiatric disorders. Many of these disorders are linked to deficits in adaptive social skills (Bhat et al., 2014; Dodell-Feder et al., 2015), therefore understanding the neural mechanisms underlying social engagement in both sexes is essential. In most mammalian species, social interactions among both sexes are governed by dominance relationships. As such, behaviors associated with these relationships (e.g. social recognition, stress, competitive aggression) are the foundation for social interactions and are highly relevant for understanding psychiatric disorders (Blanchard et al., 1993; Albers et al., 2002; Fernald, 2014). Emerging genetic and environmental evidence suggests that traits such

as dominance, competitive aggression, and active coping strategies are linked, resulting in phenotypes more resistant to psychiatric disorders (Koolhaas et al., 2010; Russo et al., 2012; Cooper et al., 2015). In contrast, subordinate status and the absence of aggression are linked to phenotypes more susceptible to adverse psychiatric outcomes (e.g., PTSD). Despite the importance of understanding the basic mechanisms underlying aggression and dominance and their role in psychiatric disorders, possible sex differences in the basic neural mechanisms underlying these phenomena have received almost no attention.

Given that social behavior is evolutionarily ancient and social strategies used by females and males evolved in response to very different selective pressures, it seems likely that there are fundamental sex differences in the neural mechanisms regulating expression of social behaviors such as dominance and competitive aggression. And yet, little is known about the neurobiology of dominance and competitive aggression, particularly in females. Studies of aggression in female rodents have focused almost exclusively on maternal aggression at least in part because female laboratory rodents rarely display spontaneous aggression. In contrast, Syrian hamsters provide an outstanding rodent model with which to investigate competitive strategies in females as well as males because aggression does not have to be induced as in many other social species (e.g., mice and rats) by artificial means (e.g., electric shock) or by mating-induced pair bonding (e.g., voles) (Potegal and Ferris, 1989; Ferris et al., 1997; Ferris and Albers., 2013). Female hamsters, like female monkeys, display a range of competitive strategies including the rapid formation of robust hierarchal dominance relationships and the ability to inhibit the reproductive capacity of other females (Rosvall, 2011; Werling and Geschwind, 2013). Both female and male hamsters also readily display a variety of social behaviors that are fundamental for social relationships. In addition, a great deal is known about the biological mechanisms controlling

social behavior in Syrian hamsters, making it a powerful model system for preclinical study of behaviors that underlie psychiatric health and illness (Cooper et al., 2009; Werling and Geschwind, 2013; Alekseyenko and Kravitz, 2014).

One of the most well-known phenomena in behavioral neuroscience is the ability of serotonin (5-HT) to inhibit impulsive behaviors, including aggression, in males in species ranging from invertebrates to primates (Cooper et al., 2005; Ferrari et al., 2005; Morrison and Melloni, 2014). In contrast to 5-HT, centrally administered arginine-vasopressin (AVP) stimulates aggression in males and antagonists of AVP V1a receptors (V1aRs) reduce aggression (Potegal and Ferris, 1989; Ferris et al., 1997; Gutzler et al., 2010). In support of the hypothesis that there are fundamental sex differences in the neural mechanisms regulating social behavior, we recently found that AVP has the opposite effect on competitive aggression in female hamsters (Villalba et al., 1997). In contrast to males, central administration of AVP in females reduces aggression, and V1aR antagonists stimulate aggression. Remarkably, despite hundreds of studies demonstrating that 5-HT inhibits aggression in males, the effects of 5-HT on aggression in females remain essentially unknown (Joppa et al., 1997; Cooper et al., 2009).

In the following experiments, we investigated the hypothesis that there are fundamental sex differences in the neural regulation of aggression and dominance. More specifically, we investigated whether 5-HT promotes and AVP inhibits aggression and dominance in females and whether 5-HT inhibits and AVP promotes aggression and dominance in males. These studies support this hypothesis by directly comparing sex-specific effects of 5-HT and AVP on aggression and by demonstrating that acquisition of dominance is associated with activation of raphe 5-HT neurons in females and hypothalamic AVP neurons in males. Together, these data provide strong support for the hypothesis that there are striking sex differences in the

mechanisms regulating social behavior. Given the emerging relationship of aggression and dominance with phenotypes that are resilient to psychiatric disorders, understanding how the 5-HT and AVP systems control aggression and dominance has the potential for significant translational impact. For example, the following experiments demonstrate that systemic administration of one of the most commonly prescribed selective 5-HT reuptake inhibitors (SSRIs) (i.e., fluoxetine) has opposite effects in females and males, suggesting that clinical efficacy of SSRIs may differ dramatically between the sexes.

Despite widespread use of SSRIs, there appear to be no studies examining gender differences in the efficacy of SSRIs to treat stress disorders and only a limited a number examining SSRI efficacy to treat depression. Of the published peer reviewed studies, five found no difference in the efficacy of SSRIs in men and women (Entsuah et al., 2001; Quitkin et al., 2002; Hildebrandt et al., 2003; Thiels et al., 2005; Pinto-Meza et al., 2006), and six found SSRIs to be more effective in women (Haykal and Akiskal, 1999; Kornstein et al., 2000; Martenyi et al., 2001; Khan et al., 2005; Berlanga and Flores-Ramos, 2006; Young et al., 2009). There are no reports of SSRIs being more effective in men. Thus, these data do not generate confidence that there is comparable clinical efficacy of SSRIs in men and women, but rather suggest that SSRIs are more effective as an antidepressant in women than in men. The lack of definitive data is remarkable but not surprising. It is not uncommon that large sex differences in the efficacy of therapies used by millions are overlooked in clinical practice (Klein et al., 2015; LeResche et al., 2015).

## 2.3 Materials and Methods

### *2.3.1 Animals and Drug Treatment:*

Adult male and female Syrian hamsters (Charles River Laboratories Inc., Wilmington, MA, USA and in-house bred), 8–12 weeks old, weighing between 110 g – 140 g, were used for all experiments. Hamsters were individually housed in polycarbonate cages (24 x 43 x 20 cm). All hamsters were kept in a 14:10 light/dark cycle with free access to food and water. Non-aggressive intruder (NAI) hamsters were group housed. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Use of Animals and were approved by the Georgia State University Institutional Animal Care and Use Committee. The following drugs were used in the microinjection experiments: 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (8-OH-DPAT; 78950-78-4; Sigma-Aldrich, St. Louis, MO, USA) and arginine-vasopressin (AVP; 065-07; Phoenix Pharmaceuticals, Burlingame, CA, USA). 1 mM and 100 µM doses of 8-OH-DPAT were used in females and males, respectively. AVP was used at a dose of 0.9 µM in both sexes. 8-OH-DPAT / AVP cocktail was used at 1 mM / 0.9 µM doses, respectively, in both sexes. Concentrations were informed by previous studies (Ferris et al., 1997; Villalba et al., 1997; Ferris et al., 1999). Drugs were dissolved in sterile physiological saline. Controls were microinjected with 200 nL of saline. Fluoxetine hydrochloride (CAS No 56296-78-7; Cayman Chemical, Ann Arbor, MI, USA) dissolved in diH2O was injected intraperitoneally (IP) to females in the following doses: 0 mg/kg (vehicle), 8 mg/kg, and 20 mg/kg. Males received vehicle or 20 mg/kg.

### *2.3.2 Injection Experiments*

Prior to surgery, hamsters were housed singly for at least 1 week. Hamsters were deeply anesthetized with 5% isoflurane in an induction chamber and maintained under gaseous

anesthesia between 3.00% – 4.00%. Hamsters were implanted unilaterally with a 4 mm, 26-gauge guide cannula aimed at the AH using a stereotaxic apparatus. Coordinates were +0.8 mm anterior to bregma, +/- 1.5 mm from the midline, and -3.5 mm from the top of skull at an 8° angle (for reference see (Morin and Wood, 2001)). Hamsters recovered at least 3 days prior to handling. The next week, hamsters were handled daily and estrous cycles were monitored in females. The next week, hamsters were tested for aggression. Five minutes prior to testing, hamsters were microinjected with 200 nL of drug or saline. Injections lasted 30 seconds and the needle remained in the guide cannula for an additional 30 seconds. Hamsters were tested in a neutral arena for 5 minutes with a smaller NAI of the same sex. Experimental females were tested during diestrus to ensure aggression during testing and NAI females during proestrus to ensure no aggression during testing. After testing, hamsters were euthanized with an overdose of sodium pentobarbital and injected with ink to verify cannula site placement. In the fluoxetine studies, hamsters were housed, handled, and tested as described above. Two hours prior to behavior testing, hamsters were injected IP with fluoxetine or vehicle.

### *2.3.3 Immunofluorescence*

Hamsters were isolated, handled, and tested in the same way as in the injection experiments. Hamsters were paired with a weight and sex-matched opponent in a resident-intruder paradigm for 15 minutes and behavior was analyzed to determine dominance as described in a previous report (Morrison et al., 2012). Controls were moved to an empty, dirty cage belonging to a same sex hamster. One hour after the start of testing, hamsters were euthanized as described above, transcardially perfused, and tissue processed as previously described (Gil et al., 2013). Brains were cut in 40 µm coronal sections on a cryostat and stored in a cryoprotectant solution (500 mL PBS, 300 g sucrose, 10 g polyvinyl pyrrolidone, 300 mL

ethylene glycol) until immunofluorescent processing. Raphe sections were processed using antibodies for 5-HT (20079; Immunostar, Hudson, WI, USA) and c-Fos (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a marker of neural activation. Hypothalamic sections were processed using antibodies for AVP (T-5048; Peninsula Laboratories, San Carlos, CA, USA) and c-Fos. All immunofluorescent procedures were conducted at room temperature. Sections were washed in PBS 4x5 minutes and blocked in 10% normal donkey serum (NDS) with 0.4% of Triton X-100 in PBS for 1 hour. Raphe sections were incubated overnight in an antibody solution (ABS: 0.4% of Triton X-100 and 2% NDS in PBS) for 5-HT (1/1000) and c-Fos (1/1000). Hypothalamic sections were incubated overnight in ABS for AVP (1/2500) and c-Fos (1/1000). Sections were washed in ABS 5x5 minutes and incubated in darkness for 2 hours in ABS containing secondary antibodies Alexa Fluor 488 and 594 (1/500; Jackson Immunoreserach, West Grove, PA, USA). All tissue was washed in PBS in darkness for 4x5 minutes. Tissue was mounted onto Colorfrost Plus Microscope Slides (12-550-17; Fisher Scientific, Waltham, MA, USA) in PBS, rinsed with diH2O, and coverslipped with Vectashield Hard Set Mounting Medium for Fluorescence (H1400; Vector Laboratories, Burlingame, CA, USA).

### *2.3.4 Confocal Microscopy and Quantification*

Digital images were acquired with a Zeiss LSM 720 confocal microscope at 8x-20x magnification, depending on region size. Using Zeiss ZEN 2012 software, 2 µm interval Z-stack images were obtained and representative images of entire regions from each subject were quantified. Overall adjustments to brightness were applied evenly to channels of all images to maximize clarity. The “Cell Counting” plugin in ImageJ was used for quantification. Cell activation was determined by quantifying cells containing staining for 5-HT or AVP and c-Fos.

Digital zooming was used to confirm colocalization. Raphe areas quantified include the anterior dorsal raphe nucleus (DRNa), dorsal and ventral sub regions of the rostral DRNa (drDRNa and vrDRNa, respectively), the posterior dorsal raphe nucleus (DRNp), and the median raphe nucleus (MRN). Hypothalamic areas quantified include the medial supraoptic nucleus (mSON), nucleus circularis (NC), and medial paraventricular nucleus (PVN). Images in figures 2.4 and 2.5 are maximum intensity projection images.

### *2.3.5 Data Analysis*

SPSS v22 was used to analyze all data. 2-way independent analysis of variance (ANOVA), single variable ANOVA, and independent t-tests were all used where appropriate. When appropriate, data were transformed using the square root before commencing with analysis. Graphs are of original data and not transformed data. All post-hoc comparisons were a priori. Tests were two tailed and differences were determined to be significant at $p < 0.05$.

## 2.4 Results

### *2.4.1 Activation of 5-HT1a receptors (5-HT1aRs) in the anterior hypothalamus (AH) stimulates aggression in females*

In view of the substantial evidence that activation of 5-HT1aRs inhibits aggression in males, we examined the effects of the 5-HT1aR agonist, 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (8-OH-DPAT), in females (Joppa et al., 1997). Microinjection of 8-OH-DPAT into the AH (Fig 2.6 A-C, see (Morin and Wood, 2001) for reference) produced a dose-dependent increase in aggression ($F(3,27) = 4.36$, $p < 0.05$; Fig 2.1 A) and decrease in latency to attack ($F(3,26) = 3.27$, $p < 0.01$; Fig 2.7 A). 500 µM and 1 mM 8-OH-DPAT increased the duration of aggression ($p < 0.05$ and $p < 0.01$, respectively; Fig 2.1 A) and decreased the latency to attack ($p < 0.05$; Fig 2.7 A) compared to controls. Because of the surprising finding

that 8-OH-DPAT stimulated aggression in females, we directly compared the effects of 8-OH-DPAT in females and males. There was a significant interaction between sex and drug treatment on the duration of aggression ($F(1,27) = 37.50$, $p < 0.01$; Fig 2.1 B). 8-OH-DPAT treatment increased the duration of aggression in females ($t(15) = 3.46$, $p = 0.01$) and decreased the duration of aggression in males ($t(13) = 6.82$, $p < 0.01$). There was a significant interaction between drug treatment and sex on latency to attack ($F(1,28) = 16.24$, $p < 0.01$; Fig 2.7 B). Although 8-OH-DPAT treatment did not affect latency to attack in females ($t(15) = 1.55$, $p > 0.05$), it increased latency to attack in males ($t(13) = 3.99$, $p < .01$).

### *2.4.2 8-OH-DPAT and AVP alter the expression of aggression in males and females in opposite ways*

Next, we directly compared the effects of 8-OH-DPAT, AVP, and 8-OH-DPAT combined with AVP (8-OH-DPAT/AVP) on aggression following injection into the AH of females and males. There was a significant interaction between sex and drug treatment on the duration of aggression ($F(3,48) = 34.14$, $p < 0.01$, Fig 2.2). 8-OH-DPAT treated females were more aggressive than control females ($p < 0.01$), whereas AVP treated females were less aggressive than control females ($p < 0.05$; Fig 2.2 A). 8-OH-DPAT/AVP treated females were more aggressive than AVP treated females ($p < 0.05$) and less aggressive than 8-OH-DPAT treated females ($p < 0.05$), but did not differ from control females ($p > 0.05$; Fig 2.2 A). 8-OH-DPAT treated males were less aggressive than control males ($p < 0.01$), whereas AVP treated males were more aggressive than control males ($p < 0.05$; Fig 2.2 A). 8-OH-DPAT/AVP treated males were less aggressive than AVP treated males ($p < 0.01$) and control males ($p < 0.01$), whereas 8-OH-DPAT/AVP treated males were more aggressive than 8-OH-DPAT treated males ($p < 0.05$; Fig 2.2 A). Direct comparisons between females and males revealed that 8-OH-DPAT

treated females were more aggressive than 8-OH-DPAT treated males ($t(13) = 10.14$, $p < 0.01$; Fig 2.2 B). 8-OH-DPAT/AVP treated females were more aggressive than 8-OH-DPAT/AVP treated males ($t(8) = 3.58$, $p < 0.01$; Fig 2.2 B). AVP treated females were less aggressive than AVP treated males ($t(12) = 7.03$, $p < 0.01$; Fig 2.2 B). There was a significant interaction between sex and drug treatment for attack latency ($F(3,49) = 12.58$, $p < 0.01$; Fig 2.8). AVP treated females had a longer latency to attack than 8-OH-DPAT treated females ($p < 0.05$) and a strong trend compared to 8-OH-DPAT/AVP treated females ($p = 0.06$; Fig 2.8 A). 8-OH-DPAT treated males had a longer latency to attack than all other male groups ($p < 0.01$; Fig 2.8 A). Direct comparisons between females and males revealed that AVP treated females had a longer latency to attack than AVP treated males ($t(12) = 3.84$, $p < 0.01$; Fig 2.8 B). 8-OH-DPAT treated females had a shorter latency to attack than 8-OH-DPAT treated males ($t(13) = 5.84$, $p < 0.01$; Fig 2.8 B).

