INVESTIGATING THE ROLE OF A NOVEL OXYTOCINERGIC NEURAL PATHWAY IN THE MODULATION OF MALE AND FEMALE JUVENILE SOCIAL PLAY

By

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ABSTRACT

Juvenile social play is a highly rewarding and motivated behavior displayed across mammalian species, including humans, non-human primates, and rats. Social play exposure has been shown to be essential in the development of social skills throughout the lifespan. Autistic children show decreased engagement in social play, which may contribute to lifelong decreases in social competency. Oxytocin (OXT) is a neuropeptide that facilitates the expression of socially rewarding behavior, but the role that OXT plays in the regulation of juvenile social play is still unknown. OXT is primarily produced in the paraventricular nucleus of the hypothalamus (PVN). PVN^{OXT} neurons interact with regions within the brain reward circuitry through direct connections to the nucleus accumbens (NAc), which in turn projects to the ventral pallidum (VP). The PVN, NAc, and VP have all been shown to regulate juvenile social play individually, but whether OXT signaling across these regions is necessary for the expression of social play is unknown. Therefore, I designed three sets of experiments to test the hypothesis that juvenile social play is modulated through this hypothalamic-striatal OXTergic pathway.

In aim 1, I used an excitatory DREADD construct to selectively stimulate PVN^{OXT} terminals in the NAc prior to social play exposure. Here, I predicted that chemogenetic stimulation of the PVN-OXT to NAc pathway would increase juvenile social play behavior in both sexes. Although there was no effect of stimulation on social play, I found that chemogenetic stimulation of PVN^{OXT} terminals in the NAc increased social investigation in males and decreased this in females. In aim 2, I determined the effects of chemogenetic inhibition of OXT receptor (OXTR) expressing neurons in the NAc, as

well as chemogenetic inhibition of NAc^{OXTR} terminals in the VP on the expression of juvenile social play behavior. As NAc inactivation has been shown to increase juvenile social play behavior, I predicted that chemogenetic inhibition of OXTR-expressing neurons in the NAc and of NAc^{OXTR} terminals in the VP would increase juvenile social play behavior in both sexes. Here, I found that chemogenetic inhibition of the NAc^{OXTR} neurons increased social play behaviors in females but had no effect on male social play behaviors. Additionally, chemogenetic inhibition of the NAc^{OXTR} terminals in the VP had no effect on social play. Lastly, in aim 3, I set out to determine the role of OXT and dopamine (DA) signaling within the NAc. DA plays a vital role in reward and the modulation of motivated behaviors, and DA receptors (DARs) are co-expressed on OXTR neurons in the NAc. Therefore, I hypothesized that both OXT and DA in the NAc modulate the expression of juvenile social play. In detail, I predicted that OXTR agonism would increase social play, while DAR antagonism in the NAc would decrease social play, and would do so in both males and females. Here, I found that OXTR agonism increased female social play behaviors but decreased male social play behaviors. Additionally, DAR-2 antagonism decreased social play in both sexes. However, DAR-1 antagonism had no effect on social play. Together, these studies indicate persistent sex differences mediated by OXT in which NAc-OXT signaling increases female social play behavior but may decrease male social play behavior. These findings could inform clinicians on the potential need for sex-specific OXT-based therapeutics in the treatment of social deficits.

This dissertation is dedicated to my partner, David Duplechain, my steadfast light throughout it all. This process never could have happened without you, and I am eternally grateful to have you in my life.

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CHAPTER 1. INTRODUCTION

The ability to adequately navigate the social world in which we live is a vital skill, and the social skills necessary for survival also change throughout development. Socialization begins in infancy and early childhood and is primarily comprised of offspring-parental interactions. As the child ages, peer-peer interactions become more commonplace. These peer-peer social interactions can manifest in a variety of ways, but arguably the most ubiquitous is social play (i.e. rough and tumble play). Social play within this early childhood and juvenile period is invaluable for the social and cognitive development of vertebrate and invertebrate animal species. In fact, low levels of social play can be a hallmark symptom in human social deficit disorders, such as attention-hyperactivity deficit disorder and autism spectrum disorder (ASD). Diagnoses of ASD are rising globally, yet there are no widely accepted and clinically proven therapeutics available to alleviate social behavior deficits displayed in ASD individuals. By improving our understanding of the neurobiological systems and pathways that contribute to the expression of juvenile social play, new treatment options may become available to improve play engagement, and later social competency, in ASD individuals.

The goal of this chapter is to introduce the concept of juvenile social play generally, as well as social play specifically in human children and laboratory rats. Next, I will discuss the diagnostic criteria and patterns of social behaviors seen in ASD individuals, specifically highlighting deficits in social play as seen in ASD children compared to neurotypical children. I will then introduce potential neurological mechanisms that may contribute to decreased social play engagement in this population. Following this background information, I will then propose that the

neuropeptide oxytocin (OXT) and the OXTergic circuitry within the brain is a likely candidate for the modulation of juvenile social play behavior based on both human and non-human animal studies. My thesis will focus on a hypothalamic to striatal pathway, specifically linking the paraventricular nucleus of the hypothalamus (PVN) to the ventral striatal pallidal system, which is comprised of the nucleus accumbens (NAc) and ventral pallidum (VP). I will discuss how these three brain regions individually modulate social behaviors, with an emphasis on social play. Finally, I will provide the background and rationale needed to link these three regions within one circuit, which I hypothesize is involved in the regulation of social play behavior. Together, this chapter will provide the adequate background and justification to explore this OXT-based circuit as a key modulator in the regulation of juvenile social play in both male and female juvenile rats.

Phylogeny and importance of social play

One of the most ubiquitous forms of peer-peer interactions in the animal kingdom is that of social play. Social play, also known as rough-and-tumble or play fighting, peaks in the juvenile to early adolescent phase of development. This behavior is prevalent across both mammalian and non-mammalian species, including humans, non-human primates, rats, hamsters, birds, and reptiles. Although the prevalence of social play has been widely noted across the animal kingdom, how best to define play, as well as the underlying function of play, is still debated.

Within the context of animal behavior, Gordon Burghardt (Burghardt, 2006; Burghardt et al., 2024; Graham & Burghardt, 2010) identified five key criteria to operationalize the definition of play. First, play is not fully functional in its current form, meaning the behavior is not necessary at the time for the animal's survival. Second,

play is done for its own sake. It is spontaneous, voluntary, rewarding and pleasurable to the animal engaging in this behavior. Third, play is incomplete, exaggerated, or otherwise modified in form or target from “serious” behaviors. Fourth, play is a repeated, but not overly stereotyped, action. And finally, play is only initiated when other survival needs are met, such as the animal is fed, healthy, and relatively free from stress (Burghardt, 2006, 2010; Burghardt et al., 2024; Palagi et al., 2016)). While these guidelines are applicable to play in general, they can also be used to help researchers differentiate social play from other social non-play behaviors.

Adding to the complexity, there are several competing theories set forth on the purpose and/or function of social play. First, there are the surplus energy and surplus resource theories of social play. The surplus energy theory is simple: the young (of any species) have more energy as they do not have to focus on gathering food, collecting resources, or protecting themselves from danger (Shiller, 1873). As such, they can use this extra energy to engage in play. The surplus energy theory is similar to the surplus resource theory, which states that in times of resource abundance there is an increase in play behavior (Burghardt, 2006). These theories imply that given the intrinsically rewarding character of play, it will almost always arise when the rest of one's needs are met. In the pre-exercise theory, play is thought to be practice for behaviors that are needed for future survival, such as aggression or mating behaviors (Groos, 1898). In recapitulation, play is thought to be a conserved behavior acted out not for a future purpose but as a holdover from previous evolutionary lines (Hall, 1906). More recently, social play has been directly linked to brain development and cognitive function (Siviy,

2016; L. J. M. J. Vanderschuren & Trezza, 2014; van den Berg et al., 1999a), which gives evidence that juvenile social play is necessary for typical development.

Social play in human children

While play has been observed in humans for centuries, the systematic study of play and the development of theories surrounding this behavior primarily began in the twentieth century. The seminal work of Jean Piaget (1962) posited that as a child develops both physically and cognitively, the types of play they engage in also change. This gave rise to Piaget's theory of cognitive development. According to Piaget, from birth to 18 months when a child is in the sensorimotor stage of development, the primary type of play displayed is "practice play". This period is characterized by repetitive sequences of actions aiding in the development of motor skills. Around two years old, children move into the preoperational stage and begin to display more purposeful actions, aiding in the transition to "symbolic play". Symbolic or pretend play typically involves the use of an object or toy that is manipulated by the child, but importantly, the child exerts meaning or symbolism to the object in question. Such examples of symbolic play include putting a doll down for a nap or playing "house". Symbolic play becomes increasingly more elaborate as the child begins the transition to the concrete operational stage around age seven. At this point, children begin to primarily play "games with rules". While play in the sensorimotor and preoperational stages are primarily isolated or object-oriented play, play in the concrete operational stage takes on a highly social character. This rule-oriented play leans on cooperation between the players, which can be facilitated by authority figures, such as adults, or by their peers.

While Piaget viewed changes in play as an indicator of social and cognitive development, Lev Vygotsky (1967) viewed play as a driving force in development. Vygotsky emphasized that play with peers, including symbolic play, has rules, which may be either implicit or explicit. Navigation and growth around these rules within a social context in turn allows for learning and practice of the appropriate response. In other words, Vygotsky hypothesized that participation in these peer-peer social interactions directly enhances cognitive flexibility.

One of the most common forms of this peer-peer interaction is social play, also termed “rough and tumble play” or “play fighting”. Social play is already seen in preschool aged children, and peaks from ages 7 to 11, constituting approximately 10% of observed recess behaviors (Anthony D. Pellegrini & Smith, 1998; A D Pellegrini, 1989). Though both boys and girls actively participate in social play, systematic studies have shown that girls participate at lower levels than boys (A D Pellegrini, 1989; VanRyzin et al., 2020; Whiting & Edwards, 1973). This sex difference in the rate of social play may in part come from societal expectations, in which girls are discouraged from participation in rough and tumble play. Indeed, when play was studied without the presence of an observer in same gender dyads, there were no differences between boys and girls in initiation of play (Scott & Panksepp, 2003). Although the levels of play were similar between the genders, in line with other studies, there were differences in play elements such as shoving (A D Pellegrini, 1989; Scott & Panksepp, 2003).

While social play may appear to mimic adult aggressive encounters in form, there are several unique aspects of this kind of play. One of the defining characteristics is that participants in social play show a positive affect (i.e. smiling, laughing) in comparison to

the negative affect seen in aggression (i.e. frowning). Additionally, social play is "imaginative" or "pretend"- there is no intent to harm, and actions are often reciprocated by the partner. This reciprocation in social play often leads to role-reversals (i.e. chaser-chased; dominate-subordinate), while aggressive encounters are typically more one-sided. Consistent with Vygotsky's hypothesis on play, because rough and tumble play requires both initiation of play and reciprocity to another child's initiation of play, it is thought to build social competence (A D Pellegrini, 1989). In fact, children that display higher levels of rough and tumble play also display more flexibility in social problem-solving (A D Pellegrini, 1992). These studies imply there is a great deferred benefit of social play, in which social play engagement can positively impact social function and competency later in life.

Children with social communication disorders such as ASD show deficits in their ability to properly navigate social interactions, such as social play with peers. ASD children often display difficulty in engaging in spontaneous social play with their peers, which can reduce the ability of autistic individuals to appropriately navigate social and emotional situations later in life (Jordan, 2003). Furthermore, ASD is characterized by diminished social interest (Sigman et al., 2004). For example, autistic children are less likely to choose a social reward than a non-social reward (Ruta et al., 2017), and ASD individuals report lower levels of satisfaction following social rather than non-social interactions (Supekar et al., 2018). These studies imply an overall decrease in social motivation in ASD. This decrease in social motivation in ASD individuals may be in part due to alterations of the reward pathways in the brain. Indeed, fMRI studies have demonstrated that ASD children show atypical connectivity between the ventral

tegmental area (VTA) and the nucleus accumbens (NAc) during social reward processing (Supekar et al., 2018). Though ASD children show high levels of engagement in rule-oriented social play, such as board games and structured play, this play lacks the implicit back and forth nature of spontaneous rough and tumble play (Jarrold et al., 1996; Morrier & Ziegler, 2018). This preference for rule-oriented play may contribute to decreased social competency later in life. Improving social play engagement of ASD individuals could improve social skills, which first requires a better understanding of the neurobiological mechanisms underlying the motivational and rewarding aspects of social play.

Social play in juvenile rats

Though play has been studied in several species under naturalistic and laboratory conditions, rough and tumble play has been most extensively characterized in the laboratory rat. Rats in captivity show high levels of social play and strong motivation to engage in play with conspecifics (Sergio M. Pellis & Pellis, 1987b; Poole & Fish, 1975; Trezza et al., 2009; L. J. M. J. Vanderschuren & Trezza, 2014). Like humans, social play in rats peaks during the juvenile period (postnatal day 25-40 in rats, roughly corresponding to ages 7-10 in children), and is easily distinguished from aggression. In rats, a play bout is typically initiated by a nape attack, in which the focal animal quickly nuzzles the nape of the partner's neck with their snout without biting, and the bout is terminated by pinning down the partner on their back (Sergio M. Pellis & Pellis, 1987b; L. J. M. J. Vanderschuren & Trezza, 2014). Aggressive encounters, however, typically involve biting, are focused more on the partner's flanks than the nape, and are rarely seen in juveniles (Sergio M. Pellis & Pellis, 1987b).

Studies in juvenile rats have demonstrated that social play is a highly motivating and rewarding behavior. For example, juvenile rats can be taught to lever press at increasingly high intervals for access to a conspecific with which to play (Achterberg, van Kerkhof, et al., 2016; Achterberg, van Swieten, et al., 2016), a measure of heightened motivation. Additionally, juvenile rats develop a conditioned place preference for chambers in which social play has occurred (Calcagnetti & Schechter, 1992; Trezza et al., 2009). Social play in rats also plays a key role in the development of social competency (Panksepp et al., 1984). Rats that are socially isolated during the juvenile period show cognitive and social deficits in adulthood, such as context-inappropriate displays of aggression (L. J. Vanderschuren et al., 1997; van den Berg et al., 1999a). Juvenile rats housed with adults or housed with other juveniles separated by a mesh screen, which allowed for auditory, olfactory, and tactile interactions except for social play, still showed decreased cognitive and emotional behaviors in adulthood (Einon & Morgan, 1977; Einon et al., 1978; Sergio M. Pellis & Pellis, 2007). Additionally, rats that were socially isolated during the juvenile period displayed increased foot-shock induced aggression in adulthood. This effect was attenuated in socially isolated rats that were allowed 1-hour of social interaction per day, most of which was comprised of social play (Einon & Morgan, 1977; Einon et al., 1978). These studies indicate that life-long social deficits are caused specifically by lack of social play, as other social interactions were unable to mitigate these effects. These parallels between humans and laboratory rats in social play and social motivation make rats an attractive model species in which to study the neurobiological mechanisms underlying the motivational and rewarding aspects of social play.

Neurobiological mechanisms of social play

Previous research has identified several brain regions involved in social play in juvenile rats. Studies using c-Fos as a marker for neuronal activation following social play exposure reported increased cellular activity within cortex and striatum of juvenile male rats (Gordon et al., 2002; van Kerkhof et al., 2014). Pharmacological inactivation via $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen of prefrontal brain regions decreases nape attacks and pins in juvenile male rats (van Kerkhof et al., 2013). As the prefrontal cortex is highly involved in executive function and decision making, activation of these regions during a socially and cognitively flexible interaction such as play may help facilitate this activity. Additionally, inactivation of the NAc core using $GABA_A$ and $GABA_B$ receptor agonists decreased duration of social play (van Kerkhof et al., 2013). This is in line with the hypothesis that reduced NAc activity is critical in reward processing (William A Carlezon & Thomas, 2009), which will be covered in more detail in later sections.

Given the rewarding and reinforcing properties of play, research into neurotransmitter systems that modulate play has primarily focused on the dopamine, opioid, and endocannabinoid systems (Trezza et al., 2010, 2011; L. J. Vanderschuren et al., 1997; L. J. M. J. Vanderschuren et al., 2016). Dopamine (DA) may be of particular importance in social play as systemic DA receptor antagonism decreases, and can virtually eliminate, this behavior in rats (Niesink & Van Ree, 1989; Siviy et al., 1996). In the opioid system, activation of specific receptor subtypes has opposing effects on social play, with μ-opioid receptor agonists increasing and κ-opioid receptor agonists decreasing social play in rats (L. J. Vanderschuren et al., 1995). Additionally, direct

agonism of endocannabinoid receptor CB1 decreases social play, while indirect agonism via endocannabinoid reuptake inhibition increases social play (Trezza et al., 2011). These studies reflect the complex mechanisms and receptor subtype specificity of opioids and endocannabinoids in the regulation of social play, though it is important to note that these studies used intraperitoneal (IP) injections and only included juvenile male rats. While there is less research outside the classic reward circuits and associated neurotransmitter systems in the neuromodulation of juvenile social play in rats, evidence suggests that the neuropeptide oxytocin (OXT) may also contribute to the regulation of social play.

Oxytocin as a potential regulator of social play

Oxytocin (OXT) -like neuropeptides are highly evolutionarily conserved peptides, which are found in mammalian (OXT), non-mammalian vertebrates (vasotocin), and avian (mesotocin) species (Grinevich et al., 2016; Gruber, 2014). The peripheral effects of OXT have been well defined, specifically through its release in the neurohypophyseal system. Here, OXT produced within the magnocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) and the supraoptic nucleus (SON) is transported to the posterior pituitary where OXT is stored in vesicle for peripheral release (Sawchenko & Swanson, 1983). Peripheral OXT plays a key role in childbirth and lactation with OXT stimulating uterine contractions and aiding in milk letdown (Sofroniew, 1983). In addition to peripheral actions, OXT is also centrally released via projections within the brain and spinal cord (Knobloch et al., 2012; Mitre et al., 2016; Tang et al., 2020). OXT has been shown to have anxiolytic properties as administration of OXT in the brain decreases anxiety-like behavior in both male and female rats and mice (Ring et al., 2006; Windle et

al., 1997). Additionally, female OXT knockout (KO) mice display higher levels of anxiety-like behaviors then their wild-type (WT) littermates in the elevate plus maze. This anxiogenic phenotype is rescued by intracerebroventricular (ICV) infusion of OXT prior to testing (Mantella et al., 2003). Interestingly, in a separate study, it was found that KO of OXT in female mice show decreased social anxiety (Choleris et al., 2003). Together, these studies demonstrate that OXT differentially modulates anxiety-like behaviors depending on the context, which may be accomplished by altering social salience. In line with this, OXT has also been found to modulate a wide variety of social behaviors across animal species, including humans, non-human primates, and rodent species (Dumais & Veenema, 2016; H.-J. Lee et al., 2009; Putnam et al., 2018; Young et al., 2001).

OXT modulates neuronal activity via binding to the OXT receptor (OXTR). The OXTR is a G-protein coupled receptor, in which the binding of OXT begins a variety of intracellular signaling cascades to alter overall cellular activity. OXTR signaling cascades are typically either net excitatory or inhibitory (paired to the G_q or G_i subunit, respectively). OXTRs are expressed widely across the brain and periphery, and subtype of the GPCR subunits varies by location (Jurek & Neumann, 2018). In rodent models, OXT and OXTR signaling is necessary for the expression of parental care, pair bonding, and social recognition (D'Cunha et al., 2011; Numan, 2007; Oettl et al., 2016; Olazábal & Young, 2006b; H E Ross et al., 2009).

The role of OXT signaling in the regulation of social behaviors in humans is more nuanced, relying primarily on correlational analyses and exogenous intranasal administration of OXT. In line with non-human research, OXT in humans has been

linked to maternal care, social bonding, and social engagement. For example, measurements of plasma OXT levels in postpartum mothers show a positive correlation between OXT levels and perceived mother-infant bonding (Levine et al., 2007). Additionally, plasma OXT levels are higher in individuals in an early-stage romantic relationship than single individuals (Schneiderman et al., 2012). These findings imply that OXT may underlie feelings of social attachment in humans. Intranasal administration studies have found that OXT increases feelings of empathy and enhances the ability to recognize the emotions of others in neurotypical individuals (Lischke et al., 2012). Likewise, OXT has also been found to improve processing of relevant social cues and social reciprocity (Andari et al., 2010; Watanabe et al., 2015) in autistic children. ASD individuals show lower plasma levels of OXT than neurotypical individuals (Green et al., 2001; Modahl et al., 1998), indicating that social deficits in ASD individuals may be linked to lower levels of OXT. As such, OXT is currently being studied as a potential therapeutic in the alleviation of social deficits in ASD.

Oxytocin in the Paraventricular Nucleus of the Hypothalamus

While both the PVN and SON produce OXT, the role of the PVN^{OXT} system is well-established for its role in the regulation of social behaviors. For example, it has been shown that PVN^{OXT} cellular activity is increased during social investigation of juvenile mice by adult male mice, and optogenetic stimulation of PVN^{OXT} cells increased social interaction with a juvenile in adult male mice (Hung et al., 2017). Evidence suggests that PVN^{OXT} neurons function similarly in females as inhibition of this cell population decreases social interaction with a conspecific in adult female rats (Tang et al., 2020). Together, these findings suggest that activation of PVN^{OXT} neurons may

modulate adult social behaviors, but the role of the SON^{OXT} and PVN^{OXT} systems in juvenile social behaviors, specifically social play, is unknown.

To investigate the role of hypothalamic OXT in juvenile social play behavior, I stimulated OXTergic neurons in either the SON or the PVN using a viral vector construct expressing an excitatory Designer Receptor Exclusively Activated by Designer Drugs (DREADDs). This viral vector (AAV1/2-OXTp-hM3Dq-mCherry) inserts a modified G-protein coupled human muscarinic receptor (hM3Dq) into the cell membrane, which can only be activated by the exogenous ligand Clozapine-*N*-oxide (CNO). When CNO is bound to its receptor, intracellular calcium levels are altered via activation of the phospholipase C signaling cascade. This increases the levels of intracellular calcium and causes the cell to fire in a burst-like pattern (Roth, 2016; K. S. Smith et al., 2016). Furthermore, this particular DREADD viral vector is under the control of the OXT promoter, which will selectively transfect and insert the excitatory DREADD specifically into the membrane of OXT synthesizing neurons. This technique allowed for chemogenetic stimulation of the SON^{OXT} or PVN^{OXT} cells of juvenile male and female Wistar rats to determine how these cell populations influence the expression of social play behavior. Here, I found that chemogenetic stimulation of SON^{OXT} cells increased social investigation duration in both sexes without altering duration or frequency of social play behaviors (Preliminary data, Figure 1). I also found that chemogenetic stimulation of PVN^{OXT} cells did not alter social investigation but modulated social play behavior sex-specifically. In detail, chemogenetic stimulation of PVN^{OXT} cells decreased social play levels in males and increased social play levels in females (Preliminary data, Figure 2). This data provides strong evidence for a role of PVN^{OXT} but not SON^{OXT}

signaling in the modulation of social play behavior, which occurs in a sex-specific manner.

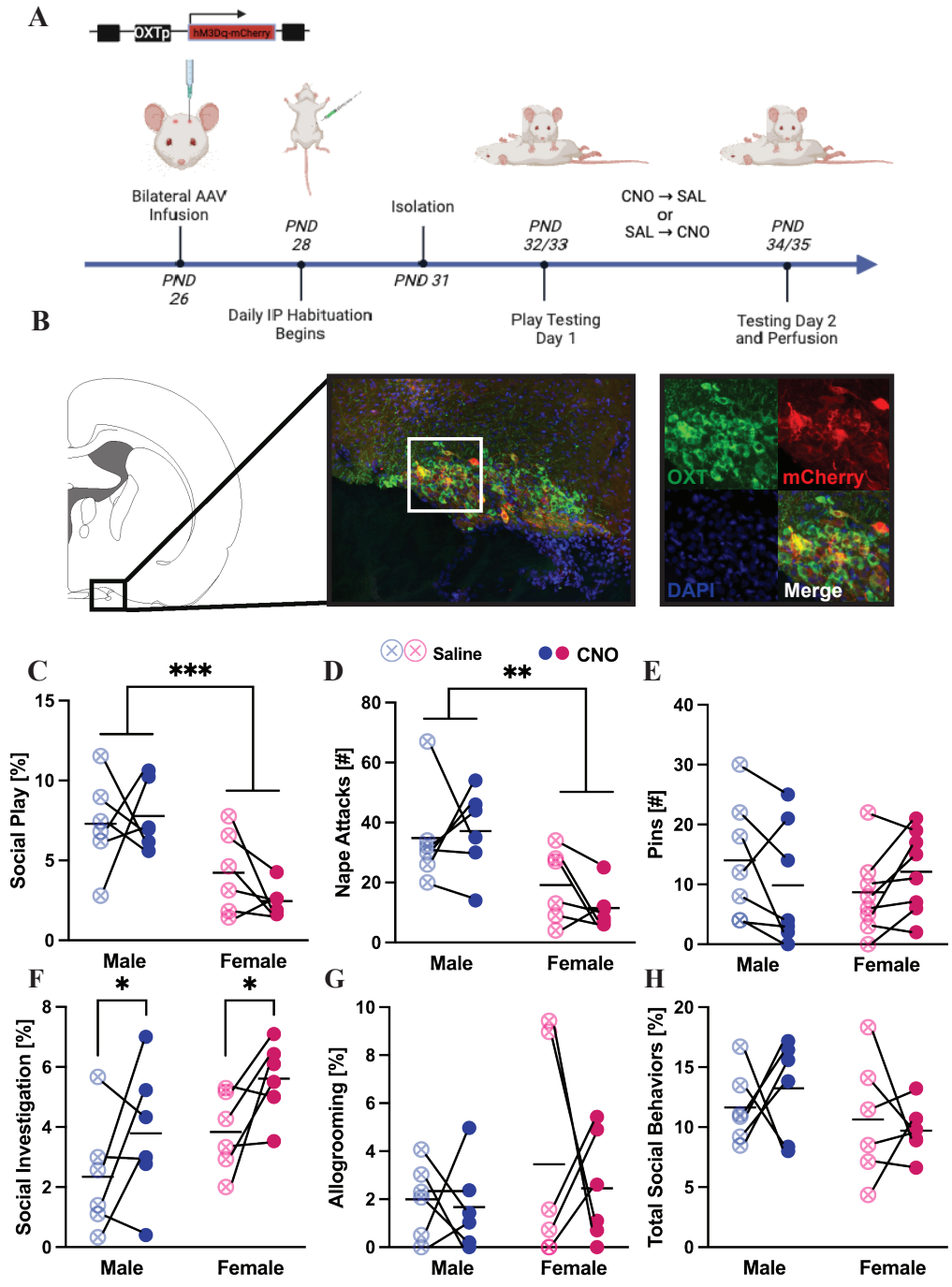

Figure 1. Chemogenetic stimulation of SON^{OXT} neurons increases social investigation in both male and female juvenile rats. (A) Timeline of experimental

Figure 1 (cont'd)

procedures. Briefly, juvenile rats underwent stereotaxic surgery and infusion of OXTp-hM3Dq-mCherry into the SON at postnatal day (PND) 26. Following recovery and habituation to intraperitoneal injections, all rats underwent two days of social play testing in which either saline or CNO was administered 30 minutes prior to testing. Drug conditions were randomly assigned and counterbalanced over the two days of testing. (B) Rat brain atlas image (modified from Paxinos and Watson, 2007) indicating the location and representative image of AAV-OXTp-hM3Dq-mCherry expression in OXT neurons of the PVN as well as magnification of image denoting mCherry (Red) and OXT (Green) positive neurons that are co-localized using DAPI (Blue) as a cellular marker. (C) Irrespective of drug treatment, females displayed lower levels social play and (D) fewer nape attacks than males. (F) CNO administration increased the duration of social investigation in both sexes compared to saline administration. Neither drug nor sex altered the duration of (G) allogrooming and (H) total duration of social behaviors. Durations of social play (B), social investigation (F), allogrooming (G), and total social behavior (H) are expressed as a percentage of total time. 2-way ANOVA; Holm-Sidak post-hoc tests, *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$.

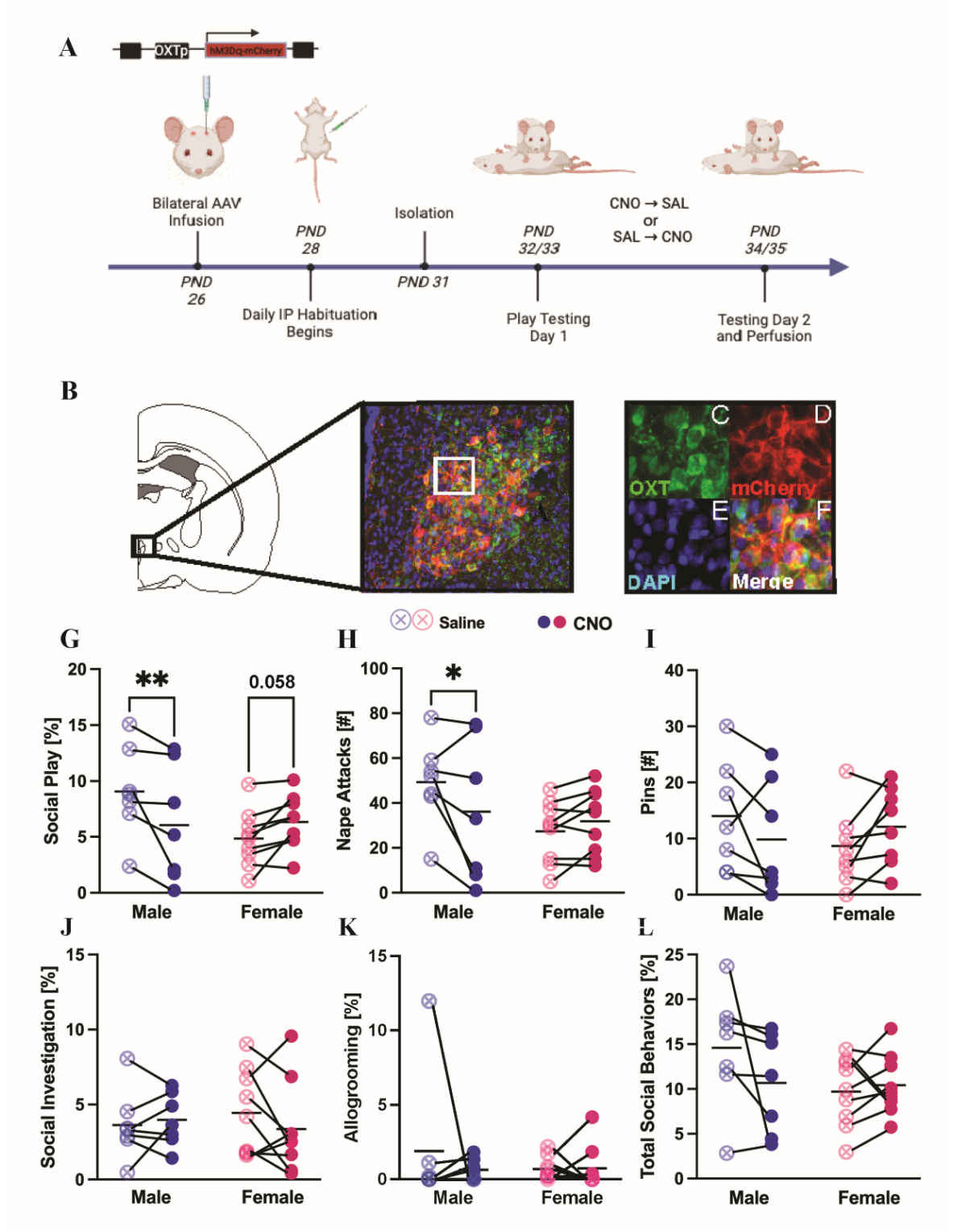

Figure 2. Chemogenetic stimulation of PVN^{OXT} neurons alters social play sex-specifically in juvenile rats. (A) Timeline of experimental procedures. Briefly, juvenile

Figure 2 (cont'd)

rats underwent stereotaxic surgery and infusion of OXTp-hM3Dq-mCherry into the PVN at postnatal day (PND) 26. Following recovery and habituation to intraperitoneal injections, all rats underwent two days of social play testing in which either saline or CNO was administered 30 minutes prior to testing. Drug conditions were randomly assigned and counterbalanced over the two days of testing. (B) Rat brain atlas image (modified from Paxinos and Watson, 2007) indicating the location and representative image of AAV-OXTp-hM3Dq-mCherry expression in OXT neurons of the PVN. (C-F) Magnification of image in B denoting mCherry (C; Red) and OXT (D; Green) positive neurons that are co-localized (F) using DAPI (E) as a cellular marker. (G-L) Administration of CNO decreased duration of social play in males and had a strong tendency towards an increased duration of social play in females (G) as well as decreased nape attacks in males (H). CNO administration did not alter number of pins (I), duration of social investigation (J), duration of allogrooming (K), or total duration of social behaviors (L). Social play (G), investigation (J), allogrooming (K), and combined social behaviors (L) are expressed as a percentage of total time. 2-way ANOVA Sex x Drug interaction; *: $p < 0.05$, **: $p < 0.01$; Holm's-Sidak post-hoc tests.

