Development of oxytocin, vasopressin V1a, and mu-opioid receptor expression in the rat brain: Implications for the regulation of juvenile social novelty-seeking behavior

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DEVELOPMENT OF OXYTOCIN, VASOPRESSIN V1a, AND μ-OPIOID RECEPTOR EXPRESSION IN THE RAT BRAIN: IMPLICATIONS FOR THE REGULATION OF JUVENILE SOCIAL NOVELTY-SEEKING BEHAVIOR

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DEVELOPMENT OF OXYTOCIN, VASOPRESSIN V1a, AND μ-OPIOID RECEPTOR EXPRESSION IN THE RAT BRAIN: IMPLICATIONS FOR THE REGULATION OF JUVENILE SOCIAL NOVELTY-SEEKING BEHAVIOR

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Across species, the juvenile period is characterized by increased social interaction with peers and heightened novelty-seeking behavior, as compared to any other life stage. These behaviors are likely to be highly adaptive during this developmental phase. Still, an excessive novelty-seeking phenotype may predispose individuals to risk-taking and substance abuse, while too little social engagement and low novelty-seeking are characteristics of neuropsychiatry disorders such as autism. The over-arching aim of this dissertation research has been to elucidate the neural mechanisms underlying juvenile social novelty-seeking behavior. Central activation of oxytocin, vasopressin V1a, and μ-opioid receptors (OTR, V1aR, and MOR, respectively) have been implicated in the regulation of adult social behavior, but our understanding of the expression and function of OTR, V1aR, and MORs in the juvenile brain is incomplete. Therefore, in Studies 1 and 2, age differences in binding density of OTR, V1aR, and MOR throughout the rat brain were identified using receptor autoradiography. Next, in Study 3, I established the social novelty preference test, a new paradigm designed to assess the preference of juvenile rats to interact with either a novel or a familiar (cage mate) conspecific. Using this social novelty preference test, in Studies 3, 4, and 5, the functional involvement of OTR, V1aR, and MOR in the regulation of juvenile social novelty preference was characterized using both intracerebroventricular and local in-vivo pharmacological

manipulations. The results of these experiments demonstrate that both OTR and MOR activation in the brain are involved in the regulation of juvenile social novelty preference, particularly acting within the nucleus accumbens. Finally, in Study 5, I investigated the impact of social isolation on juvenile social novelty preference. My findings show that social isolation potently reduces social novelty preference, which, in turn, can be restored by MOR activation in the nucleus accumbens. Taken together, this body of work significantly advances our understanding of the neural systems underlying juvenile social novelty preference, and suggests that both oxytocin and opioid systems in the brain may be potential clinical targets for restoring social novelty-seeking behavior in neurodevelopmental disorders, such as autism.

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Chapter One: General Introduction

As a highly social species, social behavior is not only critical for reproduction, it is essential to our health and wellbeing throughout the lifespan. The presence of strong social bonds and inclusion in social networks is a potent predictor of health and longevity (Yang et al., 2015; Isaacowitz et al., 2003). On the other hand, social separation and loneliness have been associated with a number of negative physical and mental health outcomes and have even been shown to increase the risk of mortality by upwards of a staggering 50% (Holt-Lunstad et al., 2010; Cacioppo & Cacioppo, 2014; Luo et al., 2012). Moreover, deficits in social functioning are observed in many, if not all, neuropsychiatric disorders including autism spectrum disorders (ASD) and schizophrenia (American Psychiatric Association, 2013). Therefore, it is imperative that we gain a better understanding of the neural mechanism underlying social behavior so that we can provide better treatment outcomes for patients suffering from these disorders.

While social behavior repertoires vary widely across species, the importance of social behavior to survival remains central. Indeed, rank within a social hierarchy determines access to food, territory, and potential mates, as well as stress levels, in species ranging from cichlid fish and mice to non-human primates and humans (Fernald & Maruska, 2012; So et al., 2015; Sapolsky, 2005; Wilkinson, 2000). Moreover, in species with colony structures, such as ants and naked-mole rats, survival depends on the combined efforts of all members of the colony (Czechowski & Godzinska, 2015; Faulkes & Bennett, 2013). Positive social relationships have also been shown to have a powerful stress buffering effect in numerous species (Ishii et al., 2016; Sullivan & Perry, 2015).

The neural structures underlying social behavior are highly conserved across species. In 1999, the social behavior neural network (SBNN) was proposed as a reciprocally interconnected set of brain regions involved in the regulation of a diverse range of social behaviors in the mammalian brain (Newman, 1999). More recent evidence suggests that the SBNN is a network designation with remarkable homology and relevance across vertebrate species (O'Connell & Hofmann, 2011; 2012). It has been proposed that activation across these interconnected nodes of the SBNN leads to the expression of social behavior (Newman, 1999; O'Connell & Hofmann, 2012) and that differences in the *relative* activation across this network lead to the display of distinct forms of social behavior, such as aggression, sexual behavior, and parental behavior (Newman, 1999; Goodson et al., 2005; Crews et al., 2006). Given the conservation across species in the neural systems underlying social behavior, studies in mammals other than humans, such as rats and mice, have the potential to lend valuable insight into the brain mechanisms underlying human social behavior.

Importantly, the expression, frequency, and regulation of social behavior may differ across the life span. The juvenile period, in particular, is one during which social interactions with peers are of critical importance (Spear et al., 2000; Somerville, 2013). Indeed, across species, this period is characterized by increased time spent in social interaction with peers as compared to younger or older ages, and increased susceptibility to social influence (Spear et al., 2000; Casey et al., 2008; Somerville, 2013). Animals also tend to be more exploratory during this life phase, as well as to engage in more risk-taking and novelty-seeking behaviors (Doremus-Fitzwater et al., 2010). While this novelty-seeking behavior is likely to be highly adaptive, too much or too little novelty-

seeking can have negative consequences. For example, an excessive novelty-seeking phenotype is associated with risk-taking and drug abuse (Wang et al., 2015; Wills et al., 1998; Dellu et al., 1996; Hittner et al., 2006; Kelly et al., 2006), while low social novelty-seeking behavior is a symptom of ASD and may contribute to deficits in social interaction in individuals with ASD (Anckarsater et al., 2006; American Psychiatric Association, 2013). Therefore, the overarching aim of my dissertation work has been to understand the neural mechanisms underlying this juvenile social novelty-seeking behavior. Of note, we here define the juvenile period as approximately postnatal day 28 to postnatal day 40 in the rat. While it is difficult to directly compare life stages between animal species as distinct as rats and humans, this timeframe has been suggested to correspond roughly to the peri-pubertal period in humans, extending from approximately 10 to 18 years of age (Spear et al., 2000).

The expression and/or regulation of social behavior is also largely influenced by the sex of the individual. Males and females differ in the forms of social behavior that they display, as well as in the neural mechanisms regulating those behaviors (Dumais & Veenema, 2016; Galea et al., 2016; Bayless & Shah, 2016). For example, females engage in maternal behavior following parturition, while, in the vast majority (95%) of mammalian species, males do not participate in parental care of offspring (Numan & Young, 2016). Similarly, during mating, males display mounting behaviors (Hull & Domingues, 2007), while females engage in lordosis (Harlan et al., 1984; Segovia & Guillamon, 1993). Even social behaviors that are displayed by both males and females can differ by degree. For example, in rats, males show greater social interest (as measured by time spent in social investigation) as compared to females (Dumais et al.,

2016). Furthermore, many neuropsychiatric disorders, such as ASD, depression, anxiety, and schizophrenia show sex differences in prevalence, symptom severity and response to therapeutic treatment (Fombonne et al., 2003; Li & Graham, 2017). Many of these disorders have a pre-pubertal or pubertal age of onset. Therefore, it is critical that we gain a better understanding of sex differences in the brain and behavior in pre-pubertal animals. Throughout my dissertation research I am to address sex differences by including both males and females in my experimental design, unless the exclusion of one sex can be justified based on current results.

Social exclusion has both acute and long-lasting effects on social behavior, particularly during the juvenile period. In humans, peer victimization or bullying during the juvenile period are risk factors for the development of anxiety and depression (Fahy et al., 2016; Merrill & Hanson, 2016; Hager & Leadbeater, 2016), while friendships and family support can provide a buffer for adolescents who are at risk for depression (van Harmelen et al., 2016). In juvenile rats, long-term isolation from social peers (on the order of weeks) leads to alterations in adult social behavior including reduced social interaction, abnormal aggression, and impaired sexual behavior (Niesink & van Ree, 1982; Van den Berg, et al., 1999a,b,c; Hol et al., 1999; Gerall, et al., 1967; Gruendel & Arnold, 1969; Toth et al., 2011). In contrast, short-duration or acute social isolation (on the order of hours to days) during the juvenile period actually stimulates both social interaction and social play behaviors, immediately after isolation in male and female juvenile rats (Panksepp & Beatty, 1980; Varlinskaya et al., 1999; Varlinskaya & Spear, 2008). These findings demonstrate that social isolation is a powerful modulator of social behavior. Yet, much less is known regarding how changes to social context, such as

separation from familiar peers, might influence behavior in situations in which individuals are provided with a choice of social partners (such as a novel or a familiar conspecific). Therefore, understanding how social separation alters social novelty preference is one of the aims of my dissertation.

Within the brain, several signaling molecules have been implicated in the pathways modulating social behavior. Among these, oxytocin (OXT) and vasopressin (AVP) have been particularly well-studied. These neuropeptides are closely related (they differ in structure by only two amino-acids) and both are synthesized mainly in the paraventricular and supraoptic nuclei of the hypothalamus (Sofroniew & Weindl, 1978; Sofroniew 1980). AVP is also synthesized in the bed nucleus of the stria terminalis and medial amygdala (Caffe et al., 1987; DeVries et al., 1981). Oxytocin receptors (OTR) and vasopressin V1a receptors (V1aR) are widely expressed in the adult brain (Gimpl & Fahrenholz, 2001; Tribollet et al., 1990, Tribollet et al., 1998; Shapiro & Insel, 1989; Dumais et al., 2013; Dumais & Veenema, 2016). By activating these receptors, OXT and AVP have been shown to regulate a wide array of social behaviors, including parental behavior, aggression, pair bond formation, and peer affiliation, across phylogeny (Veenema & Neumann, 2008; Goodson & Kabelik, 2009; Albers, 2015). Importantly, OXT and AVP are currently regarded as potential therapeutic targets in the treatment of neurodevelopmental disorders such as ASD (Modi & Young, 2012; Meyer-Lindenberg et al., 2011; Guastella & Hickie, 2016). However, our understanding of the structure and function of OXT and AVP systems in the juvenile brain is incomplete. Furthermore, while previous work from our lab has demonstrated the presence of sex differences in OTR and V1aR binding densities in the adult rat brain (Dumais et al., 2013; Dumais &

Veenema, 2016), it is unclear whether these sex differences are already present during the juvenile period. Elucidating age and sex differences in the density of OTR and V1aR in the brain may aid us in understanding the age- and sex- specific roles of activation of these receptors in the regulation of social behavior.

The opioid system in the brain has also been implicated in the neural regulation of social behavior. In particular, the μ opioid receptor (MOR) is a well-established component of the neural systems underlying the hedonic or "pleasurable' nature of rewarding stimuli, including social interactions (Selleck & Baldo, 2017; Le Merrer et al., 2009; Berridge & Kringelbach, 2015). In animal models, MOR activation has been shown to facilitate social interaction in juvenile mice (Cinque et al., 2012), social play behavior in juvenile rats (Panksepp et al., 1980, 1985; Beatty & Costello, 1982; Vanderschuren et al., 1995a,b, c; Trezza & Vanderschuren, 2008; Trezza et al., 2011), and pair-bond formation in adult prairie voles (Resendez et al., 2013). In humans, μ-opioid receptor activation enhanced pleasure derived from positive social interactions (Chelnokova et al., 2014, Hsu et al., 2013). Importantly this effect was not observed in patients with major depression (Hsu et al., 2015), suggesting that reduced opioid activity may underlie reduced pleasure derived from positive social interactions. Furthermore, alterations in opioid activity have been suggested to underlie impaired social interest in ASD (Sahley & Panksepp, 1987, Oddi et al., 2013; Moles et al., 2004; Becker et al., 2014). While MOR activation has been shown to regulate social behavior during the juvenile period as discussed above, virtually nothing is known regarding MOR expression in the juvenile rat brain (Kornblum et al., 1987).

The specific aims of this dissertation have been three-fold. In the first aim, I sought to characterize age and sex differences in the expression of OTR, V1aR, and MOR in the rat brain. To this end, in Study 1, I used receptor autoradiography to measure the binding density of OTR and V1aR in the brains of juvenile and adult, male and female rats, with a particular emphasis on brain regions that are considered part of the social decision-making network. In Study 2, I used the same methodology to assess age and sex differences in MOR binding density across regions of the rat brain.

In the second aim, I sought to determine the causal involvement of OTR, V1aR, and MOR activation in the regulation of juvenile social behavior. Therefore, in Study 3, I developed a social novelty preference test in which to assess social novelty-seeking behavior, i.e. the preference of a juvenile rat to interact with either a novel or a familiar (cage mate) conspecific. I then used pharmacological manipulations to centrally block OTRs, V1aRs, or MORs, and determine the impact of these manipulations on social novelty-seeking behavior in juvenile male rats. Subsequently, in Study 4, I used pharmacological manipulations to test the involvement of OTR in either the nucleus accumbens, lateral septum, or basolateral amygdala in the regulation of social novelty-seeking behavior in juvenile male rats. Similarly, in Study 5, I used this methodology to assess the role of MORs in the nucleus accumbens and basolateral amygdala in the regulation of this behavior.

In the third aim, I sought to characterize the impact of social context on juvenile social novelty-seeking behavior. Thus, in Study 5, I exposed juvenile male and female rats to four different acute social conditions: social housing, social isolation, social separation, and a final condition in which the subject is isolated by its cage mate

remained socially-housed to control for the social context of the stimulus cage mate. Finally, because I found a role of MOR in the regulation of juvenile social novelty-seeking behavior in my second aim, I asked whether low juvenile social novelty-seeking caused by social separation could be restored by activation of the MOR, either intracerebroventricularly or locally in the nucleus accumbens.

The rationale for this research is that gaining a better understanding of the neural mechanisms underlying normal social behavior during the juvenile period may inform our understanding of how these behaviors are disrupted in neurodevelopmental disorders, such as ASD.

Chapter Two: Age and sex differences in oxytocin and vasopressin V1a receptor binding densities in the rat brain: Focus on the social decision-making network*

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ABSTRACT: Oxytocin (OT) and vasopressin (AVP) regulate various social behaviors via activation of the OT receptor (OTR) and the AVP V1a receptor (V1aR) in the brain. Social behavior often differs across development and between the sexes, yet our understanding of age and sex differences in brain OTR and V1aR binding remains incomplete. Here, we provide an extensive analysis of OTR and V1aR binding density throughout the brain in juvenile and adult male and female rats, with a focus on regions within the social decision-making network. OTR and V1aR binding density were higher in juveniles than in adults in regions associated with reward and socio-spatial memory and higher in adults than in juveniles in key regions of the social decision-making network and in cortical regions. We discuss possible implications of these shifts in OTR and V1aR binding density for the age-specific regulation of social behavior. Furthermore, sex differences in OTR and V1aR binding density were less numerous than age differences. The direction of these sex differences was region-specific for OTR but consistently higher in females than in males for V1aR. Finally, almost all sex differences in OTR and V1aR binding density were already present in juveniles and occurred in regions with denser binding in adults compared to juveniles. Possible implications of these sex differences for the sex-specific regulation of behavior, as well potential underlying mechanisms, are discussed. Overall, these findings provide an important

framework for testing age- and sex-specific roles of OTR and V1aR in the regulation of social behavior.

Abbreviations

aAcbC *anterior nucleus accumbens core*
aAIP *anterior agranular insular cortex*
aAcbSh *anterior nucleus accumbens shell*
cAcbSh *central nucleus accumbens shell*
pAcbSh *posterior nucleus accumbens shell*
AODL *anterior olfactory nucleus, dorsolateral part*
AOM *anterior olfactory nucleus, medial part*
AOVP *anterior olfactory nucleus, ventroposterior part*
Arc *arcuate nucleus*
AVP *arginine vasopressin*
avThal *anteroventral thalamic nucleus*
BLA *basolateral amygdala*
BMA *basomedial amygdala*
BNSTld *bed nucleus of the stria terminalis, lateral division, dorsal part*
BNSTlp *bed nucleus of the stria terminalis, lateral division, posterior part*
BNSTmp *bed nucleus of the stria terminalis, posteromedial part*
BNSTp *bed nucleus of the stria terminalis, posterior part*
CeA *central amygdala*
Cl *claustrum*
dCPu *dorsal caudate putamen*
DP *dorsal peduncular cortex*
DRN *dorsal raphe nucleus*
DS *dorsal subiculum*
GrDG *granular layer of the dentate gyrus*
ICj *islands of Calleja*
IL *infralimbic cortex*
IPAC *interstitial nucleus of the posterior limb of the anterior commissure*
LH *lateral hypothalamus*
LPAG *lateral periaquaductal grey*
LSD *lateral septum, dorsal part*
LSI *lateral septum nucleus, intermediate part*
LSV *lateral septum nucleus, ventral part*
MA3 *medial accessory oculomotor nucleus*
mAIP *medial agranular insular cortex*
mCPu *medial caudate putamen*
MePD *medial amygdala, posterodorsal part*
MePV *medial amygdala, posteroventral part*
MMN *medial mammillary nucleus*
moDG *molecular layer of the dentate gyrus*
MPOA *medial preoptic area*
mThal *medial thalamic nucleus*
Nv *navicular nucleus of the basal forebrain*
OT *oxytocin*
OTR *oxytocin receptor*
pAcbSh *nucleus accumbens shell posterior part*
pCPu *posterior caudate putamen*
Pir *piriform cortex*
PRH *perirhinal cortex*
PrL *prelimbic cortex*
PVN *paraventricular hypothalamic nucleus*
PVT *paraventricular thalamic nucleus*
S1 *primary somatosensory cortex*
SCN *suprachiasmatic nucleus*
SMN *supramammillary nucleus*
SPFPC *subparafascicular nucleus*
stg *stigmoid hypothalamic nucleus*
Sug *superficial grey layer of the superior colliculus*
tuLH *tuberal region of the lateral hypothalamus*
V1aR *vasopressin V1a receptor*
VMH *ventromedial hypothalamic nucleus*
vmThal *ventromedial thalamic nucleus*
VP *ventral pallidum*
VS *ventral subiculum*

INTRODUCTION

Oxytocin (OT) and vasopressin (AVP) are neuropeptides primarily synthesized in the paraventricular and supraoptic nuclei of the hypothalamus. AVP is also synthesized in the bed nucleus of the stria terminalis (BNST) and medial amygdala (Sofroniew & Weindl, 1978; Sofroniew 1980; Caffe et al., 1987; De Vries et al., 1981; DeVries & Buijs, 1983, Rood & De Vries, 2011). These OT and AVP- synthesizing nuclei send fiber projections to a wide array of brain regions (Buijs, 1978, 1980; Knobloch & Grinevich, 2014); Rood & De Vries, 2011). Via these direct projections, as well as via dendritic release (Ludwig et al., 2002, 2005; Ludwig & Leng, 2006), OT and AVP reach the OT receptor (OTR) and V1a receptor (V1aR), which are widely distributed in the brain (Gimpl & Fahrenholz, 2001; Tribollet et al., 1990, Tribollet et al., 1998; Shapiro & Insel, 1989; Dumais et al., 2013; Dumais & Veenema, 2015). By activating these receptors, OT and AVP have been shown to modulate a broad range of social behaviors in adult rodents, including social recognition, aggression, and maternal behavior (Veenema & Neumann, 2008; Goodson & Kabelik, 2009; Albers, 2015).

In addition to regulating adult social behaviors, OT and AVP systems have been more recently implicated in the regulation of social behaviors during development. For example, OT and AVP systems were found to modulate a juvenile-typical and highly rewarding social behavior, namely social play behavior, in 35-day-old juvenile rats (Veenema et al., 2013; Bredewold et al., 2014). The juvenile period (here synonymous with the peri-pubertal or early adolescent period and spanning postnatal days 28-42 in rats (Spear, 2000) is characterized by increased time spent engaging in peer interactions, novelty-seeking, and risk-taking behavior than at younger or older ages (Doremus-

Fitzwater et al., 2010; Blakemore & Mills, 2014). Juvenile animals also show more robust conditioned place preferences for peer interactions, suggesting that these interactions are more rewarding to juveniles than they are to adults (Douglas et al, 2004; Trezza et al., 2011; Crone & Dahl, 2012). These findings suggest differences in the regulation of social behaviors between juveniles and adults. In support, the AVP system modulates social recognition differently in juvenile as compared to adult rats (Veenema et al., 2012). Such age differences in the regulation of social behavior by AVP, and possibly OT, may be due to age differences in OTR and V1aR expression in the brain.

Previous studies have characterized the developmental trajectory of OTR and V1aR binding densities in the rat brain from the prenatal period up through the peri-weaning period (as late as postnatal day 30) and compared these binding patterns with those in adult rats (Tribollet et al., 1989; Snijdewint et al., 1989; Shapiro & Insel, 1989; Tribollet et al., 1991; Tribollet et al., 1992). However, to the best of our knowledge, only two studies so far have compared OTR and/or V1aR binding densities between 35-day-old juveniles and adults, albeit only in males and in a limited number of brain regions (Tribollet et al., 1989; Lukas et al., 2010). Furthermore, Tribollet et al., 1989 included only four animals in each group and did not provide quantitative statistical analysis of age differences between 35-day-old juveniles and adults. Thus, a comparison of differences in OTR and V1aR binding densities between juvenile and adult rats of both sexes, throughout the brain, is lacking.

OT and AVP often regulate social behaviors in sex-specific ways in adult rats (Bluthe & Dantzer 1990; Dantzer, 1987; Engelmann et al., 1998; Dumais et al., 2013; Lukas & Neumann, 2014; Dumais & Veenema, 2015, 2016). This may be due to sex

differences in OTR and V1aR expression in the brain. In support, sex differences have been found in OTR and V1aR binding densities in several regions of the adult rat brain with predominantly higher binding densities in males than in females (Dumais et al., 2013; Dumais & Veenema, 2015). Importantly, OT and AVP also regulate social behaviors in sex-specific ways in juvenile rats (Veenema et al., 2013; Bredewold et al., 2014), suggesting the presence of sex differences in OTR and V1aR binding densities prior to puberty. However, whether the sex differences in OTR and V1aR binding found in adult rats are already present in juvenile rats has yet to be determined.

We herein aim to provide a comprehensive comparison between juveniles and adults and between males and females of OTR and V1aR binding densities in the rat brain. We have particularly focused on analyzing OTR and V1aR binding densities in brain regions that are part of the social decision-making network (O'Connell & Hoffman, 2011, 2012). This network combines brain regions of the mesolimbic reward system with those of the social behavior network (Newman et al., 1999; O'Connell & Hoffman, 2011, 2012) to form a reciprocally interconnected subset of brain regions involved in reward processing and behavioral regulation (O'Connell & Hoffman, 2011, 2012). OTR and V1aR are expressed in most nodes of the social decision-making network (Albers, 2015). Because there are age and sex differences in the reward value and expression of various social behaviors (Douglas et al., 2004; Varlinskaya et al., 2015; Panksepp et al., 1984; Terranova et al., 1993), we hypothesize that age and sex differences in OTR and V1aR binding densities will occur in multiple nodes of the social decision-making network. Furthermore, because sex differences in the regulation of social behavior by OT and AVP systems have been found in adults (Dantzer et al., 1987; Bluthe & Dantzer, 1990;

Veenema et al., 2012; Dumais et al., 2016), as well as in juveniles (Veenema et al., 2013; Bredewold et al., 2014), we hypothesize that sex differences in OTR and V1aR binding previously observed in adults (Dumais et al., 2013; Dumais & Veenema, 2015) will already be present in juveniles.

METHODS

Animals

Male and female Wistar rats were obtained from Charles River Laboratories (Raleigh, NC) at 22 or 56 days of age and housed under standard laboratory conditions (12 hour light/dark cycle, lights on at 7:00 am, food and water available *ad libitum*, 22° C, 60% humidity). Upon arrival at our facility, rats were housed in standard rat cages (26.7 x 48.3 x 20.3 cm). Twenty-two-day-old juvenile rats were housed in same-sex groups of 3-4 until brain collection for receptor autoradiography at 35 days of age (Juvenile group). The age of 35 days was chosen to be consistent with previous work on OTR and V1aR binding in the brains of 35-day-old male rats (Lukas et al., 2010). Furthermore, 35 days of age marks a distinctive developmental stage in rats with peak levels of social play (Panksepp, 1981; Pellis & Pellis, 1990) a behavior modulated by activation of OTR and V1aR in the brain (Veenema et al., 2012, 2013; Bredewold et al., 2014). Fifty-six-day-old rats were housed in same-sex pairs until brain collection for receptor autoradiography at 84 days of age (Adult group). All experiments were conducted in accordance with the NIH *Guide to the Care and Use of Laboratory Animals*

and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

Receptor Autoradiography

Coronal sectioning

Rats (juvenile males: n=13; juvenile females: n=13; adult males: n=12; adult females: n=12) were killed using CO_2 inhalation and brains were removed, rapidly frozen in methylbutane on dry ice, and stored at -45˚ C. Brains were cut on a cryostat into 16-µm coronal sections and mounted onto slides in eight adjacent series. Collection began at approximately 3.72 mm anterior to bregma and ended at approximately 8.52 mm posterior to bregma (Paxinos & Watson, 2007). Sections were then frozen -45˚ C until receptor autoradiography was performed. Receptor autoradiography was conducted for OTR using [^{125}I]- Ornithine Vasotocin Analog (d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]-OVTA; Perkin Elmer, Boston, MA) as tracer and for V1aR using [^{125}I]-d(CH_2)$_5$(Tyr[Me])-AVP (Perkin Elmer, Boston, MA) as tracer on adjacent series. Specificity of these tracers to bind OTR and V1aR, respectively, has been demonstrated previously (Beery et al., 2008; Campbell et al., 2009; Anacker et al., 2016). Receptor autoradiography was conducted in accordance with Lukas et al. (2010). In brief, slides were thawed and dried at room temperature followed by a short fixation in 0.1 % paraformaldehyde. The slides were then washed twice in 50 mM Tris (pH 7.4), exposed to tracer buffer (50 pM tracer, 50 mM Tris, 10 mM MgCl2, 0.01% BSA) for 60 min, and washed four times in Tris + 10 mM $MgCl_2$. Finally, slides were dipped in distilled water, air-dried, and exposed to Biomax MR films (VWR International, Pittsburgh, PA). Brain sections of both ages and sexes were processed together and balanced across incubation

chambers and exposure to films. A 3-day exposure time was used to analyze OTR binding density in brain regions with relatively high OTR binding density (total of 13 regions): the medial anterior olfactory nucleus, ventroposterior anterior olfactory nucleus, anterior nucleus accumbens core, dorsal caudate putamen, medial caudate putamen, dorsal peduncular nucleus, islands of Calleja, posterior BNST, dorsolateral BNST, ventromedial hypothalamus, central amygdala, dorsal subiculum, and ventral subiculum. OTR binding density in additional regions with lower OTR binding density (21 regions) was analyzed using a 9-day exposure time. A 4-day exposure time was used to analyze V1aR binding density in a total of 29 brain regions. See Fig. 2.1 for receptor autoradiograms and schematic diagrams indicating the brain regions in which OTR and V1aR binding was quantified. All abbreviations of brain regions are in accordance with Paxinos & Watson (2007), except for the nucleus accumbens core and nucleus accumbens shell, where we added the subdivisions anterior core, medial shell, and posterior shell to delineate the separate areas analyzed as well as for the anterior olfactory nucleus where we used the abbreviation AODL to refer to measurements including both the dorsal and lateral divisions.

Image and Data Analysis

Autoradiography films were digitized using a Northern Light Illuminator (InterFocus Imaging, U.K.) and optical densities of OTR and V1aR were measured in coronal sections using ImageJ (NIH, http://imagej.nih.gov/ij/). The data were converted to dpm/mg (disintegrations per minute/milligram tissue) using a [^{125}I] standard microscale (American Radiolabeled Chemicals Inc., St. Louis, MO). Each measurement was subtracted by film background and binding densities were calculated by taking the

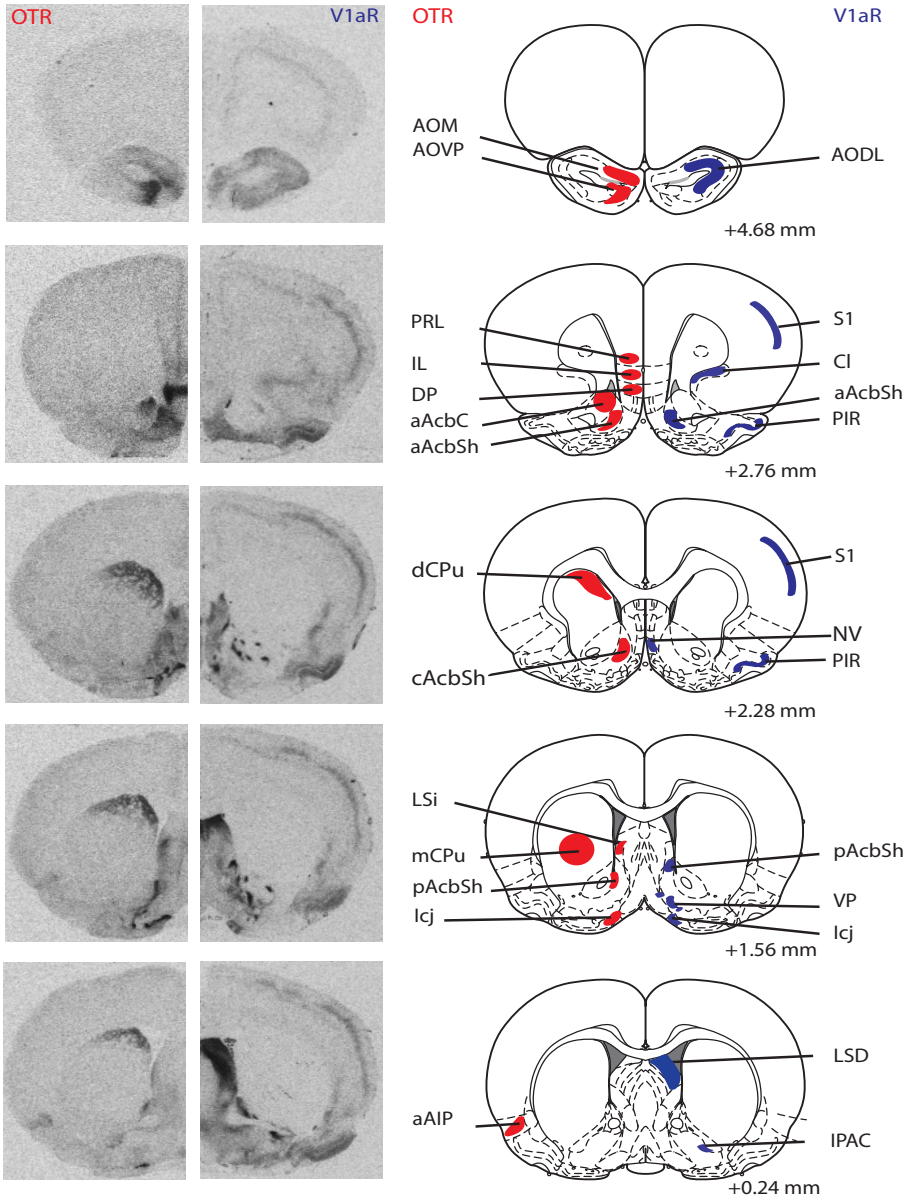


Figure 2.1. Representative autoradiograms of OTR and V1aR binding in coronal sections of the brain from the same adult male rat (on the left) with corresponding rat brain atlas images (Paxinos & Watson, 2007; on the right). Brain regions in which receptor binding was measured are highlighted in red for OTR and are highlighted in blue for V1aR. Distance is measured in millimeters from bregma according to Paxinos & Watson (2007). Note that while many brain regions were analyzed across multiple bregma distances, regions are highlighted in the most representative atlas images only.

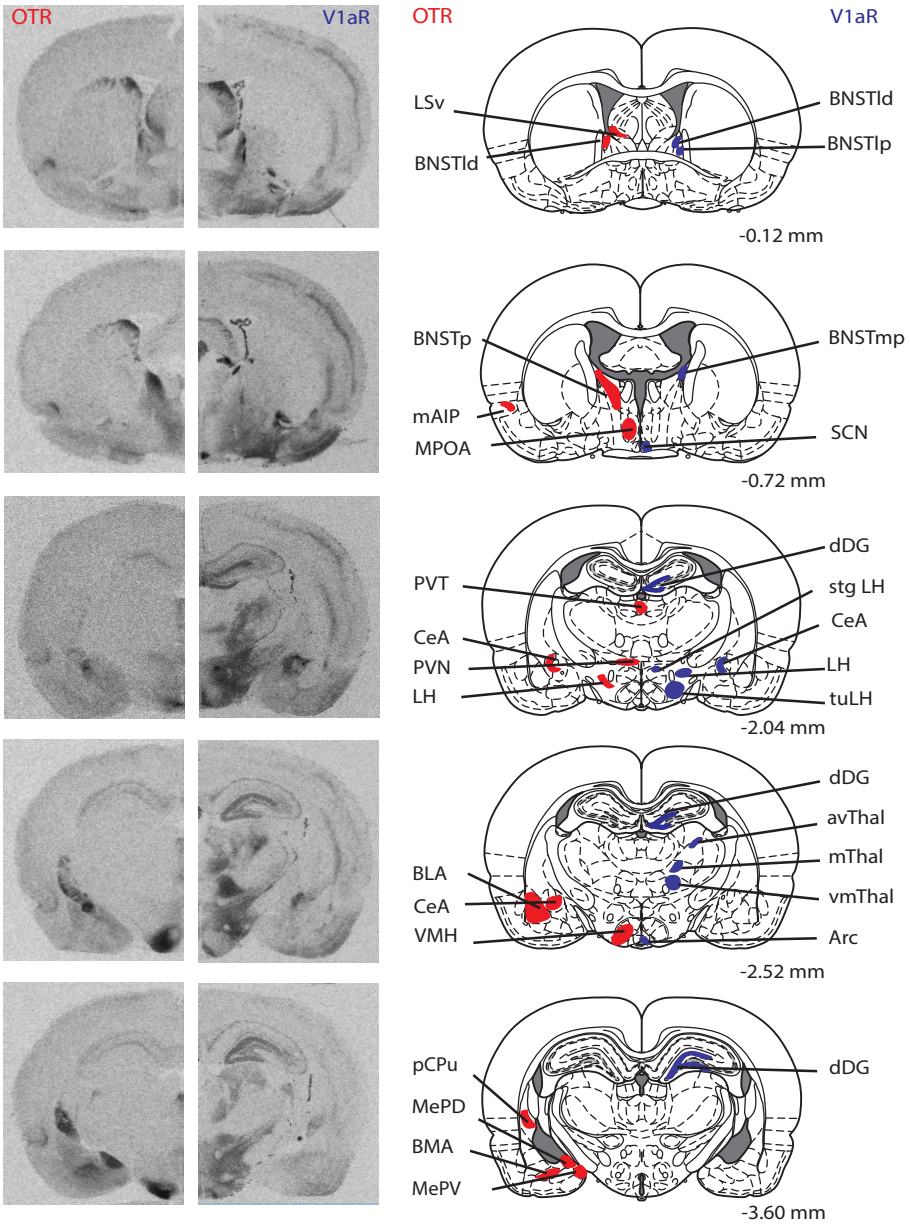


Figure 2.1. Continued...

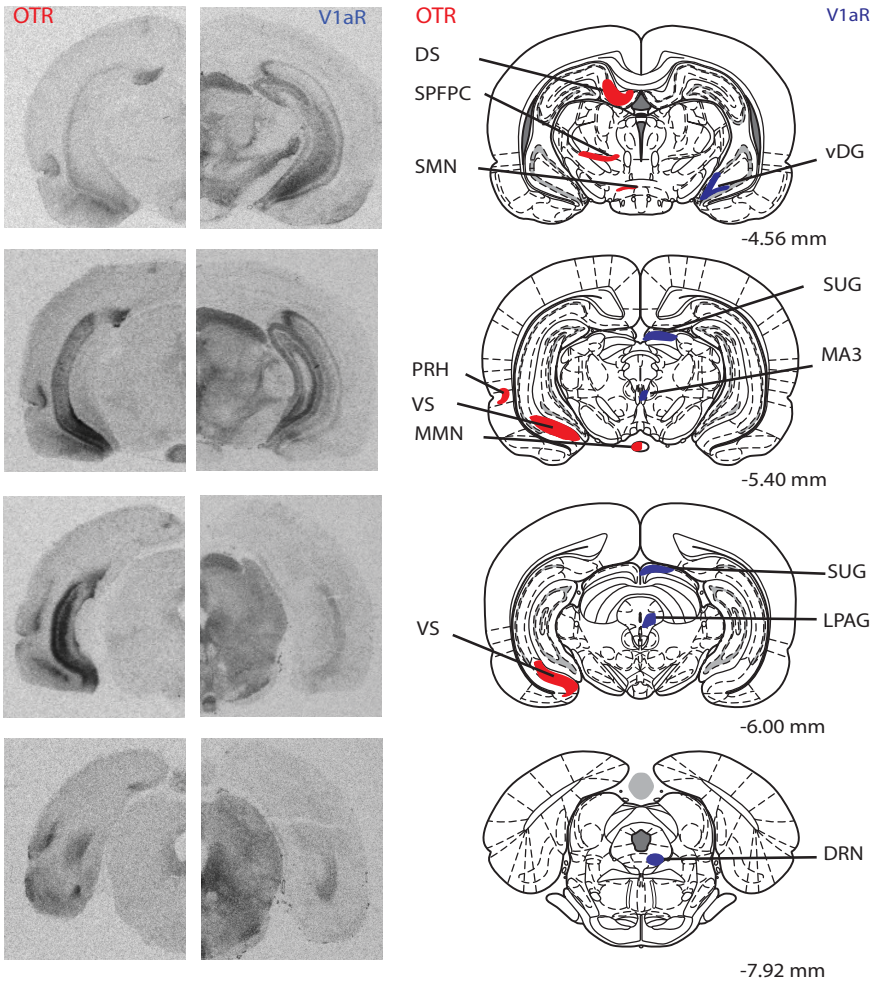


Figure 2.1. Continued...

mean of bilateral measurements in a fixed number of sections per region of interest per rat. The total number of sections included depended on the size of the region of interest with a minimum of 2 sections. Regions of interest included those of the social decision-making network (see underlined brain regions in Table 2.1) as well as additional regions with dense OTR or V1aR binding. This resulted in 35 brain regions analyzed for OTR binding and 29 brain regions analyzed for V1aR binding. See Fig 2.1 for receptor autoradiograms and schematic diagrams indicating all brain regions analyzed.

Statistics

For all statistical analysis, PASW/SPSS Statistics (Version 22.0) was used. Two-way ANOVAs were used to test for age and sex differences in OTR and V1aR binding density in each brain region. The false discovery rate (FDR) procedure was used to correct for multiple comparisons (age, sex, and interaction) for each receptor separately. This resulted in an FDR $\alpha < 0.020$ for OTR (based on 105 comparisons) and an FDR $\alpha < 0.015$ for V1aR (based on 87 comparisons) (Benjamini & Hochberg, 1995). Significant interaction effects were followed by Bonferroni post-hoc tests (reflecting *t*-tests pre-adjusted for multiple comparisons) to examine differences among groups. Significant age or sex effects were followed by Cohen's D to calculate the effect size of age differences (overall and separately for male and females) and of sex differences (overall and separately for juveniles and adults). Subsequent independent samples t-tests were run separately for OTR and V1aR to determine whether the effect size of age differences was different between males and females for all brain regions and whether the effect size of sex differences was different between juveniles and adults. Bivariate correlation analyses were used to determine correlations of OTR and/or V1aR binding densities between pre-selected brain regions based on patterns of age and/or sex differences in OTR and V1aR binding densities. Given the exploratory nature of our correlations we did not include a correction for multiple comparisons. Significance for correlation analyses was set at $p<0.05$.

