INVOLVEMENT OF THE NUCLEUS ACCUMBENS TO VENTRAL PALLIDUM PATHWAY IN THE REGULATION OF SOCIAL PLAY BEHAVIOR IN JUVENILE MALE AND FEMALE RATS

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ABSTRACT

Social play behavior is a highly rewarding behavior that is displayed in various mammalian species, including rats and humans. Engaging in social play is essential for the development of social skills necessary throughout life. Autistic children show decreased engagement in social play and fMRI studies implicate aberrations in the mesolimbic reward system that are associated with decreased social motivation and preference. The nucleus accumbens (NAc) and ventral pallidum (VP) are core brain regions of the mesolimbic reward system and individually regulate social play behavior in juvenile rats. Given that the NAc projects to the VP, I aimed to determine whether and how the NAc to VP pathway regulates social play behavior. NAc inputs to the VP are primarily GABAergic and studies show that inhibition of the NAc to VP pathway, which disinhibits the VP, promotes non-social rewarding behaviors, while stimulation of this pathway, which suppresses the VP, reduces non-social rewarding behaviors. Therefore, I hypothesized that inhibition of the NAc to VP pathway is required for the typical expression of social play behavior in juvenile male and female rats. In the first aim of my thesis (Chapter 2), I used Gad1-iCre juvenile rats, in which cre recombinase is expressed on Gad1 (glutamate decarboxylase 1, an enzyme required for GABA production) cells which allows for selective manipulation of the activity of GABAergic cells using cre-dependent DREADDs. I predicted that chemogenetic stimulation of the NAc to VP pathway would reduce social play behaviors in both sexes. I found that chemogenetic stimulation of NAc to VP pathway of Gad1-iCre juvenile rats reduced the duration of social play and other elements of social play such as the number of nape attacks, pins, and supine poses in both males and females. These effects were specific

to social play, as Gad1-icre juvenile rats showed no changes in the duration of other social behaviors measured. Chemogenetic stimulation of NAc^{GABA} terminals in the VP reduced the number of *fos*+, a marker of neuronal activation, VP cells, showing that activation of the NAc to VP pathway reduces VP activation. The second aim of my thesis (Chapter 3) determined whether social play exposure altered activation of VP-projecting cells in the two subregions of the NAc, the NAc core and shell, of juvenile male and female rats. I utilized the retrograde tracer cholera toxin subunit B (CtB) and fos *in situ* hybridization to quantify activated VP-projecting NAc cells. Although males and females showed a similar duration of social play, I found a sex-specific shift in activation of VP-projecting NAc cells following social play exposure. In both the NAc core and shell, there was a baseline sex difference in the activation of VP-projecting cells, such that males showed greater activation of VP-projecting cells compared to females. These baseline sex differences were eliminated by social play exposure in NAc subregion-specific ways. In the NAc core, social play exposure decreased the activation of VP-projecting cells in males. In the NAc shell, social play exposure tended to increase the activation of VP-projecting cells in females. Findings from Chapter 3 indicate that the equal expression of social play in males and females is associated with the elimination of a baseline sex difference in the activation of VP-projecting cells in the NAc core and shell in a sex-specific way. Overall, I provide the first evidence that inhibition of the NAc to VP pathway allows for the typical expression of social play behavior in juvenile male and female rats and this is associated with the elimination of a baseline sex difference in activation of the NAc to VP pathway.

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CHAPTER 1: Introduction

After birth, various mammalian species are thrust into complex social spheres that they must navigate in order to optimize their survival. One of the earliest peer interactions that juveniles of most mammalian species will face is social play, also known as rough-and-tumble play or play-fighting (Thor and Holloway, 1984a; Pellis and Pellis, 1987; Pellis and Iwaniuk, 2000; Himmler et al., 2014; Pellis et al., 2018). Although social play behavior is exhibited in diverse forms depending on the species (Bekoff, 1974a; West, 1974; Markus and Croft, 1995; Vanderschuren et al., 1997; Jordan, 2003; Koevoet, 2020), it can be differentiated from non-play behaviors. There are a set of criteria proposed by Burghardt (2010) that is widely used to differentiate play (including solitary play, object play, social play) from non-play behaviors (Burghardt, 2010): (i) play is not fully functional in the form or context in which it is expressed; (ii) play is a voluntary behavior that is rewarding, pleasurable, and motivating; (iii) play is different from other forms of serious behaviors in that it is incomplete, exaggerated, precocious, or involves behavioral patterns that is in modified form, sequencing, or targeting; (iv) play is a repeated behavior but not in a stereotypic way; (v) play is expressed in a low stress environment (i.e. animal is well fed, healthy, etc) although mild stress can facilitate play. This set of criteria had provided scientists guidance on how to identify play behaviors exhibited in various forms in mammalian species.

In addition, many theories regarding the phylogeny and functions of play (Thor and Holloway, 1984a; Pellis and Iwaniuk, 1999; Pellegrini and Bjorklund, 2004; Bateson, 2015; Pellis et al., 2018, 2019; Lewis, 2023) have been proposed, attesting to the complex nature of this nearly ubiquitous behavior in mammals. For example, the

"surplus resource theory" states that play occurred as a functionless behavior in juveniles during a period of abundance of resources, such as food and time, as a way to release surplus energy (Burghardt, 2005) while the "pre-exercise theory" posits that play is practicing skills, such as fighting, that are essential for survival (Karl Groos, 1898). Multiple studies demonstrate that engaging in social play behavior contributes to brain development (Bell et al., 2010; Himmler et al., 2013; Bijlsma et al., 2022; Pellis et al., 2023) as well as the context-appropriate expression of adult social behaviors (Wright et al., 1991; van den Berg et al., 1999; Lukkes et al., 2009; Meng et al., 2010; Marquardt et al., 2022), providing evidence that social play behavior is an essential part of juvenile development. Deficits in this behavior during this crucial window may have detrimental effects on the developmental trajectory of the individual.

This chapter will first discuss how social play behavior is expressed in human children and the importance of engaging in social play at a young age. I will then discuss how children diagnosed with autism spectrum disorder show changes in social play behavior and the potential brain systems that contribute to typical as well as decreased social play engagement. Following the background on social play behavior in children, I will discuss how social play behavior is studied in juvenile rats in a laboratory setting and the brain network that is likely involved in regulating social play. I will focus specifically on two brain regions, the nucleus accumbens (NAc) and ventral pallidum (VP), and how these brain regions are individually involved in regulating social play. Finally, I will propose that the NAc to VP pathway is involved in regulating social play and discuss the literature that implicates this pathway in the regulation of rewarding behaviors in adults. I will provide the rationale of why this pathway is likely involved in

regulating social behaviors, particularly social play behavior, and how I intend on testing this hypothesis. Together, these studies and literature review will provide a comprehensive background on how these two brain regions are involved in regulating social play and implicate, for the first time, pathway-specific regulation of social play behavior in juvenile male and female rats.

Social play behavior in human children

As children develop cognitively, the complexity of their play, as well as how they include peers in their play, also changes (Smilansky, 1968). In detail, from birth to around 2 years of age, children will engage in functional play, in which they perform simple, repetitive movements with or without an object. An example would be shaking a rattle or jumping up and down. As they grow older, they begin to engage in constructive play, in which they manipulate objects to create something, such as creating a tower of blocks. Following constructive play, they move onto pretend play, which can range from pretending a log is a boat or playing “house”. This stage is where there is a shift from solitary or parallel play (when two or more children play next to each other without any genuine interaction) to social play. The last stage of play development is engaging in play with rules, such as checkers or chess. This requires complex thinking for children to understand the rules of the games and requires cooperation with peers. Therefore, as children get older, the social component of play becomes more prevalent.

Social play in children is present across different cultures and often is presented in different forms such as sports, rough-and-tumble play, board games, and more (Parten, 1933; Marivate and Marwala, 2008; Bruner et al., 2014; Chou, 2017; Eriksson et al., 2021; Nery et al., 2023). Social play has elements of rules and cooperation that

children must follow to be involved in group social play. For example, a popular form of social play (referred to as pretend or imaginative play) among children is "house" (Parten, 1933). "House" is a reenactment of home situations and requires children to assume the stereotypical home roles such as "dad", "mom", and "brother/sister". Once children decide on their roles, they enact situations such as: mom goes to the doctor with her child, dad comes home from work and eats dinner with his family, and so forth (Parten, 1933). This type of social play requires the child to differentiate between different roles and correctly act out their role. Although there is an element of imagination within each child that makes this group social play possible, cooperation between all children within the group is prevalent (Parten, 1932; Rubin, 1977; Orlick, 1981). These types of peer social play offer children the opportunity to practice communication skills as well as cognitive flexibility (Bonino and Cattelino, 1999; Memari et al., 2013; Badamian and Moghaddam, 2017).

Another form of social play is spontaneous play. Spontaneous social play in children occurs without the presence of an adult or authority figure, and the pace of social play is determined by the children (Hewes, 2014). Structured play is typically social play that is guided by adults (Stahmer, 1999; Kok et al., 2002; Murata and Maeda, 2002). Structured play requires children to participate in the activities that are prepared by adults in guided exercises. On the other hand, spontaneous group social play gives children full control of what games to play, what toys to play with, and so forth in order to give children the opportunity to experience play in two ways: control and willing participation (Parten, 1932; Hewes, 2014). In addition, there are implicit cooperation rules that children follow such as knowing when it's not their turn to be in charge, or

following how the group wants to play, even if the individual doesn't want to (Hewes, 2014). The social context of spontaneous play is that all children within the group will follow these rules. Spontaneous play gives children the freedom to express their imagination that is different from what structured play offers (Wolfberg and Schuler, 1999; Lillard et al., 2013; Bateson, 2015). Therefore, this type of social play enhances children's cognitive and emotional awareness (Miller, 1974; Spinka et al., 2001; Hewes, 2014) as they learn social group dynamics without an authority figure to guide them. Overall, engaging in social play, whether it's pretend or imaginative play, structured play, or spontaneous play, contributes to the cognitive, emotional, and physical development in children (Memari et al., 2013; Nijhof et al., 2018; Koevoet, 2020; Lee et al., 2020).

Studies have also investigated whether there are gender differences in social play behaviors in children. Although some studies report that boys and girls showed no differences in rough-and-tumble play initiation (Lloyd and Smith, 1985; Reimers et al., 2018), others report that boys initiated rough-and-tumble play more than girls (Scott and Panksepp, 2003; Ostrov and Keating, 2004). These discrepancies can be partly attributed to social norms, where boys are encouraged to engage in rough-and-tumble play, while this behavior is discouraged in girls (Humphreys and Smith, 1987). Additionally, girls may be more aware of an observer and be less likely to engage in rough-and-tumble play compared to boys (Scott and Panksepp, 2003). However, in order to eliminate the presence of an observer, one study observed children's rough-and-tumble play behavior through a camera and reported no gender differences in rough-and-tumble play initiation (Scott and Panksepp, 2003). Although the actual

elements of rough-and-tumble were slightly different between boys and girls, with boys showing more pushes during social play than girls, rough-and-tumble play between boys and girls were similar (Scott and Panksepp, 2003).

Engaging in social play has been shown to have therapeutic value and can be a tool for emotional regulation in children. For example, engaging in social play decreased urinary cortisol levels by over 30% in boys and girls that were hospitalized in public pediatric hospitals (Potasz et al., 2013) and increased self-esteem (Gillis, 1989), providing evidence that social play can be used as a coping tool to alleviate severe stress, such as hospitalization in an unfamiliar environment, and encourage positive emotions in young children. This is especially important as children who grew up with chronic childhood diseases such as sickle cell anemia, heart diseases, autoimmune diseases and more are often hospitalized for extended periods of time and have fewer opportunities to interact with their peers. Over time, these children are at a greater risk for declining mental health and report greater instances of depressive symptoms, anxiety, and academic and social problems (Patenaude and Kupst, 2005; Pinquart and Shen, 2011) compared to their healthy peers. Hence, engaging in social play during childhood not only impacts the immediate emotional and physical health of children, but has lasting impacts into adolescence and adulthood.

Children diagnosed with disorders involving social communication deficits, such as autism spectrum disorder (ASD), have difficulty initiating and maintaining social play (Holmes and Willoughby, 2005; Matson and Wilkins, 2007; Buggey et al., 2011). ASD children have difficulty engaging in social play with their peers due to three dimensions of play (Jordan, 2003): (i) shared attention and emotional regulation; (ii) understanding

the cognitive rules and complexities of social interactions; (iii) evaluating the social status of peers and oneself during play interactions. Additionally, if social play is highly structured, with guided instructions and rules such as board games as a group, ASD children show engaged participation with their peers (Lewis and Boucher, 1988; Jarrold et al., 1996; Charman and Baron-Cohen, 1997; Libby et al., 1998). However, they show difficulty, and oftentimes failure, to participate in spontaneous play (Lewis and Boucher, 1988; Libby et al., 1998; Stahmer, 1999) and thus spend less time engaging in social play. Aberrations in social reward processing (Supekar et al., 2018) may be a contributing factor in the decreased engagement in social play exhibited by ASD children. ASD children show significantly lower preference for social images compared to typically developing peers and this decreased preference correlated with a lower number of eye contacts made with the experimenter (Ruta et al., 2017). These findings suggest that overall social motivation may be lower in ASD children due to decreased reward value of social interactions. Indeed, an fMRI study investigating responses in the brain's reward system in ASD children showed that ASD children exhibited structural changes in the mesolimbic reward system, as displayed by lower density of white matter tracts between the nucleus accumbens (NAc) and ventral tegmental area (VTA), two brain regions important for reward processing, compared to typically developing children (Supekar et al., 2018). These structural changes were associated with social communication deficits, such that ASD children that showed lower density of NAc-VTA tracts displayed more severe social interaction impairments (Supekar et al., 2018). Finally, ASD children showed decreased functional connectivity between the NAc and VTA compared to typically developing children when they were shown a social stimulus

(image of a face; Supekar et al., 2018). Together, these findings point towards deficits in the mesolimbic reward system that contribute to overall decrease in social motivation, play, and preference in ASD children. Furthermore, ASD is 4 times more prevalent in boys compared to girls (Baio et al., 2018; Maenner et al., 2023), suggesting that there might be sex differences in how changes in the mesolimbic reward system affect social interaction impairments in ASD children.

Overall, social play is crucial in the cognitive and emotional development of children and decreased engagement in social play contributes to difficulty in social communication later in life. A target brain system that may be involved in motivation to engage in social play is the mesolimbic reward system. However, not much is known about the involvement of the mesolimbic reward system in regulating social play behavior. Therefore, we need a better understanding of how the mesolimbic reward system is involved in regulating social play behavior in males and females in order to refine therapeutic interventions that aim to improve social communication, including social play, in ASD children.

Social play behavior in juvenile rats

Although social play is displayed in a wide variety of mammalian species, including Japanese macaques (Petit et al., 2008; Reinhart et al., 2010; Shimada and Sueur, 2018), domestic cats (West, 1974; Barrett and Bateson, 1978; Delgado and Hecht, 2019; Gajdoš Kmecová et al., 2021), and dogs (Bekoff, 1974a; Ward et al., 2008; Smuts, 2014), the laboratory rat is commonly used to study the neural mechanisms that are involved in regulating social play behavior. Social play behavior is well characterized in juvenile rats (Pellis and Pellis, 1987; Pellis and Iwaniuk, 2000; Pellis et al., 2019;

Achterberg et al., 2023) and can be used to study the impacts of social play on neural and social development. On the other hand, mice, another well used laboratory animal, cannot be used as a model to study social play because they do not show social play behavior (Wolff, 1981; Pellis and Pasztor, 1999). Juvenile male and female rats display stereotypical social play behaviors (Pellis and Pellis, 1987; Panksepp et al., 1994), which usually involves two partners that are engaging in reciprocal behaviors during the play bouts (Pellis and Pellis, 1987; Achterberg et al., 2023). In detail, juvenile rats usually aim for the nape of their partner's neck to initiate the play bout, nuzzle their snout on the nape, and then pounce on their play partner (Pellis and Pellis, 1987). The partner will then show defensive behaviors to protect their nape by assuming a supine pose and then will initiate its own attack towards the nape of their partner's neck by twisting around and pinning their partner (Pellis and Pellis, 1987). This balance and back-and-forth of attack and defensive behaviors between the two juvenile rats is important to keep the interactions playful for both rats (Palagi et al., 2016; Pellis and Pellis, 2017; Achterberg et al., 2023). It is also possible to modify the internal motivational states of juvenile rats to engage in social play by utilizing a brief isolation period (usually between 2 to 24 hours; Ikemoto and Panksepp, 1992) and measure motivation through operant conditioning in order to understand how reward and motivational processing are involved in the decision to engage in social play (Achterberg et al., 2016a, 2019).

There are various paradigms to study and measure social play behaviors in juvenile rats. Social play behaviors can be measured in a novel testing environment (Olioff and Stewart, 1978; Panksepp et al., 1994; Vanderschuren et al., 1995; Trezza et

al., 2011b; van Kerkhof et al., 2013; Manduca et al., 2016b; Argue et al., 2017; Zhao and Riters, 2023) or a familiar home cage environment (Veenema et al., 2013; Bredewold et al., 2018; Reppucci et al., 2018, 2020). Studies show that the effects of pharmacological manipulations on the expression of social play can change depending on the context in which juvenile rats are tested in (home cage vs. novel testing environment; Bredewold et al., 2014). Play partners used during social play testing range from novel or unfamiliar same-sex partners (Veenema et al., 2013; Manduca et al., 2016b; Bredewold et al., 2018; Reppucci et al., 2018) to mixed-sex group partners (Olioff and Stewart, 1978). Rats are either exposed to a brief isolation (2 hrs; Trezza et al., 2011b; van Kerkhof et al., 2013; Manduca et al., 2016b; Achterberg et al., 2023) or longer isolation (24 hrs; Panksepp et al., 1994; Vanderschuren et al., 1995; Veenema et al., 2013; Lukas and Wöhr, 2015; Bredewold et al., 2018; Reppucci et al., 2018, 2020; Kisko et al., 2021; Zhao and Riters, 2023) prior to social play testing. These differences in paradigms can influence the expression of social play behavior in juvenile male and female rats by altering the contextual environment in which juvenile rats are tested in or the internal motivational state to engage in social play by subjecting juvenile rats to a brief social isolation.

One factor that is affected by the design of social play paradigms is sex differences in social play behaviors. Some studies report that males exhibit a longer duration and increased frequency of social play behaviors (Olioff and Stewart, 1978; Meaney and Stewart, 1981; Pellis and Pellis, 1990; Northcutt and Nwankwo, 2018), as well as a greater number of social play-induced 50-kHz ultrasonic vocalization (USV; (Kisko et al., 2021; Tivey et al., 2022) compared to females. On the other hand, other

studies report no sex differences in the duration or frequency of social play behaviors (Veenema et al., 2013; Bredewold et al., 2018; Reppucci et al., 2020). The different conditions in which social play is tested, such as differences in isolation time, the environment in which social play testing is conducted, or the social play partners, contribute to the variability in sex differences, or lack thereof, reported. For example, juvenile male and female rats that were tested for social play behavior in mixed-sex groups consisting of novel conspecifics show sex differences in the number of play events (i.e. number of pinning, pouncing, boxing behaviors; Meaney et al., 1981; Argue et al., 2017). Specifically, juvenile males showed a higher number of play events compared to juvenile females (Meaney et al., 1981; Argue et al., 2017). In addition, juvenile males showed higher social play events compared to juvenile females when subjected to social play testing in a testing apparatus and exposed to a same-sex novel conspecific (Olioff and Stewart, 1978). Finally, juvenile males showed a higher number of social play events when they were tested in their home cage following a brief 1 hr isolation prior to social play testing (Taylor et al., 2012). On the other hand, juvenile male and female rats that were isolated for 24 hrs prior to social play testing and and underwent social play testing in their homecage showed a similar duration of social play and number of social play events (Veenema et al., 2013; Bredewold et al., 2014, 2018; Reppucci et al., 2018, 2020). Additionally, there are strain differences in the duration of social play that may contribute to the discrepancy of sex differences reported in social play studies (Thor and Holloway, 1984b; Manduca et al., 2014; Ku et al., 2016; Northcutt and Nwankwo, 2018). For example, when tested during the light phase and exposed to

novel stimulus animals, sex differences were only observed in Long-Evans rats and not in Sprague-Dawley or Wistar rats (Northcutt and Nwankwo, 2018).

Overall, it is still valid to consider that the neural systems underlying social play behavior may not be the same for males and females, even if there are no sex differences in the outward expression of social play behavior. Indeed, there is evidence that there are robust sex differences in the neural regulation of social play behavior under conditions when males and females show no difference in the expression of social play (Veenema et al., 2013; Bredewold et al., 2015). Therefore, it is important to include males and females in experimental designs and if there are any indications of a sex difference, then one could decide to further study those sex differences.

As mentioned earlier, fMRI studies in humans identified the mesolimbic reward system as a possible neural mechanism involved in processing the rewarding properties of social stimuli (Spreckelmeyer et al., 2009; Supekar et al., 2018). The mesolimbic reward system is comprised of multiple forebrain regions including the nucleus accumbens (NAc; Ikemoto and Panksepp, 1999; Nestler and Carlezon, 2006; Pierce and Kumaresan, 2006) and ventral pallidum (VP; Nestler and Carlezon, 2006; Pierce and Kumaresan, 2006; Smith et al., 2009). Brain regions in this system have been implicated in rewarding and motivating behaviors, such as feeding (Stratford et al., 1999; van Zessen et al., 2012; Perello and Dickson, 2015; Gendelis et al., 2021), drug-seeking (Schmidt and Pierce, 2006; Mahler et al., 2014; Golden et al., 2019; Pardo-Garcia et al., 2019; Kupchik and Prasad, 2021), and social interactions (Young et al., 2001; Dölen et al., 2013; Salti et al., 2015; Knowland et al., 2017; Kopec et al., 2018; Gómez-Gómez et al., 2019; Borie et al., 2021). The expression of social behaviors requires the interaction

between the mesolimbic reward system and the Social Behavior Network (SBN; Newman, 1999). The SBN is a network of brain regions that are interconnected and their collective activation patterns regulate social behaviors, often in a sex-specific manner (Newman, 1999). Social behaviors are rewarding in nature (Thiel et al., 2008; Krach et al., 2010; Trezza et al., 2010, 2011a; Vanderschuren et al., 2016; Borland et al., 2019; Hu et al., 2021), and the SBN and mesolimbic reward system interact in order to elicit the appropriate social behaviors. The interaction between the SBN and mesolimbic reward pathway to guide social decision-making is referred to as the Social Decision-Making Network (SDMN; (O'Connell and Hofmann, 2011; Tremblay et al., 2017). The SDMN integrates chemosensory feedback and reward processing signals in order to evaluate the valence of the social stimulus prior to eliciting the appropriate behavioral response (O'Connell and Hofmann, 2011).

Various brain regions that regulate social play behavior are a part of the SDMN (Trezza et al., 2012; Manduca et al., 2016b, 2016a; Vanderschuren et al., 2016; Reppucci et al., 2018; Lee et al., 2021). Using lesion techniques, studies determined that brain regions in the SDMN such as the amygdala (Meaney et al., 1981; Daenen et al., 2002), anterior hypothalamus (Beatty and Costello, 1983; Leedy and Hart, 1986), ventromedial hypothalamus (Beatty and Costello, 1983), and lateral septum (Beatty et al., 1982) are crucial for the typical expression of social play behavior. In detail, amygdala lesions, including the central, basolateral, and lateral amygdala, decreased social play behaviors in males only (Meaney et al., 1981; Daenen et al., 2002) while lesions to the anterior hypothalamus and ventromedial hypothalamus reduced social play behaviors in both males and females (Beatty and Costello, 1983). On the other

hand, lateral septum lesions increased social play behaviors in males and females (Beatty et al., 1982). More recent techniques using pharmacology or the early gene protein c-Fos, used as a proxy for neuronal activation, have implicated additional brain regions that are part of the SDMN in regulating social play behavior (Gordon et al., 2002; Northcutt and Nguyen, 2014; van Kerkhof et al., 2014; Reppucci et al., 2018). All these studies provide evidence that individual brain regions are important for the expression of social play behavior, but there are no studies investigating how brain regions work collectively to regulate social play in juvenile rats. I will focus on two brain regions within the reward system, the nucleus accumbens (NAc) and ventral pallidum (VP), and whether projections from the NAc to VP are involved in regulating social play in juvenile rats.

The NAc and social play behavior

The NAc is part of the mesolimbic reward system (Salamone and Correa, 2002; Salamone et al., 2005; Carlezon and Thomas, 2009; Klawonn and Malenka, 2018) and comprises mainly two subregions: the core and shell (Heimer et al., 1991; Deutch and Cameron, 1992; Brog et al., 1993; O'Donnell and Grace, 1993; Zahm and Heimer, 1993; Jones et al., 1996). Although the NAc core and shell have differential inputs and outputs (Brog et al., 1993; Zahm and Heimer, 1993; Jongen-Rêlo et al., 1994; Li et al., 2018), both subregions receive dopaminergic innervation from the ventral tegmental area (VTA; Heimer et al., 1991; Brog et al., 1993), another brain region within the mesolimbic reward system. The NAc is composed of primarily GABAergic medium spiny neurons (MSN; Preston et al. 1980; O'Donnell and Grace 1993) which express dopamine 1 (D1)-like or dopamine 2 (D2)-like receptors, both of which are important for reward

processing in the NAc (Wong et al., 1999; Schmidt and Pierce, 2006; Laviolette et al., 2008; Namvar et al., 2019).

The NAc is implicated in regulating a variety of rewarding social behaviors in rodents such as pair-bonding in adult female prairie voles (Aragona et al., 2003; Liu and Wang, 2003; Ross et al., 2009), maternal behaviors in adult female rats (Keer and Stern, 1999; Champagne et al., 2004; Numan et al., 2005b, 2005a; Olazábal and Young, 2006; Ross et al., 2009), sociosexual motivation (Beny-Shefer et al., 2017; Kondo and Hayashi, 2021), and social play in juvenile rats (Trezza et al., 2012; Manduca et al., 2016b, 2016a; Kopec et al., 2018). The involvement of the NAc in regulating social play has been studied using immunohistochemistry techniques as well as pharmacological methods (Trezza et al., 2012; van Kerkhof et al., 2013; Manduca et al., 2016a, 2016b; Kopec et al., 2018). In detail, social play exposure is associated with an increase in c-Fos expression in the NAc core and shell (van Kerkhof et al., 2014) in juvenile male rats. Pharmacological inactivation of the NAc core, but not the shell, via infusions of muscimol ($GABA_A$ receptor agonist) and (RS)-baclofen ($GABA_B$ receptor agonist) increased social play duration in juvenile male rats (van Kerkhof et al., 2013). This finding is consistent with the inhibition-reward hypothesis of NAc neural activity (Carlezon and Thomas, 2009), which argues that inhibition of the NAc is required to enhance the rewarding properties of a stimulus. Support for this hypothesis comes from electrophysiological studies that show decreased frequency of neuronal firing in the NAc before and during sucrose consumption (Krause et al., 2010). Therefore, it is likely that decreased activation of the NAc is associated with the regulation of social play behavior.

In addition, dopaminergic signaling in the NAc is involved in regulating social play in juvenile rats. Intra-NAc infusions of amphetamine, a dopamine release/reuptake inhibitor which increases extracellular presence of dopamine, and apomorphine, a non-selective dopamine receptor agonist, both increased social play behaviors (Manduca et al., 2016b) in juvenile male rats. The play-enhancing effects of amphetamine were blocked when it was co-infused with SCH-23390 (specific D1 dopamine receptor antagonist) or eticlopride (specific D2 dopamine receptor antagonist) in doses in which these antagonists did not alter social play on their own (Manduca et al., 2016b). In addition, higher doses of SCH-23390 and eticlopride reduced social play behaviors following a prolonged isolation prior to social play testing (Manduca et al., 2016b). Together, these findings suggest that activation of the dopamine receptor system in the NAc is crucial for the play-enhancing effects of dopamine and stimulation of dopamine receptors are necessary for the typical expression of social play. Importantly, all these studies were conducted in males and therefore it is unclear whether the involvement of dopamine signaling in the NAc modulates social play behavior similarly in males and females.