Nucleus Accumbens

The nucleus accumbens (NAc) is located in the ventral striatum of the brain and is part of the mesolimbic reward pathway (Hikida et al., 2016; Klawonn & Malenka, 2018; Salgado & Kaplitt, 2015). One key role of the NAc is in the modulation of motivational and rewarding behaviors, such as drug-seeking and food consumption (Chen et al., 2023; Gore & Zweifel, 2013; Ikemoto & Panksepp, 1999). The NAc receives inputs from

a wide variety of brain regions, including the hippocampus, hypothalamus, prelimbic cortex, as well as the ventral tegmental area and the substantia nigra (Salgado & Kaplitt, 2015). The medium spiny neurons (MSNs) of the NAc in turn project to motor regions of the brain, as well as ventral pallidum and extended amygdala (Salgado & Kaplitt, 2015). These inputs and outputs of the NAc have led researchers to hypothesize that the NAc behaves as an interface, receiving and processing salient information from cortical and limbic systems and integrating these signals into action via the motor regions of the brain (Mogenson & Yang, 1991; Mogenson et al., 1980).

Anatomically, the NAc can be divided into the core (NAcc) and shell (NAcSh) subregions (Groenewegen et al., 1999; Salgado & Kaplitt, 2015; Zahm & Brog, 1992), which are thought to have relatively distinct functions in the processing of reward. The NAcc is more involved with reward evaluation and motor initiation, while the NAcSh is thought to be involved in reward prediction and reward learning (Klawonn & Malenka, 2018). These distinct functions of the NAcc and NAcSh in reward processing are thought to be due to regional differences in inputs or receptor expression.

The NAc is a heterogeneous region, which receives primarily excitatory glutamateric input from cortical, hippocampal, and amygdalar regions, as well as dopaminergic (DA) input from midbrain regions (Klawonn & Malenka, 2018; Scofield et al., 2016). Additionally, the NAc receives OXTergic projections from the PVN (He et al., 2021; Knobloch et al., 2012; H E Ross et al., 2009; Tang et al., 2020). The OXT system in the NAc has been linked to the modulation of social behaviors, with the most well characterized being pair bonding behaviors in prairie voles. Prairie voles are socially monogamous rodents which form deep social attachments following mating, while non-

monogamous vole species are socially promiscuous (Heather E Ross et al., 2009; Shapiro et al., 1991). The ability to form a pair bond, which is measured by the expression of a partner preference in voles, is mediated through the OXT system in the NAc (Cho et al., 1999; Insel & Shapiro, 1992; Insel et al., 1998; H E Ross et al., 2009). For example, OXTR density in the nucleus accumbens correlates with pair bond formation in vole species, in which monogamous female prairie voles express higher levels of OXTRs in the NAc than their non-monogamous counterparts, such as the montane and meadow vole (Insel & Shapiro, 1992). Viral vector-induced overexpression of OXTR in the NAc of prepubertal female prairie voles facilitates partner preference in adulthood (Keebaugh & Young, 2011), while OXTR antagonism blocks partner preference in adult female prairie voles (Young et al., 2001). NAc-OXTR activation is necessary for the expression of various social behaviors in other rodent species as well. In adolescent male mice, OXTR antagonism within the NAc abolishes conditioned place preference for a social stimulus (Dölen et al., 2013). Additionally, NAc^{OXTR} antagonism reduces maternal behavior in adult rats (Olazábal & Young, 2006a). Furthermore, preliminary findings from our lab show that OXTR antagonism within the NAc reduces social play in juvenile rats in a sex-specific manner, with males requiring 10x more of the drug to reduce social play behavior than females (Preliminary data, Figure 3A). This is the first study directly comparing males and females, and demonstrates the necessity of OXTR activation in NAc for the typical expression of juvenile social play behavior. Additionally, the difference in dosage of the OXTR antagonist between males and females suggests a subtle sex difference in the actions of OXTRs in the NAc, likely requiring higher OXTR activation in males than in females.

Electrophysiological studies have shown that bath application of OXT reduces the excitability of NAc cells in juvenile male mice (Dölen et al., 2013). This may suggest that NAc^{OXTR} cells are directly inhibited by OXT. Given the requirement of OXTRs in the typical expression of juvenile social play and the electrophysiological data, I hypothesized that social play would alter NAc^{OXTR} cell activation. To test this, juvenile male and female rats underwent a 10-minute social play test with a sex- and age-matched conspecific. Twenty-five minutes after the conclusion of the play test, all rats were perfused to capture the peak expression of *Fos* mRNA via *in situ* hybridization. NAc tissue was processed to quantify *oxtr* mRNA and *fos* mRNA expressing cells, as well as cells that co-express *oxtr* mRNA and *fos* mRNA. I found that levels of social play negatively correlate with the total number of *fos*-expressing cells in the NAc, as well as the proportion of activated *oxtr* expressing neurons within the NAc (*oxtr+fos/oxtr*100)* of juvenile male and female rats. Furthermore, the negative correlation between social play duration and NAc^{OXTR} activity was driven by males (Preliminary data; Figure 3 A-D). These data support a model in which the expression of social play is associated with OXTR activation, which may lead to an overall suppression of NAc cell activity.

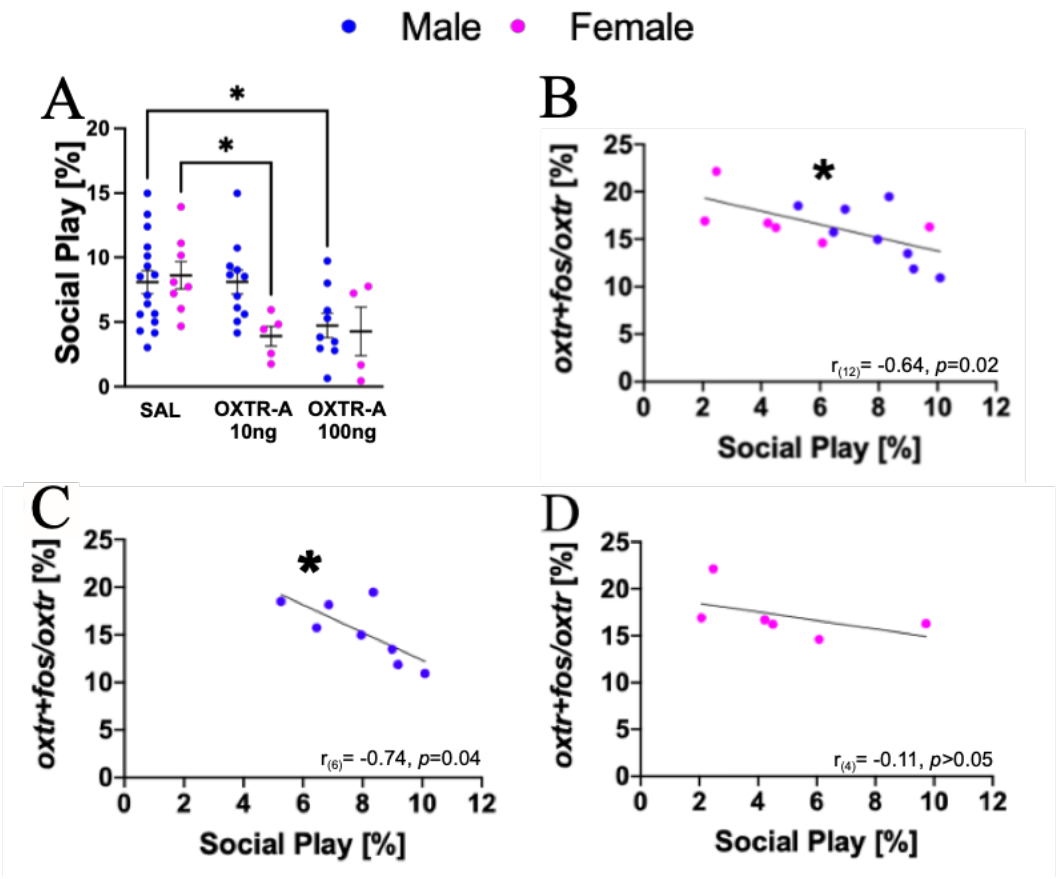

Figure 3. Effects of OXTR-antagonism as well as intrinsic NAc activity in response to social play is sex-specific. (A) Administration of an OXTR-A into the NAc reduces social play behavior at sex-dependent dosages, with males requiring 10x more of the antagonist than females to reduce social play. (B) Duration of social play negatively correlates with recruitment of *oxtr* mRNA expressing cells within the NAc (*oxtr+fos/oxtr*100*). This negative correlation is driven by (C) males, but not (D) females. (A. 2-way ANOVA, Tukey's post-hoc; B-D. Pearson's correlation; * $p < 0.05$); SAL = saline, OXTR-A = OXTR antagonist.

Oxytocin-Dopamine Interactions in the Nucleus Accumbens

The NAc is largely comprised of GABAergic MSNs, most of which express either DA 1-like (D1) or DA 2-like (D2) receptors (Yager et al., 2015). D1-like receptors are excitatory, in which activation of the G_s subunit within these D1 expressing cells will increase cellular activity, while D2-like receptors are inhibitory, and activation of the G_i subunit within these D2 expressing cells will decrease cellular activity (Rommelfanger & Wichmann, 2010; R. J. Smith et al., 2013). Selective activation of either D1-like or D2-like receptors can have vastly different behavioral outcomes. For example, D1-like receptor agonism impairs the formation of a pair bond in male and female prairie voles while D2-like receptor agonism facilitates formation (Aragona et al., 2006; Hostetler et al., 2011; Wang et al., 1999). The coordinated efforts of both OXT and DA in the NAc may facilitate social behaviors as researchers have shown that co-administration of OXTR and D2R antagonists blocks the effect of OXTR and D2R agonists on the facilitation of a partner preference in female prairie voles (Liu & Wang, 2003). We found that, in juvenile rats, approximately 70% of OXTR-expressing neurons in the NAc co-express either D1R or D2Rs (Figure 4). Given the high rate of OXTR and DAR co-expression, OXT and DA may interact in the NAc to regulate juvenile social behaviors. Additionally, we found a sex difference in OXTR neurons co-expressing either the D1R or D2R in juvenile rats. While both males and females have a higher percentage of *oxtr*+ cells co-expressing *d2r* than *d1r,* females show a higher percentage of oxtr cells co-expressing the d1r. (Preliminary data, Figure 4A). As a result, males tend to have a higher proportion of *oxtr+d2r:oxtr+d1r* compared to females (Preliminary data, Figure 4B). These differences in OXTR expression on DAR cells in the NAc suggest that

OXTR+D1R and OXTR+D2R expressing neurons may play distinct roles in the regulation of social behaviors, which may affect males and females differently.

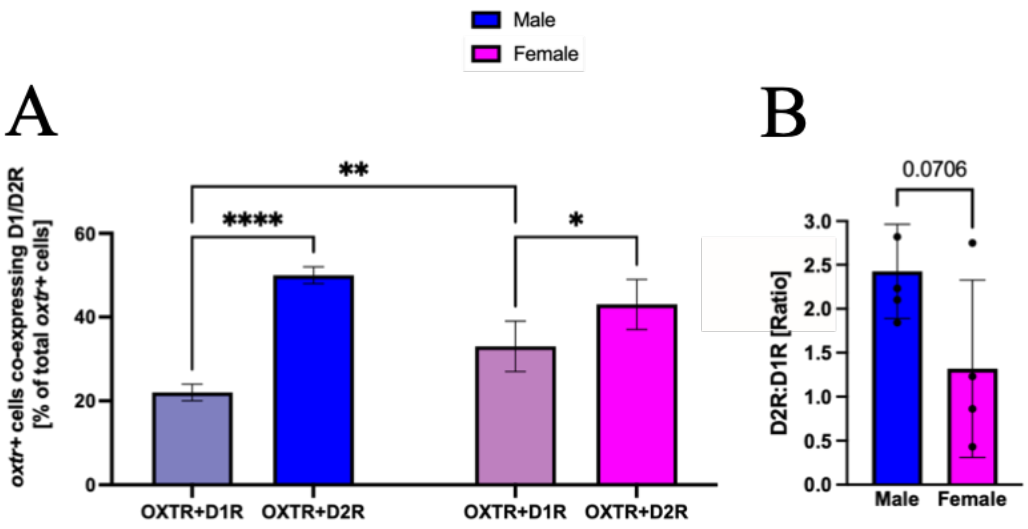

Figure 4. Percentage of *oxtr*-positive cells that co-express the *d1r* or *d2r* in the NAc of male and female juvenile rats. (A) Both sexes have significantly more *oxtr*+ cells co-expressing *d2r* compared to *d1r* in the NAc, with females having higher co-expression of *oxtr* with *d1r* than males. (B) There is a trend in which males have a greater proportion of *oxtr*+ cells that co-express the *d2r* than the *d1r* than females (2-way ANOVA, Tukey's post-hoc for multiple comparisons * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; Student's unpaired t-test).

Ventral Pallidum

While D1-like MSNs in the NAc project primarily to the ventral tegmental area and substantia nigra, D2-like MSNs in the NAc project primarily to the ventral pallidum (VP) (R. J. Smith et al., 2013; Yager et al., 2015). These D2-like MSNs are GABAergic, and as such, a decrease in activity from NAc MSNs projecting to the VP facilitates

disinhibition of the VP (Mogenson et al., 1983; Numan, 2007; Swerdlow et al., 1990). Indeed, electrophysiological studies show that infusion of DA into the NAc of adult rats resulted in a prolonged increase in VP activity (Yang & Mogenson, 1989). Additionally, VP activity is significantly reduced following optogenetic stimulation of the NAc (Chometton et al., 2020), demonstrating that NAc activation is sufficient to suppress VP activity.

Much like the NAc, the VP is known to regulate diverse motivated behaviors, including drug seeking, eating, and social behaviors (J. D. A. Lee et al., 2021; K. S. Smith et al., 2009). For example, pharmacological inactivation of the anterior VP via the $GABA_A$ receptor agonist muscimol significantly reduced sucrose intake in female rats (Chometton et al., 2020). Recently, our lab demonstrated that inactivation of the VP via muscimol decreases social play in juvenile male and female rats (J. D. A. Lee et al., 2021). Additionally, inhibition of the NAc via $GABA_A$ and $GABA_B$ receptor agonists increases social play in juvenile male rats (van Kerkhof et al., 2013), and chemogenetic excitation of NAc^{GABA} terminals in the VP significantly decreases social play in both male and female juvenile rats (Lee, 2023). Taken together, these studies demonstrate the necessity of VP activity for the typical expression of social play, which may require inhibition of NAc^{GABA} input. However, the role of OXTR-expressing projections from the NAc to the VP in juvenile social play behavior remains unknown.

Summary of Experiments and Dissertation Chapters

The overall purpose of this dissertation is to better understand how OXTergic signaling within the brain modulates juvenile social play behavior in male and female

rats. Based on the existing literature combined with my preliminary data that was discussed above, I hypothesized that the PVN, NAc, and VP connect to form a neural circuit, and that OXT and DA signaling in the NAc is required for the expression of social play behavior. More specifically, I hypothesized that OXT released from the PVN inhibits the activity of NAc^{OXTR} expressing cells projecting to the VP, thus disinhibiting the VP and thereby promoting the typical expression of social play behavior. Additionally, I hypothesized that DAR activity in the NAc may facilitate the expression of social play, primarily through activation of the inhibitory D2R. Furthermore, given the sex differences in behavior seen when stimulating PVN^{OXT} cell bodies, I hypothesized that OXTergic modulation throughout this circuit may have sex-specific effects on juvenile social play behavior. Though PVN^{OXT} neurons, OXTRs in the NAc, and the VP have been shown individually to modulate social play behavior, it is currently unclear how these regions interact to regulate social play. Therefore, I designed three sets of experiments to test this.

In Chapter 2, I first quantified the density of OXT fibers from the PVN to the NAc to determine potential sex differences in PVN-OXT innervation of the NAc. Here, I found that there were no sex differences in PVN^{OXT} fiber density within the NAc. Instead, there was a significantly higher density of OXT fibers in the anterior portion of the NAc in both males and females in comparison to other NAc subregions. I used this finding to then determine the effects of chemogenetic stimulation of PVN^{OXT} neurons projecting to the anterior NAc on the expression of social play in juvenile male and female rats. As I previously showed that chemogenetic stimulation of PVN^{OXT} neurons reduced social play in males and increases social play in females, I predicted that chemogenetic

stimulation of the PVN^{OXT} terminals in the NAc would follow this same pattern. To test this, I used an excitatory DREADD construct under the control of the OXT promoter in the PVN to selectively stimulate PVN^{OXT} neurons projecting to the anterior NAc. Contrary to my hypothesis, I found that chemogenetic excitation did not affect juvenile social play, but instead sex-specifically altered social investigation.

Previous findings of the lab showed that OXTRs in the NAc are necessary for the typical expression of juvenile social play, in which an OXTR antagonist sex-specifically decreased social play behaviors (Figure 3A). Additionally, I have previously shown that social play decreases the proportion of activated OXTRs in the NAc (*Oxtr+Fos/Oxtr[%]),* an effect that was primarily driven by males (Figure 3B-D). This led me to hypothesize that NAc^{OXTR} neuronal inhibition was necessary for the expression of juvenile social play. Therefore, in experiment 1 of chapter 3, I determined the effects of chemogenetic inhibition of NAc^{OXTR} cell bodies on the expression of social play. For this, I injected a Cre-dependent inhibitory DREADD construct in the NAc of OXTR-iCre rats. I predicted that chemogenetic inhibition of NAc^{OXTR} expressing cells would increase juvenile social play behavior in both sexes, with males showing a larger magnitude increase in play than females. As predicted, there was an increase in juvenile social play in females, but contrary to my hypothesis, social play was not affected in males. These results suggest that OXTR-expressing neurons in the NAc differentially modulate juvenile social play in males and females. Next, as chemogenetic stimulation of NAc terminals in the VP decreases juvenile social play in both sexes (Lee, 2023), I hypothesized that OXTR-expressing neurons in the NAc projecting to the VP are involved in the regulation of social play. Specifically, I predicted that chemogenetic inhibition of the NAc^{OXTR}

terminals in the VP would increase juvenile social play in males and females. Using OXTR-iCre rats, I chemogenetically inhibited the NAc^{OXTR} terminals within the VP, where I found no effects on social play. Instead, males showed decreased levels of social investigation.

Finally, as juvenile male and female rats show OXTR co-expression on either D1Rs or D2Rs neurons, I aimed to better understand the roles of both DA and OXT in the NAc in the regulation of juvenile social play. Previously, our lab has demonstrated that NAc^{OXTR} expressing neurons co-express either D1R or D2R, with males showing a higher D2R+OXTR:D1R+OXTR ratio than females (Figure 4B). Additionally, D1R and D2R activation is necessary in males (Manduca et al., 2016) and OXTR activation is necessary in both sexes (Fig. 3A) for the typical expression of juvenile social play behavior. Therefore, I hypothesized that the DA and OXT system within the NAc may both modulate juvenile social play in rats. To test this, juvenile rats were bilaterally cannulated in the NAc and were infused with either a D1R antagonist, D2R antagonist, or synthetic OXT prior to social play testing. I predicted that D1R or D2R antagonism would decrease social play in both sexes, while OXT would increase social play. Furthermore, I predicted that males may require a higher dose of both the DAR antagonists and OXT to show similar alterations in play compared to females. Contrary to my prediction, D1R antagonism had no effect on social play in either males or females, although D2R antagonism decreased social play in both sexes. On the other hand, OXTR agonism altered social play sex-specifically, decreasing social play in males and eliminating a baseline sex differences between males and females.

Taken together, these findings point to a sex-specific role of OXT signaling in a circuit spanning the PVN, NAc, and VP in the regulation of social behaviors in juvenile rats. Generally, OXT signaling either decreased or had no effect on the expression of social behaviors in males and increased social behaviors in females, though the specific behavior and direction of change varied by brain region or pathway. Therefore, chapter 5 will discuss potential mechanisms and alternative pathways that may underlie the differential regulation of juvenile social play in male versus female rats. This thesis will end with addressing the implications of this research within the broader field of social behavior.

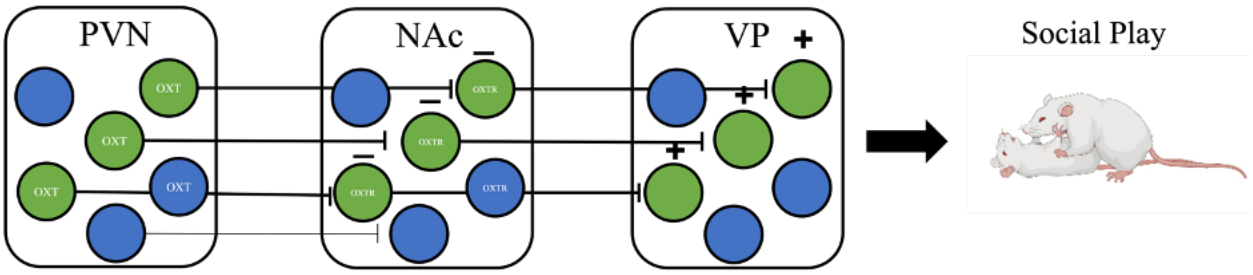

Figure 5. Proposed oxytocinergic pathway in the regulation of juvenile social play. OXT is released from PVN^{OXT} terminals in the NAc. Binding of OXT activates the OXTR coupled to the G_i subunit and/or OXTR+D2R complexes in the NAc, leading to inhibition of NAc^{OXTR} expressing cells projecting to the VP. OXT-induced inhibition of NAc^{OXTR} neurons projecting to the VP then disinhibits the VP which is required for the expression of juvenile social play behavior.

CHAPTER 2. DETERMINE THE ROLE OF THE PVN^{OXT} TO NAC PATHWAY IN THE EXPRESSION OF JUVENILE SOCIAL PLAY

Abstract

Social play is a highly rewarding and motivated behavior expressed during the juvenile period of many mammalian species, including humans and laboratory rats. Social play is invaluable in the development and maintenance of social competency throughout the lifespan, though the neurobiological mechanisms modulating social play are still not well understood. Oxytocin (OXT) is a neuropeptide that regulates a variety of social behaviors, including social motivation, social investigation, pair bonding, and maternal care. In humans, OXT has also been used in clinical trials to help alleviate symptoms in children with social deficit disorders, such as autism spectrum disorder (ASD). ASD children show reduced participation in social play, but whether OXT signaling could improve social play engagement is unknown. OXT is primarily produced within the paraventricular nucleus of the hypothalamus (PVN), and I have previously shown that chemogenetic stimulation of PVN^{OXT} neurons sex-specifically alters juvenile social play. In detail, PVN^{OXT} stimulation decreased social play in males and increased social play in females. The PVN^{OXT} sends projections to the nucleus accumbens (NAc), a key region within the brains reward circuitry. Given the motivating and rewarding properties of social play, I hypothesized that OXT signaling between the PVN and NAc would modulate juvenile social play sex-specifically, which could be due to sex differences in PVN^{OXT} fiber innervation to the NAc. Therefore, in experiment 1, I determined whether there were sex differences in PVN^{OXT} fiber density within subregions of the NAc. While I found no differences in fiber innervation between male

and female juvenile rats, I found significantly higher OXT fiber density in the anterior portion of the NAc. Next, in experiment 2, I chemogenetically stimulated PVN^{OXT} fibers in the anterior NAc to determine the effects of this pathway on the regulation of juvenile social play. I predicted that stimulation would follow the pattern of PVN^{OXT} cell body stimulation in which males decreased and females increased juvenile social play. Here, I found no effect of chemogenetic stimulation on the expression of juvenile social play. Instead, stimulation of PVN^{OXT} terminals in the NAc increased social investigation in males and decreased social investigation in females. Males and females show increased PVN^{OXT} fiber innervation in the anterior NAc, with similar innervation patterns across both sexes. Despite this the similar patterns in innervation, there was a sex difference in behavioral regulation by OXT in the PVN to anterior NAc pathway. Together, these findings indicate there may be sex differences in how release of OXT into the NAc regulates the expression of social investigation.

1. **Introduction**

Social play is a highly motivated and rewarding behavior (Trezza et al., 2010; L. J. M. J. Vanderschuren et al., 2016) displayed by many mammalian species, including rats, non-human primates, and humans (Calcagnetti & Schechter, 1992; Panksepp, 1981; Anthony D. Pellegrini & Smith, 2005; S M Pellis & Pellis, 1997; Shimada & Sueur, 2018). Social play (a.k.a rough and tumble play or play fighting) peaks during the juvenile period and is necessary for the expression of context-appropriate social behaviors throughout life (Anthony D. Pellegrini & Smith, 1998; A D Pellegrini & Smith, 1998; van den Berg et al., 1999a). Social play is a collaborative interaction between peers and as such does not have strict rules. Instead, social play relies on the interpretation of actions and implicit cues in the moment (Burghardt, 2010). As this requires both the initiation of and appropriate reciprocity to another's initiation of play, engagement in social play is thought to build social competency and help establish flexible social problem-solving skills (A D Pellegrini, 1989). Deficits in social play are seen in children with social disorders, such as autism spectrum disorder (ASD). ASD children are less likely to initiate or to be included in social play, which can reduce the ability of ASD individuals to learn how to appropriately navigate social and emotional situations later in life (Chevallier, Kohls, et al., 2012; Chevallier et al., 2013; Jordan, 2003). ASD is further characterized by reduced interest in social interactions and reduced social reward seeking. For example, when given the choice between a social (child smiling) picture or non-social (toy) picture, ASD children are less likely to choose the social picture compared to neurotypical children (Ruta et al., 2017). ASD individuals also report lower levels of satisfaction following social rather than non-social

interactions, and fMRI analyses have demonstrated that ASD children show structural differences in connectivity within the brain reward system (Supekar et al., 2018). These studies indicate both behavioral and structural differences in social reward processing in ASD, which may in turn alter motivation to engage in social play with peers.

The neuropeptide oxytocin (OXT) has been implicated as a potential treatment option to improve social play engagement in ASD children. In animal models, OXT has been extensively studied in the modulation various social behaviors such as social investigation, social recognition, pair bonding, and parental behavior (D'Cunha et al., 2011; Numan, 2007; Oettl et al., 2016; Olazábal & Young, 2006a; H E Ross et al., 2009). While the role of OXT in human social behaviors has been studied to a lesser degree than in animal models, this field of research has grown recently. Studies show that in humans, plasma OXT levels are positively correlated with the degree of perceived mother-infant bonding (Levine et al., 2007), and plasma OXT is elevated in individuals in a romantic relationship (Schneiderman et al., 2012). Additionally, increasing available OXT via intranasal administration enhances the ability of neurotypical individuals to recognize the emotions of others (Lischke et al., 2012). ASD individuals, who show deficits in social communication and decreased social behaviors, have lower levels of plasma OXT in comparison to neurotypical individuals (Green et al., 2001; Modahl et al., 1998). As such, researchers have begun trials to test whether intranasal OXT administration may improve social skills in ASD individuals. It was shown that ASD children given OXT were able distinguish between cooperative and non-cooperative players in a Cyber Ball game, demonstrating an increase in social discrimination (Andari et al., 2010). Additionally, both chronic (6-week) and single-dose administration of OXT

increases the ability of autistic adults to correctly interpret a picture of a person as either "friend" or "foe" based solely on implicit visual cues (Watanabe et al., 2014, 2015). Together, these studies demonstrate that intranasal OXT administration increases the processing of socially relevant cues and social competency in autistic children. However, whether OXT may be used to improve social play engagement in ASD children, or in any animals, is currently unknown. Therefore, understanding how OXT circuits within the brain may modulate juvenile social play is the first step towards improving treatments for ASD children, potentially improving social competency throughout the lifespan.

OXT in the brain is primarily produced within the hypothalamus, specifically within the paraventricular nucleus of the hypothalamus (PVN) and the supra optic nucleus (SON) (Sawchenko & Swanson, 1983). The PVN and SON send axonal projections to the posterior pituitary, which in turn prompts peripheral release of OXT throughout the body. Additionally, PVN^{OXT} neurons send axonal projections to a wide variety of regions within the brain, such as the bed nucleus of the stria terminalis (BNST), lateral septum (LS), medial amygdala (MeA), and the nucleus accumbens (NAc) (Knobloch et al., 2012; Tang et al., 2020). Specifically, PVN^{OXT} neurons have been implicated in the modulation of social investigation in adult mice and rats (Grund et al., 2019; Hung et al., 2017; Oettl et al., 2016; Tang et al., 2020). For example, PVN^{OXT} neuronal activity increases during social investigation and interaction of juvenile mice by adult male mice (Hung et al., 2017). Additionally, optogenetic stimulation of PVN^{OXT} cells increased social investigation of juvenile mice by adult male mice (females were not tested; Hung et al. 2017). Furthermore, optogenetic inhibition of PVN-OXT cells

decreased social interaction of a female conspecific by adult female rats (males were not tested; Tang et al., 2020). PVN ^{OXT} neurons have also been directly implicated in the regulation of juvenile social play in rats. Previously, I found that chemogenetic stimulation of PVN ^{OXT} cell bodies alters juvenile social play sex-specifically, with males decreasing and females increasing duration and frequency of social play behaviors (Figure 2). Modulation of juvenile social play is specific to the PVN^{OXT} neuronal population as chemogenetic stimulation of SON^{OXT} neurons did not alter social play behaviors in either sex but did increase social investigation in both males and females (Figure 1). Despite this, it remains unclear how PVN^{OXT} neurons modulate social play through central projections within the brain.