### *2.4.3 Systemically administered fluoxetine alters aggression in opposite ways in males and females*

We tested the hypothesis that systemically administered fluoxetine increases aggression in females. There was a dose-dependent increase in duration of aggression ($F(2,34) = 3.70$, $p < 0.05$; Fig 2.3 A). Treatment with 20 mg/kg fluoxetine increased aggression when compared with vehicle ($p < 0.05$). There was also a dose-dependent decrease in the latency to attack ($F(2,34) = 3.65$, $p =$ Fig 2.9 A). Treatment with 20 mg/kg fluoxetine significantly decreased attack latency when compared with vehicle ($p < 0.05$). We directly compared the effect of fluoxetine on aggression in females and males. There was an interaction between fluoxetine treatment and sex on the duration of aggression ($F(1,37) = 47.62$, $p < 0.01$; 2.3 B). 20 mg/kg fluoxetine increased the duration of aggression in females ($t(29) = 2.57$, $p < 0.05$) and decreased the duration of

aggression in males ($t(8) = 5.66$, $p < 0.01$). There was an interaction between fluoxetine treatment and sex on latency to attack ($F(1,37) = 35.77$, $p < 0.01$; Fig 2.9 B). 20 mg/kg fluoxetine decreased latency to attack in females ($t(29) = 2.63$, $p < 0.05$) and increased latency to attack in males ($t(8) = 4.160$, $p < 0.01$).

### *2.4.4 Dominance and the activation of 5-HT neurons*

We hypothesized that activity of 5-HT cells in the raphe, as measured by colocalization of 5-HT-immunoreactivity (ir) and fos-ir, is upregulated by acquisition of dominance in female hamsters. In the DRNa (Fig 2.4 A-B) there was a significant effect of dominance status ($F(2,54) = 3.081$, $p = 0.05$) but not sex ($F(1,54) = 0.686$, $p > 0.05$) nor an interaction ($F(2,52) = 1.311$, $p > 0.05$) in 5-HT-ir/fos-ir . In the DRNp (Fig 2.4 C-D), there was a significant effect of dominance status ($F(2,50) = 3.084$, $p = 0.05$) and a trend toward an effect of sex ($F(1,50) = 3.165$, $p = 0.08$) but no interaction ($F(2,50) = 0.383$, $p > 0.05$) in 5-HT-ir/fos-ir. Within-sex a priori comparisons revealed that dominant females had more 5-HT-ir/fos-ir in the DRNa and DRNp than subordinate females ($p < 0.05$; Fig 2.4 B, 2.4 D). Dominant females had more 5-HT-ir/fos-ir in the DRNa and a trend toward more 5-HT-ir/fos-ir in the DRNp than control females ($p < 0.05$ and $p = 0.08$, respectively; Fig 2.4 B, 2.4 D). There was no effect of dominance status on 5-HT-ir/fos-ir in the DRNa and DRNp of males ($p > 0.05$; Fig 2.4 B, 2.4 D). In the MRN (Fig 2.4 E-F), there was no effect of dominance status ($F(2,48) = 1.736$, $p > 0.05$) but a strong trend toward an effect of sex ($F(1,48) = 3.758$, $p = 0.06$) and no interaction ($F(2,48) = 1.648$, $p > 0.05$) in 5-HT-ir/fos-ir. Within-sex a priori comparisons revealed no differences in 5-HT-ir/fos-ir for either females or males ($p > .05$; Fig 2.4 F).

We also examined whether dominance status altered 5-HT-ir/fos-ir in the ventral (vrDRNa) and dorsal (drDRNa) subdivisions of the most rostral portion of the DRNa in female

and male hamsters because a previous study in male hamsters found subordinates have significantly more 5-HT-ir/fos-ir in the vrDRNa than dominant and control hamsters. In the vrDRNa (Fig 2.4 G-H) there was an effect of dominance status on 5-HT-ir/fos-ir in males ($F(2,23) = 3.902$, $p < 0.05$), with subordinate males having more 5-HT-ir/fos-ir compared to dominants and controls ($p < 0.05$; Fig 2.4 H). In females, there no effect of dominance status on 5-HT-ir/fos-ir in the vrDRNa ($F(2,26) = 0.723$, $p > 0.05$) nor were there differences between dominant, subordinate, and control females ($p > 0.05$; Fig 2.4 H). In the drDRNa (Fig 2.4 I-J), there was an effect of dominance status on 5-HT-ir/fos-ir in males ($F(2,23) = 6.355$, $p < 0.01$), with dominant males having more 5-HT-ir/fos-ir than controls or subordinates ($p < 0.05$ and $p < 0.01$, respectively; Fig 2.4 J). In females, there was an effect of dominance status on the number of 5-HT-ir/fos-ir cells in the drDRNa ($F(2,26) = 6.431$, $p < 0.01$). Dominant females had more 5-HT-ir/fos-ir than controls and subordinates ($p < 0.01$ and $p < 0.05$, respectively; Fig 2.4 J).

### *2.4.5 Dominance and the activation of AVP neurons*

In males, we hypothesized that the activity of AVP containing cells, as measured by the colocalization of AVP-ir/fos-ir, is upregulated in hypothalamic nuclei by the acquisition of dominance. In the mSON (Fig 2.5 A-B), there was an effect of dominance status on AVP-ir/fos-ir ($F(1,55) = 31.51$, $p < .01$) but no effect of sex ($F(1,55) = 0.32$, $p > 0.05$) and no interaction ($F(2, 55) = 1.37$, $p > 0.05$). Within-sex a priori comparisons revealed subordinate males had more AVP-ir/fos-ir than control males ($p < 0.01$) and dominant males had more AVP-ir/fos-ir than subordinate males ($p < 0.05$) and control males ($p < 0.01$; Fig 2.5 B). In females, both subordinate and dominant animals had more AVP-ir/fos-ir than control females ($p < 0.01$), but there was no difference between dominants and subordinates ($p > .05$; Fig 2.5 B). In the NC (Fig 2.5 C-D), there was no effect of sex ($F(1,46) = 1.69$, $p > 0.05$), but a main effect of dominance

status ($F(1,46) = 15.75$, $p < 0.01$), and a trend towards an interaction ($F(2,46) = 2.81$, $p = 0.07$). Within-sex a priori comparisons revealed that dominant females had more AVP-ir/fos-ir than control females ($p < 0.01$) but not subordinate females ($p > 0.05$; Fig 2.5 D). Dominant males had more AVP-ir/fos-ir than control males ($p < 0.01$) and subordinate males ($p < 0.01$; Fig 2.5 D). In the medial PVN (Fig 2.5 E-F), there was an effect of dominance status ($F(2, 51) = 13.46$, $p < 0.01$), sex ($F(1,51) = 14.91$, $p < 0.01$), and a trend towards an interaction ($F(2,51) = 2.70$, $p = 0.07$). Within-sex a priori comparisons in females revealed no effect of dominance status on AVP-ir/fos-ir ($p > 0.05$; Fig 2.5 F). In males, both subordinate and dominant hamsters ($p < 0.01$; Fig 2.5 F) had significantly more AVP-ir/fos-ir than controls.

## 2.5 Discussion

These data support the hypothesis that there are fundamental sex differences in the neural regulation of aggression and dominance. Direct comparisons of 5-HT and AVP indicate that these neurochemical signals act in opposite ways within the same brain site to regulate aggression and dominance in females and males. Hypothalamic injection of a 5-HT-1a agonist stimulated aggression in females and inhibited aggression in males, whereas injection of AVP inhibited aggression in females and stimulated aggression in males. These data also provide the first evidence that there are striking differences in the neural mechanisms regulating the acquisition of dominance in females and males. Acquisition of dominance was associated with activation of 5-HT cell bodies within the DRN of females and activation of hypothalamic AVP neurons in males. Finally, systemic administration of fluoxetine increased aggression in females and substantially decreased aggression in males, suggesting that sex differences may be a critical factor in the clinical efficacy of 5-HT-active drugs.

The present data reinforce previous work indicating that the AH is a critical element within the neural circuitry controlling agonistic behavior (Delville et al., 2000; Albers, 2012; Ferris and Albers., 2013; Albers, 2015). Not only do 5-HT and AVP fibers project to this region (Morin and Meyer-Bernstein, 1999; Delville et al., 2000), the present data demonstrate that activation of 5-HT and AVP cell bodies are associated with acquisition of dominance in a sex-dependent manner. Dominant females displayed significantly more activation of 5-HT cell bodies within the DRN than subordinate or control females. In contrast, there were no significant differences in the number of 5-HT activated cell bodies in the DRN among dominant, subordinate or control males. Despite these dramatic sex differences, more subtle effects of dominance status were observed in subregions of the DRNa in both females and males. Interestingly, subordinate males, but not females, displayed significantly more activation of 5-HT cell bodies in the vrDRNa than dominant or control males, as has been reported previously, and dominance status altered the activation of 5-HT cell bodies in the drDRNa in both females and males (Alekseyenko and Kravitz, 2014). Therefore, although activation of 5-HT cell bodies throughout the DRN is associated with the acquisition of dominance in females and not males, more subtle changes within DRNa subregions can be related to dominance status in males as well as females.

In males, acquisition of dominance was strongly associated with activation of AVP cell bodies in the mSON and NC. Dominant males displayed significantly more activation of AVP cell bodies within both the mSON and NC than did subordinate or control males. In contrast, in females, a significantly larger number of AVP cell bodies were activated in the mSON and NC in both dominant and subordinate females than in controls. Thus, there is a substantial sex difference in the relationship between dominance and activation of AVP cell bodies in the mSON and NC. In males, activation of AVP cell bodies in these sites was associated with

dominance, while in females activation of these neurons was independent of dominance status but was associated with social interaction. Interestingly, in the PVN, activation of AVP cell bodies in males was associated with social interaction while no such relationship was observed in females.

Studies of the neural mechanisms underlying competitive aggression have been conducted almost exclusively in males probably because of the longstanding emphasis on male-male competition in intrasexual selection (Darwin, 1871). The present findings are consistent with the large body of previous work on the roles of 5-HT and AVP in regulating competitive aggression in males. In contrast, studies of the neural mechanisms underlying aggression in females have focused almost exclusively on maternal aggression (Lonstein and Gammie, 2002). While the importance of female competitive behaviors in achieving reproductive benefits has been long been recognized, particularly in primates, little attention has been paid to their underlying neural mechanisms (Rosvall, 2011; Stockley and Bro-Jorgensen, 2011). The present data are the first to demonstrate that, although the behavioral expression of aggression and dominance in females and males is quite similar, 5-HT and AVP act in opposite ways within the same brain site to regulate these behaviors.

The strong but opposite relationship between activation of the 5-HT and AVP systems and the promotion of aggression/dominance in females and males may have significant clinical importance. For example, because dominance imparts a resistance to the adverse consequences of social stress, the findings of major sex differences in the neurochemical regulation of aggression/dominance may be important for our understanding of sex differences in the incidence and treatment of stress-related psychiatric disorders (Ferrari et al., 2005; Morrison et al., 2012; Cooper et al., 2015). Indeed, it is possible that 5-HT-active drugs are more efficacious

in treating some psychiatric disorders in females and AVP-active drugs are more efficacious in males. One of the most commonly prescribed 5-HT-active drugs for treatment of psychiatric disorders is fluoxetine (Prozac), and the possibility that there are sex differences in its efficacy is reinforced by our findings that the systemic administration of fluoxetine increases aggression in females and reduces it in males. In support of this possibility, systemic administration of fluoxetine to rats reduced negative effects of stress on learning in females but not males (Leuner et al., 2004). Further support comes from the finding that the severity of PTSD symptoms was negatively correlated with urinary levels of AVP in men but not women (Marshall, 2013). Furthermore, intranasal AVP improved PTSD symptoms in men but not women (Marshall, 2013). Determination of possible sex differences in the efficacy of 5-HT- and AVP-active drugs to treat of stress-related psychiatric disorders would have an almost immediate clinical impact by guiding drug treatment and development, emphasizing the role of 5-HT-targeted drugs in females and AVP-targeted drugs in males.

## 2.6 Acknowledgements

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## 2.7 Chapter 2 Figures

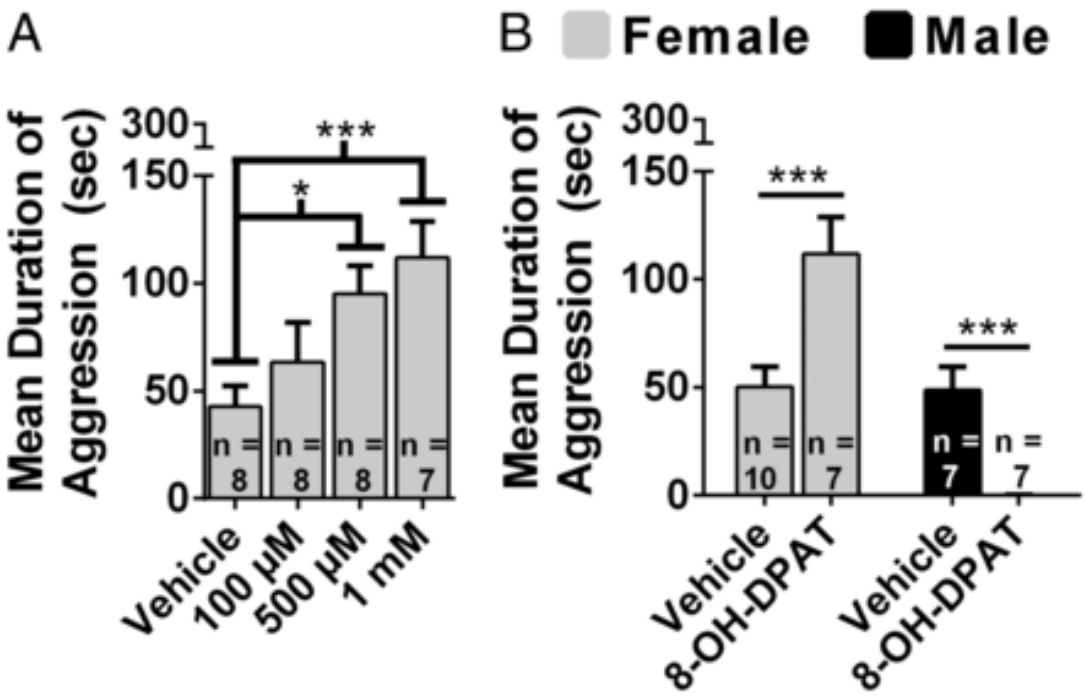


***Figure 2.1 Sex differences in the effects of 8-OH-DPAT on aggression***

(A) Dose-dependent effects of 8-OH-DPAT injected into the AH of female hamsters on the duration of aggression. (B) Direct comparisons between females and males of the effect of 8-OH-DPAT (1 mM and 100 µM, respectively) on duration of aggression. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

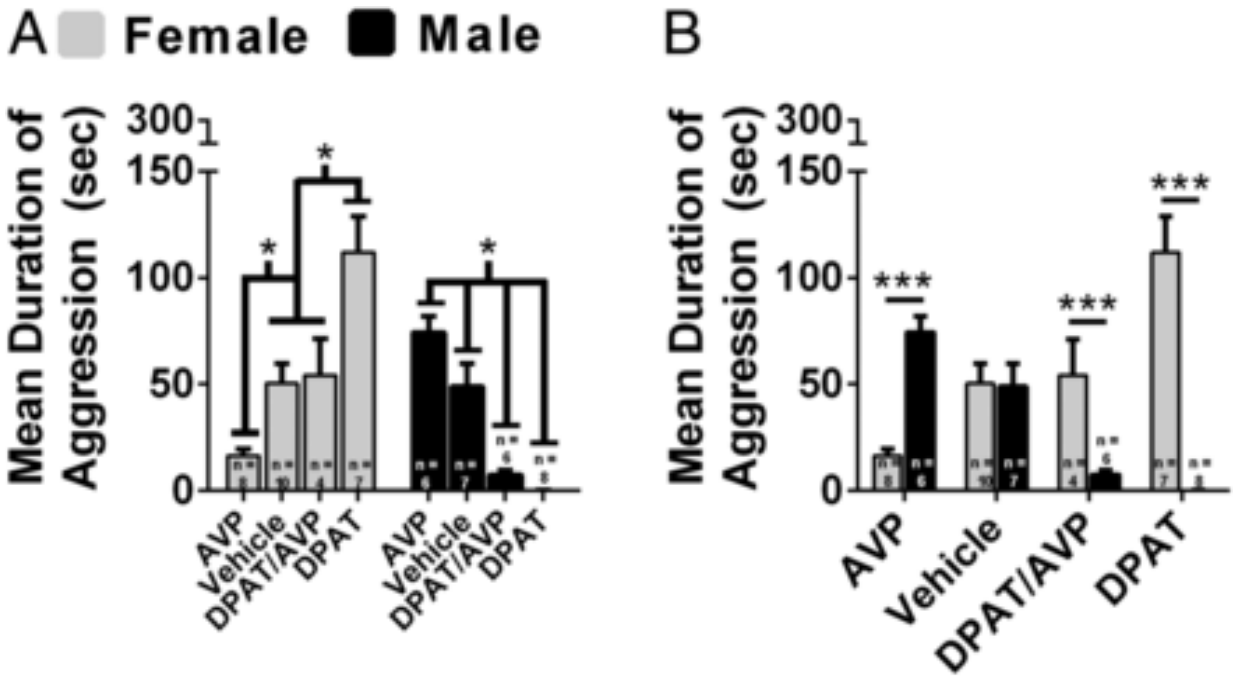


***Figure 2.2 Effects of 8-OH-DPAT (DPAT), AVP, and their combined injection into the AH on aggression in females and males***

