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Behavioral Sex Differences Caused by Distinct Vasopressin Sources

by

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Under the Direction of Aras Petrulis, PhD

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ABSTRACT

Dysfunction in social communication is a prominent aspect of many psychopathologies and social disorders including autism, schizophrenia, and social anxiety. Consequently, development of clinical treatment for these disorders requires an understanding of neural circuitry underlying social communication. Sex differences are a persistent feature of social disorders, where autism is more prevalent in males, while social anxiety occurs more frequently in females. A critical gap in knowledge exists in understanding the role of sex-differences in the control of social behavior and communication. A reasonable hypothesis is that differences in neural circuitry underlie sex-differentiated dysfunctions in social behavior and communication. A well-studied circuit in this regard is the sexually dimorphic expression of the neuropeptide arginine vasopressin (AVP). AVP in the nervous system originates from several distinct sources which are, in turn, regulated by different inputs and regulatory factors. Using modern molecular approaches, we can begin to define the specific role of AVP cell populations in social behavior. We demonstrate a behavioral function for the sexually dimorphic AVP neurons in the bed nucleus of the stria terminalis (BNST) and in the paraventricular nucleus of the hypothalamus (PVN). Collectively, our results indicate that AVP cell groups appear to play opposite roles in social investigation by males and females, as BNST-AVP cell ablations and BNST AVP knockdown reduced male social approach, while PVN-AVP cell ablations increased female social approach. We next utilized circuit level tracing techniques to map the inputs and outputs of BNST and medial amygdala (MeA) AVP cells, which are the major source of sexually dimorphic AVP expression. Finally, we tested the function of several sexually dimorphic BNST-AVP projection areas, such as, the lateral septum (LS), lateral habenula (LHb), and dorsal raphe (DR). In male mice, but not female mice, optogenetic stimulation of the BNST AVP terminals in

the LS increased their social investigation and anxiety-like behavior in the elevated-zero maze. Antagonism of V1aR in the LS blocked optogenetic-mediated increases in male social investigation and anxiety-like behavior. Therefore, activation of a distinct BNST-LS AVP circuit modulates sex-specific social approach and anxiety-like behavior, which is mediated by V1aR within the LS. This work suggests that sex differences in the neurochemical underpinnings of social behavior may contribute to sex differences in disorders of social behavior and communication.

INDEX WORDS: Vasopressin, Sex differences, Social behavior, Mice, Bed nucleus of the stria terminals, Medial amygdala, Paraventricular nucleus of the hypothalamus, Lateral septum

Sex-Specific Modulation of Social Behavior by Distinct Vasopressin Sources

by

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Georgia State University

May 2023

DEDICATION

I dedicate this work to those who have faced the struggles of drug addiction, anxiety, and other mental health disorders. It is a testament to our collective resilience and strength, and a reminder that we are not defined by our struggles but by our ability to overcome them. As someone who has faced these challenges, I know firsthand the impact they can have on one's life, but I also know that with the right support and resources, we can achieve great things. May this work serve as a beacon of hope for anyone facing similar challenges, and a reminder that we can achieve great things when we believe in ourselves and our ability to overcome.

ACKNOWLEDGEMENTS

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- My co-mentor and friend, Dr. Geert de Vries. Geert has an infectious positive attitude that has made me feel like I can take on the world. He has been an inspiration not only in science, but in life, as he emphasizes the importance of staying in tune with your passions in and outside of work.
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- I would like to express my deepest gratitude to my family for their support.
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LIST OF ABBREVIATIONS

AOB	Accessory olfactory bulb
AHiPM/al	amygdalohippocampal area
APir	amygdalopiriform transition area
AA	anterior amygdala
ACo	anterior cortical amygdala
AHA	anterior hypothalamic area
AHC	anterior hypothalamic area, central part
AHP	anterior hypothalamic area, posterior part
AVPe	anteroventral periventricular nucleus
Arc	arcuate hypothalamic nucleus
BLA	basolateral amygdala
BLP	basolateral amygdala, posterior
BMA	basomedial amygdala
BNSTa	bed nucleus of the stria terminalis (anterior)
BNSTp	bed nucleus of the stria terminalis (posterior)
CA1	CA1 hippocampus
CeM	central amygdala
EAM	extended amygdala, medial part
DLEnt	dorsolateral entorhinal cortex
DM	dorsomedial hypothalamic nucleus
DR	dorsal raphe

DM	dorsomedial hypothalamic nucleus
HDB	horizontal diagonal band
LHb	lateral habenula
LH	Lateral hypothalamus
LM	lateral mammillary nucleus
LPO	lateral preoptic nucleus
Lsi	Lateral septum (intermediate)
LSv	Lateral septum (ventral)
MD	mediodorsal thalamus
MeAD	medial amygdala (dorsal)
MePD	medial amygdala (posterior dorsal)
MeAV/PV	medial amygdala (ventral, posterior ventral)
MO/DTT	medial orbital cortex
MPOA	Medial preoptic area
MS	medial septum
Mtu	medial tuberal nucleus
m. accessory	mouse accessory
NAcc core	Nucleus Accumbens (core)
Nacc shell	Nucleus Accumbens (shell)
PVN	paraventricular nucleus of the hypothalamus
PVT	paraventricular thalamus
PLH	peduncular part of lateral hypothalamus
PAG	periaqueductal gray
Pir	piriform cortex
PLCo	posterolateral cortical amygdala

PMCo	posteromedial cortical amygdala
PMD/PMV	premammillary nucleus
RCh	retrochiasmatic area
RM/SuM	retromammillary/supramammillary nucleus
Shy	septohypothalamic nucleus
SIB	substantia innominata
SNR	substantia nigra, reticular part
ESO/SON	supraoptic nucleus
VDB	ventral diagonal band
VMH	ventromedial hypothalamus
VP	ventral pallidum
VTA	ventral tegmental area
VLPO	ventrolateral preoptic nucleus
VMPO	ventromedial preoptic nucleus

1 INTRODUCTION

1.1 Dissertation Introduction

The neuropeptide arginine-vasopressin (AVP) has many peripheral functions, such as maintenance of blood pressure and antidiuresis (Bourque et al., 2007; Cunningham and Sawchenko, 1991). In addition to these well-established physiological effects, AVP (and its non-mammalian analogue vasotocin) has long been implicated in the regulation of social behavior and communication in vertebrates, including humans (Donaldson and Young, 2013; Goodson and Bass, 2001; Guastella et al., 2011; Insel, 2010; Kelly and Goodson, 2013a; Rigney et al., 2022). AVP acts on various brain regions to regulate social recognition (Bluthe et al., 1993; Dantzer et al., 1988; Veenema et al., 2012), communication (Albers, 2015; Goodson and Bass, 2001; Rigney et al., 2020a), aggression (Ferris et al., 1997), maternal care (Bayerl and Bosch, 2019), pair bonding (Johnson and Young, 2015), and cognition (Landgraf and Neumann, 2004). Additionally, AVP contributes to avoidance and anxiety-like behavior in response to stressful situations (Harper et al., 2019; Kovács et al., 1986). In humans, AVP has been implicated in psychopathology, as variations in the vasopressin V1a receptor (V1aR) gene and AVP serum levels are associated with autism spectrum disorder (ASD) (Meyer-Lindenberg et al., 2009; Parker, 2022). As such, there has been increasing interest in AVP mechanisms that regulate social behavior, thus opening translational opportunities (Rigney et al., 2022).

AVP-like-peptides contain conserved features that evolved over half a billion years ago and can be traced to similar molecules in invertebrates (Johnson and Young, 2018; Theofanopoulou et al., 2021). In non-mammalian vertebrates, vasotocin (AVT) is the most common ancestral AVP-like molecule and is produced in birds, fish, amphibians, and reptiles, while AVP is produced in most mammalian species. Other variants of AVP, such as lysine

vasopressin and phenypressin, are found in marsupials and a limited number of eutherian mammals (Chauvet et al., 1985, 1984, 1980; Rouillé et al., 1988). In my dissertation, I will use the term "AVP" for all these peptides. In mammals, AVP acts on three canonical receptor types: V1aR, V1bR, and V2R (Frank and Landgraf, 2008), as well as on the oxytocin receptor (OXTR) (Manning et al., 2012; Song and Albers, 2017), with V1aR and OXTR being the predominant receptors expressed in the nervous system. AVP acts on three canonical receptor types: V1aR, V1bR, and V2R (Frank and Landgraf, 2008), as well as on the oxytocin receptor (OXTR) (Manning et al., 2012; Song and Albers, 2017), with V1aR and OXTR being the predominant receptors expressed in the nervous system. Furthermore, AVP can have dual actions on cells in target zones via V1aR action. For example, AVP causes both direct excitation and indirect inhibition within the lateral septum (LS), in the latter case via excitation of inhibitory interneurons (Allaman-Exertier and Reymond-Marron, 2007). In mammals, AVP is produced primarily in the paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), and the suprachiasmatic nucleus (SCN). AVP expressed in these nuclei regulate homeostatic functions, such as water/salt balance, blood pressure (i.e., PVN/SON), and circadian rhythms (i.e., SCN). Additional AVP-producing cells are found in the anterior hypothalamus (AH), nucleus circularis (NC), preoptic area (POA), as well as within sensory systems, such as the olfactory bulb (OB) and retina (Wacker and Ludwig, 2019). Most mammals also produce AVP within the extended amygdala (bed nucleus of the stria terminalis (BNST), medial amygdala (MeA)), and do so in a steroid-dependent and sex-specific manner (De Vries and Panzica, 2006). Other non-mammalian species (e.g., roughskin newt) have AVP-expressing cells in other brain regions (Lowry et al., 1997), and fish may lack the BNST/MeA AVP system altogether (but see (Rodriguez-Santiago et al., 2017)).

While the role of brain AVP on social and other behaviors has been well-described, the linkage of specific AVP sources to specific effects of AVP on social behavior has been, until recently, been based predominantly on circumstantial and correlational evidence, often with varying results sometimes in contradiction with each other. For example, AVP immunoreactivity, measured by immunohistochemical detection of AVP fiber density, and V1aR expression in the septum, detected by autoradiography, is positively correlated with aggression levels in California mice (*Peromyscus*) (Bester-Meredith et al., 1999), but the density of septal AVP-immunoreactive (AVP-ir) fibers is negatively correlated with male aggression in laboratory mice (*M. musculus*) and rats (Compaan et al., 1993; Everts et al., 1997). Additionally, more aggressive rats release less (Beiderbeck et al., 2007) or more (Veenema et al., 2010) AVP in the septum, and bouts of aggression in deer mice (*Peromyscus*) are positively correlated with BNST AVP-ir cell number (Steinman et al., 2015). In addition, BNST AVP neurons of male laboratory mice increase Fos expression in response to sexual-, but not aggression-related stimuli (Ho et al., 2010). Direct comparisons of correlational measures and RNA-interference manipulations in birds have yielded different interpretations as to how AVP influences avian social behavior (Kelly and Goodson, 2014a). These differences in the relationships between AVP expression, release, cell activity, and aggression, while possibly due to species and other differences, make it clear that studies that investigate the causal nature of AVP action are needed, such as those that delineate which AVP cell populations contribute to AVP effects on social behavior.

As most of our foundational knowledge of AVP's influence on social behavior has been gained from pharmacological targeting of AVP receptors within different brain regions as well as from microdialysis measurements of local AVP release during social behavior (Albers, 2015; Bayerl and Bosch, 2019; Dumais and Veenema, 2016; Smith et al., 2019; Wotjak et al., 1996),

we have incomplete understanding about which AVP cell populations are directly regulating social behavior. Indeed, the diverse anatomy of AVP projections across species suggests that AVP control of social behavior is complex (Kelly and Goodson, 2014a). An additional complication is that distinct AVP sources overlap in their projections to some terminal zones. For example, the LS receives AVP input from both the BNST and PVN (Otero-Garcia et al., 2014; Rood et al., 2013) (Figure 1-1), and so pharmacological manipulations within the LS may influence multiple AVP pathways, each with potentially different functions. This may be one reason for inconsistent results of manipulations of V1aR in the LS on intermale aggression and anxiety (Beiderbeck et al., 2007; Koolhaas et al., 1991) (Figure 1-1). Similarly, microdialysis measurements of AVP release in LS similarly cannot distinguish between AVP derived from different sources (Beiderbeck et al., 2007; Gobrogge et al., 2017; Veenema et al., 2010; Veenema and Neumann, 2008).

In addition to the adjacent or overlapping innervation of target structures from different AVP sources, complications arise from primarily focusing on targets of AVP action and not on the sources of AVP. For example, somatodendritic release of AVP from magnocellular PVN and SON neurons may generate both local autocrine/paracrine effects and as well as influencing distal regions (Ludwig and Stern, 2015), but see (Grinevich and Ludwig, 2021). Thus, AVP action on a particular brain region can originate from multiple sources, both synaptic and extrasynaptic. Moreover, the known cross-talk between AVP and OXT, a closely related peptide (Rae et al., 2022; Song and Albers, 2017), on their cognate receptors suggests that AVP may have some of its behavioral action via OXTR, further complicating the analysis of sources and sites of AVP action (Albers, 2015; Smith et al., 2019; Tan et al., 2019; Theofanopoulou et al., 2021).

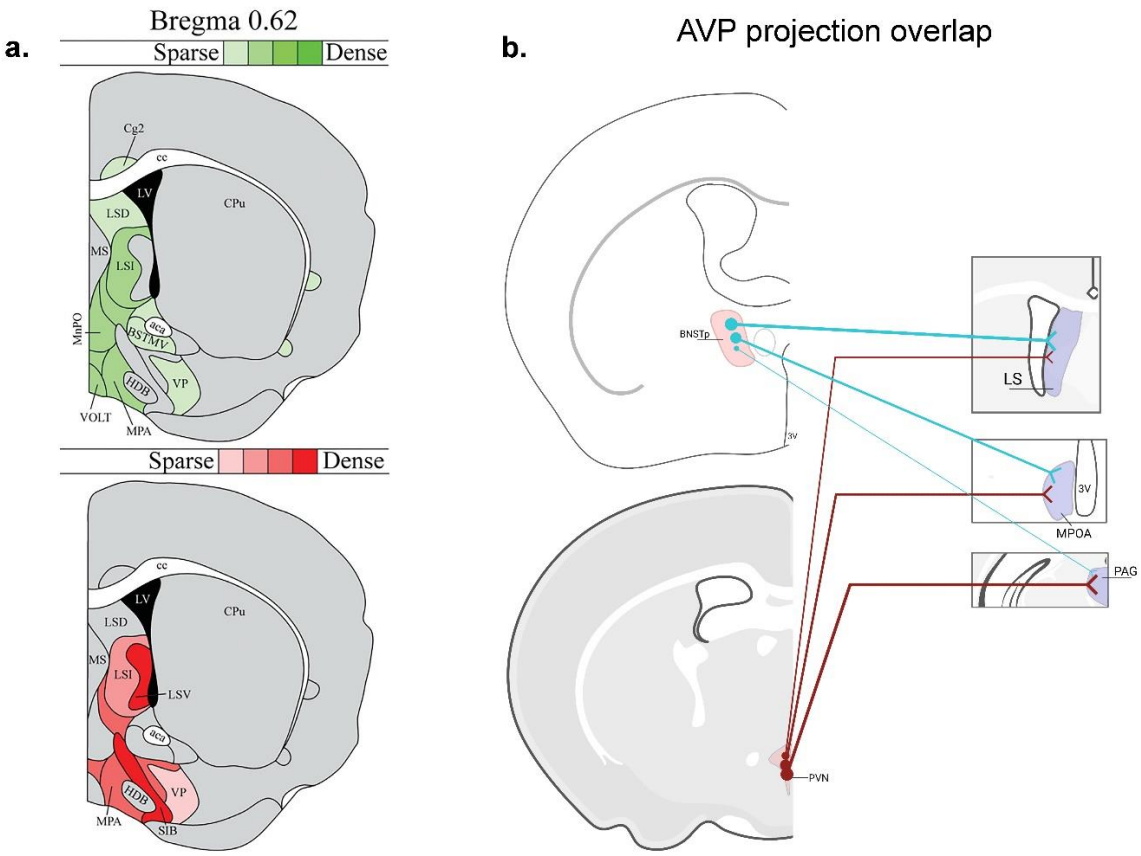

Figure 1-1 - Vasopressin (AVP) projection site overlap in mice. *(a) Image modified from (Rood et al., 2013) demonstrating that the LS and ventral forebrain receives steroid-dependent AVP signal likely from BNST/MeA as well as from the hypothalamus. Red shading indicates location of AVP-ir fibers that decrease after gonadectomy in males. Green shading indicates location of AVP-ir fibers likely originating from PVN, SON, or accessory cells (i.e., AVP-ir remained after gonadectomy and SCN lesions). AVP-ir fiber density is indicated by color shade. (b) Examples of several regions from (Rood et al., 2013) that receive both BNST and PVN AVP input: lateral septum (LS), medial preoptic area (MPOA), periaqueductal gray (PAG) (not all overlap regions are listed). Thickness of lines represents the strength of AVP fiber projections.*

1.2 Sexually differentiated vasopressin expression within the bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA)

The BNST and MeA, which is where the sexually-dimorphic and steroid-sensitive AVP cell populations are found, belong to the "extended amygdala" (de Olmos and Heimer, 1999; Shammah-Lagnado et al., 2000) and are key nodes connecting the Social Behavioral Neural

Network (SBNN) and the Social Decision-Making Network (Newman, 1999; O'Connell and Hofmann, 2011). Work by de Vries and others demonstrated that these cells are greater in number and express more AVP per cell in males than in females (De Vries et al., 1983; Vries et al., 1984). They are also steroid-dependent and express only AVP in the presence of gonadal hormones (de Vries et al., 1984b), which may act directly on these cells, as they express both androgen receptor (AR) and estrogen receptor alpha (ERα) (Axelson and Leeuwen, 1990; Zhou et al., 1994). AVP-expressing cells in the posterior BNST/MeA are the most likely sources of sexually-dimorphic and steroid-dependent AVP innervation of specific brain regions that regulate various aspects of social behavior (e.g., the lateral septum, ventral pallidum, dorsal raphe, lateral habenula, and other areas; see Figure 1-2) (De Vries and Panzica, 2006). Although the BNST and MeA are complex structures with numerous subdivisions (Flanigan and Kash 2022, Petrulis, 2020), AVP cell populations are generally limited to the intermediate and principal posterior sections of BNST and the posterodorsal part of MeA (Otero-Garcia et al., 2014; Rood et al., 2013). Steroidal effects on AVP peptide and AVP mRNA expression occur during early development as well as in adulthood, reflecting organizational and activational effects of steroid hormones, respectively (De Vries et al., 1994; Wang et al., 1994b). Additionally, phenotypically female mice with XY sex chromosomes (compared to XX) have denser AVP-ir fibers in the LS compared to XX female mice, demonstrating that genes on sex chromosomes, independent of gonadal development, partially regulate the sexually differentiation of BNST/MeA AVP expression (De Vries et al., 2002).

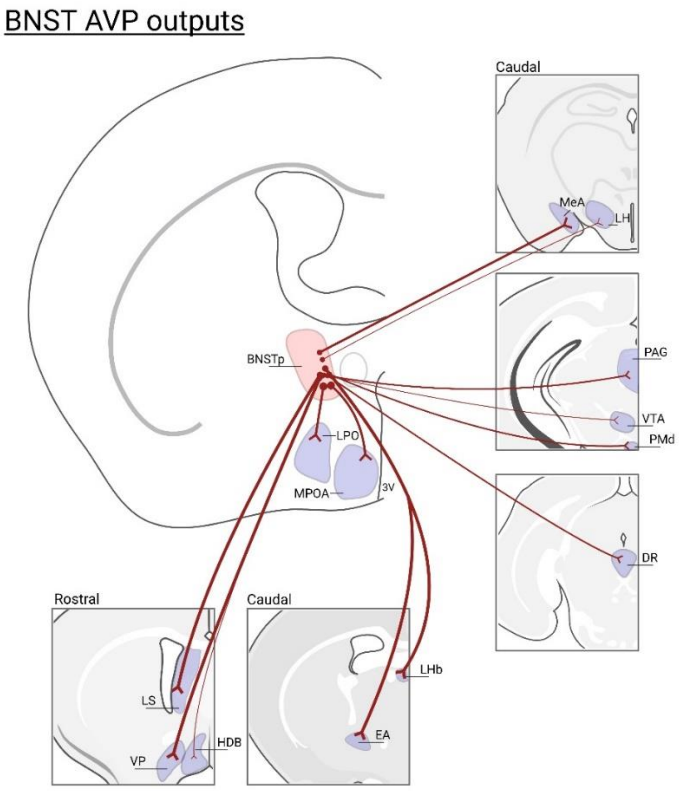

Figure 1-2- BNST AVP cell outputs. *Brain regions that lose AVP innervation following gonadectomy (Rood et al., 2013) or BNST lesions (De Vries and Buijs, 1983; Otero-Garcia et al., 2014): lateral septum (LS), ventral pallidum (VP), horizontal diagonal band (HDB), medial preoptic area (MPOA), lateral preoptic area (LPO), lateral habenula (LHb), extended amygdala (EA), medial amygdala (MeA), lateral hypothalamus (LH), periaqueductal gray (PAG), ventral tegmental area (VTA), dorsal premammillary nucleus (PMd), dorsal raphe (DR). Not all BNST AVP cell output regions are listed. Thickness of lines represents the density of AVP-ir fiber innervation.*

1.2.1 Function of BNST AVP cells

Opposite-sex interactions (affiliative behavior)

The BNST/MeA AVP cells have been linked to control of affiliative or prosocial behavior, primarily in males. For example, when male zebra finches and prairie voles pair-bond with females, Avp mRNA increases in the BNST (Lowrey and Tomaszycki, 2014; Wang et al., 1994b), and male finches that fail to court females have fewer BNST AVP-ir cells (Goodson et al., 2009b). Based on these findings and on the observation that BNST AVP cells show Fos expression in the presence of females, but not other rewarding stimuli, Goodson and colleagues

argued that BNST AVP is important for positively-valenced social interactions in birds (Goodson et al., 2009b). This idea was further supported by BNST AVP cells in roosters and male mice expressing more Fos after copulation, but not, or less so, following aggressive interactions (Ho et al., 2010; Xie et al., 2011). Similarly, BNST AVP neurons of male brown anole lizards show Fos activation during sexual behavior, while PVN AVP neurons show more Fos activation during aggressive encounters (Kabelik et al., 2013).

The monogamous prairie vole expresses higher levels of V1aR in the VP and LS compared to other, non-monogamous, vole species, suggesting that this difference in receptor density may contribute to the differences in social behavior between vole species (Insel and Young, 2001; Young and Wang, 2004; Johnson and Young, 2015). Indeed, blockade or knockdown of V1aR in these brain regions prevents pair bonding and bi-parental behavior in male prairie voles (Barrett et al., 2013; Lim and Young, 2004; Wang et al., 1994a) and overexpression of Avpr1a in the VP of both prairie and meadow voles facilitates pair bonding behavior (Lim et al., 2004; Pitkow et al., 2001). Although rats do not form monogamous pair bonds, inserting the prairie vole Avpr1a transgene in cells in the LS increased social interactions (Landgraf et al., 2003). Overall, it appears that AVP action within the LS and VP facilitates pair bonding and social interactions. However, the AVP sources regulating these behaviors have not been conclusively demonstrated.

Same-sex social interactions (mate competition and aggression)

In addition to its effects on prosocial behaviors, AVP can influence male aggression, but in a way that depends on social context and often differs between species. For example, AVP release in the lateral habenula, a major BNST target, may regulate territorial scent marking in mice (Higuchi et al., 2023; Rigney et al., 2020a) and AVP release in the lateral septum (LS),

another major BNST target, facilitates aggression in male mice (Leroy et al., 2018) as well as in gregarious zebra finches that compete for a mate, whereas LS AVP infusions inhibit resident-intruder aggression in territorial bird species (Kelly and Goodson, 2014a). However, blocking V1aR reduces competition for mates in territorial bird species, while not affecting territorial aggression (Goodson et al., 2009a). Indeed, AVP cell groups in the BNST may be responding to female stimuli in the context of mate competition, but not to other social stimuli in the context of territorial aggression or social aversion (Goodson and Wang, 2006).The activity patterns in BNST AVP cells may also depend on social organization, as BNST AVP-Fos colocalization increases after male-male interactions in gregarious bird species, but decreases after such interactions in more territorial/asocial bird species (Goodson and Wang, 2006). This pattern suggests that increased BNST AVP cell activity in more social birds may reflect positively-valenced male-male interactions compared to typically more negatively-valenced interactions in more territorial species (Goodson and Wang, 2006). In addition, the number of BNST AVP cells positively correlate with aggression in California mice (Bester-Meredith et al., 1999; Bester-Meredith and Marler, 2001; Steinman et al., 2015). In this species, increased paternal care can increase both territorial aggression and the number of BNST AVP-ir cells (Yohn et al., 2017). BNST AVP may also influence female aggressive behavior as AVP injections within the LS reduces female resident-intruder aggression (Oliveira et al., 2021).

Social memory

AVP can also influence social memory through its actions on the putative targets of BNST/MeA AVP cells, such as the LS (Aspesi and Choleris, 2021). In rats, V1aR antagonist injected into the LS impaired social memory in males but failed to do so in females when injected peripherally or intracerebroventricularly (Bluthé and Dantzer, 1990; Dantzer et al.,

1988; Engelmann et al., 1998). V1aR antagonist injected directly into the LS, however, can block social recognition in adult males and female rats (Veenema et al., 2012), but only in males while juvenile (Veenema et al., 2012). These effects of V1aR antagonist are modulated by gonadal steroids in both rats and mice (Bluthe et al., 1993; Dantzer et al., 1988). This sex- and steroid-dependency of AVP action suggests that projections from BNST (and/or MeA) cells are the most relevant sources of AVP acting on social recognition (Bluthe et al., 1993; Bluthé and Dantzer, 1990; Dantzer et al., 1988; de Vries et al., 1984a). Indeed, removal of BNST AVP cells in mice show that these cells are necessary for social recognition in males, but not females (see below) (Whylings et al., 2020).

Developmental aspects of social behavior (social play)

Social play fighting in juvenile rats, an important behavior for social skill development, is mediated by V1aR action within the LS in a sex-specific manner. Specifically, pharmacological blockade of V1aR in the LS increased social play behavior in juvenile male rats but reduced social play behavior in females (Bredewold and Veenema, 2018; Veenema et al., 2013), and a similar effect was found in the ventral pallidum (Lee et al., 2021). Therefore, in rats, AVP action within the LS and ventral pallidum may prevent social play in juvenile males, while facilitating social play in juvenile females. Additionally, Avp mRNA expression in the BNST correlates negatively with social play behavior in male, but not female, juvenile rats (Paul et al., 2014). Although the BNST and MeA (as well as PVN) send AVP projections to the LS and VP (De Vries and Panzica, 2006; DiBenedictis et al., 2020), future studies are needed to dissect precisely which AVP sources regulate social play.

Anxiety and social interactions

Since AVP has been implicated in the physiological stress response (Gibbs, 1986), studies have examined AVP's role in generating anxiety-like behavior, which can greatly impact how animals interact socially (Beery and Kaufer 2015). Indeed, V1aR knockdown within the LS reduced anxiety-like behavior in rats (Landgraf et al., 1995) and anxiety-like behavior is reduced only in V1aR KO male, but not female, mice (Bielsky et al., 2005b, 2004). These results suggest that the sexually dimorphic BNST/MeA AVP system regulates anxiety-like states primarily in males (Bielsky et al., 2005a). Since AVP plays a larger role in the regulation of male anxiety-like behavior, this system may influence how males interact with conspecifics, coping strategies, and social memory formation (Albers, 2012).

Direct manipulation of BNST AVP cells

Despite the substantial indirect evidence implicating the sexually-dimorphic BNST AVP cells in the control of male-typical social behavior, direct evidence for their involvement has been limited. To address this issue, I used intersectional genetic techniques to selectively delete BNST AVP cells in mice and found that such lesions strongly reduced male-male social investigation, modulated aspects of male social communication (i.e., urine marking), and impaired female sexual behavior, without altering resident-intruder aggression, ultrasonic vocalizations, male copulation, or anxiety-related behaviors (Rigney et al., 2019). Similarly, shRNA knockdown of AVP within the BNST also reduced male-male social investigation but, unlike BNST AVP cell ablations, decreased consummatory aspects of male copulatory behavior (Rigney et al., 2021). Some of these effects align with the effects of similar manipulations in birds (Kelly and Goodson, 2014a). For example, in territorial Angolan blue Waxbills, knockdown of BNST AVP reduces social contact primarily between males but does not affect

anxiety-like behavior (Kelly and Goodson, 2013b). However, in the more social zebra finch, knockdown of BNST AVP reduces preference for larger flocking groups (gregariousness), and increases anxiety-like behavior (Kelly et al., 2011). In housing situations that promote mate-guarding, BNST AVP knockdown in zebra finches increases aggression and reduces courtship communication, primarily in males (Kelly et al., 2011; Kelly and Goodson, 2013a). Thus, although species/context differences are apparent, reducing BNST AVP seems to primarily influence male social interactions, mostly (but not exclusively) in the context of male-male competition, communication, and courtship behavior. This suggests that BNST AVP neurons normally play a prominent role in driving specific aspects of male-male competitive behavior and investigation as well as affiliation (i.e., copulatory behavior; gregariousness in birds). In addition, BNST AVP cells are also critical for male social recognition, as deletion of these cells reduces social recognition in males, but not females (Jack Whylings et al., 2020) This is consistent with AVP effects within LS on social recognition (see above) and the recent finding that stimulation of BNST AVP terminals in LS restores social recognition in male mutant mice (Magel2-KO) with pre-existing social recognition deficits (Borie et al., 2021).

1.2.2 Function of MeA AVP cells

The MeA is a heterogeneous structure that is implicated in the control of defensive responses, aggression, parental behavior, play behavior, social communication, sexual behavior, as well as social recognition (Petrulis, 2020; Raam and Hong, 2021). The posterior dorsal part of the MeA (MeApd) contains the steroid-sensitive and sexually dimorphic AVP cell population (De Vries and Panzica, 2006; Otero-Garcia et al., 2014). The MeA and BNST may form an integrated system originating from a singular structure as there is developmental continuity between these two cell groups around the internal capsule (de Olmos and Heimer, 1999; Marler

et al., 1999) and AVP expression is similarly modulated by sex-steroids in these two structures (Rood et al., 2013). Despite having been described several decades ago (Caffe and Van Leeuwen, 1987; van Leeuwen et al., 1985), few studies have tested the functional role of this AVP cell population. Most of the existing work has focused on the role of MeA AVP cells in regulating defensive responses to stressors, such as predator odors, as well as reproductive behavior. For example, MeApd AVP cell activation (Fos expression) in rats positively correlates with male investigation of female conspecifics and copulatory behavior (Hari Dass and Vyas, 2014) and reduced methylation of the Avp promoter in MeA/BNST, which promotes gene transcription, correlates with reduced aversion to predator odors (Tong et al., 2019). Similarly, ablation of MeA AVP cells increases aversion to predator odors whereas overexpression of AVP in MeA reduces aversion to predator odors (Tong et al., 2021a). Counter-intuitively, over-expression of AVP in MeA increases activation of PVN-AVP neurons following predator odor exposure, suggesting that MeA AVP may facilitate PVN AVP cell responses during an acute stressor (Tong et al., 2021b). However, while the functional nature of this interaction is unknown, deletion of PVN AVP cells increases anxiety-like behavior in male mice ((Rigney et al., 2020b); see below), suggesting that activation of PVN AVP cells may be normally anxiolytic, and therefore anti-defensive, in males. Taken together, this data suggests that MeA AVP cells normally suppress defensive behavior, perhaps in situations where defensive behavior would be counter-productive, such as in reproductive contexts or during competitive behavior (Wang et al., 2013). More experiments targeting these cells are clearly needed to determine the social role of MeA AVP cells, their projections, and neurochemical interactions.

1.3 Vasopressin cells of the paraventricular nucleus of the hypothalamus (PVN)

AVP is produced in magnocellular PVN and SON neurons that project to the posterior pituitary where they release AVP into the periphery to regulate, e.g., blood pressure and water balance (Kortenoeven et al., 2015; Rocha E Silva and Rosenberg, 1969). AVP is also expressed in parvocellular PVN neurons that project to the median eminence, where AVP is released into a portal system between the median eminence and the anterior pituitary to regulate adrenocorticotropic hormone (ACTH) from the corticotrophs, and thereby indirectly release of glucocorticoids from the adrenal cortex (Gillies et al., 1982). A separate group of parvocellular neurons project centrally to brain regions to modulate physiology as well as social and other motivated behaviors (Albers, 2015; Buijs and Swaab, 1979; Rigney et al., 2022). PVN AVP neurons, while not differing in cell number in males and females (Rood et al., 2013), do display projection patterns that partially differ by sex (Freda et al., 2022). In females, PVN AVP cells send stronger projections to striatal areas (i.e., nucleus accumbens; NAcc) whereas males have denser innervation of mid- and hind-brain regions (Freda et al., 2022). Moreover, PVN AVP cell projections are generally denser in females compared to males (Rood et al., 2013). As many of these regions contain both V1aR and OXTR (Froemke and Young, 2021), AVP released from PVN cells may act on both V1aR and OXTR-expressing neurons to drive behavior, given the known cross-talk between OXT/AVP receptors (Song and Albers, 2017). Not only do PVN AVP cells project to a variety of regions, they also receive input from brain regions that regulate aspects of social and emotional behavior, such as the ventral tegmental area (VTA), NAcc, dorsal raphe (DR), BNST, LS, VP, and preoptic area (POA) (Wei et al., 2021) (Figure 1-3). Even though PVN and BNST/MeA AVP cell projections sometimes overlap, regions that regulate prediction of aversive and reward outcomes (i.e., lateral habenula and lateral septum) receive

much stronger AVP input from the BNST/MeA compared to the PVN (Lammel et al., 2012; Rood et al., 2013) (Figure 1-1b).

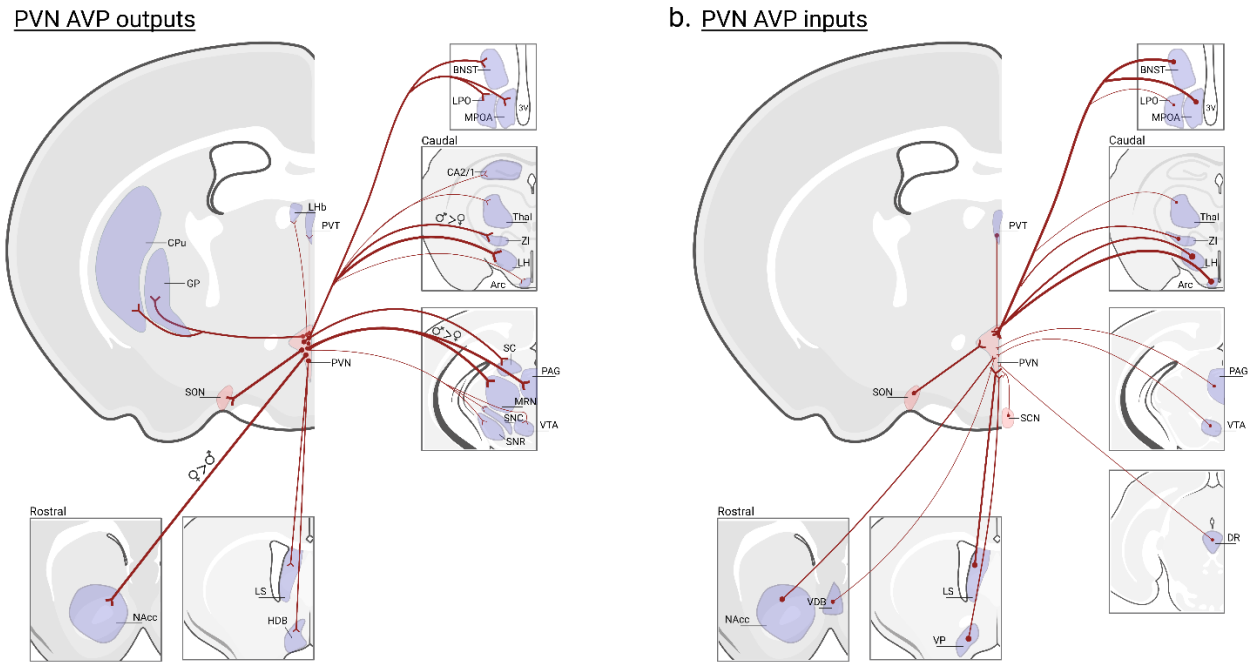

Figure 1-3 - PVN AVP cell inputs and outputs in mice *(a) Regions that receive prominent input from vasopressin cells in the PVN: nucleus accumbens (NAcc)*, lateral septum (LS), horizontal diagonal band (HDB), medial preoptic area (MPOA), lateral preoptic area (LPO), bed nucleus of the stria terminalis (BNST), lateral habenula (LHb), paraventricular nucleus of the thalamus (PVT), globus pallidus (GP), caudate putamen (CPu), supra optic nucleus (SON), hippocampus (CA2/1), thalamus (Thal), zona interna (ZI)†, lateral hypothalamus (LH), arcuate nucleus (Arc), superior colliculus (SC), periaqueductal gray (PAG)†, median raphe nucleus (MRN)†, substantia nigra pars compacta (SNC), substantia nigra reticulata (SNR), ventral tegmental area (VTA); Data based on PVN AVP cell-specific tracing (Freda et al., 2022) and the location of AVP-ir fibers remaining after male gonadectomy or SCN lesions (Rood et al., 2013). Note that fiber measurements do not determine AVP cell synapses in labeled regions. (b) Regions that send input to vasopressin cells in the PVN: NAcc, vertical diagonal band (VDB), LS, ventral pallidum (VP), BNST, MPOA, LPO, PVT, SON, suprachiasmatic nucleus (SCN), Thal, ZI, LH, Arc, PAG, VTA, dorsal raphe (DR); data based on transsynaptic-monosynaptic retrograde tracing from PVN AVP cells (Wei et al., 2021). Not all PVN AVP cell input/output regions are listed. Thickness of lines represents the strength of projections. * Indicates greater projections in females, † indicates greater projections in males.*

1.3.1 Function of PVN AVP cells

Opposite-sex interactions (affiliation)

Forming pair bonds increases AVP-ir in the PVN of male and female zebra finches (Lowrey and Tomaszycki, 2014), whereas pair-bond breakage with concomitant increases in anxiety-like and affiliative behavior also increases PVN AVP-ir in prairie voles (Sun et al., 2014). In birds and prairie voles, parenting and nesting behavior are consistently positively correlated with levels of PVN AVP expression and cellular activity (Kenkel et al., 2012; Klatt and Goodson, 2013; Wang et al., 2000).

Same-sex interactions (aggression)

The relationship between PVN AVP dynamics and aggression are inconsistent. For example, dominant male, but not female, Mandarin voles have greater PVN-AVP-ir than subordinates (Qiao et al., 2014), and aggressive behavior is positively correlated with this increased AVP expression in Brandt's voles (Huang et al., 2021). In male song sparrows, PVN AVP cells are more active after agonistic encounters and, in lizards, PVN AVP cell activity is positively correlated with aggression and can predict subordinate status (Goodson and Evans, 2004; Kabelik et al., 2013). However, PVN AVP-ir levels do not differ between males of aggressive and less aggressive Peromyscus species (Bester-Meredith and Marler, 2001), and, in male mice, PVN AVP-ir is reduced following aggressive interactions (Veenema et al., 2007). It is clear that responses of PVN AVP cells in agonistic contexts are complex, varying with social context, sex, personality, and their interactions (Kelly and Goodson, 2014b).

Social stress and aggression

A number of studies have focused on AVP interactions with the hypothalamic–pituitary–adrenal (HPA) axis (Aguilera et al., 2008; Engelmann et al., 2004). The HPA axis is activated during aversive situations to facilitate both physiological and behavioral coping responses/strategies (Ebner et al., 2005). Following HPA activation, animals may display

increased vigilance and arousal, and chronic HPA activation can lead to psychiatric disorders (i.e., anxiety and depression) in humans (Faravelli et al., 2012; Juruena, 2014). During stress, hypothalamic and systemic release of AVP are regulated in a site- and stressor-specific manner. For example, parvocellular PVN AVP cells can regulate ACTH secretion, which ultimately increases corticosteroid release (Engelmann et al., 2004). In addition to its role in the regulation of general stress physiology, several lines of evidence link PVN AVP cells to behavioral responses to social stress and the regulation of anxiety-related behaviors. AVP is released within the PVN of rats following social defeat, without stimulating the hypothalamic–neurohypophysial system or HPA axis (Ebner et al., 2005; Wotjak et al., 1996), implicating local somatodendritic AVP release. In resident-intruder tests, intruder rats that used active, but not passive (e.g., freezing behavior) coping strategies, increased AVP release in the PVN (Ebner et al., 2005). In contrast, the effects of chronic social stress on AVP expression within PVN are mixed, if not contradictory. For example, following long-term social defeat, both increases and reductions in PVN *Avp* mRNA (or no change in PVN AVP-ir) are observed in male mice (Keeney et al., 2006; Neumann and Landgraf, 2012). Moreover, changes in PVN AVP expression following chronic social defeat have not been detected in male rats (Albeck et al., 1997), and even acute social defeat has little effect on PVN *Avp* gene expression in mice (Keeney et al., 2006; Neumann and Landgraf, 2012). However, acute social defeat does reduce PVN *Avp* mRNA and AVP immunoreactivity in male, but not female, California mice (Steinman et al., 2015). These changes in California mice are not correlated with changes in aggression but are correlated with a reduction in social investigation. Overall, it is unclear if species differences or differences in short versus long-term AVP action are responsible for these conflicting results about the role of

PVN AVP cells in social stress

The species-specific nature of PVN AVP signaling following social stress are paralleled by findings that PVN AVP responses following non-social stress also depend heavily on the species investigated. Rats and mice bred for high anxiety traits (i.e., passive coping) had greater Avp gene and AVP peptide expression within the PVN compared to low-anxiety lines (Aubry et al., 1995; Bosch and Neumann, 2008; Bunck et al., 2009; Keck et al., 2003; Murgatroyd et al., 2004; Wigger et al., 2004); an effect which is found in male, but not female rats (Bosch and Neumann, 2008; Wigger et al., 2004). Additionally, Avp mRNA increases in the PVN after acute stress in rats (Wotjak et al., 2001, 1996). However, this type of increase is not observed in prairie voles following swim stress (Liu et al., 2001), and, in fact, reductions in Avp mRNA occur in female mice following acute restraint (Borrow et al., 2019), indicating that species, sex, and type of acute non-social stressor determine the level of *Avp* gene expression in PVN. Similar species differences are also apparent in PVN AVP responses to chronic stress. Female mice, but not male mice, show decreased *Avp* mRNA (Borrow et al., 2018), whereas California mice show increases in *Avp* mRNA after chronic variable stress (De Jong et al., 2013). Notably, neither acute nor chronic restraint in male rats or chronic variable stress in mice alters AVP immunoreactivity (Borrow et al., 2018; Sánchez et al., 1998). This may be due to significant heterogeneity in PVN AVP cell responses such that different AVP cell groups within the PVN may respond in opposite directions to chronic stressors (Grassi et al., 2014).

The lack of agreement about PVN AVP stress-related signaling across studies might reflect not only known sex- and species-differences but also unresolved mechanisms. For example, because PVN AVP cells can release AVP from soma and dendrites independently from synaptic release (Ludwig and Stern, 2015), AVP levels within the PVN may reflect non-synaptic,

possibly autoregulatory, local release, rather than release of AVP in PVN target structures. Indeed, high anxiety rat lines do not increase AVP release in the PVN during maternal aggression but do increase it in the central amygdala (CeA), where V1aR antagonists can reduce maternal aggression (Bosch and Neumann, 2010). This may explain the conflicting effects of AVP agonists/antagonists injected into PVN on anxiety-like behavior (Blume et al., 2008; Wigger et al., 2004; Wotjak et al., 1996). Additionally, changes in PVN AVP cell activity and AVP production may reflect physiological adaptations linked to behavioral changes but may not be causally involved in driving these behaviors. Lastly, because the PVN contains several different AVP cell types (i.e., magnocellular and parvocellular), it is likely that these different cell populations could have different effects on behavioral responses to social and non-social stressors and may themselves be composed of heterogeneous subpopulations, such as the PVN AVP cell population that expresses estrogen-receptor beta primarily in females (Alves et al., 1998; Oyola et al., 2017). Consequently, intersectional genetic approaches, such as those used to study PVN OXT (Froemke and Young, 2021; Qian et al., 2022), may be useful in identifying the function of specific PVN AVP cell subpopulations in social stress.

Developmental aspects of social behavior (social play) and parental behavior

In addition to their responses following social stress and aggression, PVN AVP neurons also respond in other social contexts. *Avp* mRNA levels in the PVN are positively correlated with social play in male, but not female, juvenile rats (Paul et al., 2014; Veenema and Neumann, 2009). Likewise, increases in PVN *Avp* mRNA are positively correlated with levels of adult male-male interactions in prairie voles and mice (Murakami et al., 2011; Pan et al., 2009) and with parental care (both sexes) in prairie, but not montane, voles (Wang et al., 2000). Taken together, PVN AVP levels/activity appears to be correlated with changes in both agonistic and

affiliative interactions, with the direction of the correlation largely depending on the species examined.

Direct manipulation of PVN AVP cells:

Opposite- and same-sex interactions (aggression and social investigation)

Although there have been a number of correlational studies suggesting PVN AVP's involvement in aggression (see above), direct manipulations of PVN AVP cells suggest that these cells are not required for aggressive behavior. In mice, ablations of AVP-expressing cells in the PVN did not alter resident-intruder aggression (Rigney et al., 2020b), and PVN AVP knockdown in zebra finches did not alter same-sex aggression (Kelly and Goodson, 2014b). Therefore, other hypothalamic AVP-expressing cell groups, such as the nucleus circularis (NC) (see below), may be driving pro-aggressive effects of AVP (Cheng et al., 2008; Ferris et al., 1989; Gobrogge et al., 2007).

PVN AVP cells may have a greater influence on female social interactions compared to males. For example, PVN AVP cell ablations increase female, but not male, social approach and investigation in mice (Rigney et al., 2020b). Similarly, chronic variable stress increases social investigation in female, but not male, mice while concomitantly reducing Avp mRNA levels in PVN (Borrow et al., 2018). In addition, PVN AVP action on CRF cells can mediate the effects of social buffering in female mice, effectively "erasing" the synaptic effects of a stressful experience (Loewen et al., 2020). Therefore, AVP within the PVN may regulate, among other things, female stress resilience.

Social memory

Unlike the effects of BNST AVP cell deletions, removing PVN AVP cells did not affect the ability of mice to distinguish between familiar and unfamiliar mice (Whylings et al. 2020).

However, activation of PVN AVP afferents to the CA2 subfield of the hippocampus enhances social memory in male mice (Smith et al., 2016). This discrepancy between results may be due to procedural differences in behavioral testing but may also indicate that, while this circuit is sufficient to modulate memory, it may not be absolutely required. Alternatively, critical PVN AVP sub-circuits, such as those projecting to CA2, may have been spared in the lesion experiment.

Parental behavior

Recent work in monogamous mice has demonstrated that chemogenetic inhibition of PVN AVP cells increases nest building in male mice, while excitation reduces nest building in females, without altering any other aspects of parental behavior (Bendesky et al., 2017). These sex differences in PVN AVP function may be examples of compensation for other biological sex differences as monogamous male and female mice display similarly high levels of nest building and parental care (De Vries 2004).

Anxiety and social interactions

In addition to social behavior, PVN AVP cells contribute to sexually differentiated aspects of anxiety-like behavior. For example, removal of these cells increases non-social, anxiety-related behaviors in males, but not in females (Rigney et al., 2020b). In contrast, administration or knockdown of AVP in the PVN does not alter anxiety-like behavior in male rats or male and female zebra finches (Blume et al., 2008; Kelly and Goodson, 2014b). The lesion results also diverge from some correlational studies showing that PVN AVP-ir positively correlates with levels of anxiety-like behavior in rats and mice (Bunck et al., 2009; Murgatroyd et al., 2004; Wigger et al., 2004), and that reductions of PVN AVP expression correlated with increases in anxiety in juvenile male, but not female, rats (Tanaka et al., 2010). Collectively,

however, these data suggest that AVP within the PVN may influence anxiety-like states primarily in males, while not affecting their ability to socially interact with male and female conspecifics, suggesting a lack of linkage between social- and non-social anxiety states (Rigney et al., 2020b).

1.4 Vasopressin cells of the supraoptic nucleus (SON) and nucleus circularis

Although the peripheral effects of SON AVP on physiology are well known (Carter et al., 2008), much less is known about the behavioral function of these cells. Changes in measures of cellular activity in SON AVP cells suggest that they may modulate aggressive behavior or aggression-related physiology in rats and hamsters (Cheng et al., 2008; Delville et al., 2000; Veenema et al., 2006). More directly, non-selective lesions of the medial SON impaired odor-induced flank marking in hamsters (Ferris et al., 1990), suggesting that a subpopulation of SON AVP cells may drive aspects of social communication.

The nucleus circularis (NC) is a small cluster of cells that contain AVP located within the anterior hypothalamus between the SON and PVN and has been historically implicated in osmotic thirst (Price Peterson, 1966; Wallace and Harrell, 1983). The discovery that AVP release within the anterior hypothalamus (AH) drives social communication (i.e., flank marking) in male hamsters (Ferris et al., 1986) and that non-selective NC lesions reduces flank marking, led to the suggestion that these cells could be one of the AVP sources important for flank marking (Ferris et al., 1990). Indeed, in male hamsters, the number of NC AVP-ir neurons positively correlates with offensive aggression, social dominance status, and flank marking (Delville et al., 2000; Ferris et al., 1989; Melloni and Ricci, 2010), and, in prairie voles, the number of cells correlates with the level of mating-induced aggression (Gobrogge et al., 2017, 2009, 2007). This may explain why PVN and BNST AVP cell lesions in mice did not affect aggressive behavior in

resident-intruder settings (Rigney et al., 2020b, 2019). However, as lesions to NC were non-specific, damaging both NC and the nearby AH, i.e., the location where AVP drives flank marking in males (Ferris et al., 1986; Terranova et al., 2017), it is difficult to discern whether AVP production within the NC drives flank marking. Specific manipulations of NC AVP cells, such as those used to assess BNST AVP function (Rigney et al., 2019), will be necessary for determining their role in social behavior.

1.5 Vasopressin cells of the suprachiasmatic nucleus (SCN) and retina

AVP is also produced within the suprachiasmatic nucleus (SCN), a key driver of circadian rhythms in behavioral and physiological regulation (Kalsbeek et al., 2010) (Buijs et al., 2021; Mieda et al., 2016). The AVP cells, located in the dorsomedial SCN, receive their inhibitory inputs from the ventrolateral SCN, which, in turn, is targeted by a subpopulation of retinal ganglion cells some of which also express AVP (Tsuji et al., 2017). SCN AVP release, as well as the expression of genes involved in photo-entrainment of biological rhythms, likely mediate aspects of jetlag (Tsuji et al., 2017; Yamaguchi et al., 2013). SCN AVP neurons project locally as well as to the PVN, the subparaventricular zone, MPOA, BNST, PVT, ARC, dorsal medial hypothalamus, NTS, and into the cerebrospinal fluid (CSF) (Buijs et al., 2021; Kalsbeek et al., 2010; Rood et al., 2013; Taub et al., 2021).

Although it has been suggested that SCN AVP may interact with sex hormones to influence sexual preference in humans (Swaab et al., 1995), the direct influence of SCN AVP on social behavior is much less clear. Non-specific SCN lesions in male Syrian hamsters did not influence AVP-sensitive social communication (i.e., flank marking) (Delville et al., 1998). Lesions of AVP cells in SCN of mice did not influence their social behavior but did increase anxiety-like behavior and sucrose consumption in both sexes (Whylings et al., 2021). However,

since these lesions only reduced the number of SCN AVP cells by about 50%, greater reductions in the population of SCN AVP cells may reveal additional effects on social behavior.

1.6 Vasopressin cells in the olfactory system

In rats, the main (MOB) and accessory (AOB) olfactory bulbs contain projection neurons that express AVP (Tobin et al., 2010; Wacker et al., 2010). These AVP cells are non-bursting, tufted cells (Wacker and Ludwig, 2019), which extend their primary dendrites toward glomeruli, thereby receiving information from olfactory sensory neurons (Tobin et al., 2010). Some of these AVP cells also co-express V1bR and may therefore be strongly autoregulated (Wacker et al., 2010). In the AOB, AVP cells project to MePD, which may influence kisspeptin release within this region (which also contains AVP cells) (Pineda et al., 2017). In addition, AVP neurons have also been found in the olfactory cortex (anterior olfactory nucleus, piriform cortex), indicating their involvement in complex odor recognition (Tobin et al., 2010; Wacker and Ludwig, 2019).

In most mammals, olfactory processing is critical for appropriate social communication via chemosignals produced by a conspecific (Wyatt, 2003). Pharmacological work suggests that AVP is released in the OB to modulate early stages of sensory processing and social recognition (Tobin et al., 2010) via feedback inhibition, a key component in sensory processing (Namba et al., 2016). As with other OB projection neurons, AVP cells are modulated by cholinergic systems to ultimately regulate social odor processing (Suyama et al., 2021). More work is needed to determine the specific excitation, inhibition, or disinhibition pathways within which OB AVP cells function to modulate social interactions.

1.7 AVP action in humans and other primates

Like humans, many non-human primates are highly social and capable of complex social cognition, making them desirable models for studies of social competence. As in other species,

AVP and V1aR is expressed in social brain regions of primates (Freeman et al., 2014; French et al., 2016; Grebe et al., 2021; Rogers Flattery et al., 2021) and is likely to influence their social behavior. For example, AVP concentration in CSF, but not CSF OXT or blood AVP concentration, predicts social functioning in Rhesus monkeys and positively correlates with time spent in social grooming (Parker, 2022; Parker et al., 2018). In humans, manipulation of AVP and V1aR systems have been explored as therapies for ASD, schizophrenia, and drug-abuse (Bolognani et al., 2019; Clarke et al., 2022; Parker, 2022; Rae et al., 2022). Indeed, CSF AVP is linked to ASD symptom severity in children (Oztan et al., 2018) and blood AVP concentration may be useful in predicting social development outcomes in newborns (Parker, 2022). Intranasal AVP also increases risky cooperative behavior in men (Brunnlieb et al., 2016) and enhanced social skills in autistic children (Parker et al., 2019). Additionally, intranasal AVP has been shown to modulate emotional responses to faces (Price et al., 2017; Thompson et al., 2006), improve memory for emotional faces, and identification of social words (Guastella et al., 2011, 2010; Parker, 2022). Although intranasal administration of AVP may offer a promising future therapeutic, the mechanisms by which AVP acts to modulate social behavior in humans remains to be seen. This will be especially important because of sex differences in ASD diagnoses (Parker, 2022) and sex-specific effects observed in animal models. In none of these cases, it is clear which source of AVP contributes to AVP effects on social behavior.

Dissertation goals: My dissertation will assess the role of specific AVP cell groups in social behavior by directly manipulating AVP cell populations. Specifically, I test the functional role of sexually differentiated vasopressin expression within the BNST **(Chapter 2 and 3**), AVP cells within the hypothalamus (**Chapter 4**), map the inputs and outputs of sexually dimorphic BNST

and MeA AVP cells (**Chapter 5**), and test the functional role of BNST AVP projections to specific sites, such as the lateral habenula, dorsal raphe (*indirect*) (**Chapter 6**), and lateral septum (*direct*) (**Chapter 7**). For more in-depth information on AVP action within specific target brain regions, see: (Albers, 2015; Dumais and Veenema, 2016; Goodson and Bass, 2001; Johnson and Young, 2017; Veenema and Neumann, 2008).

2 SEXUALLY DIMORPHIC VASOPRESSIN CELLS MODULATE SOCIAL INVESTIGATION AND COMMUNICATION IN SEX-SPECIFIC WAYS

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2.1 Introduction

Social and communication show profound sex-differences in many species (Darwin, 1871; Bradbury and Vehrencamp, 1998) and, in humans, dysfunction in social behavior and communication is prominent in chronic, debilitating, and pervasive psychopathologies (Insel, 2010) that show sex-differences in prevalence and clinical outcome (Halladay et al., 2015), such as autism (Schultz, 2005). One reasonable hypothesis is that sex differences in the underlying neural circuitry contribute to sexually differentiated function and dysfunction in social behavior and communication. A particularly well-positioned circuit in this respect is the vasopressin (AVP) innervation of the brain, which shows marked sex differences across many species, including humans (Goodson and Bass, 2001; De Vries and Panzica, 2006; de Vries, 2008). Research across taxa confirms an important role for AVP in social behavior. For example, AVP has been implicated in aggression, pair bonding, maternal behavior, and communication across vertebrates (Goodson and Bass, 2001; Albers, 2012). However, the anatomy of AVP projections suggests that AVP control of social behavior is complex and, in most cases, the anatomical

substrate of AVP's control of social behavior is unclear (Kelly and Goodson, 2013a; Ludwig and Stern, 2015; Dumais and Veenema, 2016). In most animals, AVP is synthesized in several cell groups, each of which projecting to distinct brain areas (De Vries and Boyle, 1998; Rood and De Vries, 2011; Rood et al., 2013). AVP cells in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) contribute to the most pronounced sex differences in AVP innervation in brain (De Vries and Boyle, 1998). For example, male rats and mice have about two to three times as many AVP cells as females in these nuclei and the projections of these cells to areas such as lateral septum (LS) are denser as well (van Leeuwen et al., 1985; Rood and De Vries, 2011; Rood et al., 2013; Otero-Garcia et al., 2014).

Various studies suggest that AVP cells in the BNST and MeA modulate pro-social as well as antagonistic behaviors. In birds, for example, partial knockdown of AVP gene expression in the BNST reduces prosocial vocalizations and social interactions in birds while increasing male-male aggression (Kelly et al., 2011; Kelly and Goodson, 2013a, 2013b). Evidence for involvement of AVP projections from the BNST and MeA in social behavior in mammals is less direct. For example, the density of AVP fibers in BNST and MeA projection areas and c-Fos activation in AVP cells in the BNST correlate negatively with aggression in male mice and rats (Compaan et al., 1993; Everts et al., 1997; Ebner et al., 2000; Beiderbeck et al., 2007; Veenema et al., 2010) but positively with prosocial behavior (Goodson et al., 2009; Ho et al., 2010). In addition, injecting specific V1a receptor agonists or boosting V1a receptor expression in target areas of AVP cells in the BNST and MeA promotes affiliation in voles (Wang et al., 1994; Liu et al., 2001; Pitkow et al., 2001; Lim and Young, 2004; Lim et al., 2004) and social recognition and active social behaviors in rats (Dantzer et al., 1988; Veenema et al., 2012). In rats, where AVP release in the septum, one of the most prominent projection areas of BNST and MeA AVP cells

(De Vries and Buijs, 1983; De Vries and Panzica, 2006), correlates positively with intermale aggression in a resident-intruder test, a behavioral response could be blocked by retro-dialysis of an AVP antagonist (Veenema et al., 2010). None of these results, however, can be tied with certainty to AVP cells in the BNST and MeA, as all these areas receive AVP input from other sources as well, most importantly the PVN (e.g., Rood et al. 2013). In addition, AVP released dendritically from neurosecretory neurons in the hypothalamus may reach these areas as well (Ludwig and Stern 2015). To directly test the hypothesis that AVP cells in the BNST modulate social behavior, I injected an adeno-associated virus (AAV) with a Cre-dependent, genetically modified executioner caspase-3 complex (Yang et al., 2013; Unger et al., 2015b) into the BNST of adult AVP-iCre+ and AVP-iCre- (Mieda et al., 2015) male and female mice, which specifically deleted AVP cells in the area, and tested the effects of these deletions on social investigation, courtship ultrasonic vocalizations (USV; (Chabout et al., 2015)), and territorial urine marking (UM; (Arakawa et al., 2008b)), all aspects of mouse communication known to show pronounced sex differences (Crawley, 2012; Lehmann et al., 2013; Wöhr, 2014).

2.2 Methods

2.2.1 Animals and Husbandry

All mice were maintained at 22°C on a 12:12 reverse light cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems, Centennial, CO, USA), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were performed in accordance with the Georgia State University animal care committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects:

Founding AVP-iCre mice were obtained from Dr. Michihiro Mieda (Kanazawa University, Japan). These mice were generated using a bacterial artificial chromosome (BAC) that expressed codon-improved Cre recombinase (Shimshek et al., 2002) under the transcriptional control of the AVP promoter (AVP-iCre mice). In these animals, iCre expression is found in the bed nucleus of the stria terminalis and the medial amygdala, as well as in hypothalamic areas (Mieda et al., 2015). Subjects were derived by crossing heterozygous iCre+ mutants to wildtype C57Bl/6J mice and genotyped (ear punch) by polymerase chain reaction (PCR) at 21-24 days of age (Transnetyx, Cordova, TN, USA). Both iCre+ and iCre- littermates were used in behavioral experiments. All subject mice were singly-housed for a minimum of one week.

Stimulus animals:

CD1(ICR) (Charles River Laboratories, Wilmington, MA, USA) mice were used as stimuli for behavioral testing and to provide male and female subjects with social experience because strain differences between subjects and stimulus mice increase social investigation(Gheusi et al., 1994). Mice were used at 9-16 weeks of age and were novel and unrelated to the subject to which they were exposed.

Female stimulus mice were grouped-housed, ovariectomized and implanted with an estradiol capsule (GDX+E), and given two sexual experiences before testing. Two groups of stimulus males were used for behavioral testing. Mice that were used as subordinate mice in the home cage aggression tests and for providing aggressive experience to subjects, were grouped-housed, gonadectomized (GDX) and subjected to two aggressive encounters with a dominant male. Mice in the second group, which provided sexual experience to female subjects and served

as sexual partners during copulatory tests and as stimulus animal in the 3-chamber social test, were singly-housed, gonadectomized and implanted with testosterone (GDX+T), and given two sexual experiences before testing.

2.2.2 Viral Vector and Surgery

Viral Vector

AVP driven-, Cre-expressing-BNST neurons were ablated using an adeno-associated virus (AAV-flex-taCasp3-TEVp; serotype 2/1; 3×1012 IU/mL; University of North Carolina at Chapel Hill Vector Core) that encodes, in a Cre-dependent fashion, a mutated pro-caspase-3 and its activator (TEVp) (Figure 2-1a). This system activates an apoptotic signaling cascade, cleaving multiple structural and regulatory proteins critical for cell survival and maintenance (Yang et al., 2013; Unger et al., 2015a) and thereby inducing far less inflammation than other lesion approaches (Morgan et al., 2014).

Surgery

All surgeries were carried out using 1.5-3% isoflurane gas anesthesia in 100% oxygen; 3 mg/kg of carprofen was given before surgery to reduce pain.

Stereotaxic surgery

Mice were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand operated drill was used to make holes in the skull exposing the dura. For all subjects, 500 nl of AAV-flex-taCasp3-TEVp was delivered bilaterally to the BNST (coordinates: AP -0.01 mm; ML ± 0.75 mm; DV 4.8 mm (Paxinos and Franklin, 2012) at a rate of 100 nl/min using a 5 µl Hamilton syringe with a 30-gauge beveled needle mounted on a stereotaxic injector.

Following virus delivery, the syringe was left in place for 15 minutes and slowly withdrawn from the brain.

Gonadectomy and Hormone Treatment

Testes were cauterized and removed at the ductus deferens via a midline abdominal incision. Silastic capsules (1.5 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) were filled with crystalline T (Sigma, St. Louis, MO, USA) and inserted subcutaneously between the scapulae after gonadectomy; this procedure leads to physiological levels of T (Barkley and Goldman, 1977; Matochik et al., 1994). To further reduce aggression in stimulus animals (Beeman, 1947), some males were gonadectomized, but did not receive a T implant (GDX).

The ovaries of stimulus female mice were removed by cauterization at the uterine horn and attendant blood vessels. Silastic capsules (0.7 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) containing estradiol benzoate (E) (diluted 1:1 with cholesterol) were implanted subcutaneously in the scapular region immediately following ovariectomy (Bakker et al., 2002; Ström et al., 2012) (GDX + E). To induce sexual receptivity, stimulus females were injected subcutaneously with 0.1 ml of progesterone (500 μg dissolved in sesame oil, Sigma, St. Louis, MO, USA) four hours preceding sexual experience, urine collection, and behavioral testing (Veyrac et al., 2011).

2.2.3 Social Experience

As opposite-sex sexual experience and attaining competitive status ("social dominance") promote male and female communicative behaviors (Lumley et al., 1999; Roullet et al., 2011), mice received social experience over five consecutive days (sexual encounters on days 1 and 4, aggressive encounters on days 2 and 5, and no encounters on day 3).

Sexual Experience

Subjects were given two opportunities to interact with either a stimulus female (for male subjects) or a stimulus male (for female subjects). A sexually-experienced stimulus mouse was placed in the subject's home cage and removed five minutes after one ejaculation or ninety minutes in the absence of ejaculation. Subjects that did not show ejaculation (2 iCre- males) or did not elicit ejaculation (1 iCre+ female) on either trial were removed from further testing.

Aggressive Experience

Male subjects were exposed to two interactions with subordinate males treated with 40µl of GDX+T male urine applied to their backs. Gonadectomy, group housing, and social defeat of our subordinates reduce offensive aggression in mice, while GDX+T male urine provides subjects with a male urinary cue that elicits offensive aggression (Beeman, 1947; Connor and Winston, 1972; Van Loo et al., 2001). Subordinate stimulus males were placed in the subject's home cage and removed after the subject's first offensive attack (biting) within a ten-minute period. All subject males attacked the intruder male stimulus by the second encounter, and all subordinate stimulus males displayed submissive behavior, defined as defensive postures (e.g. on-back) , fleeing, and non-social exploring (Koolhaas et al., 2013). Female subjects were exposed to a female intruder; however, this did not elicit any attacks from either animal.