The VP and social play behavior

The VP was originally identified as the primary output region for motivated goal-directed movement by incorporating reward processing signals from the striatum (Swerdlow and Koob, 1987). However, more recent studies have demonstrated that the VP itself encodes reward (Richard et al., 2016; Ottenheimer et al., 2018; Vento and Jhou, 2020) and incentive values (Smith et al., 2011; Richard et al., 2016) in adult male rats. There is evidence that the VP receives dopaminergic (Klitenick et al., 1992) and

GABAergic (Zhou et al., 2022) input from the VTA, and these inputs are associated with modulation of reward-driven behaviors (Campbell and Lobo, 2021; Pribiag et al., 2021; Zhou et al., 2022). The idea that the VP is more than just an integration hub for locomotor output of the mesolimbic reward system was further demonstrated when it was reported that modulation of opioid signaling in the VP can alter hedonic "liking" responses to sucrose (Smith and Berridge, 2005, 2007; Peciña et al., 2006; Tindell et al., 2006) and subsequently change feeding behaviors (Smith and Berridge, 2005, 2007) in adult rats. Therefore, direct modulation of neurosignaling in the VP can change the encoding of rewarding stimulus and alter the expression of rewarding behaviors. Studies now provide evidence that the VP is involved in regulating various rewarding behaviors such as food-seeking (Smith and Berridge, 2007; Gendelis et al., 2021; Doucette et al., 2022) and drug-seeking (Skoubis and Maidment, 2003; Mahler et al., 2014; Farrell et al., 2019; Prasad et al., 2020; Kupchik and Prasad, 2021) behaviors. More relevant to this thesis is that the VP is also involved in regulating rewarding social behaviors.

Similar to the NAc, the VP is also known for regulating pair-bonding (Lim and Young, 2004; Lim et al., 2004a, 2004b), parental behaviors (Numan et al., 2005b; Akther et al., 2014), and opposite sex preference (DiBenedictis et al., 2020). As part of my Master's thesis, I showed the first evidence that the VP is involved in regulating social play behavior in juvenile male and female rats. Specifically, I infused muscimol, the $GABA_A$ receptor agonist, into the VP and found that muscimol-treated subjects displayed lower social play duration (Fig 1A) and a fewer number of nape attacks (Fig 1B) and pins (Fig 1C) compared to vehicle-treated subjects. In addition, I found no sex

differences in the effects of muscimol on social play behavior (Fig 1A-C), demonstrating that activation of the VP is required for the typical expression of social play in both males and females. In addition, considering that the VP is involved in regulating goal-directed locomotor movement (Austin and Kalivas, 1989, 1991; Gong et al., 1996; Fletcher et al., 1998), it would be possible that the decrease in social play behaviors due to muscimol infusions can be attributed to overall decrease in movement. However, I found the opposite, such that muscimol-treated subjects showed a higher duration of non-social cage exploration compared to vehicle-treated subjects (Fig 1F), demonstrating that the decrease in social play behaviors is not due to an overall decrease in locomotion. Finally, there was no difference in the duration of other social behaviors measured in the test, such as social investigation (Fig 1D) or allogrooming (Fig 1E), providing evidence that the decrease in social play behaviors was not due to a decrease in overall social motivation or interest. Together, these findings show that VP activation is required for the typical expression of social play behavior in juvenile male and female rats.

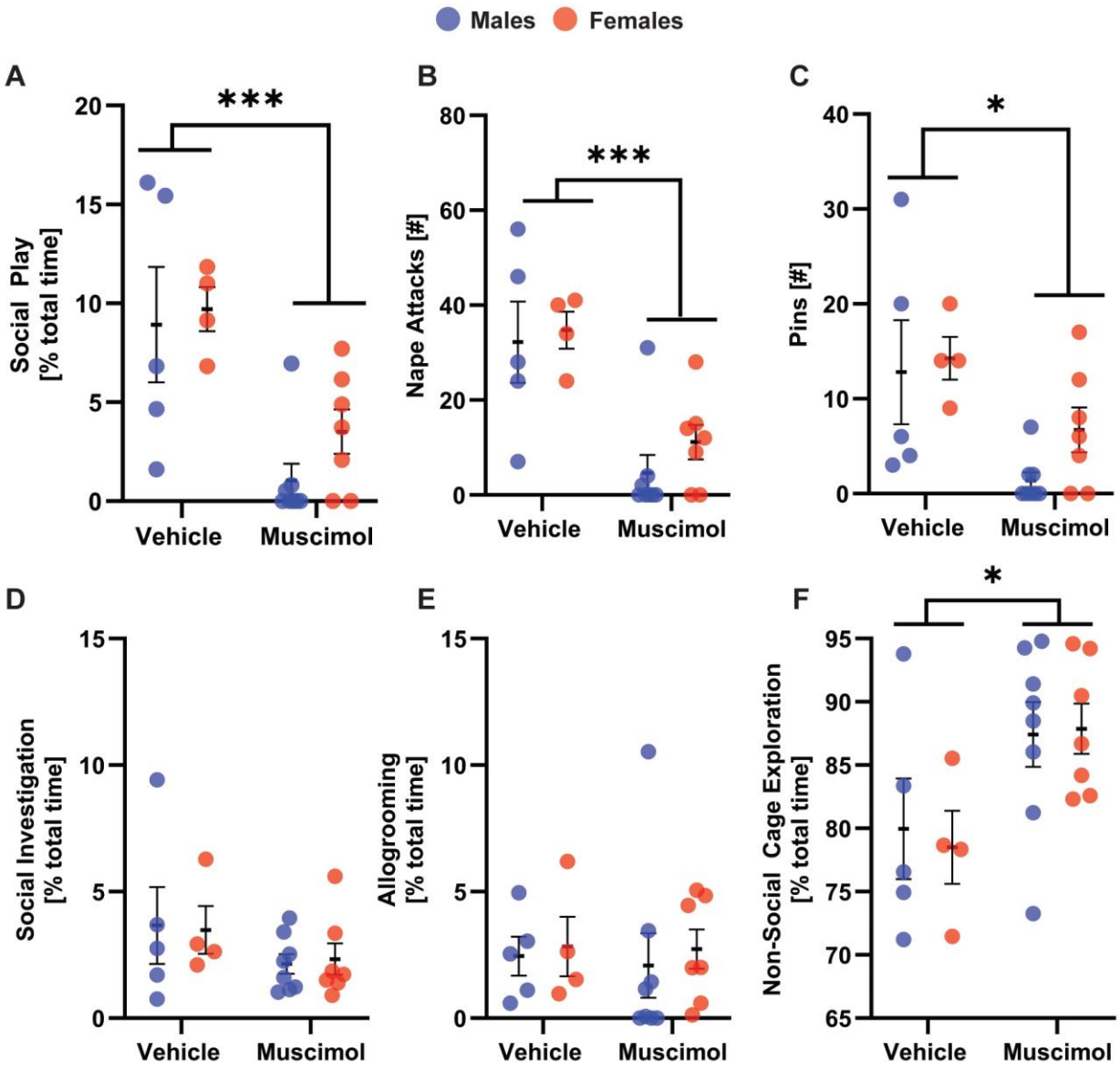

Figure 1. Bilateral infusions of muscimol into the VP decreased social play behaviors in male and female rats. Bilateral ventral pallidal infusions of the $GABA_A$ receptor agonist muscimol decreased the duration of social play **(A)**, the number of nape attacks **(B)**, and the number of pins **(C)** in male and female rats compared to their vehicle-treated counterparts. Muscimol increased the duration of non-social cage exploration in male and female rats compared to vehicle-treated counterparts **(F)** but did

Figure 1 (cont'd)

not alter the duration of social investigation **(D)** or allogrooming **(E)**. Black bars indicate mean ± SEM; *$p < 0.05$, ***$p < 0.001$, main effect two-way ANOVA.

The involvement of the NAc to VP pathway in regulating rewarding behaviors in adult rodents

One of the main sources of input to the VP comes from the NAc (Williams et al., 1977; Groenewegen and Russchen, 1984; Usuda et al., 1998). Both the NAc core and shell project to the VP (Zahm and Heimer, 1990, 1993; Heimer et al., 1991), and there is evidence to support that the nature of this projection is GABAergic (Churchill and Kalivas, 1994; Root et al., 2015). For example, stimulation of NAc cells reduced spontaneous activity of VP cells (Jones and Mogenson, 1980; Chrobak and Napier, 1993) and NAc lesions reduced the presence of Gad1- (glutamate decarboxylase, enzyme that catalyzes the production of GABA) immunoreactive fibers in the VP (Root et al., 2015) in adult rats. Furthermore, NAc neurons were found to form symmetric synapses onto VP neurons (Zahm et al., 1985; Bolam et al., 1986), which are indicative of inhibitory GABAergic axons (Hendry et al., 1983; Ribak and Roberts, 1990; Boyes and Bolam, 2007). Since both brain regions are individually implicated in reward processing and modulation of reward-driven behaviors, studies then focused on how NAc inputs to the VP may be involved in regulating such behaviors. Researchers utilized optogenetics (Stefanik et al., 2013; Wang et al., 2014; Chometton et al., 2020; Yao et al., 2021; Correia et al., 2023) and chemogenetics (Gallo et al., 2018; Pardo-Garcia et al., 2019) in order to either stimulate or inhibit NAc inputs to the VP to see whether these change rewarding behaviors. Optogenetic stimulation of NAc terminals in the VP decreased

sucrose intake in adult female rats (Chometton et al., 2020). Moreover, chemogenetic inhibition of NAc terminals in the VP increased lever press responses and breakpoints in response to a food reward in adult mice (Gallo et al., 2018). *In vitro* studies demonstrate that optogenetic stimulation of NAc terminals in the VP decreased *fos* mRNA expression in the VP (Wang et al., 2014) and chemogenetic inhibition of these terminals decreased inhibitory postsynaptic currents in the VP (Gallo et al., 2018). These findings show firstly that NAc inputs to the VP are inhibitory, and inhibition or stimulation of these inputs regulates the expression of reward-driven behaviors. In support, inhibition of NAc inputs to the VP disinhibits the VP, and increases rewarding behaviors, suggesting that reduction of inhibitory tone from the NAc to VP serves as a regulatory mechanism for the expression of rewarding behaviors. Although the mentioned studies used sucrose as the rewarding stimulus, it is likely that this regulatory mechanism is engaged for overall rewarding behaviors, and not just sucrose-driven behaviors. Therefore, I propose that inhibition of NAc inputs to the VP also occurs when animals engage in social behaviors (Fig 2B), from juvenile to adulthood.

Indeed, the proposal that disinhibition of the VP via inhibition of NAc^{GABA} inputs is required for the typical expression of rewarding social behaviors was indirectly shown in the context of maternal behaviors (Numan et al., 2005b; Numan, 2007; Numan and Stolzenberg, 2009). Infusions of muscimol in the NAc did not alter pup retrieval scores or nursing duration in postpartum adult females (Numan et al., 2005b). Contrary, infusions of muscimol in the VP significantly reduced pup retrieval scores and nursing duration and significantly increased the latency to approach and sniff pups in postpartum females (Numan et al., 2005b). Combined, these findings suggest that VP

activation is required for the typical expression of social behaviors. Furthermore, VP activation is achieved via decreased inhibitory inputs from the NAc in response to a social stimulus, which allows the animal to display the appropriate social response. In addition, although all these studies were conducted studying maternal behaviors, I showed that activation of the VP is required for the typical expression of social play, and therefore further propose that disinhibition of the NAc to VP pathway is also engaged when juvenile rats play.

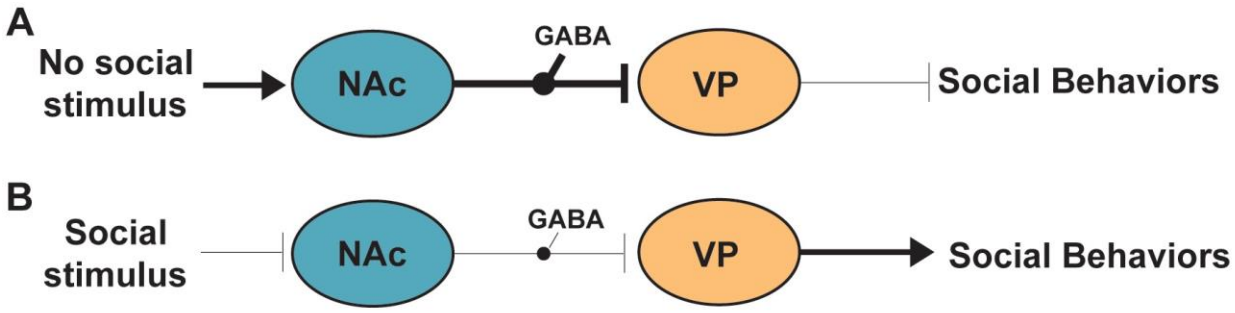

Figure 2. Visual schematic of modulation of social behaviors via change in activity of the NAc to VP pathway. (A) Without the presence of a rewarding social stimulus, GABAergic inputs from the NAc to the VP are active, thereby inhibiting activation of the VP to weaken or prevent the expression of social behaviors. (B) In the presence of a rewarding social stimulus, GABAergic inputs from the NAc to the VP are inhibited, therefore disinhibiting the VP, promoting VP activation in order to support the expression of social behaviors.

Overview of dissertation chapters

Based on the past findings that demonstrate inactivation of the NAc core increased social play behaviors while inactivation of the VP decreased social play behaviors, I hypothesize that the NAc to VP pathway is involved in regulating social play behavior in juvenile male and female rats. To test this hypothesis, I determined whether

the involvement of the NAc^{GABA} to VP pathway in regulating social play behavior in juvenile male and female rats (Chapter 2). I utilized Gad1-iCre juvenile rats, in which cre recombinase is expressed on all cells that express Gad1, in order to chemogenetically stimulate NAc^{GABA} terminals in the VP using a cre dependent excitatory DREADD (designer receptors activated by designer drugs). I predicted that chemogenetic stimulation of NAc^{GABA} terminals in the VP would increase GABA signaling in the VP, thereby inhibiting the VP, and decrease social play behaviors in both juvenile male and female rats. I did not expect to see sex differences in social play behaviors following chemogenetic stimulation of the NAc to VP pathway because VP inactivation decreased social play behaviors for both males and females (Fig 1A). Therefore, I expected that stimulating GABAergic inputs to the VP would have the same effects on males and females. As predicted, chemogenetic stimulation of the NAc^{GABA} to VP pathway decreased social play behaviors in both juvenile males and females. In addition, chemogenetic stimulation of the NAc^{GABA} to VP pathway was associated with a decrease in VP neuronal activation, suggesting that our manipulation increased extracellular GABA concentration in the VP by stimulating NAc^{GABA} terminals in the VP, thereby inhibiting the VP, and resulting in a decrease in social play behaviors.

Findings from my Master's thesis data showed that although males and females showed similar levels of social play (Fig 3A), the degree of activation from the VP was sex-specific. In detail, social play exposure was associated with an increase in VP activation in males only (Fig 3C). On the other hand, females showed similar activation of the VP following social play exposure (Fig 3C). This led me to hypothesize that while the social play behavioral phenotype is the same between males and females, the

underlying neural mechanisms that modulate social play are sex-specific. Specifically, I hypothesized that sex differences in activation of NAc inputs to the VP contribute to the sex difference in VP activation following social play exposure. To test this hypothesis, I determined whether social play exposure altered activation of VP-projecting NAc cells in juvenile male and female rats (Chapter 3) using a combination of retrograde tracing and *in situ* hybridization. I predicted that social play exposure would be associated with a sex-specific decrease in activation of VP-projecting NAc cells (Fig 3C). Specifically, I expected social play exposure to be associated with a decrease in activation of VP-projecting NAc cells in males only. In females, I expected no change in activation of VP-projecting NAc cells following social play exposure. This decrease in activation of VP-projecting NAc cells in males, which could indicate a decrease in inhibitory inputs to the VP, would be consistent with the corresponding increase in VP activation observed following social play exposure (Fig 3C). As predicted, I found that social play exposure decreased activation of VP-projecting NAc cells in both the NAc core and NAc shell of males, while there was no change in activation observed in the females. These results point towards a sex difference in the neural mechanisms regulating social play in order to allow for the equal expression of social play between males and females.

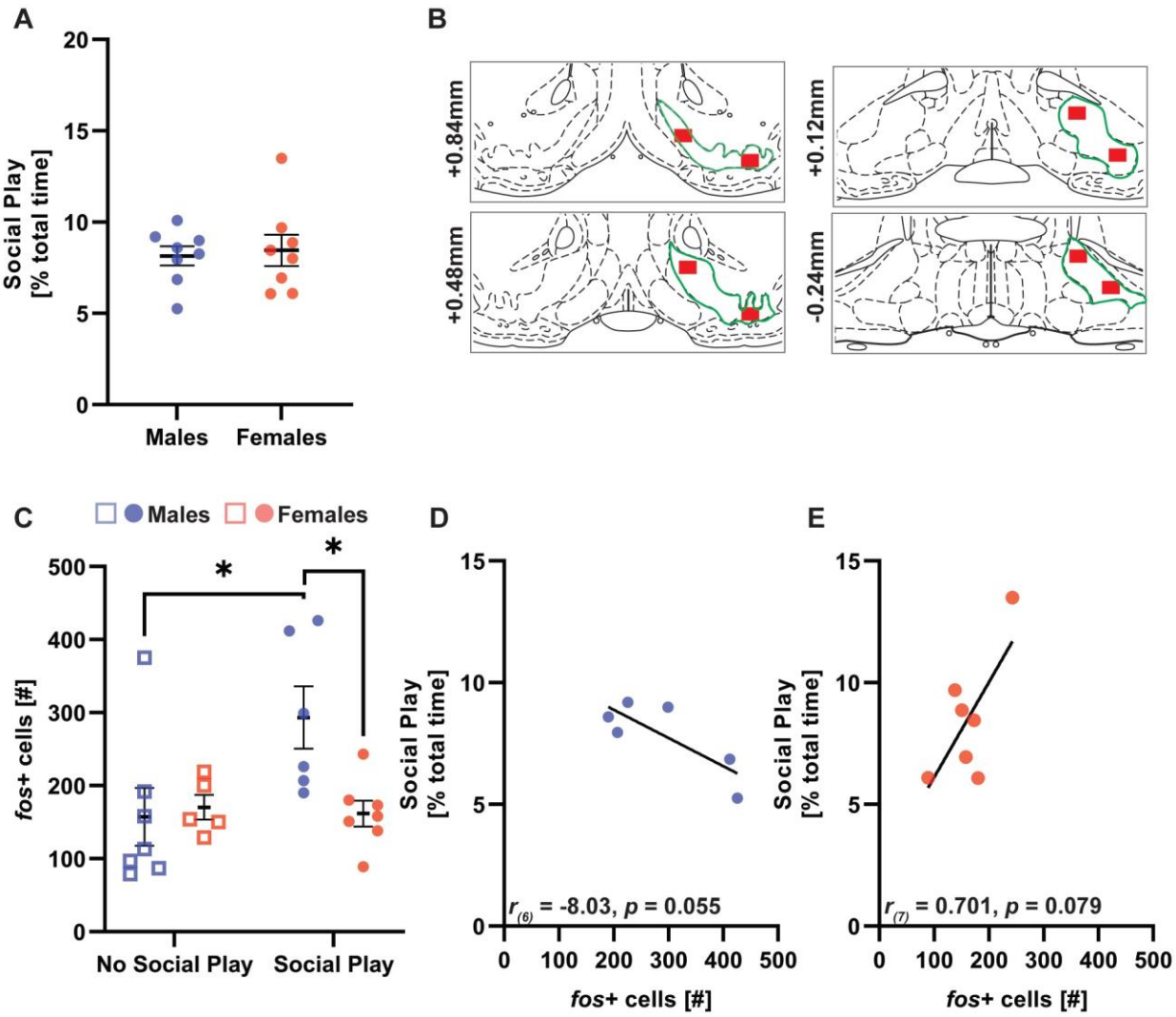

Figure 3. VP cells were activated in a sex-specific manner following social play exposure. (A) Males and females spent a similar amount of time engaging in social play during the 10-minute social play test. **(B)** Image sampling locations in the VP shown in modified rat brain atlas templates (Paxinos and Watson, 2007). VP is outlined in green; numbers to the left of the atlas templates represent distance from Bregma; red boxes represent the sampling location where images were taken. **(C)** Exposure to social play was associated with an increase in *fos*+ cells in males but not in females. Time

Figure 3 (cont'd)

spent engaging in social play did not significantly correlate with the number of *fos*+ cells in males **(D)** or females **(E)**. The number of *fos*+ cells was similar across sampling regions in all groups and thus data was collapsed across sampling regions to highlight sex and play condition effects. Black bars indicate mean ± SEM; *$p < 0.05$, Bonferroni *post hoc* comparisons.

Although I found no sex difference in social play behaviors following chemogenetic stimulation of the NAc to VP pathway, I observed sex differences in VP activation and activation of NAc inputs to the VP following social play exposure. These findings suggest that although baseline activation of the VP is required for the typical expression of social play in juvenile rats, the underlying mechanisms that regulate VP activation during social play behavior are sex-specific. Chapter 4 will discuss mechanisms that contribute to this sex difference in GABA transmission in the NAc as well as implications of sex differences in VP activation on outputs to other brain regions involved in the SDMN.

CHAPTER 2: Determine whether chemogenetic stimulation of NAc^{GABA} to VP pathway alters social play behavior in juvenile male and female rats

Abstract

Social play behavior is a highly rewarding and motivating behavior shown by juveniles of various mammalian species, including humans and rats. Engagement in social play is crucial for the development of social skills necessary for social competence throughout life. Children diagnosed with autism spectrum disorder show reduced engagement in social play with their peers and report social interactions as less rewarding and pleasant compared to non-social activities. Therefore, it is possible that alterations in the way the brain processes social rewards may underlie decreased social play engagement observed in autistic children. The mesolimbic reward system is the brain's main reward processing network and several brain regions within the mesolimbic reward system have been implicated in the regulation of social play behavior in juvenile male and female rats. Specifically, the nucleus accumbens (NAc) and the ventral pallidum (VP) are both individually involved in modulating social play behavior but it is unclear whether projections from the NAc to VP are important for social play. To determine whether the NAc to VP pathway is involved in regulating social play in juvenile male and female rats, I manipulated NAc inputs to the VP using an excitatory DREADD and locally infused CNO into the VP 20 min prior to social play testing. Chemogenetic stimulation of NAc terminals in the VP reduced social play behavior in both juvenile male and female rats. These findings are the first to indicate a pathway-specific modulation of social play behavior in juvenile rats. Next, I determined DREADDs-dependent changes in VP activation by infusing CNO in one hemisphere

and vehicle in the contralateral hemisphere of DREADDs-expressing juvenile male and female rats. I showed that chemogenetic stimulation of NAc terminals in the VP reduced neuronal activation of the VP, as measured by *fos* mRNA expression. Together, these findings support a model in which inhibitory inputs from the NAc to the VP suppresses VP activation, subsequently reducing the expression of social play behavior. Therefore, in order to allow for the expression of social play, NAc inputs to the VP must be inhibited and thereby disinhibiting the VP. These findings provide insights in the coordination of activity within a larger network involving the mesolimbic reward system to support the expression of social play in juvenile rats. Furthermore, there were no sex differences in the effects of chemogenetic stimulation of the NAc to VP pathway, suggesting that activation of the VP, through inactivation of NAc inputs, is crucial for the expression of social play in both males and females.

Introduction

Social play behavior is rewarding (Mason et al., 1963; Calcagnetti and Schechter, 1992; Ikemoto and Panksepp, 1992; Trezza et al., 2009; Achterberg et al., 2019) and motivating (Mason et al., 1962; Achterberg et al., 2016a, 2016b) behavior that is displayed by juveniles of various mammalian species, including humans (Parten, 1933; Scott and Panksepp, 2003) and rats (Thor and Holloway, 1984a; Marquardt et al., 2022; Pellis et al., 2022). Social play is the earliest form of peer social interaction (Eckerman et al., 1975; Panksepp, 1981) and provides the opportunity for individuals to acquire skills that promote social communication (Craig-Unkefer and Kaiser, 2002; Hewes, 2014; Chester et al., 2019), cognitive flexibility (Bateson, 2015; Bijlsma et al., 2022), and emotional regulation (Ginsburg, 2007; Potasz et al., 2013). Additionally, social play also has consequences on the development of the appropriate expression of adult social behaviors (Hol et al., 1999; van den Berg et al., 1999; Lesscher et al., 2015; Marquardt et al., 2022). Social play is well characterized in laboratory rats (Olioff and Stewart, 1978; Taylor, 1980; Thor and Holloway, 1984a; Northcutt and Nwankwo, 2018; VanRyzin et al., 2020; Kisko et al., 2021; Pellis et al., 2022; Achterberg et al., 2023), and studies using juvenile rats have investigated the importance of engaging in social play on social development. In detail, juvenile rats that underwent social play deprivation during the juvenile period (typically between 22 to 35 days after birth) showed changes in social behaviors, such as decreases in social investigation and approach behaviors compared to juvenile rats that were not deprived of social play (Einon et al., 1978; Hol et al., 1999; van den Berg et al., 1999; Marquardt et al., 2022). Social play-deprived rats also showed inappropriate social behaviors as adults, such as continued aggression

even after experiencing social defeat and incurring more injuries (van den Berg et al., 1999; Von Frijtag et al., 2002). In addition, as adults, social play-deprived rats displayed deficits in executive functioning such as slower habituation to a novel environment (Einon and Morgan, 1977) and slower acquisition of the rat gambling task (Baarendse et al., 2013).

Adult rats that were deprived of social play as juveniles also showed enhanced consummatory behaviors for drugs of abuse such as cocaine (Baarendse et al., 2014) and alcohol (Lesscher et al., 2021), suggesting that social play deprivation may have altered functional connectivity within the mesolimbic reward system. Indeed, an fMRI study comparing functional connectivity within the mesolimbic reward system in typically developing children and autistic children (who show decreased social play engagement compared to their typically developing peers; Supekar et al., 2018) reported that autistic children displayed decreased functional connectivity between the nucleus accumbens (NAc) and ventral tegmental area (VTA) compared to their typically developing peers when shown a picture of a face (social stimulus; Supekar et al., 2018).

Brain regions that are part of the mesolimbic reward system have been implicated in regulating social play behavior in juvenile male and female rats (Meaney et al., 1981; van Kerkhof et al., 2013, 2014; Manduca et al., 2016b; Argue et al., 2017; Zhao and Riters, 2023), specifically the NAc (van Kerkhof et al., 2013, 2014; Manduca et al., 2016b) and ventral pallidum (VP; Lee et al., 2021). In detail, pharmacological inactivation of the NAc core via infusions of muscimol ($GABA_A$ receptor agonist) and (RS)-baclofen ($GABA_B$ receptor agonist) increased social play behaviors, as reflected by an increase in the duration of social play, in juvenile male rats (van Kerkhof et al.,

2013). Furthermore, social play exposure was associated with an increase in c-Fos protein expression in the NAc core and shell in juvenile male rats (van Kerkhof et al., 2014). These findings suggest that modulation of NAc neuronal activity regulates the expression of social play behavior. More recently as part of my Master's thesis, I showed that pharmacological inactivation of the VP via bilateral infusions of muscimol decreased social play behaviors in juvenile male and female rats. In detail, muscimol-treated rats showed a shorter duration, fewer number of nape attacks, and fewer numbers of pins compared to vehicle-treated rats. In addition, I found that social play exposure was associated with a sex-specific change in VP neuronal activation, such that social play exposure was associated with an increase in the number of *fos*+ cells in males, but no change in the number of *fos*+ cells in females. Therefore, while VP activation is required for the typical expression of social play, the degree of VP neuronal activation required to support the expression of social play is different between males and females. Although these studies indicate that the NAc and VP are individually involved in regulating social play, it is unclear how interactions between these two brain regions contribute to modulation of social play behavior in juvenile male and female rats.

The NAc is a major source of GABAergic input to the VP (Walaas and Fonnum, 1979; Jones and Mogenson, 1980; Swerdlow et al., 1990; Root et al., 2015) and this pathway has been implicated in the regulation of rewarding behaviors such as food-seeking (Gallo et al., 2018; Chometton et al., 2020) in adult rodents. In detail, optogenetic stimulation of NAc terminals in the VP, which reduced *fos* mRNA expression in the VP, decreased sucrose intake in adult female rats (Chometton et al.,

2020). Moreover, chemogenetic inhibition of NAc terminals in the VP, which was associated with a decrease in inhibitory postsynaptic currents in the VP, increased lever press responses and breakpoints in response to a food reward in adult mice (Gallo et al., 2018). These studies provide direct evidence that the NAc to VP pathway is involved in regulating rewarding behaviors in adult rats. In addition, these findings show that inhibition of NAc inputs to the VP disinhibits the VP, and this disinhibition is associated with the enhancement of the expression of rewarding behaviors.

Given that activation of the VP is required for the typical expression of social play behavior, inhibition of NAc GABAergic inputs to the VP may allow for juvenile male and female rats to display this typical expression. Therefore, I hypothesized that inhibition of the NAc to VP pathway is required for the expression of social play for both males and females. In order to test this hypothesis, I utilized Gad1-iCre juvenile male and female rats and chemogenetic techniques in order to stimulate the NAc to VP pathway. I predicted that stimulation of this pathway would decrease VP neuronal activation, through increased GABAergic inputs from the NAc, and would decrease expression of social play behaviors in both males and females.