RESULTS

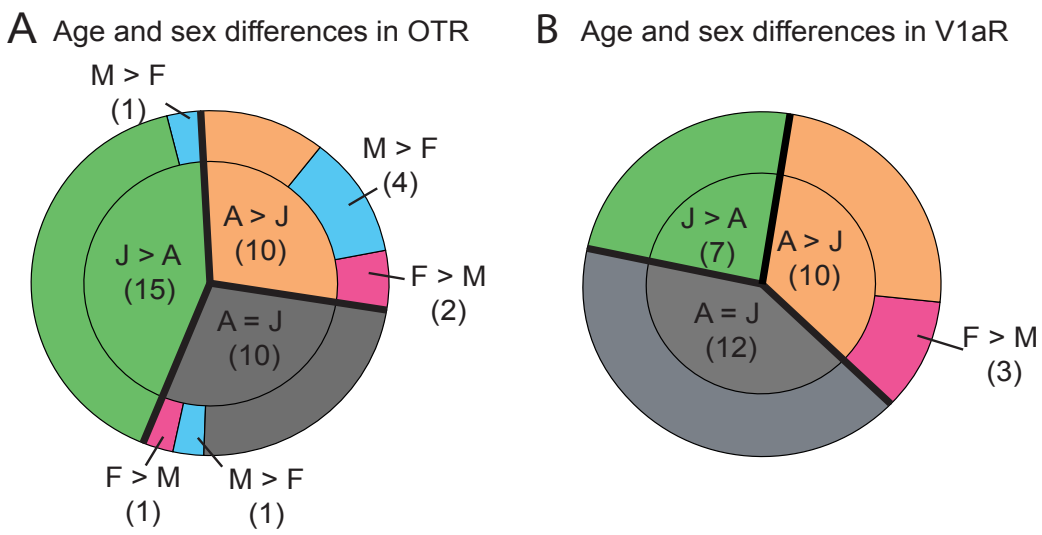


Figure 2.2. Overview of age and sex differences in OTR and V1aR binding densities in the rat brain. Age differences (green and orange) are more prevalent than sex differences (blue and pink) in both OTR binding density (A) and V1aR binding density (B). Data represent proportions of brain regions that significantly differ by age and sex for OTR or V1aR determined by two-way ANOVA with FDR correction for multiple comparisons. The number of brain regions is indicated in parentheses. J: juveniles; A: adults; M: males; F: females.

Age differences in OTR binding density

Age differences in OTR binding density were found in 25 of the 35 brain regions analyzed (Fig. 2.2A; see Table 2.1 for complete statistics & Fig. 2.5 for representative images). OTR binding density was significantly higher in juveniles than in adults in 15 brain regions, consisting of subregions in the olfactory nucleus (medial and posterior-ventral anterior), striatum (anterior nucleus accumbens core, dorsal caudate putamen, medial caudate putamen), hypothalamus (paraventricular, medial mammillary, and supramammillary nuclei), amygdala (basolateral and basomedial), septum (ventral lateral), hippocampus (dorsal and ventral subiculum), and thalamus (paraventricular

nucleus, subparafascicular nucleus) (Fig. 2.3A). OTR binding density was higher in adults as compared to juveniles in 10 brain regions: the islands of Calleja, ventromedial hypothalamus, posterior BNST, posterodorsal medial amygdala, medial preoptic area, and prelimbic, infralimbic, anterior and medial insular, and perirhinal cortices (Fig. 2.4A). While the size of individual age differences sometimes differed between males and females (see Table 2.2 for details), we found no overall difference in the effect sizes of age differences in OTR binding between the sexes ($t_{(48)} = -0.51$; $p=0.61$).

Age differences in V1aR binding density

Age differences in V1aR binding density were found in 17 of the 29 brain regions analyzed (Fig. 2.2B; see Table 2.1 for complete statistics & Fig. 2.5 for representative images). V1aR binding density was higher in juveniles as compared to adults in 7 brain regions: the dorsolateral anterior olfactory nucleus, anterior claustrum, islands of Calleja, central amygdala, dentate gyrus (granular layer and molecular layer), and oculomotor nucleus (Fig. 2.3B). Adults had higher V1aR binding density than juveniles in 10 brain regions: the primary somatosensory and piriform cortices, posterior nucleus accumbens shell, ventral pallidum, arcuate nucleus, navicular nucleus, dorsal lateral septum, stigmoid hypothalamic nucleus, ventromedial thalamic nucleus, and interstitial nucleus of the posterior limb of the anterior commissure (Fig. 2.4B). While the size of individual age differences sometimes differed between males and females (see Table 2.2 for details), we found no overall difference in the effect sizes of age differences in V1aR binding between the sexes ($t_{(32)} = -0.007$; $p=0.99$).

Table 2.1. Statistical details of age, sex, and interaction effects for OTR and V1aR binding densities in the rat brain. Significant effects (two-way ANOVA with FDR correction: $\alpha<0.020$ for OTR and $\alpha<0.015$ for V1aR) are bolded. Underlined brain regions are part of the social decision-making network according to O'Connell & Hoffman (2011, 2012).

OTR/V1aR	Direction		Age Effect	Sex Effect	Interaction Effect
Cortical areas					
aAIP	OTR	Higher in adults & females	**$F_{(1,41)}$=48.2; p<0.001**	**$F_{(1,41)}$=7.80; p<0.005**	$F_{(1,41)}$=0.64; p=0.43
mAIP	OTR	Higher in adults	**$F_{(1,45)}$=21.0; p<0.001**	$F_{(1,45)}$=4.14; p=0.05	$F_{(1,45)}$=0.03;p=0.86
DP	OTR		$F_{(1,46)}$=0.97; p=0.33	$F_{(1,46)}$=0.67; p=0.42	$F_{(1,46)}$=2.60; p=0.11
Il	OTR	Higher in adults	**$F_{(1,46)}$=7.54; p<0.001**	$F_{(1,46)}$=0.01; p=0.99	$F_{(1,46)}$=0.01; p=0.94
Pir	V1aR	Higher in adults	**$F_{(1,44)}$=11.7; p<0.001**	$F_{(1,44)}$=0.10; p=0.75	$F_{(1,44)}$=0.02; p=0.90
PRh	OTR	Higher in adults & females	**$F_{(1,44)}$=15.2; p<0.001**	**$F_{(1,44)}$=7.80; p<0.001**	$F_{(1,44)}$=1.41; p=0.24
PRL	OTR	Higher in adults	**$F_{(1,46)}$=18.8; p<0.001**	$F_{(1,46)}$=3.14; p=0.08	$F_{(1,46)}$=0.01; p=0.99
S1	V1aR	Higher in adults	**$F_{(1,44)}$=23.5; p<0.001**	$F_{(1,44)}$=2.65; p=0.11	$F_{(1,44)}$=0.26; p=0.62
Olfactory Areas					
AOM	OTR	Higher in juveniles	**$F_{(1,23)}$=16.4; p<0.001**	$F_{(1,23)}$=0.88; p=0.36	$F_{(1,23)}$=0.71; p=0.41
AOPV	OTR	Higher in juveniles	**$F_{(1,22)}$=29.6; p<0.001**	$F_{(1,22)}$=3.54; p=0.07	$F_{(1,22)}$=0.15; p=0.71
AODL	V1aR	Higher in juveniles	**$F_{(1,20)}$=8.59; p<0.01**	$F_{(1,20)}$=0.06; p=0.80	$F_{(1,20)}$=0.33; p=0.57
Nv	V1aR	Higher in adults	**$F_{(1,44)}$=12.5; p<0.001**	$F_{(1,44)}$=0.49; p=0.49	$F_{(1,44)}$=3.26; p=0.08
Striatal areas					
aAcbC	OTR	Higher in juveniles	**$F_{(1,46)}$=84.3; p<0.001**	$F_{(1,46)}$=0.02; p=0.88	$F_{(1,46)}$=1.84; p=0.18
aAcbSh	OTR		$F_{(1,44)}$=4.02; p=0.05	$F_{(1,44)}$=0.31; p=0.58	$F_{(1,44)}$=0.13; p=0.72
aAcbSh	V1aR		$F_{(1,39)}$=0.01; p=0.97	$F_{(1,39)}$=1.03; p=0.32	$F_{(1,39)}$=4.35; p=0.04
cAcbSh	OTR		$F_{(1,45)}$=1.18; p=0.28	$F_{(1,45)}$=0.41; p=0.53	$F_{(1,45)}$=0.44; p=0.51
pAcbSh	OTR		$F_{(1,46)}$=0.77; p=0.39	$F_{(1,46)}$=0.01; p=0.99	$F_{(1,46)}$=0.05; p=0.83
pAcbSh	V1aR	Higher in adults	**$F_{(1,44)}$=47.4; p<0.001**	$F_{(1,44)}$=1.35; p=0.25	$F_{(1,44)}$=0.64; p=0.43
dCPu	OTR	Higher in juveniles	**$F_{(1,40)}$=80.0; p<0.001**	$F_{(1,40)}$=1.89; p=0.18	$F_{(1,40)}$=0.32; p=0.57
mCPu	OTR	Higher in juveniles	**$F_{(1,46)}$=6.35; p=0.015**	$F_{(1,46)}$=0.02; p=0.88	$F_{(1,46)}$=0.04; p=0.84
pCPu	OTR	Higher in adult females	$F_{(1,46)}$=3.54; p=0.07	$F_{(1,46)}$=0.26; p=0.61	**$F_{(1,46)}$=6.34, p=0.02**
ICj	OTR	Higher in adult males	**$F_{(1,45)}$=175; p<0.001**	**$F_{(1,45)}$=80.2; p<0.001**	**$F_{(1,45)}$=99.6, p<0.001**
ICj	V1aR	Higher in juveniles	**$F_{(1,44)}$=11.3; p<0.005**	$F_{(1,44)}$=1.79; p=0.19	$F_{(1,44)}$=0.42; p=0.52
VP	V1aR	Higher in adults	**$F_{(1,44)}$=26.7; p<0.001**	$F_{(1,44)}$=1.72; p=0.20	$F_{(1,44)}$=0.42; p=0.52
Lateral septum					
LSD	V1aR	Higher in adults & females	**$F_{(1,42)}$=22.8; p<0.001**	**$F_{(1,42)}$=9.14; p<0.005**	$F_{(1,42)}$=0.92; p=0.34
LSI	OTR	Higher in females	$F_{(1,46)}$=4.0; p=0.50	**$F_{(1,46)}$=8.80; p<0.01**	$F_{(1,46)}$=1.34; p=0.25
LSV	OTR	Higher in juveniles	**$F_{(1,46)}$=34.3; p<0.001**	$F_{(1,46)}$=0.02; p=0.88	$F_{(1,46)}$=0.53; p=0.47
BNST					
BNSTld	OTR		$F_{(1,45)}$=2.53; p=0.12	$F_{(1,45)}$=1.62; p=0.21	$F_{(1,45)}$=1.22; p=0.27
BNSTld	V1aR		$F_{(1,43)}$=3.83; p=0.06	$F_{(1,43)}$=1.91; p=0.17	$F_{(1,43)}$=0.04; p=0.85
BNSTlp	V1aR		$F_{(1,43)}$=5.25; p=0.03	$F_{(1,43)}$=5.42; p=0.03	$F_{(1,43)}$=0.16; p=0.69
BNSTmp	V1aR		$F_{(1,43)}$=0.55; p=0.47	$F_{(1,43)}$=0.01; p=0.98	$F_{(1,43)}$=0.16; p=0.69
BNSTp	OTR	Higher in adults & males	**$F_{(1,41)}$=6.89; p<0.05**	**$F_{(1,41)}$=136; p<0.001**	$F_{(1,41)}$=4.07; p=0.05
Amygdala					
BLA	OTR	Higher in juveniles	**$F_{(1,45)}$=7.99; p<0.005**	$F_{(1,45)}$=3.50; p=0.07	$F_{(1,45)}$=2.61; p=0.11
BMA	OTR	Higher in juveniles	**$F_{(1,45)}$=8.96; p<0.005**	$F_{(1,45)}$=0.03; p=0.86	$F_{(1,45)}$=5.54; p=0.02
CeA	OTR		$F_{(1,46)}$=0.01; p=0.96	$F_{(1,46)}$=1.84; p=0.18	$F_{(1,46)}$=2.38; p=0.13
CeA	V1aR	Higher in juveniles	**$F_{(1,44)}$=10.3; p<0.005**	$F_{(1,44)}$=1.99; p=0.17	$F_{(1,44)}$=0.40; p=0.53

Cl	V1aR	Higher in juveniles	**$F_{(1,44)}$=26.1; p<0.001**	$F_{(1,44)}$=0.49; p=0.49	$F_{(1,44)}$=0.09; p=0.77
IPAC	V1aR	Higher in adults	**$F_{(1,44)}$=7.08; p=0.011**	$F_{(1,44)}$=0.04; p=0.85	$F_{(1,44)}$=0.46; p=0.50
MePD	OTR	Higher in adult males	**$F_{(1,46)}$=23.0; p<0.001**	**$F_{(1,46)}$=252; p<0.001**	**$F_{(1,46)}$=26.4, p<0.001**
MePV	OTR	Higher in males	$F_{(1,46)}$=0.34; p=0.56	**$F_{(1,46)}$=35.1; p<0.001**	$F_{(1,46)}$=0.42; p=0.52
Hypothalamus					
Arc	V1aR	Higher in adults & females	**$F_{(1,44)}$=50.5; p<0.001**	**$F_{(1,44)}$=30.6; p<0.001**	$F_{(1,44)}$=0.29; p=0.60
LH	OTR		$F_{(1,41)}$=0.35; p=0.56	$F_{(1,41)}$=0.29; p=0.59	$F_{(1,41)}$=0.01; p=0.99
LH	V1aR		$F_{(1,42)}$=0.13; p=0.72	$F_{(1,42)}$=0.30; p=0.58	$F_{(1,42)}$=0.06; p=0.81
MMN	OTR	Higher in juveniles	**$F_{(1,40)}$=36.0; p<0.001**	$F_{(1,40)}$=3.50; p=0.07	$F_{(1,40)}$=2.82; p=0.10
MPOA	OTR	Higher in adults	**$F_{(1,43)}$=50.0; p<0.001**	$F_{(1,43)}$=4.32; p=0.04	$F_{(1,43)}$=0.57; p=0.46
PVN	OTR	Higher in juveniles & males	**$F_{(1,41)}$=18.2; p<0.001**	**$F_{(1,41)}$=11.5; p<0.005**	$F_{(1,41)}$=0.01; p=0.91
SCN	V1aR		$F_{(1,44)}$=1.06; p=0.31	$F_{(1,44)}$=2.31; p=0.14	$F_{(1,44)}$=1.71; p=0.20
SMN	OTR	Higher in juveniles	**$F_{(1,45)}$=20.6; p<0.001**	$F_{(1,45)}$=0.86; p=0.36	$F_{(1,45)}$=4.61; p=0.04
Stg	V1aR	Higher in juveniles	**$F_{(1,44)}$=14.0; p<0.005**	$F_{(1,44)}$=0.38; p=0.54	$F_{(1,44)}$=0.06; p=0.81
tuLH	V1aR		$F_{(1,42)}$=0.06; p=0.82	$F_{(1,42)}$=2.41; p=0.13	$F_{(1,42)}$=1.70; p=0.20
VMH	OTR	Higher in adult males	**$F_{(1,46)}$=269; p<0.001**	**$F_{(1,46)}$=15.5; p<0.001**	**$F_{(1,46)}$=12.2, p<0.001**
Thalamus					
avThal	V1aR		$F_{(1,44)}$=0.66; p=0.42	$F_{(1,44)}$=5.57; p=0.02	$F_{(1,44)}$=1.43; p=0.24
mThal	V1aR		$F_{(1,44)}$=2.40; p=0.13	$F_{(1,44)}$=0.06; p=0.80	$F_{(1,44)}$=0.14; p=0.71
PVT	OTR	Higher in juveniles	**$F_{(1,41)}$=40.1; p<0.001**	$F_{(1,41)}$=3.34; p=0.08	$F_{(1,41)}$=0.04; p=0.85
SPFPC	OTR	Higher in juveniles	**$F_{(1,39)}$=13.5; p<0.005**	$F_{(1,39)}$=0.07; p=0.79	$F_{(1,39)}$=0.77; p=0.39
vmThal	V1aR	Higher in adults & females	**$F_{(1,44)}$=130; p<0.001**	**$F_{(1,44)}$=29.3; p<0.001**	$F_{(1,44)}$=4.95; p=0.03
Hippocampus					
DS	OTR	Higher in juveniles	**$F_{(1,46)}$=89.0; p<0.001**	$F_{(1,46)}$=0.90; p=0.35	$F_{(1,46)}$=0.19; p=0.67
VS	OTR	Higher in juveniles	**$F_{(1,43)}$=37.1; p<0.001**	$F_{(1,43)}$=1.88; p=0.18	$F_{(1,43)}$=3.69; p=0.06
dDG	V1aR	Higher in juveniles	**$F_{(1,44)}$=79.9; p<0.001**	$F_{(1,44)}$=0.26; p=0.61	$F_{(1,44)}$=0.30; p=0.59
vDG	V1aR	Higher in juveniles	**$F_{(1,38)}$=16.3; p<0.001**	$F_{(1,38)}$=0.56; p=0.46	$F_{(1,38)}$=0.05; p=0.83
Midbrain					
DRN	V1aR		$F_{(1,36)}$=2.66; p=0.11	$F_{(1,36)}$=5.77; p=0.02	$F_{(1,36)}$=0.55; p=0.46
LPAG	V1aR		$F_{(1,44)}$=3.75; p=0.06	$F_{(1,44)}$=2.36; p=0.13	$F_{(1,44)}$=2.06; p=0.16
MA3	V1aR	Higher in juveniles	**$F_{(1,44)}$=9.75; p<0.005**	$F_{(1,44)}$=0.01; p=0.94	$F_{(1,44)}$=1.04; p=0.31
Sug	V1aR		$F_{(1,44)}$=4.79; p=0.03	$F_{(1,44)}$=0.01; p=0.91	$F_{(1,44)}$=3.97; p=0.05

Sex differences in OTR binding density

Sex differences in OTR binding density were found in 9 of the 35 brain regions analyzed (Fig. 2.2A; See Table 2.1 for complete statistics). Males had higher OTR binding density than females in 6 brain regions: the islands of Calleja, posterior BNST, ventromedial hypothalamus, posterior-dorsal and posterior-ventral medial amygdala, and paraventricular nucleus of the hypothalamus (Fig. 2.7A). Females had higher OTR binding than males in 3 brain regions: the anterior insular cortex, perirhinal cortex, and intermediate lateral septum (Fig. 2.7A). While the size of individual sex differences sometimes differed between juveniles and adults (see Table 2.3 for details), we found no overall difference in the effect sizes of sex differences in OTR binding between ages ($t_{(18)} = -1.35$; $p=0.19$).

Sex differences in V1aR binding density

Sex differences in V1aR binding density were found in 3 of 29 brain regions analyzed (Fig. 2.2B; see Table 2.1 for complete statistics). Females had higher V1aR binding density than males in the arcuate nucleus, dorsal lateral septum and ventromedial thalamus (Fig. 2.7B). While the size of individual sex differences sometimes differed between juveniles and adults (see Table 2.3 for details), we found no overall difference in the effect sizes of sex differences in V1aR binding between ages ($t_{(4)} = -2.04$; $p=0.11$).

Age x Sex interaction effects

Significant age x sex interaction effects were found for OTR binding density in the islands of Calleja, posterior caudate putamen, ventromedial hypothalamus, and posterodorsal medial amygdala (Table 2.1). Bonferroni post-hoc testing revealed that OTR binding density in the islands of Calleja was higher in adults as compared to

juveniles in both sexes (males: $p<0.001$; females: $p<0.05$) and was higher in adult males as compared to adult females ($p<0.001$). OTR binding density in the posterior caudate putamen was higher in adult females compared to juvenile females ($p <0.01$) and compared to adult males ($p <0.05$). OTR binding density in the ventromedial hypothalamus was higher in adult males compared to juvenile males ($p <0.001$) and compared to adult females ($p <0.001$; Fig. 2.8B). Finally, OTR binding density in the posterodorsal medial amygdala was higher in juvenile males compared to juvenile females ($p < 0.001$) and was higher in adult males compared to juvenile males ($p <0.001$) and compared to adult females ($p <0.001$; Fig. 2.8B). No significant age x sex effects were found for V1aR binding density.

Similar OTR and V1aR binding density between the ages and sexes

No significant sex or age differences were found in 8 out of 35 brain regions analyzed for OTR binding density : the anterior nucleus accumbens shell, central nucleus accumbens shell, posterior nucleus accumbens shell, dorsal peduncular nucleus, lateral-dorsal BNST, central amygdala, lateral hypothalamus, and posterior caudate putamen (Fig. S2.1; see Table 2.1 for statistics) and in 12 out of 29 brain regions analyzed for V1aR binding density: the anterior nucleus accumbens shell, suprachiasmatic nucleus, medial-posterior, lateral-dorsal, and lateral-posterior BNST, lateral periaqueductal grey, lateral hypothalamus, tuberal hypothalamus, anteroventral thalamus, medial thalamus, superficial grey layer of the superior colliculus, and dorsal raphe nucleus (Fig. S2.1; see Table 2.1 for statistics).

Table 2.2. **Cohen's D effect size measurements for age differences in OTR and V1aR binding densities overall, and analyzed separately in males and females.** Only brain regions showing significant main effects of age or interaction (see Table 1) are included.

AGE DIFFERENCES					
	OTR/V1aR	Direction	Both sexes	Males	Females
Cortical areas					
aAIP	OTR	Higher in adults	-1.92	-2.64	-1.89
mAIP	OTR	Higher in adults	-1.29	-1.66	-1.09
Il	OTR	Higher in adults	-0.79	-0.72	-0.82
Pir	V1aR	Higher in adults	-1.03	-0.93	-1.15
PRh	OTR	Higher in adults	-1.08	-1.49	-0.78
PRL	OTR	Higher in adults	-1.21	-1.05	-1.56
S1	V1aR	Higher in adults	-1.35	-1.38	-1.40
Olfactory Areas					
AOM	OTR	Higher in juveniles	1.56	1.90	1.26
AOPV	OTR	Higher in juveniles	2.01	2.07	2.30
AODL	V1aR	Higher in juveniles	1.19	1.00	1.37
NV	V1aR	Higher in adults	-1.03	-1.32	-0.65
Striatal Areas					
aAcbC	OTR	Higher in juveniles	2.62	2.29	2.92
pAcbSh	V1aR	Higher in adults	-2.00	-1.52	-2.79
dCPu	OTR	Higher in juveniles	2.84	2.71	3.10
mCPu	OTR	Higher in juveniles	0.73	0.69	0.73
pCPu	OTR	Higher in adult females	-0.51	0.20	-1.17
Icj	OTR	Higher in adult males	-1.73	-6.79	-0.90
Icj	V1aR	Higher in juveniles	1.01	1.35	0.71
VP	V1aR	Higher in adults	-1.50	-1.50	-1.49
Lateral Septum					
LSD	V1aR	Higher in adults	-1.25	-1.46	-1.54
LSV	OTR	Higher in juveniles	1.70	2.62	1.20
BNST					
BNSTp	OTR	Higher in adults	-0.44	-1.28	-0.21
Amygdala					
BLA	OTR	Higher in juveniles	0.76	1.19	0.38
BMA	OTR	Higher in juveniles	0.82	1.34	0.23
CeA	V1aR	Higher in juveniles	0.95	1.07	0.91
Cl	V1aR	Higher in juveniles	1.52	1.54	1.48
IPAC	V1aR	Higher in adults	-0.78	-0.69	-0.83
MePD	OTR	Higher in adults	-0.52	-2.81	0.10

Hypothalamus					
Arc	V1aR	Higher in adults	-1.56	-2.31	-1.8
MMN	OTR	Higher in juveniles	1.76	2.95	1.17
MPOA	OTR	Higher in adults	-1.99	-2.16	-2.04
PVN	OTR	Higher in juveniles	1.13	1.13	1.44
SMN	OTR	Higher in juveniles	1.27	1.78	0.76
Stg	V1aR	Higher in adults	-1.13	-1.17	-1.04
VMH	OTR	Higher in adults	-3.75	-5.25	-3.99
Thalamus					
PVT	OTR	Higher in juveniles	1.93	1.82	2.07
SPFPC	OTR	Higher in juveniles	1.20	1.38	0.92
vmThal	V1aR	Higher in adults	-2.51	-3.99	-2.73
Hippocampus					
DS	OTR	Higher in juveniles	2.70	2.87	2.49
VS	OTR	Higher in juveniles	1.72	1.25	2.31
dDG	V1aR	Higher in juveniles	2.66	2.41	2.88
vDG	V1aR	Higher in juveniles	1.30	1.44	1.14
Midbrain					
MA3	V1aR	Higher in juveniles	0.94	1.14	0.66

Correlational analyses

Correlational analyses were performed to further explore the relationships between OTR and V1aR binding densities across preselected brain regions based on the observed age and sex differences in binding densities.

Regions with age differences in OTR and V1aR binding: social and spatial memory

Age differences in OTR binding densities were found in the ventral lateral septum, dorsal subiculum, ventral subiculum, medial mammillary nucleus and supramammillary nucleus and in V1aR binding densities in the lateral septum and dorsal and ventral dentate gyrus. These brain regions are part of a hypothesized neural network underlying social and spatial memory (Risold & Swanson, 1997; Pan & McNaughton, 2004; Allen & Hopkins, 1989; Meibach & Siegel, 1977). Given the importance of social and spatial memory as prerequisites for social decision-making, correlational analyses were run by age to investigate age-specific associations between binding density patterns in these brain regions. Data were collapsed across sexes because no sex differences in binding density were found in these regions. Based on the observed age differences in binding densities in these regions, we hypothesized that OTR and V1aR would show stronger correlations across this neural network in juveniles than in adults. Indeed, eight significant correlations were found across this network in juveniles while none were found in adults (Fig. 2.6).

Regions with both age and sex differences in OTR binding: core nodes of the social behavior network

We found both age and sex differences in OTR binding density in the posterior BNST, ventromedial hypothalamus, and posterodorsal and posteroventral medial amygdala (Fig. 2.8A). These regions are core nodes of the social behavior network (Newman, 1999). Because OTR binding density was higher in males and higher in adults in these regions, we hypothesized that OTR binding densities across these regions would correlate more strongly in adults than in juveniles and more strongly in males than in females. Correlational analyses were run separately by age and sex to investigate associations between OTR binding densities in these regions. Out of six possible correlations, five were significant in juvenile males, three were significant in adult males, and one was significant in juvenile and in adult females (Fig. 2.9).

Table 2.3. Cohen's D effect size measurements for sex differences in OTR and V1aR binding densities overall, and analyzed separately in juveniles and adults. Only brain regions showing significant main effects of sex or interaction (see Table 1) are included.

		SEX DIFFERENCES			
	OTR/V1aR	Direction	Both sexes	Juveniles	Adults
Cortical areas					
aAIP	OTR	Higher in females	-0.56	-0.58	-1.11
PRh	OTR	Higher in females	-0.82	-1.15	-0.59
Striatal areas					
pCPu	OTR	Higher in adult females	-0.11	-0.49	-1.10
Icj	OTR	Higher in adult males	0.93	-0.41	4.38
Lateral Septum					
LSD	V1aR	Higher in females	-0.65	-1.14	-0.68
LSI	OTR	Higher in females	-0.82	-1.25	-0.48
BNST					
BNSTp	OTR	Higher in males	3.19	2.57	4.93
Amygdala					
MePD	OTR	Higher in males	3.15	2.64	7.39
MePV	OTR	Higher in males	1.69	1.36	2.10
Hypothalamus					
Arc	V1aR	Higher in females	-1.06	-1.93	-1.32
PVN	OTR	Higher in males	0.84	1.06	0.97
VMH	OTR	Higher in males	0.41	0.12	2.16
Thalamus					
vmThal	V1aR	Higher in females	-0.75	-2.10	-1.02

DISCUSSION

We hypothesized that age and sex differences in OTR and V1aR binding density would occur in brain regions within the social decision-making network. Moreover, we hypothesized that sex differences in OTR and V1aR binding density would already be present in juveniles. In line with our hypotheses, our analysis revealed a wide array of brain regions, including several within the social decision-making network, in which OTR and V1aR binding densities differ between juvenile and adult rats, as well as between the sexes. More regions displayed denser binding in juveniles than in adults for the OTR, while the opposite was true for the V1aR. Interestingly, sex differences in OTR and V1aR binding densities were less numerous than age differences. The direction of these sex differences was region-specific for the OTR (i.e., OTR binding density was higher in some regions in males and higher in other regions in females), but consistently higher in females for the V1aR. Finally, again in line with our hypothesis, the majority of sex differences in OTR and V1aR binding density were already present in juveniles. Overall, these findings demonstrate that OTR and V1aR binding densities vary greatly between juveniles and adults as well as between the sexes, and highlight the importance of considering developmental stage and sex when making inferences as to the functional roles of OTR and V1aR in the regulation of social behaviors.

Below, we highlight age- and sex-specific patterns of OTR and V1aR binding density with potential relevance to age and sex differences in the regulation of social behavior. Based on previous literature (Young et al., 1999; Knobloch et al., 2012; Caughey et al., 2011; Calcagnoli et al., 2014; Johnson et al., 2016), we make the

assumption that higher receptor binding density reflects the likelihood of higher receptor activation. Furthermore, if OTR or V1aR in a given brain region has previously been shown to be involved in the facilitation of a particular behavior, we assume that higher receptor binding density will enhance this facilitation. This assumption also finds support in previous studies (Popik & van Ree, 1991; Engelmann & Landgraf, 1994; Everts & Koolhaas, 1999; Tobin et al., 2010; Veenema et al., 2012; Dumais et al., 2016). However, we realize that these two assumptions might be too simplistic. For example, central OT and AVP system function is intrinsically dependent on OT and AVP release in the brain. Few studies have measured local extracellular OT or AVP release in specific brain regions (Bosch & Neumann, 2010; Bosch et al., 2010; Veenema et al., 2010; Lukas et al., 2011) and only one study thus far compared this release between the sexes (Dumais et al., 2016). A proxy measure for OT and AVP release is the analysis of OT and AVP fiber density. Therefore, where possible, we will refer to studies measuring release or fiber density at different ages and in both sexes. Furthermore, OTR and V1aR binding density patterns vary widely across rodent species (Beery et al., 2008; Kelly & Ophir, 2015; Albers et al., 2015; Hammock et al., 2015). Therefore, we will focus on discussing relevant research in rats, but we will reference other species where appropriate. With these limitations in mind, the main purpose of this discussion is to provide a conceptual framework in which to further scrutinize the functional roles of age and sex differences in OTR and V1aR binding density.

Age differences in OTR and V1aR binding density

To the best of our knowledge, only one other study has compared OTR and V1aR binding densities between 35-day-old juvenile rats and adult rats, albeit only in males and

in a limited number of brain regions (Lukas et al., 2010). Our results confirm the age differences in OTR and V1aR binding densities reported by Lukas et al (2010) in the dorsal caudate putamen, ventral lateral septum, and ventromedial hypothalamus for the OTR and dorsal lateral septum, dentate gyrus, and central amygdala for the V1aR. Furthermore, we demonstrate the presence of additional age differences in forebrain and midbrain regions not previously analyzed. Importantly, our design included both males and females, and thus, demonstrates for the first time that age differences in OTR and V1aR binding density are for the most part expressed in both sexes. Although we did not assess vaginal opening or preputial separation in the juvenile rats to determine their reproductive status, these findings demonstrate that OTR and V1aR binding densities at these two life stages are very different and may have important functional consequences for juvenile- versus adult-specific regulation of behavior.

We found that more brain regions displayed higher OTR binding density in juveniles (15 regions) than in adults (10 regions). The age differences we observe in OTR binding densities are largely in line with what is known regarding the developmental trajectory of the OTR in the rat brain (Shapiro & Insel, 1989). For example, OTR binding densities were found to be higher in pre-weaning versus adult male rats in the anterior and ventroposterior olfactory nucleus, nucleus accumbens, dorsal caudate putamen, basolateral and basomedial amygdala, dorsal subiculum, and lateral septum (Shapiro & Insel, 1989). Our data demonstrate that these age differences in OTR binding density are maintained into the juvenile period. A contrasting pattern of age differences emerged for the V1aR. Here, more brain regions in which V1aR binding differed with age displayed higher binding in adults (10 regions) than in juveniles (7 regions). Previous findings

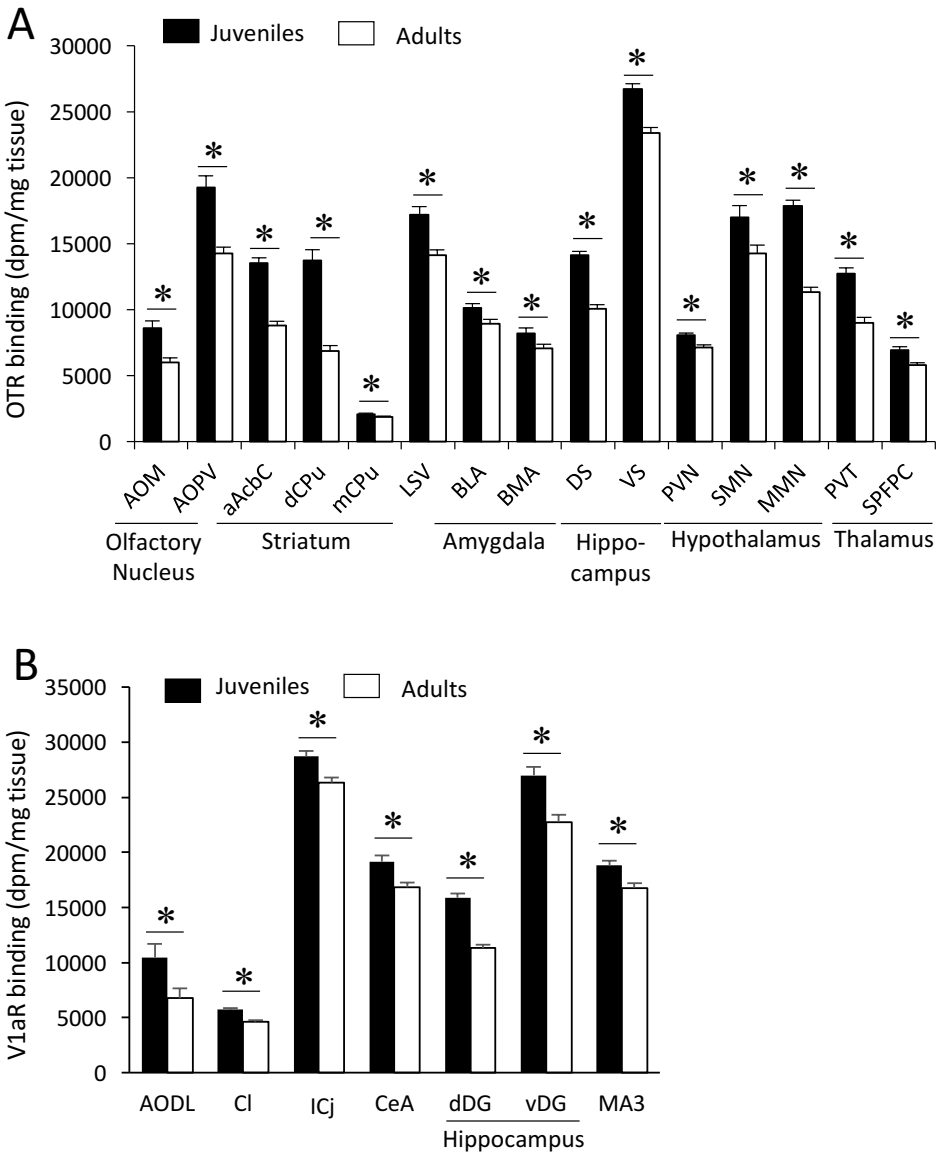


Figure 2.3. Brain regions in which OTR (**A**) and V1aR (**B**) binding density is higher in juveniles than in adults. OTR binding was analyzed on three-day exposure films for subregions of the olfactory nucleus, striatum, and hippocampus and on nine-day exposure films for all other regions. V1aR binding was analyzed on four-day exposure films. Bars indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons; data are collapsed across sexes to highlight main effects of age: *FDR $\alpha < 0.020$ (**A**) and *FDR $\alpha < 0.015$ (**B**).

suggest that AVP binding density reaches adult levels in the rat brain by the time of weaning (Tribollet et al., 1991; Snijdewint et al., 1989). Our results challenge that notion by showing that in most brain regions V1aR binding is still less dense in juveniles as compared to adults. Taken together, these results reveal a large network of brain areas in which OTR and V1aR binding densities differ between juveniles and adults of both sexes. The possible functional implications of these age differences are further discussed below.