Drug concentrations used were determined by dose–response studies (Fig 2.1, Fig 2.7, and (Ferris et al., 1999)). Duration of aggression was compared between females and males that received AVP (0.9 µM), vehicle, 8-OH-DPAT/AVP mixture (1 mM and 0.9 µM, respectively), or 8-OH-DPAT (1 mM for females and 100 µM for males). Female N: AVP = 8; vehicle = 10; DPAT/AVP = 4; DPAT = 7. Male N: AVP = 6; vehicle = 7; DPAT/AVP = 6; DPAT = 8. (A) Within-sex comparisons of drug treatments on duration of aggression. (B) Direct comparisons of drug treatments on duration of aggression between females and males. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

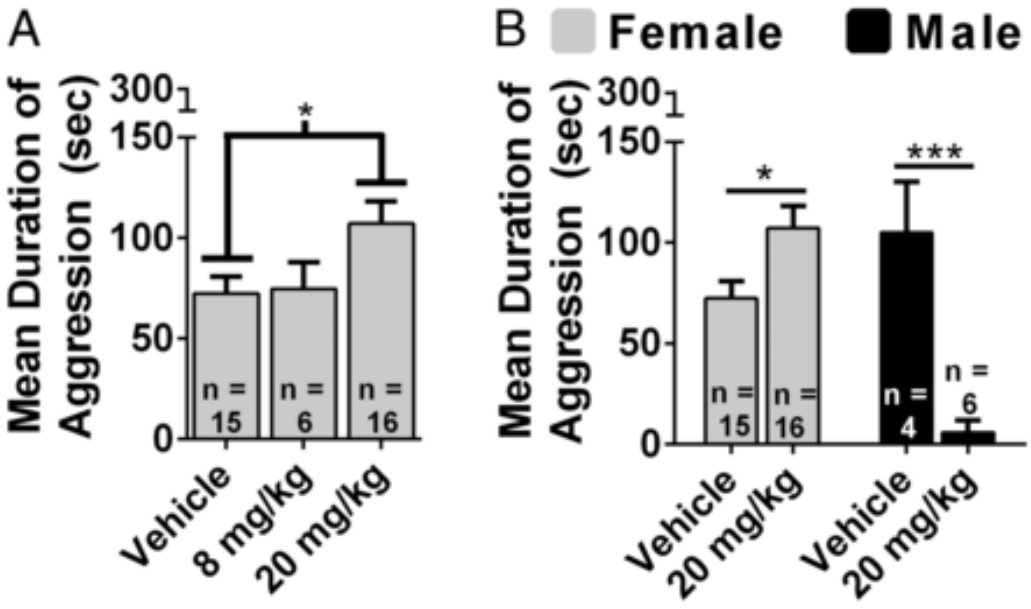


***Figure 2.3 Sex differences in the effects of fluoxetine on aggression***

(A) Dose–response study of the effects of systemically injected fluoxetine on duration of aggression in females. (B) Direct comparisons between females and males of the effects of fluoxetine on duration of aggression. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

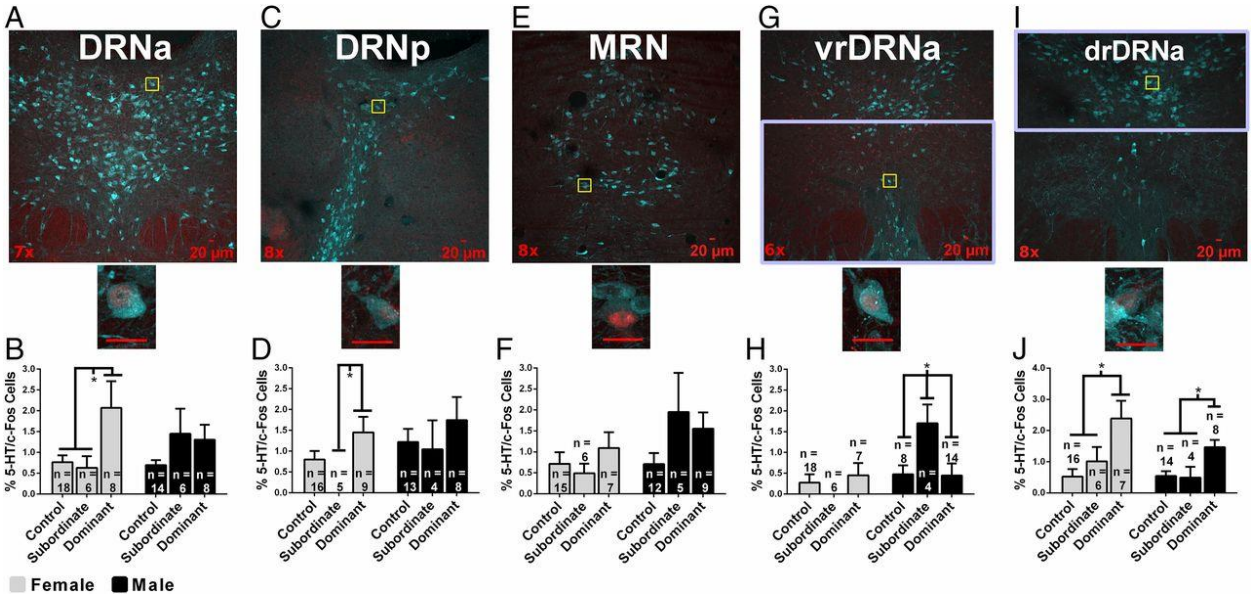


***Figure 2.4 Immunofluorescent colocalization of 5-HT-ir and fos-ir in cells within regions of the raphe***

Immunofluorescent colocalization of 5-HT-ir (teal) and fos-ir (red) in cells within regions of the raphe. DRNa (A and B), DRNp (C and D), MRN (E and F), vrDRNa (G and H), and drDRNa (I and J). Magnification is indicated in the lower left corner. Yellow boxes indicate subregions magnified to 40×. (Scale bars, 20 µM.) Purple boxes in G and I represent region boundaries for the vrDRNa and drDRNa, where 5-HT-ir/fos-ir cells were quantified. Graphs indicate the percentage of 5-HT-ir cells that colocalize fos-ir (percentage of activated 5-HT cells) as a function of dominance status and sex in DRNa, DRNp, MRN, vrDRNa, or drDRNa. Error bars indicate SEM. * $p < 0.05$.

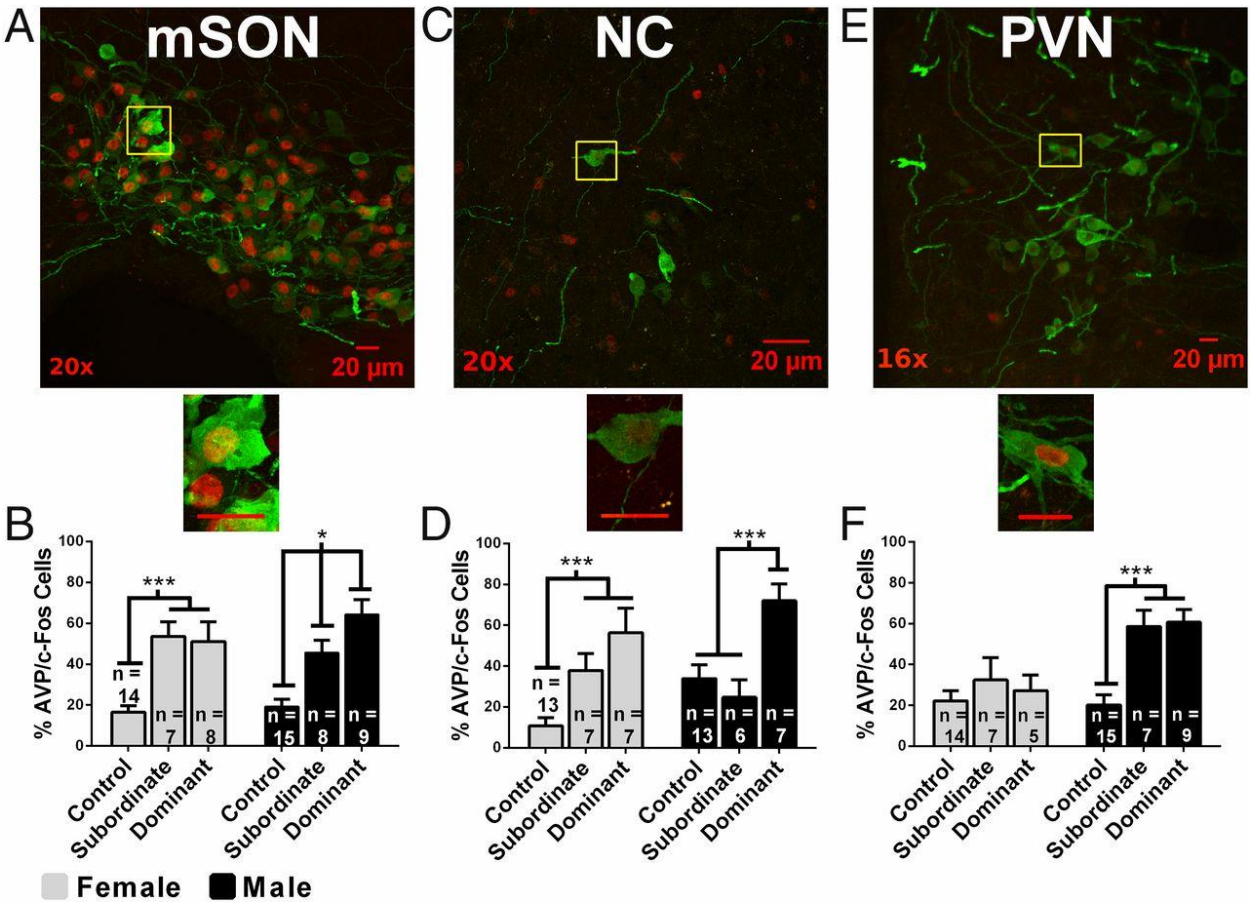


***Figure 2.5 Immunofluorescent colocalization of AVP-ir and fos-ir in cells within hypothalamic regions***

Immunofluorescent colocalization of AVP-ir (green) and fos-ir (red) in cells within hypothalamic regions. The mSON (A and B), NC (C and D), and medial PVN (E and F). Magnification is indicated in the lower left corner. Yellow boxes indicate subregions magnified to 40×. (Scale bars, 20 µM.) Graphs indicate the percentage of AVP-ir cells that colocalize fos-ir (percentage of activated AVP cells) as a function of dominance status and sex in mSON, NC, or PVN. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

## 2.8 Chapter 2 Supplemental Figures

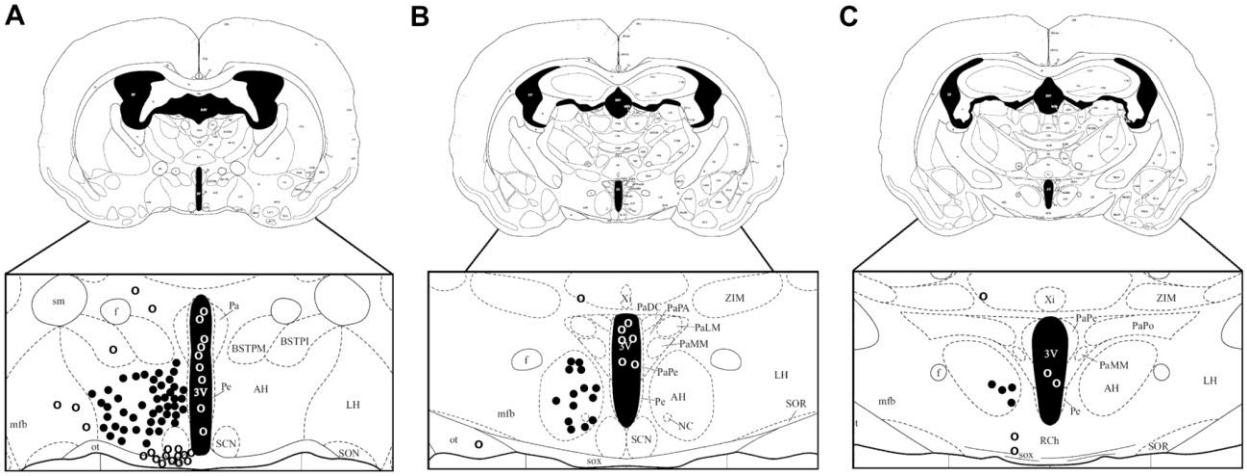


***Figure 2.6 Microinjection sites***

Sites of drug injections into the AH at (A) –0.3 mm, (B) –0.6 mm, and (C) –1.2 mm from bregma as determined by postmortem histological analysis. Closed circles are considered "hits" and open circles "misses" (figure modified from (Morin and Wood, 2001)).

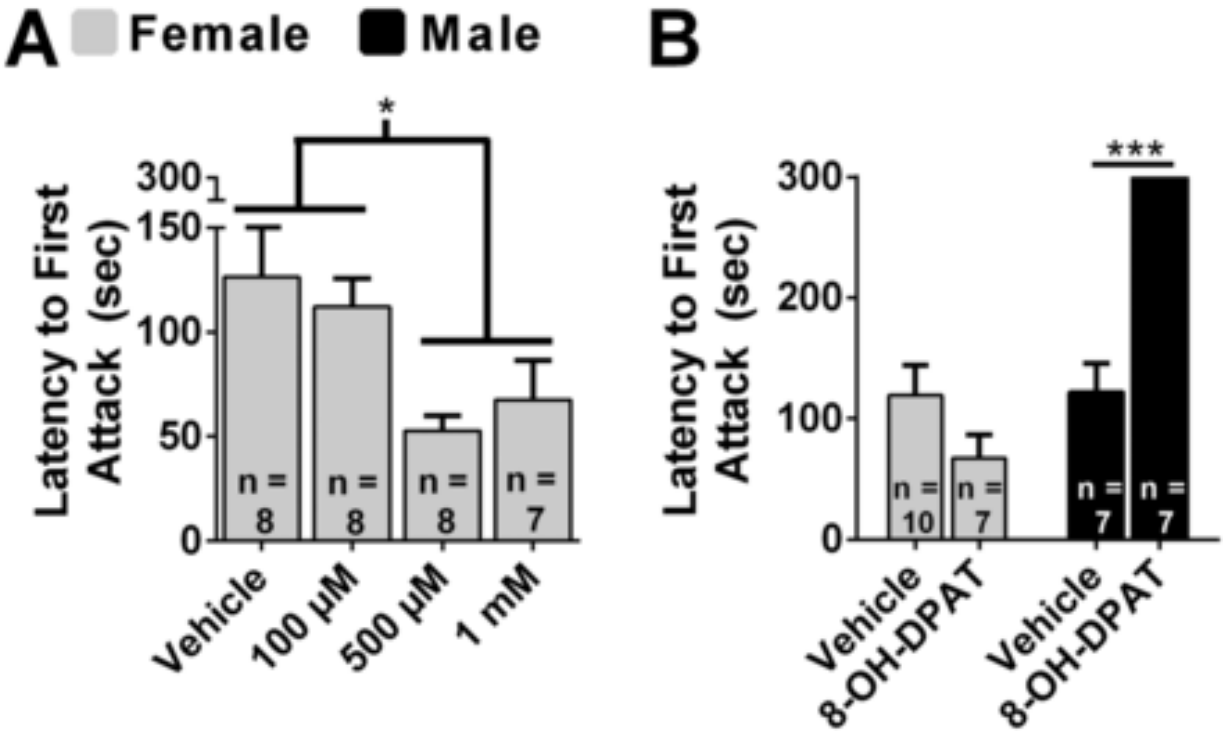


***Figure 2.7 Sex differences in the effects of 8-OH-DPAT on attack latency***

(A) Dose-dependent effects of 8-OH-DPAT injected into the AH of female hamsters on attack latency. (B) Direct comparisons between females and males of the effect of 8-OH-DPAT (1 mM for females and 100 µM for males) on attack latency. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