2.2.4 Experimental Procedure

All testing occurred within the first six hours of the dark cycle under red light illumination, except for the elevated plus maze. All tests were scored by an experimenter blind to the genotype of the subject. Three to four weeks after viral injections, subjects were habituated to the testing room and apparatus by handling and placing mice (for five minutes) in the 3-chamber apparatus (see below) each day for three days. On experimental days, subjects were adapted to

the experimental room for fifteen minutes prior to testing. First, we tested mice on an elevated plus maze to test for anxiety-related behavior (Lister, 1987). Mice were then tested in the 3-chamber apparatus over six consecutive days with a day off on the fourth day. Lastly, odor discrimination, copulatory, and aggressive behavior were measured in the subject's home cage (Figure 2-1a). Female subjects were tested irrespective of estrous cycle day, except during copulation testing, when they were in behavioral estrus. Prior research indicates minimal effects of estrous cycle on female mouse communicative behavior (Maggio and Whitney, 1985; Coquelin, 1992; Moncho-Bogani et al., 2004). Following testing, subjects were killed and their brain tissue was processed for in situ hybridization to detect AVP expression in BNST and nearby hypothalamic areas.

Social Behavior

USV, UM, and social investigation were recorded in an acrylic three-chamber apparatus (Crawley, 2007; Arakawa et al., 2008a; Moy et al., 2009) (Harvard Apparatus, Holliston, MA, USA; dimensions: 20.3 x 42 x 22 cm). Instead of a solid floor, the apparatus was placed on absorbent paper (Nalgene Versi-dry paper, Thermo Fisher Scientific, Waltham, MA, USA) so as to accurately measure UM. Animals were also tracked using motion detection software (ANY-maze, San Diego Instruments, RRID:SCR_014289). During testing with stimulus animals, subjects had access to either a stimulus animal in a cage (8 cm (D), 18 cm (H); 3 mm diameter steel bars, 7.4 mm spacing) or an empty cage placed at opposite corners of the outermost chambers of the apparatus. For testing with social odors, subjects had access to 50 µl of fresh urine from a stimulus animal or 50 µl saline pipetted onto a clean piece of filter paper (3 cm2), that was taped on the outside of cages. The location of stimulus and the "clean" cage were counterbalanced across animals. After placing the subject in the center of the middle chamber,

we measured, across a 5-minute trial, close investigation of clean and stimulus cages, distance traveled throughout the apparatus, time spent in the stimulus and clean cage chambers as well as USV and UMs. After testing, the apparatus and cages were thoroughly cleaned with 70% ethanol and allowed to dry before further testing. In all cases, urine stimulus from one sex was presented first followed by a live stimulus of that same sex; this order was then repeated for the opposite sex. In this fashion, mice experienced first weak (urine) then stronger social stimuli (stimulus animal; the order of male and female stimuli presentation was counterbalanced.

Investigation and Ultrasonic Vocalizations

Close investigation was defined as time spent sniffing within 2 cm of the stimulus or clean cage; climbing on the cage was not scored as investigation. USV were detected using a condenser microphone connected to an amplifier (UltraSoundGate CM16/CMPA, 10 kHz - 200 kHz, frequency range) placed 4 cm inside the apparatus and directly above the center compartment. USV were sampled at 200 kHz (16-bit) with target frequency set to 70 kHz (UltraSoundGate 116Hb, Avisoft Bioacoustics, Berlin, Germany). Recordings were then analyzed using a MATLAB (MATLAB, Mathworks, RRID:SCR_001622) plug-in that automates USV analysis (Van Segbroeck et al., 2017). Using this program, sonograms were generated by calculating the power spectrum on Hamming windowed data and then transformed into compact acoustic feature representations (Gammatone Filterbank). Each 200-millisecond window containing the maximum USV syllable duration was then clustered, via machine learning algorithms, into USV syllable types (repertoire units) based on time-frequency USV shape. Repertoire units that appeared as background noise were discarded. We counted the number of all USV produced by each subject. USV syllable types were identified from a subset of males (iCre- n = 6; iCre+ n = 7) using criterion previously described: short, composite,

downward, upward, 1 frequency jump, modulated, multiple frequency jumps, u-shape, flat, chevron (Hanson and Hurley, 2012).

Urine Marking

Following testing, the substrate sheet was allowed to dry for one hour and then sprayed with ninhydrin fixative (LC-NIN-16; Tritech Forensics Inc., Southport, NC, USA) to visualize urine marks (Meyer, 1957; Lehmann et al., 2013). After twenty-four hours, sheets were imaged (Sony DSC-S700 camera), binarized and analyzed using a computer-aided imaging software (ImageJ, RRID:SCR_003070). Urine marking was measured as the total area (cm2) of visualized ninhydrin urine marks in the entire arena. Urine marks that were larger than 6 cm2 and directed toward corners were counted as eliminative 'pools' and were counted separately (Bishop and Chevins, 1987).

Copulatory and Aggressive Behavior

To measure copulatory behavior, the stimulus mouse was placed in the subject's home cage and then removed five minutes after one ejaculation had occurred or if ninety minutes had elapsed without copulation. The latency and total time investigating the anogenital region, latency to mount, percent of females that were mounted, percent of male ejaculations, and number of mount rejections (female kicking male off during mounting attempt) in female subjects was recorded. To measure territorial aggression, subordinate stimulus males were placed in the subject's home cage and then removed after the subject's first offensive attack (biting) within a ten-minute period; the latency to first bite was recorded.

Odor Discrimination

We used a habituation-discrimination procedure on a subset of subjects to test whether they could distinguish between social odors (Baum and Keverne, 2002). Subjects were given five

consecutive 2-min presentations of an odor stimulus with one min intervals between presentations. Subjects were first presented with deionized water, followed by two non-social odors (100% almond, lemon, or vanilla extract, Frontier Natural Products, Norway, IA, USA), and then two urine samples (one from each sex) within their home cage. The sequence of odor presentation was counterbalanced within non-social or social odor category. Each odor stimulus (30 μl) was placed onto a clean piece of filter paper (3 cm2) taped to an empty food hopper such that subjects could contact the urine samples. Time spent sniffing within 2 cm of the filter paper was recorded. Food hoppers were cleaned with 70% ethanol and allowed to dry between each odor presentation.

Elevated-Plus Maze

The elevated plus maze (EPM) consisted of two open arms (30 x 5 cm) and two closed arms (30 x 25 x 5 cm) crossed perpendicularly and raised 60 cm above the floor. Subjects were placed at the arm intersection facing the open arm and were allowed to habituate to the apparatus for one minute; subjects were then observed for an additional five minutes. Animals were tracked by ANY-maze so that measurements of time spent in open and closed arms were recorded automatically whereas the number of risk assessment behaviors (stretch-attend posture, head-dips) were manually scored from video (Cole and Rodgers, 1993).

Urine Collection

Pooled urine samples were collected from stimulus females induced into estrus and from stimulus males (5-8 mice per sample). Estrous state was verified by color, swelling, and expanded size of vaginal opening (Caligioni, 2009). To collect urine, mice were picked up by the tail base and held by dorsal neck skin; this method was often sufficient to induce urination. If the mouse did not urinate, stroking its belly from an anterior to posterior direction stimulated bladder

voiding. Each mouse provided 15-50 µl of urine that was pooled into a 1.5 ml Eppendorf tube. Urine samples were used fresh within one hour of collection to prevent chemosignal degradation (Roullet et al., 2011).

2.2.5 Histology and In Situ Hybridization

Following testing, subjects were killed via CO2 asphyxiation. Brains were extracted and flash-frozen via submersion in 2-methyl-2-butanol (Sigma, St. Louis, MO, USA) for 10-20 s and stored at −80°C until sectioned. Coronal sections (20 µm) were cut with a cryostat (Leica CM3050 S, Leica Biosystems, Heidelberg, Germany) into three series and stored at −80°C. All tissue was handled in a RNase-free environment. Tissue was post-fixed in paraformaldehyde, followed by a wash in 2X saline-sodium citrate (SSC) and acetylation in a triethanolamine/acetic anhydride solution, rinsed in dH20, washed in acetone/methanol solutions (1:1), and again in 2X SSC. Tissue was first incubated at 65°C in hybridization buffer (50% deionized formamide, 1% yeast tRNA, 10% dextran sulphate, 1x Denhardt's solution, 5% 20x SSC) for 30 minutes before probe application. Riboprobes were developed from linearized PK Bluescript SK(+) with inserted mouse-vasopressin gene (NM_027106.4, Genscript) using digoxygenin (DIG)-conjugated uracil. Riboprobe synthesized from this plasmid was added to hybridization buffer at a concentration of 100 ng/100 µl and denatured at 90°C for five minutes. Tissue was then hybridized at 65°C for 24 hours in a humid chamber. The tissue was then subjected to two ten-minute washes in 2x SSC at room temperature followed by a fifteen-minute digestion with RNase A (10 g/ml in 2x SSC) at 37°C. This was followed by a thirty-minute 2x SSC wash at 56°C and two ten-minute 2x SSC washes at room temperature. The tissue was then quenched in 1% H2O2 in 1x SSC for fifteen minutes, rinsed twice in 1x SSC with .1% Tween followed by one five-minute TBS (20 mM Tris, 150 mM NaCl, pH 7.6) wash. Blocking solution (Normal

sheep serum and bovine caesin) was applied and tissue was incubated for thirty minutes followed by two-hour, room temperature incubation with anti-DIG-HRP (1:200, Roche Applied Sciences, Penzberg, Germany). Unbound antibody was washed away with three ten-minute washes in TBS-T (0.05% Tween in TBS). DIG-labeled probe signal was amplified and visualized using a TSA Plus Fluorescein kit (Perkin Elmer, Waltham, MA, USA) by incubating sections in a 1:50 dilution of the Fluorescein working solution for twelve minutes followed by three ten-minute washes in TBS. Tissue was then cover-slipped using Prolong Gold (Life Technologies, Carlsbad, CA, USA) for subsequent imaging and tissue analysis. Tissue processed using sense RNA probe generated no specific labeling. A subset of brain sections was Nissl stained to determine if viral vector injections resulted in a non-specific loss of BNST cells.

Tissue Analysis

Bilateral images were taken at 10x magnification using a Zeiss Axio Imager.M2 microscope (Carl Zeiss Microimaging, Göttingen, Germany), which transferred fluorescent images (FITC contrast reflector) to image analysis software (Stereo Investigator, MicroBrightField, RRID:SCR_002526). Imaging domains (2 mm2) were placed with reference to anatomical landmarks (ventricles, fiber tracts)(Paxinos and Franklin, 2012). Fluorescently labeled AVP mRNA-expressing cells were counted in the BNST in both hemispheres and averaged over three sections covering the extent of the AVP cell population in the BNST. In addition, we counted nearby accessory AVP mRNA expressing cells (Rood and De Vries, 2011) as well as average label intensity for AVP mRNA in the paraventricular nucleus of the hypothalamus (PVN) (ImageJ) to determine any possible off-target effects of our injections. Although we counted AVP mRNA-expressing cells in the PVN as well, AVP mRNA label intensity was chosen as the preferred method for quantification due to the difficulty of

discriminating between overlapping AVP cells in PVN. Lastly, we Nissl stained and imaged BNST tissue at 20x magnification to confirm no significant cell loss.

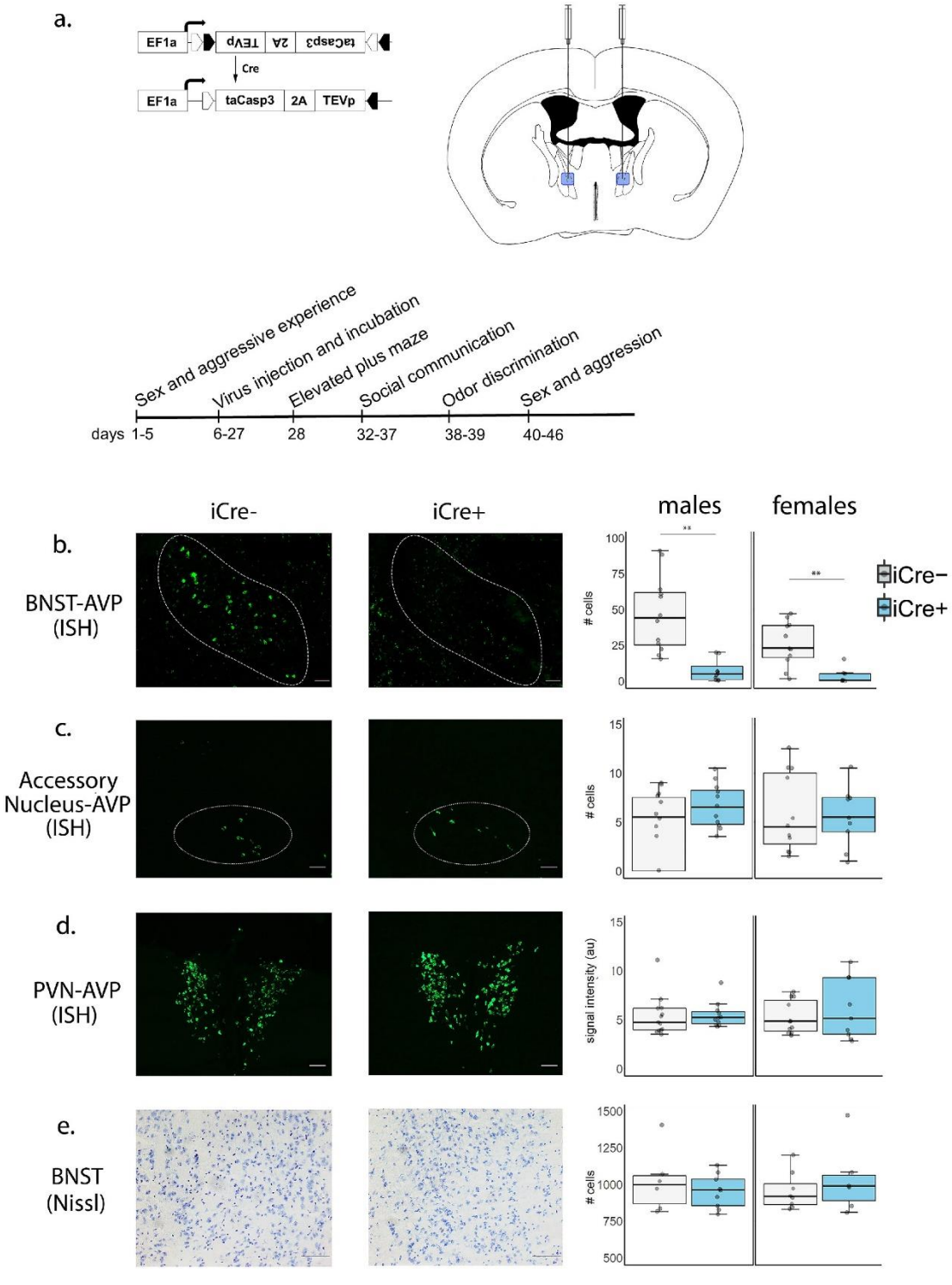

Figure 2-1- AVP histology and experiment timeline *(a) Cre-dependent adeno-associated virus (AAV-flex-taCasp3-TEVp) and location of bilateral BNST injection site; coordinates: AP -0.01 mm; ML ± 0.75 mm; DV 4.8 mm; modified from (Paxinos and Franklin, 2012). Timeline of experimental manipulations. (b) Example images of fluorescent in situ hybridization (ISH) labeled BNST AVP cells and boxplot of cell number. Within the BNST, a significant decrease in AVP cell label was observed in both iCre+ male and female mice compared to iCre- control animals (males: p = 0.00014; females: p = 0.0025). iCre- (n = 13) and iCre+ (n = 11) males and iCre- (n = 13) and iCre+ (n = 8) females. (c) Example images of fluorescent ISH-labeled accessory nucleus-AVP cells and boxplot of cell number. No significant AVP cell loss was observed between iCre+ and iCre- subjects (males: p = 0.98; females: p = 0.89). iCre- (n = 13) and iCre+ (n = 11) males and iCre- (n = 13) and iCre+ (n = 8) females. (d) Example images of fluorescent ISH-labeled PVN and boxplot of image intensity (arbitrary units). iCre+ and iCre- subjects did not differ in PVN signal intensity (males: t(20) = 0.66, p = 0.947; females: p = 0.29). iCre- (n = 13) and iCre+ (n = 10) males and iCre- (n = 13) and iCre+ (n = 8) females. (e) Example images of Nissl-stained BNST tissue and boxplot of cell number. No difference in BNST cell number between iCre+ and iCre- subjects was observed (males: p = 0.439; females: p = 0.44) iCre- (n = 6) and iCre+ (n = 9) males and iCre- (n = 8) and iCre+ (n = 6) females. In boxplots, dots indicate individual data points, bold horizontal lines illustrate the median, the areas above and below the lines show the 1st/3rd quartile. The vertical bars range from the minimal to the maximal values excluding outliers (± 1.35 standard deviations from interquartile range). Images were taken at 10x for fluorescent material and 20x for Nissl-stained tissue. Scale bar = 50 µm. ** indicates significant effect of genotype, p < 0.005.*

2.2.6 Statistical Analysis

All data were analyzed and graphed in R (3.4.4; R Core Team, 2017). Histology, social investigation, movement, UM (in male subjects), odor discrimination, and EPM data met the assumptions of parametric statistical tests. Therefore, we analyzed histological data with t-tests and data on social investigation, movement (distance travelled, time in chambers containing stimulus and clean cages) and male UM with mixed-model ANOVAs (between-subject factor: genotype (iCre+, iCre-); within-subject factors: sex of stimulus (male, female); preference for stimulus (stimulus, clean) followed by t-tests assessing genotype effects. Total distance travelled within the apparatus was analyzed using a mixed-model ANOVA (between-subject factor: genotype; within-subject factor: sex of stimulus) as was time spent in open/closed arms in the EPM test (between-subject factor: genotype; within-subject factor: arm); effects of genotype on

additional anxiety behaviors (stretch-attend, head-dips) were analyzed using t-tests. We determined whether subjects could discriminate between odors by comparing odor investigation on the last trial for one odor and odor investigation on the first trial of the subsequent odor using paired t-tests. The number of female UMs, USV, USV syllable type, measures of copulatory behavior, and aggression behavior were not normally distributed and could not be transformed, therefore, we analyzed genotype effects using pairwise Mann-Whitney U tests. Differences in proportion of animals engaging in copulatory/aggressive behaviors across genotype was assessed using chi-square tests. All post-hoc pairwise comparisons report Bonferroni-corrected p-values and Cohen's D for effect size when statistically significant. Results were considered significant if $p < 0.05$.

2.3 Results

2.3.1 Histology

Injection of a viral vector encoding a Cre-dependent cell-death construct into the BNST highly effectively reduced AVP cell numbers in both iCre+ males and females, which had only 10% of the number found in iCre- subjects (males: $t(22) = 4.57$, $p = 0.00014$, $d = 2.64$; females: $t(19) = 3.58$, $p = 0.0025$, $d = 2.02$, Figure 2-1b), without significantly reducing the number of nearby accessory AVP mRNA-expressing cells (males: $t(22) = -0.16$, $p = 0.98$; females: $t(19) = -0.15$, $p = 0.89$, Figure 2-1c) or total level of AVP mRNA label in the PVN (males: $t(20) = 0.66$, $p = 0.947$; females: $t(19) = -1.10$, $p = 0.29$, Figure 2-1d), suggesting that there were no significant off-target effects. We also observed no difference in the number of AVP mRNA-expressing cells in the PVN between genotype (males: $t(20) = 0.66$, $p = 0.514$; females: $t(19) = 0.79$, $p = 0.82$). In addition, Nissl-stained tissue from a subset of subjects revealed no overall cell loss in the BNST (males: $t(13) = 0.79$, $p = 0.439$; females: $t(12) = -0.80$, $p = 0.44$ (Figure 2-1e). Two iCre+

males and two iCre+ females were removed from the analysis as their AVP cell numbers in the BNST were more than 3 standard deviations above the mean, which we interpreted as off-target injections. Two additional iCre+ females were removed due to unilateral AVP cell loss in the PVN.

2.3.2 BNST AVP cell ablations in iCre+ males reduced male-male social investigation

Mice from both genotypes investigated female stimulus animals more than male stimulus animals (males subjects: $F(1,22) = 261.34$, $p < 0.00001$; female subjects: $F(1,19) = 55.92$, $p < 0.00001$) and had similar overall levels of investigation (male subjects: $F(1,22) = 0.62$, $p = 0.438$; female subjects: $F(1,19) = 1.60$, $p = 0.29$). However, iCre- and iCre+ males differed in preference for investigating the stimulus animal depending on the sex of stimulus ($F(1,22) = 11.16$, $p = 0.003$). Post-hoc comparisons revealed that iCre+ males significantly decreased investigation of male animals compared to iCre- littermates ($t(22) = 3.75$, $p = 0.004$, $d = 1.52$; Figure 2-2a) but not female stimulus animals ($t(22) = -0.70$, $p = 1.0$), while ablation of these cells in females did not affect social investigation ($F(1,19) = 0.004$, $p = 0.94$; Figure 2-2b).

Males of both genotypes investigated female urine more than male urine ($F(1,22) = 117.39$, $p < 0.00001$), whereas female subjects investigated male urine more than female urine ($F(1,19) = 60.33$, $p < 0.00001$). iCre+ mice did not investigate urine differently from iCre- littermates (males: $F(1,22) = 0.22$, $p = 0.64$; females: $F(1,19) = 2.91$, $p = 0.10$; Figure 2-2c-d).

Overall, mice of both genotypes traveled similar distances throughout the three-chamber apparatus (males: $F(1,22) = 3.48$, $p = 0.33$; females: $F(1,19) = 3.47$, $p = 0.08$); this pattern did not differ when presented with female or male stimulus animals (males: $F(1,22) = 0.01$, $p = 0.92$; females: $F(1,19) = 0.01$, $p = 0.91$). There were no differences between genotypes in the amount

of time mice spent in the stimulus and clean chamber zones (males: F(1,22) = 0.13, p = 0.72; females: F(1,19) = 0.04, p = 0.85, Table 1).

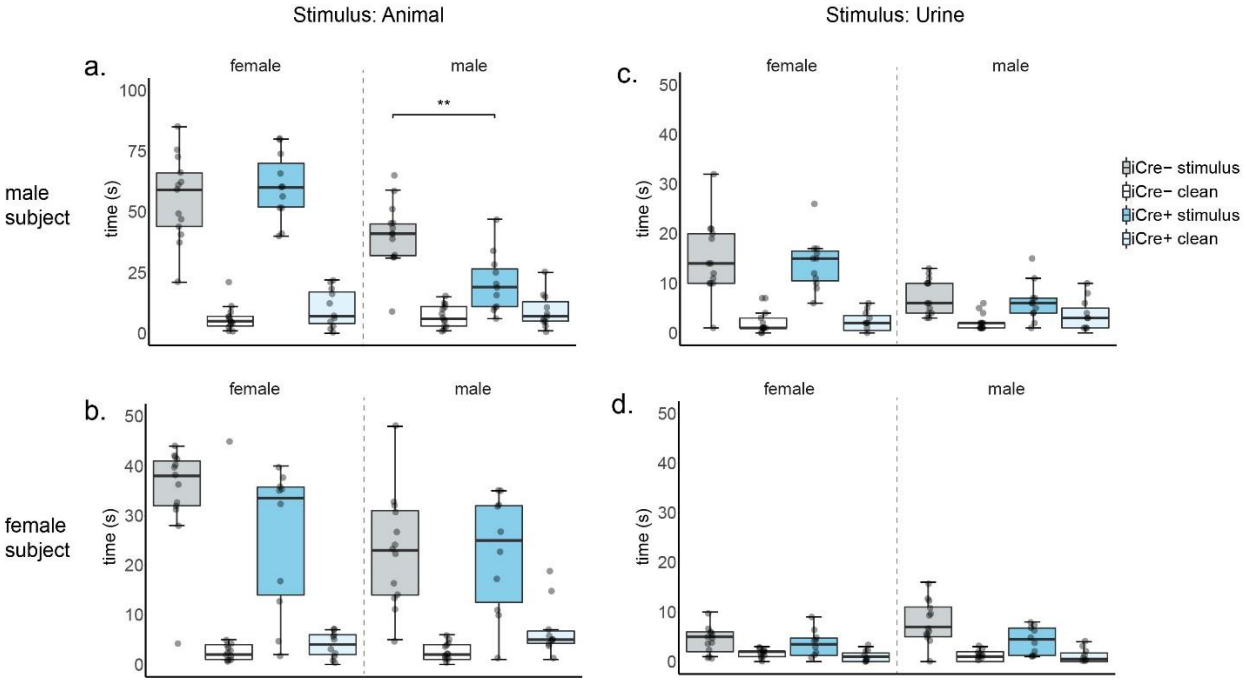

Figure 2-2- Boxplot and individual data points of time spent investigating male or female animals or their urine versus clean control stimuli within the three-chamber apparatus *(a-b) Time spent investigating either a caged female vs. a clean cage or a caged male vs. clean cage. (a) iCre- (males: n = 13, females: n = 13) and iCre+ mice (males: n = 11, females: n = 8) differed in preference for investigating the stimulus depending on the sex of stimulus (p = 0.003). Post-hoc analysis revealed iCre+ males significantly decreased investigation of the male animal compared to iCre- littermates p = 0.004 (b) iCre- and iCre+ females did not differ in investigation (p = 0.94). (c-d) Time spent investigating either female urine or male urine vs. saline control placed on filter paper. iCre- and iCre+ subjects did not differ in their investigation of female or male urine. (c) male subjects: p = 0.64. (d) female subjects: p = 0.10. Note scale difference in animal investigation time between male and female subjects. ** indicates significant effect of genotype, p = 0.004.*

2.3.3 BNST AVP cell ablated iCre+ males increased urine marking to females

Male mice urine marked (UM) more in the presence of females than males (F(1,22) = 52.62, p < 0.00001. iCre- and iCre+ males differed in overall UM (F(1,22) = 23.72, p = 0.00007) and UM depending on the sex of stimulus (F(1,22) = 21.02, p = 0.00015). Post-hoc comparisons

revealed that iCre+ males significantly increased UM to the female stimulus compared to iCre- littermates ($t(22) = 4.6$, $p = 0.000112$, $d = 2.04$; Figure 2-3a) but not to the male stimulus ($t(22) = 1.45$, $p = 0.16$). iCre- and iCre+ females did not differ in UM in the presence of males ($U = 32$, $p = 0.32$) or females ($U = 20$, $p = 0.15$, Figure 2-3a). Neither male nor female subjects differed in UM to urine depending on the sex of stimulus (male subjects: $F(1,22) = 1.16$, $p = 0.70$; female subjects: $U = 52$, $p = 0.467$ (female stimulus), $U = 32$, $p = 0.858$ (male stimulus)). Only one male (iCre+) pooled urine in the male condition, therefore we did not analyze pooled urine.

Table 1- Table of median (interquartile range) distance traveled and time spent in stimulus or clean cage chamber. iCre- and iCre+ mice did not differ in distance traveled, time spent in animal stimulus or clean stimulus chambers.

	Male Subjects				**Female Subjects**			
	iCre-		**iCre+**		**iCre-**		**iCre+**	
stimulus	***female***	***male***	***female***	***male***	***female***	***male***	***female***	***male***
distance traveled (m)	.33 (.22-.54)	.32 (.16-.5)	.34 (.19-.48)	.29 (.13-.44)	.28 (.08-.38)	.23 (.01-.47)	.35 (.16-.42)	.34 (.04-.5)
time in stimulus chamber (s)	185 (129-248)	127 (78-195)	175 (111-229)	97 (15-209)	174 (102-246)	147 (30-227)	155.75 (23-198)	119 (91-155)
time in clean chamber (s)	86 (45-122)	127 (73-155)	90 (49-158)	130 (67-231)	90 (43-176)	113 (52-240)	106 (48-170)	136 (74-282)

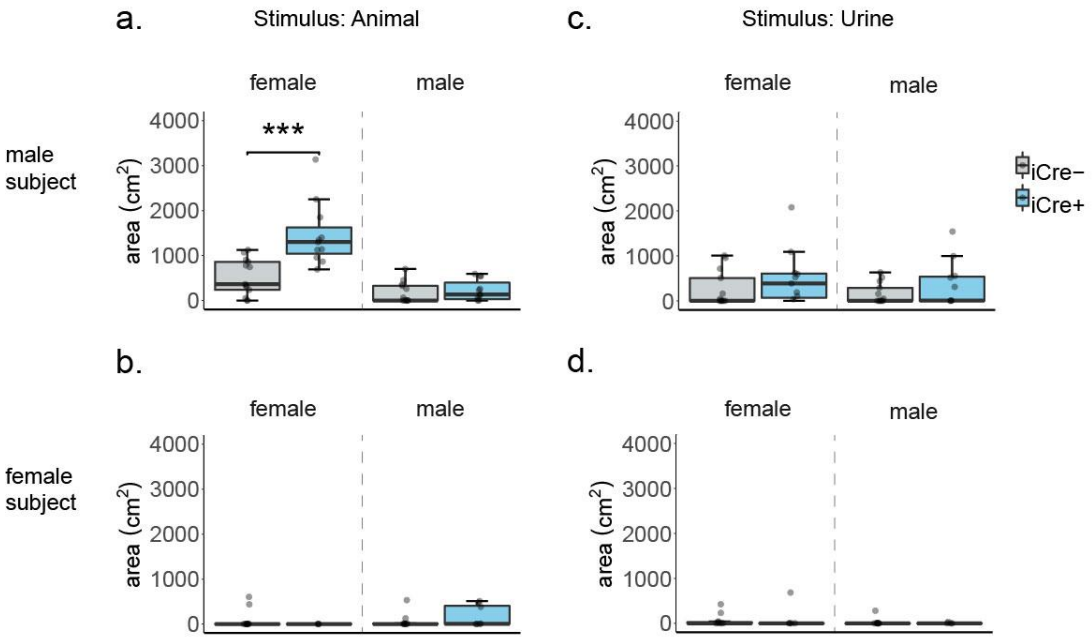

Figure 2-3- BNST AVP cell ablations in iCre+ males increased urine marking to females. *Boxplot and individual data points of UM in presence of males or females or their urine within the three-chamber apparatus. (a) iCre- (males: n = 13, females: n = 13) and iCre+ mice (males: n = 11, females: n = 8) differed in UM depending on the sex of stimulus (p = 0.00015). Post-hoc analysis revealed iCre+ males significantly increased UM to the female stimulus compared to iCre- littermates (p = 0.000112) (b) iCre- and iCre+ females did not differ in UM to stimulus animals (p = 0.32 (males), p = 0.15 (females)). (c-d) UM with either female urine or male urine present. iCre- and iCre+ subjects did not differ in UM to female or male urine. (c) male subjects: (p = 0.70). (d) female subjects: (p = 0.467) (female stimulus), (p = 0.858) (male stimulus). *** indicates significant effect of genotype, p = 0.00015.*

2.3.4 BNST AVP cell ablations in iCre+ animals did not alter ultrasonic vocalizations

The total number of USVs emitted when iCre- and iCre+ mice were placed with females (male subjects: U = 64, p = 1.0; female subjects: U = 58, p = 0.18) or males animals did not differ by genotype (male subjects: U = 47, p = 0.33; female subjects: U = 52, p = 0.16, Figure 2-4a-b). Mice from both genotypes also did not differ in USVs to female urine (male subjects: U = 60, p = 0.77; female subjects: U = 53, p = 0.26) or male urine (male subjects: U = 52, p = 0.5; female subjects: U = 60, p = 0.49, Figure 2-4c-d). Additionally, BNST AVP cell ablations did not change the percentage of USV syllable types produced between male genotypes (short: U =

10, p = 0.14, composite: U = 22, p = 1.0, downward: U = 17, p = 0.63, upward: U = 17, p = 0.63, 1 frequency jump: U = 15, p = 0.45, modulated: U = 27, p = 0.37, multiple frequency jumps: U = 24.5, p = 0.63, u-shape: U = 19.5, p = 0.83, flat: U = 18, p = 0.73, chevron: U = 39, p = 0.08; Figure 2-4e-f).

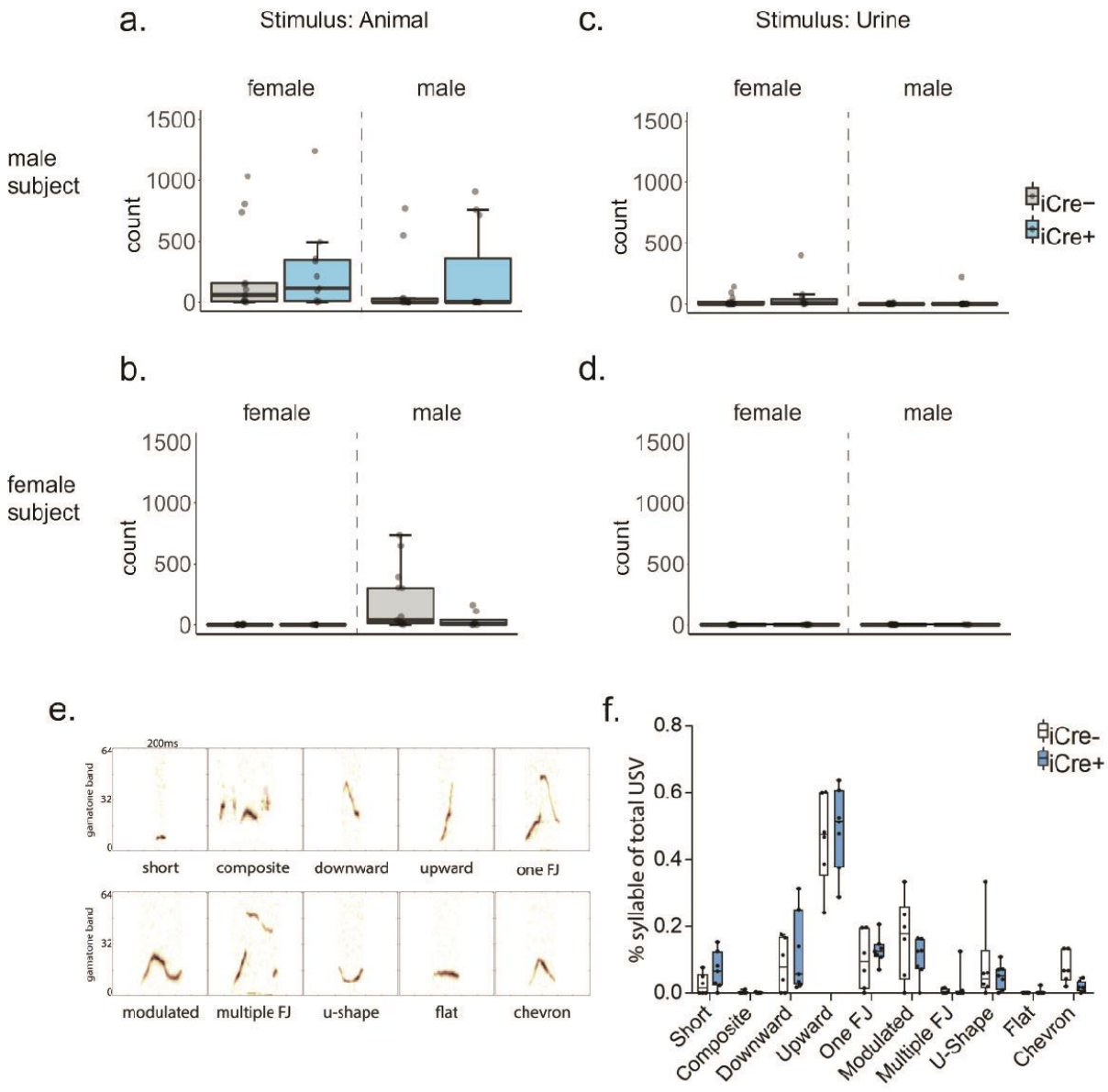

Figure 2-4- BNST AVP cell ablations in iCre+ animals did not alter ultrasonic vocalizations. *Boxplot and individual data points of USV in presence of a male or female or their urine within the three-chamber apparatus. (a-b) iCre- (males: n = 13, females: n = 13) and*

iCre+ mice (males: n = 11, females: n = 8) did not differ by genotype in USV production. (a) male subjects: (p = 1.0) (female stimulus), (p = 0.33) (male stimulus) (b) female subjects: (p = 0.18) (female stimulus), (p = 0.16) (male stimulus) (c-d) USV with either female urine or male urine present. iCre- and iCre+ subjects did not differ in USVs to female or male urine. (c) male subjects:(p = 0.77) (female stimulus), (p = 0.5) (male stimulus). (d) female subjects: (p = 0.26) (female stimulus), (p = 0.49) (male stimulus). (e) USV emitted by male mice were gammatone-transformed (200 ms window) and divided into 10 categories of calls based on spectrographic parameters. (f) Male USV syllable type (iCre- n = 6; iCre+ n = 7). BNST-AVP ablations did not change the percentage of USV syllable types produced between genotypes.

2.3.5 BNST AVP cell ablations in iCre+ animals did not influence anxiety

All mice spent less time in the open arm than the closed arm of the EPM (male subjects: F = (1,22) 51.74, p < 0.000001; female subjects:(1,19) = 89.41, p < 0.000001). iCre- and iCre+ mice did not differ in the time spent in either arm of the EPM (male subjects: F(1,22) = 2.81, p = 0.11; female subjects: F(1,19) = 1.30, p = 0.59, Figure 2-5a-b). Additionally, both genotypes did not differ in frequency of stretch attend postures or head dips (male subjects: F(1,22) = 3.90, p = 0.16; female subjects: F(1,19) = 0.80, p = 0.38, Figure 2-5c-d).

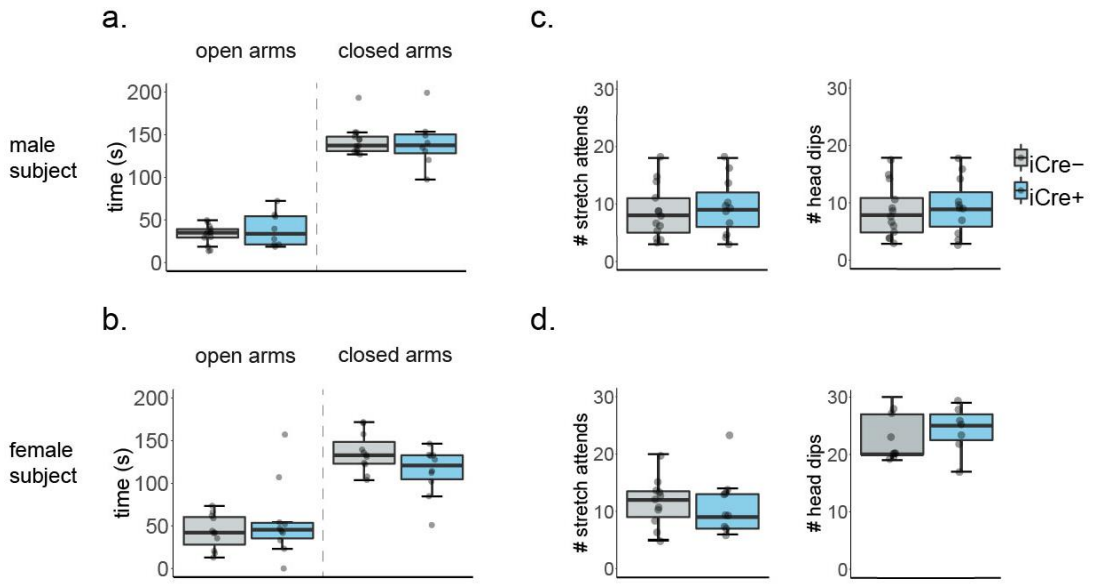

Figure 2-5- BNST AVP cell ablations in iCre+ animals did not influence anxiety-like behavior. *Boxplot and individual data points of time spent in the open and closed arms within the EPM, number of stretch attends, and number of head dips (a-b) iCre- (males: n = 13, females: n = 13) and iCre+ mice (males: n = 11, females: n = 8) did not differ by genotype in*

time spent in open and closed arms. (a) male subjects: (p = 0.11) (b) female subjects: (p = 0.59) (c-d) iCre- (males: n = 13, females: n = 13) and iCre+ mice (males: n = 11, females: n = 8) did not differ by genotype in number of stretch attends or head dips (c) male subjects: (p = 0.16) (d) female subjects: (p = 0.38).

2.3.6 BNST AVP cell ablations in iCre+ animals did not alter male copulatory behavior but did reduce mounting of females

iCre+ and iCre- males mounted females with similar latencies and did not differ in the percentage of subjects mounting and/or ejaculating (t(22) = 1.04, p = 0.31 Figure 2-6a, c). However, it took longer for males to mount iCre+ females (U = 22, p = 0.03) and fewer iCre+ females were mounted overall as compared to iCre- females (X2 (2), p < 0.000001). However, female iCre+ mice did not reject stimulus males more frequently than did iCre- females (t(19) = 0.52, p = 0.61). One iCre+ female was removed during the sex behavior test and sex behavior analysis because the stimulus male attacked the female.

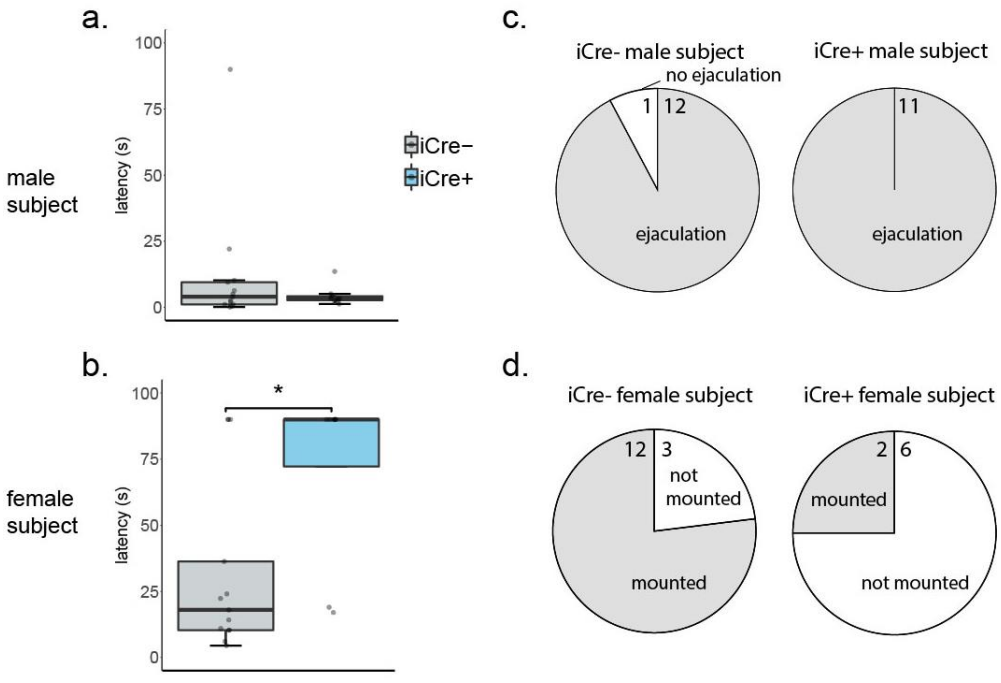

Figure 2-6- BNST AVP cell ablations in iCre+ animals did not alter male copulatory behavior but did reduce mounting of females. *Boxplot and individual data points of male subject's latency to mount a female (a) or female subject's latency to be mounted (b). Pie chart summarizing proportion of male subjects that ejaculated (c) or the proportion of female subjects*

*mounted by a male (d) with number of subjects in each category indicated. (a, c) iCre- (n = 13) and iCre+ (n = 11) male mice did not differ by genotype in their latency to mount females or in the percentage of subjects ejaculating. (b, d) iCre+ (n = 8) female mice were mounted at longer latencies (p = 0.03) and proportionally less (p < 0.000001) than iCre- (n = 13) females. * indicates significant effect of genotype, p = 0.03.*

2.3.7 BNST AVP cell ablation did not alter territorial aggression

The proportion of male subjects that attacked the subordinate intruder in their home cage did not differ between genotypes (X2 (2), p = 0.85) nor did they differ in attack latency (U = 60, p = 0.955, Figure 2-7a-b). Female subjects did not attack female intruders.

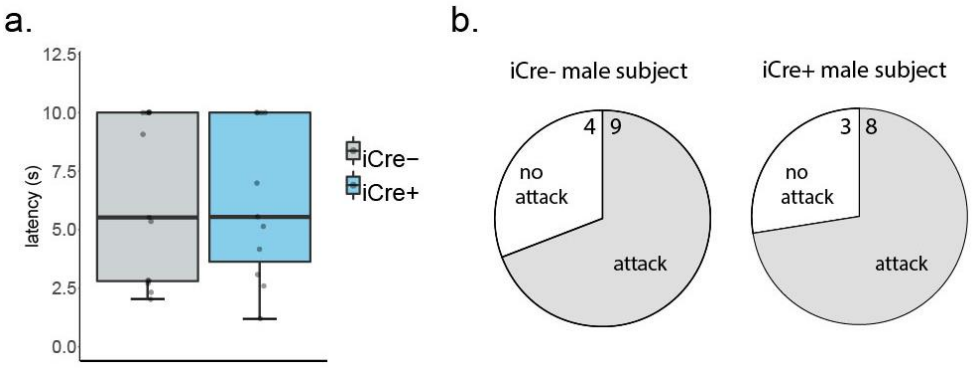

Figure 2-7 - BNST AVP cell ablation did not alter territorial aggression.*(a) Boxplot and individual data points of male subject's latency to attack a subordinate intruder male. iCre- (n = 13) and iCre+ (n = 11) male mice did not differ by genotype in latency to attack the intruder (p = 0.955). (b) Pie chart summarizing proportion of male subjects that attacked the subordinate intruder in their home cage with number of subjects in each category indicated. Subjects did not differ between genotypes (p = 0.85).*

2.3.8 BNST AVP cell ablations did not change the ability to discriminate between social odors

Males and females of both genotypes were able to discriminate between male and female urine odors (males: t(10) = 7.936, p = 0.00001 (iCre-), t(4) = 8.313, p = 0.001 (iCre+); females: t(4) = 7.071, p = 0.002 (iCre-), t(6) = 5.211, p = 0.002 (iCre+)) and could distinguish between non-social and social odors (males: t(10) = 11.41, p < 0.00001 (iCre-), t(4) = 6.675, p = 0.003

(iCre+); females: t(4) = 6.197, p = 0.003 (iCre-), t(6) = 7.454, p = 0.0003 (iCre+)). However, subjects' ability to discriminate between non-social odors was not robust. Although both iCre+ and iCre- males discriminated between water and almond odor, females did not, and no subjects discriminated between the two non-social odors (Figure 2-8).

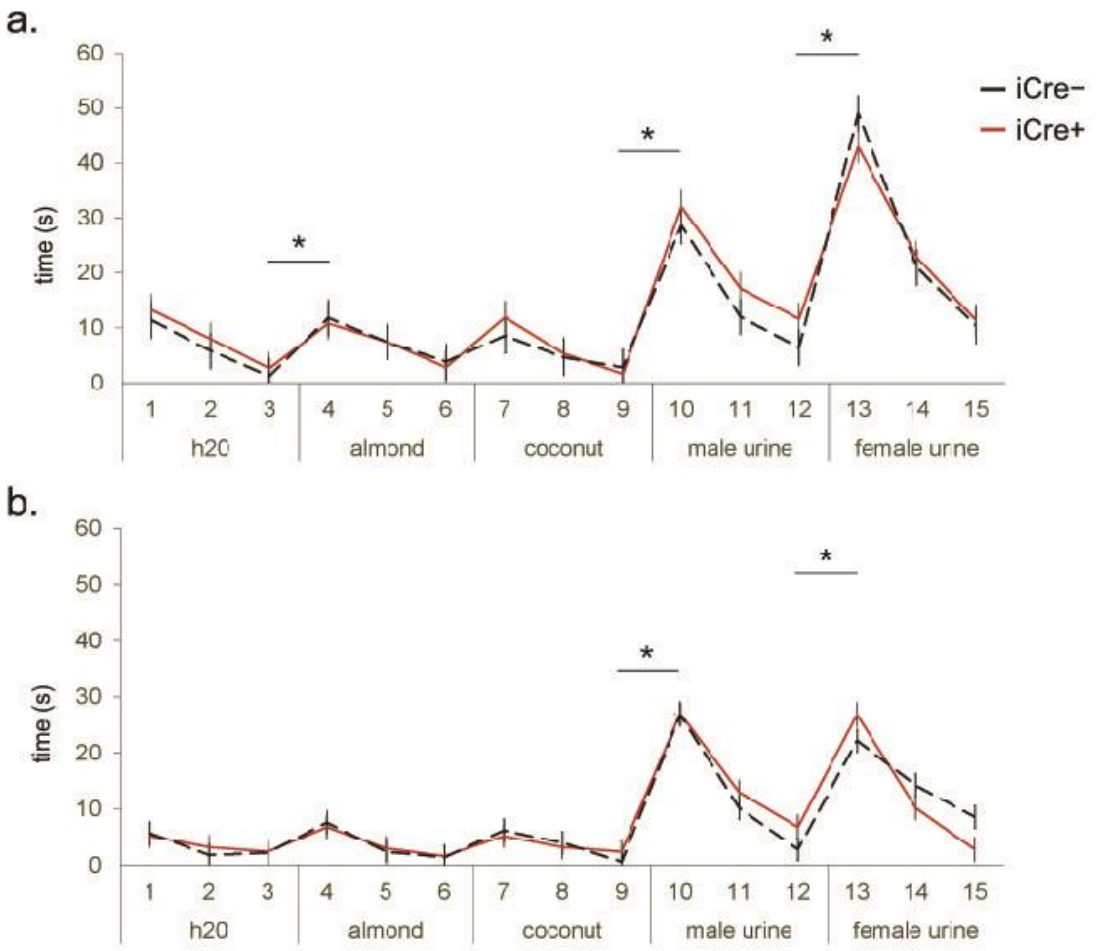

Figure 2-8- BNST AVP cell ablations did not change the ability to discriminate between social odors. *Time spent investigating water, almond or coconut extract, male urine, or female urine. Males and females of both genotypes were able to discriminate between male and female urine odors (males: (p = 0.00001) (iCre-), (p = 0.001) (iCre+); females: (p = 0.002) (iCre-), (p = 0.002) (iCre+)) and could distinguish between non-social and social odors (males: (p < 0.00001) (iCre-), (p = 0.003) (iCre+); (p = 0.003) (iCre-), (p = 0.0003) (iCre+)). However, subjects' ability to discriminate between non-social odors was not robust. Although both iCre+ and iCre- males discriminated between water and almond odor, females did not, and no subjects discriminated between the two non-social odors. Data are expressed as mean ± SEM; trial*

*numbers are given on the x-axis. * indicates significant difference (all $p < 0.005$) between investigation of odors, irrespective of genotype.*

2.4 Discussion

We found that deletion of the sexually dimorphic AVP cell group in the bed nucleus of the stria terminalis (BNST) significantly affected social behavior in males, reducing social investigation of other males and increasing urine marking (UM) in the presence of a female. In females, which have significantly fewer AVP BNST cells, similar deletions minimally affected social behavior and communication. This is the first time that direct alteration of a male-biased, sexually-dimorphic population of neuropeptidergic cells has caused a sex-difference in mammalian social responses. While several studies have directly tested the social function of sexually-dimorphic cell populations (Yang et al., 2013; Scott et al., 2015; Unger et al., 2015b; Wei et al., 2018), only one has found a strong sex-difference in social behavior tied to an anatomical sexual dimorphism, in this case a female-biased one (Scott et al., 2015)). Our results indicate that sex differences in AVP cells in the BNST contributes to sexually dimorphic components of social communication.

Our experiments do not allow us to conclude that removal of AVP production in the BNST, by and of itself, caused the behavioral effects observed in this study. Lesioning AVP cells also removed all other neuroactive substances co-expressed by these cells, for example, galanin (Miller et al., 1993), which also has been implicated in control of social behavior (Wu et al., 2014). Consequently, our behavioral results may be due to depletion of AVP, or of co-transmitters from BNST projections, or both. Our finding of reduced male-male investigation following BNST AVP cell deletion, however, is strikingly similar to the effects of RNA interference for the homologous peptide arginine vasotocin (AVT) in the BNST of territorial

finches (Kelly and Goodson, 2013b) and suggests that this effect is mainly due to a reduction of AVP signal from the BNST.

Our cell deletion approach is, by design, permanent, and so behavioral effects may reflect molecular, cellular, and anatomical adjustments or compensations to chronic depletion of AVP cells in the BNST. Indeed, long-term pharmacological reduction of V1a receptor activity in the lateral septum, a key target of AVP cells in the BNST (De Vries and Panzica, 2006), produces different changes in behavior than acute receptor blockade (Liebsch et al., 1996; Everts and Koolhaas, 1999). A lack of an effect on a behavior, therefore, does not exclude involvement of these cells in that behavior. Nevertheless, our findings of male-specific alterations in social interactions and communication following deletion of AVP cells in the BNST strongly indicate that in males these cells are critical for these functions.

One of the strongest effects of deletion of AVP cells in the BNST was the reduction of same-sex social investigation, which we found in males but not in females. Importantly, this reduction was not due to a lack of general social interest, as investigation of females was unchanged. It was not due to changes in general chemosensory function or motor behavior either, as odor habituation or discrimination of non-social odors and detection of social odors (male or female urine) was unaffected and measures of general activity were also not changed. This reduction can also not be explained by a general increase in anxiety-like behaviors, because the lesions did not affect behavior in the anxiogenic elevated plus-maze (EPM). Instead, it suggests that one function of AVP cells in the BNST is to generate male-typical approach, investigation, and assessment of potential territorial competitors (Oldfield et al., 2015), which is consistent with the observation that knocking down AVT mRNA in the BNST of territorial birds reduces social contact with other males (Kelly and Goodson, 2013b), and that over-expression of V1a

receptors (Landgraf et al., 2003) or AVP injections (Koolhaas et al, 1991) in the lateral septum, a key target for AVP projections from the BNST (De Vries and Panzica, 2006), increases active male-male interactions and aggressive behavior in rats. Indeed, retrodialysis of V1aR antagonist into the lateral septum of male rats blocks further territorial aggression during repeated resident-intruder tests (Veenema et al., 2010). Our finding that deleting AVP cells in the BNST reduced investigation of potential competitors is consistent with these findings. Our failure to see a change in aggressive behavior is not. One explanation for this apparent discrepancy is that AVP from other sources contribute to stimulating effects on aggressive behavior in areas such as the lateral septum. For example, AVP cells in the MeA show similar dimorphisms as BNST cells and project to overlapping areas (De Vries and Panzica, 2006). Lesioning both cell groups may be necessary to identify all behaviors in which these sexually dimorphic cell groups are critically involved. Nevertheless, AVP cells in the BNST appear to be required for the proactive investigation of credible social threats rather than for offensive aggression per se.

Ablation of AVP cells in the BNST of male, but not female, mice strongly increased urine marking (UM) toward females, suggesting that these cells normally suppress male scent marking toward females. This increase in UM is not due to excessive eliminative urination because it was not observed toward male stimuli nor was it manifested as an increase in pooled urine. It is possible that this increase is simply due to increased proximity and interest in females. However, males with deletions of AVP cells in the BNST did not differ from control animals in time spent investigating female stimuli, making this explanation less likely. Although UM strongly depends on chemosensory processing (Maruniak et al., 1986; Labov and Wysocki, 1989), the increase in UM by lesioned males was not driven primarily by female urine cues because marking to female urine was not increased, suggesting that other cues, chemosensory as

well as non-chemosensory, may drive this increase in scent marking. Given the metabolic costs of urination (Gosling et al., 2000) and the increased risks associated with attraction of aggressive competitors and predators toward urine marks (Desjardins et al., 1973; Roberts et al., 2001), UM should be strongly regulated so that signaling occurs in only specific social contexts. The present results indicate that the BNST-AVP cells may be a key part of the neural system that adaptively regulates this critical signaling behavior.

Although AVP/AVT has been implicated in the production of vocalizations across taxa (Goodson and Bass, 2001), deleting the AVP cells in the BNST did not significantly alter the production of ultrasonic vocalizations in a reproductive context. This is consistent with the few studies examining male courtship vocalizations (in birds) that report no effect of central infusions of AVT or V1a antagonists on female-directed singing (Goodson and Adkins-Regan, 1999; Goodson and Evans, 2004) or got mixed results from antisense knockdown of BNST-AVP, with one study finding no effect (Kelly et al., 2011) and another observing decreased singing (Kelly and Goodson, 2013b). Most other studies supporting a role for AVP/AVT in vocalizations have not identified the relevant neuroanatomical source of the peptide or its locus of action (Wilczynski et al., 2017) or tended to focus on stress-related or aggressive vocalizations across development (Winslow and Insel, 1993; Bleickardt et al., 2009; Iijima et al., 2014; Lukas and Wöhr, 2015; Paul et al., 2016; Freeman et al., 2018).

Our study also points to a limited, sexually dimorphic role of BNST AVP cells in reproductive behavior. Removal of these cells in males did not alter copulatory behavior, which is consistent with the absence of copulatory deficits in whole-animal knockouts of V1a or V1b receptors (Wersinger et al., 2004, 2007). However, unexpectedly, ablating these cells in females delayed, and reduced the number of times they were mounted by males. This effect may

represent reduced attractiveness of the female or an increased rejection of the male. The latter possibility seems less likely, as we did not detect any change in male investigation toward lesioned females, nor did such females reject males more frequently. It is possible that these females show decreases in cryptic proceptive and/or receptive behaviors. Although AVP has been implicated in female rat sexual behavior, the pattern of prior results is opposite to our findings: in rats, AVP reduced, whereas V1a receptor antagonists increased, female sexual behavior in rats (Södersten et al., 1983; Pedersen and Boccia, 2006). However, as in these studies peptides were injected into the lateral ventricle, it is unclear with what AVP system they interacted.

Although central AVP has been repeatedly implicated in the generation of anxiety states (Ebner et al., 2002; Bielsky et al., 2004; Raggenbass, 2008), we did not observe changes in anxiety-like behavior of lesioned males or females in a non-social anxiogenic environment (Hogg, 1996). This is somewhat surprising because AVP in the LS — an important target of the BNST AVP cells (De Vries and Panzica, 2006) — controls anxious states. For example, injections of AVP or V1aR antagonists, or V1aR knockdown within LS all support the idea that AVP is anxiogenic at this site (Landgraf et al., 1995; Liebsch et al., 1996; Beiderbeck et al., 2007). However, since large lesions of BNST have no effect on anxiety-like behavior in the EPM task either (Treit et al., 1998), it may simply mean that, as with aggressive behavior, AVP cells in the BNST are not critically involved in anxiety-like behaviors, and that AVP derived from other sources, such as the the paraventricular nucleus of the hypothalamus or the amygdala (Rood et al., 2013), also drive AVP's anxiogenic action in the septum and elsewhere.

Conclusions

A growing body of literature indicates that vasopressin plays a sexually dimorphic role in control of social and anxiety-related behaviors. In humans, for example, intranasal vasopressin stimulates reciprocation of cooperation in males and conciliatory behavior in females, while activating brain areas implicated in reward, social bonding, arousal and memory in males, but not in females (Rilling et al., 2014). In hamsters, hypothalamic injections of vasopressin stimulate aggression in males, while reducing it in females (Terranova et al., 2016). In mice, a genetic knockout of the vasopressin V1a receptor gene reduces anxiety-related behaviors in males, but not in females (Bielsky et al., 2004, 2005). In rats, a V1a antagonist reduces social play in males while increasing them in females when injected intracerebroventricularly, but has the exact opposite effect when injected into the septum (Veenema et al., 2013). Although, these studies clearly point at a sexually dimorphic role of vasopressin and its cognate receptor in behavior, they do not identify which AVP system is involved, and the opposite effects in play behavior, depending on the area injected, suggests that more than one system may be involved. By targeting a specific AVP cell group directly, our study has identified the sexually dimorphic AVP cells in the BNST as contributing to sex differences in social behavior, and has shown the feasibility of following a similar approach in identifying the contributions of other AVP systems in the brain as well.

3 KNOCKDOWN OF SEXUALLY DIFFERENTIATED VASOPRESSIN EXPRESSION IN THE BED NUCLEUS OF THE STRIA TERMINALIS REDUCES SOCIAL AND SEXUAL BEHAVIOR IN MALE, BUT NOT FEMALE, MICE

Previously published in Journal of Neuroendocrinology, doi: 10.1111/jne.13083 (*PMID: 34978098)*

3.1 Introduction

The neuropeptide arginine-vasopressin (AVP) has been strongly implicated in the regulation of social behavior and communication across species(Caldwell, 2017; Donaldson and Young, 2008; Goodson and Bass, 2001; Insel, 2010; Kelly and Goodson, 2013a). Many studies report that vasopressin, acting via V1a receptors (V1aR), modulate these behaviors in sexually different ways (Donaldson and Young, 2008; Dumais and Veenema, 2016; Duque-Wilckens et al., 2016); however, the sources that drive these sex differences are not well described. In most animals, AVP is synthesized in several cell groups, each of which project to distinct brain areas (De Vries and Boyle, 1998; Rood et al., 2013; Rood and De Vries, 2011). Two sources likely to contribute to sexually differentiated effects of AVP are the neurons in the bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA), which contribute to the most pronounced sex differences in AVP innervation of the brain and are exquisitely responsive to gonadal steroids(De Vries and Panzica, 2006).

Several studies suggest a sexually differentiated role for AVP cells in the BNST in social behavior. For example, injections of AVP or AVP antagonists in BNST projection sites such as the lateral septum, lateral habenular nucleus, and ventral pallidum (Rood et al., 2013) affect social behavior differently in males and females in rats and mice (DiBenedictis et al., 2020; Rigney et al., 2020a; Veenema et al., 2013, 2012). In addition, partial knockdown of AVP gene

expression in the BNST reduces male, but not female, social interactions, while increasing male, but not female, aggression in finches (Kelly et al., 2011; Kelly and Goodson, 2013a, 2013b). In rats, intermale aggression correlates with AVP release in the septum and is reduced by intra-septal AVP antagonist application (Veenema et al., 2010). Overall, these studies indicate that BNST AVP is important for male-male interactions and for certain aspects of male prosocial communication, while playing a lesser role in female social behavior and communication.

Recently, we directly tested whether cells expressing AVP in the BNST are involved in social behaviors in mice (Rigney et al., 2019; Whylings et al., 2020). We found that ablations of these cells reduced male-male investigation and increased male scent marking toward female stimuli (Rigney et al., 2019). These studies, however, left unresolved the critical question as to whether AVP or other neuroactive substances are responsible for the effects seen after removal of BNST AVP cells. Consequently, in order to specifically target AVP release from these cells, we reduced AVP-expression in the BNST using viral expression of a short hairpin RNA (shRNA) targeted against AVP mRNA and tested the effects of this manipulation on social investigation, ultrasonic vocalization (USV), and urine marking, all aspects of mouse communication that show pronounced sex differences (Crawley, 2012; Lehmann et al., 2013; Wöhr, 2014).

3.2 Methods

3.2.1 Animals and Husbandry

All mice were maintained at 22°C on a 12:12 reverse light cycle with food and water available ad libitum and housed in individually ventilated cages (Animal Care Systems, Centennial, CO, USA) with ALPHA-dri bedding (Shepherd Specialty Papers, Watertown, TN), a nestlet square, and a housing tube. All animal procedures were performed in accordance with the

Georgia State University Animal Care and Use Committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects:

Forty-eight male and female C57BL/6J mice between 8 and 12 weeks of age were obtained from Jackson Laboratories (stock # 000664) and were singly-housed for a minimum of one week prior to testing. We singly-housed subjects to increase territorial/competitive behavior as well as to avoid variations in social status due to group housing (Poole and Morgan, 1973).

Stimulus animals:

CD1(ICR) mice (Charles River Laboratories, Wilmington, MA, USA) were used as stimuli for behavioral testing and to provide male and female subjects with social experience, because strain differences between subjects and stimulus mice increase social interactions (Gheusi et al., 1994). Mice were used at 9-16 weeks of age and were novel to the subject to which they were exposed.

Female stimulus mice were grouped-housed, ovariectomized, and implanted with an estradiol capsule (GDX+E; see below), and given two sexual experiences before testing. Two groups of stimulus males were used for behavioral testing. Mice that were used as subordinates in the home cage aggression tests and to provide aggressive experience to subjects, were grouped-housed, gonadectomized (GDX), and subjected to two aggressive encounters with a dominant male (see below). Mice in the second group, which provided sexual experience to female subjects and served as sexual partners during copulatory tests as well as stimulus animals in the three-chamber social test, were singly-housed, gonadectomized, implanted with testosterone (GDX+T; see below), and then given two sexual experiences before testing.

3.2.2 shRNA virus

We used an adeno-associated virus (AAV) expressing a short hairpin RNA (shRNA) to target Avp mRNA (AAV8-GFP-U6-mAVP-shRNA; titer: 1.0 x 1013 GC/mL; lot: 190930#33; sense target sequence: GGATGCTCAACACTACGCTCTCTCGAG AGAGCGTAGTGTTGAGCATCC; Vector Biolabs, Malvern, PA) and an AAV-expressing a scramble shRNA (AAV8-GFP-U6-scramble-shRNA; titer: 4 x 1013 GC/mL; lot: 19044-190820; Vector Biolabs, Malvern, PA), which does not target any known sequence as a control. The Avp-shRNA target sequence demonstrated 91% knockdown in vitro as tested by the manufacturer. BLAST searches did not reveal significant target alignment between the Avp-shRNA sequence and other coding mRNAs, including oxytocin, indicating that the shRNA targeted Avp mRNA specifically. Both scramble (control) and Avp-shRNA vectors express green fluorescent protein (GFP) to allow for visualization of the infected neurons.

3.2.3 Surgery

All surgeries were carried out using 1.5-2.5% isoflurane gas anesthesia in 100% oxygen; 3 mg/kg of carprofen was given subcutaneously before surgery to reduce pain.

Stereotaxic surgery

Mice were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand-operated drill was used to make holes in the skull, exposing the dura. 200 nl of Avp-shRNA or scramble-sequence virus was delivered bilaterally to the BNST (coordinates: AP +0.15 mm; ML ± 0.8 mm; DV 4.4 mm) at a rate of 100 nl/min using a 5 µl Hamilton syringe with a 30-gauge beveled needle mounted on a stereotaxic injector with a graduated control knob

for precise delivery of viruses to brain regions. Following virus delivery, the syringe was left in place for 10 minutes before slowly withdrawing it from the brain.