Methods

Subjects

Three-week-old male and female Long Evans Gad1-iCre rats (Sharpe et al., 2017; Gibson et al., 2018; Farrell et al., 2021, 2022) and their Cre-negative wildtype littermates were housed in single-sex groups of four in standard rat cages (48 X 27 X 20 cm or 41.2 X 30 X 23.4 X cm) and maintained under standard laboratory conditions (12 hr light/dark cycle, lights off at 14:00, food and water available *ad libitum*) unless

otherwise mentioned. Stimulus animals for the experimental animals were age- and sex-matched rats that were either bred in-house from a different litter than the experimental animals or acquired through a breeding facility (Envigo). The 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

Infusion of the cre-dependent excitatory DREADDs in the NAc

Juvenile male and female rats (22 days old) were anesthetized with isoflurane (2-4% as needed; Henry Schein, Melville, NY) and mounted on a stereotaxic frame. A 1 µL, 7000 series Hamilton syringe (Hamilton, Reno, NV) was attached to a motorized stereotaxic injector system (Stoelting, Wood Dale, IL) and bilateral injections of an excitatory DREADD-containing vector (0.2 µL/hemisphere; AAV-DJ-EF1a-DIO-hM3D(Gq)-mCherry; Gene Vector and Virus Core, Stanford University, CA) was directed to the NAc. Coordinates for the NAc from bregma were: 1.2 mm rostral to bregma, 2.7 mm lateral to the midline, and 6.7 mm ventral to the surface of the skull (Paxinos and Watson, 2007). The syringe was injected at a 10° angle from the midsagittal plane to avoid damage to the sagittal sinus. Subjects were given an s.c. injection of meloxicam (2mg/kg; Covetrus, 049759) immediately after surgery and once a day for an additional two days. Subjects were pair-housed with two novel age- and sex-matched stimulus animals after surgery and remained pair-housed until their second stereotaxic surgery. All subjects' body weights were monitored for three days post surgery to ensure normal recovery.

Bilateral cannulation targeting the VP

Seven days later, all subjects (29 days old) underwent a second stereotaxic surgery in order to bilaterally implant guide cannulae aimed at the VP, allowing for local administration of the synthetic ligand clozapine-n-oxide (CNO). Guide cannulae (22 gauge, 9mm; Plastics One, Roanoke, VA) were bilaterally implanted 2 mm dorsal to the VP. Coordinates for the VP from bregma were: 0.23 rostral to bregma, 2.4 mm lateral to the midline, and 5.8 mm ventral to the surface of the skull (Paxinos and Watson, 2007). Cannulae were also implanted under a 10° angle in order 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). Post-operative monitoring was the same as the first stereotaxic surgery, with the exception that all subjects were also given an s.c. injection of Enrofloxacin (22.7mg/kg; Covetrus, 074743) immediately after surgery. Following surgery, all subjects were returned to their group-housed homecage and remained there until the start of behavioral testing.

Social play testing

Previous social play testing in our lab revealed that Long Evans juvenile rats displayed optimal levels of social play behavior when exposed to a familiar stimulus rat as opposed to a novel stimulus rat. Therefore, the two age- and sex-matched rats that were housed with each experimental subject after the first stereotaxic surgery were used as stimulus rats for the social play test. 24 hours prior to social play testing, all experimental subjects (30 days old) were socially isolated by removing the stimulus rats from the homecage. The two stimulus rats were placed into a novel homecage and remained pair-housed for the duration of the behavioral experiment.

Habituation day

In order to familiarize experimental subjects to the infusion procedures and social play testing, all experimental subjects (31 days old) received one habituation session. During the first hour of the dark phase, 20 min prior to their social play test, the experimental subjects were removed from their homecage and their dummy cannulas were tightened and re-tightened while the microinfusion pump (GenieTouch, Kent Scientific, Torrington, CT) was turned on in order to acclimate experimental subjects to the removal of the dummy cannulas and the sound of the infusion pump. No drugs were infused through the cannulas for the habituation sessions. After the mock infusions were completed, animals were returned to their homecage until behavioral testing.

20 min following the mock infusions, the experimental subject's homecage was removed from the cage rack, the cage lid and food corral was removed and replaced with a Plexiglass lid. A tripod and video camera were set up above each cage to record the tests. One of the familiar stimulus rats was then placed into the cage. The social play test lasted 10 minutes, during which time, the experimental subject was allowed to freely interact with the stimulus rat. Stimulus rats were stripped with a permanent marker 60 min prior to the start of social play testing in order to distinguish between the experimental subject and stimulus rat during later video analysis. Food and water were not available during the 10 min test but were immediately returned upon completion of each session. After the end of the session, the stimulus rat was removed and returned to its homecage. The experimental subject remained single-housed until the end of the behavioral experiment.

Chemogenetic stimulation of NAc^{GABA} terminals in the VP (Testing days 1-2)

In order to determine the effects of chemogenetic stimulation of NAc^{GABA} terminals in the VP on the expression of social play in juvenile male and female rats, the vehicle and clozapine-n-oxide (CNO) were bilaterally infused into the VP in a randomized and counterbalanced order using a randomizer program (http://www.jerrydallal.com/random/permute.htm). 20 min prior to the social play test, either the vehicle (0.9% sterile saline; Medline, 533-JB1301P) or CNO (1mM; CNO was dissolved in 0.2M sterile PBS with 10% (2-Hydroxypropyl)-β-cyclodextrin)) were infused into the VP of all experimental subjects (32-33 days old). The infusions (0.3 µL per hemisphere) were given over a course of 45 sec via injector cannulas (28 gauge; Plastic One, Roanoke, VA) that extended 2 mm beyond the guide cannula and were connected via polyethylene tubing to a 2 µL syringe (Hamilton Company #88400) mounted onto a microinfusion pump (GenieTouch, Kent Scientific, Torrington, CT). The injector cannulas were kept in place for an additional 30 sec following infusion to allow for tissue uptake before being replaced by the dummy cannulas.

20 min after the bilateral infusions, experimental subjects underwent social play testing as described on habituation day. In addition, the stimulus rats for each experimental subject were rotated so that the experimental subjects did not interact with the same stimulus rat on consecutive days of testing. All social play tests were videotaped and behavior was measured by the experimenter blind to sex, genotype, and drug treatment conditions of the experimental subjects using Solomon Coder (https://solomon.andraspeter.com/). The following behaviors were scored for the experimental subjects according to Veenema and Neumann (2009): duration of social

play (the total amount of time spent in playful social interactions including nape attacks, pinning, and supine poses), duration of social investigation (the experimental subject is sniffing the anogenital and head/neck regions of the stimulus rat), duration of allogrooming (the experimental subject is grooming the stimulus rat), duration of non-social cage exploration (the experimental subject is walking, rearing, sitting, or engaging in other neutral behaviors), number of nape attacks (the experimental subject displays nose attacks or nose contacts toward the nape of the neck of the stimulus rat), number of pins (the experimental subject holds the stimulus rat on its back in a supine position), and number of supine poses (the experimental subject is pinned by the stimulus rat).

DREADD-dependent stimulation of NAc^{GABA} terminals in the VP by CNO (Testing day 3)

In order to determine the effects of stimulation of NAc^{GABA} terminals in the VP on neuronal activation of the VP, all experimental subjects were infused with the vehicle in one hemisphere and CNO on the contralateral hemisphere using the same infusion procedures from testing days 1 and 2. 20 min following the infusions, experimental subjects underwent social play testing as described above. Although we are utilizing an excitatory DREADD, we expect to see a decrease in VP neuronal activation by increasing GABAergic input to the VP from the NAc. Therefore, in order to detect a decrease in activation, we used social play exposure as a stimulant. Thirty minutes after the start of the social play test, experimental subjects were euthanized via transcardial perfusions. This time course was chosen because stimulus-induced *c-fos* mRNA expression is at its peak 30 min after stimulation (Morgan and Curran, 1991). Experimental subjects (34 days old) were deeply anesthetized with isoflurane before

being transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH: 9.5). Brains were then extracted and post-fixed for 24 hrs in 12% sucrose in 4% paraformaldehyde solution. Afterwards, brains were rapidly frozen in methylbutane and stored at 80°C until further histological processing.

Histological procedures

Brains were mounted in the cryostat (Leica CM3050 S) and sliced into 30 µm coronal sections containing the NAc (corresponding to distances +3.24 mm to +1.08 mm from bregma; Paxinos and Watson, 2007) and VP (corresponding to distances +0.96 mm to -0.24 mm from bregma; Paxinos and Watson, 2007) into 4 series. All 4 series were put into a cryoprotectant solution (0.05 mol L^{-1} sodium phosphate buffer, 30% ethylene glycol, 20% glycerol) and stored at -20°C until further histological processing.

Localization of DREADD expression in the NAc and cannula placement in the VP

For Gad1-iCre experimental subjects, the first series of the NAc was processed using fluorescence mCherry immunohistochemistry to determine DREADDs expression and co-stained with a fluorescent Nissl in order to identify cytoarchitectonic borders of brain regions. Briefly, tissue sections were thoroughly rinsed in tris buffered saline (TBS; pH: 7.4) and incubated for 24 hrs at 4°C in a blocking solution (TBS with 0.3% Triton X-100 and 2% normal donkey serum; 017-000-121; Jackson ImmunoResearch, West Grove, PA) with the primary antibody anti-mCherry raised in chicken (1:2000 concentration; AB205402, ABCam). Afterwards, tissue sections were rinsed in TBS and incubated for 1 hr in the blocking solution containing the secondary antibody Alexa Fluor 594 anti-chicken raised in donkey (1:500 concentration; 703-585-155, Jackson

ImmunoResearch). After the secondary antibody incubation, tissue sections were rinsed in TBS and stained with a fluorescent Nissl (1:500, 1 hr; NeuroTrace^{TM}; N21479, 435/455nm, Thermo Fisher Scientific). Sections were then mounted onto gelatin-coated slides, air-dried, and coverslipped with Vectashield hardset antifade mounting medium with a DAPI counterstain (H-1500-10, Vector Laboratories) and stored at 4°C. DREADDs expression was visualized with a 4x objective on a Keyence BZ-X700E/BZ-X710 fluorescent microscope and associated BZ-H3AE software (Keyence Corporation of America).

For all WT experimental subjects and Gad1-iCre experimental subjects with DREADDs expression in the NAc, the first series of the VP was stained using a fluorescent Nissl (as described above) in order to map cannula placements using the Rat Brain Atlas (Paxinos and Watson, 2007). Only Gad1-iCre experimental subjects with bilateral DREADDs expression in the NAc (Fig 4B) and bilateral cannula tracks terminating in the VP (Fig 5E) and WT experimental subjects with bilateral cannula tracks terminating in the VP (Fig 5C) will be included in behavioral scoring and statistical analysis.

Change in VP neuronal activation following chemogenetic stimulation of NAc^{GABA} terminal in the VP by CNO

Gad1-iCre experimental subjects with bilateral DREADDs expression in the NAc and bilateral cannula tracks terminating in the VP and WT experimental subjects with bilateral cannula tracks terminating in the VP were processed for *in situ* hybridization in order to visualize *fos* mRNA in the VP. In detail, the tissue sections from the second series of the VP were blocked using a razor to only contain the left and right

hemispheres of the VP in smaller sections. These blocked tissue sections were then mounted onto separate slides (Superfrost Plus; Fisher Scientific) and processed via an *in situ* hybridization using an RNAScope™ Multiplex Fluorescent Reagent Kit v2 (323100, Advanced Bell Diagnostics) to quantify *fos* mRNA expressing cells according to user manual from the supplier (Document Number 323100-USM, Advanced Cell Diagnostics). Briefly, tissue sections were washed in phosphate buffer solution (pH: 7.6), dried at 60°C (30 min), then post-fixed in 4% paraformaldehyde followed by dehydration in an ethanol series. Following hydrogen peroxide incubation and target retrieval in a steamer at 99°C, tissue was then treated with protease III (30 min; 322340, Advanced Cell Diagnostics) at room temperature. The *fos*-C1 (403591, Advanced Cell Diagnostics) probe was then hybridized in a HybEZ™ oven (2 hrs; Advanced Cell Diagnostics) at 40°C. After probe hybridization, tissue sections were incubated with amplifier probes (AMP1, 40°C, 30 min; AMP2, 40°C, 30 min; AMP3, 40°C, 15 min). *fos* mRNA was tagged to the fluorophore fluorescein (1:1500; NEL741E001KT, Akoya Biosciences). Slides were then rinsed in TBS and stained with a fluorescent Nissl (1:500, 1 hr; NeuroTrace™; N21479, 435/455nm, Thermo Fisher Scientific). Slides were then coverslipped with Vectashield hardset antifade mounting medium with a DAPI counterstain (H-1500-10, Vector Laboratories) and stored at 4°C.

All images were acquired with a 40X objective on a Keyence BZ-X700E/BZ-X710 fluorescent microscope and associated BZ-H3AE software (Keyence Corporation of America). Cells were counted as *fos*+ if they had five or more puncta (Farrell et al., 2021). In each image, the total number of *fos*+ cells were counted by the experimenter blind to sex, genotype, and drug treatment of each hemisphere.

Statistical Analysis

A mixed-effects ANOVA (REML) was used to determine the effects of sex (between-subjects factor) and drug treatment (vehicle vs CNO; within-subjects factor) on the expression of social play behavior. A separate ANOVA was used for WT and Gad1-iCre experimental subjects. When significant interactions were found in the mixed-effects models, Bonferroni *post hoc* multiple comparison tests were conducted to clarify the effects. A paired t-test was used to compare VP neuronal activation following infusions of vehicle and CNO. A separate paired t-test was used for WT and Gad1-iCre experimental subjects.

All data were analyzed using GraphPad Prism 10 or IBN SPSS 28, and statistical significance was set at $p < 0.05$. Partial eta squared (η_p^2) for all mixed-effects models and Cohen's d (*d*) effect sizes for all t-tests were manually computed when significant main effects or interactions were found.

Results

Gad1-iCre male and female subjects showed hM3Dq-mCherry expression localized in the NAc core and shell (Fig 4B). As expected, WT juvenile male and female rats did not show mCherry expression in the NAc (not shown). Additionally, the presence of mCherry-positive fibers was detected in Gad1-iCre subjects (Fig 7B), while no mCherry-fibers were found in WT subjects (not shown).

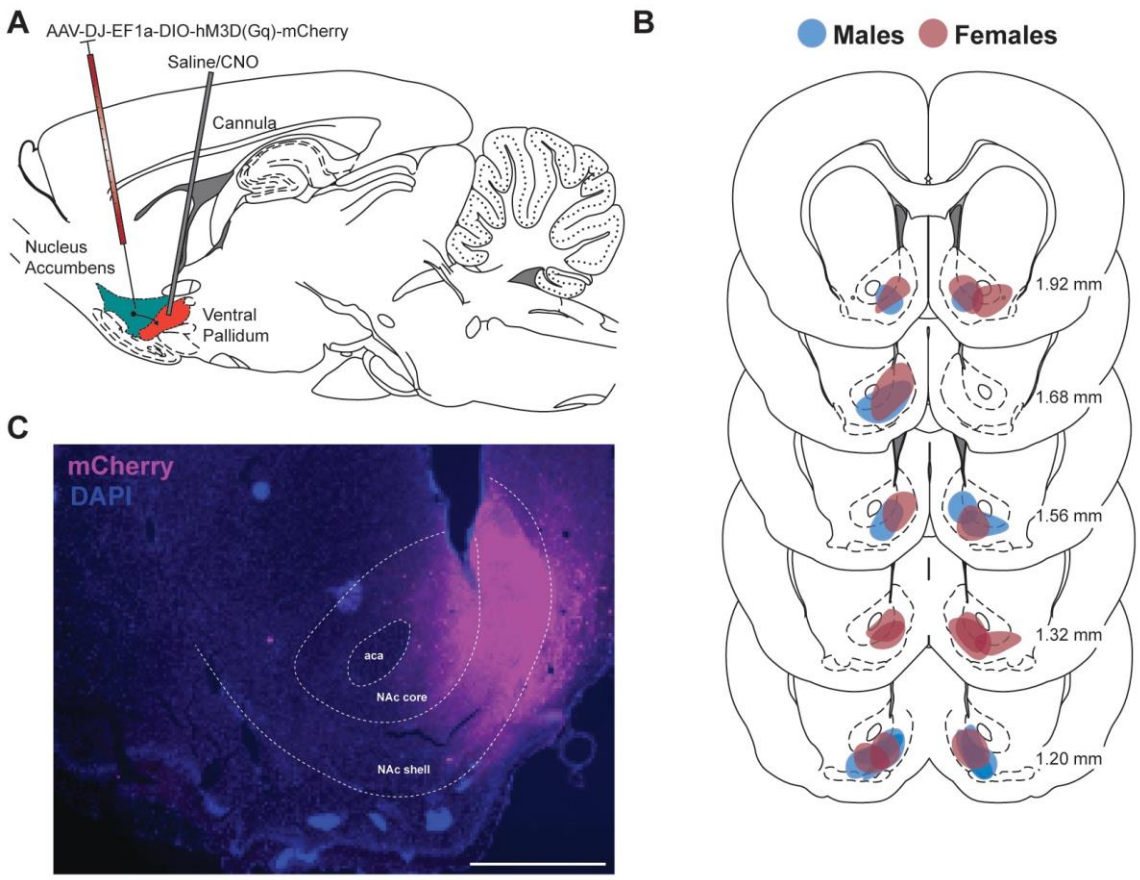

Figure 4. DREADDs expression in Gad1-iCre juvenile male and female rats. (A) Schematic illustrating bilateral infusions of the cre-dependent excitatory DREADDs (AAV-DJ-EF1a-DIO-hM3D(Gq)-mCherry) targeting the NAc and bilateral cannula implantation targeting the VP. (B) Coronal sections depicting the center of DREADDs infusions in Gad1-iCre males and females. Numbers at the right refer to distance from Bregma (in mm; Paxinos and Watson, 2007). (C) DREADDs expression (purple) is localized within the borders of the NAc (NAc subregions outlined in white). Scale bar = 500 µm.

Chemogenetic stimulation of the NAc to VP pathway reduces social play behaviors in juvenile male and female rats

There was a main effect of drug condition on social play behaviors in Gad1-iCre subjects (Table 1). CNO treatment decreased social play behaviors in both males and females compared to saline treatment. Gad1-iCre subjects treated with CNO showed shorter duration of social play (Fig 5A), fewer number of nape attacks (Fig 5B), fewer number of pins (Fig 5C), and fewer number of supine poses (Fig 5D) compared to saline treatment. However, CNO treatment increased the duration of non-social cage exploration (Fig 5H) compared to saline treatment, suggesting that the decrease in social play behaviors was not due to a decrease in locomotion, but potentially a decrease in social interest or motivation. The effects of CNO were specific to social play, as CNO treatment did not alter the duration of social investigation (Fig 5E) or allogrooming (Fig 5F).

Table 1. Two-way ANOVA statistics for Gad1-iCre subjects. Significant effects are indicated in **bold**.

	Sex	Drug Condition	Interactions
Social play duration	$F_{(1,11)} = 0.68$, $p = 0.42$	**$F_{(1,11)} = 58.67$, $p < 0.0001$, $\eta_p^2 = 0.84$**	$F_{(1,11)} = 1.01$, $p = 0.33$
Nape attacks [#]	$F_{(1,11)} = 1.23$, $p = 0.29$	**$F_{(1,11)} = 49.05$, $p < 0.0001$, $\eta_p^2 = 0.82$**	$F_{(1,11)} = 1.21$, $p = 0.29$
Pins [#]	$F_{(1,11)} = 4.02$, $p = 0.07$	**$F_{(1,11)} = 13.31$, $p = 0.003$, $\eta_p^2 = 0.54$**	$F_{(1,11)} = 2.98$, $p = 0.11$
Supine poses [#]	$F_{(1,11)} = 1.70$, $p = 0.21$	**$F_{(1,11)} = 7.11$, $p = 0.02$, $\eta_p^2 = 0.39$**	$F_{(1,11)} = 1.70$, $p = 0.21$
Social investigation duration	$F_{(1,11)} = 1.83$, $p = 0.20$	$F_{(1,11)} = 0.54$, $p = 0.47$	$F_{(1,11)} = 0.32$, $p = 0.58$
Allogrooming duration	$F_{(1,11)} = 0.55$, $p = 0.47$	$F_{(1,11)} = 1.30$, $p = 0.27$	$F_{(1,11)} = 0.56$, $p = 0.46$

Table 1 (cont'd)

Non-social cage exploration duration	$F_{(1,11)} = 1.19$, $p = 0.29$	**$F_{(1,11)} = 13.26$, $p = 0.003$, $\eta_p^2 = 0.55$**	$F_{(1,11)} = 2.84$, $p = 0.12$

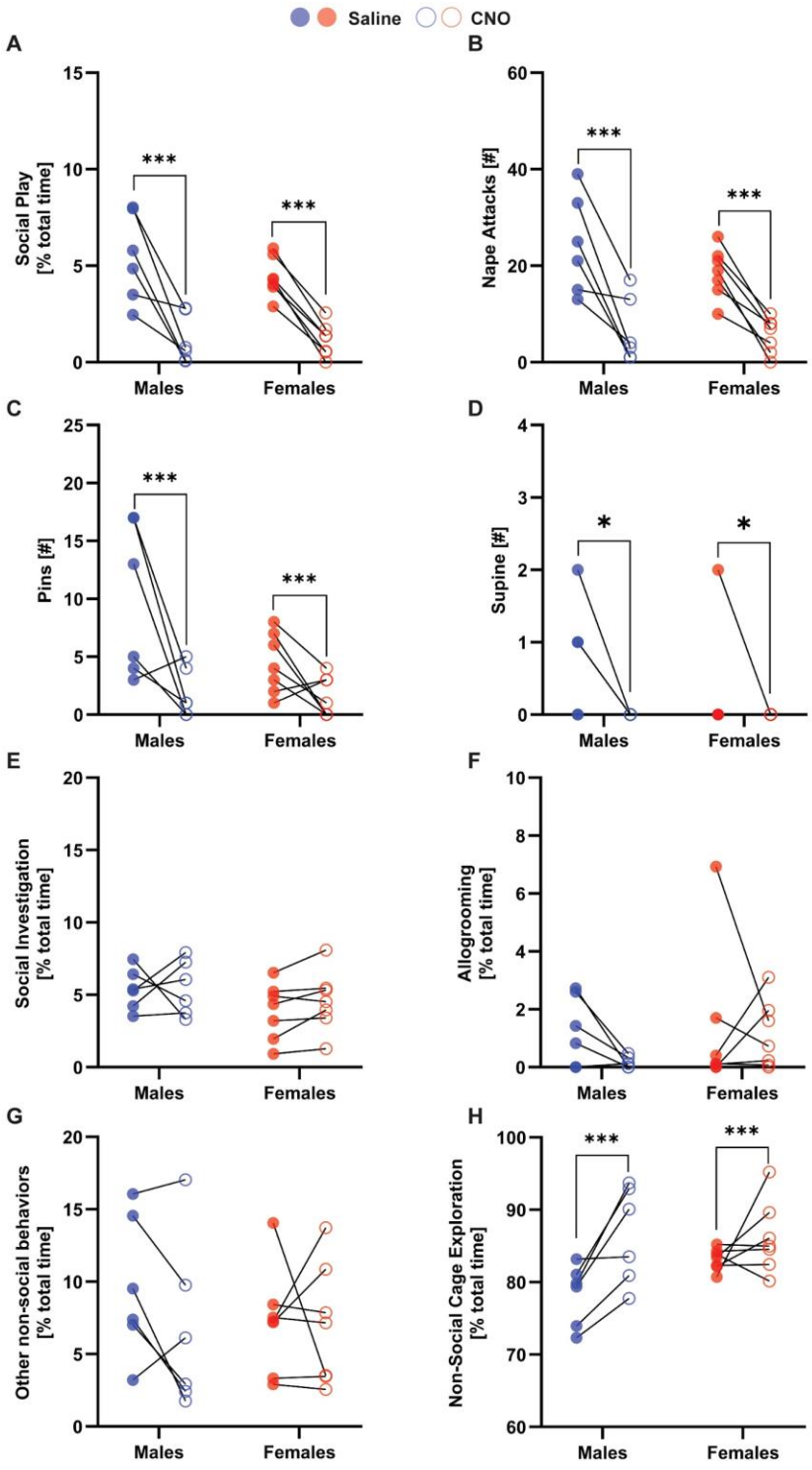

Figure 5. CNO decreased social play behaviors in Gad1-iCre males and females.

Gad1-iCre subjects showed a lower duration of social play (A), number of nape attacks

Figure 5 (cont'd)

(B), number of pins (C), and number of supine poses (D) when treated with CNO compared to when they were treated with saline. However, Gad1-iCre subjects showed a similar duration of social investigation (E), allogrooming (F), and non-social behaviors (G) following saline and CNO treatments. (H) Gad1-iCre subjects showed a higher duration of non-social cage exploration when treated with CNO compared to when they were treated with saline. *$p < 0.05$, ***$p < 0.0001$, main effect repeated measures ANOVA.

Several studies report that CNO in the absence of DREADDs expression has behavioral effects on rats (MacLaren et al., 2016; Ilg et al., 2018; Rodd et al., 2022). In order to confirm that CNO infused into the VP does not alter social play behaviors in WT subjects, CNO and 0.9% sterile saline were infused in a randomized, counterbalanced order. WT subjects displayed similar social play behaviors following saline and CNO infusions (Table 2). In detail, WT male and female subjects showed a similar duration of social play (Fig 6A), number of nape attacks (Fig 6B), number of pins (Fig 6C), and number of supine poses (Fig 6D) when treated with saline and CNO. Additionally, there were no effects of drug treatment on other social behaviors measured during the social play test such as social investigation (Fig 6E) or allogrooming (Fig 6F).

There was no main effect of sex on any social play behaviors (Table 2). WT males and females showed a similar duration of social play (Fig 6A), number of nape attacks (Fig 6B), number of pins (Fig 6C), and number of supine poses (Fig 6D). However, WT females showed a longer duration of non-social cage exploration compared to WT males, regardless of drug treatment (Fig 6H).

Table 2. Two-way ANOVA statistics for WT subjects. Significant effects are indicated in **bold**.

	Sex	Drug Condition	Interactions
Social play duration	$F_{(1,10)} = 3.55$, $p = 0.08$	$F_{(1,10)} = 0.09$, $p = 0.76$	$F_{(1,10)} = 0.005$, $p = 0.94$
Nape attacks [#]	$F_{(1,10)} = 2.41$, $p = 0.15$	$F_{(1,10)} = 0.92$, $p = 0.35$	$F_{(1,10)} = 0.56$, $p = 0.47$
Pins [#]	$F_{(1,10)} = 4.79$, $p = 0.06$	$F_{(1,10)} = 1.58$, $p = 0.23$	$F_{(1,10)} = 0.52$, $p = 0.48$
Supine poses [#]	$F_{(1,10)} = 0.29$, $p = 0.59$	$F_{(1,10)} = 4.00$, $p = 0.07$	$F_{(1,10)} = 1.00$, $p = 0.34$
Social investigation duration	$F_{(1,10)} = 2.01$, $p = 0.18$	$F_{(1,10)} = 0.02$, $p = 0.87$	$F_{(1,10)} = 0.45$, $p = 0.51$
Allogrooming duration	$F_{(1,10)} = 1.83$, $p = 0.21$	$F_{(1,10)} = 1.02$, $p = 0.33$	$F_{(1,10)} = 0.01$, $p = 0.89$
Non-social cage exploration duration	**$F_{(1,10)} = 7.18$, $p = 0.02$, $\eta_p^2 = 0.39$**	$F_{(1,10)} = 1.17$, $p = 0.30$	$F_{(1,10)} = 0.16$, $p = 0.69$

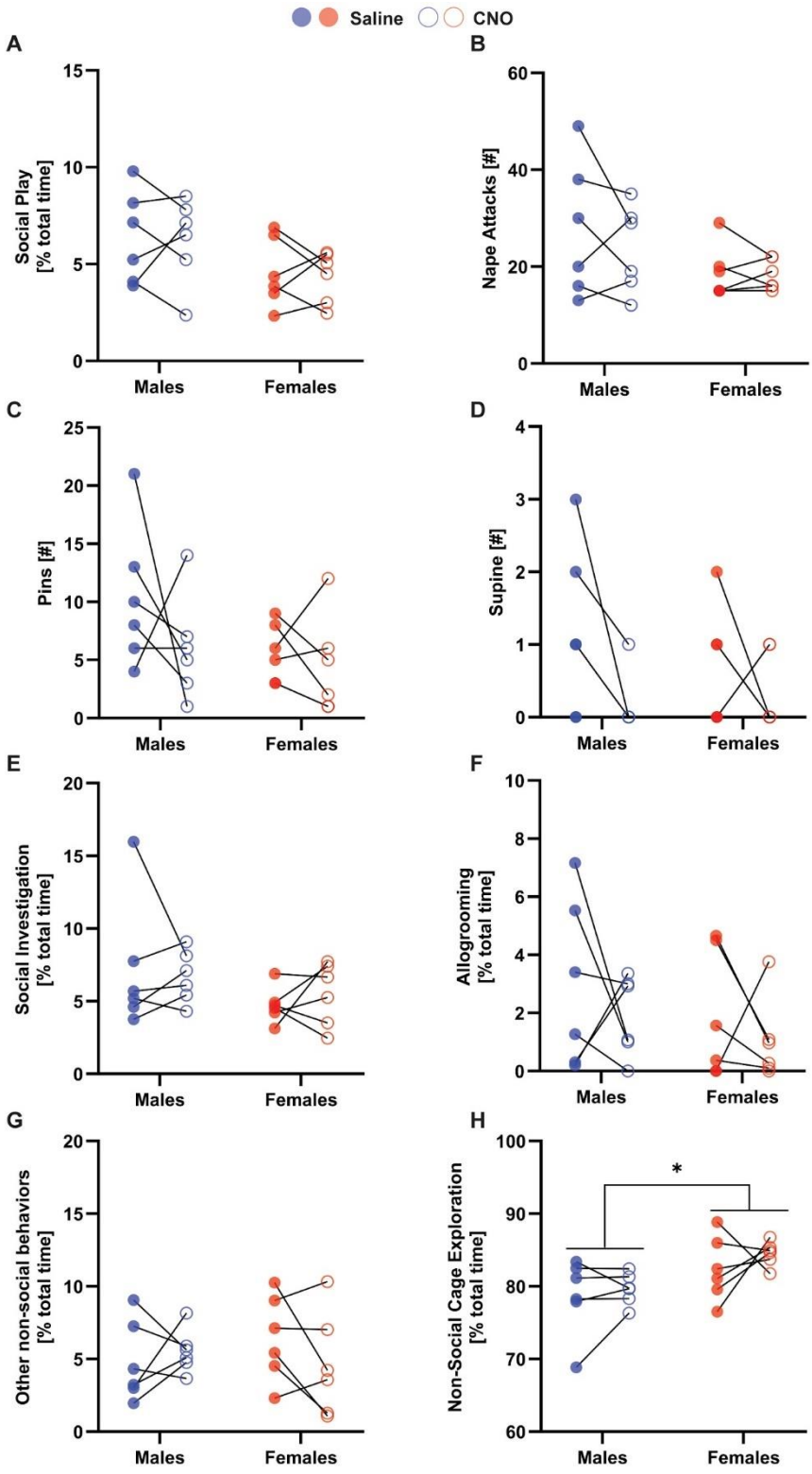

Figure 6. CNO by itself did not alter any behaviors during the social play test in WT males and females. Males and females showed a similar duration of social play

Figure 6 (cont'd)

(A), social investigation (E), allogrooming (F), and non-social behaviors (G) when treated with saline and CNO. In addition, WT subjects showed a similar number of nape attacks (B), pins (C), and supine poses (D) following saline and CNO treatments. However, females showed a higher duration of non-social cage exploration compared to males, regardless of drug treatment (H). *$p < 0.05$, main effect repeated measures ANOVA.