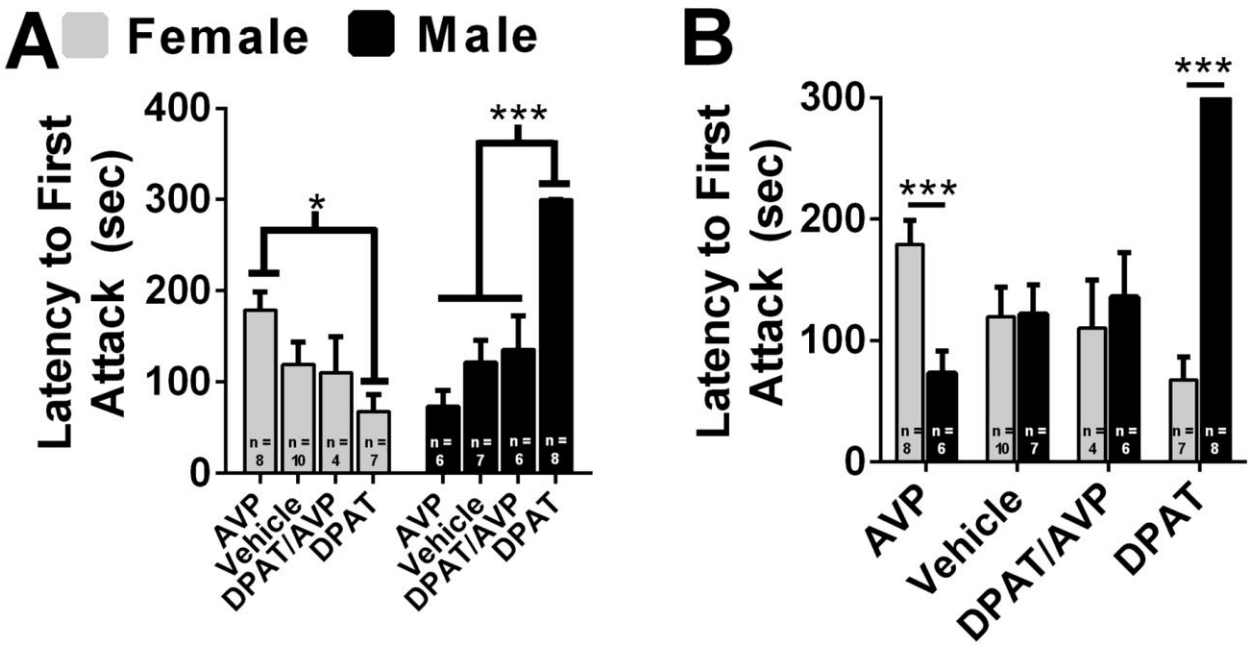


***Figure 2.8 Effects of 8-OH-DPAT (DPAT), AVP, and their combined injection into the AH on attack latency in females and males***

Drug concentrations used were determined by dose–response studies (Fig 2.1, Fig 2.7, and (Ferris et al., 1999)). Attack latency was compared between females and males that received AVP (0.9 µM), vehicle, a mixture containing 8-OH-DPAT/AVP (1 mM and 0.9 µM, respectively), or 8-OH-DPAT (1 mM for females and 100 µM for males). (A) Within-sex comparisons of different drug treatments on attack latency. (B) Direct comparisons of drug treatment on attack latency between females and males. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

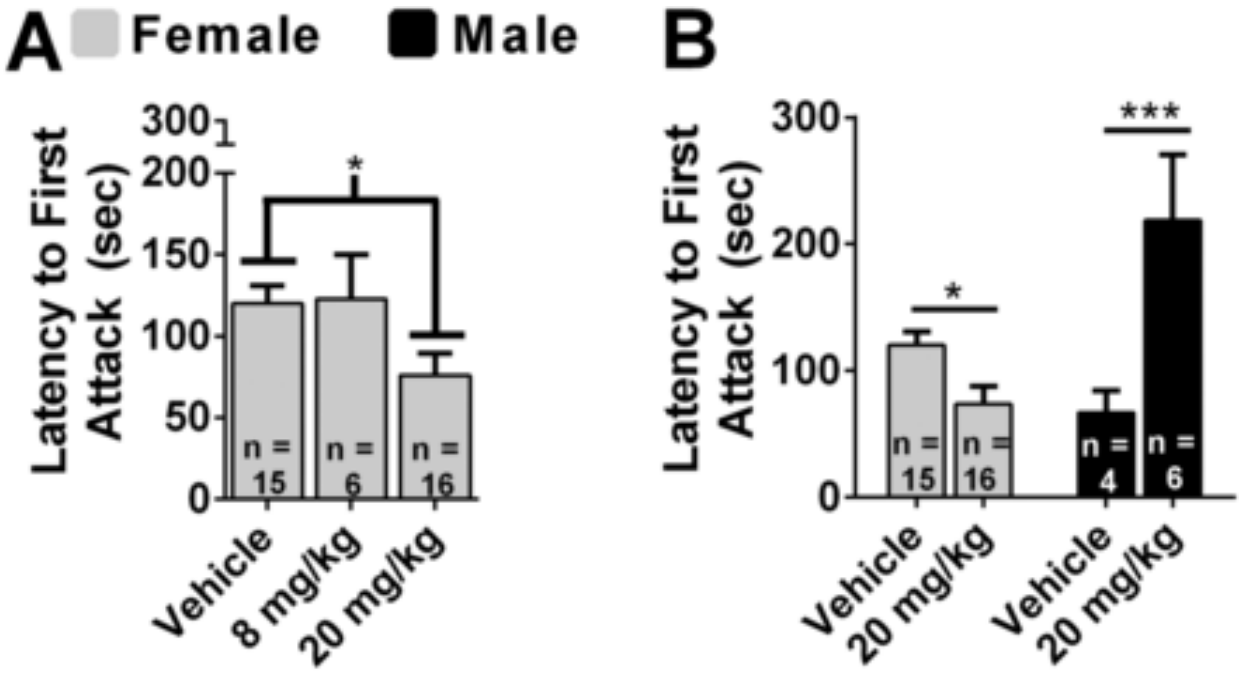


***Figure 2.9 Sex differences in the effects of fluoxetine on attack latency***

(A) Dose–response study of the effects of systemically injected fluoxetine on attack latency in female hamsters. (B) Direct comparisons between females and males of the effects of fluoxetine on attack latency. Error bars indicate SEM. * $p < 0.05$; *** $p < 0.01$.

# 3 THE RELATIONSHIP BETWEEN SEX, DOMINANCE STATUS, AND ACTIVATION OF SEROTONIN AND ARGININE-VASOPRESSIN CELLS AFTER EXPOSURE TO SOCIAL STRESS

Joseph I. Terranova, Eric Yan, Alan Emerson, Corey Andrews, Kim L. Huhman, H. Elliott Albers

## 3.1 Abstract

Several lines of evidence suggest that aggressiveness/dominance are traits that are associated with resistance to social stress. However, the specific neural mechanisms that may underlie these relationships are not well understood. In Syrian hamsters, serotonin (5-HT) and arginine vasopressin (AVP) are critical regulators of aggression and acquisition of dominance. Moreover, there are striking sex differences in how 5-HT and AVP regulate aggression and the acquisition of dominance. Central injection of a 5-HT1a receptor (5-HT1aR) agonist into the anterior hypothalamus (AH) increases aggression in female hamsters but decreases aggression in male hamsters. Central injection of AVP into the AH decreases aggression in female hamsters but increases aggression in male hamsters. Systemic injection of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, increases aggression in female hamsters but decreases aggression in male hamsters. 5-HT cell activation in the raphe is associated with acquisition of dominance in female hamsters and AVP cell activation in the hypothalamus is associated with acquisition of dominance in male hamsters. Although hamsters rapidly acquire dominance status, recent evidence suggests that in male hamsters the acquisition of a resistance to social defeat stress requires dominance maintained for at least 14 days (Morrison et al., 2012; Morrison et al., 2014a). Because our previous findings identified major sex differences in the neural mechanisms associated with the acquisition of dominance, in the present study, we explored whether there are

also sex differences in how these neural mechanisms respond to social defeat stress in males and females that have been dominant for 14 days. More specifically, we hypothesize that the development of resistance to social stress is associated with activation of 5-HT neurons in dominant females compared to subordinate females or males, and that social stress is associated with activation of AVP neurons in dominant males compared to subordinate males or females. To test this hypothesis, we allowed same-sex, weight-matched pairs of female and male hamsters to establish stable dominance relationships for 14 days, and then subsequently defeated both pair members on the day after the last training session. One hour after social defeat, tissue was processed for 5-HT-ir/fos-ir in the raphe and AVP-ir/fos-ir in the hypothalamus. The findings that activation of AVP-containing neurons was lower in established dominant males compared to defeated control males, does not support our hypothesis. Activation of 5-HT-containing neurons, on the other hand, was higher in established dominant females compared to established subordinate, defeated control, and non-defeated control females, which supports our hypothesis. Together, these data suggest there are sex differences in how 5-HT- and AVP-containing neurons respond to social stress.

## 3.2 Introduction

Aggression and dominance are positively associated with resistance to social stressors in many rodent species. Dominant rats exhibit lower basal corticosterone levels and have longer lifespans than subordinate colony members (Adams and Boice, 1983; Blanchard et al., 1993). Mice bred for a short attack latency (SAL) are more aggressive and dominant than mice bred for a long attack latency (LAL) (Benus et al., 1991a; Veenema et al., 2003). When both SAL and LAL mice are subjected to repeated social defeat stress, LAL mice display more anxiety than SAL mice (Veenema et al., 2003). In male Syrian hamsters with established, 14-day dominance

relationships, their social status determines their resistance to social stressors (Morrison et al., 2012; Morrison et al., 2014a). Established dominant male hamsters that are socially defeated by a larger resident aggressor (RA) are less submissive following their defeat experience than socially defeated subordinates (Morrison et al., 2012; Morrison et al., 2014a). Although aggression and dominance are associated with increased resistance to social stress, the specific neural mechanisms that underlie the relationship between aggression, dominance, and resistance to social stress are not well understood.

5-HT and AVP are two neurochemical signals that are important for the neural control of offensive aggression and acquisition dominance in Syrian hamsters. Moreover, there are striking sex differences in how 5-HT and AVP regulate aggression and dominance. Central administration of a 5-HT1aR agonist into the AH of female hamsters increases aggression, whereas 5-HT1aR stimulation in the AH of male hamsters profoundly decreases aggression (Ferris et al., 1999; Morrison and Melloni, 2014; Terranova et al., 2016). Systemic administration of the SSRI, fluoxetine, increases aggression in females and decreases aggression in males (Ferris et al., 1997; Ferris et al., 1999; Morrison and Melloni, 2014; Terranova et al., 2016). Central administration of AVP into the AH of female hamsters decreases aggression, whereas it increases aggression in male hamsters (Ferris et al., 1997; Caldwell and Albers, 2004; Gutzler et al., 2010; Morrison and Melloni, 2014; Terranova et al., 2016). With regards to acquisition of dominance, 5-HT-containing neurons are associated with acquisition of dominance in female hamsters, whereas AVP-containing neurons are associated with acquisition of dominance in male hamsters (Terranova et al., 2016). Because of their extensive role regulating aggression and acquisition of dominance, 5-HT and AVP may also be associated with resistance to social stress in hamsters in established dominance relationships.

The goal of this study is to explore whether sex differences exist in 5-HT neuronal activation, and AVP neuronal activation after social defeat stress in hamsters in well-established dominance relationships. We hypothesize that the development of resistance to social stress is associated with activation of 5-HT neurons in dominant females compared to subordinate females or males and that social stress is associated with activation of AVP neurons in dominant males compared to subordinate males or females. To test this hypothesis we paired hamsters in same-sex, weight-matched pairs, once a day for 14 days (Morrison et al., 2014a). We then socially defeated established dominant, established subordinate, and control hamsters with a larger, trained RA and measured activation of 5-HT- and AVP-containing neurons. Our data partially supports our hypothesis; activation of 5-HT-containing neurons was higher in established dominant females compared to other female groups. In contrast to our predictions, activation of AVP-containing neurons was reduced in established dominant males compared to defeated control males. These data provide evidence suggesting that there are sex differences in how 5-HT and AVP contribute to the neural control of resistance to social stress.

## 3.3 Methods

### *3.3.1 Animals*

Adult male and female Syrian hamsters (Charles River Laboratories Inc. and in-house bred), 8–12 wk old, weighing between 110 and 150 g, were used for all experiments. Experimental hamsters were individually housed in polycarbonate cages (24 × 43 × 20 cm). A colony of larger resident aggressor (RA) hamsters, weighing between 160 g and 175 g was used to socially defeat experimental hamsters. All hamsters were kept in a 14:10 light/dark cycle with free access to food and water. All experiments were conducted in accordance with the National

Institutes of Health Guidelines for the Use of Animals and were approved by the Georgia State University Institutional Animal Care and Use Committee.

### *3.3.2 Behavioral experiment*

Hamsters were housed singly and handled as described in Chapter 2 (Terranova et al., 2016). A 14-day training period was used in which, after acquiring dominant or subordinate status, hamsters were established as dominants or subordinates, as previously described by (Morrison et al., 2012; Morrison et al., 2014a). On the first day of training, same sex dyads of weight-matched male and female hamsters were paired together for 10 minutes in a resident-intruder test, so that each member of the pair could acquire dominant or subordinate status. Hamsters that attacked consistently throughout an agonistic encounter were considered dominant, and hamsters that exhibited submissive behaviors throughout an agonistic encounter were considered subordinate. Hamsters were randomly assigned to be the resident or intruder and this designation lasted throughout the course of the training period. Resident or intruder status did not predict dominance status. In subsequent pairings, days 2-14, subjects were paired for 5 minutes to maintain their dominance relationship and establish their role as a dominant or subordinate. Dominant and subordinate behaviors were identified by the agonistic behaviors exhibited by each experimental hamster. Dyads were filmed during each training day and the dominant hamster was assessed for each training day. Dyads in which in the dominance relationship was reversed were excluded from analysis. 4 female pairs and 2 male pairings were excluded from analysis because of dominance status reversal. Females were matched for day of estrous cycle and began the training period on the first day of diestrus. Control hamsters were handled in their home cages during the training period. Control females began their training period on the first day of diestrus.

After the 14-day training period, established dominant hamsters, established subordinate hamsters, and handled control hamsters of both sexes were socially defeated by RAs for 15 minutes as previously described (McCann and Huhman, 2012). Briefly, hamsters were placed in the home cage of a larger RA of the same sex for 15 minutes. The RA reliably attacked and defeated the experimental and defeated control hamsters. All social defeats were filmed. Overall duration of submissive behaviors was scored for all defeated hamsters according to criteria similar to that of previous experiments, which included fleeing, upright, tail up, full submissive posture, stretch attend, and attempted escape from cage (McDonald et al., 2012; Gray et al., 2015). The social defeats were monitored to make sure no injuries occurred to either animal. Non-defeated control hamsters were placed in an empty cage belonging to an RA of the same sex for 15 minutes.