Higher OTR in the juvenile dorsal and ventral striatum may support higher social motivation

Of the regions in which OTR binding was denser in juveniles than in adults, the most robust differences were observed in the anterior nucleus accumbens core and dorsal caudate putamen. These striatal regions have been implicated in the regulation of socially rewarding behaviors (Trezza et al., 2011; Burkett et al., 2011; Resendez et al., 2013), and form key nodes in the social decision-making network (O'Connell & Hoffman, 2011, 2012). Moreover, OTR in these regions is involved in mediating socially rewarding behaviors. In detail, activation of the OTR in the striatum promotes partner preference formation and alloparental care in adult female prairie voles (Olazabal & Young, 2006; Keebaugh et al., 2015; Ross et al., 2009; Liu & Wang, 2003) and conditioned place preference for social stimuli in adult male mice (Dolen et al., 2013). Furthermore, OTR binding is higher in the nucleus accumbens of eusocial naked mole-rats as compared to antisocial cape mole-rats, which may suggest that accumbal OTRs play a role in the non-reproductive social behaviors displayed by the former species (Kalamatianos et al., 2010). It should be noted, however, that OTR in the nucleus accumbens is not always

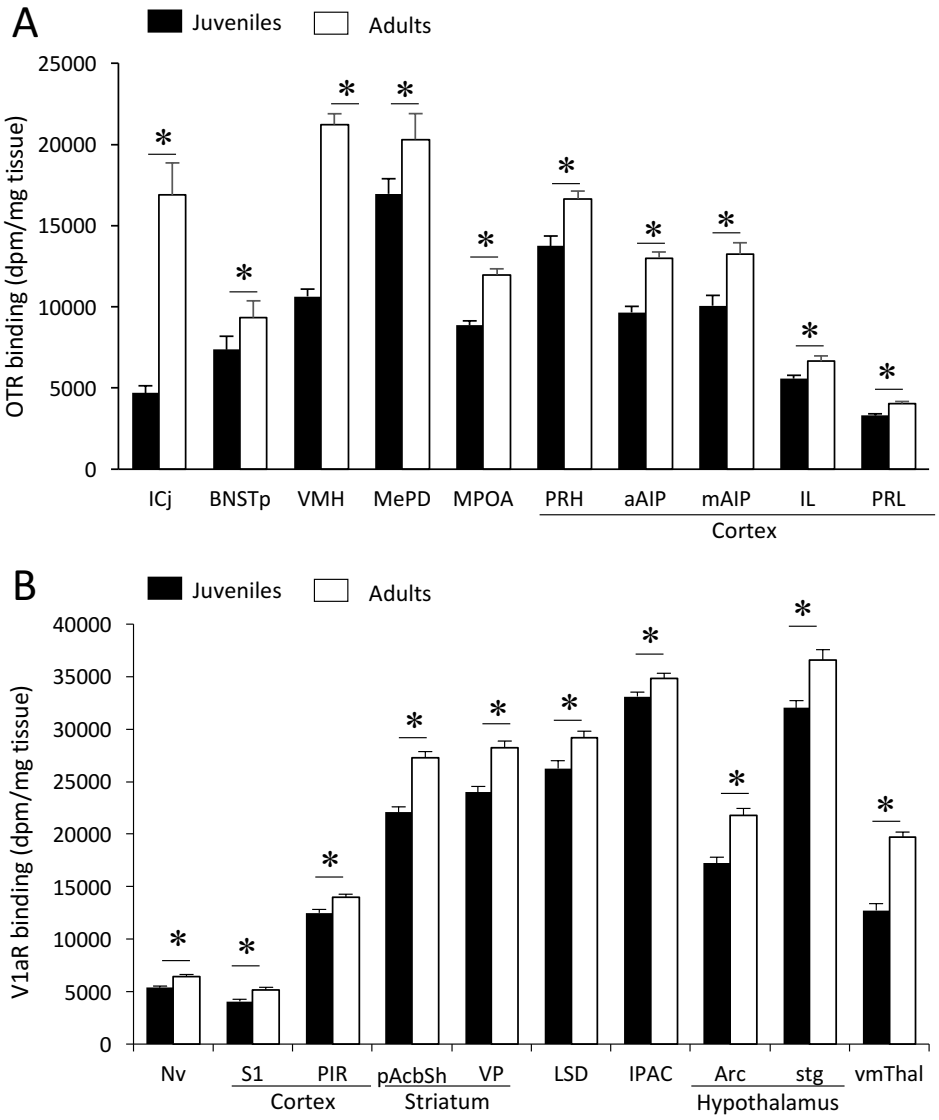


Figure 2.4. Brain regions in which OTR (**A**) and V1aR (**B**) binding density is higher in adults than in juveniles. OTR binding was analyzed on three-day exposure films for the ICj, BNSTp and VMH and on nine-day exposure films for all other regions. V1aR binding was analyzed on four-day exposure films. Bars indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons; data are collapsed across sexes to highlight main effects of age: *FDR $\alpha < 0.020$ (**A**) and *FDR $\alpha < 0.015$ (**B**).

associated with more affiliative social behavior repertoires, as evidenced by comparative studies in species such as tuco-tucos (Beery et al., 2008). Thus, cross-species comparisons regarding the potential role of OTR in the striatum should be made cautiously. Importantly, across species, juvenile animals spend more time engaging in peer interactions than do younger or older animals (Doremus-Fitzwater et al., 2010; Blakemore & Mills, 2014; Larson et al. 1996). These interactions seem to be more rewarding to juveniles, as indicated by the formation of more robust conditioned place preferences for social interaction during the juvenile period as compared to adulthood (Douglas et al, 2004; Trezza et al., 2011; Crone & Dahl, 2012). Therefore, we propose that higher OTR binding density in these regions allows for higher OTR activation which may serve to promote enhanced engagement in peer interactions as observed in juveniles, a hypothesis that remains to be tested.

The juvenile period is also characterized by increases in risk-taking, novelty-seeking behavior, and drug abuse (Steinberg et al., 2008), behaviors that have been shown to be mediated by the striatum (Yager et al., 2015; Tops et al., 2014). Additionally, these behaviors are highly sensitive to social context, which can have either a positive or negative influence. For example, the presence of peers can increase alcohol intake and risk-taking behavior in adolescent rats, mice and humans (Varlinskaya et al., 2015; Logue et al., 2014; Chein et al., 2011, Smith et al., 2015), while strong social attachments can reduce the risk of developing a drug addiction in humans (Tops et al., 2014; Young et al., 2014; Buijman-Pijlman et al., 2014; McGregor et al., 2008; Baumgartner et al., 2008). Interestingly, OT has been suggested to play a prominent role in decreasing vulnerability to risk-taking, novelty-seeking, and drug abuse by acting on

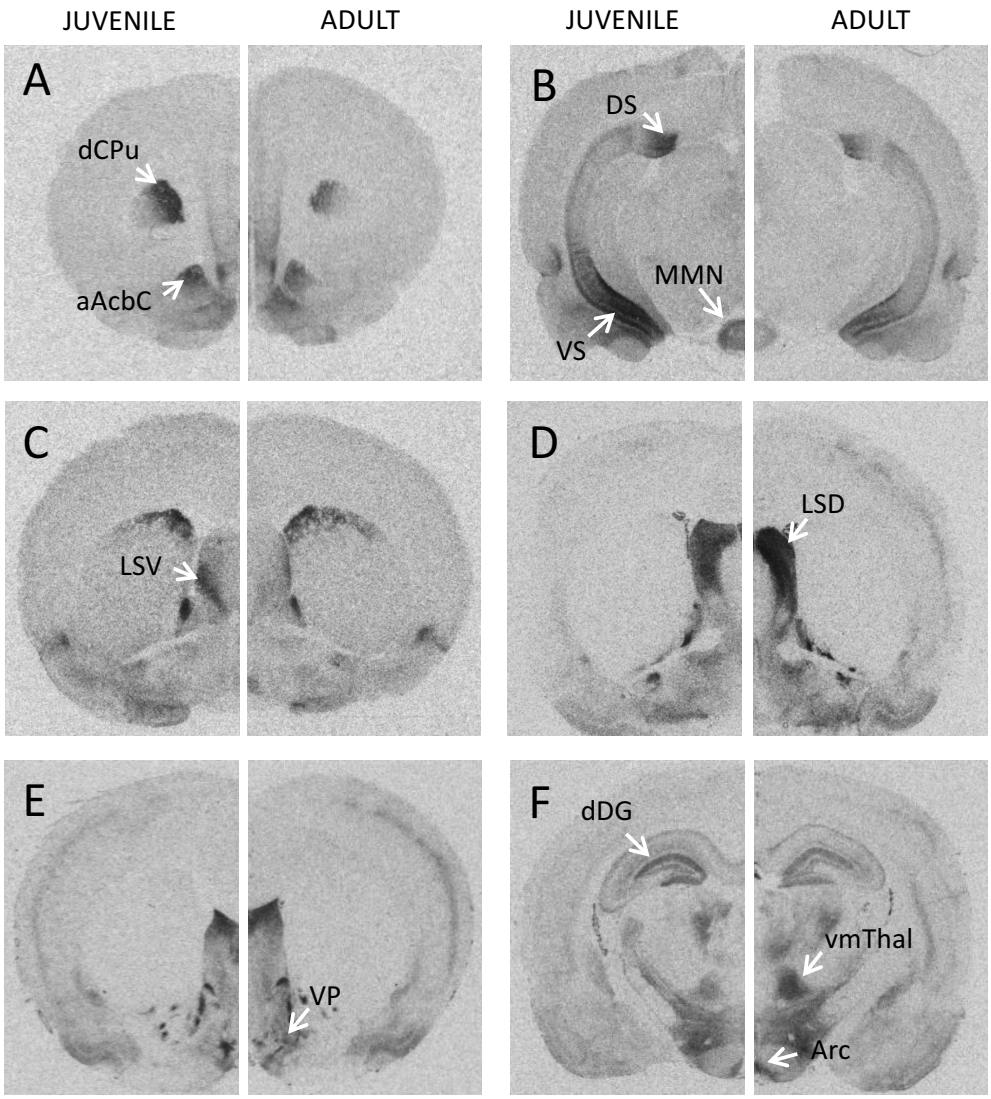


Figure 2.5. Representative images of age differences in OTR (**A**, **B**, **C**) and V1aR (**D**, **E**, **F**) binding densities. OTR binding density is higher in juveniles than adults in the dCPu and aAcbC (**A**), in the DS, VS, and MMN (**B**), and in the LSV (**C**). V1aR binding density is higher in adults than juveniles in the LSD (**D**), VP (**E**), Arc, and vmThal (**F**), but higher in juveniles than adults in the dDG (**F**). Images represent autoradiograms of a juvenile male and an adult male.

the striatum to promote social attachments (Tops et al., 2014). It is therefore possible that higher OTR binding density in striatal subregions of juveniles compared to adults provides a mechanism for the enhanced influence of social context on risk-taking and novelty-seeking behaviors in juveniles.

Age differences in OTR and V1aR in hippocampus, lateral septum, and mammillary nuclei may underlie age differences in social and spatial memory performance

Age differences in OTR and V1aR binding densities were found in all sub-regions of the hippocampus, with higher OTR in juveniles in the dorsal and ventral subiculum and higher V1aR in juveniles in the dorsal and ventral dentate gyrus. Age differences were also found in sub-regions of the lateral septum (ventral lateral septum with higher OTR in juveniles and dorsal lateral septum with lower V1aR in juveniles) and in the mammillary nuclei (medial mammillary and supramammillary nuclei with higher OTR in juveniles). The hippocampus, lateral septum, and mammillary nuclei are highly interconnected brain structures (Risold & Swanson, 1997; Pann & McNaughton, 2004; Allen & Hopkins, 1989; Meibach & Siegel, 1977). Furthermore, previous work has shown that both OT and AVP systems regulate social and spatial memory by acting on sub-regions of the lateral septum and the hippocampal formation. For example, OTR or V1aR blockade in the lateral septum impair social recognition in adult rats (Lukas et al., 2013; Veenema et al., 2012). Moreover, infusion of anti-OT serum into the ventral hippocampus or anti-AVP serum into the dorsal or ventral hippocampus impairs social memory performance in adult male rats (van Wimersma-Graidanus & Maigret, 1996). Furthermore, AVP injection into the dorsal hippocampus facilitates spatial memory in adult male mice (Paban et al., 2003). Finally, OTR binding densities in the hippocampus,

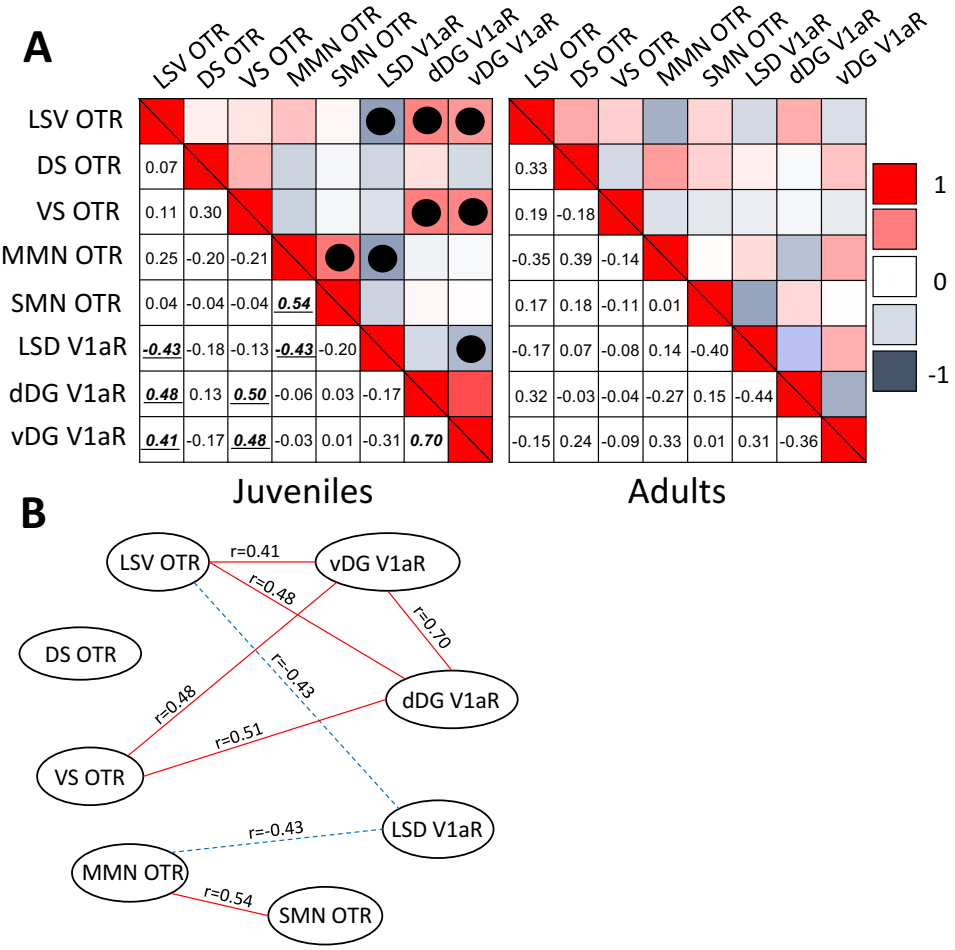


Figure 2.6. Patterns of covariation between OTR and V1aR binding densities within a network of brain regions involved in social and spatial memory. (**A**) Heat maps are shown for juveniles (left) and for adults (right) of both sexes combined. The upper and lower triangle in each heat map show the same data. The hue in the upper triangle represent the strength of the correlation and color indicates the direction of the correlation (red = positive; blue= negative). The numbers in the lower triangle represent the correlation coefficient. Significant correlations are marked with a solid dot in the upper triangle and bolded, italicized, and underlined in the lower triangle. Note the presence of eight significant correlations in this network in juveniles and none in adults. (**B**) Visualization of the network in juveniles showing the correlation coefficients with solid lines indicating positive correlations and dashed lines indicating negative correlations. Significance set at $p<0.05$.

septohippocampal nucleus, and lateral septum correlate with each other and predict socio-spatial memory in adult male prairie voles (Ophir et al., 2012). The mammillary nuclei have also been implicated in spatial memory formation (Mendez-Lopez et al., 2009), although it is currently unknown whether this involves OTR activation. Based on these findings it is plausible that these brain regions form an interconnected network of structures in which OTR and V1aR activation modulates social and spatial memory performance.

Interestingly, correlational analyses revealed that OTR and V1aR binding densities were more strongly correlated across this interconnected network in juveniles than in adults, with eight significant correlations in juveniles and none in adults. Along with the established roles of OTR and V1aR in these brain regions in adults as discussed above, this finding suggests that age differences in OTR and V1aR binding densities in the hippocampus, lateral septum, and mammillary nuclei may regulate social and spatial memory performance differently in juveniles than in adults. In line with this hypothesis, exposure to a spatial learning task (water maze) evoked neuronal activation in the hippocampus and medial mammillary nucleus in juvenile, but not adult rats (Mendez-Lopez et al., 2009). Furthermore, V1aR blockade in the lateral septum impaired social recognition in adult, but not in juvenile, male and female rats (Veenema et al., 2012). These two studies are the first to suggest age differences in the regulation of social and spatial memory performance by OTR and V1aR in these regions. Further research is needed to provide a causal link between age differences in OTR and V1aR binding density in this network of brain regions and differential regulation of social and spatial memory in juveniles and adults.

Higher OTR and V1aR in adulthood in core nodes of the social decision-making network may facilitate adult-specific social behaviors

We found denser OTR binding in adults as compared to juveniles in the ventromedial hypothalamus, posterior BNST, posterodorsal medial amygdala, and medial preoptic area. These brain regions represent core nodes of the social behavior network (Newman, 1999) as well as the more expanded, social decision-making network (O'Connell &Hofmann, 2011, 2012). Several studies suggest that OTR activation in these regions may enhance the processing of social odor cues. For example, OT facilitates social recognition in adult male and female rats by acting on OTR in the posterior BNST (Dumais et al., 2016) and in adult male rats by acting on OTR in both the medial amygdala (Lukas et al., 2013; Gur et al., 2014) and medial preoptic area (Popik & Van Ree, 1991). Additionally, social investigation time correlated positively with OTR binding density in the medial amygdala in female rats (Dumais et al., 2013). These brain regions are also critical to the regulation of reproductive behaviors in both adult males and females (Schulze & Gorzalka, 1991; Witt & Insel, 1991; McCarthy et al., 1994; Emery and Sachs, 1976; Claro et al., 1995; Patil and Brid, 2010; Masugi-Tokita et al., 2015; Noack et al., 2015, Kondo, 1993; Vochteloo & Koolhaas, 1987; Dobolyi et al., 2014) and OTR in each of these brain regions has been implicated in the regulation of adult-specific social behaviors. For example, OT facilitates lordosis responding in adult female rats by acting on OTR in the ventromedial hypothalamus (McCarthy et al., 1994; Schulze & Gorzalka, 1991) and facilitates maternal aggression in lactating rats by acting on OTR in the posterior BNST and medial preoptic area (Consiglio et al., 2005; Pedersen et al., 1994). Furthermore, in adult male rats, duration of aggressive behavior correlated

positively with OTR binding density in the posterior BNST (Calcagnoli et al., 2014). To the best of our knowledge, the role of OTR in these brain regions is unknown in juveniles. Therefore, it would be of interest to determine whether higher OTR binding density in these brain regions in adults as compared to juveniles serves to enhance the processing of social odor cues and promote adult reproductive behaviors.

We also found higher V1aR binding density in the ventral pallidum of adults as compared to juveniles. The ventral pallidum is part of the mesolimbic reward system and forms a node in the social decision-making network (O'Connell & Hoffman, 2011, 2012). V1aR in the ventral pallidum has been associated with mating-induced social affiliation in adult voles. Here, V1aR binding is denser in monogamous male prairie voles than in polygynous male montane voles (Young et al., 2001). Furthermore, V1aR activation in the ventral pallidum facilitates partner preference formation in monogamous male prairie voles (Lim & Young, 2004; Pitkow et al., 2001) and experimentally increased V1aR in the ventral pallidum induces monogamous-like partner preference formation in polygynous male meadow voles (Lim et al., 2004). Increased V1aR expression in the ventral pallidum of male prairie voles enhanced mating-induced neuronal activation, as evidenced by Fos induction (Lim & Young, 2004). Interestingly, paired female prairie voles show higher V1aR binding density in the ventral pallidum than do their single counterparts (Zheng et al., 2013), suggesting that ventral pallidum V1aR may also play a role in the regulation of mating-induced partner preference formation in females. Moreover, ventral pallidum V1aR binding density is higher in the early stages of pregnancy as compared to both the later stages of pregnancy and non-pregnancy (Ophir et al., 2013), which may also reflect mating-induced changes in the

V1aR. Ophir et al., (2013) has suggested that high ventral pallidum V1aR binding density may facilitate social bonding and that low ventral pallidum V1aR binding density may accommodate social promiscuity (Ophir et al., 2013). Although rats are not known as a monogamous species, under certain conditions male rats do form mating preferences for a familiar female rat (Ismail et al., 2009). Taken together, it is plausible that the V1aR in the ventral pallidum of adult rats facilitates preferences for the opposite sex and/or mating behaviors. This hypothesis has yet to be explored in rats and thus offers an interesting avenue for future research.

Higher OTR and V1aR in cortical areas in adulthood may facilitate social behavior by improving signal-to-noise ratio and, therefore, enhancing the salience of social cues

Adult rats showed higher OTR and V1aR binding density than juvenile rats in seven out of eight cortical regions analyzed. These were the infralimbic, prelimbic, anterior insular, medial insular, and perirhinal cortices for the OTR and the piriform and sensory (S1) cortices for V1aR. Infralimbic and prelimbic OTR binding was very low as compared to OTR binding in any of the other regions analyzed. Nevertheless, recent studies indicate that OTR in these areas are functional and play a role in reproduction-related social behaviors. For example, impairing OTR function in the medial prefrontal cortex (including the infralimbic and prelimbic cortices; either by pharmacological inhibition of the OTR, deletion of the OTR gene, or chronic silencing of OTR expressed on interneurons) reduced social investigation of adult male mice by adult female mice, especially when the subject females were in estrus (Nakajima et al., 2014). Additionally, OTR blockade in the prelimbic cortex impaired maternal care behavior in lactating female rats (Sabihi et al., 2014). Little is known about the mechanisms and neural circuits

by which activation of OTR modulates these behaviors. However, a recent study focusing on the auditory cortex may shed light on a potential mechanism of action. Here, OT facilitated pup retrieval in adult virgin female mice by balancing excitatory and inhibitory signals in the auditory cortex, thereby improving the signal-to-noise ratio (Marlin et al., 2015). Improved excitatory/inhibitory balance is a feature naturally observed in the auditory cortex of lactating female mice (Cohen et al., 2011; Liu et al., 2006; Rothschild et al., 2013). Marlin et al (2015) speculate that this reshaping of neuronal responses by OT may enhance the salience of pup distress calls and allow for the appropriate behavioral response, i.e., pup retrieval. Interestingly, the signal-to-noise ratio of cortical electrical signals is lower in adolescents as compared to adults in rats and humans (Segalowitz & Davies, 2004; Sturman & Moghaddam, 2011b). Moreover, OT acting on OTR was shown to balance excitatory and inhibitory input in the hippocampus in juvenile rats (Owen et al., 2013). These findings may suggest that cortical OTR play a role in mediating an optimal excitatory/inhibitory balance irrespective of age, while our current findings may suggest that this function is heightened in adults compared to juveniles because of higher OTR, and perhaps V1aR, binding density in cortical regions.

Sex differences in OTR and V1aR binding densities

We are the first to directly compare OTR and V1aR binding densities in males and females and juveniles and adults in one design. Although less numerous than age differences, we found a number of sex differences in OTR and V1aR binding density. Importantly, we are the first to demonstrate that in most brain regions, sex differences in OTR and V1aR binding density are present at postnatal day 35, and that sex differences in OTR and V1aR occur in brain areas in which binding density is higher in adults than in

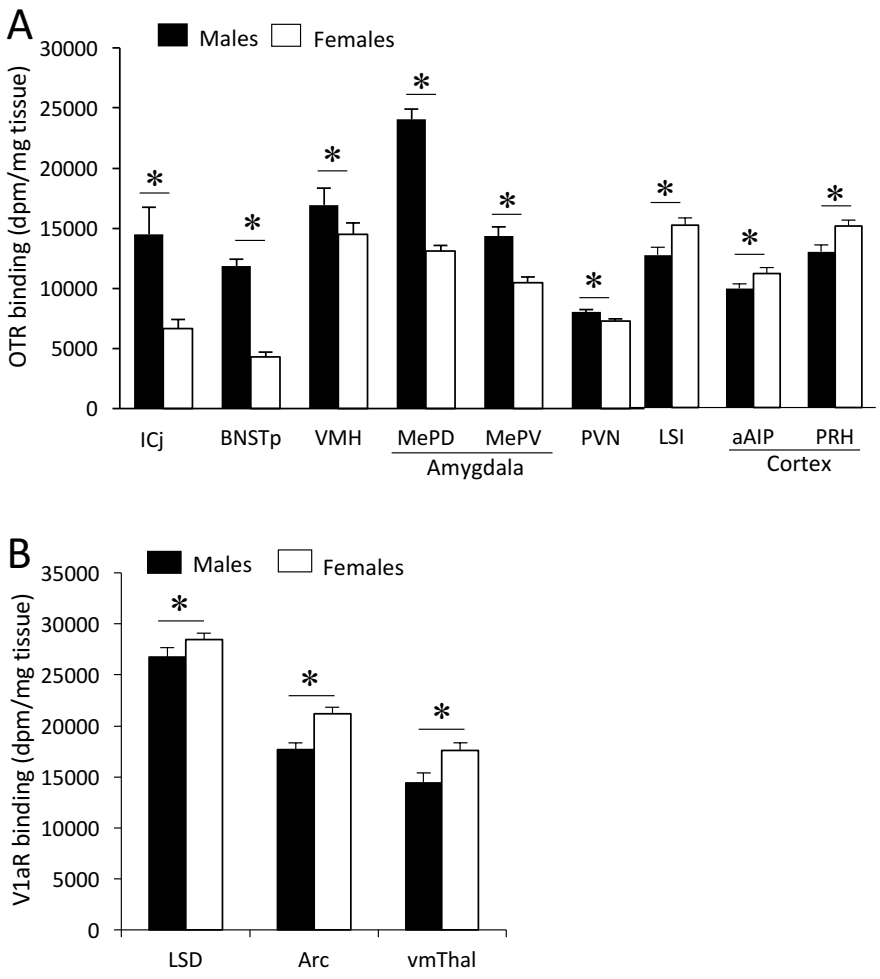


Figure 2.7. Brain regions in which OTR (A) and V1aR (B) binding densities show sex differences. OTR binding was analyzed on three-day exposure films for the ICj, BNSTp and VMH and on nine-day exposure films for all other regions. V1aR binding was analyzed on four-day exposure films. Bars indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons; data collapsed across ages to highlight main effects of sex: *FDR $\alpha < 0.020$ (**A**) and *FDR $\alpha < 0.015$ (**B**).

juveniles. It should be noted that we did not control for possible effects of estrous cycle phase on OTR and V1aR binding density. However, previous findings in adult rats suggest that estrous phase has limited impact on sex differences in OTR and V1aR binding density (Dumais et al., 2013; Dumais & Veenema, 2016). Moreover, OTR and V1aR binding density variability (as interpreted by the average standard deviation of binding density in all brain regions) was no greater in females than in males, suggesting that it is unlikely that estrous phase had a large impact on the observed sex differences in OTR and V1aR binding density.

OTR binding was denser in males than in females in the posterior BNST, ventromedial hypothalamus, medial preoptic area and posterodorsal and posteroventral medial amygdala. These sex differences confirm those of previous studies in rats (Uhl-Bronner et al., 2005; Dumais et al., 2013), suggesting that these are highly robust and persistent sex differences. On the other hand, we also found sex differences in OTR and V1aR binding density (such as in the islands of Calleja, intermediate lateral septum, anterior insular cortex, and perirhinal cortex for the OTR and in the dorsal lateral septum, arcuate nucleus, and ventromedial thalamus for the V1aR) that were either not found or not characterized in previous studies of receptor binding (Dumais et al., 2013; Dumais & Veenema, 2016) or mRNA (Szot et al., 1994). Importantly, one notable difference between our current study and previous work (Dumais et al., 2013; Dumais & Veenema, 2015) is the behavioral experience of the subjects. In the current study, rats were socially housed but underwent no behavioral testing prior to brain tissue collection for receptor binding. In the previous studies, rats were singly housed and exposed to several social behavioral tests two weeks prior to brain tissue collection (Dumais et al., 2013; Dumais

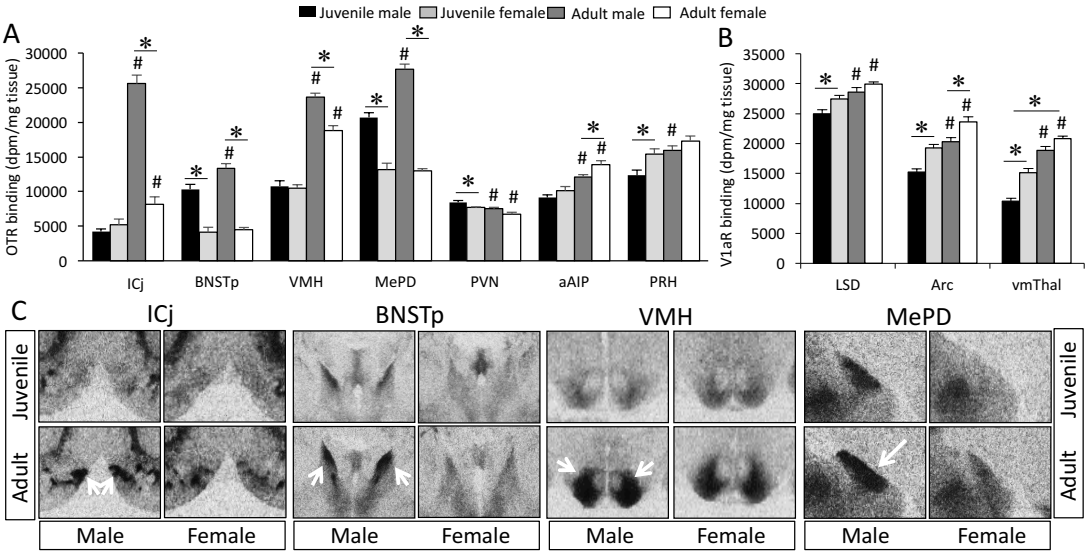


Figure 2.8. Age and sex differences in OTR (A) and V1aR (B) binding. OTR binding density in the ICj, BNSTp, VMH, and MePD (**C**) of juvenile and adult male and female rats. OTR binding is higher in adults compared to juveniles in males only in the ICj, BNSTp, and MePD, but in both sexes in the VMH. OTR binding is higher in males than in females at both ages in the BNSTp and MePD, but in adults only in the ICj and VMH. Bars indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons: * <0.05 versus opposite sex; # < 0.05 versus respective juvenile group.

& Veenema, 2015). Both OTR and V1aR binding densities can be altered by life experiences such as parenthood and early life stress (Lukas et al., 2010; Bales & Perkeybile, 2012; Bosch et al., 2010; Bosch & Neumann, 2008; Lukas et al., 2010; Dumais & Veenema, 2015), and this may occur in sex-specific ways (Curley et al., 2009). Given this plasticity, it is likely that differences in social experiences contribute to the inconsistencies in sex differences in OTR and V1aR binding densities between these studies. Notably, those sex differences in OTR binding density that were replicated were of higher magnitude, making it possible that, if binding density were to be changed due to experience, that this may not have obscured the sex difference in these regions. Conversely, experience-induced plasticity may be able to change the presence or direction of sex differences in OTR and V1aR binding density in those brain regions showing smaller magnitude sex differences.

Sex differences in correlations between OTR binding densities in the posterior BNST, ventromedial hypothalamus, and medial amygdala: Implications for the regulation of sex differences in behavior

OTR binding densities across the posterior BNST, ventromedial hypothalamus, posterodorsal medial amygdala, and posteroventral medial amygdala correlated more strongly in juvenile and adult males than in females. It has been suggested that the relative activation of these regions, along with other regions within the social behavior network, determine the type of social behavior expressed (Newman, 1999). Accordingly, OTR in the posterior BNST, ventromedial hypothalamus, and medial amygdala may play a key role in this relative activation. It would be interesting to test whether higher and more strongly correlated OTR binding densities in these regions in males than in females provides a mechanism to mediate sex-specific regulation of particular social behaviors in both juveniles and adults.

Most sex differences in OTR and V1aR binding density are already present at juvenile age: Role for gonadal hormones early in life?

A novel and important finding of this study is the early emergence of sex differences in OTR and V1aR binding density. With the notable exceptions of the ventromedial hypothalamus and anterior insula, all sex differences found were already present at the juvenile age. The function of the early presence of these sex differences is unclear, but may suggest a role for OTR and V1aR in the sex-specific regulation of juvenile, in addition to adult, social behaviors. Interestingly, OTR and V1aR were found

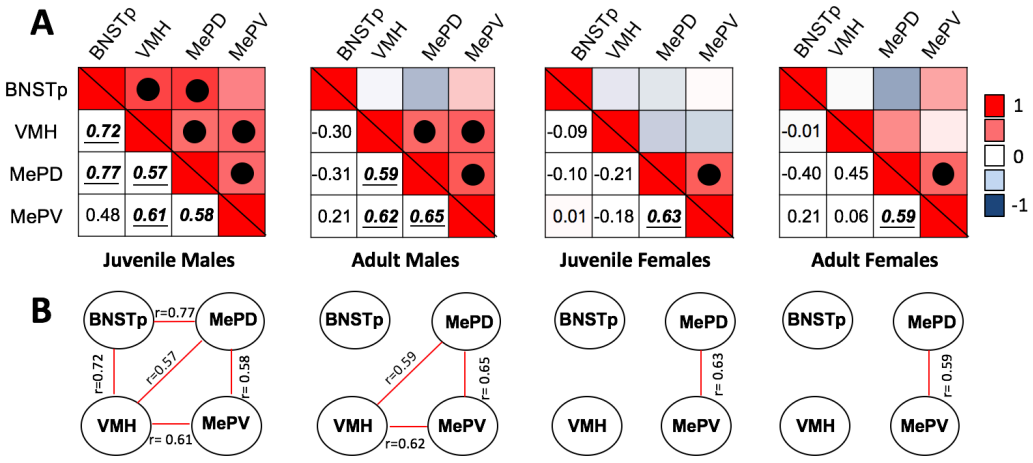


Figure 2.9. Patterns of covariation between OTR binding densities in the BNSTp, VMH, MePD, and MePV, core brain regions in the social behavior network. Note the strong correlational network in juvenile males and the much weaker correlational network in juvenile and adult females. (**A**) Heat maps are shown for each age and sex separately with hue representing the strength of the correlation and color indicating the direction of the correlation (red = positive; blue= negative). The upper and lower triangle in each heat map shows the same data. The hue in the upper triangle represent the strength of the correlation and color indicates the direction of the correlation (red = positive; blue= negative). The numbers in the lower triangle represent the correlation coefficient. Significant correlations are marked with a solid dot in the upper triangle and bolded, italicized, and underlined in the lower triangle. (**B**) Visualization of the network for each age and sex showing the correlation coefficients with solid red lines indicating positive correlations. Significance set at $p<0.05$.

to be involved in the sex-specific regulation of juvenile social play behavior (Veenema et al., 2013; Bredewold et al., 2014). Further research in juveniles is required in order to understand, more completely, the sex-specific roles of OT and AVP systems in the regulation of social behavior. Moreover, further research is also needed to clarify the mechanisms by which sex differences in OTR and V1aR binding density in the brain are created.

Sex differences in OTR and V1aR binding density occur in brain regions with denser binding in adults than in juveniles: 1. Role in both age- and sex-specific regulation of social behavior?

We found that in most of the brain regions in which OTR and V1aR binding density differs between the sexes, binding is denser in adult as compared to juvenile rats. These brain regions are the islands of Calleja, ventromedial hypothalamus, posterior BNST, posterodorsal medial amygdala, anterior insular and perirhinal cortex for the OTR and the dorsal lateral septum, arcuate nucleus, and ventromedial thalamus for the V1aR. These may be regions in which OTR and V1aR play a role in both age and sex differences in the regulation of social behavior. For example, the AVP system in the lateral septum is involved in both age- and sex-specific regulation of social recognition in rats (Veenema et al., 2012). Interestingly, the age difference in V1aR binding density in the lateral septum corresponds with a similar age difference in AVP fiber density in the lateral septum (De Vries et al., 1981), further supporting an age-specific role of the AVP system in the lateral septum. However, the sex difference in V1aR binding density (higher in females) is opposite to the sex difference in AVP fiber density (higher in males) in the lateral septum (De Vries et al., 1981), making it more difficult to understand

the behavioral consequences in either sex. Furthermore, we recently showed that the OTR in the posterior BNST plays a role in the sex-specific regulation of social recognition of adult rats, with higher OTR binding density associated with higher OT release in males than in females (Dumais et al., 2016). These studies highlight the importance of determining whether age and sex differences in OTR and V1aR binding density are accompanied by age and sex differences in OT and AVP innervation/release in the same regions. This knowledge may help to understand the functional consequences of age and sex differences in OTR and V1aR binding density.

Sex differences in OTR and V1aR binding density occur in brain regions with denser binding in adults than in juveniles: 2. Role for circulating gonadal hormones after puberty?

The age-dependent increase in OTR and V1aR binding density predominantly occurs in regions that also show sex differences in receptor binding density. It is therefore likely that these regions are sensitive to gonadal steroids and that the age-dependent increases are induced by increases in gonadal hormone levels at the onset of puberty and thereafter. Indeed, evidence suggests that the expression of OTR, and to some extent V1aR, requires circulating gonadal hormones. For example, adult gonadectomy reduced the amount of [^{3}H] OT binding in the islands of Calleja and ventromedial hypothalamus in both male and female rats (Tribollet et al., 1990). Furthermore, testosterone or estradiol replacement after gonadectomy restored [^{3}H] OT binding in the islands of Calleja and ventromedial hypothalamus to pre-gonadectomy levels in female rats (Tribollet et al. 1990). Moreover, adult treatment with an aromatase inhibitor (which prevents the conversion of testosterone to estradiol) decreased [^{3}H] OT binding in the

ventromedial hypothalamus, albeit not as robustly as gonadectomy, in male rats (Tribollet et al. 1990). It should be noted that the above discussed findings are based on the use of a nonselective OT-radiolabeled ligand and a relatively low numbers of animals (3-4) per group (Tribollet et al., 1990). These factors may also explain why adult gonadectomy was not found to alter [^{3}H] AVP binding in rats (Tribollet et al., 1990). In contrast, using a more specific [^{125}I] V1aR-radiolabeled ligand, adult gonadectomy decreased V1aR binding density in the BNST and ventromedial hypothalamus, among other regions, in male hamsters (Delville & Ferris, 1995; Johnson et al., 1995; Young et al., 2000). These results lend support to the idea that gonadal hormones are important for the maintenance of OTR binding density. However, comparative studies will be required to demonstrate that gonadal hormone removal in adulthood reduces OTR or V1aR binding density to juvenile levels.

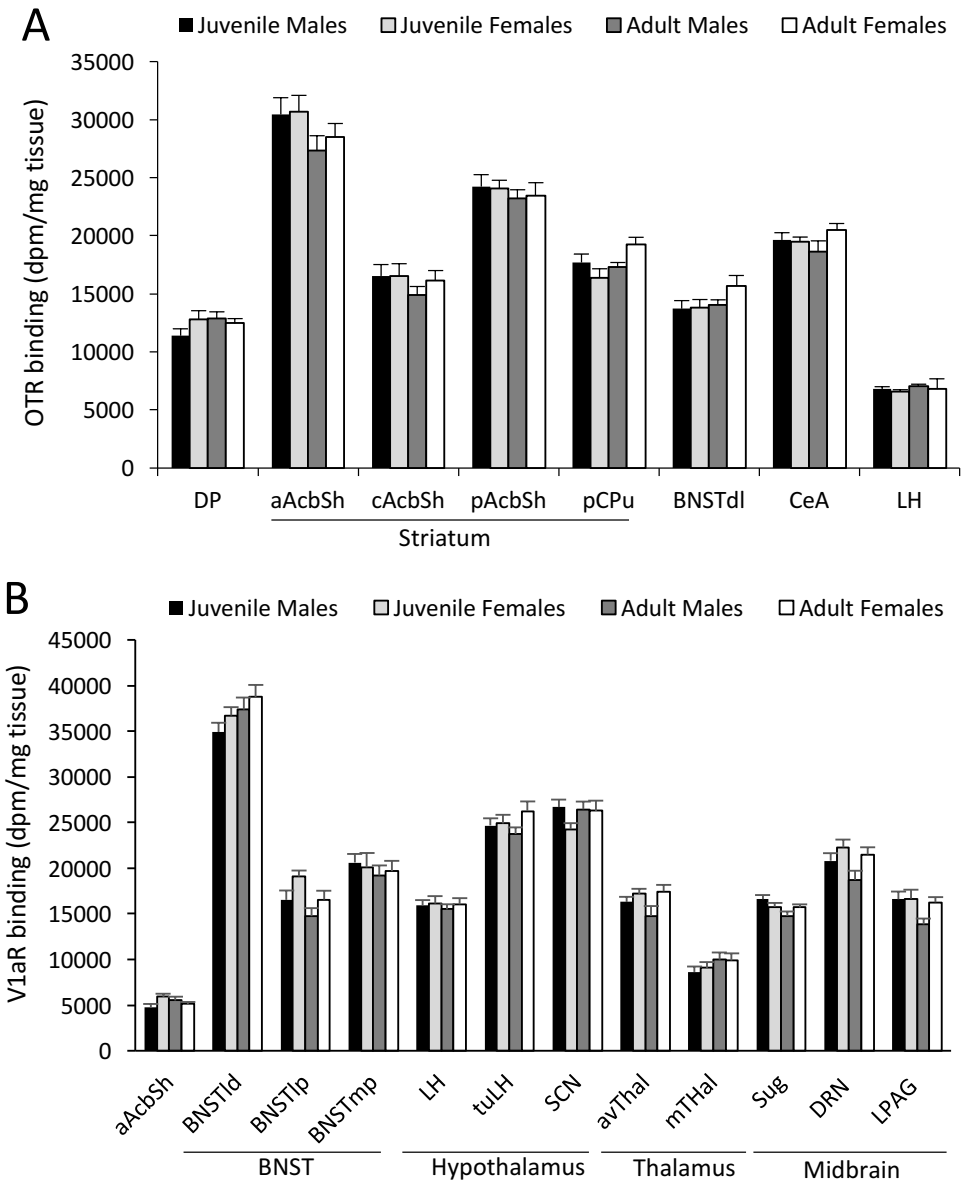
Regions of functional significance, despite a lack of age and sex differences in OTR and V1aR binding density

It is important to note that many brain regions show dense OTR and V1aR binding without age and sex differences. For the OTR, these areas include the dorsal peduncular nucleus, anterior, central and posterior nucleus accumbens shell, posterior caudate putamen, laterodorsal BNST, central amygdala and lateral hypothalamus. For the V1aR these areas include all subdivisions of the BNST, the anterior nucleus accumbens shell, the lateral and tuberal hypothalamus, the suprachiasmatic nucleus, the anteroventral and medial thalamus, the lateral periaqueductal grey, the superficial grey layer of the superior colliculus, and the dorsal raphe nucleus. The latter two represent regions in

which, to the best of our knowledge, V1aR binding has not been analyzed before. Interestingly, V1aR binding has been observed in the periaqueductal grey of *Scotinomys teguina*, a species of singing mouse, where it may play a role in the generation of vocalizations (Campbell et al., 2009). OTR and V1aR in these brain regions likely serve important functions that may not differ across development or between the sexes. Yet, the absence of an age or sex difference in OTR and V1aR binding density does not exclude the possibility of age- or sex-specific involvement of OTR and V1aR in the regulation of social behavior. It would therefore be of interest to further explore the functions of OTR and V1aR in these brain regions at both juvenile and adult ages and in both males and females.