Gonadectomy and Hormone Treatment

Testes were removed after cauterizing the ductus deferens and blood supply via a midline abdominal incision. Silastic capsules (1.5 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) were filled with crystalline testosterone (T; Sigma, St. Louis, MO, USA) and inserted subcutaneously between the scapulae after gonadectomy; this procedure leads to physiological levels of T (Barkley and Goldman, 1977; Matochik et al., 1994). To further reduce aggression in stimulus animals (Beeman, 1947), males used for providing aggressive experience (see below) were gonadectomized, but did not receive a T implant (GDX).

The ovaries of stimulus female mice were removed after cauterizing its blood supply via an abdominal incision at the uterine horn. Silastic capsules (0.7 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) containing estradiol benzoate (E; diluted 1:1 with cholesterol) were implanted subcutaneously in the scapular region immediately following ovariectomy (GDX + E) (Bakker et al., 2002; Ström et al., 2012). To induce sexual receptivity, stimulus females were injected subcutaneously with 0.1 ml of progesterone (500 μg dissolved in sesame oil, Sigma, St. Louis, MO, USA) four hours preceding sexual experience, urine collection, and behavioral testing (Veyrac et al., 2011).

3.2.4 Social Experience

As opposite-sex sexual experience and attaining competitive status (“social dominance”) promote communicative behaviors(Lumley et al., 1999; Roullet et al., 2011), mice received

social experience over five consecutive days (sexual encounters on days 1 and 4, aggressive encounters on days 2 and 5; no encounters on day 3).

Sexual Experience

Subjects were given two opportunities to interact with either a stimulus female (for male subjects) or a stimulus male (for female subjects). A sexually-experienced stimulus mouse was placed in the subject's home cage and removed the next day (first experience) or after ninety minutes (second experience). Subjects that did not ejaculate or elicit ejaculation (females) during the second sexual experience were removed from further testing.

Aggressive Experience

Male subjects were exposed to two interactions with a group housed, socially defeated and gonadectomized stimulus male treated with 50 μl of GDX+T male urine applied to its back, a manipulation which reduces aggression from stimulus males while eliciting offensive aggression in subjects(Beeman, 1947; Connor and Winston, 1972; Van Loo et al., 2001). Stimulus males were placed in the subject's home cage and removed after the subject's first offensive attack (biting) within a ten-minute period. All subject males attacked the intruder stimulus male, and all stimulus males displayed submissive behavior, defined as defensive postures (e.g. on-back postures, fleeing, and non-social exploring(Koolhaas et al., 2013)) and no attacking behavior. Female subjects were exposed to a female intruder; however, this did not elicit attacks from either animal.

Urine Collection

Pooled urine samples were collected from stimulus females induced into estrous and from stimulus males (5-10 mice per sample). Estrous state was verified by color, swelling, and expanded size of the vaginal opening(Caligioni, 2009) and collection of vaginal smears three

hours prior to testing(Cora et al., 2015). To collect urine, mice were picked up by the tail base and held by dorsal neck skin; this method typically induced urination. If the mouse did not urinate, stroking its belly from an anterior to posterior direction stimulated bladder voiding. Each mouse provided 40-80 µl of urine that was pooled into a 1.5 ml Eppendorf tube. Urine samples were used fresh within one hour of collection to prevent chemosignal degradation(Roullet et al., 2011).

3.2.5 Experimental Procedure

All testing occurred within the first five hours of the dark cycle under red light illumination (27 lux), with the exception of the elevated plus maze (EPM), which took place in bright illumination (435 lux). All tests were scored by an experimenter blind to the treatment of the subject and testing occurred across multiple cohorts of subjects. Three weeks after viral injections, subjects were habituated to the testing room and apparatus by handling and placing mice for five minutes in the three-chamber apparatus (see below) each day for three days. On experimental days, subjects were adapted to the experimental room for fifteen minutes prior to testing. First, we tested mice on an EPM to test for anxiety-related behavior(Lister, 1987). Mice were then tested in the three-chamber apparatus over six days with a one day break on the fourth day. Lastly, copulatory and aggressive behavior were measured sequentially, with a day in between, in the subject's home cage. Female subjects were tested irrespective of estrous cycle day, except during copulation testing, when estrous state was determined prior to testing by vaginal smear analysis. Prior research indicates minimal effects of estrous cycle on female mouse communicative behavior(Coquelin, 1992; Maggio and Whitney, 1985; Moncho-Bogani et al., 2004). Following testing, subjects were sacrificed and their brain tissue was processed using immunohistochemistry to detect whether AVP knockdown resulted in a reduction of AVP levels

in the BNST and its target areas, specifically the lateral septum (LS). Also, in order to detect potential off-target effects of the shRNA, we analyzed a subset of BNST brain sections for oxytocin (OT) and galanin (GAL)-immunoreactive (-ir) cells. To determine if the viral spread was limited to the BNST, we analyzed GFP-ir cells in the BNST and paraventricular nucleus of the hypothalamus (PVN). Subjects with viral spread outside the BNST were considered 'misses' and were excluded from the study (males: n=2; females: n=4).

Social Behavior

USV, urine marking, and social investigation were recorded in an acrylic three-chamber apparatus (Ugo Basile, Gemonio (VA) Italy; dimensions: 60 x 40 x 22 cm)(Arakawa et al., 2008; Crawley, 2007; Moy et al., 2009). Instead of a solid floor, the apparatus was placed on absorbent paper (Nalgene Versi-dry paper, Thermo Fisher Scientific, Waltham, MA, USA) to allow for accurate measurement of urine marking. One corner of the apparatus had a cylindrical cage (8 cm (D), 18 cm (H); 3 mm diameter steel bars, 7.4 mm spacing) that housed a stimulus animal, the opposite corner had a similar cage that was left empty. For testing with social odors, subjects had access to 50 µl of fresh urine from a stimulus animal or 50 µl saline pipetted onto a clean piece of filter paper (3 cm2) that was taped on the outside of cages. The location of stimulus and the "clean" cage were counterbalanced across animals. After placing the subject in the center of the middle chamber, we measured, across a 5-minute trial, close investigation of clean and stimulus cages as well as USV and urine marking, as described below. After testing, the apparatus and cages were thoroughly cleaned with 70% ethanol and allowed to dry before further testing. In all cases, male or female urine stimulus was presented first (day one), followed by exposure to a stimulus animal of the same sex the following day (day two); this order was then repeated one day later for the opposite sex. In this fashion, mice first experienced a weak

stimulus (urine), then a stronger social stimulus (live animal). The order of male and female stimuli presentation was counterbalanced across subjects.

Investigation and Ultrasonic Vocalizations

Close investigation was defined as time spent sniffing within 2 cm of the stimulus or clean cage; climbing on the cage was not scored as investigation. USVs were detected using a condenser microphone connected to an amplifier (UltraSoundGate CM16/CMPA, 10 kHz - 200 kHz, frequency range) placed 4 cm inside the apparatus and directly above the center compartment. USVs were sampled at 200 kHz (16-bit) with target frequency set to 70 kHz (UltraSoundGate 116Hb, Avisoft Bioacoustics, Berlin, Germany). Recordings were then analyzed using a MATLAB (MATLAB, Mathworks, RRID:SCR_001622) plug-in that automates USV analysis(Van Segbroeck et al., 2017). Using this program, sonograms were generated by calculating the power spectrum on Hamming-windowed data and then transformed into compact acoustic feature representations (Gammatone Filterbank). Each 200-millisecond window containing the maximum USV syllable duration was then clustered via machine learning algorithms into USV syllable types (repertoire units) based on time-frequency USV shape. Repertoire units that appeared as background noise were discarded. We collapsed and counted across all syllable types and analyzed the total number USVs produced by each subject.

Urine Marking

Following testing, the substrate sheet was allowed to dry for one hour and then sprayed with ninhydrin fixative (LC-NIN-16; Tritech Forensics Inc., Southport, NC, USA) to visualize urine marks(Lehmann et al., 2013; Meyer, 1957). After twenty-four hours, sheets were imaged (Sony DSC-S700 camera), binarized and analyzed using computer-aided imaging software

(ImageJ, RRID:SCR_003070). Urine marking was measured as the total area (pixels) of visualized ninhydrin urine marks in the entire arena.

Copulatory and Aggressive Behavior

To measure copulatory behavior, a stimulus mouse was placed in the subject's home cage and then removed after ninety minutes had elapsed. The number of mounts, intromissions, and ejaculations and their latencies were recorded, along with mount rejections (female kicking male off during mounting attempt) by female subjects. To measure territorial aggression, subordinate stimulus males were placed in the subject's home cage and then removed after the subject's first offensive attack (biting and rolling) within a ten-minute period; the latency to first bite and to first rolling-attack was recorded.

Elevated Plus Maze

The elevated plus maze (EPM) consisted of two open arms (30 x 5 x 0 cm) and two closed arms (30 x 5 x 25 cm) crossed perpendicularly and raised 60 cm above the floor. Subjects were placed at the arm intersection facing the open arm and were habituated to the apparatus for one minute; subjects were then observed for an additional five minutes. Time spent in open and closed arms and the number of risk assessment behaviors (stretch-attend posture, head-dips) were manually scored from video(Cole and Rodgers, 1993). Subjects were removed from EPM data analysis if they fell off the EPM during testing.

3.2.6 Histology and immunohistochemistry

Mice were transcardially perfused with 50 ml of 0.1 M PBS (pH 7.4), followed by 50 ml of 4% paraformaldehyde in phosphate buffer (pH 7.2). Brains were immediately removed and post-fixed in 4% paraformaldehyde overnight (4 °C) and then cryoprotected for 48 h in 30% sucrose. Coronal sections (30μm) of brain tissue were sectioned on a cryostat (Leica CM3050 S,

Leica Biosystems, Heidelberg, Germany) into four series and stored in cryoprotectant until immunohistochemical processing. Tissue from all subjects (n=48) was stained for GFP, and AVP, whereas sections from a subset of subjects were stained for GAL (n=10) or OT (n=8). Sections were removed from cryoprotectant and rinsed thoroughly in PBS. For optimal AVP/OT/GAL staining, we used an antigen retrieval step in which sections were incubated in 0.05M sodium citrate in PBS at 70°C for 30 min and allowed to cool for 10 min prior to primary antibody incubation. To reduce endogenous peroxidase activity, tissue sections were incubated in 0.5% hydrogen peroxide in PBS for 15 min, followed by a blocking step for 1 hr (10% normal goat or donkey serum). Sections were incubated in the appropriate primary antibody to AVP (1:50,000, guinea pig polyclonal, lot: A17901), OT (1:100,000, guinea pig polyclonal, lot: A17698), or GAL (1:40,000, rabbit polyclonal, lot: A17602) (Peninsula Laboratories International, Inc., Switzerland) in 0.4% Triton-X 100 for 24 hr at room temperature. After incubation in primary antibody, sections were rinsed in PBS and then incubated for 1 hr in goat anti-guinea pig (lot: 151056) or goat anti-rabbit (lot: 151508) biotinylated secondary antibody (1:600 Jackson ImmunoResearch, West Grove, PA, USA) in PBS with 0.4% Triton-X 100. Sections were rinsed again in PBS and then incubated for 1 hr in avidin–biotin complex (18 µl each of A and B reagents/ml PBS with 0.4% Triton-X 100, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS and then in 0.175 M sodium acetate, sections were incubated in 3,3′-diaminobenzidine HCl (0.2 mg/ml, Sigma) and hydrogen peroxide (1 µl/ml, Sigma) in a nickel-sulfate solution (25 mg/ml, Sigma) for 15 min. The reaction was stopped by rinsing sections in sodium acetate. Stained tissue sections were mounted onto subbed glass slides and allowed to air-dry overnight. Slides were then dehydrated in alcohols, cleared in xylenes, and coverslipped using Permount (Fisher Scientific).

For GFP staining, sections were removed from cryoprotectant and rinsed thoroughly in PBS. After several rinses, sections were incubated in primary antibody against GFP (1:5,000, chicken polyclonal, lot: ab13970, Abcam, Cambridge MA) in 0.4% Triton-X 100 for 24 hr at room temperature. After incubation in primary antibody, sections were rinsed in PBS and then incubated for 2 hr in goat anti-chicken fluorescent (Alexa Fluor® 488) secondary antibody (1:600, lot: ab150169, Abcam, Cambridge MA). Stained tissue sections were mounted onto subbed glass slides and cover-slipped using Prolong Gold (Fisher Scientific).

Tissue Analysis

Bilateral BNST images were taken at 10x magnification using a Zeiss Axio Imager M2 microscope (Carl Zeiss Microimaging), which transferred fluorescent images (FITC contrast reflector) to image analysis software (Stereo Investigator, MicroBrightField, RRID:SCR_002526). Imaging domains (2 mm2) were placed with reference to anatomic landmarks (ventricles, fiber tracts; Paxinos and Franklin, 2012). Only subjects with fluorescent labeled GFP cells limited to the BNST were included in the analysis (Fig. 1a); subjects with viral spread, as determined by GFP label, to other regions (e.g. PVN) or with only unilateral infection were removed from analysis (males: n=2; females: n=4). AVP-, OT-, and GAL-expressing cells in the BNST, AVP-expressing cells in the PVN, and AVP fiber density in the LS, a prominent target for sex-different BNST AVP-expressing cells(Rood et al., 2013; van Leeuwen et al., 1985), were measured and averaged across both hemispheres and over three sections (BNST and LS) or four sections (PVN). Subjects with <50% AVP reduction in the BNST were removed from analysis (males: n=1; females: n=3). Density of AVP-ir fibers in the LS was measured by gray-level thresholding of digitally captured images using ImageJ (NIH, http://imagej.nih.gov/ij). Background measurements were taken from an adjacent area with no AVP label and averaged

across all brains. Specific AVP-ir fiber density was calculated by subtracting the average non-specific background from the AVP-ir density measurement (pixels).

3.2.7 DeepLabCut and SimBA Analysis

Deeplabcut (DLC) 2.2b8 was used to track 8 body points (nose, ears, center, left flank, right flank, tail base, and tail end) on the subject mice. A tracking dataset was created by labeling 20 frames from 10 randomly chosen male and female subject videos for a total of 200 labeled frames. To adequately train a network that could track mice movements, the generated dataset underwent 200,000 iterations of analysis under the default neural network, resnet_50(Mathis et al., 2018). The resulting network was then applied to each subject's 305-second video of their time spent in the 3-chamber apparatus. Tracking point coordinates (CSV file) were produced for each video analyzed and used for further analysis.

CSV files generated from DLC analysis and their corresponding videos were further analyzed with Simple Behavioral Analysis (SimBA 0.84.1)(Nilsson et al., 2020), an open source program that enables the automated classification of complex social behaviors. First, pixel/millimeter ratios were established for each video, and a region of interest analysis was used to calculate features related to distance, directionality, and time spent in each chamber of the 3-chamber apparatus. The center body marker was the point of interest used to track subjects' distance covered in the 3-chamber apparatus to detect if BNST-AVP knockdown influenced locomotor activity. Second, three rectangular regions of interest, each one outlining a different chamber in the apparatus, were generated to calculate the subject's time spent in each chamber. Lastly, a circular region of interest was outlined around the stimulus cage to measure head orientation, and therefore attention towards a distal stimulus, similar to social vigilance measurements that indicate increased anxiety in stressed animals(Duque-Wilckens et al., 2018).

We measured this orientation behavior when the subject's nose was oriented toward the center of the circular region of interest and was a distance of 9-30cm away from the center of the circular region of interest.

3.2.8 Statistical Analysis

Data were analyzed and graphed in R (3.4.4; R Core Team, 2017) using tidyverse and rstatix packages, "anova_test()", "t.test"), "wilcox.test(y,x)", "chisq.test". Data on histology, social investigation, movement, head orientation, time spent in chambers, urine marking, copulatory behavior, and EPM met the assumptions of parametric statistical tests (i.e. normal distribution, common variance, independence of observation). Therefore, we analyzed these data using a 2x3 mixed-model ANOVA with treatment (injections with Avp-shRNA, scramble control) and sex (male, female) as between-subject factors, and sex of stimulus (male, female) and stimulus location (stimulus cage, empty cage) as within-subject factors when appropriate; these were followed by planned t-tests comparing the treatment effects. For copulatory behavior, which was measured for males and females differently, we used t-tests. Differences in proportion of animals engaging in copulatory behaviors across treatments was assessed using a chi-square ($\chi 2$) test. Measures of USVs and aggressive behavior were not normally distributed and could not be transformed. Therefore, we analyzed treatment effects on these behaviors using Mann-Whitney U tests. Power analyses for each sample size were above 0.7 and post-hoc comparisons report Bonferroni-corrected p-values. Exact p values are reported, except when p exceeds a significance threshold of 0.00001. Eta-squared ($\eta 2$), Cohen's D (d), and Phi (φ) are reported for standardized effect sizes. Degrees of freedom are mentioned for ANOVAs, t-tests, and Mann-Whitney U tests.

3.3 Results

3.3.1 shRNA effectively reduced AVP expression in BNST and LS

Males had more AVP-immunoreactive (-ir) cells in the BNST than females ($F(1,45) = 30.34$, $p < 0.00001$, $\eta 2 = 0.41$). AVP knockdown caused an overall reduction in the number of AVP-ir cells ($F(1,45) = 154.46$, $p < 0.00001$, $\eta 2 = 0.8$). There was an interaction between treatment and sex ($F(1,45) = 23.01$, $p = 0.00002$, $\eta 2 = 0.3$), which may be related to the knockdown reducing the number of AVP-ir cells more in males than in females (82% in males ($t(25) = 14.41$, $p < 0.00001$, $d = 5.8$) and 72% in females ($t(19) = 4.56$, $p = 0.0002$, $d = 2.1$)) (Fig. 1c-d).

Males had a higher density of AVP-ir fibers in the LS than females ($F(1,45) = 64.2$, $p < 0.00001$, $\eta 2 = 0.61$). AVP knockdown caused an overall reduction in the density of LS AVP-ir fibers ($F(1,45) = 24.74$, $p = 0.00002$, $\eta 2 = 0.38$). There was an interaction between treatment and sex $F(1,45) = 5.29$, $p = 0.027$, $\eta 2 = 0.11$), with the reduction being larger in males than in females (Figure 3-1e-f).

The number of AVP-ir cells was not altered in the PVN of male or female subjects ($F(1,45) = 0.5$, $p = 0.47$, $\eta 2 = 0.01$), nor was there a reduction in the number of GAL-ir ($F(1,8) = 0.51$, $p = 0.5$, $\eta 2 = 0.05$) or OT-ir cells ($F(1,6) = 0.04$, $p = 0.85$, $\eta 2 = 0.006$) in the BNST in a subset of male subjects, indicating that the shRNA targeted Avp mRNA specifically and did not have off-target effects (Figure 3-2).

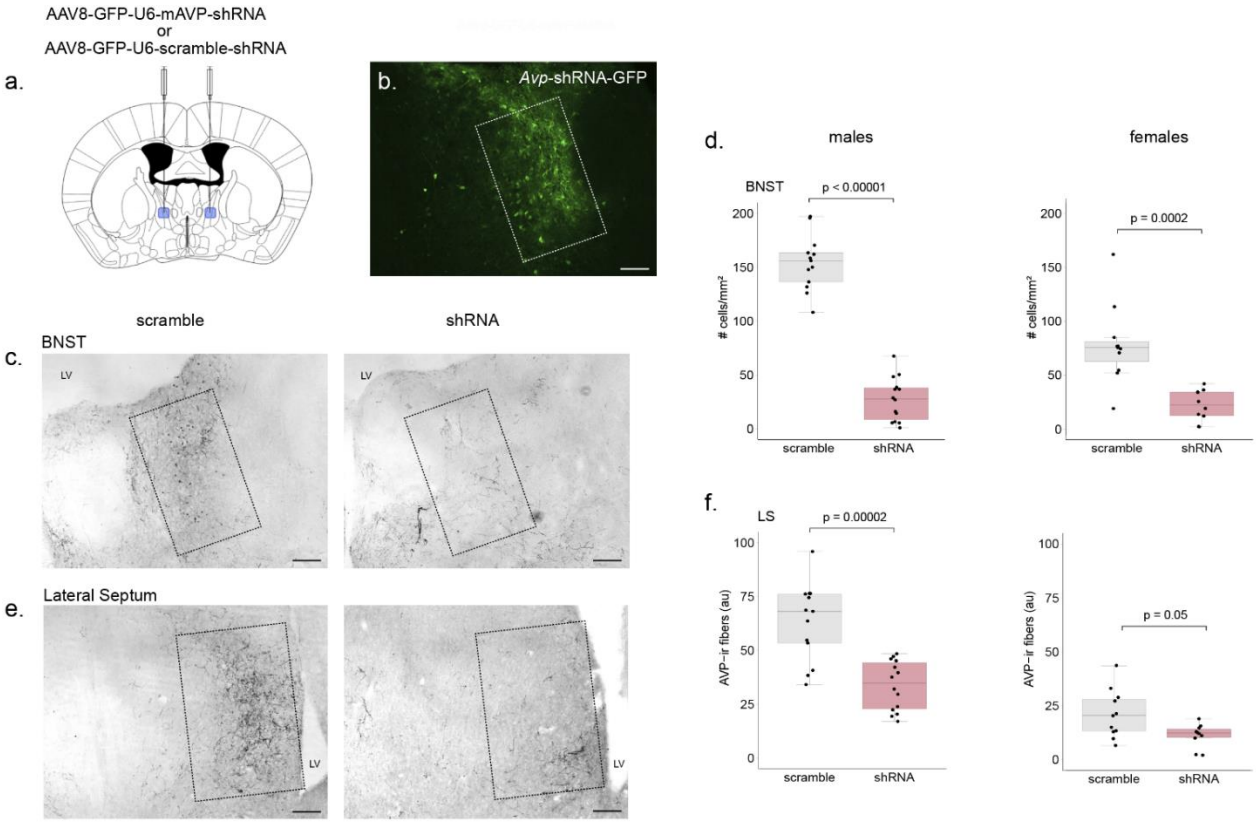

Figure 3-1- Histology. *(a) adeno-associated virus (AAV) expressing a short hairpin RNA (shRNA) to target Avp mRNA (AAV8-GFP-U6-mAVP-shRNA) or a control AAV expressing a scramble shRNA sequence (AAV8-GFP-U6-scramble-shRNA) and location of bilateral injection site in the bed nucleus of the stria terminalis (BNST). (b) Example image showing AAV-GFP-labeled cells in the BNST. (c) Example images of AVP-immunoreactive (ir) cells within the BNST following a scramble shRNA AAV (left) or Avp-shRNA AAV injections (right). Rectangles indicate position of AVP-ir cells (d) Boxplots showing that AVP-ir cell number is significantly lower in AVP shRNA-injected male (n = 14, p < 0.00001) and female (n = 10, p = 0.0002) subjects compared to scramble shRNA-injected controls (males: n = 13; females n = 11). (e) Example images of AVP-immunoreactive (ir) fibers within the LS following scramble shRNA AAV (left) or Avp-shRNA AAV (right) injections into the BNST. Images were taken at 10x magnification from sections from the same scramble shRNA or Avp-shRNA AAV-injected male subjects. Rectangles indicate position of AVP-ir fibers. Scale bar = 50 µm (f) Boxplots showing that AVP-ir fiber density is significantly lower in Avp-shRNA-injected male (p = 0.00002) and female (p = 0.05) subjects compared to scramble shRNA-injected controls. Boxplots indicate individual data points, median, first, and third quartiles.*

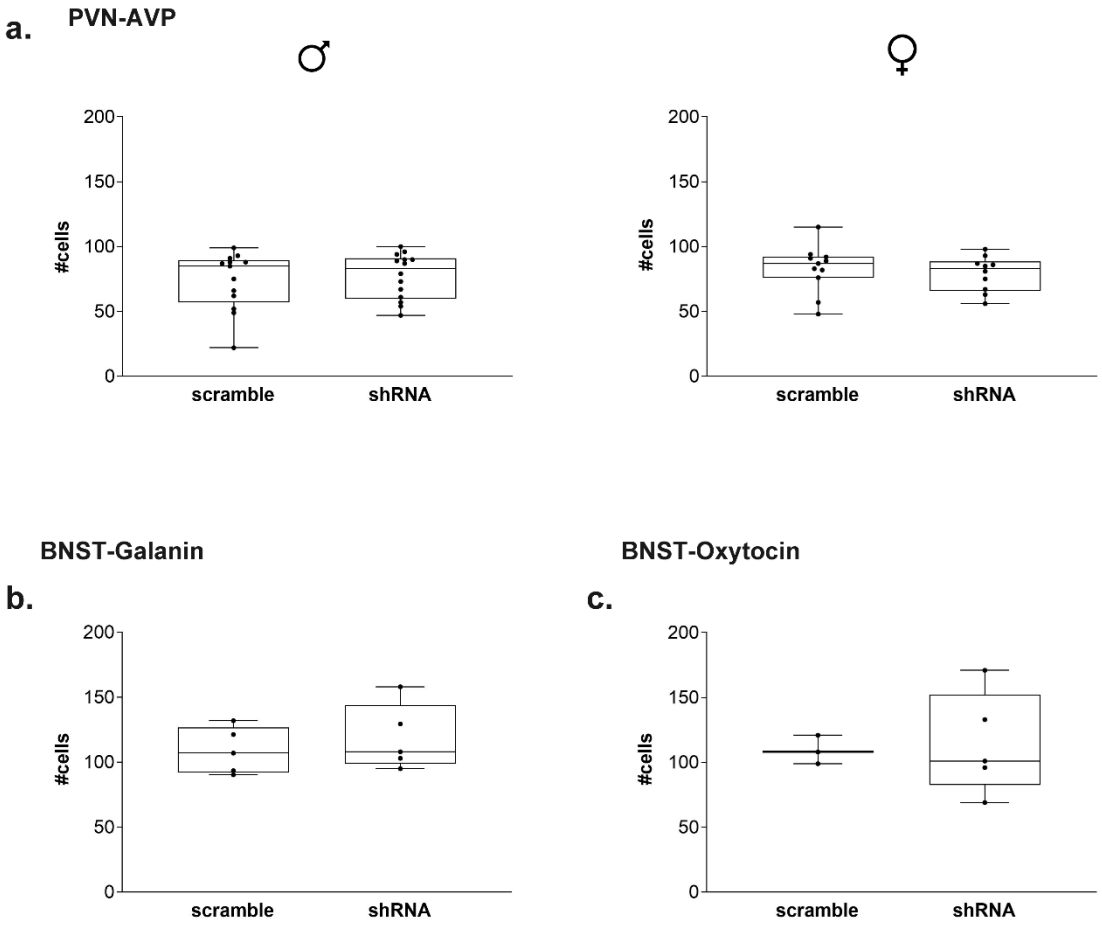

Figure 3-2- Additional Histology. *(a) Boxplot of AVP-ir cell number within the paraventricular nucleus of the hypothalamus (PVN). There was no difference in AVP-ir cells within the PVN in both Avp-shRNA-injected male and female mice compared to scramble shRNA- injected control mice (males: p = 0.6; females: p = 0.58). Avp-shRNA (n = 14) and scramble (n = 13) injected males and AVP-shRNA (n = 10) and scramble (n = 11) injected females. (b) Boxplot of galanin-ir cell number within the BNST. There were no differences in the number of galanin-ir cells in AVP shRNA-injected male mice compared to scramble shRNA-injected control mice, p = 0.5. Avp-shRNA (n = 5) and scramble (n = 5) injected males. (c) Boxplot of oxytocin (OT)-ir cell number within the BNST. There were no differences in the number of OT-ir cells in shRNA injected male mice compared to scramble injected control mice, p = 0.85. Avp-shRNA (n = 5) and scramble (n = 3) injected males. Boxplots indicate individual data points, median, first, and third quartiles.*

3.3.2 BNST AVP knockdown reduced social investigation of unfamiliar males in males but not in females

All subjects investigated cages with stimulus animals more than the empty cages ($F(1,45) = 30.05$, $p < 0.00001$, $\eta 2 = 0.6$). Overall, AVP knockdown decreased the time animals investigated cages with stimulus animals ($F(1,45) = 8$, $p = 0.007$, $\eta 2 = 0.3$). However, effects were limited to male animals, causing an interaction between sex and treatment ($F(1,45) = 10.57$, $p = 0.002$, $\eta 2 = 0.2$). While control males investigated male stimuli more than did control females (male stimuli: $t(21) = 5.2$, $p = 0.0001$, $d = 2.27$; female stimuli: $t(21) = 4.22$, $p = 0.001$, $d = 1.8$), BNST AVP knockdown specifically reduced the time males spent investigating cages with stimulus males ($t(25) = 6.01$, $p < 0.00001$, $d = 2.43$). This eliminated a sex difference in the time that control males and females spent investigating male stimuli ($t(21) = 1.06$, $p = 0.3$, $d = 0.2$). AVP knockdown did not affect male subjects' investigation of female stimulus animals ($t(25) = 1.9$, $p = 0.14$, $d = 0.7$) nor did it affect female subjects' investigation of male ($t(20) = 1.45$, $p = 0.32$, $d = 0.6$) or female ($t(20) = 1.66$, $p = 0.23$, $d = 0.7$) stimulus animals (Fig. 2a-b).

All subjects investigated cages with urine samples more than the empty cages ($F(1,45) = 19.67$, $p = 0.00006$, $\eta 2 = 0.4$). Overall, AVP knockdown decreased the time animals investigated cages with urine samples ($F(1,45) = 14.42$, $p = 0.0004$, $\eta 2 = 0.3$). However, as with stimulus animals, AVP knockdown effects were limited to male animals, causing an interaction between sex and treatment ($F(1,45) = 6.08$, $p = 0.018$, $\eta 2 = 0.18$). While control males investigated urine more than did control females (male urine: $t(21) = 3.7$, $p = 0.002$, $d = 1.6$; female urine: $t(21) = 4.3$, $p = 0.0006$, $d = 1.8$), BNST AVP knockdown specifically reduced the time males spent investigating both male and female urine (male urine: $t(25) = 4.42$, $p = 0.0003$, $d = 1.77$; female urine: $t(25) = 2.99$, $p = 0.012$, $d = 1.2$). This eliminated a sex difference in the time that control

males and females spent investigating male urine ($t(21) = 2.1$, $p = 0.094$, $d = 0.6$) but not for investigating female urine ($t(21) = 2.5$, $p = 0.04$, $d = 0.8$). AVP knockdown did not affect female subjects' investigation of male ($t(20) = 1.13$, $p = 0.27$, $d = 0.4$) or female ($t(20) = 0.75$, $p = 0.46$, $d = 0.3$) urine (Figure 3-3c-d).

DeepLabCut and SimBA analysis revealed that subjects differed in time spent in the chamber containing stimulus animals ($F(2,82) = 0.5$, $p = 0.44$, $\eta 2 = 0.007$). Overall, AVP knockdown increased the time animals spent in the chamber furthest away from the stimulus animals ($F(2,82) = 4.35$, $p = 0.016$, $\eta 2 = 0.09$). There was no interaction between sex, treatment, and chamber location ($F(2,82) = 1.3$, $p = 0.27$, $\eta 2 = 0.02$). However, post hoc analysis revealed that BNST AVP knockdown specifically increased the time males spent in the furthest chamber of the apparatus with stimulus males (male stimuli: $t(25) = 2.78$, $p = 0.01$, $d = 1.1$; female stimuli: $t(25) = 0.9$, $p = 0.37$, $d = 0.3$). There was also a trend toward BNST AVP knockdown decreasing the time males spent in the chamber with the stimulus male compared to controls (male stimuli: $t(25) = 2.0$, $p = 0.056$, $d = 0.8$; female stimuli: $t(25) = 0.4$, $p = 0.73$, $d = 0.1$). AVP knockdown did not affect female subject's time spent in the clean chambers (male stimuli: $t(20) = 1.7$, $p = 0.11$, $d = 0.7$; female stimuli: $t(20) = 0.03$, $p = 0.98$, $d = 0.01$) or the chamber with the stimulus animals compared to controls (male stimuli: $t(20) = 1.3$, $p = 0.18$, $d = 0.6$; female stimuli: $t(20) = 0.9$, $p = 0.36$, $d = 0.3$)(Figure 3-4).

BNST AVP knockdown did not affect overall locomotion within the 3-chamber apparatus (($F(1,45) = 1.55$, $p = 0.22$, $\eta 2 = 0.018$, Figure 3-5), and there was no interaction between treatment and sex ($F(1,45) = 0.04$, $p = 0.85$, $\eta 2 = 0.001$). Additionally, BNST AVP knockdown did not affect the amount of time subjects spent oriented toward the stimulus cages at a 9-30cm distance (Figure 3-6).

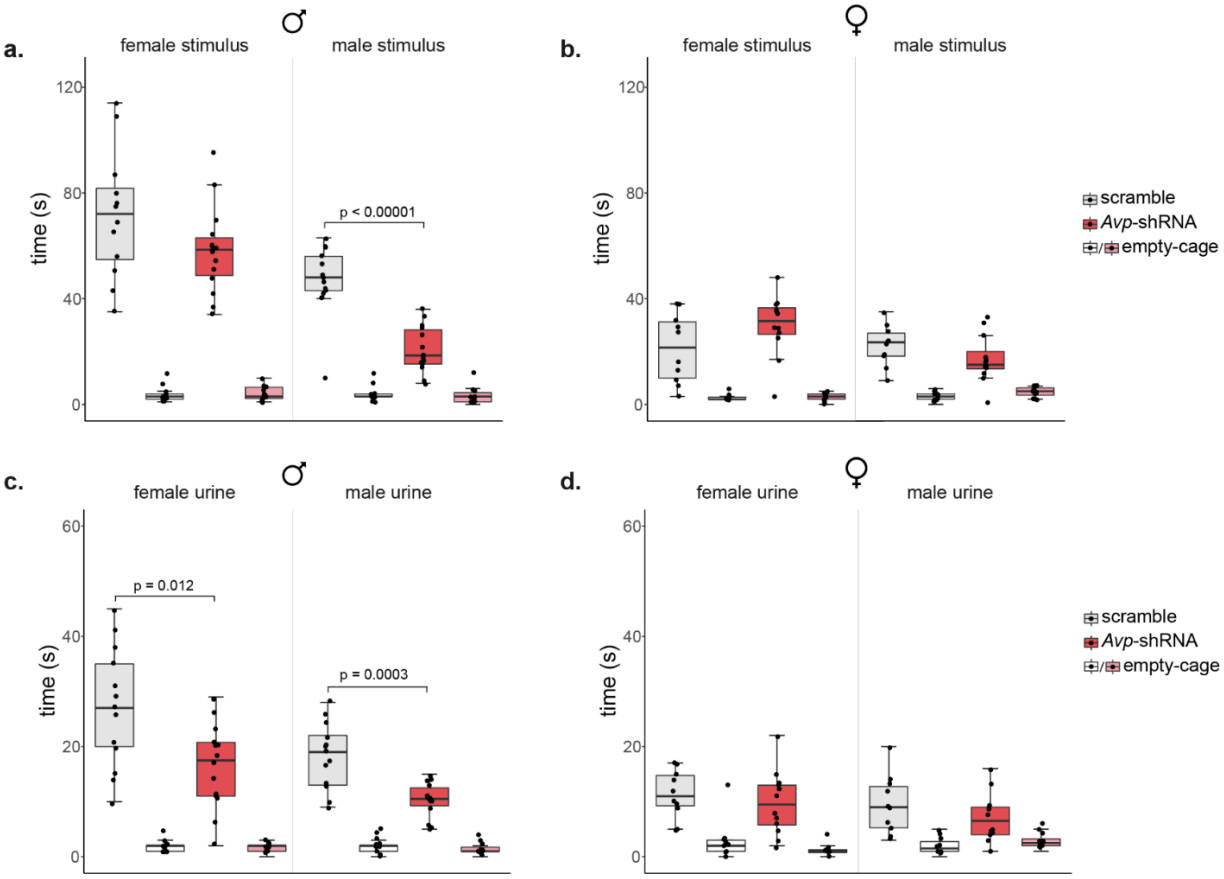

Figure 3-3- Social investigation. *Boxplots indicate individual data points, median, first and third quartiles for time spent investigating wire cages containing male or female stimulus animals, or an empty wire cage within the three-chamber apparatus. BNST AVP knockdown in males (a), but not females (b), decreased investigation of male ($p < 0.00001$) stimuli compared to controls. BNST AVP knockdown in males (c), but not females (d), decreased investigation of male urine ($p = 0.012$) and female urine ($p = 0.0003$) compared to controls.*

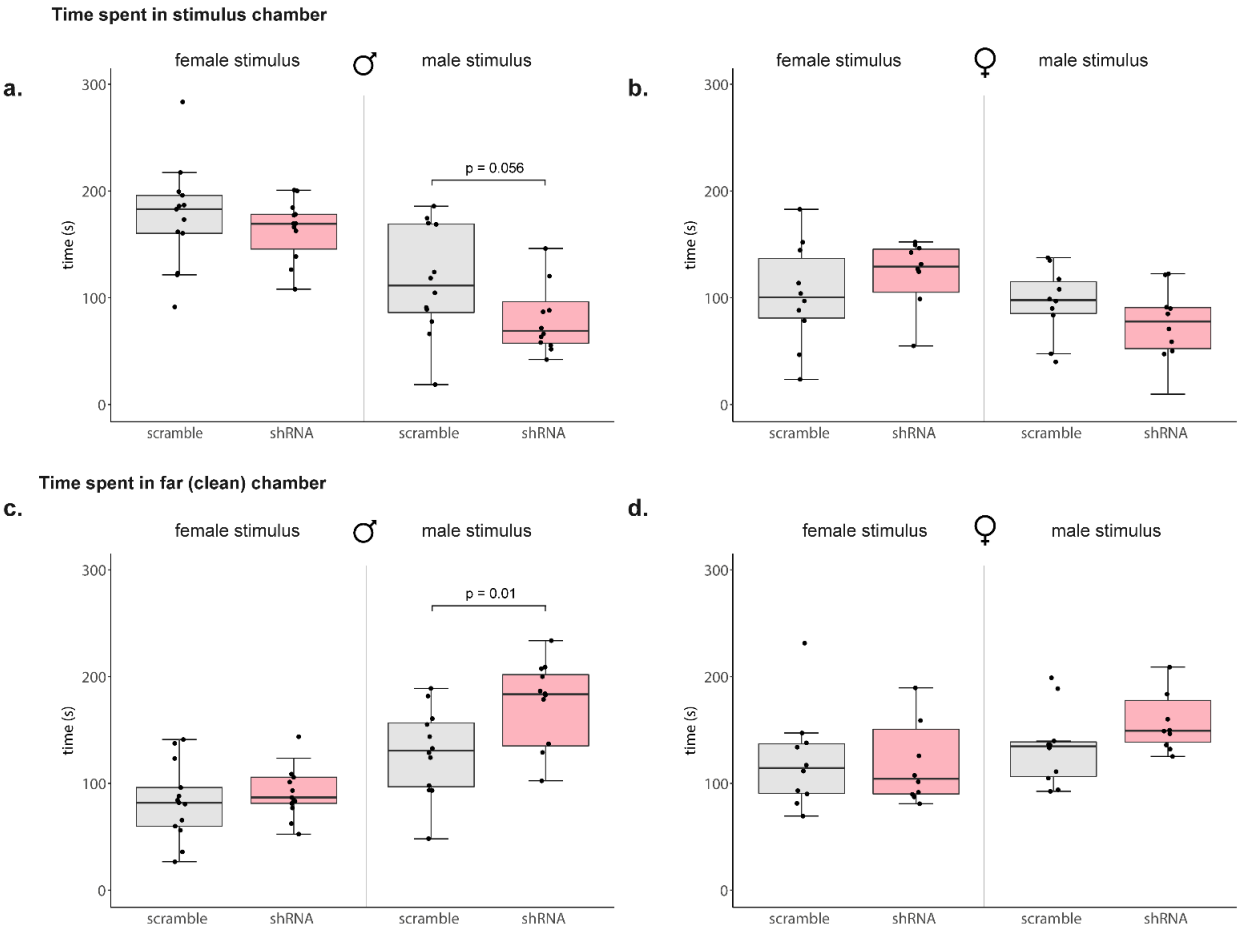

Figure 3-4- Time spent in the stimulus or clean chamber of the three-chamber apparatus. *(a) There was a trend toward Avp-shRNA-injected males to spend less time in the chamber containing a male stimulus compared to scramble shRNA control (p = 0.056), but there was no difference between Avp-shRNA and scramble shRNA-injected females (b). (c) Avp-shRNA-injected males spent more time in the chamber furthest away from the male stimulus (p = 0.01), and again, (d) there was no difference between Avp-shRNA and scramble shRNA-injected females. Boxplots indicate individual data points, median, first, and third quartiles.*

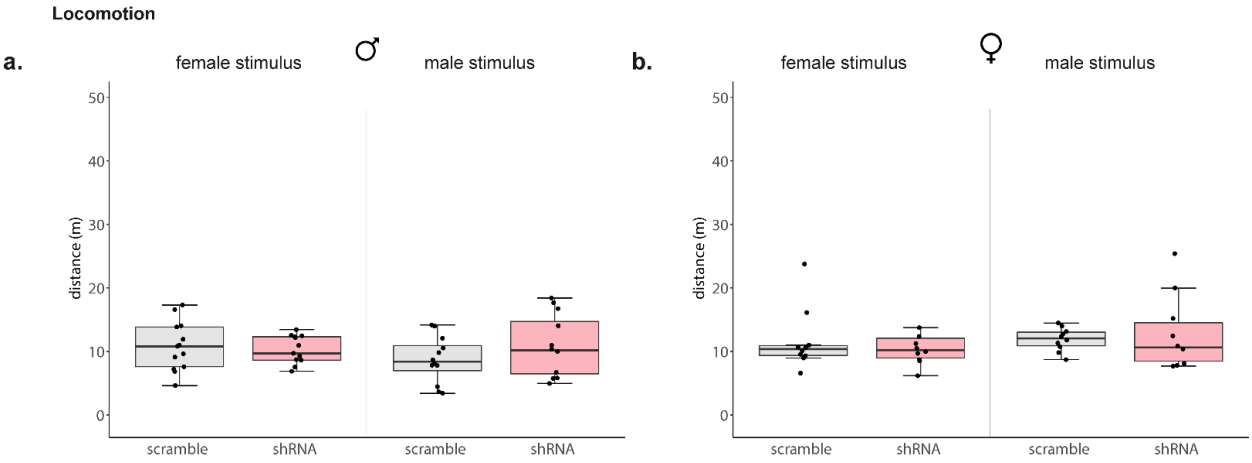

Figure 3-5- Locomotion *(a, b) Scramble shRNA control and Avp-shRNA subjects did not differ in the distance traveled within the three-chamber apparatus. Boxplots indicate individual data points, median, first, and third quartiles.*

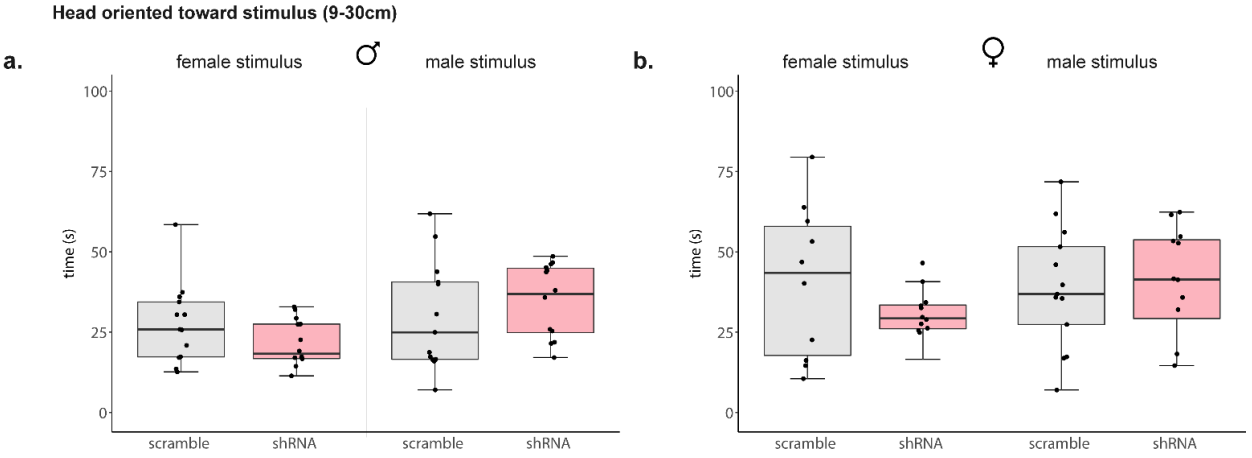

Figure 3-6- Head orientation toward male and female stimuli. *(a, b) Scramble control and Avp-shRNA subjects did not differ in the time spent oriented toward stimuli at a 9-30cm distance within the three-chamber apparatus. Boxplots indicate individual data points, median, first, and third quartiles.*

3.3.3 BNST AVP knockdown reduced male-male USVs

Most vocalizations were produced during male-female interactions and least during female-female interactions (Figure 3-7). BNST AVP knockdown reduced overall USVs produced during social interactions (U = 234, p = 0.024, d = 0.55), However, this effect seems to be driven primarily by a reduction of USVs produced during male-male interactions (male

subjects with male stimuli: U = 45.5, p = 0.05, d = 0.68; male subjects with female stimuli: U = 57, p = 0.11, d = 0.6). USVs produced during interactions of female subjects and stimulus animals were much lower and unaltered following BNST AVP knockdown (female subjects and female stimuli: U = 45, p = 0.35, d = 0.1; female subjects with male stimuli: U = 67, p = 0.67, d = 0.04) (Fig. 3a-b). AVP knockdown did not alter USVs produced during exposure to female urine (males: U = 77, p = 0.52, d = 0.1; females: U = 34, p = 0.09, d = 0.2) or male urine (males: U = 47, p = 0.42, d = 0.09; females: U = 77, p = 0.52 d = 0.2) (Figure 3-8).

Overall, males, but not females, produced urine marks (Fig. 3c-d). Males produced more urine marks and covered a larger area with urine in the presence of a female than of a male stimulus (number of marks: $F(1,25) = 16.53$, $p = 0.0004$, $\eta 2 = 0.1$; area: ($F(1,25) = 29.713$, $p = 0.00001$, $\eta 2 = 0.19$)). BNST AVP knockdown did not affect the number of urine marks nor the area covered with those marks in the presence of stimulus animals (number of marks: $F(1,25) = 0.4$, $p = 0.56$, $\eta 2 = 0.001$; area: ($F(1,25) = 0.1$, $p = 0.76$, $\eta 2 = 0.01$))(Fig. 3c-d) or urine samples ($F(1,25) = 0.5$, $p = 0.51$, $\eta 2 = 0.02$)(Figure 3-8).

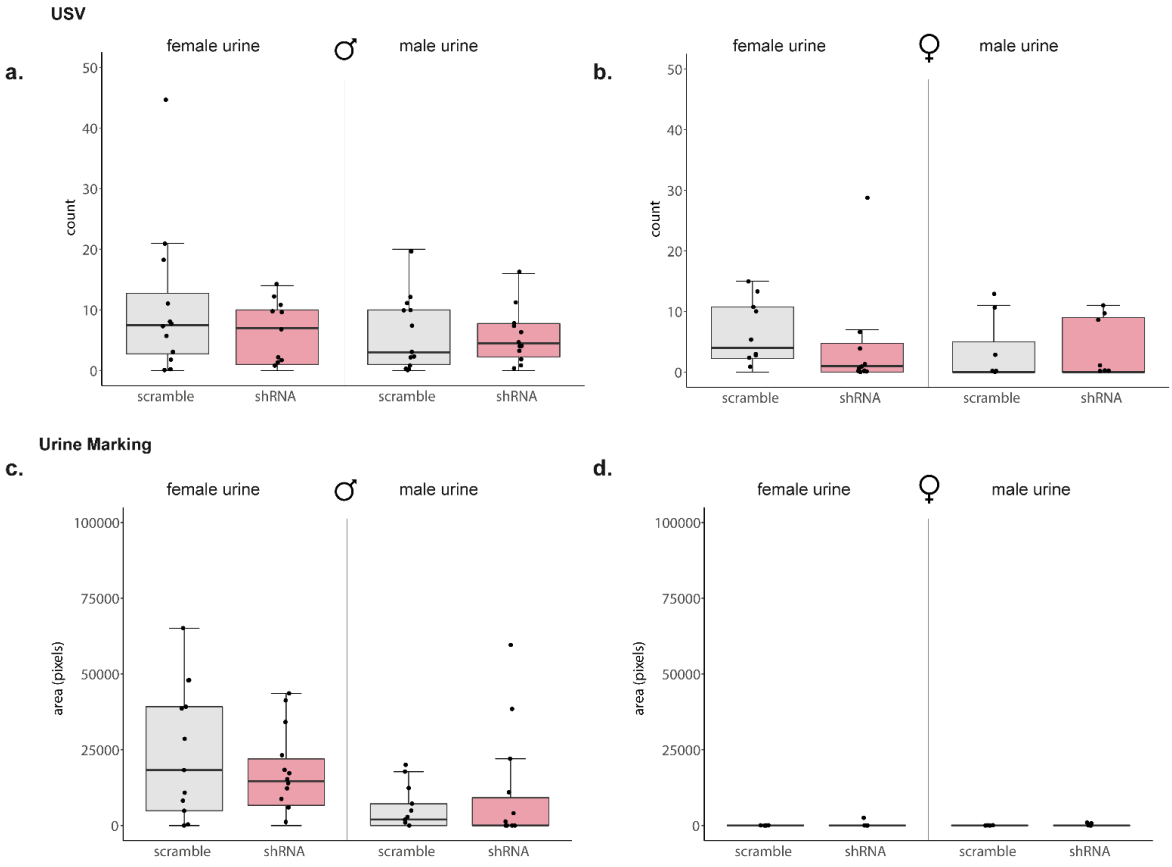

Figure 3-7- Ultrasonic vocalizations (USV) and urine marking toward male and female urine stimuli within the three-chamber apparatus. *(a-b) Avp-shRNA and scramble shRNA-injected males (a) and females (b) did not differ in USVs produced toward urine stimuli. (c-d) Avp-shRNA and scramble shRNA-injected males (c) and females (d) did not differ in urine marking toward urine stimuli. Boxplots indicate individual data points, median, first and third quartiles.*

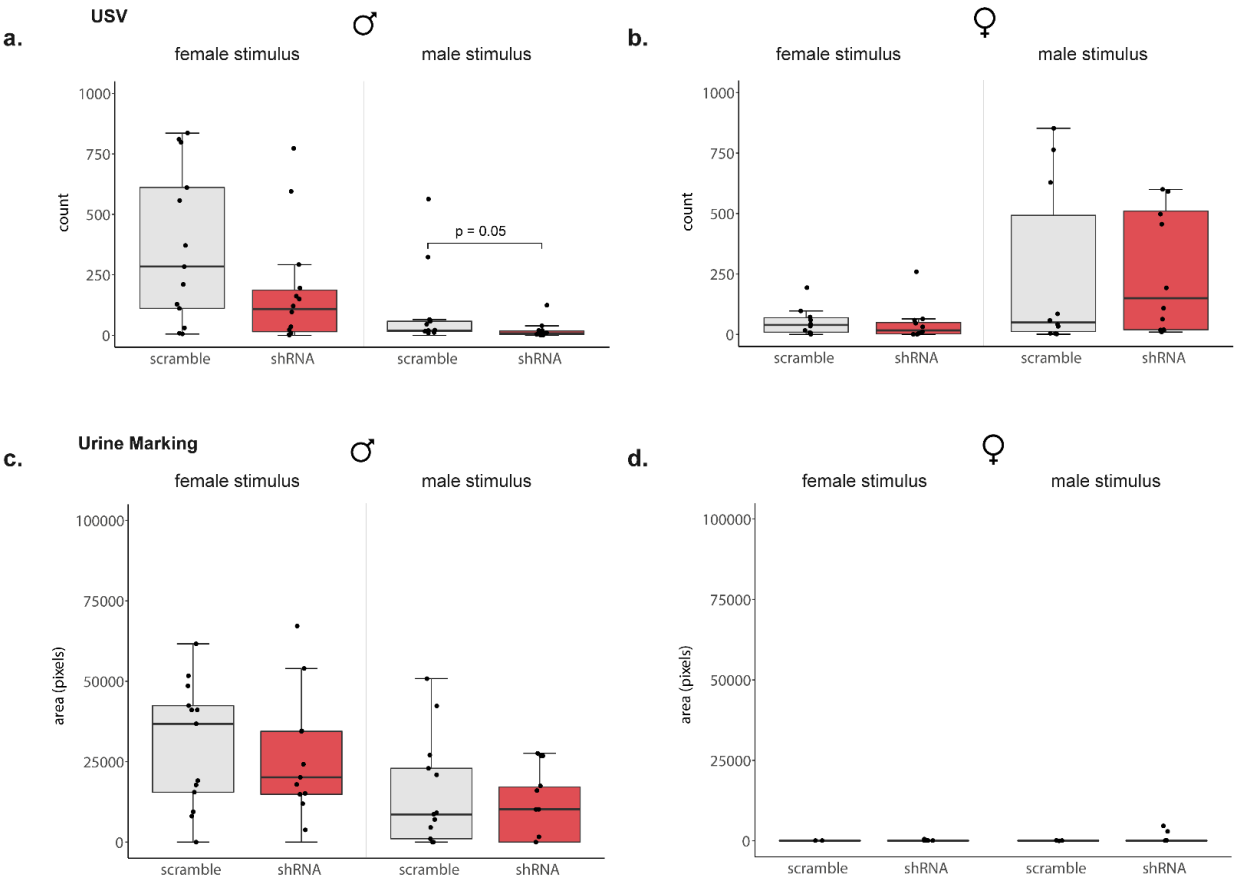

Figure 3-8- Ultrasonic vocalizations (USV) and urine marking within the three-chamber apparatus. *(a) BNST AVP knockdown reduced USVs produced during male-male conditions (p = 0.05). (b) Avp-shRNA and scramble shRNA- injected females did not differ in USVs produced during three-chamber testing. (c-d) Avp-shRNA and scramble shRNA-injected males (c) and females (d) did not differ in urine marking (area covered) toward male or female stimuli during three-chamber testing. Boxplots indicate individual data points, median, first and third quartiles.*

3.3.4 BNST AVP knockdown reduced offensive signaling

Male subjects were the only ones to engage in aggressive attacks and tail rattling during the aggression tests. BNST AVP knockdown reduced tail rattles of subjects toward male intruders ($U = 50$, $p = 0.048$, $d = 0.83$); however, the latency to bite ($U = 89$, $p = 0.9$, $d = 0.1$) and attack ($U = 73.5$, $p = 0.4$, $d = 0.1$) the male intruder was similar between control and shRNA-injected males (Figure 3-9).

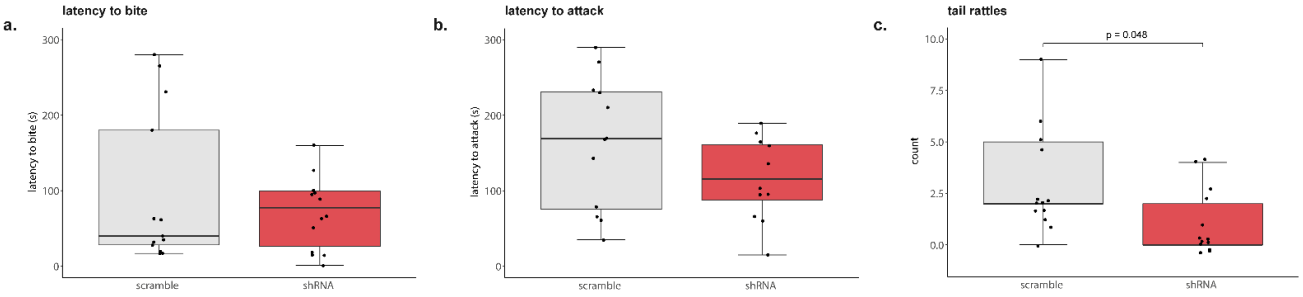

Figure 3-9- Aggressive behavior. *In males, BNST AVP knockdown did not alter the latency to bite (a) or latency to rolling attack (b) but did reduce the number of tail rattles during encounters with male intruders (c), p = 0.048. Boxplots indicate individual data points, median, first and third quartiles.*

3.3.5 BNST AVP knockdown reduced copulatory behavior in males

In males, BNST AVP knockdown reduced the number of intromissions ($t(25) = 3.13$, $p = 0.002$, $d = 0.9$) but did not affect the number of mounts ($t(25) = 0.7$, $p = 0.49$, $d = 0.2$). Similarly, BNST AVP knockdown increased the latency to intromit ($t(25) = 2.6$, $p = 0.002$, $d = 0.9$), but did not affect the latency to mount ($t(25) = 1.1$, $p = 0.28$, $d = 0.3$) or ejaculate ($t(25) = 1.2$, $p = 0.23$, $d = 0.2$), Fig. 5a-d). Furthermore, BNST AVP knockdown caused fewer males to ejaculate with a receptive female ($x2\ (2)\ p < 0.00001$, $\varphi = 1.06$, Figure 3-10). In females, BNST AVP knockdown did not affect the number of mounts received ($t(20) = 1.3$, $p = 0.19$, $d = 0.5$), intromissions received ($t(20) = 1.1$, $p = 0.27$, $d = 0.5$), or the latency to receive mounts ($t(20) = 0.2$, $p = 0.82$, $d = 0.1$), intromissions ($t(20) = 0.97$, $p = 0.35$, $d = 0.4$), or ejaculations ($t(20) = 0.3$, $p = 0.78$, $d = 0.1$)(Figure 3-10).

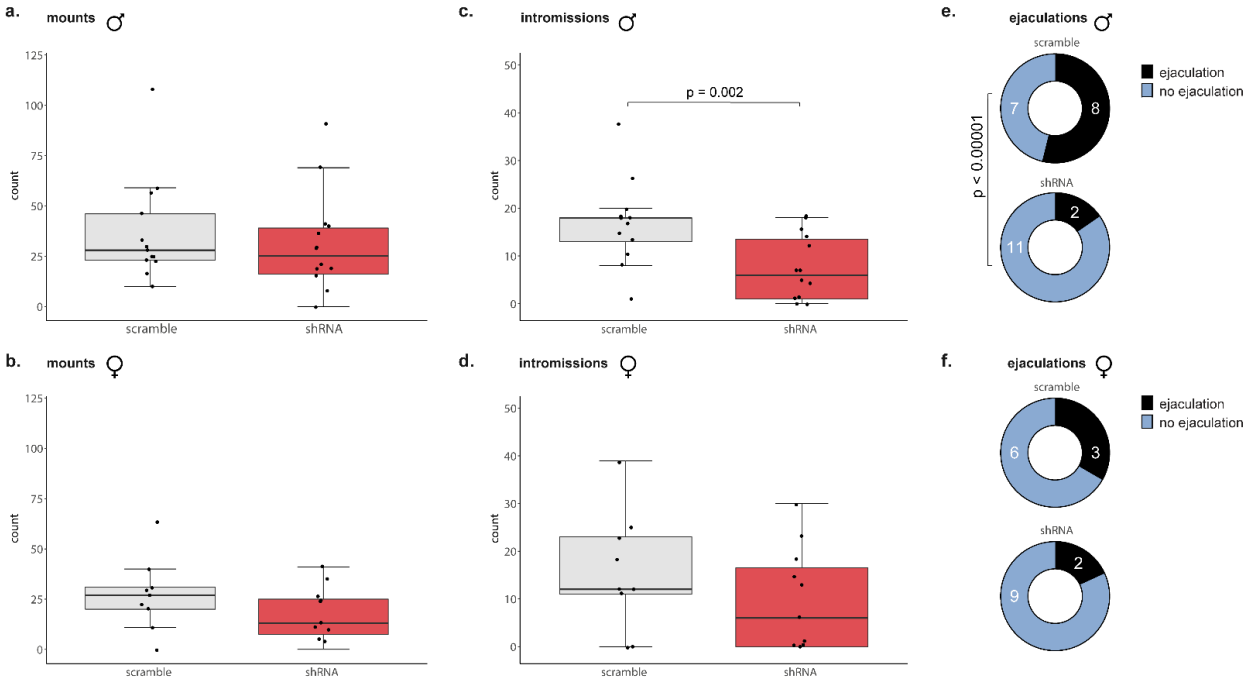

Figure 3-10- Copulatory behavior. *Avp-shRNA and scramble shRNA- injected males (a) and females (b) did not differ in number of mounts performed (males) and number of times mounted (females). (c) Avp-shRNA reduced the number of intromissions performed by males compared to controls (p = 0.002). (d) The number of intromissions received by females was unaltered. Pie chart summarizing the proportion of male subjects that ejaculated (e) and the proportion of male stimulus animals that ejaculated with female subjects (f). BNST AVP knockdown resulted in fewer males ejaculating (p < 0.00001). Boxplots indicate individual data points, median, first and third quartiles.*

3.3.6 BNST AVP knockdown did not alter anxiety-like behavior

We did not find sex differences in time spent in the open arms ($F(1,45) = 1.12$, $p = 0.3$, $\eta 2 = 0.02$), the number of stretch-attend postures ($F(1,45) = 0.4$, $p = 0.53$, $\eta 2 = 0.002$) or head dips ($F(1,45) = 2.05$, $p = 0.16$, $\eta 2 = 0.02$) observed in the EPM. BNST AVP knockdown did not affect any of these measures (time spent in the open arms: ($F(1,45) = 0.01$, $p = 0.9$, $\eta 2 = 0.001$; stretch-attend postures: ($F(1,45) = 2.8$, $p = 0.097$, $\eta 2 = 0.01$); head dips: ($F(1,45) = 0.1$, $p = 0.75$, $\eta 2 = 0.001$)) (Figure 3-11).

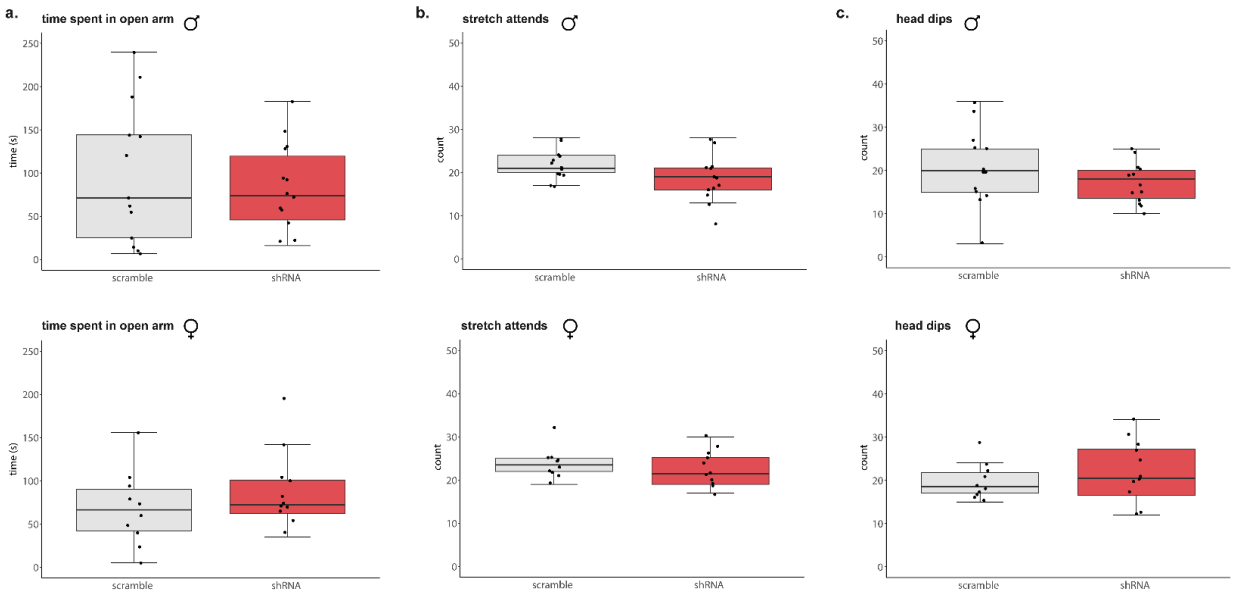

Figure 3-11- Anxiety-like behavior in the elevated plus maze (EPM). *BNST AVP knockdown did not alter anxiety-like behavior. (a) Avp-shRNA and scramble AAV-injected males and females did not differ in time spent in the open arm (a), the number of stretch attend postures (b), or head dips (c). Boxplots indicate individual data points, median, first and third quartiles.*

3.4 Discussion

Here, we report the first direct test of the function of sexually-differentiated AVP expression in the BNST. Previously, we reported that removal of AVP-expressing cells in the BNST of male, but not female, mice reduced investigation of same-sex conspecifics and altered social communication (Rigney et al., 2019). What remained unknown was whether it was removal of AVP or removal of other factors, such as other neuropeptides and neurotransmitters produced by the same cells, that caused changes in social investigation and communication in male mice. Using shRNA viral strategy, we found that knockdown of AVP synthesis in BNST cells, and thereby presumably of AVP release from these cells and its terminals, reduced male-male social investigation, male communicative behavior (USV, tail rattles) toward same-sex conspecifics, and male copulatory behavior. We did not find any behavioral effects in females. Overall, these results suggest that AVP produced in, and released by BNST cells plays a more

prominent role in social behavior in males than in females, and that AVP expression in the BNST drives specific aspects of social behavior in a sexually differentiated way.

AVP produced by BNST cells may play an even more impactful role in behavior than our results indicate, as not all AVP was eliminated, and because AVP cells in the MeA, which show similar sex differences, project to overlapping areas(De Vries and Panzica, 2006). Therefore, it may be necessary to knockdown AVP expression in both groups of cells (BNST, MeA) to identify all behaviors modulated by AVP released from these cells. Furthermore, testing started three weeks after BNST AVP knockdown was initiated. As a result, additional behavioral effects of this knockdown may have disappeared due to the system adapting to chronic depletion of AVP. Such adaptation may explain the opposite effects of acute versus chronic V1aR manipulations in the lateral septum (LS) on anxiety-like behavior(Everts and Koolhaas, 1999; Liebsch et al., 1996).