### *3.3.3 Immunofluorescence*

One hour after the start of social defeat, all hamsters were euthanized as described earlier, transcardially perfused, and tissue processed as previously described (Gil et al., 2013; Terranova et al., 2016). Brains were cut in 40-μm coronal sections on a cryostat and stored in a cryoprotectant solution (500 mL PBS, 300 g sucrose, 10 g polyvinyl pyrrolidone, 300 mL ethylene glycol) until immunofluorescent processing. Raphe sections were processed using antibodies for 5-HT (20079; Immunostar) and c-Fos (sc-166940; Santa Cruz Biotechnology), a marker of neural activation. Hypothalamic sections were processed using antibodies for AVP (T-5048; Peninsula Laboratories) and c-Fos. All immunofluorescent procedures were conducted at room temperature. Sections were washed in PBS five times for 5 minutes and blocked in 10% normal donkey serum (NDS) with 0.4% of Triton X-100 in PBS for 1 h. Raphe sections were incubated overnight in an antibody solution (ABS; 0.4% of Triton X-100 and 2% NDS in PBS)

for 5-HT (1/1,000) and c-Fos (1/100). Hypothalamic sections were incubated overnight in ABS for AVP (1/2,500) and c-Fos (1/100). Sections were washed in ABS five times for 5 min and incubated in darkness for 2 h in ABS containing secondary antibodies Alexa Fluor 488 (1/500) and 555 (1/500; Fisher Scientific). All tissue was washed in PBS in darkness four times for 5 min. Tissue was mounted onto Colorfrost Plus Microscope Slides (12-550-17; Fisher Scientific) in PBS, rinsed with diH2O, and coverslipped with Vectashield Hard Set Mounting Medium for Fluorescence with DAPI (H1500; Vector Laboratories).

### *3.3.4 Confocal Microscopy and Quantification*

Digital images were acquired with a Zeiss LSM 700 confocal microscope at 5–20× magnification, depending on region size. Using Zeiss ZEN 2012 software, 2-μm interval *Z* stack images were obtained, and representative images of entire regions from each subject were quantified. Overall adjustments to brightness were applied evenly to channels of all images to maximize clarity. The "Cell Counting" plugin in ImageJ was used for quantification. Cell activation was determined by quantifying cells containing staining for 5-HT or AVP and c-Fos. Digital zooming was used to confirm colocalization. Raphe areas quantified include the DRNa, DRNp, vrDRNa, and drDRNa. Hypothalamic areas quantified include mSON, NC, and medial PVN. Images in Figs. 3.2 and 3.3 are maximum intensity projection images.

### *3.3.5 Data Analysis*

SPSS v22 was used to analyze all data. 2-way independent analysis of variance (ANOVA), single variable ANOVA, and independent t-tests were all used where appropriate. When appropriate, data were transformed using the square root before commencing with analysis. Graphs are of original data and not transformed data. All post-hoc comparisons were a priori. Tests were two tailed and differences were determined to be significant at $p < 0.05$.

## 3.4 Results

### *3.4.1 Dominance and duration of submission during social defeat*

There were no between group differences in the duration of submission in established dominant hamsters (DOM), established subordinate hamsters (SUB) and defeated control hamsters (DC) during their defeat by the RA. Analysis of the duration of submission during social defeat (Fig. 3.1) revealed that there was no effect of sex ($F(1, 36) = 0.95$, $p > 0.05$), no effect of dominance status ($F(2, 36) = 0.02$, $p > 0.05$), nor was there an interaction between sex and dominance status ($F(2, 36) = 1.739$, $p > 0.05$). Within-sex a priori comparisons did not reveal any differences between groups ($p < 0.05$, Fig. 3.1). Therefore, all groups of hamsters received similar intensities of social defeat.

### *3.4.2 Effects of chronic dominance status on the activation of 5-HT-containing neurons after social defeat*

In females, we hypothesized that the activity of 5-HT containing neurons, as measured by the colocalization of 5-HT-ir/fos-ir, is higher in the raphe nuclei of established dominant females after social defeat compared to established subordinates, defeated controls, non-defeated controls (NDC). In the anterior dorsal raphe nucleus (DRNa) (Fig. 3.2 A and B) there was a significant interaction between sex and dominance status ($F(3, 53) = 5.68$, $p < 0.01$). Within-sex a priori comparisons revealed that NDC females had more 5-HT-ir/fos-ir than SUB and DC females ($p < 0.05$, Fig. 3.2 B). There were no differences in 5-HT-ir/fos-ir between any of the male groups ($p > 0.05$, Fig. 3.2 B). In the posterior dorsal raphe nucleus (DRNp) (Fig. 3.2 C and D) there was no effect of sex ($F(1, 54) = 0.456$, $p > 0.05$), no effect of dominance status ($F(3, 54) = 1.036$, $p > 0.05$), nor was there an interaction between sex and dominance status ($F(3, 54) = 0.527$, $p > 0.05$). Within-sex a priori comparisons did not reveal any differences between groups in 5-HT-

ir/fos-ir ($p > 0.05$, Fig. 3.2D). In the median raphe nucleus (MRN) (Fig. 3.2 E and F) there was a trend toward a significant effect of sex ($F(1, 48) = 3.39$, $p = 0.07$), no effect of dominance status ($F(3,48) = 0.44$, $p > 0.05$), and a trend toward a significant interaction ($F(3, 48) = 2.03$, $p = 0.12$). Within-sex a priori comparisons revealed a decrease 5-HT-ir/fos-ir in DC females compared to NDC females ($p < 0.05$, Fig. 3F). There were no differences in 5-HT-ir/fos-ir between any of the anatomical areas examined in males.

We also examined the relationship between sex, dominance status, and social defeat in 5-HT-ir/fos-ir in the ventral (vrDRNa) and dorsal (drDRNa) subdivisions of the most rostral portion of the DRNa in female and male hamsters because previous studies found effects of dominance in these DRNa subregions on 5-HT-ir/fos-ir in both female and male hamsters (Cooper et al., 2009; Terranova et al., 2016). In the vrDRNa (Fig. 3.2 G and H), there was no effect of sex ($F(1, 42) = 0.01$, $p > 0.05$), no effect of dominance status ($F(3, 42) = 0.42$, $p > 0.05$), and no interaction between sex and dominance status ($F(3, 42) = 0.06$, $p > 0.05$). Within sex a priori comparisons did not reveal any differences between groups for both females and males ($p > 0.05$, Fig. 3H). In the drDRNa (Fig. 3I and 3J), there was a trend for a significant effect of sex ($F(1, 40) = 2.70$, $p = 0.11$), no effect of dominance status ($F(3, 40) = 1.13$, $p > 0.35$), and a trend toward a significant interaction between sex and dominance status ($F(3,40) = 2.63$, $p = 0.06$). Within-sex a priori comparisons revealed that DOM females had higher 5-HT-ir/fos-ir compared to all other female groups ($p < 0.05$, Fig 3.2 J). There were no differences in 5-HT-ir/fos-ir between any of the male groups ($p > 0.05$, Fig 3.2 J).

### *3.4.3 Effects of chronic dominance and subordination on activation of AVP-containing neurons after social defeat*

In males, we hypothesized that the activity of AVP-containing neurons, as measured by the colocalization of AVP-ir/fos-ir, is higher in hypothalamic nuclei of DOM males after social defeat compared to SUB, DC, NDC males. In the medial supraoptic nucleus (mSON) (Fig 3.3 A and B) there was a significant interaction between sex and dominance status ($F(3, 42) = 3.34$, $p < 0.05$). Within-sex a priori comparisons revealed that DOM males had less AVP-ir/fos-ir than DC males ($p < 0.05$, Fig. 3.3 B). There were no significant differences in AVP-ir/fos-ir between any of the female groups ($p > 0.05$, Fig. 3.3 B). In the nucleus circularis (NC) (Fig. 3.3 C and D) there was a trend towards an effect of sex ($F(1, 44) = 2.47$, $p = 0.12$), no effect of dominance status ($F(3, 44) = 0.75$, $p > 0.05$), and a trend towards a significant interaction ($F(3, 44) = 2.14$, $p = 0.11$). Within-sex a priori comparisons revealed that DOM males had less AVP-ir/fos-ir than DC males ($p < 0.05$, Fig. 3.3 D). There were no differences in AVP-ir/fos-ir between any of the female groups ($p > 0.05$, Fig. 3.3 D). In the medial paraventricular nucleus (PVN) (Fig. 3.3 E and F) there was a significant main effect of sex ($F(1, 50) = 14.01$, $p < 0.01$), no effect of dominance status ($F(3, 50) = 0.55$, $p > 0.05$) and a trend toward a significant interaction between sex and dominance status ($F(3, 50) = 2.01$, $p = 0.12$). With-sex a priori comparisons revealed that DOM males had less AVP-ir/fos-ir than DC males ($p = 0.05$, Fig. 3.3 F). There were no differences in AVP-ir/fos-ir between any of the female groups ($p > 0.05$, Fig. 3.3 F).

## 3.5 Discussion

The hypothesis that activation of 5-HT-containing neurons is higher in socially defeated established dominant female hamsters compared to established subordinates, defeated controls, and non-defeated controls was supported. The hypothesis that activation of AVP-containing

neurons is higher in socially defeated established dominant male hamsters compared to established subordinates, defeated controls, and non-defeated controls was not supported. Instead, socially defeated established dominant males had lower AVP-ir/fos-ir compared to defeated control males. These findings demonstrate that there are sex differences in the activation of 5-HT and AVP neurons in response to social defeat stress, suggesting that that there are sex differences in how 5-HT and AVP contribute to the resistance to social stress. Importantly, there were no differences in the duration of submissive behavior across groups during social defeat suggesting that all groups experienced similar levels of social defeat stress.

Established dominant females had higher 5-HT-ir/fos-ir in the drDRNa compared to the other female groups. Dominant females did not have higher 5-HT-ir/fos-ir in the DRNp, MRN, or vrDRNa compared to other female groups. These data indicate that 5-HT-containing neurons in the DRNa respond differently to social defeat stress in established dominant female hamsters when compared to dominant males as well as subordinates and controls.

As expected, there was a sex difference in the activation of AVP-containing neurons after social defeat, such that we observed effects of established dominance on AVP-ir/fos-ir in males but not females. Surprisingly, we found that socially defeated established dominant males that had lower AVP-ir/fos-ir compared to defeated control males in all AVP brain regions examined. These findings are perplexing, given that AVP in males increases aggression and is associated with acquisition of dominance (Terranova et al., 2016). Notably, for all AVP brain regions observed, socially defeated established dominant males had consistently lower levels of AVP-ir/fos-ir compared to defeated controls. Thus, reduction of AVP-containing neuron activity in male hamsters during social defeat stress is associated with established dominance. Future work will need to explicitly investigate the neural mechanisms for this reduction in activation of AVP-

containing neuron activity and the implications of these mechanisms in the neural control of social stress.

While 14 days of dominance or subordinance did not alter activation of 5-HT-containing neurons in males, pharmacological manipulation of 5-HT1a autoreceptors of male hamsters with no prior dominance status is sufficient to alter their response to social defeat stress. Microinjection of a 5-HT1aR agonist into the DRN of male hamsters, either prior to social defeat or prior to testing with an NAI, decreases submissive and defensive behaviors (Cooper et al., 2008). Conversely, microinjection of a 5-HT1aR antagonist into the DRN of male hamsters, either prior to social defeat or prior to testing with an NAI, increases submissive and defensive behaviors (Cooper et al., 2008). Furthermore, 5-HT1aR mRNA is increased in the DRN of dominant male hamsters compared to subordinates (Cooper et al., 2009). Therefore, in males, reducing the activation of presynaptic DRN 5-HT neurons by stimulating inhibitory 5-HT1a autoreceptors decreases susceptibility to social stress. Increasing activation of presynaptic DRN 5-HT neurons by blocking inhibitory 5-HT1a autoreceptors increases susceptibility to social stress.

While previous studies have demonstrated a resistance to social stress in dominant male hamsters allowed to interact with their subordinate partners for 14 days, no comparable data are available for females (Morrison, et al, 2014). One immediate future direction will be to determine whether there are sex differences in the resistance to social stress or whether females and males display the same patterns of stress resistance. The present data indicate that the existence of sex differences in the neuronal responses to stress in the hypothalamus and in the raphe. However, as we have seen previously in females and males the underlying neural mechanisms controlling the same behavior (e.g., aggression) can be sexually differentiated.

The present study demonstrates that activation of 5-HT neurons is greater in established dominant females after a social defeat experience compared to other female groups, and that activation of AVP neurons is reduced in established dominant males after social defeat compared to defeated control males. Our findings indicate that sex and social status influence how social stress modulates the activity of 5-HT and AVP. The next chapter of this dissertation will explore how 5-HT and AVP regulate sex differences in resistance to social stress but from a different perspective. Several lines of evidence have demonstrated that there is a positive relationship between aggressiveness and resistance to social stress. Because 5-HT and AVP increases aggression in females and males, respectively, we investigated whether 5-HT-active drugs would increase resistance to social defeat stress in females but not males, whereas AVP-active drugs would increase resistance to social defeat stress in males but not females.

### 3.6 Acknowledgements

The authors would like to acknowledge Sarah Clark, Ravi Das, Atit Patel, and Daniel Cox for their advice regarding optimization of the immunofluorescent procedures in this study. This work is supported by MH11978526 to H.E.A. and MH062044 to K.L.H.

## 3.7 Chapter 3 Figures

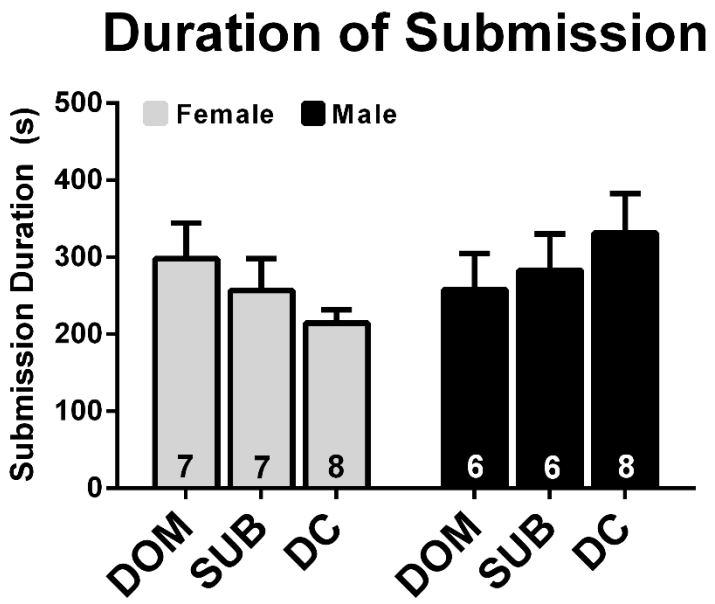


***Figure 3.1 Submissive behaviors of defeated hamsters***

Overall of submissive behaviors (see methods for definition) of established dominant hamsters (DOM), established subordinate hamsters (SUB), defeated control hamsters (DC), and non-defeated control hamsters (NDC). Error bars indicate SEM. Numbers inside bars represent N for each group.

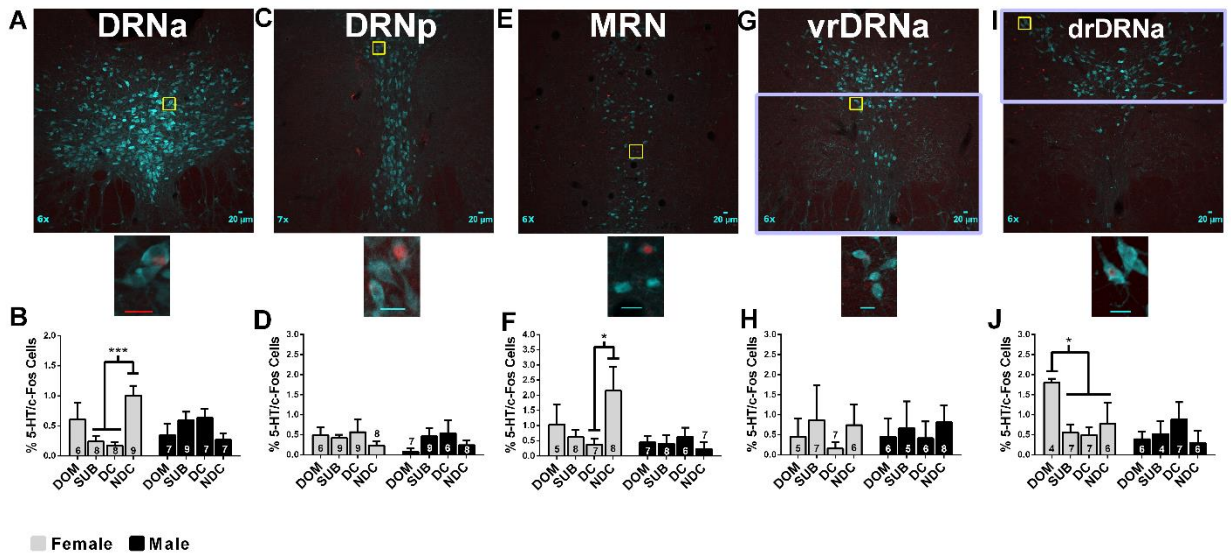


***Figure 3.2 Immunofluorescent colocalization of 5-HT-ir and fos-ir in cells within raphe subregions***

Immunofluorescent colocalization of 5-HT-ir (teal) and fos-ir (red) in cells within regions of the raphe of established dominant hamsters (DOM), established subordinate hamsters (SUB), defeated control hamsters (DC), and non-defeated control hamsters (NDC). DRNa (A and B), DRNp (C and D), vrDRNa (E and F), and drDRNa (G and H). Magnification is indicated in the lower left corner. Yellow boxes indicate subregions magnified to 40×, except the vrDRNa, which is scaled to 30x. (Scale bars, 20 µM.) Purple boxes in G and I represent region boundaries for the vrDRNa and drDRNa, where 5-HT-ir/fos-ir cells were quantified. Graphs indicate the percentage of 5-HT-ir cells that colocalize fos-ir (percentage of activated 5-HT cells) as a function of dominance status and sex in DRNa, DRNp, vrDRNa, or drDRNa. Error bars indicate SEM. Numbers inside bars or above bars represent N for each group. * $p < 0.05$; *** $p < 0.01$.