Chemogenetic stimulation of NAc^{GABA} terminals in the VP reduces VP neuronal activation

To verify that: (i) CNO itself did not alter neuronal activation of the VP and (ii) CNO via activation of hM3Dq receptors located on NAc terminals decreased VP neuronal activation, CNO was infused into one hemisphere and 0.9% sterile saline into the contralateral hemisphere of the VP in WT (no DREADDs-positive fibers in the VP) and Gad1-iCre (DREADDs-positive fibers) juvenile rats. The number of *fos*+ cells was similar between the saline-infused and CNO-infused hemispheres in WT subjects (Table 3; Fig 7D). However, in Gad1-iCre subjects, the CNO-infused hemisphere showed a fewer number of *fos*+ cells compared to the saline-infused hemisphere (Table 3; Fig 7F). These results confirm that CNO, via activation of hM3Dq receptors located on NAc^{GABA} terminals in the VP, suppresses VP activity, presumably through the release of GABA at the synapse by recruiting endogenous G_q signaling.

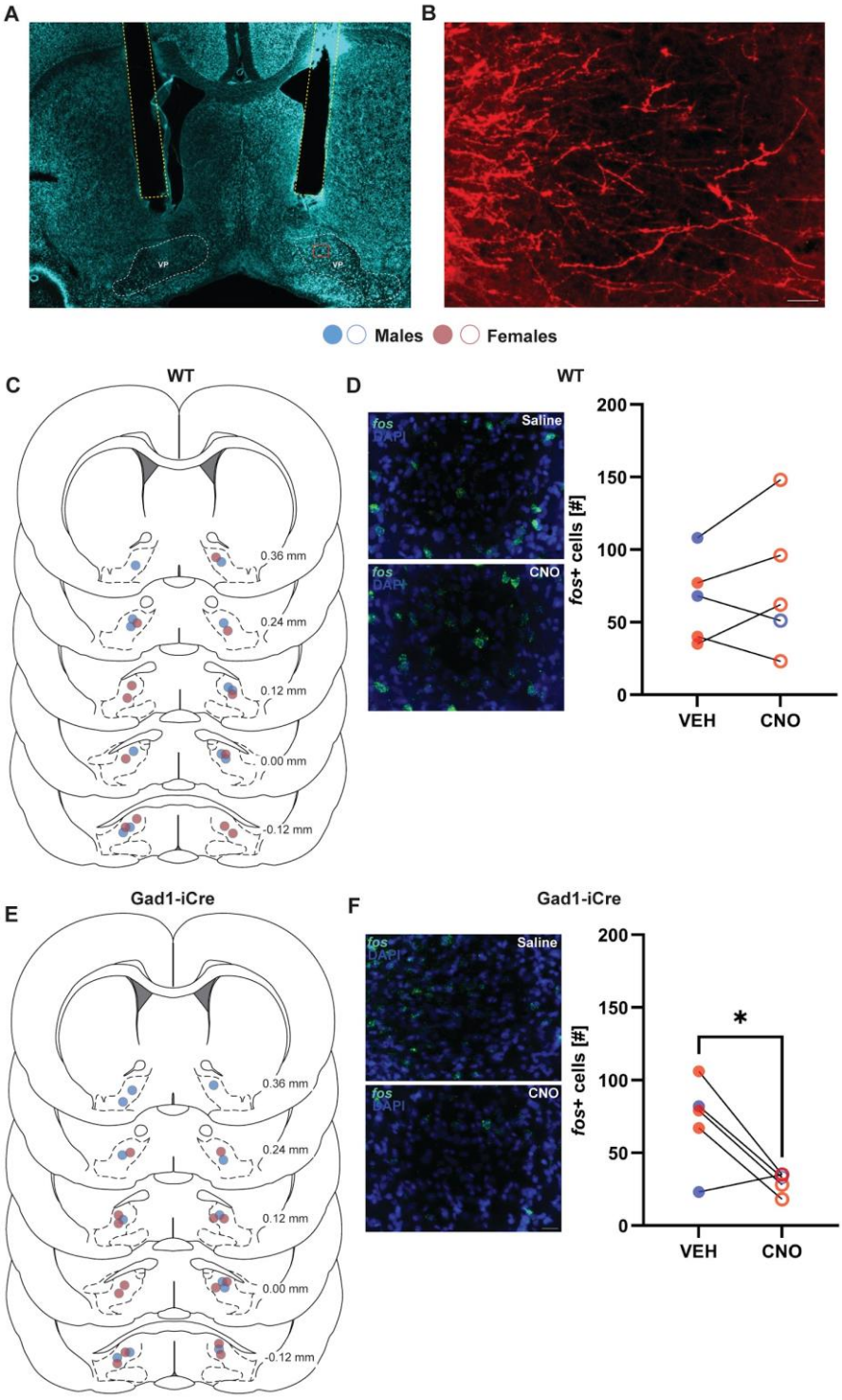

Figure 7. CNO reduced VP neuronal activation in Gad1-iCre rats. (A) Fluorescent Nissl photomicrograph of a coronal brain section depicting bilateral cannula placement

Figure 7 (cont'd)

(outlined in yellow) targeting the VP. Cannulas are implanted 2 mm above the VP (outlined in white). **(B)** Representative photomicrograph of DREADDs-positive fibers (shown in red) in the VP originating from NAc^{GABA} cells. Coronal sections depicting bilateral cannula placements for WT **(C)** and Gad1-iCre **(E)** males and females. Numbers at the right refer to distance from Bregma (in mm; Paxinos and Watson, 2007). Representative photomicrographs showing *fos* mRNA staining in green in the VP following infusion of either saline (D, top left; F, top left) or CNO (D, bottom left; F, bottom left). **(D)** WT subjects displayed a similar number of *fos*+ cells in the VP in the hemispheres infused with saline and CNO. **(F)** Gad1-iCre subjects displayed a lower number of *fos*+ cells in the hemisphere infused with CNO compared to the hemisphere infused with saline. Scale bar = 15 µm. *$p < 0.05$, paired samples t-test.

Table 3. T-test statistics for *fos* mRNA expression in saline- and CNO-infused hemispheres in WT and Gad1-iCre subjects. Significant effects are indicated in **bold**.

	Sex
WT subjects	$t_{(4)} = 0.89$, $p = 0.42$
Gad1-iCre subjects	$\mathbf{t_{(4)} = 2.96}$, $\mathbf{p = 0.04}$, $\mathbf{d = 1.34}$

Discussion

Here, I showed for the first time, evidence of a pathway-specific neural mechanism that regulates social play behavior in juvenile male and female rats. Specifically, I showed that chemogenetic stimulation of the NAc to VP pathway reduced the duration of social play, number of nape attacks, number of pins, and number of

supine poses in both males and females, demonstrating that inhibition of the NAc to VP pathway is required for the typical expression levels of social play in both sexes.

Inputs from the NAc to the VP are inhibitory (Walaas and Fonnum, 1979; Mogenson et al., 1983; Swerdlow et al., 1990; Root et al., 2015) and suppress activation of the VP (Chrobak and Napier, 1993; Clark, 2020). Using G_q-coupled DREADDs receptors, I demonstrated that chemogenetic stimulation of NAc^{GABA} terminals in the VP is able to suppress VP activation (Fig 7F). Chemogenetic manipulation of axonal terminals has been performed in previous studies (Mahler et al., 2014; Stachniak et al., 2014; Venniro et al., 2017; Gallo et al., 2018; Pardo-Garcia et al., 2019) and provides evidence that terminal manipulation using chemogenetics is capable of altering the expression of rewarding behaviors, such as sucrose consumption (Gallo et al., 2018; Chometton et al., 2020) and cocaine-seeking behaviors (Stefanik et al., 2013; Mahler et al., 2014; Pardo-Garcia et al., 2019; Inbar et al., 2022). Furthermore, I demonstrated that 10 days after DREADDs infusion in juvenile rats is sufficient for axonal transport and functional expression of DREADDs receptors at the terminal to allow for terminal stimulation.

Chemogenetic stimulation of the NAc to VP pathway decreased the duration of social play, number of nape attacks, number of pins, and number of supine poses in both juvenile male and female rats. This finding confirms the hypothesis that VP activation, via inhibition of NAc inputs, is required to allow for the typical expression of social play behavior. This further suggests that activation of VP outputs is critical for social play expression in juvenile male and female rats. I propose that disinhibition of the VP, through inhibition of NAc inputs, is involved in the regulation of rewarding

behaviors, including social and non-social behaviors. In support of this, optogenetic stimulation of NAc terminals in the VP reduced sucrose intake in adult female rats (Chometton et al., 2020), while chemogenetic inhibition of NAc terminals in the VP increased lever press responses and breakpoint for a food reward (Gallo et al., 2018). These studies, along with the current study, indicate that inhibition of the NAc to VP pathway is critical to support rewarding behaviors in response to any type of rewarding stimulus, either social or non-social.

The VP is a core brain region in the mesolimbic reward system (Berridge and Robinson, 2003; Smith et al., 2009; Berridge and Kringelbach, 2015) and projects to other regions within the mesolimbic reward system, such as the ventral tegmental area (VTA; (Groenewegen et al., 1993; Kalivas et al., 1993; Hjelmstad et al., 2013; Mahler et al., 2014; Tooley et al., 2018). VP inputs to the VTA are predominantly GABAergic (Kalivas et al., 1993; Hjelmstad et al., 2013) and synapse onto dopaminergic and non-dopaminergic cells in the VTA (Floresco et al., 2003). Inactivation of the VP via infusions of muscimol increased dopamine neuron population activity in the VTA (Floresco et al., 2003), suggesting that inputs from the VP can influence VTA dopamine neuronal firing and release within the brain. VP to VTA projections have also been shown to regulate drug seeking behaviors (Mahler et al., 2014; Faget et al., 2018; Tooley et al., 2018), such that inhibition of the VP to VTA pathway blocked cocaine-seeking during cued reinstatement (Mahler et al., 2014). The VTA itself is involved in regulating social play (Northcutt and Nguyen, 2014; van Kerkhof et al., 2014; Zhao and Riters, 2023) and it is possible that modulation of VTA activity, through activation of VP inputs, is an important mechanism that contributes to social play behavior in juvenile rats. It would be of further

interest to determine whether downstream projections from the NAc to VP to VTA forms a circuit within the mesolimbic reward system in order to process rewarding and motivational values of social play behavior, and whether coordination of this circuit, potentially mediated through dopamine signaling, is required for social play in juvenile male and female rats.

The VP is a heterogeneous brain structure and is composed of various cellular phenotypes (Knowland et al., 2017; Faget et al., 2018; Prasad et al., 2020), with GABAergic and glutamatergic cells being the most predominant (Heinsbroek et al., 2020). Both GABAergic (Grove et al., 1986; Záborszky and Cullinan, 1992; Yao et al., 2021) and glutamatergic (Tooley et al., 2018; Yao et al., 2021) VP cells receive inputs from the NAc, and these two cell types have seemingly opposite effects on motivational behaviors (Faget et al., 2018; Heinsbroek et al., 2020; Stephenson-Jones et al., 2020). Stimulating VP^{GABA} neurons augmented remifentanil, a mu-opioid receptor agonist, seeking in adult male and female rats (Farrell et al., 2022). On the other hand, stimulation of $VP^{Glutamate}$ neurons induced a place aversion in an otherwise neutral environment (Tooley et al., 2018), and ablation of $VP^{Glutamate}$ neurons increased lever press responses and breakpoints for a sucrose reward in adult male and female mice (Tooley et al., 2018). Together, these findings point towards opposing roles of GABA and glutamate neurons in the VP, with GABAergic neurons facilitating rewarding and motivational behaviors, while glutamate neurons attenuate these behaviors. Although these studies implicate VP GABA and glutamate neurons in regulating rewarding behaviors in adult rodents, it's unknown whether these neuronal populations also have similar opposing roles in regulating rewarding social play behaviors in juvenile rats. In

addition, it is unclear whether NAc inputs that were manipulated in the current study synapsed onto GABAergic or glutamatergic cells in the VP. Investigating the role of VP GABA and glutamate neurons in regulating social play will elucidate mechanisms within the VP that potentially could contribute to both the facilitation (via VP GABAergic cells) and the suppression (via VP glutamatergic cells) of social play behavior in juvenile male and female rats.

GABAergic fibers in the VP are co-localized with enkephalin (Zahm et al., 1985; Reiner and Anderson, 1990; Kalivas et al., 1993), and both GABA and enkephalin form symmetrical synapses onto VP neurons (Zahm et al., 1985; Ribak and Roberts, 1990; Boyes and Bolam, 2007). Furthermore, one source of enkephalin release in the VP is from the NAc (Kalivas et al., 1993), suggesting a potential co-release of GABA and enkephalin that regulates inhibitory tone in the VP. Enkephalin is a neuropeptide system in the central nervous system (Hökfelt et al., 1977; Simantov et al., 1977; Finley et al., 1981) and is widely present within the mesolimbic reward system (Phillips et al., 1983; Kalivas, 1985; Fibiger and Phillips, 1988). Enkephalin binds to mu-opioid, delta-opioid, and kappa-opioid receptors (Pollard et al., 1977; Chang and Cuatrecasas, 1979; Reisine, 1995), which have been implicated in the regulation of social play behavior (Vanderschuren et al., 1995; Trezza et al., 2011b; Achterberg et al., 2019; Zhao et al., 2020). In detail, subcutaneous injections of morphine, a mu-opioid receptor agonist, at a high dose decrease motivation to engage in social play, as evidenced by decrease in lever press responses and breakpoints for access to a play partner (Achterberg et al., 2019). It should be noted that morphine at low doses selectively binds with mu-opioid receptors, but when administered in high doses, will bind non-selectively with opioid

receptors, including mu-opioid, delta-opioid, and kappa-opioid receptors (Chang and Cuatrecasas, 1979; Trescot et al., 2008). Therefore, exogenous stimulation of opioid receptors decreased the motivation to engage in social play in juvenile male rats. However, morphine administered in a low dose, which would selectively target mu-opioid receptors, induced a conditioned-place preference to a social play-associated compartment (Achterberg et al., 2019), suggesting that morphine working selectively on mu-opioid receptors reinforces the rewarding properties of social play behavior. This is further supported by the role of mu-opioid receptors in the medial preoptic area (MPOA) and NAc. Viral-vector mediated knockdown of mu-opioid receptors in the MPOA reduced social play behavior in juvenile male and female rats (Zhao et al., 2020). In addition, NAc infusions of morphine increased social play behaviors in juvenile male rats (Trezza et al., 2011b), further supporting the facilitating and reward-reinforcing role of mu-opioids in regulating social play behavior.

Mu-opioid receptors are also expressed in the VP (Mansour et al., 1994b, 1994a; Mitrovic and Napier, 1995) and the regional expression of mu-opioids within the VP creates a "hedonic hotspot" (Smith and Berridge, 2005, 2007; Peciña et al., 2006; Smith et al., 2009; Khan et al., 2020). The VP hedonic hotspot, located primarily in the posterior VP (Smith and Berridge, 2005, 2007; Peciña et al., 2006), has been studied for the enhancing effects of mu-opioid stimulation on rewarding behaviors, specifically sucrose intake (Smith and Berridge, 2005). Infusions of DAMGO, a specific mu-opioid receptor agonist, enhanced positive orofacial reactions, indicated by rhythmic tongue protrusions and paw licking, and sucrose intake in adult rats (Smith and Berridge, 2005). Specifically, increased positive orofacial responses have been interpreted as "liking"

(Smith and Berridge, 2005) and suggests stimulation of mu-opioid receptors in the hedonic hotspot of the VP enhances the rewarding properties of an already rewarding stimulus. Interestingly, the anterior VP also expresses mu-opioid receptors but creates a "hedonic coldspot" (Smith and Berridge, 2005). Stimulation of mu-opioid receptors in the VP hedonic cold spot has been shown to have an opposing effect on "liking" than the VP hedonic hotspot. For example, infusions of DAMGO into the anterior VP actually decreased positive orofacial reactions to sucrose (Smith and Berridge, 2005), suggesting that opioid signaling to the anterior VP may dampen the reward value of stimuli. Although the involvement of mu-opioid receptors in the VP in regulating social play in juvenile rats is still unknown, the decrease in social play following stimulation observed in the current study could be due to activation of mu-opioid receptors in the anterior VP, or the hedonic coldspot, through the co-release of enkephalin with GABA from the NAc.

Conclusions

Here, I report for the first time, a pathway-specific neural mechanism in the regulation of social play behavior in juvenile male and female rats. Specifically, I showed that stimulation of the NAc to VP pathway reduced social play behaviors in both juvenile male and female rats. This finding further supports the idea that VP activation, achieved through inhibition of NAc inputs, is required for the typical expression of social play behaviors in juvenile male and female rats. Although previous studies have implicated individual brain regions involved in the mesolimbic reward pathway in regulating social play behavior (Meaney et al., 1981; Gordon et al., 2002; Trezza et al., 2011b; van Kerkhof et al., 2013, 2014; Achterberg et al., 2016b, 2016a; Manduca et al.,

2016b; Argue et al., 2017; Zhao and Riters, 2023), this study demonstrates that coordinated signaling within the mesolimbic reward system is critical to support the expression of social play. Future research should focus on the interaction between the NAc, VP, and VTA and whether they form a unidirectional neural circuit (VTA→NAc→VP) or if they form a feedback loop within this circuit and integrate signals bidirectionally. Findings from these studies will provide a better understanding of how signal integration within the mesolimbic reward system is coordinated to allow for the expression of social play behavior in juvenile male and female rats.

CHAPTER 3: Determine whether social play exposure alters activation of VP-projecting cells in the NAc in juvenile male and female rats

Abstract

The nucleus accumbens (NAc) and ventral pallidum (VP) are two regions that are a part of the mesolimbic reward pathway, the brain's reward system. Both the NAc and VP are involved in regulating social play behavior, a highly rewarding behavior that is displayed by many mammalian species, including humans and rats. Previous studies have shown that social play exposure is associated with an increase in activation of the NAc core and NAc shell in juvenile male rats. More recently, I showed that social play exposure is associated with a sex-specific change in VP activation in juvenile rats. Specifically, I reported a social play-induced increase in VP activation in males, but no change in VP activation in females. The VP receives inputs from both subregions NAc (NAc core and NAc shell) and it is possible that sex-specific activation of VP-projecting cells in these two subregions of the NAc may be a mechanism that modulates VP activity differently in males and females. In this study, I determined whether social play exposure is associated with sex-specific activation of the NAc to VP pathway. To do this, I utilized retrograde tract-tracing (cholera toxin B; CtB) in combination with *in situ* hybridization (*fos* mRNA) in order to visualize VP-projecting cells in the NAc core and shell of rats that were exposed to social play. Males and females were either assigned to the "No Social Play" group, where subjects were undisturbed until transcardial perfusions, or the "Social Play" group, where subjects were exposed to social play prior to perfusions. First, I found that social play exposure was associated with an increase in the number of *fos*+ cells in the NAc core in both males and females. However, social

play exposure was associated with a sex-specific change in the number of *fos*+ cells of the NAc shell, with females, but not males, showing an increase. I then found a sex difference in activation of VP-projecting cells in the NAc core and shell in the "No Social Play" group, with males showing a greater number of *fos*+ VP-projecting NAc core cells compared to females. However, this sex difference in the NAc core was eliminated following social play exposure, likely due to an increase in the number of *fos*+ cells that project to the VP in females. The sex difference in the NAc shell was also eliminated following social play exposure, but this was due to a reduction in the activation of VP-projecting cells in the NAc shell in males only. Finally, there was a sex difference in the proportion of *fos*+ cells projecting to both the NAc core and NAc shell in the "No Social Play" group, with males showing a greater percentage of *fos*+ cells projecting to the VP compared to females. These sex differences in the NAc core and shell were eliminated following social play exposure due to a decrease in the percentage of *fos*+ cells that co-expressed CtB in males in the "Social Play" group. In summary, social play exposure was associated with sex-specific activation of the NAc core and shell as well as sex-specific changes in activation of VP-projecting cells in the NAc core and shell. Furthermore, although there was a baseline sex difference in the activation of VP-projecting cells in the NAc core and shell, following social play exposure, males and females showed similar activation of VP-projecting cells in both subregions. This suggests that social play exposure is associated with similar activational patterns in the NAc core and shell, including their projections to the VP, in order to support the equal expression of social play in juvenile male and female rats.

Introduction

Social reward is reflected by the motivation and the pleasure to engage in social interactions, and is essential in reinforcing positive social interactions from juvenile rats (Trezza et al., 2011b; Nardou et al., 2019) to adult rats (Mason et al., 1963; Douglas et al., 2004; Dölen et al., 2013). Indeed, social interactions are sufficient to induce a conditioned place preference (Calcagnetti and Schechter, 1992; Fleming et al., 1994; Paredes and Alonso, 1997; Thiel et al., 2008; Kummer et al., 2011; Peartree et al., 2012) and increase motivation in an operant lever pressing task for access to a social stimulus (Mason et al., 1962, 1963; Wilsoncroft, 1968; Strobel, 1972; Everitt, 1990; Achterberg et al., 2019) in juvenile and adult rats. The mesolimbic reward system is activated during rewarding and motivating behaviors (Woodward et al., 2000; Wightman and Robinson, 2002; Schott et al., 2008; Numan and Stolzenberg, 2009; Trainor, 2011). It has been proposed that the mesolimbic reward system, along with the Social Behavior Network (Newman, 1999), a proposed network of brain regions involved in regulating social behaviors, integrate into a larger network called the Social Decision-Making Network (SDMN; (O'Connell and Hofmann, 2011). The SDMN is involved in evaluating the reward value of the external stimuli and coordinating the appropriate social response with this information (O'Connell and Hofmann, 2011). Therefore, involvement of the mesolimbic reward system in the SDMN is critical for adaptive behaviors in response to the reward value of the social stimulus (Paredes, 2009; Steinman et al., 2019), and facilitates the continued seeking and enjoyment of positive social companionship.

The mesolimbic reward system is composed of various brain regions, including the nucleus accumbens (NAc; (Ikemoto and Panksepp, 1999; Carlezon and Thomas,

2009; West and Carelli, 2016; Soares-Cunha et al., 2020)) and ventral pallidum (VP; (Smith et al., 2009; Richard et al., 2018; Ottenheimer et al., 2020b, 2020a)). The NAc is involved in regulating various rewarding social behaviors across the lifespan, such as social play behavior in juvenile male rats (Trezza et al., 2011b, 2012; Manduca et al., 2016b, 2016a), maternal behaviors in adult female rats (Keer and Stern, 1999; Champagne et al., 2004; Numan et al., 2005a, 2005b), and aggressive behaviors in adult male rats (Couppis and Kennedy, 2008; Beiderbeck et al., 2012; Aleyasin et al., 2018; Golden et al., 2019). The NAc can be divided into two subregions: the NAc core, which surrounds the anterior commissure, and the NAc shell, which surrounds the medial, lateral, and ventral borders of the NAc core (Záborszky et al., 1985; Zahm and Brog, 1992; O'Donnell and Grace, 1993; Jongen-Rêlo et al., 1994; Kelley, 1999; Maria-Rios et al., 2023). The core and shell have distinct inputs and outputs (Heimer et al., 1991; Zahm and Brog, 1992; Brog et al., 1993; Wright et al., 1996), and show differential regulation of rewarding behaviors (Corbit et al., 2001; Chaudhri et al., 2010; Ambroggi et al., 2011; Ito and Hayen, 2011). Although it is unclear whether the NAc core and shell have different roles in regulating social behaviors, as most studies either do not differentiate between the two subregions or only focus on one of the subregions, it is likely that the distinct anatomical projections of the NAc subregions within the SDMN may contribute to different modulation of social behaviors.

The VP was originally thought of as an integration hub within the mesolimbic reward system (Haber and Nauta, 1983; Bengtson and Osborne, 1999; de Olmos and Heimer, 1999) and primarily responsible for the facilitation of motor output of reward-driven behaviors (Mogenson et al., 1980; Swerdlow and Koob, 1987; Groenewegen et

al., 1993; Groenewegen and Trimble, 2007). However, recent studies demonstrate that the VP also provides information of the reward value of stimuli (Tindell et al., 2004; Richard et al., 2016; Ottenheimer et al., 2018; Vento and Jhou, 2020) and is not solely an integration hub that regulates locomotion. The VP is also involved in regulating rewarding social behaviors across the lifespan, including social play behavior in juvenile male and female rats (van Kerkhof et al., 2014; Lee et al., 2021), maternal behaviors in adult female rats (Numan et al., 2005b; Akther et al., 2014), and sociosexual motivation in adult male and female rats (DiBenedictis et al., 2020). Additionally, some studies show that activation of the VP is required for the typical expression of social behaviors. In detail, inactivation of the VP via infusions of muscimol, the $GABA_A$ receptor agonist, reduced maternal behaviors, while muscimol infusions into the NAc did not alter the expression of maternal behaviors (Numan et al., 2005b). I recently showed that activation of the VP is also required for the typical expression of social play behavior in juvenile male and female rats (Lee et al., 2021). There are also studies that demonstrate that the VP is involved in encoding the rewarding properties of stimuli. For example, modulation of different regions in the VP can either enhance or attenuate the rewarding properties of sucrose (Smith and Berridge, 2005; Peciña et al., 2006; Khan et al., 2020), which alters consumption (Smith and Berridge, 2005). In addition, VP calcium signaling ramped up when adult mice approached the sucrose reward and peaked after sucrose consumption (Vachez et al., 2021). Therefore, it is also possible that the VP is involved in encoding the rewarding properties of social behaviors, and by either enhancing or attenuating the reward value of the social stimulus, can modulate the expression of rewarding social behaviors.

Social play behavior is predominantly shown by juveniles of various mammalian species (Bekoff, 1974b, 1974a; West, 1974; Thor and Holloway, 1984a; Markus and Croft, 1995; Ginsburg, 2007), including rats (Thor and Holloway, 1984a; Ikemoto and Panksepp, 1992; Hol et al., 1999; Lukas and Wöhr, 2015; Vanderschuren et al., 2016). Immediate early gene studies have associated both the NAc (Gordon et al., 2002; van Kerkhof et al., 2014) and VP (van Kerkhof et al., 2014) in the regulation of social play behavior. In detail, social play exposure was associated with an increase in neuronal activation, measured by c-Fos expression, in the NAc core and NAc shell in juvenile male rats (van Kerkhof et al., 2014). This study did not include females, and thus, it is unclear whether juvenile female rats show the same change in NAc neuronal activation following social play exposure. On the other hand, as part of my Master's thesis, I showed that social play exposure was associated with a sex-specific change in VP neuronal activation. Males showed enhanced VP activation following social play exposure while there was no change in VP activation in females following social play exposure (Fig 3C).