One of the largest effects we saw after Avp-shRNA knockdown of AVP in the BNST was a reduction in male investigation of male conspecifics. AVP knockdown also increased the time males spent in the chamber furthest away from stimulus males, suggesting active avoidance of these males. This reduction in investigation of stimulus males was not due to deficits in overall social interest, activity level, or generalized anxiety, as investigation of female stimuli and anxiety-like behaviors as measured in the EPM were unaltered. Also, head orientation toward the stimulus animals within the three-chamber apparatus was not affected by AVP knockdown, indicating a specific deficit in close social investigation and not in overall attention toward the stimulus(Defensor et al., 2011; Duque-Wilckens et al., 2018). Additionally, we found that AVP knockdown strongly reduced male investigation of urine samples, with the largest effect seen on investigation of male urine. Together, our findings suggest that, in males, AVP

produced in the BNST stimulates investigation of potentially territorial competitors and their social odors, which aligns with prior work showing that vasotocin knockdown in birds reduced social contact between males(Kelly and Goodson, 2013b) and that AVP injections into the LS, a downstream projection site for BNST AVP cells(De Vries and Panzica, 2006), increased male-male interactions in rats(Koolhaas et al., 1991).

AVP knockdown did not affect male aggressive behavior toward subordinate stimulus males. While these results align with our previous finding that BNST AVP cell ablation affected investigative, but not aggressive, behavior (Rigney et al., 2019), they are somewhat unexpected, as knockdown of BNST AVP also reduced AVP-ir fibers in the LS, a site where AVP acts on aggressive behavior in males(Leroy et al., 2018; Veenema et al., 2010) and females(de Moura Oliveira et al., 2021), but see reference(Beiderbeck et al., 2007). This inconsistency may be due to differences in testing and/or procedural conditions (i.e., exposure to different types of intruders) as well as to species and strain differences (Chalfin et al., 2014; Veenema et al., 2010). Nevertheless, our results leave open the possibility that AVP in the LS from sources other than the BNST contribute to modulation of aggressive behavior, as BNST AVP knockdown did not eliminate all LS AVP-ir fibers. Therefore, AVP inputs from sources such as the medial amygdala and PVN (Rood et al., 2013) may contribute to AVP effects within the LS on aggressive behavior.

Although BNST AVP knockdown did not change offensive attack behavior, it still modestly reduced male signaling behavior in potentially antagonistic settings. Specifically, AVP knockdown reduced tail rattling, a known component of aggressive behavior by dominant males(Lee et al., 2019), and USVs in the presence of other males. However, knockdown did not reduce USVs produced in the presence of receptive females. Typically, males vocalize more than

females(Warren et al., 2018) and primarily direct their vocalizations toward females(Matsumoto and Okanoya, 2018); however, males do produce USVs in male-male territorial contexts(Matsumoto and Okanoya, 2018; Sangiamo et al., 2020). Therefore, our results suggest that BNST AVP is involved in some aspects of male offensive/territorial signaling (e.g., USVs and tail rattling).

We observed no changes in anxiety-like behavior following BNST AVP knockdown. Although this was in agreement with the effects of BNST AVP cell ablation (Rigney et al., 2019), this was somewhat unexpected, as central AVP has been implicated in the modulation of anxiety(Bielsky et al., 2004; Ebner et al., 2002; Raggenbass, 2008). Moreover, pharmacological studies suggest that AVP in the LS, a target of BNST AVP cells in mice, where we noticed a reduction in AVP immunoreactivity after AVP knockdown in the present study, may modulate anxiety(Beiderbeck et al., 2007; Landgraf et al., 1995; Liebsch et al., 1996). However, the present results are consistent with observations that electrolytic lesions of the BNST or BNST AVP cell deletions had no effect on anxiety-like behavior in the EPM in rodents(Rigney et al., 2019; Treit et al., 1998). Therefore, AVP derived from other sources may modulate anxiety action when released in the septum. We do note, however, that ablation of AVP cell groups in the PVN in males, or in the suprachiasmatic nucleus (SCN) in both sexes, increased anxiety-like behavior in the EPM (Rigney et al., 2020b; Whylings et al., 2021). Consequently, the LS may require AVP signals from both BNST and MeA to increase anxiety-like behaviors, while other sources of AVP may work to inhibit it.

While BNST AVP knockdown and BNST AVP cell ablation (REF 19) have similar effects or lack thereof on male-male social investigation, aggression, and anxiety, AVP knockdown did not replicate other behavioral effects of BNST AVP cell deletion. For example,

BNST AVP cell ablation in males increased urine marking toward females, whereas BNST AVP knockdown did not. It may be that BNST AVP cell ablations are more effective in reducing AVP levels than AVP knockdown. However, the percentage of reduction of AVP-ir cells in the BNST was high in both studies (shRNA: ~70-80% average; cell ablations: ~90%+ average). A limitation in comparing results between these two studies, however, is that these experiments were carried out at different times and on different strains of mice, so there may be hidden confounds that caused differences in behavioral outcome between these two studies. The most parsimonious explanation, however, may be that AVP cell ablation may have removed more signaling molecules than just AVP, each of which may have effects on behavior.

Our results demonstrate a distinct, sexually differentiated role of BNST AVP in male copulatory behavior. Knockdown of AVP within the BNST reduced intromissions and ejaculations in males, but did not alter copulatory behavior in females. These results are broadly consistent with the observation that BNST and MeA AVP cells are active during male copulatory behavior (Hari Dass and Vyas, 2014; Ho et al., 2010). As we did not observe changes in precopulatory (male investigation, USVs, urine marking) or mounting behaviors toward receptive females after AVP knockdown, the effects on copulatory behavior are more likely to be due to deficits in bridging the appetitive and consummatory phases of sexual behavior, rather than to changes in sexual motivation(Petrulis, 2013). These results are not in agreement with the effects of BNST AVP cell ablations, which reduced female, but not male, copulatory behavior (Rigney et al., 2019). One possible explanation for this discrepancy is that AVP cell ablations reduced the expression of galanin, a neuropeptide colocalized with AVP in the BNST(Miller et al., 1993), whereas shRNA knockdown may have increased overall inhibitory signaling to targets of BNST AVP cells, as galanin promotes neuronal inhibition(Kask et al., 1995) while AVP

promotes excitation(Raggenbass, 2008). Indeed, ICV injections of galanin strongly inhibited male copulatory behavior in rats(Poggioli et al., 1992), and galanin has been shown to block AVP-induced flank marking in golden hamsters(Ferris et al., 1999). Future studies may help unravel the physiological and behavioral significance of co-expression of AVP and other signaling molecules.

Our results indicate that sexually dimorphic AVP expression in BNST cells contributes to sex differences in the control of social behavior. More specifically, BNST AVP knockdown in male, but not female, mice reduced investigation and communicative behaviors directed toward same-sex conspecifics as well as male sexual behavior, without changing offensive attacks, anxiety-related behaviors, and overall activity. The most parsimonious explanation for seeing these larger effects in males than in females appears to be the much denser innervation of AVP in BNST target areas in males than in females (De Vries and Panzica, 2006). This sex difference has been linked to AVP playing a larger role in controlling functions in males than in females, even in cases where there are no obvious sex differences in those functions (De Vries, 2004). Another explanation may be that BNST cells receive neural and hormonal signals that differ between males and females. This is certainly true for gonadal steroid effects on these cells, as practically all of them co-express estrogen, androgen, and progesterone receptors (Axelson and Leeuwen, 1990; Zhou et al., 1994). Alternatively, these cells may innervate circuits that are sexually differentiated in form and function. In either case, disrupting AVP signaling will interrupt a flow of information that is inherently sexually differentiated.

Our current results are similar to those of other studies that have shown a sex-specific role of AVP in behavior. For example, AVP and its antagonists have effects that differ by sex on aggressive play behavior in rats (Bredewold et al., 2014), territorial aggression in hamsters(Grieb

et al., 2021; Terranova et al., 2017, 2016), and social communication in humans(Feng et al., 2015; Rilling et al., 2014; Thompson et al., 2006). Together, these studies point toward a sexually differentiated role of AVP in vertebrate social behavior. By ablating specific AVP cell groups in the BNST, PVN, SCN (Rigney et al., 2020b, 2019; Whylings et al., 2021), or knocking down AVP specifically in the BNST (present results), we have started addressing directly the question as to which AVP cell groups contribute to sex differences in social behavior and its regulation.

4 SEX DIFFERENCES IN THE CONTROL OF SOCIAL INVESTIGATION AND ANXIETY BY VASOPRESSIN CELLS OF THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS.

Previously published in Neuroendocrinology, doi: 10.1159/000509421 (PMID: 32541145)

4.1 Introduction

The neuropeptide arginine-vasopressin (AVP) (vasotocin in non-mammalian vertebrates) is an important modulator of vertebrate social behavior (Caldwell, 2017; Choleris et al., 2009; Donaldson & Young, 2008; Insel, 2010), including that of humans (Guastella et al., 2010; Rilling et al., 2014). AVP acts on several receptors and in various brain regions to alter social communication (Goodson & Bass, 2001; Kelly & Goodson, 2013; Rigney et al., 2020), maternal care (Bosch & Neumann, 2008), pair bonding (Carter et al., 1995; Jarcho et al., 2011; Young & Wang, 2004), cognition (Landgraf & Neumann, 2004), and social recognition (Dantzer et al., 1988; Johnson & Young, 2017; Veenema et al., 2012). Studies involving blockade or stimulation of central vasopressin receptors and vasopressin knockout also implicate AVP in the generation

of anxiety states and generally point at AVP acting as an anxiogenic neuromodulator (Bielsky et al., 2004; Fabio et al., 2012; Landgraf, 2006; Mak et al., 2012; Neumann & Landgraf, 2012, Caldwell et al., 2008). Consequently, it is possible that AVP system effects on social behavior and communication are mediated by alterations in anxiety.

Although species differences are evident in the patterning of brain AVP receptor expression (Albers, 2015; Goodson & Bass, 2001), most brain sources of AVP exhibit a conserved anatomical distribution (Johnson & Young, 2017). AVP is made in classical neurosecretory cells in the paraventricular (PVN), supraoptic (SON), and accessory nuclei of the hypothalamus (de Vries, 2008; De Vries & Panzica, 2006; Farina-Lipari & Valentino, 1993; Ring, 2005; Rood & De Vries, 2011; Sawchenko & Swanson, 1982). AVP is also made in cells that project to target areas in the brain, most notably cells in the suprachiasmatic nucleus, the bed nucleus of the stria terminalis (BNST) and medial amygdala (MA), as well as the PVN (de Vries, 2008). Recently, other areas, such as the accessory olfactory bulb have shown to contain AVP-expressing cells involved in social behavior (Tobin et al., 2010). The projections from the BNST and MA are sexually dimorphic, with male rodents having about twice as many AVP-expressing cells as females in these nuclei (Otero-Garcia et al., 2014; Rood & De Vries, 2011; van Leeuwen et al., 1985). We have recently demonstrated that elimination of BNST AVP-expressing cells reduced specific male communicative behaviors, but that other social behaviors known to be influenced by AVP remained intact (i.e., male social investigation of female conspecifics) (Rigney et al., 2019; Whylings et al., 2020). It is plausible that other centrally projecting AVP systems, such as the PVN, are involved in regulating these aspects of social behavior. Indeed, hypothalamic and extrahypothalamic AVP inputs overlap in brain regions, such as the periaqueductal gray (Tschida et al., 2019), that are involved in social behavior, communication

and emotion (Goodson & Bass, 2001; Herman et al., 2005; Rood & De Vries, 2011; Rood et al., 2013).

AVP-expressing cells in the PVN are strong candidates for regulation of social communication as they have been implicated in both social- and stress-related behaviors and in sex-different ways. For example, high levels of AVP mRNA in the PVN coincide with increased male social interactions, but with decreased female social investigation under stress conditions (Borrow et al., 2018; Murakami et al., 2011). However, few studies have directly tested the role of PVN AVP-expressing cells on social behavior. One study showed that PVN AVP knockdown impaired gregariousness in zebra finches (Kelly & Goodson, 2014), whereas another indicated that stimulating PVN AVP projections to the hippocampus increases social memory (A. S. Smith et al., 2016). The effects of manipulating PVN AVP-expressing cells on social communication and behavior have not been tested in rodents.

Here we test the hypothesis that PVN AVP is necessary for social communication and behavior in both male and female mice by deleting PVN AVP-expressing cells using a viral-mediated, cell-death construct (activated caspase) and then measuring communicative behaviors (ultrasonic vocalizations, urine marking) and social interactions (social investigation, copulatory behavior, aggression). In addition, we also measured anxiety-related behaviors in a non-social context so as to clarify if any observed changes in social behavior were due to changes in anxiety.

4.2 Methods

4.2.1 Animals and Husbandry

All mice were maintained at 22°C on a 12:12 reverse light cycle with food and water available ad libitum and housed in individually ventilated cages (Animal Care Systems, Centennial, CO, USA) with corncob bedding, a nestlet square, and a housing tube. All animal procedures were performed in accordance with the Georgia State University animal care committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects

AVP-IRES2-Cre-D (AVP-Cre) mice were obtained from The Jackson Laboratory (Stock No: 023530; Bar Harbor, Maine, USA). AVP-Cre knockin mice have Cre recombinase expression directed to vasopressin-expressing cells that are restricted to populations within the hypothalamus. Subjects were derived by crossing heterozygous Cre+ mutants to wildtype C57Bl/6J mice and genotyped (ear punch) by polymerase chain reaction (PCR) at 21-24 days of age (Transnetyx, Cordova, TN, USA). Both Cre+ and Cre- littermates were used in behavioral experiments. All subjects were adult mice (2-4 months old) that were singly-housed for 1-2 weeks prior to testing to prevent the formation of dominant-subordinate relationships and to increase male-typical territorial behavior (Kappel et al., 2017).

Stimulus animals

CD1(ICR) (Charles River Laboratories, Wilmington, MA, USA) mice were used as stimuli for behavioral testing and to provide male and female subjects with social experience because strain differences between subjects and stimulus mice increase social investigation

(Gheusi et al., 1994). Mice were used at 9-16 weeks of age and were novel and unrelated to the subject to which they were exposed.

Female stimulus mice were grouped-housed, ovariectomized, and implanted with an estradiol capsule (GDX+E), and given two sexual experiences before testing. Two groups of stimulus males were used for behavioral testing. Mice that were used as subordinate mice in the home cage aggression tests and for providing aggressive experience to subjects, were grouped-housed, gonadectomized (GDX), and subjected to two aggressive encounters with a dominant male (see below). Mice in the second group, which provided sexual experience to female subjects and served as sexual partners during copulatory tests and as stimulus animal in the three-chamber social test, were singly-housed, gonadectomized, implanted with testosterone (GDX+T), and then given two sexual experiences before testing.

4.2.2 Viral Vector

AVP driven-, Cre-expressing PVN neurons were ablated using an adeno-associated virus (AAV-flex-taCasp3-TEVp; In-Stock AAV Vector – Dr. Nirao Shah; serotype 2/1; 3×10^{12} IU/mL; University of North Carolina at Chapel Hill Vector Core) that encodes, in a Cre-dependent fashion, a mutated pro-caspase-3 and its activator (TEVp) (Figure 4-2a). This system activates an apoptotic signaling cascade, cleaving multiple structural and regulatory proteins critical for cell survival and maintenance (Unger et al., 2015; Yang et al., 2013) and killing cells with far less collateral inflammation than other lesion approaches (Morgan et al., 2014).

4.2.3 Surgery

All surgeries were carried out using 1.5-3% isoflurane gas anesthesia in 100% oxygen; 3 mg/kg of carprofen was given subcutaneously before surgery to reduce pain.

Stereotaxic surgery

Mice were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand-operated drill was used to make holes in the skull, exposing the dura. For all subjects, 700 nl of AAV-flex-taCasp3-TEVp was delivered bilaterally to the PVN (coordinates: AP -0.42 mm; ML ± 0.35 mm; DV 5.2 mm (Paxinos & Franklin, 2012)) at a rate of 100 nl/min using a 5 µl Hamilton syringe with a 30-gauge beveled needle mounted on a stereotaxic injector. Following virus delivery, the syringe was left in place for 15 minutes before slowly withdrawing it from the brain.

Gonadectomy and Hormone Treatment

Testes were removed after cauterizing the ductus deferens and blood supply via a midline abdominal incision. Silastic capsules (1.5 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) were filled with crystalline testosterone (T; Sigma, St. Louis, MO, USA) and inserted subcutaneously between the scapulae after gonadectomy; this procedure leads to physiological levels of T (Barkley & Goldman, 1977; Matochik et al., 1994). To further reduce aggression in stimulus animals (Beeman, 1947), some males were gonadectomized, but did not receive a T implant (GDX).

The ovaries of stimulus female mice were removed after cauterizing its blood supply via an abdominal incision at the uterine horn. Silastic capsules (0.7 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter; Dow Corning Corporation, Midland, MI, USA) containing estradiol benzoate (E; diluted 1:1 with cholesterol) were implanted subcutaneously in the scapular region immediately following ovariectomy (GDX + E) (Bakker et al., 2002; Ström et al., 2012). To induce sexual receptivity, stimulus females were injected subcutaneously with 0.1

ml of progesterone (500 μg dissolved in sesame oil, Sigma, St. Louis, MO, USA) four hours preceding sexual experience, urine collection, and behavioral testing (Veyrac et al., 2011).

Urine Collection

Pooled urine samples were collected from stimulus females induced into estrus and from stimulus males (3-8 mice per sample). Estrous state was verified by color, swelling, and expanded size of the vaginal opening (Caligioni, 2009). To collect urine, mice were picked up by the tail base and held by dorsal neck skin; this method typically induced urination. If the mouse did not urinate, stroking its belly from an anterior to posterior direction stimulated bladder voiding. Each mouse provided 15-50 μl of urine that was pooled into a 1.5 ml Eppendorf tube. Urine samples were used fresh within one hour of collection to prevent chemosignal degradation (Roullet et al., 2011).

4.2.4 Social Experience

As opposite-sex sexual experience and attaining competitive status ("social dominance") promote communicative behaviors (Lumley et al., 1999; Roullet et al., 2011), mice received social experience over five consecutive days (sexual encounters on days 1 and 4, aggressive encounters on days 2 and 5; no encounters on day 3).

Sexual Experience

Subjects were given two opportunities to interact with either a stimulus female (for male subjects) or a stimulus male (for female subjects). A sexually-experienced stimulus mouse was placed in the subject's home cage and removed the next day (first experience) or after ninety minutes (second experience). Subjects that did not ejaculate or elicit ejaculation (females) during the second sexual experience were removed from further testing.

Aggressive Experience

Male subjects were exposed to two interactions with subordinate males treated with 50 µl of GDX+T male urine applied to their backs. Gonadectomy, group housing, and social defeat of subordinates reduce offensive aggression in mice, while GDX+T male urine provides subjects with a cue that elicits offensive aggression (Beeman, 1947; Connor & Winston, 1972; Van Loo et al., 2001). Subordinate stimulus males were placed in the subject's home cage and removed after the subject's first offensive attack (biting) within a ten-minute period. All subject males attacked the intruder male stimulus by the second encounter, and all subordinate stimulus males displayed submissive behavior, defined as defensive postures (e.g. on-back postures, fleeing, and non-social exploring (Koolhaas et al., 2013)). Female subjects were exposed to a female intruder; however, this did not elicit attacks from either animal.

4.2.5 Experimental Procedure

All testing occurred within the first eight hours of the dark cycle under red light illumination, with the exception of the elevated plus maze. All tests were scored by an experimenter blind to the genotype of the subject and testing occurred across multiple cohorts of subjects. Three to four weeks after viral injections, subjects were habituated to the testing room and apparatus by handling and placing mice (for five minutes) in the three-chamber apparatus (see below) each day for three days. On experimental days, subjects were adapted to the experimental room for fifteen minutes prior to testing. First, we tested mice on an elevated plus maze to test for anxiety-related behavior (Lister, 1987). Mice were then tested in the three-chamber apparatus (Figure 4-1a) over six days with 2-7 days between testing. Lastly, odor discrimination, copulatory, and aggressive behavior were measured sequentially, with a day in between, in the subject's home cage (Figure 4-1b, timeline). Female subjects were tested irrespective of estrous cycle day, except during copulation testing, when they were in behavioral

estrus. Prior research indicates minimal effects of estrous cycle on female mouse communicative behavior (Coquelin, 1992; Maggio & Whitney, 1985; Moncho-Bogani et al., 2004). Following testing, subjects were sacrificed, and their brain tissue was processed for in situ hybridization to detect AVP- or OT-expressing cells within the PVN.

Social Behavior

USV, urine marking, and social investigation were recorded in an acrylic three-chamber apparatus (Harvard Apparatus, Holliston, MA, USA; dimensions: 20.3 x 42 x 22 cm) (Arakawa et al., 2008; Crawley, 2007; Moy et al., 2009). Instead of a solid floor, the apparatus was placed on absorbent paper (Nalgene Versi-dry paper, Thermo Fisher Scientific, Waltham, MA, USA) so as to accurately measure urine marking. Animals were also tracked using motion detection software (ANY-maze, San Diego Instruments, RRID:SCR_014289). During testing with stimulus animals, subjects had access to either a stimulus animal in a cylindrical cage (8 cm (D), 18 cm (H); 3 mm diameter steel bars, 7.4 mm spacing) or an empty cage placed at opposite corners of the outermost chambers of the apparatus. For testing with social odors, subjects had access to 50 µl of fresh urine from a stimulus animal or 50 µl saline pipetted onto a clean piece of filter paper (3 cm2) that was taped on the outside of cages. The location of stimulus and the "clean" cage were counterbalanced across animals. After placing the subject in the center of the middle chamber, we measured, across a 5-minute trial, close investigation of clean and stimulus cages, distance traveled throughout the apparatus, as well as USV and urine marking, as described below. After testing, the apparatus and cages were thoroughly cleaned with 70% ethanol and allowed to dry before further testing. In all cases, urine stimulus from one sex was presented first (day 1), followed by a live stimulus of that same sex on the following day (day 2); this order was then repeated 3-5 days later for the opposite sex. In this fashion, mice first

experienced a weak stimulus (urine), then a stronger social stimulus (live animal). The order of male and female stimuli presentation was counterbalanced across subjects.

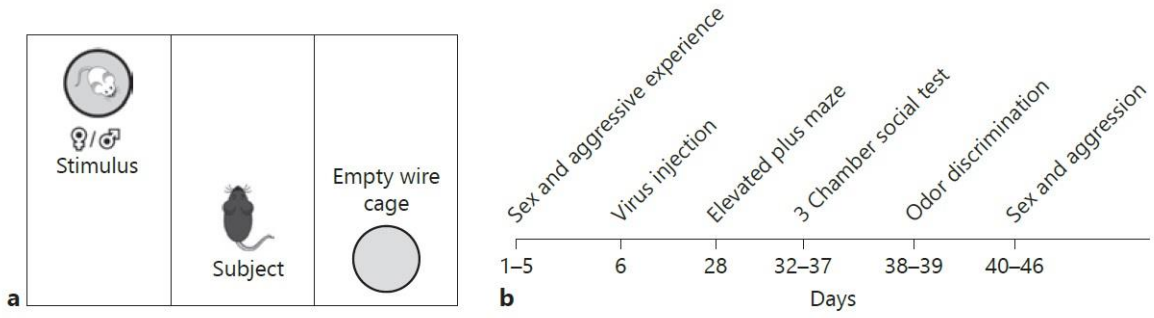

Figure 4-1– (a) Three-chamber social testing (b) Experimental timeline.

Investigation and Ultrasonic Vocalizations

Close investigation was defined as time spent sniffing within 2 cm of the stimulus or clean cage; climbing on the cage was not scored as investigation. USV were detected using a condenser microphone connected to an amplifier (UltraSoundGate CM16/CMPA, 10 kHz - 200 kHz, frequency range) placed 4 cm inside the apparatus and directly above the center compartment. USV were sampled at 200 kHz (16-bit) with target frequency set to 70 kHz (UltraSoundGate 116Hb, Avisoft Bioacoustics, Berlin, Germany). Recordings were then analyzed using a MATLAB (MATLAB, Mathworks, RRID:SCR_001622) plug-in that automates USV analysis (Van Segbroeck et al., 2017). Using this program, sonograms were generated by calculating the power spectrum on Hamming-windowed data and then transformed into compact acoustic feature representations (Gammatone Filterbank). Each 200-millisecond window containing the maximum USV syllable duration was then clustered, via machine learning algorithms, into USV syllable types (repertoire units) based on time-frequency USV

shape. Repertoire units that appeared as background noise were discarded. We counted the number of all USV produced by each subject. USV syllable types were identified using criteria previously described: short, composite, downward, upward, 1 frequency jump, modulated, multiple frequency jumps, u-shape, flat, chevron (Hanson & Hurley, 2012).

Urine Marking

Following testing, the substrate sheet was allowed to dry for one hour and then sprayed with ninhydrin fixative (LC-NIN-16; Tritech Forensics Inc., Southport, NC, USA) to visualize urine marks (Lehmann et al., 2013; Meyer, 1957). After twenty-four hours, sheets were imaged (Sony DSC-S700 camera), binarized and analyzed using computer-aided imaging software (ImageJ, RRID:SCR_003070). Urine marking was measured as the total area (pixels) of visualized ninhydrin urine marks in the entire arena. Urine marks that were larger than 6 cm^2 and directed toward corners were counted as elimination 'pools' and were counted separately (Bishop & Chevins, 1987).

Copulatory and Aggressive Behavior

To measure copulatory behavior, the stimulus mouse was placed in the subject's home cage and then removed after ninety minutes had elapsed. The latency to mount, percent of females that were mounted, percent of male ejaculations, and number of mount rejections (female kicking male off during mounting attempt) in female subjects was recorded. To measure territorial aggression, subordinate stimulus males were placed in the subject's home cage and then removed after the subject's first offensive attack (biting) within a ten-minute period; the latency to first bite was recorded.

Elevated-Plus Maze

The elevated plus maze (EPM) consisted of two open arms (30 x 5 x 0 cm) and two closed arms (30 x 5 x 25 cm) crossed perpendicularly and raised 60 cm above the floor. Subjects were placed at the arm intersection facing the open arm and were habituated to the apparatus for one minute; subjects were then observed for an additional five minutes. Time spent in open and closed arms and the number of risk assessment behaviors (stretch-attend posture, head-dips) were manually scored from video (Cole & Rodgers, 1993). Subjects were removed from EPM data analysis if they fell off the EPM during testing.

Odor Discrimination

We used a habituation-discrimination approach to test whether subjects could distinguish between social and non-social odors, as described previously (Baum & Keverne, 2002; Rigney et al., 2019). Briefly, we used the subject's natural tendency to decrease investigation to repeated, familiar odors and to then increase it toward novel odors. We operationally defined odor discrimination as a statistically significant difference between time sniffing the last familiar odor and the novel odor sample.

4.2.6 Histology and In Situ Hybridization

Following testing, subjects were killed via CO2 asphyxiation. Brains were extracted and flash-frozen via submersion in 2-methyl-2-butanol (Sigma, St. Louis, MO, USA) for 10-20 s and stored at −80°C until sectioned. Coronal sections (20 μm) were cut with a cryostat (Leica CM3050 S, Leica Biosystems, Heidelberg, Germany) into three series and stored at −80°C. All tissue was handled in a RNase-free environment. Tissue was post-fixed in paraformaldehyde, followed by a wash in 2X saline-sodium citrate (SSC) and acetylation in a triethanolamine/acetic anhydride solution, rinsed in dH20, washed in acetone/methanol solutions (1:1), and again in 2X

SSC. Tissue was first incubated at 65°C in hybridization buffer (50% deionized formamide, 1% yeast tRNA, 10% dextran sulphate, 1x Denhardt's solution, 5% 20x SSC) for 30 minutes before probe application. Riboprobes were developed from linearized PK Bluescript SK(+) with inserted mouse-vasopressin or oxytocin gene (AVP: NM_027106.4, OT: NM_011025.3, Genscript) using digoxygenin (DIG)-conjugated uracil. Riboprobe synthesized from this plasmid was added to the hybridization buffer at a concentration of 100 ng/100 µl and denatured at 90°C for five minutes. Tissue was then hybridized at 65°C for 24 hours in a humid chamber. The tissue was then subjected to two ten-minute washes in 2x SSC at room temperature followed by a fifteen-minute digestion with RNase A (10 g/ml in 2x SSC) at 37°C. This was followed by a thirty-minute 2x SSC wash at 56°C and two ten-minute 2x SSC washes at room temperature. The tissue was then quenched in 1% H2O2 in 1x SSC for fifteen minutes, rinsed twice in 1x SSC with 0.1% Tween followed by one five-minute TBS (20 mM Tris, 150 mM NaCl, pH 7.6) wash. Blocking solution (Normal sheep serum and bovine caesin) was applied and tissue was incubated for thirty minutes followed by two-hour, room temperature incubation with anti-DIG-HRP (1:200, Roche Applied Sciences, Penzberg, Germany). Unbound antibody was washed away with three ten-minute washes in TBS-T (0.05% Tween in TBS). DIG-labeled probe signal was amplified and visualized using a TSA Plus Fluorescein kit (Perkin Elmer, Waltham, MA, USA) by incubating sections in a 1:50 dilution of the fluorescein working solution for twelve minutes followed by three ten-minute washes in TBS. Tissue was then cover-slipped using Prolong Gold (Life Technologies, Carlsbad, CA, USA) for subsequent imaging and tissue analysis. Tissue processed using sense RNA probe generated no specific labeling.

Tissue Analysis

Bilateral images were taken at 10x magnification using a Zeiss Axio Imager.M2 microscope (Carl Zeiss Microimaging, Göttingen, Germany), which transferred fluorescent images (FITC contrast reflector) to image analysis software (Stereo Investigator, MicroBrightField, RRID:SCR_002526). Imaging domains (2 mm2) were placed with reference to anatomical landmarks (ventricles) (Paxinos & Franklin, 2012) and the number of fluorescent cells per mm2 was reported. For each subject, fluorescently labeled AVP or OT mRNA-expressing cells were counted in the PVN in both hemispheres across four sections covering the extent of the AVP or (in a smaller subset of subjects: Cre- (n = 3) and Cre+ (n = 4) males and Cre- (n = 4) and Cre+ (n = 3) females) OT cell population (from bregma: -0.58 mm, -0.70 mm, -0.82 mm, -0.94 mm), summed and averaged across each hemisphere, with left and right hemisphere cell averages combined into an overall PVN cell averages and compared across ablated and control subjects. Ablated subjects were included in the analysis if they had > 60% AVP-expressing cell ablation bilaterally throughout the entire rostral-caudal extent of the nucleus. In addition, we counted AVP-labeled cells in the nearby mouse accessory area to determine if AVP-expressing cell deletion spread beyond the PVN.

4.2.7 Statistical Analysis

All data were analyzed and graphed in R (3.4.4; R Core Team, 2017). Histology, social investigation, movement, urine marking, USV, and EPM data met the assumptions of parametric statistical tests. Therefore, we analyzed data on histology, social investigation, movement (distance travelled), time spent in open/closed arms in the EPM test, and urine marking with mixed-model ANOVAs with genotype (Cre+, Cre-) and sex (male, female) as between-subject factors; and sex of stimulus (male, female) and stimulus location (stimulus cage, clean cage) as

within-subject factors: followed by t-tests using Bonferroni-correction assessing genotype effects. Effects of genotype on additional anxiety behaviors (stretch-attend, head-dips) were analyzed using t-tests. Measures of copulatory behavior, and aggression behavior were not normally distributed and could not be transformed, therefore, we analyzed genotype effects on these behaviors using Kruskal Wallis tests. All post-hoc comparisons report Bonferroni-corrected p-values and Cohen's D for effect size when statistically significant ($p < 0.05$).

4.3 Results

4.3.1 Histology

Injections of a viral vector encoding a Cre-dependent cell-death construct into the PVN substantially reduced AVP mRNA-expressing cells in both Cre+ males and females compared to Cre- subjects ($F(1,44) = 183.9$, $p < 0.00001$). Cre+ males showed a 79% reduction in AVP cells ($t(25) = 9.85$, $p < 0.00001$, $d = 3.62$) and Cre+ females showed a 85% reduction in AVP cells ($t(18) = 9.132$, $p < 0.00001$, $d = 4.29$) compared to Cre- controls (Figure 4-2b). Although there was a trend toward a significant interaction ($F(1,44) = 3.86$, $p = 0.056$), probably due to females having more AVP mRNA-expressing cells in the Cre- controls and fewer in the Cre+ mice, post hoc tests did not reveal a significant difference in the amount of cells deleted in males compared to females ($t(22) = 1.89$, $p = 0.19$), and no sex difference in the number of PVN AVP-expressing cells in control animals ($t(21) = 1.47$, $p = 0.31$). Ablations of PVN AVP-expressing cells did not significantly reduce the number of oxytocin (OT) mRNA-expressing cells ($F(1,10) = 0.7$, $p = 0.36$; males: $t(5) = 1.03$, $p = 0.35$; females: $t(5) = 0.12$, $p = 0.9$, Figure 4-2c,e) or reduce the number of AVP mRNA-expressing cells in the nearby hypothalamic/mouse accessory area (data not shown), suggesting no significant off-target cell deletions. Additionally, all anterior-posterior levels of the PVN had significant bilateral reduction in AVP cell number (Figure 4-3), indicating

no systematic bias in targeted cell death within the PVN. Cre+ subjects that had less than 60% cell deletion (bilaterally) were removed from the analysis (five males and four females).

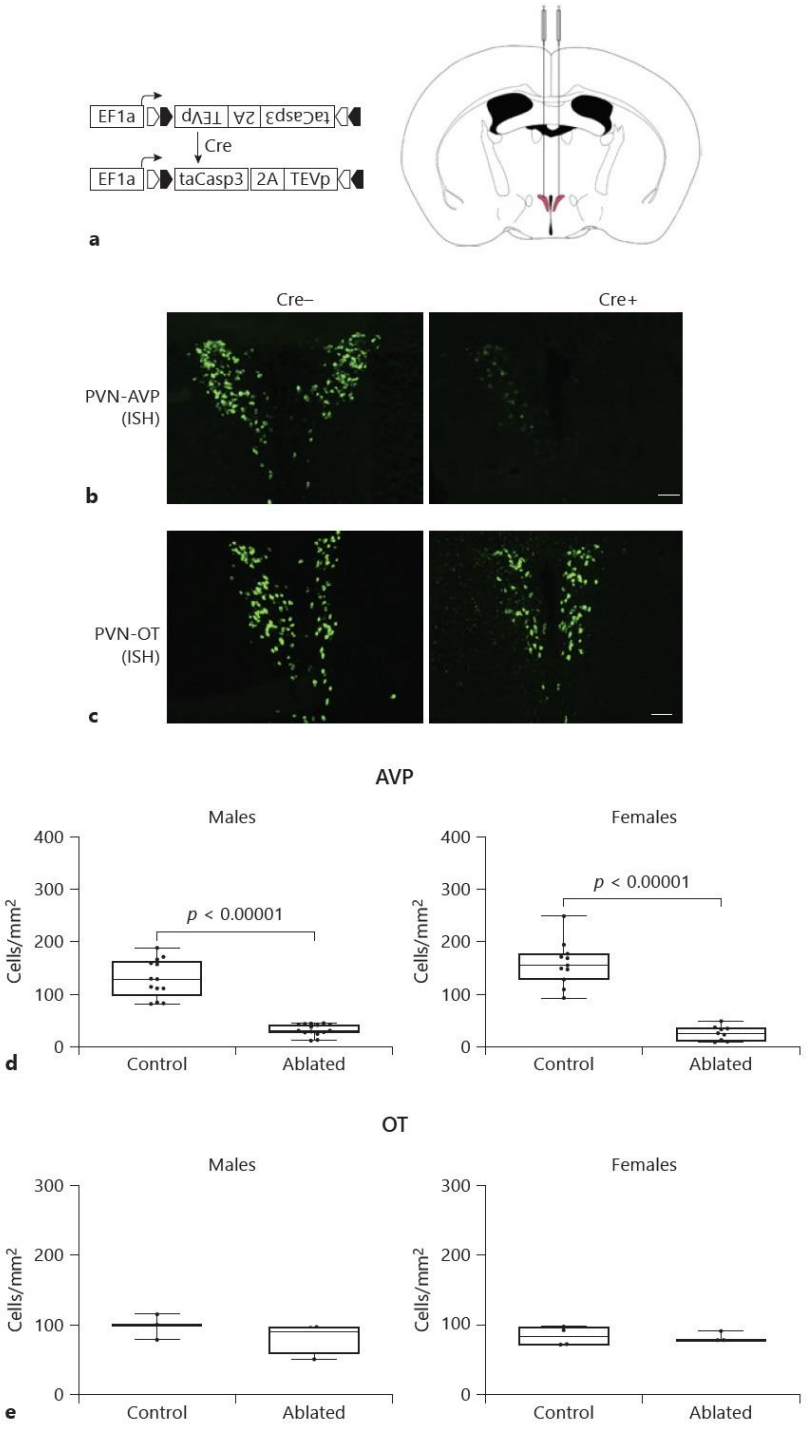

Figure 4-2- Histology. *(a) Cre-dependent adeno-associated virus (AAV-flex-taCasp3-TEVp) construct and location of bilateral PVN injection site; AP -0.42 mm; ML ± 0.35 mm; DV*

5.2 mm; modified from [48]. (b) Representative images of fluorescent in situ hybridization-labeled PVN AVP-expressing cells and (c) OT-expressing cells. (d) Boxplots indicating individual data points, median, first and third quartiles for PVN AVP-expressing cell number across four anterior-posterior sections in male and female subjects. Within the PVN, we found a significant decrease in AVP-expressing cell number in both Cre+ male and female subjects compared to Cre- controls (males: p < 0.00001; females: p < 0.00001). Cre- (n = 13) and Cre+ (n = 15) males and Cre- (n = 11) and Cre+ (n = 9) females. (e) We found no significant OT-expressing cell loss between Cre+ and Cre- subjects (males: p = 0.35; females: p = 0.9). Cre- (n = 3) and Cre+ (n = 4) males and Cre- (n = 4) and Cre+ (n = 3) females. Images were taken at 10x, fluorescent cells per mm2, scale bar = 50 µm.

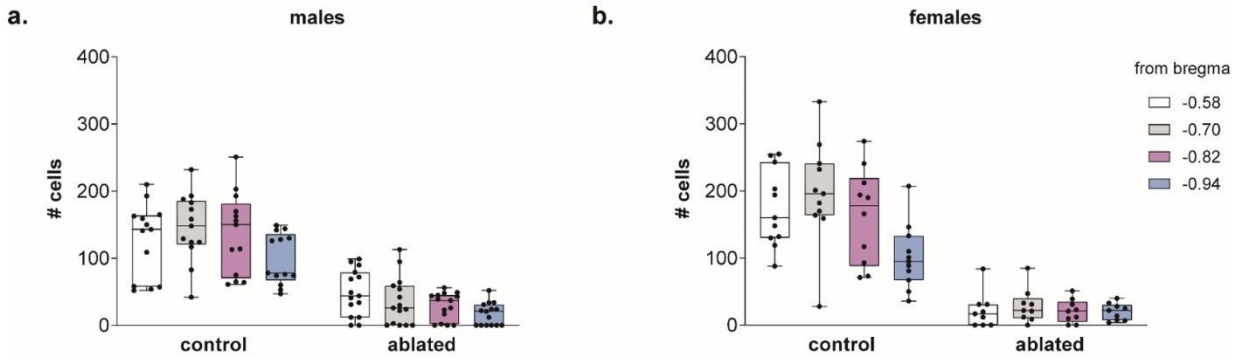

***Figure 4-3– Additional Histology**. Boxplot indicating individual data points, median, first and third quartiles for PVN AVP-expressing cell counts throughout four anterior-posterior sections of the PVN of control and cell-ablated animals. A significant decrease in AVP-expressing cell number within each PVN section was observed in cell-deleted males and females, compared to controls.*

4.3.2 PVN AVP-expressing cell ablation in females increases social investigation

All subjects investigated cages with stimulus animals more than the empty cages ($F(1,44) = 208.5$, $p < 0.00001$). Overall, there was a significant difference between genotypes ($F(1,44) = 4.38$, $p = 0.04$) as well an interaction between sex and genotype ($F(1,44) = 5.33$, $p = 0.02$). Control males showed higher levels of investigation than did control females (female stimulus: $t(22) = 2.15$, $p = 0.046$, $d = 0.9$; male stimulus: ($t(22) = 2.90$, $p = 0.001$, $d = 1.22$); ablation of PVN AVP-expressing cells, however, removed this sex difference (female stimulus: $t(22) = 0.1$, $p = 0.9$; male stimulus: $t(22) = 0.8$, $p = 0.41$). Removing PVN AVP-expressing cells in females

increased investigation of cages with male as well as female stimulus animals (female stimulus: t(26) = 2.92, p = 0.009, d = 1.3; male stimulus: (t(18) = 3.36, p = 0.004, d = 1.4; Figure 4-4b), whereas ablations in males did not (male stimulus: (t(26) = 0.1, p = 0.93); female stimulus: t(26) = 0.13, p = 0.9) Figure 4-4a). AVP-expressing cell deletion did not alter investigation of filter paper with urine (F(1,44) = 0.002, p = 0.97). Males and females of both genotypes were able to discriminate between male and female urine odors (data not shown).

Although males were more active than females (F(1,44) = 4.6, p = 0.038), there were no significant overall differences in activity between control and ablated subjects (F(1,44) = 3, p = 0.09, Figure 4-4c-d), nor were there interactions between genotype and sex (F(1,44) = 0.7, p = 0.4), or genotype and sex of stimulus animal (F(1,44) = 0.15, p = 0.7). Consequently, PVN AVP-expressing cell ablations do not grossly alter locomotor behavior.

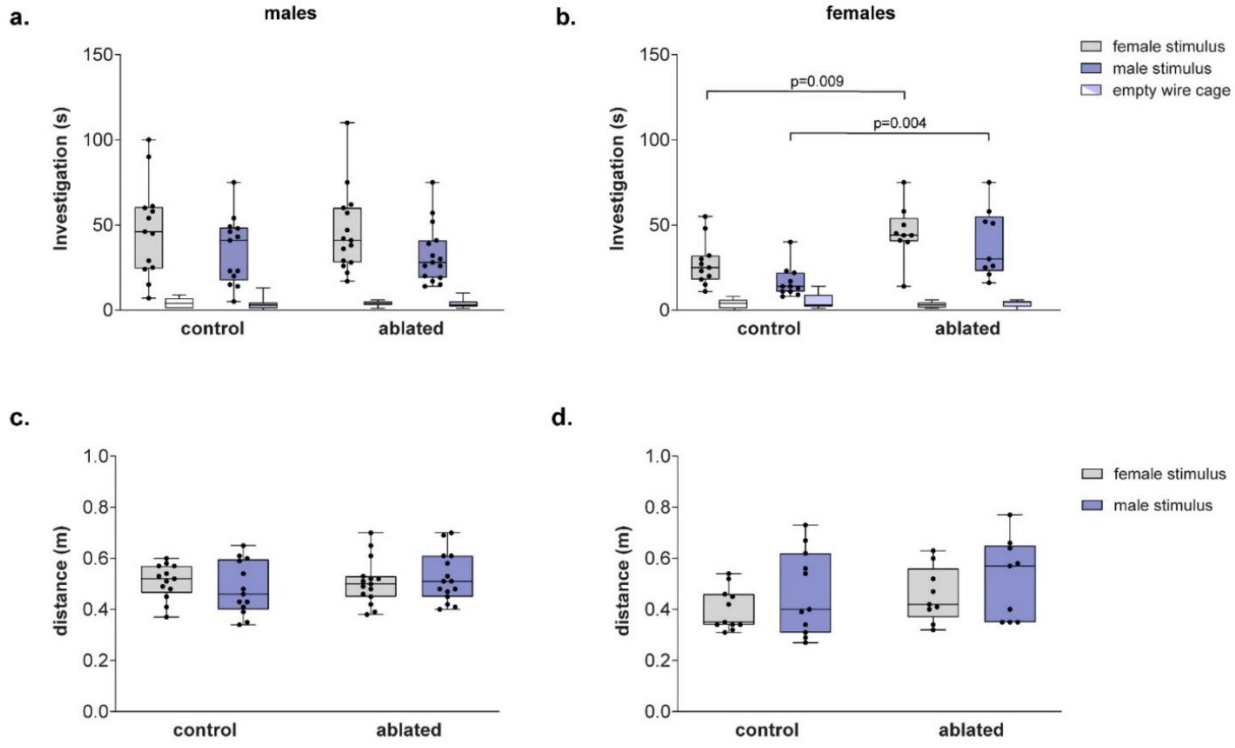

Figure 4-4- PVN AVP-expressing cell ablations increases social investigation by females. *(a-b) Boxplot indicating individual data points, median, first and third quartiles for time spent investigating wire cages with male or female stimulus animals, or an empty wire cage within the three-chamber apparatus. PVN AVP-expressing cell ablations in females (b), but not males (a), significantly increased investigation of female (p = 0.009) and male (p = 0.004) stimuli compared to controls. (c-d) Control and AVP-expressing cell-ablated subjects did not differ in distance traveled within the three-chamber apparatus.*

4.3.3 PVN AVP-expressing cell ablations did not alter social communication

Male subjects urine-marked more than females, but PVN AVP-ablated and control did not differ in urine marking within the three-chamber apparatus ($F(1,44) = 2.01$, $p = 0.16$, Table 2). Only two male mice (both Cre- controls) urinated in an elimination 'pool' (one to a female stimulus, the other to a male stimulus; both in a corner); these pools were not counted as urine marks. Similarly, males produced more USVs than females, specifically toward female stimuli ($F(1,44) = 6.78$, $p = 0.01$), but overall AVP cell-ablated and control subjects did not differ in the amount of USVs emitted ($F(1,44) = 0.02$, $p = 0.88$, Table 2) or in the types of syllables produced ($F(1,44 = 0.4$, $p = 0.54$, Figure 4-5). Subjects did not differ in urine marking ($F(1,44) = 0.81$, $p = 0.37$) or USVs ($F(1,44) = 1.4$, $p = 0.24$) in the presence of male or female urine (Table 3).

Table 2-Median and range of urine marked area and number of USV produced within the 3 chamber apparatus. Subjects with PVN AVP-expressing cell deletions did not differ from controls in urine marking or USV production in response to male or female stimuli

	male subjects				female subjects			
genotype	control		ablated		control		ablated	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
urine marking (pixels)	39621 (0-2047224	32988 (0-2043559)	10364 (0-2197340)	2071 (0-1906592)	3848 (0-2024380)	8612 (0-1626100)	1087800 (0-1982604)	0 (0-1976011)
USV (count)	33 (0-992)	2 (0-30)	20 (0-1097)	0 (0-45)	0 (0-21)	4 (0-386)	0 (0-9)	6 (0-280)

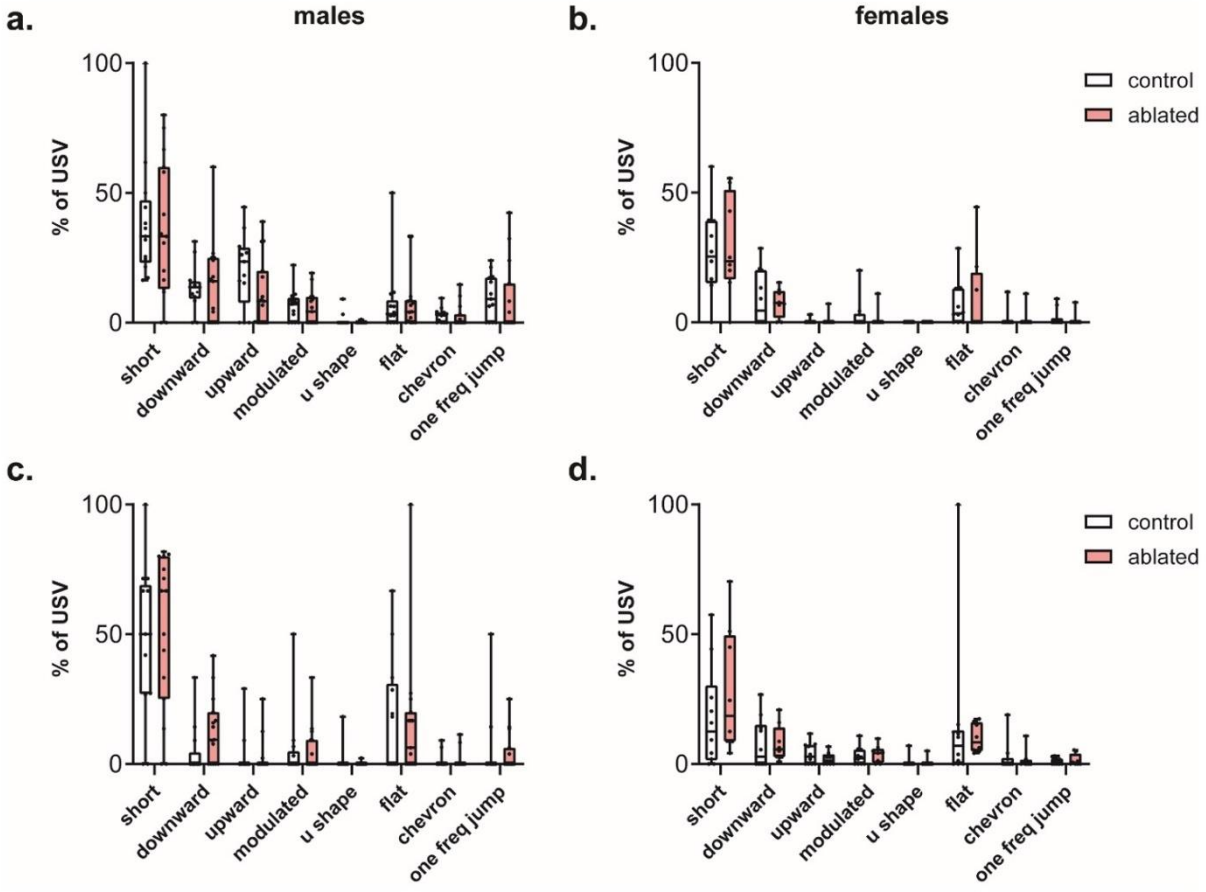

Figure 4-5– USV syllables*. Boxplot indicating individual data points, median, first and third quartiles for USV syllable types emitted by male and female subjects in response to male (a, b) and female (c,d) stimuli. PVN AVP-expressing cell ablation did not alter the percentage of USV syllable types produced.*

Table 3-Median and range of investigatory and communicative responses within the 3 chamber apparatus to male or female urine stimuli within the three-chamber apparatus. Cell-deleted and control subjects did not differ in social investigation, urine mark.

	male subjects				female subjects			
genotype	control		ablated		control		ablated	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
social investigation (s)	18 (6-27)	10 (3-18)	14 (1-32)	11 (1-27)	6 (1-22)	4 (2-15)	5 (0-26)	6 (1-17)
urine marking (pixels)	17815 (0-2068194)	5826 (0-749476)	6285 (0-1998000	300 (0-1710944)	0 (0-11693)	0 (0-791312)	0 (0-1898424)	0 (0-1735449)
USV (count)	5 (0-290)	2 (0-29)	0 (0-69)	0 (0-9)	0 (0-5)	0 (0-14)	0 (0-5)	0 (0-9)

4.3.4 PVN AVP-expressing cell ablations in males increases anxiety-like beahvior

We measured time spent in the open, anxiogenic, arms along with risk assessment postures (stretch attends, head dips) in the elevated plus maze (EPM). Six males (2 control, 4 cell-deleted) and one control female fell off the EPM during testing and so their data are not included in the EPM analysis. Overall, there were no sex differences in time spent in the open arms ($F(1,37) = 0.3$, $p = 0.57$) or in frequency of risk assessment behaviors (stretch attends: $F(1,37) = 0.03$, $p = 0.85$; head dips: ($F(1,37) = 3.1$, $p = 0.09$). There was a trend toward a main effect of genotype in time spent in the open arms ($F(1,37) = 3.4$, $p = 0.07$) with an interaction between genotype and sex of subject for stretch attends ($F(1,37) = 5.9$, $p = 0.02$). Post-hoc analysis revealed male subjects with PVN AVP cell ablations decreased the amount of time spent in the open arms of the EPM ($t(20) = 2.27$, $p = 0.034$, $d = 0.96$, Figure 4-6), increased the amount of stretch attend postures ($t(20) = 3.66$, $p = 0.002$, $d = 1.56$, Figure 4-6c), and trended toward an increase in the amount of head dips ($t(20) = 1.82$, $p = 0.08$, $d = 0.78$, Figure 4-6e) compared to controls. PVN AVP cell-ablated female subjects did not differ from controls on any anxiety-like

behaviors in the EPM (time spent in open arm: (t(17) = 0.49, p = 0.63; stretch attends: (t(17) = 1.38, p = 0.19; head dips: (t(17) = 0.42, p = 0.68), Figure 4-6b, d, f).

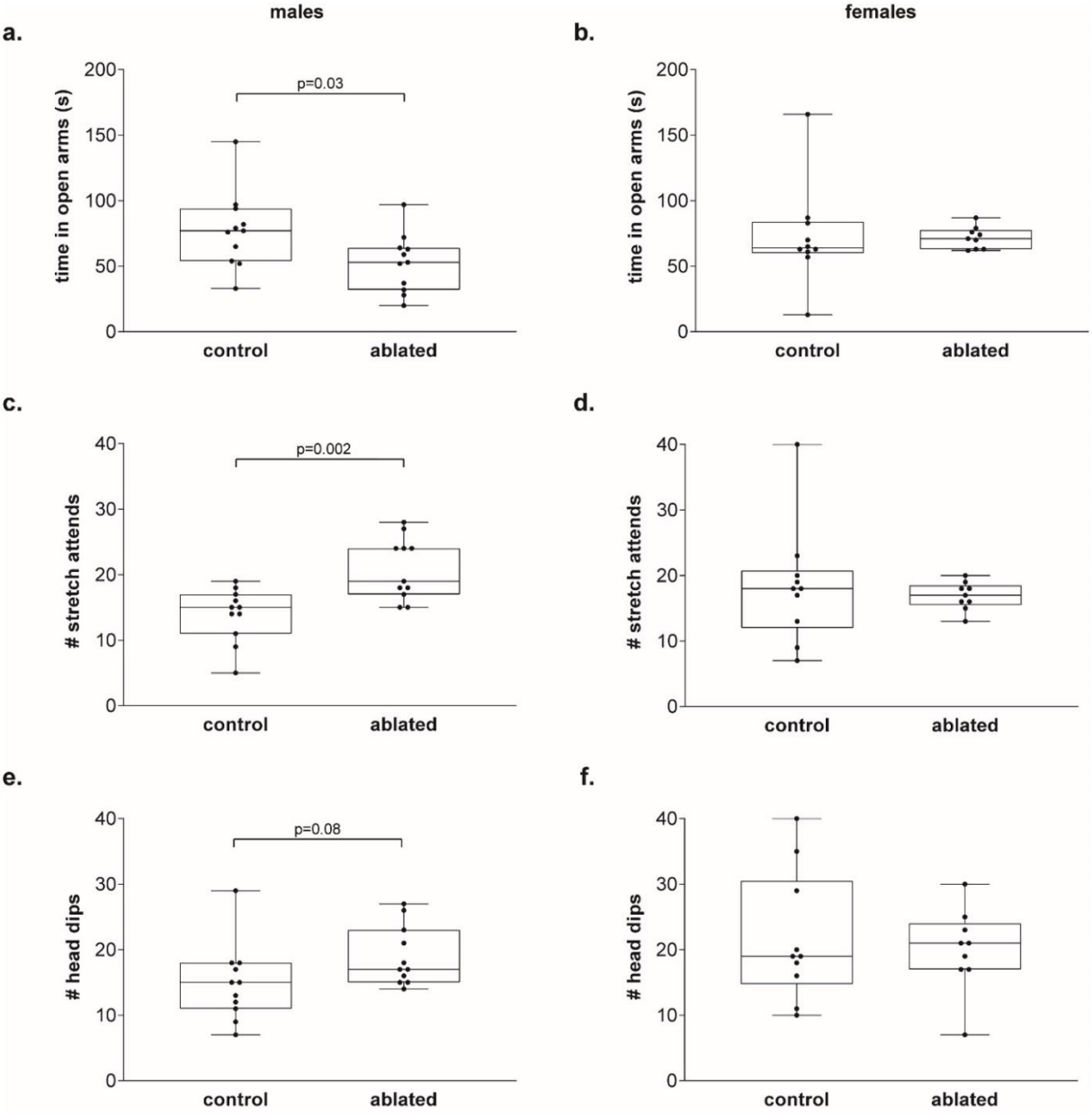

Figure 4-6– PVN AVP-expressing cell ablations in males increases anxiety-like behavior. *Boxplot indicating individual data points, median, first and third quartiles for time spent in the open arms within the EPM, number of stretch attend postures, and number of head dips. (a) males with PVN AVP-expressing cell ablations increased time spent in open arms (p = 0.03) whereas females with PVN AVP-expressing cell removal did not differ from controls (b). Similarly, lesioned males (c), but not females (d), produced more stretch attend postures than*

controls (p = 0.002). Cell ablated males (e), but not females (f), trended toward increasing head dips (p = 0.08) compared to controls.

4.3.5 Cell ablation does not alter copulatory or aggressive behavior

Cell-ablated males did not differ from controls in the number of mounts (H = 0.02, p = 0.89) or intromissions (H = 0.4, p = 0.52) targeted toward females (Figure 4a). Similarly, lesioned females did not differ from controls in the number of mounts (H = 1, p = 0.32) or intromissions (H = 0.2, p = 0.89) received from males (Figure 4b). There were no differences between controls and PVN AVP-expressing cell-ablated males in their latency to attack intruders (H = 0.6, p = 0.81, Figure 4c). Females, regardless of condition, did not fight during same-sex interactions.

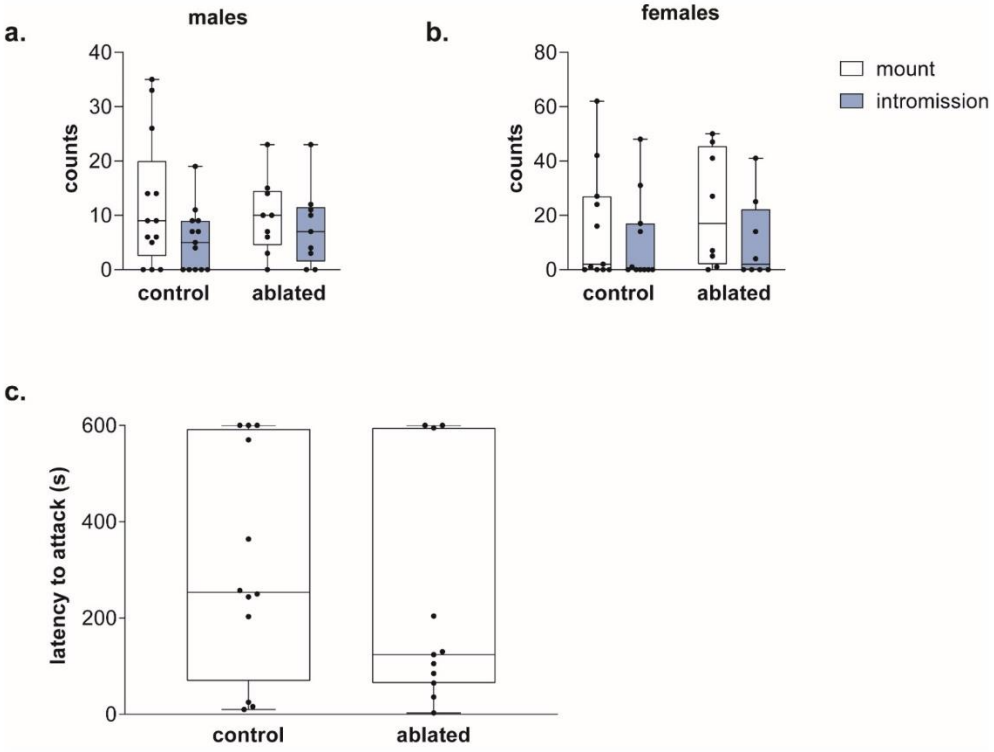

Figure 4-7– PVN AVP-expressing cell ablations did not alter copulatory or aggressive behavior. *Boxplot indicating individual data points, median, first and third quartiles for the number of mounts and intromissions performed by male subjects (a) and the number of mounts*

and intromissions received by females (b). No differences were found in these measures. No differences were found either in latency to attack an intruder male between control and cell-deleted males (c).

4.4 Discussion

Our results indicate that PVN AVP-expressing cells may contribute to sexually dimorphic components of social behavior and anxiety, because PVN AVP-expressing cell ablations increased social investigation (of same as well as opposite-sex conspecifics) in females, but not in males. However, in males but not in females, these lesions increased non-social, anxiety-related behaviors. These results point to differential involvement of PVN AVP-expressing cells in the control of social and emotional behavior in the two sexes.

The effect of PVN AVP lesions on social investigation and anxiety-like behaviors may be the result of other, more general, changes in behavior. This is unlikely, however, because these lesions did not affect other communicative behaviors, general activity, territorial aggression, and sexual behavior. Moreover, the effects of our PVN AVP-expressing cell ablations are not likely to be caused by off-target effects, as we did not observe differences in expression of oxytocin (OT), a related neuropeptide, in the PVN between lesioned and control animals or AVP-expressing cell-deletion in nearby hypothalamic areas.

Our experiments do not allow us to conclude that removal of AVP itself within the PVN caused the behavioral effects observed in this study. Ablating AVP-expressing cells in the PVN also removed all other neuroactive substances co-expressed, produced, and secreted by these cells, which may have caused the behavioral results we observed. For example, female rodents have more ERβ in PVN AVP-expressing cells than males (Hrabovszky et al., 1998)-(Oyola et al., 2017) and global ERβ knockout increases social investigation by females preferentially (Handa

et al., 2012; Tsuda et al., 2014). Consequently, our effects may be due to removal of sexually dimorphic signaling mediated by ERβ or other molecules in PVN cells.

Our experiments also do not allow us to identify which AVP-expressing cells may have contributed to our results. The PVN contains at least three different sets of AVP-expressing cells, magnocellular cells that project to the posterior pituitary, corticotropin releasing factor (CRF) cells that may co-express AVP and project to the median eminence, and non-neurosecretory AVP-expressing cells that project to other brain areas (Biag et al., 2012; De Vries & Panzica, 2006). In principle, all of these cells may have been deleted in our Cre+ mice, and possible effects may therefore be attributed to central as well as peripheral effects. As our animals did not show differences in water uptake (data not shown) or in urination (urine marking) patterns, the reduction in peripheral AVP derived from the PVN may have been compensated by AVP secreted from SON neurosecretory cells. A loss of CRF cells may in principle have changed the function of the HPA axis, which may have contributed to our results. However, the overlap of CRF and AVP is about 10% in unstressed mice (Biag et al., 2012), suggesting that ablation of these cells might have minimal effect on the HPA axis. In addition, mice genetically altered to specifically remove CRF cells in the PVN show reduced anxiety (Zhang et al., 2017), while removal of AVP-expressing cells in the PVN in the current study produced an increase in anxiety. It is also possible that AVP within the PVN can be released by dendrites and therefore our results may be due to disruption of local dendritic release (Ludwig & Leng, 2006). Lastly, centrally projecting AVP cells of the PVN innervate a distinct set of targets within the brain (Rood & De Vries, 2011) and removal of these cells would deprive their targets of AVP input. Therefore, both removal of dendritically and axonally released AVP and potentially other

neuroactive substances produced by AVP-expressing cells in the PVN may have contributed to our results.

An additional caveat, is that our results may reflect molecular, cellular, and anatomic adjustments or compensations to chronic depletion of AVP-expressing cells in the PVN. Indeed, long-term pharmacological reduction of V1a receptor (V1aR) activity in the lateral septum, a target of AVP-expressing cells in the PVN (Rood et al., 2013), increases anxiety-like behavior, while acute V1aR blockade in this region has the opposite effect (Everts & Koolhaas, 1999; Liebsch et al., 1996). A lack of an effect on a behavior, therefore, does not completely exclude involvement of these cells in acute regulation of these behaviors; this could be addressed in future studies using acute chemogenetic or optogenetic techniques.

It is surprising that ablation of AVP-expressing cells in the PVN increased anxiety-like behaviors in the elevated plus maze in males, since most studies point at central AVP stimulating, not inhibiting, these behaviors. In addition, male or female rats and mice bred for high levels of trait anxiety show higher AVP expression in the PVN (Bunck et al., 2009; Murgatroyd et al., 2004; Wigger et al., 2004). However, we note that these findings supporting a positive relationship between brain AVP and anxiety are correlational. In fact, other findings indicate that decreased PVN AVP expression correlates with increased anxiety in isolation-reared male, but not female rats (Tanaka et al., 2010). Importantly, direct AVP manipulations such as intracerebroventricular AVP administration or knockdown of AVP expression in the PVN have produced no effects on anxiety-like behavior (Blume et al., 2008; Kelly & Goodson, 2014). Our results, however, indicate that PVN AVP-expressing cells appear to be important for the regulation of anxiety, but more so in males than in females.

Deleting PVN AVP-expressing cells elevated conspecific investigation in females, but not males, indicating that these cells are more important modulators of social investigation in females than in males. The fact that PVN AVP-expressing cell deletion had no effect on male social investigation is somewhat surprising given that low AVP mRNA within PVN is correlated with less time social investigating other males (Murakami et al., 2011). However, other findings indicate that chronic variable stress decreases female AVP mRNA in PVN and increases female social investigation of other females, while not affecting males, perhaps due to higher stress resilience in males (Borrow et al., 2018). In combination with our previous work demonstrating that BNST AVP-expressing cells are important for male, but not female, social investigation of other males (Rigney et al., 2019), the present findings suggest that males and females may use different AVP circuitry for modulating social investigation. Interestingly, they also point at AVP-expressing cells in the PVN as an important factor in the sex difference in social investigation, with males showing higher levels of investigation than do females, observed in the present study and in others (Bluthé & Dantzer, 1990; Holmes et al., 2011; Karlsson et al., 2015; Markham & Juraska, 2007), as removal of these cells removed the sex difference as well.