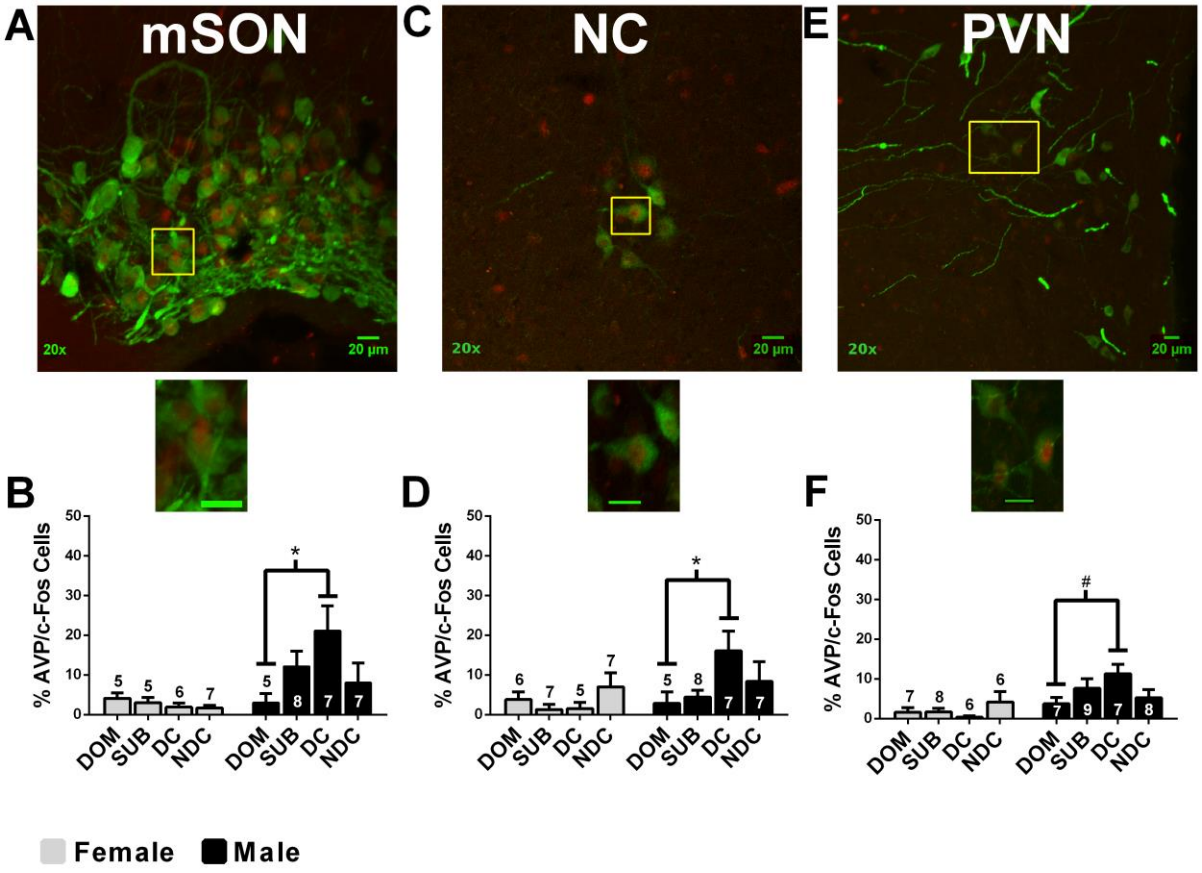


***Figure 3.3 Immunofluorescent colocalization of AVP-ir and fos-ir in cells within hypothalamic subregions***

Immunofluorescent colocalization of AVP-ir (green) and fos-ir (red) in cells within hypothalamic regions of established dominant hamsters (DOM), established subordinate hamsters (SUB), defeated control hamsters (DC), and non-defeated control hamsters (NDC). The mSON (A and B), NC (C and D), and medial PVN (E and F). Magnification is indicated in the lower left corner. Yellow boxes indicate subregions magnified to 40×. (Scale bars, 20 µM.) Graphs indicate the percentage of AVP-ir cells that colocalize fos-ir (percentage of activated AVP cells) as a function of dominance status and sex in mSON, NC, or PVN. Error bars indicate SEM. Numbers inside bars or above bars represent N for each group. * $p < 0.05$; # $p = 0.05$.

# 4 THE ROLE OF SEROTONIN (5-HT) AND ARGININE VASOPRESSIN (AVP) IN RESISTANCE TO SOCIAL STRESS

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## 4.1 Abstract

Social stress is one of the most common stressors experienced by humans and is an important risk factor for debilitating psychiatric disorders, such as post-traumatic stress disorder (PTSD) and major depressive disorder. However, only a subset of individuals who experience stressful life events will develop psychiatric disorders such as PTSD and major depressive disorder. An important question is why are some individuals resistant to the negative effects of social stress and others are more susceptible? In rodents, increased aggression and dominance is associated with increased resistance to social stress. Recently, our lab has shown that there are sex differences in how 5-HT and AVP control aggression and the acquisition of dominance in Syrian hamsters. 5-HT increases aggression and is associated with acquisition of dominance in females, whereas it decreases aggression in males. AVP, on the other hand, increases aggression and is associated with acquisition of dominance in males, whereas it decreases aggression in females. Recent data indicates that the resistance to social stress occurs in dominant males only after 14 days of daily social interaction with their subordinate partner. We have also examined how social defeat affects activation of 5-HT- and AVP-containing neurons in hamsters that are in established, 14-day dominant/subordinate relationships. Socially defeated dominant females in established dominance relationships have greater activation of 5-HT-containing neurons in subregions of the dorsal raphe nucleus, whereas socially defeated dominant males in established dominance relationships have less activation of AVP-containing neurons. Given that there are

sex differences in how 5-HT and AVP regulate aggression and dominance, we investigated whether there are sex differences in how 5-HT and AVP regulate the resistance to social stress using a social avoidance model. Because we have previously shown that a single administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, stimulates aggression in females and inhibits aggression in males we examined whether a single administration of fluoxetine could alter social avoidance induced by social defeat stress. We found that a single systemic administration of fluoxetine significantly increased social avoidance in male hamsters after social defeat. In contrast, fluoxetine had no effect on the duration of social avoidance in females after social defeat. Given these sex differences in the response to a single systemic injection of fluoxetine we investigated whether a single injection of AVP or 8-OH-DPAT, a serotonin 1a receptor agonist (5-HT1aR) into the anterior hypothalamus (AH) influenced the duration of social avoidance following social defeat in female and male hamsters. Microinjection of AVP into the AH significantly increased social avoidance in females, but not males or controls, while injection of 8-OH-DPAT into the AH significantly decreased social avoidance in males but not females or controls. These data indicate that there are sex differences in how 5-HT and AVP influence social avoidance in response to stress induced by social defeat.

## 4.2 Introduction

Social stress is an important risk factor for psychiatric disorders such as PTSD and major depressive disorder (Björkqvist, 2001; Almeida et al., 2002; Ozer et al., 2003). However, only a subset of individuals exposed to traumatic stressors develop psychiatric disorders such as PTSD and major depressive disorder (Merikangas et al., 2010; Cooper et al., 2015; Horn et al., 2016). One explanation that has been proposed is that aggressive, dominant individuals are more resistant to the social stress, whereas docile, subordinate individuals are more susceptible to

social stress (Koolhaas et al., 1999; Koolhaas et al., 2010; Cooper et al., 2015; de Boer et al., 2017). Indeed, rodents that are aggressive and dominant tend to be more resistant to social defeat stress than their docile, subordinate counterparts (Adams and Boice, 1983; Huhman et al., 1991; Blanchard et al., 1993; Veenema et al., 2003; Morrison et al., 2012; Morrison et al., 2014a). Thus, the neural mechanisms that facilitate aggression and dominance may underlie resistance to social stress.

Syrian hamsters are an excellent model species to explore the neural mechanisms that underlie the linkage between aggression, dominance, and resistance to social stress because they reliably attack intruders, engage in highly ritualized agonistic behaviors, and rapidly form stable dominance relationships (Payne and Swanson, 1970; Potegal et al., 1993; Olivier and Young, 2002; McCann and Huhman, 2012). Both female and male hamsters engage in spontaneous aggression, which is unlike other rodent species where males are more aggressive (Payne and Swanson, 1970; Blanchard et al., 1988). Furthermore, female hamsters, like female primates, display a rich range of competitive strategies including offensive aggression and dominance behaviors (Albers et al., 2002; Rosvall, 2011). When socially defeated, hamsters are no longer spontaneously aggressive. Instead, they avoid a caged intruder and are submissive to a smaller, weaker NAI hamster. This response to social defeat stress is termed *conditioned defeat* (Potegal et al., 1993; McCann and Huhman, 2012). Importantly, the behavioral changes induced by conditioned defeat are quantifiable and both sexes express these changes, thus providing a robust behavioral model to investigate sex differences in the neural mechanisms of resistance to social stress (Huhman et al., 2003).

There are striking sex differences in how 5-HT and AVP regulate aggression and dominance. In female hamsters, central administration of a 5-HT1aR agonist into the AH

increases aggression, whereas, in males, 5-HT1aR stimulation in the AH decreases aggression (Ferris et al., 1999; Morrison and Melloni, 2014; Terranova et al., 2016). Systemic injection of the SSRI fluoxetine increases aggression in females and decreases aggression in males (Ferris et al., 1997; Ferris et al., 1999; Morrison and Melloni, 2014; Terranova et al., 2016). Increased activation of 5-HT-containing neurons is associated with acquisition of dominance in female hamsters but not male hamsters (Terranova et al., 2016). Central administration of AVP into the AH, on the other hand, decreases aggression in females and increases aggression in males (Ferris et al., 1997; Caldwell and Albers, 2004; Gutzler et al., 2010; Morrison and Melloni, 2014; Terranova et al., 2016). Increased activation of AVP-containing neurons is associated with acquisition of dominance in male hamsters but not female hamsters (Terranova et al., 2016).

Recent data demonstrate that resistance to social stress in dominant males only occurs after 14 days of repeated social interactions with their subordinate partner (Morrison et al., 2012; Morrison et al., 2014a). In the previous chapter, we identified changes in activation of 5-HT-containing neuron and activation of AVP-containing neuron after social defeat in both female and male hamsters that are in established, 14-day dominant/subordinate relationships. Socially defeated dominant females have greater activation of 5-HT-containing neurons in dorsal raphe nucleus subregions, whereas socially defeated dominant males have less activation of AVP-containing neurons.

Because there are sex differences in how 5-HT and AVP regulate aggression and dominance, we used a model of social avoidance in Syrian hamsters (for reference, see (McCann and Huhman, 2012)) to investigate whether there are sex differences in how 5-HT and AVP respond to social stress. In this study, we test the hypothesis that 5-HT and AVP are critical mechanisms in regulating sex differences in resistance to social stress by manipulating 5-HT and

AVP circuits that control sex differences in offensive aggression. In females, we predict that 5-HT increases resistance to social stress, whereas AVP decreases resistance to social stress. In males, we predict that the 5-HT decreases resistance to social stress, whereas AVP increases resistance to social stress. Our data partially support our predictions. A single systemic injection of fluoxetine increases social avoidance in male hamsters after social defeat stress but has no effect on female hamsters. Microinjection of AVP into the AH of defeated female hamsters, but not non-defeated female hamsters and male hamsters, increases social avoidance. Microinjection of 8-OH-DPAT, a 5-HT1aR agonist, into the AH of defeated male hamsters, but not non-defeated male hamsters and female hamsters, decreases social avoidance. These findings indicate that there are nuanced, sex-specific effects of how 5-HT and AVP influence the resistance to social stress.

## 4.3 Materials and Methods

### *4.3.1 Animals*

Adult male and female Syrian hamsters (Charles River Laboratories Inc. and in-house bred), 8–12 wk old, weighing between 110 and 150 g, were used for all experiments. Experimental hamsters were individually housed in polycarbonate cages (24 × 43 × 20 cm). A colony of larger resident aggressor (RA) hamsters, weighing between 160 g and 175 g was used to socially defeat experimental hamsters. All hamsters were kept in a 14:10 light/dark cycle with free access to food and water. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Use of Animals and were approved by the Georgia State University Institutional Animal Care and Use Committee.

### *4.3.2 Drugs*

The following drugs were used in the microinjection experiments: 8-OH-DPAT (78950–78-4; Sigma-Aldrich) and AVP (065–07; Phoenix Pharmaceuticals). Doses of 1 mM 8-OH-DPAT and 0.9 μM AVP dissolved in sterile physiological saline were used in both sexes. Concentrations were informed by previous studies (Ferris et al., 1997; Ferris et al., 1999; Gutzler et al., 2010; Terranova et al., 2016). Controls were microinjected with 200 nL of saline. The following drugs were used in the intraperitoneal (IP) injection experiments: Fluoxetine hydrochloride (Spectrum Chemical) dissolved in diH2O was injected IP to both sexes in the following doses: 0 mg/kg (vehicle) and 20 mg/kg. 8-OH-DPAT dissolved in saline was injected IP to males in the following doses: 0 mg/kg and 0.5 mg/kg.

### *4.3.3 Social Defeat Model*

The social defeat model used was adapted from (McCann and Huhman, 2012). During social defeat, hamsters were placed in the home cage of a larger, RA for 15 minutes. The RA reliably attacked and defeated the experimental hamsters. The social defeats were monitored to make sure no injuries occurred to either animal. Non-defeated control animals were placed in an empty home cage belonging to an RA of the same sex.

The following day, experimental hamsters were tested in a neutral testing arena with an unfamiliar RA. The RA was placed behind a plastic caged mesh (13.5 × 13.5 × 7 cm), so the experimental hamsters could see, smell, and hear the RA but not come into physical contact. The caged mesh was placed in the home cage of the RAs during social defeat training for both defeated hamsters and non-defeated controls. The caged mesh was cleaned with 70% ethanol and allowed to dry between RA changes. Experimental hamsters were placed facing the arena wall

opposite the caged RA and subsequently explored the arena for 5 minutes. Duration of time spent on the far side of the arena was defined as avoidance and quantified.

### *4.3.4 Injection Experiments*

For the fluoxetine experiments, hamsters singly housed for two weeks prior to handling. Hamsters were handled for one week and estrous cycle was monitored in females. During the following week, hamsters were then socially defeated and tested for avoidance the following day as described above. Experimental females were defeated and tested during diestrus. RA females were ovariectomized to ensure continuous aggression regardless of day. 2 hours prior to social avoidance testing, hamsters were injected IP with fluoxetine or vehicle.

For cannulation experiments, hamsters were housed singly for at least 1 wk. Hamsters were deeply anesthetized with a gaseous solution of 5% isoflurane mixed with oxygen in an induction chamber and maintained under gaseous anesthesia between 3.00% and 4.00% isoflurane mixed with oxygen. Hamsters were implanted unilaterally with a 4-mm, 26-gauge guide cannula aimed at the AH using a stereotaxic apparatus. Coordinates were +0.8mm anterior to bregma, ±1.5mm from the midline, and –3.5mm from the top of the skull at an 8° angle (for reference see (Morin and Wood, 2001). Hamsters recovered at least 3 days before handling. The next week, hamsters were handled daily, and estrous cycles were monitored in females. The next week, hamsters were socially defeated and tested for avoidance.