Although these studies provide evidence that social play is associated with changes in neuronal activation of the NAc and of the VP, it is unknown how the NAc affects VP activation when rats are exposed to social play. The NAc is a major source of GABAergic input to the VP (Walaas and Fonnum, 1979; Jones and Mogenson, 1980; Swerdlow et al., 1990; Root et al., 2015), and alterations in NAc inputs to the VP can have effects on the activity of the VP. Additionally, the NAc core projects primarily to the dorsolateral region of the VP (Zahm and Brog, 1992; Zahm et al., 1996), while the NAc shell projects primarily to the ventromedial (Zahm and Heimer, 1990; Heimer et al.,

1991) and ventrolateral (Zahm and Brog, 1992; Zahm et al., 1996) regions of the VP. Therefore, the focus of this chapter is on determining whether social play exposure is associated with a change in activation of VP-projecting cells in the NAc core and NAc shell. Given the sex-specific changes in VP activation following social play exposure, I hypothesized that social play exposure is associated with sex-specific activation of VP-projecting cells in the NAc. To test this hypothesis, I combined retrograde tract-tracing with *in situ* hybridization to identify VP-projecting cells in the NAc core and NAc shell that expressed *fos* mRNA following social play exposure. I expected that males exposed to social play will show a decrease in activation of VP-projecting cells in both the NAc core and shell. This decrease in activation of VP-projecting cells in the NAc core and shell, which may reflect a decrease in GABAergic inputs to the VP, could correspond with the increase of VP activation observed in Fig 3C in males. On the other hand, I expected to see no change in activation of VP-projecting cells in the NAc core and shell in females. This would correspond with no change in activation of the VP following social play exposure observed in Fig 3C in females, since the GABAergic inputs from the NAc core and shell to the VP would be unaffected by social play exposure.

Methods

Subjects

Three-week-old male and female Wistar rats were obtained from Charles River Laboratories (Raleigh, NC) and maintained under standard laboratory conditions (12 h light/dark cycle, lights off at 14:00 h, food and water available *ad libitum*). Experimental subjects were housed in single-sex groups of four in standard rat cages (48 X 27 X 20

cm or 41.2 X 30 X 23.4 X cm) unless otherwise mentioned. The 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

Retrograde tracer injections

Juvenile male and female Wistar rats (25 days old) were anesthetized with isoflurane (2-4% as needed; Henry Schein, Melville, NY) and mounted on a stereotaxic frame. A 1 µL, 7000 series Hamilton syringe (Hamilton, Reno, NV) was attached to a motorized stereotaxic injector system (Stoelting, Wood Dale, IL) and a 0.2 µL unilateral injection of the retrograde tracer cholera toxin-B (CTB) conjugated to a fluorescent fluorophore (Alexa Fluor 594, Molecular Probes, dissolved in 0.1M PBS, resulting in 1% CTB solution) was directed to the left hemisphere of the VP at a rate of 0.1 µL/min using the following coordinates: 0.23 mm rostral to bregma, 2.5 mm lateral to the midline, and 7.7 mm ventral to the surface of the skull (Paxinos and Watson, 2007). Injections were made under an angle of 10° from the midsagittal plane to avoid damage to the sagittal sinus. The needle was left in place for 10 min following the injection to allow time for tissue uptake of the tracer. Experimental subjects were given an s.c. injection of meloxicam (2mg/kg; Covetrus, 049759) immediately after surgery and once a day for an additional two days. Immediately after surgery, experimental subjects were pair-housed with a same-sex subject after surgery and remained pair-housed until behavioral testing. All subjects remained undisturbed for seven days to allow the tracer to be taken up by axon terminals and transported back to the cells bodies of origin.

Social play exposure

In order to determine whether there are sex differences in the recruitment of NAc cells that project to the VP in juvenile rats following exposure to social play, experimental subjects were divided into “No Social Play” and “Social Play” conditions. All experimental subjects (33 days old) were single-housed in new cages, and the pair-housed cages of the subjects in the “Social Play” condition were kept in order to be used as the social play testing environment. The following day, experimental subjects (34 days old) in the “Social Play” condition were rejoined with their previous cagemate in their original pair-housed cage for the social play test. Social play testing was performed as described in Chapter 2. One of the subjects in each pair was striped with a permanent marker 30-60 min prior to social play testing in order to distinguish the two subjects during later video analysis. After the end of the 10 min session, subjects were returned to their single-housed cages and remained there until transcardial perfusions.

Thirty minutes after the start of the social play test, subjects in the “Social Play” condition were euthanized via transcardial perfusions. This time course was chosen because stimulus-induced *c-fos* mRNA expression is at its peak 30 min after stimulation (Morgan and Curran, 1991; Cullinan et al., 1995). Subjects in the “No Social Play” condition remained single-housed and undisturbed until euthanasia via transcardial perfusions. Perfusion and post-fixation procedures were performed as described in Chapter 2.

***In situ* hybridization**

Brains were mounted in the cryostat (Leica CM3050 S) and blocked using a razor blade in order to collect tissue containing the left hemisphere for the NAc

(corresponding to distances +3.24 mm to +1.08 mm from bregma; Paxinos and Watson, 2007) and VP (corresponding to distances +0.96 mm to -0.24 mm from bregma; Paxinos and Watson, 2007). Brains were sliced in 30 µm coronal sections into 4 series on a cryostat and each series was collected into TBS (50mM, pH: 7.4) and mounted on separate slides (Superfrost Plus; Fisher Scientific) on the same day, which were then stored at -80°C until subsequent histological processing.

The first series from the NAc and VP was stained with a fluorescent Nissl (1:500, 1 hr; NeuroTrace™; N21479, 435/455nm, ThermoFisherScientific) in order to assess CTB infusion sites. Slides were examined using a Keyence BZ-X700E/BZ-X710 fluorescent microscope and CTB infusion sites were mapped using The Rat Brain Atlas of Paxinos and Watson (2007; Fig 9C). Only rats with infusion sites restricted to the VP were included in the NAc analysis.

The second series of the NAc was processed for *in situ* hybridization using an RNAScope™ Multiplex Fluorescent Reagent Kit v2 (323100, Advanced Cell Diagnostics) to quantify *fos* mRNA expressing cells according to the user manual from the supplier (Document Number 323100-USM, Advanced Cell Diagnostics). Briefly, tissue sections were washed in phosphate buffer solution (pH: 7.6), dried at 60°C (30 min), then post-fixed in 4% paraformaldehyde followed by a dehydration in an ethanol series. Following hydrogen peroxide incubation and target retrieval in a steamer, tissue sections were treated with protease III (20 min; 322340, Advanced Cell Diagnostics) at room temperature. The *fos*-C1 (403591, Advanced Cell Diagnostics) probe was hybridized in a HybEZ™ oven (2 hrs; Advanced Cell Diagnostics) at 40°C. After probe hybridization, tissue sections were incubated with amplifier probes (AMP1, 40°C, 30

min; AMP2, 40°C, 30 min; AMP3, 40°C, 15 min). *fos* mRNA was tagged to the fluorophore fluorescein (1:1500; NEL741E001KT, Akoya Biosciences). Slides were rinsed in TBS and stained with a fluorescent Nissl (as described in Chapter 2). Slides were then coverslipped with Vectashield hardset antifade mounting medium with a DAPI counterstain (H-1500-10, Vector Laboratories) and stored at 4°C.

All images were acquired with a 40x objective on a Keyence BZ-X700E/BZ-X710 fluorescent microscope and associated BZ-H3AE software (Keyence Corporation of America). Images were taken at three sampling locations across the NAc core and shell in order to assess the distribution of *fos*+ cells, CTB+ cells, and *fos*+ cells that co-express CTB. Specifically, the following anterior-posterior distances were imaged (distances refer to mm from bregma; Paxinos and Watson, 2007): +1.68 mm, +1.32 mm and +1.08 mm. At each anterior-posterior location, a dorsal and ventral image was taken (for imaging plan, please see Fig 8). Cells were counted as *fos*+ if they have five or more puncta and CTB+ if they have one or more puncta. In addition to counting the number of *fos*+ cells, CTB+ cells, and *fos*+ cells that co-express CTB, the percent of *fos*+ cells that co-expressed CTB was quantified as [(# of double-labelled cells/total number of *fos*+ cells)*100] and the percent of CTB+ cells that co-express *fos* was quantified as [(# of double-labelled cells/total number of CTB+ cells)*100].

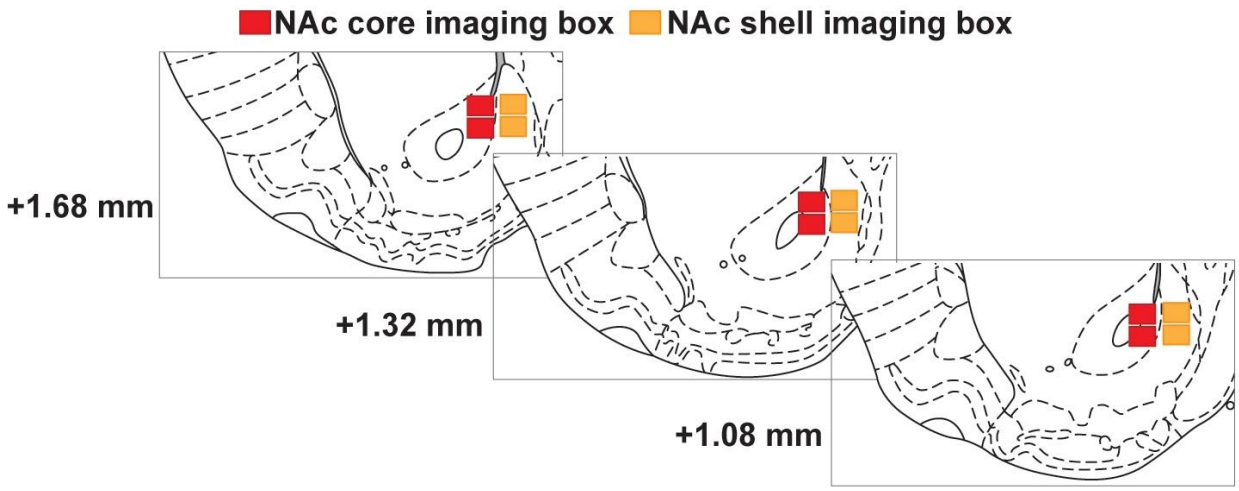

Figure 8. Image sampling locations in the NAc core and NAc shell shown on modified rat brain atlas templates (Paxinos and Wayson, 2007). Numbers to the left of the atlas templates represent distance from Bregma; red boxes represent sampling location where images were taken in the NAc core; yellow boxes represent sampling location where images were taken in the NAc shell.

Statistical analysis

An independent sample t-test was used to analyze the effect of sex on behaviors during the social play test. A mixed-effects [anterior-posterior location (within-subjects factor) x sex (between-subjects factor) x social play condition (between-subjects factor)] ANOVAs were used to assess the effects of sex and social play exposure on the number of VP-projecting cells in the NAc core and NAc shell, activation of NAc core and NAc shell as well as VP-projecting cells in both subregions of the NAc. For all ANOVAs, there was no main effect of, or interaction with, sampling location and thus the data is represented collapsed across sampling locations in order to highlight sex and social play condition effects. Pearson correlations were used to determine whether activation in the NAc core, NAc shell, and VP-projecting cells in both subregions correlated with

the percent of time experimental subjects in the "Social Play" condition engaged in social play. When significant interactions were found in the mixed-effects ANOVAs, Bonferroni *post hoc* multiple comparison tests were conducted to clarify the effects.

All data were analyzed using GraphPad Prism 10 or IBN SPSS 28, and statistical significance was set at $p < 0.05$. Partial eta squared (η_p^2) for all mixed-effects models and Cohen's d (*d*) effect sizes for the t-test were manually computed when significant main effects or interactions were found.

Results

Males and females show a similar number of VP-projecting cells in the NAc core and NAc shell

In order to determine whether there were sex differences in the number of VP-projecting cells in the NAc core and shell, the number of CtB+ cells were counted in males and females in the "No Social Play" and "Social Play" groups. Males and females in the "No Social Play" and "Social Play" groups showed a similar number of CtB+ cells in both the NAc core (Table 4; Fig 9D) and NAc shell (Table 4; Fig 9E).

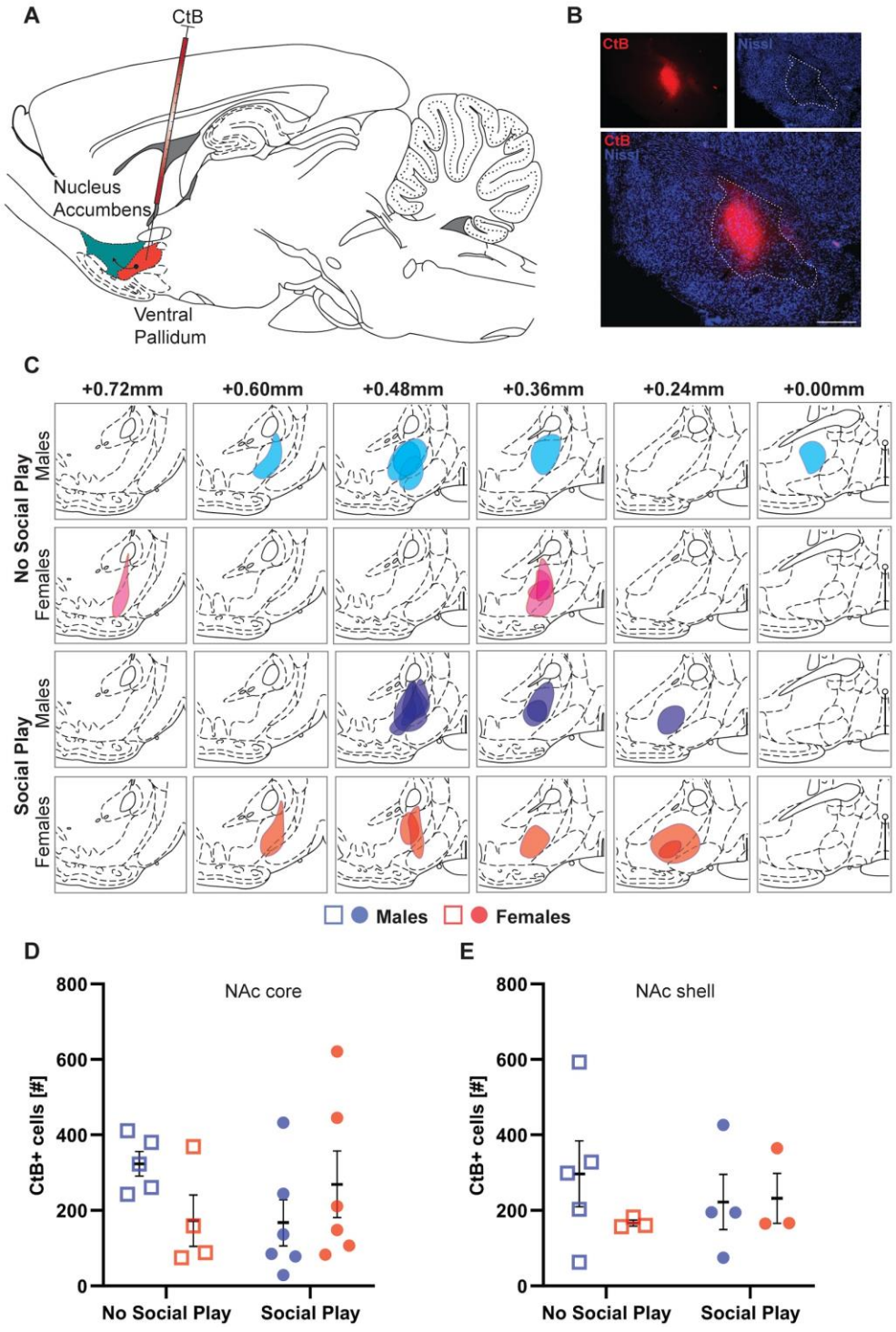

Figure 9. No sex difference in the number of VP-projecting cells in the NAc core and shell. (A) Schematic illustration of unilateral infusion of the retrograde tracer CtB (AF 594) targeting the VP. (B) CtB expression (red) is localized within the borders of the

Figure 9 (cont'd)

VP (outlined in white) defined using a fluorescent Nissl (blue). (C) Coronal sections depicting the center of CtB infusions in males and females in the "No Social Play" and "Social Play" groups. Numbers at the top refer to distance from Bregma (in mm; Paxinos and Watson, 2007). Males and females in the "No Social Play" and "Social Play" groups show similar numbers of VP-projecting cells in the NAc core (D) and NAc shell (E). Scale bar = 150 µm.

Table 4. Two-way ANOVA statistics for the number of CTB+ cells in the NAc core and shell between males and females in the "No Social Play" and "Social Play" groups. Significant effects are indicated in **bold**.

	Sex	Social play condition	Interactions
NAc core CtB+ cells [#]	$F_{(1,17)} = 0.0003$, $p = 0.98$	$F_{(1,17)} = 0.003$, $p = 0.95$	$F_{(1,17)} = 1.93$, $p = 0.18$
NAc shell CtB+ cells [#]	$F_{(1,11)} = 0.59$, $p = 0.45$	$F_{(1,11)} = 0.003$, $p = 0.95$	$F_{(1,11)} = 0.79$, $p = 0.39$

Social play exposure increased in NAc core activation in both males and females

Males and females in the "Social Play" group displayed a similar duration of social play (Table 5; Fig 10A) during the social play test. In addition, there was a main effect of social play condition (Table 6), such that social play exposure increased in activation in the NAc core in both males and females (Fig 10C). In detail, males and females in the "Social Play" group expressed a greater number of *fos*+ cells compared to their "No Social Play" counterparts (Fig 10C). There was no main effect, or interaction with, sex (Table 6). In addition, the time spent engaging in social play did not significantly correlate with the number of *fos*+ cells (Fig 10D) in either males (Fig 10E)

or females (Fig 10F). Overall, engagement in social play is associated with enhanced activation of the NAc core in both males and females.

Table 5. T-test statistics for the effect of sex on social play behaviors. Significant effects are indicated in **bold**.

	Sex
Social play duration	$t_{(10)} = 0.78$, $p = 0.78$

Table 6. Two-way ANOVA statistics for the number of *fos*+ cells in the NAc core and shell between males and females in the “No Social Play” and “Social Play” groups. Significant effects are indicated in **bold**.

	Sex	Social play condition	Interactions
NAc core *fos*+ cells [#]	$F_{(1,17)} = 0.21$, $p = 0.65$	**$F_{(1,17)} = 4.83$, $p = 0.04$, $\eta_p^2 = 0.22$**	$F_{(1,17)} = 1.13$, $p = 0.30$
NAc shell *fos*+ cells [#]	$F_{(1,11)} = 0.68$, $p = 0.42$	**$F_{(1,11)} = 7.06$, $p = 0.02$, $\eta_p^2 = 0.39$**	**$F_{(1,11)} = 6.54$, $p = 0.02$, $\eta_p^2 = 0.37$**

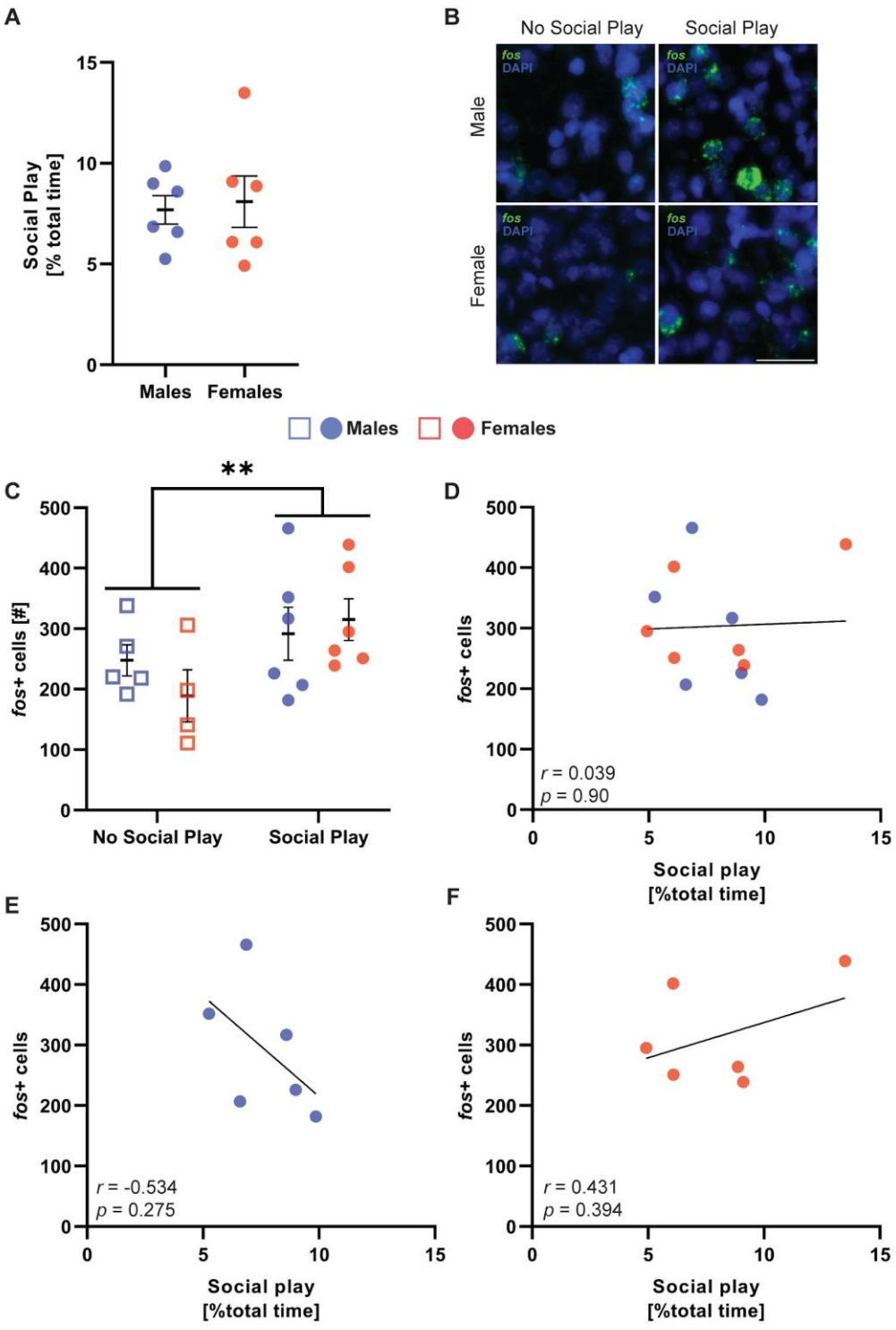

Figure 10. Social play exposure enhanced activation of the NAc core in both males and females. (A) Juvenile male and female rats spent a similar amount of time engaging in social play. (B) Example photomicrographs showing *fos* mRNA staining in

Figure 10 (cont'd)

green in NAc core cells. The number of single-labelled *fos*+ cells were counted. (C) Exposure to social play increased activation of NAc core cells in both males and females. Time spend engaging in social play did not significantly correlate with the number of *fos*+ cells (D) in males (E) or females (F). Black bars indicate mean ± SEM; *$p < 0.05$, two-way ANOVA main effect. Scale bar = 15 µm.

Social play exposure altered activation of the NAc shell in a sex-specific manner

There was a main effect of social play condition (Table 6) and a significant sex X social play condition interaction (Table 6) on the number of *fos*+ cells in the NAc shell. Bonferroni *post hoc* comparisons reveal that males in the "No Social Play" group show a higher number of *fos*+ cells in the NAc shell compared to females in the "No Social Play" group (Fig 11A). In addition, females in the "Social Play" group show a greater number of *fos*+ cells compared to females in the "No Social Play" group (Fig 11A). Males in the "No Social Play" and "Social Play" groups expressed a similar number of *fos*+ cells in the NAc shell. Time spent engaging in social play did not significantly correlate with the number of *fos*+ cells in the NAc shell (Fig 11B) in either males (Fig 11C) or females (Fig 11D). Therefore, social play exposure enhanced activation of the NAc shell in females but not in males. In addition, there is a sex difference in baseline activation of the NAc shell, and social play exposure eliminates this sex difference.

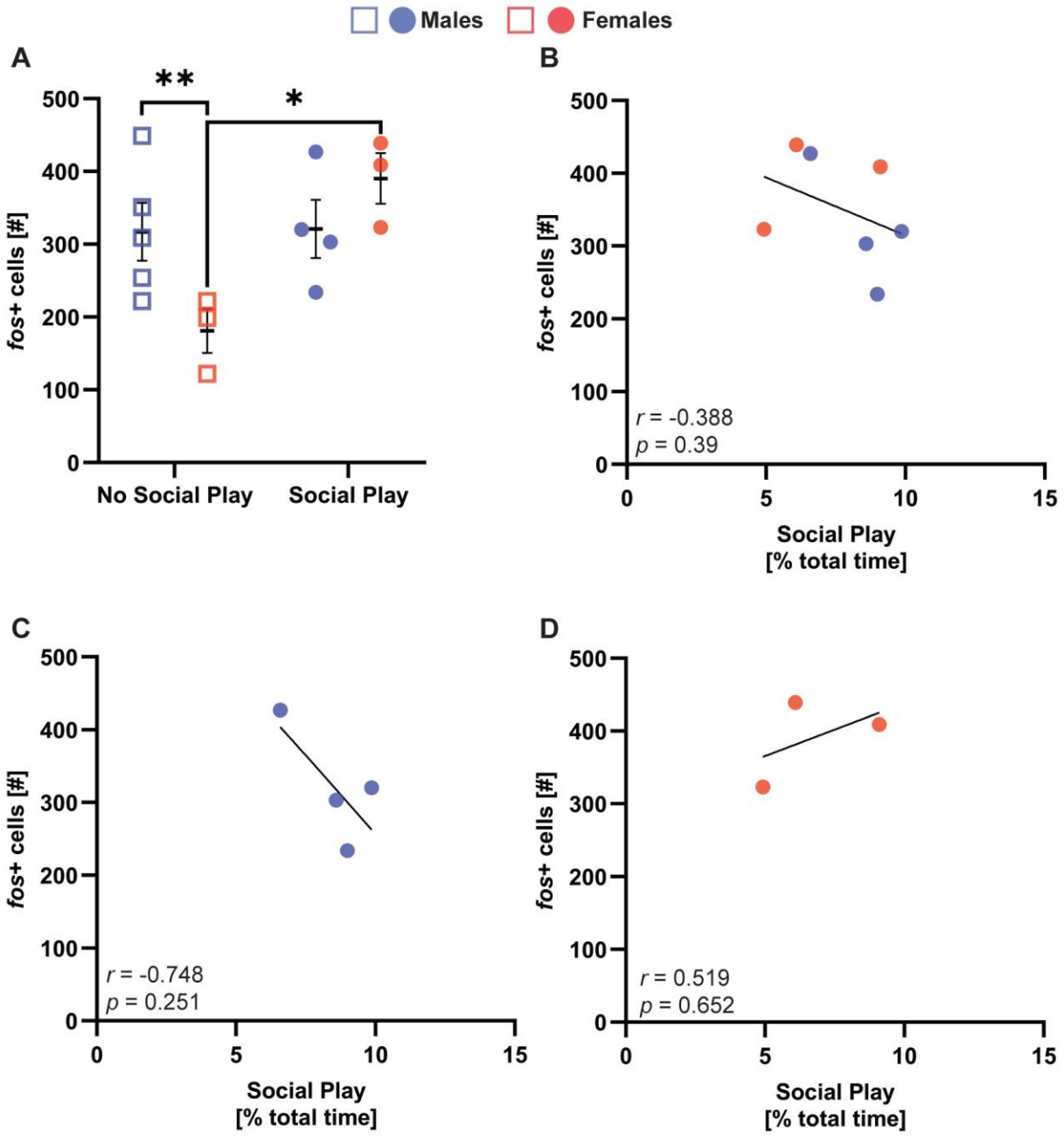

Figure 11. Social play exposure enhanced activation of the NAc shell in females but not in males. (A) Males in the "No Social Play" group showed a greater number of fos+ cells compared to females in the "No Social Play" group. In addition, females in the "Social Play" group showed a greater number of *fos*+ cells than females in the "No Social Play" group. Males in the "No Social Play" group and "Social Play" group showed

Figure 11 (cont'd)

a similar number of *fos*+ cells. Time spent engaging in social play did not significantly correlate with the number of *fos*+ cells (B) in males (C) or females (D). Black bars indicate mean ± SEM; *$p < 0.05$,Bonferroni *post hoc* multiple comparisons.