Opposing roles of OTR and V1aR in the central amygdala: Role in age-specific anxiety or fear responding?

OT in the central amygdala has been shown to decrease fear responding in adult male and female rats (Viviani et al., 2011; Knobloch et al., 2012). This is thought to be mediated through local network activity involving both OTR and V1aR. In detail, activation of OTR-expressing central amygdala neurons inhibits the activation of V1aR-expressing central amygdala neurons (Huber et al., 2005). This pathway may allow for OTR and V1aR within the central amygdala to have opposing responses on anxiety and fear. Interestingly, OTR binding density in the central amygdala doesn't show age or sex differences, but V1aR binding density in the central amygdala is higher in juveniles than in adults. This may result in more V1aR relative to OTR activation in juveniles as compared to adults. Although the role of V1aR and OTR in the central amygdala in



Supplementary Figure 2.1. Brain regions in which no age or sex differences in OTR (**A**) or V1aR (**B**) binding densities in the rat brain were found. OTR binding was analyzed on three-day exposure films for the DP, BNSTdl and CeA and on nine-day exposure films for all other regions. V1aR binding was analyzed on four-day exposure films. Bars indicate mean + SEM; two-way ANOVA (age x sex) with FDR $\alpha < 0.020$ for OTR binding (**A**) and FDR $\alpha < 0.015$ for V1aR binding (**B**) .

juvenile rats in unknown, this could indicate that the V1aR plays a larger functional role than the OTR in juveniles, and may result in age-specific anxiety/fear responses. This remains to be tested.

Conclusion

In conclusion, our work demonstrates the presence of age and sex differences in OTR and V1aR binding densities in the rat brain, particularly in brain regions that form the social decision-making network (O'Connell & Hoffman, 2011, 2012). We discussed that age differences in OTR and V1aR binding densities may have implications for the regulation of social motivation (through higher OTR binding in striatal areas) and social and spatial memory (through age differences in OTR and V1aR in the hippocampus, lateral septum and mammillary nuclei) in juveniles and for the regulation of adult-typical social behaviors (through higher OTR and V1aR in nodes of the social decision-making network and higher OTR in cortical areas). We further showed, for the first time, that most sex differences in OTR and V1aR binding are already present at juvenile age and are found in regions showing a further increase in binding density in adults. We discussed that these regions represent a unique network in which to expect both age and sex differences in the regulation of social behavior. Further research is needed to determine a) the behavioral relevance of age and sex differences in OTR and V1aR and b) the neural and molecular mechanisms that underlie these age and sex differences.

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Chapter Three: Age, but not sex, differences in mu-opioid receptors in the rat brain: Relevance for reward and drug seeking in juveniles*

**manuscript in preparation:*
Smith C.J.W., Ratnaseelan A.M., Veenema A.H. (2017) Brain region-specific age and sex differences in μ-opioid receptor binding in the rat

ABSTRACT: The μ-opioid receptor (MOR) in the brain is involved in reward-seeking behaviors and plays a pivotal role in the mediation of opioid use disorders. Furthermore, reward-seeking behaviors and susceptibility to opioid addiction are particularly evident during the juvenile period, with a higher incidence of opioid use in males and higher sensitivity to opioids in females. Despite these age and sex differences in MOR-mediated behaviors, little is known regarding potential age and sex differences in the expression of MORs in the brain. Here, we used receptor autoradiography to compare MOR binding densities between juveniles and adults and males and females throughout the rat brain. Age differences were found in MOR binding density in 12 out of 33 brain regions analyzed, with 11 regions showing higher MOR binding density in juveniles as compared to adults. These include sub-regions of the lateral septum, bed nucleus of the stria terminalis, hippocampus, and thalamus. Sex differences in MOR binding density were observed in only two brain regions, namely, the lateral septum (higher in males) and the posterior cortical nucleus of the amygdala (higher in females). Overall, these findings provide an important foundation for the generation of novel hypotheses regarding the age-specific role of MORs. In particular, juveniles may show increased MOR activation which could modulate behaviors that are heightened during the juvenile period, such as reward-seeking and drug abuse.

Abbreviations

aAcbC	*anterior nucleus accumbens core*
aAcbSh	*anterior nucleus accumbens shell*
aCPu	*anterior caudate putamen*
BLA	*basolateral amygdala*
BNSTmp	*bed nucleus of the stria terminalis, posteromedial part*
BNSTp	*bed nucleus of the stria terminalis, posterior part*
CA1	*CA1 layer of the hippocampus*
CA2/3	*CA2/3 layers of the hippocampus*
CIC	*central nucleus of the inferior colliculus*
CPu	*caudate putamen*
DCIC	*dorsal cortex of the inferior colliculus*
DLG	*dorsal lateral geniculate nucleus*
dmAcbSh	*dorsomedial nucleus accumbens shell*
IPA	*apical subnucleus of the of the interpeduncular nucleus*
IPAC	*interstitial nucleus of the posterior limb of the anterior commissure*
IPC	*caudal subnucleus of the of the interpeduncular nucleus*
IPL	*lateral subnucleus of the of the interpeduncular nucleus*
LDTN	*laterodorsal thalamic nucleus*
LPAG	*lateral periaqueductal grey*
LPTN	*lateroposterior thalamic nucleus*
LS	*lateral septum*
MDT	*mediodorsal thalamus, lateral part*
MePD	*medial amygdala, posterodorsal part*
moDGp	*molecular layer of the dentate gyrus, posterior part*
MOR	*μ opioid receptor*
PMCo	*posteromedial cortical amygdaloid nucleus*
Po	*posterior thalamic nucleur group*
PV	*paraventricular thalamic nucleus*
Re	*reuniens nucleus of the thalamus*
Rt	*reticular nucleus of the thalamus*
sm	*stria medularis of the thalamus*
SNCD	*substantia nigra, dorsal tier, compact part*
SUG	*superficial grey layer of the superior colliculus*
vAcbSh	*ventral nucleus accumbens shell*

INTRODUCTION

The juvenile period is one during which individuals are particularly driven to seek rewards and to engage in risk-taking behaviors and drug abuse (Spear et al., 2000; Foulkes & Blakemore, 2016; Casey et al., 2008; Compton & Volkow, 2006). Moreover, juvenile animals are more likely to engage in social interactions with peers, and find these social interactions to be more rewarding than at younger or older ages (Spear, 2000; Doremus-Fitzwater et al., 2010). Previous work in humans and rodents suggests an important role for the mu opioid receptor (MOR) in the regulation of these behaviors. For example, in adolescent boys and girls, polymorphisms in the MOR gene (*OPRM1)* are associated with differences in neural activation to reward cues (Nees et al., 2017). These gene variants are also associated with differences in alcohol use (Miranda et al., 2010) and attention to alcohol-related environmental cues (Pieters et al., 2011). In juvenile male rats, central MOR activation facilitates social play behavior (Vanderschuren et al., 1995a; Trezza et al., 2011) and the preference to interact with a novel as opposed to a familiar conspecific (Smith et al., 2015). Furthermore, central MOR antagonism blocks the reinforcing properties of ethanol in juvenile rats (Pautassi et al., 2011). Based on this literature, we propose that the juvenile propensity to engage in social interaction, novelty-seeking, and risk-taking may be due to heightened MOR activation in the brain as compared to younger and older ages. Yet, little is known regarding age differences in MOR expression in the brain. Although some studies have charted the pre-weaning development of MORs in the rat brain (Recht et al., 1985; Kornblum et al., 1987; Moon-Edley & Herkenham, 1984; Spain et al., 1985), a quantitative comparison of MOR binding densities between juveniles and adults is lacking. Therefore, our first aim was to

determine MOR binding densities in the brains of juvenile and adult rats. We hypothesized that MOR binding density would be higher in juveniles as compared to adults in brain regions involved in regulating reward-seeking behaviors.

Several studies suggest that there are sex differences in reward-seeking behaviors and susceptibility to drug abuse. For example, boys are more likely to engage in reward and sensation seeking behaviors than girls (Steinberg et al., 2008; Romer & Hennessey, 2007), while girls are more likely to consume alcohol than boys (Johnston et al., 2015). Moreover, men are more likely than women to engage in substance abuse (Lynch et al., 2002), while women become addicted to opiates more quickly following first use (Lex et al., 1991; Roth et al., 2004). Similarly, female rats acquired heroin self-administration more quickly than their male counterparts, and subsequently, self-administered larger amounts of the drug (Lynch & Carroll, 1999; Cicero et al., 2003). It is plausible that sex differences in MOR activation underlie sex differences in these behaviors. In support, a PET scanning study demonstrated higher MOR binding in the amygdala, thalamus, and caudate in women as compared to men (Zubieta et al., 1999). Likewise, higher MOR binding density was found in female rats as compared to male rats in the nucleus accumbens, posterior cortical nucleus of the amygdala, basolateral amygdala, and bed nucleus of the stria terminalis (Vathy et al., 2003). However, these rats were gonadectomized, leaving it unclear whether there are sex differences in MOR binding densities in intact rats. Therefore, our second aim was to compare MOR binding density between intact male and female rats. We hypothesized that MOR binding density would be higher in females as compared to males, based on previous findings in humans and gonadectomized adult rats (Zubieta et al., 1999; Vathy et al.,2003).

METHODS

Animals

Male and female Wistar rats were obtained from Charles River Laboratories (Raleigh, NC) at 22 or 56 days of age and housed under standard laboratory conditions (12-hour light/dark cycle, lights on at 7:00 am, food and water available *ad libitum*, 22° C, 60% humidity). Upon arrival at our facility, rats were housed in standard rat cages (26.7 x 48.3 x 20.3 cm). Twenty-two-day-old rats were housed in same-sex groups of 3-4 until brain collection for receptor autoradiography at 35 days of age (Juvenile group). Fifty-six-day-old rats were housed in same-sex pairs until brain collection for receptor autoradiography at 84 days of age (Adult group). All experiments were conducted in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

Receptor Autoradiography

Rats (juvenile males: n=13; juvenile females: n=13; adult males: n=12; adult females: n=12) were killed using CO_2 inhalation and brains were removed, rapidly frozen in methylbutane on dry ice, and stored at -45˚ C. Brains were cut on a cryostat into 16-µm coronal sections and mounted onto slides in eight adjacent series. Collection began at approximately 3.72 mm anterior to bregma and ended at approximately 8.52 mm posterior to bregma (Paxinos & Watson, 2007). Sections were then frozen -45˚ C until receptor autoradiography was performed. MOR autoradiography was conducted using

[^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin (DAMGO; Perkin Elmer, Boston, MA) as tracer. In brief, slides were thawed and air-dried at room temperature followed by pre-incubation for 30 min in 50 nM Tris-HCl (pH 7.4) containing 0.9% NaCl. The slides were then exposed to tracer buffer (4 nM [^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin and 50 mM Tris) for 60 min. Non-specific binding was accessed in adjacent brain sections by incubation in tracer buffer with the addition of 1 μM naloxone (Sigma-Aldrich, St. Louis, MO). All slides were then washed three times, for 5 min each, in ice-cold Tris-HCl, air-dried, and exposed to Biomax MR films (VWR International, Pittsburgh, PA) for 16 weeks. Brain sections from animals of both sexes were processed together and balanced across incubation chambers and exposure to films.

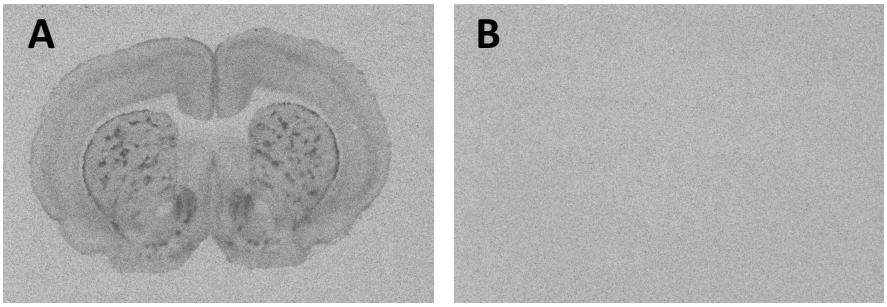

Figure 3.1. MOR binding in 16 μm coronal brain sections in the absence and presence of the selective MOR receptor antagonist naloxone. (**A**) Incubation with the radioligand [^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin yielded MOR binding in the striatum and cortex. (**B**) Incubation with the same radioligand and an excess of unlabeled naloxone yielded no binding, indicating that binding in (**A**) is specific to the MOR.

Image and Data Analysis

Autoradiography films were digitized using a Northern Light Illuminator (InterFocus Imaging, Cambridge, UK) and optical densities of MOR binding were measured in coronal sections using ImageJ (NIH, http://imagej.nih.gov/ij/). The data were converted to dpm/mg tissue (disintegrations per minute/milligram tissue) using a [^{3}H] standard microscale (American Radiolabeled Chemicals Inc., St. Louis, MO). Because non-specific binding was undetectable (Fig. 3.1), film background values were subtracted from total binding values to yield specific binding values. Binding densities were calculated by taking the mean of bilateral measurements in a fixed number of sections per region of interest per rat. The total number of sections included depended on the size of the region of interest with a minimum of 2 sections. MOR binding density was measured in a total of 33 brain regions (see Fig. 3.2 for receptor autoradiograms and schematic diagrams indicating the brain regions in which MOR binding was quantified. All abbreviations of brain regions are in accordance with Paxinos & Watson (2007), except for the nucleus accumbens core and nucleus accumbens shell, where we added the subdivisions anterior core, anterior shell, dorsomedial shell and ventral shell to delineate the separate areas analyzed as well as for the laterodorsal thalamic nucleus where we used the abbreviation LDTN to refer to the dorsomedial and ventrolateral parts of the nucleus combined, the lateral posterior thalamic nucleus where we used the abbreviation DPTN to refer to the mediorostral and laterorostral parts combined, and the molecular layer of the dentate gyrus where we used the abbreviation moDGp to refer to the more posterior part of the region.

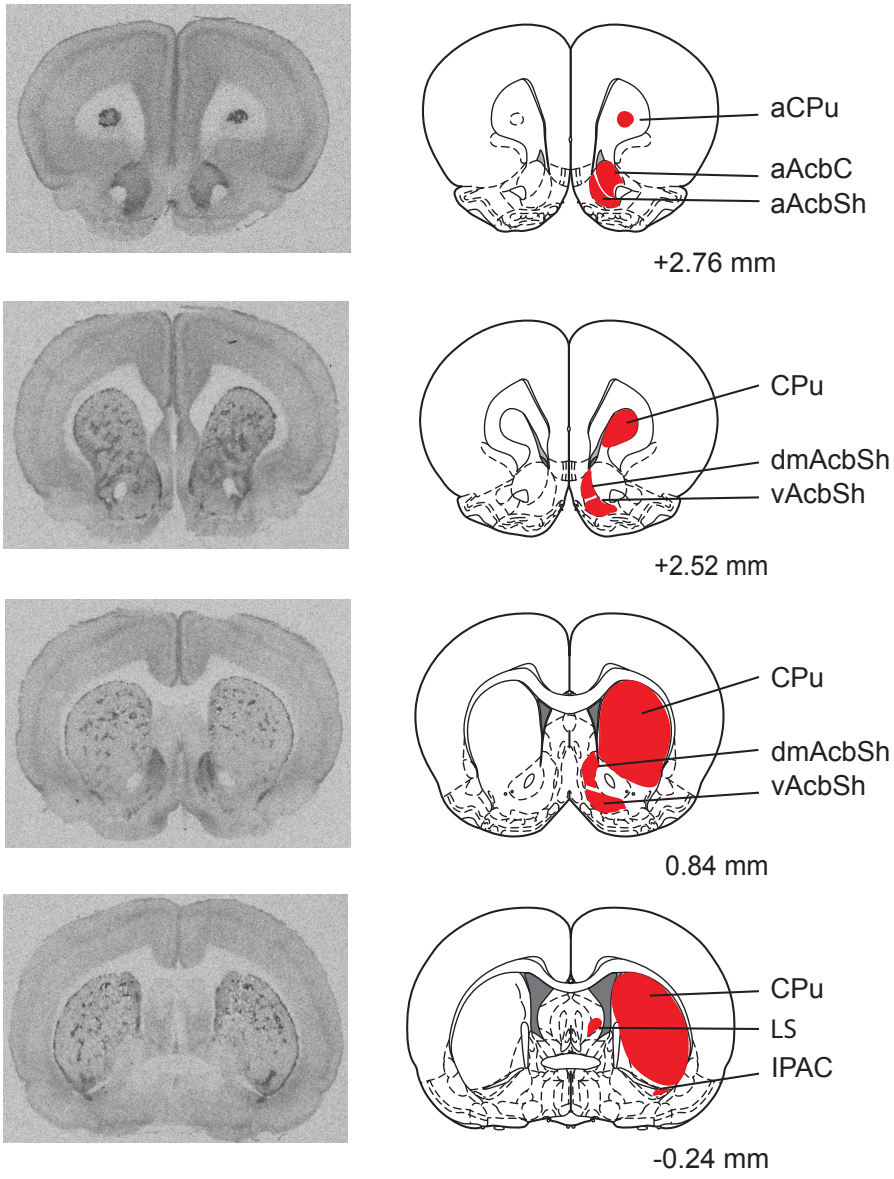

Figure 3.2. Representative autoradiograms of MOR binding in coronal rat brain sections. Brain regions in which MOR binding was measured are highlighted in red. Distances are measured in millimeters from bregma, according to Paxinos & Watson (2007). Note that while many brain regions are analyzed across multiple bregma distances, regions are highlighted in the most representative atlas images only.

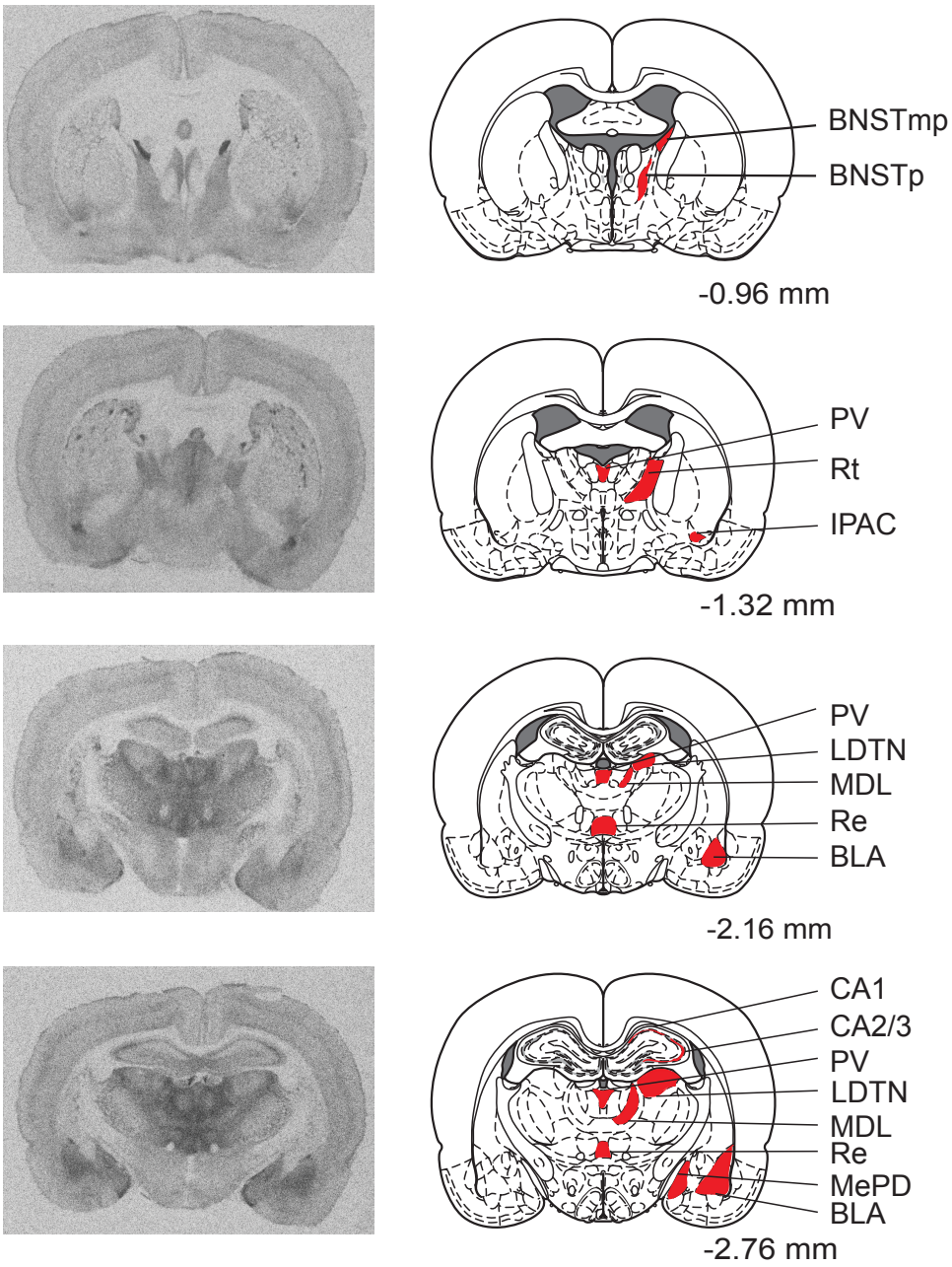

Figure 3.2. Continued...

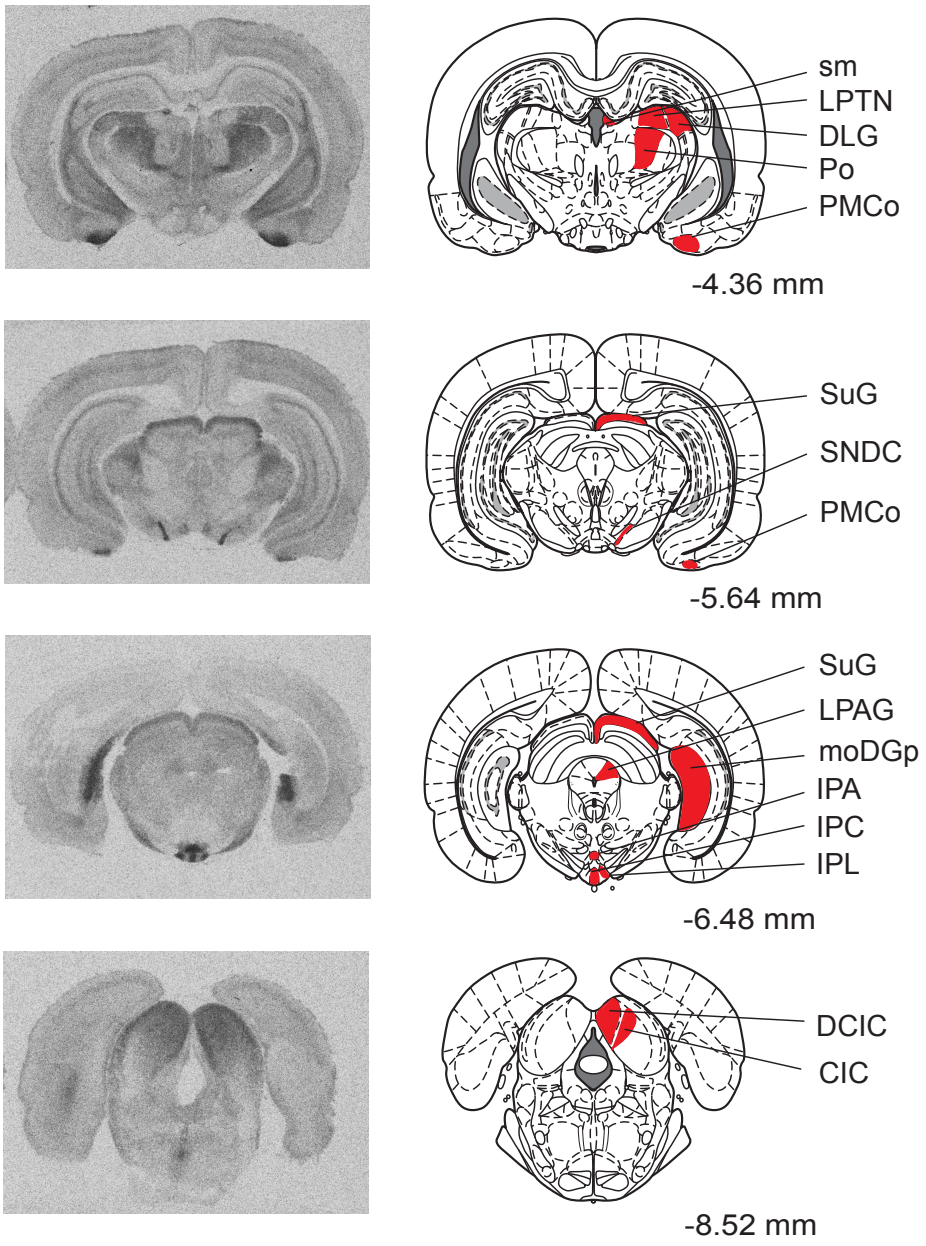

Figure 3.2. Continued...

Statistics

For all statistical analysis, PASW/SPSS Statistics (Version 22.0) was used. A one-way ANOVA followed by Bonferroni posthoc testing was used to compare MOR binding density across all brain regions analyzed, independent of age and sex. Two-way ANOVAs were used to test for age and sex differences in OTR and V1aR binding density in each brain region. The false discovery rate (FDR) procedure was used to correct for multiple comparisons (age, sex, and interaction) for each receptor separately. This resulted in an FDR $\alpha < 0.0130$ (based on 99 comparisons) (Benjamini & Hochberg, 1990). Significant interaction effects were followed by Bonferroni post-hoc tests (reflecting *t*-tests pre-adjusted for multiple comparisons) to examine differences among groups. Significant age or sex effects were followed by Cohen's D to calculate the effect size of age and sex differences (overall and separately for male and females) and of sex differences (overall and separately for juveniles and adults). A subsequent independent samples t-test was run to determine whether the effect size of age differences was different between males and females for all brain regions. Significance for independent samples t-tests was set at $p<0.05$.

RESULTS

Brain region specific-patterns of MOR binding density

MOR binding density varied greatly by brain region ($F_{(32, 1527)} = 260.8$; $p<0.001$; Fig. 3.3), with an approximate 10 fold difference between the highest and lowest MOR binding density. Binding density was highest in the apical subnucleus of the

Table 3.1. Statistical details of age, sex, and interaction effects on MOR binding densities in the rat brain. Significant effects (two-way ANOVA with FDR correction: p<0.0130) are bolded.

Direction	Age Effect	Sex Effect	Interaction Effect
Telencephalon			
Striatal Areas			
aCPu	$F_{(1,40)}$=0.34; p=0.56	$F_{(1,40)}$=3.52; p=0.07	$F_{(1,40)}$=0.69; p=0.41
CPu	$F_{(1,46)}$=0.23; p=0.63	$F_{(1,46)}$=0.48; p=0.49	$F_{(1,46)}$=0.03; p=0.86
aAcbC	$F_{(1,46)}$=0.30; p=0.59	$F_{(1,46)}$=1.73; p=0.19	$F_{(1,46)}$=0.22; p=0.64
aAcbSh	$F_{(1,46)}$=0.19; p=0.66	$F_{(1,46)}$=1.16; p=0.29	$F_{(1,46)}$=4.01; p=0.05
dmAcbSh	$F_{(1,46)}$=0.14; p=0.71	$F_{(1,46)}$=1.24; p=0.27	$F_{(1,46)}$=0.68; p=0.41
vAcbSh	$F_{(1,46)}$=4.59; p=0.04	$F_{(1,46)}$=0.10; p=0.75	$F_{(1,46)}$=0.16; p=0.69
Septal Areas			
LS Higher in juveniles & males	**$F_{(1,42)}$=86.2; p<0.001**	**$F_{(1,42)}$=6.73; p=0.013**	$F_{(1,42)}$=1.63; p=0.21
Bed Nucleus of the Stria Terminalis Areas			
BNSTpm	$F_{(1,45)}$=4.32; p=0.04	$F_{(1,45)}$=0.14; p=0.71	$F_{(1,45)}$=1.54; p=0.22
BNSTp Higher in juveniles	**$F_{(1,41)}$=38.29; p<0.001**	$F_{(1,41)}$=0.00; p=0.95	$F_{(1,41)}$=5.95; p=0.02
Amygdala Areas			
IPAC	$F_{(1,46)}$=0.46; p=0.50	$F_{(1,46)}$=1.12; p=0.30	$F_{(1,46)}$=0.05; p=0.83
MePD	$F_{(1,46)}$=0.93; p=0.34	$F_{(1,46)}$=0.26; p=0.61	$F_{(1,46)}$=2.76; p=0.10
BLA	$F_{(1,46)}$=5.84; p=0.02	$F_{(1,46)}$=0.04; p=0.83	$F_{(1,46)}$=0.06; p=0.80
PMCo Higher in females	$F_{(1,46)}$=0.38; p=0.54	**$F_{(1,46)}$=9.56; p<0.005**	$F_{(1,46)}$=6.60; p=0.01
Hippocampal Areas			
CA1 Higher in juveniles	**$F_{(1,46)}$=11.0; p<0.002**	$F_{(1,46)}$=0.53; p=0.47	$F_{(1,46)}$=0.13; p=0.72
CA2/3 Higher in juveniles	**$F_{(1,46)}$=12.5; p<0.001**	$F_{(1,46)}$=1.85; p=0.18	$F_{(1,46)}$=0.08; p=0.77
MoDGp Higher in adults	**$F_{(1,40)}$=7.02; p=0.012**	$F_{(1,40)}$=0.46; p=0.50	$F_{(1,40)}$=1.42; p=0.24
Diencephalon			
Thalamic Areas			
DLG Higher in juveniles	**$F_{(1,46)}$=32.1; p<0.001**	$F_{(1,46)}$=1.83; p=0.18	$F_{(1,46)}$=0.49; p=0.49
LDTN	$F_{(1,40)}$=0.18; p=0.67	$F_{(1,40)}$=4.02; p=0.05	$F_{(1,40)}$=3.16; p=0.08
LPTN Higher in juveniles	**$F_{(1,38)}$=12.8; p<0.001**	$F_{(1,38)}$=0.39; p=0.54	$F_{(1,38)}$=1.05; p=0.31
MDL	$F_{(1,40)}$=0.09; p=0.77	$F_{(1,40)}$=0.25; p=0.62	$F_{(1,40)}$=0.65; p=0.42
Po Higher in juveniles	**$F_{(1,45)}$=35.1; p<0.001**	$F_{(1,45)}$=0.09; p=0.76	**$F_{(1,45)}$=6.63; p=0.01**
PV Higher in juveniles	**$F_{(1,40)}$=41.4; p<0.001**	$F_{(1,40)}$=1.70; p=0.20	$F_{(1,40)}$=0.05; p=0.82
Re Higher in juveniles	**$F_{(1,46)}$=84.0; p<0.001**	$F_{(1,46)}$=0.10; p=0.75	$F_{(1,46)}$=3.07; p=0.09
Rt Higher in juveniles	**$F_{(1,46)}$=26.2; p<0.001**	$F_{(1,46)}$=1.52; p=0.22	$F_{(1,46)}$=0.02; p=0.89
Sm	$F_{(1,46)}$=4.64; p=0.04	$F_{(1,46)}$=0.23; p=0.63	$F_{(1,46)}$=0.38; p=0.54

Mesencephalon				
Tectal Areas				
SuG		$F_{(1,46)}$=4.39; p=0.04	$F_{(1,46)}$=2.32; p=0.13	$F_{(1,46)}$=0.37; p=0.54
CIC		$F_{(1,36)}$=2.72; p=0.11	$F_{(1,36)}$=0.40; p=0.53	$F_{(1,36)}$=0.10; p=0.75
DCIC		$F_{(1,46)}$=4.66; p=0.04	$F_{(1,46)}$=1.83; p=0.18	$F_{(1,46)}$=0.49; p=0.49
Tegmental Areas				
LPAG		$F_{(1,39)}$=4.56; p=0.04	$F_{(1,39)}$=0.15; p=0.70	$F_{(1,39)}$=2.90; p=0.10
SNCD		$F_{(1,44)}$=3.97; p=0.05	$F_{(1,44)}$=0.09; p=0.77	$F_{(1,44)}$=0.12; p=0.73
IPA		$F_{(1,42)}$=0.57; p=0.45	$F_{(1,42)}$=0.03; p=0.87	$F_{(1,42)}$=3.56; p=0.07
IPC	Higher in juveniles	**$F_{(1,42)}$=9.13; p<0.005**	$F_{(1,42)}$=0.30; p=0.59	$F_{(1,42)}$=2.27; p=0.14
IPL	Higher in juvenile males	$F_{(1,42)}$=5.26; p=0.03	$F_{(1,42)}$=0.85; p=0.36	**$F_{(1,42)}$=10.1; p<0.005**

interpeduncular nucleus and stria medularis of the thalamus and lowest in the lateral periaqueductal grey, CA1 region of the hippocampus, and lateral septum (Fig. 3.3). Notably, age, sex, and age x sex interaction effects were found across the spectrum of MOR binding densities.

Age differences in MOR binding density

Age differences in MOR binding density were found in 12 of the 33 brain regions analyzed (see Table 3.1 for complete statistics). MOR binding density was higher in juveniles as compared to adults in 11 brain regions, namely four telencephalic brain regions (the lateral septum, the CA1 and CA2/3 subregions of the hippocampus, and the posterior BNST; Fig. 3.4A), six diencephalic brain regions (the dorsal lateral geniculate nucleus, the lateroposterior thalamic nucleus, the posterior thalamic nucleur group, the paraventricular thalamic nucleus, the reticular thalamic nucleus, and the nucleus reuniens; Fig. 3.5A), and the caudal subnucleus of the interpeduncular nucleus in the mesencephalon (Fig. 3.5B). MOR binding density was higher in adults than juveniles in only one brain region, namely the posterior molecular layer of the dentate gyrus (Fig.

3.4B). There was no overall difference in the effect sizes of age differences between the sexes ($t_{(1, 22)}$ =-1.21; p=0.24; Fig. 3.6).

Sex differences in MOR binding density

Sex differences in MOR binding density were observed in two of 33 brain regions analyzed (see Table 3.1 for complete statistics). MOR binding density was higher in males than in females in the lateral septum and higher in females as compared to males in the posteromedial cortical amygdaloid nucleus (Fig. 3.7).

Age x Sex interaction effects

Significant age x sex interaction effects on MOR binding density were found in two brain regions: the lateral subnucleus of the interpeduncular nucleus and the posterior thalamic nucleur group (see Table 3.1 for complete statistics). In the lateral subnucleus of the interpeduncular nucleus, MOR binding density was higher in juveniles as compared to adults in males (p<0.001), but not in females (p=0.534; Fig.3.8A). In the posterior thalamic nucleus group, juveniles of both sexes had significantly higher MOR binding density than their adult counterparts (males: juveniles vs. adults, p<0.001; females: juveniles vs. adults, p=0.021). Furthermore, adult males had significantly lower MOR binding density than adult females (p<0.05), while there was no such sex difference in juveniles (p=0.112; Fig 3.8B).

Similar MOR binding density between the ages and sexes

No age or sex differences were found in 19 of the 33 brain regions analyzed, despite robust MOR binding. In the telencephalon, these brain regions consisted of several sub-regions of the striatum (anterior caudate putamen, caudate putamen, anterior nucleus accumbens core, and anterior, dorsomedial, and ventral nucleus accumbens

shell), the posteromedial bed nucleus of the stria terminalis, the interstitial nucleus of the posterior limb of the anterior commissure, and sub-regions of the amygdala (posterodorsal medial amygdala and basolateral amygdala). In the diencephalon, these consisted of sub-regions of the thalamus (laterodorsal thalamic nucleus, stria medularis of the thalamus, and lateral part of the mediodorsal thalamus). Finally, in the mesencephalon, they consisted of tectal regions (the superficial grey layer of the superior colliculus and the central and dorsal cortex of the inferior colliculus) and tegmental regions (lateral periaqueductal grey, dorsal tier of the compact part of the substantia nigra, and apical sub-nucleus of the interpeduncular nucleus (see Table 3.1 for statistics).

DISCUSSION

Using receptor autoradiography, we show that MOR binding is found in numerous regions throughout the rat brain, but that the density of MOR binding varies considerably across brain regions. Importantly, age differences in MOR binding density were found in 12 out of 33 brain regions analyzed. All but one of these age differences reflected higher MOR binding density in juveniles as compared to adults and differences were predominantly seen in thalamic sub-regions. Interestingly, sex differences in MOR binding density were found in only two of the 33 brain regions assessed. Taken together, these findings demonstrate that while MOR binding varies considerably with age in many brain regions, sex may not be an important determining variable of MOR binding density. The higher MOR binding density in juveniles as compared to adults observed here may

allow for enhanced MOR activation. This, in turn, may be required for the regulation of juvenile-typical behavior. This hypothesis is discussed further below.