In the first set of experiments, five minutes before social defeat, hamsters were microinjected with 200 nL of drug or saline. Injections lasted 30 seconds, and the needle remained in the guide cannula for an additional 30 seconds. Hamsters were then socially defeated and tested for avoidance the following day as described above. After testing, experimental hamsters were euthanized with an overdose of sodium pentobarbital and injected

with ink to verify cannula site placement. The second of experiments used the same design as the first set, except drugs were microinjected 5 minutes before social avoidance testing and non-defeat controls were used, because there were effects of drug treatment on duration of avoidance behavior.

In the 8-OH-DPAT experiment, hamsters were housed, handled, and tested as described in the fluoxetine experiment. The day following social defeat, hamsters were injected IP with 8-OH-DPAT or vehicle 25 minutes before social avoidance testing

#### *4.3.5 Data Analysis*

SPSS v22 was used to analyze all data. 2-way independent analysis of variance (ANOVA), single variable ANOVA, and independent t-tests were all used where appropriate. When appropriate, data were transformed using the square root before commencing with analysis. Graphs are of original data and not transformed data. All post-hoc comparisons were a priori. Tests were two tailed and differences were determined to be significant at $p < 0.05$.

### 4.4 Results

#### *4.4.1 IP injection of Fluoxetine after social defeat and prior to social avoidance testing increased social avoidance in males*

Given there are sex-specific effects of systemically administered fluoxetine on aggression, we tested the hypothesis that fluoxetine systemically administered prior to social avoidance testing decreases social avoidance in females and increases social avoidance in males. There was a main effect of drug treatment ($F(1, 30) = 6.67$, $p < 0.05$), but no effect of sex ($F(1, 30) = 0.31$, $p > 0.05$), nor was there an interaction between drug treatment and sex ($F(1, 30) = 1.52$, $p > 0.05$, Fig 4.1 A). Within-sex comparisons revealed that males who were socially defeated and given fluoxetine IP prior to avoidance testing had higher levels of avoidance

compared to vehicle treated males ($p < 0.05$, Fig 4.1 A). There was a strong trend towards a significantly increased duration of avoidance in fluoxetine-treated non-defeated control males compared to vehicle-treated non-defeated control males ($p < 0.05$, Fig 4.1 B).

### *4.4.2 Microinjection of 8-OH-DPAT and AVP into the AH immediately prior to social defeat did not affect social avoidance*

To test the hypothesis that increased aggressive drive enhances resistance to social stress, we microinjected drugs in the AH known to increase or decrease offensive aggression in both females and males (8-OH-DPAT and AVP, for reference see (Terranova et al., 2016)). There was a trend toward a significant effect of sex ($F(1,32) = 4.12$, $p = 0.09$, Fig 4.2 A) but not of drug ($F(2,32) = 0.06$, $p > 0.05$), nor was there an interaction between drug treatment and sex on the duration of duration ($F(2,32) = 0.06$, $p > 0.05$). With-in sex a priori post-hoc comparisons did not reveal any effect of drug treatment on avoidance duration ($p > 0.05$, Fig 4.2 A). For both sexes, there was no effect of drug treatment on the duration of aggression of the RA towards the experimental animal ($p > 0.05$, Fig 4.2 B) and there was no effect of drug treatment on the duration of submissive behavior of the experimental animal ($p > 0.05$ Fig 4.2 C).

### *4.4.3 Microinjection of 8-OH-DPAT and AVP into the AH after social defeat and prior to social avoidance testing affected social avoidance in both males and females*

To further test the hypothesis that the neural mechanisms that drive aggressiveness facilitate the resistance to social stress, we microinjected 8-OH-DPAT and AVP into the AH of both females and males 24 hours after social defeat and 5 minutes prior to social avoidance testing. There was a main effect of drug treatment ($F(2, 45) = 5.224$ $p < 0.01$, Fig 4.3 A) but not of sex ($F(1,45) = 0.001$, $p > 0.05$), nor was there an interaction between drug treatment and sex on the duration of avoidance ($F(2,45) = 1.11$, $p > 0.05$). With-in sex *a priori* post-hoc

comparisons revealed that, in females, AVP increased the duration of avoidance compared to saline treatment ($p < 0.05$) and 8-OH-DPAT treatment ($p < 0.05$) (Fig 4.3 A). In males, 8-OH-DPAT decreased the duration of avoidance compared to saline treatment ($p < 0.05$) and 8-OH-DPAT treatment ($p < 0.05$) (Fig 4.3 A). There was no effect of AVP treatment in non-defeated females or of 8-OH-DPAT treatment in non-defeated male controls ($p > 0.05$, Fig 4.3 B).

### *4.4.4 IP injection of 8-OH-DPAT after social defeat and prior to social avoidance testing did not affect the duration of social avoidance in males*

Given the different effects of centrally administered 8-OH-DPAT and the peripheral effects of fluoxetine, we tested whether IP injection of 8-OH-DPAT after social defeat and prior to avoidance testing affects social avoidance in males. There was a trend toward a significant reduction in social avoidance in males who received 8-OH-DPAT IP 25 minutes before avoidance testing ($t(21) = 1.43$, $p = 0.17$, Fig 4.4 A). While 8-OH-DPAT microinjection in the AH of males was sufficient to reduce social avoidance, peripheral injection of 8-OH-DPAT failed to alter duration of avoidance.

## 4.5 Discussion

These data support the hypothesis that there are sex differences in the 5-HT and AVP mechanisms that contribute to the resistance to social stress. However, these data only partially support our predictions that 5-HT increases and AVP decreases resistance to social stress in female hamsters and that AVP increases and 5-HT decreases resistance to social stress in male hamsters. We first administered fluoxetine systemically to both female and male hamsters after social defeat and 2 hours prior to avoidance testing. Because fluoxetine increases aggression in female hamsters and profoundly decreases aggression in male hamsters, we predicted that systemic fluoxetine would reduce social avoidance in females and increase social avoidance in

males. Systemic administration of fluoxetine prior to social avoidance testing increased duration of avoidance in defeated males and there was a trend toward a significant increase in duration of avoidance in non-defeated males. Fluoxetine injection prior to social avoidance testing had no effect in female hamsters. These data suggest that the neural systems that regulate aggression, at least in males, also regulate resistance to social stress.

We were surprised by the lack of effect of fluoxetine on duration of avoidance in female hamsters, given the effects we have observed of fluoxetine on female aggression and the on duration of avoidance in male hamsters (Terranova et al., 2016). Because the effects of fluoxetine on aggression in female hamsters are less pronounced than the effects of fluoxetine in male hamsters (Terranova et al., 2016), it is possible that the lack effect of fluoxetine on duration of avoidance is a floor effect. Currently, we are subjecting females to a more intense bout of social defeat stress and then testing their avoidance behaviors.

Given the sex differences we observed with a single injection of systemic fluoxetine, we then proceeded to inject 8-OH-DPAT or AVP into the AH of female and male hamsters to measure their effects on the duration of avoidance. Central injection of 8-OH-DPAT and AVP into the AH prior to social defeat failed to affect the duration of avoidance in either females or males. Central injection of AVP into the AH prior to social avoidance testing, however, increased the duration of avoidance in defeated females but not in males or non-defeated females. Central injection of 8-OH-DPAT into the AH prior to social avoidance testing decreased the duration of avoidance in defeated males but not females or non-defeated males. These findings demonstrate that there are sex differences in how 5-HT and AVP regulate the resistance to social stress. However, the actions of 5-HT and AVP at the level of the AH to regulate resistance to social stress are more nuanced than their actions to regulate aggression.

Neither central injection of 8-OH-DPAT nor AVP into the AH altered social avoidance in either sex when injected prior to a social defeat experience. Because 5-HT1aRs and V1aRs within the AH regulate aggression in a sex-specific manner (Ferris et al., 1999; Gutzler et al., 2010; Terranova et al., 2016), it is surprising that 8-OH-DPAT and AVP microinjections prior to social defeat had no effect on social avoidance. Interestingly, 8-OH-DPAT injected systemically in males prior to social defeat is sufficient to decrease submissive behaviors when tested on the following day (Bader et al., 2014). One interpretation of these data is that 5-HT and AVP are involved in modulating the severity of social stress during the social defeat experience, but not at the level of the AH. This hypothesis can be tested by investigating other brain regions that contain 5-HT1aRs and V1aRs besides the AH, such as the lateral hypothalamus, central amygdala, lateral septum, and bed nucleus of the stria terminalis (Albers et al., 2006; Ricci et al., 2006). In addition, 5-HT receptors other than 5-HT1aRs within the AH may play a role in modulating defeat-induced social anxiety. For example, 5-HT3aRs are densely expressed in within the AH (Carrillo et al., 2010) and microinjection of a 5-HT3 receptor agonist in male hamsters decreases the number of entries into the open arms of an elevated plus maze test and the duration of time spent in the open arms (Morrison et al., 2015b). Thus, 5-HT3Rs could mediate serotoninergic effects that contribute to the resistance to social stress.

8-OH-DPAT microinjection into the AH prior to social avoidance testing reduced social avoidance in males. This result is surprising, given that 8-OH-DPAT in the AH profoundly reduces aggression in males and reductions in aggression have been associated with decreased resistance to social stress (Veenema et al., 2003; Terranova et al., 2016). Furthermore, we found that a single systemic injection of fluoxetine prior to social avoidance testing was sufficient to increase the duration of avoidance in both defeated and non-defeated males. To address this

discrepancy between the global effects of fluoxetine on 5-HT release and the site-specific effects of 8-OH-DPAT within the AH, we injected 8-OH-DPAT IP in socially defeated and non-defeated males. When 8-OH-DPAT was systemically administered prior to avoidance testing in male hamsters, we failed to observe a significant decrease in duration of avoidance in males. This suggests that the actions of 5-HT1aRs on male avoidance are site-specific, at least at the level of the AH.

Taken together, the data in this chapter supports the notion that there is a nuanced relationship between the neural mechanisms that control aggression and resistance to social stress. For example, systemic injection of fluoxetine in male hamsters reduces aggression and increases social avoidance. However, microinjection of 8-OH-DPAT prior to social defeat in male hamsters, which profoundly decreases aggression, has no effect on social avoidance. Furthermore, microinjection of 8-OH-DPAT into the AH of male hamsters after social defeat and prior to social avoidance testing, decreases social avoidance. All 5-HT-active drugs used in these experiments decrease aggression, and yet these drugs have different effects on resistance to social stress depending on the type of drug (e.g. fluoxetine or 8-OH-DPAT) and the time point when the drug was administered (e.g. before social defeat or before social avoidance testing). These findings do not generate confidence that there is a simple inverse relationship between the neural mechanisms that regulate aggressiveness, dominance, and resistance social stress. Instead, these findings suggest that the neural mechanisms that regulate aggression, dominance, and resistance to social stress are overlapping but not entirely the same.

While the 5-HT and AVP systems play a role in how social stress is regulated in hamsters, these neurochemicals do not fully explain the mechanisms that underlie resistance to social stress. Other neurochemicals and receptor subtypes warrant further study. One candidate is

the 5-HT3 receptor, which is implicated in both the neural control of aggression and anxiety in male hamsters (Ricci et al., 2004; Morrison et al., 2015b). Another is the GABA-A receptor, whose activation in both the LS and AH increases aggression in male hamsters and stimulation in the LS decreases conditioned defeat (McDonald et al., 2012; Morrison et al., 2014b). Finally, stimulation of D2 receptors in the AH increases aggression in male hamsters (Morrison et al., 2015a). Given the effect of 5-HT3Rs, GABA-ARs, and D2Rs on aggression, it is possible that these receptor subtypes may act to regulate social stress in female and male Syrian hamsters. Future studies will need to consider additional neurochemicals and receptor subtypes to obtain a more complete understanding of the neural circuity that underlies resistance to social stress.

## 4.6 Acknowledgements

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## 4.7 Chapter 4 Figures

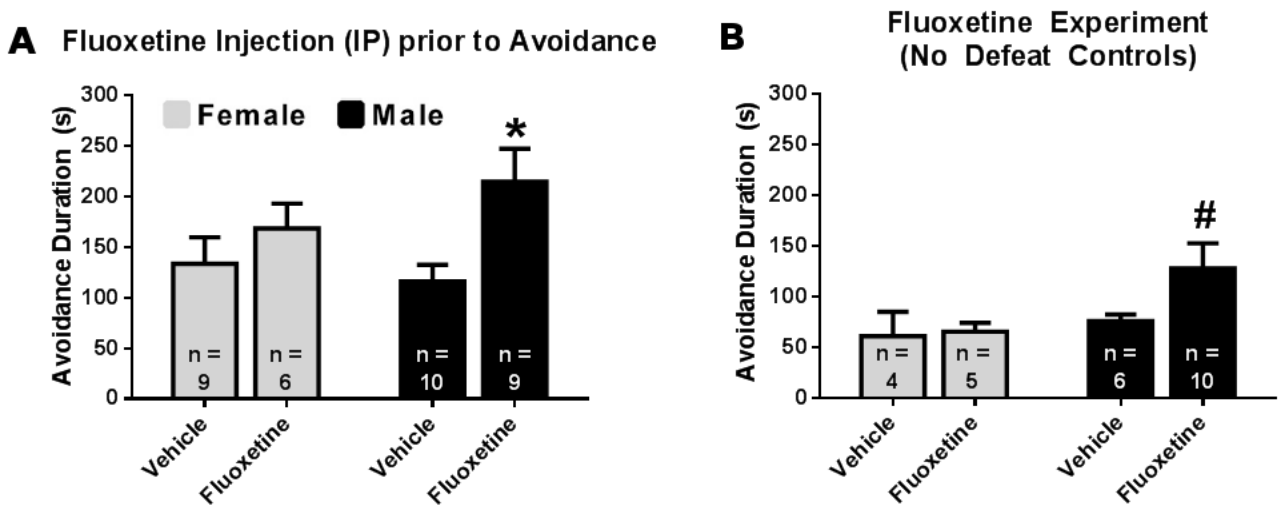


***Figure 4.1 Effect of IP injection of fluoxetine 2 hours prior to social avoidance testing in females and males***

(A) Effect of drug treatment on duration of avoidance of socially defeated experimental animals. (B) Effect of drug treatment on duration of avoidance in non-defeated controls. Error bars indicate SEM. * $p < 0.05$ for with-in sex comparisons. # $p = 0.06$ for within-sex comparisons

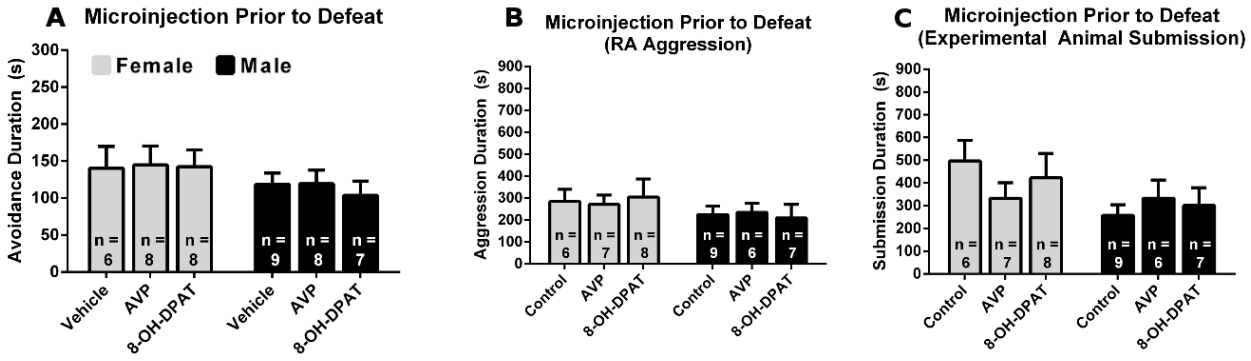


***Figure 4.2 Effects of 8-OH-DPAT and AVP microinjection into the AH prior to social defeat on social avoidance***

Effects of microinjection of 8-OH-DPAT and AVP into the AH of males and females 5 minutes prior to social defeat on social avoidance 24 hr later. (A) Effect of drug treatment on duration of avoidance of experimental animals. (B) Effect of drug treatment on duration of aggression of RA towards experimental animals. (C) Effect of drug treatment on duration of submission of experimental animals towards RA. Error bars indicate SEM.