Social play exposure altered activation of VP-projecting cells in the NAc shell but not the NAc core in a sex-specific manner

There was a significant sex X social play condition interaction on the number of CtB+ cells that co-expressed *fos* in the NAc core (Table 7). Bonferroni *post hoc* tests show that males in the "No Social Play" group expressed a greater number of CtB+ cells that co-expressed *fos* compared to females in the "No Social Play" group (Fig 12B). However, males and females in the "Social Play" group expressed a similar number of CtB+ cells that co-expressed *fos* (Fig 12B), suggesting that social play exposure eliminated the baseline sex difference in activation of VP-projecting cells in the NAc core. This elimination can be due to an increase in the number of CtB+ cells that co-expressed *fos* in females ($p = 0.053$), pointing towards a sex-specific change in activation of VP-projecting cells in the NAc core. Time spent engaging in social play did not significantly correlate with the number of CtB+ NAc core cells that co-expressed *fos* (Fig 12C) in males (Fig 12D) or females (Fig 12E).

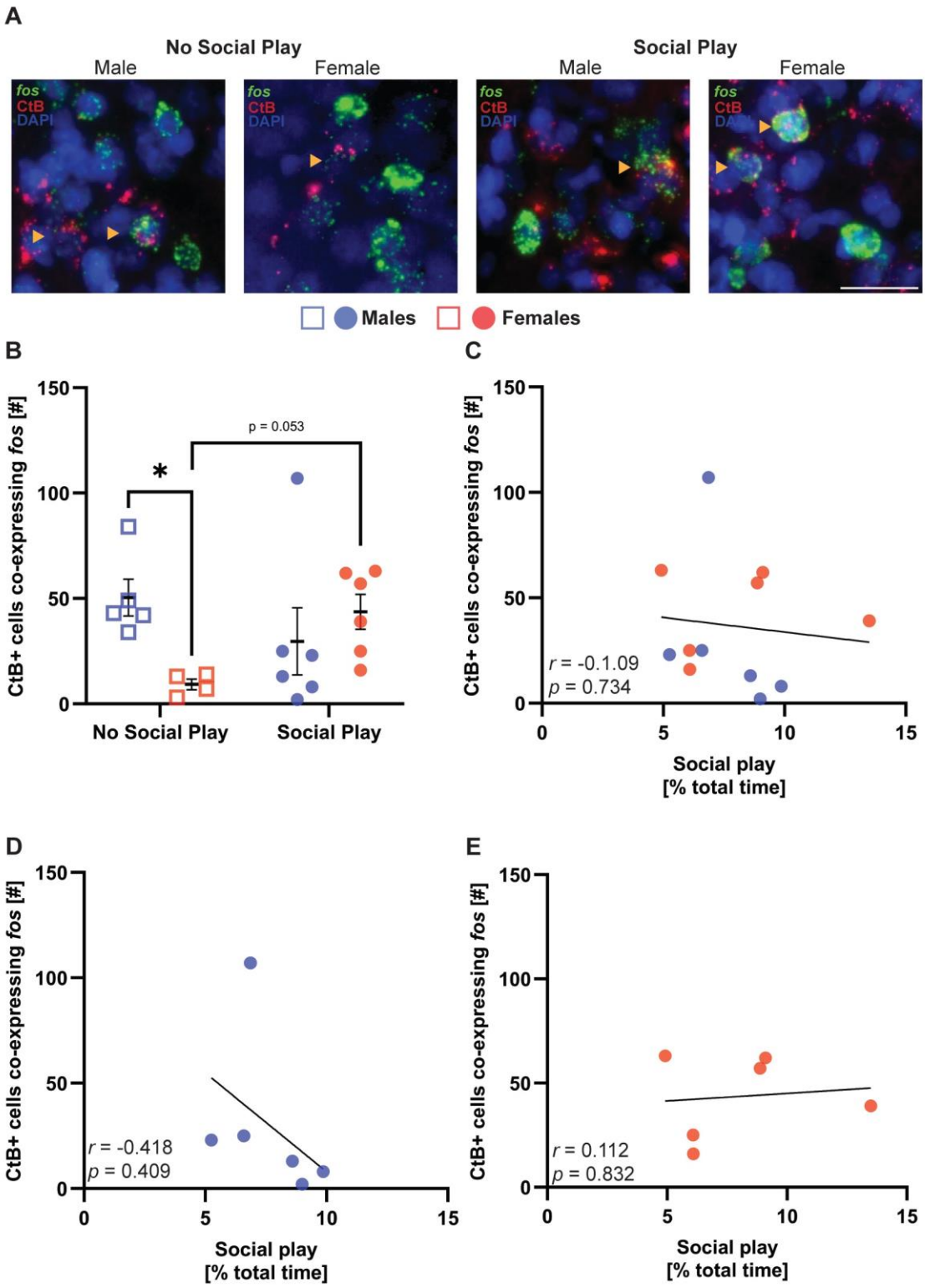

Figure 12. Sex difference in the number of activated VP-projecting cells in the NAc core was eliminated after social play exposure. (A) Example photomicrograph

Figure 12 (cont'd)

showing *fos* mRNA staining in green and CtB staining in red. Yellow arrowheads indicate *fos*+ cell co-expressing CtB. (B) Males that were not exposed to social play expressed a greater number of VP-projecting cells that expressed *fos* compared to females that were not exposed to social play. Following social play exposure, males and females express a similar number of activated VP-projecting cells in the NAc core. Time spent engaging in social play did not significantly correlate with the number of activated VP-projecting NAc core cells (C) in males (D) or females (E). Black bars indicate mean ± SEM; **p* = 0.05, Bonferroni *post hoc* multiple comparisons. Scale bar = 15 µm.

Table 7. Two-way ANOVA statistics for the number of *fos*+ cells co-expressing CtB in the NAc core and shell between males and females in the "No Social Play" and "Social Play" groups. Significant effects are indicated in **bold**.

	Sex	Social play condition	Interactions
NAc core *fos*+ cells co-expresing CtB [#]	$F_{(1,17)} = 1.43$, $p = 0.24$	$F_{(1,17)} = 0.36$, $p = 0.55$	**$F_{(1,17)} = 5.90$, $p = 0.02$, $\eta_p^2 = 0.25$**
NAc shell *fos*+ cells co-expressing CtB [#]	$F_{(1,11)} = 1.27$, $p = 0.28$	$F_{(1,11)} = 0.45$, $p = 0.51$	**$F_{(1,11)} = 7.23$, $p = 0.02$, $\eta_p^2 = 0.39$**

There was a significant sex X social play condition interaction on the activation of VP-projecting cells in the NAc shell (Table 7). Bonferroni *post hoc* tests reveal that males in the "No Social Play" group displayed a greater number of CtB+ NAc shell cells that co-expressed *fos* compared to females in the "No Social Play" group (Fig 13A). In addition, males in the "No Social Play" showed a greater number of CtB+ NAc shell cells that co-expressed *fos* compared to males in the "Social Play" group (Fig 13A).

Therefore, social play exposure decreased the activation of VP-projecting cells in the NAc shell in males but not in females. In addition, a baseline sex difference was eliminated by a social play-induced decrease in activation of VP-projecting NAc shell cells in males. Time spent engaging in social play did not significantly correlate with the number of CtB+ cells that co-expressed *fos* in the NAc shell (Fig 13B) in males (Fig 13C) or females (Fig 13D).

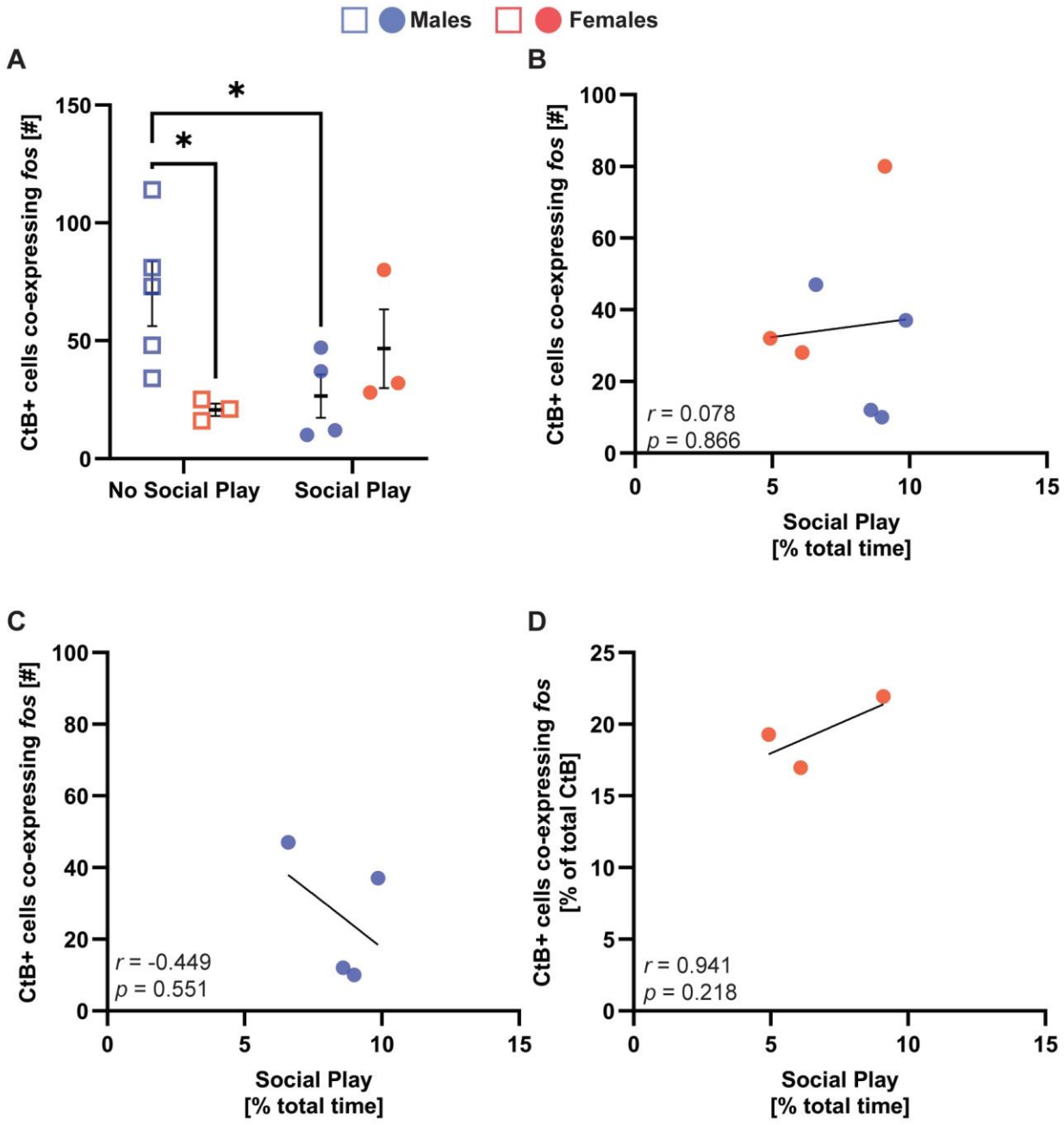

Figure 13. Social play exposure is associated with a sex-specific decrease in the activation of VP-projecting NAc shell cells. (A) Males exposed to social play showed a fewer number of VP-projecting NAc shell cells that were activated compared to males that were not exposed to social play. Time spent engaging in social play did not significantly correlate with the number of activated VP-projecting cells in the NAc shell

Figure 13 (cont'd)

(B) in males (C) or females (D). Black bars indicate mean ± SEM; *$p < 0.05$, Bonferroni *post hoc* multiple comparisons.

Table 8. Two-way ANOVA statistics for the percentage of *fos*+ cells co-expressing CtB in the NAc core and shell between males and females in the "No Social Play" and "Social Play" groups. Significant effects are indicated in **bold**.

	Sex	Social play condition	Interactions
NAc core % of *fos*+ cells co-expresing CtB [#]	$F_{(1,17)} = 1.97$, $p = 0.17$	$F_{(1,17)} = 0.07$, $p = 0.78$	**$F_{(1,17)} = 12.49$, $p = 0.002$, $\eta_p^2 = 0.42$**
NAc shell % of *fos*+ cells co-expressing CtB [#]	$F_{(1,11)} = 0.73$, $p = 0.41$	**$F_{(1,11)} = 5.36$, $p = 0.04$, $\eta_p^2 = 0.32$**	**$F_{(1,11)} = 5.03$, $p = 0.04$, $\eta_p^2 = 0.31$**

Sex-specific change in the proportion of activated cells that project to the VP in the NAc core and NAc cell following social play exposure

There was a significant sex X social play condition interaction on the proportion of *fos*+ cells that co-expressed CtB in the NAc core (Table 8). Bonferroni *post hoc* tests revealed that females in the "No Social Play" group showed a lower proportion of *fos*+ cells that co-expressed CtB compared to males in the "No Social Play" group (Fig 14A). In addition, males in the "No Social Play" group showed a higher proportion of *fos*+ cells that co-expressed CtB compared to males in the "Social Play" group (Fig 14A). On the other hand, females in the "No Social Play" group were trending to show a lower proportion of *fos*+ cells that co-expressed CtB compared to females in the "Social Play" group ($p = 0.07$; Fig 14A). Time spent engaging in social play did not significantly

correlate with the proportion of *fos*+ cells that co-expressed CtB (Fig 14B) in males (Fig 14C) or females (Fig 14D).

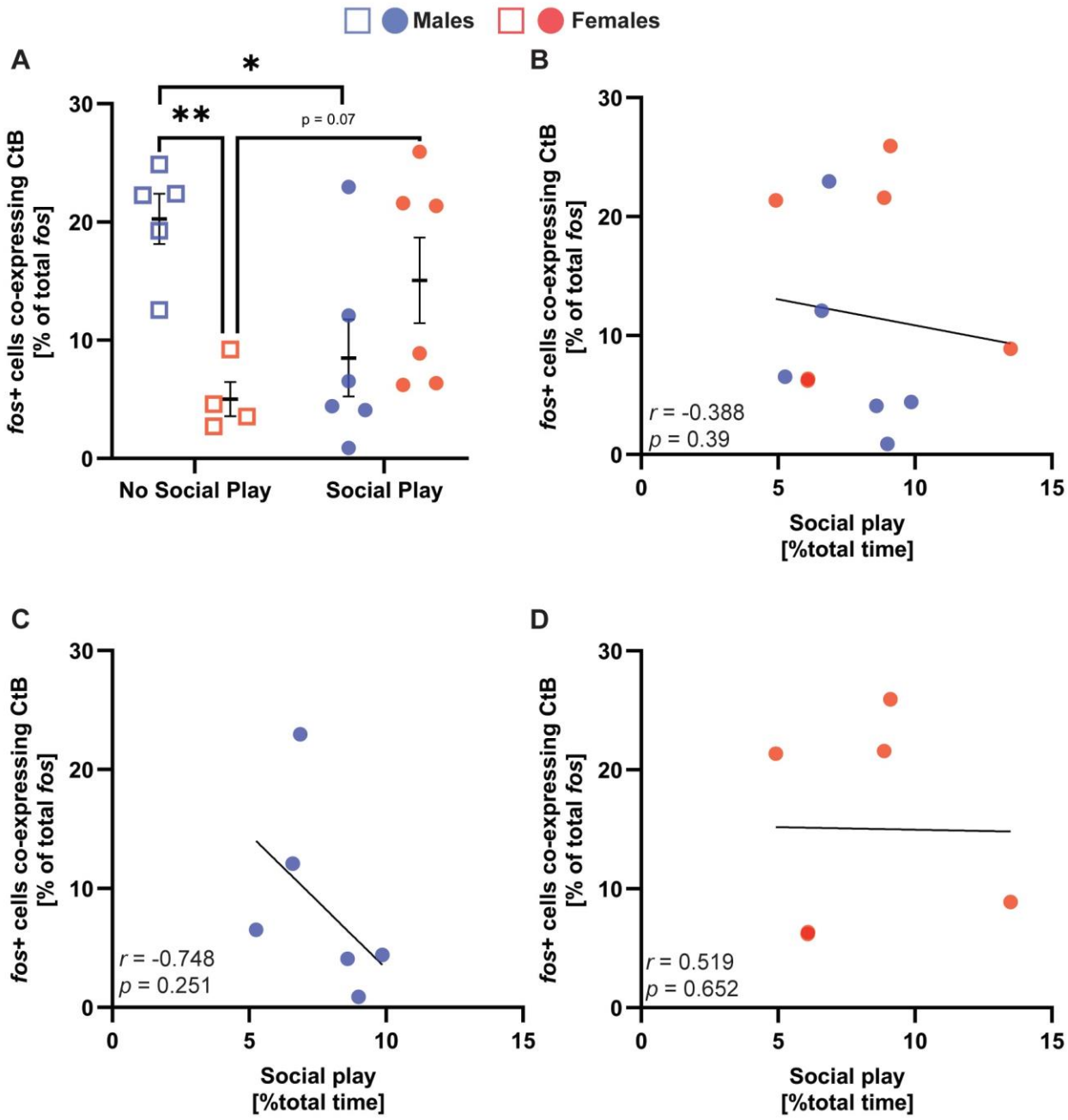

Figure 14. Social play exposure altered the proportion of activated NAc core cells that project to the VP in a sex-specific manner. (A) Males in the "No Social Play" group showed a greater proportion of *fos*+ cells that co-expressed CtB compared to

Figure 14 (cont'd)

females in the "No Social Play" group and males in the "Social Play" group. Time spent engaging in social play did not significantly correlate with the proportion of *fos*+ cells that co-expressed CtB (B) in males (C) or females (D). Black bars indicate mean ± SEM; *$p < 0.05$,**$p < 0.001$, Bonferroni *post hoc* multiple comparisons.

Social play exposure is associated with a decrease in the proportion of activated cells that projects to the VP in the NAc shell in males but not in females

There was a main effect of social play condition (Table 8) as well as a significant sex X social play condition interaction (Table 8) on the proportion of *fos*+ cells that co-expressed CtB in the NAc shell. Bonferroni *post hoc* tests revealed that males in the "No Social Play" group displayed a higher proportion of *fos*+ cells that co-expressed CtB compared to males in the "Social Play" group (Fig 15A). Females in the "No Social Play" and "Social Play" groups displayed a similar proportion of *fos*+ cells that co-expressed CtB in the NAc shell (Fig 15A). Time spent engaging in social play did not significantly correlate with the proportion of *fos*+ cells that co-expressed CtB (Fig 15B) in males (Fig 15C) or females (Fig 15D).

In combination with the findings from the NAc core, social play exposure is associated with sex-specific changes in the population shift of activated NAc cells that project to the VP. Specifically, in both the NAc core and NAc shell, social play exposure lowered the proportion of activated cells that project to the VP in males, suggesting that social play requires decreased input to the VP from the NAc in males, but not in females. On the other hand, baseline inputs from the NAc to the VP is sufficient to support social play in females and does not require a shift in the intensity of the inputs.

These findings suggest a sex difference in the modulation of NAc inputs to the VP in order to support the equal expression of social play behavior in males and females.

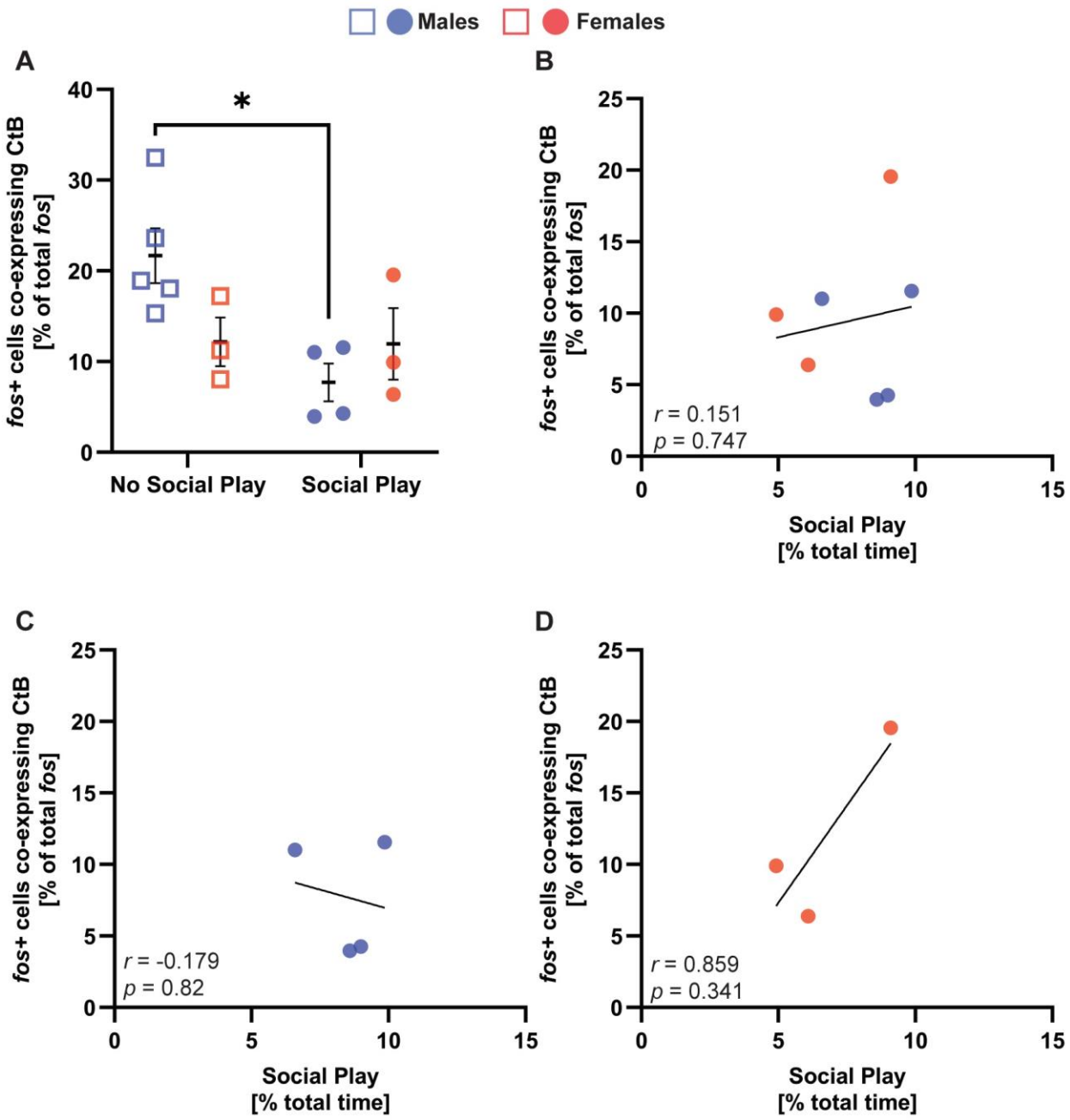

Figure 15. Social play exposure was associated with a decrease in the proportion of activated VP-projecting cells in the NAc shell in males only. (A) Males in the "No Social Play" group showed a greater proportion of VP-projecting cells that were active

Figure 15 (cont'd)

compared to males in the "Social Play" group. Females in the "No Social Play" and "Social Play" groups showed a similar proportion of VP-projecting cells in the NAc shell that were active. Time spent engaging in social play did not significantly correlate with the proportion of CTB+ cells that co-expressed *fos* (B) in males (C) or females (D). Black bars indicate mean ± SEM; *$p < 0.05$, Bonferroni *post hoc* multiple comparisons.

No change in the proportion of VP-projecting cells that were activated following social play exposure in either the NAc core or NAc shell of juvenile male and female rats

There were no main effects of, or interactions with, sex and social play condition on the proportion of CtB+ cells that co-expressed *fos* in the NAc core (Table 9; Fig 16A) or NAc shell (Table 9; Fig 17A). Males and females in the "No Social Play" and "Social Play" groups showed similar proportions of CtB+ cells that co-expressed *fos* in the NAc core (Fig 16A) and the NAc shell (Fig 17A). In addition, time spent engaging in social play did not significantly correlate with the proportion of CtB+ cells that co-expressed *fos* in the NAc core (Fig 16B-D) and NAc shell (Fig 17B-D).

Table 9. Two-way ANOVA statistics for the percentage of CtB+ cells co-expressing *fos* in the NAc core and shell between males and females in the "No Social Play" and "Social Play" groups. Significant effects are indicated in **bold**.

	Sex	Social play condition	Interactions
NAc core % of CtB+ cells co-expresing *fos* [#]	$F_{(1,17)} = 1.28$, $p = 0.27$	$F_{(1,17)} = 0.59$, $p = 0.45$	$F_{(1,17)} = 2.32$, $p = 0.14$

Table 9 (cont'd)

NAc shell % of CtB+ cells co-expressing *fos* [#]	$F_{(1,11)} = 2.35$, $p = 0.15$	$F_{(1,11)} = 3.47$, $p = 0.08$	$F_{(1,11)} = 3.23$, $p = 0.09$

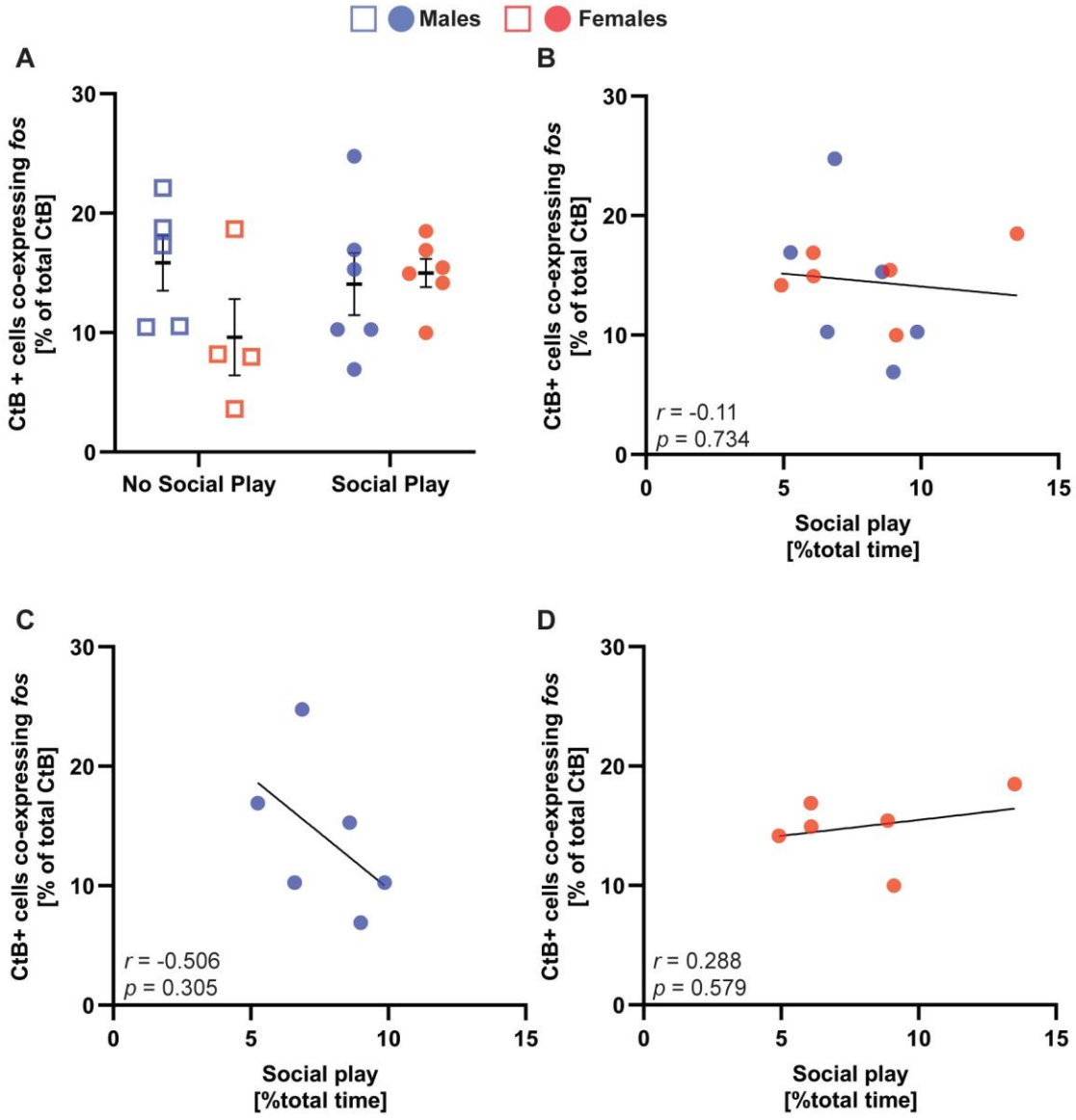

Figure 16. Social play exposure did not change activation of VP-projecting cells in the NAc core. (A) Males and females in the "No Social Play" and "Social Play" groups showed a similar proportion of CTB+ cells that co-expressed *fos*. Time spent engaging

Figure 16 (cont'd)

in social play did not significantly correlate with the proportion of CTB+ cells that co-expressed *fos* (B) in males (C) or females (D). Black bars indicate mean ± SEM.