MOR binding density across the rat forebrain and midbrain

MOR binding density varied substantially across regions of the rat brain, independent of age and sex. In general, MOR binding was observed in the same brain regions as previously reported in the adult male rat using MOR autoradiography (Mansour et al., 1986, 1987; Temple & Zukin, 1987; McLean et al., 1986; Mansour et al., 1994) or MOR immunohistochemistry (Moriwaki et al., 1996), with the latter suggesting that MOR binding density resembles protein expression. Moreover, the patterns of MOR binding in the rat brain are largely consistent with those reported in other mammalian species (Daunais et al., 2001; Hurd & Herkenham; Voorn et al., 1996; Ragen et al., 2015a). In particular, MOR binding in the striatum and amygdala has been found in species ranging from rats, voles, and guinea pigs to non-human primates and humans (Daunais et al., 2001; Hurd & Herkenham; Voorn et al., 1996; Ragen et al., 2015a; Inoue et al., 2013; Resendez et al., 2013; Sharif & Hughes 1989). Furthermore, the notable absence of MOR binding in the central nucleus of the amygdala observed in rats is consistent with reports in titi monkeys and macaques (Daunais et al., 2001; Ragen et al., 2015a). Still, our current study is the first to quantitatively compare MOR binding density across brain regions in the rat. We find that MOR binding density is highest in sub-regions of the interpeduncular nucleus, in the stria medularis of the thalamus, and in the posterior cortical nucleus of the amygdala. MOR binding density is lowest in the lateral periaqueductal grey, the CA1 region of the hippocampus, and in the lateral septum. We also find novel differences in MOR binding density between sub-regions of several brain

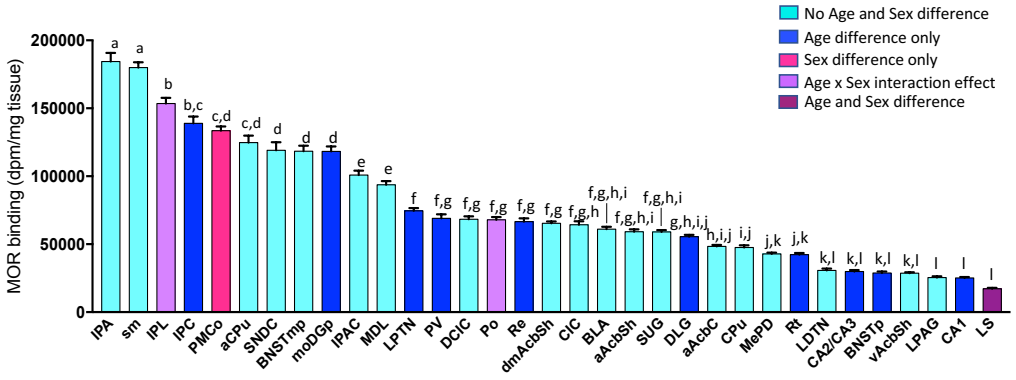

Figure 3.3. MOR binding density levels across brain regions. Brain regions in which MOR binding density was analyzed, are organized from highest (left) to lowest (right) MOR binding density. MOR binding densities are collapsed for both ages and sexes per brain region. Data represent mean + SEM; bars without letters in common differ significantly ($p<0.05$) based on one-way ANOVA (brain region) followed by Bonferroni post-hoc comparisons. Color coding indicates brain regions in which age, sex, age x sex, or no effects for MOR binding density were observed.

areas. For example, MOR binding density was higher in the anterior as compared to the more medial portions of the caudate putamen and MOR binding density was lower in the ventral part as compared to the dorsomedial or anterior parts of the nucleus accumbens shell. Interestingly, this pattern of MOR binding in the striatum is consistent with findings in other species. For example, MOR binding density also follows a rostro-caudal gradient within the dorsal striatum, with higher binding in the more anterior portion of the region, in both prairie and meadow voles (Resendez et al., 2013), and in macaques (Daunais et al., 2001). Similarly, MOR binding density is significantly lower in the ventral as compared to the dorsomedial nucleus accumbens shell in both prairie and meadow voles (Resendez et al., 2013). These quantitative differences in MOR binding densities within a brain region may reflect differences in MOR activation, which, in turn, can have an impact on the regulation of behavior by MORs. This may be especially relevant in those instances where MORs can have opposite functional roles. For example, MOR activation in the dorsomedial nucleus accumbens shell increases the hedonic

reward value of sucrose (Pecina & Berridge, 2005; Castro & Berridge, 2014) while MOR activation in caudal portions of the nucleus accumbens core decreases hedonic reactions to sucrose (Pecina & Berridge, 2005). Finally, the consistency of these MOR binding density patterns across species could indicate that the functions of MORs in these sub-regions are conserved. Indeed, the neural circuitry underlying pleasure and reward is evolutionarily ancient, and evidence for the involvement of MORs in such processes can be found in all of the above-mentioned species (Berridge & Kringelbach; 2015; Resendez et al., 2013; Trezza et al., 2011; Hsu et al., 2013; Ragen et al., 2015b; Barr et al., 2010).

Age differences in MOR binding density

Age differences in MOR binding density were observed in 12 out of 33 brain regions analyzed, including sub-regions of the lateral septum, hippocampus, BNST, thalamus, and interpeduncular nucleus. Importantly, the direction of these age differences was uniform with denser binding in juveniles as compared to adults (except for one brain region, the posterior molecular layer of the dentate gyrus). Moreover, the effect sizes of these age differences in MOR binding density were similar between males and females, indicating that these age differences are independent of sex. Previous studies in pre-weaning and adult rats have shown that in many brain regions, MOR binding density is highest around postnatal day 12 compared to earlier ages and compared to adulthood (Recht et al., 1985; Kornblum et al., 1987; Moon-Edley & Herkenham, 1984; Spain et al., 1985). The decline in MOR binding density between postnatal day 12 and adulthood has been suggested to be due to increased synaptic pruning in the third and fourth weeks of postnatal life in the rat (Kornblum et al., 1987). However, synaptic elimination is a developmental process that continues well into the pubertal period (Andersen 2000;

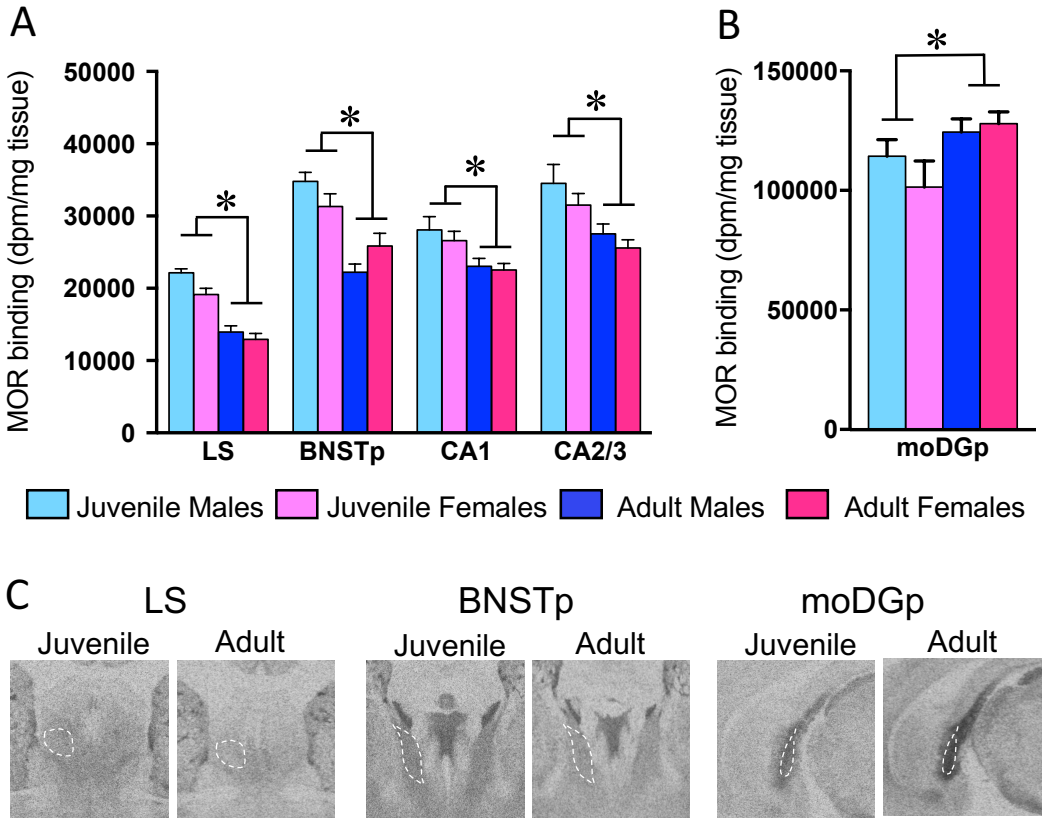

Figure 3.4. Age differences in MOR binding density in the telencephalon. Brain regions in which MOR binding density is higher (**A**) or lower (**B**) in juveniles as compared to adults within the telencephalon. Representative autoradiograms of age differences in MOR binding density in the LS, BNSTp, and moDGp (**C**). Bars in **A** and **B** indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons: ✻FDR $\alpha<0.013$.

Huttenlocher & Dabholkar, 1997; Gerocs et al. 1986). Therefore, it is plausible that the brain regions in which we observed higher MOR binding densities in juvenile as compared to adult rats are those in which synaptic pruning is not complete until later in development. If so, then one might expect to see a similar decline in binding density between juveniles and adults for other types of receptors in the same brain regions. However, using adjacent sections of the same brains as in the current study, we recently

reported very different patterns of age differences in binding density for the oxytocin receptor (Smith et al., 2016). For example, oxytocin receptor binding density is higher in adults as compared to juveniles in the posterior BNST (Smith et al., 2016), a brain region in which we now report that MOR binding density is higher in juveniles as compared to adults. Furthermore, binding densities of the dopamine D1, D2, and D4 receptors are higher in juvenile as compared to adult rats in the nucleus accumbens (Tarazi & Baldessarini, 2000), while we find no age difference in MOR binding density in this region. If it is pruning that is causing this decline in receptors, then these findings suggest that pruning is specific to synapses expressing only certain types of receptors, such as MORs in the posterior BNST and dopamine receptors in the nucleus accumbens. Irrespective of the underlying cause, it is possible that higher MOR binding density in juveniles has relevance to the facilitation of juvenile-specific behaviors.

Indeed, we hypothesized that MOR binding density might be higher in juveniles as compared to adults in reward-related brain regions. One region of particular interest is the lateral septum, because it shows the most robust age difference in MOR binding density (denser binding in juveniles), is reciprocally connected to the mesolimbic reward system (Swanson, 1982), is involved in socially rewarding behaviors (Veenema et al., 2013; Bredewold et al., 2014, 2015), and plays a role in the age-specific regulation of social behavior (Veenema et al., 2012). Surprisingly, the role of MORs in the lateral septum in the regulation of reward and social behavior has not been studied. Instead, MOR activation in the lateral septum has been shown to increase anxiety-related behavior in adult mice (Le Merrer et al., 2006). This corresponds with the overall role of the lateral septum, with activation of this region having anxiogenic effects (Anthony et al., 2014;

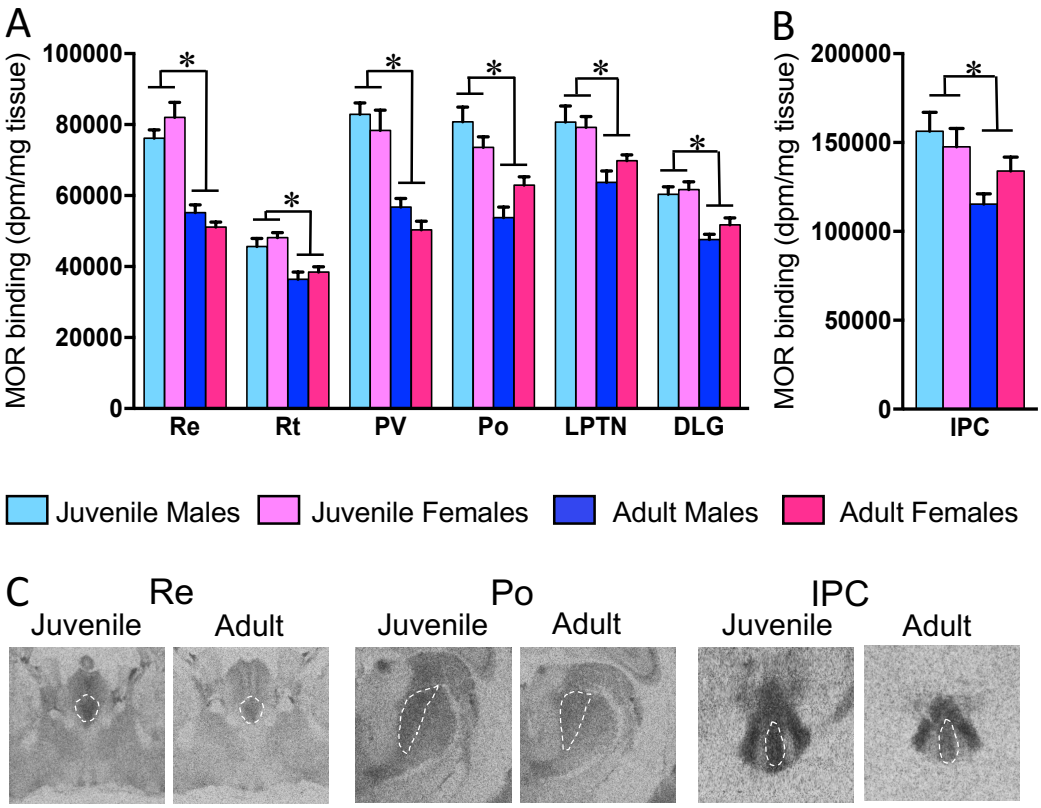

Figure 3.5. Age differences in MOR binding density in the diencephalon and mesencephalon. Brain regions in which MOR binding density is higher in juveniles as compared to adults within the diencephalon (**A**) and mesencephalon (**B**). Representative autoradiograms of age differences in MOR binding density in the Re, Po, and IPC (**C**). Bars in **A** and **B** indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons: ✻FDR α<0.013.

Veening et al., 2009). It would therefore be interesting to determine whether the age difference in lateral septum MORs has implications for the age-specific regulation of anxiety-like behaviors, in addition to reward-related social behaviors.

Importantly, age differences in MOR binding density were particularly evident in many sub-divisions of the thalamus, including the nucleus reuniens, the reticular thalamus, the paraventricular thalamic nucleus, the posterior thalamic nuclear group, the lateroposterior thalamic nucleus, and the lateral geniculate nucleus. Although the

functional role of MORs in these sub-regions is unknown, interesting and testable hypotheses can be generated based on the function of each of these sub-regions. For example, the nucleus reuniens receives input from the prefrontal cortex and relaying it, via glutamatergic outputs, to the hippocampus (Ito et al., 2015; Hallock et al., 2016). Disruption of this pathway by blockade of the reuniens impairs spatial navigation, learning, and memory (Ito et al., 2015; Davoodi et al., 2009). Because MOR activation has an overall inhibitory effect on the thalamus (Brunton & Charpak, 1998; Nakahama et al., 1981; Benoist et al., 1986), it is possible that denser MOR binding in the nucleus reuniens allows for higher MOR activation, which, in turn may mediate a greater inhibition of this circuit in juveniles compared to adults. Furthermore, the paraventricular nucleus of the thalamus mediates the aversive effects of opiate withdrawal through connections with the nucleus accumbens (Zhu et al., 2016). Because juveniles are less sensitive to the aversive effects of withdrawal than adults (Doremus-Fitzwater et al., 2007; Hodgson et al., 2010) it is plausible that denser MOR binding allows for higher MOR activation in the paraventricular thalamic nucleus, which in turn may inhibit signaling in this pathway, resulting in reduced withdrawal symptoms in juveniles. These hypotheses will need to be tested in future studies.

Finally, age differences in MOR binding density were found in the caudal sub-region (higher in juveniles of both sexes) and in the lateral sub-region (higher in juveniles, but only in males) of the interpeduncular nucleus. This nucleus is densely interconnected with the lateral habenula (Sutherland, 1982). This habenulo-interpeduncular pathway exerts a chronic inhibitory influence over the mesolimbic reward pathway (Nishikawa et al., 1986). In fact, it has been suggested that these two

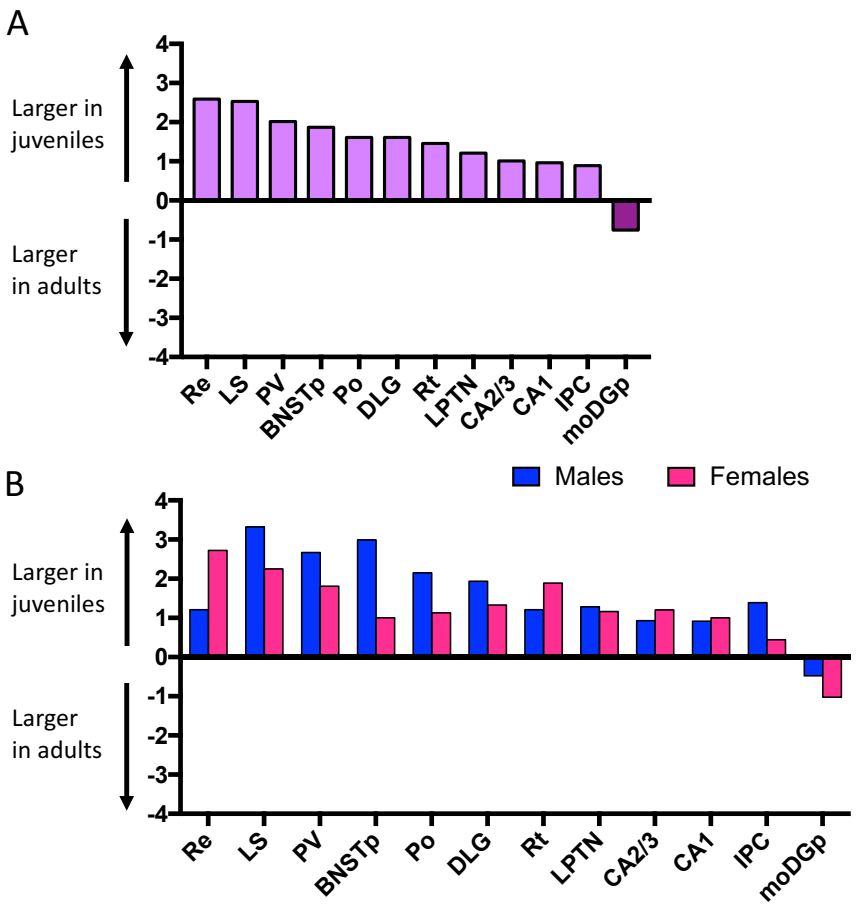

Figure 3.6. Cohen's D effect size measurements for all significant age differences in MOR binding density, collapsed across sexes (**A**) and separately in males and females (**B**).

pathways jointly regulate the rewarding properties of drugs (Ellison et al., 1994). Given the increased susceptibility to drug seeking behavior in the juvenile period (Spear et al., 2000), it would be of interest to determine whether higher MOR binding density in the interpeduncular nucleus leads to higher susceptibility to MOR activation within the habenulo-interpeduncular pathway in juveniles versus adults. This, in turn, might result in higher susceptibility to drug-seeking behaviors in juveniles.

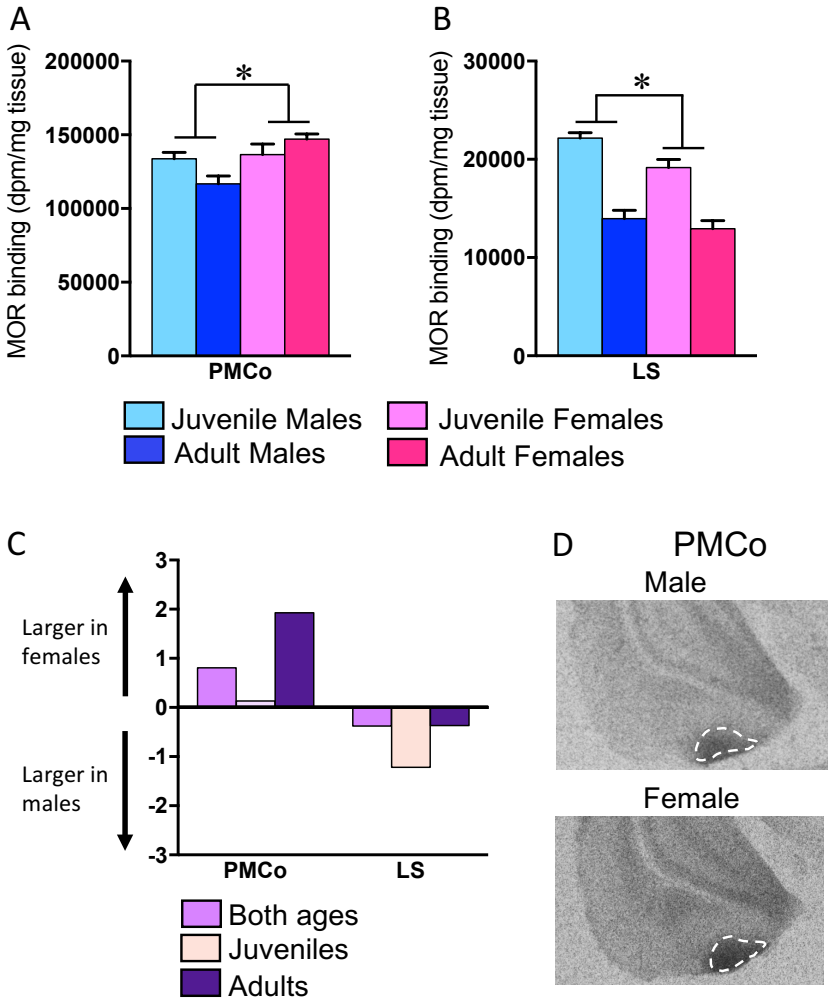

Figure 3.7. Sex differences in MOR binding density. MOR binding density is higher in females than in males in the PMCo (**A**) and higher in males than in females in the LS (**B**). Cohen's D effect size measurements for significant sex differences collapsed across age and separately in juveniles and adults (**C**). Representative autoradiograms of MOR binding in the PMCo in an adult male and an adult female (**D**). Bars in **A** and **B** indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons: ✻FDR α<0.013.

Sex differences in MOR binding density

We hypothesized that MOR binding density would be higher in females as compared to males in reward-related brain regions, based on the observation that females are more likely to become addicted to opiates than males (Lynch et al., 2002; Lynch & Carroll, 1999; Cicero et al., 2003) and based on previously reported sex differences in MOR binding density in gonadectomized rats (Vathy et al., 2003). To our surprise, MOR binding density was similar between males and females in 31 out of 33 analyzed brain regions. One limitation of the current study is that we did not measure the effect of estrous phase on MOR binding density. However, MOR binding density variability (as interpreted by the average standard deviation of binding density in each brain region) was no greater in females than in males, suggesting that it is unlikely that estrous phase had a large impact on the absence of sex differences in MOR binding density. This suggests that sex differences in opioid sensitivity and response are not due to sex differences in the density of MORs. However, it is possible that sex differences occur in the downstream signaling pathways of the MOR. In support, estrogens have been shown to inhibit MOR-mediated signaling via a protein kinase A pathway (Wagner et al., 1998). Further work is needed to determine whether other aspects of the MOR system show sex differences and if so, whether these underlie the observed sex differences in MOR-mediated addictive behaviors.

The two regions in which we found sex differences in MOR binding density were the lateral septum (higher in males) and the posterior cortical nucleus of the amygdala (higher in females). These sex differences were already present in juveniles, demonstrating a pre-pubertal age of onset. Interestingly, both brain regions have sexually

dimorphic features. The intermediate sub-region of the lateral septum, in which MOR binding was measured, contains more cells in females than in males (Segovia et al., 2009), while the posterior cortical nucleus of the amygdala contains more cells in males than in females (Vinader-Caerols et al., 1998). Thus, the direction of the sex difference in

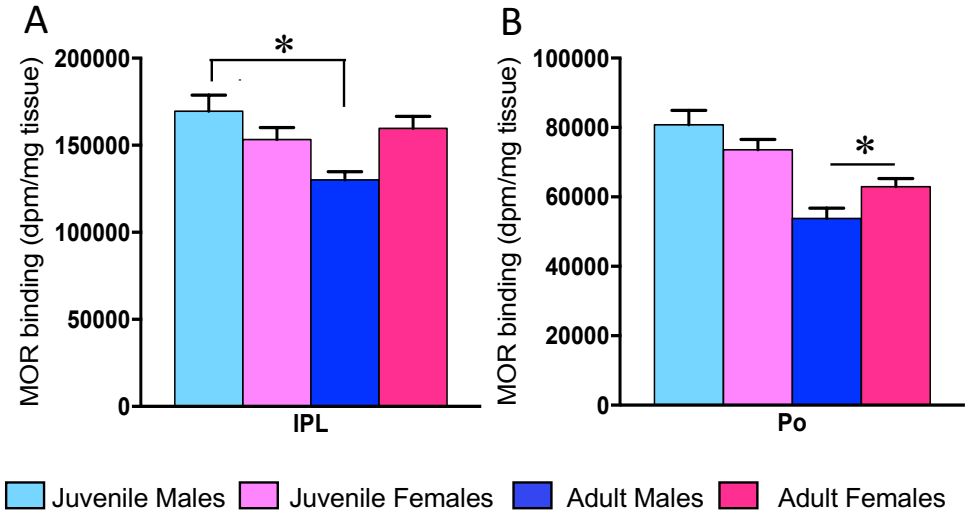

Figure 3.8. Age x sex interaction effects for MOR binding density. In the IPL, MOR binding density is significantly higher in juvenile males as compared to adult males (**A**). In the Po, MOR binding density is higher in adult females as compared to adult males (**B**). Bars indicate mean + SEM; two-way ANOVA (age x sex) with FDR correction for multiple comparisons (α<0.013) followed by Bonferroni post-hoc tests (* $p < 0.05$).

cell number is opposite to the direction of the sex difference in MOR binding density in both regions. Interestingly, similar to MOR binding density, the sex difference in posterior cortical nucleus volume and cell number is present prior to puberty (Akhmadeev & Kalimullina et al., 2014) and in gonadectomized rats (Vathy et al., 2003), suggesting that this sex difference does not depend on circulating gonadal hormones. Further research is needed to determine any functional implications of the sex differences in MOR binding density in these sexually dimorphic brain regions.

Conclusion

In conclusion, our results demonstrate that age differences in MOR binding density in the rat brain are highly prevalent, while sex differences are not. We find numerous brain regions in which MOR binding density is higher in juveniles as compared to adults, providing a potential mechanism for heightened MOR activation in the juvenile period that might then be linked to higher engagement in reward and drug-seeking behaviors. Overall, the observation of these age differences in MOR binding density provides an important first step in generating and testing novel hypotheses as to the involvement of MORs in the age-specific regulation of reward and drug-seeking behaviors.

Chapter Four: Social novelty investigation in the juvenile rat: modulation by the mu-opioid system*

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ABSTRACT: The drive to approach and explore novel conspecifics is inherent to social animals and may promote optimal social functioning. Juvenile animals seek out interactions with novel peers more frequently and find these interactions more rewarding than their adult counterparts. Here, we aim to establish a behavioral paradigm to measure social novelty-seeking in juvenile rats and to determine the involvement of opioid, dopamine, oxytocin, and vasopressin systems in this behavior. To this end, we developed the social novelty preference test to assess the preference of a rat to investigate a novel over a familiar (cage mate) conspecific. We show that across the juvenile period both male and female rats spend more time investigating a novel conspecific than a cage mate, independent of subject sex or repeated exposure to the test. We hypothesized that brain systems subserving social information processing and social motivation/reward, i.e., the opioid, dopamine, oxytocin, vasopressin systems might support social novelty preference. To test this, receptor antagonists of each of these systems were administered intracerebroventricularly prior to exposure to the social novelty preference test and, subsequently, to the social preference test, to examine the specificity of these effects. We find that μ-opioid receptor antagonism reduces novel social investigation in both the social novelty preference and social preference tests while leaving investigation of a cage mate (social novelty preference test) or an object (social preference test) unaffected. In contrast, central blockade of dopamine D_2 receptors (with eticlopride), oxytocin receptors

(with des-Gly-NH_2,d$(CH_2)_5$[Tyr(Me)^2,Thr_4]OVT) or vasopressin V1a receptors [with $(CH_2)_5$Tyr(Me^2)AVP] failed to alter social novelty preference or social preference. Overall, we have established a new behavioral test to study social novelty-seeking behavior in the juvenile rat and showed that the μ-opioid system facilitates this behavior, possibly by reducing risk avoidance and enhancing the hedonic and/or motivational value of social novelty.

INTRODUCTION

Across species, the juvenile period is uniquely characterized by heightened engagement in peer-directed social interactions, novelty-seeking, and risk-taking behaviors (Spear, 2000; Varlinskaya et al., 2013; Somerville, 2013; Doremus-Fitzwater et l., 2010; Casey, 2008). These behaviors are likely to be highly adaptive; as individuals transition from juvenility to adulthood, it is essential that they venture out into the world in search of new territories, novel food supplies, and potential mates (Trimpop et al., 1999; Zuckerman, 1986; Spear, 2000). Furthermore, the shift in focus from parental to peer interaction is critical to the development of adult social skills and social competence across species (Blakemore, 2008; Larson et al., 2007).

On the other hand, very high or low levels of novelty-seeking may be maladaptive. For example, a high novelty-seeking behavioral phenotype has been shown to correlate with substance abuse, risk preference, and sensation seeking in rodents and humans (Wang et al., 2015; Wills et al., 1998; Dellu et al., 1996; Hittner & Swickert, 2006; Kelly et al., 2006), suggesting that excessive novelty-seeking may predispose individuals to potentially harmful behaviors and/or environments. Reduced novelty-seeking is a characteristic of autism spectrum disorder (ASD; Anckarsäter et al., 2006), and may contribute to the low social interest and poor social reciprocity in ASD (American Psychiatric Association, 2013). These findings may suggest overlap between non-social novelty-seeking and social novelty-seeking. Therefore, elucidating the mechanisms underlying this behavior may enhance our understanding of the normal expression of novelty-seeking as well as how it contributes to, or is altered in, drug addiction and ASD.

Novelty-seeking behavior in juvenile rats is reflected by robust preferences for both novel environments (Bronstein & Spear, 1972; Philpot & Wecker, 2008) and novel objects (Douglas et al., 2003; Reger et al., 2009; Cyrenne & Brown, 2011; Philpot & Wecker, 2008) over ones which are familiar. However, surprisingly little is known regarding preferences for novelty of a social nature in juvenile animals. What little we do know comes from studies which test for social discrimination by presenting the subject with a previously briefly encountered social stimulus along with one that is novel. In this test, male juvenile rats spent more time investigating a novel social stimulus over one to which they have previously been exposed (Lukas et al., 2011, Veenema et al., 2012), suggesting a preference for social novelty. Remarkably, female juvenile rats did not spend more time interacting with a novel as compared to a previously encountered stimulus rat (Veenema et al., 2012). It is yet unclear whether this indicates a sex difference in social recognition memory or the motivation to explore novel social stimuli. Interestingly, non-social novelty-seeking behavior is higher in males than in females in juvenile rats (Cyrenne & Brown, 2011a,b) and humans (Zuckerman, 2007).

In this study, we tested the hypothesis that the heightened non-social novelty preference seen in juveniles would extend to social stimuli, but that there might be a sex difference in the motivation to explore novel social stimuli.

Furthermore, we hypothesized that candidate neural systems modulating social novelty preference are those involved in social information processing and social motivation/reward, i.e. the opioid, dopamine, oxytocin (OT), and vasopressin (VP) systems in the brain. Opioid action in the brain has been suggested to mediate the hedonic or “pleasurable” nature of stimuli and the rewarding aspects of social interaction (Niesink

& van Ree, 1989; Trezza et al., 2011). The μ-opioid receptor subtype in particular may be of relevance as it has been implicated in non-social novelty preference and novelty exploration in mice (Cinque et al., 2012; Yoo et al., 2004). Dopamine acting through the D_2 receptor (D_2R) has been shown to facilitate conditioned place preference for novel objects (Besheer et al., 1999) as well as social novelty discrimination and novel object recognition in the rat (Watson et al., 2012). Finally, a large body of evidence suggests that OT and VP, acting via the oxytocin receptor (OTR) and vasopressin V1a receptor (V1aR) respectively, modulate a broad range of social behaviors (Alber et al., 2014; Caldwell, 2012; Veenema & Neumann, 2008; Donaldson, 2008), including social preference in adult rats (Lukas et al., 2011) and social play in juvenile rats (Veenema et al., 2013; Bredewold et al., 2014).

In the present study, our first aim was to develop a paradigm to assess social novelty preference in the juvenile rat. In this social novelty preference test, each rat was given the option to investigate either a novel conspecific or one which was highly familiar (a cage mate). We hypothesized that juvenile rats would display a preference to investigate a novel conspecific as opposed to a familiar cage mate, but that this might only be the case in males. Our second aim was to identify the neurotransmitter systems that facilitate social novelty preference. To this end, we administered intracerebroventricularly (i.c.v.) specific receptor antagonists targeting the μ-opioid receptor, D_2R, OTR, or V1aR and determined the effects on social novelty preference. We further examined whether or not these effects were specific to social novelty preference rather than social preference (the preference to explore a novel conspecific over a novel object). Given their role in motivational and social behaviors, we predicted

that central blockade of each of these receptors would lead to a reduction in social novelty preference.

METHODS

Animals

Male and female Wistar rats (22 or 23 days of age) were obtained from Charles River (Wilmington, MA) and housed under standard laboratory conditions (12 hour light/dark cycle, lights on at 6:00 am, food and water available *ad libitum*, 22° C, 60% humidity). Upon arrival, rats were housed in standard rat cages (26.7 x 48.3 x 20.3 cm) and given at least five days to acclimate to our facilities prior to testing. Experimental rats (23 days of age) were housed in same-sex pairs. Stimulus rats (22 days of age) were housed in same-sex groups of 3-4 per cage. Experimental rats were one day older than the stimulus rats to ensure that they were unrelated. All experiments were conducted in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

Behavioral Testing

Social Novelty Preference Test (Experiments 1 and 2)

We established the social novelty preference test to assess the preference of a juvenile rat to investigate a novel or familiar (cage mate) sex- and age-matched conspecific. This test is based on Crawley's social interaction test (Moy et al., 2004; Nadler et al., 2004), but with an important modification to the familiar social stimulus. To make it unlikely that any change in social novelty preference following system

manipulations might be due to impairments in memory for the familiar rat, we used a cage mate as a familiar stimulus animal rather than a stimulus animal regarded as familiar due to a brief previous exposure. Social novelty preference was tested in a Plexiglas apparatus consisting of a rectangular three-chambered box. Each chamber (40 x 27 x 40 cm) is separated by a Plexiglas partition with a rectangular opening to allow for passage between chambers. A novel stimulus rat is placed in one end chamber, while the subject's cage mate (familiar stimulus) is placed in the other (Fig. 4.4.1 1A & C). Both rats are confined to small containers (18 x 10 x 21 cm) composed of translucent Plexiglas bars to restrict their movement while still allowing for social investigation by the experimental rat. Experimental and stimulus rats were moved to the testing room at least 1 hour prior to the onset of behavioral testing. All testing took place in the latter half of the light phase. Light intensity in the testing apparatus was ~240 lux. The apparatus was cleaned with a dilute soap solution prior to each test. The experimental rat was placed in the middle chamber and was allowed to freely explore the three chambers and the social stimuli for 10 min. Behavior was video recorded and later scored using the behavioral analysis program JWatcher (http://www.jwatcher.ucla.edu) by an experimenter blind to the sex of the rat and the experimental treatment. Time spent investigating each stimulus rat, frequency of investigation, time spent in each chamber, and number of entries into the middle chamber were measured. Investigation of the stimulus rats was defined as direct nose poking through the bars of the containers holding the stimulus rats. Number of entries into the middle chamber was taken as a measure of general locomotor activity. To obtain a measure of social novelty preference, the percentage of time investigating the novel stimulus rat (time investigating the novel stimulus rat/time investigating the novel

+ familiar rat x 100) was calculated. Experimental animals were considered to exhibit a preference for social novelty when the percentage of time spent investigating the novel conspecific was significantly different from chance (50%). The sum of novel and cage mate investigation times was calculated as a measure of total social investigation time. Exclusion criteria from further analysis included failure to cross between the chambers (as no preference score can then be calculated) and spending greater than 25% of total test duration climbing the containers in which the stimulus rats were housed.

Social Preference Test (Experiment 3)

Rats were exposed to the social preference test to determine whether the effects of i.c.v. receptor blockade on social novelty preference were specific or also pertain to general sociability. The preference of a juvenile rat to investigate a social stimulus or a non-social stimulus was measured in the same three-chambered box as described above (Fig. 4.1B). All testing procedures and behavioral analysis were the same as described above, except that instead of investigating either a cage mate or novel social stimulus, subjects were given the choice to investigate either a novel social stimulus (sex- and age-matched conspecific) or a novel inanimate object (a rubber bath turtle). Exclusion criteria from further analysis included failure to ever cross between the chambers (as no preference score can then be calculated) and spending greater than 25% of total test duration climbing the containers in which stimulus animals were housed.

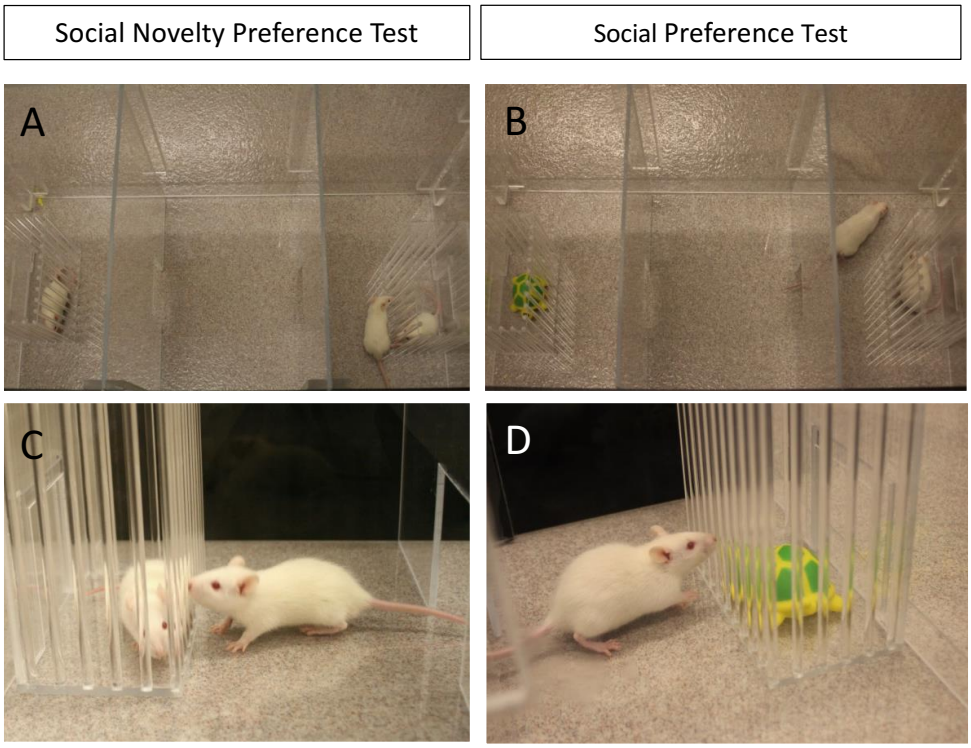

Figure 4.1. Social novelty & Social preference tests. In the social novelty preference test (**A** & **C**) juvenile experimental animals are given the choice to interact with either a novel stimulus juvenile or a familiar cage mate. In the social preference test (**B** & **D**) juvenile experimental animals are given the choice to interact with either a novel stimulus juvenile or a novel inanimate object (a rubber bath turtle).