Ablations of AVP-expressing cells in the PVN did not significantly alter the production of USVs or urine marking, suggesting these cells are not necessary for all social communicative behaviors. Although AVP has been implicated in the production of vocalizations (Goodson & Bass, 2001), few studies have identified the relevant neuroanatomical source of AVP or its locus of action. Recently, we found that blockade of V1aR in the lateral habenula reduced male courtship USVs in mice (Rigney et al., 2020) but that BNST AVP-expressing cell deletion did not affect USVs (Rigney et al., 2019). Therefore, it is likely that AVP sources outside the PVN and the BNST, such as the MA, play the lead role in driving USV production. We did find BNST

AVP-expressing cell ablations influence other communicative behaviors, such as urine marking, therefore AVP signals originating from sexually dimorphic structures such as the BNST or MA may be more important for male communication than AVP from the PVN (Rigney et al., 2019).

Ablations of AVP-expressing cells in the PVN did not significantly alter aggression. While pharmacological studies suggest that several structures, such as the anterior hypothalamus, ventromedial hypothalamus, and lateral septum receive AVP input that facilitate aggressive behavior (Leroy et al., 2018; Veenema et al., 2010; Veenema & Neumann, 2008), our finding that PVN AVP-expressing cell deletions did not alter territorial aggression does not support the idea that the PVN is the source of AVP that facilitates aggression. This is in agreement with the lack of a reduction in aggression following AVP knockdown in the PVN of birds (Kelly & Goodson, 2014). Other hypothalamic AVP-expressing cell groups unaffected by our lesions, like the nucleus circularis, may be driving pro-aggressive effects of AVP (Cheng et al., 2008; Ferris et al., 1989; Gobrogge et al., 2007).

There are several reasons why the effects of PVN AVP-expressing cell ablations may have had sexually dimorphic effects on anxiety and social behavior. The first is that these cells are inherently dimorphic. For example, AVP projections from the PVN are denser in females than in males (Rood et al., 2013). One may therefore expect that removal of these projections would have a larger effect on behavior in females than in males. Although this is true for social behavior, it was the opposite for anxiety related-behavior. Another reason for sex-different effects is that these cells may project to sexually differentiated targets or to areas whose function may differ in males and females. Indeed, PVN AVP cells project to areas that show sex differences in V1a and OT receptor binding (Rood et al., 2013), (C. J. W. Smith et al., 2017), the two receptors that mediate behavioral effects of AVP (Song & Albers, 2017). In addition, some

target areas of PVN AVP-expressing cells, such as the locus coeruleus (Rood et al., 2013), which has been implicated in the control of anxiety (McCall et al., 2015), show different physiological responses, even to similar afferent stimulation (Valentino & Bangasser, 2016). Sex differences like this may have contributed to the sex-biased effects of removal of PVN AVP-expressing cells on anxiety, which were present in males, but not in females.

Our previous work has identified the sexually dimorphic AVP-expressing cells in the BNST as contributors to sex differences in social behavior, especially for social investigation and communication (Rigney et al., 2019). The present study demonstrates that AVP-expressing cells in the PVN can also modulate social interactions, as well as anxiety, but do so independently and in a sex-dependent manner.

5 THE CONNECTIONAL ARCHITECTURE OF VASOPRESSIN NEURONS IN THE BED NUCLEUS OF THE STRIA TERMINALIS AND MEDIAL AMYGDALA

5.1 Introduction

Arginine vasopressin (AVP) is a neuromodulatory peptide that regulates many aspects of social behavior in vertebrates (Donaldson and Young, 2013; Goodson and Bass, 2001; Guastella et al., 2011; Insel, 2010; Kelly and Goodson, 2013a; Rigney et al., 2022) and contains features that evolved over half a billion years ago which remain conserved among most vertebrate species today (Johnson and Young, 2018; Theofanopoulou et al., 2021). AVP acts on various brain regions to regulate social recognition (Bluthe et al., 1993; Dantzer et al., 1988; Veenema et al., 2012), communication (Albers, 2015; Goodson and Bass, 2001; Rigney et al., 2020a), aggression (Ferris et al., 1997), maternal care (Bayerl and Bosch, 2019), pair bonding (Johnson and Young, 2015), and cognition (Landgraf and Neumann, 2004). In mammals, AVP is produced primarily in the paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), and the suprachiasmatic nucleus (SCN) (Grinevich and Ludwig 2021; Rohr et al. 2021). AVP expressed in these nuclei regulate homeostatic functions, such as water/salt balance, blood pressure (i.e., PVN/SON), and circadian rhythms (i.e., SCN). Most mammals also produce AVP within the extended amygdala (bed nucleus of the stria terminalis (BNST), medial amygdala (MeA)), regions which are key nodes connecting the Social Behavioral Neural Network (SBNN) (Newman, 1999; O'Connell and Hofmann, 2011). Importantly, AVP cells in the BNST and MeA contribute to the pronounced steroid-dependent, sex difference in AVP innervation in the brain (De Vries and Boyle 1998; De Vries and Panzica 2006). For example, male rodents have about two to three times as many AVP cells as females in the BNST/MeA and the projections of these cells to areas such as the lateral septum (LS), dorsal raphe (DR), and lateral habenula (LHb) are

denser as well (Rigney et al., 2019; Rood et al. 2013; Rood & de Vries 2011). Additionally, AVP has been shown to act in these downstream regions, oftentimes sex-specifically, to regulate social communication and other aspects of social behavior (Rigney et al. 2020; Veenema et al. 2012; Borie et al. 2021).

The BNST AVP cells have emerged as the primary AVP cell population associated with social behavior, demonstrated by their significant role in male-male competitive behavior, social investigation, and affiliation (Rigney et al., 2023). In addition, BNST AVP cells are critical for male social recognition, as deletion of these cells reduces social recognition in males, but not females (Whylings et al. 2020).

In contrast, far less is known about the function of the steroid-sensitive and sexually dimorphic AVP cell population in the MeA, despite being described decades ago (Caffe and Van Leeuwen, 1987; van Leeuwen et al., 1985). What limited research exists focuses on their role in regulating defensive responses to stressors, such as predator odors, as well as aspects of reproductive behavior (Hari Dass and Vyas 2014; Tong et al. 2019; Tong et al. 2021). Given that AVP expression in both BNST and MeA are modulated by sex-steroids and are located in two regions that are thought to be developmentally and functionally related as the extended medial amygdala (de Olmos and Heimer 1999), it is plausible that these two AVP cell groups interact to form an integrated sexually-dimorphic AVP system that together regulate social and emotional behavior.

However, the distinct functional roles of AVP projections from the BNST, MeA, as well as the hypothalamus remain largely unknown, with most of our foundational knowledge about AVP's effect on social behavior coming from pharmacological targeting of AVP receptors in various brain regions and measurements of local AVP release during social behavior (Rigney et

al., 2023; Albers, 2015; Bayerl and Bosch, 2019; Dumais and Veenema, 2016; Smith et al., 2019; Wotjak et al., 1996). Therefore, our understanding of which specific AVP circuits directly regulate social behavior is incomplete. A recent study has described the input and output architecture of PVN AVP neurons (Freda et al. 2022), and so greatly improves our understanding of how this AVP cell population may regulate physiology and behavior. However, while the general connectivity of the BNST and MeA is well-known (Petrulis 2020; Flanigan and Kash 2022), we currently have no information about the specific inputs to, and direct outputs of, BNST and MeA AVP cells in the mouse brain. Although previous studies have explored the impact of gonadectomy on AVP-immunoreactive BNST and MeA AVP fibers and have used retrograde tracing with immunocytochemistry to confirm the specific BNST AVP projections to the lateral septum and pallidum or MeA AVP projections to the hippocampus and lateral septum, a comprehensive analysis of the presynaptic outputs of these cells has yet to be performed (Rood et al. 2013; Caffe et al. 1987; van Leeuwen et al. 1985). To address this, we use a modified rabies virus (RV) approach to identify the cells that provide monosynaptic input to BNST and MeA AVP cells and an adeno-associated viral (AAV) anterograde tracer strategy to map the outputs of these two cell populations. A comprehensive understanding of how AVP systems are structured and function may lead to more effective therapeutic interventions for psychiatric disorders characterized by significant social deficits (Rigney et al. 2022).

5.2 Methods

5.2.1 Animals

Founding AVP-iCre mice were obtained from Dr. Michihiro Mieda (Kanazawa University, Japan). These mice were generated using a bacterial artificial chromosome (BAC) that expressed codon-improved Cre recombinase (Shimshek et al. 2002) under the transcriptional

control of the AVP promoter (AVP-iCre mice). In these animals, iCre expression is found in the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA), as well as in hypothalamic areas (Mieda et al. 2015). Subjects were derived by crossing heterozygous iCre mutants to wildtype C57Bl/6J mice and genotyped (ear punch) by polymerase chain reaction (PCR) at 21–24 days of age (Transnetyx). AVP-iCre positive male and female mice were used for tracing BNST and MeA AVP neuron projections and neuronal inputs. AVP-iCre negative littermates were used as controls.

All mice were maintained at 22°C on a 12/12 hr reverse light/dark cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems, Centennial, CO, USA), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were performed in accordance with the Georgia State University Institutional Animal Care and Use Committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

5.2.2 Viral vectors

Monosynaptic rabies virus (BNST/MeA AVP cell inputs)

To trace the monosynaptic inputs of BNST and MeA AVP cells, we used a modified rabies virus tracing strategy (Kim et al. 2016). Briefly, the monosynaptic tracing system uses a rabies virus from which the "G" gene, encoding the envelope glycoprotein, has been deleted, resulting in the inability for the virus to spread (Wickersham et al. 2007) as well as pseudotyping the rabies virus with the avian sarcoma leukosis virus glycoprotein (EnvA). Monosynaptic tracing therefore requires engineered expression of two genes in the targeted population of starting neurons: the gene encoding TVA, the avian receptor protein for EnvA, and the G gene encoding the rabies virus glycoprotein. The rabies virus can then infect the starting cells via the

receptor TVA and, complemented by the expression of G, infect input neurons, but will not spread further due to lack of G-expressing virus in input neurons (Lavin et al. 2019).

For this experiment we injected two helper viruses, AAV2/1-syn-FLEX-splitTVA-EGFP-tTA (Addgene 100798) and AAV2/1-TREtight-mTagBFP2-B19G (Addgene 100799). These viruses express the tetracycline transactivator system (Gossen and Bujard 1992) so as to conjointly express proteins. The TVA virus (Jha et al. 2011) is Cre-dependent and expresses, in Cre-expressing cells, EGFP and the tetracycline transactivator ("tTA"); The G virus is not Cre-dependent, but expresses both G and the blue fluorophore mTagBFP2 (Subach et al. 2011) under the control of the tetracycline response element. Consequently, expression of tTA from the TVA AAV drives expression of G from the other AAV in the co-infected cells (Lavin et al. 2020). The EnvA pseudotyped G-deleted Rabies (SADB19) mCherry (RVdG-mCherry) was obtained from the Vector Core at Kavli Institute for Systems Neuroscience (Trondheim, Norway).

Synaptophysin virus (BNST/MeA AVP cell outputs)

For anterograde tracing, we used a synaptophysin-tagging virus, in which the vector differentially labels cytoplasm and presynaptic terminals. We used an AAV(2/1) hSyn-FLEx-mGFP-2A-Synaptophysin-mRuby viral vector (Addgene 71760) to cre-dependently label fibers/cell bodies (GFP) and presynaptic terminals (mRuby) projecting from BNST and MeA AVP cells in AVP-iCre mice (or control cre-negative littermates). However, it is important to note that the presence of a labeled presynaptic terminal does not necessarily indicate a functional synapse (Lerner et al. 2015).

5.2.3 Stereotaxic Surgery

All surgeries were conducted using 1.5–3% isoflurane gas anesthesia in 80% oxygen and 20% nitrous oxide; 3 mg/kg of carprofen was given before surgery to reduce pain. Mice were

positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand-operated drill was used to make holes in the skull, exposing the dura. BNST coordinates: DV: -4.3, AP: +0.13, ML: ±0.8; MeA coordinates: (15 degree angle) DV:, AP:, ML: ;(Paxinos and Franklin 2012). For retrograde rabies virus tracing, 250 nl of 1:1 volume mixture of AAV2/1-syn-FLEX-splitTVA-EGFP-tTA and a diluted (1:20 in Dulbecco's phosphate-buffered saline; Fisher, 14-190-250) AAV2/1-TREtight-mTagBFP2-B19G was injected unilaterally (side randomized across animals). Ten days later, 200 nl of RVdG-mCherry was injected into the same location and the mice were sacrificed seven days later. For the anterograde tracing, mice were injected unilaterally (side randomized across animals) with 150 nl of AAV2/1-hSyn-FLEx-mGFP-2A-Synaptophysin-mRuby vector, and the mice were sacrificed six weeks later.

5.2.4 Perfusions and Histology

Mice were transcardially perfused with 50 ml of PBS (pH 7.4), followed by 50 ml of 4% paraformaldehyde in phosphate buffer (pH 7.2). Brains were immediately removed and post-fixed in 4% paraformaldehyde overnight (4 °C) and then cryoprotected for 48 h in 30% sucrose. For rabies virus tracing experiments, brain tissue outside of the injection site was sectioned (50 µm; 3 series) on a cryostat (Leica CM3050 S, Leica Biosystems), mounted on glass slides, and coverslipped using ProLong Diamond Antifade Mountant with DAPI (ThermoFisher Scientific). The region injected with RVdG-mCherry (i.e., BNST or MeA) was sectioned at 25µm, placed in cryoprotectant until immunohistochemical processing. For the anterograde tracing experiments,, coronal sections (30µm; 1:3 series) of brain tissue were taken and stored in cryoprotectant until immunohistochemical processing.

Sections for immunohistochemical processing were removed from cryoprotectant and rinsed five times in PBS. Sections were then incubated in primary antibodies against GFP and/or RFP (GFP: 1:5,000, chicken polyclonal, lot: ab13970; RFP: 1:1,000, rabbit polyclonal, lot: ab124754, Abcam) in 0.4% Triton-X 100 for 12-24 h at room temperature. After incubation in primary antibody, sections were rinsed in PBS and then incubated for 2 h in goat anti-chicken and/or anti-rabbit fluorescent (Alexa Fluor 488, 594) secondary antibody (chicken polyclonal: 1:600, lot: ab150169; rabbit polyclonal: 1:600, lot: ab150080, Abcam). Stained tissue sections were mounted onto subbed glass slides and coverslipped using Prolong Gold with DAPI (ThermoFisher Scientific).

5.2.5 Tissue Analysis

Unilateral images were taken at 10x and 20x magnification using a Zeiss Axio Imager M2 microscope (Carl Zeiss Microimaging), which transferred fluorescent images (FITC contrast reflector) to image analysis software (Stereo Investigator, MicroBrightField, RRID:SCR_002526). a 40x objective was used to quantify RNAscope tissue. For atlas mapping, brain sections were chosen from bregma +3.56 to −6.72 and anatomical borders and landmarks were identified by DAPI staining in reference to the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin 2012).

Monosynaptic retrograde tracing: Multichannel immunofluorescence microscopy was used to identify starter cells (expressing both TVA (GFP) and RV (mCherry)) in BNST and MeA AVP cell populations. To quantify monosynaptic inputs to BNST/MeA, we manually counted mCherry+ cells (every other section) in brain regions and expressed them as the total percentage of all retrogradely labeled cells in order to control for differences in number of starter cells across animals.

Anterograde tracing: Multichannel immunofluorescence microscopy was used to identify infected BNST and MeA AVP cells and the distribution of GFP-labeled fibers and synaptophysin–mCherry-labeled putative synaptic terminals every third section. Each analyzed brain region was assigned an axon labeling score of using the following scoring categories: + (very low), very few fibers; ++ (low), few dispersed fibers +++ (moderate), part of area densely covered by fibers; and ++++ (strong), most of area densely covered by fibers. In most cases, brain areas containing GFP+ fibers were spread over multiple brain sections, and separate scores were assigned for each section containing an individual region.

To quantify synaptophysin-labeled puncta, areas within each brain region with the densest fiber/puncta label were imaged at 40x magnification. If multiple subregions were labeled (e.g., the ventral or lateral portion of the lateral septum), each subregion was analyzed both separately and in combination with other subregions. Quantification was automated using the Analyze Particles Fiji plugin in ImageJ (Schindelin et al. 2012) with manual counting as confirmation. The results were averaged across mice within genotype (AVP-iCre+ and AVP-Cre-) and separated by sex.

RNAscope: To visualize if BNST/MeA input cells originated from AVP+ cells, an RNAscope fluorescent multiplex assay (V2, Advanced Cell Diagnostics, Newark, CA, USA) was used. Brains with BNST or MeA AVP RV cell infection were flash-frozen and sectioned into three 20 μm-thick series and mounted onto SuperFrost Plus slides (Fisher Scientific). In situ hybridization was performed according to the RNAscope fluorescent multiplex kit V2 user manual for frozen tissue (Advanced Cell Diagnostics) using RNAscope Probe-mm-AVP-C1 (cat#472261) and Probe-V-RABV-gp1-C2 (cat#456781). Slides were coverslipped using ProLong mounting medium (Life Technologies).

5.2.6 Statistical Analysis

All data were analyzed and graphed using R or GraphPad Prism 9. T-tests were used to compare the number of starter cells in AVP-iCre+ mice versus AVP-iCre- controls and to compare starter cell, total cell, and input per starter number across sex. We used a two-way repeated-measures ANOVA to compare labeling in each brain region across sex and genotype (iCre+, iCre-). We used t tests when comparing overall labeling between the sexes. Results are expressed as the means ± SEM. The sample sizes used are comparable to published anatomical experiments (Schwarz et al. 2015; Wall et al. 2016). The criterion for statistical significance was set at $p < .05$. Benjamini-Hochberg (False Discovery Rate, 5%) was used to control for multiple comparisons for all statistical analyses and so, after applying this correction, the adjusted significance threshold was set at: $p < 0.0125$ (BNST-retrograde tracing), $p < 0.006$ (MeA-retrograde tracing), $p < 0.023$ (BNST-anterograde tracing), $p < 0.003$ (MeA-anterograde tracing). Effects sizes were reported if the comparison was statistically significant (Eta squared, Cohen's d).

5.3 Results

5.3.1 Afferent inputs to BNST AVP neurons

Our study used the retrograde monosynaptic rabies virus tracing approach (Callaway and Luo 2015; Lavin et al. 2019) to identify the regions providing afferent input to BNST AVP cells. To ensure that our viral injections were specific to AVP-expressing cells, we included a control group of AVP-iCre negative injected animals, in which we observed a very small number of mCherry-labeled (RV-infected) cells, consistent with previous reports of Cre-independent TVA expression (Faget et al., 2016; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012). As the number of mCherry-labeled cells in control animals was less than 1% of the total averaged

labeled cells in AVP-iCre positive animals, we are confident that our identified inputs to BNST AVP cells are specific for this population (Supplementary Figure 1, data not shown). We also confirmed that injections were restricted to the BNST, with the exception that some cells of the nearby reticular thalamus (Rt) were also labeled in some animals, revealing a cryptic population of AVP-expressing thalamic cells. To control for this off-target labeling, we compared the pattern of inputs of animals with Rt label with those that brains did not have Rt infection as well as including an additional control group, where we injected the Rt but not the BNST. This analysis revealed that most input cells to the Rt AVP originate in the cortex and thalamus, as has been reported for Rt neurons generally (Guillery and Harting 2003; Lozsádi 1994). In addition, we excluded the posterior BNST from our analysis of input cells due to the well-known difficulty in distinguishing local inputs from cryptic TVA expression using the rabies virus tracing approach (Lavin et al. 2020). Although males have substantially greater BNST AVP expression than females (De Vries and Panzica 2006), only a small portion of these starter cells were co-infected (~30%) (see (Rigney et al. 2021) for comparison), with variation across animals. Consequently, we did not see a sex difference in the amount of BNST AVP starter cells, the number of total input cells, or the percentage of inputs per starter cells (Figure 5-1). Despite this, we observed a sex difference in the proportion of BNST AVP inputs (see below; Figure 5-2).

Olfactory

The structures of the olfactory area provided very sparse input to BNST AVP neurons (~3%), with no more than 10 cells per animal. The very sparsely labeled olfactory areas included the medial orbital cortex/dorsal tenia tecta (MO/DTT) (males: 1.5% ± 0.85%, females: 1.69% ± 1.5%), posterolateral cortical amygdala (PLCo) (males: 0.2% ± 0.14%, females: 0.01% ±

0.01%), posteromedial cortical amygdala (PMCo) (males: 0.66% ± 0.27%, females: 0% ± 0%), and piriform cortex (Pir) (males: 0.62% ± 0.85%, females: 0% ± 0%). The proportion of inputs from olfactory areas did not differ between the sexes.

Striatum

Inputs from the striatum provided roughly 15% of the inputs to BNST AVP cells, primarily consisting of inputs from the lateral septum (LS) (males: 5.4% ± 1 %, females: 2% ± 0.73%) and the nucleus accumbens (NAcc) (males: 5.6% ± 0.14%, females: 16.3% ± 4.5%). A substantial sex difference was found, where male mice had greater input from the lateral septum than females (LS: $F(1,6) = 26.55$, $p = 0.0012$, $\eta 2 = 0.88$) and females had greater input from the nucleus accumbens than males (NAcc: $F(1,6) = 26.62$, $p = 0.001$, $\eta 2 = 0.9$; Figure 5-2). Most of the LS label was found in the intermediate, ventral region, with no label seen in the dorsal LS. NAcc label varied between the core and shell.

Pallidum

Nearly 10% of inputs to BNST AVP cells originate from the pallidum, but with varying input strength. The strongest inputs come from the ventral pallidum (VP) (males: 3.4% ± 1%, females: 6.7% ± 0.3%) and anterior BNST (males: 3.6% ± 1%, females: 3.4% ± 0.3%). Sparse input originated from the ventral diagonal band (VDB) (males: 1.5% ± 0.5%, females: 0.5% ± 0.3%), horizontal diagonal band (HDB) (males: 0.6% ± 0.3%, females: 1.8% ± 1%), medial septum (MS) (males: 0.8% ± 0.2%, females: 0.3% ± 0.3%), and substantia innominata (SIB) (males: 0.3% ± 0.2%, females: 0.3% ± 0.29%). Of these regions, we found sex differences in VP inputs, where female mice had greater input from the VP cells than did males ($F(1,6) = 26.32$, $p = 0.001$, $\eta 2 = 0.9$; Figure 5-2).

Hypothalamus

The majority, (~50%) of BNST AVP cell inputs originated from the hypothalamus (Figure 5-2). Strong hypothalamic inputs originated from the medial preoptic area (MPOA) (males: 10.2% ± 5%, females: 19.6% ± 1.8%), lateral preoptic area (LPO) (males: 6.9% ± 2%, females: 7.1% ± 1%), paraventricular nucleus of the hypothalamus (PVN) (males: 5.9% ± 2%, females: 9.3% ± 2.7%), lateral hypothalamus (LH) (males: 5% ± 2%, females: 1.2% ± 1%), the peduncular sub-region of the LH (PLH) (males: 6.5% ± 2%, females: 3.8% ± 1.1%) (total LH:(males: 12% ± 2%, females: 5% ± 1%)), and the ventromedial hypothalamus (VMH) (males: 4.3% ± 2%, females: 3.7% ± 0.05%). Other hypothalamic areas with less input included the ventromedial-lateral preoptic nucleus (VMPO/VLPO) (males: 0.6% ± 0.4%, females: 0.65% ± 0.5%), septohypothalamic nucleus (SHy) (males: 0.8% ± 0.3%, females: 2.4% ± 1.5%), anterior hypothalamic area (AHA) (males: 1.3% ± 1%, females: 1.7% ± 0.16%), anterior hypothalamic area, central part (AHC) (males: 2.7% ± 1.9%, females: 0% ± 0%) (total AH:(males: 4% ± 1.5%, females: 1.7% ± 0.16%)), mouse accessory (males: 0.8% ± 0.3%, females: 1.1% ± 0.2%), retrochiasmatic area (RCh) (males: 1% ± 0.5%, females: 1.6% ± 0.6%), arcuate hypothalamic nucleus (Arc) (males: 1.3% ± 1.4%, females: 0.3% ± 0.2%), anterior hypothalamic area, posterior part (AHP) (males: 0.7% ± 0.7%, females: 0% ± 0%), dorsomedial hypothalamic nucleus (DM) (males: 1.6% ± 1.4%, females: 1.5% ± 0.9%), premammillary nucleus, dorsal/ventral (PMD/PMV) (males: 0.3% ± 0.2%, females: 0% ± 0%), supramammillary nucleus (SuM) (males: 1.2% ± 0.6%, females: 0% ± 0%), and medial tuberal nucleus (Mtu) (males: 1.6% ± 0.8%, females: 1% ± 1%).

We found a sex difference in the proportion of BNST AVP inputs from the lateral hypothalamus ($F(1,6) = 13.956$, $p = 0.0055$, $\eta 2 = 0.82$), and particularly the PLH, from which males received greater input than females ($F(1,6) = 48.66$, $p = 0.0001$, $\eta 2 = 0.95$). The lateral hypothalamus label was throughout the entire LH. We found a female-bias sex difference in MPOA inputs ($F(1,6) = 46.31$, $p = 0.00023$, $\eta 2 = 0.94$) and PVN inputs ($F(1,6) = 14.063$, $p = 0.005$, $\eta 2 = 0.82$), in which females had greater input than males (Figure 5-2).

Thalamus

We saw few inputs to BNST AVP cells from thalamus, with the exception of the lateral habenula (LHb) (males: 0.71% ± 0.5%, females: 5.4% ± 1%), in which inputs were more female-biased ($F(1,6) = 43.1$, $p = 0.00028$, $\eta 2 = 0.93$).

Amygdala

Near 10% of input cells originated from within the amygdala. Amygdaloid regions that projected to BNST AVP cells included the medial amygdala (posterodorsa subregionl; MeApd) (males: 6% ± 1%, females: 0.6% ± 0.5%), medial amygdala (ventral; MeAV) (males: 0.7 ± 0.3%, females: 0.1% ± 0.09%), medial amygdala (anterodorsal; MeAD) (males: 2.2% ± 1.5%, females: 0.5% ± 0.3%), amygdalohippocampal area (AHiPM) (males: 0.4% ± 0.2%, females: 0% ± 0%), basolateral amygdala (BLA) (males: 0.2% ± 0.1%, females: 3% ± 2%), basomedial amygdala (BMA) (males: 1.7% ± 0.8%, females: 0.2% ± 0.2%), anterior amygdala (AA) (males: 1% ± 0.8%, females: 0% ± 0%), central amygdala (CeM) (males: 1.4% ± 1%, females: 0% ± 0%), and amygdalopiriform transition area (APir) (males: 0.1% ± 0.1%, females: 0% ± 0%). We observed a sex difference within the MeApd, where males had greater inputs than females ($F(1,6) = 26.16$, $p = 0.001$, $\eta 2 = 0.9$). Furthermore, these cells are likely to express AVP themselves (see below).

Midbrain

Few BNST AVP input cells (~3%) originated from the midbrain; however, we did label areas such as the ventral tegmental area (VTA) (males: 0.14% ± 0.08%, females: 0% ± 0%), substantia nigra (SNR) (males: 2.4% ± 0.2%, females: 1% ± 1%), periaqueductal gray (PAG) (males: 1.5% ± 0.8%, females: 0% ± 0%), and dorsal raphe (DR) (males: 0.6% ± 0.4%, females: 0% ± 0%). The proportion of midbrain BNST AVP cell inputs did not differ significantly between the sexes.

Hippocampus

The hippocampal system provided very sparse input to BNST AVP neurons, with no more than 9 cells per animal. The very sparse inputs included ventral CA1 (males: 0.63% ± 0.3%, females: 0.14% ± 0.14%) and did not differ between the sexes.

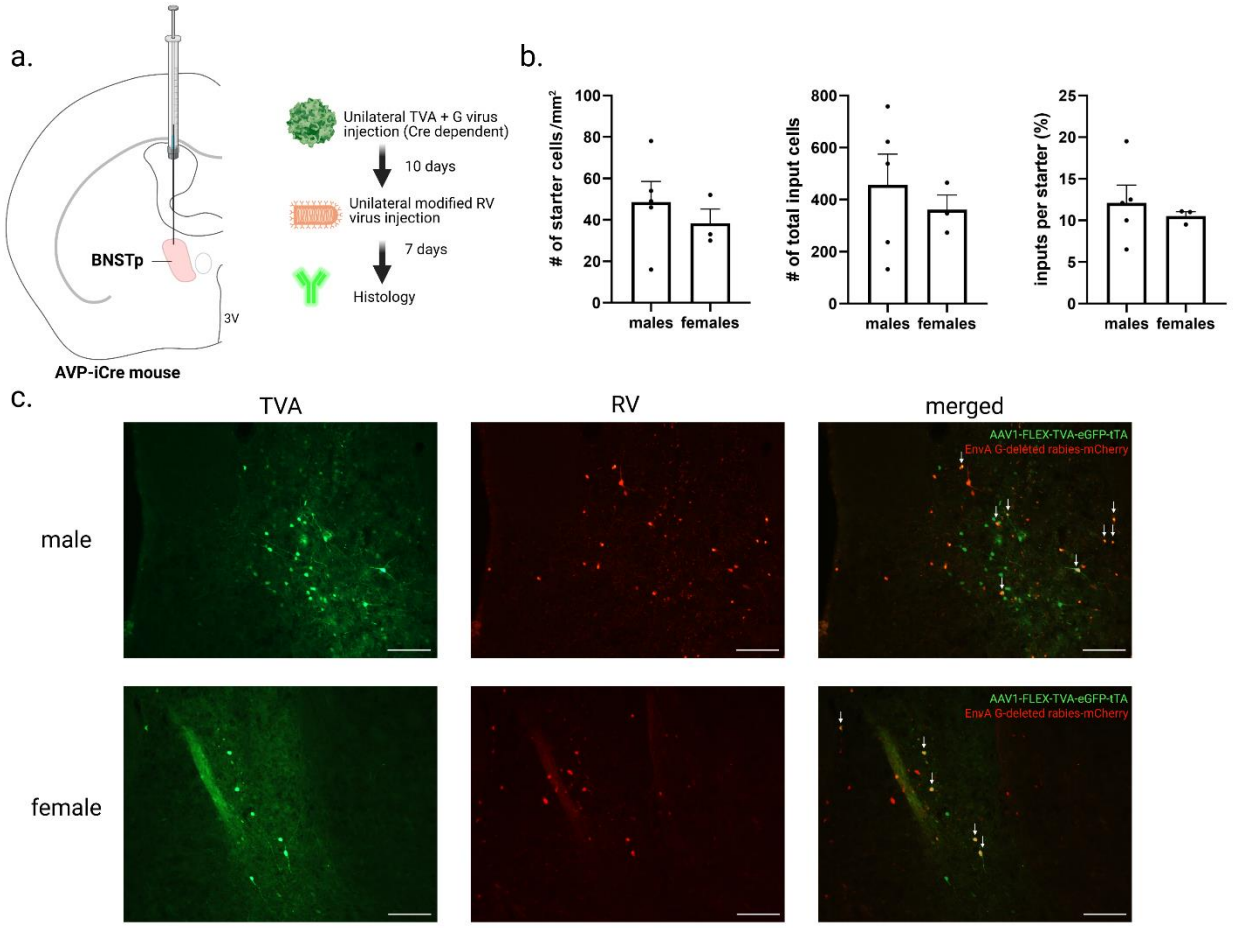

Figure 5-1– BNST AVP starter cells. *(a) BNST injection site and timeline. (b) Number of starter cells, number of total input cells, and percent of inputs per starter cells. (c) Example image of starter fluorescent TVA labeled BNST-AVP cells (green), EnvA g-deleted rabies virus (red), and merged images in both sexes. Scale bar = 50 µm.*

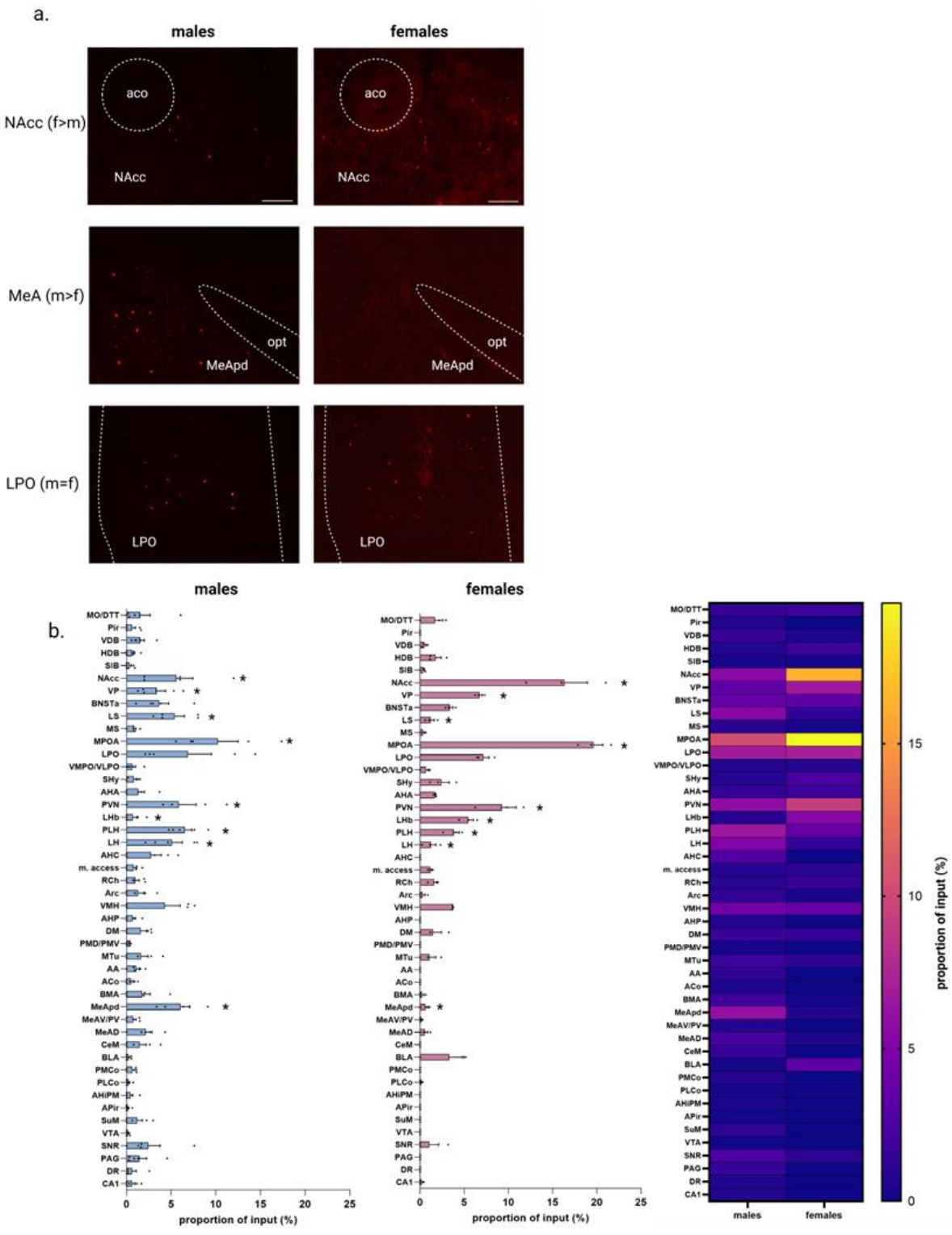

Figure 5-2- BNST AVP cell inputs. *(a) Example images of areas that contained female-bias input cells (Nacc), male-bias input cells (MeA), or sex-invariant input cells (LPO). Cells labeled with EnvA g-deleted rabies virus (mCherry) are in red. (b) Bar graph and heatmap of*

*retrogradely labeled mCherry+ inputs by brain region, quantified as percentage of all retrogradely labeled cells. Male (n=4) and female (n=3) data showed a significant sex difference in areas such as the NAcc, VP, LS, MPOA, PVN, LHb, PLH, LH, and MeApd (two-way ANOVA using Benjamini-Hochberg (False Discovery Rate, 5%) was used to control for multiple comparisons) Mean ± SEM data represented. Scale bar = 50 µm. *p < 0.05*

5.3.2 Afferent inputs to MeA AVP neurons

We confirmed that starter cell injections were restricted to the MeA, with the exception that some cells of the dorsally-located reticular thalamus (Rt) were also labeled in some animals, revealing a population of thalamic cells that express low level of AVP, similar to those seen after BNST injections. To control for this off-target labeling, we compared the pattern of inputs of animals with Rt label with those that brains did not have Rt infection as well as including an additional control group, where we injected the Rt but not the MeA. This analysis revealed that most input cells to the Rt AVP originate in the cortex and thalamus, as has been reported for Rt neurons generally (Guillery and Harting 2003; Lozsádi 1994). In addition, we excluded the MeApd from our analysis of input cells due to the well-known difficulty in distinguishing local inputs from cryptic TVA expression using the rabies virus tracing approach (Lavin et al. 2020). We observed no mCherry-labeled cells in AVP-iCre negative control animals.

As was the case with the BNST input-tracing experiment, males have substantially greater MeA AVP expression than females (De Vries and Panzica 2006), but only a small portion of these starter cells were co-infected, with variation across animals. Consequently, we did not see a sex difference in the amount of MeA AVP starter cells, the number of total input cells, or the percentage of inputs per starter cells (Figure 5-3). Despite this, we observed some sex differences in the proportion of MeA AVP inputs (see below; Figure 5-4).

Olfactory

Olfactory areas provided the strongest input to MeA AVP cells representing approximately 35% of the total input. The piriform cortex (Pir) sent the strongest input to MeA AVP cells (males: 6% ± 1%, females: 19% ± 4%), with the majority of the label in the rostral Pir (cortical layers 1-2). Other regions that sent moderate input to MeA AVP cells were the anterior cortical amygdala (ACo) (males: 3.1% ± 0.9%, females: 6.9% ± 3%), posteromedial cortical amygdala (PMCo) (males: 3.9% ± 3%, females: 2.5% ± 1%), posterolateral cortical amygdala area (PLCo) (males: 5% ± 0.5%, females: 0.03% ± 0.02%), and olfactory tubercle (Tu) (males: 2.6% ± 2%, females: 9.4% ± 2.7%). Sparse label was detected in the accessory olfactory bulbs (AOB) (males: 0.07% ± 0%, females: 3.2% ± 2.7%) and medial orbital cortex/dorsal tenia tecta (MO/DTT) (males: 0% ± 0%, females: 0.4% ± 02%).

A significant sex difference was found, where male mice had greater input from the PLCo than did females ($F(1,6) = 126.3$, $p = 0001$ $\eta 2 = 0.96$) and females had greater input from the Pir than did males and a trend toward greater inputs from the AOB (Pir: $F(1,6) = 34.55$, $p = 0.002$, $\eta 2 = 0.87$; AOB: $F(1,6) = 35.29$, $p = 0.002$, $\eta 2 = 0.87$; Figure 5-4).

Striatum

Structures of the striatum supplied very little input to the MeA AVP neurons, providing only 3% of the total input. The nucleus accumbens (NAcc) and caudate putamen (CPu) contained sparse RVdG-mCherry-labeled cells (NAcc: males: 0.7% ± 0.3%, females: 1.3% ± 0.8%; Cpu: males: 2.9% ± 1%, females: 1% ± 0.6%). No sex differences in inputs were found originating from these regions.

Pallidum

About 16% of MeA AVP cell inputs originated from the pallidum. The strongest of these was from the posterior BNST (males: 9.8% ± 1%, females: 2.4% ± 0.7%) and ventral pallidum (VP) (males: 5.1% ± 2.9%, females: 4.8% ± 1.3%), followed by the horizontal diagonal band (HDB) (males: 1.8% ± 1.3%, females: 7.2% ± 2.9%), ventral diagonal band (VDB) (males: 1.6% ± 0.7%, females: 0.05% ± 0.04%), and the substantia innominata (SI) (males: 0.2% ± 0.2%, females: 0.2% ± 0.2%). We observed a sex difference within the posterior BNST, where males had greater inputs than females ($F(1,5) = 33.15$, $p = 0.002$, $\eta 2 = 0.87$). Furthermore, these cells likely express AVP (see below).

Hypothalamus

The hypothalamus contributed to nearly 12% of the MeA AVP cell inputs; however, this contribution was distributed among several regions each exhibiting moderate-to-low label. These regions included the medial preoptic area (MPOA) (males: 3.2% ± 0.4%, females: 1.3% ± 0.9%), lateral preoptic area (LPO) (males: 1.3% ± 0.1%, females: 1.3% ± 0.5%), lateral hypothalamus (LH) (males: 2.2% ± 0.7%, females: 2% ± 1%), the peduncular part of lateral hypothalamus (PLH) (males: 4.2% ± 0.7%, females: 4.2% ± 1.2%) (total LH:(males: 7% ± 1%, females: 6% ± 1%)), paraventricular nucleus of the hypothalamus (PVN) (males: 1% ± 0.6%, females: 0.5% ± 0.2%), and ventromedial hypothalamus (VMH) (males: 1.3% ± 1%, females: 0.5% ± 0.2%). No discernible differences were found between the sexes in the proportion of hypothalamic inputs to MeA AVP cells.

Thalamus

Thalamic structures provided very little input to MeA AVP neurons, contributing 2% to the total input. The lateral habenula (LHb) (males: 1% ± 0.6%, females: 0% ± 0%) and the anterior

portion of the paraventricular thalamus (PVT) (males: 0.9% ± 0.2%, females: 0.5% ± 0.3%) exhibited sparse RVdG-mCherry-labeled cells. No sex differences in inputs were found in these regions.

Amygdala

The amygdala was found to contribute significantly to the MeA AVP cells, providing about a quarter of total input. Notably, ventral and anterior dorsal subregions of the medial amygdala (MeAV: males: 0.6% ± 0.5%, females: 0.6% ± 0.5%; MeAD: males: 2% ± 1%, females: 1.8% ± 0.7%) sent inputs to the AVP cells in the MeApd. Additionally, other amygdala structures such as the central amygdala (medial region) (CeM) (males: 9% ± 2%, females: 8% ± 4%), basolateral amygdala (BLA) (males: 2.7% ± 2%, females: 1.1% ± 0.3%), basomedial amygdala (BMA) (males: 3.9% ± 0.4%, females: 3% ± 1.9%), anterior amygdala (AA) (males: 4.6% ± 1%, females: 4.1% ± 1%), extended amygdala (medial) (EAM) (males: 6.5% ± 2%, females: 1.9% ± 0.8%), and the amygdalohippocampal area (AHiPM) (males: 2% ± 1.1%, females: 0.2% ± 0.01%) were found to contribute inputs to the MeA AVP cells. No sex differences in inputs were observed in these regions.

Midbrain

The midbrain provided nearly 6% of input to the MeA AVP neurons. Labeled cells were split between two midbrain structures: the anterior portion of the ventral tegmental area (VTA) (males: 1.9% ± 0.05%, females: 2.7% ± 2.1%) and the substantia nigra, reticular part (SNR) (males: 1.4% ± 1%, females: 2.9% ± 2.4%). No sex differences were found in these regions.

Hippocampus

Similar to inputs of BNST AVP cells, hippocampal areas provided very sparse input to MeA AVP neurons (~2-3%), with no more than 20 cells per animal. The very sparsely labeled area

included the ventral CA1 (males: 0.4% ± 0.6%, females: 0.25% ± 0.2%) and ventral CA2 (males: 0% ±0%, females: 1.3% ± 0.7%); neither input differed between the sexes.

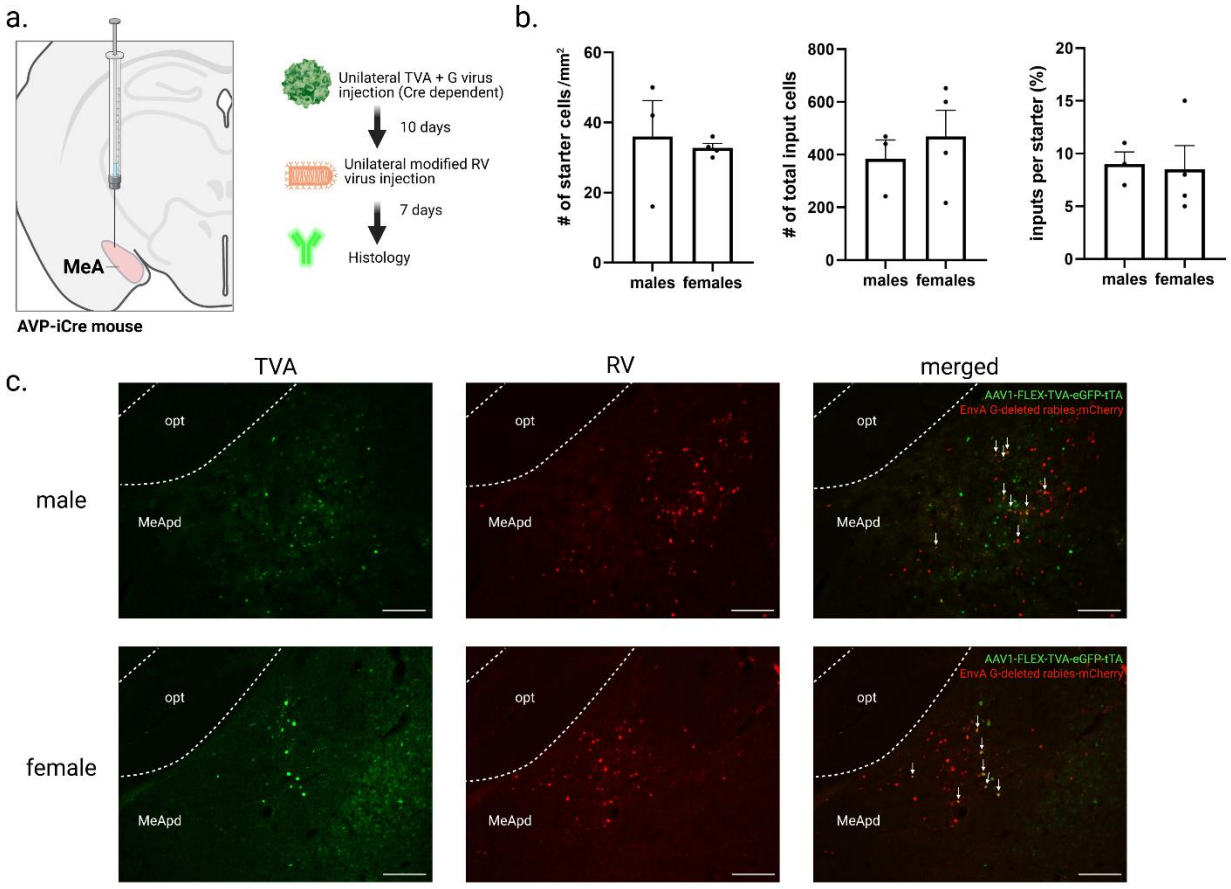

Figure 5-3– MeA AVP starter cells. *(a) MeA injection site and timeline. (b) Number of starter cells, number of total input cells, and percent of inputs per starter cells. (c) Example image of starter fluorescent TVA labeled MeA-AVP cells (green), EnvA g-deleted rabies virus (red), and merged images in both sexes. Scale bar = 50 µm.*

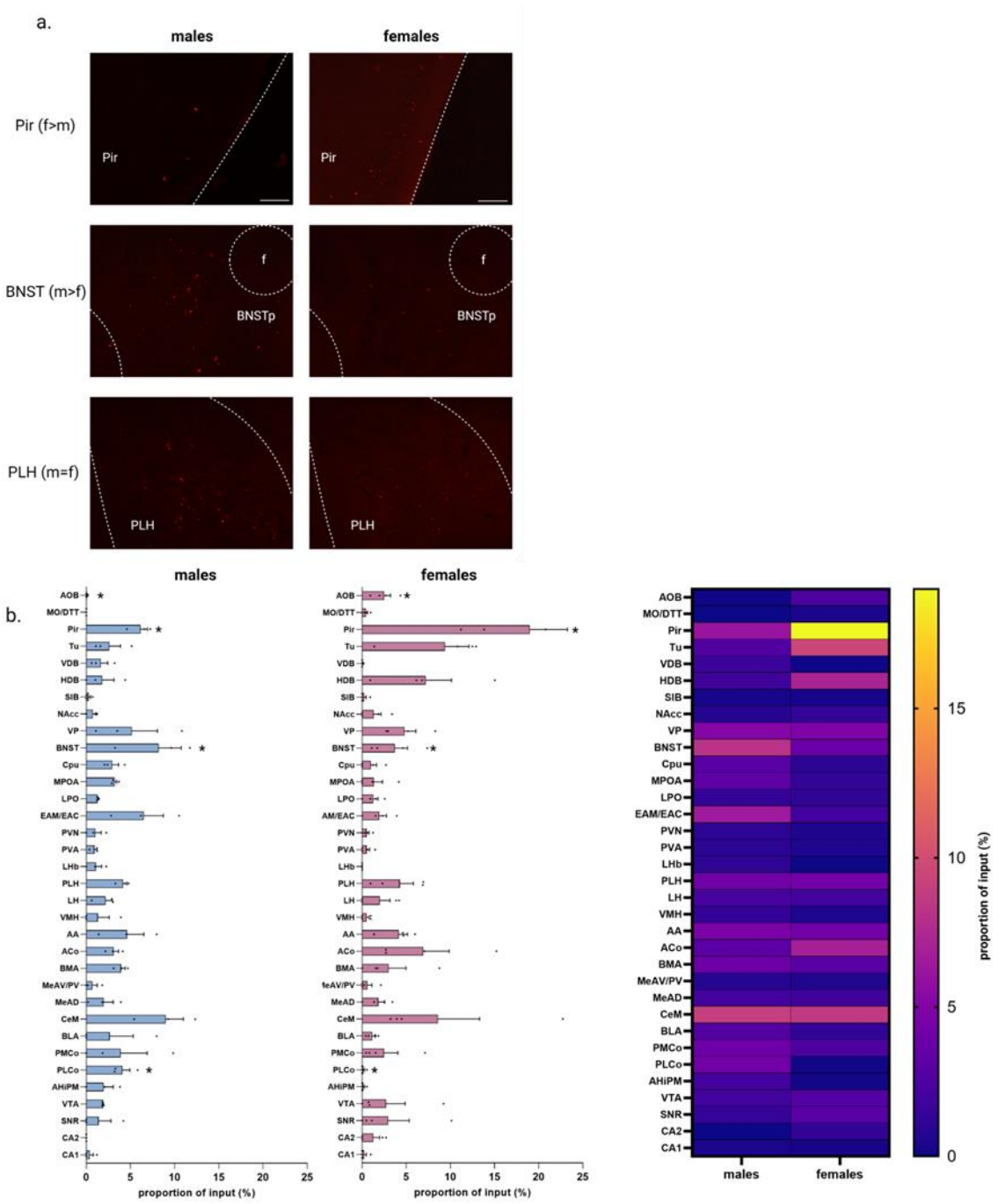

Figure 5-4- MeA AVP cell inputs. *(a) Example images of areas that contained female-bias input cells (Pir), male-bias input cells (BNST), or sex-invariant input cells (PLH). Cells labeled with EnvA g-deleted rabies virus (mCherry) are in red. (b) Bar graph and heatmap of retrogradely labeled mCherry+ inputs by brain region, quantified as percentage of all retrogradely labeled cells. Male (n=3) and female (n=4) data showed a significant sex difference in areas such as the AOB, Pir, BNST, and PLCo (two-way ANOVA using Benjamini-Hochberg (False Discovery Rate, 5%) was used to control for multiple comparisons) Mean ± SEM data represented. Scale bar = 50 µm. *p < 0.05*

AVP inputs to BNST and MeA AVP neurons

As we detected BNST and MeA reciprocal inputs, to directly test if BNST and MeA AVP cells receive inputs from each other and from other major AVP-producing sources, such as the PVN, we used single-molecule, *in situ* hybridization (RNAscope) fluorescent multiplex assay in RVdG-mCherry injected male brains, to detect *Avp* and *RV* mRNA labeling (Figure 5-5). Our findings indicate that the BNST and MeA AVP cells exhibit strong AVP inputs from each other, with approximately 70-80% of the *Avp*+ cells showing *RV* mRNA co-label, while 10-20% of PVN *Avp*+ cells showed *RV* mRNA co-label (Figure 5). Moreover, the RV labeled inputs originating from the PVN were typically situated in the dorsal lateral subsection of the PVN, suggesting the potential existence of a subset of PVN AVP cells that communicate with the sexually dimorphic AVP system.

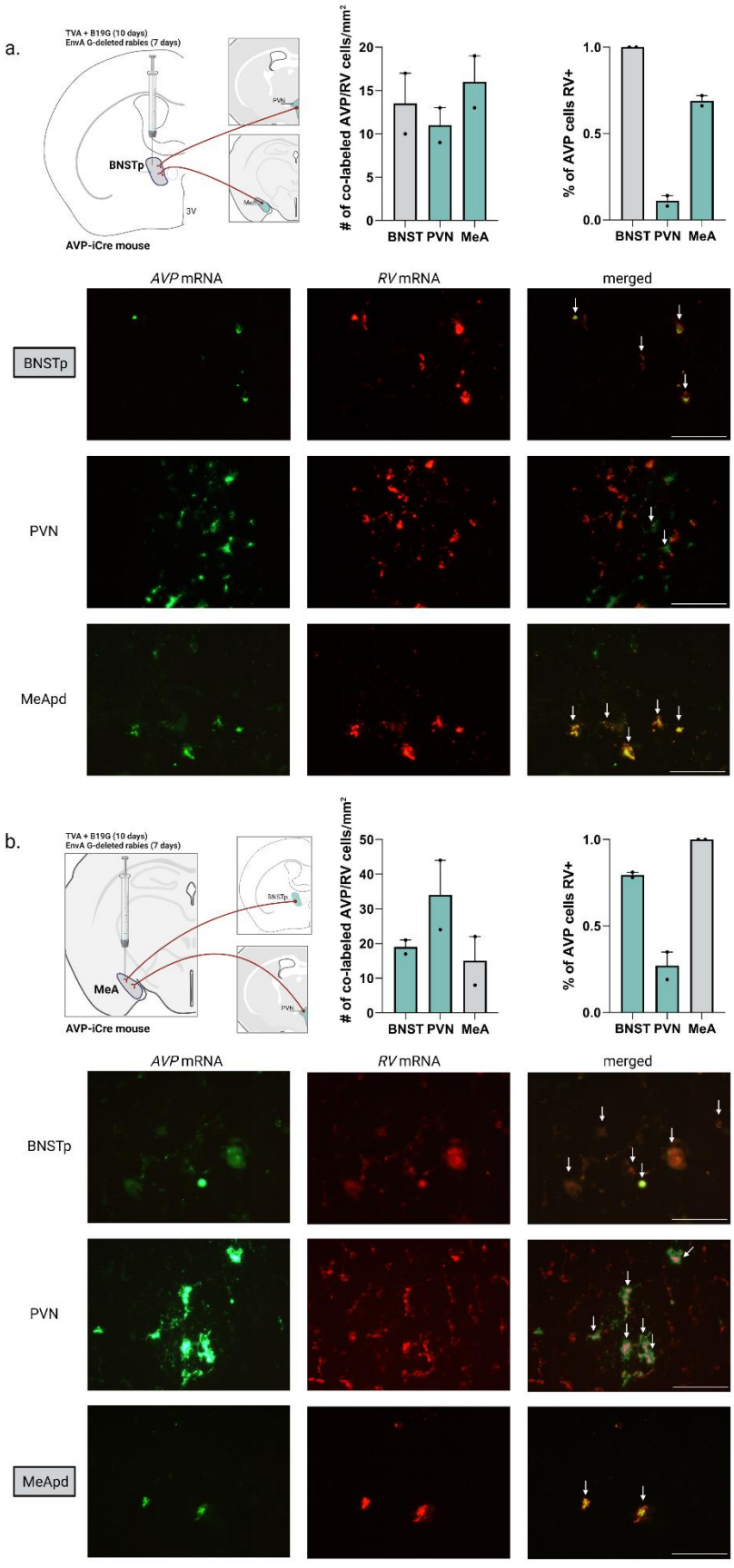
a.
TVA + B19G (10 days)
EnvA G-deleted rabies (7 days)
BNSTp
PVN
MeA
3V
AVP-iCre mouse
# of co-labeled AVP/RV cells/mm²
% of AVP cells RV+
BNST PVN MeA
AVP mRNA
RV mRNA
merged
BNSTp
PVN
MeApd
b.
TVA + B19G (10 days)
EnvA G-deleted rabies (7 days)
MeA
BNSTp
PVN
AVP-iCre mouse
# of co-labeled AVP/RV cells/mm²
% of AVP cells RV+
BNST PVN MeA
AVP mRNA
RV mRNA
merged
BNSTp
PVN
MeApd

Figure 5-5– BNST and MeA AVP cell inputs contain Avp mRNA. *(a) BNST injection site, bar graph of retrogradely labeled mCherry+ inputs that were co-labeled with Avp mRNA, and the percent of AVP cells that contained RV mCherry+ label. Example images of each region measured (posterior BNST, PVN, MeApd) and Avp mRNA (green), EnvA g-deleted rabies virus (red), and merged images. (b) MeA injection site, bar graph of retrogradely labeled mCherry+ inputs that were co-labeled with Avp mRNA, and the percent of AVP cells that contained RV mCherry+ label. Example images of each region measured (posterior BNST, PVN, MeApd) and Avp mRNA (green), EnvA g-deleted rabies virus (red), and merged images. Scale bar = 200 µm.*

5.3.3 Efferent projections from BNST AVP neurons

To examine the projections of BNST AVP neurons, we unilaterally injected the BNST of both male and female AVP-iCre+ mice with a Cre-dependent AAV expressing a mGFP-2A-Synaptophysin-mRuby fusion protein which can be used to identify both fibers (GFP) and putative synapses (RFP) (Beier 2016) (Figure 6a-b). In all mice, GFP+ starter cells were largely restricted to the BNST and no label was observed in AVP-iCre negative control animals. We were able to achieve a greater level of AAV expression in BNST AVP cells, as compared to the RVdG-mCherry virus, which enabled us to observe the expected sexual dimorphism, with males exhibiting a higher number of starter cells than females ($t(5) = 13.72$, $p = 0.000037$, $d = 2.3$; Figure 5-6e). As anticipated, our findings revealed that males exhibited a higher total puncta count in comparison to females ($t(5) = 4.9$, $p = 0.0045$, $d = 2.2$; Figure 5-6e) and there were no regions where females had greater outputs than males. We also qualitatively defined the fiber density of each BNST AVP cell output region, which generally matched the amount of puncta quantified (i.e., denser fibers = larger amounts of puncta, with a few exceptions (e.g. mediodorsal thalamus); Supplementary Table 1, data not shown).

<u>Striatum</u>

The lateral septum (LS) received the largest projection from BNST AVP cells (Figure 5-6c-d). Within the LS, the intermediate and ventral segments received the strongest projections, similar to previous reports of AVP fiber density in mice (Rood and De Vries 2011). A considerable sex difference was found, where male mice had greater mRuby+ puncta in the lateral septum than females ($F(1,5) = 18.46$, $p = 0.008$, $\eta 2 = 0.79$; Figure 5-6c-d).

Pallidum

Approximately a quarter (~25%) of all BNST AVP mRuby+ puncta were observed in pallidal regions. The strongest projections were observed in the ventral and horizontal diagonal band (VDB/HDB), the substantia innominata (SIB), and the ventral pallidum (VP). Although no statistically significant sex differences were found among these regions, males had a greater overall pallidal labeling compared to females ($t(5) = 2.75$, $p = 0.041$, $d = 2.09$). The regions with the highest density of fibers and puncta are primarily located in two pallidal areas. The first area, referred to as the ventral septal area by Rood and de Vries (2011) and medioventral striato-pallidum by Otero-Garcia et al. (2014), is situated within and/or between the diagonal band and the nucleus accumbens (+1.18mm from bregma). To match the Paxinos and Franklin atlas, we have labeled this area as VDB. The second area, located between the VP and HDB (+0.74mm from bregma), is referred to as the SIB by Paxinos and Franklin (2012) and Rood and de Vries (2011), and is considered a continuation of the medioventral striato-pallidum by Otero-Garcia et al. Previous studies have used substance P to define pallidal territories, which have shown that AVP-immunoreactive fibers innervate the region between the ventral striatum and ventral pallidum. More detailed studies are needed to precisely determine the specific cell populations of the basal forebrain that receive BNST AVP inputs as well as their relationships with each other.

Hypothalamus

While the BNST AVP cells receive substantial input from the hypothalamus, outputs to the hypothalamus are primarily directed to six regions: medial preoptic area (MPOA), lateral preoptic area (LPO), the peduncular part of lateral hypothalamus (PLH), dorsomedial hypothalamic nucleus (DM), dorsal premammillary nucleus (PMD), and the supramammillary nucleus (SuM). The PLH exhibited the highest level of BNST AVP cell output, not only within the hypothalamus, but across all regions, with levels comparable to the LS and LHb. Our findings also revealed a significant sex difference in outputs to hypothalamus, with males exhibiting a greater number of BNST AVP puncta and fibers within the PLH as compared to females ($F(1,5) = 13.9$, $p = 0.014$, $\eta 2 = 0.74$; Figure 5-6c-d). Moreover, the preoptic area contained more puncta in both lateral and medial zones in males compared to females (MPOA: ($F(1,5) = 14.63$, $p = 0.012$, $\eta 2 = 0.75$; LPO:($F(1,5) = 14.68$, $p = 0.012$, $\eta 2 = 0.75$; Figure 5-6c-d). Although the DM, PMD, and SuM received fewer fibers and puncta compared to the MPOA, LPO, and PLH, the SuM also exhibited greater fiber density and significantly higher puncta number in males when compared to females ($F(1,5) = 22.53$, $p = 0.005$, $\eta 2 = 0.82$; Figure 5-6c-d; Supplementary Table 1).

Thalamus

The lateral habenula (LHb) received the largest projection from BNST AVP cells (Figure 5-6c-d) and these were much greater in males compared to females ($F(1,5) = 13.97$, $p = 0.013$, $\eta 2 = 0.74$; Figure 5-6c-d). Notably, while dense fibers were observed throughout the entire LHb, presynaptic puncta were more concentrated in the posterior sections of LHb (-1.94 to -2.18 from bregma). Although we observed moderate to dense fibers within the mediodorsal thalamus (MD) and the paraventricular thalamus (PVT), we observed few presynaptic puncta in these areas,

indicating that BNST sends fibers through the region without making substantial synaptic contact.

Amygdala

The medial amygdala (MeA) and extended amygdala (EAM) each contained ~6-7% of the total puncta count of BNST AVP cell outputs. While moderate to dense fibers were present in each of the observed regions, we noted a marked difference between males and females in terms of the number of fibers and puncta within the posterior dorsal MeA (MeApd). Specifically, males exhibited significantly more fibers and puncta in the MeApd compared to their female counterparts ($F(1,5) = 21.94$, $p = 0.005$, $\eta 2 = 0.81$; Figure 5-6c-d). We also found limited presence of fibers/puncta in the posterior region of the basolateral amygdala (BLP), without discernible sex differences in innervation.

Cortex

While most of the BNST AVP cell projections were restricted to subcortical regions, we did observe some fibers and puncta located in the dorsolateral entorhinal cortex (DLEnt). These outputs were more concentrated in the anterior portion of this structure (-2.54mm to -2.80mm from bregma) and in deep cortical layers (4-6). We also observed a sex difference, where males had greater numbers of labeled puncta than females ($F(1,5) = 15.85$, $p = 0.011$, $\eta 2 = 0.76$; Figure 5-6c-d).

Midbrain

The BNST AVP cells projected to four midbrain structures: the ventral tegmental area (VTA), substantia nigra, reticular part (SNR), periaqueductal gray (PAG), and dorsal raphe (DR). Of these structures, the PAG and DR received the most fibers and puncta, with these being present throughout most of the DR, and more concentrated in the lateral and dorsal lateral subregions of

the PAG (Figure 5-7). No significant sex differences in innervation were found in these individual regions.

Hippocampus

Although sparse, we did observe some BNST AVP fibers and puncta within the hippocampus, specifically in the ventral CA1, near or within the pyramidal layer (Figure 5-7). We did not see any label in other hippocampal regions and we did not detect any discernible sex differences in this innervation of ventral CA1.