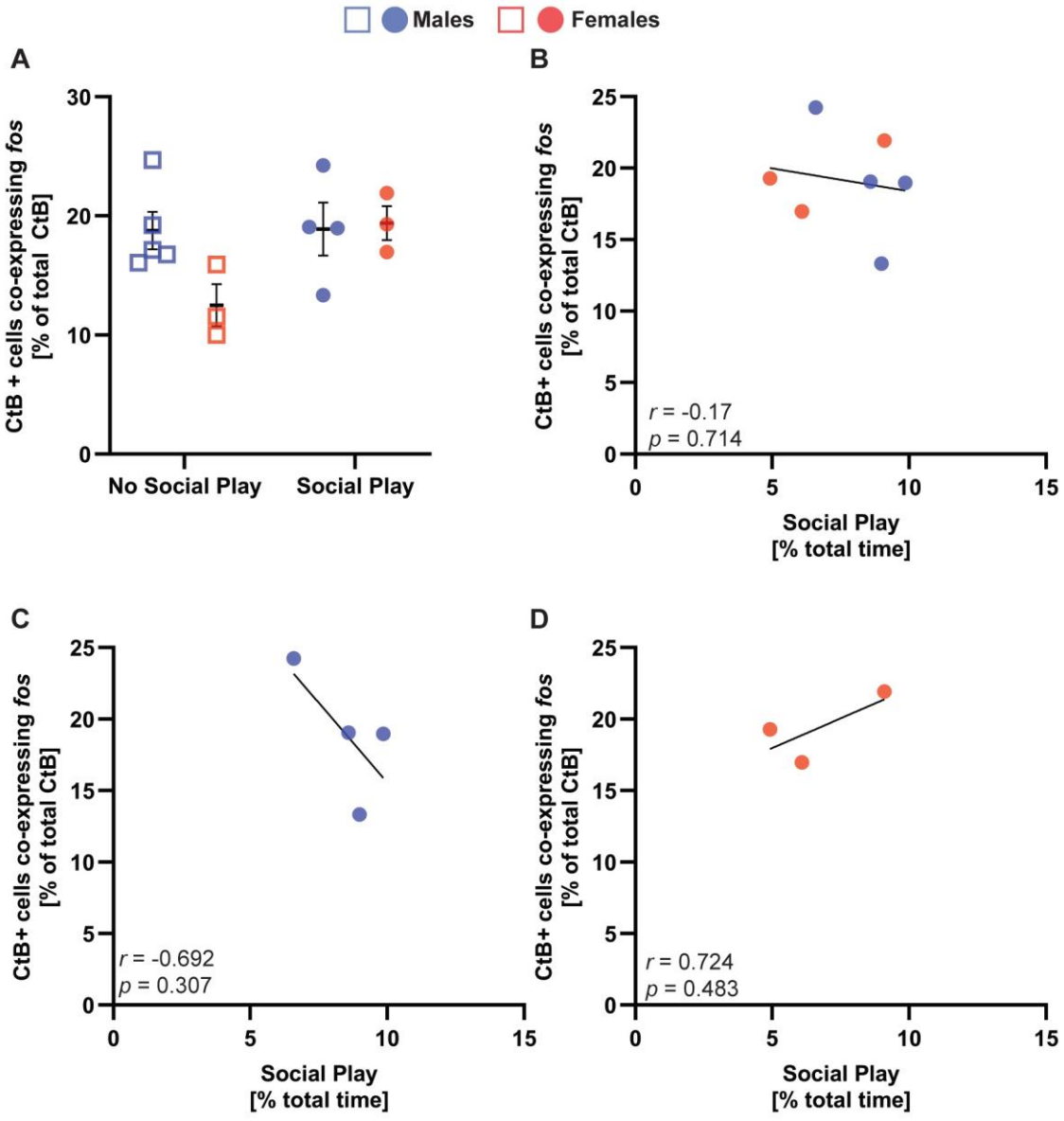

Figure 17. Social play exposure did not change activation of VP-projecting cells in the NAc shell. (A) Males and females in the "No Social Play" and "Social Play" groups showed a similar proportion of CTB+ cells that co-expressed *fos*. Time spent engaging

Figure 17 (cont'd)

in social play did not significantly correlate with the proportion of CTB+ cells that co-expressed *fos* (B) in males (C) or females (D). Black bars indicate mean ± SEM.

Discussion

In the present study, I demonstrated that social play exposure changed activation of the NAc core and shell as well as VP-projecting cells in both subregions in juvenile male and female rats. In detail, I found that social play exposure increased activation of the NAc core in both males and females. On the other hand, I found that social play exposure eliminates the sex difference in baseline activation of the NAc shell. This was due to an increase in NAc shell activation in females following social play testing. In line with my prediction, I then demonstrated that males and females show differential changes in the number of activated VP-projecting cells in the NAc core and NAc shell following social play exposure. Specifically, males displayed a decrease in activation of NAc shell inputs to the VP following social play exposure, while females displayed a tendency toward an increased activation of NAc core inputs to the VP following social play exposure. These findings indicate that sex-specific modulation of NAc inputs to the VP is associated with the regulation of social play behavior in juvenile rats. Furthermore, the findings point towards a sex-specific pathway engagement within the mesolimbic reward system that may underlie the equal expression of social play behavior in juvenile male and female rats.

Increased activation of the NAc core in both sexes and female-specific activation of the NAc shell following social play exposure

I observed an increase in activation of the NAc core following social play exposure in juvenile male and female rats. This is in line with a previous study that also reported social play-induced increased activation of the NAc core in juvenile male rats. I show for the first time a similar engagement of the NAc core in females compared to males upon social play exposure.

On the other hand, I observed a sex-specific change in activation of the NAc shell following social play exposure. In the absence of social play exposure, there was a sex difference in the activation of the NAc shell, such that males show greater NAc shell activation compared to females. Following social play exposure, this sex difference was eliminated due to an increase in NAc shell activation in females and no change in NAc shell activation in males. These findings in the males are not in line with a previous study showing that social play exposure increased NAc shell activation in juvenile male rats (van Kerkhof et al., 2014). There are some methodological differences to consider. For example, the van Kerkhof study tested experimental subjects in a testing cage during the light phase and exposed subjects to a familiar cagemate. On the other hand, the current study tested experimental subjects in their home cage during the dark phase and exposed subjects to a novel conspecific. In addition, the van Kerkhof study measured c-Fos-positive cell density while the current study measured the number of *fos* mRNA-expressing cells. These differences in testing methodology and quantification may account for the discrepancy between the van Kerkhof study and the current study. It should also be noted that these methodological differences did not have an effect on

the findings for the NAc core, suggesting that there might be differences in the robustness of the involvement of the NAc core versus the shell in social play behavior in juvenile rats.

The involvement of the NAc in modulating rewarding behaviors has been studied widely (Cardinal and Howes, 2005; Floresco et al., 2008; Schott et al., 2008; Trezza et al., 2012; Hart et al., 2014; Salti et al., 2015) and it is generally accepted that the NAc plays a key role in encoding reward saliency of a stimulus and regulating the motivation to work towards access to a rewarding stimulus (Day et al., 2011; Hart et al., 2014; West and Carelli, 2016). One theory that encapsulates how the NAc is involved in reward states to regulate reward-driven behavior is the "Nucleus accumbens activity hypothesis" (Carlezon and Thomas, 2009). This hypothesis proposes that inhibition of NAc activity occurs in rewarding states while enhancement of NAc activity occurs in aversive states (Carlezon and Thomas, 2009). In support of this hypothesis, studies show transient inhibition of NAc firing in rats during self-administration of cocaine (Carelli and Deadwyler, 1994; Peoples and West, 1996), heroin (Chang et al., 1997; Lee et al., 1999), and ethanol (Janak et al., 1999). In addition, there is a similar inhibition of NAc activity when animals are presented with a sucrose reward without having to complete an operant response (Roitman et al., 2005), suggesting that NAc inhibition in response to reward is not directly related to the motor output (i.e. completing an operant task for a reward) but more likely related to the motivation to seek the reward. Based on the nucleus accumbens activity hypothesis, NAc firing should increase in order to encode aversive states. In support, viral vector-mediated overexpression of CREB, which increased neuronal excitability of medium spiny neurons (MSN's) in the NAc,

induced a conditioned place aversion to cocaine (Pliakas et al., 2001), when normally, cocaine induces a conditioned place preference (Masukawa et al., 1993; Busse and Riley, 2004). This finding suggests that elevated neuronal activity in the NAc is associated with drug aversion. Additionally, NAc neurons exhibited increased firing rate in response to the aversive taste of quinine (Roitman et al., 2005), suggesting that enhanced neuronal firing in the NAc encodes aversive stimuli. Together, there is evidence to suggest that modulation of NAc activity encodes information about the rewarding and aversive nature of the stimuli, and thus the NAc plays a crucial role in choosing the appropriate behavioral response.

Studies have shown that juvenile rats will work for access to a play partner responding more to the active lever associated with a play partner compared to the inactive lever (Achterberg et al., 2016a, 2019) and display social play-induced place preference (Calcagnetti and Schechter, 1992; Thiel et al., 2008; Trezza et al., 2009; Achterberg et al., 2016a, 2019), demonstrating that social play is a rewarding behavior for juvenile rats. According to the nucleus accumbens activity hypothesis, I predicted a decrease in NAc activation in response to social play, but instead, I observed an increase in NAc core and shell activation. However, we cannot know whether the increase in the number of *fos+ cells* is indicative of an increase in excitatory or inhibitory transmissions in the NAc. This is an important distinction because it is possible that of the NAc cells that were activated following social play exposure, some of them were GABAergic interneurons, which suppress activation of NAc MSN when stimulated (Qi et al., 2016; Wright et al., 2017; Yu et al., 2017). Therefore, although I observed an increase in NAc core (males and females) and shell (females only) activation following

social play exposure, this may reflect increased engagement of inhibitory local circuits that ultimately attenuate MSN outputs. Future experiments should focus on determining the phenotype of *fos*+ cells in the NAc core and shell since it is unclear whether the activated cells in the current study are MSN output neurons or interneurons.

Alternatively, if the observed increase in NAc core (males and females) and shell (females only) activation is reflective of an increase in activation of MSN outputs, then this suggests that NAc outputs to other brain regions within the SDMN besides the VP are involved in regulating social play behavior. In support, the NAc also projects to the ventral tegmental area (Heimer et al., 1991; Kalivas et al., 1993; O'Connell and Hofmann, 2011; Xia et al., 2011) and lateral septum (Groenewegen and Russchen, 1984; Heimer et al., 1991; Zahm et al., 2013), and both of these regions have been implicated in the regulation of social play behavior in juvenile rats. In detail, social play exposure increased activation of dopaminergic cells in the VTA in females but not in males (Northcutt and Nguyen, 2014). In the LS, social play behavior is modulated in a sex-specific manner through vasopressinergic signaling in juvenile rats (Veenema et al., 2013; Bredewold et al., 2014). Furthermore, extracellular GABA concentrations in the LS were higher in both males and females during social play compared to baseline (Bredewold et al., 2015). Therefore, if the observed social play associated increased activation of NAc core/shell in male and female juvenile rats represents GABAergic outputs to the VTA, then these outputs may inhibit VTA GABAergic interneurons, which suppress activation of VTA dopaminergic neurons (Gysling and Wang, 1983; Johnson and North, 1992; Mathon et al., 2003), thereby disinhibiting dopamine neurons in the VTA, which in turn may support the expression of social play. Furthermore, if the

observed social play associated increased activation of NAc core/shell in male and female juvenile rats represents GABAergic outputs to the LS, then this may contribute to the increased extracellular GABAergic concentrations as observed in males and females engaging in social play (Bredewold et al., 2015). Together, increased activation of NAc outputs to the LS and VTA may support the expression of social play behavior in juvenile male and female rats. Future studies should test whether modulation of NAc inputs to the LS or VTA changes extracellular concentrations of GABA and if so, determine whether NAc inputs to the LS/VTA are required for social play behavior in juvenile rats.

In the current study, the equal expression of social play in juvenile rats corresponds with sex-specific changes in the activation of the NAc core and shell. This is not the first study to show that sex-specific involvement of the NAc supports the equal expression of rewarding behaviors in adult rodents. For example, cocaine exposure was associated with a higher release of dopamine in the NAc in adult male rats compared to adult female rats (Cummings et al., 2014). Similarly, adult male mice displayed higher extracellular concentration of dopamine in the NAc shell under baseline and following social defeat stress compared to adult female mice (Campi et al., 2014). Additionally, infusions of the D1R agonist SKF38393 into the NAc shell altered social interaction with a novel mouse differently in adult male and female mice. In detail, infusions of SKF38393 did not alter the time adult male mice spent in the interaction zone with a novel mouse present (Campi et al., 2014). However, infusions of SKF38393 in adult female mice reduced the time spent in the interaction zone with a novel mouse present (Campi et al., 2014). These studies along with the current study show that in juvenile

and adult rodents, the NAc is engaged differently in males versus females and this supports the similar expression of social and non-social rewarding behaviors.

Baseline sex differences in activation of NAc inputs to VP are eliminated following social play exposure

I found that there was a baseline sex difference in the activation of VP-projecting cells in the NAc core and NAc shell. Males showed a greater number of VP-projecting cells that were active compared to females in the absence of any stimulation. Although this may suggest a sex difference in baseline activation of the VP, I found that males and females showed similar VP activation at baseline (Fig 3C). Therefore, it may be that sex-specific activation of NAc inputs to the VP supports similar VP activation at baseline. Although there are currently no studies that have reported sex differences in NAc inputs to the VP in rodents, there is evidence to suggest that there are sex differences in afferents to the NAc, potentially modulating NAc outputs in a sex-specific manner. Adult female rats showed higher spine density in the NAc core and a trend towards higher spine density in the NAc shell compared to adult males (Forlano and Woolley, 2010; Wissman et al., 2012). Adult females also showed larger spine heads in the NAc core and shell compared to adult males (Forlano and Woolley, 2010). Therefore, sex differences in cellular morphology in the NAc core and shell may suggest that adult females receive denser inputs from upstream regions than adult males, which can ultimately affect NAc outputs to downstream targets. These studies were performed in adult male and female rats and it is unclear whether the sex differences in NAc spine density observed is present in juvenile rats. However, if sex differences in cellular morphology are present in juvenile rats, then it is possible that denser inputs to the NAc

in juvenile females compared to juvenile males may contribute to the sex difference in baseline activation of VP-projecting cells observed in NAc core and shell observed in the current study.

Following social play exposure, these baseline sex differences in activation of NAc core and shell inputs to the VP are eliminated in a sex-specific manner. In the NAc core, females showed a tendency towards an increase in activation of NAc inputs to the VP following social play exposure while males showed no change. On the other hand, in the NAc shell, males showed a decrease in the activation of NAc inputs to the VP following social play exposure while females showed no change. Furthermore, for both the NAc core and shell, males showed a decrease in the percentage of activated cells that project to the VP, while females showed no change. Therefore, although there were sex differences in the activation of VP-projecting cells in the NAc core and shell under baseline conditions, these sex differences were no longer seen following social play exposure. These findings suggest that equal expression of social play behavior in juvenile rats corresponds with the similar activation of VP-projecting cells in the NAc core and shell that is achieved by increased activation of NAc core inputs to the VP in females and decreased activation of NAc shell inputs to the VP in males.

NAc subregion projections to the VP are topographically organized (Nauta et al., 1978; Heimer et al., 1991; Zahm and Heimer, 1993; Usuda et al., 1998) and reveals that the NAc core and NAc shell innervate different regions of the VP. In detail, the NAc core projects to the dorsolateral region of the VP (VPdl) while the NAc shell projects to the ventromedial (VPvm) and ventrolateral (VPvl) regions of the VP (Heimer et al., 1991; Zahm and Heimer, 1993; Zahm et al., 1999; Root et al., 2015). In turn, these subregions

of the VP also have distinct outputs. For example, the VPdl projects to is the substantia nigra (Haber et al., 1985; Groenewegen and Berendse, 1990) while the VPvm projects to the VTA (Kalivas et al., 1993; Zahm et al., 2011), and the VPvl projects to the basolateral amygdala (BLA; Carlsen et al., 1985; Haber et al., 1985; Mascagni and McDonald, 2009). Therefore, based on the sex-specific changes in activation of VP-projecting cells in the NAc core and shell, it is possible that social play behavior is regulated through two different pathways involving the NAc and VP in juvenile rats. In detail, in females, enhanced activation of the NAc core→VPdl→SN pathway may be involved in regulating social play behavior. In males, decreased activation of the NAc shell→VPvm/VPvl→VTA/BLA pathway may be involved in regulating social play behavior.

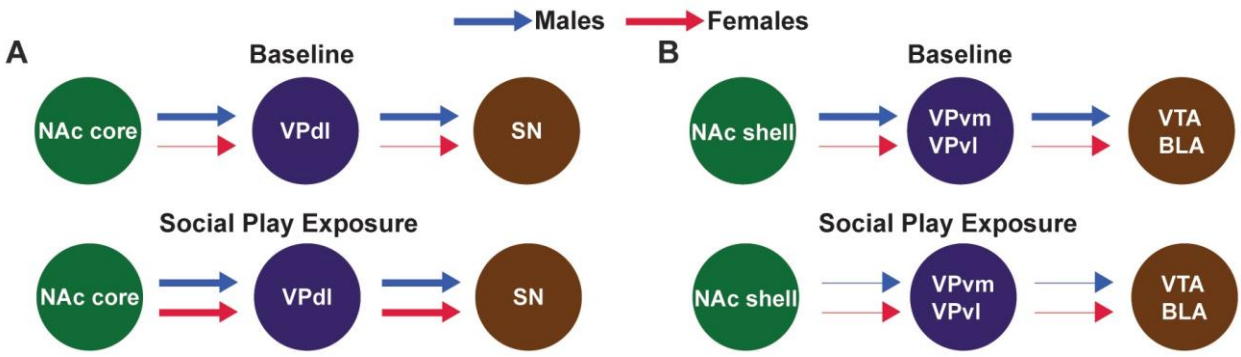

Figure 18. Proposed model of sex-specific changes in activation of VP-projecting cells in the NAc core and shell to eliminate sex differences in baseline activation of VP-projecting cells in the NAc core and shell. (A) Under baseline conditions, males show greater activation of the NAc core→VPdl→SN pathway compared to females. Following social play exposure, females show increased activation of the NAc core→VPdl→SN pathway to show similar activation of this pathway as males. (B) Under baseline conditions, males show greater activation of the NAc

Figure 18 (cont'd)

shell→VPvm/VPvl→VTA/BLA pathway compared to females. Following social play exposure, males show decreased activation of the NAc shell→VPvm/VPvl→VTA/BLA pathway to show similar activation of this pathway as females. Thickness of lines represents strength of inputs.

There are also functional differences between the NAc core and shell in regulating rewarding behaviors. For example, pharmacological inactivation of the NAc core impaired cue-induced reinstatement of food-seeking behavior, while pharmacological inactivation of the NAc shell had the opposite effect and enhanced cue-induced reinstatement of food-seeking behavior in adult male rats (Floresco et al., 2008; Ambroggi et al., 2011). In juvenile male rats, pharmacological inactivation of the NAc core increased social play behaviors while pharmacological inactivation of the NAc shell did not alter social play behaviors (van Kerkhof et al., 2013). These behavioral studies provide evidence that the NAc core and NAc shell have different roles in regulating rewarding behaviors, potentially through innervation of different brain regions.

All of these studies were conducted using juvenile and adult male rats, and based on the findings from the current study, it is likely that the involvement of the NAc in regulating rewarding non-social and social behaviors is different between males and females. Therefore, it would be essential to conduct further research including juvenile and adult females in order to gain a better understanding of how the NAc regulates rewarding behaviors in both sexes.

Conclusions

In summary, while social play exposure was associated with a similar increase in activation of the NAc core in males and females, it was associated with a sex-specific increase in the NAc shell, suggesting differential engagement of the NAc core versus shell in social play behavior. In addition, social play exposed males showed a decrease in the number of VP-projecting NAc shell cells and a decrease in the percentage of activated VP-projecting NAc core and shell cells, while females showed a tendency towards an increase in the number and the percentage of activated VP-projecting NAc core cells. Together, these findings demonstrate that the engagement of NAc inputs to the VP is different between males and females and this may have implications for the activation of the VP. Future studies should focus on elucidating the two pathways initiated by NAc core and shell outputs and how they are involved in regulating social play behavior. In addition, it would be interesting to determine whether dopaminergic inputs differentially affect the NAc core or NAc shell initiated pathways in juvenile males and females. If there are differences in the release of dopamine into the NAc core and shell, then this could be a mechanism that regulates the activation of NAc core and shell initiated pathways. Furthermore, based on the current study, it is possible that there might be sex differences in the release of other neurotransmitters such as GABA into the NAc core and shell that underlies the differential change in activation of the VP-projecting cells in these two subregions. Future studies should investigate social play-induced changes in extracellular neurotransmitter concentrations in the NAc core and shell in order to gain further understanding of how changes in neurotransmitter release can modulate the NAc core→VPdl→SN and NAc shell→VPvm/VPvl→VTA/BLA

pathways differently and whether they do so in a sex-specific manner in juvenile rats to support the expression of social play behavior.

CHAPTER 4: Overall discussion

Summary of findings

Previously for my Master's thesis, I showed that inhibition of the VP via infusions of the $GABA_A$ receptor agonist muscimol reduced social play behaviors in both juvenile male and female rats (Fig 1A-C), providing evidence that activation of the VP is required for the typical expression of social play in juvenile rats. In order to understand the mechanisms that modulate VP activity during social play, I focused on inputs from the NAc. NAc inputs to the VP are GABAergic (Walaas and Fonnum, 1979; Jones and Mogenson, 1980; Sugimoto and Mizuno, 1987) and activation of these inputs suppresses VP activity (Wang et al., 2014; Chometton et al., 2020; Clark, 2020). The NAc is divided into two subregions: the NAc core and NAc shell (Záborszky et al., 1985; Zahm and Brog, 1992), and they project to different regions of the VP (Zahm and Heimer, 1990; Heimer et al., 1991; Zahm et al., 1996). Therefore, based on my previous findings and the literature on how NAc inputs modulate VP activity, I hypothesized that inhibition of the NAc to VP pathway is required to support the typical expression of social play behavior in both males and females. Furthermore, I predicted that chemogenetic stimulation of the NAc to VP pathway would result in decreased expression of social play behaviors in both males and females and be associated with a decrease in VP activity. Confirming my prediction, I found that chemogenetic stimulation of the NAc to VP pathway reduced the duration of social play, the number of nape attacks, the number of pins, and the number of supine poses (Fig 5A-D) in both males and females. Chemogenetic stimulation of the NAc to VP pathway also decreased

activation of the VP (Fig 7F), suggesting that chemogenetic stimulation of NAc inputs to the VP suppresses VP activity.

I was then interested in determining how activation of VP-projecting cells in the NAc core and shell changed during social play exposure. My findings from my Master's thesis showed that social play exposure changed activation of the VP in a sex-specific manner. Males showed increased activation of the VP following social play exposure while females showed no change in VP activation (Fig 3C). Therefore, I hypothesized that social play would also be associated with a sex-specific change in the activation of VP-projecting cells in the NAc core and shell. Specifically, I predicted that social play exposure would decrease activation of VP-projecting cells in the NAc core and shell in males, which would reflect an increase in VP activation previously reported. On the other hand, I predicted that social play exposure would not change activation of VP-projecting cells in the NAc core and shell in females. Firstly, I found that there were no sex differences in social play behavior, with males and females showing a similar duration of social play (Fig 10A). Next, I found that social play exposure increased activation of the NAc core in both males and females (Fig 10C). On the other hand, I found a sex-specific change in activation of the NAc shell, with females showing an increase in NAc shell activation following social play exposure, while males showed no change in activation (Fig 11A). Contrary to my prediction, I found that social play exposure did not change the number of activated VP-projecting cells in the NAc core in males (Fig 12B). However, when considering the total population of activated cells in the NAc core following social play exposure, males showed a decrease in the percentage of activated cells in the NAc that project to the VP (Fig 14A). These findings

together suggest that males show an increase in activation of non-VP-projecting NAc core cells following social play exposure. On the other hand, females show a non-significant trend towards an increase in activation of VP-projecting cells in the NAc core (Fig 12B). This shows that there are sex differences in the activation of NAc core inputs to the VP during social play exposure.

Confirming my prediction, I found that social play exposure decreased the number of activated VP-projecting cells in the NAc shell in males, while no change in number of activation VP-projecting cells in the NAc shell was observed in females. In detail, social play exposure decreased the number of activated VP-projecting cells in males, and no change in the number of activated VP-projecting cells in females (Fig 13A). Likewise, there was a decrease in the percentage of activated NAc shell cells that project to the VP in males, while no change in this percentage was observed in females (Fig 15A). These findings suggest that reduced activation of NAc shell inputs to the VP is associated with the equal expression of social play in males and females.

Taken all together, although the chemogenetic manipulation of the NAc to VP pathway yielded the same effect on social play behavior in males and females, the neural mechanisms underlying the NAc to VP pathway regulation of social play behavior are sex-specific. This suggests that a potential difference in the activation of NAc inputs to the VP between males and females supports the equal expression of social play behavior. Specifically, I propose that a decrease in activation of NAc shell inputs to the VP in males and an increase in activation of NAc core inputs to the VP in females underlie the equal expression of social play behavior. Numan and colleagues were the first to propose that decreased activation of NAc inputs to the VP supports the

expression of rewarding social behaviors (Numan, 2007; Numan and Stolzenberg, 2009). This idea was based on findings from adult female rats, showing that pharmacological inactivation of the VP, but not the NAc, reduced the expression of maternal behaviors (Numan et al., 2005b). In the current study, I provide the first evidence to support the hypothesis proposed by Numan and colleagues by showing that enhanced activation of the NAc to VP pathway reduces the expression of social play behavior in juvenile male and female rats. In addition, I showed that the NAc to VP pathway is involved in the sex-specific regulation of social play behavior in juvenile rats. My findings suggest that sex-specific modulation of the NAc to VP pathway is a crucial mechanism to support the equal expression of social play behavior between males and females. Indeed, it has been proposed that sex differences within the brain may prevent sex differences in the overt expression of behaviors (De Vries, 2004).

D1- and D2-medium spiny neuron projections from the NAc to the VP: involvement in regulating rewarding behaviors

Dopaminergic (DA) signaling in the NAc has been shown to regulate social play behaviors in juvenile rats via activation of dopamine 1 (D1) or dopamine 2 (D2) receptors (Manduca et al., 2016b; Kopec et al., 2018). Intra-NAc infusions of amphetamine and apomorphine, both of which enhance DA signaling, increased the expression of social play behavior in juvenile male rats, as reflected by an increase in the frequency of pinning and pouncing (Manduca et al., 2016b). Intra-Nac infusions of either SCH-23390, the specific D1 receptor antagonist, or eticlopride, the specific D2 receptor antagonist, into the NAc decreased social play behaviors in juvenile male rats (Manduca et al., 2016b). A limitation from the above mentioned study is that the drugs

were infused at the border of the NAc core and shell (Manduca et al., 2016b), and therefore it is likely that the drugs affected DA receptors expressed in both NAc core and shell. Furthermore, this study was conducted only in juvenile male rats, and therefore it is unknown whether manipulation of DA signaling in the NAc will have the same effects in juvenile female rats. Hence, it is difficult to determine whether the role of D1 and D2 receptors in the NAc core and shell are similar or different between sexes.