Cannulation

At postnatal day (PND) 27 or 28, experimental rats were anesthetized with isoflurane (Henry Schein, Dublin OH) and positioned into a stereotaxic frame with the incisor bar set at -4.5 mm. Throughout surgery, a heating pad was used to maintain body temperature. Guide cannulae (21 gauge, Plastics One, Roanoke VA) were implanted unilaterally to target the lateral ventricle. Coordinates were determined using the Paxinos & Watson Rat Brain Atlas (Paxinos & Watson, 2007) and adapted for use in juveniles. Guide cannulae were implanted 2 mm dorsal to the right lateral ventricle, 1.0 mm rostral

to bregma, -1.6 mm lateral to the midline, and 2 mm ventral to the surface of the skull. Guide cannulae were secured via stainless steel screws and dental acrylic adhesive and were closed with a dummy cannula (Plastics One). Following surgery, rats were given an injection of Rimadyl analgesic (Henry Schein, Dublin OH) and singly housed for one hour before rehousing with their cage mate.

Microinjection Procedures and Histology

Experimental rats were handled daily for four days prior to testing to habituate them to the injection procedure. Injection systems were composed of polyethylene tubing connected to an injector cannula and a 25 µl Hamilton syringe (Hamilton, Bonaduz, Switzerland). The injector cannula (26 gauge) extended 2 mm beyond the guide cannula, was kept in place for 30 s following injection to allow for tissue uptake, and was then replaced by a dummy cannula. At the end of the experiments, rats were euthanized with CO_2. Blue ink was injected through the cannula system to verify introduction into the ventricular system, and hence correct cannula placement. Brains were removed, cut coronally with a razor blade, and visually inspected for ink in the ventricular system. Rats with incorrect cannula placement were excluded from further analysis.

Experimental Procedures

Experiment 1: Establishing the Social Novelty Preference Test

In rats, the juvenile period spans approximately PND 28-40 (Spear & Brake, 1983; Meaney & Stewart; 1981). Additionally, previous research suggests the potential for sex differences in social novelty preference in juvenile rats (Veenema et al., 2012; Cyrenne & Brown, 2011a,b). We therefore tested both male and female juvenile rats for social novelty preference at multiple time points during the juvenile period. To test

whether or not social novelty preference differs with exact day of age, two separate groups of male and female juveniles were tested at either PND 28 or PND 35. To test for effects of repeated exposure to the social novelty preference test, a group of male and female juveniles were tested at PND 28 (Exposure 1), and subsequently, at PND 35 (Exposure 2) and PND 40 (Exposure 3). This allowed us to dissociate effects of age from repeated exposures to the test. The final number of rats for each group is indicated in the graphs in Fig. 4.2.

Experiment 2: Neuromodulation of Social Novelty Preference

Male juvenile rats were used in experiments 2 and 3. Rats were exposed to the social novelty preference test two days after cannulation, at PND 29 or 30. Each system manipulation (μ-opioid receptor, D_2R, OTR, or V1aR blockade) was conducted as a separate experiment with its own vehicle control comparison (see Fig. 4.4 for final animal numbers per group). Specifically, rats received an injection into the lateral ventricle with either Ringer's solution (Vehicle; 3μl; Butler-Schein, Albany, NY), the specific μ-opioid receptor antagonist CTAP (4μg/ 3μl or 10μg/ 3μl; Sigma-Aldrich, St. Louis, MO; 43), the specific dopamine D_2R antagonist Eticlopride (40ng/ 3μl or 400ng/ 3μl; Sigma-Aldrich; St. Louis, MO; Wang et al., 1999), the specific OTR antagonist desGly-NH2,d(CH2)5[Tyr(Me)2,Thr4]OVT (OTRa, 75ng/ 3μl or 750ng/ 3μl; Manning et al., 2008), or the specific V1aR antagonist d(CH2)5[Tyr(Me)2]AVP (V1aRa; 75ng/ 3μl or 750ng/ 3μl; 45) 20 min prior to exposure to the social novelty preference test. Drugs and drug doses were chosen based on their effectiveness in altering social behavior (Veenema et al., 2012; Lukas et al., 2011; Schroeder et al., 2007; Besheer et al., 1999;

Bevins et al., 2002; Trezza et al., 2011; Liu & Wang, 2003). The final number of rats for each group is indicated in the graphs in Fig.4.3.

Experiment 3: Neuromodulation of Social Preference

To determine the impact of μ-opioid receptor, D_2R, OTR, or V1aR blockade on general sociability, rats used in experiment 2 were exposed to the social preference test two days after exposure to the social novelty preference test, at PND 31 or 32. Rats received the same drug treatment as in experiment 2, 20 min. prior to exposure to the social preference test. The final number of animals per group is represented in Fig. 4.4.

Statistical Analysis

For all statistical analysis, PASW/SPSS Statistics (Version 19.0) was used. Statistical significance was set at $p < 0.05$. A two-way ANOVA (sex x age) was used to test for effects of sex and age on all parameters in the social novelty preference test in experiment 1. A two-way mixed design ANOVA (sex x number of exposures to the test) was used to test for effects of sex and repeated exposure to the test in experiment 1. Bonferroni post-hoc tests were used to test for differences between the number of exposures to the test. Single sample *t*-tests compared to 50% (chance level) were used to determine the presence of social novelty preference (experiments 1 and 2) and social preference (experiment 3). Finally, one-way ANOVAs followed by Bonferroni post-hoc tests were used in experiments 2 and 3 to test for differences between drug treatment conditions on all parameters in the social novelty preference and social preference tests.

RESULTS

Experiment 1: Establishing the Social Novelty Preference Test

Effects of sex on social novelty preference

Overall, males spent more time in novel investigation ($F_{(1,28)}$=5.93, p<0.03; Fig. 4.2A) and in total social investigation ($F_{(1,28)}$=8.84, p<0.005; Fig. 4.2A) than females. Males also made fewer entries into the middle chamber ($F_{(1,28)}$=4.87, p<0.05; Table 4.1) than females. Together, these findings indicate that juvenile males show higher social interest towards novel conspecifics, while juvenile females show higher locomotor activity. Importantly, there was no main effect of sex on the percentage of novel investigation ($F_{(1,28)}$=1.59, p=0.22; Fig. 4.2A). Indeed, both males and females spent a significantly higher proportion of time investigating the novel social stimulus than would be predicted by chance (males: $t_{(15)}$=6.33, p<0.001; females: $t_{(15)}$ =4.11, p<0.005, collapsed across ages; Fig. 4.2D). This indicates that both males and females show robust social novelty preference.

Table 4.1. Establishing the social novelty preference test – sex, age and repeated exposure effects. Number of entries into the middle chamber was lower in males than in females (**A**). Number of entries into the middle chamber was lower at PND 28 than at PND 35 (**B**). Repeated exposure to the test led to an increase in entries into the middle chamber (**C**). Data are mean ± SEM; *p<0.05, main effect of sex or age in two-way ANOVA (sex x age) (**A**) or mixed-design two-way ANOVA (sex by exposure) followed by Bonferroni posthoc (**C**); s, seconds; N, number; Exp., Exposure.

A: Sex	Males	Females
Novel Chamber Time (s)	342 ± 16	318 ± 15
Cage mate Chamber Time (s)	205 ± 15	220 ± 13
Middle Chamber Time (s)	53.0 ± 3.9	61.9 ± 4.0
Entries Middle Chamber (N)	8.5 ± 0.9	22.3 ± 1.7*

B: Age	PND 28	PND 35
Novel Chamber Time (s)	337 ± 16	323 ± 16
Cage mate Chamber Time (s)	211 ± 15	214 ± 14
Middle Chamber Time (s)	52.5 ± 3.7	62.3 ± 4.1
Entries Middle Chamber (N)	18.0 ± 1.1	22.7 ± 1.5*

C: Repeated Exposure	Exp. 1	Exp. 2	Exp. 3
Novel Chamber Time (s)	335 ± 28	306 ± 12	333± 14
Cage mate Chamber Time (s)	205 ± 16	228 ± 11	210 ± 14
Middle Chamber Time (s)	50.4 ± 42	64.6 ± 4.3	56.5 ± 5.4
Entries Middle Chamber (N)	17.5 ± 1.1	27.9 ± 1.8*	31.1 ± 3.2*

Effects of age on social novelty preference

We found no differences between PND 28 and PND 35 in novel investigation time ($F_{(1,28)}$=2.14, p=0.15; Fig. 4.2B), cage mate investigation time ($F_{(1,28)}$=0.01, p=0.91; Fig. 4.2B), total social investigation time ($F_{(1,28)}$=0.31, p=0.08; Fig. 4.2B), or social novelty preference ($F_{(1,28)}$=0.82, p=0.37; Fig. 4.2E). There was, however, a significant increase between PND 28 and PND 35 in locomotor behavior as reflected by more entries

made into the middle chamber at PND 35 compared to PND 28 ($F_{(1,28)}$=7.41, p<0.05; Table 4.1). At both ages, juvenile rats investigated the novel stimulus rats more than predicted by chance (PND 28: $t_{(15)}$=7.02, p<0.001; PND 35: $t_{(15)}$ =3.87, p<0.01; collapsed across sexes; Fig. 4.2E). Taken together, these results indicate that social investigation and social novelty preference remain constant, but that locomotor activity is higher at PND 35 than PND 28.

Effects of repeated test exposure on behavior in the social novelty preference test

To test for effects of repeated exposure to the test on performance in the social novelty preference paradigm, behavior was tested first at PND 28 (Exposure 1), and then again at PND 35 (Exposure 2) and 40 (Exposure 3) in the same cohort of male and female juvenile rats.

The sex differences in novel and total social investigation time seen upon first exposure to the test persisted across multiple exposures. In detail, males spent more time in novel investigation ($F_{(1,12)}$=7.14, p<0.05) and in total investigation ($F_{(1,12)}$=18.28, p<0.001) than females.

Repeated exposures to the test led to significant decreases in novel investigation time ($F_{(2,24)}$=22.13, p<0.001; Bonferroni post-hoc: Exposure 1 vs. Exposure 2: p<0.005, Exposure 1 vs. Exposure 3: p<0.001, Exposure 2 vs. Exposure 3: p=0.11; Fig. 4.2C), cage mate investigation time ($F_{(2,24)}$=8.42, p<0.005; Bonferroni post-hoc: Exposure 1 vs. Exposure 2: p=1.00, Exposure 1 vs. Exposure 3: p<0.01, Exposure 2 vs. Exposure 3: p<0.05; Fig.4.2C), and total social investigation ($F_{(2,24)}$=58.11, p<0.001; Bonferroni post-hoc: Exposure 1 vs. Exposure 2: p<0.005, Exposure 1 vs. Exposure 3: p<0.001, Exposure 2 vs. Exposure 3: p<0.001; Fig. 4.2C). Repeated testing increased the number of entries

made into the middle chamber ($F_{(2,24)}$=18.41, p<0.001; Bonferroni post-hoc: Exposure 1 vs. Exposure 2: p<0.005, Exposure 1 vs. Exposure 3: p<0.005, Exposure 2 vs. Exposure 3: p=0.66; Table 4.2). These results suggest that investigatory social behavior decreases while locomotor activity increases with repeated exposure to the social preference test.

Importantly, repeated exposure to the test did not alter the percentage of novel investigation ($F_{(2, 24)}$=1.61, p=0.22;). Indeed, rats showed significant social novelty preference regardless of number of exposures to the test (Exposure 1: $t_{(13)}$ =6.73, p<0.001; Exposure 2: $t_{(13)}$ =5.84, p<0.001; Exposure 3: $t_{(13)}$ =7.32, p<0.001, collapsed across sexes; Fig. 4.2F). Overall, these findings indicate that social interaction decreases with repeated exposure, but social novelty preference does not.

Experiment 2: Neuromodulation of Social Novelty Preference

Administration of the μ-opioid receptor antagonist CTAP significantly reduced time spent investigating the novel social stimulus ($F_{(2,40)}$ = 6.82, p<0.005; Fig. 4.3A), while leaving cage mate investigation unaffected ($F_{(2,40)}$ = 0.66, p=0.52; Fig. 4.3A). Post-hoc analysis revealed that this was driven by a significant decrease in novel social investigation in rats administered the higher dose of CTAP (10μg) compared to vehicle-treated rats (Bonferroni post-hoc: p<0.005). Furthermore, CTAP administration reduced total social investigation at the higher dose (Main effect: $F_{(2,40)}$ = 5.50, p<0.01; Bonferroni post-hoc p<0.01; Fig. 4.3A). Finally, the higher dose of CTAP significantly decreased time spent in the chamber containing the novel social stimulus (Main effect: $F_{(2,40)}$ =3.93, p<0.05; Bonferroni post-hoc: p<0.05; Table 4.2). All groups investigated the novel stimulus rat more than predicted by chance (Fig. 4.3; see Table 4.3 for statistics).

Table 4.2. Neuromodulation of Social Novelty Preference. CTAP administration reduced the amount of time spent in the novel chamber at the 10μg dose as compared to vehicle. Both Eticlopride and V1aR-A treatment increased the number of entries into the middle chamber at the lower dose, as compared to both vehicle and the higher dose. Data are mean ± SEM; *p<0.05, one-way ANOVA (treatment) followed by Bonferroni posthoc; s, seconds; N, number.

CTAP	Vehicle	4μg	10μg
Novel Chamber Time (s)	371 ± 12	339 ± 14	315 ± 19*
Cage mate Chamber Time (s)	167 ± 10	189 ± 14	206 ± 26
Middle Chamber Time (s)	61.1 ± 4.1	71.8± 4.3	78.2 ± 10
Middle Entries (N)	23.3 ± 1.6	23.2 ± 2.0	18.8 ± 2.7
Eticlopride	Vehicle	40ng	400ng
Novel Chamber Time (s)	375 ± 15	375 ± 13	348 ± 10
Cage mate Chamber Time (s)	166 ± 14	161 ± 10	188 ± 13
Middle Chamber Time (s)	57.8 ± 3.2	62.8 ± 3.8	63.2 ± 4.1
Middle Entries (N)	29.1 ± 2.3	38.1 ± 2.9*	27.1 ± 2.0
OTR-A	Vehicle	75ng	750ng
Novel Chamber Time (s)	352 ± 16	379 ± 13	332 ± 16
Cage mate Chamber Time (s)	181 ± 13	158 ± 12	196 ± 13
Middle Chamber Time (s)	65.4 ± 3.8	61.9 ± 4.5	70.8 ± 7.3
Middle Entries (N)	28.7 ± 1.8	35.8 ± 4.9	28.5 ± 12
V1aR-A	Vehicle	75ng	750ng
Novel Chamber Time (s)	331 ± 24	353 ± 21	354 ± 21
Cage mate Chamber Time (s)	208 ± 23	183 ± 18	185 ± 21
Middle Chamber Time (s)	59.6 ± 4.2	61.5± 9.3	59.3 ± 3.7
Middle Entries (N)	25.1 ± 2.9	37.1± 4.4*	25.3 ± 2.3

Administration of D_2R, OTR, or V1aR antagonists had no effect on novel (Main effects: D_2R: $F_{(2,27)}$ =0.14, p=0.87; OTR: $F_{(2,28)}$ =1.10, p=0.35, V1aR: $F_{(2,28)}$ =0.30, p=0.74), cage mate (Main effects: D_2R: $F_{(2,27)}$ =0.20, p=0.82; OTR: $F_{(2,28)}$ =2.29, p=0.12, V1aR: $F_{(2,28)}$ =1.04, p=0.37), or total (Main effects: D_2R: $F_{(2,27)}$ =0.05, p=0.95; OTR: $F_{(2,28)}$ =1.49, p=0.24, V1aR: $F_{(2,28)}$ =0.21, p=0.81) investigation time, nor on social novelty preference (Main effects: D_2R: $F_{(2,27)}$ =0.27, p=0.76; OTR: $F_{(2,28)}$ =1.73, p=0.20, V1aR: $F_{(2,28)}$ =0.52, p=0.60) (Fig. 4.3B-D) . All groups investigated the novel stimulus rat more than predicted by chance (Fig. 4.3; see Table 4.3 for statistics).

Taken together, these results suggest that blockade of μ-opioid receptors, but not D_2R, OTR, or V1aR, reduces novel social investigation and total social investigation, while leaving cage mate investigation unaffected.

Table 4.3. Single sample t-test results for social novelty preference. All groups showed significant social novelty preference as compared to 50% (chance level), regardless of drug treatment or dose.

CTAP	Mean ±SEM	*t*	p
Vehicle	79 ± 2	14.6	<0.001
4µg	72 ± 4	6.32	<0.001
10µg	72 ± 3	6.57	<0.001
Eticlopride	Mean ±SEM	*t*	p
Vehicle	76 ± 4	7.15	<0.001
40ng	79 ± 3	9.76	<0.001
400ng	76 ± 4	12.3	<0.001
OTR-A	Mean ±SEM	*t*	p
Vehicle	73 ± 3	7.34	<0.001
75ng	79 ± 2	12.8	<0.001
750ng	71 ± 3	6.58	<0.001
V1aR-A	Mean ±SEM	*t*	p
Vehicle	69 ± 5	3.99	<0.005
75ng	72 ± 5	4.52	<0.005
750ng	75 ± 4	6.82	<0.001

Experiment 3: Neuromodulation of Social Preference

Administration of the highest dose of µ-opioid receptor antagonist CTAP significantly decreased the time spent investigating the social stimulus (Main effect: $F_{(2,39)}$ =14.36, p<0.001; Bonferroni post-hoc: 10µg CTAP vs. vehicle: p<0.001, 10µg CTAP vs. 4µg: p<0.001; Fig. 4.4A) while having no impact on time spent investigating the inanimate object (Main effect: $F_{(2,39)}$ =0.72, p=0.49; Fig. 4.4A). This further resulted in a decrease in total time spent investigating both stimuli (Main effect: $F_{(2,39)}$ =13.31, p<0.001; Bonferroni post-hoc: 10µg CTAP vs. vehicle: p<0.001, 10µg CTAP vs. 4µg

Table 4.4. Neuromodulation of Social Preference. CTAP administration reduced time spent in the social chamber and increased time spent in the middle chamber at the 10µg dose as compared to vehicle. Both Eticlopride and V1aR-A treatment increased the number of entries into the middle chamber at the lower dose, as compared to both vehicle and the higher dose. Data are mean ± SEM; *p<0.05, one-way ANOVA (treatment) followed by Bonferroni posthoc; s, seconds; N, number.

CTAP	Vehicle	4µg	10µg
Social Chamber Time (s)	408 ± 14	363 ± 9	333 ± 27*
Object Chamber Time (s)	117 ± 11	144 ± 10	158 ± 20
Middle Chamber Time (s)	74.7 ± 5.3	93.3 ± 6.6	108 ± 14*
Middle Entries (N)	26.4 ± 2.5	28.8 ± 2.9	21.3 ± 2.8
Eticlopride	Vehicle	40ng	400ng
Social Chamber Time (s)	389 ± 14	361 ± 21	381 ± 18
Object Chamber Time (s)	147 ± 12	156 ± 24	156 ± 15
Middle Chamber Time (s)	63.1 ± 5.7	82.8± 6.4	62.5 ± 6.0
Middle Entries (N)	29.1 ± 2.3	38.1 ± 2.9*	27.1 ± 2.0
OTR-A	Vehicle	75ng	750ng
Social Chamber Time (s)	373 ± 15	366 ± 19	352 ± 26
Object Chamber Time (s)	159 ± 12	148 ± 11	172 ± 19
Middle Chamber Time (s)	66.8 ± 7.3	86.1 ± 12	75.4 ± 9.5
Middle Entries (N)	36.8 ± 4.7	40.9 ± 3.5	30.7 ± 2.9
V1aR-A	Vehicle	75ng	750ng
Social Chamber Time (s)	404 ± 20	408 ± 23	406 ± 21
Object Chamber Time (s)	136 ± 16	123 ± 21	140 ± 20
Middle Chamber Time (s)	59.6 ± 5.7	68.9 ± 4.9	53.7 ± 4.0
Middle Entries (N)	29.7 ± 3.1	39.4 ± 0.8 *	28.6 ± 1.9

CTAP: $p<0.001$; Fig. 4.4A). Moreover, CTAP significantly decreased social preference, as reflected in a decrease in the percentage of social investigation time (Main effect: $F_{(2,39)} =10.01$, $p<0.001$; Bonferroni post-hoc: 10µg CTAP vs. vehicle: $p<0.001$, 10µg CTAP vs. 4µg CTAP: $p<0.005$; Fig. 4.4A). Finally, CTAP administration significantly decreased the time spent in the chamber in which the social stimulus was housed (Main effect: $F_{(2,39)} =4.98$, $p<0.05$; Bonferroni post-hoc: 10µg CTAP vs. vehicle: $p<0.05$; Table 4.4) and increased time spent in the middle chamber of the testing apparatus (Main effect: $F_{(2,39)} =4.43$, $p<0.05$, Bonferroni post-hoc: 10µg CTAP vs. vehicle: $p<0.05$; Table 4.4).

Administration of D_2R, OTR, or V1aR antagonists had no effect on social (Main effects: D_2R: $F_{(2,28)} =0.53$, $p=0.59$; OTR: $F_{(2,29)} =1.23$, $p=0.31$, V1aR: $F_{(2,28)} =0.82$,

p=0.45), object (Main effects: D_2R: $F_{(2,28)}$ =1.07, p=0.35; OTR: $F_{(2,29)}$ =2.11, p=0.14, V1aR: $F_{(2,28)}$ =1.99, p=0.16), or total (Main effects: D_2R: $F_{(2,28)}$ =1.31, p=0.29; OTR: $F_{(2,29)}$ =2.30, p=0.12, V1aR: $F_{(2,28)}$ =1.57, p=0.23) investigation time, nor on social preference (Main effects: D_2R: $F_{(2,28)}$ =0.52, p=0.60; OTR: $F_{(2,29)}$ =1.46, p=0.25, V1aR: $F_{(2,28)}$ =1.37, p=0.26) (Fig. 4.4B-D). All groups investigated the social stimulus more than predicted by chance (Fig. 4.4; see Table 4.5 for statistics).

Taken together, these results suggest that blockade of μ-opioid receptors, but not D_2R, OTR, or V1aR, reduces social investigation and total investigation time, while leaving object investigation unaffected.

Table 4.5. Single sample t-test results for social preference. All groups showed significant social preference as compared to 50% (chance level), regardless of drug treatment or dose.

CTAP	Mean ±SEM	*t*	p
Vehicle	86 ± 2	19.8	<0.001
4μg	85 ± 2	17.9	<0.001
10μg	71 ± 4	5.61	<0.001
Eticlopride	**Mean ±SEM**	***t***	**p**
Vehicle	82 ± 2	16.6	<0.001
40ng	80 ± 6	4.86	<0.005
400ng	86 ± 4	10.2	<0.001
OTR-A	**Mean ±SEM**	***t***	**p**
Vehicle	74 ± 3	8.83	<0.001
75ng	82 ± 3	11.5	<0.001
750ng	78 ± 4	7.48	<0.001
V1aR-A	**Mean ±SEM**	***t***	**p**
Vehicle	80 ± 2	12.7	<0.001
75ng	86 ± 3	12.4	<0.001
750ng	84 ± 3	13.3	<0.001

DISCUSSION

In this study, we first sought to establish a behavioral paradigm in which to assess the preference of a juvenile rat to investigate either a novel conspecific or a familiar cage mate. We found that, throughout the juvenile period and irrespective of repeated exposure to the test, both male and female rats robustly display a preference for social novelty. Given this finding, our second aim was to examine the involvement of major neural systems in the mediation of social novelty preference. We found that in the social novelty preference test central blockade of μ-opioid receptors, but not of D_2R, OTR, or V1aR, reduced both duration and frequency of novel conspecific, but not cage mate, investigation. Central blockade of μ-opioid receptors also decreased investigation of a novel social stimulus, but not of a novel object in the social preference test. Taken together, these findings indicate that male and female juvenile rats show robust social novelty preference as well as social preference and suggest an important role of the brain μ-opioid system in the mediation of both behaviors.

It is well known that juvenile rats show a robust preference for both novel objects and novel environments (Bronstein & Spear, 1972; Philpot & Wecker, 2008; Douglas et al., 2003; Reger et al., 2009; Cyrenne & Brown, 2011a). Moreover, previous work from our group suggests that this juvenile novelty preference extends to social stimuli (Lukas et al., 2011), but that it might do so in sex-specific ways (Veenema et al., 2012). To the best of our knowledge, we are the first to directly assess preference for a novel conspecific as compared to a cage mate in juvenile rats. Our findings demonstrate that juvenile rats indeed show a robust social novelty preference and that this preference does

not differ between the sexes, suggesting that both males and females are highly motivated to explore novel conspecifics over familiar ones.

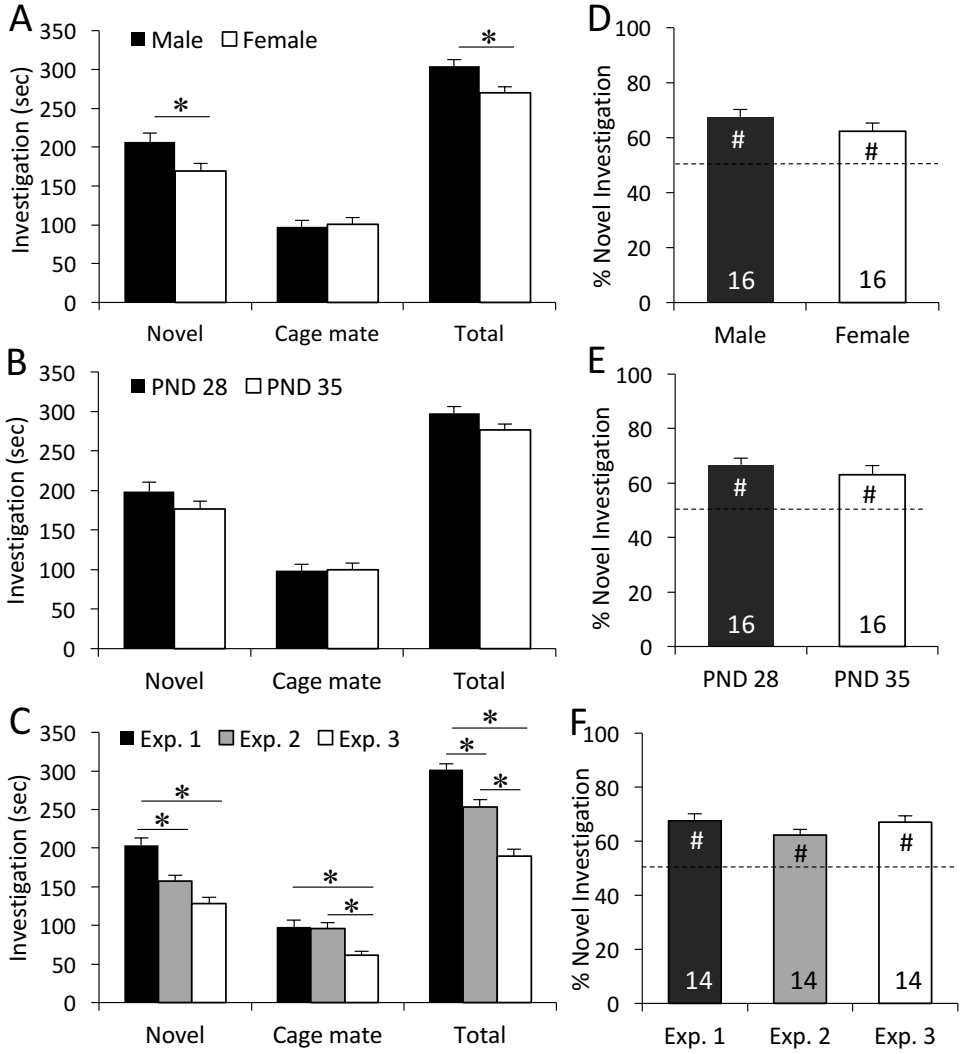

Figure 4.2. Establishing the social novelty preference test in male and female juvenile rats – results by sex, age and repeated exposure to the test. Main effects of sex (**A**), age (**B**), and repeated exposure to the test (**C**) were compared for novel, cage mate, and total social investigation time. Females showed lower novel and total investigation times than males (**A**). No age effects were found between PND 28 and PND 35 for any of these parameters (**B**). However, repeated exposure to the test led to significant decreases in novel, cage mate, and total investigation time (**C**). Independent of sex, age or repeated exposure to the test, rats showed a significant preference for social novelty (**D, E, F**). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. Dashed line indicates chance level (50%). Bars indicate means + SEM. * $p<0.05$, two-way ANOVA in (**A**), one-way ANOVA in (B), two-way mixed-design ANOVA followed by Bonferroni post-hoc tests in (**C**), # $p<0.05$ one sample *t*-test against 50%. Exp., Exposure.

A sex difference was found, however, in social investigation time, which was higher in males. This is in line with studies in adult rats in which males spent more time investigating a stimulus rat than females (Thor, 1980; Thor et al., 1988; Tejada & Rissman, 2012; Dumais et al., 2013). Furthermore, we found that females showed higher locomotor activity, as reflected by more entries into the middle chamber of the testing apparatus. This agrees with other studies showing that female rats are generally more active than males (Slob et al., 1986; Van Haaren, 1991). Thus, irrespective of age, female rats show less interest in exploring social stimuli, but higher locomotor activity than male rats.

Our findings further demonstrate a link between exploration of novel social stimuli and the brain opioid system in juvenile rats. Specifically, we found that central administration of the μ-opioid receptor antagonist CTAP decreased the time male juvenile rats spent investigating a novel stimulus rat without altering the time spent investigating a familiar rat (a cage mate in the social novelty preference test). Our findings are the first to demonstrate a role for the μ-opioid that is specific to a novel social stimulus as compared to one which is familiar. This is in line with findings outside the social domain suggesting that μ-opioid blockade alters non-social novelty-seeking. For example, in adult male mice, peripheral administration of a μ-opioid receptor antagonist decreased novelty-induced locomotion (Radcliffe & Erwin, 1998) and μ-opioid receptor knockout mice showed reduced novelty exploration (Yoo et al., 2004). Moreover, we found that central administration of the μ-opioid receptor antagonist CTAP decreased the time male juvenile rats spent investigating a novel stimulus rat as compared to a novel object (in the social preference test). These results confirm and extend previous

findings in rodents suggesting a role for the μ-opioid receptor in the facilitation of social interaction in juvenile animals. For example, juvenile mice lacking the μ-opioid receptor gene (*Oprm1*$^{-/-}$) show a reduced preference to interact with a conspecific over an object as well as reduced preference for environments previously associated with a social stimulus (Cinque et al., 2012). Additionally, blockade of μ-opioid receptors either peripherally or in the nucleus accumbens of male juvenile rats decreased social play behaviors as well as conditioned place preference for social play (Beatty & Costello, 1982; Panksepp et al., 1985; Vanderschuren et al., 1995; Trezza et al., 2011).

Novelty exploration can be risky, as it may increase exposure to predators or sources of infection (Lima & Dill, 1990). Approaching novel stimuli may therefore require not only the motivation to approach and explore, but also the suppression of risk avoidance. Interestingly, the μ-opioid receptor plays a crucial role in the neuromodulation of fear and anxiety (Colasanti et al, 2011). For example, the μ-opioid receptor agonist morphine reduces anxiety-related behavior when administered into the nucleus accumbens, ventral hippocampus, or lateral septum in adult male mice or rats (Le Merrer et al., 2007; Zarrindast et al., 208) while CTAP increases anxiety-related behavior when administered into the central amygdala in adult male rats (Wilson & Junor, 2008). It would be of interest to determine whether central μ-opioid receptor blockade decreases social novelty exploration in juvenile rats by increasing anxiety and thereby enhancing risk aversion.

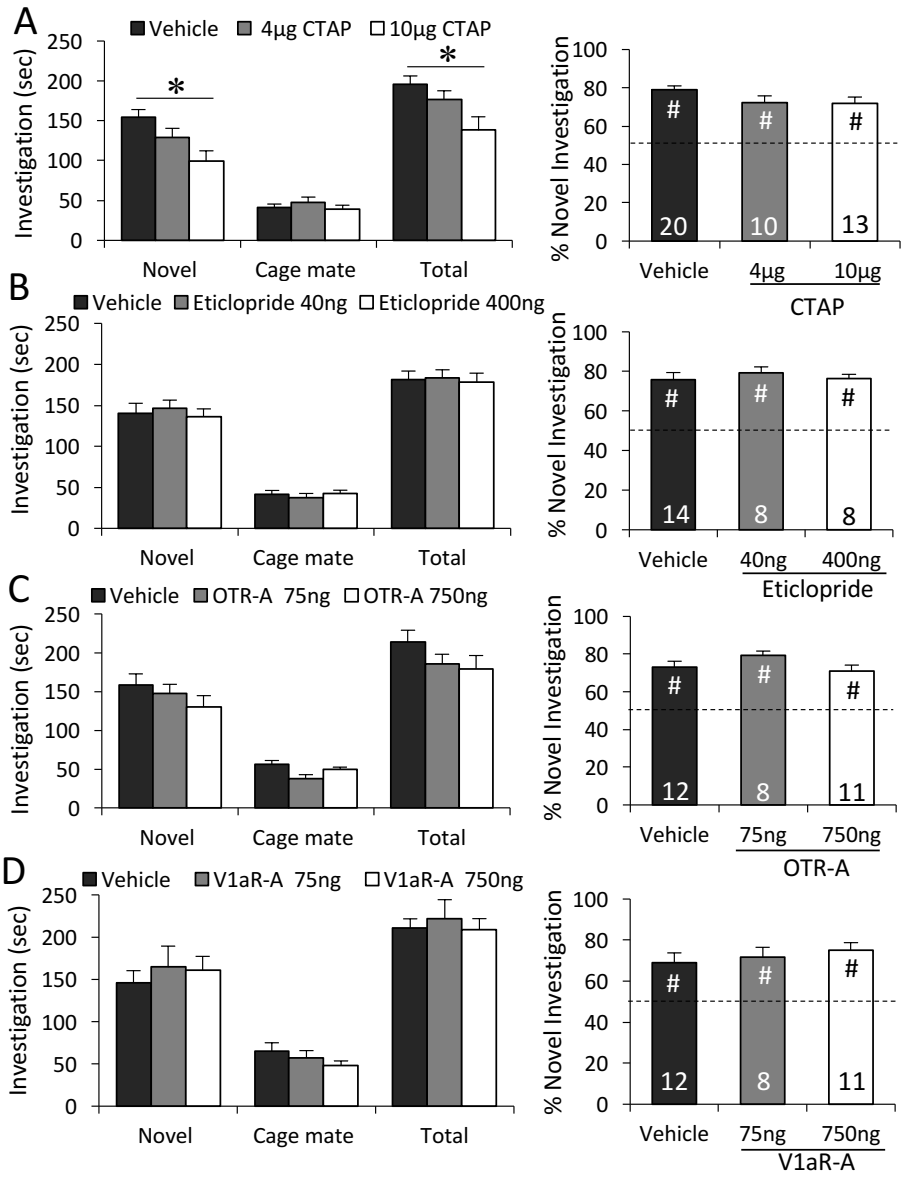

Figure 4.3. Neuromodulation of Social Novelty Preference. Central administration of the µ-opioid receptor antagonist CTAP significantly reduces novel investigation time and total social investigation time at the higher dose of 10µg **(A)**. Central administration of an antagonist specific for the D_2R (Eticlopride), OTR (OTR-A), or V1aR (V1aR-A) does not alter novel, cage mate or total social investigation time or the percentage of novel investigation time **(B, C, D)**. The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. Dashed line indicates chance level (50 %). Bars indicate means + SEM. * $p<0.05$, one-way ANOVA followed by Bonferroni post-hoc tests, # $p<0.05$ one-sample *t*-test against 50 %.

Previous work has suggested that µ-opioid receptor activation facilitates juvenile social play and adult partner preference formation, by acting on the ventral and dorsal

striatum, respectively. Specifically, μ-opioid receptor agonist administration into the nucleus accumbens, but not the dorsal striatum, increased social play behavior in juvenile male rats (Trezza et al., 2011). Furthermore, CTAP administered into the nucleus accumbens decreased social play as well as play-conditioned place preference (Trezza et al., 2011). In contrast, CTAP administration into the dorsal striatum and dorsomedial nucleus accumbens shell, but not the nucleus accumbens core or ventral nucleus accumbens shell, inhibited partner preference formation in female prairie voles (Burkett et al., 2011; Resendez et al., 2013). Interestingly, it has recently been proposed that the ventral striatum may mediate the reward value of novel social interactions while the dorsal striatum is important for the transition to familiarity and habit formation (Tops et al., 2014). This might explain why μ-opioid receptor activation in the nucleus accumbens promotes rewarding social interactions with novel conspecifics (as in the case of social play), while μ-opioid receptors in the dorsal striatum would mediate partner preference (familiarity and habit formation). It is possible, therefore, that activation of μ-opioid receptors in ventral striatal regions such as the nucleus accumbens, would facilitate social novelty interaction. Further brain region-specific manipulation studies are needed to determine where in the brain μ-opioid signaling mediates social novelty preference in juvenile rats.

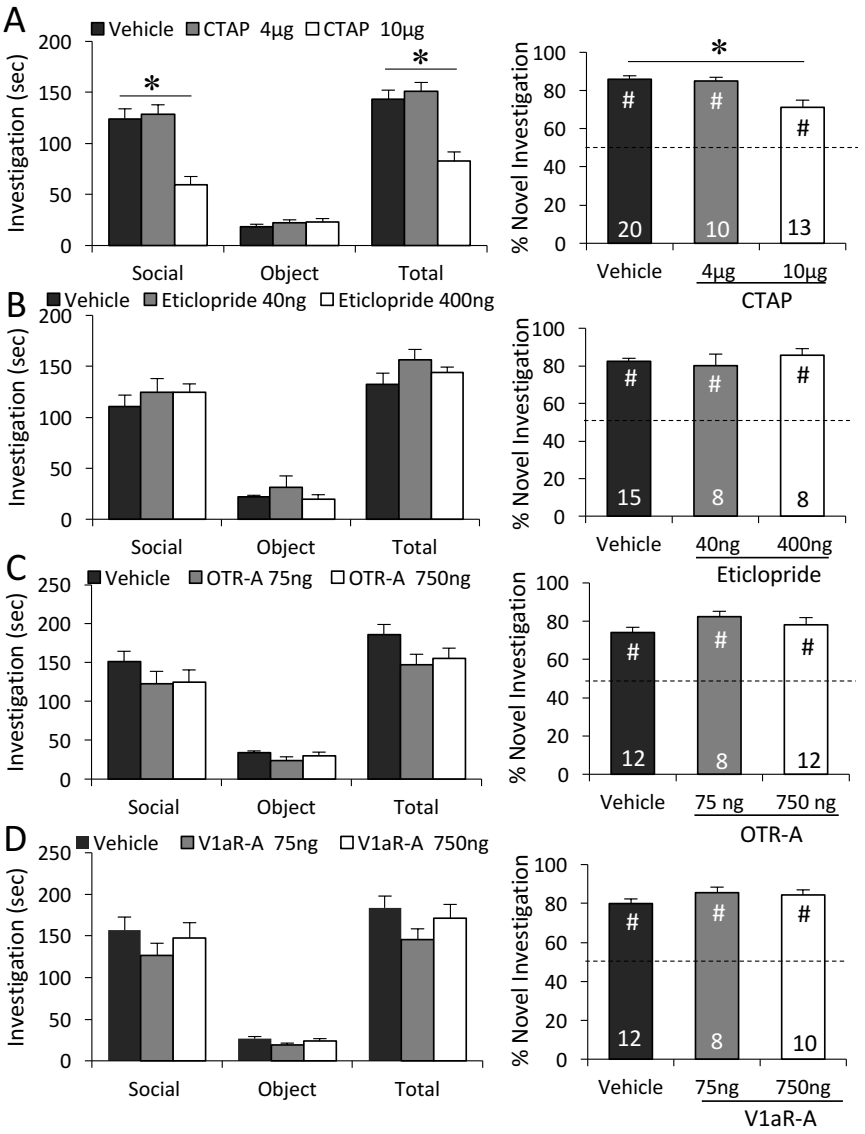

Figure 4.4. Neuromodulation of Social Preference. Central administration of the µ-opioid receptor antagonist CTAP significantly reduces social investigation time, total investigation time, and the percentage of social investigation time at the higher dose of 10µg **(A)**. Central administration of an antagonist specific for the D_2R (Eticlopride), OTR (OTR-A), or V1aR (V1aR-A) does not alter social, object, or total interaction time or the percentage of social investigation time **(B, C, D)**. The percentage of social investigation reflects the proportion of total investigation time spent investigating the social stimulus. Dashed line indicates chance level (50 %). Bars indicate means + SEM. * p <0.05, one-way ANOVA followed by Bonferroni post-hoc tests, # p<0.05 one-sample *t*-test against 50 %.