The NAc is a key area within the mesolimbic reward system which receives OXTergic projections from the PVN (He et al., 2021; Knobloch et al., 2012; H E Ross et al., 2009; Tang et al., 2020), and is involved in the regulation of rewarding and motivated behaviors, such as feeding, drug-seeking, and socio-sexual behaviors (Klawonn & Malenka, 2018; Matsumoto et al., 2012; Olaniran et al., 2022; Salgado & Kaplitt, 2015; Stratford & Kelley, 1999). NAc neurons express receptors for OXT as well as for other neurochemicals including dopamine, GABA, glutamate, and serotonin (Mitre et al., 2016; Shirayama & Chaki, 2006; Yager et al., 2015). In rodent species, the NAc OXT receptor (OXTR) in particular have been studied in the context of social behaviors. For example, NAc OXTR activation is required for pair bond formation in female prairie voles, as direct administration of an OXTR antagonist concurrently with OXT into the NAc during cohabitation with a male prevents the formation of a partner preference (Liu & Wang, 2003). OXTR antagonism within the NAc also abolishes social conditioned

place preference for same-sex social stimuli in adolescent male mice (Dölen et al., 2013), and decreases social novelty seeking in juvenile rats (C. J. W. Smith, Mogavero, et al., 2017). These studies imply that NAc^{OXTR} activity is necessary for the expression of a variety of social behaviors across rodent species. Additionally, the PVN^{OXT} to NAc pathway has been implicated in the regulation of social behaviors in adult monogamous vole species. For example, PVN^{OXT} neurons projecting to the NAc are activated in male mandarin voles when interacting with their pups (He et al., 2021), suggesting a role of this pathway in paternal behaviors. Indeed, chemogenetic stimulation of PVN^{OXT} neurons projecting to the NAc increases, while chemogenetic inhibition decreases, the expression of paternal behaviors in mandarin voles including licking of pups and latency to retrieve pups (He et al., 2021). Additionally, optogenetic stimulation of PVN^{OXT} axonal fibers in the NAc increased time spent investigating a novel age- and sex-matched conspecific in male and female adult mandarin voles (W. Hou et al., 2023). These data suggest a pivotal role of the PVN^{OXT} to NAc pathway in the regulation of adult social behaviors, but the role of this pathway for juvenile social behaviors, specifically social play, is unknown.

The overall goal of this chapter is to elucidate the role of the PVN^{OXT} to NAc pathway in the regulation of juvenile social play behavior. I have previously shown that chemogenetic stimulation of PVN^{OXT} cell bodies alters levels of social play behavior sex specifically in juvenile rats, without altering other social behaviors. Therefore, I hypothesize that the PVN^{OXT} to NAc pathway regulates social play behavior sex specifically, which may occur due to sex differences in PVN^{OXT} innervation to the NAc. In experiment 1, I determined whether there are sex differences in the projections of

PVN^{OXT} neurons to the NAc by quantifying PVN^{OXT} fiber density in the NAc of juvenile male and female rats. In experiment 2, I determined whether the PVN^{OXT} to NAc pathway regulates social play behaviors and does so sex-specifically. This was accomplished by chemogenetic stimulation of PVN^{OXT} fiber terminals in the NAc in juvenile male and female rats. As I have found that stimulation of PVN^{OXT} cell bodies decreases social play in males and increases social play in females (Figure 2G), I predicted that chemogenetic stimulation of PVN^{OXT} terminals in the NAc will follow this same pattern. In detail, I predict that activation of the PVN^{OXT} to NAc pathway will decrease the expression of social play behaviors in juvenile males, while increasing the expression of social play behaviors in juvenile females.

2. **Methods**

Animals

Subjects were experimentally naïve male and female Wistar rats bred in-house using animal stock previously obtained from Charles River Laboratories (Raleigh, NC, USA), and maintained under standard laboratory conditions (12:12h light/dark cycle, lights off at 13:00h, 22°C, 50% humidity, food, and water available *ad libitum*). Experimental rats were weaned at post-natal day (PND) 21 and housed in single-sex groups of 2-4 in standard Allentown cages (48x27x20cm). Male and female Wistar rats used as stimulus rats for social play testing were obtained from Charles River Laboratories (Raleigh, NC, USA) at PND 26-30 and were housed under the same conditions as in-house bred experimental rats following arrival in our animal facilities. All experiments were conducted in accordance with the National Institute of Health *Guidelines for Care and*

Use of Laboratory Animals and approved by the Michigan State University Institutional Animal Care and Use Committee.

Experiment 1: Determine potential sex differences in PVN^{OXT} fiber density in the NAc of juvenile rats

On PND22, a mCherry or Venus fluorescent fluorophore reporter virus under the control of the OXT promoter (AAV1/2-OXTp-mCherry [n=6/sex] or AAV1/2-OXTp-Venus [n=6/sex]) into the PVN of male and female rats. Rats were anesthetized with isoflurane (2-4% as needed; Henry Schein, Melville, NY, USA) and mounted onto a stereotaxic frame (Stoelting, Wood Dale, IL, USA). A 1ml 7000 series Hamilton syringe (Hamilton, Reno, NV, USA) was attached to a stereotaxic injector system (Stoelting, Wood Dale, IL, USA) and loaded with the viral vector. The syringe was aimed at the PVN (AP: -1.70mm ML: ±1.70mm DV: -7.70mm from bregma in accordance with the Rat Brain Atlas; Paxinos and Watson, 2007) at a 10° angle to avoid damage to the sagittal sinus. Viral vectors were infused at a rate of 100nl/minute, and the needle remained in place for 10 min to allow adequate time for diffusion and uptake of the viral vector. The syringe was slowly withdrawn, and the process was repeated in the contralateral PVN. All rats received a subcutaneous injection of meloxicam (2mg/kg; Covetrus) following infusion and once a day for two days. All rats were singly housed and monitored post-surgery until fully ambulatory. Following recovery, rats were housed in same-sex groups of two or three until transcardial perfusion and brain extraction at PND 35.

Brains were sliced into 30 µm coronal sections on a cryostat (Leica CM3050, Buffalo Grove, IL) and collected into cryoprotectant solution (0.05 M sodium phosphate

buffer, 30 % ethylene glycol, 20 % glycerol) in four consecutive series containing both the NAc and PVN (Bregma -1.08mm to -1.72mm Paxinos and Watson, 2007). One tissue series of rats injected with AAV1/2-OXTp-mCherry containing the PVN was processed for mCherry cell body detection, and one tissue series of rats injected with AAV1/2-OXTp-Venus containing the PVN was processed for green fluorescent protein (Venus) cell body detection using fluorescent IHC. Briefly, tissue was washed in tris-buffered saline (TBS; 0.1M; pH=7.4) and incubated overnight at 4°C in a primary antibody solution containing either chicken anti-mCherry (1:2000; Abcam, Cambridge, UK) or Chicken anti-GFP (1:2000; Invitrogen, Waltham, MA) in a blocking solution with 2% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% triton-X (Sigma-Aldrich, Burlington, VA). The following day, tissue was washed in TBS and incubated in a secondary antibody solution containing either AlexaFluor 594 donkey anti-chicken (1:500; Jackson ImmunoResearch, West Grove, PA) or AlexaFluor 488 donkey anti-chicken (1:500; Jackson Immunoresearch, West Grove, PA) at room temperature for 1h. After rinsing in TBS, tissue was mounted onto gelatin-subbed slides, air-dried, and coverslipped with a Vectashield hardset antifade mounting medium with a DAPI counterstain (Vector Laboratories, Burlingame, CA) and stored at 4°C. Tissue with either AAV1/2-OXTp-mCherry or AAV1/2-OXTp-Venus bilaterally expressed within the PVN was used for subsequent fiber density analysis in the NAc (Bregma +3.24mm to +1.08mm; Paxinos and Watson, 2007). Fluorescent IHC was used as described above to detect mCherry-positive or Venus-positive fibers in the NAc.

Images were acquired with a 20x objective on a Keyence BZ-X700E/BZ-X710 fluorescent microscope and associated BZ-H3AE software (Keyence Corporation of

America, Elmwood Park, NJ). A total of five images were acquired per rat for fiber density measurements in the NAc, including the anterior NAc shell+core, intermediate NAc shell, intermediate NAc core, posterior NAc shell, and posterior NAc core (Figure 1A; Bregma +2.76mm to +2.28mm, +1.80mm to +1.56mm, and +1.08mm to +0.84mm, respectively; Paxinos and Watson, 2007). Quantitative integrated density analysis was performed to determine relative density of mCherry-positive or Venus-positive fibers in the NAc. Briefly, fiber density was measured by converting images of mCherry-positive or Venus-positive fibers in the NAc into 8-bit in ImageJ (NIH; imagej.nih.gov/ij). The number of pixels representing mCherry or Venus-positive fibers was calculated using the integrated density (IntDen) function. An average background density (BGD) measurement was acquired per image, and adjusted integrated density was calculated as (IntDen – (Area of Image*Average BGD)).

Experiment 2A: Validation of AAV1/2-OXTp-hM3Dq-mCherry in the PVN of juvenile rats

PND 26 male and female Wistar rats (n=10) received an AAV1/2-OXTp-hM3Dq-mCherry (200nl/hemisphere) injection into the PVN using stereotaxic surgery as described in experiment 1. After a 9-day transfection period, all rats received an intraperitoneal (IP) injection of either Clozapine-*N*-oxide (CNO) or saline 120 minutes prior to transcardic perfusion to quantify activation of mCherry+ neurons in the PVN following drug injection. This time period was chosen to capture the peak of c-Fos protein expression (~90 min) after activation of the DREADD receptors by CNO (~30 min) to verify that CNO increased activation of mCherry neurons in comparison to saline (Xu et al 2006; Aparico et al 2022). Brains were sliced into 30 µm coronal sections on a

cryostat (Leica CM3050, Buffalo Grove, IL) and collected into cryoprotectant solution (0.05 M sodium phosphate buffer, 30 % ethylene glycol, 20 % glycerol) in four consecutive series containing the PVN (Bregma -1.08mm to -1.72mm Paxinos and Watson, 2007). One tissue series of rats injected with AAV1/2-OXTp-hM3Dq-mCherry containing the PVN was processed for mCherry and OXT cell body detection using fluorescent IHC to determine the efficiency and specificity of viral vector transfection. Briefly, tissue was treated as described in Experiment 1 for mCherry protein amplification. Sequentially, the same tissue sections were then washed in tris-buffered saline (TBS; 0.1M; pH=7.4) and incubated overnight at 4°C in a primary antibody solution containing mouse anti-OXT (1:5000; PS38, Gift from Dr. Harold Gainer) in a blocking solution with 2% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% triton-X (Sigma-Aldrich, Burlington, VA). The following day, tissue was washed in TBS and incubated in a secondary antibody solution containing AlexaFluor 488 donkey anti-mouse (1:500; Jackson Immunoresearch, West Grove, PA) at room temperature for 1h. After rinsing in TBS, tissue was mounted onto gelatin-subbed slides, air-dried, and coverslipped with a Vectashield hardset antifade mounting medium with a DAPI counterstain (Vector Laboratories, Burlingame, CA) and stored at 4°C. Additionally, a second tissue series of rats injected with AAV1/2-OXTp-hM3Dq-mCherry containing the PVN was processed for mCherry and c-Fos protein detection using fluorescent IHC to determine the activation of mCherry+ neurons following either CNO or saline IP injection. Briefly, tissue was treated as described in Experiment 1 for mCherry protein amplification. Sequentially, the same tissue sections were then washed in tris-buffered saline (TBS; 0.1M; pH=7.4) and incubated overnight at 4°C in a primary

antibody solution containing rabbit anti-Fos (1:1000; Synaptic Systems, Goettingen, Germany) in a blocking solution with 2% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% triton-X (Sigma-Aldrich, Burlington, VA). The following day, tissue was washed in TBS and incubated in a secondary antibody solution containing AlexaFluor 488 donkey anti-rabbit (1:250; Jackson Immunoresearch, West Grove, PA) at room temperature for 1h. After rinsing in TBS, tissue was mounted onto gelatin-subbed slides, air-dried, and coverslipped with a Vectashield hardset antifade mounting medium with a DAPI counterstain (Vector Laboratories, Burlingame, CA) and stored at 4°C.

Images were acquired with a 20x objective on a Keyence BZ-X700E/BZ-X710 fluorescent microscope and associated BZ-H3AE software (Keyence Corporation of America, Elmwood Park, NJ). For efficiency and specificity analysis, verification of AAV1/2-OXTp-hM3Dq-mCherry injection placement aimed at the PVN was assessed based on anatomical features and in reference to The Rat Brain Atlas (Paxinos and Watson, 2007). Images were acquired anterior to posterior throughout the entirety of the PVN. Analysis for specificity and efficiency was conducted using triple-merged images containing OXT immunoreactive (ir), mCherry-ir, and DAPI and quantified in Photoshop (Adobe; San Jose, CA). Viral specificity (Mean: 78.80%, SEM± 3.06; percent of mCherry cells that co-express OXT; mCherry-ir+OXT-ir/mCherry*100) and efficiency (Mean: 22.27%, SEM± 4.03; percent of OXT cells that co-express mCherry; mCherry+OXT-ir/OXT*100) were calculated.Additionally, activation of mCherry-ir neurons following CNO or saline administration was quantified. In each image, the percentage of activated mCherry neurons (mCherry-ir/mCherry+Fos double labeled

cells*100) was quantified for both saline (Mean: 35.72%, SEM± 3.85) and CNO (Mean: 57.43%; SEM± 10.02) conditions (Figure 6).

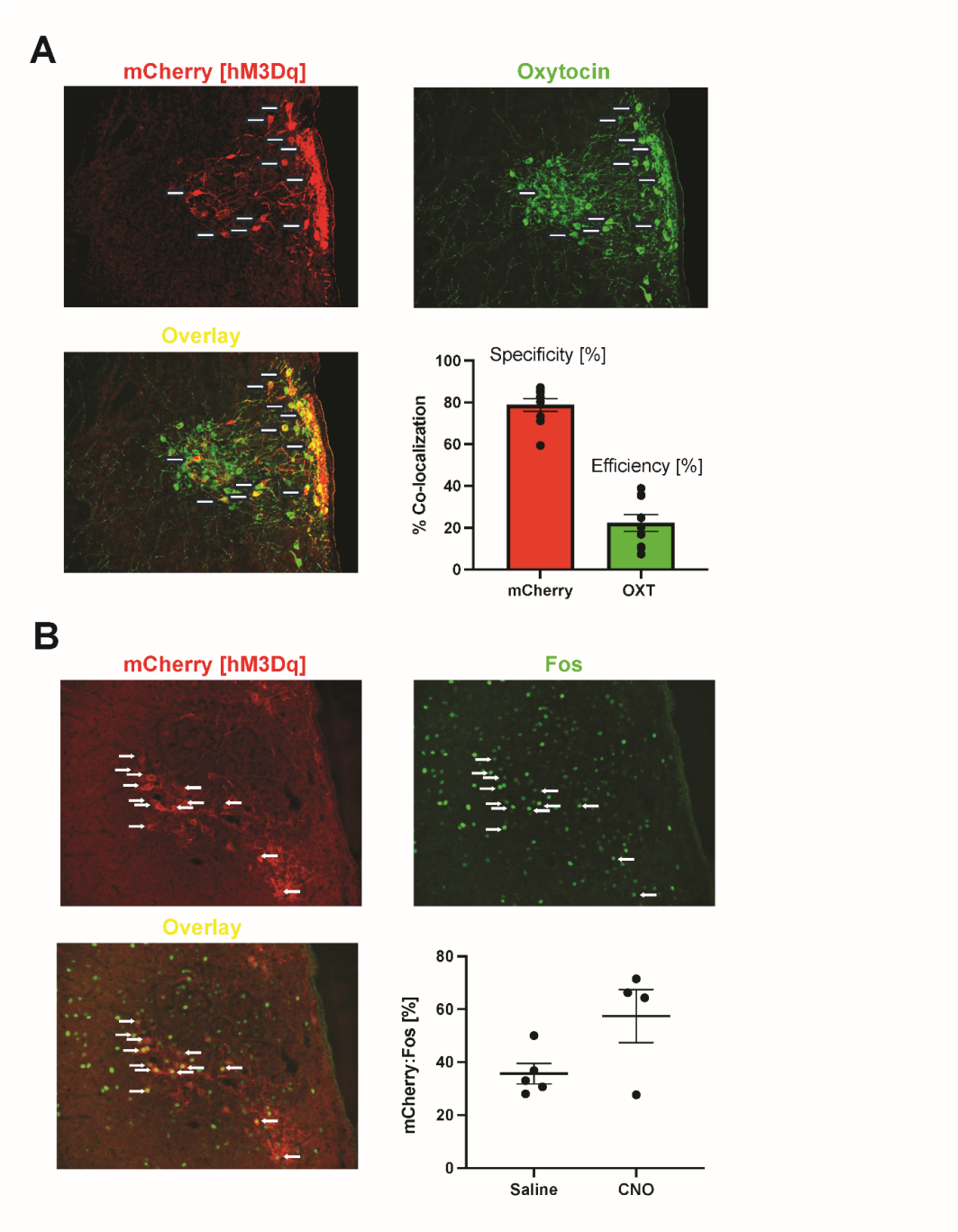

Figure 6. Validation of AAV1/2-OXTp-hM3Dq-mCherry in the PVN. (A) Representative image of mCherry (red), OXT (green), and co-localization of mCherry

Figure 6 (cont'd)

and OXT (yellow) in the PVN used to determine the specificity (% of mCherry-ir cells co-localized in OXT-ir cells; 78.8%, SEM± 3.06) and efficiency (% of OXT-ir cells co-localized in mCherry-ir cells; 22.27%, SEM± 4.03). (B) Representative image of mCherry (red), Fos (green), and co-localization of mCherry and Fos (yellow) used to determine percentage of activated mCherry-ir+ neurons following administration of either saline (35.72%, SEM± 3.85) or CNO (57.43%, SEM± 10.02).

Experiment 2B: Determine the effects of chemogenetic stimulation of PVN^{OXT} terminals in the NAc on social play behavior in juvenile male and female rats

On PND 22, subject male and female rats received an AAV1/2-OXTp-hM3Dq-mCherry (200nl/hemisphere) infused into the PVN using stereotaxic surgery as described in experiment 1. At PND 28-29, subjects underwent a second stereotaxic surgery for bilateral implantation of 6mm guide cannulae aimed at the NAc (AP:-2.6mm, ML:+/-2.5mm, DV:-3.25mm from Bregma, Paxinos and Watson, 2007). This allowed for local administration CNO into the NAc to chemogenetically stimulate the PVN^{OXT} axonal fiber terminals in the NAc. All subjects were pair-housed with same-sex littermates that underwent stereotaxic surgery until behavioral testing.

Beginning the day after cannula surgery, experimental rats began habituation to the handling and microinjection procedure. Once a day, subjects were removed from their homecage, and wrapped tightly in a towel. The dummy cannula was removed, and the internal cannula was placed in the guide and left for 30s to acclimate the subject to the microinjection procedure. At PND 31-32, approximately 24 prior to the start of social

play testing, all subjects were singly housed in clean cages and remained housed like this until the conclusion of testing. Social play testing occurred in the early dark phase (13:00-16:00h) over two days from PND 32-35. Twenty min prior to social play testing, subjects received bilateral infusions of either sterile 0.9% saline or 1mM CNO into the NAc. All experimental rats received both drugs, which were randomly assigned in a counterbalanced manner. The infusions (0.3 µL/hemisphere) were given over the course of 45 seconds via an internal cannula (28 gauge; Plastics One, Roanoke, VA) that extends 2 mm beyond the guide cannula and was connected via polyethylene tubing to a 2 µL syringe (Hamilton Company #88400) mounted onto a microinfusion pump (GenieTouch, Kent Scientific, Torrington, CT). The internal cannula was kept in place for an additional 30s following infusion to allow for tissue uptake before being replaced by the dummy cannula. Drug conditions were randomly assigned in a counterbalanced manner over the two days of testing. During the test, a group-housed sex- and age-matched conspecific (stimulus rat) was introduced into the subjects' home cage and they were allowed to freely interact for 10 min. After the conclusion of testing, the stimulus rat was removed, and the subjects cage was placed back on the housing rack. Play sessions were video recorded and behavior of the experimental rat was later scored using the video analysis program Solomon Coder (Solomon.andraspeter.com) by a researcher blind to the experimental conditions. Duration of social play (time spent engaging in playful interactions with the stimulus rat including chasing, nape attacks, wrestling, and pinning), social investigation (sniffing the anogenital region of the stimulus rat), allogrooming (grooming head and neck of stimulus rat), and cage exploration, along with number of pins (experimental rat holds stimulus rat in supine

position), nape attacks (experimental rat attacks or makes nose contact with the nape of the neck of the stimulus rat), and supine positions (experimental rat rolls on its back or is pinned on its back by stimulus rat) was scored according to Bredewold et al. (Bredewold & Veenema, 2018).

At the conclusion of the second day of testing, rats were deeply anesthetized with isoflurane (5%) before transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1M borate buffer (pH= 9.5). Brains were extracted and post-fixed in 4% paraformaldehyde with 12% sucrose for 24h. The olfactory bulb and cerebellum with removed using a brain matrix. Brains were flash frozen in 2-methlybutane and stored at -80°C until histological processing.

Brains were sliced into 30 µm coronal sections on a cryostat (Leica CM3050, Buffalo Grove, IL) and collected into cryoprotectant solution (0.05 M sodium phosphate buffer, 30 % ethylene glycol, 20 % glycerol) in four consecutive series containing both the NAc and PVN (Bregma -1.08mm to -1.72mm Paxinos and Watson, 2007). Series 1 of the NAc was mounted onto slides and counterstained with thionin to determine cannula placement. Additionally, series 1 of the PVN was processed for mCherry-ir cells via fluorescent IHC as described in Experiment 1 to confirm viral transfection within PVN cell bodies. For rats with at least 100 labeled neurons (approximately 10-20% of OXT neurons; Figure 8, series 2 of the NAc was processed for mCherry-ir fibers projecting from the PVN. Only rats with bilateral mCherry staining contained within the PVN, as well as evidence of mCherry fibers within the NAc, were used in the subsequent behavioral analysis. Additionally, rats expressing bilateral PVN mCherry with cannula placement outside the anterior NAc (NAc misses) were used as control

group to account for effects of potential chemogenetic stimulation of PVN^{OXT} fibers outside of the region of interest within the NAc.

Statistical Analysis

For experiment 1, effects of sex (male v female), fluorophore (Venus v mCherry), and NAc subregion (Anterior v Intermediate Core v Intermediate Shell v Posterior Core v Posterior Shell) in fiber density were assessed using a 3-way analysis of variance (ANOVA). For experiment 2, the effects of sex (male versus female) and drug (SAL v CNO) on behavior were analyzed using a 2-way repeated measures ANOVA. Additionally, a control group with cannula placement outside of the NAc was analyzed using a 2-way repeated measures ANOVA to determine that CNO administration outside of the area of interest does not alter behavior. For both experiments, Holm-Sidak post-hoc tests were performed when significant interactions occurred. Effect sizes were calculated using partial η^2 ($\eta^2_p = SS_{effect}/SS_{residual}$) for repeated measures and mixed effect ANOVAs. Analyses were performed in GraphPad Prism (version 9) and significance was set at $p < 0.05$.

3. Results

3.1 Characterization of PVN^{OXT} fibers projecting to the NAc

There was a main effect of NAc subregion on PVN^{OXT} labeled fibers (Fig. 7; $F_{4,20} = 9.26$, $p = 0.0002$). Post-hoc Holm-Sidak's test for multiple comparisons revealed that the anterior region of the NAc core/shell showed a higher density of PVN^{OXT} labeled fibers compared to the intermediate NAc core ($p = 0.003$), intermediate NAc shell ($p = 0.0004$), and posterior NAc shell ($p = 0.001$). Due to the higher density of PVN^{OXT} fibers

in the anterior portion of the NAc, this region was specifically targeted in experiment 2. Additionally, there was a NAc subregion x AAV interaction (Fig. 7; $F_{4,20} = 3.705$, $p = 0.02$). Post-hoc tests revealed a trend towards higher density of mCherry fibers versus Venus fibers in the anterior and posterior core of the NAc, though this did not quite reach significance ($p = 0.06$ for both). Given that fluorophore may alter transfection of both PVN^{OXT} neurons, and therefore PVN^{OXT} fibers in the NAc, all subsequent experiments used only mCherry-tagged viral vectors.

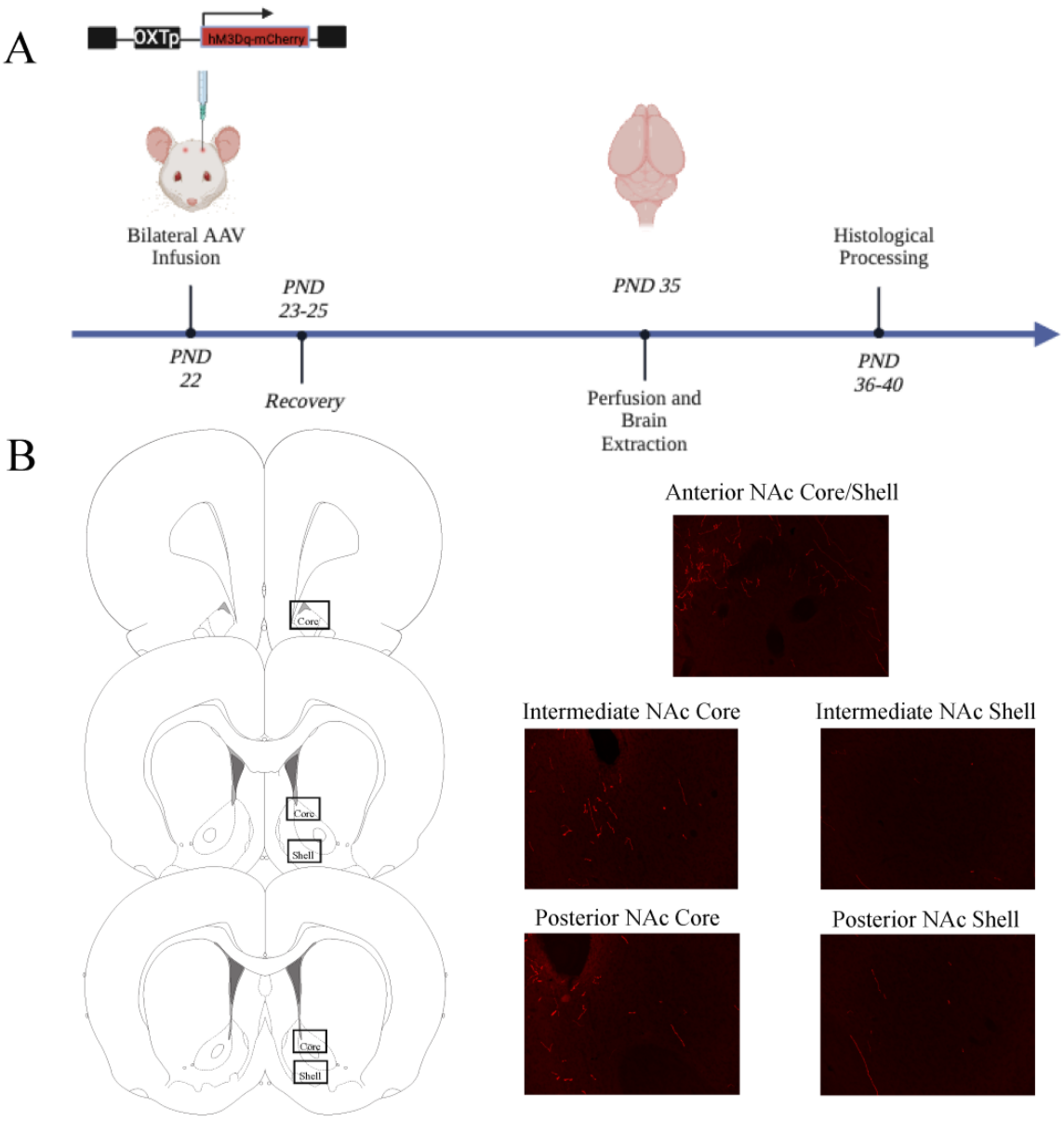

Figure 7. PVN^{OXT} fiber expression in the NAc. (A) Schematic of experimental timeline. Briefly, juvenile male and female rats were infused with an OXTp-reporter AAV tagged to either the fluorophore mCherry or Venus at PND22. All rats were perfused and brains were collected at PND35 for histological processing and fiber density analysis. (B) Imaging parameters and representative images for each of the five levels of the NAc that were analyzed.

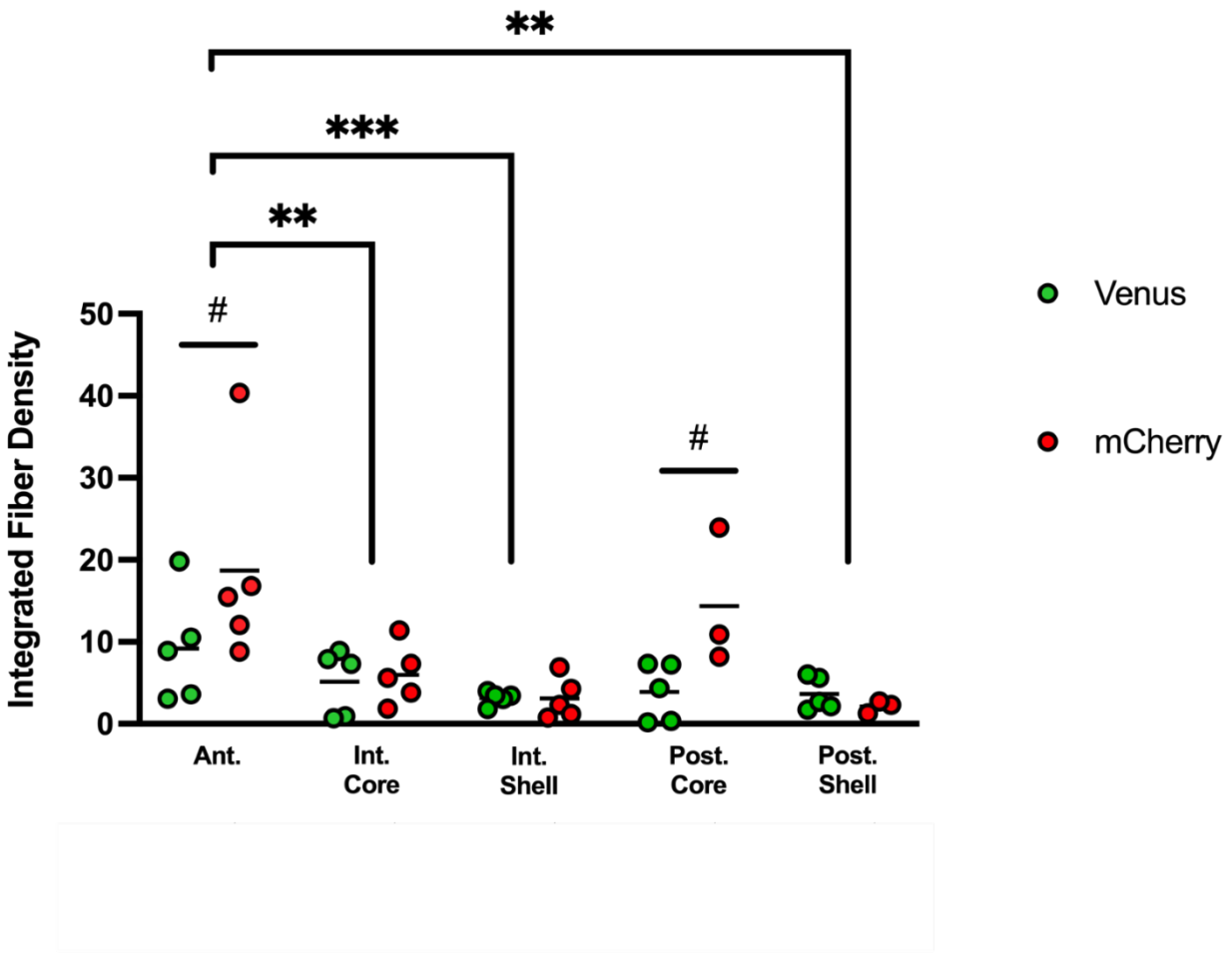

Figure 8. The anterior subregion of the NAc shows a higher integrated density of PVN^{OXT} fibers compared to the intermediate and posterior subregions. The anterior portion of the NAc has a higher density of OXT fibers projecting from the PVN in comparison to the intermediate core, intermediate shell, and the posterior shell. In addition, there was a trend towards increased integrated fiber density in the mCherry-expressing fibers compared to Venus-expressing fibers in the anterior and posterior core of the NAc. 2-way ANOVA; main effect of region, **: $p < 0.01$, ***: $p < 0.001$; region x AAV interaction, #: $p < 0.1$, Holm's-Sidak post-hoc tests.