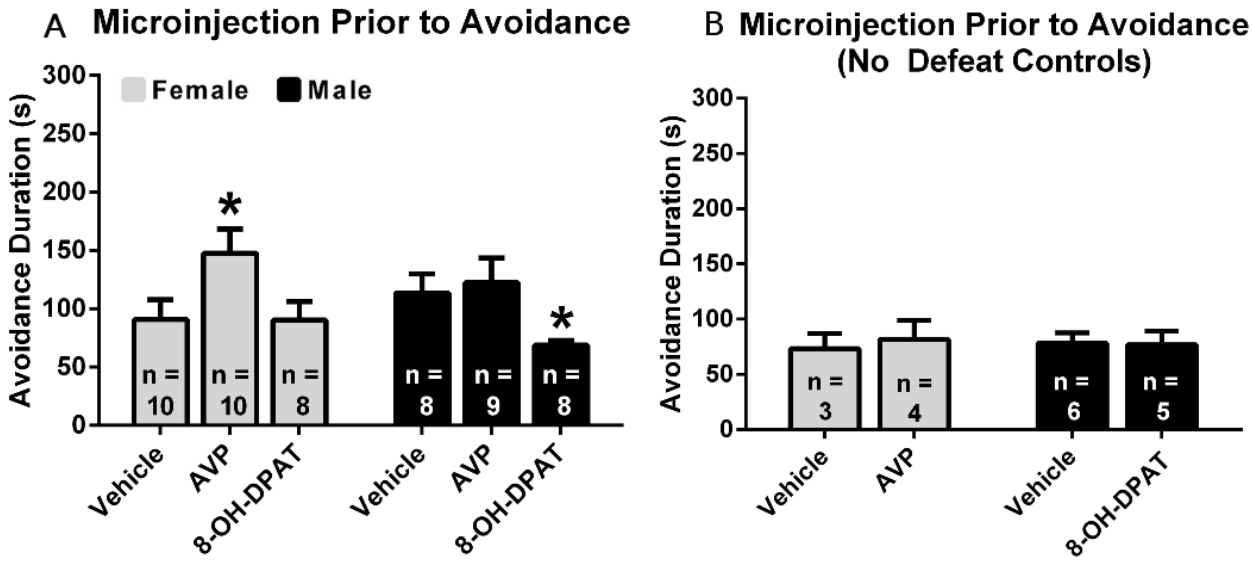


***Figure 4.3 Effects of 8-OH-DPAT and AVP microinjection into the AH prior to social avoidance testing***

Effects of microinjection of 8-OH-DPAT and AVP into the AH 5 minutes prior to social avoidance testing in females and males. (A) Effect of drug treatment on duration of avoidance of experimental animals. (B) Effect of drug treatment on duration of avoidance in non-defeated controls. Error bars indicate SEM. *$P < 0.05$ for with-in sex comparisons

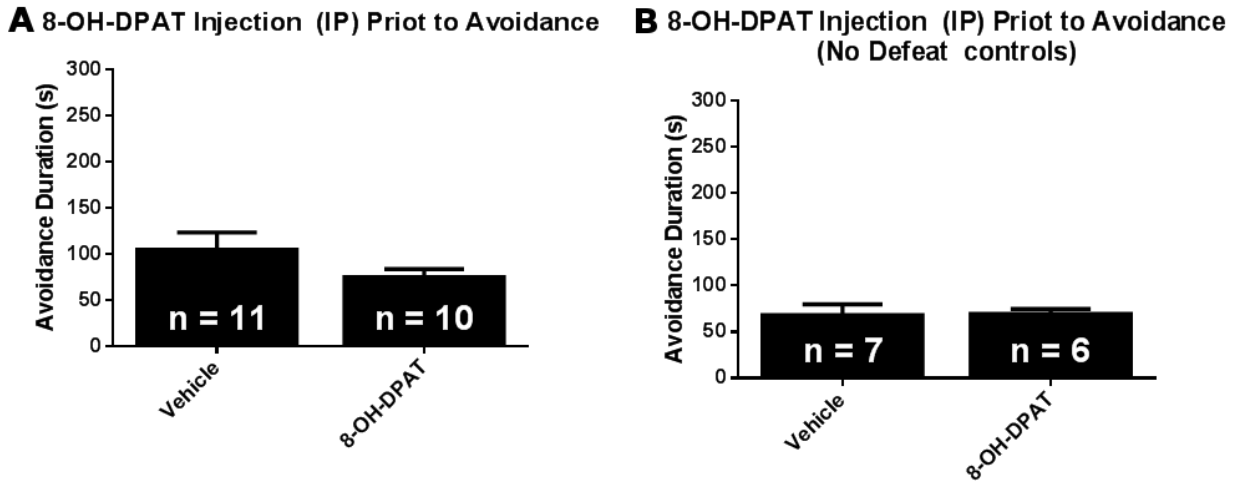


***Figure 4.4 Effect of 8-OH-DPAT injection IP 25 minutes prior to social avoidance testing in males***

(A) Effect of drug treatment on duration of avoidance of socially defeated experimental male hamsters. (B) Effect of drug treatment on duration of avoidance in non-defeated male control hamsters. Error bars indicate SEM.

# 5 CONCLUSIONS

The goal of this dissertation was to investigate sex differences in the neural mechanisms that control aggression and dominance, and then apply these mechanisms to investigate sex differences in the neural control of resistance to social stress. Chapter 2 demonstrated that there are profound sex differences in how 5-HT and AVP regulate aggression and acquisition of dominance. In male Syrian hamsters, 5-HT profoundly decreased aggression, which is in-line with the majority of studies on 5-HT and aggression in males (Morrison and Melloni, 2014). Microinjection of 8-OH-DPAT, a 5-HT1a receptor (5-HT1aR) agonist, decreased aggression in male hamsters (Terranova et al., 2016). Systemic administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, is sufficient to reduce aggression in male hamsters, as demonstrated in Chapter 2 and in previous reports (Ferris et al., 1997; Morrison and Melloni, 2014; Terranova et al., 2016). In contrast to the long-standing dogma that 5-HT decreases aggression, we showed that 5-HT potently increased aggression in female hamsters (Terranova et al., 2016). Infusion of 8-OH-DPAT into the AH of female hamsters dose-dependently increases aggression (Terranova et al., 2016). Furthermore, systemic injection of fluoxetine is sufficient to increase aggression in females (Terranova et al., 2016).

We investigated the role of AVP in the neural control of aggression in male and female hamsters. Microinjection of AVP into the AH increases aggression in males and decreases aggression in females, which is consistent with previous reports (Ferris et al., 1997; Ferris et al., 1999; Gutzler et al., 2010; Terranova et al., 2016). Chapter 2 also explored the role of 5-HT and AVP in the acquisition of dominance. Higher activation of 5-HT neurons in the dorsal raphe nucleus (DRN) is associated with acquisition of dominance in female hamsters but not male hamsters. Interestingly, in the most rostral subregions of the DRN nucleus, there are changes in

activation of 5-HT neurons in male hamsters. Subordinate males demonstrate higher activation of 5-HT neurons compared to dominant and control males in the ventral rostral DRNa (vrDRNa) subregion, as found in a previous report (Cooper et al., 2009; Terranova et al., 2016). The higher activation of 5-HT-containing neurons in the vrDRNa of subordinate males suggests that this DRNa subregion is associated with the acquisition of subordinace in males. In the dorsal rostral DRNa (drDRNa) subregion, dominant females had higher activation of 5-HT neurons, which is in concordance with the higher activation of 5-HT neurons observed in the DRNa and DRNp subregions of dominant females (Terranova et al., 2016). Strikingly, dominant males had increased activation of 5-HT-containing neurons in the drDRNa compared to subordinate and control males (Terranova et al., 2016). Additional mechanistic work is needed to further characterize the function of 5-HT neurons in the drDRNa in males.

In the hypothalamus, activation of AVP-containing neurons was associated with acquisition of dominance in male but not female hamsters. In the medial supraoptic nucleus (mSON) and nucleus circularis (NC), dominant males had higher activation of AVP-containing neurons compared to subordinates and controls (Terranova et al., 2016). Both dominant and subordinate females had higher activation of AVP-containing neurons compared to controls (Terranova et al., 2016). Taken together, these data suggest that activation of AVP-containing neurons is associated with acquisition of dominance in male hamsters, whereas activation of AVP-containing neurons is associated with social interaction in female hamsters. Interestingly, in the PVN, both dominant and subordinate males had higher activation of AVP-containing neurons compared to control males, whereas there were no differences reported between any of the female groups. In summary, the experiments in Chapter 2 demonstrate that, although both

female and male hamsters are capable of offensive aggression and acquisition of dominance, the neural mechanisms that regulate these behaviors are fundamentally different between the sexes.

Chapter 3 explored sex differences in the association between established dominant or subordinate status and the activation of 5-HT and AVP neurons after social defeat. Recent data show that, in dominant male hamsters, resistance to social stress only occurs after 14 days of daily interaction with a subordinate partner (Morrison et al., 2014a). Because we found sex differences in how 5-HT and AVP regulate aggression and acquisition of dominance, we investigated whether there are sex differences in how 5-HT and AVP respond to social stress in male and female hamsters that have been dominant or subordinate for 14 days. We socially defeated hamsters that had been in stable, 14-day dominant/subordinate relationships and then measured 5-HT and AVP neuron activation in dominant, subordinate, and control hamsters. Established dominant females had higher 5-HT-ir/fos-ir in the drDRNa subregion compared to established subordinate, defeated control, and non-defeated control females. There was no effect of established dominant or subordinate status on 5-HT-ir/fos-ir in males. Established dominant males had lower AVP-ir/fos-ir than defeated control males in the mSON, NC, and PVN. We did not observe any effects of established dominant or subordinate status on activation of AVP neurons in female hamsters. Taken together, these findings support the notion that there are sex differences in association between social status and activation of 5-HT and AVP neurons.

Chapter 4 builds on the findings from the previous two chapters to test how 5-HT and AVP regulate resistance to social stress, using a model of social avoidance in Syrian hamsters (McCann and Huhman, 2012). Because aggression is positively associated with resistance to social stress (de Boer et al., 2017), we hypothesized that neural mechanisms that increase aggression would increase resistance to social stress and, conversely, neural mechanisms that

decrease aggression would increase susceptibility to social stress. Therefore, based on the findings of Chapter 2 and Chapter 3, we predicted that the 5-HT system increases resistance to social stress in females and decreases resistance to social stress in males, whereas the AVP system increases resistance to social stress in males and decreases resistance to social stress in females. First, because a single systemic injection of fluoxetine increases aggression in female hamsters and decreases aggression in male hamsters, we administered a single systemic injection of fluoxetine in defeated female and male hamsters prior to social avoidance testing. While peripheral administration of fluoxetine did not affect duration of avoidance in females, both defeated males and non-defeated males had increased duration of avoidance. Because we found that there were sex differences with a single systemic injection of fluoxetine, we proceeded to microinject 8-OH-DPAT or AVP into the AH of female and male hamsters and then measure duration of avoidance. Microinjection of 8-OH-DPAT and AVP into the AH of female and male hamsters prior to social defeat had no effect on duration of avoidance. Microinjection of AVP after social defeat and prior to social avoidance testing increased duration of avoidance in females but not males or non-defeated females. Microinjection of 8-OH-DPAT after social defeat and prior to social avoidance testing decreased duration of avoidance in males but not females or non-defeated males. These data demonstrate that there are sex differences in how 5-HT and AVP regulate resistance to social stress. However, the actions of 5-HT and AVP to control resistance to social stress are more nuanced than their actions to control aggression.

The coping style literature on rodents argues that there is an inverse relationship between aggression and resistance to social stress and dominance and resistance to social stress (Koolhaas et al., 1999; Koolhaas et al., 2010; Cooper et al., 2015; de Boer et al., 2017). Indeed, there are several lines of evidence support this hypothesis. One study utilized strains of mice bred for a

short attack latency (SAL) or long attack latency (LAL) (Veenema et al., 2003). When both strains of mice are subjected to repeated social defeat stress, the SAL mice perform better on physiological and psychological measures of anxiety than their LAL counterparts, suggesting that the SAL mice are more resistant to social defeat stress. In another study, rats bred for low anxiety behavior demonstrate increased aggression and reduced hypothalamic-pituitary-adrenal axis (HPA) activation in response to a stressor compared to rats bred for high anxiety behavior (Veenema and Neumann, 2007). In hamsters, males that are in established dominance relationships have altered responses to social stress, such that established dominant males are more resistant to social defeat stress and established subordinate males are more susceptible to social defeat stress (Morrison et al., 2012; Morrison et al., 2014a). Beyond rodents, aggression, dominance, and resistance to social stress are linked. Stress coping style in rainbow trout predicts aggressiveness and dominance (Øverli et al., 2004). Great tits, a species of wild bird, demonstrate increased social avoidance immediately after social defeat stress (Carere et al., 2001). Taken together, the literature on coping style suggests that there is an inverse relationship between aggression/dominance and resistance to social stress.

While there is a straightforward relationship between aggression, dominance status, and resistance to social stress, this dissertation demonstrates that the neural mechanisms underlying this relationship are not straightforward. 5-HT and AVP clearly regulate aggression and acquisition of dominance in female and male hamsters. 5-HT increases aggression and is associated with acquisition of dominance in female hamsters, whereas it decreases aggression in male hamsters. Conversely, AVP increases aggression and is associated with acquisition of dominance in male hamsters, whereas it decreases aggression in female hamsters. However, this straightforward relationship between neural mechanism and behavioral output breaks down when

5-HT and AVP are examined in the context of social stress. For example, 5-HT profoundly decreases aggression in males, whether in the context of systemic administration of fluoxetine or by microinjecting 8-OH-DPAT directly into the AH. In the context of social stress, however, 5-HT has conflicting effects on males depending on the type of pharmacological treatment. While systemic administration of fluoxetine decreases resistance to social stress in males, microinjection of 8-OH-DPAT into the AH *increases* resistance to social stress. This increase in resistance to social stress is notable because, as demonstrated in Chapter 2, 8-OH-DPAT microinjection into the AH virtually abolishes aggression in males. These findings highlight that straightforward correlations in behavior (e.g. aggressive/dominant = resistant to social stress; docile/subordinate = susceptible to social stress) do not always equal straightforward correlations in neural mechanism. Thus, there is a nuanced relationship between 5-HT, AVP, and resistance to social stress that warrants further study.

There are several neural mechanisms, beyond 5-HT1aRs and V1aRs, that have been implicated in the neural control of aggression, dominance, and resistance to social stress. 5-HT3Rs are implicated in the neural control of aggression, are densely expressed within the AH, and can decrease anxiety behavior in hamsters when microinjected into the AH (Ricci et al., 2005; Carrillo et al., 2010; Morrison et al., 2015b). Dopamine is another candidate mechanism linking aggression, dominance, and resistance to social stress. Microinjection of a dopamine D2 receptor agonist into the AH increases aggression in male hamsters (Morrison et al., 2015a). Subcutaneous injection of apomorphine, a non-selective dopamine receptor agonist, increases stereotyped behaviors in SAL mice compared to LAL mice, suggesting that dopamine controls behavior differently depending on the coping style of an individual (Benus et al., 1991b). Systemic administration of a D2 antagonist in both male macaques and male mice reduces the

dominance status of high-ranking individuals (Yamaguchi et al., 2017). Given that dopamine, through the D2 receptor, is sufficient to increase aggression and is necessary for a high dominance ranking, it is possible that D2 receptor stimulation or inhibition modulates resistance to social stress. Finally, GABA-A receptors are an additional mechanism that may link aggression, dominance, and resistance to social stress. Microinjection of a muscimol, a GABA-A receptor agonist, into the lateral septum (LS) and AH increases aggression in male hamsters (McDonald et al., 2012; Morrison et al., 2014b). Moreover, GABA-A receptor stimulation in the LS and amygdala decreases conditioned defeat (Jasnow and Huhman, 2001; McDonald et al., 2012). Besides 5-HT1aRs and V1aRs within the AH, there are several potential neural that link aggression, dominance, and resistance to social stress.

In summary, this dissertation has demonstrated that there are sex differences in the neural mechanisms that underlie aggression, dominance, and resistance to social stress. 5-HT is important for aggression and acquisition of dominance in female hamsters, whereas AVP is important for aggression and acquisition of dominance in male hamsters. Although 5-HT and AVP are important for regulating resistance to social stress, their actions in the control of social stress are more nuanced than their actions in the control of aggression and acquisition of dominance. The data in this dissertation has important implications not just for understanding sex differences in the basic neural mechanisms of aggression, dominance, and resistance to social stress, but for better understanding sex differences in the pathogenesis and treatment of psychiatric disorders like PTSD and depression.

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