Our suggestion that activation of the µ-opioid receptor may suppress risk avoidance and enhance the rewarding value of novel social interactions, may find support in recent studies in humans. Healthy subjects who scored high on harm avoidance

(defined as increased sensitivity to aversive and novel stimuli) displayed lower endogenous μ-opioid activity in several cortical brain regions (Tuominen et al., 2012). Additionally, μ-opioid receptor activation facilitated pleasure derived from positive novel social interactions (Chelnokova et al., 2014), especially by acting on the ventral striatum (Hsu et al., 2013; Hsu et al., 2015). Interestingly, this effect was not seen in patients with major depression (Hsu et al., 2015), suggesting that reduced opioid activity may underlie reduced pleasure derived from positive social interactions. Furthermore, alterations in opioid activity have been suggested to underlie impaired social interest in ASD (Sahley & Panksepp, 1987). Given these observations and our current findings, it would be of interest to further explore the role of the μ-opioid receptor in mediating both harm avoidance and social novelty seeking in healthy humans and in those showing aberrations in these behaviors including ADHD, drug addiction, social anxiety disorder, and ASD (Anckarsäter et al., 2006; Mortberg et al., 2007; Kerekes et al., 2013; Kampman et al., 2014; Binelli et al., 2015).

Based on an extensive literature demonstrating a role for the D_2R, OTR, and V1aR in the regulation of social behavior (Aragona et al., 2006, Gingrich et al., 2000; Cibrian-Llanderal et al., 2012; Lukas et al., 2011; Wang et al., 1999), we predicted that central blockade of any of these receptors would reduce social novelty preference. However, we found no effect of any of these manipulations on either social novelty preference or social preference, despite the use of two drug doses. Interestingly, we are not the first to report null effects of manipulations to these systems in the regulation of social behavior. While a role for OT in the regulation of social preference has been shown in adult male rats and mice (Lukas et al., 2011; Sala et al., 2013), this finding is

not consistent. For example, using OTR knockout mice, one study reported impairments (Sala et al., 2013) in social preference, while another found no effects (Crawley et al., 2007). Furthermore, while OTR blockade has been shown to reduce social preference in adult male rats (Lukas et al., 2011), a recent study found no effects of either OTR or V1aR blockade on social preference in adult female rats (Lukas & Neumann et al., 2014). Finally, there is abundant evidence suggesting age differences in neuropeptide system parameters (such as receptor binding densities) and behavioral function (for review: Hammock, 2015; Grinevich et al., 2015). Indeed, age differences have been shown in the role of V1aR in the regulation of social recognition (Veenema et al., 2012). These and our current findings suggest that findings in adult male rats may not translate between the sexes and across development. This adds to a growing body of evidence that the role of the OT system in the modulation of social behavior in animals and humans depends on a variety of factors, including social context and individual differences (Lukas et al., 2013; Bredewold et al., 2014; Bartz et al., 2011).

Moreover, the regulation of social behavior has often been shown to be highly brain region-specific. For example, eticlopride reduces partner preference formation when injected into the nucleus accumbens, but not the prelimbic cortex, in female prairie voles (Gingrich et al., 2000). Adult male rats given an OTR antagonist into the lateral septum, but not the medial amygdala, showed impaired memory for juvenile rats (Lukas et al., 2013). When administered centrally, a V1aR antagonist reduced social play behavior in juvenile male rats, but when administered into the lateral septum, it had the opposite effect (Veenema et al., 2013, Bredewold et al., 2014). It is therefore possible that the null effects of central D_2R, OTR, and V1aR blockade represent a net effect across

brain regions and therefore produce no behavioral change. Thus, we cannot exclude a potential brain region-specific role for any of these systems in the regulation of social novelty preference.

In summary, our results indicate the robust presence of social novelty preference in juvenile rats of both sexes. Moreover, our findings implicate the brain opioid system, acting via the μ-opioid receptor, as a facilitator of this behavior. Although several rodent studies have indicated a role of the μ-opioid receptor in novelty exploration, our study is the first, to our knowledge, demonstrating a role for the μ-opioid receptor in the regulation of social novelty exploration. We discussed that the μ-opioid system might promote social novelty exploration both by reducing anxiety and risk avoidance and by increasing the motivation to explore novel conspecifics. Using the social novelty preference test, further research will focus on revealing the brain regions and pathways by which the μ-opioid receptor reduces social novelty exploration in juvenile rats.

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Chapter Five: Involvement of the oxytocin system in the nucleus accumbens in the regulation of juvenile social novelty-seeking behavior*

*Submitted Manuscript:
Smith C.J.W., Mogavero J.N., Tulimieri M.T., Veenema A.H. (2016) Involvement of the oxytocin system in the nucleus accumbens in the regulation of juvenile social novelty-seeking behavior, Hormones & Behavior ~ Short Communication

ABSTRACT: Exploration of novel environments, stimuli, and conspecifics is highly adaptive during the juvenile period, as individuals transition from immaturity to adulthood. We recently showed that juvenile rats prefer to interact with a novel individual over a familiar cage mate. However, the neural mechanisms underlying this juvenile social novelty-seeking behavior remain largely unknown. One potential candidate is the oxytocin (OXT) system, given its involvement in various motivated social behaviors. Here, we show that administration of the specific oxytocin receptor antagonist desGly-NH_2,d$(CH_2)_5$-[Tyr$(Me)^2$,Thr^4]OVT reduces social novelty seeking-behavior in juvenile male rats when injected into the nucleus accumbens (10ng/0.5 µl/side). The same drug dose was ineffective at altering social novelty-seeking behavior when administered into the lateral septum or basolateral amygdala. These results are the first to suggest the involvement of the OXT system in the nucleus accumbens in the regulation of social novelty-seeking, a behavior which is impaired in children diagnosed with autism spectrum disorders.

INTRODUCTION

Across species, the juvenile period is characterized by heightened engagement in novelty-seeking behavior and increased social interaction with peers as compared to both younger and older ages (Spear, 2000). These behaviors are likely to be highly adaptive as individuals transition from immaturity to adulthood (Spear, 2000). However, a high novelty-seeking behavioral phenotype may predispose individuals to risk-taking and substance use (Dellu et al., 1996). Conversely, reduced social novelty-seeking is a characteristic of autism spectrum disorders (ASD) and may contribute to low social reciprocity and social interest in individuals with ASD (Anckarsäter et al., 2006; American Psychiatric Assoc., 2013). Thus, understanding the neural mechanisms underlying social novelty-seeking behavior may be a first step towards understanding how this behavior is disrupted in individuals diagnosed with substance use disorders or ASD.

We recently developed the 'social novelty preference test' to assess social novelty-seeking behavior and demonstrated that juvenile rats prefer to interact with novel over familiar (i.e., cage mate) conspecifics (Smith et al., 2015). Here, we aim to determine whether social novelty-seeking behavior is modulated by the brain oxytocin (OXT) system. OXT has been shown to regulate numerous social behaviors in various species via activation of the OXT receptor (OTR) in the brain (Anacker & Beery, 2013; Meyer-Lindenberg et al., 2011). However, most of this research has been conducted in adult animals and much less is known regarding the regulation of social behavior in juveniles. We focus here on the OXT system in the nucleus accumbens (NAc), lateral septum (LS) and basolateral amygdala (BLA), because these brain regions show higher

OTR binding density in juvenile rats as compared to adult rats (Smith et al., 2016) and because the OXT system in these brain regions has been implicated in the regulation of various social behaviors (Guzman et al., 2013; 2014; Lukas et al., 2013; Dolen et al., 2013; Chang et al., 2015). We hypothesize that OTR activation in either the NAc, LS, or BLA will facilitate social novelty-seeking behavior.

METHODS

Animals

Male Wistar rats were obtained from Charles River Laboratories (Raleigh, NC) and housed in standard rat cages (26.7 x 48.3 x 20.3 cm) under standard laboratory conditions (12-hour light/dark cycle, lights on at 7:00 am, food and water available ad libitum, 22° C, 60% humidity). Experimental rats (23 days of age at arrival) were housed in same-sex pairs. Stimulus rats (22 days of age at arrival) were housed in same-sex groups of 3-4 and were one day younger than experimental rats to ensure that they were unrelated. All experiments were conducted in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

Cannulation and Injection Procedures

At 27 or 28 days of age, experimental rats were anesthetized with isoflurane (Henry Schein, Dublin OH) and positioned into a stereotaxic frame with the incisor bar set at -4.5 mm. Throughout surgery, a heating pad was used to maintain body temperature. Coordinates were based on Paxinos & Watson (2007) and adapted for use in

juveniles to hit target brain regions (See Fig. 5.1). Guide cannulae (22 gauge, Plastics One, Roanoke, VA) were implanted 2 mm dorsal to the target region, which was either the NAc (2.5 mm rostral to bregma, +/- 2.5 mm lateral to the midline, 4.6 mm ventral to the surface of the skull, angle of 10° from the midline), LS (1.0 mm rostral to bregma, +1.0 mm lateral to the midline, -3.6 mm ventral to the surface of the skull, angle of 10° from the midline), or BLA (2.7 mm caudal to bregma, +/- 4.3 mm lateral to the midline, 6.3 mm ventral to the surface of the skull). Guide cannulae were secured via stainless steel screws and dental acrylic adhesive and were closed with a dummy cannula (28 gauge; Plastics One). Following surgery, rats were given an injection of Rimadyl analgesic (Henry Schein, Dublin OH) and singly housed for one hour before rehousing with their cage mate.

Experimental rats were handled daily for four days prior to testing to habituate them to the injection procedure. Injection systems were composed of polyethylene tubing connected to an injector cannula and a 10 µl Hamilton syringe. The injector cannula (28 gauge; Plastics One) extended 2 mm beyond the guide cannula, was kept in place for 30 s following injection to allow for tissue uptake, and was then replaced by a dummy cannula. After the experiments, rats were euthanized with CO_2, and charcoal was injected through the guide cannula as a marker to check for proper cannula placement on Nissl-stained coronal brain sections (Fig. 5.1A-F).

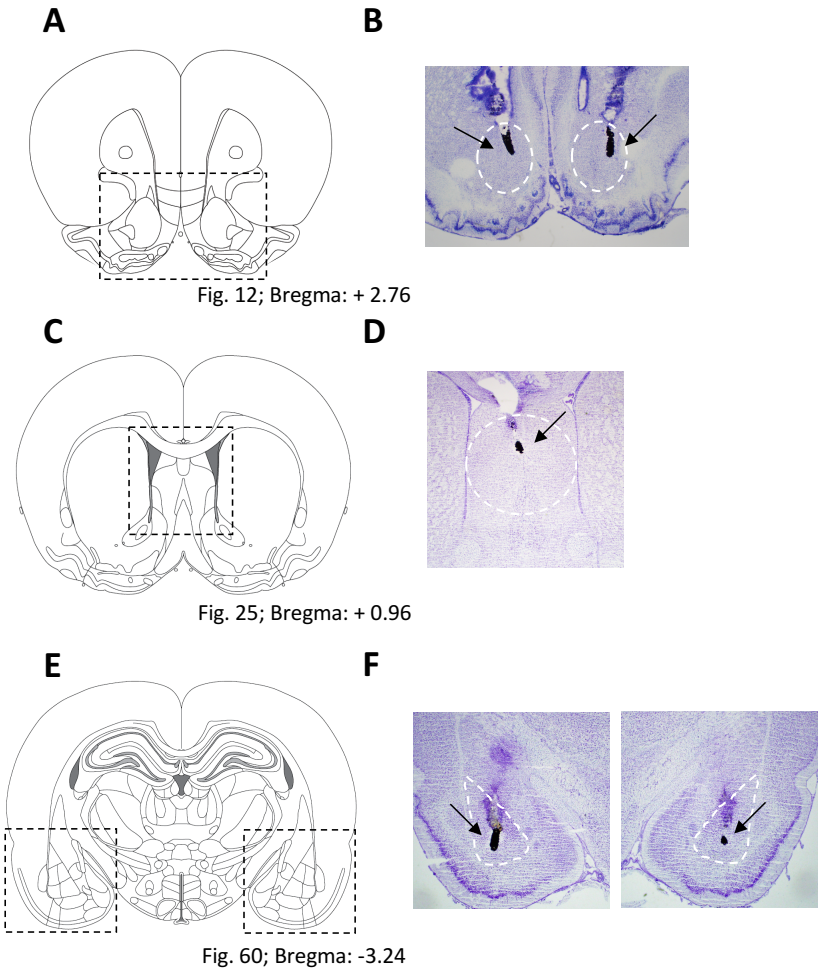

Figure 5.1. Representative cannula placements in the NAc, LS, and BLA. Schematic drawings of the rat brain adapted from Paxinos and Watson (2007) illustrating the NAc (**A**), LS (**C**) and BLA (**E**), as well as Nissl-stained coronal brain sections indicating with arrows the location of microinjections using charcoal as a marker (**B**,**D**,**F**). Bilateral cannulae were implanted in the NAc and in the BLA, while one cannula was implanted in the center of the LS. Animals with incorrect cannula placements were excluded from analysis. Dashed squares in schematic drawings represent the area of enlargement in Nissl images; Dashed outlines represent target brain areas.

Social Novelty Preference Test

The social novelty preference test was conducted according to Smith et al. (2015). Briefly, one day prior to testing, all experimental and stimulus rats were moved to the behavioral testing room for 1 hour and then placed in the testing apparatus for 10 min., to

acclimate them to each. On the day of testing, experimental and stimulus rats were placed in the behavioral testing room 1 hour before behavioral testing. Social novelty preference was assessed in a Plexiglas apparatus consisting of a rectangular three-chambered box. Each chamber (40 x 27 x 40 cm) is separated by a Plexiglas partition with a rectangular opening to allow for passage between chambers. A novel stimulus rat was placed in one end chamber, while the subject's cage mate (familiar stimulus) was placed in the other. Both stimulus rats were confined to small containers (18 x 10 x 21 cm) composed of translucent Plexiglas bars to restrict their movement while still allowing for social investigation by the experimental rat. The experimental rat was placed in the middle chamber and allowed to freely explore the three chambers and the social stimuli for 10 min. Behavior was video recorded and later scored using the behavioral analysis program JWatcher (http://www.jwatcher.ucla.edu) by an experimenter blind to experimental treatment. Investigation of the stimulus rats was defined as direct nose poking through the bars of the containers holding the stimulus rats. To obtain a measure of social novelty preference, the percentage of time spent investigating the novel stimulus rat (time investigating the novel stimulus rat/time investigating the novel + familiar rat x 100) was calculated. Experimental rats were considered to exhibit social novelty preference when the percentage of time spent investigating the novel conspecific was significantly different from chance (50%). The sum of novel and cage mate investigation times was calculated as a measure of total social investigation time.

Experimental Procedures

A within-subjects design was used to determine the effects of local OTR blockade in the NAc (n=12), the LS (n=14), or the BLA (n=11) on social novelty preference in 30-

34-day-old male rats. We limited our investigation to males because there were no sex differences in social novelty preference (Smith et al., 2015) nor in OTR binding density in these brain regions (Smith et al., 2016). Separate cohorts of rats were used to test the effects of OTR blockade on social novelty-seeking behavior in each brain region. Experimental rats received an injection of either vehicle (0.9% saline; 0.5 µl/side) or the selective OTR antagonist desGly-NH_2,d$(CH_2)_5$-[Tyr(Me)^2,Thr^4]OVT (10ng/0.5 µl/side; Manning et al., 2008) 20 min prior to exposure to the social novelty preference test. One to two days later, rats were again exposed to the social novelty preference test, this time with the opposite drug treatment, in counter-balanced order. The dose of the oxytocin receptor antagonist was based on previous literature showing its effectiveness in altering social behavior in mice and rats (Guzman et al., 2013, 2014; Lukas et al., 2013).

Statistical Analysis

Single-sample t-tests compared to 50% (chance level) were used to determine the presence of social novelty preference. Paired-samples t-tests were used to determine the effect of OTR antagonist treatment on all other measures of the social novelty preference test.

RESULTS

OTR antagonist administration into the NAc significantly decreased social novelty preference ($t_{(11)}$=2.71; $p<0.05$; Fig. 5.2A). Subjects spent more time investigating the novel conspecific than would be expected by chance following both vehicle ($t_{(11)}$=12.5; $p<0.001$) and OTR antagonist ($t_{(11)}$=8.03; $p<0.001$) administration into the

NAc (Fig. 5.2A). There was no significant effect of OTR antagonist treatment on novel investigation time ($t_{(11)}$=0.13; p=0.90), cage mate investigation time ($t_{(11)}$=-1.33; p=0.21), or total social investigation time ($t_{(11)}$=0.36; p=0.73) (Fig. 5.2A).

No significant effect of OTR antagonist administration into the LS or BLA was observed for any measure in the social novelty preference test. In detail, subjects spent more time investigating the novel conspecific than would be expected by chance following vehicle treatment (LS: $t_{(13)}$=7.67; p<0.001; BLA: $t_{(10)}$=14.14; p<0.001) and OTR antagonist treatment (LS: $t_{(13)}$=8.91; p<0.001; Fig. 5.2B; BLA: $t_{(10)}$=13.11; p<0.001; Fig. 5.2C). OTR-A administration had no effect on social novelty preference (LS: $t_{(13)}$=-0.75; p=0.47; Fig. 5.2B; BLA: $t_{(10)}$=0.76; p=0.46; Fig 5.2C), novel investigation time (LS: $t_{(13)}$=-0.38; p=0.71; BLA: ($t_{(10)}$=1.76; p=0.11), cage mate investigation time (LS: $t_{(13)}$=0.96; p=0.35; BLA: $t_{(13)}$=-0.02; p=0.99), or total social investigation time (LS: $t_{(13)}$=-0.1; p=0.92; Fig. 5.2B; BLA: $t_{(10)}$=1.85; p=0.095; Fig.5.2C).

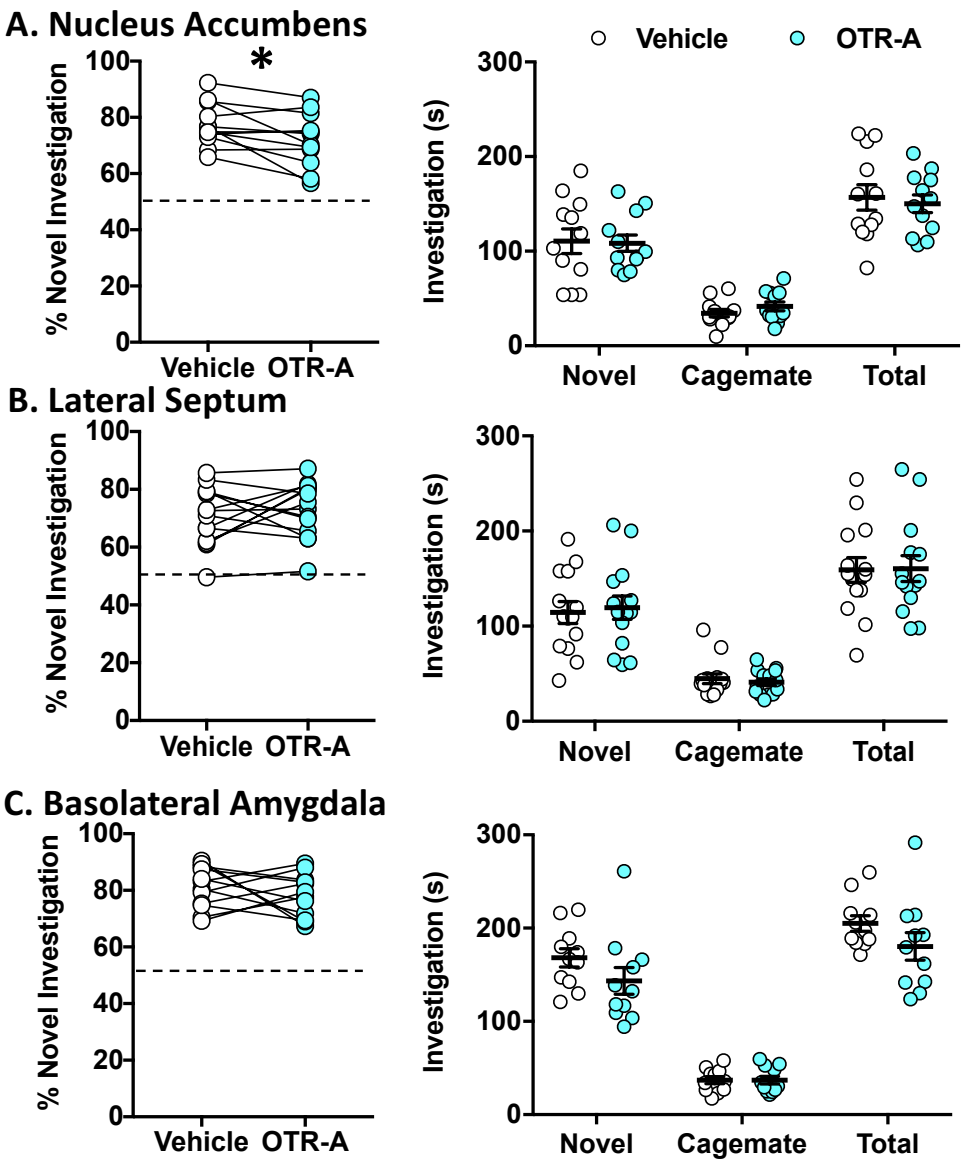

Figure 5.2. OTR antagonist (OTR-A) administration in the NAc, but not in the LS or BLA, reduced social novelty-seeking behavior in juvenile male rats. Administration of the specific OTR-A des-Gly-NH_2,d$(CH_2)_5$[Tyr(Me)^2,Thr_4]OVT (10ng/0.5 µl/side) into the NAc significantly reduced social novelty preference, but did not alter novel, cage mate, or total social investigation time (**A**). OTR-A administration into the LS (10ng/0.5 µl) or the BLA (10ng/0.5 µl/side) had no effect on social novelty preference, nor on novel, cage mate, or total social investigation time (**B**,**C**). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. A dashed line indicates the chance level (50%). Dots represent individual subjects, with mean + SEM. *$p < 0.05$, paired-sample t-tests.

DISCUSSION

Our results demonstrate that OTR blockade in the NAc reduces social novelty preference in juvenile male rats. This finding in rats expands upon previous work in prairie voles and mice demonstrating that OTR activation in the NAc facilitates motivated social behaviors in juvenile animals. Specifically, OTR blockade or OTR downregulation in the NAc reduced spontaneous alloparental behavior in 5-week-old female prairie voles (Keebaugh et al., 2015). Furthermore, OTR blockade in the NAc reduced conditioned place preference for a conspecific in 4-6-week-old male mice (Dolen et al., 2013). NAc-OTR activation has also been shown to facilitate motivated social behaviors in adult animals. For example, viral vector-induced over-expression of OTRs in the NAc facilitated pair-bond formation (Liu & Wang, 2003; Ross et al., 2009), while viral vector-induced OTR downregulation or OTR antagonist administration into the NAc impaired pair-bond formation (Liu & Wang, 2003; Keebaugh et al., 2015) in adult female prairie voles. Together, these findings indicate that the NAc is an important locus of action for the OXT system in the regulation of motivated social behaviors across ages and species.

Other neuromodulatory systems in the NAc, such as opioids, serotonin, endocannabinoids, and dopamine, have been shown to regulate motivated social behaviors, in some cases in concert with the OXT system (Liu & Wang, 2003; Dolen et al., 2013; Wei et al., 2015). Within the NAc, several sub-regions have been identified with differing functional roles (Voorn et al., 2004; Mannella et al., 2014). Interestingly, opioid receptor activation has been shown to be necessary for partner preference

formation in female prairie voles in the dorsomedial NAc shell, but not the NAc core, or the ventral NAc shell (Resendez et al., 2013). Here, we targeted the anterior portion of the NAc core because it contains the highest density of OTRs compared to other NAc sub-regions in the rat (Smith et al., 2016). Studies in voles and mice have targeted more posterior NAc sub-regions, possibly because of a different distribution of OTRs within the NAc in those species (Ross et al., 2009; Dolen et al., 2013). Therefore, it would be interesting to determine in future studies whether the involvement of OTR in the regulation of social novelty seeking behavior is specific to certain NAc sub-regions, as well as whether and how the OXT system interacts with other systems in the NAc to facilitate juvenile social novelty-seeking behavior.

In contrast to the NAc, the same dose of OTR antagonist did not alter social novelty preference when administered into the LS or BLA. This suggests brain region-specific involvement of the OXT system in modulating social novelty-seeking behavior. However, we cannot exclude the possibility that higher doses of the OTR antagonist administered into the LS or BLA might be effective at changing social novelty-seeking behavior. Yet, similar or lower doses of the same OTR antagonist have been shown to alter other social behaviors. For example, a similar dose of the OTR antagonist injected into the LS impaired social recognition in male rats (Lukas et al., 2013) and reduced the buffering effect of prior social interaction on freezing behavior in response to fear conditioning in male mice (Guzman et al., 2013, 2014). A lower dose of the OTR antagonist impaired social recognition in male and female rats when injected in the posterior bed nucleus of the stria terminalis (Dumais et al., 2016), a brain region with one of the highest OTR binding densities in rats (Dumais et al., 2013; Smith et al., 2016).

Interestingly, all of these studies were performed in adult rodents. Therefore, more research is required to elucidate this potentially brain region-specific role of the OXT system in the regulation of juvenile social novelty-seeking behavior.

In conclusion, our results demonstrate that OTR activation in the NAc facilitates social novelty-seeking behavior in juvenile male rats. Future studies are needed to determine the neural mechanisms and pathways by which NAc-OTRs facilitate this behavior. This may be of relevance to our understanding of disorders that are characterized by altered social novelty-seeking behavior, such as ASD.

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Chapter Six: Mu opioid receptors in the nucleus accumbens mediate interaction preference for novel over familiar social stimuli in juvenile rats: Implications for restoring social novelty preference following social isolation *

**Manuscript in preparation:*
Smith C.J.W., Wilkins K.B., Li S., Tulimieri M., Veenema A.H. (2017) Nucleus accumbens μ-opioid receptors and changes to social context modulate juvenile social novelty preference

ABSTRACT: The μ opioid receptor (MOR) in the nucleus accumbens is involved in assigning 'hedonic' value to rewarding (including social) stimuli. Importantly, the hedonic value of a given rewarding stimulus likely depends on an individual's current motivational state. Here, we examined the involvement of MORs in two distinct motivational drives, that is, the motivation to interact with either a novel or with a familiar conspecific. To this end, we used the recently established social novelty preference test, in which juvenile rats have the choice to interact with either a novel or a familiar (cage mate) conspecific. First, we demonstrate that the selective MOR antagonist CTAP administered into the nucleus accumbens reduces social novelty preference in juvenile male rats. Interestingly, this effect is mediated by a decrease in time spent interacting with the novel conspecific as well as an increase in time spent interacting with the cage mate. The same dose of CTAP had no effect on social novelty preference when administered into the basolateral amygdala, suggesting that the effect is specific to the nucleus accumbens. Second, we hypothesized that a brief (3 h) separation from its cage mate would alter the motivational state of the subject, resulting in a decrease in social novelty preference. Indeed, a 3 h separation from the cage mate reduces social novelty preference, an effect which is primarily driven by an increase in interaction with that cage mate. This effect was found in both males and females and regardless of whether subjects

were isolated or remained socially housed while being separated from the cage mate. Last, we find that MOR agonism, either centrally or locally in the nucleus accumbens, restores social novelty preference in those rats that did not show social novelty preference following social isolation. Taken together, these data support a model in which endogenous MOR activation in the nucleus accumbens mediates the hedonic value of novel social stimuli, while exogenous MOR activation is sufficient to reduce the hedonic value of familiar social stimuli in rats susceptible to social separation.

INTRODUCTION

The μ-opioid receptor (MOR) has been implicated in the regulation of the hedonic value of rewarding stimuli (Berridge & Kringelbach, 2015; Laurent et al., 2015), including those that are social. For example, juvenile male and female MOR knockout mice showed reduced interest in peers and no preference for socially rewarding environments (Cinque et al., 2012). Furthermore, systemic administration of MOR agonists enhanced, while MOR antagonists reduced, social play behavior (a highly rewarding behavior) in juvenile male rats (Panksepp et al., 1980, 1985; Beatty & Costello, 1982; Vanderschuren et al., 1995a,b). Moreover, we recently showed that central MOR blockade reduced the preference of juvenile male and female rats to interact with a novel over a familiar conspecific (Smith et al., 2015). The latter finding suggests that MOR activation regulates the choice between two distinct social stimuli (i.e., novel versus familiar) that both have hedonic value. Here, we aimed to determine where in the brain these effects are mediated. The nucleus accumbens (NAc) and basolateral amygdala (BLA) are two candidate regions because of their well-established roles in the regulation of reward-related behaviors (Stuber et al., 2011; Ambrogge et al., 2008; Britt et al., 2012; Pecina & Berridge, 2000; Katayama et al., 2009; Dolen et al., 2013) and the abundant expression of MORs in these brain regions (Kornblum et al., 1987). In addition, activation of MORs in the NAc mediates the reward value of social play behavior in juvenile male rats (Trezza et al., 2011) and pair-bond formation in adult female prairie voles (Resendez et al., 2013). Similarly, PET-imaging studies in humans have revealed that MOR activation in the ventral striatum (which encompasses the NAc) predicts motivation to engage in social interaction with unfamiliar individuals (Hsu et al., 2013).

MOR activation in the BLA has been implicated in the regulation of motivated behaviors associated with sucrose reward in rats (Wassum et al., 2009; Wassum et al., 2011; Lichtenberg & Wassum, 2016). Surprisingly, to the best of our knowledge, the role of BLA-MORs in the regulation of social behavior has not been explored so far. Therefore, we aimed to determine whether MOR activation in the NAc and/or BLA is causally involved in the regulation of social novelty preference in juvenile male rats. We hypothesized that MOR activation in the NAc and BLA would facilitate social novelty-seeking behavior, and predicted that pharmacological blockade of MORs in either the NAc or the BLA would reduce social novelty preference.

Importantly, some social stimuli may have more hedonic value than others. For example, when juvenile male and female rats were housed with a cage mate, and then given the choice to interact with a novel conspecific or their cage mate, they interacted more with the novel conspecific (Smith et al., 2015). However, when juvenile male and female rats were isolated from their cage mate for 24 hours, they engaged more in affiliative social interactions with that cage mate upon reunion than did juveniles that were united with a novel individual following social isolation (Circulli et al., 1996; Terranova et al., 1999). These findings suggest that the hedonic value of a conspecific, and therefore the motivation to engage with that conspecific, depends on an individuals' current social context. Importantly, the MOR system plays a role in the motivation to seek out social interaction upon social separation. For example, peripheral MOR agonism reduced distress vocalizations upon separation from their mothers in puppies, chicks, rat pups, and infant rhesus monkeys, and this effect was reversed by administration of an opioid antagonist (Panksepp et al., 1978; Panksepp et al., 1980; Carden & Hofer, 1990;

Kalin et al., 1988). Therefore, our second aim was to determine the impact of acute separation from familiar peers on social novelty preference in juvenile male and female rats. We hypothesized that social separation would decrease social novelty preference and that this would be restored by MOR agonist administration.

METHODS

Animals

Male and female Wistar rats were obtained from Charles River Laboratories (Raleigh, NC) at 22 or 23 days of age and were housed in standard rat cages (26.7 x 48.3 x 20.3 cm) under standard laboratory conditions (12-hour light/dark cycle, lights on at 7:00 am, food and water available ad libitum, 22° C, 60% humidity). In *Experiment 1*, male and female rats were housed in same-sex groups of 3-4 per cage until brain collection for receptor autoradiography at 35 days of age. In *Experiments 2, 4,* and *5,* experimental male rats (23 days of age) were housed in pairs and stimulus male rats (22 days of age) were housed in groups of 3-4 per cage. In *Experiment 3*, experimental male and female rats (23 days of age) were housed in same-sex pairs or in same-sex triads and stimulus rats (22 days of age were housed in same-sex groups of 3-4 per cage. All experiments were conducted in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

MOR autoradiography

Juvenile rats (35 days of age) were killed using CO_2 inhalation and brains were removed, rapidly frozen in methylbutane on dry ice, and stored at -45˚C. Brains were cut on a cryostat into 16-µm coronal sections containing the NAc and BLA and mounted onto slides in eight adjacent series. Sections were then stored at -45˚C until MOR autoradiography was performed. MOR autoradiography was conducted using [^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin (DAMGO; Perkin Elmer, Boston, MA) as tracer. In brief, slides were thawed and air-dried at room temperature followed by pre-incubation for 30 min in 50 nM Tris-HCl (pH 7.4) containing 0.9% NaCl. The slides were then exposed to tracer buffer (4 nM [^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin and 50 mM Tris) for 60 min. Non-specific binding was accessed in adjacent brain sections by incubation in tracer buffer with the addition of 1 µM naloxone (Sigma-Aldrich, St. Louis, MO). All slides were then washed three times, for 5 min each, in ice-cold Tris-HCl, air-dried, and exposed to Biomax MR films (VWR International, Pittsburgh, PA) for 16 weeks. Brain sections from animals of both sexes were processed together and balanced across incubation chambers and exposure to films. Autoradiography films were digitized using a Northern Light Illuminator (InterFocus Imaging, Cambridge, UK) and optical densities of MOR binding were measured in coronal sections using ImageJ (NIH, http://imagej.nih.gov/ij/). The data were converted to dpm/mg tissue (disintegrations per minute/milligram tissue) using a [^{3}H] standard microscale (American Radiolabeled Chemicals Inc., St. Louis, MO). Because non-specific binding was undetectable, film background values were subtracted from total binding values to yield specific binding values. Binding densities were calculated by taking the mean of bilateral measurements in 4 sections (8 measurements) per region of interest per rat. MOR binding density was

measured in the dorsomedial NAc (based on previous work suggesting that MORs in the dorsomedial, but not the ventral, NAc shell are involved in social motivation in prairie voles; Resendez et al., 2013; Fig. 6.2A) and in the BLA (Fig. 6.3A).

Social Novelty Preference Test

The social novelty preference test was used to assess the preference of a juvenile rat to investigate a novel or familiar (cage mate) sex- and age-matched conspecific (Smith et al., 2015). Experimental rats were one day older than novel stimulus rats to ensure that they were unrelated. One day prior to the beginning of behavioral testing, all experimental and stimulus animals were acclimated to the behavioral testing room for 1 hour and then to the behavioral testing apparatus for 10 min. On the day of testing, experimental and stimulus rats were moved to the testing room at least 1 hour prior to the onset of behavioral testing. All testing took place in the latter half of the light phase. Light intensity in the testing apparatus was ~240 lux. The apparatus was cleaned with a dilute soap solution prior to each test. Social novelty preference was tested in a Plexiglas apparatus consisting of a rectangular three-chambered box. Each chamber (40 x 27 x 40 cm) is separated by a Plexiglas partition with a rectangular opening to allow for passage between chambers. A novel stimulus rat is placed in one end chamber, while the subject's cage mate (familiar stimulus) is placed in the other. Both stimulus rats are confined to small containers (18 x 10 x 21 cm) composed of translucent Plexiglas bars to restrict their movement while still allowing for social investigation by the experimental rat. The experimental rat was placed in the middle chamber and was allowed to freely explore the three chambers and the social stimuli for 10 min. Behavior was video recorded and later scored using the behavioral analysis program JWatcher (http://www.jwatcher.ucla.edu)

by an experimenter blind to the sex of the rat and the experimental treatment. Time spent investigating each stimulus rat was measured. Investigation of the stimulus rats was defined as direct nose poking through the bars of the containers holding the stimulus rats. To obtain a measure of social novelty preference, the percentage of time investigating the novel stimulus rat (time investigating the novel stimulus rat/time investigating the novel + familiar rat x 100) was calculated. Experimental animals were considered to exhibit a preference for social novelty when the percentage of time spent investigating the novel conspecific was significantly different from chance (50%). A difference score between novel and cage mate investigation was also taken by subtracting time spent investigation the cage mate from time spent investigation the novel rat. The sum of novel and cage mate investigation times was calculated as a measure of total social investigation time.

Cannulation and Injection Procedures

At postnatal day (PND) 27 or 28, experimental rats were anesthetized with isoflurane (Henry Schein, Dublin, OH) and positioned into a stereotaxic frame with the incisor bar set at -4.5 mm. Throughout surgery, a heating pad was used to maintain body temperature. Guide cannulas (Plastics One, Roanoke, VA) were implanted bilaterally 2 mm dorsal to the target region using coordinates based on Paxinos & Watson Rat Brain Atlas (Paxinos & Watson, 2007) and adapted for use in juveniles. To target the NAc (*Experiments 2 & 5*), guide cannulae (22 gauge) were implanted bilaterally 1.6 mm rostral to bregma, +/- 2.4 mm lateral to the midline, and 4.3 mm ventral to the surface of the skull. Both cannulae were implanted at an angle of 10^{o} from the midline. To target the BLA (*Experiment 2*), guide cannulae (22 gauge) were implanted bilaterally 2.6 mm caudal to bregma, +/- 4.3 mm lateral to the midline, and 6.0 mm ventral to the surface of

the skull. To target the lateral ventricle for intracerebroventricular (ICV) injections, a guide cannula (21 gauge) was implanted unilaterally 1.0 mm caudal to bregma, +1.6 mm lateral to the midline, and 2.0 mm ventral to the surface of the skull. Guide cannulae were secured via stainless steel screws and dental acrylic adhesive and were closed with a dummy cannula (28 gauge for local injections and 26 gauge for ICV injections; Plastics One) that extended 1 mm beyond the guide cannula (local injections) or had no extension (ICV injections). Following surgery, rats were given an injection of Rimadyl analgesic (Henry Schein, Dublin OH) and singly housed for one hour before rehousing with their cage mate.

Experimental rats were handled daily for four days prior to testing to habituate them to the injection procedure. Injection systems were composed of polyethylene tubing connected to an injector cannula and a 10 µl (local injections) or a 25 µl (ICV injections) Hamilton syringe. The injector cannula (26 gauge for local and 28 gauge for ICV injections; Plastics One) extended 2 mm beyond the guide cannula, was kept in place for 30 s following injection to allow for tissue uptake, and was then replaced by a dummy cannula. At the end of the experiments, rats were euthanized with CO_2, and either charcoal (local injections) or blue ink (ICV) was injected through the guide cannula as a marker to check proper placement of the cannula histologically on Nissl stained coronal brain sections for local placement or visually for ICV placement.

Experimental Procedures

Experiment 1: MOR binding density in the NAc and BLA of juvenile male and female rats

MOR binding density was measured in the dorsomedial NAc shell and in the BLA of 35-day-old rats (males: n=13; females: n=13).