3.2 Chemogenetic stimulation of PVN^{OXT} terminals alters social investigation sex-specifically

There was a main effect of sex on the number of pins, in which females displayed fewer acts of pinning a play partner in comparison to males, regardless of drug condition (Figure 8F). Additionally, there was a significant sex x drug interaction on social investigation following stimulation of PVN^{OXT} terminals via local infusion of CNO in the NAc (Figure 8G). Holm-Sidak post-hoc tests revealed that CNO administration increased social investigation in juvenile males ($p = 0.03$), as well as a tendency towards a decrease in social investigation in females ($p = 0.06$). There were no effects of sex or drug administration on the duration of social play, number of nape attacks, duration of allogrooming, or cumulative duration of social behaviors (Figure 8; see Table 1 for full ANOVA results). Local infusion of CNO outside of the NAc did not alter any social or non-social behaviors in either sex (Figure 9 A-F; Table 1, $p > 0.05$ for all).

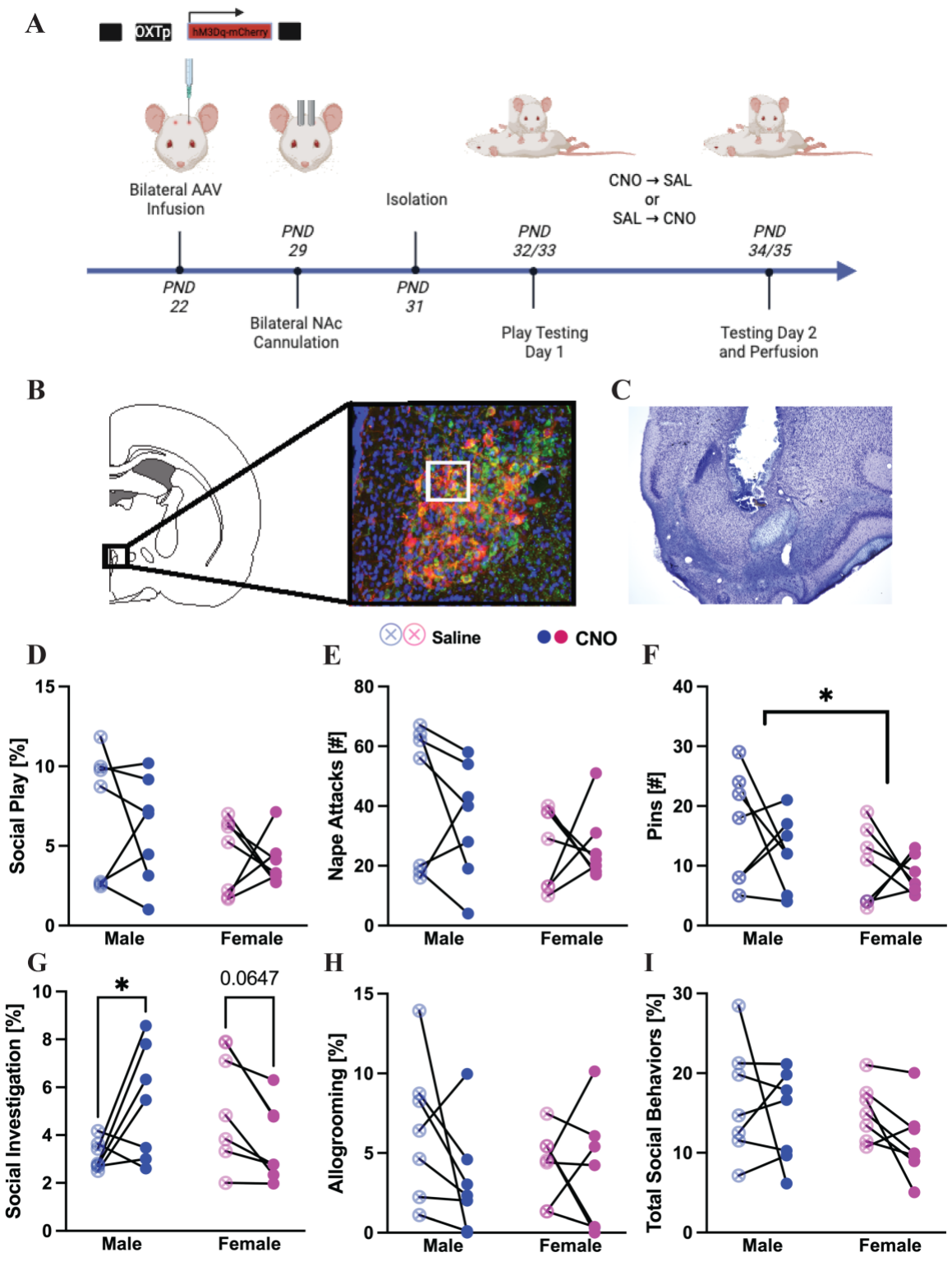

Figure 9. Terminal stimulation of PVN^{OXT} fibers in the NAc does not alter juvenile

Figure 9 (cont'd)

social play behavior in either male or female rats, but sex-specifically alters social investigation. (A) Timeline of experimental procedures. Briefly, juvenile rats underwent stereotaxic surgery and infusion of OXTp-hM3Dq-mCherry into the PVN at postnatal day (PND) 22 with bilateral cannulation of the NAc at PND 29. Following recovery and habituation to cannulation infusion procedures, all rats underwent two days of social play testing in which either saline or CNO was locally infused 20 minutes prior to testing. Drug conditions were randomly assigned and counterbalanced over the two days of testing. (B) Rat brain atlas image (modified from Paxinos and Watson, 2007) indicating the location and representative image of AAV-OXTp-hM3Dq-mCherry expression in OXT neurons of the PVN. Magnification of image in B denoting mCherry (Red) and OXT (Green) positive neurons that are co-localized using DAPI as a cellular marker. (C) Nissl-stained image of a brain section showing cannula placement within the anterior NAc. (D-F) CNO administration did not alter the duration of social play (D) number of nape attacks (E), number of pins (F), duration of allogrooming (H), or total duration of social behaviors (I), though females displayed fewer pins than males regardless of drug (F). Administration of CNO sex-specifically altered social investigation, wherein males showed a significant increase, and females had a strong tendency towards a decrease, in duration of social investigation (G). Durations of social play (G), investigation (J), allogrooming (K), and combined social behaviors (L) are expressed as a percentage of total time. 2-way ANOVA; main effect of sex, *: $p < 0.05$; Sex x Drug interaction, *: $p < 0.05$, Holm's-Sidak post-hoc tests.

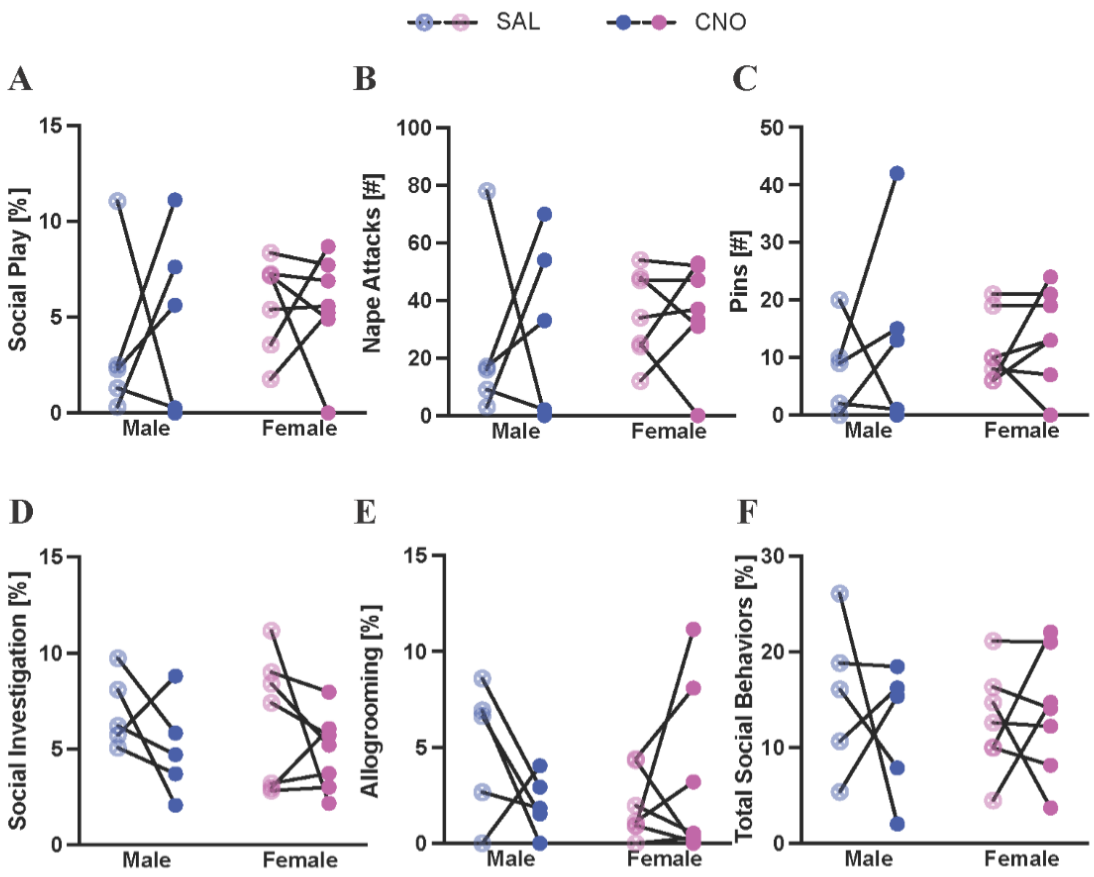

Figure 10. Local infusion of CNO outside of the NAc does not alter any social behaviors in juvenile male or female rats. Males and females showed similar duration of social play (A), social investigation (D), allogrooming (E), and total social behaviors (F), as well as frequency of nape attacks (B) and pins (C), when treated with saline and CNO; repeated measures 2-way ANOVA, $p > 0.05$ for all.

Table 1: 2-way ANOVA statistics for behaviors quantified in social play testing. Significant main effects or interactions are notated in **bold.**

Effects of chemogenetic stimulation of PVN^{OXT} terminals in the NAc			
	Drug	Sex	Drug x Sex

Table 1 (cont'd)

% Social Play (Fig 9D)	$F_{1,12}$ = 0.37, p = 0.56, η^2_p = 0.03	$F_{1,12.}$ = 3.52, p = 0.09, η^2_p = 0.29	$F_{1,12}$ = 04, p = 0.84, η^2_p = 0.004
Nape Attacks (Fig 9E)	$F_{1,12}$ = 0.43, p = 0.52, η^2_p = 0.03	$F_{1,12}$ = 3.19, p = 0.10, η^2_p = 0.29	$F_{1,12}$ = 0.50, p = 0.50, η^2_p = 0.04
Pins (Fig 9F)	$F_{1,12}$ = 1.12, p = 0.31, η^2_p = 0.09	**$F_{1,12}$ = 5.02, p = 0.04, η^2_p = 0.23**	$F_{1,12}$ = 0.18, p = 0.68, η^2_p = 0.01
% Investigation (Fig 9G)	$F_{1,12}$ = 0.29, p = 0.60, η^2_p = 0.02	$F_{1,12}$ = 0.08, p = 0.78, η^2_p = 0.02	**$F_{1,12}$ = 11.67, p = 0.005, η^2_p = 0.49**
% Allogrooming (Fig 9H)	$F_{1,12}$ = 2.14, p = 0.17, η^2_p = 0.15	$F_{1,12}$ = 0.32, p = 0.58, η^2_p = 0.03	$F_{1,12}$ = 1.19, p = 0.30, η^2_p = 0.09
% Total Social Behavior (Fig 9I)	$F_{1,12}$ = 2.05, p = 0.18, η^2_p = 0.15	$F_{1,12}$ = 1.08, p = 0.32, η^2_p = 0.10	$F_{1,12}$ = 0.19, p = 0.67, η^2_p = 0.02

Effects of CNO administration outside of the NAc

	Drug	Sex	Drug x Sex
% Social Play (Fig 10A)	$F_{1,10}$ = 0.12, p = 0.73, η^2_p = 0.01	$F_{1,10}$ = 1.72, p = 0.22, η^2_p = 0.07	$F_{1,10}$ = 0.23, p = 0.64, η^2_p = 0.02
Nape Attacks (Fig 10B)	$F_{1,10}$ = 0.15, p = 0.71, η^2_p = 0.01	$F_{1,10}$ = 0.77, p = 0.40, η^2_p = 0.04	$F_{1,10}$ = 0.07, p = 0.79, η^2_p = 0.01
Pins (Fig 10C)	$F_{1,10}$ = 1.10, p = 0.32, η^2_p = 0.10	$F_{1,10}$ = 0.11, p = 0.75, η^2_p = 0.01	$F_{1,10}$ = 0.20, p = 0.67, η^2_p = 0.02

Table 1 (cont'd)

% Investigation (Fig 10D)	$F_{1,10} = 2.73$, $p = 0.13$, $\eta^2_p = 0.21$	$F_{1,10} = 0.11$, $p = 0.74$, $\eta^2_p = 0.01$	$F_{1,10} = 0.03$, $p = 0.87$, $\eta^2_p = 0.002$
% Allogrooming (Fig 10E)	$F_{1,10} = 0.31$, $p = 0.59$, $\eta^2_p = 0.03$	$F_{1,10} = 0.47$, $p = 0.51$, $\eta^2_p = 0.04$	$F_{1,10} = 2.52$, $p = 0.14$, $\eta^2_p = 0.20$
% Total Social Behavior (Fig 10F)	$F_{1,10} = 0.16$, $p = 0.70$, $\eta^2_p = 0.02$	$F_{1,10} = 0.04$, $p = 0.85$, $\eta^2_p = 0.002$	$F_{1,10} = 0.51$, $p = 0.49$, $\eta^2_p = 0.05$

4. **Discussion**

Here, I demonstrated that OXTergic neurons of the PVN differentially innervate the NAc, with the anterior portion of the NAc showing significantly higher fiber density measurements than the intermediate or posterior portions. There were no sex differences found within any of the NAc subregions analyzed, indicating that males and females do not differ in the distribution patterns of PVN^{OXT} fibers within the NAc. Additionally, I showed that chemogenetic stimulation of the PVN-OXT to anterior NAc pathway modulates juvenile social investigation sex-specifically, without altering juvenile social play. In detail, chemogenetic stimulation of PVN-OXT terminals in the anterior NAc increased social investigation of an age- and sex-matched conspecific in males and tended to decrease social investigation in females. These findings imply that while there are no sex differences in OXTergic innervation from the PVN to the NAc , there may be sex differences in the function of this pathway.

The anterior portion of the NAc shows higher density of PVN^{OXT} fibers than other NAc subregions in both male and female juvenile rats

No differences in fiber density between males and females were detected in any subregion of the NAc analyzed. I instead found that the anterior NAc shows higher integrated density of PVN-OXT fibers compared to the intermediate core/shell, as well as the posterior shell. This was unexpected given behavioral differences between the sexes when stimulating PVN-OXT terminals in the NAc (Figure 9). However, there are few sex differences within the OXT system, specifically in OXT synthesis, though this varies by species (Dumais & Veenema, 2016). In fact, quantification of OXT fibers within 22 different nodes of the social behavior neural network (SBNN) in adult Wistar rats also showed no sex differences in innervation (DiBenedictis et al., 2017). Furthermore, this finding is in line with a lack of a sex difference in OXTR binding density studies in the anterior NAc (Dumais et al., 2013). Despite quantitative measurements of OXTergic projections (DiBenedictis et al., 2017), as well as PVN^{OXT} projections specifically (Knobloch et al., 2012) throughout the rat brain, this study is the first to quantify PVN^{OXT} projections throughout different subregions of the NAc.

It is important to note that though there was an increase in fiber density to the anterior NAc compared to other subregions, it is unclear whether higher fiber density in the anterior NAc compared to other regions equates to higher OXT release. Additionally, a limitation to this AAV mediated tracing technique is that it is unclear whether fibers represent axonal terminals, fibers of passage, or dendrites. The use of retrograde tracing techniques, such as cholera toxin subunit B (CtB), could be used to verify synaptic connections between PVN^{OXT} fibers and neurons within the NAc. CtB is

taken up by axon terminals and actively transported back to the soma of the cell (Lai et al., 2015; Saleeba et al., 2019), thus verifying that synapses are made between these two regions of interest. In support, I previously found CtB and OXT co-labeling within the PVN, indicating the potential for neurotransmission between these two regions (Bowden et al., 2021), though CtB infusion was contained only within the intermediate portion of the NAc. As such, future studies should be conducted to confirm the connection between the PVN and the anterior NAc specifically. This could be accomplished using retrograde tracing in the NAc and co-labeling with OXT in the PVN, or histological detection of synaptic connections using synaptophysin staining within PVN-OXT fibers of the NAc. Additionally, it would be of interest to combine PVN^{OXT} fiber density measures with CtB tracing within the NAc subregions. This could be used to determine whether different populations of OXT neurons project to different subregions of the NAc. This information could then be used to target specific populations of PVN^{OXT} to NAc projecting neurons to determine whether they regulate discrete social behaviors, such as social investigation or social play. Furthermore, this current study utilized a bilateral AAV infusion approach to quantify fiber density measurements within the NAc. Future studies could instead employ a unilateral approach to better understand the ipsilateral, and potential for contralateral, innervation of the NAc by PVN^{OXT} fibers.

Chemogenetic stimulation of PVN^{OXT} terminals in the anterior NAc sex-specifically modulates social investigation without altering social play

I did not find evidence to support my hypothesis that the PVN^{OXT} to NAc pathway regulates social play behavior, nor that it did so sex specifically. However, I found that chemogenetic stimulation of PVN^{OXT} terminals in the NAc altered social investigation in

a sex-specific manner. In detail, PVN^{OXT} terminal stimulation in the NAc increases social investigation in males and has a strong tendency to decrease social investigation in females. Thus, like chemogenetic stimulation of PVN^{OXT} cell bodies, chemogenetic stimulation of PVN^{OXT} terminals in the NAc altered juvenile social behavior sex-specifically.

Chemogenetic stimulation of either PVN^{OXT} cell bodies or PVN^{OXT} fibers in the NAc has been shown to modulate the expression of social behaviors, specifically in male rodents. For example, optogenetic stimulation of PVN^{OXT} neurons in adult male mice induced a real-time CPP for a chamber paired with novel juvenile male mouse (Hung et al., 2017), suggesting that PVN^{OXT} neurons enhances social motivation. Additionally, calcium imaging studies have shown that PVN^{OXT} neurons projecting to the NAc are activated in adult male mandarin voles when investigating their pups (He et al., 2021). Furthermore, chemogenetic stimulation of the PVN^{OXT} to NAc pathway in these mandarin voles increased paternal behaviors such as decreased latency for pup retrieval and increased licking (He et al., 2021). These data are in line with my findings that chemogenetic stimulation of PVN^{OXT} terminals in the NAc increases social investigation in juvenile male rats. In contrast to the male data, I found that chemogenetic stimulation of the PVN^{OXT} terminals in the NAc decreased social investigation in females (Figure 8), indicating a sex-specific role of OXT in social investigation. While I have previously shown that PVN^{OXT} cell body stimulation does not alter social investigation in juvenile female rats (Figure 2), these opposing effects on social investigation could be due in part to large differences in methodology. For example, cell body manipulations used intraperitoneal (IP) injections of CNO, which

would theoretically stimulate PVN^{OXT} cell bodies and terminals in the NAc as well as terminals elsewhere in the brain. Therefore, it would be expected for global increase in OXT due to IP CNO injections to differentially modulate behaviors in comparison to discrete pathway manipulations. While I found that PVN^{OXT} cell body stimulation increased social play in juvenile females, Tang et al. (Tang et al., 2020) found that chemogenetic stimulation of PVN^{OXT} cell bodies increased social investigation in adult female rats to a conspecific. These studies together suggest that global increases in OXT via PVN cell body stimulation increases the expression of social behaviors in females, including social play in juveniles and social investigation in adults, though the downstream pathway regulating these behaviors are currently unknown.

In the second experiment, my goal was to directly determine the role of OXT projections from the PVN to the NAc in juvenile social play. However, my results show that chemogenetic stimulation of PVN^{OXT} terminals in the NAc had no effect on juvenile social play behaviors, including duration of social play, frequency of nape attacks, or frequency of pins in either sex. These findings are in contrast with my previous report in which chemogenetic activation of PVN^{OXT} cell bodies decreased juvenile social play in males and increased social play in females (Figure 2). In both juvenile and adult rats, OXTR activation in the NAc is necessary for the typical expression of social behaviors, with antagonism of OXTRs in the NAc generally decreasing expression of these behaviors. For example, OXTR antagonism in the NAc eliminates the formation of a social conditioned place preference (CPP) in juvenile male mice (Dölen et al., 2013). Additionally, NAc-OXTR antagonism following parturition in female rats impairs the onset of maternal behavior (D'Cunha et al., 2011). Furthermore, preliminary data from

our lab has shown that OXTR-A in the NAc decreases juvenile social play sex-specifically, with males requiring a 10x higher dose of the OXTR-A than females to show a similar reduction in play (Figure 3A). Importantly, while these studies demonstrate the necessity of NAc-OXTR signaling in the expression of social behaviors, they did not test whether increased OXT signaling was sufficient to further increase social behaviors. Therefore, the null effects on juvenile social play could be due to the insufficiency of artificially increasing OXT within the NAc via PVN^{OXT} terminal stimulation. Future studies should be done to determine the necessity of the PVN^{OXT} to NAc pathway in juvenile social play, which could be accomplished via PVN^{OXT} terminal inhibition in the NAc.

The use of chemogenetic tools, such as the OXT promotor inhibitory DREADD AAV used in this study, has been well validated to target and manipulate discrete neuronal populations to determine alterations in behavioral outcomes (Knobloch et al 2012; Roth et al 2016; Smith et al 2016; Grund et al 2019; Tang et al 2020). Despite this, there are several limitations of the use of DREADDs that may have influenced the behavioral effects of chemogenetic stimulation of PVN^{OXT} terminals within the NAc. First, the ligand CNO has shown the potential to back metabolize to clozapine, which can affect both the serotonergic and dopaminergic system, though this is rarely seen in rodents at the dosages utilized in this experiment (Roth 2016; Whissell 2016). While there was no effect found on any social behaviors measured following CNO administration in regions outside of the anterior portion of the NAc, validity of these findings could be improved through testing the effects of CNO administration within the NAc in the absence of DREADD receptors. This could be accomplished by intracranial

administration of CNO into the NAc of WT rats that instead received an OXT promotor reporter AAV in the PVN. Alternatively, use of newly developed DREADD receptor ligands which are less likely or unable to back metabolize to clozapine could be used in the future (Roth 2016). Additionally, chemogenetic methods are restricted by the efficiency of transfection of the AAV into the cell type or region of interest. For these experiments, approximately 10-20% of the total PVN^{OXT} neurons expressed the fluorophore mCherry, a proxy measure of DREADD receptor transfection. Given this lower transfection rate of PVN^{OXT} neurons, of which only a subset may in turn project to the NAc, stimulation of the fibers within the anterior NAc may have been insufficient to induce robust behavioral changes in the expression of social play. In the future, multiple injections of the OXT promotor excitatory DREADD construct across the anterior-posterior axis of the PVN could instead be infused to improve efficiency of OXT cell body transfection, as well as increase DREADD receptor expressing terminals in the NAc.

In addition to the NAc, PVN^{OXT} neurons project to several other brain regions involved in the modulation of social behaviors, such as the lateral septum, the bed nucleus of the stria terminalis, and the ventral tegmental area (VTA) (He et al., 2021; Hung et al., 2017; Knobloch et al., 2012). The PVN to VTA pathway in particular has been implicated in the modulation of rewarding behaviors, including social reward. For example, optogenetic excitation of PVN^{OXT} fibers in the VTA is sufficient to induce a social CPP, while optogenetic inhibition creates a social conditioned place aversion in adult male mice (Hung et al., 2017). Furthermore, chemogenetic stimulation of this pathway increases paternal behaviors, such as pup licking, in male mandarin voles (He

et al., 2021). OXTR antagonism in the VTA in male and female Syrian hamsters prior to social CPP attenuates the time spent in a social chamber compared to saline controls (Borland, Aiani, et al., 2019; Borland, Rilling, et al., 2019). Together, these studies suggest a key role of VTA^{OXTR} signaling in the modulation of social reward.

PVN^{OXT} fibers in the VTA are located in close proximity to VTA dopamine (DA) neurons, which also express the OXTR (Melis et al., 2007; Rappeneau & Castillo Díaz, 2024). These $VTA^{DA+OXTR}$ neurons in turn project to the NAc, which may modulate social behaviors through the release of DA (He et al., 2021; Hung et al., 2017; Peris et al., 2017). Indeed, research shows that optogenetic inhibition of DAergic VTA neurons projecting to the NAc decreases levels of paternal care in male mandarin voles (He et al., 2021). Additionally, juvenile female, but not male, rats show an increase in VTA^{DA} neuronal activity following social play (Northcutt & Nguyen, 2014). Given the necessity of OXTR signaling in both the NAc and VTA individually in the expression of social behaviors, these data together lead to the potential of coordinated actions of OXT release from the PVN in their regulation. In support, PVN^{OXT} cell body stimulation, which would in theory release OXT into both the NAc and VTA, is sufficient to modulate social play, while PVN^{OXT} terminal stimulation in the NAc alone is not. But, whether co-release of OXT from the PVN in both the VTA and the NAc is necessary is yet unknown. Future studies should be conducted to determine potential interactions between the PVN, VTA, and NAc in the regulation of juvenile social play.

CHAPTER 3. DETERMINE THE ROLE OF THE NAC^{OXTR} TO VP PATHWAY IN THE EXPRESSION OF JUVENILE SOCIAL PLAY BEHAVIOR

Abstract

Social play, also called rough and tumble play, is an integral part of mammalian behavior most commonly displayed during the juvenile period. Social play is indispensable for the development of social competency, which is needed for the expression of context appropriate social behaviors throughout life. Children with social developmental disorders, such as autism spectrum disorder, show reduced engagement in social play and report social interactions as less rewarding than non-social interactions. As such, it is possible that aberrations within the brain reward circuitry may contribute to decreased engagement in social play in autistic children. The nucleus accumbens (NAc) and ventral pallidum (VP) are two key regions within the mesolimbic reward pathway, both of which have been shown individually to be involved in the regulation of juvenile social play. Additionally, the neuropeptide oxytocin (OXT) has been implicated in the regulation of social behaviors within the NAc, but the role of OXT receptor (OXTR)-expressing neurons in the NAc on the expression of juvenile social play is unknown. Therefore, I determined the role of NAc^{OXTR} cell body activity on social play behavior using an inhibitory DREADD construct that was only expressed in OXTR neurons within the NAc. Here, I found that chemogenetic inhibition of NAc^{OXTR} cell bodies sex-specifically altered juvenile social play behavior, increasing social play in females with a tendency towards a reduction in males. Next, I wanted to determine whether NAc^{OXTR} neuronal projections to the VP was involved in the regulation of social play. To test this, inhibitory DREADD constructs were injected into the NAc of juvenile rats and the terminals within the VP were selectively activated via local infusion of the

ligand clozapine-N-oxide. I found that chemogenetic inhibition of NAc^{OXTR} terminals did not alter the expression of juvenile social play or social investigation, although this study lacked the power to test for potential sex differences in behavioral effects. Together, these studies demonstrate sex differences in the regulation of social play by OXT in the NAc, though more research is necessary to understand the role of the NAc^{OXTR} to VP pathway in the regulation of juvenile social behavior. Additionally, findings support a model in which inhibition of the NAc^{OXTR} neurons facilitates the expression of juvenile social play only in females, suggesting a different mechanism of OXT action within the NAc of males and females in the regulation of social play.

1. Introduction

Social interactions are integral for the development and maintenance of social competency, or the ability to generate the appropriate response and adequately adapt within the social environment (Rose-Krasnor, 1997). While different types of social interactions vary across the life span, one of the most predominately displayed social behaviors in juveniles is social play (Palagi, 2018; Anthony D. Pellegrini & Smith, 1998; A D Pellegrini, 1989; Sergio M. Pellis & Pellis, 1987a; Shimada & Sueur, 2018). Social play behavior is one of the first forms of peer-peer interaction, and these playful interactions during the juvenile period help to build social competency and enhance cognitive flexibility. Researchers have found that social isolation during the juvenile period in rats caused social, emotional, and cognitive deficits in adulthood (Fone & Porkess, 2008; L. J. Vanderschuren et al., 1997; van den Berg et al., 1999b), including context-inappropriate aggression (Byrd & Briner, 1999; Hol et al., 1999) and increased anxiety-like behavior (Wright et al., 1991; Weiss et al., 2004; Cuesta et al., 2020). These behavioral deficits seem to be specific to a lack of social play, as juvenile rats housed with a mesh barrier to separate them from a conspecific (thus allowing olfactory, visual, and tactile information; but not for free-moving social interaction i.e. social play) still show social and cognitive deficits later in life (Sergio M. Pellis & Pellis, 2007). This suggests that active engagement in social play is critical for typical development.

In humans, children diagnosed with social deficit disorders such as autism spectrum disorder (ASD) are less likely to engage in social play than their neurotypical peers (Jordan, 2003). This behavioral deficit may contribute to the lifelong social communication and competency issues commonly seen in ASD adults. ASD children

show reduced interest in social interactions and rate social interactions as less pleasurable than neurotypical children (Chevallier, Grèzes, et al., 2012; Ruta et al., 2017). This observed decrease in social motivation could be due to alterations in signaling within the reward pathway of the brain in ASD individuals. Indeed, fMRI results indicate that there is a reduction in functional connectivity within the mesolimbic reward pathway, specifically between the ventral tegmental area (VTA) and the nucleus accumbens (NAc), in ASD children in reaction to a social stimulus (Supekar et al., 2018). These studies suggest a key role for the mesolimbic reward system in the regulation of social behaviors. The nucleus accumbens (NAc) in particular has been implicated in the regulation of juvenile social play in rats (Manduca et al., 2016; van Kerkhof et al., 2013, 2014). For example, NAc core and shell activity is increased during spontaneous play sessions in male juvenile rats (van Kerkhof et al., 2014). Additionally, inactivation of the NAc core via site-specific infusion of GABA-A and -B receptor agonists increased duration of juvenile social play (van Kerkhof et al., 2013). These studies indicate that modulation of NAc activity may be involved in the regulation of juvenile social play, but the neurochemical mechanisms underlying social play are unclear.