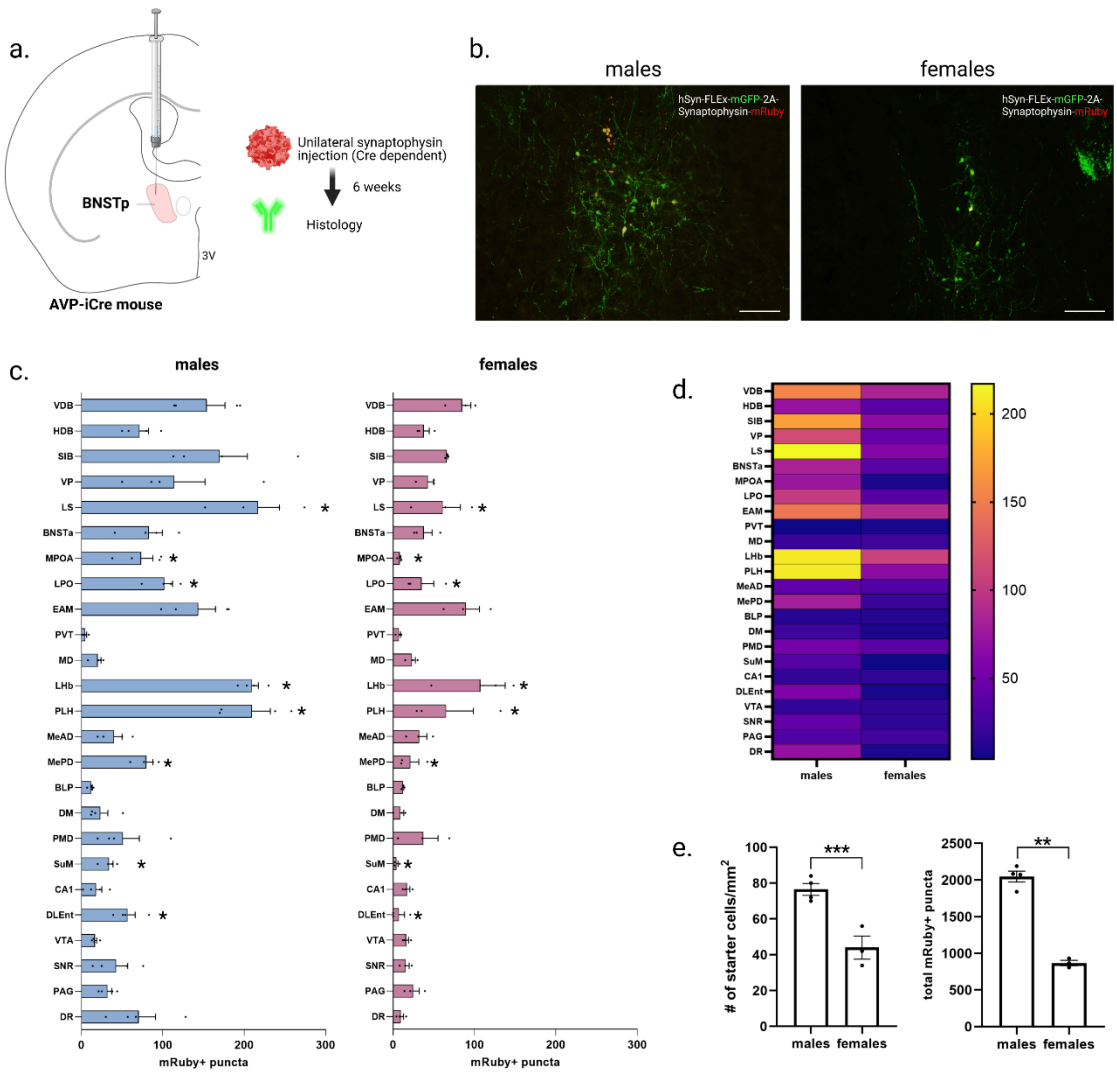

Figure 5-6– BNST AVP cell outputs. *(a) BNST injection site and timeline. (b) Example merged image of BNST-AVP cell starter region labeled with GFP-2A-Synaptophysin-mRuby virus. Scale bar = 50 µm. (c) Bar graph and (d) heatmap of the number of anterogradely labeled mRuby+ puncta by brain region. Male (n=4) and female (n=3) data showed a significant sex difference in areas such as the LS, MPOA, LPO, LHb, PLH, MeApd, SuM, and DLEnt (two-way ANOVA using Benjamini-Hochberg (False Discovery Rate, 5%) was used to control for multiple comparisons). (e) Number of labeled BNST AVP starter cells and total mRuby puncta labeled. Mean ± SEM data represented. *p < 0.05, ** p < 0.01, ***p < 0.001*

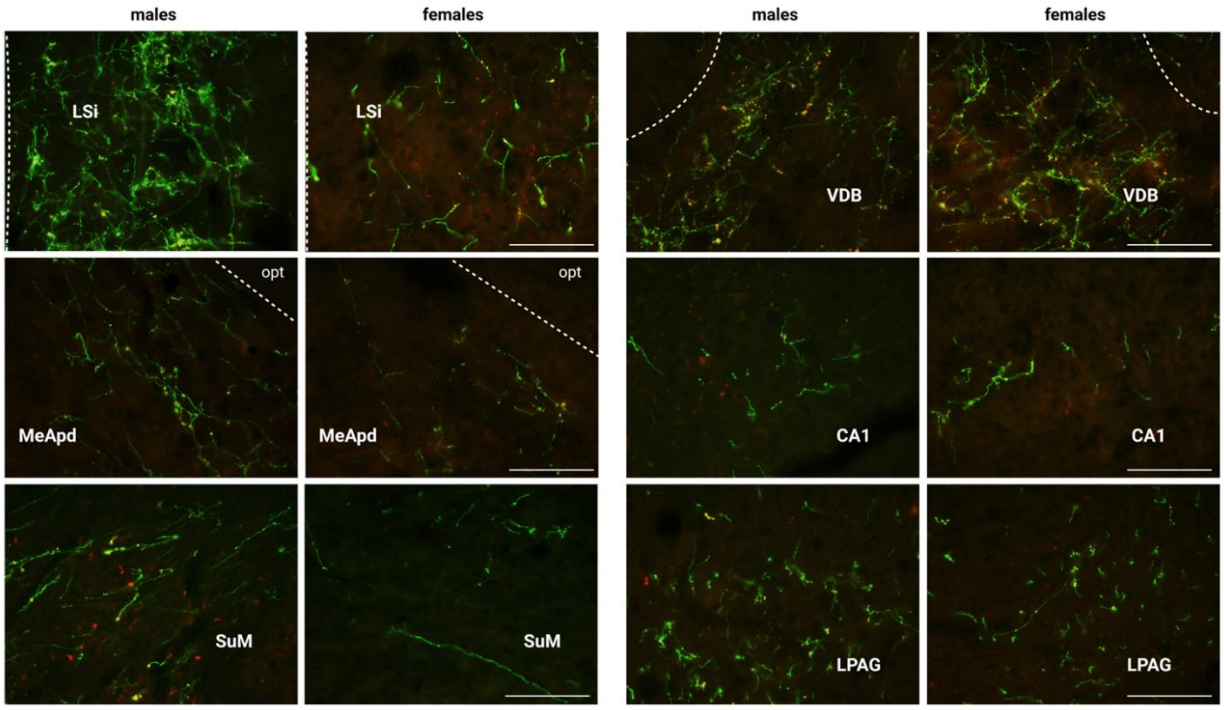

Figure 5-7– BNST AVP cell outputs in males and females. *(a) Example of merged images of areas that received BNST AVP cell output fibers (green) and presynaptic puncta (red) labeled with GFP-2A-Synaptophysin-mRuby virus at 40x magnification. Example images include the LS, MeApd, SuM, VDB (or ventral septal area (Rood et al, 2011), medioventral striato-pallidum (Otero-Garcia et al., 2014), CA1, and lateral PAG. Scale bar = 200 µm.*

5.3.4 Efferent projections from MeA AVP neurons

To examine the efferent projections of MeA AVP neurons, we unilaterally injected the MeA of both male and female AVP-iCre+ mice with Cre-dependent AAV expressing a mGFP-2A-Synaptophysin-mRuby fusion protein (Figure 5-8a-b). In all mice, GFP+ starter cells were largely restricted to the MeA and no label was observed in AVP-iCre negative control animals. Similar to the BNST anterograde tracing experiment, we were able to achieve a greater level of AAV expression in MeA AVP cells, as compared to the RVdG-mCherry virus, which enabled us to observe the expected sexual dimorphism, with males exhibiting a higher number of starter cells than females ($t(4) = 3.81$, $p = 0.019$, $d = 1.9$; Figure 5-8e). Unexpectedly, no differences were found between males and females in total puncta count (Figure 5-8e), despite the sex

difference in number of MeA AVP cells. In contrast to the BNST, we did not observe sex differences in the number of presynaptic puncta within any of the regions labeled following correction for multiple comparisons, although many of these regions were similar to those targeted by BNST AVP cells, with the exception of MeA AVP inputs to central amygdala (CeM) and basomedial amygdala (BMA). The fiber density of each MeA AVP cell output region generally matched the amount of puncta quantified (i.e., denser fibers = larger amounts of puncta; Supplementary Table 2, data not shown).

Striatum

The lateral septum (LS) received projections from MeA AVP cells (Figure 5-8c-d), although these outputs were not as strong as those originating from BNST AVP cells. Within the LS, the intermediate and ventral segments received the strongest projections, similar to previous reports of AVP fiber density in mice (Rood and De Vries 2011). However, in contrast to the BNST, no sex differences were found in the number of mRuby+ puncta in LS (Figure 5-8c-d).

Pallidum

Similar to the BNST, a quarter (~25%) of total MeA AVP mRuby+ puncta were observed in pallidal regions. The strongest connections were observed with the ventral and horizontal diagonal band (VDB/HDB), the substantia innominata (SIB), and the ventral pallidum (VP). No statistically significant sex differences in innervation were found among these regions (Figure 5-8c-d).

Hypothalamus

The output from the MeA AVP cells extended to five hypothalamic regions, similar to those targeted by the BNST AVP cells. These regions include the medial preoptic area (MPOA), lateral preoptic area (LPO), the peduncular part of the lateral hypothalamus (PLH), ventral

premammillary nucleus (PMV), and supramammillary nucleus (SuM). Notably, the MeA AVP inputs to the premammillary nucleus slightly differed from those of the BNST, in that MeA AVP fibers/puncta were found in the ventral subregion instead of the dorsal region. As was observed in BNST AVP outputs, the PLH exhibited the highest level of innervation from the MeA AVP cells as well as containing more fibers and puncta in the lateral (vs. medial) zones (i.e., LPO) (Figure 5-8c-d). No sex differences were found in the number of puncta counts within any area of the hypothalamus, however males tended to have more puncta overall in the hypothalamus ($t(4) = 2.92$, $p = 0.043$, $d = 1.3$).

Thalamus

The lateral habenula (LHb) received strong fiber projections and moderate presynaptic puncta from MeA AVP cells (Figure 5-6c-d). Similar to BNST, these fibers were dense throughout the entire LHb but presynaptic puncta were more concentrated in the posterior sections (-1.94 to -2.18 from bregma). We observed qualitatively more fibers in males compared to females, but did not find a sex difference in puncta counts. Similar to the BNST, we observed moderate fibers within the mediodorsal thalamus (MD) and the paraventricular thalamus (PVT), but with few presynaptic puncta in these areas, indicating that these are likely fibers of passage (Figure 5-8c-d).

Amygdala

The MeA AVP cells primarily projected to other amygdala structures including the dorsal MeA (MeAD), extended amygdala (EAM), basomedial amygdala (BMA), central amygdala (CeM), and basolateral amygdala (BLP). The EAM stands out as one of the main targets of the MeA AVP cells, with males showing a numerically, but not significantly, higher number of puncta

than females (Figure 5-8c-d). The BMA and CeM stand out as regions that receive MeA, but not BNST, AVP cell output.

Cortex

While most of the MeA AVP cell projections were restricted to subcortical regions, we observed some fibers and puncta located in the dorsolateral entorhinal cortex (DLEnt), similar to BNST AVP outputs. This innervation was concentrated in the anterior portion of this structure (-2.54mm to -2.80mm from bregma) and in deep cortical layers (4-6); however, unlike BNST AVP outputs, males and females had similar levels of innervation.

Midbrain

Similar to the BNST, MeA AVP cells projected to three midbrain structures: the ventral tegmental area (VTA), periaqueductal gray (PAG), and dorsal raphe (DR), but unlike the BNST AVP outputs, the substantia nigra, reticular part (SNR) did not receive input from MeA AVP cells. Of these structures, the PAG and DR received the most fibers and puncta, with fibers/puncta present throughout most of the DR, and fibers/puncta more concentrated in the lateral and dorsal lateral subregions of the PAG, similar to that observed from BNST AVP inputs. No significant sex differences in innervation were found in any of these regions.

Hippocampus

As with BNST, we observed sparse MeA AVP cell fibers and puncta within the hippocampus, specifically in the ventral CA1, near or within the pyramidal layer. We did not see any label in other hippocampal regions and we did not detect any discernible sex differences in innervation.

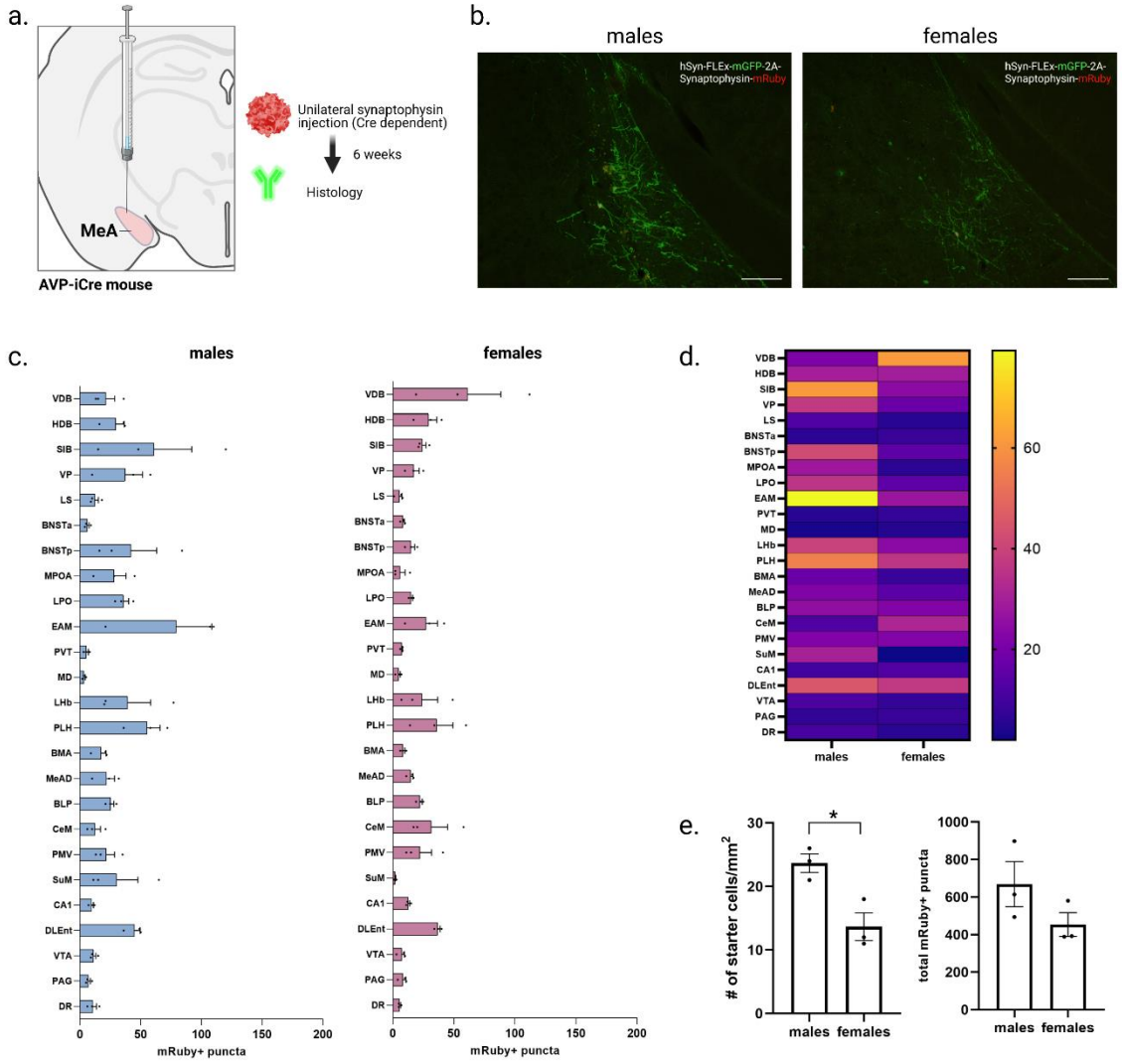

Figure 5-8– MeA AVP cell outputs. *(a) MeA injection site and timeline. (b) Example merged image of MeA-AVP cell starter region labeled with GFP-2A-Synaptophysin-mRuby virus. Scale bar = 50 µm. (c) Bar graph and (d) heatmap of the number of anterogradely labeled mRuby+ puncta by brain region. Male (n=3) and female (n=3) data showed no significant sex differences in all areas (two-way ANOVA using Benjamini-Hochberg (False Discovery Rate, 5%) was used to control for multiple comparisons). (e) Number of labeled BNST AVP starter cells and total mRuby puncta labeled. Mean ± SEM data represented. *p < 0.05*

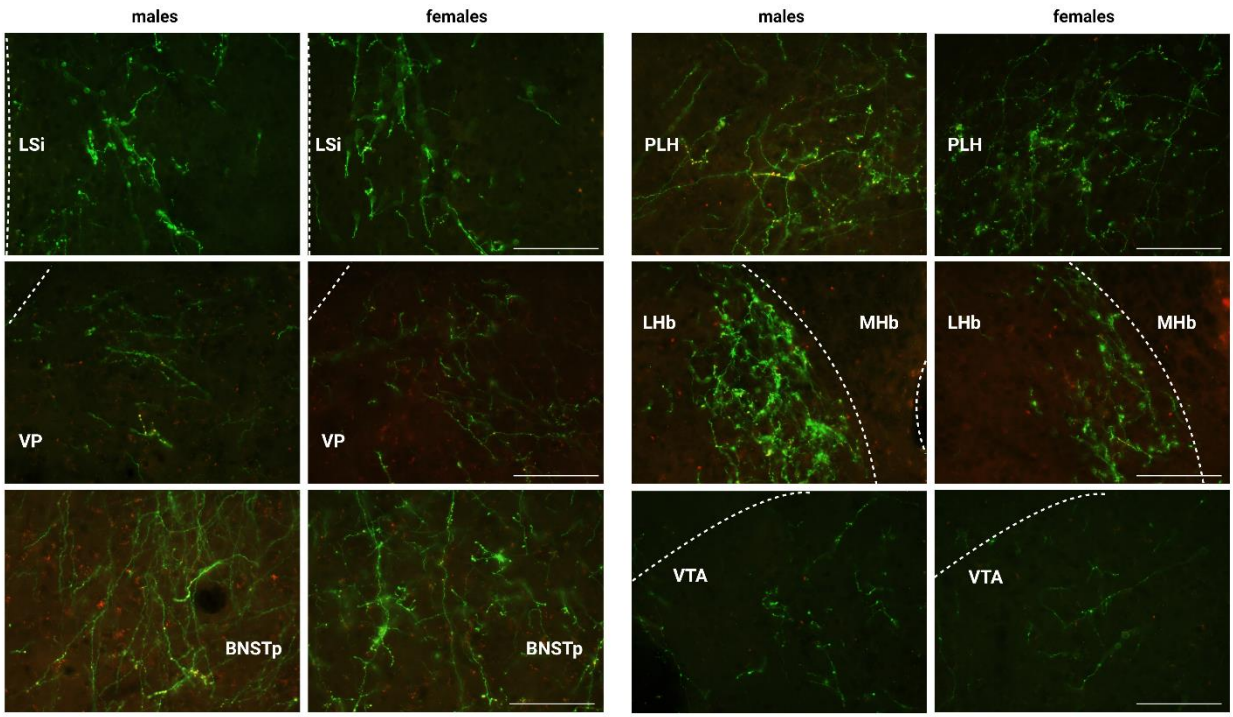

Figure 5-9– MeA AVP cell outputs in males and females. *(a) Example of merged images of areas that received MeA AVP cell output fibers (green) and presynaptic puncta (red) labeled with GFP-2A-Synaptophysin-mRuby virus at 40x magnification. Example images include the LS, VP, posterior BNST, PLH, LHb, and VTA. Scale bar = 200 µm.*

5.4 Discussion

The neuropeptide AVP has been identified as playing a key role in sex-specific social behavior depending on the AVP source (Rigney et al. 2023). Although inputs and outputs to the BNST and MeA as a whole have been described in rats (Myers et al. 2014; Dong et al. 2001; Weller and Smith 1982; Gu et al. 2003), hamsters (Wood and Swann 2005; Coolen and Wood 1998), and mice (DiBenedictis et al. 2014; Pardo-Bellver et al. 2012; Cádiz-Moretti et al. 2016), inputs specific to BNST and MeA AVP cells are completely unknown. Furthermore, outputs of these cells have been analyzed indirectly and in isolation,with often failing to distinguish between fibers of passage and presynaptic terminal areas (Rood et al. 2013; Caffe et al. 1987; van Leeuwen et al. 1985). To address this gap in the literature, we used a viral tract-tracing

strategy to specifically target AVP cells in AVP-iCre male and female mice for monosynaptic-transsynaptic retrograde labeling (using a cre-dependent rabies virus strategy) and anterograde terminal tracing (using a cre-dependent synaptophysin-tagged virus) so as to identify the neural inputs to, and outputs from, BNST and MeA-AVP cells. We found that these cells receive and send signals to areas that drive motivation and social behavior in mice (i.e., olfactory regions, striatum, pallidum, thalamus, hypothalamus, amygdala, and midbrain structures). Our findings also indicate that these sexually differentiated AVP systems exhibit both commonalities and divergences in its connections between the sexes. Furthermore, the AVP cells located in both the BNST and MeA are in direct contact with each other as well as sharing numerous similarities in projection patterns. These findings suggest the existence of an integrated, sexually dimorphic, AVP system within the extended medial amygdala. The results of these experiments provide, for the first time, a comprehensive mapping of the connectional architecture of sexually dimorphic and steroid-sensitive vasopressin neurons of the BNST and MeA in the mouse brain.

Experimental considerations

Although the modified rabies virus tracing approach identifies monosynaptic inputs onto targeted cells, it is known that the virus may not cross all synapses with the same efficiency (Lavin et al. 2019). As a result, it is possible that certain cell types may be overrepresented in the presynaptic connectome. Additionally, the extent of spread of rabies virus from neuron to neuron is influenced by several factors, including the level of expression of Cre from driver lines, the titer of the AAV helper virus, and the titer of the rabies virus (Lavin et al. 2019; Lavin et al. 2020). As we did not infect all BNST and MeA AVP cells, it is possible that these variables may have influenced our final results. However, despite observing no sex difference in the number of

starter cells (due to this partial labeling), we did detect differences in the percentage of labeled input neurons between the sexes, the total number of input cells, and the percentage of inputs per starter cell. Nevertheless, it is possible that had we labeled all or most BNST and MeA AVP cells, these sex differences may have been either more pronounced or weaker.

While the vast majority of our initially infected cells ('starter cells') were confined to the BNST and MeA regions using either the antero- or retrograde tracers, some caution is warranted when interpreting our results, for several reasons. First, as the reticular thalamus and central amygdala, two areas located in very close proximity to the target regions, may express low levels of AVP and therefore Cre, some of our input and output labeling may be due to additional infection of these areas. However, we believe these issues are minimal as: (a) we were able to identify reticular thalamus inputs and outputs as distinct from BNST/MeA using control injections limited to thalamus, (b) we observed a lack of labeling external to known inputs and outputs of BNST and MeA cells in the majority of animals (Gu et al. 2003; Cádiz-Moretti et al. 2016; Weller and Smith 1982; Pardo-Bellver et al. 2012). Second, as previous reports have shown Cre-independent TVA expression (Faget et al., 2016; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012), our retrograde label may also reflect non-AVP cell inputs. However, the number of rabies virus labeled (mCherry+) cells in control animals was less than 1% of the total averaged labeled cells in AVP-iCre positive animals, therefore supporting labeling specificity of inputs to BNST and MeA AVP cells.

Adeno-associated virus (AAV) vectors encoding tagged-synaptophysin are commonly used to label presynaptic terminals, which express synaptophysin (Beier et al. 2015). However, because this approach depends on targeting synaptophysin, a constitutively-present presynaptic protein (Jahn and Fasshauer 2012; Diao and Ma 2020), we cannot distinguish between active and

inactive synapses. Despite these limitations, the tagged-synaptophysin viral approach remains a valuable method for distinguishing between putative synaptic terminals and their corresponding fibers. Future studies will be needed for a more precise detection of synaptic patency, such as using channelrhodopsin-mediated circuit mapping (Petreanu et al. 2007).

Sex and region-dependent differences in afferent inputs and efferent outputs to BNST and MeA AVP cells

The expression of AVP in BNST and MeA neurons is sexually dimorphic, with males showing a higher number of AVP-expressing cells than females, and is dependent on both developmental and adult levels of gonadal steroid, an effect documented in multiple species, including mice (de Vries et al. 1984; De Vries et al. 1994; De Vries and Panzica 2006; Rood et al. 2013). The BNST and MeA AVP cells have been considered related populations due to their sexually dimorphism and steroid-sensitivity as well as their neurochemical phenotype. Here we show that these two cell populations, with few exceptions, send and receive similar projections, indicating that they are likely both functionally and anatomically (see below) interconnected, which fits well with the more general idea that the medial and intermediate parts of the BNST and MeA together form an integrated “extended (medial) amygdala” (de Olmos and Heimer 1999).

The lateral septum (LS) has long been known to be the major target of sexually-differentiated AVP innervation and has been implicated in the sex-specific regulation of social recognition, social play, social affiliation, aggression, and anxiety-related behavior (De Vries and Panzica 2006; Bredewold and Veenema 2018; Rigney et al. 2023). While more indirect approaches have supported the BNST and MeA as the sources of this AVP innervation, here we

confirm that both BNST and MeA AVP cells send strong inputs to the LS. Projections originating from the BNST are sexually dimorphic, with males showing a greater number of BNST AVP presynaptic puncta than females, MeA AVP cell outputs to the LS in male subjects were comparatively less intense than those originating from the BNST, and, consequently, there were no discernible sex differences observed. This difference could be attributed to the lower number of starter cells reflecting the smaller number of AVP cells located in the MeA compared to the BNST. We also show that the LS is reciprocally connected with BNST, but not MeA, AVP cells (see Figures 2 and 4) and that these inputs are sexually dimorphic, with males receiving significantly more inputs than females. This finding of increased BNST-LS interconnectivity in males supports the idea that AVP cells within the BNST, rather than the MeA, play a prominent role in driving specific aspects of male social behavior (i.e., competition, investigation, and affiliation) through their connections with the LS (Rigney et al. 2023).

Similarly, the lateral habenula (LHb) is another region that receives substantially more output from BNST AVP cells in males than in females. The LHb acts as a hub for integrating value-based, sensory, and experience-dependent information to regulate a variety of motivational, cognitive, and motor processes and targets all midbrain neuromodulatory cells, such as those of the noradrenergic, serotonergic, and dopaminergic systems (Hu et al. 2020). Thus, AVP may be interacting with the LHb, primarily in males, to encode negative or positive valence. Indeed, prior work has suggested that the hypothalamic-to-LHb AVP pathway may play a role in regulating motivational state via thirst (Zhang et al. 2016), while V1aR blockade in the LHb reduces communicative behaviors in male mice, suggesting that AVP in the LHb plays an important role in promoting male-typical active responses to social competition and biological threats (Rigney et al. 2020; Zhang et al. 2016). Further experiments are required to reveal the

functional significance of the BNST AVP-LHb pathway and its role in behavioral and physiological sex differences.

We observed that females had a higher number of inputs to BNST AVP cells in several regions compared to males. One notable difference was that females had greater inputs from the paraventricular nucleus of the hypothalamus (PVN) compared to males. As the PVN and BNST work together to inhibit stress response (Choi et al. 2007; Choi et al. 2008), and AVP exerts a sex-specific influence on stress and anxiety (Bielsky et al. 2005), this suggests that PVN AVP inputs to BNST may impact coping mechanisms and stress resilience between the sexes (Borrow et al. 2018; Rigney et al., 2023); further research is necessary to directly test this idea.
In female mice, the medial preoptic area (MPOA) also sent a greater number of inputs to BNST AVP cells compared to males. Conversely, the BNST AVP cells had more outputs to MPOA in males than in females. Although the functional role of this sex difference in reciprocal architecture remains unclear, we do know that the MPOA is an important region for regulating male mating (Baum and Everitt 1992), female parental care (Numan and Numan 1997; Tsuneoka et al. 2013; McHenry et al. 2015), and, in a subset of MPOA galanin neurons, parental behaviors in both male and female mice (Wu et al. 2014). Furthermore, activation of estrogen receptor (Esr1)-expressing neurons in the MPOA resulted in male-typical mating and pup retrieval in both males and females (Wei et al. 2018) and activation of MPOA neurotensin cells, which are highly co-localized with Esr1, increases preference for opposite sex stimuli in both sexes (McHenry et al. 2017). Since the BNST AVP cells are sexually-differentiated in their MPOA connectivity, it is possible that they also modulate socially-directed behavior in sex-specific ways, but further experiments are necessary to demonstrate this possibility.

Other sex differences in input to BNST AVP cells favoring females originated from the nucleus accumbens (NAcc) and ventral pallidum (VP). These areas have similar neurochemistry and are part of the mesolimbic reward system that form key nodes in the social decision-making network (O'Connell and Hofmann 2011; O'Connell and Hofmann 2012; Zahm and Heimer 1990). Indeed, the NAcc and VP regulates both reward and aversion (Yao et al. 2021; Chometton et al. 2020; Gallo et al. 2018) and it remains to be seen if the inputs from these areas are dopamine sensitive or express V1aR. As a result, BNST AVP cells in females may be more strongly regulated by motivational or reward signals generated by NAcc/VP than are males. These pathways may also be targets for AVP action on social motivation and reward in females at particular life stages as higher V1aR density in the VP, an area implicated in maternal behavior (Numan et al. 2005), are observed after partner formation and pregnancy in female prairie voles (Zheng et al. 2013; Ophir et al. 2013). This may further explain why the NAcc, MPOA, and VP together send stronger inputs to BNST AVP cells, as these structures are functionally interconnected (Numan et al. 2005).

It has also been argued that sex differences in brain structure may not only cause sex differences in behavior and physiology but also prevent them by compensating for sex differences in physiology (De Vries 2004). Given that there are fewer vasopressin AVP-expressing cells in the BNST in females, the NAcc, MPOA, and VP may be compensating for these sex differences by sending stronger inhibitory signal to BNST AVP cells, which are likely GABAergic (Lebow and Chen 2016). Indeed, our lab has shown that stimulating BNST AVP cells can increase social investigation in females, while inhibiting the cells had no effect (Rigney et al, 2023, unpublished), suggesting that artificial stimulation of BNST AVP cells in females may override natural inhibitory input that could be biasing females away from social

investigation. Using this same logic, females may also be receiving more PVN inputs to compensate for less BNST AVP expression. For example, PVN AVP cells may function to inhibit social investigation in females, but not males (Rigney et al. 2020).

Although the MeA and BNST AVP cells display strong similarities in their inputs and outputs, important differences exist in olfactory-related inputs to these cells, with MeA, but not BNST, AVP neurons receiving more inputs from both main and accessory olfactory structures. This finding is not entirely unexpected, as the MeA receives numerous olfactory inputs (Petrulis 2020), whereas the BNST receives considerably less (Kang et al. 2009; Kang et al. 2011). Specifically, the MeA AVP cells, but not the BNST ones, receive inputs from both accessory (accessory olfactory bulb (AOB); posteromedial cortical amygdala (PMCo) and main olfactory structures (piriform cortex (Pir), olfactory tubercle (Tu), posterolateral cortical amygdala (PLCo)). Furthermore, inputs to MeA AVP cells are sex-biased with females receiving more input from AOB, Pir, and PLCo compared to males. While the functional significance of this sex difference in input is unknown, it may mean that the role of MeA AVP cells in pheromone-directed reproductive behaviors (Hari Dass and Vyas 2014) may be sexually-differentiated as well.

Similarities in BNST and MeA AVP cell connectivity

The perifornical lateral hypothalamus (PLH) receives sexually-differentiated and steroid-dependent AVP fibers in mice (Rood et al. 2013) and here we demonstrate that this area has strong bidirectional connectivity with MeA and BNST AVP cells, and that males have greater BNST-PLH connectivity compared to females. While the lateral hypothalamus regulates many aspects of behavior such as feeding, reward, arousal and stress (Stuber and Wise 2016) via

connections to other regions that the BNST/MeA AVP cells communicate with (Bonnavion et al. 2016), the exact role of AVP in this region is not well understood. Recent studies suggest that AVP neurons in the paraventricular nucleus (PVN) send signals to orexin neurons in the PLH to regulate sleep and wakefulness (Islam et al. 2022), and in hamsters, gonadal hormones have an impact on how AVP affects aggression in the ventrolateral hypothalamus (VLH) (Delville et al. 1996). The bidirectional connections between MeA and BNST AVP cells and the PLH as well as the observed sex differences in BNST-PLH connectivity suggest a potential role for AVP signaling in sex-dependent regulation of behavior through lateral hypothalamic circuits.

As previously reported, the AVP cell populations in the BNST and MeA share similar features such as steroid-sensitive and sexually dimorphic expression of AVP, in contrast to other AVP-producing regions (de Vries et al. 1984; De Vries and Panzica 2006), suggesting that BNST and MeA AVP are related cell populations. Our results support this idea by demonstrating that BNST and MeA AVP cells send strong reciprocal connections to each other and that these connections are also sexually differentiated, with males having more robust connections than females. This provides compelling evidence that these two cell groups function as a coherent unit for the regulation of social and emotional behavior, with perhaps different functions between the sexes.

Conclusions

Our findings demonstrate that the sexually dimorphic AVP cell populations in the BNST and MeA exhibit substantial interconnectivity with discrete brain regions that play critical roles in regulating social behavior, motivation, reward, aversion, anxiety, and stress-related behaviors (O’Connell and Hofmann, 2012, 2011) and, more specifically, may help explain AVP's effects

on prosocial behavior, pair bond maintenance, aggression, and communication (Albers, 2012; Beiderbeck et al., 2007; Compaan et al., 1993; Everts et al., 1997; Forero et al., 2023; Veenema et al., 2010). However, the BNST and MeA AVP cells may not act completely independently from hypothalamic AVP sources, as they do receive input from the PVN (Freda et al., 2022) and even from PVN AVP cells, indicating some interconnectedness between these brain regions.

The anatomical connections outlined here will set the foundation for a better understanding of the organizational principles of the sexually dimorphic AVP system which will, in turn, enable future circuit-based analyses that explore their function. For example, investigating the connectivity between the BNST AVP cells and the lateral septum, which is more pronounced in males than in females, could elucidate how this connection shapes sex-specific social and anxiety-related behaviors (Aspesi and Choleris, 2021; Beiderbeck et al., 2007; Veenema et al., 2013). Finally, these specific connections could also be examined in relation to V1aR contact and the possibility of reciprocal communication between V1aR-expressing cells or other cell types involved in regulating social behavior.

6 SEX DIFFERENCES IN VASOPRESSIN 1A RECEPTOR REGULATION OF SOCIAL COMMUNICATION WITHIN THE LATERAL HABENULA AND DORSAL RAPHE OF MICE.

Previously published in Hormones and Behavior, 121:104715. doi: 10.1016/j.yhbeh.2020.104715. (PMID: 32067962)

6.1 Introduction

Animals often display profound sex differences in social behavior and communication and especially in reproductively-oriented behaviors such as courtship displays, territorial marking, parenting, and copulation (Darwin 1871; Bradbury and Vehrencamp 1998; Scott et al. 2015; Song et al. 2018). In humans, dysfunction in social communication is a common feature of several psychopathologies, such as autism, that show substantial sex differences in prevalence and impact (Halladay et al., 2015). It is therefore plausible that sex differences in neural circuitry may contribute to sexually differentiated function and dysfunction in social behavior and communication. One of the largest and most evolutionarily conserved sex-different systems in the vertebrate brain is the male-biased and steroid-dependent expression of the neuropeptide vasopressin/vasotocin (AVP) (de Vries, 2008; de Vries and Panzica, 2006; Goodson and Bass, 2001). AVP, working through the vasopressin V1a receptor (V1aR), has been repeatedly implicated in modulation of social behaviors across vertebrate species, including humans, and often in a sex-different way (Choleris et al. 2009; Donaldson and Young 2008; Insel 2010; Duque-Wilckens et al. 2016; Guastella et al., 2010; Rilling et al., 2014). For example, AVP acts in various brain regions to alter vertebrate social communication (Goodson and Bass, 2001; Kelly and Goodson, 2013), maternal care (Bosch and Neumann, 2008), pair bonding (Carter et al., 1995; Jarcho et al., 2011; Young and Wang, 2004), and social recognition (Dantzer et al.

1988; Everts and Koolhaas 1999; Bielsky and Young 2004; Bielsky et al. 2004; Veenema et al. 2012; Johnson and Young 2017).

A number of brain regions involved in social and emotional behavior contain sex-different and steroid-sensitive AVP innervation and V1aR expression, such as the lateral septum (LS), ventral pallidum (VP), lateral habenula (LHb) and several hypothalamic and midbrain areas, including the dorsal raphe nuclei (DR) (Rood et al. 2013; Dumais and Veenema 2016). However, most examinations of the social role of AVP have focused on LS and, to a lesser degree, the VP and hypothalamus (Lim and Young 2004; Bielsky et al. 2005; Ophir et al. 2008; Gobrogge et al. 2009; Bredewold et al. 2014; Otero-Garcia et al. 2014; DiBenedictis et al. 2020). Consequently, we know little about the behavioral role of AVP in other areas previously implicated in social and emotional behavior, such as LHb and DR nuclei (Nagayasu 2017; Balázsfi et al. 2018; Yang et al. 2018; Congiu et al. 2019; Russo et al. 2019) even though they also contain dense, steroid-sensitive and sexually dimorphic AVP innervation (Rood et al. 2013). Moreover, AVP, acting through V1aR, indirectly excites DR serotonin neurons (Rood and Beck 2014) and recent work suggests that increased AVP is associated with decreased LHb output and decreased fear response in stressful situations (Zhang et al. 2016).

Most sex-different brain AVP innervation, including that of LHb and DR, likely originates from sex-different AVP-expressing neurons within the bed nucleus of the stria terminalis (BNST) (de Vries and Panzica, 2006), a key structure in the social behavior neural network. Therefore, we hypothesized that BNST-AVP inputs to DR and LHb regulate sexually differentiated effects of AVP on social communication. Indeed, selective lesions of BNST-AVP neurons reduces social investigation and alters social communication in males, but not females (Rigney et al. 2019). Consequently, we predict that blocking V1aR action in DR or LHb will

recapitulate the sex-specific (male, but not female) deficits in social investigation and communication caused by BNST-AVP lesions. To test this, we assessed the effects of injecting a highly-selective V1aR antagonist (Manning et al. 2012) in the LHb or DR on male and female mouse communicative (ultrasonic vocalizations, urine marking, social investigation) and aggressive behaviors that are sexually differentiated (Kimura and Hagiwara 1985; Yang et al. 2013; Palanza and Parmigiani 2017; Zala et al. 2017).

6.2 Methods

6.2.1 Animals

All mice were maintained at 22°C on a 12/12 h reverse light/dark cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were performed in accordance with the Georgia State University animal care and use committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects

Fifty-six male and female C57BL/6J mice between 4-8 weeks of age were obtained from Jackson Laboratories (stock # 000664) and were singly-housed for a minimum of one week prior to testing.

Stimulus animals

Adult CD1(ICR) mice obtained from Charles River Laboratories were used as stimuli for behavioral testing and to provide male and female subjects with social experience as strain differences between subjects and stimulus mice increase social investigation (Gheusi et al.,

1994). Mice were used at 9 –16 weeks of age and were novel and unrelated to the subject to which they were exposed.

Female stimulus mice were group-housed, ovariectomized, implanted with an estradiol capsule (GDX+E), and given two sexual experiences before testing. Two groups of stimulus males were used for behavioral testing differed in their social experience, to either render them more competitive ("dominant") or less competitive ("subordinate"). Male subordinate mice that were used in the home cage aggression tests and for providing aggressive experience to subjects were group-housed, gonadectomized (GDX), and subjected to two aggressive encounters as an intruder in a resident male's home cage. Mice in the second group (dominant) were singly-housed, GDX, implanted with testosterone (GDX+T), and given two sexual experiences before testing; these males provided sexual experience to female subjects and served as stimulus animals in the three-chamber social tests.

6.2.2 Surgery

All surgeries were conducted using 1.5–3% isoflurane gas anesthesia in 100% oxygen with 3 mg/kg of carprofen given prior to surgery to reduce pain.

Gonadectomy and hormone treatment

Testes were cauterized and removed at the ductus deferens via a midline abdominal incision. SILASTIC capsules (1.5-cm active length; 1.02-mm inner diameter, 2.16-mm outer diameter; Dow Corning Corporation) were filled with crystalline T (Sigma) and inserted subcutaneously between the scapulae after gonadectomy; this procedure leads to physiologic levels of T (Barkley and Goldman, 1977; Matochik et al., 1994). To reduce aggression and promote submissive behavior in stimulus males (Beeman 1947), these mice were GDX but did not receive a T implant (GDX).

The ovaries of stimulus female mice were removed by cauterization at the uterine horn and attendant blood vessels. SILASTIC capsules (0.7-cm active length; 1.02-mm inner diameter, 2.16-mm outer diameter; Dow Corning Corporation) containing estradiol benzoate (E; diluted 1:1 with cholesterol) were implanted subcutaneously in the scapular region immediately following ovariectomy (GDX+E; Bakker et al. 2002; Ström et al. 2012). To induce sexual receptivity, stimulus females were injected subcutaneously with 0.1 mL of progesterone (500 µg dissolved in sesame oil, Sigma) 4 hours preceding sexual experience and behavioral testing (Veyrac et al. 2011).

Stereotaxic surgery and cannula implantation

Mice were positioned in a stereotaxic frame (David Kopf Instruments) with ear and incisor bars holding the skull level relative to bregma and lambda. After a midline scalp incision, a hand operated drill was used to make holes in the skull, exposing the dura. Subjects were then implanted (LHb animals bilaterally, DR animals unilaterally) with 26-gauge guide cannulas (Plastics One, Roanoke, VA) with a 1mm projection below the pedestal, fixed at a 15° angle 0.99mm anterior to bregma and 1.19mm lateral to the midline suture (LHb) or 4.3mm anterior to bregma and 0.78mm lateral to the midline suture (DR). DR injections were placed at a 15° angle to reduce the potential for V1aR antagonist crossing into the cerebral ventricle. Since crossing the ventricles was unavoidable in LHb injections, we analyzed a separate ‘miss’ group with cannula tracks ending in the lateral ventricle. Guide cannula were secured to the skull with dental cement and a skull screw, and dummy cannula of length equal to the guide cannula were inserted and screwed into place. All subjects were allowed to recover for at least 10 days prior to behavioral testing.

6.2.3 Drug injections

The highly-specific V1aR antagonist (d(CH2)5[Tyr(Me)2,Dab5] AVP; (a generous gift from M. Manning, University of Toledo)) was diluted in sterile saline to a final injected dose of 450ng/300nL and stored at −20°C until use. This antagonist, modified from the original Manning compound with the addition of diaminobutyric acid (Dab), is exceptionally selective for V1aR, eliciting no detectable anti-OT activity in vitro or in vivo (Chan et al. 1996; Manning et al. 2012); the dose selected here is effective in blocking AVP action in rodents (Lozić et al. 2016).

Forty five to sixty minutes prior to behavioral testing, subjects were briefly anesthetized (1.5–3% isoflurane gas) and a 33 gauge needle was inserted through the guide cannula, extending a total distance of 2.4 mm and 2.3 mm ventral from dura for LHb and DR animals, respectively. Subjects were then injected with 300 nL sterile saline (vehicle) or V1aR antagonist at 100nL/min (10 μL Hamilton syringe; Harvard Apparatus PHD 22/2000 syringe pump) via the guide cannula. The injection needle was left in place for one minute to allow the drug to diffuse away from the tip of the injection needle.

6.2.4 Social experience

As opposite-sex sexual experience and attaining competitive status (“social dominance”) promotes male and female communicative behaviors (Lumley et al. 1999; Roullet et al. 2011), male and female (adjusted for stage of estrous cycle; determined by vaginal lavage) mice received social experience (two sexual and two aggressive encounters on separate days) prior to testing.

Sexual experience

Subjects were given two opportunities to copulate with either a novel stimulus female (for male subjects) or a novel stimulus male (for female subjects). In the first interaction, a

sexually experienced stimulus mouse was placed in the subject's home cage overnight. In the second interaction, a novel stimulus mouse was placed in the subject's home cage and removed 5 minutes after one ejaculation or 90 minutes in the absence of ejaculation. Subjects that did not engage in any sexual behavior (mounting, intromission, or ejaculation) during the second trial were removed from further testing.

Same-sex (aggressive) experience

Male and female subjects were exposed to two daily interactions with a stimulus animal of the same sex. Male subjects received two interactions with subordinate stimulus males that had 40 µL of freshly-collected GDX+T male urine (pooled from five males) applied to their backs to provide aggression-promoting cues to otherwise non-aggressive stimulus males (Beeman 1947; Connor and Winston 1972; Van Loo et al. 2001).

Stimulus males were placed in the subject's home cage and removed after the subject's first offensive attack (lunge with bite) within a 10 minute period. All subject males attacked the intruder male stimulus by the second encounter, and all subordinate stimulus males displayed submissive behavior, defined by defensive postures (e.g., on-back), fleeing, and non-social exploration (Koolhaas et al. 2013). Female subjects received two interactions with a group housed GDX stimulus female in their home cage over a 10 minute period. Female encounters rarely resulted in aggressive responses from either animal; however, if any aggressive behavior, though limited, occurred, the female was removed after the first offensive attack, much like following male-male interactions.

6.2.5 Experimental design

All testing occurred within the first 6 hours of the dark cycle under red light illumination. Subjects were habituated to the testing room and apparatus by handling and placing mice for 5

minutes in the three-chamber apparatus (see below) once a day for 3 days. On experimental days, subjects were adapted to the experimental room for 15 minutes before testing. All tests were scored by an experimenter blind to the drug treatment of the subject.

Subjects received a total of five injections of V1aR antagonist/vehicle across four tests for social communication in the three-chamber apparatus (two antagonist and two vehicle injections) and during one final test (antagonist or vehicle injection) within the subject's home cage to measure any aggression during same-sex interactions. Each test was separated by at least three days. The order of treatment (saline, V1aR antagonist) and stimulus condition (male, female) was counterbalanced across subjects, except that subjects exposed to a stimulus type on the first test were then given that same stimulus type on the second test (Figure 6-1). Female subjects were tested irrespective of estrous cycle day; prior research indicates minimal effects of estrous cycle on female mouse communicative behavior (Maggio and Whitney 1985; Coquelin 1992; Moncho-Bogani et al. 2004). Following the final test, subjects were euthanized and injected through guide cannula with dilute India ink.

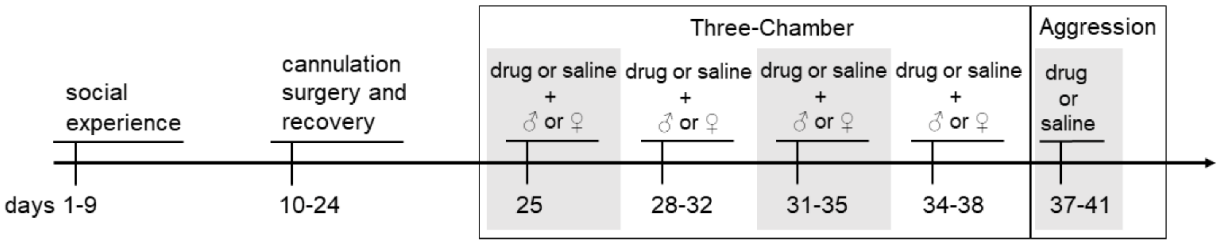

Figure 6-1- Experimental timeline.

6.2.6 Social communication and behavior

Ultrasonic vocalizations (USV), urine marking, and social investigation by subjects was recorded in an acrylic three-chamber apparatus (Crawley 2007; Arakawa et al. 2008; Moy et al.

2009; Harvard Apparatus; dimensions: 20.3 x 42 x 22 cm). Instead of a solid floor, the apparatus was placed on absorbent paper (Nalgene Versi-dry paper, Thermo Fisher Scientific) so as to retain and accurately measure urine marking. Animals were tracked using motion detection software (ANY-maze, San Diego Instruments, RRID:SCR_014289). During testing with stimulus animals, subjects had access to a stimulus animal in a cage (8 cm (D), 18 cm (H); 3-mm diameter steel bars, 7.4 mm spacing) and an empty "clean" cage placed at opposite corners of the outermost chambers of the apparatus. The location of stimulus and clean cages was counterbalanced across animals. After placing the subject in the center of the middle chamber, we measured investigation of clean and stimulus cages, distance traveled throughout the apparatus, time spent in the chambers containing stimulus and clean cages as well as USVs and urine marking across a 5 minute trial. After testing, the apparatus and cages were thoroughly cleaned with 70% ethanol and allowed to dry before further testing.

Social investigation and USVs

Social investigation was defined as time spent sniffing within 2 cm of the stimulus or clean cage; climbing on the cage was not scored as investigation. USVs of subject and stimulus interactions were detected using a condenser microphone connected to an amplifier (UltraSoundGate CM16/CMPA, 10 –200 kHz, frequency range) placed 4 cm inside the apparatus and directly above the center compartment. USVs were sampled at 200 kHz (16-bit) with target frequency set to 70 kHz (UltraSoundGate 116Hb, Avisoft Bioacoustics). Recordings were then analyzed using a MATLAB (MathWorks, RRID:SCR_001622) plug-in that automates USV analysis (Van Segbroeck et al. 2017). Using this program, sonograms were generated by calculating the power spectrum on Hamming windowed data and then transformed into compact

acoustic feature representations (Gammatone Filterbank). Each 200-ms window containing the maximum USV syllable duration was then clustered, via machine learning algorithms, into USV syllable types (repertoire units) based on time-frequency USV shape. Repertoire units that appeared as background noise were discarded. Both the number of all USV produced in each condition as well as the number of USV syllable types (using criteria previously described: short, composite, downward, upward, 1 frequency jump, modulated, multiple frequency jumps, u-shape, flat, chevron (Hanson and Hurley 2012)) were counted.

Urine marking

Following testing, the substrate sheet was allowed to dry for 1 hour and then sprayed with ninhydrin fixative (LC-NIN-16; Tritech Forensics Inc.) to visualize urine marks (Meyer 1957; Lehmann et al. 2013). After 24 hours, sheets were imaged (Sony DSC-S700 camera), binarized and analyzed using a computer-aided imaging software (ImageJ, RRID:SCR_003070). Urine marking was measured as both as the total area (cm2) covered and the total count of individual marks throughout the entire arena. Urine spots that were larger than 6 cm2 and directed toward corners were counted as eliminative “pools” and were excluded from analysis (Bishop and Chevins 1987).

Same-sex interactions and aggressive behavior

To measure territorial aggression, a subordinate male stimulus animal with applied urine (see above) was placed in a male subject’s home cage and then removed after the subject’s first offensive attack (lunge with bite) within a 10 minute period; the latency to first bite was recorded. Each female subject was presented with a GDX, group housed, female stimulus animal within the subject’s home cage for a 10 minute period. Though rare, if an offensive attack occurred, the stimulus animal was removed, much like following male-male interactions.

6.2.7 Tissue collection and histological analysis

Animals were euthanized by intraperitoneal injection of Beuthanasia-D (150 mg/kg) and subsequently injected through guide cannula with 300nL of dilute India ink. Brains were extracted and flash frozen on dry ice and stored at -80 °C until further processing. Coronal sections were cut at a thickness of 30 µm with a cryostat (Leica CM3050 S, Leica Biosystems) and analyzed for injection placement with reference to anatomical landmarks (ventricles, fiber tracts) and plotted on anatomical plates (Paxinos and Franklin 2012).

6.2.8 Statistical Analysis

All data were analyzed and graphed in R (3.4.4; R Core Team, 2017). Social investigation, movement, USV, and urine marking data met the assumptions of parametric statistical tests. Therefore, we analyzed data on social investigation, movement (distance traveled, time in chambers containing stimulus and clean cages), number of USVs, and urine marking with mixed-model ANOVAs [between-subject factor: sex; within-subject factors: treatment (V1aR antagonist, vehicle), sex of stimulus (male, female)] followed by paired t tests assessing treatment effects. The number of specific USV syllables and aggressive behavior (latency to bite) were not normally distributed and could not be transformed, therefore, we analyzed treatment effects using pairwise Mann–Whitney U tests. All post hoc pairwise comparisons report Bonferroni-corrected p values and Cohen's d for effect size when statistically significant. Results were considered statistically significant if $p < 0.05$.

6.3 Results

6.3.1 Histology

Subjects were included in the main LHb/DR analysis if the tip of the injection cannula was located within LHb bilaterally (10 males, 9 females; Fig. 6-2a) or the DR (10 males, 8

females; Fig. 2b). Eleven LHb targeted subjects (6 males, 5 females) were analyzed in a separate 'miss' group due to placement of injection needle tip outside the LHb, including subjects with only unilateral LHb cannulation (Fig. 6-2a, Table 4). Similarly, eight DR targeted subjects (males = 4, females = 4) were analyzed in a separate 'miss' group due to injection needle placement within the periaqueductal gray (PAG) (Fig. 6-2b, Table 4).

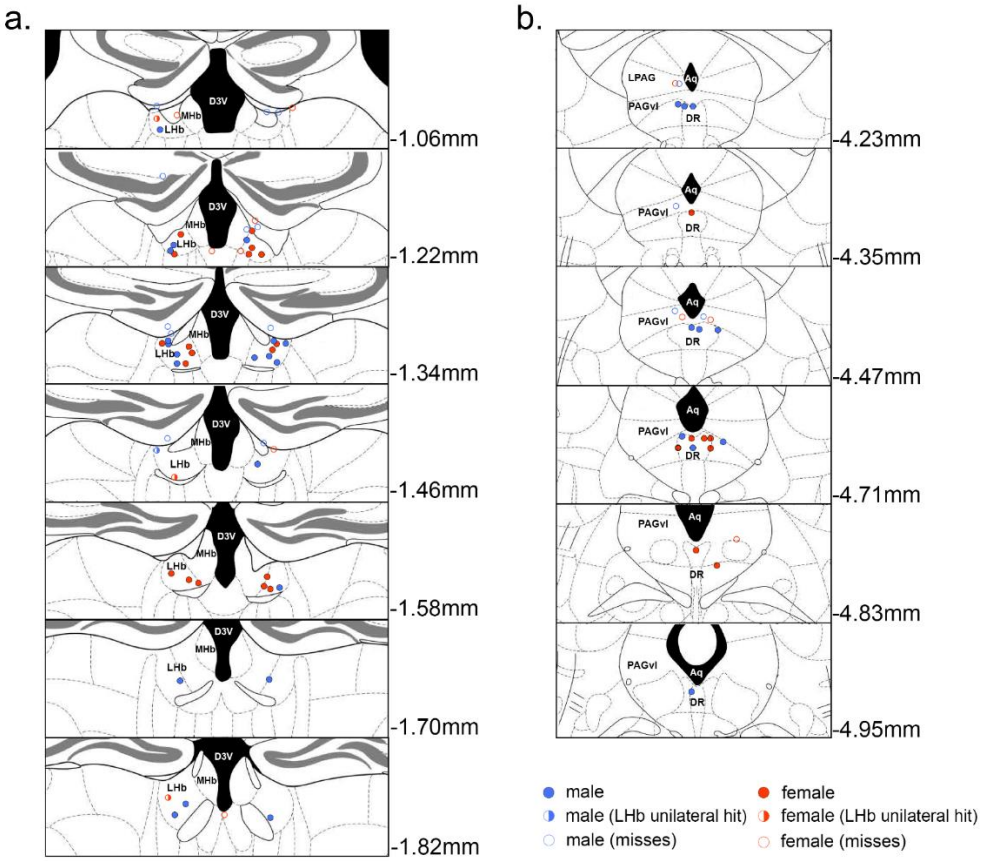

Figure 6-2– Histological identification of injection sites. *Coronal sections through the rostral-caudal extent of the mouse brain (relative to bregma) referenced to Paxinos and Franklin (2012). a. Lateral habenula (LHb) targeted subjects. Blue dots represent male subjects with bilateral injections into LHb ('hits', n=10), half-filled blue dots represent a subject with a unilateral LHb injection ('unilateral hit', n=1), and open blue dots represent bilateral injections outside of LHb ('bilateral misses', n=5). Orange dots represent female subjects with bilateral injections into LHb ('hits', n=9), half-filled orange dots represent a subject with a unilateral LHb injection ('unilateral hit', n=3), and open orange dots represent bilateral injections outside of LHb ('bilateral misses', n=5). b. Dorsal raphe (DR) targeted subjects. Blue dots represent male subjects with injections into DR ('hits', n=10) and open blue dots represent injections*

outside of DR ('misses', n=4). Orange dots represent female subjects with injections into DR ('hits', n=8) and open orange dots represent injections outside of DR ('misses', n=4).

Table 4 - Table of median (interquartile range) of urine mark (UM) counts, UM area (in pixels), USV counts, and social investigation (SI, seconds) produced by LHb 'miss' and DR 'miss' subjects. Male LHb 'miss' subjects (n=6) reduced the number of UM (counts, p=0.007) to female stimuli following infusion of V1aR antagonist compared to vehicle injections. Male DR 'miss' subjects (n=4) reduced the number of UM (counts, p=0.03) to male stimuli following infusion of V1aR antagonist compared to vehicle injections. Bold numbers represent significant differences between treatment groups.

	male subjects				female subjects			
treatment	vehicle		V1aR antagonist		vehicle		V1aR antagonist	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
LHb 'miss' subjects								
UM (count)	**70 (30-118)**	49 (31-134)	**(p=0.007) 14 (4-85)**	53 (0-112)	0 (0-0)	0 (0-16)	0 (0-112)	0 (0-0)
UM (pixels)	7640 (3560-19277)	19871 (536-28798)	12042 (851-35031)	8934 (0-60452)	0 (0-0)	0 (0-3888)	0 (0-323)	0 (0-0)
USV (count)	463 (187-848)	24 (9-132)	400 (7-1256)	23 (16-58)	16 (7-70)	24 (17-25)	257 (1-398)	16 (14-21)
SI (s)	37 (25-64)	23 (13-39)	56 (26-91)	16 (11-40)	20 (17-26)	8 (5-113)	18 (12-33)	13 (5-118)
DR 'miss' subjects								
UM (count)	83 (34-118)	**101 (31-136)**	30 (0-179)	**(p=0.03) 28 (0-57)**	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-7)
UM (pixels)	24044 (12388-62580)	18865 (5568-32421)	9509 (0-23348)	4481 (0-8962)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1975)
USV (count)	25 (11-155)	1 (0-2)	68 (4-461)	4 (1-14)	5 (2-12)	30 (1-49)	7 (3-17)	8 (4-17)
SI (s)	45 (17-89)	24 (6-36)	39 (23-66)	27 (3-30)	26 (20-34)	18 (11-25)	24 (23-26)	8 (7-19)

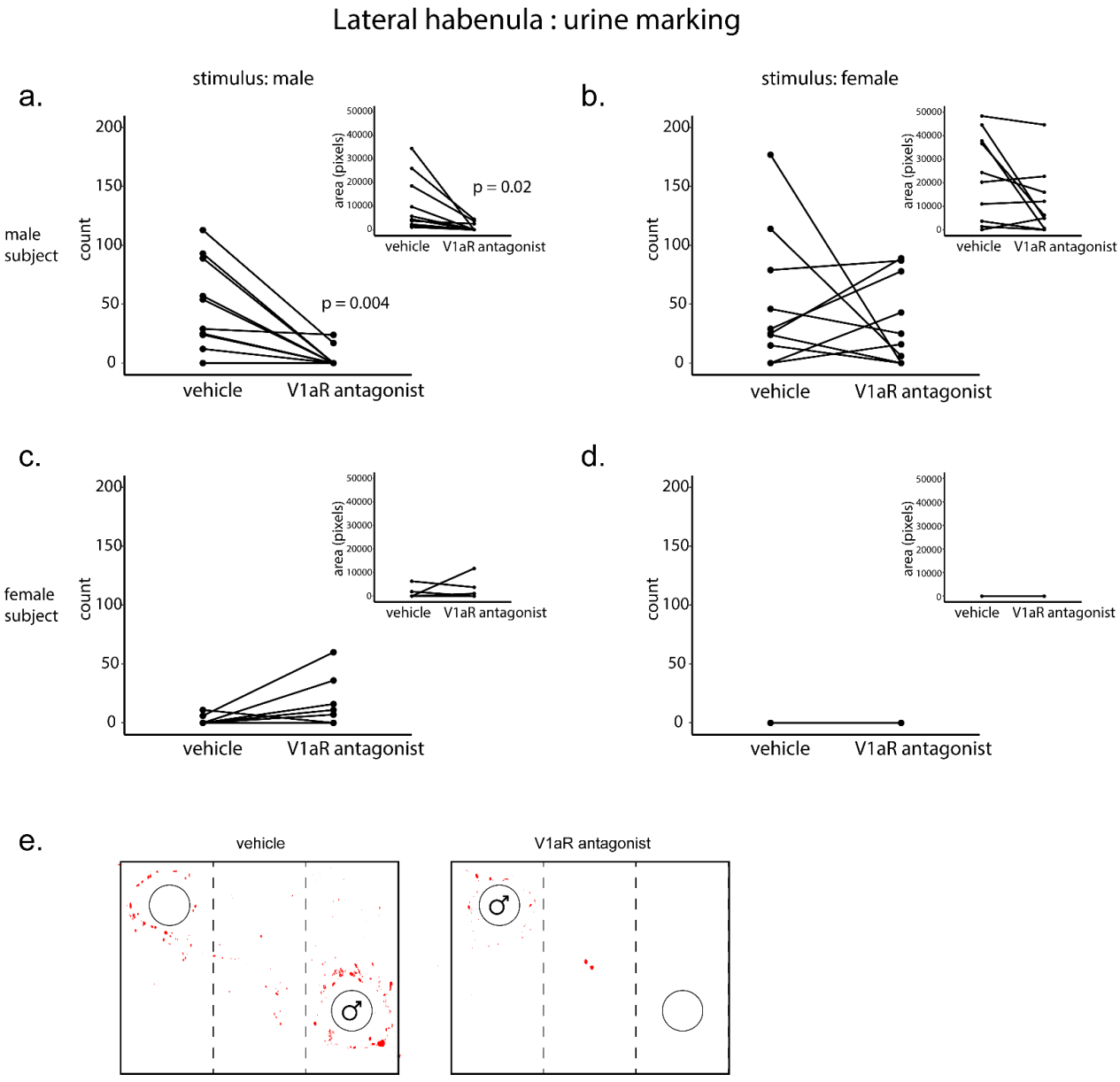

Figure 6-3– Effects of V1aR blockade in the lateral habenula (LHb) on urine marking in the presence of a confined male (a, c) or female (b, d) within a three-chamber apparatus. *Urine marking was evaluated following infusion of V1aR antagonist (450ng/300nL) or vehicle (saline), counterbalanced. a-b. Male subjects (n=10) reduced the number of urine marks (counts, p=0.004) as well as area covered by urine marking (pixels, inset; p = 0.02) to (a) male, but not (b) female, stimuli following infusion of V1aR antagonist compared to vehicle injections. c-d. Female subjects (n=9) did not alter the number of urine marks or area covered by urine marking (inset) to male (c) or female (d) stimuli following infusion of V1aR antagonist compared to vehicle injections. e. Example images of reduced urine marking by a male subject (toward a male stimulus) following V1aR antagonist or vehicle injections. Each point and horizonal line represents individual within-subject data. Overlapping data are represented as one point/line.*

V1aR antagonism within lateral habenula

6.3.2 LHb V1aR antagonism decreased male urine marking to males

Male subjects produced more urine marks than female subjects ($F(1,17) = 21.25$, $p = 0.0003$) with an interaction between sex of subject and treatment ($F(1,17) = 5.37$, $p = 0.03$) and a trend toward an interaction between sex of subject and sex of stimulus ($F(1,17) = 4.0$, $p = 0.06$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased urine marks to male stimuli compared to when given vehicle (saline) injections ($t(9) = 3.84$, $p = 0.004$, $d = 1.22$; Figure 6-3a); this effect was not observed in response to female stimuli ($t(9) = 0.70$, $p = 0.50$; Figure 6-3b). Injections of V1aR antagonist in female subjects did not alter the number of urine marks to male stimuli ($t(8) = 1.84$, $p = 0.10$; Figure 6-3c) or female stimuli (no marking; Figure 6-3d) compared to vehicle injections.

Male subject's urine marking covered more area than female subjects ($F(1,17) = 14.63$, $p = 0.001$) with interactions between sex of subject and sex of stimulus ($F(1,17) = 7.41$, $p = 0.01$) and sex of subject and treatment ($F(1,17) = 6.37$, $p = 0.02$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased urine marking area to male stimuli compared to when given vehicle injections ($t(9) = 2.79$, $p = 0.02$, $d = 0.9$; Figure 6-3a inset); this effect was not observed in response to female stimuli ($t(9) = 2.13$, $p = 0.06$; Figure 6-3b inset). Injections of V1aR antagonist in the LHb of female subjects did not alter the urine marking area in the presence of male stimuli ($t(8) = 0.83$, $p = 0.43$; Figure 6-3c inset) or female stimuli (no marking; Figure 6-3d inset).

6.3.3 LHb V1aR antagonism decreased male ultrasonic vocalizations to females

There was no overall difference in USV production by sex of subject ($F(1,17) = 0.13$, $p = 0.73$; but there was an interaction between sex of subject and treatment ($F(1,17) = 6.57$, $p = 0.02$) and sex of stimulus and treatment ($F(1,17) = 5.42$, $p = 0.03$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased USVs to female stimuli compared to when given vehicle injection ($t(9) = 3.18$, $p = 0.01$, $d = 0.99$; Figure 6-4b); this effect was not observed in response to male stimuli ($t(9) = 0.67$), $p = 0.52$, Figure 6-4a). As expected, males that received vehicle injections produced more USVs to female stimuli than to male stimuli ($t(9) = 2.59$, $p = 0.029$; however, when males received the V1aR antagonist, this difference disappeared ($t(9) = 1.26$, $p = 0.24$). V1aR injections into LHb of females did not change USV production in the presence of male stimuli ($t(8) = 0.68$, $p = 0.52$, Figure 6-4c) or female stimuli ($t(8) = 1.1$, $p = 0.30$, Figure 6-4d) compared to vehicle treatment. Additionally, V1aR antagonism did not change the distribution of USV syllable types in either males or females (Table 5).