The NAc is primarily composed of medium spiny neurons (MSN's; (Wilson and Groves, 1980; Gerfen, 1992), which express D1 or D2 receptors (Gerfen, 1992; Curran and Watson, 1995; Lu et al., 1998). DA receptors in the NAc are G protein-coupled receptors that mediate responses to neurotransmitter stimulation (Rosenbaum et al., 2009). D1 receptors in the NAc are paired with stimulatory G_s and G_{olf} proteins (Iwamoto et al., 2003; Neve et al., 2004; Yano et al., 2018) that increase calcium currents in the cells leading to depolarization (Surmeier et al., 1995; Neve et al., 2004). Furthermore, D1 receptor stimulation in the NAc decreases activation of GABA receptors expressed on MSN's (Nicola and Malenka, 1998; Flores-Hernandez et al., 2000). Therefore, activation of D1 receptors expressed on accumbens MSN has an overall stimulatory/excitatory effect. On the other hand, D2 receptors in the NAc are paired with G_i proteins (Neve et al., 2004) that attenuate calcium currents in cells leading to hyperpolarization (Hernandez-Lopez et al., 2000; Neve et al., 2004). Hence, stimulation of D2 receptors expressed on accumbens MSN has an overall inhibitory effect. Both D1-MSN's and D2-MSN's project to the VP (Lu et al., 1998; Smith et al., 2013; Gallo et al., 2018; Pardo-Garcia et al., 2019; Yao et al., 2021; Correia et al., 2023), and stimulation of D1-MSN and D2-MSN terminals in the VP decrease the firing rate of VP

GABAergic neurons (Correia et al., 2023). D1-MSN and D2-MSN projections to the VP are also involved in regulating a variety of behaviors, such as anxiety-like behaviors (Correia et al., 2023), sucrose consumption and preference (Yao et al., 2021), cocaine-seeking (Pardo-Garcia et al., 2019; Inbar et al., 2022) and food-seeking behaviors (Gallo et al., 2018). Optogenetic stimulation of D1-MSN terminals in the VP induced a conditioned place aversion to the chamber associated with laser stimulation, while the same manipulation on D1-MSN terminals in the ventral mesencephalon induced a conditioned place preference for the laser-associated chamber (Liu et al., 2022). Furthermore, optogenetic stimulation of D1-MSN terminals in the VP reduced cocaine conditioned place preference (Liu et al., 2022). Therefore, stimulation of D1-MSN projections to the VP induces aversive states. Optogenetic stimulation of D2-MSN terminals in the VP, originating from the ventromedial (vmNAc shell) and ventrolateral (vlNAc shell) NAc shell, decreased sucrose consumption (Yao et al., 2021). Furthermore, optogenetic stimulation of D2-MSN terminals in the VP, originating from the NAc core, increased anxiety-like behaviors such as less time spent in the open arm during the open arm test and light zone during the light-dark box test (Correia et al., 2023) in adult mice. On the other hand, chemogenetic silencing of D2-MSN terminals in the VP increased the motivation for food-seeking behaviors, as evidenced by an increase in lever press responses and breakpoint for a food reward (Gallo et al., 2018) in adult mice. Based on these studies, it is possible that DA acting on D1R expressed on VP-projecting MSN attenuates rewarding behaviors presumably by suppressing VP activity through increased GABAergic inputs. On the other hand, DA acting on D2R

expressed on VP-projecting MSN facilitates the expression of rewarding behaviors by enhancing VP activation through inhibiting GABAergic inputs (Fig 19B).

GABAergic neurons in the VP receive inputs from both D1- and D2-MSN's (Yao et al., 2021). Chemogenetic inhibition of GABAergic neurons in the VP reduced sucrose consumption (Yao et al., 2021), induced a real time place avoidance to laser stimulation (Faget et al., 2018; Yao et al., 2021), and attenuated remifentanil seeking (Farrell et al., 2022). Chemogenetic stimulation of VP GABAergic neurons augmented remifentanil seeking (Farrell et al., 2022). Therefore, inhibition of GABAergic VP neurons is capable of inducing aversion to rewarding stimuli while stimulation of these neurons reinforce the rewarding properties of the stimuli. In the current study, I found that chemogenetic stimulation of NAc terminals in the VP reduced social play behavior in both juvenile male and female rats, possibly due to inhibition of VP^{GABA} neurons. Therefore, I propose that activation of D2R's expressed on VP^{GABA}-projecting MSNs will attenuate inhibitory input to VP^{GABA} neurons, thereby disinhibiting them in order to facilitate the expression of social play behavior in juvenile male and female rats. Future studies should determine whether NAc to VP pathway regulation of social play is mediated by D1R or D2R expressed on VP-projecting MSNs. Furthermore, studies should investigate the phenotype of VP cells that receive inputs from NAc D2R-MSNs and using genetic tools, specifically manipulate the NAc^{D2R} to VP pathway in order to determine whether this pathway regulates the expression of social play behavior. Furthermore, there should be focus on whether this pathway regulates social play in a sex-specific manner in juvenile rats.

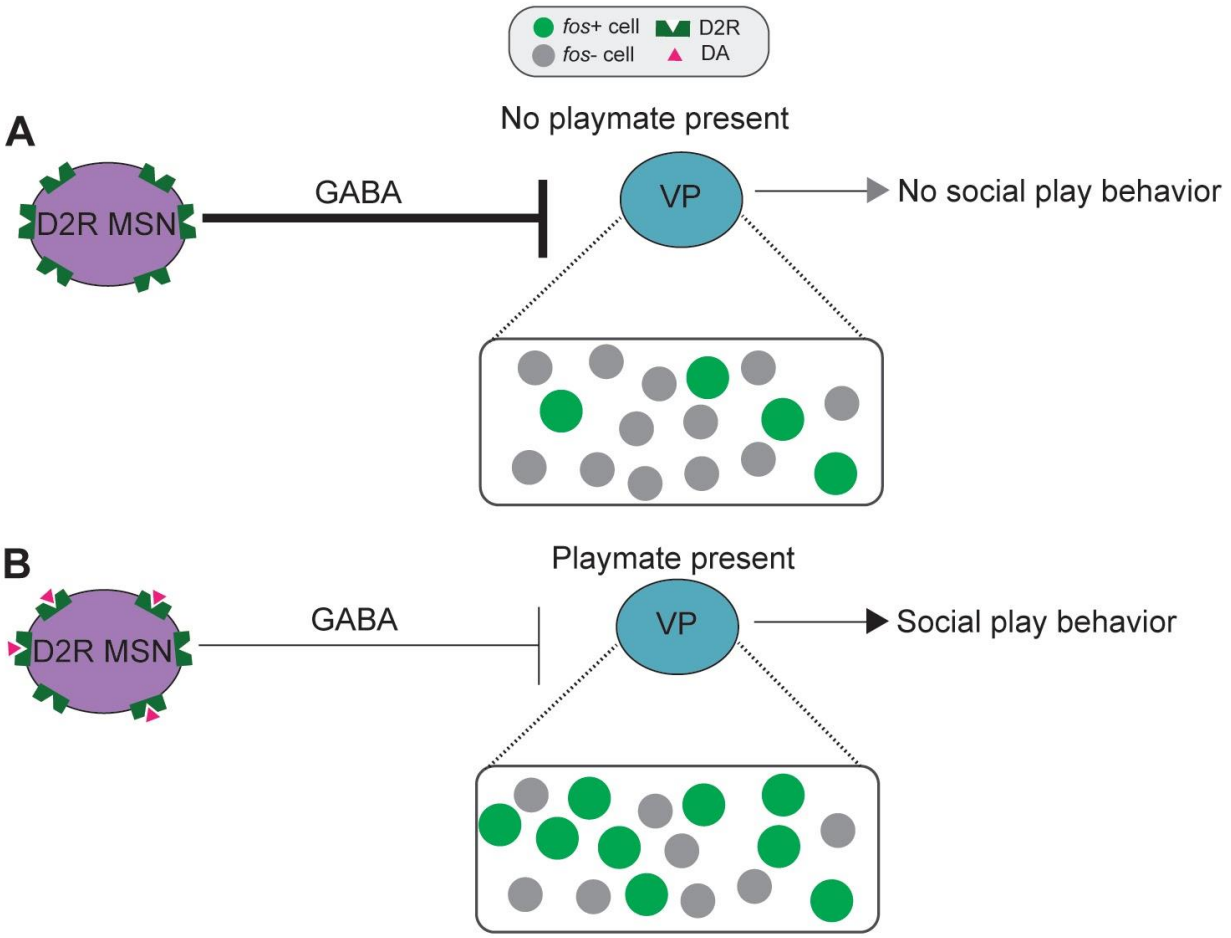

Figure 19. Proposed model of the involvement of the NAc^{D2R} to VP pathway in regulating social play behavior in juvenile male and female rats. (A) Under baseline conditions, inactivation of D2R expressed on VP-projecting MSNs maintains GABAergic inputs to the VP, thereby suppressing VP activity. Suppression of VP activity prevents the typical expression of social play in juvenile rats. (B) The facilitation of the typical expression of social play behavior is supported through activation of D2R expressed on VP-projecting MSNs. Activation of D2Rs will hyperpolarize VP-projecting MSNs and presumably decrease GABAergic inputs to the VP. This will disinhibit the VP and support the expression of social play behavior in juvenile male and female rats.

VP^{GABA} cells make synaptic connections with VTA DA cells and decrease population activity of VTA^{DA} cells (Floresco et al., 2003). Inactivation of VP^{GABA} inputs to the VTA caused an increase in extracellular DA levels in the NAc (Floresco et al., 2003), demonstrating that VP^{GABA} inputs to the VTA affects tonic DA signaling in the NAc. Given that DA signaling in the NAc supports the expression of social play (Manduca et al., 2016b), it is possible that inhibition of the VP^{GABA} to VTA pathway, which increases extracellular DA concentration in the NAc, is involved in regulating social play behaviors in juvenile male and female rats. Furthermore, it is possible that sex-specific modulation of DA signaling in VP^{GABA}-VTA^{DA}-NAc circuit may be an essential mechanism that underlies the regulation of social play in juvenile rats. Although there are no current studies demonstrating sex differences in DA release in the NAc in juvenile rats, there are some studies that suggest DA release may be different between adult male and female rats. Estradiol benzoate acting on estradiol receptor beta (ER_{β}) enhanced DA release in the NAc shell in females, but not in males (Yoest et al., 2019). In addition, DA release is sensitive to housing conditions in females, such that pair-housed females showed greater DA release in response to electrical stimulation in the NAc shell compared to single-housed females (Gonzalez et al., 2023). On the other hand, housing conditions had no effect on DA release in males (Gonzalez et al., 2023). These findings show that although adult male and female rats were exposed to similar manipulations, there were differences in DA release in the NAc, suggesting that the sex-specific release may underlie the equal expression of rewarding behaviors. Therefore, sex-specific modulation of DA release in the NAc may underlie the equal expression of social play behavior in juvenile rats. In detail, I propose that my previous observation of

increased VP activation in males, but not females, following social play may lead to decreased DA release in the NAc in males (Fig 20A), while preserving tonic DA release in the NAc in females (Fig 20B).

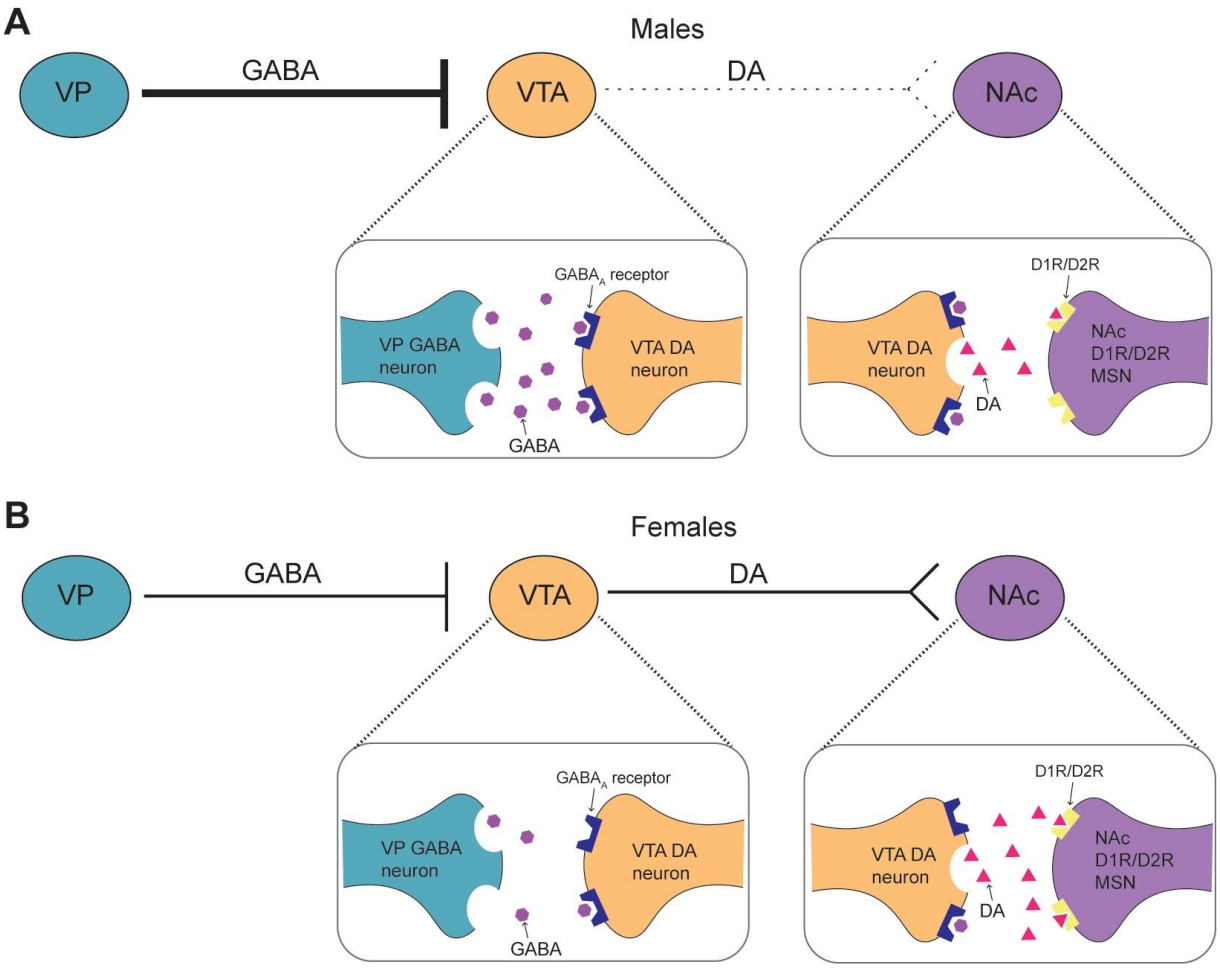

Figure 20. Proposed sex-specific circuit within the mesolimbic reward system involved supporting the equal expression of social play behavior in juvenile male and female rats. (A) In order to facilitate the similar expression of social play behavior to females, increased activation of VP^{GABA} neurons that project to the VTA in males decreases activation of VTA^{DA} neurons. This in turn results in decreased extracellular release of DA in the NAc. (B) For females, baseline activation of VP^{GABA} neurons that project to the VTA maintains baseline activation of VTA^{DA} neurons. This will in turn

Figure 20 (cont'd)

result in no change in extracellular release of DA in the NAc compared to baseline levels. Therefore, sex-specific modulation of the VP^{GABA}→VTA^{DA}→NAc pathway, resulting in decreased DA release in the NAc of males only, may be involved in supporting the equal expression of social play in juvenile rats.

Arkypallidal neurons in the VP suppress NAc shell neuron activity

Findings from Chapter 3 show that in the NAc shell, males displayed decreased activation of VP-projecting cells following social play exposure. In my Master's thesis, I showed that males displayed an increase in VP activation following social play exposure (Fig 3C). Together, these findings provide evidence suggesting that social play exposure is associated with decreased GABAergic input from the NAc shell, resulting in disinhibition of the VP. In the current study, although the number of *fos*+ cells is a proxy for the number of cells that were activated following social play exposure, it is unclear what specific cell types were activated. Hence, the *fos*+ cells in the VP could have represented activated interneurons, activated output neurons, or activated ventral arkypallidal neurons (vArky). vArky neurons in the VP project to the NAc shell (Vachez et al., 2021) and it remains unclear whether vArky neurons also project to the NAc core.

VP vArky neurons are predominantly GABAergic (86%; Vachez et al., 2021) and provide greater monosynaptic inputs to NAc shell MSNs than to NAc shell interneurons (Vachez et al., 2021). Furthermore, 84% of D1-MSNs and 79% of D2-MSNs in the NAc shell receive monosynaptic inputs from vArky neurons (Vachez et al., 2021). Optogenetic inhibition of the NAc shell increased drinking time and drinking bout length of a sucrose solution in adult male and female mice (Vachez et al., 2021), providing

evidence that inhibition of the NAc shell is associated with reward consumption. Therefore, inhibitory inputs from vArky neurons to the NAc shell may be a source of NAc shell inhibition that facilitates reward consumption. Indeed, optogenetic inhibition of vArky terminals in the NAc shell, which increased NAc shell firing rate, decreased total drinking time and drinking bout lengths of a sucrose solution in adult male and female mice (Vachez et al., 2021). Together, these findings suggest that reward-related inhibition of the NAc shell can be facilitated through increasing vArky inputs to the NAc shell.

Although this study was performed in adult mice using a sucrose reward (Vachez et al., 2021), it is possible that inhibition of NAc shell cells through VP vArky inputs may be involved in the regulation of overall rewarding behaviors. Based on the findings from my Master's thesis, I propose that in juvenile male rats, social play-induced increase in activation of the VP may reflect an increased activation of vArky neurons, which inhibits VP-projecting cells in the NAc shell in order to support the expression of social play (Fig 21B). If so, this would suggest that the VP vArky neurons receive information about the rewarding stimulus earlier than the NAc in order to suppress NAc inputs to the VP to support the expression of rewarding behaviors. Indeed, there is evidence that shows reward-specific firing is present earlier in the VP than in the NAc (Ottenheimer et al., 2018). Future studies should determine whether VP vArky neurons display earlier firing activity than VP-projecting NAc cells when exposed to social play in order to provide evidence of the involvement of this pathway in regulating social play behavior in juvenile rats. Furthermore, I propose that this mechanism functions in a sex-specific manner. Findings from Chapter 3 show that females displayed no change in activation of VP-

projecting cells in the NAc shell following social play exposure. In addition, in my Master's thesis, I showed that social play exposure did not alter the activation of VP in females. Therefore, the vArky to NAc shell pathway may function in a sex-specific manner, such that activation of this pathway is involved in the expression of social play in males (Fig 21B), but not in females (Fig 21C).

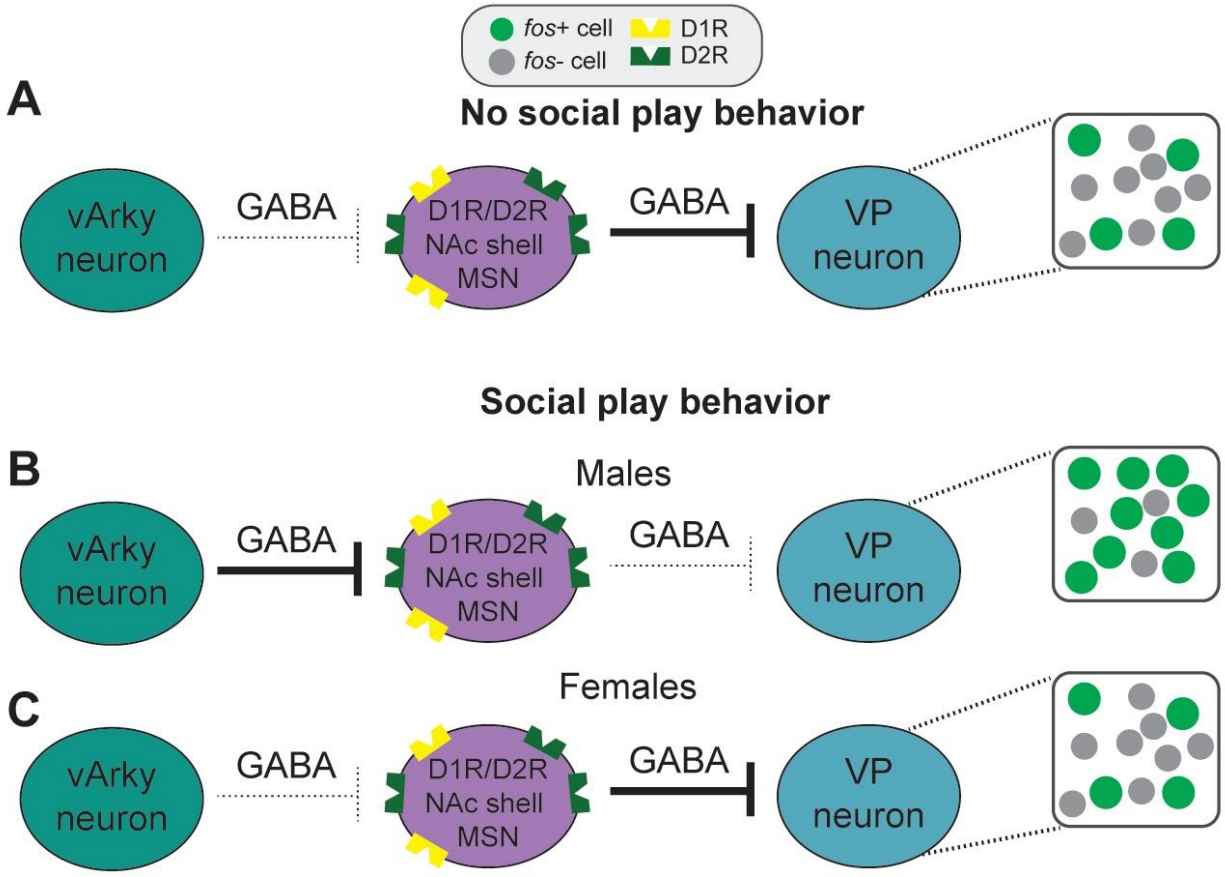

Figure 21. Proposed vArky to NAc shell pathway in the sex-specific regulation of social play behavior in juvenile rats. (A) NAc shell MSN suppresses VP activation through GABAergic inputs when there is no rewarding social stimulus present in juvenile male and female rats. (B) In males, reward-related inhibition of the NAc shell in the presence of a rewarding social stimulus is facilitated through increased GABAergic inputs to the NAc shell from vArky neurons. Inhibition of NAc shell MSNs disinhibits the

Figure 21 (cont'd)

VP, resulting in increased VP activation compared to baseline conditions, in order to support the expression of social play. (C) On the other hand, in the presence of a rewarding social stimulus, females show no change in activation of vArky inputs to the NAc shell, thereby maintaining similar VP activation compared to baseline conditions in order to facilitate the equal expression of social play behavior as males.

Other inputs to the VP: involvement of vasopressin inputs to the VP in the sex-specific regulation of social play behavior in juvenile rats

The neuropeptide arginine vasopressin (AVP) is involved in regulating a wide variety of social behaviors across the lifespan in many species across including juvenile (Veenema et al., 2012, 2013; Bredewold et al., 2014; Paul et al., 2014) and adult (Le Moal et al., 1987; Dantzer et al., 1988; Dluzen et al., 1998; Bychowski et al., 2013; DiBenedictis et al., 2020) rats. In juvenile rats, AVP acting in the lateral septum (LS) regulates social play behavior in a sex-specific manner (Veenema et al., 2013; Bredewold et al., 2014), such that pharmacological blockade of AVP 1a receptors (V1aR) increased social play behaviors in juvenile males, but decreased these behaviors in juvenile females. AVP inputs to the LS originate from the bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA) in rats (de Vries et al., 1981; Caffé et al., 1987; De Vries and al-Shamma, 1990; Compaan et al., 1993), with robust sex differences in LS-AVP innervation observed in juvenile and adult rats (de Vries et al., 1981; DiBenedictis et al., 2017; Smith et al., 2017). These sex differences in AVP innervation to the LS may contribute to the observed sex differences in LS-AVP-mediated regulation of social play behavior.

Recently, I found that *avp*-expressing cells in the posterior BNST and posterodorsal medial amygdala (MePD) project to the VP in juvenile rats, suggesting that AVP signaling in the VP may also regulate social play behaviors. Furthermore, AVP acting in the VP regulates social play behavior sex-specifically in juvenile rats. Indeed, I showed that pharmacological blockade of V1aR's in the VP (Lee et al., 2021) had a similar sex-specific effect on social play behaviors in juvenile rats as seen in the LS (Veenema et al., 2013; Bredewold et al., 2014). That is, V1aR blockade in the VP increased social play behaviors in males but decreased them in females. Additionally, I found robust sex differences in the structural organization of the AVP system in the VP, suggesting that similar to the LS, these structural sex differences may be functionally associated with the sex-specific regulation of social play in juvenile rats.

It is possible that AVP is co-released with glutamate or GABA within the brain. In adult male rats, AVP cells in the BNST and MePD express *slc32a1*, a marker for the GABA transporter VGAT (Zhang et al., 2020). AVP cells in the BNST and MePD did not express *slc17a7* or *slc17a6*, that are markers for the glutamate transporters VGLUT1 and VGLUT2, respectively (Zhang et al., 2020). These findings suggest that there is a high probability that AVP originating in the BNST and MePD is co-released with GABA in the VP. If so, then BNST/MePD inputs to the VP may have a similar effect as NAc inputs to the VP, in that they suppress VP activation through AVP and GABA co-release. Therefore, sex-specific modulation of the BNST/MePD to VP pathway may support the typical expression of social play behavior in juvenile rats. Based on the effects of V1aR blockade in the VP found in juvenile rats (Lee et al., 2021), it is likely that AVP signaling in the VP suppresses social play behavior in males in order to

support the equal expression of social play behavior. Therefore, I propose that activation of the BNST/MePD to VP pathway in males (Fig 22B) facilitates the similar expression of social play behavior compared to females (Fig 22C). Indeed, social play exposure increased activation of *v1aR*+ cells in the VP of males, but induced no change in activation of *v1aR*+ cells in females (Lee et al., 2021). This finding suggests that social play may be associated with a greater release of AVP, and co-release of GABA, in the VP of males but not in females. Future studies should determine whether BNST/MePD terminals co-release AVP and GABA and whether the extracellular concentrations of AVP and GABA change during social play in males and females. These studies will further our understanding of how GABAergic and vasopressinergic inputs in the VP from various sources, including the NAc, BNST, and MePD, contribute to the sex-specific regulation of social play behavior in juvenile rats.

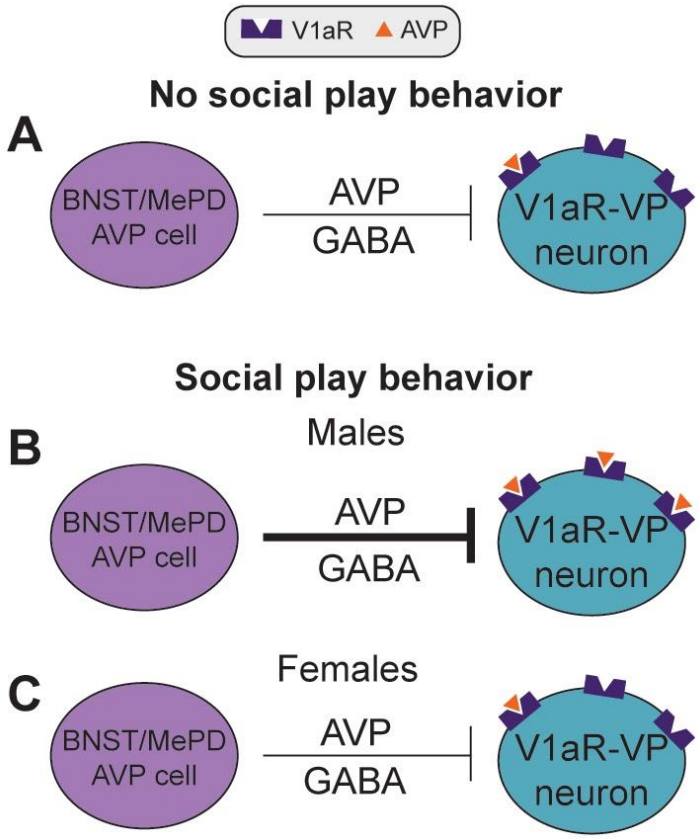

Figure 22. Proposed involvement of BNST/MePD^{AVP} to VP pathway in the sex-specific regulation of social play behavior in juvenile male and female rats. (A) BNST/MePD cells that project to the VP may co-release AVP and GABA into the VP. In order to support the equal expression of social play behavior, a sex-specific change in activation of the BNST/MePD^{AVP} to VP pathway may be involved. (B) In males, increased activation of this pathway may result in increased extracellular concentrations of AVP and GABA. In turn, this may lead to enhanced activation of V1aR-expressing cells in the VP. (C) In females, baseline activation of the BNST/MePD^{AVP} to VP pathway may be sufficient to support the expression of social play.

Conclusions

This body of work provides evidence that the NAc to VP pathway regulates social play behavior in juvenile male and female rats. I found that chemogenetic stimulation of the NAc to VP pathway reduced social play behaviors in both males and females, suggesting that inhibition of this pathway is required for the typical and similar expression of social play in both sexes (Chapter 2). I further found that there were baseline sex differences in the activation of VP-projecting cells in the NAc core and shell, which were eliminated following social play exposure (Chapter 3). This suggests that the equal expression of social play behavior is facilitated through equalizing the activation of VP-projecting cells in the NAc core and shell in males and females. However, the elimination of the sex differences is achieved in sex-specific ways, such that females showed a trend towards an increase in activation of VP-projecting cells in the NAc core, while males showed a decrease in activation of VP-projecting cells in the NAc shell. These findings suggest that NAc inputs to the VP may regulate social play behavior in males and females through two different pathways: in males, a predominant NAc shell to VP pathway is involved while in females, a predominant NAc core to VP pathway is involved. The NAc core and shell project to different subregions in the VP (Heimer et al., 1991; Zahm and Heimer, 1993), which modulate subregion-specific outputs of the VP (Carlsen et al., 1985; Haber et al., 1985; Zahm et al., 2011). Therefore, future studies should determine whether social play behavior is regulated by subregional NAc to VP pathways in juvenile male and female rats, and whether there are distinct mechanisms, such as DA, AVP, or GABA, involved in regulating NAc core and NAc shell initiated pathways.

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