The NAc is composed of primarily GABAergic neurons which express receptors for oxytocin (OXT), as well as other neurochemicals (Mitre et al., 2016; Shirayama & Chaki, 2006; Yager et al., 2015). OXT receptor (OXTR) activation in the NAc is critical for the expression of social behaviors, including social approach (Williams et al., 2020; Yu et al., 2016), pair bonding (Keebaugh et al., 2015; Liu & Wang, 2003; Young et al., 2001), maternal care (D'Cunha et al., 2011; Keebaugh & Young, 2011; Keebaugh et al.,

2015; Witchey et al., 2024) and social reward (Dölen et al., 2013; Nardou et al., 2019; Wei et al., 2015). In both male and female California mice, for example, OXTR antagonism in the NAc decreased investigation to a sex- and age-matched stimulus mouse (Williams et al., 2020). Additionally, viral vector mediated selective knockdown of OXTRs the NAc reduced the expression of alloparental and pair bonding behaviors in female prairie voles (Keebaugh & Young, 2011). OXTR antagonism in the NAc also inhibited the formation of a conditioned place preference (a measure of social reward processing) in adolescent male mice to a chamber previously associated with a social stimulus (Dölen et al., 2013). Furthermore, OXTR antagonism reduces social play sex-specifically in rats, with males requiring a ten-fold higher dose than females to show equivalent reductions in social play (Figure 3A). This suggests the necessity of NAc^{OXTR} signaling in the regulation of juvenile social play, but the actions of OXTRs in the NAc may differ slightly between the sexes. Indeed, I have found that juvenile social play duration negatively correlates to activation of *Oxtr*-expressing neurons in the NAc, an effect that was primarily driven by males (Figure 3B-D). Together, these data support a model in which inhibition of OXTR-expressing neurons in the NAc may facilitate the expression of juvenile social play behavior in both sexes, but to a greater extent in males than females.

This model finds support in the overall hypothesis that reward processing is mediated via inhibition of the NAc (William A Carlezon & Thomas, 2009). In support, electrophysiological recordings of NAc neurons show an inhibitory response to intraoral administration of sucrose in adult male rats (Roitman et al., 2005), demonstrating that rewarding stimuli induces inhibition of the NAc *in vivo.* Additionally, indiscriminate

inhibition of the NAc is sufficient to induce reward-like behavior, as rats readily learn to self-administer the NMDA receptor antagonist dizocilpine directly into the NAc (W A Carlezon & Wise, 1996). As the NAc is primarily comprised of GABAergic medium spiny neurons (MSNs), inhibition of these cells during reward processing could disinhibit NAc MSNs projecting to regions that facilitate reward directed behaviors, like the ventral pallidum (VP) (Kretschmer, 2000; K. S. Smith et al., 2009; Soares-Cunha & Heinsbroek, 2023). Indeed, the VP has directly been implicated in the regulation of rewarding and motivated behaviors (Faget et al., 2024; Heimer et al., 1991; Kretschmer, 2000; K. S. Smith et al., 2009). For example, pharmacological inhibition of the VP via infusion of the $GABA_A$ receptor agonist muscimol decreases juvenile social play in both male and female rats (J. D. A. Lee et al., 2021). Additionally, chemogenetic excitation of NAc^{GABA} terminals in the VP decreases social play in juvenile rats (Lee, 2023). Together, these studies indicate that VP activity is necessary for the typical expression of juvenile social play. This process may be accomplished via inhibition of NAc^{GABA} inputs to the VP, thus allowing for the disinhibiting the VP. However, the mechanisms at the level of the NAc that would accomplish disinhibition of the VP are currently unknown.

Given the modulatory effects of OXTRs in the NAc on social play, I hypothesized that one possible mechanism could be that NAc^{OXTR} expressing neurons projecting to the VP regulates the expression of juvenile social play. To test this hypothesis, I utilized juvenile OXTR-iCre male and female rats, which allowed for selective chemogenetic targeting of OXTR-expressing neurons in the NAc. As correlational data suggests inhibition of NAc^{OXTR} neurons occurs during spontaneous social play (Figure 3B), I predicted that inhibition of NAc^{OXTR} expressing neurons would facilitate the expression

of juvenile social play. Additionally, as the negative correlation between NAc^{OXTR} activity and social play was primarily driven by males, I predicted that inhibition of NAc^{OXTR} neuronswould further increase social play in males compared to females. Additionally, I predicted that inhibition of fiber terminals originating from the NAc within the VP would increase the expression of juvenile social play behavior.

2. Methods

Animals

Subjects were experimentally naïve male and female heterozygous OXTR-iCre + rats bred in-house by crossing OXTR-iCre + rats with wild-type (WT) Wistar animal stock previously obtained from Charles River Laboratories (Raleigh, NC, USA). Rats were maintained under standard laboratory conditions (12:12h light/dark cycle, lights off at 13:00h, 22°C, 50% humidity, food, and water available *ad libitum*). Subject rats were weaned at post-natal day (PND) 21 and housed in single-sex groups of 2-4 in standard Allentown cages (48x27x20cm). Stimulus male and female Wistar rats were used for social play testing (PND 22-30 at arrival; Charles River Laboratories, Raleigh, NC, USA) and were housed in the same conditions as in-house bred rats following arrival. WT littermates were used as controls for all experiments. All experiments were conducted in accordance with the National Institute of Health *Guidelines for Care and Use of Laboratory Animals* and approved by the Michigan State University Institutional Animal Care and Use Committee.

Social Play Testing

Social play testing occurred between post-natal day (PND) 31-35 for all experiments. Subjects were singly housed in clean cages twenty-four hours prior to social play testing. Social play testing began at lights off (13:00h) for all experiments. During the test, a sex- and age-matched conspecific was introduced into the subjects' home cage and they were allowed to freely interact for 10 min. Play sessions were video recorded and behavior of the experimental rat was later scored using the video analysis program Solomon Coder (Solomon.andraspeter.com) by a researcher blind to the experimental conditions. Duration of social play (time spent engaging in playful interactions with the stimulus rat including chasing, nape attacks, wrestling, and pinning), social investigation (sniffing the anogenital region of the stimulus rat), allogrooming (grooming head and neck of stimulus rat), and cage exploration, along with number of pins (experimental rat holds stimulus rat in supine position), nape attacks (experimental rat attacks or makes nose contact with the nape of the neck of the stimulus rat), and supine positions (experimental rat rolls on its back or is pinned on its back by stimulus rat) was scored according to Bredewold et al. (Bredewold & Veenema, 2018).

Experiment 1: Determine the effects of NAc^{OXTR} cell body inhibition in the modulation of juvenile social play behavior

PND 26 male and female OXTR-iCre (n=7 males and n=5 females) or wild-type (WT) Wistar rats (n=6/sex) were anesthetized with isoflurane (2-4% as needed; Henry Schein, Melville, NY, USA) and mounted onto a stereotaxic frame (Stoelting, Wood Dale, IL, USA). A 1ul 7000 series Hamilton syringe (Hamilton, Reno, NV) was attached

to a stereotaxic injector system (Stoelting, Wood Dale, IL, USA) and loaded with AAVDJ-hSyn-DIO-hM4Di-mCherry (DREADD). This Cre-dependent DREADD viral vector allows for the selective targeting and transfection of the mutated human muscarinic receptor M4 tagged with a red fluorescent protein (mCherry) into neurons that express Cre (i.e. OXTR). When the exogenous ligand Clozapine-*N*-oxide (CNO) is bound, cellular inhibition occurs via two simultaneous processes: the cell is hyperpolarized via activation of inward-rectifying potassium channels and presynaptic neurotransmitter release is inhibited (Roth, 2016). The infusion of the Cre-dependent DREADD construct into WT rats was done to monitor the effects of systemic injections of CNO without its receptor present. The syringe was aimed at the NAc (AP: +2.4mm ML: ± 2.5mm DV: -4.6mm from bregma in accordance with the Rat Brain Atlas; Paxinos and Watson, 2007) at a 10°, and the virus was infused at a rate of 100nl/minute. Following infusion, the needle remained in place for 10 minutes to allow adequate time for diffusion and uptake of the viral vector. The syringe was then slowly withdrawn, and the process wase repeated in the opposite hemisphere. After surgery, all rats received a subcutaneous injection of meloxicam (2mg/kg; Covetrus). Rats were singly housed and monitored post-surgery until fully ambulatory, after which they were paired-housed with same-sex littermates until the start of play testing.

To determine the effects of NAc^{OXTR} cell body inhibition on the expression of social play behavior, juvenile male and female rats underwent two social play tests. Beginning at PND 30, rats were handled daily to acclimate the subject to the IP injection procedure. At PND 32, approximately 24hrs prior to social play testing, all rats were singly housed as this has been shown to increase levels of social play initiation

(Panksepp, 1981; Panksepp et al., 1984). 30 min prior to social play testing, subjects received IP injections of either vehicle (0.9% saline; 0.02ml) or CNO (0.3mg/kg in 0.9% saline). Social play testing occurred on PND 33 and PND 35.

Twenty-five-minutes after play testing on PND 35, experimental rats were deeply anesthetized with isoflurane (5%; Henry Schein, Melville, NY, USA) before being transcardially perfused with 0.9% physiological saline followed by 4% paraformaldehyde in 0.1M borate buffer (pH= 9.5). Brains were then extracted and post-fixed in a 4% paraformaldehyde solution with 12% sucrose for 18-24 hours. Afterwards, brains were rapidly frozen in 2-Methlybutane for 30 seconds and stored at -80°C until processing.

Brains were sectioned at 30um into four series, including the NAc. Series 1 of the NAc was processed for mCherry immunoreactive (ir) cells via immunohistochemistry (IHC) to confirm viral transfection. Briefly, tissue was washed in tris-buffered saline (TBS; 0.1M; pH=7.4) and incubated overnight at 4°C in a primary antibody solution containing chicken anti-mCherry (1:2000; Abcam AB205402; Cambridge, UK) in a blocking solution with 2% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% triton-X (Sigma-Aldrich, Burlington, VA). The following day, tissue was washed in TBS and incubated in a secondary antibody solution containing AlexaFluor 594 donkey anti-chicken (1:500; Jackson Immunoresearch, West Grove, PA) at room temperature for 1h. After rinsing in TBS, tissue was mounted onto gelatin-subbed slides, air-dried, and coverslipped with a Vectashield hardset antifade mounting medium with a DAPI counterstain (Vector Laboratories, Burlingame, CA) and stored at 4°C.

All images were acquired with a 20x objective on a Keyence BZ-X700E/BZ-X710 fluorescent microscope and associated BZ-H3AE software (Keyence Corporation of America, Elmwood Park, NJ). Verification of DREADD injection placement aimed at the NAc was assessed based on anatomical features and in reference to The Rat Brain Atlas (Paxinos and Watson, 2007). OXTR-iCre+ rats with bilateral mCherry staining that was contained only within in the NAc were used for behavioral analysis. Duration and frequency of social (duration of social play, social investigation, and allogrooming, as well as frequency of nape attacks and pins) and non-social (non-social exploration and self-grooming) behaviors above were scored by a researcher blind to the experimental conditions.

Experiment 2: Determine the effects of chemogenetic inhibition of the NAc^{OXT} to VP pathway in the modulation of juvenile social play

PND 22 male and female OXTR-iCre rats (n=3 males and n=6 females) underwent stereotaxic surgery for viral vector infusion as described in Experiment 1. Following recovery, rats were paired-housed until cannulation. At PND 29, all subjects were again anesthetized with isoflurane (2-4% as needed; Henry Schein, Melville, NY, USA) and mounted onto a stereotaxic frame (Stoelting, Wood Dale, IL, USA) for bilateral implantation of 9mm guide cannula aimed at the VP. This allowed for local administration of CNO into the VP to stimulate the NAc^{OXTR} fibers projecting to the VP. All subjects were pair-housed with same-sex littermates until behavioral testing.

To determine the effects of NAc^{OXTR} terminal inhibition in the VP on the expression of social play behavior, juvenile male and female rats underwent two social

play tests. Beginning at PDN 30, all rats were handled daily and habituated to the microinfusion procedure. Once a day, subjects were removed from their homecage, and wrapped tightly in a towel. The dummy cannula was removed, and the internal cannula was placed in the guide and left for 30s to acclimate the subject to the microinjection procedure. At PND 32, approximately 24hrs prior to social play testing, all rats were singly housed as described in experiment 1. Social play testing occurred on PND 33 and PND 35. 20 min prior to social play testing, subjects received bilateral infusions of either vehicle or CNO into the VP. The infusions (0.3 µL/hemisphere) were given over the course of 1 min via an internal cannula (28 gauge; Plastics One, Roanoke, VA) that extends 2 mm beyond the guide cannula and is connected via polyethylene tubing to a 2 µL syringe (Hamilton Company #88400) mounted onto a microinfusion pump (GenieTouch, Kent Scientific, Torrington, CT). The internal cannula was kept in place for an additional 30s following infusion to allow for tissue uptake before being replaced by the dummy cannula.

Twenty-five-minutes after the 10-min social play test on PND 35, experimental rats were deeply anesthetized with isoflurane (5%; Henry Schein, Melville, NY, USA) before being transcardially perfused with 0.9% physiological saline followed by 4% paraformaldehyde in 0.1M borate buffer (pH= 9.5). Brains were extracted and post-fixed in a 4% paraformaldehyde solution with 12% sucrose for 18-24 hours. Afterwards, brains were rapidly frozen in 2-Methlybutane for 30 seconds and stored at -80°C until processing.

Brains were sectioned at 30µm into four series, including the NAc and the VP. Series 1 of the VP was mounted onto slides and counterstained with thionin to

determine cannula placement within the VP. Then, series 1 of the NAc of rats with bilateral cannula placement within the VP were processed for mCherry-ir cells via IHC to confirm viral transfection as described in Experiment 1. Rats with bilateral mCherry staining that was contained only within in the NAc as well as bilateral cannula placement in the VP were used for behavioral analysis. Duration and frequency of social and non-social behaviors described in experiment 1 were scored by a researcher blind to the experimental conditions.

Statistical Analysis

For experiment 1, group differences between drug condition (saline/CNO) and sex (male/female) were assessed using a repeated measures 2-way ANOVA. Holm-Sidak post-hoc tests were performed when significant interactions occurred. For experiment 2, effects of drug condition (saline/CNO) were assessed using a paired T-test as this experiment was not adequately powered to detect sex differences in behavior. Effect sizes were calculated using partial η^2 ($\eta^2_p = SS_{effect}/SS_{residual}$) for repeated measures ANOVAs or Cohen' *d* (*d*= $(M_2-M_1)/SD_{pooled}$) for paired t-tests. Analyses were performed in GraphPad Prism (version 9) and significance was set at $p < 0.05$.

3. Results

3.1 Chemogenetic inhibition of NAc^{OXTR} cell bodies increases juvenile social play behaviors in females, but not in males

There was a sex x drug interaction on the duration of social play, the number of nape attacks, and the number of pins (Table 2; $p < 0.05$ for all). Post-hoc analyses revealed that females played at lower levels than males ($p = 0.03$; Figure 10E) and

displayed fewer nape attacks than males (p = 0.049; Figure 10F) under saline conditions. Additionally, females significantly increased duration of social play (p = 0.007; Figure 10E), number of nape attacks (p = 0.02; Figure 10F), and number of pins (p = 0.01; Figure 10G) following CNO treatment. Moreover, CNO eliminated the baseline sex difference in play between males and females in play duration (Figure 10E) and nape attacks (Figure 10F). These effects were specific to juvenile social play as there were no effects of CNO administration on the duration of social investigation (Figure 10H), duration of allogrooming (Figure 10I), or cumulative duration of social behaviors (Figure 10J) in either sex. CNO administration in WT rats did not alter any social or non-social behaviors in either sex (Figure 11; Table 2, p > 0.05 for all).

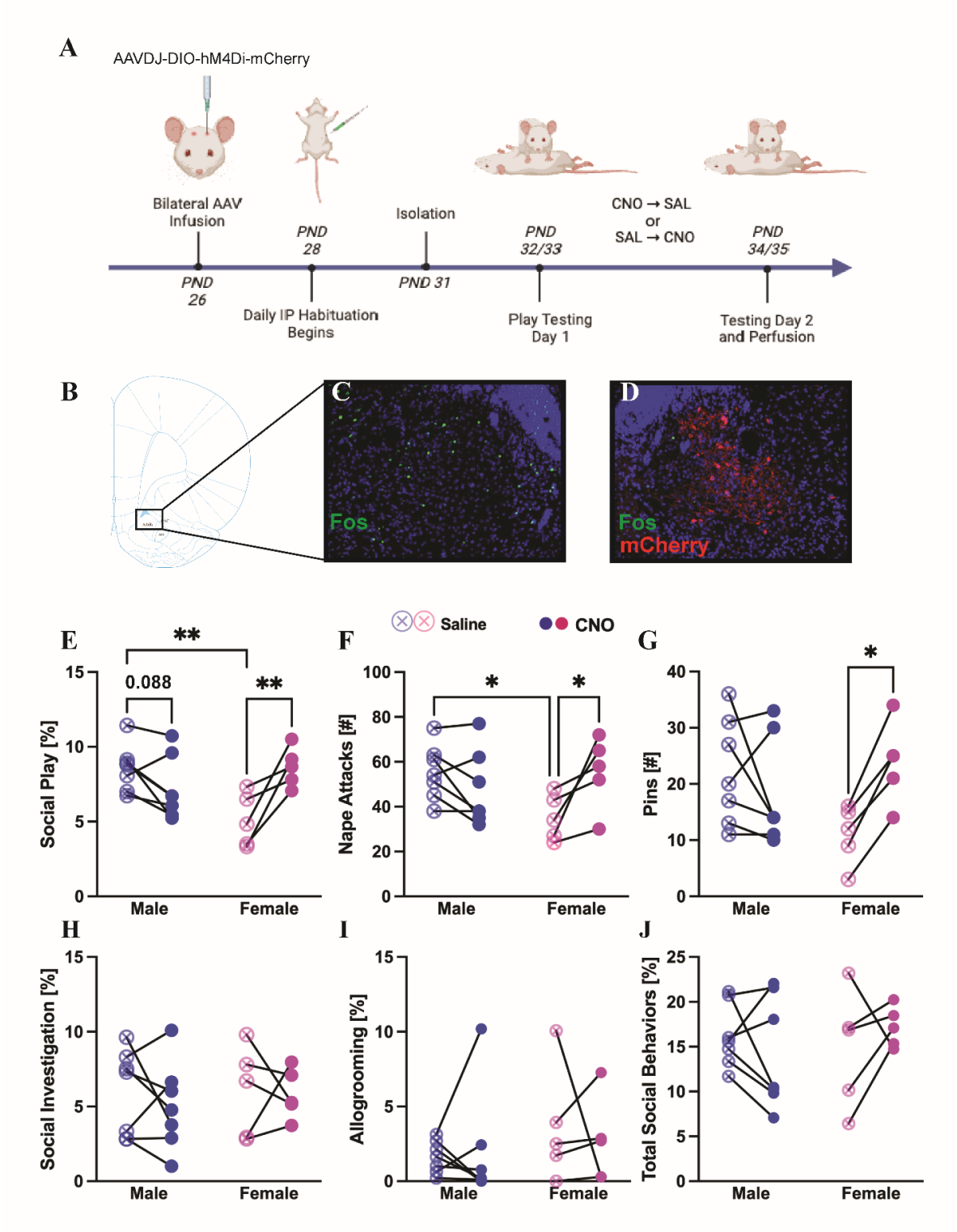

Figure 11. Chemogenetic inhibition of NAc^{OXTR} cell bodies sex-specifically alters juvenile social play, increasing female play to male-typical levels. (A) Timeline of

Figure 11 (cont'd)

experimental procedures. Briefly, juvenile OXTR-iCre+ rats underwent stereotaxic surgery and infusion of AAVDJ-hSyn-hM4Di-mCherry into the NAc at PND 26. Following recovery and habituation to intraperitoneal injections, all rats underwent two days of social play testing in which either saline or CNO was administered 30 minutes prior to testing. Drug conditions were randomly assigned and counterbalanced over the two days of testing. (B) Rat brain atlas image (modified from Paxinos and Watson, 2007) indicating the location targeted within the NAc for DREADD transfection. (C-D) Representative images of the neuronal activation marker Fos (Green) in non-DREADD transfected (C) and DREADD transfected (mCherry; D) NAc tissue 120 minutes after CNO administration. Duration of social play (E) and frequency of nape attacks (F) during the saline trials is significantly lower in females than in males. Additionally, CNO administration significantly increases duration of social play (E), frequency of nape attacks (F), and frequency of pins (G) in females, with no effect of drug administration on male social play behaviors. There was no effect of sex or drug on social investigation (H), allogrooming (I), or total duration of social behaviors (J). Social play (E), investigation (H), allogrooming (I), and combined social behaviors (J) are expressed as a percentage of total time. 2-way ANOVA Sex x Drug interaction; *: $p < 0.05$, **: $p < 0.01$; Holm's-Sidak post-hoc tests.

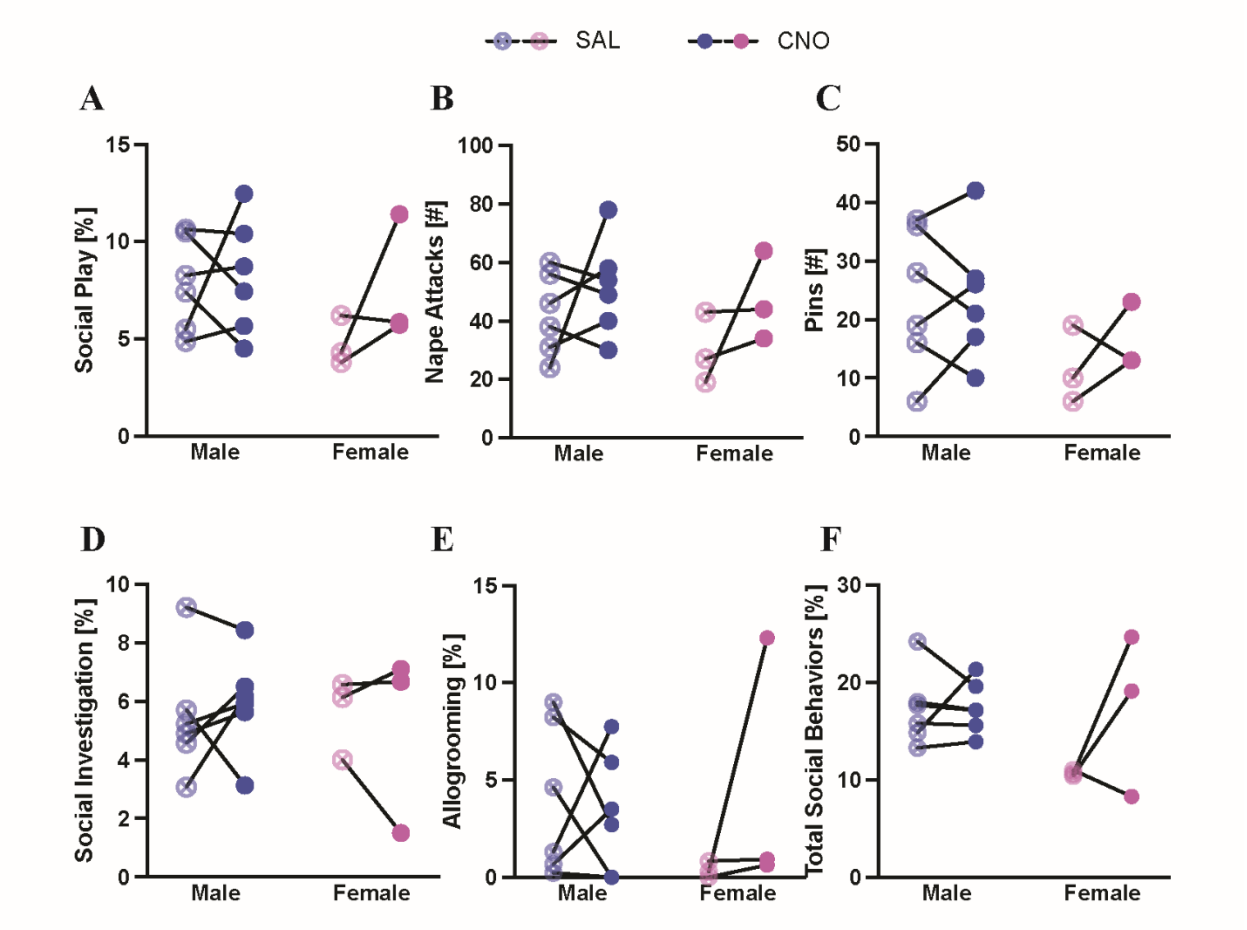

Figure 12. Systemic injections of CNO in WT juvenile rats does not alter any social behaviors in either sex. Males and females showed similar duration of social play (A), social investigation (D), allogrooming (E), and total social behaviors (F), as well as frequency of nape attacks (B) and pins (C), when treated with saline and CNO; repeated measures 2-way ANOVA, $p > 0.05$ for all.

Chemogenetic inhibition of NAc^{OXTR} terminals in the VP does not alter social play behavior

There was no effect of CNO administration on any social behaviors, including duration of social play ($t_{(8)}= 0.09$, $p = 0.93$, $d = 0.03$), number of nape attacks ($t_{(8)}= 0.17$, $p = 0.87$, $d = 0.07$), number of pins ($t_{(8)} = 0.23$, $p =0.83$, $d = 0.13$), social investigation

($t_{(8)}$ = 2.144, p = 0.06, d = 0.96), allogrooming ($t_{(8)}$ = 1.772, p = 0.11, d = 0.83), or total duration of social behaviors ($t_{(8)}$ = 0.28, p =0.79, d = 0.15). Sex was not included as a factor for this analysis (Figure 13).

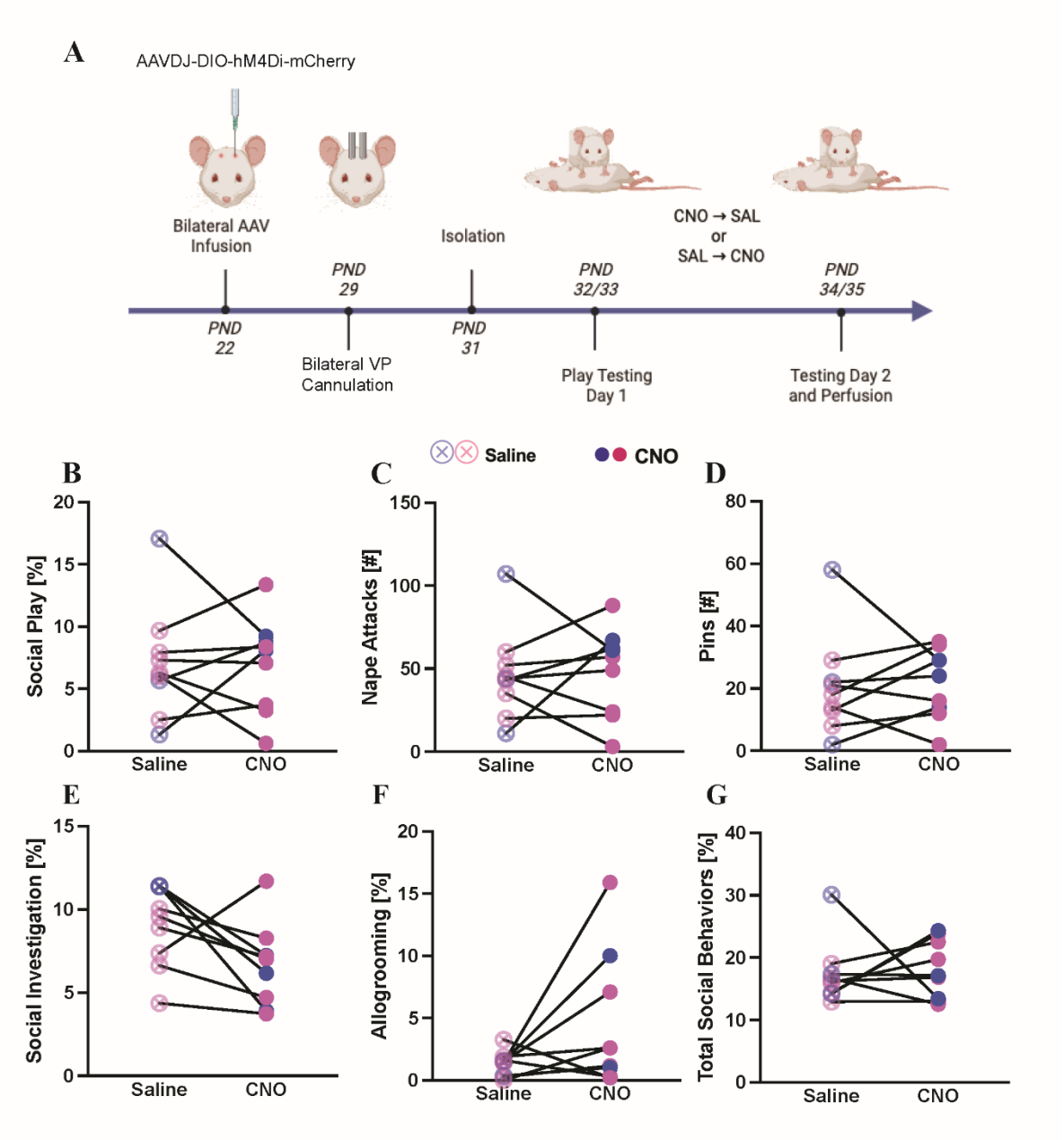

Figure 13. Chemogenetic inhibition of NAc^{OXTR} terminals does not alter social play in juvenile rats. (A) Timeline of experimental procedures. Briefly, juvenile OXTR-iCre+

Figure 13 (cont'd)

rats underwent stereotaxic surgery and infusion of AAVDJ-hSyn-hM4Di-mCherry into the NAc at PND 22 with bilateral cannulation of the VP at PND 29. Following recovery and habituation to cannulation infusion procedures, all rats underwent two days of social play testing in which either saline or CNO was locally infused 20 minutes prior to testing. Drug conditions were randomly assigned and counterbalanced over the two days of testing. There were no effects of CNO administration on the duration of social play (B), number of nape attacks (C), number of pins (D), duration of social investigation (E), duration of allogrooming (F), or total duration of social behavior (G). Duration of social play (B), investigation (E), allogrooming (F), and total duration of social behaviors (G) are expressed as a percentage of total time. Paired T-test; $p > 0.05$ for all.