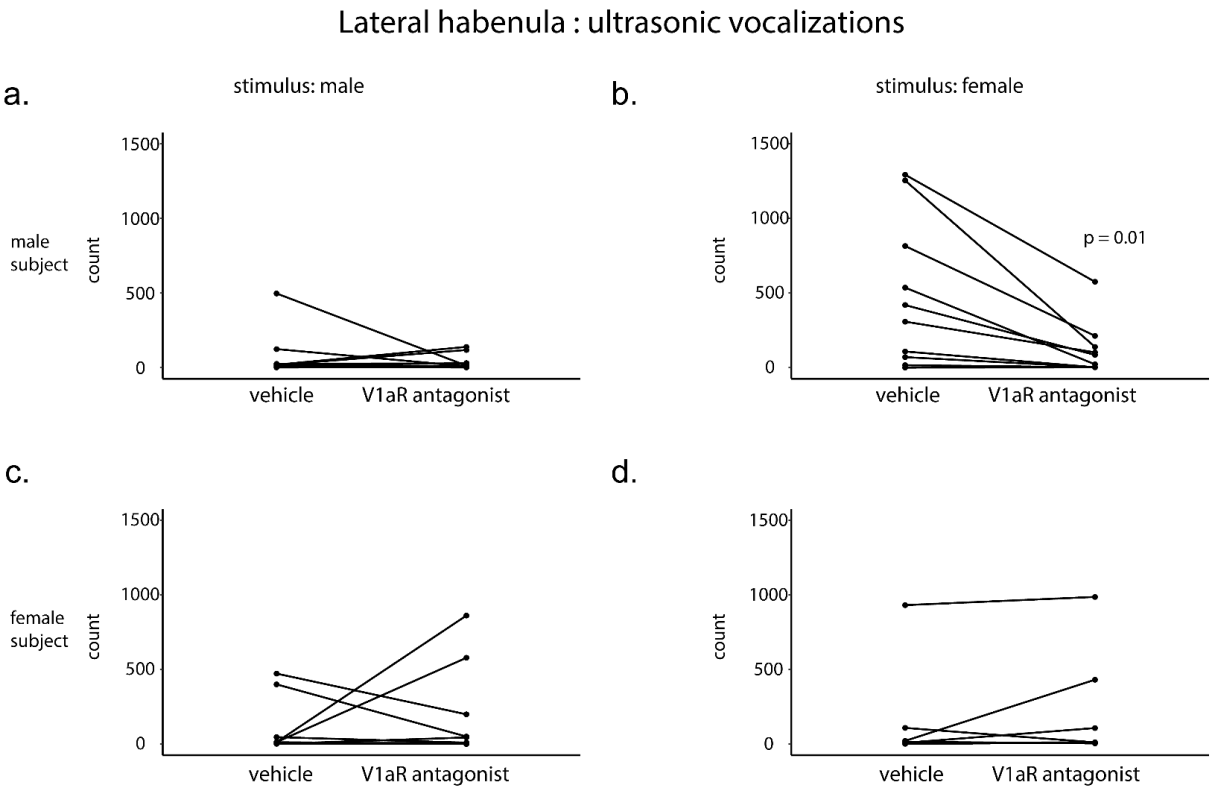

Figure 6-4– Effects of V1aR blockade in the lateral habenula (LHb) on USVs in the presence of a confined male (a, c) or female (b, d) within a three-chamber apparatus. *USVs were evaluated following infusion of V1aR antagonist (450ng/300nL) or vehicle (saline), counterbalanced. a-b. Male subjects (n=10) did not alter USVs to (a) male stimuli, but did decrease USVs to (b) female stimuli (p=0.01), following infusion of V1aR antagonist into LHb compared to vehicle injections. c-d. Female subjects (n=9) did not alter USVs to male (c) or female (d) stimuli following infusion of V1aR antagonist compared to vehicle injections. Each point and horizonal line represents individual within-subject data. Overlapping data are represented as one point/line.*

Table 5 - Table of median (interquartile range) counts of different USV syllable types in male and female subjects. V1aR antagonism in the LHb did not change the composition of USV syllable types produced compared to vehicle treatment.

	male subjects				female subjects			
treatment	vehicle		V1aR antagonist		vehicle		V1aR	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
short	17 (0-44)	22 (0-56)	24 (0-49)	32 (0-72)	13 (0-38)	13 (0-38)	11 (0-37)	10 (0-32)
down	6 (0-25)	5 (0-23)	6 (0-27)	2 (0-13)	0 (0-13)	0 (0-10)	1 (0-13)	0 (0-9)
upward	22 (0-54)	5 (0-40)	23 (0-50)	3 (0-35)	0 (0-29)	0 (0-38)	8 (0-46)	0 (0-40)
flat	7 (0-33)	20 (0-45)	7 (0-27)	9 (0-50)	25 (7-100)	28 (0-100)	8 (0-100)	27 (0-100)
modulated	7 (0-15)	0 (0-8)	4 (0-18)	3 (0-14)	0 (0-25)	2 (0-8)	4 (0-13)	4 (0-15)
u shape	7 (0-15)	9 (0-19)	5 (0-13)	3 (0-23)	4 (0-25)	0 (0-8)	5 (0-42)	0 (0-8)
chevron	5 (0-10)	0 (0-8)	3 (0-15)	0 (0-5)	0 (0-25)	0 (0-29)	4 (0-26)	4 (0-29)
one frequency jump	12 (0-23)	12 (0-28)	9 (0-18)	3 (0-18)	0 (0-20)	3 (0-20)	3 (0-20)	0 (0-16)
composite	3 (0-10)	2 (0-14)	2 (0-6)	2 (0-9)	0 (0-14)	0 (0-2)	0 (0-7)	0 (0-4)

6.3.4 LHb V1aR antagonism did not alter social investigation in males or females

Subjects spent more time investigating female stimuli than male stimuli ($F(1,17) = 25.56$, $p = 0.001$), regardless of treatment ($F(1,17) = 0.01$, $p = 0.92$) with no interaction between sex of subject and treatment ($F(1,17) = 0.05$, $p = 0.82$), or sex of stimulus and treatment ($F(1,17) < 0.01$, $p = 0.99$; Fig. 6-5a-d).

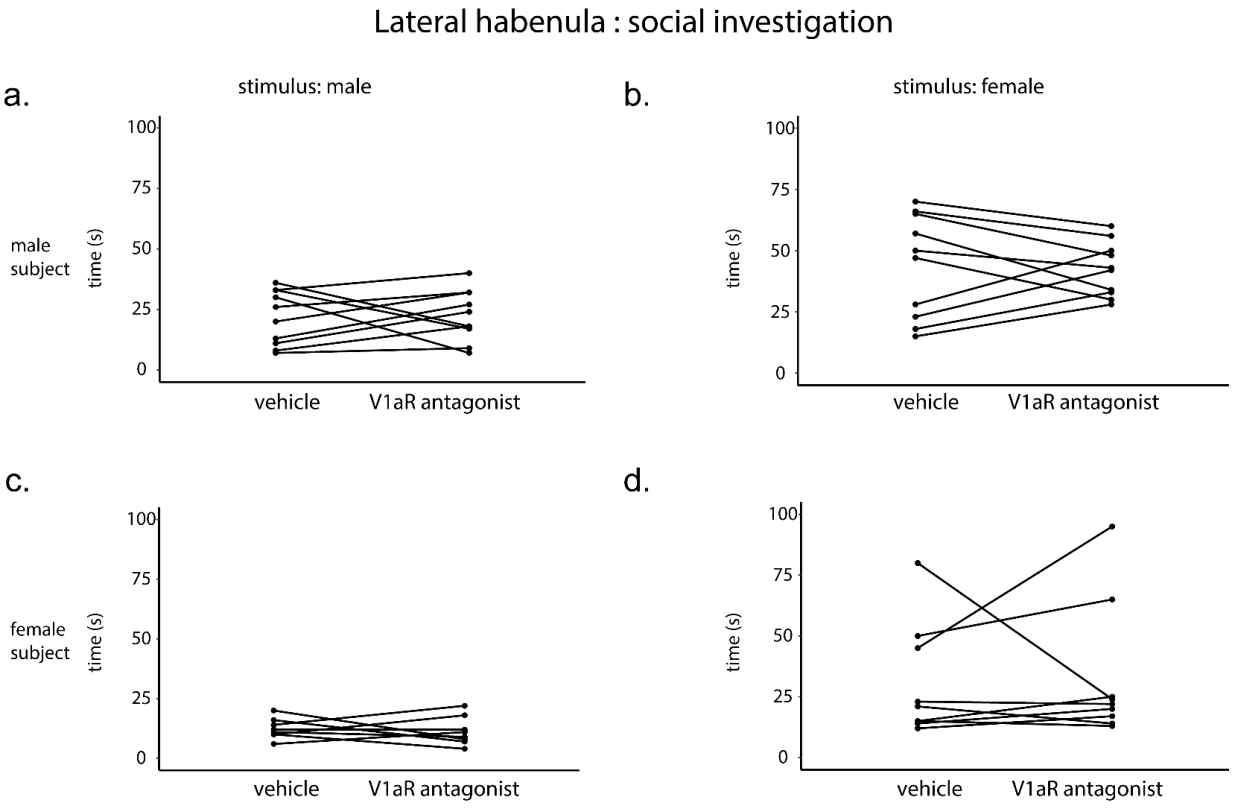

Figure 6-5- Effects of V1aR blockade in the lateral habenula (LHb) on urine marking in the presence of a confined male (a, c) or female (b, d) within a three-chamber apparatus. *Social investigation was evaluated following infusion of V1aR antagonist (450ng/300nL) or vehicle (saline), counterbalanced.* ***a-b.*** *Male subjects (n=10) did not alter social investigation to male* ***(a)*** *or female* ***(b)*** *stimuli following infusion of V1aR antagonist compared to vehicle injections.* ***c-d.*** *Female subjects (n=9) did not alter social investigation to male* ***(c)*** *or female* ***(d)*** *stimuli following infusion of V1aR antagonist compared to vehicle injections. Each point and horizonal line represents individual within-subject data. Overlapping data are represented as one point/line.*

6.3.5 V1aR antagonism in LHb did not alter the amount or spatial distribution of activity

Overall, subjects in both treatment conditions traveled similar distances throughout the three-chamber apparatus (F(1,17) = 3.56 p = 0.08) with no evident differences between treatment conditions in time spent in the stimulus chambers (F(1,17) = 0.96, p = 0.34) but with an interaction between treatment and sex in time spent in the clean chamber (F(1,17) = 6.43, p = 0.02). Post hoc comparisons revealed that males receiving V1aR antagonist spent more time in the clean chamber zone in presence of female stimuli compared to when given vehicle injections (t(9) = 2.85, p = 0.01, d = 0.9; Table 2); this effect was not observed in response to male stimuli (t(9) = 0.65, p = 0.53; Table 2) or in female subjects (to male stimuli: (t(8) = 1.65, p = 0.14; to female stimuli: (t(8) = 1.65, p = 0.14; Table 6)).

Table 6 - Table of median (interquartile range) distance traveled (meters) and time spent (seconds) in stimulus or clean cage chamber. Subjects with V1aR antagonist infused into the LHb did not differ in distance traveled or time spent in stimulus chambers compared to when injected with vehicle (saline). However, males receiving V1aR antagonist spent more time in clean chambers during tests with female stimuli compared to when injected with vehicle. Bold numbers represent significant differences between treatment groups.

	male subjects				female subjects			
treatment	vehicle		V1aR antagonist		vehicle		V1aR antagonist	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
distance traveled (m)	0.56 (0.3-0.74)	0.5 (0.4-0.66)	0.57 (0.34-0.73)	0.5 (0.39-0.83)	0.52 (0.34-0.83)	0.52 (0.34-0.83)	0.68 (0.54-0.86)	0.68 (0.54-0.86)
time in stimulus chamber (s)	112 (52-199)	101 (15-138)	174 (62-216)	96 (13-143)	151 (54-188)	151 (54-188)	140 (112-160)	140 (112-160)
time in clean chamber (s)	111 (70-186)	115 (49-193)	**p=0.02 82 (21-189)**	146 (99-222)	72 (27-117)	73 (27-116)	92 (70-111)	93 (70-111)

V1aR antagonism within dorsal raphe

6.3.6 DR V1aR antagonism decreased male urine marking to males

Male subjects produced more urine marks than female subjects ($F(1,16) = 15.02$, $p = 0.001$) with a trend toward an interaction between sex of stimulus and treatment ($F(1,16) = 4.4$, $p = 0.052$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased urine marks to male stimuli compared to when given vehicle injections ($t(9) = 3.0$, $p = 0.015$, $d = 0.95$; Figure 6-6a); this effect was not observed in response to female stimuli ($t(9) = 0.5$, $p = 0.63$; Figure 6-6b). Injections of V1aR antagonist in the DR of female subjects did not alter the number of urine marks in the presence of male stimuli ($t(7) = 1.02$, $p = 0.92$; Figure 6-6c) or female stimuli ($t(7) = 1.54$, $p = 0.17$; Figure 6-6d) compared to vehicle injections.

Male subject's urine marking covered more area than female subjects ($F(1,16) = 13.61$, $p = 0.002$) with an interaction between sex of stimulus and treatment ($F(1,16) = 9.99$, $p = 0.006$) and sex of subject and sex of stimulus and treatment ($F(1,16) = 6.69$, $p = 0.02$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased urine marking area to male stimuli compared to when given vehicle injections ($t(9) = 2.57$, $p = 0.03$, $d = 0.81$; Figure 6-6a inset); this effect was not observed in response to female stimuli ($t(9) = 1.15$, $p = 0.28$; Figure 6-6b inset). Injections of V1aR antagonist in the DR of female subjects did not alter the urine marking area in the presence of male stimuli ($t(7) = 0.98$, $p = 0.36$; Figure 6-6c inset) or female stimuli ($t(7) = 1.11$, $p = 0.3$; Figure 6-6d inset).

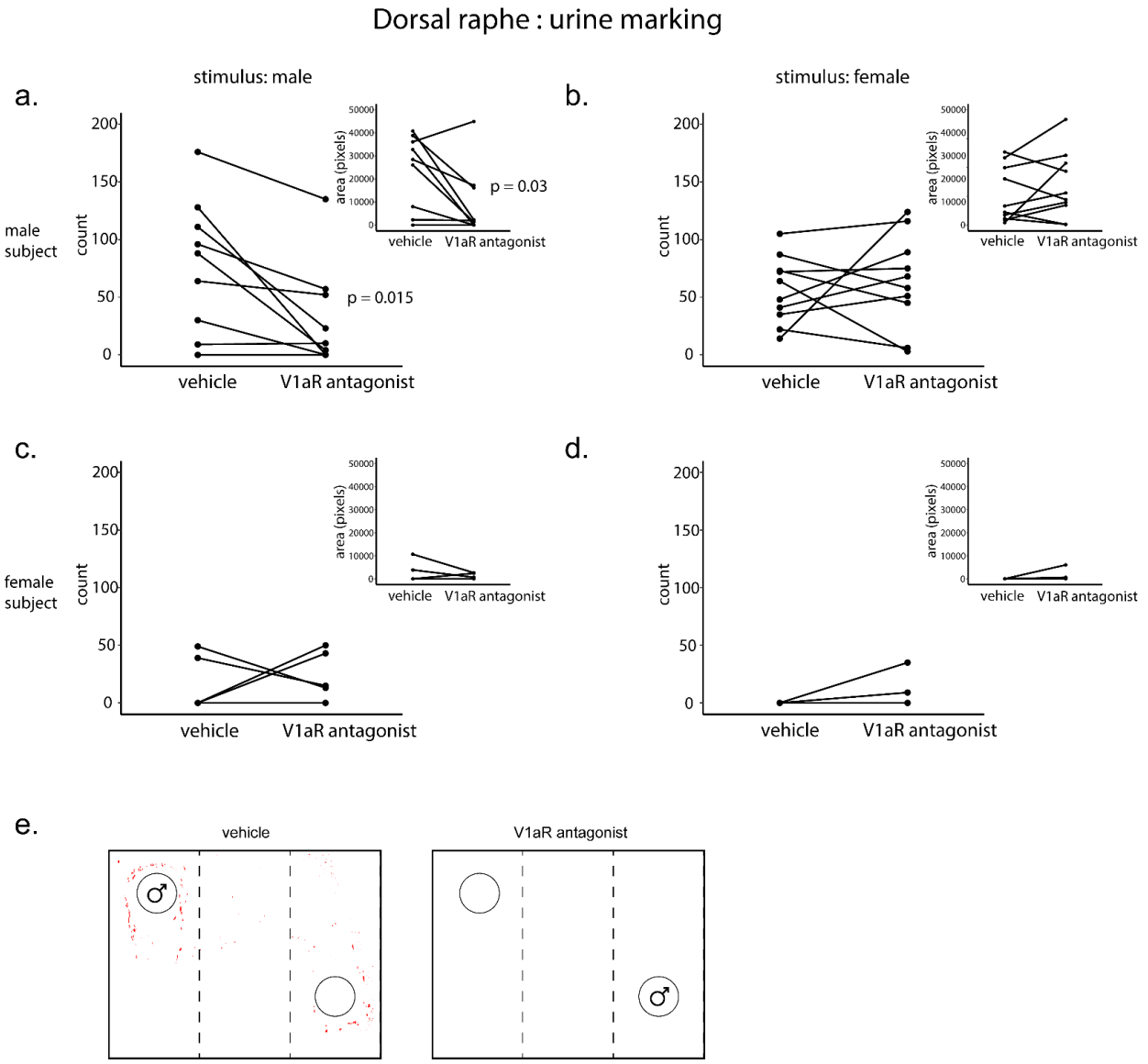

Figure 6-6- Effects of V1aR blockade in the dorsal raphe (DR) on urine marking in the presence of a confined male (a, c) or female (b, d) within a three-chamber apparatus. *Urine marking was evaluated following infusion of V1aR antagonist (450ng/300nL) or vehicle (saline), counterbalanced. a-b. Male subjects (n=10) reduced the number of urine marks (counts, p=0.015) as well as area covered by urine marking (pixels, inset; p = 0.03) to (a) male, but not (b) female, stimuli following infusion of V1aR antagonist compared to vehicle injections. c-d. Female subjects (n=8) did not alter the number of urine marks (counts) or area covered by urine marking (pixels, inset) to male (c) or female (d) stimuli following infusion of V1aR antagonist compared to vehicle injections. e. Example images of reduced urine marking by a male subject (toward a male stimulus) following V1aR antagonist or vehicle injections. Each point and horizonal line represents individual within-subject data. Overlapping data are represented as one point/line.*

6.3.7 DR V1aR antagonism did not alter USVs in males or females

V1aR antagonism within DR did not change the number of USVs emitted in either female or male stimulus conditions (treatment: $F(1,16) = 0.1$, $p=0.73$; sex: $F(1,16) = 4.07$, $p = 0.06$; Figure 6-7a-d). Additionally, injections of V1aR antagonist did not change the distribution of USV syllable types produced in these conditions (Table 3).

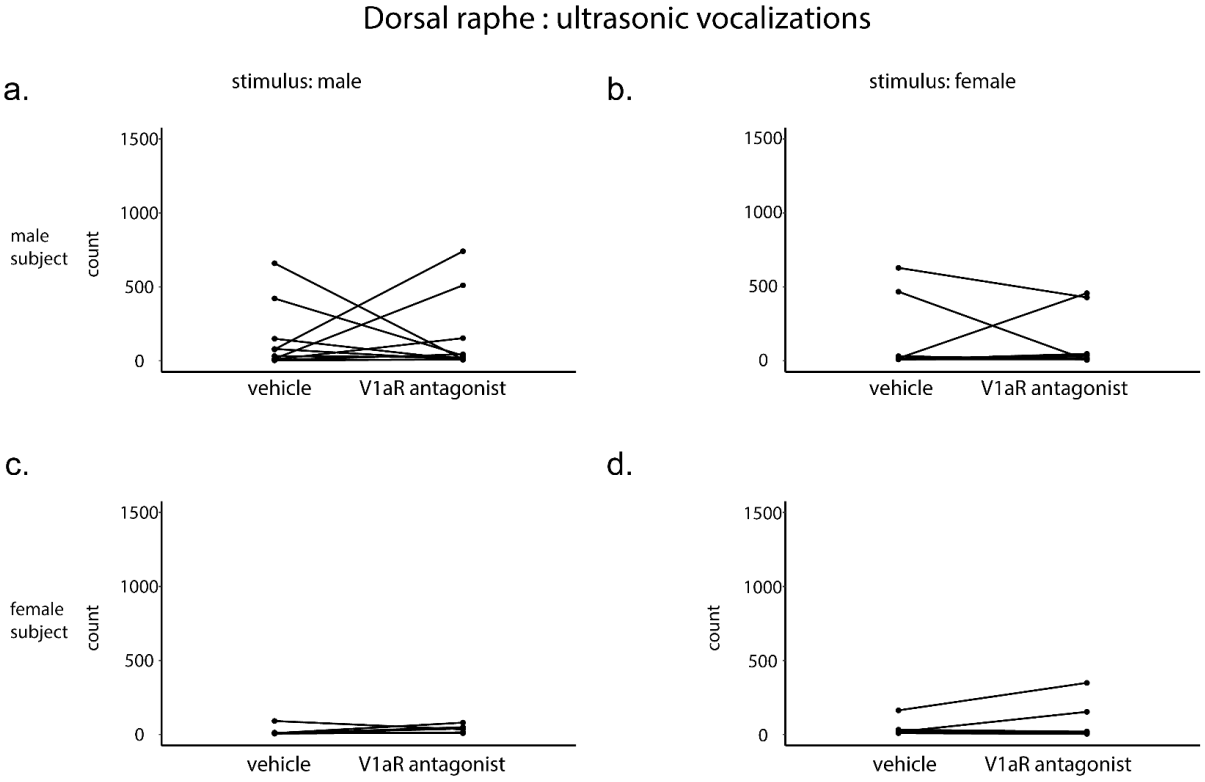

Figure 6-7- Effects of V1aR blockade in the dorsal raphe (DR) on USVs in the presence of a confined male (a, c) or female (b, d) within a three-chamber apparatus. *USVs were evaluated following infusion of V1aR antagonist (450ng/300nL) or vehicle (saline), counterbalanced.* ***a-b.*** *Male subjects (n=10) did not alter USVs to male* ***(a)*** *or female* ***(b)*** *stimuli following infusion of V1aR antagonist compared to vehicle injections.* ***c-d.*** *Female subjects (n=8) did not alter USVs to male* ***(c)*** *or female* ***(d)*** *stimuli following infusion of V1aR antagonist compared to vehicle injections. Each point and horizonal line represents individual within-subject data. Overlapping data are represented as one point/line.*

Table 7 - *Table of median (interquartile range) number of different USV syllable types in male and female subjects. V1aR antagonism in the DR did not change the distribution of USV syllable types produced compared to vehicle treatment.*

	male subjects		female subjects					
treatment	vehicle antagonist		V1aR antagonist		vehicle		V1aR	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
short	19 (0-56)	33 (0-73)	17 (0-67)	35 (0-78)	13 (0-29)	13 (0-22)	15 (0-100)	25 (3-89)
down	10 (0-33)	6 (0-14)	9 (0-21)	0 (0-9)	0 (0-25)	0 (0-11)	0 (0-11)	0 (0-6)
upward	24 (0-49)	9 (0-25)	25 (0-63)	4 (0-36)	10 (0-42)	0 (0-40)	0 (0-32)	6 (0-38)
flat	6 (0-45)	25 (3-51)	14(7-33)	18 (0-75)	47 (0-63)	56 (0-91)	39 (0-100)	25 (0-68)
modulated	1 (0-11)	0 (0-11)	0 (0-12)	0 (0-0)	0 (0-17)	0 (0-11)	0 (0-8)	0 (0-17)
u shape	0 (0-14)	0 (0-17)	0 (0-8)	0 (0-5)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
chevron	0 (0-5)	0 (0-4)	0 (0-4)	0 (0-0)	0 (0-0)	0 (0-18)	0 (0-0)	0 (0-18)
one frequency jump	1 (0-40)	0 (0-29)	0 (0-29)	0 (0-14)	24 (0-67)	2 (0-20)	11 (0-31)	8 (0-21)
composite	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)

6.3.8 DR V1aR antagonism did not alter social investigation in males or females

Subjects spent more time investigating female stimuli than male stimuli ($F(1,16) = 15.82$, $p = 0.001$), regardless of treatment ($F(1,16) = 3.01$, $p = 0.10$); there was no interaction between sex of subject and treatment ($F(1,16) = 0.07$, $p = 0.79$), or sex of stimulus and treatment ($F(1,16) = 0.04$, $p = 0.85$; Figure 6-8a-d).

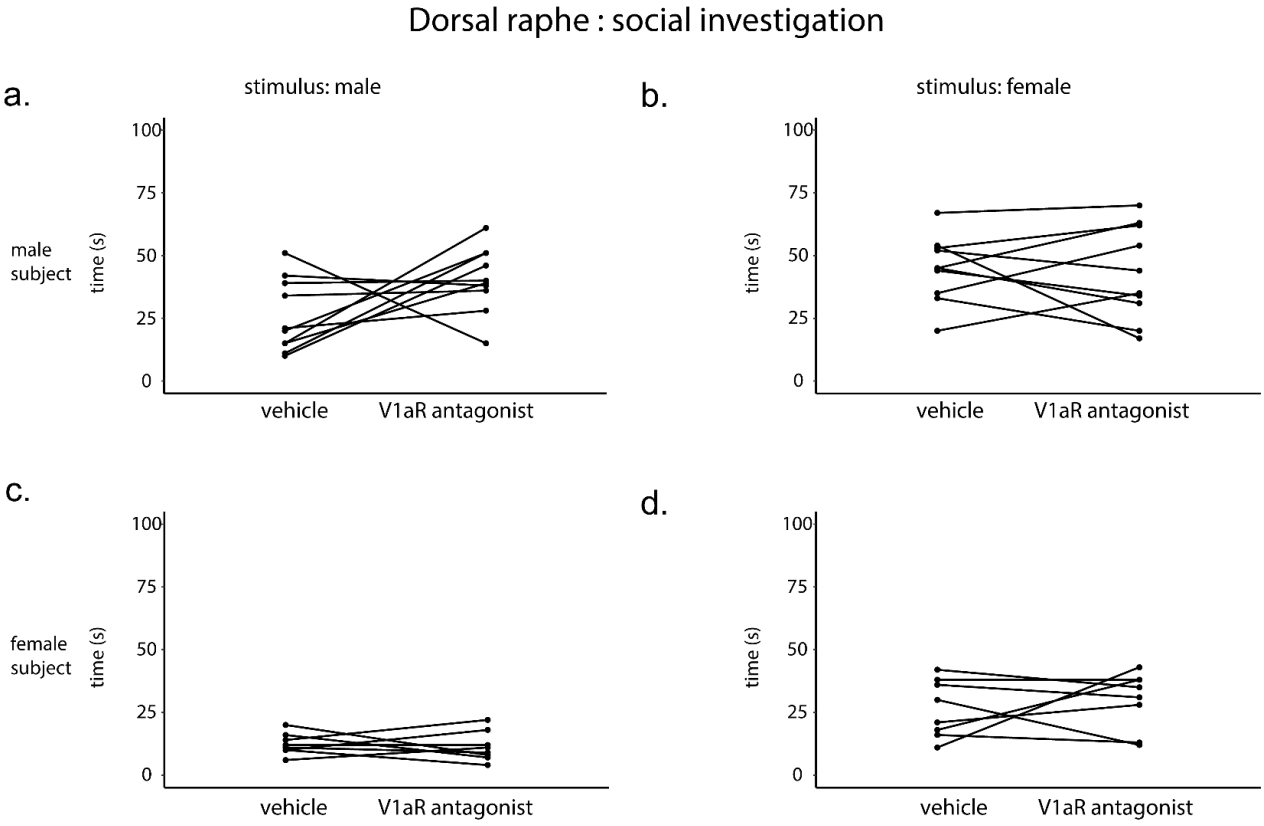

Figure 6-8- Effects of V1aR blockade in the dorsal raphe (DR) on social investigation in the presence of a confined male (a, c) or female (b, d) within a three-chamber apparatus. *Social investigation was evaluated following infusion of V1aR antagonist (450ng/300nL) or vehicle (saline), counterbalanced.* ***a-b.*** *Male subjects (n=10) did not alter social investigation to male* ***(a)*** *or female* ***(b)*** *stimuli following infusion of V1aR antagonist compared to vehicle injections.* ***c-d.*** *Female subjects (n=8) did not alter social investigation to male* ***(c)*** *or female* ***(d)*** *stimuli following infusion of V1aR antagonist compared to vehicle injections. Each point and horizonal line represents individual within-subject data. Overlapping data are represented as one point/line.*

6.3.9 V1aR antagonism in DR did not alter spatial activity

Overall, subjects in both treatment groups traveled similar distances throughout the three-chamber apparatus ($F(1,16) = 0.01$ $p = 0.12$); there were no differences between treatment groups in the amount of time subjects spent in the stimulus chamber zones ($F(1,16) = 0.36$, $p = 0.56$) or clean chamber zones ($F(1,16) = 0.74$, $p = 0.40$; Table 4).

Table 8 *- Table of median (interquartile range) distance traveled (meters) and time spent (seconds) in stimulus or clean cage chamber. Subjects with V1aR antagonist infused into the DR did not differ in distance traveled, time spent in stimulus or clean chambers compared to when injected with vehicle (saline).*

	male subjects				female subjects			
treatment	vehicle		V1aR antagonist		vehicle		V1aR antagonist	
stimulus	*female*	*male*	*female*	*male*	*female*	*male*	*female*	*male*
distance traveled (m)	0.35 (0.22-0.5)	0.34 (0.3-0.46)	0.36 (0.3-0.56)	0.3 (0.28-0.53)	0.47 (0.34-0.67)	0.43 (0.38-0.66)	0.55(0.43-0.6)	0.49 (0.26-0.69)
time in stimulus chamber (s)	194 (151-237)	145 (88-208)	189 (135-229)	97 (29-181)	167 (105-171)	158 (98-184)	162 (142-186)	130 (105-202)
time in clean chamber (s)	78 (45-114)	97 (29-181)	78 (47-120)	134 (59-164)	94 (50-104)	70 (55-147)	78 (57-116)	94 (74-119)

6.3.10 V1aR antagonism in LHb or DR did not alter territorial aggression

The attack latency did not differ between treatment groups for LHb or DR animals (LHb: U = 14, p = 0.84; DR: U = 11, p = 0.84; Figure 6-9). Female subjects did not attack female intruders.

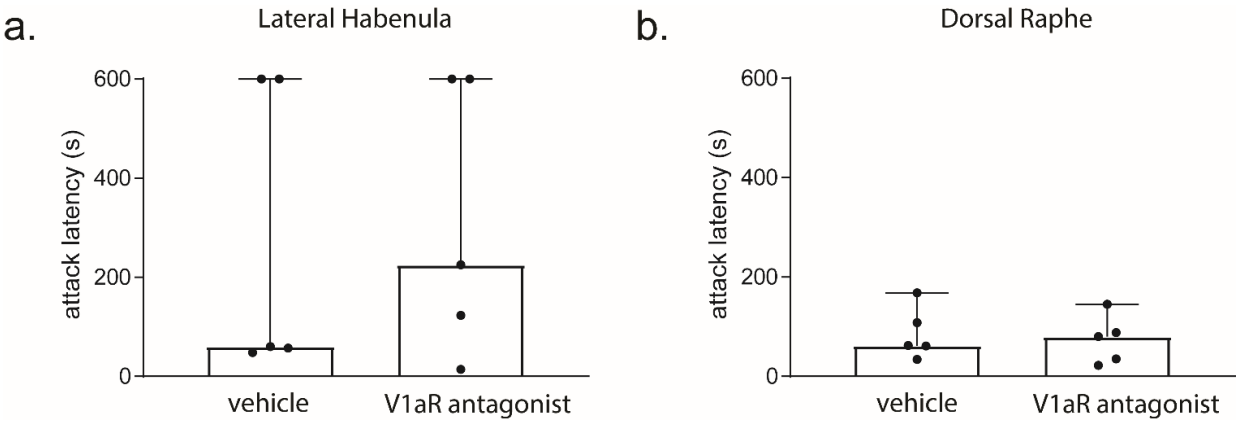

Figure 6-9 - V1aR antagonism in LHb (n=5) (A) or DR (n=5) (B) did not alter onset of male-male aggression compared to vehicle (saline) injected subjects (LHb: n=5; DR: n=5). *Bar graph and individual data points represent median and range of male subject's latency to attack a subordinate intruder male.*

6.3.11 Effects of V1aR antagonism on areas outside of LHb or DR

LHb or DR targeted subjects with placement of injection needle tip outside of LHb (unilateral hits included) or DR were analysed in a separate 'miss' group for urine marking, ultrasonic vocalization (USV), and social investigation behavior. In male subjects with LHb 'misses', a treatment and stimulus interaction was found in number of urine marks produced ($F(1,9) = 5.76$, $p = 0.04$) but not in urine marking area covered ($F(1,9) = .001$, $p = 0.97$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased urine marks to female stimuli compared to when given vehicle injections ($t(5) = 4.45$, $p = 0.007$, $d = 1.82$; Table 4; this effect was not observed in response to male stimuli ($t(5) = 1.57$, $p = 0.18$; Table 4).

Injections of V1aR antagonist in female subjects with LHb ‘misses’ did not alter urine mark number in the presence of male stimuli ($t(4) = 1$, $p = 0.37$) or female stimuli ($t(4) = 1.07$, $p = 0.35$; Table 4). No treatment effect was found in USV production ($F(1,9) = 0.58$, $p = 0.47$) or social investigation ($F(1,9) = 0.03$, $p = 0.87$; Table 4). Therefore, data from LHb ‘misses’ did not match the pattern of behavior following direct LHb injections (reduced urine marking to males and reduced USVs to females).

Injections in subjects that missed the DR were all located above the DR and within the ventrolateral PAG and resulted in a treatment and sex interaction in number of urine marks produced ($F(1,6) = 9.11$, $p = 0.02$) as well as urine marking area ($F(1,6) = 6.97$, $p = 0.04$). Post hoc comparisons revealed that males receiving V1aR antagonist significantly decreased urine marks to male stimuli compared to when given vehicle injections ($t(3) = 3.89$, $p = 0.03$, $d = 1.94$; Table 4); this effect was not observed in response to female stimuli ($t(3) = 0.65$, $p = 0.50$) or urine marking area (to male stimuli: $t(3) = 2.53$, $p = 0.09$; to female stimuli: $t(3) = 1.38$, $p = 0.26$; Table 4). These results are partially similar to effects of direct DR injections: both reduced male urine marks to other males. V1aR injections in female subjects that missed the DR did not alter urine mark number in the presence of male stimuli ($t(3) = 1$, $p = 0.39$) or female stimuli (no marking; Table 4). No treatment effect was observed in USVs ($F(1,6) = 0.82$, $p = 0.40$) or SI responses ($F(1,6) = 3.56$, $p = 0.11$; Table 4).

6.4 Discussion

The importance of central AVP acting on V1aR in the modulation of rodent social behavior has been well-established (Lukas and Neumann 2013) and is often sexually-differentiated (Veenema et al. 2013; Dumais and Veenema 2016; Duque-Wilckens et al. 2016;

Rigney et al. 2019). Here, we pharmacologically blocked V1aR in LHb or DR, areas known to have sex-different (male-biased) AVP expression (Rood et al. 2013) and broad involvement in social behavior (Luo et al. 2017; Soutschek 2018), and assessed changes in social behavior and communication. Our results indicate that sex differences in AVP innervation of LHb and DR may contribute to sexually dimorphic expression of social communication, as V1aR blockade in the LHb of males, but not females (who have less LHb and DR AVP innervation), reduced urine marking to unfamiliar males and production of ultrasonic vocalizations (USV) to unfamiliar, sexually receptive females, while V1aR antagonism in the DR of males, but not females, reduced urine marking to unfamiliar males. These changes occurred even though social investigation, locomotion, and territorial aggression were unaffected by V1aR blockade in either area, indicating specific effects on communicative behavior.

V1aR action in lateral habenula

The LHb has long been known to receive sex-different, hormone-sensitive AVP innervation (de Vries and Panzica 2006; de Vries 2008), likely from the BNST cells that show sexually dimorphic AVP expression (de Vries and Panzica 2006). Our previous findings demonstrate that BNST-AVP cells are important for male urine marking and social investigation of unfamiliar male (Rigney et al. 2019) as well as for detection of social novelty (Whylings et al. 2020). Consequently, our present results suggest that BNST-AVP action on social communication is potentially mediated by the LHb, although the LHb does receive AVP axonal projections from other sources as well (Rood and De Vries, 2011, Zhang et al. 2018). We found that V1aR blockade in the LHb reduced communicative behaviors typical of dominant, territorial male mice (urine marking to unfamiliar males and USVs to unfamiliar females) without concomitant changes in social investigation, locomotion or territorial aggression toward

subordinate males. This suggests that AVP may normally act in the LHb to promote specifically male-typical communication, rather than changing all aspects of social behavior. Our experiments do not allow us to fully exclude the possibility that changes in USVs following V1aR blockade in LHb in male subjects are due to changes in USV production by female stimuli. However, given the substantial male bias in USVs in opposite-sex interactions, it is likely that male subjects produced most of the USVs (Warren et al., 2018). Importantly, we did not observe V1aR antagonist effects on social investigation suggesting that AVP/V1aR action does not appear to alter the broader aversive function of LHb (Sparta et al. 2013; Golden et al. 2016; Benekareddy et al. 2018, but see Lecourtier et al. 2004). Moreover, the behavioral effects of V1aR antagonism are likely due to action on LHb directly as injections outside of LHb did not recapitulate the effects of LHb injections in that they did not reduce urine marking to unfamiliar males or production of USVs to unfamiliar, sexually receptive females. It should be noted that our misses were all very close to LHb and so it is possible that observed urine marking reductions in these offsite injections may still have allowed spread of antagonist to LHb.

Recent work on AVP action in LHb suggests that AVP may decrease defensive behavior in response to water deprivation (Zhang et al. 2016), potentially through hypothalamic AVP inputs (Zhang et al. 2018). This reduction in fear-responses, although correlative, combined with the present results, suggests an important role of AVP in LHb in promoting male-typical active responses to social competition and other biological threats. The exact source and nature of AVP action within LHb (BNST, medial amygdala, or hypothalamic) requires further examination.

V1aR action in dorsal raphe

The DR is interconnected with the BNST (Weissbourd et al. 2014; Garcia-Garcia et al. 2018) and contains more steroid-sensitive AVP expression in males than in females, suggesting

that BNST AVP and/or medial amygdala AVP cells are the main source of AVP here (Rood et al. 2013). Similarly, sex differences in V1aR expression in DR have been noted in other species (Ross et al., 2019) and AVP has been found to indirectly excite DR serotonin neurons via V1aR action (Rood and Beck 2014), possibly through intrinsic connections of glutamatergic DR cells (Soiza-Reilly and Commons 2011). Our study is the first to examine the role of DR V1aR on social behavior and here we demonstrate that V1aR blockade within DR in males reduces urine marking toward unfamiliar males, but not unfamiliar females, without altering social investigation and other behaviors. The lack of social avoidance following V1aR antagonism is in contrast with previous studies showing that AVP injections within DR facilitate fear-motivated passive avoidance in rats (Kovács et al. 1979; Kovács et al. 1986). This discrepancy may reflect differences in task parameters (social vs. non-social; memory processes), species (rat vs. mouse) as well as the known differences in behavioral effect between exogenous stimulation of the AVP system verses blockade of endogenous action of V1aR action (Engelmann 2008).

V1aR blockade in the ventrolateral periaqueductal gray (PAG) overlying DR also reduced urine marks toward unfamiliar males. As this area is the only nearby region to also contain AVP fibers and V1aR expression, is it possible that V1aR activation in PAG can generate appropriate levels of urine marking in male mice. Although AVP innervation of this relatively caudal region of the PAG is likely of hypothalamic origin and not sex-different or steroid sensitive (Rood et al. 2013), it may play a role in social communication as AVP injected into the PAG stimulates scent marking in hamsters, a species lacking extrahypothalamic AVP (Hennessey et al. 1992). Indeed, the PAG is broadly involved in other aspects of mouse social communication, such as USVs (Tschida et al. 2019). It is also possible that V1aR antagonism could be acting in DR and PAG together to alter behavior since these structures are close in

proximity. Additionally, our cannulation approach in LHb or DR does not avoid the possibility that the V1aR antagonist spread into the lateral ventricle (near LHb) or cerebral aqueduct (near DR). Yet, we feel that this is unlikely to fully account for our effects since LHb 'misses' did not replicate effects seen in LHb 'hit' subjects and a scenario of ventricular leak would require further downstream sex-differences in V1aR responsiveness. Nevertheless, additional experiments targeting different AVP cell populations (hypothalamic vs. limbic) will be required to disambiguate AVP/V1aR action in PAG from DR.

As sex differences in LHb and DR AVP fiber density likely originate from BNST cell bodies (de Vries 2008; Rood et al. 2013; Otero-Garcia et al. 2014), it is surprising that we did not observe alterations in male-male investigation following LHb/DR V1aR antagonism because deletion of BNST-AVP cells reduces male investigation of other males (Rigney et al. 2019). Similarly, BNST-AVP lesions did not alter USVs whereas V1aR blockade within LHb did. These apparent discrepancies may be due to several factors. First, AVP action at other BNST target structures, such as ventral pallidum, may drive social investigation, as V1aR activation in this area regulates male investigatory behavior and partner preference (Lim and Young 2004; DiBenedictis et al. 2020). Second, the source of AVP that regulates USVs in LHb may originate from medial amygdala AVP or hypothalamic AVP cells (Browne et al. 2018). Lastly, it is possible, given the cross-talk between OT and V1aR, that OT projections may be responsible for behavioral action (Song and Albers, 2017).

Conclusions

Our results indicate that the V1aR system plays a sexually dimorphic role in control of social communicative behaviors via LHb and, to a lesser extent, DR. This is largely in keeping with other findings that the AVP/V1aR system is sexually-differentiated not just in anatomy, but

also across various domains of behavioral function and across species (Albers, 2015; Dumais and Veenema, 2016; Hammock, 2015), including humans (Meyer-Lindenberg et al. 2011; Rilling et al. 2014). Given that the LHb and DR are strongly interconnected (Pasquier et al. 1976; Aghajanian and Wang 1977; Kalén et al. 1986; Ferraro et al. 1996; Yang et al. 2008) and appear to work as a system to regulate emotional responding (Zhao et al. 2015; Dolzani et al. 2016), concurrent AVP action on V1aR on LHb and DR may coordinate strongly-competitive aspects of social communication, such as territorial scent marking. In contrast, less-competitive aspects of communication, such as sexually-motivated vocalizations, may require direct AVP/V1aR action more specifically at LHb (and perhaps other AVP targets). The present work highlights the need to explore less-investigated targets of sex-different AVP inputs as they may play significant roles in sex-different social and emotional behaviors.

7 A BNST-TO-LATERAL SEPTUM VASOPRESSIN CIRCUIT THAT MODULATES SEX-SPECIFIC SOCIAL APPROACH, COMMUNICATION, AND ANXIETY-LIKE BEHAVIOR IN MICE

7.1 Introduction

Dysfunction in social communication is a prominent aspect of many psychopathologies and social disorders including autism, schizophrenia, and social anxiety (Bahrami & Yousefi, 2011; Insel, 2010; Tamminga & Medoff, 2000). Consequently, development of clinical treatment for these disorders requires an understanding of neural circuitry underlying social interactions. Sex differences are a persistent feature of social disorders, where autism and schizophrenia is more prevalent in males, while social anxiety occurs more frequently in females (Bahrami & Yousefi, 2011; Halladay et al., 2015). A critical gap in knowledge exists in understanding the role of sex-differences in the control of social behavior and communication. For example, there is no clear understanding of how sex differences in the neurobiology of normal and disordered social behavior occur (Lai et al., 2015). A reasonable hypothesis is that differences in neural circuitry underlie sex-differentiated dysfunctions in social behavior and communication. A well-studied circuit in this regard is the sexually dimorphic expression of the neuropeptide arginine vasopressin (AVP) (de Vries, 2008). AVP neurons are prominent in areas such as the extended amygdala (bed nucleus of the stria terminalis (BNST), medial amygdala), and hypothalamic structures. These areas and their projection sites control multiple social behaviors, including social communication (O'Connell & Hofmann, 2012). The contributions of AVP neurons in each of these cell groups to social behavior, however, is relatively unknown.

One of the largest and most evolutionarily conserved sex-different systems in the vertebrate brain is the male-biased and steroid-dependent expression of AVP (de Vries, 2008; De

Vries & Panzica, 2006; Goodson & Bass, 2001). AVP has been repeatedly implicated in modulation of social behaviors in sex-different ways (Choleris et al., 2009; Donaldson & Young, 2008; Duque-Wilckens et al., 2016; Guastella et al., 2010; Insel, 2010; Rilling et al., 2014) and is an important modulator for animal and human sociality (Guastella et al., 2010; Rilling et al., 2014). In humans, AVP has been implicated in psychopathology (Meyer-Lindenberg et al., 2011). For example, variations in the vasopressin V1a receptor (V1aR) gene and AVP serum levels are associated with autism spectrum disorder (ASD) (Miller et al., 2013; Tansey et al., 2011; Wassink et al., 2004; Xu et al., 2013; Yang et al., 2010; Yirmiya et al., 2006). AVP acts on various brain regions that regulate social communication (Goodson & Bass, 2001; Kelly & Goodson, 2013b), aggression (Albers, 2012) maternal care (Bosch & Neumann, 2008), pair bonding (Carter et al., 1995; Jarcho et al., 2011; Young & Wang, 2004), cognition(Landgraf & Neumann, 2004), and social recognition (Bielsky et al., 2004; Bielsky & Young, 2004; Everts & Koolhaas, 1999; Johnson & Young, 2017; Veenema et al., 2012). Additionally, AVP contributes to avoidance-anxiety related behavior (Griebel et al., 2002; Hammock et al., 2005) and passive avoidant behavior (Bohus et al., 1978) as well as in the evaluation of stressful situations (Hari Dass & Vyas, 2014b; Tong, 2018). However, behavioral effects of direct manipulation of AVP cell populations have yet to be fully explored.

The anatomy of AVP projections suggests that AVP control of social behavior is complex and the anatomical substrate of AVP's control of social behavior is unclear (Dumais & Veenema, 2016; Kelly & Goodson, 2013a; Ludwig & Stern, 2015). AVP is synthesized in several cell populations, each of which project to distinct brain areas (De Vries & Boyle, 1998; Rood et al., 2013; Rood & De Vries, 2011). Neurons of the paraventricular and supraoptic nuclei (PVN and SON) release AVP as a hormone whereas other PVN neurons project to

hypothalamus, hindbrain and spinal cord to regulate autonomic function (Sawchenko & Swanson, 1982). The suprachiasmatic nucleus (SCN) projects to midline areas and contributes to circadian functions (Hoorneman & Buijs, 1982). AVP cells in the BNST contribute to the most pronounced sex difference in AVP innervation in the brain (De Vries & Boyle, 1998). For example, male rodents have 2-3 times as many AVP cells as females in the BNST and their projections to areas such as the lateral septum (LS) are denser as well (De Vries and Buijs 1983).

BNST AVP cells are thought to be involved in prosocial behavior and communication (Goodson & Bass, 2001) since release of AVP in areas which receive BNST input correlate positively with prosocial behavior (Goodson et al., 2009; Hari Dass & Vyas, 2014a; Ho et al., 2010). Additionally, there are sex differences with partial knockdown of the AVP gene in the BNST, where prosocial interactions are reduced more in males than in females and male-male aggression is increased in birds (Kelly & Goodson, 2013a, 2013b). A recent cell ablation study indicates that BNST AVP cell groups are linked to male, but not female, social investigation and communication (Rigney et al., 2019). However, these behavioral effects may reflect molecular, cellular, and anatomic adjustments or compensations to chronic depletion of AVP cells in the BNST. To tackle this problem, we acutely stimulate or inhibit BNST AVP cells using optogenetics and test whether these manipulations influence male and female social behavior.

The lateral septum (LS) has long been known to be the major target of sexually-differentiated AVP innervation and has been implicated in the sex-specific regulation of social recognition, social play, social affiliation, aggression, and anxiety-related behavior (Bredewold & Veenema, 2018; De Vries & Buijs, 1983; De Vries & Panzica, 2006; Rigney et al., 2023). We therefore test the hypothesis that the sexually-differentiated BNST AVP projections to the LS

play a prominent role in driving male, but not female, social approach and communication. Since pharmacological studies suggest that AVP in the LS can modulate anxiety (Beiderbeck et al., 2007; Landgraf et al., 1995; Liebsch et al., 1996), we further tested anxiety-like behavior in all optogenetic manipulations. These results will help elucidate if the sexually-dimorphic AVP cells in the BNST acutely drive specific aspects of male social investigation and communication and if these cells project to the LS to facilitate these behaviors.

7.2 Methods

7.2.1 Animals and Husbandry

All mice were maintained at 22°C on a 12/12 hr reverse light/dark cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems, Centennial, CO, USA), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were performed in accordance with the Georgia State University Institutional Animal Care and Use Committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects

Founding AVP-iCre mice were obtained from Dr. Michihiro Mieda (Kanazawa University, Japan). These mice were generated using a bacterial artificial chromosome (BAC) that expressed codon-improved Cre recombinase (Shimshek et al., 2002) under the transcriptional control of the AVP promoter (AVP-iCre mice). In these animals, iCre expression is found in the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA), as well as in hypothalamic areas (Mieda et al., 2015). Subjects were derived by crossing heterozygous iCre mutants to wildtype C57Bl/6J mice and genotyped (ear punch) by polymerase chain reaction (PCR) at 21–24 days of age (Transnetyx). A total of 170 iCre+ mice were used for

in vivo behavioral experiments (91 males, 79 females); Fos experiment: n = 15 males, n = 15 females; BNST AVP cell stimulation experiment: n = 18 males, n = 22 females; BNST AVP cell inhibition experiment: n = 20 males, n = 21 females; BNST-LS terminal stimulation experiment: n = 20 males, n = 21 females; BNST-LS terminal stimulation with V1aR antagonist experiment: n = 18 males. All subject mice were singly housed for a minimum of one week prior to experimental use.

Stimulus Animals

CD1 (ICR; Charles River Laboratories, Wilmington, MA, USA) mice were used as stimuli for behavioral testing and to provide male and female subjects with social experience because strain differences between subjects and stimulus mice increase social investigation (Gheusi et al., 1994). Mice were used at 9–16 weeks of age and were novel and unrelated to the subject to which they were exposed. Female stimulus mice were grouped-housed, ovariectomized, and hormonally primed with subcutaneous injection of estradiol benzoate (E; 5ug/0.1mL sesame oil) followed by subcutaneous injection of progesterone (P; 250ug/0.1mL sesame oil) 44-48 hr later and approximately 4 hours prior to use. Female stimulus mice were given two sexual experiences before use in social experience and behavioral testing of subjects.

Two groups of stimulus males were used for social experience and behavioral testing. Males that were used as subordinate mice to provide aggressive experience to subjects, were grouped-housed, gonadectomized (GDX), and subjected to two aggressive encounters with a dominant male (see below). Mice in the second group, which provided sexual experience to female subjects and were used as stimulus animals in the three-chamber social task, were singly housed, gonadectomized, implanted with testosterone (GDX + T), and then given two sexual experiences before use in social experience/behavioral testing.

7.2.2 Viral Vector

Cell and terminal excitation: AVP driven-, Cre-expressing-BNST neurons were induced to express blue-light activated excitatory opsin, channelrhodopsin-2 (ChR2) fused to eYFP, using an adeno-associated virus (AAV-EF1a-double floxed-hChR2; serotype 5; Addgene plasmid #20298; http://n2t.net/addgene:20298 ; RRID:Addgene_20298) or eYFP alone (AAV-EF1a-DIO-eYFP; serotype 5; Dr. Karl Deisseroth; Addgene plasmid #27056; http://n2t.net/addgene:27056; RRID:Addgene_27056).

Cell inhibition: AVP driven-, Cre-expressing-BNST neurons were induced to express blue-light activated inhibitory opsin, guillardia theta anion-conducting channelrhodopsins (stGtACR; hSyn1-SIO-stGtACR2-FusionRed; serotype 1; Addgene plasmid #81070; https://www.addgene.org/105677/; RRID:Addgene_105677) or eYFP alone (AAV-EF1a-DIO-eYFP; serotype 5; Dr. Karl Deisseroth; Addgene plasmid #27056; http://n2t.net/addgene:27056; RRID:Addgene_27056).

7.2.3 Surgical Procedures

All surgeries were conducted using 1.5–3% isoflurane gas anesthesia in 80% oxygen and 20% nitrous oxide; 3 mg/kg of carprofen was given before surgery to reduce pain.

Stereotaxic Surgery

Mice were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand-operated drill was used to make holes in the skull, exposing the dura. For all subjects, 200nL of AAV-EF1a-double floxed-hChR2 (ChR2) or AAV-Ef1a-DIO-eYFP (YFP controls) was delivered bilaterally to the BNST (coordinates: DV: -4.3, AP: +0.13, ML: ±0.8; (Paxinos and Franklin, 2012)) at a rate of 100 nL/min using a 5-μL Hamilton syringe with a 30-gauge

beveled needle mounted on a stereotaxic injector. Following virus delivery, the syringe was left in place for 5 minutes before slowly withdrawing it from the brain. Dual optic fiber cannulas (Doric Lenses, DFC_200/230-0.48_4mm_DF1.6_DFL) were chronically implanted immediately following viral injections using the same coordinates. For dual delivery of V1aR antagonist and light stimulation in the lateral septum, Dual Optofluid cannulas with interchangeable injectors were implanted. The dual fiber optic implants and cannulas were secured to the scalp with 1 bone screw, 1 layer of dental cement (Calk Dentsply) and 2 layers of Ortho-jet powder and liquid mix (Lang Dental Manufacturing Co Inc). Mice were allowed to recover for at least 14 days prior to handling to allow for optimal viral expression.

Gonadectomy and Hormone Treatment (stimulus animals)

Testes were cauterized and removed at the ductus deferens via a midline abdominal incision. Silastic capsules (1.5-cm active length; 1.02-mm inner diameter, 2.16-mm outer diameter; Dow Corning Corporation, Midland, MI, USA) were filled with crystalline testosterone (T; Sigma, St. Louis, MO, USA) and inserted subcutaneously between the scapulae after gonadectomy (GDX + T); this procedure leads to physiologic levels of T (Barkley and Goldman, 1977; Matochik et al., 1994). To further reduce aggression in stimulus animals, some males were gonadectomized, but did not receive a T implant (GDX).

The ovaries of stimulus female mice were removed by cauterization at the uterine horn and attendant blood vessels. To induce sexual receptivity, stimulus females were sequentially (E 44-48 hr prior to P injection 4 hr before use) injected subcutaneously with E and P.

7.2.4 Social Experience

As opposite-sex sexual experience and attaining competitive status (“social dominance”) promote male and female communicative behaviors (Lumley et al., 1999; Roullet et al., 2011),

subject mice received social experience. Social experience consisted of two sets of the following sequence: an opposite-sex encounter (sexual experience) followed by a same-sex encounter (aggressive experience) the following day with at least one day break in between sets of encounters.

Opposite-sex (sexual) Experience

Subjects were given two opportunities to interact with either a stimulus female (for male subjects) or a stimulus male (for female subjects). A sexually experienced stimulus mouse was placed in the subject's home cage and removed the next day (overnight, first experience) or after 90 minutes (second experience). Subjects that did not engage in any sexual behavior (mounting, intromission, or ejaculation) during the second experience were removed from further testing.

Same-sex (aggressive) Experience

Male subjects were exposed to two interactions with subordinate males (GDX) treated with 40 uL of GDX + T stimulus male urine applied to their backs. Gonadectomy, group housing, and social defeat of our subordinates reduce offensive aggression in mice, while GDX + T male urine provides subjects with a male urinary cue that elicits offensive aggression (Beeman, 1947; Connor and Winston, 1972; Van Loo et al., 2001). Subordinate stimulus males were placed in the subject's home cage and removed after the subject's first offensive attack (biting) within a 10-minute period. All subject males attacked the intruder male stimulus by the second encounter, and all subordinate stimulus males displayed submissive behavior, defined as defensive postures (e.g., on-back), fleeing, and non-social exploring (Koolhaas et al., 2013). Female subjects were exposed to a female intruder for a 10-minute period; however, this did not elicit any attacks from either animal.

7.2.5 Experimental Procedure

All testing occurred during the dark cycle under red light illumination, except for the elevated zero maze (EZM). Two weeks after viral injection and implantation surgery, subjects were habituated to the testing room and apparatus by handling and placing mice (for 3-5 min) in the three-chamber apparatus (see below) each day for 3 days. On experimental days, subjects were adapted to the experimental room for 15 minutes before testing. All tests were scored by an experimenter blind to the viral and drug manipulation of the subject.

Subjects underwent a total of four tests for social communication and social approach in the three-chamber apparatus (24.5x16x9in) with at least 4 days off in between test days. Each subject received two test days with light stimulation and two test days without light stimulation with each stimulus type (male and female live conspecifics). The order of treatment (light-ON, light-OFF) and stimulus condition (male, female) was counterbalanced across subjects, except that subjects exposed to a stimulus type on the first test were then given that same stimulus type on the second test with the opposite treatment condition. Female subjects were tested irrespective of estrous cycle day. Prior research indicates minimal effects of estrous cycle on female mouse communicative behavior (Maggio and Whitney, 1985; Coquelin, 1992; Moncho-Bogani et al., 2002). Finally, we tested mice on an EZM to test for anxiety-like behavior or tested mice within the 3-chamber with a novel object (Lego or Hot Wheel car). After behavior testing, subjects in the BNST AVP cell stimulation experiment underwent 10 minutes of light stimulation in their home cage and were sacrificed and perfused 70 minutes later. Brain tissue was extracted and processed for immunohistochemistry to verify viral targeting of BNST (mice in all experiments) and sufficient activation of ChR2-infected AVP cells by quantifying colocalization of YFP and Fos, an immediate early gene used as a marker for neural activity.

Blue Light Stimulation and Inhibition

A dual fiber optic patch cord (Doric Lenses, DFP_200/230/900-0.48_1m_DF1.6-2FC) was coupled to dual optic fiber cannulas chronically implanted in subjects with a zirconia sleeve (Doric Lenses, SLEEVE_ZR_2.5). The dual fiber optic patch cord was connected to a rotary joint (Doric Lenses, FRJ_1x2i_FC-2FC_0.22) to minimize torque on the animal's head during testing via a FC/PC connector. The rotary joint was mounted above the approximate center of testing arenas using a gimbal holder (Doric Lenses, GH_FRJ) to further minimize torsional stress on subjects during behavior testing. A mono-fiber optic patch cord (Doric Lenses, MFP_200/240/LWMJ-0.22_1m_FC-FC) connected the rotary joint to a 473nm blue diode pumped solid-state laser (Shanghai Laser & Optics Century Co, BL473T8-150FC) via FC/PC connectors. Blue light laser pulses were generated via a controller (PlexBright 4-Channel Optogenetics Controller) and Plexon Radiant Software (2.2.0.19). During all simulations, 10Hz pulses of blue light (20ms pulse width, 473nm, 5-6mW light power) were delivered at 5s on/off intervals over a 10-minute (three-chamber tests) or 5-minute period (EZM tests). Mice that were in the cell inhibition experiment received 10Hz of constant light (473nm, 5-6mW light power). The power of optic fiber light was verified before each test day, using a light sensor (Thor Labs, S140C), and light power ranged from 5-6mW at the tip of the dual optic fiber implant.

V1aR antagonist infusions into the LS

The highly-specific V1aR antagonist (d(CH2)5[Tyr(Me)2,Dab5] AVP; (Bachem) was diluted in sterile saline and 0.1% acetic acid to a final injected dose of 2.5uM and stored at −20 °C until use. This antagonist, modified from the original Manning compound with the addition of diaminobutyric acid (Dab), is exceptionally selective for V1aR, eliciting no detectable anti-OT activity in vitro or in vivo (Chan et al., 1996; Manning et al., 2012). 30-45 minutes before

behavioral testing, subjects were briefly anesthetized (1.5–3% isoflurane gas) and a 33 gauge needle was inserted through the guide cannula, extending a total length of 3.7 mm. Subjects were then injected with 300 nL sterile saline (vehicle) or V1aR antagonist at 100 nL/min (10 μL Hamilton syringe; Harvard Apparatus PHD 22/2000 syringe pump) via the guide cannula. The injection needle was left in place for 1 minute to allow the drug to diffuse away from the tip of the injection needle followed by placement of interchangeable optical fibers.

Three-Chamber

Ultrasonic vocalizations (USV), urine marking, and social investigation were recorded in an acrylic three-chamber apparatus (Crawley, 2007; Arakawa et al., 2008; Moy et al., 2009). Instead of a solid floor, the apparatus was placed on absorbent paper (Nalgene Versi-dry paper, Thermo Fisher Scientific) to accurately measure urine marking. During testing with stimulus animals, subjects had access to either a stimulus animal in a triangular cage [7.5in (hypotenuse), 6in (triangle legs), 9in (height)] or an empty (clean) cage placed at opposite corners of the outermost chambers of the apparatus. The location of the stimulus and clean cage were counterbalanced across subjects.

Subjects were placed in the center of the middle chamber after being connected to the dual fiber optic patch cord and allowed to acclimate to the apparatus for 1 minute. After 1 minute, we measured close investigation of clean and stimulus cages, distance traveled throughout the apparatus, time spent in the stimulus and clean cage chambers as well as USVs and urine marking across a 10-minute trial period. After testing, the apparatus and cages were thoroughly cleaned with 70% ethanol and allowed to dry before further testing.

Social Investigation and USVs

Close social investigation was defined as time spent sniffing within 2 cm of the stimulus or clean cage; climbing on the cage was not scored as investigation. USVs were detected using a condenser microphone connected to an amplifier (UltraSoundGate CM16/CMPA, 10 –200 kHz, frequency range) placed adjacent to the three-chamber apparatus and directly above the center chamber. USVs were sampled at 200 kHz (16-bit) with target frequency set to 70 kHz (UltraSoundGate 116Hb, Avisoft Bioacoustics). Recordings were then analyzed using a MATLAB (MathWorks, RRID:SCR_001622) plug-in that automates USV analysis (Van Segbroeck et al., 2017). Using this program, sonograms were generated by calculating the power spectrum on Hamming windowed data and then transformed into compact acoustic feature representations (Gammatone Filterbank). Each 200-ms window containing the maximum USV syllable duration was then clustered, via machine learning algorithms, into USV syllable types (repertoire units) based on time-frequency USV shape. Repertoire units that appeared as background noise were discarded.

Urine Marking

Following testing, the substrate sheet was allowed to dry for 1 h and then sprayed with ninhydrin fixative (LCNIN-16; Tritech Forensics Inc.) to visualize urine marks (Lehmann et al., 2013). After 24 h, sheets were imaged (Sony DSC-S700 camera), binarized and analyzed using a computer-aided imaging software (ImageJ, RRID:SCR_003070). Urine marking was measured as the total area (cm2) or number of visualized ninhydrin urine marks in the entire arena.

To attach the fiber optic patch cord to implants, subjects needed to be restrained by “scruffing” briefly which often elicited urination. While attaching the patch cord, subjects were placed over a separate sheet of absorbent paper from that used to collect urine marks during

three-chamber testing. Total area of urine pools during scruffing was measured and counted separately to determine whether increased restraint-related urination affected urine marks during testing.

Elevated Zero Maze (EZM)

The EZM consisted of 5.5 cm wide circular platform of internal diameter 35 cm raised 50 cm off the ground. The circular platform consisted of two equally spaced enclosed compartments covering half of the platform and two open compartments. Subjects were connected to the fiber optic patch cord and placed at the start of a closed compartment in the EZM and observed after a 1-minute acclimation period for 5-minute while receiving light stimulation (light-ON) or not (light-OFF). Each subject underwent 2 tests in EZM, one with light stimulation and one without, with the order of treatment (light-ON, light-OFF) counterbalanced across subjects. Time spent in open and closed compartments of the EZM was manually scored.

Real-time place preference

To investigate the inherent rewarding or aversive nature of BNST AVP cell stimulation or inhibition, the subjects underwent a real-time place preference (RTPP) test. The test involved habituating the subjects to a clear plastic box divided into two equal-sized chambers (50cm x 50cm x 25cm) and allowing them to explore freely for 1 minute without any light stimulation. Subsequently, subjects were given a 10-minute period to explore either a chamber with light stimulation or a chamber without any light stimulation (randomized), and the time spent in each chamber was recorded. The recorded data was used to generate a preference score to determine if the light stimulation had any effect on the time spent in each chamber (Stamatakis and Stuber 2012).

7.2.6 Histology

Approximately 70 minutes after 10-minute home-cage light stimulation, animals were anesthetized with intraperitoneal injection of Beuthanasia-D (150mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were extracted and post-fixed in 4% paraformaldehyde for approximately 12 hours overnight. Brains were then transferred to a 30% sucrose solution and stored at 4C before cryosectioning. Coronal sections were cut at a thickness of 30um with a cryostat (Leica CM3050 S, Leica Biosystems) into 12-well plates filled with cryoprotectant and stored at -20C before immunohistochemistry processing.

Sections were washed in 0.1M PBS (sequence of 5 washes for 5 minute each) before being incubated with agitation overnight at room temperature with a primary antibody for cFos (rabbit anti-cFos, 1:1000, ab214672) and primary antibody for eYFP/GFP (chicken anti-GFP; 1:5000; ab13970) in 0.4% Triton-X in 0.1M PBS solution. The following day, sections were washed in 0.1M PBS again with the same sequence and incubated with agitation in the dark for 2 hr in 0.4% Triton-X in 0.1M PBS with secondary antibodies: goat anti-rabbit IgG (Alexa Fluor 594; 1:600; Invitrogen) and goat anti-chicken IgG (Alexa Fluor 488; 1:600; Invitrogen). Sections were washed in 0.1M PBS a final time using the same sequence then mounted onto microscope slides (Fisherbrand Superfrost Plus) and cover-slipped using Prolong Gold (Invitrogen) for subsequent tissue analysis.