Table 2: 2-way ANOVA statistics for behaviors quantified in social play testing. Significant main effects or interactions are notated in **bold.**

Effects of chemogenetic inhibition of NAC^{OXTR} cell bodies			
	Drug	Sex	Drug x Sex
% Social Play (Fig 11E)	$F_{1,10}$ = 3.77, p = 0.08, η^2_p = 0.27	$F_{1,10.}$ = 1.43, p = 0.26, η^2_p = 0.25	**$F_{1,10}$ = 19.19, p = 0.001, η^2_p = 0.66**
Nape Attacks (Fig 11F)	$F_{1,10}$ = 2.31, p = 0.16, η^2_p = 0.19	$F_{1,10}$ = 0.72, p = 0.42, η^2_p = 0.18	**$F_{1,10}$ = 11.52, p = 0.007, η^2_p = 0.54**
Pins (Fig 11G)	$F_{1,10}$ = 3.05, p = 0.11, η^2_p = 0.23	$F_{1,10}$ = 0.41, p = 0.54, η^2_p = 0.10	**$F_{1,10}$ = 11.67, p = 0.007, η^2_p = 0.54**

Table 2 (cont'd)

% Investigation (Fig 11H)	$F_{1,10} = 0.34$, p = 0.57, $\eta^2_p = 0.03$	$F_{1,10} = 0.11$, p = 0.75, $\eta^2_p = 0.03$	$F_{1,10} = 0.17$, p = 0.69, $\eta^2_p = 0.02$
% Allogrooming (Fig 11I)	$F_{1,10} = 0.07$, p = 0.80, $\eta^2_p = 0.007$	$F_{1,10} = 1.12$, p = 0.32, $\eta^2_p = 0.11$	$F_{1,10} = 0.28$, p = 0.61, $\eta^2_p = 0.03$
% Total Social Behavior (Fig 11J)	$F_{1,10} = 0.02$, p = 0.90, $\eta^2_p = 0.002$	$F_{1,10} = 0.11$, p = 0.74, $\eta^2_p = 0.02$	$F_{1,10} = 1.53$, p = 0.24, $\eta^2_p = 0.13$

Effects of systemic CNO administration in the absence of DREADD receptors

	Drug	Sex	Drug x Sex
% Social Play (Fig 12A)	$F_{1,7} = 1.54$, p = 0.26, $\eta^2_p = 0.18$	$F_{1,7} = 1.85$, p = 0.22, $\eta^2_p = 0.22$	$F_{1,7} = 0.96$, p = 0.36, $\eta^2_p = 0.12$
Nape Attacks (Fig 12B)	$F_{1,7} = 2.53$, p = 0.16, $\eta^2_p = 0.27$	$F_{1,7} = 1.77$, p = 0.23, $\eta^2_p = 0.13$	$F_{1,7} = 0.27$, p = 0.62, $\eta^2_p = 0.04$
Pins (Fig 12C)	$F_{1,7} = 0.60$, p = 0.47, $\eta^2_p = 0.08$	$F_{1,7} = 2.20$, p = 0.18, $\eta^2_p = 0.58$	$F_{1,7} = 0.52$, p = 0.50, $\eta^2_p = 0.07$
% Investigation (Fig 12D)	$F_{1,7} = 0.001$, p = 0.98, $\eta^2_p < 0.001$	$F_{1,7} = 0.09$, p = 0.78, $\eta^2_p = 0.04$	$F_{1,7} = 0.53$, p = 0.49, $\eta^2_p = 0.07$
% Allogrooming (Fig 12E)	$F_{1,7} = 0.86$, p = 0.38, $\eta^2_p = 0.11$	$F_{1,7} = 0.34$, p = 0.58, $\eta^2_p = 0.05$	$F_{1,7} = 1.68$, p = 0.24, $\eta^2_p = 0.19$
% Total Social Behavior (Fig 12F)	$F_{1,7} = 3.08$, p = 0.12, $\eta^2_p = 0.31$	$F_{1,7} = 2.22$, p = 0.18, $\eta^2_p = 0.3$	$F_{1,7} = 0.2.81$, p = 0.14, $\eta^2_p = 0.29$

4. Discussion

Here, I found first evidence of a sex-specific role of NAc^{OXTR} neurons and the NAc^{OXTR} to VP pathway in the regulation of social behaviors in juvenile rats. Using chemogenetics, I found that NAc^{OXTR} cell body inhibition, as well as NAc^{OXTR} terminal inhibition in the VP, sex-specifically alters discrete social behaviors in juvenile rats. Specifically, I showed that inhibition of OXTR-expressing neurons in the NAc increases juvenile social play behaviors, including duration of social play, number of nape attacks, and number of pins, in females without altering social play in males (Figure 10). Additionally, I found that chemogenetic inhibition of NAc^{OXTR} terminals in the VP reduces social investigation in males only (Figure 12) demonstrating the necessity of this pathway in males, but not females, in the typical expression of social investigation. Together, these findings suggest that NAc^{OXTR} expressing neurons in the NAc, as well as their projections to the VP, sex-specifically modulate social behaviors in juvenile rats, demonstrating sex differences in the regulation of social behaviors by OXT.

Chemogenetic inhibition of NAc^{OXTR} neurons increases social play in juvenile female, but not male, rats

I hypothesized that chemogenetic inhibition of NAc^{OXTR} expressing neurons would increase the expression of juvenile social play behavior in both male and female rats, with males potentially showing a greater increase in expression of social play than females. However, I found that inhibition of NAc^{OXTR} neurons increased social play behaviors in females without altering any social play behaviors in males (Figure 10E-G). Furthermore, there was a baseline sex difference in social play behaviors, in which males had a higher duration of social play, higher number of nape attacks, and higher

number of pins than females (Figure 10E-G). This sex difference was eliminated following chemogenetic inhibition of NAc^{OXTR} cell bodies. Taken together these findings with my previous findings of chemogenetically stimulating PVN^{OXT} cell bodies (Figure 2) and PVN^{OXT} to NAc terminals (Figure 8), I continue to find a pattern of sex-specific modulation of juvenile social behaviors by stimulating OXT signaling.

There are very few documented sex differences within the OXT system, specifically regarding OXT synthesis (Caldwell, 2018; Dumais et al., 2013; C. J. W. Smith, Poehlmann, et al., 2017). Despite this, several researchers have found sex differences in behavior that are modulated by OXT, including pair bonding (Bales & Carter, 2003; Bales et al., 2007, 2013), social recognition (Dumais et al., 2016), and juvenile social play (Bredewold et al., 2014). For example, chronic intranasal OXT administration in adult prairie voles blocks the formation of a partner preference in males without affecting females (Bales et al., 2013). Additionally, OXT infusion into the bed nucleus of the stria terminalis prolonged the interval of social recognition in adult male, but not female, rats (Dumais et al., 2016). Furthermore, both OXTR agonism and antagonism in the lateral septum (LS) induce sex-specific alterations in social play depending on the context (homecage versus novel cage; (Bredewold et al., 2014). In detail, neither OXT nor OXTR antagonism in the LS altered play behaviors in juvenile males, regardless of context. Conversely, OXT administration into the LS in females decreased duration of social play in their homecage without affecting play levels when tested in a novel cage (Bredewold et al., 2014). These findings demonstrate that OXT administration can exert divergent effects on males and females, some facilitating social behaviors and some attenuating social behaviors. These studies, along with the current study, suggest that

the neuromodulatory role of OXT is sex-specific across rodent species, brain regions and types of social behavior.

In the present study, inhibitory DREADD transfection was limited to the anterior region of the NAc (Figure 10B-D). I previously reported that there are no sex differences in OXT fiber density within any subregion of the NAc (Figure 7), nor are there sex differences in OXTR binding density within the anterior region of the NAc (Dumais et al., 2013). Therefore, the sex differences observed in social behavior following chemogenetic inhibition of NAc^{OXTR} expressing neurons may instead be due to sex differences in OXTR signaling pathways and/or in other signaling mechanisms within the NAc, such as the dopaminergic (DA) system.

DAergic signaling in the NAc is critical for the processing of reward and motivation to engage in reward-directed behaviors (Berridge, 2007; Floresco, 2015; Salamone & Correa, 2012). In juvenile rats, approximately 70% of NAc^{OXTR} expressing neurons co-express either DA 1-like (D1R) or DA 2-like (D2R) receptors (Figure 4A), which suggests that DARs and OXTRs may interact to modulate social behaviors, including social play. Indeed, DA and OXT interactions in the NAc underlie the expression of a partner preference in adult female prairie voles (Liu & Wang, 2003). Additionally, outside of the NAc, coordinated efforts of OXT with DA are implicated in the expression of sexual and parental behavior (reviewed in (Baskerville & Douglas, 2010). Therefore, future studies should be conducted to investigate the potential interplay of DAR and OXTR signaling in the NAc and how this affects the regulation of juvenile social play. These experiments could also provide insight into the mechanisms underlying the sex-specific modulation social play following OXTR neuronal inhibition.

While use of the cre-dependent inhibitory DREADD vector has been independently validated via electrophysiological recordings of mCherry+ neurons within the NAc (Veenema and Robison Lab, Unpublished Data), there is the potential that the efficiency of transfection of OXTR neurons in the NAc was insufficient to induce statistically significant behavioral changes in males. Histological analysis was limited to confirmation of mCherry+ cell transfection of the NAc given technical issues in combining immunohistochemistry (for mCherry) with *in situ* hybridization (for *Oxtr*). Additionally, direct measures of specificity were not calculated in the rats included in this study, though previously the OXTR-iCre line has shown high levels (~90%) of specific Cre-production within OXTR-expressing neurons in the NAc (Veenema Lab, Unpublished Data). Future studies will employ this combinatorial technique to calculate the efficiency and give further evidence for the specificity of DREADD transfection within the OXTR neurons in the NAc. Additionally, while there were no behavioral effects found following injection of CNO in WT littermates (Figure 12), this group was underpowered to detect potential effects of sex or potential interaction effects of sex and drug administration. Therefore, more females should be added to as a control to verify the sex-specific behavioral changes following chemogenetic inhibition of NAc^{OXTR} cells are not due to sex-specific off-target effects of CNO administration.

Chemogenetic inhibition of NAc^{OXTR} terminals in the VP did not alter social play behaviors in juvenile rats

The NAc is primarily comprised of inhibitory GABAergic neurons, which send projections to the VP (Mogenson et al., 1983; Yager et al., 2015). Suppression of VP activity by either pharmacological inactivation or chemogenetic excitation of GABAergic

input from the NAc decreases social play behavior in both male and female juvenile rats (J. D. A. Lee et al., 2021), indicating that activation of the VP is necessary for this behavior. Therefore, I predicted that chemogenetic inhibition of NAc^{OXTR} terminals in the VP would decrease GABAergic input to the VP and thus lead to an increase in the duration of juvenile social play. Instead, I found that inhibition of NAc^{OXTR} terminals did not alter any social play behaviors (Figure 13).

Together, this data indicates that while inhibition of NAc-GABAergic input to the VP is necessary for the typical expression of juvenile social play, inhibition of NAc-OXTR-expressing neurons projecting to the VP is not sufficient to alter this behavior. This null effect could be because OXTR-expressing neurons are only a subset of NAc MSNs that project to the VP. Therefore, chemogenetically inhibiting this small portion of projections may have been insufficient to alter social play. In the future, it would be of interest to chemogenetically stimulate the NAc^{OXTR} terminals in the VP to determine whether this population is sufficient to reduce the expression of social play, or if again, a larger population of neurons is required. Alternatively, this could be due to differences in co-expression of other receptors on NAc^{OXTR} neurons. OXTRs in the NAc are co-expressed on both D1R and D2R-expressing neurons (Figure 4), both of which project to the VP (Lu et al., 1998). These DA receptor subtypes elicit different cellular responses in which D1R binding increases cellular activity while D2R binding decreases cellular activity (Rommelfanger & Wichmann, 2010; R. J. Smith et al., 2013). In juvenile rats, OXTRs show higher levels of co-expression on D2R than D1R expressing neurons in the NAc (Figure 4A), implying that NAc^{OXTR} terminal inhibition in the VP would predominately target the D2R-expressing projection neurons. As D2Rs are inhibitory,

chemogenetic inhibition of these neurons may mimic the endogenous action of DA binding during engagement in social behaviors. If true, chemogenetic inhibition of these neurons may not have further decreased GABAergic activity in the VP, and therefore maintains the same level of expression of juvenile social play. Future studies could instead look at the necessity of inhibiting NAc^{OXTR} projections to the VP by chemogenetically stimulating this population. Furthermore, it would be of interest to confirm the DAR subtype co-expression of NAc^{OXTR} neurons projecting to VP.

Critically, this experiment lacked the adequate number of subjects to test for potential sex differences in the expression of juvenile social behaviors, including social play and social investigation. Given the sex specific modulation of these behaviors through chemogenetic manipulations within the OXT system previously shown (Figure 2; Figure 9; Figure 11), an important next step is to include more male subjects to determine whether inhibition of NAc^{OXTR} terminals within the VP alter juvenile social behaviors sex-specifically.

CHAPTER 4. DETERMINE THE ROLE OF OXT AND DA SIGNALING IN THE NAC IN THE EXPRESSION OF JUVENILE SOCIAL PLAY

Abstract

Social play is a motivated and rewarding behavior primarily displayed during the juvenile period of many mammalian species. Participation in social play aids in the development of social competency and flexibility while deficits in social play, as seen in autistic children, may contribute to life-long difficulties with social interactions. Additionally, autistic children find social interactions less pleasurable and are less motivated to seek out these social interactions. This indicates the need for preclinical research into the neural basis of the motivating and rewarding aspects of social play, which may lead to therapeutic strategies to improve social play engagement in autistic children. Here, I focused on the role of the nucleus accumbens (NAc) in social play behavior in juvenile rats, as the NAc is involved in the regulation of motivated and rewarding behaviors. Oxytocin (OXT) is well known for its modulation of social behaviors, and the NAc highly expresses the OXT receptor (OXTR). Additionally, the majority of NAc neurons express either dopamine (DA) 1 receptor (D1Rs) and DA 2 receptor (D2Rs), and selective activation of D1Rs or D2Rs has been shown to differentially modulate rewarding social behaviors in adult rodents. Importantly, both the D1Rs and D2Rs are expressed on OXTR-expressing neurons in the NAc. Furthermore, interactions between D2R and OXTR in the NAc facilitate adult rewarding social behaviors such as pair bonding. However, the role of DARs and OXT in the NAc in the regulation of juvenile social play is unknown. Therefore, the purpose of this study was to investigate the role of OXT, D1R, and D2R individually within the NAc on social play in

juvenile male and female rats. To accomplish this, rats were bilaterally cannulated in the NAc and infused with either OXT, a D1R antagonist, or a D2R antagonist 20 minutes prior to play testing. I predicted that OXTR agonism would increase social play in both sexes, while D1R and D2R antagonism would both decrease juvenile social play. I found that infusion of OXT into the NAc decreased social play duration and frequency in males, while OXT infusion eliminated a baseline sex difference in social play between males and females. Addionally, D1R antagonism did not alter juvenile social play. However, D2R antagonism decreased duration and frequency of social play behaviors in both males and females. This research provides evidence for a sex-specific role of OXT in the NAc in the regulation of social play behavior, in which activation of the OXT system in the NAc attenuates play behaviors in males. Additionally, research implies that D2R signaling but not D1R signaling, is necessary for the expression of social play.

1. Introduction

Social motivation is critical for the expression of adaptive social behaviors and survival of the species. The rewarding aspects of social interactions provide positive reinforcement, which aid in the development of social relationships (Borland et al., 2017; Burghardt, 2010; Krach et al., 2010). One such rewarding social behavior is that of social play (Trezza et al., 2010; L. J. Vanderschuren et al., 1997; L. J. M. J. Vanderschuren et al., 2016). Social play, or rough-and-tumble play, is most prominently displayed during the juvenile period (Achterberg, van Kerkhof, et al., 2016; Achterberg, van Swieten, et al., 2016; L. J. M. J. Vanderschuren & Trezza, 2014; L. J. M. J. Vanderschuren et al., 2016). Both juvenile rats and human children develop a social conditioned place preference (CPP) to a room previously paired with a playful interaction (Baron et al., 2020; Calcagnetti & Schechter, 1992; Trezza et al., 2009). Additionally, laboratory rats will learn to lever press to gain access to a playmate (Achterberg, van Kerkhof, et al., 2016), and brief social isolation increases play solicitation (Thor & Holloway, 1984) and social-seeking behaviors (Olaniran et al., 2022). Thus, social play is understood to be an innately rewarding behavior. Yet, in children diagnosed with autism spectrum disorder (ASD) there is a decrease in spontaneous social play in comparison to their neurotypical peers, in which ASD children are less likely to initiate social play or reciprocate appropriately when approached (Jarrold et al., 1996; Jordan, 2003; Morrier & Ziegler, 2018). Furthermore, ASD individuals find social interactions less pleasurable or rewarding than non-social interactions (Ruta et al., 2017; Supekar et al., 2018). This decrease in motivation to initiate social play and pleasurable aspects of social interactions in ASD individuals may be due to alterations

in social reward processing. Indeed, ASD children show aberrations in the brain reward circuitry when processing social stimuli. In detail, ASD children show decreased functional connectivity between the ventral tegmental area (VTA) and the nucleus accumbens (NAc) compared to neurotypical peers in response to a social stimulus (Supekar et al., 2018). These aberrations in social reward processing within the brain of ASD individuals correlate to known decreases in social engagement, but the causality remains unclear.

The VTA and NAc comprise two of the key nodes within the mesolimbic reward system and have been studied for their role in the regulation of various rewarding behaviors, including drug-seeking (Pierce & Kumaresan, 2006; Self, 2004), food intake (Bond et al., 2020; Skibicka et al., 2013), and social interactions (Barbier et al., 2023; He et al., 2021; Le Merrer et al., 2024). The NAc is thought to act as the interface between the corticolimbic and motor systems of the brain, which integrates the motivational and emotional state of an animal and relays this into action (Mogenson & Yang, 1991). The NAc specifically has been implicated in the in the modulation of juvenile social play (Manduca et al., 2016; van Kerkhof et al., 2013, 2014). For example, pharmacological inactivation of the NAc via $GABA_A$ and $GABA_B$ receptor agonists increases social play in juvenile male rats (females not tested, (van Kerkhof et al., 2013). This implies that activation of the NAc inhibits the expression of juvenile social play behavior, but the mechanisms regulating NAc activity during social play are unknown.

Within the mesolimbic reward system, the NAc receives Dopaminergic (DA) projections from the VTA, and binding of DA to DA receptors in the NAc is thought to be a key signaling mechanism that underlies the encoding of motivation and reward

(William A Carlezon & Thomas, 2009; G. Hou et al., 2024; Soares-Cunha et al., 2020). Therefore, it has been theorized that an increase in DA release within the NAc may be a key modulator of juvenile social play. In support, direct infusions of either amphetamine, which promotes DA release and inhibits DA reuptake, or a nonselective DA agonist into the NAc increases social play behaviors in juvenile male rats (females not tested, (Manduca et al., 2016). This effect was mediated by DA binding to DA receptors in the NAc as pretreatment with a nonselective DA receptor antagonist attenuated the amphetamine-induced increase in social play (Manduca et al., 2016). These experiments point to a key role of DA signaling in the NAc in the regulation of juvenile social play, but the DA antagonists used were nonselective and therefore did not discriminate between different DA receptor subtypes.

The NAc is primarily composed of GABAergic medium spiny neurons (MSNs), most of which express either the DA 1-like (D1R) or DA 2-like (D2R) receptors (Yager et al., 2015). D1-like receptors are excitatory, in which activation of the G_s subunit within these D1 expressing cells will increase cellular activity. Conversely, D2-like receptors are inhibitory, and activation of the G_i subunit within these D2 expressing cells will decrease cellular activity (Rommelfanger & Wichmann, 2010; R. J. Smith et al., 2013).While pharmacological blockade of either the D1R or D2R in the NAc decreases juvenile social play in male rats, this occurred only in a state of heightened motivation due to a prolonged period of isolation for both play partners in the testing dyad (Manduca et al., 2016). When dyads were tested after a short isolation period (<2 hrs), D1R or D2R antagonism did not alter social play behaviors within these pairs (Manduca et al., 2016). This suggests that NAc^{DAR} activation supports the expression of social play

only under highly motivated conditions. Therefore, DAergic regulation of social play may be dependent on the motivational state of the subject and of the perceived salience of the playmate.

Oxytocin (OXT) is a neuropeptide that has been proposed to modulate behavior by altering social salience (Averbeck, 2010; Shamay-Tsoory & Abu-Akel, 2016). For example, deficits in OXT synthesis and release via OXT knockout (KO) rodents can have either an anxiogenic or anxiolytic effect depending on the context. In detail, female OXT KO mice show increased anxiety-like behavior in the elevated plus maze, a test of general anxiety-like behavior, which was alleviated when administered intracerebroventricular OXT (Mantella et al., 2003). In contrast, female OXT KO mice show a decrease specifically in measures of social anxiety (Choleris et al., 2003), showing that alterations in OXT signaling can have opposing effects on anxiety depending on the social context. OXT is also critical for social recognition and memory in rodents, in which OXT KO mice are unable to discriminate between a familiar and a novel mouse (Ferguson et al., 2000). Critically, OXT KO mice were still able to discriminate between, and habituate to, non-social olfactory stimuli (Ferguson et al., 2000), indicating that OXT is necessary specifically for the recognition of social olfactory stimuli. In humans, intranasal OXT administration enhances recognition and memory recall for pictures of faces, but not pictures of non-social objects (Rimmele et al., 2009). This could be accomplished by OXT increasing the salience of social images over nonsocial images, biasing attention towards social stimuli.

OXT modulates activity via binding to the OXT receptor (OXTR). OXTRs are expressed widely across the brain, including within the NAc (Dumais et al., 2013; Gimpl

& Fahrenholz, 2001; Insel & Shapiro, 1992; Jurek & Neumann, 2018). OXTR signaling has been linked to the reward pathway within the brain through its effects on pair bond formation and social reward. For example, OXTR antagonism blocks the expression of a partner preference in monogamous female prairie voles (Liu & Wang, 2003; Young et al., 2001). Additionally, NAc^{OXTR} binding is necessary for the formation of social CPP in adolescent male mice (Dölen et al., 2013), implying that OXT signaling may play an important role in within the reward system. Previous studies have also found that both OXT and DA interact in the NAc to further facilitate social behavior. In detail, while infusion of a D2R agonist into the NAc was sufficient to induce a partner preference in virgin female prairie voles, this effect was blocked by infusion of either a D2R or OXTR antagonist (Liu & Wang, 2003). These findings suggest that simultaneous access to both receptors is necessary in females for partner preference formation, but the role of coordinated actions of OXT and DA in the regulation of other social behaviors remains unknown. In the NAc, OXTRs are co-expressed on both D1R and D2R-expressing neurons, and there is evidence of OXTR and D2R heteromeric complexes within the striatum (de la Mora et al., 2016; Romero-Fernandez et al., 2013). Preliminary findings from our lab show that in juvenile rats, both males and females have a higher percentage of OXTRs on D2R-expressing neurons compared to D1R-expressing neurons (Figure 4A). Additionally, there is a tendency towards males having a higher proportion of OXTR neurons co-expressing D2R than females (Figure 4B). This data suggests that OXTR and D2R-expressing neurons in the NAc play a key role in the modulation of social behaviors, which may occur sex-specifically.

Although these studies together provide evidence for the modulation of social behaviors via NAc OXTR and/or DAR activation juvenile males, a comprehensive analysis of these two systems have yet to be assessed in the regulation of juvenile social play behavior. Manduca et al. (Manduca et al., 2016) showed the necessity of both D1R and D2R receptor signaling in NAc on the expression of juvenile social play, but only in highly motivated dyads of rats. This design could be problematic, as it alters the motivational state of both the subject and the stimulus rat. As such, it is yet to be determined how DARs in the NAc regulate social play when only the experimental rat has been isolated. Furthermore, these studies did not include females nor determined the potential role of the OXT system in the NAc in the regulation of play. Therefore, the focus of this chapter is to determine the roles of OXT and DA signaling in the NAc on the expression of juvenile social play in both males and females. I hypothesized that both DARs and OXTRs in the NAc play a crucial role in the modulation of social play in juvenile males and females. Additionally, given the sex-bias in OXTR co-localization on D2R-expressing neurons in the NAc in juveniles, I hypothesized that OXTR and D2R signaling the NAc would alter social play sex-specifically. To test this, juvenile male and female rats were bilaterally cannulated in the NAc and infused with an OXTR agonist or DAR specific antagonists prior to social play testing. I predicted that OXTR agonism would increase levels of juvenile social play in both males and females, while D1R and D2R antagonism would decrease level of juvenile social play. Additionally, I expected that males would require a higher dose of the D2R antagonist to show comparable reduction in juvenile social play to females.

2. **Methods**

Animals

Subjects were experimentally naïve male and female Wistar rats obtained from Charles River Laboratories (Raleigh, NC, USA), and maintained under standard laboratory conditions (12:12h light/dark cycle, lights off at 13:00h, 22°C, 50% humidity, food, and water available *ad libitum*). Subject rats arrived between post-natal day (PND) 25-27 and were housed in same sex groups of 2-4 in standard Allentown cages (48x27x20cm). Stimulus male and female Wistar rats were used for social play testing (PND 24-30 at arrival; Charles River Laboratories, Raleigh, NC, USA) and were housed in the same conditions as subject rats following arrival. All experiments were conducted in accordance with the National Institute of Health *Guidelines for Care and Use of Laboratory Animals* and approved by the Michigan State University Institutional Animal Care and Use Committee.

Stereotaxic Surgery

At PND 28-29, subjects were anesthetized with isoflurane (2-4% as needed; Henry Schein, Melville, NY, USA) and mounted onto a stereotaxic frame (Stoelting, Wood Dale, IL, USA) for bilateral implantation of 6 mm guide cannulae aimed at the NAc (AP:-2.6mm, ML:+/-2.5mm, DV:-3.25mm from Bregma, Paxinos and Watson, 2007). Cannulae were implanted at a 10° angle from the midsagittal plane to avoid damage to the sagittal sinus. Cannulae were fixed to the skull with four stainless steel screws and dental cement and closed with a dummy cannula (Plastics One, Roanoke,

VA). Following a brief recovery, all subjects were pair-housed with same-sex littermates that underwent stereotaxic surgery until behavioral testing.

Handling and Habituation to Experimental Procedures

Beginning two days prior to behavioral testing, experimental subjects were habituated to the handling and microinjection procedure. Subjects were removed from their homecage, and wrapped tightly in a towel. The dummy cannula was removed, and the internal cannula was placed in the guide and left for 30s to acclimate the subject to the microinjection procedure. After the second day of microinjection habituation, approximately 24 prior to the start of social play testing, all subjects were singly housed in clean cages and remained housed like this until the conclusion of testing.

Microinjections

Twenty min prior to social play testing, subjects received bilateral infusions of either sterile 0.9% saline, OXT (1μM/0.3μl [low dose], 5μM/0.3μl [high dose]; Exp. 1), the D1R specific antagonist SCH-23390 (0.1μg/0.3μl [low dose], 0.3μg/0.3μl [high dose]; Exp. 2), or the D2R specific antagonist eticlopride (5μg/0.3μl [low dose], 15μg/0.3μl [high dose]; Exp. 3) into the NAc. DA receptor antagonist drug concentrations were chosen as they were previously shown to be effective in altering social play in juvenile male Wistar rats (Manduca et al., 2016). All drug conditions were randomly assigned and counterbalanced over the three days of testing for each experiment. The infusions (0.3 μL/hemisphere) were given over the course of 45 secs via an internal cannula (28 gauge; Plastics One, Roanoke, VA) that extends 2 mm beyond the guide cannula and was connected via polyethylene tubing to a 2 μL syringe

(Hamilton Company #88400) mounted onto a microinfusion pump (GenieTouch, Kent Scientific, Torrington, CT). The internal cannula was kept in place for an additional 30s following infusion to allow for tissue uptake before being replaced by the dummy cannula.

Social Play Testing

Social play testing occurred between PND 30-35 for all experiments. Subjects were singly housed in clean cages twenty-four hours prior to the social play test. Social play testing began at lights off (13:00h/14:00h) for all experiments. During the test, a sex- and age-matched conspecific was introduced into the subjects' home cage, and they were allowed to freely interact for 10 min. At the end of the testing session, the stimulus rat was removed, and the subjects cage was placed back on the housing rack. Play sessions were video recorded and behavior of the experimental rat was later scored using the video analysis program Solomon Coder (Solomon.andraspeter.com) by a researcher blind to the experimental conditions. Duration of social play (time spent engaging in playful interactions with the stimulus rat including chasing, nape attacks, wrestling, and pinning), social investigation (sniffing the anogenital region of the stimulus rat), allogrooming (grooming head and neck of stimulus rat), and cage exploration, along with number of pins (experimental rat holds stimulus rat in supine position), nape attacks (experimental rat attacks or makes nose contact with the nape of the neck of the stimulus rat), and supine positions (experimental rat rolls on its back or is pinned on its back by stimulus rat) was scored according to Bredewold et al. (Bredewold & Veenema, 2018).

Brain Extractions and Verification of Cannulae Placement

At the conclusion of the third day of testing, rats were euthanized with CO_2, and charcoal was injected as a marker to check proper placement of the cannulae into the NAc. Brains were then extracted, flash frozen in 2-methlybutane, and stored at -80°C until histological processing. The NAc was sectioned at 16um onto Superfrost Plus slides (Fisher Scientific, Waltham, MA, USA) using a cryostat (Leica CM3050, Buffalo Grove, IL). Slides were counterstained with thionin to determine cannula placement. Rats with bilateral charcoal placement within the NAc were used in subsequent behavioral analyses.

Statistical Analysis

For experiments 1 and 3, group differences between drug condition (saline/low dose/high dose) and sex (male/female) were assessed using a repeated measures 2-way ANOVA. Holm-Sidak post-hoc tests were performed when significant interactions or a significant effect of drug occurred. For experiment 2, effect of drug condition was assessed using a repeated measures 1-way ANOVA. Effect sizes were calculated using partial η^2 ($\eta^2_p = SS_{effect}/SS_{residual}$) for repeated measures ANOVAs. Analyses were performed in GraphPad Prism (version 9) and significance was set at $p < 0.05$.

3. **Results**

3.1 OXT administration in the NAc induces sex-specific effects on juvenile social play behaviors

A significant sex x drug interaction was found on the duration of social play ($F_{2,16} = 5.90$, $p = 0.01$, $\eta^2_p = 0.42$), number of nape attacks ($F_{2,16} = 5.23$, $p = 0.02$, $\eta^2_p = 0.40$),

and number of pins ($F_{2,16} = 4.45$, $p = 0.03$, $\eta^2_p = 0.36$). Holm-Sidak post-hoc test showed that, in males only, infusion of OXT into the NAc significantly decreased the duration of social play (1μM: *p*=0.058, 5μM: *p*=0.0018), number of nape attacks (5μM: *p*=0.0052), and number of pins (1μM: *p*=0.021, 5μM: *p*=0.0126) compared to the saline condition. The male specific effects of OXT administration eliminated the baseline sex difference in duration of social play and number of nape attacks, in which females played less than males (*p*=0.011 and *p*=0.013, respectively). In contrast, OXT induced a sex difference in the number of pins, with OXT-treated females showing more pins than males (1μM: =0.050, 5μM: *p*=0.045). No effect of sex or drug was detected on durations of social investigation, allogrooming, and total social behavior (*p* > 0.05 for all; Figure 13; see Table 3.1 for full details).

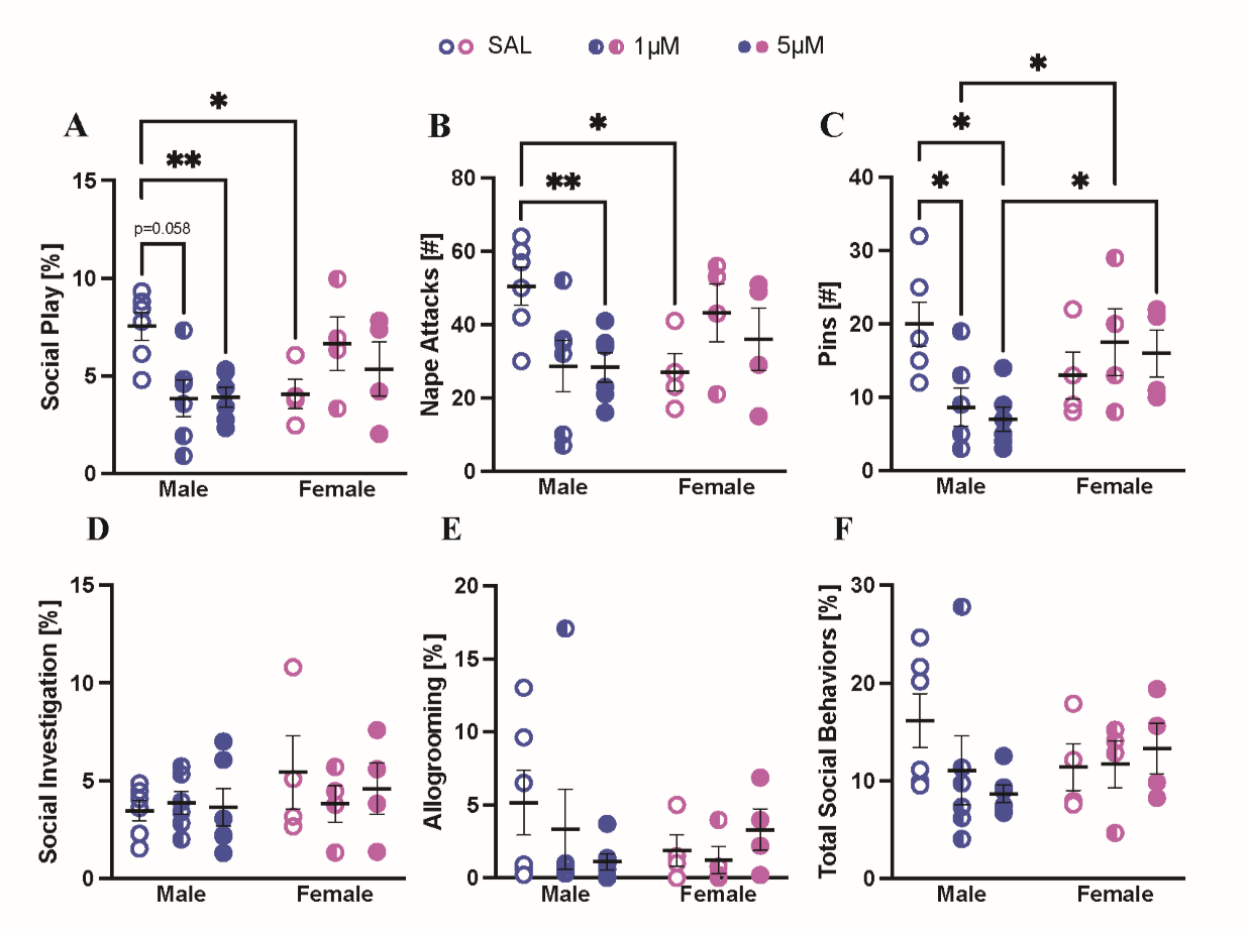

Figure 14. Infusion of OXT into the NAc sex specifically alters juvenile social play behaviors. There was a baseline sex difference in the saline condition in which females had a lower duration of social play (A), fewer nape attacks (B) fewer pins (C) than males. This sex difference was eliminated for all social play behaviors following OXT administration. Additionally, males showed a decrease in duration of social play (A) and number of nape attacks (B) at the highest dose of OXT, as well as a decrease in the number of pins (C) at both the high and low dose of OXT. There were no effects of OXT administration in either sex for duration of social investigation (D), duration of allogrooming (E), or cumulative duration of social behaviors (F). 2-way repeated measures ANOVA, Holm-Sidak post-hoc *: $p < 0.05$; **: $p < 0.01$.

3.2 Antagonism of D1Rs via SCH-29930 infusion in the NAc does not alter juvenile social play behavior in either sex

There was no effect of drug following infusion of SCH-29930 into the NAc on duration of social play ($F_{(2,16)} = 0.80$, $p = 0.47$, $\eta^2_p = 0.03$), number of nape attacks ($F_{(2, 16)} = 0.84$, $p = 0.42$, $\eta^2_p = 0.04$), number of pins ($F_{(2, 16)} = 0.08$, $p = 0.87$, $\eta^2_p = 0.004$), social investigation ($F_{(2,16)} = 0.58$, $p = 0.57$, $\eta^2_p = 0.03$), allogrooming ($F_{(2,16)} = 1.36$, $p = 0.29$, $\eta^2_p = 0.08$), or total duration of social behaviors ($F_{(2, 16)} = 0.40$, $p = 55$, $\eta^2_p = 0.03$) (Fig 15).

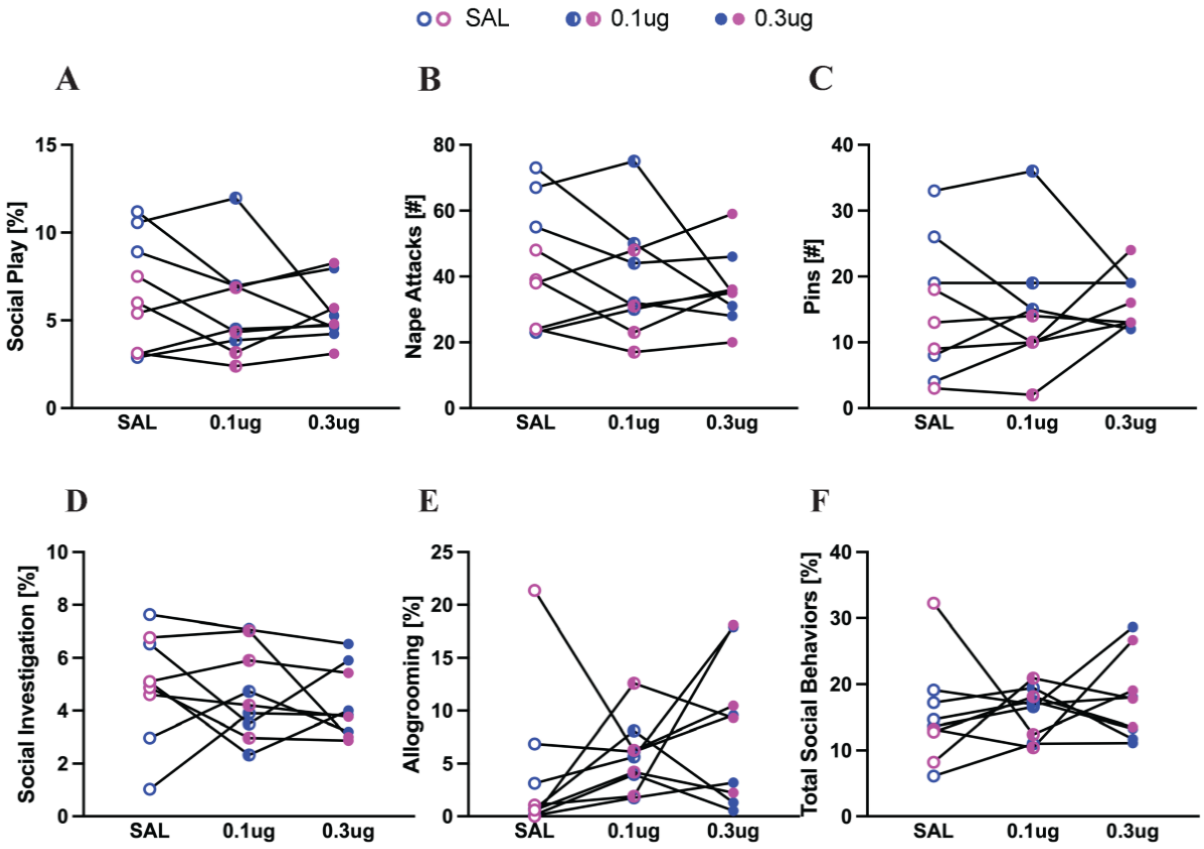

Figure 15. Antagonism of D1R in the NAc does not alter social behavior in juvenile rats. No effect of drug condition was found on duration of social play (A),

Figure 15 (cont'd)

number of nape attacks (B), number of pins (C), duration of social investigation (D), duration of allogrooming (E), or cumulative duration of social behaviors (F); 1-way repeated measures ANOVA, $p > 0.05$ for all.

3.3 Administration of the D2R antagonist Eticlopride in the NAc decreases juvenile social behaviors in both males and females

While there was no main effect of sex on any behaviors measured, there was a significant effect of drug condition on social play behaviors, including duration of social play ($F_{(2,18)} = 16.54$, $p < 0.0001$, $\eta^2 = 0.65$), frequency of nape attacks ($F_{2,18} = 17.82$, $p = 0.0004$, $\eta^2_p = 0.66$), frequency of pins ($F_{12,18} = 9.83$, $p = 0.003$, $\eta^2_p = 0.52$). Holm-Sidak post-hoc tests for multiple comparisons revealed that eticlopride reduced duration of social play (5µg: $p = 0.001$; 15µg: $p = 0.0012$), number of nape attacks (5µg: $p = 0.0004$ and 15µg: $p = 0.0010$), and number of pins (5µg and 15µg: $p = 0.0074$). Additionally, there was a strong trend towards a significant effect of D2R antagonism on the duration of social investigation ($F_{2,18} = 3.35$, $p = 0.06$, $\eta^2_p = 0.27$) or duration of allogrooming ($F_{2,18} = 3.40$, $p = 0.06$, $\eta^2_p = 0.27$) in which the highest dose of eticlopride had a tendency to reduce social investigation ($p = 0.08$) and allogrooming ($p = 0.05$) compared to the saline condition. Finally, there was a main effect of drug on total duration of social behaviors ($F_{2,18} = 22.17$, $p < 0.0001$, $\eta^2_p = 0.71$), in which eticlopride reduced the total duration of social behaviors (5µg: $p = 0.0224$ and 15µg: $p = 0.0003$) compared to saline. Additionally, the high dose of eticlopride further decreased the total duration of social behaviors compared to the low dose of eticlopride ($p = 0.0003$).

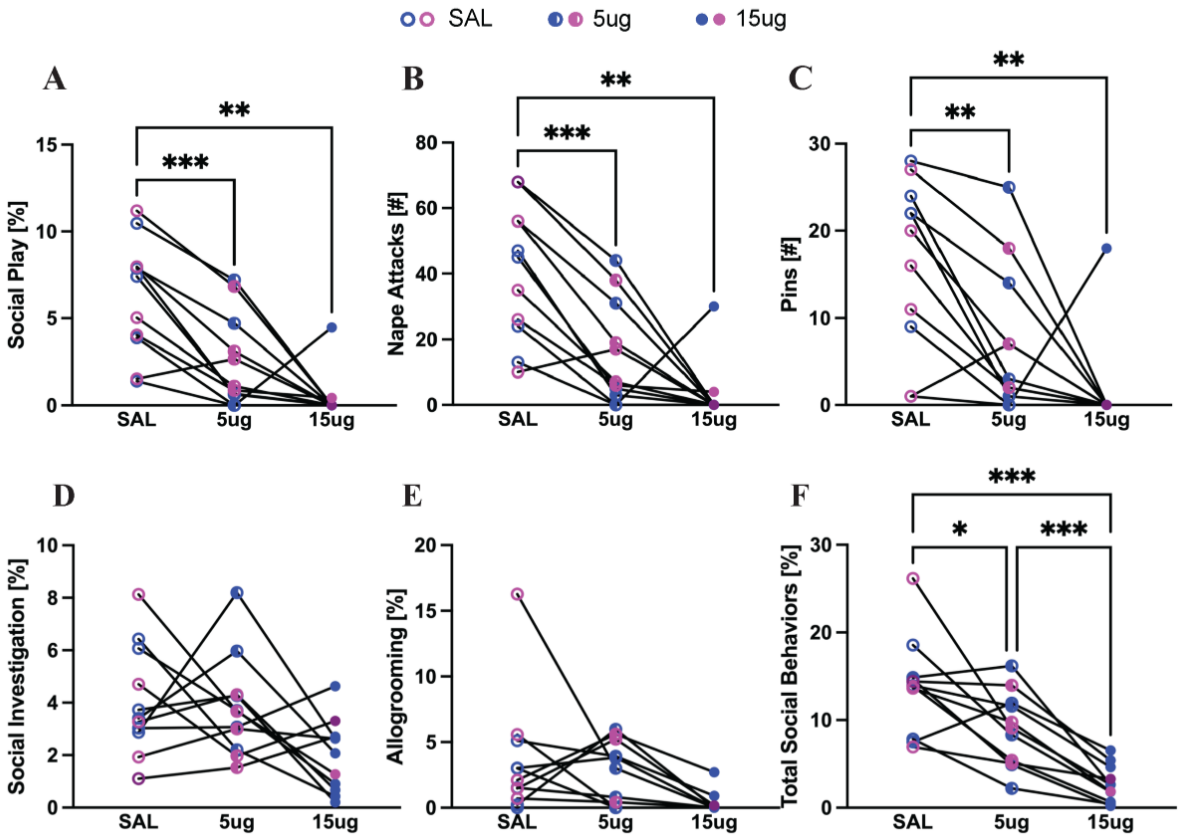

Figure 16. Infusion of a D2R antagonist into the NAc reduces social play behaviors in male and female juvenile rats. Both the low (5µg) and high dose (15µg) of eticlopride reduced the duration of social play (A), number of nape attacks (B), number of pins (C), and the cumulative duration of all social behaviors (F) compared to the saline trial. There was no effect of eticlopride infusion on duration of social investigation (D), or allogrooming (E). 2-way repeated measures ANOVA, Holm-Sidak post-hoc *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

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Table 3: 2-way ANOVA statistics for behaviors quantified in social play testing. Significant main effects or interactions are notated in bold.

3.1: Effects of OXT administration in the NAc

	Drug	Sex	Drug x Sex
% Social Play (Fig 14A)	$F_{2,16} = 0.75$, $p = 0.48$, $\eta^2_p = 0.09$	$F_{1,8} = 0.12$, $p = 0.74$, $\eta^2_p = 0.01$	**$F_{2,16} = 5.90$, $p = 0.01$, $\eta^2_p = 0.42$**
Nape Attacks (Fig 14B)	$F_{2,16} = 0.56$, $p = 0.58$, $\eta^2_p = 0.06$	$F_{1,8} = 0.006$, $p = 0.94$, $\eta^2_p = 0.00004$	**$F_{2,16} = 5.23$, $p = 0.02$, $\eta^2_p = 0.40$**
Pins (Fig 14C)	$F_{2,16} = 1.38$, $p = 0.28$, $\eta^2_p = 0.15$	$F_{1,8} = 2.39$, $p = 0.16$, $\eta^2_p = 0.11$	**$F_{2,16} = 4.45$, $p = 0.03$, $\eta^2_p = 0.36$**
% Investigation (Fig 14D)	$F_{2,16} = 0.24$, $p = 0.79$, $\eta^2_p = 0.03$	$F_{1,8} = 0.88$, $p = 0.38$, $\eta^2_p = 0.10$	$F_{2,16} = 0.66$, $p = 0.53$, $\eta^2_p = 0.08$
% Allogrooming (Fig 14E)	$F_{2,16} = 0.39$, $p = 0.69$, $\eta^2_p = 0.05$	$F_{1,8} = 0.30$, $p = 0.60$, $\eta^2_p = 0.04$	$F_{2,16} = 1.48$, $p = 0.26$, $\eta^2_p = 0.16$
% Total Social Behavior (Fig 14F)	$F_{2,16} = 0.79$, $p = 0.47$, $\eta^2_p = 0.09$	$F_{1,8} = 0.003$, $p = 0.96$, $\eta^2_p = 0.0003$	$F_{2,16} = 1.91$, $p = 0.18$, $\eta^2_p = 0.19$

3.3: Effects of D2R antagonism (Eticlopride) in the NAc

	Drug	Sex	Drug x Sex

Table 3 (cont'd)

% Social Play (Fig 16A)	**$F_{2,18}$ = 16.54, p < 0.0001, η^2_p = 0.65**	$F_{1,9}$ = 0.02, p = 0.89, η^2_p = 0.002	$F_{2,18}$ = 0.28, p = 0.76, η^2_p = 0.03
Nape Attacks (Fig 16B)	**$F_{2,18}$ = 17.82, p = 0.0004, η^2_p = 0.66**	$F_{1,9}$ = 0.03, p = 0.86, η^2_p = 0.003	$F_{2,18}$ = 0.22, p = 0.81, η^2_p = 0.02
Pins (Fig 16C)	**$F_{12,18}$ = 9.83, p = 0.003, η^2_p = 0.52**	$F_{1,9}$ = 0.34, p = 0.57, η^2_p = 0.02	$F_{2,18}$ = 0.12, p = 0.57, η^2_p = 0.01
% Investigation (Fig 16D)	$F_{2,18}$ = 3.35, p = 0.06, η^2_p = 0.27	$F_{1,9}$ = 1.53, p = 0.25, η^2_p = 0.04	$F_{2,18}$ = 0.67, p = 0.53, η^2_p = 0.07
% Allogrooming (Fig 16E)	$F_{2,18}$ = 3.40, p = 0.06, η^2_p = 0.27	$F_{1,9}$ = 1.52, p = 0.25, η^2_p = 0.08	$F_{2,18}$ = 0.69, p = 0.52, η^2_p = 0.07
% Total Social Behavior (Fig 16F)	**$F_{2,18}$ = 22.17, p < 0.0001, η^2_p = 0. 71**	$F_{1,9}$ = 0.09, p = 0.77, η^2_p = 0.009	$F_{2,18}$ = 0.04, p = 0.96, η^2_p = 0.005

4. Discussion

In this study, I demonstrated that both OXT and DA signaling in the NAc are involved in the modulation of juvenile social play behavior. In detail, I found that OXT sex-specifically altered social play, in which infusion of OXT into the NAc decreased juvenile social play behaviors in males, but not females. Additionally, OXT administration in females eliminated a baseline sex difference in social play between the sexes. Furthermore, there was no effect of D1R antagonism on social play behaviors, although D2R antagonism reduced social play in both males and females. For both OXTR agonism and D2R antagonism, the effects found were specific to social play, as

drug administration did not alter any other social behaviors, such as investigation or allogrooming. These findings suggest that OXT modulates social play differently in juvenile males and females, while D2Rs are necessary for the expression of social play in both sexes.

OXT administration in the NAc sex-specifically alters juvenile social play behavior

The highest dose of OXT administered in the NAc decreased the expression of social play behaviors in males, including the duration of social play and number of nape attacks, while both the high and low dose of OXT decreased the number of pins. Additionally, females showed lower levels of social play during their saline trial in comparison to males, an effect that was eliminated following OXT administration of either dose. Together, this suggests sex differences in the regulation of juvenile social play through activity within the OXT system.

The relative increase of social play in females compared to males following OXT administration in the NAc is also in line with my previous research, which showed an increase in social play in females following PVN^{OXT} cell body stimulation (Figure 2). Additionally, inhibition of NAc^{OXTR} neurons increased social play in females, also eliminating a baseline sex difference in play between males and females (Figure 10). Together, these findings give support to the model in which social play in females is facilitated through inhibition of the NAc, specifically through OXT signaling. This is in contrast to what is seen in males, in which OXT in the NAc decreased the expression of juvenile social play. Several studies have demonstrated the necessity of OXTR signaling in the NAc in the expression of adult-typical social behaviors. For example, OXTR antagonism in the NAc for female prairie voles blocks the expression of a partner

preference following mating (Young et al., 2001). Additionally, blockade of NAc^{OXTR}s decreased social investigation towards a conspecific in male and female California mice (Williams et al., 2020). Regarding social behavior in juveniles, OXTR antagonism in the NAc decreases social novelty seeking in male rats (C. J. W. Smith, Mogavero, et al., 2017), as well as decreases social play in both males and females (Figure 3A). While these findings demonstrate that OXTR binding in the NAc is essential for the expression of various social behaviors, they do not speak to how OXTR binding itself may alter neuronal activity to promote, or inhibit, the expression of social behaviors.

OXTRs are G protein receptors, which can be coupled to both the excitatory G_q and the inhibitory G_i subunit (Busnelli & Chini, 2018; Busnelli et al., 2012; Williams et al., 2020). Therefore, OXT binding activating either the excitatory or inhibitory subunit may have distinct behavioral effects. In support, infusion of a selective OXTR G_q antagonist/G_i agonist in the NAc of stressed female California mice decreased social interaction with a conspecific, while infusion of a selective OXTR G_q agonist increased social interaction (Williams et al., 2020). Therefore, the distinct behavioral effects seen between juvenile males and females in the expression of social play with OXT infusion in the NAc may be due to differences in activation of either the G_q or G_i subunits of OXTRs. Future research could include selective agonism of OXTR G_q or G_i subunits to determine their effects on the expression of juvenile social play, and if this differs between males and females.

It is important to note that in the current study, post-hoc tests did not reach significance for a direct increase in social play in females following OXT administration. Instead OXT in females increased expression of juvenile social play to male typical

levels (Figure 13 A). Furthermore, low dose of OXT (5μM) in males did not decrease the duration of social play or number of pins, though there was a trend towards a decrease (Figure 13A). Therefore, testing a wider range of dosages of OXT on the effects of social play would be of interest in the future. Infusions of OXT have dose- and sex-dependent effects on social preference in male and female mandarin voles (Yu et al., 2016). In detail, an intermediate dose of 1ng OXT into the NAc increased preference to investigate a social stimulus over an object, while a 0.1ng and 10ng of OXT did not alter this behavior. Additionally, the intermediate dose of OXT further increased social interaction in females compared to males (Yu et al., 2016). This suggests that specific concentrations of OXT in the NAc are critical in the regulation of social behaviors, which may induce sex-specific effects. Furthermore, the concentration of OXT alters binding affinity to OXTRs expressing different G protein subtypes. OXT in lower concentrations favors binding to the receptors coupled with the stimulatory G_q protein, while higher concentrations (<10nM) of OXT preferentially activate inhibitory $G_{i/o}$ proteins (Busnelli & Chini, 2018). This indicates that OXT could dose-dependently alter social play via changes in activation from stimulatory to inhibitory signaling mechanisms, though whether changes in binding affinity for OXT G protein subtypes is the same in juveniles as adults is unknown.

D2R antagonism reduces juvenile social play behaviors in both sexes, while D1R antagonism does not alter social play behavior

Here, I did not find evidence in support of my hypothesis that D1R activity modulates the expression of social play in juvenile rats. I found no effect of D1R antagonism on any social behaviors, including social play, social investigation, or

allogrooming (Figure 13). On the other hand, D2R antagonism decreased duration of social play, number of nape attacks and number of pins in both males and females, as predicted (Figure 14). These results show a robust, dose-dependent decrease in juvenile social play of both sexes following D2R antagonism, demonstrating the necessity of D2R signaling in the regulation of social play.

Previous reports found that in juvenile males, D1R antagonism in the NAc decreased social play behavior, as seen by a decrease in the number of pins and pounces (i.e. nape attacks) (Manduca et al., 2016). While contrary to my results, this discrepancy could be due to differences in methodology between the studies. In the Manduca et al. (2016) study, the play dyads were both subjected to the same isolation periods and drug manipulations. Conversely, my current study used an unmanipulated, group-housed stimulus rat as the play partner paired with a socially isolated subject. Therefore, these discrepancies in results could be due to differences in motivation to play between the subject and the stimulus. In support, Manduca et al. (2016) found that in play pairs with lower motivation to engage in play (due to only a 2 hr social isolation period), there were no effects of D1R antagonism. Therefore, D1R signaling may be sufficient to enhance the expression of social play behavior, but only in highly motivated dyads.

Manduca et al. (2016) reported similar results for antagonism of D2Rs in the NAc as antagonism of D1Rs. In detail, D2R antagonism did not alter social play in briefly isolated pairs of rats, but significantly reduced pins and pounces in pairs of rats isolated for at least 24 hours (Manduca et al., 2016). Likewise, I found that D2R antagonism decreased social play behavior in both male and female juvenile rats that had been

socially isolated for 24 hours prior to testing (Figure 14). Note that again, my design used group-housed, unmanipulated stimulus rats, which may have resulted in lower motivation from the stimulus rat to engage in play with the subject rat. Taken with the D1R antagonist data, this implies that D2R signaling may mediate enhancements in motivation.

Selective activation of either the D1-like or D2-like receptors in the NAc can have opposing effects on the expression of social behaviors. For example, D1R agonism impairs the formation of a pair bond in male and female monogamous prairie voles while D2R agonism facilitates the formation (Aragona et al., 2006; Wang et al., 1999). Additionally, chemogenetic stimulation of D1Rs in the NAc reduces social dominance while chemogenetic stimulation of D2Rs enhances social dominance in male mice (Shan et al., 2022). Furthermore, diphtheria toxin mediated selective ablation of D1R-expressing neurons in the NAc decreased social contact and interaction in adolescent male mice, while D2R-expressing neuronal ablation increased social contact and interaction (Le Merrer et al., 2024). These studies demonstrate distinct roles for D1R and D2R neurons in the regulation of social behavior.

These opposing roles of D1R and D2R activity in NAc are due in part to the different mechanisms of neuronal signaling following the binding of DA to D1Rs or D2Rs. D1R activation is excitatory, while D2R activation is inhibitory (Rommelfanger & Wichmann, 2010; R. J. Smith et al., 2013). OXTRs are more abundantly expressed on D2R neurons within the NAc than on D1Rs (Figure 3A). Therefore, while not examined in the present study, I hypothesize that D2Rs and OXTRs in the NAc may interact to facilitate the expression of juvenile social play. Few studies have directly tested the

coordinated actions of OXT and DA within the same brain region, though co-activation of both OXTRs and D2Rs are necessary for the expression of a partner preference in female prairie voles (Liu & Wang, 2003). Additionally, there is evidence that D2Rs and OXTRs can form heteroreceptor complexes within the ventral striatum in rats (Misganaw, 2021; Romero-Fernandez et al., 2013). Binding of OXTR to these heteromeric complexes enhances the recognition of DA to D2Rs, which would increase D2R signaling (Romero-Fernandez et al., 2013). Thus, increases in OXT binding in the NAc could enhance the inhibition of NAc^{D2R} neurons, promoting the expression of social play. Future experiments should be conducted to test this hypothesis, which could be accomplished by infusing both OXT and D2R antagonists into the NAc to determine whether blockade of D2R binding inhibits the behavioral effects in males and females following OXT administration in the NAc. Furthermore, it would be of interested to understand how OXTR binding, D2R binding, as well as simultaneous activation of OXTRs and D2Rs in the NAc alter neuronal excitability. Fiber photometry could be used with a calcium sensor in NAc neurons to record changes in activity following infusion of OXT, a selective D2R agonist, or OXT+D2R agonist in juvenile males and females. This information could uncover potential sex differences in NAc activity mediated by OXT signaling.

CHAPTER 5. DISCUSSION

Summary of findings

Previous research from our lab have shown that alterations in OXT signaling within the PVN and NAc sex-specifically modulated social play behavior in juvenile rats. Specifically, I found that chemogenetic stimulation of PVN^{OXT} neurons decreased juvenile social play in males and had a strong tendency to increase juvenile social play in females (Figure 2). Additionally, OXTR antagonism in the NAc, which receives OXTergic input from the PVN, decreases social play behavior in both male and female juvenile rats, with males requiring a 10x higher dose than females to show comparable reductions in social play levels (Figure 3A). OXTRs in the NAc of juvenile rats are co-expressed on DAR-expressing neurons, with a bias towards co-expression on inhibitory D2Rs (Figure 4A). These D2R-expressing neurons in the NAc primarily project to the VP (Yager et al., 2015; Yang & Mogenson, 1984, 1989), and activation of the VP is necessary for the expression of juvenile social play in both males and females (J. D. A. Lee et al., 2021). While the evidence presented implicates a key role for the PVN, NAc, and VP individually in the expression of social play, it is unknown how connections between these three regions may regulate juvenile social play, and whether OXT and/or DA transmission within these regions may modulate this behavior. Therefore, the overall goal of this dissertation was to determine how the OXTergic signaling in and between the PVN, NAc, and VP, as well as DAergic signaling in the NAc, regulates the expression of juvenile social play, and if this occurs sex-specifically. In detail, I hypothesized that upon presentation with a play partner, OXT is released from the PVN into the NAc, which inhibits OXTR-expressing neurons that co-express

D2Rs projecting to the VP, promoting the typical expression of juvenile social play behavior. To test this, I determined how the PVN^{OXT} to NAc pathway (Chapter 2), the NAc^{OXTR} to VP pathway (Chapter 3), and how OXT and DA in the NAc (Chapter 4) regulates juvenile social play in rats.

In chapter 2, I aimed to determine the distribution of PVN^{OXT} fibers within the NAc, as well as the role of PVN^{OXT} projections to the NAc in the regulation of juvenile social play. Given the sex differences in social play expression with PVN^{OXT} cell body stimulation (Figure 2) and OXTR antagonism in the NAc (Figure 3A), I hypothesized that these sex differences in behavior would be due to sex differences in PVN^{OXT} innervation patterns within the NAc. Additionally, I hypothesized that chemogenetic stimulation of the PVN^{OXT} terminals within the NAc would sex-specifically alter juvenile social play behavior. In detail, I predicted that stimulation would decrease the expression of juvenile social play in males and increase the expression of juvenile social play in females. In contrast to this, I found that there were no sex differences in PVN^{OXT} fibers in the NAc, though fiber density was significantly higher in the anterior portion of the NAc in comparison to intermediate and posterior portions of the NAc (Figure 8). Furthermore, there was no effect of PVN^{OXT} terminal stimulation on the expression of juvenile social play behaviors, but there was a sex-specific alteration of social investigation. In detail, chemogenetic stimulation of PVN^{OXT} terminals in the NAc increased social investigation in males, with a strong trend towards a decrease in social investigation in females (Figure 9). These data suggest that while terminal stimulation of PVN^{OXT} fibers in the NAc is not sufficient to induce changes in juvenile social play, it sex-specifically regulates social investigation. Furthermore, this sex difference in investigation is not

associated with sex differences in PVN^{OXT} fiber innervation to the NAc, implying there may be potential sex differences in the activation of this pathway upon exposure to a social stimulus.

As OXTR antagonism in the NAc sex-specifically decreases social play in juveniles (Figure 3), in chapter 3 I was interested in determining the role of NAc^{OXTR} expressing cells in the regulation of social play. Previous evidence shows that social play is negatively correlated with activation of OXTR-expressing neurons in the NAc (Figure 4), implying inhibition of OXTR-expressing neurons in the NAc may facilitate juvenile social play. Therefore, I predicted that inhibition of NAc^{OXTR} neurons would increase social play in both male and female juvenile rats. Additionally, as the negative correlation in OXTR activity and social play was primarily driven by males, I predicted that inhibition of NAc^{OXTR} neurons would further increase social play in males compared to females (Figure 4). As predicted, NAc^{OXTR} inhibition significantly decreased the duration of social play, as well as the number of nape attacks and the number of pins in females, although there was no effect on any social behaviors in males (Figure 10).

I was next interested in determining how NAc^{OXTR}-expressing neurons projecting to downstream brain regions may modulate social play. The NAc sends GABAergic projections to the VP (Chometton et al., 2020; Salgado & Kaplitt, 2015; Swerdlow et al., 1990). Pharmacological inactivation of the VP decreases social play in both male and female rats (J. D. A. Lee et al., 2021), demonstrating that activation of the VP is necessary for the expression of juvenile social play. Furthermore, stimulation of NAc-VP projecting neurons results in a decreased c-fos expression in the VP (Chometton et al., 2020) and decreased levels of social play (Lee, 2023). This suggests that inhibition of

the GABAergic input from the NAc to the VP is necessary for the typical expression of social play, but the role OXTR-expressing neurons in the NAc projecting to the VP was unknown. I hypothesized that inhibition of OXTR-expressing neurons projecting from the NAc to the VP are necessary for the expression of social play. Specifically, I predicted that chemogenetic inhibition of NAc^{OXTR} neurons projecting from the NAc to the VP would increase juvenile social play. In contrast with my hypothesis, I found that inhibition of NAc^{OXTR} terminals in the VP did not alter social play behavior (Figure 13A-C), although this study was unable to detect potential sex differences or interactions between sex and drug administration. These findings support a model in which inhibition of the NAc facilitates the expression of juvenile social play in females but may not include the VP as a downstream pathway of NAc^{OXTR} neurons or may not be sufficient on its own to induce changes in social play.

Lastly, in chapter 4, I aimed to determine the role of OXT and DA signaling in the NAc in the regulation of juvenile social play. As OXTR antagonism reduces social play in both males and females (Figure 3), and D1R and D2R antagonism reduces social play in males (females not tested; Manduca et al., 2016), I hypothesized that OXT and DA signaling in the NAc are independently required for the expression of juvenile social play. I predicted that infusion of OXT into the NAc would increase social play in both sexes, while D1R and D2R antagonism into the NAc would decrease social play. Here, I found that OXT administration in the NAc decreased social play in males and eliminated a baseline sex difference in play behaviors between males and females (Figure 14 A-C). This effect was specific to social play, as there were no changes in social investigation or allogrooming in response to OXT administration (Figure 14 D-E).