7.2.7 in vitro electrophysiological recordings

Ex vivo Slice Preparation

On the day of the experiment, a mouse injected with either AAV-EF1a-DIO-mCherry or AAV-EF1a-DIO-hChR2-mCherry was anesthetized with pentobarbital (50mg kg-1, i.p.) and then transcardially perfused with 20 ml of ice-cold sucrose artificial cerebrospinal fluid (aCSF)

solution. This sucrose aCSF solution contained (in mM): 200 sucrose, 2.5 KCl, 1 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, 20 D-glucose, 0.4 ascorbic acid, and 2.0 CaCl2, pH 7.2, 300-305 mOsmol l-1. The mouse was then rapidly decapitated, and the brain was subsequently dissected, mounted in the chamber of a vibrotome with superglue (Leica VT1200s, Leica Microsystems), and submerged into sucrose aCSF and bubbled constantly with 95% O2/5% CO2. Coronal slices containing LS and BNST were cut at 300 μm thickness and placed in a holding chamber containing aCSF (in mM): 119 NaCl, 2.5 KCl, 1 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, 20 D-Glucose, 0.4 ascorbic acid, 2 CaCl2, 2 Na+ Pyruvate. bubbled with 95% O2/5% CO2. Slices rested in a water bath at 32° C for 20 minutes before transfer to room temperature for a minimum of 40 minutes before recording.

Whole Cell Patch Clamp

Slices were placed on the stage of a Dragonfly 200 spinning disk confocal microscope system (Andor Technologies, USA). We targeted LS neurons in close vicinity to mCherry-labeled terminals projecting from the adjacent BNST. Current clamp recordings were acquired using an MultiClamp 700A and digitized with a Digidata 1440 (Molecular Devices, Sunnyvale, CA, USA). Electrodes were pulled using a Flaming Brown horizontal puller (Sutter Instruments, Novato, CA, USA) from borosilicate capillaries (4-7 MΩ) and filled with internal solution (in mM): 135 KMeSO4, 8 KCl, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 6 Phosphocreatine, as well as [mM] AlexaFluor 488 for visualization of neurons after recording (7.2 pH; 285-295 mosmol (kg H2O)-1. Once we achieved whole cell configuration, we allowed the cell to rest for 3 minutes to ensure stability of the patch and equilibration between internal solution and cytosol. In the current clamp, we adjusted the holding current to bring the cell to a resting membrane potential where firing was present but steady (~0.2-1 Hz). Data was acquired at 10 kHz. ChR2 stimulation

was driven by an Andor mosaic using 5s On/5s Off pulse pattern at 10 Hz (20 ms pulse width, 80 ms interpulse interval) lasting 60 seconds. Cells that displayed a shift in series resistance that exceeded 20 MΩ or a 25% change from the start of the recording were discarded. Data was analyzed using ClampFit v10.7. Firing frequency was calculated from spiking data acquired in the current clamp. Example traces were generated in Igor Pro 9 (WaveMetrics Inc., Portland , OR, USA). Statistics and graphs were generated with Prism (GraphPad, Boston, MA, USA).

7.2.8 Tissue Analysis

Bilateral images were taken at 10× magnification using a Zeiss Axio Imager.M2 microscope (Carl Zeiss Microimaging), which transferred fluorescent images (FITC contrast reflector) to image analysis software (Stereo Investigator, MicroBrightField, RRID:SCR_002526). Imaging domains (2 mm2) were placed with reference to anatomic landmarks (ventricles, fiber tracts; Paxinos and Franklin, 2012). Images taken under green fluorescence were overlaid with images taken under red fluorescence and cells were counted using ImageJ software (1.8.0_172). AVP cells expressing YFP (green fluorescence), Fos, (red fluorescence), or both (YFP/Fos colocalization; yellow-orange) were counted in the BNST in both hemispheres over four sections and averaged. Percent of cells expressing colocalized YFP and Fos were determined to verify increased activation of AVP-BNST cells in ChR2-YFP expressing animals under blue light stimulation compared to controls (YFP alone). Subjects with off-target ChR2 expression or implants were considered "misses" and excluded from analyses.

7.2.9 Statistical Analysis

All data were analyzed and graphed in R. Social investigation, USV, urine marking, and EZM data met the assumptions of parametric statistical tests. Therefore, we analyzed this data

with mixed-model ANOVAs [between-subject factor: sex, virus (ChR2, YFP); within-subject factors: light stimulation (OFF, ON), sex of stimulus (male, female)] followed by paired t-tests assessing treatment effects. All post hoc pairwise comparisons report Bonferroni-corrected p values and Cohen's d for effect size when statistically significant. Results were considered statistically significant if $p < 0.05$.

7.3 Results

7.3.1 BNST AVP cell and c-Fos colocalization during social exposure in the three-chamber test

The BNST has long been implicated in regulating social behaviors (Flanigan and Kash 2022), and lesions of AVP cells within the BNST affected male, but not female, social investigation and communication (Rigney et al. 2019). As a first step towards dissecting the acute function of BNST AVP cells in males and females, we focused on social approach and communicative behaviors (i.e. urine marking and ultrasonic vocalizations) toward both male and female conspecifics. Fos induction studies in mice have shown that AVP cells in the BNST are activated during direct male-female interactions, but not other rewarding stimuli, suggesting these cells may encode positively-valenced social interactions (Ho et al. 2010; Goodson et al. 2009). In addition, BNST AVP-Fos colocalization is positively associated with the number of bites received and/or negatively associated with dominance in male-male interactions (Ho et al. 2010). We confirmed that, in male mice, BNST AVP cells exhibit elevated expression of c-Fos in the presence of a male or female conspecific in the three-chamber apparatus, where direct physical contact was limited to nose-to-nose interactions ($F(1,12) = 5.8$, $p = 0.017$, $\eta^2 = 0.4$; clean-male: $p = 0.05$, clean-female: $p = 0.025$; Figure 7-1a-c). Furthermore, female mice had greater BNST AVP-Fos colocalization in the presence of a male or female conspecific compared

to controls that explored the three-chamber with no social stimulus ($F(1,12) = 13.23$, $p = 0.0009$, $\eta^2 = 0.69$; clean-male: $p = 0.05$, clean-female: $p = 0.0007$; Figure 7-1a-c). We noticed that females had less BNST AVP-Fos colocalization in both social conditions compared to males; therefore, we collapsed the data to reveal that males had higher BNST AVP-Fos colocalization than females during social interactions ($F(1,24) = 10.69$, $p = 0.03$, $\eta^2 = 0.4$), which is likely due to the sexual dimorphism in BNST AVP expression (De Vries and Panzica 2006; Rood et al. 2013).

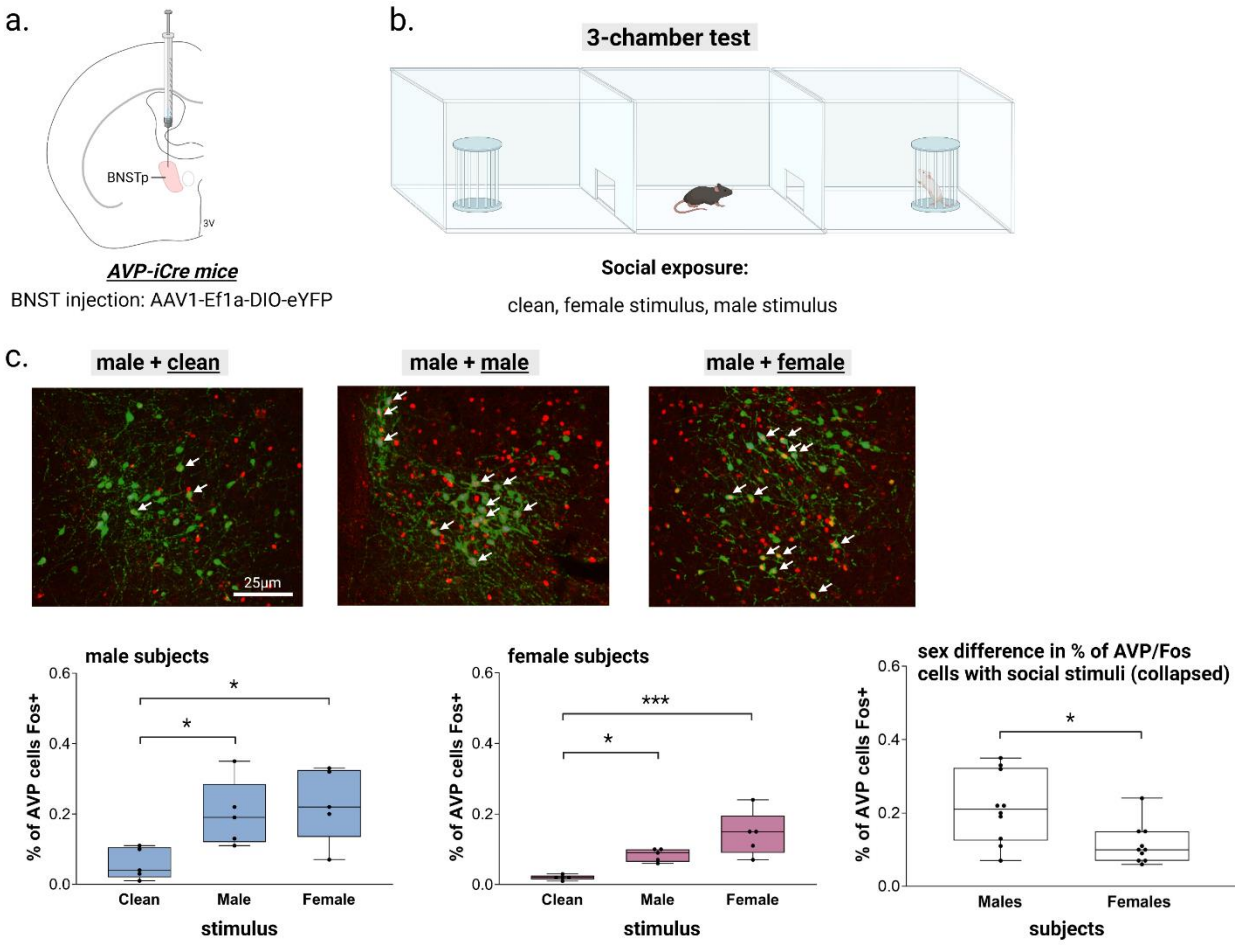

Figure 7-1– BNST AVP cell and c-Fos colocalization during social exposure in the 3-chamber test. *(a) BNST injection site and virus. (b) Three-chamber social testing. (c) (top)*

*Example images of merged BNST-AVP cells (green) and c-Fos+ cells (red). (bottom) Boxplots of the percentage of AVP cells colocalized with c-Fos in each 3-chamber stimulus condition (clean, male, female) for male and female subjects and boxplot of the percentage of BNST AVP cells colocalized with c-Fos in both social stimulus conditions collapsed (male+female stimulus). Mean ± SEM data represented. Dots indicate individual data points. Scale bar = 25 µm. *p < 0.05, ** p < 0.01, ***p < 0.001.*

7.3.2 BNST AVP cell inhibition reduces male social investigation of other males, without affecting social communicative behaviors

Although BNST AVP cell lesions and AVP knockdown studies in mice and birds have suggested that these cells are involved with male-male investigation and competitive behavior, it is also argued that these cells play a role in male affiliative behavior (Rigney et al. 2019; Rigney et al. 2021; Kelly et al. 2011; Kelly and Goodson 2013; Kelly and Goodson 2013). Therefore, it was not clear whether acute inhibition of BNST AVP cells would promote or inhibit approach to male and female conspecifics in the three-chamber apparatus. To distinguish if BNST AVP cells are necessary for social approach in males and females, we bilaterally injected Cre-dependent AAVs for cell inhibition and implanted fibers into the BNST of adult AVP-iCre+ male and female mice and assessed social investigation and communicative behaviors (Figure 7-2a-b).

We confirmed in vitro that stGTACR2-expressing cells within the BNST are silenced by light application at 10Hz (Figure 7-2c). In males, inhibition of BNST AVP cells significantly decreased time spent investigating male stimuli compared to investigation during the light-off condition (Mixed model ANOVA, treatment*light*stimulus interaction ($F(1,37) = 4.11$, $p = 0.05$, $\eta^2 = 0.6$; post hoc: $p = 0.001$; Figure 7-2e). Inhibition of BNST AVP cells in females did not affect social investigation time (Figure 7-2e) and social investigation was unaltered in YFP controls (Figure 7-2e). Additionally, BNST AVP cell inhibition did not

affect social communicative behaviors (i.e., urine marking and ultrasonic vocalizations) (Supplementary Figure 1, data not shown).

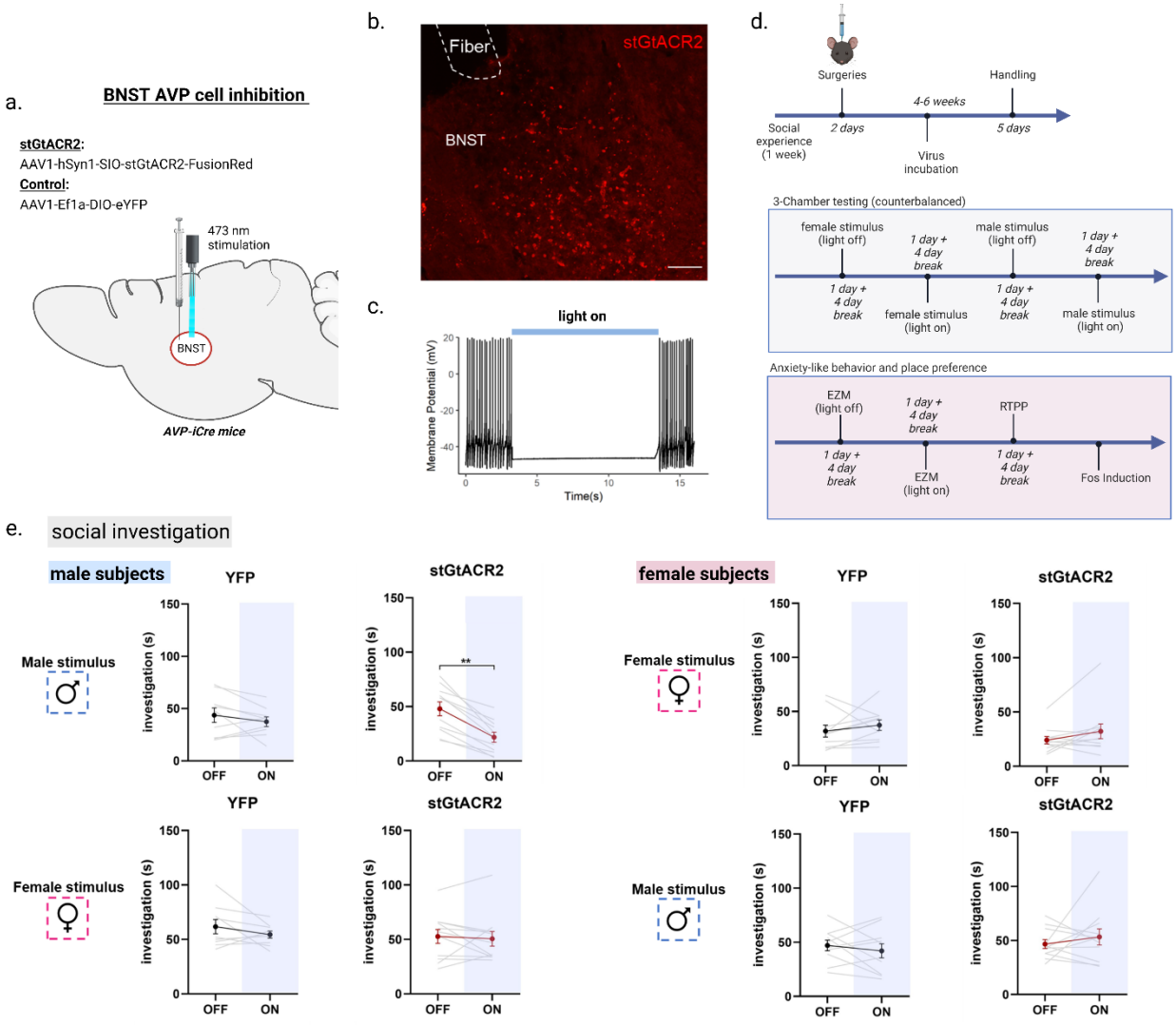

Figure 7-2– Optogenetic inhibition of AVP-BNST cells decreases male-male social investigation. *(a-b) Bilateral BNST injection and fiber implantation site; coordinates: DV: -4.4, AP: +0.15, ML: ±0.8; modified from Paxinos and Franklin (2012) and example image of BNST AVP cells infected with the inhibitory stGtACR adeno-associated virus (red). (c) Representative trace from whole-cell current-clamp recording of stGTACR2-expressing cell silenced by light application at 10Hz for 10 seconds (d) Experimental timeline (e) Investigation (in seconds) by male and female subjects during the three-chamber test (male subjects: YFP, n=9 and stGtACR2, n=11 female subjects (YFP, n=10 and stGtACR2, n=11) during light-OFF and light-ON conditions, counterbalanced. Blue light inhibition (ON) of AVP-BNST cells in stGtACR2*

*males significantly decreased time spent investigating male stimuli compared to investigation during light-OFF condition. Blue light inhibition (ON) of AVP-BNST cells in stGtACR2 females did not affect time spent investigating male or female stimuli. Light stimulation did not affect investigation times of YFP male and female subjects to either stimulus type (female or male). Each point and horizontal line represent individual within-subject data. Overlapping data are represented as one point/line. **p<0.01.*

7.3.3 BNST AVP cell inhibition triggers real-time place preference in females, but not anxiety-like behavior

Several studies point at the role of central AVP in stimulating anxiety-like behavior (Bielsky et al. 2004; Neumann and Landgraf 2012; Mak et al. 2012; Fabio et al. 2012). Therefore, we measured anxiety-like behavior within the elevated-zero maze (EZM). Blue light inhibition of BNST AVP cells did not alter time spent in the open arms of the EZM (Figure 7-3a). We next tested whether optogenetic inhibition of BNST AVP cells was rewarding or aversive within a real-time place preference test, where mice could choose to spend time in a chamber with either light stimulation or no light stimulation. stGtACR females preferred to spend more time in the 'light-on' chamber compared to YFP control females, in which BNST AVP cells were inhibited (One-Way ANOVA, treatment*sex interaction, ($F(1,37) = 4.65$, $p = 0.038$, $\eta^2 = 0.11$; post hoc: $p = 0.024$; Figure 7-3b).

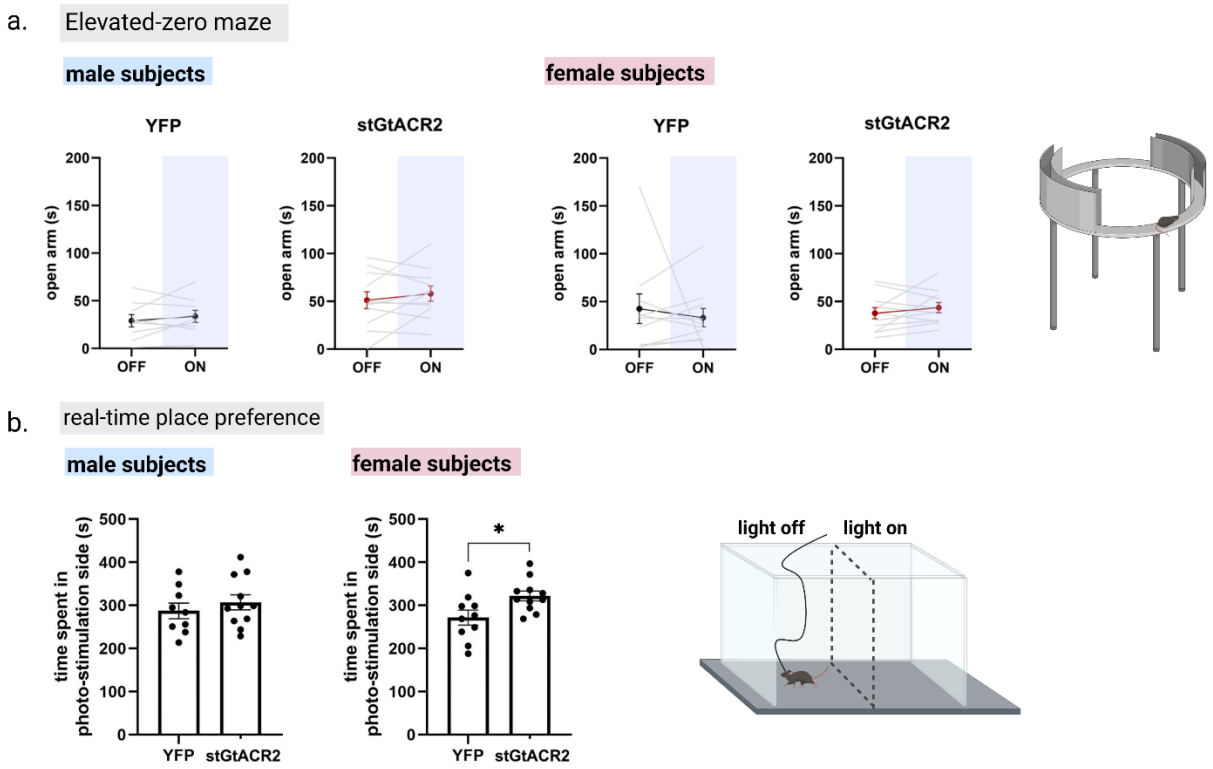

Figure 7-3– BNST AVP cell inhibition triggers real-time place preference in females, but not anxiety-like behavior. *(a) Time spent in the open arm of the elevated-zero maze (EZM). Blue light stimulation (ON) of stGtACR and YPF males and females did not alter time spent in the open arms of the EZM. (b) Real-time place preference. stGtACR females preferred to spend more time in the "light on" chamber* compared to YFP control females, *in which BNST AVP cells were inhibited. Light stimulation did not affect* time spent in the "light on" chamber *of* ChR2 and *YFP male subjects. *p<0.05.*

7.3.4 Optogenetic activation of BNST AVP cells increases social investigation in both sexes and male urine marking toward female stimuli

We next examined the behavioral effects of optogenetic stimulation of BNST AVP cells. We focused on testing social investigation and communication (i.e., urine marking and ultrasonic vocalizations) toward male and female conspecifics in the three-chamber apparatus, anxiety-like behavior in the EZM, and real-time place preference. To distinguish if BNST AVP cells are sufficient for these behaviors, we bilaterally injected Cre-dependent

AAVs for cell activation into the BNST of adult AVP-iCre+ male and female mice and implanted fibers above the BNST (Figure 7-4a-c).

We confirmed that optogenetic activation of BNST AVP cells significantly increased c-Fos expression in these cells compared to YFP controls ($F(1,35) = 234.17$, $p < 0.000001$; $\eta^2 = 0.87$; Figure 7-4b-c). We also confirmed *in vitro* that ChR2-expressing cells in the BNST are activated by light application at 10Hz (Figure 7-4d). Optogenetic activation of BNST AVP cells in males elicited profound increased time spent investigating male and female stimuli compared to investigation during the light-off condition (Mixed model ANOVA, treatment*light*sex interaction, $F(1,36) = 7.02$, $p = 0.012$; $\eta^2 = 0.5$; *post hoc*: $p = 0.002$ (male stimuli), $p = 0.004$ (female stimuli); Figure 7-4e). While inhibiting BNST AVP cells did not have an impact on female social investigation, activating these cells significantly increased the time females spent investigating male stimuli (*post hoc*: $p = 0.002$ (male stimuli); Figure 7-4e).

Urine marking is used for both mate attraction and territorial demarcation by dominant males (Kimura and Hagiwara 1985; Hurst 1990; Arakawa et al. 2008; Roberts et al. 2010). Although urine marks are predominantly produced by males in lab mice, we tested if stimulation of BNST AVP cells affected urine marking in both sexes in the presence of male or female conspecifics. We found that BNST AVP cell stimulation only affected male urine marking (total area) in the presence of a female stimulus (Mixed model ANOVA, treatment*light*sex interaction, ($F(1,36) = 4.5$, $p = 0.04$; $\eta^2 = 0.4$; *post hoc*: $p = 0.009$; Figure 7-4f). Male urine marking toward male stimuli and female urine marking was unaffected (Figure 7-4f; Supplementary Figure 2, data not shown).

Male mice produce more ultrasonic vocalizations (USVs) during investigation of females than do females; therefore, USVs are presumed to be male-typical promoters of opposite-sex

affiliation (Warburton et al. 1989; Chabout et al. 2015; Sugimoto et al. 2011). Although activation of BNST AVP cells increased male social investigation and urine marking toward female conspecifics, it did not affect USVs (Supplementary Figure 2, data not shown).

Since several studies point at the role of central AVP stimulating anxiety-like behavior (Bielsky et al. 2004; Neumann and Landgraf 2012; Mak et al. 2012; Fabio et al. 2012), we next tested if activation of BNST AVP cells affected anxiety-like behavior in the EZM. We did not find behavioral differences between light-on and light-off conditions in the EZM (Supplementary Figure 3). Since BNST AVP cell inhibition caused a place-preference in females, we next tested if optogenetic stimulation of BNST AVP cells was rewarding or aversive within the real-time place preference test. However, we found no differences in the time spent in the light-on chamber compared to YFP controls in both sexes (Supplementary Figure 3, data not shown).

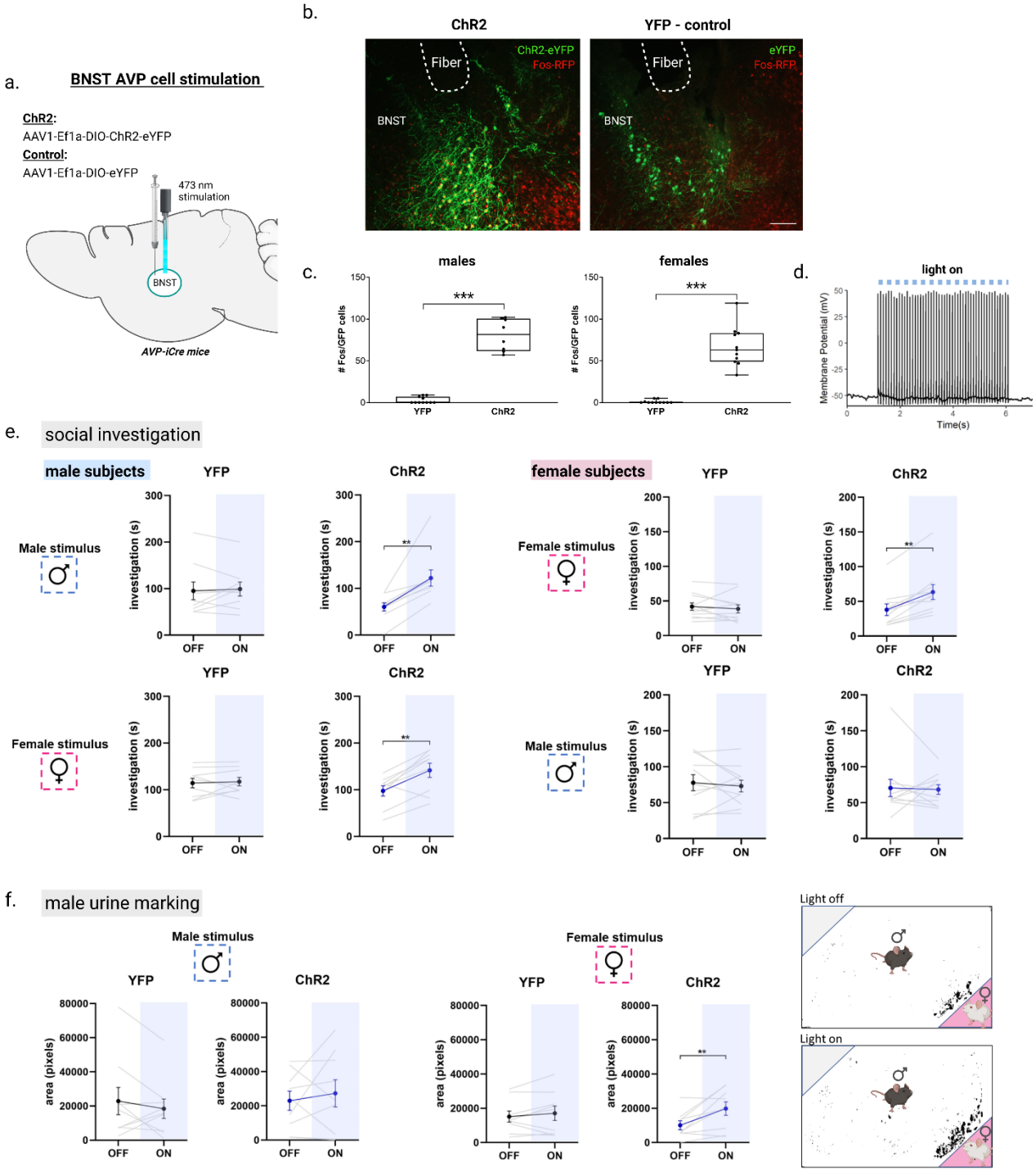

Figure 7-4– Optogenetic activation of AVP-BNST cells increases social investigation in both sexes and male urine marking toward female stimuli. *(a-b) Bilateral BNST injection and fiber implantation site; coordinates: DV: -4.4, AP: +0.15, ML: ±0.8; modified from Paxinos and Franklin (2012). (b) Example images of merged BNST-AVP cells infected with either the excitatory ChR2 adeno-associated virus (ChR2) or YFP control virus, both colocalized with c-Fos+ cells (red). (c) Boxplots of the number of BNST AVP ChR2/YFP cells colocalized with c-*

*Fos. Blue light stimulation robustly increased the number of BNST AVP labeled cells colocalized with c-Fos. (d) Representative trace from whole-cell current-clamp recording of ChR2-mCherry-expressing cells activated by light application at 10Hz for 5 seconds (e) Investigation (in seconds) by male and female subjects during the three-chamber test (male subjects: YFP, n=9 and ChR2, n=9; female subjects (YFP, n=10 and ChR2, n=11) during light-OFF and light-ON conditions, counterbalanced. Blue light stimulation (ON) of AVP-BNST cells in ChR2 males significantly increased time spent investigating male and female stimuli compared to investigation during light-OFF condition. Blue light stimulation (ON) of AVP-BNST cells in ChR2 females significantly increased time spent investigating male stimuli compared to investigation during light-OFF condition. Light stimulation did not affect investigation times of YFP male and female subjects to either stimulus type (female or male). (f) Total area of urine marking by male subjects during the three-chamber test. Blue light stimulation (ON) of AVP-BNST cells in ChR2 males significantly increased urine marking in the presence of a female stimulus. Each point and horizontal line represent individual within-subject data. Overlapping data are represented as one point/line. **p<0.01.*

7.3.5 Stimulation of LS terminals originating from BNST AVP cells increases inhibitory LS signal

The lateral septum (LS) has long been known to be the major target of sexually-differentiated AVP innervation and has been implicated in the sex-specific regulation of social recognition, social play, social affiliation, aggression, and anxiety-related behavior (De Vries and Panzica 2006; Bredewold and Veenema 2018; Rigney et al. 2023). While more indirect approaches have supported the BNST as the major source of this AVP innervation, we recently confirmed that the BNST AVP cells send their strongest projections to the LS (Rigney et al. 2023). However, it was not clear whether BNST AVP cell-to-LS projections represented functional synapses or if this connectivity promoted inhibitory or excitatory responses within the LS. Our results are similar to what was found previously in-vitro (Raggenbass 2008), where optogenetic stimulation of BNST AVP cell-to-LS terminals directly excited a neuronal subpopulation, but caused overall inhibition in virtually all LS neurons (Figure 7-5a-d). Since optogenetic stimulation of LS terminals can possibly cause the release of other neurotransmitters, such as GABA, we further tested that our results were mediated by

vasopressin receptors (V1aR). We found that stimulation of AVP terminals originating from BNST AVP cells in the LS failed to inhibit patched neurons in the presence of a selective V1aR antagonist (d(CH2)5[Tyr(Me)2,Dab5]), suggesting that optogenetic stimulation of BNST AVP-LS terminals increases inhibition within the LS, which is mediated by V1aR (Figure 6a-b).

7.3.6 Optogenetic activation of AVP-BNST cell projections to the lateral septum (LS) increases social investigation and anxiety-like behavior in males, but not in females

We next examined the behavioral effects of BNST AVP cell-to-LS terminal stimulation in male and female mice. To evaluate if BNST AVP cells project to the LS to regulate social investigation, communication, or anxiety-like behavior, we bilaterally injected Cre-dependent AAVs for LS terminal activation into the BNST of adult AVP-iCre+ male and female mice and implanted fibers above the LS (Figure 7-5a). Blue light stimulation of BNST AVP cell-to-LS terminals in ChR2 males significantly increased time spent investigating male and female stimuli compared to investigation during the light-off conditions (Mixed model ANOVA, treatment*light interaction, (F (1,17) = 6.9, p = 0.01; η2 = 0.6; post hoc: p = 0.001 (male stimuli), p = 0.02 (female stimuli); Figure 7-5e). Blue light stimulation of LS terminals in ChR2 females did not affect social investigation time compared to investigation during light-off conditions (Figure 7-5e).

Similar to stimulating BNST AVP cells, blue light stimulation of BNST AVP cell-to-LS terminals had a modest impact on male urine marking behavior. However, in contrast to the prior experiment where cell stimulation increased the total amount of urine deposited, BNST AVP-LS terminal stimulation only increased the number of marks males deposited in the presence of a female (Mixed model ANOVA, treatment*light*stimulus interaction, (F (1,17) = 5.44, p =

0.038; $\eta 2 = 0.4$; post hoc: $p = 0.03$; Supplementary Figure 4, data not shown). Blue light stimulation of BNST AVP-LS terminals did not affect other aspects of social communication, such as USVs (Supplementary Figure 4, data not shown).

Although BNST AVP cell stimulation or inhibition did not influence anxiety-like behavior within the EZM, BNST AVP-LS terminal stimulation in ChR2 males significantly decreased time spent in the open arm of the EZM (Mixed model ANOVA, treatment*light*sex interaction, ($F(1,17) = 6.2$, $p = 0.024$; $\eta 2 = 0.5$; post hoc: $p = 0.008$; Figure 5f). In females, blue light stimulation had no effect on time spent in the open arm of the EZM (Figure 5f). We found no significant differences in the real-time place preference test (Supplementary Figure 4, data not shown).

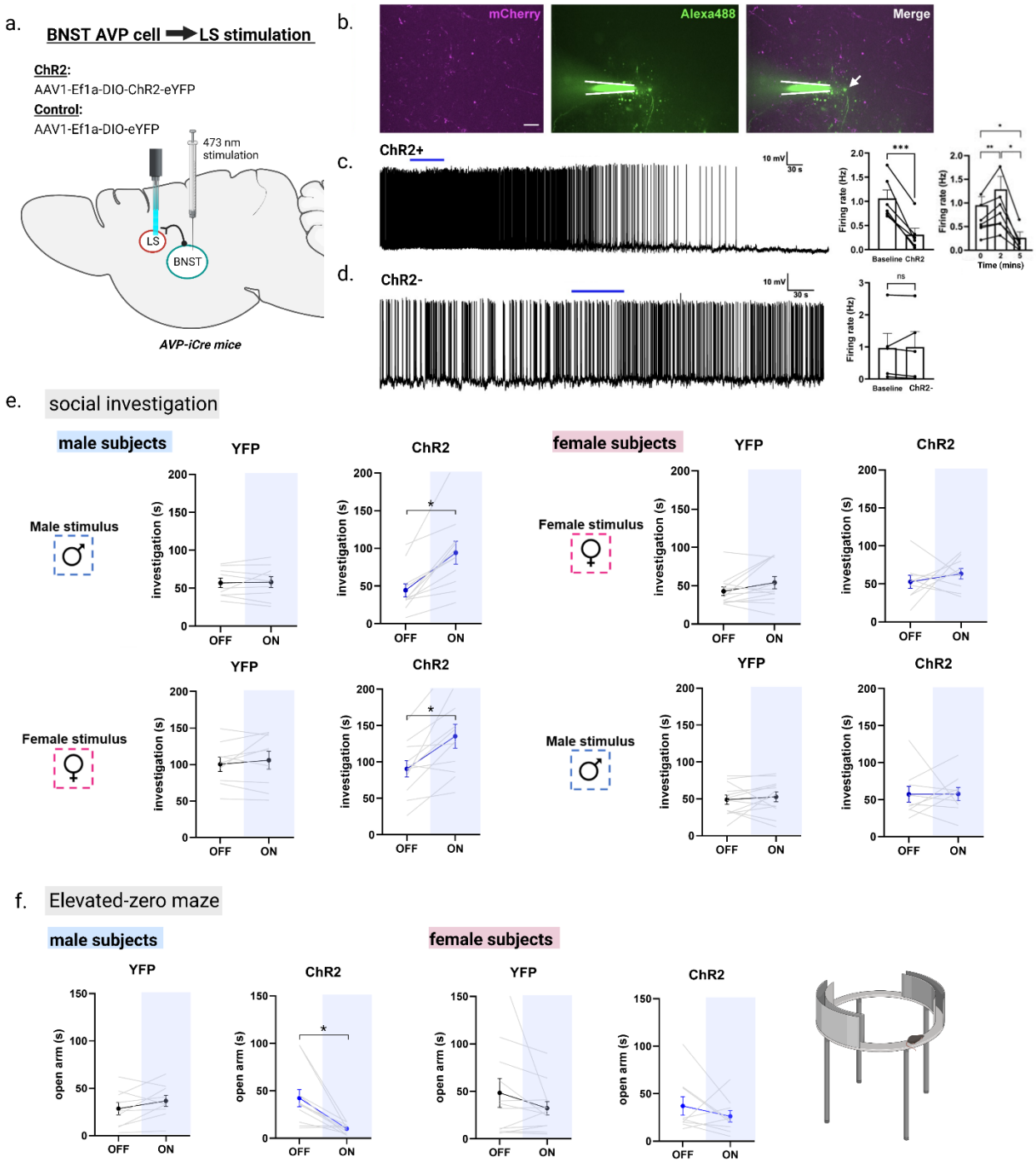

Figure 7-5– Optogenetic activation of AVP-BNST cell projections to the lateral septum (LS) increases social investigation and anxiety-like behavior in males, but not females. Stimulation of LS terminals originating from BNST AVP cells increases inhibitory LS signal *(a) Bilateral BNST injection of ChR2 adeno-associated virus (ChR2) and fiber implantation within the lateral septum (intermediate zone). (b) Images of patch clamped LS neurons filled with Alexa 488 dye (green) surrounded by mCherry-labeled ChR2 fibers afferents from BNST (magenta). Scale bar= 25µm. (c) (left) ChR2+/+ stimulation* (blue line) *of AVP terminals*

*originating from BNST AVP cells inhibits patched neurons in the vicinity. Summary data of ChR2-induced inhibition of firing rate in LS neurons (***p <0.001, paired t test). ChR2+/+ stimulation of AVP-BSNT terminals (blue line) evoked an initial increase followed by a robust inhibition of the patched LS neuron. Summary data showing mean changes in firing discharge of LS neurons at times 0, 2 and 5 mins following optogenetic activation of AVP-BNST fibers in the LS. *p< 0.05 and ** p< 0.01, Tukey multiple comparison test, RM one-way ANOVA. (d) (left) Stimulation of AVP terminals originating from BNST AVP cells that express mCherry but not ChR2. An identical stimulus fails to evoke a firing change. (right) Summary data of firing rate of ChR2-/- neurons before and after stimulation (p >0.05, paired t test). (e) Investigation (in seconds) by male and female subjects during the three-chamber test (male subjects: YFP, n=9 and ChR2, n=11; female subjects (YFP, n=12 and ChR2, n=9) during light-OFF and light-ON conditions, counterbalanced. Blue light stimulation (ON) of AVP-BNST-LS terminals in ChR2 males significantly increased time spent investigating male and female stimuli compared to investigation during light-OFF condition. Blue light stimulation (ON) of AVP-BNST* LS terminals *in ChR2 females did not affect investigation times compared to investigation during light-OFF condition. Light stimulation did not affect investigation times of YFP male and female subjects to either stimulus type (female or male). (f) Time spent in the open arm of the elevated-zero maze (EZM). Blue light stimulation (ON) of AVP-BNST-LS terminals in ChR2 males significantly decreased time spent in the open arm of the EZM. In females, blue light stimulation had no effect on time spent in the open arm of the EZM. Each point and horizontal line represent individual within-subject data. Overlapping data are represented as one point/line. *p<0.05.*

7.3.7 Antagonism of V1aR in the LS blocked optogenetic-mediated increases in male social investigation and anxiety-like behavior

Since optogenetic LS terminal stimulation may release other neuroactive substances co-expressed by BNST AVP cells, we next tested if V1aR was required for optogenetic-mediated increases in male social investigation and anxiety-like behavior. For dual delivery of V1aR antagonist and light stimulation in the LS, Dual Optofluid cannulas with interchangeable injectors were implanted. We ran two groups of ChR2+ injected males, where each group was assigned to receive either novel male or female stimuli. Mice were tested with their respective stimuli in four separate counterbalanced tests: light-off + saline, light-on + saline, light-off + V1aR antagonist, light-on + V1aR antagonist. Blue light stimulation of AVP-BNST-LS terminals in both ChR2 male groups significantly increased time spent investigating male and

female stimuli compared to investigation during the light-off condition (Mixed model ANOVA, drug*light interaction, ($F(1,16) = 11.76$, $p = 0.003$; $\eta^2 = 0.42$; *post hoc*: $p = 0.01$ (male stimuli), $p = 0.006$ (female stimuli); Figure 7-6c). However, when a V1aR antagonist was injected into the LS, optogenetic-mediated increases in male social investigation were blocked (Figure 7-6c). We were unable to replicate the effect that BNST AVP-LS terminal stimulation had on male urine marking and our manipulation did not affect USVs (Supplementary Figure 5, data not shown). BNST AVP-LS terminal stimulation in ChR2 males significantly decreased time spent in the open arm of the EZM (Mixed model ANOVA, treatment*light*sex interaction, ($F(1,17) = 6.2$, $p = 0.024$; $\eta^2 = 0.5$; *post hoc*: $p = 0.008$; Figure 7-6d) and antagonism of V1aR in the LS did not affect male anxiety-like behavior in the EZM when paired with optogenetic LS terminal stimulation (Figure 7-6d).

We examined whether administering the V1aR antagonist into the LS, without any light stimulation, resulted in behavioral changes in male mice compared to when they received saline injections. We found a main effect with the antagonist, where it reduced male social investigation time of both male and female stimuli ($F(1,16) = 13.05$, $p = 0.002$, $\eta^2 = 0.45$). However, V1aR antagonism within the LS did not alter male social communication or anxiety-like behavior within the EZM.

Finally, we wanted to confirm that optogenetic-mediated increases in male social investigation was limited to social interest and not novelty interest. We therefore stimulated BNST AVP cell-to-LS terminals in male mice and tested the investigation of a novel object (i.e., lego or hot wheel car) within the three-chamber apparatus. Although LS terminal stimulation increased social investigation in males, it did not increase investigation of a novel object (Supplementary Figure 5, data not shown).

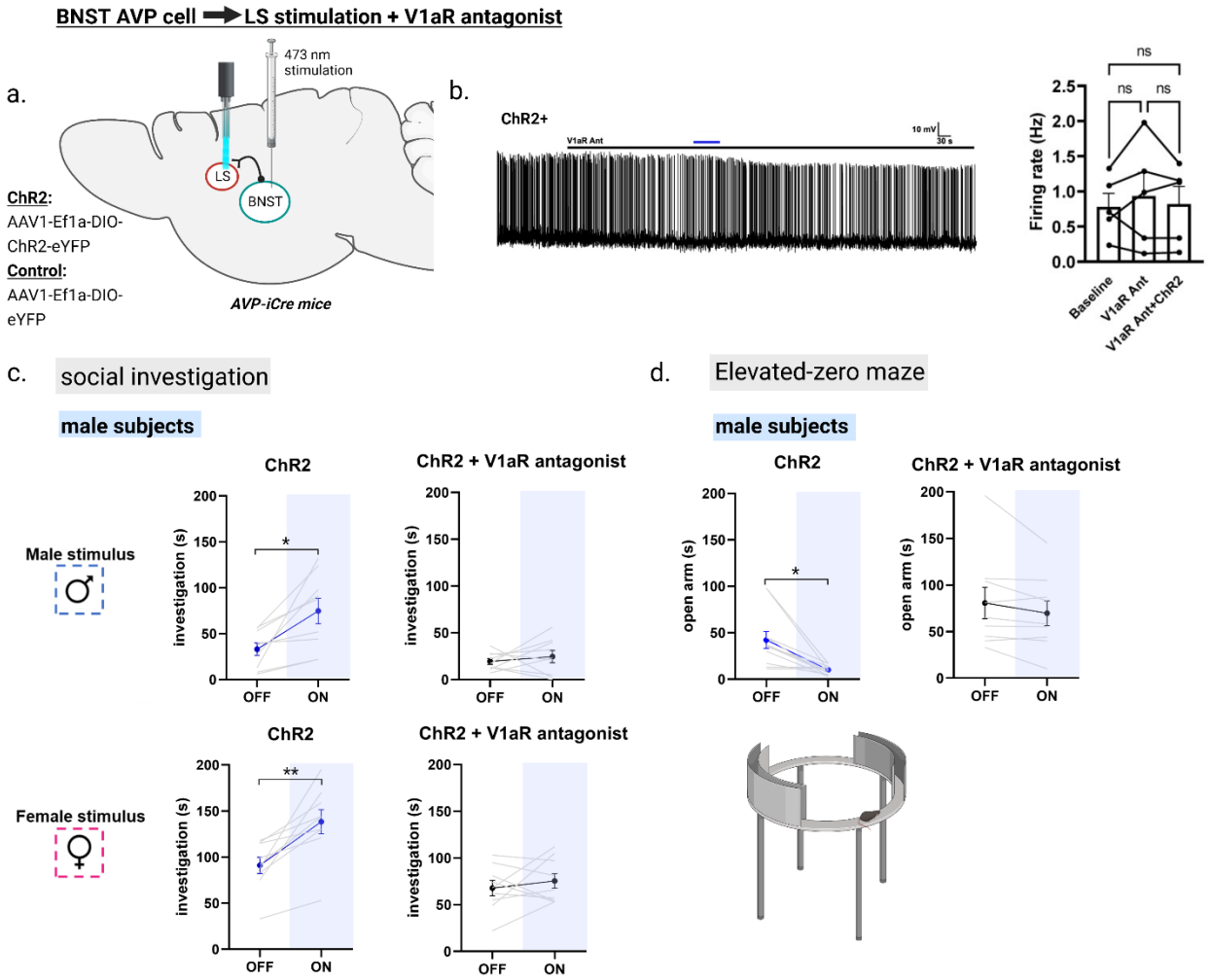

Figure 7-6– Antagonism of V1aR in the LS blocked optogenetic-mediated increases in male social investigation and anxiety-like behavior. *(a) Bilateral BNST injection of ChR2 adeno-associated virus (ChR2) and fiber implantation within the lateral septum (intermediate zone). (b) (left) ChR2+/+ stimulation of AVP terminals originating from BNST AVP cells fail to inhibit patched neurons in the presence of a selective V1aR antagonist (d(CH2)5[Tyr(Me)2,Dab5]). (right) Summary data of firing rate at baseline, in the presence of the V1aR antagonist, and then after subsequent stimulation of ChR2+/+ (p <0.05, One-Way repeated measures ANOVA). (c) Investigation (in seconds) by male subjects during the three-chamber test. Two groups of ChR2+ injected males were tested with either a male or female stimulus (LIGHT OFF/ON),* and *received the same type of stimulus (i.e., novel male or female) with LIGHT OFF/ON + a highly selective V1aR antagonist (*subjects tested with a *male stimulus: n=9;* subjects tested with a *female stimulus: n=9). All conditions were counterbalanced. Blue light stimulation (ON) of AVP-BNST-LS terminals in both ChR2 male groups significantly increased time spent investigating male and female stimuli compared to investigation during light-OFF condition. In the same male subjects, antagonism of V1aR in the LS blocked optogenetic-mediated increases in male social investigation. (d) Time spent in the open arm of the elevated-zero maze (EZM). Blue light stimulation (ON) of AVP-BNST-LS*

*terminals in ChR2 males significantly decreased time spent in the open arm of the EZM, and in the same males, antagonism of V1aR in the LS blocked optogenetic-mediated increases in male anxiety-like behavior. *p<0.05.*

7.4 Discussion

Using cell-type specific optogenetic manipulations, we identified a circuit that promotes sex-specific behaviors. This is the first time that immediate and targeted alteration of a sexually-dimorphic population of neuropeptidergic cells has resulted in a sex-specific variation in mammalian social behaviors, with a particular focus on male-biased responses. We found that inhibition of the sexually dimorphic AVP cell group in the bed nucleus of the stria terminalis (BNST) in males reduced social investigation of other males, while stimulation of these cells increased overall male social investigation and communication toward females. In females, which have significantly fewer AVP BNST cells, similar manipulations minimally affected social investigation, where BNST AVP cell stimulation only caused an increase in female-to-male investigation. We found that activation of BNST AVP cell axon terminals in the lateral septum (LS) increased male, but not female, social investigation and anxiety-like behavior in the elevated-zero maze (EZM). This effect is likely mediated by V1aR since V1aR antagonism in the LS blocked optogenetic-mediated increases in male social investigation and anxiety-like behavior. Our findings suggest that the sexually dimorphic AVP cells in the BNST contribute to sex-specific aspects of social approach and anxiety-like behavior through their connections with the LS, which are mediated by V1aR receptors.

Although optogenetic activation carries limitations in terms of mimicking physiological BNST AVP cell activity, we speculate that the ability of photostimulation to increase social and anxiety-related behaviors in males suggests that these cells, in some degree, are important drivers for these behaviors. However, the native, real-time activity of the BNST-LS AVP pathway

during social interactions has yet to be established. Despite this limitation, we demonstrate that BNST AVP cells are highly colocalized with c-Fos, a marker for neuronal activity, in the presence of social stimuli in both sexes as compared to mice that did not receive any social stimuli.

The strongest, most consistent effect of optogenetic manipulations of BNST AVP cells was the modulation of same-sex social investigation, which we found in males but not females. Activation of BNST AVP cells and BNST AVP cell axon terminals in the LS substantially increased male social investigation, with minimal effects seen in females. These effects were not due to increased interest in novelty since investigation of novel objects was unaltered. Furthermore, our results were specific to social interest, as real-time place preference scores were largely unaffected. Since BNST AVP cells and the BNST-LS AVP circuit is involved in social recognition (Borie et al. 2021; Whylings et al. 2020), it is possible we affected the animal's ability discriminate between social cues; however, this is unlikely since males investigated and communicated more with female stimuli compared to male stimuli. Our results suggest that one function of AVP cells in the BNST is to generate male-typical approach, investigation, and assessment of potential territorial competitors (Oldfield et al. 2015), which is consistent with the observation that knocking down AVP in the BNST of territorial birds and mice reduces social contact with other males (Kelly and Goodson 2013; Rigney et al. 2021), and that over-expression of V1a receptors (Landgraf et al. 2003) increases active male-male interactions and aggressive behavior in rats. Our results further suggest that the LS is a key target for AVP projections from the BNST that function to regulate social approach in males. This is somewhat consistent with prior research demonstrating that V1aR within the LS is important for territorial aggression (Veenema et al. 2010).

Stimulation of BNST AVP cells increased the total area males urine marked toward females. These results are paradoxical from our prior work where BNST AVP cell ablations caused the same behavioral effect. It is possible that BNST AVP cell ablations caused compensatory pathways to activate due to the loss of AVP cells. Additionally, stimulation of BNST-LS AVP terminals only modestly increased male urine marking behavior toward females (i.e., number of marks) and this effect was not replicated in our final V1aR antagonist experiment. Our manipulations in these experiments, along with prior chronic BNST AVP depletion (Rigney et al. 2019; Rigney et al. 2021), did not affect ultrasonic vocalizations (USVs), suggesting that sources other than BNST AVP cells are responsible for mediating this form of social signaling behavior. Together, these results indicate that the BNST AVP cells regulate a specific social signaling behavior in males (i.e., urine marking), but not through projections to the LS.

Central AVP has been repeatedly implicated in the generation of anxiety states (Ebner et al. 2002; Bielsky and Young 2004; Raggenbass 2008). We did not observe changes in anxiety-like behavior during BNST AVP cell inhibition or stimulation. These results were somewhat surprising because AVP in the LS, an important target of the BNST AVP cell, controls anxious states (De Vries and Panzica 2006; Landgraf et al. 1995; Beiderbeck et al. 2007). However, when we specifically stimulated BNST-LS AVP terminals, males, but not females, increased anxiety-like behavior within the EZM and these results were mediated through V1aR within the LS. This would indicate that stimulation of BNST AVP cells influenced other downstream structures from the LS that blocked BNST-LS AVP's anxiogenic effect. Intriguingly, BNST-LS AVP terminal stimulation increased male social interest and anxiety-like behavior in separate

tests. Further research is necessary to investigate if our manipulations may have raised alertness levels, resulting in heightened vigilance among male mice in the EZM.

Optogenetic LS terminal stimulation may have released other neuroactive substances co-expressed by BNST AVP cells, such as galanin (Miller et al. 1993; Planas et al. 1995). Our last experiment aimed to test if V1aR action was required for optogenetic-mediated increases in male social investigation and anxiety-like behavior. We found that V1aR blockade in the LS paired with BNST-LS AVP terminal stimulation blocked increases in male social investigation and anxiety-like behavior. In vitro recordings revealed that optogenetic stimulation of BNST-LS AVP terminals initially excited a neuronal subpopulation, but caused overall inhibition in virtually all LS neurons, similar to what has been found previously (Raggenbass 2008). The presence of a selective V1aR antagonist prevented the inhibition of patched neurons, indicating that the optogenetic stimulation of BNST AVP-LS terminals produces an increase in inhibition within the LS that is reliant on V1aR mediation.

Our findings indicate that stimulating BNST-LS AVP terminals results in a rise in inhibition throughout the LS, mediated by V1aR, leading to increased male social investigation and anxiety-like behavior. Our research has demonstrated the possibility of using modern molecular approaches to identify the contributions of AVP systems on social behavior and highlighting the sexually dimorphic AVP cells in the BNST, and their projections to the LS, as contributors to sex differences in social behavior.

8 CONCLUSION

8.1 Concluding remarks

AVP in the nervous system originates from several distinct sources which are, in turn, regulated by different inputs and regulatory factors. Based on both direct and indirect evidence, we can begin to define the specific roles of AVP cell populations in social behavior (Figure 8-1). The cell population that has been most directly linked to social behavior is the sexually differentiated, steroid-sensitive BNST AVP cells. This population appears to be a likely driver of male-typical social investigation (of potential competitors) and social recognition through its projections to the lateral septum, as well as regulators of affiliative responses (pair bonding, flocking, sexual behavior). Although much less is known about the other sex-different, steroid-sensitive AVP population in the MeA, it too may be involved in social investigation as well as defensive behavior. Paradoxically, one of the largest AVP cell populations, the PVN, has the least amount of direct evidence for its role in social behavior. The indirect evidence that is available presents a rather inconsistent picture of the role of PVN AVP cells in social behavior and may, at best, suggest a modest role for PVN AVP cells in the behavioral responses to stress. In contrast, direct manipulations of PVN AVP cells in mice suggest that these cells normally suppress inappropriate social/emotional behavior: social investigation in females and anxiety-like behavior in males. However, the specific role that PVN AVP cells play in social behavior may depend heavily on species- and social system- differences. For example, PVN AVP may promote social affiliation in gregarious bird species of both sexes as well as suppressing inappropriate (mate-directed) male aggression. While the available evidence suggests a minor role for PVN, BNST, and MeA AVP cells in aggressive behavior, the reputation of AVP as a pro-aggressive neuropeptide in males rests almost exclusively on AVP action within the anterior

hypothalamus, with the NC and parts of the SON likely contributors of behaviorally-relevant AVP. More recently, AVP expression within early segments of the olfactory systems has been shown to facilitate odor-based social recognition. Consequently, AVP may facilitate social odor memory by both biasing social odor processing (olfactory bulb and cortex) and by modulating memory consolidation through more central circuits (BNST-LS, PVN-hippocampus). Even though AVP in the SCN plays an important role in adjusting circadian rhythms, it does not appear to play a critical role in social behavior but may, instead, regulate reward and anxiety-like behavior. How (or whether) these different AVP systems interact in influencing social behavior is completely unknown.

Several themes and conclusions emerge from examining the evidence implicating different AVP sources in social and emotional behavior. First, because different AVP systems project to overlapping or contiguous brain regions, limiting analysis to sites of AVP action prevents a complete understanding of how AVP regulates social behavior (Kelly and Goodson, 2014a, 2014c). Similarly, as indirect measures of AVP action often show contradictory results (particularly in the PVN), direct tests of AVP cell population function are needed to clarify how different systems function across the neural axis. Second, the few studies that have tested the function of specific AVP cell projection zones have only examined a limited set of social behaviors (social investigation, communication, and recognition). Consequently, the role of AVP sources in social processing must be expanded to include other aspects of social behavior, different contexts, and other model organisms, preferably using comparable and robust measures of function (i.e. real-time activity of these circuits). Lastly, it is clear that sex differences in function may be apparent in both sexually-dimorphic structures (BNST/MeA) as well as ones without prominent structural differences (PVN). For example, PVN and BNST AVP cells in

mice control social and emotional behavior differently in the two sexes (Rigney et al., 2021, 2020b, 2019) (Figure 8-2), such that BNST AVP cell ablations decrease male social investigation of other males (Rigney et al., 2019) (Figure 8-2a), whereas PVN AVP cell ablations increase only female social investigation of males and females (Rigney et al., 2020b) (Figure 8-2b). This suggests that the PVN may contribute to baseline sex differences in social investigation, since males show higher levels of investigation than do females (Bluthé and Dantzer, 1990; Holmes et al., 2011; Karlsson et al., 2015; Markham and Juraska, 2007) and removal of these cells removes the sex difference in social investigation (Rigney et al., 2020b).

Translational opportunities

Because of the effects of AVP on social behaviors, AVP is a potential therapeutic target for treating disorders such as autism spectrum disorder (ASD), where social functioning is compromised. Oxytocin (OXT), a closely related neuropeptide, has been more intensively investigated in this regard, and its therapeutic potential is related to its modulation of social motivation and the salience of social stimuli (DeMayo et al., 2019; Ford & Young, 2021a, 2022). Single cell transcriptomics studies show that PVN OXT neurons projecting within the brain that mediate social reward behaviors are disrupted in a mouse model of ASD, and those neurons are enriched for ASD risk genes(Lewis et al., 2020). Further transcriptomic work exploring the function of subtypes of OXT and AVP-expressing cells (Romanov et al., 2017) would be useful in delineating relevant sub-circuits driving social reward.

Both AVP and V1aR antagonists have been explored as potential ASD, schizophrenia, and drug abuse therapies (Rae et al., 2022). Based on a subset of preclinical studies demonstrating anxiogenic and agonistic effects of AVP, the V1aR antagonist balovaptan has been tested in

clinical trials in ASD subjects with mixed effects on social endpoints (Bolognani et al., 2019; Jacob et al., 2022). Similarly, male rhesus macaques in large groups that have naturally impaired social functioning have decreased AVP in cerebrospinal fluid (Parker et al., 2018). This led to investigations in pediatric ASD and control samples that also found decreased CSF AVP (Oztan et al., 2018, 2020). Finally, intranasal AVP treatment considerably improved social abilities in children with ASD (Parker et al., 2019).

Translational opportunities for AVP may extend beyond mental health as polymorphisms in V1aR have been linked to variations in normal as well as disordered human behavior (Aspé-Sánchez et al., 2015). Social relationships are important for physical well-being as well (Bosch & Young, 2018). A recent lung cancer study found that a factor in sera of bonded monogamous California mice decreased oncogenic potential of tumor cells when compared to sera from bond-disrupted animals (Naderi et al., 2021). Identifying the molecular mechanisms by which positive social relationships are transduced to improved health and reduce social stress could lead to novel approaches for improving well-being, and as AVP and OXT are inextricably involved in social relationships, they may have unforeseen translational opportunities based on their function in the brain (Ford & Young, 2021b).

Modern molecular approaches are greatly enhancing our understanding of how and where AVP act to regulate social processes. Despite this progress, considerably more attention has been directed toward the OXT system than the AVP system in the past decade due to its perceived translational applications. However, more recent translational research suggests that both peptides have important clinical potential. Consequently, it will be important to rebalance OXT/AVP research in behavioral neuroscience. Specifically, more attention needs to be devoted to understanding the contribution of different peptide sources and their specific targets,

particularly with respect to AVP. The application of modern molecular approaches will greatly increase our basic understanding of how and where AVP acts to regulate social behavior, much in the same way as it has transformed research on oxytocin systems (Froemke and Young, 2021). This increasingly precise understanding of how AVP systems are organized and function may ultimately lead to better therapeutic interventions for psychiatric disorders characterized by substantial social deficits.

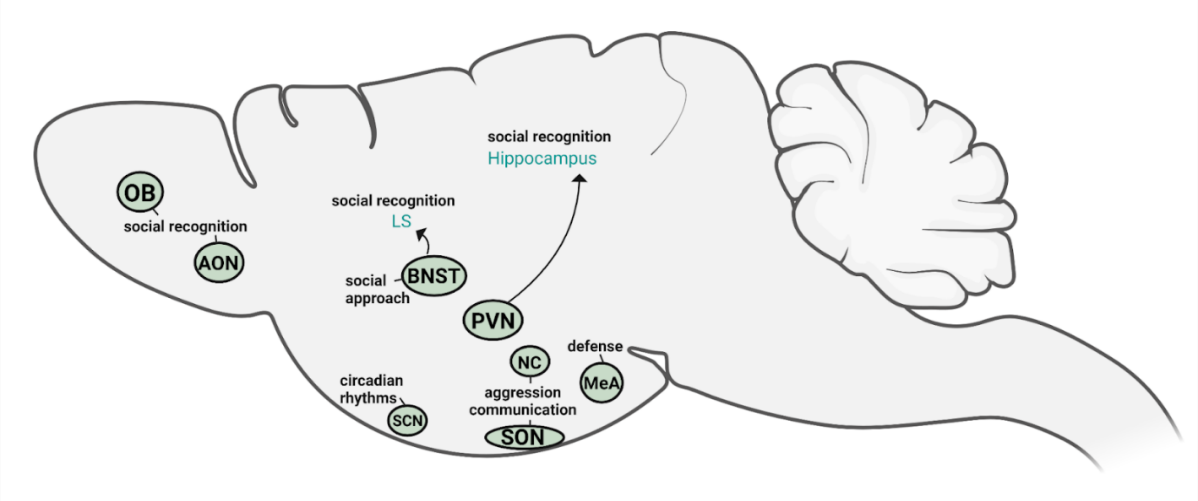

Figure 8-1- Sources of vasopressin relevant for social behavior. *Based on the available direct evidence, the AVP cells within PVN, BNST, OB, and AON directly influence social recognition and social approach, whereas NC and SON AVP cells modulate aggression and competitive signaling. MeA AVP cells primarily regulate defensive responses. SCN AVP cells regulate circadian rhythms, but are not directly linked to social behavior.*

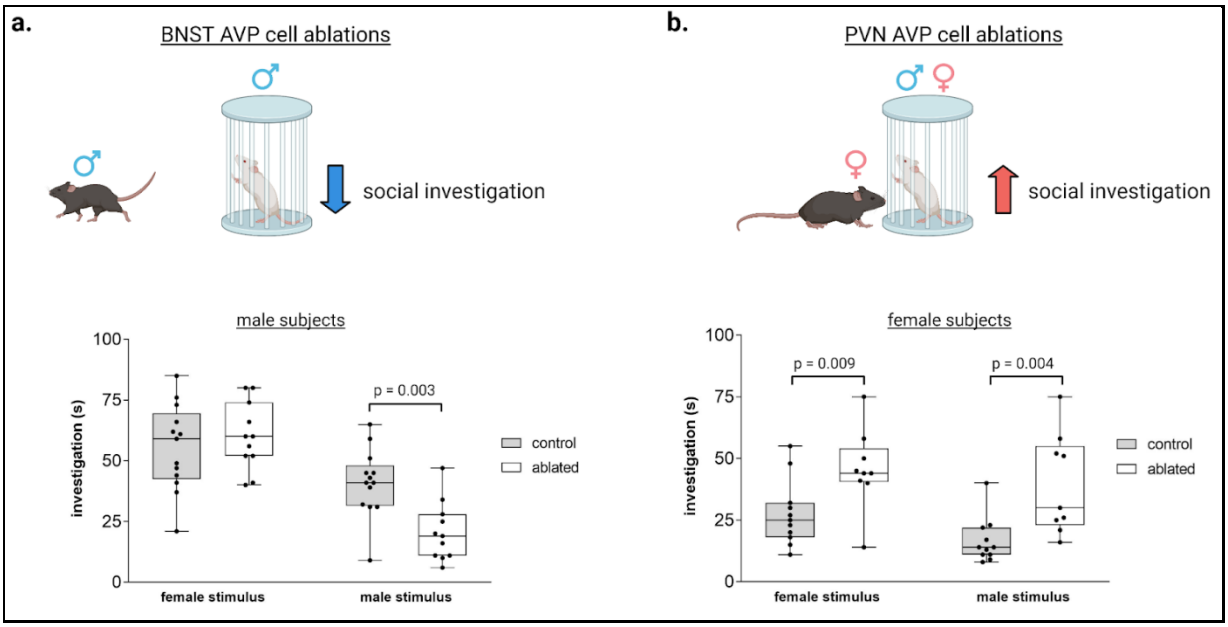

Figure 8-2- Distinct vasopressin cells modulate social investigation in a sex-specific manner. *(a) BNST AVP cell ablations in males reduce male-male social investigation (redrawn from (Rigney et al., 2019)). Boxplot and individual data points of time spent investigating male or female stimulus versus clean empty cage within the three-chamber apparatus. p = 0.003. A significant interaction was found between genotype and sex (F(1,41) = 4.9, p = 0.03. (b) PVN AVP cell ablations increase female social investigation of male (p = 0.004) and female (p = 0.009) stimuli compared to controls to similar levels as male social investigation (redrawn from (Rigney et al., 2020b)). A significant interaction was found between genotype and sex (F(1,44) = 5.33, p = 0.02).*

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