Additionally, D1R antagonism did not alter social play, or any other social behaviors (Figure 15). Finally, I found that D2R antagonism decreased juvenile social play in both sexes (Figure 16 A-C), with a trend toward a decrease in social investigation and allogrooming (Figure 16 D-E). Again, there are robust sex differences on the expression of play behavior with OXT administration, suggesting there may be sex differences in activation or the involvement of the OXT system in the regulation of play. These results are in contrast with my D2R antagonist findings, in which D2R antagonism in the NAc equally decreased social play behavior in both males and females.

Taken together these experiments demonstrate OXT differentially regulates the expression of juvenile social play in males and females, wherein OXT generally facilitates social play in females and attenuates social play in males. Additionally, these findings in females largely support my proposed model in which OXT facilitates the expression of juvenile social play via inhibition of OXTR-expressing neurons within the NAc. Given these findings, I propose that different neuronal mechanisms within the OXT system may underlie the regulation of juvenile social play in males and females. While there are few sex differences within the OXT systems of rats, including OXT synthesis within the PVN or OXT fiber density within the anterior NAc (Caldwell, 2018; Dumais et al., 2013; C. J. W. Smith, Mogavero, et al., 2017), there may be sex differences in the activation of the OXT system with consequences for the sex-specific regulation of social play. Furthermore, D2R activation in the NAc is necessary for the expression of social play in both sexes, but OXTR agonisms alters social play sex-specifically. This suggests that OXT may differentially interact with D2R in males and females, which could drive the opposing actions of OXT in the expression of social play.

OXT signaling in the NAc sex-specifically modulates juvenile social play behavior

Together, the findings in females throughout this thesis support my proposed model in which OXT binding to OXTRs in the NAc has an inhibitory effect on OXTR-expressing neurons in the NAc, which will in turn facilitate social play. GABAergic medium spiny neurons (MSNs) comprise approximately 95% of all neurons of the NAc (William A Carlezon & Thomas, 2009; Meredith et al., 1993). Activation of NAc projection neurons will release GABA, eliciting inhibition of downstream brain regions. As the NAc projects to several regions involved in the motivational aspects and motor output of reward-directed behavior, inhibition of the NAc may be necessary for the expression of reward behavior (William A Carlezon & Thomas, 2009). Indeed, oral sucrose administration is associated with a reduction of NAc activity *in vivo* (Roitman et al., 2005), suggesting that NAc activity decreases following reward consumption. Regarding juvenile social play, pharmacological inhibition of the NAc via GABA receptor agonists increases play in male rats (van Kerkhof et al., 2013). While this, along with the NAc^{OXTR} cell body inhibition data of Chapter 3, gives further evidence for a role of NAc^{OXTR} inhibition in the facilitation of social play, more research is needed to better understand intrinsic activity of NAc^{OXTR} neurons during social play. Future studies are needed to determine whether NAc^{OXTR} cells are naturally inhibited during the expression of social play, and whether these changes in activation are different between males and females. This would determine if inhibition is necessary, or if NAc inhibition is only sufficient to further facilitate this behavior. This could be accomplished by recording from OXTR neurons using fiber photometry during social play. Additionally, it would be of interest to

directly test the necessity of NAc^{OXTR} inhibition on the expression of juvenile social play in females by chemogenetically stimulating NAc^{OXTR} neurons.

In contrast to females, juvenile males show a tendency to decrease social play following chemogenetic inhibition of NAc^{OXTR} expressing cells, and show a significant decrease in social play following infusions of OXT into the NAc. This demonstrates the possibility of sex-specific mechanisms within the OXT system, both globally via PVN^{OXT} cell body release and site specifically within the NAc that regulate social play differently between males and females. OXTRs are G-coupled protein receptors (GPCRs), which are coupled to either G_q or G_i subunits (Busnelli & Chini, 2018; Jurek & Neumann, 2018). While both G_q and G_i subunits are found throughout the brain, the distribution of these subunits, or whether there are sex differences in location or affinity, is unknown within the NAc (Jurek & Neumann, 2018). Therefore, it is possible that sex differences in signal transduction following biased activation of either G_q or G_i subunits of the OXTR could be driving these behavioral differences in social play expression. Future research should include infusions of either Atosiban (a selective OXTR-G_q antagonist/OXTR-G_i agonist) or Carbetocin (a biased OXTR-G_q agonist) into the NAc to determine how selective activation of either the OXTR-G_q or OXTR-G_i signaling pathways regulates juvenile social play differently in males and females. Alternatively, one could combine calcium imaging of NAc^{OXTR} neurons and the use of OXT sensors ($GRAB_{OT1.0}$) to determine how OXT binding in the NAc alters OXTR-expressing neuronal activity at baseline, when exposed to a play partner, and during engagement in social play. Outside of sex-specific mechanisms within the OXT system, there could be sex differences in downstream pathways of NAc^{OXTR} neurons. Future studies could determine potential

sex differences in innervation of OXTR-expressing NAc neurons to regions known to modulate the expression of rewarding behaviors, such as the VP or the ventral tegmental area (VTA).

While there were robust sex differences in the expression of juvenile social play following NAc^{OXTR} cell body stimulation and OXT administration in the NAc, there were no effects found on social play following chemogenetic terminal manipulations in either experiment. These null effects on social play could be due to several factors. First, there may have been technical issues with the use of pathway specific chemogenetic manipulations. Several research groups have independently verified that terminal application of CNO in DREADD transfected neurons induces neurotransmitter release in excitatory DREADDs or a decrease in firing rates accompanied by a decrease in neurotransmitter release in inhibitory DREADDs (Mahler et al., 2014; Martinez et al., 2023; Roth, 2016; K. S. Smith et al., 2016). Although, there is the possibility that the excitatory or inhibitory DREADD constructs used in the current experiments did not elicit the expected changes in neural activity. While this is unlikely given the effects of chemogenetic terminal stimulation/inhibition on social investigation, follow up histological studies should be conducted to confirm alterations in post-synaptic cellular activity in the regions of interest. Alternatively, targeting a smaller portion of projection neurons may not be sufficient to drive the same behavioral changes as cell bodies manipulations, deeming terminal stimulation sufficient to alter social investigation but not social play. Furthermore, as both PVN^{OXT} and NAc^{OXTR} neurons project to multiple discrete brain regions, the regulation of social play may require coordinated activation of other brain regions outside of the targeted terminals.

Both PVN^{OXT} neurons and the NAc send projections to a wide variety of brain regions involved in the regulation of social behaviors, such as the VTA, the bed nucleus of the stria terminalis (BNST) and lateral septum (LS) (Knobloch et al 2012; Salgado and Kaplitt 2015; Liao et al 2020; He et al 2021). These regions are part of an interconnected system termed the social behavior neural network (SBNN) (Newman 1999; O'Connel et al 2012; Smith et al, 2019), The reciprocal innervation of these brain regions suggests that each node within the SBNN does not work in isolation to modulate a specific social behavior, but instead alterations in activity across many (or all) regions may be necessary. Furthermore, the nodes of the SBNN are each involved in the regulation of multiple social behaviors, indicating that precise alterations in activity across these regions may bias the display of specific behaviors. Social behaviors, including social play, are complex and involve a large behavioral repertoire. Therefore, discrete activation patterns across these regions in a temporally precise manner may facilitate the transition from appetitive (social approach, social investigation) to consummatory (social play, nape attacks, pins) behaviors. Terminal stimulation of a specific pathway may only be sufficient to induce changes in one aspect of juvenile social behavior, or none, as this technique may ignore the necessity of activity within other nodes of the SBNN. These possibilities will be discussed in more detail below in "Alternative pathways".

Lastly, in two experiments of this dissertation, there were baseline sex differences in juvenile social play in which females played at lower levels than males in the saline condition (Figure 9 and Figure 14). Findings are relatively mixed as to whether there is an inherent baseline sex difference between males and females in the

expression of juvenile social play in rats (Panksepp, 1981; Poole & Fish, 1975; Thor & Holloway, 1984; VanRyzin et al., 2020). Under group-housed conditions in which juvenile rats do not experience any social isolation, males display more social play behavior than females (Panksepp, 1981; Poole & Fish, 1975). Conversely, in states of higher motivation following social isolation, males and females show similar levels of social play (Bredewold et al., 2014; J. D. A. Lee et al., 2021; Thor & Holloway, 1984; VanRyzin et al., 2020). Other studies from our lab using the same social play paradigm as the current studies do not find sex differences in baseline social play (Bredewold et al., 2014; J. D. A. Lee et al., 2021; Reppucci et al., 2018; Veenema et al., 2013). There are several theories reasons for the emergence of sex differences in social play in rats, including differences in the motivation to play a dominance-related changes (S M Pellis et al., 1997), though these are unlikely to be the driving force behind the sexes differences here. For example, both males and females are socially isolated prior to testing, a paradigm which typically eliminated this sex difference. Alternatively, as play occurs in dyads, the motivation and likelihood of the stimulus rat to engage in social play could alter play levels seen in the subject animals. It could be that low motivated stimulus females decreased baseline levels of social play in some, but not all, of the female cohorts. It would be interesting to pre-screen stimulus rats prior to testing to determine their baseline level of social play initiation and reciprocation, and whether this correlates to play levels seen in their partner.

OXT sex-specifically modulates social investigation in juveniles

Although previous chemogenetic experiments have determined the effects of PVN^{OXT} to NAc pathway stimulation on the expression of social behaviors (W. Hou et

al., 2023; Hung et al., 2017), this is the first study to use juvenile subjects. Findings in juvenile males were consistent with adults, in which stimulation of the PVN^{OXT} to NAc pathway increased social investigation to a stimulus rat (Hung et al., 2017). In contrast, I found that juvenile females decreased social investigation following stimulation of the PVN^{OXT} to NAc pathway. This sex difference in social investigation is not due to a sex difference in PVN^{OXT} fiber density in the NAc (Figure 8), though a potential sex difference in OXT release following stimulation of PVN^{OXT} terminals in the NAc cannot be excluded. Therefore, it would be of interest to determine release patterns of OXT from the PVN into the NAc. This could be accomplished by coupling PVN^{OXT} optogenetic terminal stimulation in the NAc with $GRAB_{OT}$ sensors in OXTR-expressing neurons. This would allow researchers to determine changes in OXTR binding in the NAc in response to PVN^{OXT} release, and if these changes are sex specific.

Additionally, social interactions can be subdivided into appetitive and consummatory phases. Social investigation would be considered as an appetitive behavior, which would then lead to engagement in social play, the consummatory behavior. As chemogenetic stimulation of PVN^{OXT} terminals in the NAc alters the expression of social investigation, but not social play, it stands to reason that this PVN to NAc pathway may play an important role in the regulation of appetitive social behaviors. Calcium imaging of PVN^{OXT} neurons that project to the NAc during social approach and investigation, as well as social play, would be of interest to better understand intrinsic activity of this pathway during the appetitive and consummatory phases of social behavior in both males and females. Additionally, one could employ chemogenetic or optogenetic stimulation of this pathway during an operant conditioning

paradigm in which rats lever press to gain access to a playmate. If manipulations of this pathway alters lever pressing and social investigation following access to a playmate without altering social play, these data would provide more evidence towards the role of the PVN^{OXT} to NAc pathway in the regulation of appetitive social behaviors in juvenile rats. Alternatively, the PVN^{OXT} to NAc pathway could

DA signaling in the NAc modulates juvenile social play in both sexes

Unlike OXT, DA signaling in the NAc had similar effects in both males and females. In detail, D2R antagonism decreased social play behavior in both sexes, while D1R antagonism had no effect on social play. Additionally, there was a trend towards a decrease in social investigation and allogrooming at the highest dose of the D2R antagonist, suggesting that high levels of D2R antagonism may suppress the expression of social behaviors generally. Previously it was shown that both D1R and D2R receptor activation is necessary for the expression of social play in socially isolated, and thus highly motivated, dyads of rats (Manduca et al., 2016). In the current paradigm in which only the focal rat is highly motivated, only D2R antagonism significantly decreased play. This suggests that D2Rs in the NAc may play an important role in mediating the motivation to engage in juvenile social play in both sexes.

The observation that D2R antagonism decreasing social play is in line with my proposed model in which NAc inhibition promotes the expression of juvenile social play. DA receptors are G protein coupled receptors, with D1Rs coupled to the G_s protein and D2Rs coupled to the G_i protein (Rommelfanger & Wichmann, 2010). Therefore, activation of D1-like receptors has an overall stimulatory effect while D2-like receptor activation has an overall inhibitory effect on neuronal activity. Thus, DA binding to D2Rs

in the NAc may lead to an overall decrease in NAc activity, which promotes the expression of juvenile social play. Indeed, optogenetic stimulation of NAc^{D2R} neurons projecting to the VP is sufficient to decrease sucrose intake in adult female rats (Chometton et al., 2020), providing evidence that D2R inhibition facilitates the expression of rewarding behavior. Future work could determine how participation in social play alters general NAc activity, as well as alterations in D2R activity. These studies could provide insights into whether social play reduces the overall activity of the NAc, and whether this is mediated primarily through inhibition of D2R-expressing neurons.

The NAc is a large region within the ventral striatum, which can be further subdivided into the core and shell (Floresco, 2015; Klawonn & Malenka, 2018; Meredith et al., 1993; Salgado & Kaplitt, 2015; van Kerkhof et al., 2013). There is evidence that these subdivisions within the NAc differentially regulate juvenile social play. In detail, inactivation of the NAc core via the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen increases the overall duration of social play in males, while inactivation of the shell does not alter play behavior (van Kerkhof et al., 2013). In my current study, I targeted an anterior portion of the NAc (AP: +2.6mm, ML: +/- 2.5mm, DV: -5.25mm from Bregma, corresponding to atlas figures 11-12 in Paxinos and Watson, 2007), and I did not differentiate between the core and the shell of the NAc. Therefore, the effects of D2R but not D1R antagonism on social play could be due to the region of the NAc that was targeted. It would be of interest in the future to cannulate different regions within the NAc, as well as select targeting of the NAc core versus the NAc shell, to determine if D1R and D2R antagonism differentially regulates social play across the NAc.

Furthermore, D1Rs and D2Rs are co-expressed on OXTR-expressing neurons in the NAc (Figure 4). Given the individual effects of D2R antagonism and OXT agonism in the NAc in the regulation of juvenile social play, I propose that OXTR and D2Rs may interact in the NAc to facilitate, or potentially inhibit, the expression of juvenile social play. Coordinated activation of both OXTRs and D2Rs in the NAc are necessary for the formation of a partner preference in female prairie voles (Liu & Wang, 2003), but interactions between these systems have not been explored within the context of juvenile social play. Therefore, future studies should test the necessity of both OXTR and D2R signaling in the NAc in the regulation of juvenile social play. This could be done through co-infusion of OXT with a D2R antagonist in the NAc to determine if inhibition of D2Rs would block the enhancing effects of OXT on social play in females and/or block the reduction of play in males.

Rigor of studies and sex as a biological variable

A critical limitation to this thesis work is underpowered studied that lacked the number of subjects to accurately and confidently assess the potential for sex differences in the expression of social behavior following chemogenetic and pharmacological manipulations. Specifically, the study in which the effects of chemogenetic inhibition of NAc^{OXTR} terminals in the VP was investigated (Figure 13) included only 3 male subjects. Therefore, this data was collapsed by sex and only assessed for potential effects of drug condition. To properly assess by sex with an effect size of 0.5, at least 6 subjects per sex should have been utilized (calculated using G*power; https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower). Given sex differences observed in the expression of

juvenile social play and social investigation following manipulations of the OXT system within the brain, increasing the number of male subjects to assess for sex differences is of high interest. Additionally, males and females were pooled for the analysis using the D1R antagonist SCH-23390 (Figure 15). Although I did not anticipate sex differences in the effects of D1R antagonism in the NAc on the expression of juvenile social play, the testing of this hypothesis should have been conducted to verify this prediction.

Sex as a biological variable has only recently received more attention in pre-clinical science, with the majority of studies previously using only male subjects (Hayden 2010; Wald and Wu 2010; Becegato and Silva 2024), This lack of testing for sex differences in pre-clinical settings has had particularly adverse effects on the efficacy of drug treatments in humans, as males and females may respond differently to the same drug treatments. Testing for and understanding of potential sex differences are especially important given the subject matter of this thesis. Many studies in the field of social play demonstrates sex differences in the neurobiological mechanisms underlying social play behavior in rats (Bredewold et al., 2014, 2023; J. D. A. Lee et al., 2021; Reppucci et al., 2018; Veenema et al., 2013). Additionally, these pre-clinical findings have implications for potential therapeutics to mitigate social play deficits seen in ASD children. Importantly, there is a sex-bias in ASD diagnoses, with higher rates of males receiving diagnoses than females (Ferri et al., 2018; Werling & Geschwind, 2013a, 2013b). There is also evidence of sex differences in ASD symptomology (de la Roche & Kelley, 2024), although more human research is needed that accounts for sex as a factor in ASD symptom severity and expression. As such, given the sex differences in diagnoses and genetic risk factors for ASD (Ferri et al., 2018; Werling & Geschwind,

2013b), the use of both sexes in pre-clinical research is essential for the potential development of sex-specific therapeutics for ASD children.

Alternative pathways: Involvement of the VTA

Given that chemogenetic stimulation of PVN^{OXT} cell bodies sex increased play in females, but chemogenetic stimulation of PVN^{OXT} terminals was insufficient to alter social play in either sex, it is possible that co-activation of PVN^{OXT} projections to other brain regions is needed to modulate social play. In addition to the NAc, PVN^{OXT} neurons send dense projections to the VTA (He et al., 2021; Hung et al., 2017; Knobloch et al., 2012). DAergic neurons within the VTA express OXT, which in turn project to the NAc (He et al., 2021; Hung et al., 2017; Peris et al., 2017). Additionally, activation of OXTRs in the VTA can directly influence DAergic signaling in the NAc, as evidenced by increases the release of DA into the NAc following injections of OXT into VTA (Melis et al., 2007). Together, this suggests that activation of PVN^{OXT} cell bodies may increase OXT and DA signaling in the NAc through direct and indirect pathways. Given the necessity of both OXTR and D2R signaling in the NAc for the expression of juvenile social play, I therefore propose that OXT from the PVN needs to be co-released into both the NAc and VTA to facilitate this behavior. Specifically, following release from PVN terminals, OXT binds to OXTRS in the NAc as well as OXTRs in the VTA, which increases the release of DA into NAc. This coordinated release of OXT and DA into the NAc could be necessary for the typical expression of juvenile social play.

There is evidence that OXTRs and D2Rs form heteromeric receptor complexes within the striatum, in which OXT increases the binding affinity of DA to D2Rs (Romero-Fernandez et al., 2012). As D2Rs are paired with G_i proteins (Rommelfanger &

Wichmann, 2010), an increase in OXTR binding to the allosteric site on OXTR+D2R heteromers may further decrease the activity of the NAc through facilitation of D2R binding. Given that pharmacological inhibition of the NAc increases the expression of juvenile social play (van Kerkhof et al., 2013), this OXTR+D2R mediated inhibition of NAc neurons may drive the expression of juvenile social play in females. The NAc sends GABAergic projections to the VP, and activation of these inputs inhibits VP activity (Chometton et al., 2020; Mogenson et al., 1983; Yang & Mogenson, 1989). Inactivation of the VP results in a reduction of reward-oriented behaviors, including sucrose consumption (Chometton et al., 2020) and social play (J. D. A. Lee et al., 2021). Furthermore, chemogenetic stimulation of NAc^{GABA} terminals within the VP decreased juvenile social play in both male and female rats (Lee, 2023), suggesting that inhibition of GABAergic neurotransmission from the NAc to the VP is necessary for the expression of juvenile social play. Therefore, it is possible that the PVN, VTA, NAc, and VP form a pathway in which OXT and DA are released into the NAc and synergistically bind to their corresponding inhibitory receptors, thereby disinhibiting the VP to promote the expression of social play (Figure 16A). In detail, I propose that exposure to a playmate increases PVN^{OXT} activity, which releases OXT into the VTA and NAc. OXT binds to OXTRs in the VTA, promoting the release of DA into the NAc, where OXT is bound to the allosteric site of OXTR+D2R heteromers. OXTR binding in the NAc increases the affinity of DA binding to D2Rs, which inhibits NAc^{GABA} neurons projecting to the VP. This decrease in inhibitory signaling disinhibits the VP, which facilitates the expression of juvenile social play (Figure 16 C).

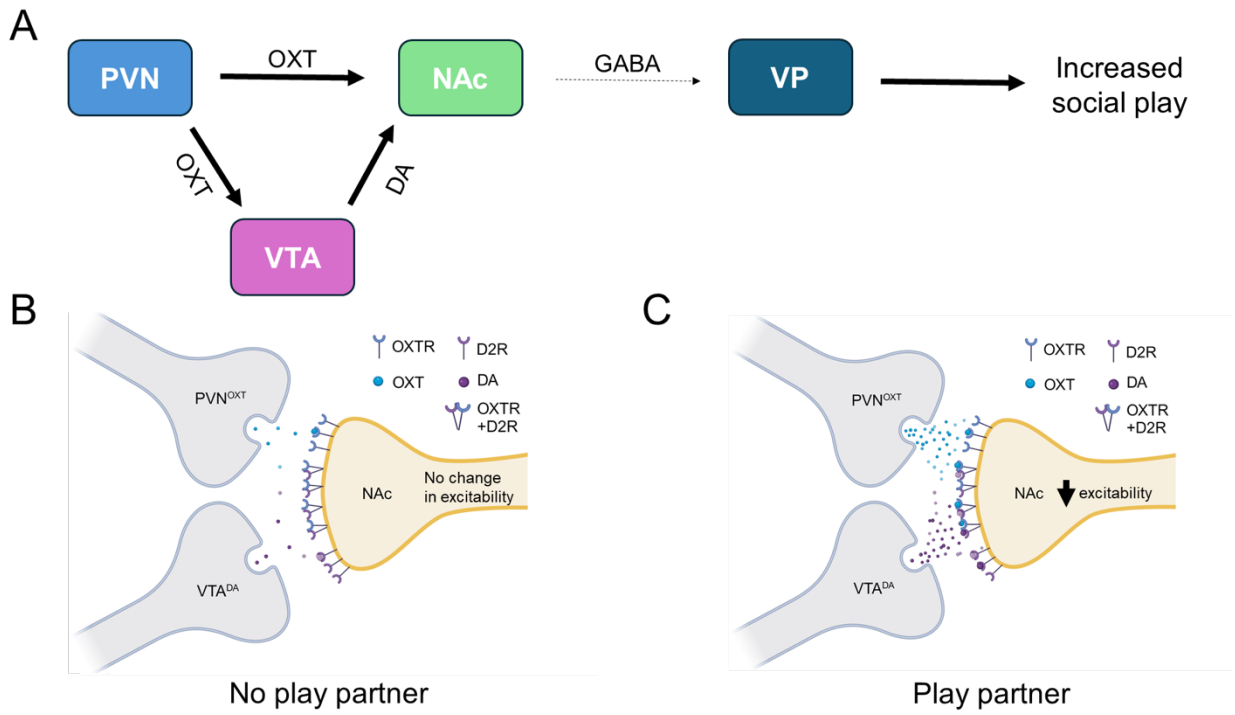

Figure 17. Proposed OXT and DA pathway involved in the regulation of juvenile social play in rats. (A) Juvenile social play is facilitated through release of OXT from the PVN into the NAc and VTA, where it binds to OXTRs. OXTR binding in the VTA stimulates the release of DA into the NAc. OXTR binding to OXTR+D2R heteromers in the NAc facilitates D2R mediated inhibition GABA release into the VP, thus disinhibiting the VP and promoting the expression of juvenile social play. (B) In the absence of a playmate, there is no increase in OXT or DA release into the NAc, and thus no change in NAc or VP activity. (C) In the presence of a playmate, OXT from the PVN and DA from the VTA are released in the NAc. Coordinated actions of OXT and DA binding to OXTRs+D2Rs in the NAc decreases $NAc^{OXTR+D2R}$ neuronal excitability. This in turn decreases GABA release in the VP, facilitating social play. Made with Biorender.

While this model works well based on the findings of this thesis in females, the generally attenuating effects of OXT signaling in males suggests that OXT sex-specifically alters activity within this pathway. These findings imply that while OXTR activation in the NAc is necessary for the expression of juvenile social play, lower levels of OXT in the NAc are required for the facilitation of social play in males compared to females. Indeed, supraphysiological enhancement of OXT signaling in the NAc of males decreases social play behavior. Therefore, I propose that under typical conditions, a similar pathway may be underlying the equivalent expression of social play between males and females. However, increases in OXT signaling in males, but not females, decreases the expression of juvenile play. This could be due to sex-specific alterations in activity of the NAc following excess OXT, potentially through excitatory mechanisms such as activation of OXTR G_q subunits on either D1Rs or D2Rs within the NAc in males but not females (Figure 18). On the other hand, although GABA receptor mediated inactivation of the NAc increased social play in juvenile males (van Kerkhof et al., 2013), I found that inhibition of NAc^{OXTR} expressing neurons specifically did not alter social play behavior (Figure 10). This data could suggest that inhibition of OXTR-expressing neurons in the NAc alone is not sufficient to increase social play in males or may not be necessary for the expression of this behavior. Therefore, there is also the possibility of another mechanism driving NAc inhibition for the facilitation of social play in males, which may be independent of OXT.

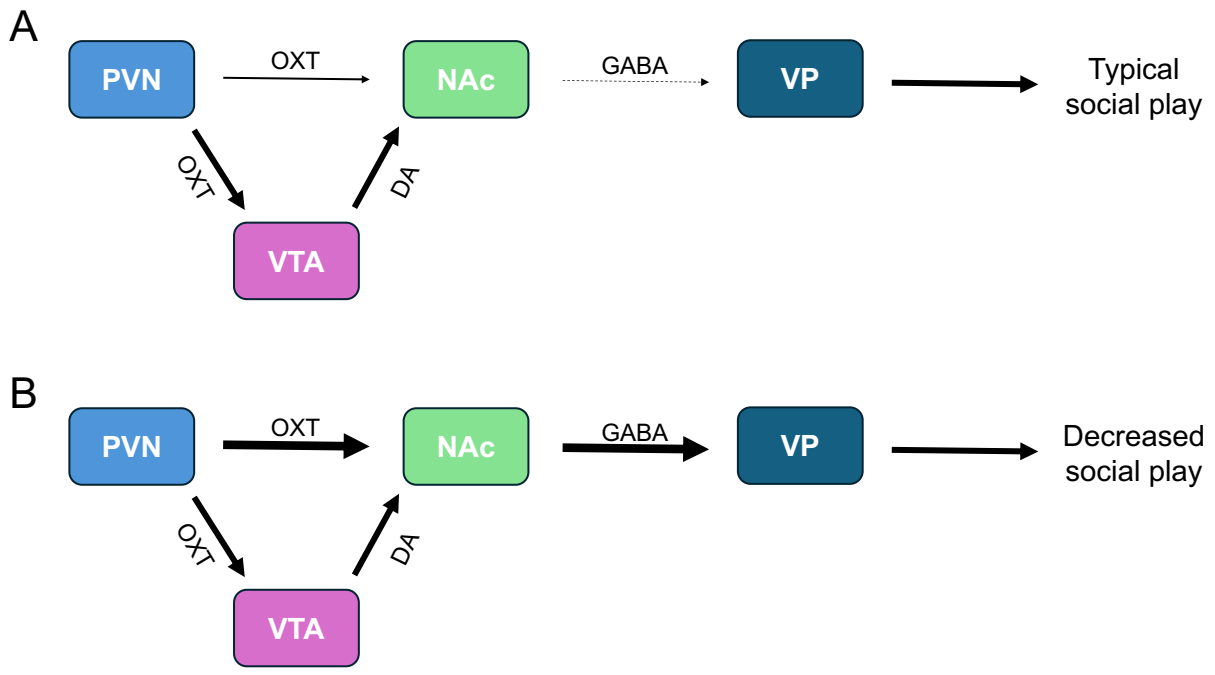

Figure 18. Proposed mechanism in males in which excess OXT signaling within the NAc decreases social play. (A) Under typical play conditions, PVN-OXT neurons release OXT into the VTA, simulating the release of DA in the NAc. Simultaneously, low levels of OXT in the NAc facilitates the binding of DA to D2Rs via heteromeric receptor interactions. D2R receptor activation in the NAc inhibits GABA release into the VP, disinhibiting the VP to promote the typical expression of social play. (B) Under enhanced OXT signaling in the NAc, OXT binds to Gq coupled receptors on both D2Rs and D1Rs in the NAc, stimulating these neurons and promoting release of GABA within the VP. Inactivation of the VP via increased GABAergic transmission then decreases the expression of juvenile social play in males.

For future studies, using fiber photometry to record the intrinsic changes in activity of the NAc during social play, as well as following OXTergic manipulations, is necessary to better understand whether the sex specific effects on social play are

indeed due to OXT induced alterations on neuronal activity. Additionally, emphasis should be placed on determining whether OXT and DA interact within the NAc in the regulation of social play, and if this sex-specifically alters the expression of play. Also, it would be of interest to determine whether stimulation of VTA^{OXTR} neurons projecting to the NAc facilitate the expression of social play in both sexes. This could be accomplished by infusing a retrograde cre-dependent flippase (Flp) AAV into the NAc of OXTR-iCre juvenile rats in combination with an infusion of a Flp-dependent excitatory DREADD construct into the VTA. This would allow for selective stimulation of VTA^{OXTR} expressing neurons that project to the NAc. Furthermore, it would be of interest to determine whether the same PVN^{OXT} neurons send axon collaterals to both the NAc and VTA using retrograde tracing techniques. Confirmation that depolarization of PVN^{OXT} neurons could simultaneously release OXT into both the VTA and NAc would provide further support for the coordinated efforts of OXT and DA in the regulation of juvenile social play.

Conclusions

This dissertation provides evidence that OXT signaling and OXTR-expressing neuronal projections within the PVN, NAc, and VP induce robust sex differences in the expression of juvenile social behavior, including social play and social investigation. I found that PVN^{OXT} neurons primarily innervate the anterior portion of the NAc, and chemogenetic stimulation of this pathway increases social investigation in males and increases social investigation in females (Chapter 2). Next, I found that chemogenetic inhibition of NAc OXTR-expressing neurons sex-specifically altered social play, while chemogenetic inhibition of NAc^{OXTR} terminals in the VP sex-specifically altered social

investigation (Chapter 3). Additionally, I found that OXT administration in the NAc decreased social play in males, with a tendency towards an increase in play in females (Chapter 4). Taken together, this demonstrates sex differences in the regulation of social play by OXT. These data suggest that increases in OXT may play a more attenuating role on social play in males and a more faciliatory role in females. Finally, I found that D2R antagonism, but not D1R antagonism, decreased social play in both sexes (Chapter 4). Given the sex-specific alterations in social play following OXT administration along with the necessity for D2R activation in both sexes, this suggests that increased OXT may differentially affect activity of OXTR and D2R-expressing neurons in the NAc in juvenile males and females. Indeed, OXTRs and D2Rs can form heteromers in the NAc (Romero-Fernandez et al., 2013), suggesting critical interactions between these systems in the regulation of behavior. Additionally, OXTR and D2R interactions in the NAc are necessary for the facilitation of pair bonding in female prairie voles (Liu & Wang, 2003), although it is unknown if the same if true in males. Therefore, future studies could focus on the potential of sex-specific interactions of the OXT and D2R system in the regulation of social play. Specifically, it could be determined if increases in OXT signaling alters the activity and function of the DA system, and if this activity is differentially altered in males and females.

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