Experiment 2: Effects of MOR blockade in the NAc or BLA on social novelty preference in juvenile male rats

A within-subjects design was used to assess the effect of MOR blockade in either the NAc or the BLA on social novelty preference in juvenile male rats (separate cohorts; NAc, n=19; BLA, n=21). We limited our investigation to males because we found no sex difference in social novelty preference behavior (Smith et al., 2015) nor in MOR binding density in the NAc and BLA, (Exp. 1). Two to three days after cannulation, rats received bilateral injections of either vehicle (0.9% saline; 0.3 µl/side) or the MOR antagonist CTAP (10 µg/µl, 0.3 µl/side dissolved in 0.9% physiological saline; Sigma-Aldrich, St. Louis, MO, USA) into either the NAc or the BLA 20 min prior to exposure to the social novelty preference test. One to two days later, rats were again exposed to the social novelty preference test, this time with the opposite drug treatment and in counter-balanced order. The dose of CTAP used was consistent with a previous study demonstrating an effect of CTAP in the NAc on social play behavior in juvenile male rats (Trezza et al., 2011).

Experiment 3: Effects of social context on social novelty preference in juvenile male and female rats

To investigate the impact of social context on social novelty-seeking behavior, 23-day-old male and female rats were randomly assigned to one of four groups (Fig. 6.1). In the control condition, rats (males n=6, females n=8) were housed in same-sex pairs for 6 days prior to exposure to the social novelty preference test (Social group). A second group of rats (males n=11, females n=11) was pair-housed for 6 days and subsequently exposed to a single 3-h period of social isolation immediately prior to exposure to the

social novelty preference test (Isolated group). To test whether changes in social novelty preference were due to isolation or separation from the cage mate, a third group of rats (males n=8, females n=8) was housed in same-sex triads for 6 days followed by a 3-h social isolation of one cage mate, while the experimental rat remained co-housed with another cage mate, prior to exposure to the social novelty preference. The experimental rat was then exposed to the social novelty preference test containing a novel conspecific and the cage mate from which it had been separated (Separated group). Finally, in order to control for possible isolation effects on the stimulus cage mate, a fourth group of rats (males n=8, females n=8) was housed in same-sex triads for 6 days, followed by a 3-h social isolation of the experimental rat prior to exposure to the social novelty preference test (Isolation + Cage mate Social group). Thus, in this condition, the stimulus cage mate remained socially housed.

On the day of testing, all experimental and stimulus animals were moved to the behavioral testing room. Subsequently, experimental rats remained either socially housed (Social group), were socially isolated (Isolated group), were separated from one cage mate but remained socially housed (Separated group), or were socially isolated while their cage mate remained socially housed (Isolated + Cage mate Social group). In all manipulations, the experimental rat remained in its home cage, so as not to introduce a novel social environment as a potential confounding factor. Three hours later, all experimental rats were exposed to the social novelty preference test (See Fig. 6.1 for experimental design).

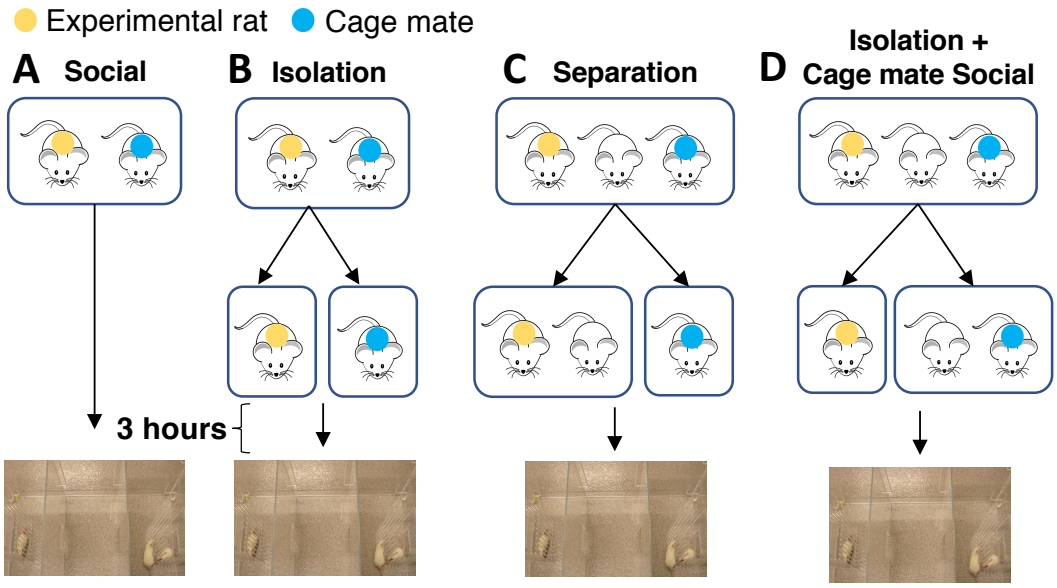

Figure 6.1. Illustration of experimental design of social context manipulations (Exp. 3) in male and female juvenile rats. In the social condition (**A**) rats were pair-housed and then placed directly into the social novelty preference test. In the isolation condition (**B**) rats were pair-housed and then subsequently isolated for 3 hours prior to exposure to the social novelty preference test. In the separation condition (**C**) rats were housed in triads. The stimulus cage mate was then isolated, while the experimental rat remained socially housed for 3 hours prior to exposure to the social novelty preference test containing the cage mate from which it had been separated. In the Isolation + Cage mate-Social condition (**D**) rats were housed in triads. The experimental rat was then isolated while the cage mates remained socially housed for 3 hours prior to exposure to the social novelty preference test.

Experiment 4: Effect of ICV MOR activation on social novelty preference following social isolation

Because the effects of social isolation on social novelty-seeking behavior were more robust in males, we limited our investigation to male juveniles. A within-subjects design was used to determine whether the reduction in social novelty preference seen following social isolation could be reversed by ICV MOR activation using the MOR

agonist DAMGO (n=14). Two to three days after cannulation, experimental and stimulus rats were moved to the behavioral testing room and all experimental rats were socially isolated for 3 h prior to exposure to the social novelty preference test. Twenty min prior to behavioral testing, experimental rats received an ICV injection of vehicle (3 µl 0.9 % saline) or the MOR agonist DAMGO (15 ng/3 µl; Sigma-Aldrich, St. Louis, MO). This dose was chosen based on pilot experiments in which a higher dose of DAMGO (150ng/3µl) caused nonspecific immobility behavior. One to two days later, rats were again exposed to the social novelty preference test, this time with the opposite drug treatment and in counter-balanced order.

Experiment 5: Effect of MOR activation in the NAc on social novelty preference following social isolation

We next determined where in the brain MOR activation restores social novelty preference after social isolation. We used a within-subjects design to investigate the effects of local MOR agonism in the NAc on social novelty preference in socially isolated juvenile male rats (n=16). Methods were identical to those described for Experiment 4, only in this case, 20 min prior to behavioral testing, experimental rats received a bilateral injection of vehicle (0.9% physiological saline; 0.3 µl/side) or the MOR agonist DAMGO (10 ng/0.3 µl/side; Sigma-Aldrich, St. Louis, MO) into the NAc. One to two days later, rats were again exposed to the social novelty preference test, this time with the opposite drug treatment and in counter-balanced order. The dose of DAMGO used was consistent with a previous study demonstrating an effect of DAMGO in the NAc on social play behavior in juvenile male rats (Trezza et al., 2011).

Statistical Analysis

For all statistical analyses, PASW/SPSS, version 22.0 (IBM Corp., Armonk, NY, USA) was used. Significance was set at $p \leq 0.05$. In Experiment 1, independent sample *t*-tests were used to compare MOR binding density between males and females in the dorsomedial NAc and BLA. In Experiments 2-5, single-sample *t*-tests compared to 50 % (chance level) were used to determine the presence of social novelty preference. In Experiment 2, paired sample *t*-tests were used to determine the effects of drug treatment on behavior in the social novelty preference test. In Experiment 3, two-way ANOVAs (sex x social context) were used to analyze the effects of sex and social context on behavior in the social novelty preference test. When a main effect of sex x social context or a main effect of social context was found, Bonferroni post-hoc tests were run. Because significant posthoc effects of social context were found for cage mate investigation, subsequent one-way ANOVAs (social context) were run separately for males and females. This was followed by Bonferroni post-hoc tests to determine effects of social context on cage mate investigation by sex. In Experiments 4 and 5, paired sample *t*-tests were used to analyze the effect of drug treatment on social novelty preference in socially isolated rats. In addition, a median split of the social novelty preference score was used to group the socially isolated rats into those "susceptible" to social isolation (Experiment 4: < 64.4 %, n= 7; Experiment 5: < 57.5 %, n=8) and those "non-susceptible" to social isolation (Experiment 4: > 64.4 %, n= 7; Experiment 5: > 57.5 %, n=8). This was followed by paired sample *t*-tests to analyze the effects of drug treatment on behavior in the social novelty preference test in "susceptible" and "non-susceptible" rats.

RESULTS

Experiment 1: MOR binding density in the NAc and BLA of juvenile male and female rats

We found no difference in MOR binding density between the sexes in either the dorsomedial NAc shell ($t_{(24)}$=1.41, p=0.17; Fig. 6.2B) or the BLA ($t_{(24)}$=-0.03, p=0.98; Fig. 6.3B).

Experiment 2: Effects of MOR blockade in the NAc or BLA on social novelty preference in juvenile male rats

MOR antagonist administration in the NAc significantly reduced social novelty-seeking behavior as reflected by a decrease in social novelty preference ($t_{(18)}$=3.54, p<0.01; Fig. 6.2C), a decrease in the difference score between novel and cage mate investigation time ($t_{(18)}$=2.95, p<0.01; Fig. 6.2D), a decrease in novel investigation time ($t_{(18)}$=2.17, p<0.05), and an increase in cage mate investigation time ($t_{(18)}$=-2.61, p<0.05). MOR blockade did not eliminate social novelty preference, because experimental rats spent significantly more time investigating the novel conspecific than would be expected by chance following both vehicle treatment ($t_{(18)}$=15.3, p<0.001) and MOR antagonist treatment ($t_{(18)}$=15.7; p<0.001; Fig. 6.2C). Finally, MOR antagonist administration in the NAc had no effect on total social investigation time ($t_{(18)}$=1.01; p=0.29; Fig. 6.2E).
In contrast to the NAc, MOR blockade in the BLA had no significant effect on any behavioral parameters in the social novelty preference test (social novelty preference: $t_{(20)}$=0.94, p=0.36; difference score between novel and cage mate investigation: $t_{(20)}$=1.20, p=0.24; novel investigation time: $t_{(20)}$=1.45, p=0.16; cage mate investigation time:

$t_{(20)}$=0.05, p=0.96: total social investigation time: $t_{(20)}$=1.46, p=0.16; Fig. 6.3C-E). Finally, experimental rats spent more time investigating the novel conspecific than would be expected by chance following both vehicle treatment ($t_{(20)}$=11.2; p<0.001) and MOR antagonist treatment ($t_{(20)}$=5.10, p<0.001; Fig. 6.3C).

Experiment 3: Effects of social context on social novelty preference in juvenile male and female rats

Social novelty preference was significantly affected by social context, with more robust effects in males than in females. In detail, significant sex x social context effects were observed for social novelty preference, the difference score between novel and cage mate investigation time, and novel investigation time (for complete statistics, see Tables 6.1 and 6.2). Posthoc testing revealed that social novelty preference (Fig. 6.4A) and novel-cage mate investigation (Fig. 6.4B) were significantly reduced in all treatment groups in males, but only in the isolated + cage mate social group in females. Interestingly, in contrast to novel investigation time (Fig. 6.5C), cage mate investigation time (Fig. 6.5D) was significantly higher in all treatment groups (isolated, separated, and isolated + cage mate social) and in both sexes compared to the control (social) group (for statistics, see Table 6.2).

Table 6.1. Effects of social context and sex on investigation behavior in the social novelty preference test. Results represent two-way ANOVAs (social context x sex) followed by Bonferroni post-hoc tests for those behaviors showing significant interaction effects. Significant (p<0.05) differences are bolded.

Two-way ANOVA	**Social context x Sex Effect**	**Social Context Effect**	**Sex Effect**
Social Novelty Preference	**$F_{(3,60)}$=3.04; p<0.05**	**$F_{(3,60)}$=14.6; p<0.001**	$F_{(1,60)}$ =0.80; p=0.37
Novel-Cage mate Investigation	**$F_{(3,60)}$ =3.17; p<0.05**	**$F_{(3,60)}$ =10.8; p<0.001**	$F_{(1,60)}$ =0.75; p=0.39
Novel Investigation	**$F_{(3,60)}$ =3.04; p<0.05**	**$F_{(3,60)}$ =4.66; p<0.005**	$F_{(1,60)}$ =0.05; p=0.82
Cage mate Investigation	$F_{(3,60)}$ =2.09; p=0.11	**$F_{(3,60)}$ =18.1; p<0.001**	$F_{(1,60)}$ =2.25; p=0.14
Total Investigation	$F_{(3,60)}$ =1.64; p=0.19	**$F_{(3,60)}$ =4.21; p<0.01**	$F_{(1,60)}$ =0.77; p=0.39

Post-hoc: Social context x Sex Effect

	Social vs. Isolated		**Social vs. Separated**		**Social vs. Isolated + Cage mate Social**	
Social context	***Males***	***Females***	***Males***	***Females***	***Males***	***Females***
Social Novelty Preference	**p<0.005**	p=0.11	**p<0.001**	p=0.06	**p<0.005**	**p<0.001**
Novel-Cage mate Investigation	**p<0.01**	p=0.34	**p<0.001**	p=0.18	**p<0.031**	**p<0.005**
Novel Investigation	p=0.34	p=0.99	**p<0.05**	p=0.99	p=0.60	**p<0.01**

Sex	**Social (M vs. F)**	**Isolated (M vs. F)**	**Separated (M vs. F)**	**Isolated + Cage-mate Social (M vs. F)**
Social Novelty Preference	p=0.50	p=0.17	**p<0.05**	p=0.23
Novel-Cage mate Investigation	p=0.60	p=0.18	**p<0.05**	p=0.16
Novel Investigation	p=0.71	p=0.20	p=0.08	**p<0.05**

Post-hoc: Social Context	**Social vs. Isolated**	**Social vs. Separated**	**Social vs. Isolated + Cage mate Social**
Cage mate Investigation	**p<0.001**	**p<0.001**	**p<0.001**
Total Investigation	p=0.15	p=0.10	p=0.99

M, male; F, female. Social novelty preference was calculated in percentage investigation time; all other parameters are in seconds.

Table 6.2. Effects of social context on cage mate investigation in the social novelty preference test tested separately for sex. Results represent one-way ANOVAs (social context) followed by Bonferroni post-hoc tests for those behaviors that show a main effect. Significant (p<0.05) differences are bolded.

One-Way ANOVA	**Males**	**Females**
Cage mate Investigation	**$F_{(3,29)}$=11.6; p<0.001**	**$F_{(3,31)}$=7.02; p=0.001**

Post-hoc: Social context	**Social vs. Isolated**		**Social vs. Separated**		**Social vs. Isolated + Cage mate Social**	
	Males	***Females***	***Males***	***Females***	***Males***	***Females***
Cage mate Investigation	**p<0.01**	**p<0.01**	**p<0.001**	**p<0.005**	**p<0.05**	**p<0.005**

Experiment 4: Effect of ICV MOR activation on social novelty preference following social isolation

Because of individual variability in the effects of social isolation on social novelty preference, a median split was used based on the social novelty preference score in order to group the socially isolated male rats into "susceptible" or "non-susceptible" to social isolation (Fig. 6.5B). Males that were "susceptible" to social isolation did not show social novelty preference under vehicle conditions, i.e., the time they spent investigating the novel conspecific was not different than would be expected by chance ($t_{(6)}$=1.51; p=0.18; Fig. 6.5F). Importantly, ICV MOR agonist administration restored a preference for social novelty, as juvenile males spent significantly more time investigating the novel stimulus rat than would be expected by chance following MOR agonism ($t_{(6)}$=3.11; p<0.05; Fig. 6.5F). As a result, social novelty preference was significantly higher following MOR agonism compared to vehicle in "susceptible" rats ($t_{(6)}$=2.53, p<0.05; Fig. 6.5F). Moreover, MOR agonism significantly increased the difference score between novel and cage mate investigation as compared to vehicle ($t_{(6)}$=2.40, p=0.05; Fig. 6.5G). There was no significant effect of ICV MOR agonism on novel investigation ($t_{(6)}$=0.12; p=0.91), cage mate investigation ($t_{(6)}$=1.33; p=0.23), or total social investigation ($t_{(6)}$=0.73; p=0.50) time (Fig. 6.5H).

"Non-susceptible" male rats showed normal social novelty preference after social isolation, i.e., they spent significantly more time investigating the novel conspecific than would be expected by chance after either ICV vehicle ($t_{(6)}$=11.8, p<0.001) or ICV MOR agonist ($t_{(6)}$=3.29, p<0.05) treatment (Fig. 6.5C). Likewise, ICV MOR agonism in "non-susceptible" males did not have an effect on other parameters in the social novelty

preference test (social novelty preference: $t_{(6)}$=0.70, p=0.51; difference score between novel and cage mate investigation: $t_{(6)}$=0.15; p=0.89; novel investigation time: $t_{(6)}$=0.39, p=0.71; cage mate investigation time: $t_{(6)}$=0.74, p=0.49; total social investigation time: $t_{(6)}$=0.93, p=0.39; Fig. 6.5C-E).

Experiment 5: Effect of MOR activation in the NAc on social novelty preference following social isolation

Using a median split based on the social novelty preference score, male rats were grouped into "susceptible" or "non-susceptible" to social isolation (Fig. 6.6B). Males that were "susceptible" to social isolation did not show social novelty preference under vehicle conditions, i.e., the time they spent investigating the novel conspecific was no different than would be expected by chance ($t_{(7)}$=1.55, p=0.16; Fig. 6.6F). Importantly, MOR agonism in the NAc restored their social novelty preference, i.e., "susceptible" rats spent significantly more time investigating the novel stimulus rat than would be expected by chance ($t_{(7)}$=4.22, p<0.01; Fig. 6.6F). MOR agonism also increased social novelty preference ($t_{(7)}$=2.26; p=0.058; Fig. 6.6F) and increased the difference score between novel and cage mate investigation ($t_{(7)}$=2.10; p=0.07; Fig. 6.6G), although this just missed the significance mark. Finally, MOR agonism significantly decreased time spent investigating the cage mate ($t_{(7)}$=4.91; p<0.01) while not significantly altering novel social investigation ($t_{(7)}$=0.32; p=0.76) or total social investigation ($t_{(7)}$=2.06; p=0.08) time (Fig 6.6H).

"Non-susceptible" male rats showed normal social novelty preference after social isolation, i.e., they spent significantly more time investigating the novel conspecific than would be expected by chance after both ICV vehicle ($t_{(7)}$=6.38; p<0.001) and MOR

agonism ($t_{(7)}$=2.37; p=0.05) treatment in the Nac (Fig. 6.6C). In line with this, MOR agonism in the NAc of "non-susceptible" males did not have an effect on other parameters in the social novelty preference test (social novelty preference: $t_{(7)}$=1.05, p=0.33; difference score between novel and cage mate investigation: $t_{(7)}$=1.54, p=0.17; novel investigation time: $t_{(7)}$=1.83, p=0.12; cage mate investigation time: $t_{(6)}$=0.15, p=0.89; total social investigation time: $t_{(7)}$=1.37, p=0.21; Fig. 6.6C-E).

DISCUSSION

We previously showed that MOR activation in the brain facilitates social novelty preference in juvenile male rats (Smith et al., 2015). Here, we demonstrate that this effect is located in the NAc, because pharmacological blockade of MORs in the NAc reduces social novelty preference. Interestingly, this effect was mediated not only by a decrease in time spent with a novel conspecific but also by an increase in time spent with a cage mate. This finding suggests a shift in the hedonic value of novel and familiar social stimuli upon NAc-MOR inactivation. A reduction in social novelty preference was also seen after a brief (3 h) separation from the cage mate, regardless of whether the subject was isolated or socially housed. This effect was primarily driven by an increase in time spent with that cage mate. Finally, we demonstrate that administration of a MOR agonist (either centrally or in the NAc) restored social novelty preference (by decreasing the time spent with the cage mate) in subjects that were "susceptible" to a brief separation from their cage mate. Taken together, these results suggest that the hedonic value of familiar

conspecifics may increase following social isolation or separation and that this is mediated by MOR activation in the NAc.

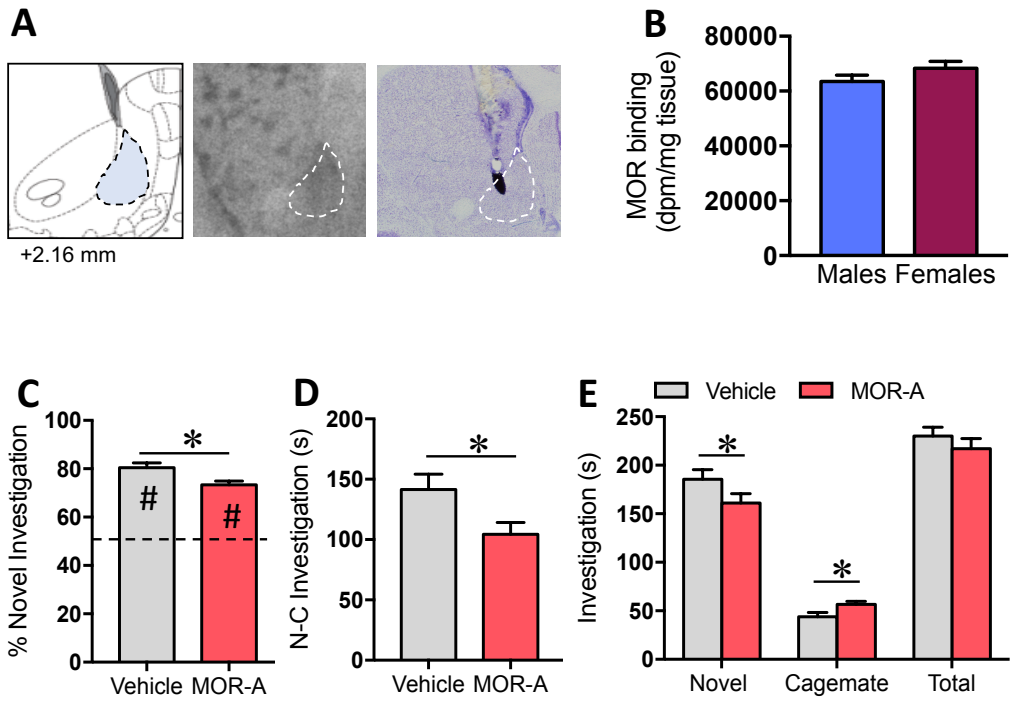

Figure 6.2. MOR blockade in the NAc reduces social novelty-seeking behavior. Schematic drawings of the rat brain, adapted from Paxinos and Watson (2007), illustrating the dorsomedial NAc shell along with corresponding autoradiograms depicting MOR binding density and Nissl-stained coronal sections indicating the location of microinjection using charcoal as a marker (**A**). Dashed outlines represent the region analysed for MOR binding density. No sex difference was observed in MOR binding in the dmNAc shell (**B**). MOR blockade in the NAc using the specific MOR antagonist CTAP (MOR-A) reduces social novelty preference (**C**), as well as the difference in time spent in novel and cage mate investigation (**D**) in juvenile male rats. MOR-A administration in the NAc also reduced time spent in novel investigation and increased time spent in cage mate investigation, but did not alter total investigation (**E**). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. A dashed line indicates the chance level (50%). Bars indicate the mean + SEM. *p < 0.05, paired-sample t-tests; # p<0.05 one-sample *t*-test against 50 %.

We show that MOR blockade in the NAc reduces social novelty preference by shifting social interaction towards a familiar as opposed to a novel conspecific. Interestingly, MORs in the NAc play a role in the regulation of the hedonic or pleasurable nature of rewarding interactions, rather than the motivation to engage in those behaviors, per se (Berridge & Kringelbach, 2015; Le Merrer et al., 2009). For example, MOR blockade in the dorsomedial NAc shell disrupts pair-bond formation in adult female prairie voles (Resendez et al., 2013). However, it does so without altering mating, a behavior on which the formation of the pair-bond depends. Therefore, it has been suggested that NAc-MOR blockade reduces the hedonic value associated with the mating experience (Resendez et al., 2013). Moreover, MOR blockade in the NAc reduces conditioned place preference for environments in which juvenile rats have had the opportunity to engage in social play with an unfamiliar peer (Trezza et al., 2011). This may further provide evidence suggesting that NAc-MOR blockade decreases the hedonic value of social play. It is therefore plausible that MOR blockade in the NAc reduces the hedonic value of social novelty, resulting in the observed decrease in time spent investigating a novel conspecific in juvenile rats.

Furthermore, we find that MOR blockade in the NAc increased interaction with a familiar conspecific. This could be the consequence of the decrease in time spent with the novel conspecific, resulting in similar total social interaction time between MOR antagonist and vehicle groups. However, it is also possible that MOR blockade in the NAc causes a state of discomfort, which then increases the hedonic value of familiar social cues (i.e., the cage mate). In support, in both rodents and primates, MOR blockade has been shown to increase the release of the stress hormone cortisol (Fabre-Nys, 1982;

Jezova et al., 1982; Eisenberg, 1984; Hayes & Stewart, 1985) as well as to increase social comfort-seeking behaviors, such as distress calls and grooming solicitation (Carden & Hofer, 1990; Schino & Troisi, 1992; Keverne et al., 1989). Therefore, it is possible that juvenile male rats spend more time interacting with a familiar conspecific following MOR blockade for the purposes of alleviating discomfort.

In contrast to the NAc, the same dose of MOR antagonist did not alter social novelty preference when administered into the BLA. It is possible that this lack of an effect is dose dependent. Yet, lower doses of CTAP (1-5 μg/side versus 10 μg/side in the current study) have been shown to alter several other behaviors in various brain regions, such as reinstatement of alcohol-seeking behavior in the NAc and ventral pallidum (Tang et al., 2005; Perry & McNally, 2013a,b), cocaine-induced locomotor activity in the NAc, caudate putamen, and ventral tegmental area (Soderman & Unterwald, 2008), and anxiety-related behavior in the central amygdala (Wilson & Junor, 2008). Furthermore, MOR antagonists applied to the BLA blocked 'incentive learning', i.e. the encoding of changes to the incentive value of sucrose, after food deprivation in adult rats (Wassum et al., 2009; Wassum et al., 2011). MOR blockade in the BLA also prevented pavlovian-to-instrumental transfer in the context of food reward (Lichtenberg & Wassum, 2016). These findings suggest that MOR activation in the BLA primarily supports encoding of the incentive value of reward-associated cues, as well as the recall of memory for those cues, rather than motivation to obtain reward, per se. In light of these findings it may not be surprising that blockade of MOR neurotransmission in the BLA does not alter spontaneous social novelty preference. However, it would be of interest to explore the

role of BLA-MORs in other aspects of socially rewarding behaviors, which is an understudied area of research.

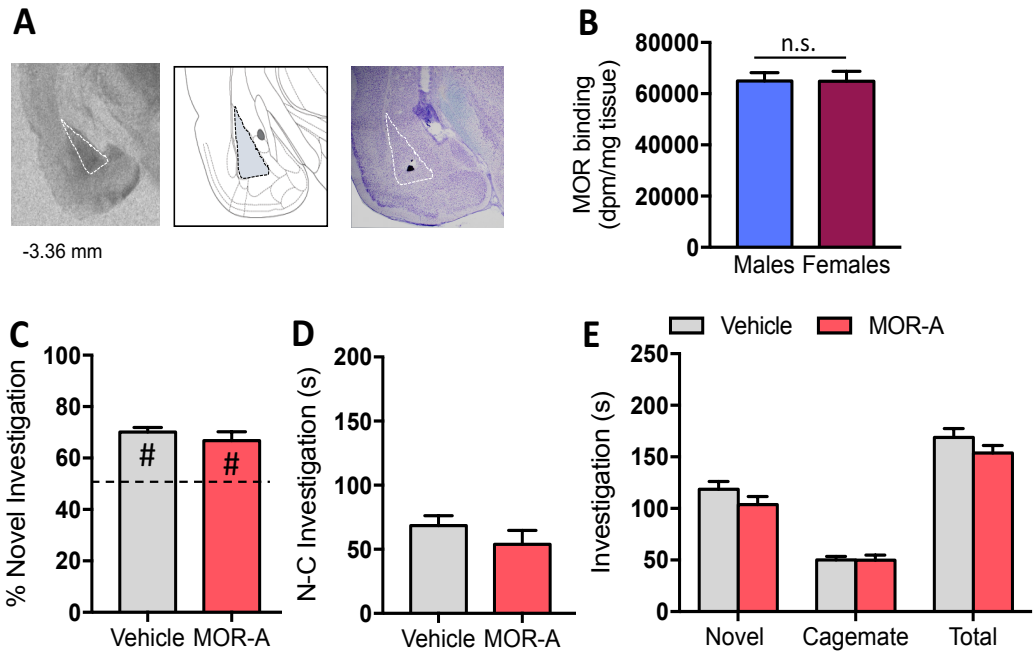

Figure 6.3. MOR blockade in the BLA does not alter social novelty-seeking behavior. Schematic drawings of the rat brain, adapted from Paxinos and Watson (2007), illustrating the BLA along with corresponding autoradiograms depicting MOR binding density and Nissl-stained coronal sections indicating the location of microinjection using charcoal as a marker (**A**). Dashed outlines represent the region analysed for MOR binding density. No sex difference was observed in MOR binding in the BLA (**B**). MOR blockade in the BLA using the specific MOR antagonist CTAP (MOR-A) had no effect on social novelty preference (**C**), or on the difference in time spent in novel and cage mate investigation (**D**) in juvenile male rats. MOR-A administration in the BLA also did not alter time spent in novel investigation, cage mate investigation, or total investigation (**E**). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. A dashed line indicates the chance level (50%). Bars indicate the mean + SEM. *p < 0.05, paired-sample t-tests; # p<0.05 one-sample *t*-test against 50 %.

We further show that a brief 3-h separation from a cage mate leads to an increase in interaction with that cage mate upon reunion, but does not increase interaction with a novel conspecific. This effect is seen in both male and female juvenile rats, but is particularly robust in males, leading to a significant decrease in social novelty preference. It is well known that social isolation, lasting anywhere from 8 hours to 8 days, during the

juvenile period stimulates social interaction and social play behaviors in male and female juvenile rats (Panksepp & Beatty, 1980; Varlinskaya et al., 1999; Varlinskaya & Spear, 2008). However, no previous studies have provided subjects with a choice of social partners. Therefore, our study is the first to assess the effect of social isolation on social preferences in a paradigm that provides a choice between a novel and a familiar social stimulus. The observed increase in cage mate interaction following social isolation may reflect a desire to seek social comfort, preferentially with a familiar conspecific. For example, in adult mice, the presence of siblings had a greater buffering effect on pain sensitivity than the presence of unrelated cage mates (D'Amato & Pavone 1993; 1996), suggesting that there may be similar differences with degree of familiarity in the capacity of social conspecifics to alleviate isolation-induced discomfort. Importantly, however, we find that the increase in interaction with the cage mate is observed even when individuals remain socially-housed during the period of separation from the cage mate. This demonstrates that seemingly subtle changes to the social housing condition can have a major impact on subsequent social preferences. Therefore, it is also possible that the social separation-induced increase in cage mate interaction reflects a type of reacquaintance or reunion behavior. For example, when juvenile male and female rats were reunited with a cage mate from whom they had been isolated for 24 hours, they played more and engaged more in huddling behaviors and allogrooming with those cage mates than did juveniles that were united with a novel individual following social isolation (Circulli et al., 1996; Terranova et al., 1999). Determining which of these motivational drives (comfort versus reacquaintance) underlies this increase in cage mate

interaction following social separation represents an interesting avenue for future research.

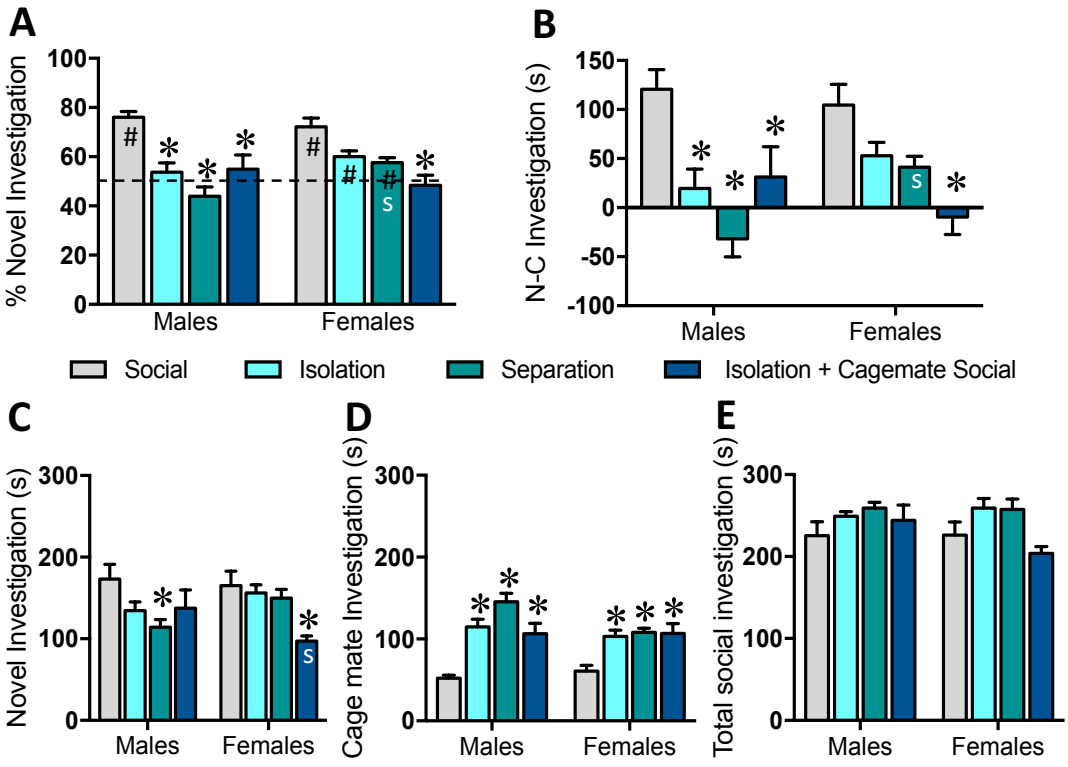

Figure 6.4. Changes to social context alter social novelty-seeking behavior in juvenile male and female rats. Isolation, separation, or isolation with cage mates socially-housed reduces social novelty preference (**A**) as well as the difference in time spent in novel and cage mate investigation (**B**) in juvenile male and female rats. However, these differences are not significant in the isolation and separation groups in females (**A**, **B**). Novel investigation is reduced by separation in males and isolation with cage mates socially-housed in females (**C**). All changes to social context significantly increase cage mate investigation in both males and females (**D**). Finally, no changes are observed in total social investigation (**E**). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. Dashed line indicates chance level (50 %). Bars indicate means + SEM. * $p<0.05$, two-way ANOVA (social context x sex) followed by Bonferroni post-hoc tests; # $p<0.05$ one-sample *t*-test against 50 %. s $p<0.05$ as compared to same group of the opposite sex.

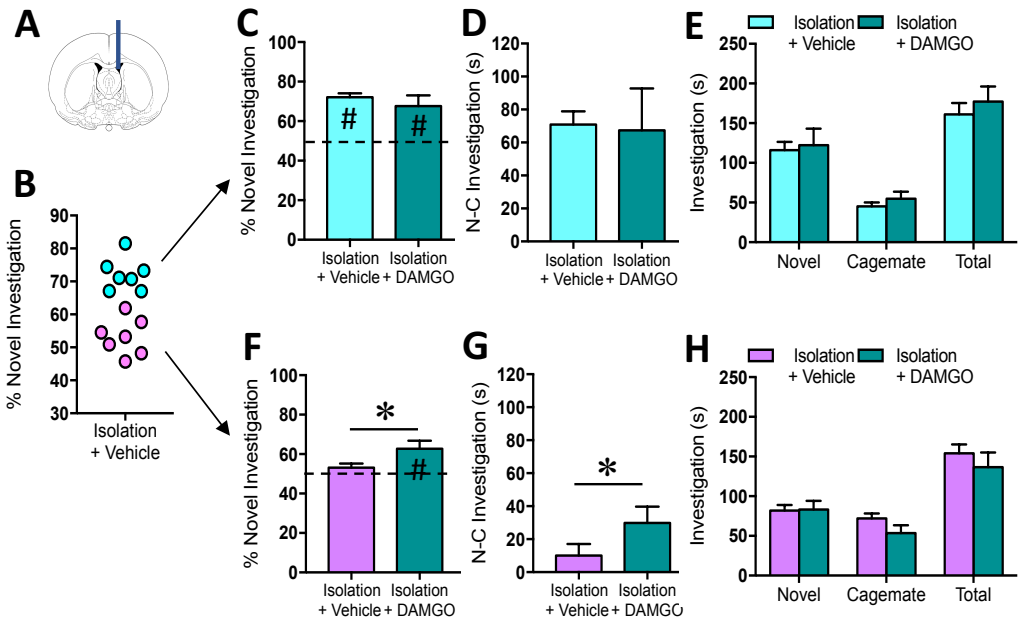

Figure 6.5. Central MOR agonism following social isolation restores social novelty preference in susceptible male juvenile rats. Vehicle or the specific MOR agonist DAMGO were administered ICV (**A**). Under vehicle conditions, not all experimental subjects are "susceptible" to social isolation, i.e. display a reduction in social novelty preference following social isolation (**B**). In experimental rats not susceptible to social isolation, MOR activation using DAMGO has no effect on social novelty preference (**C**), the difference between novel and cage mate investigation (**D**) or on novel, cage mate, or total social investigation (**E**). However, in experimental subjects susceptible to social isolation, I.C.V. DAMGO administration increases social novelty preference, restoring a preference for social novelty (**F**). No effect was observed on the difference in novel and cage mate investigation (**G**) or on novel, cage mate, or total social investigation (**H**). Subjects were divided into "not susceptible" and "susceptible" using a median split (64.4% social novelty preference). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. Dashed line indicates chance level (50 %). Bars indicate means + SEM. * $p<0.05$, paired-samples *t*-tests, # $p<0.05$ one-sample *t*-test against 50 %.

We hypothesized that MOR activation would reinstate social novelty preference following social isolation. Indeed, we found that administration of the MOR agonist DAMGO, either centrally or locally in the NAc, restored social novelty preference in juvenile male rats, but only in those that were "susceptible" to social isolation, i.e. rats that did not show a preference for social novelty after social isolation. Importantly, MOR agonism in the NAc restored social novelty preference by significantly decreasing the

time spent investigating the cage mate in rats "susceptible" to social isolation. This is opposite to NAc-MOR antagonism which enhanced cage mate exploration. Overall, this suggests that MORs in the NAc modulate social novelty preference by altering the hedonic value of the familiar, rather than novel, conspecific. It is therefore possible that the mechanism by which social separation enhances cage mate exploration involves suppression of NAc-MOR. This hypothesis is in line with previous theories that have been proposed to explain the role of MOR activation in the regulation of social comfort-seeking behavior following social isolation. In detail, the "state-dependent μ-opioid modulation of social motivation" (SOMSOM) theory (Loseth et al., 2014), building on the "brain opioid theory of social attachment" (BOTSA, Panksepp, 1978), proposes that, following social separation or social isolation, individuals are motivated to seek out social contact in order to induce an increase in neural MOR activation, thereby alleviating the distress associated with social separation. These theories are largely based on findings that peripheral MOR agonism reduces distress calls in infants of a number of species upon separation from their mothers, and these effects are blockade by administration of MOR antagonists (Panksepp et al., 1978; Panksepp et al., 1980; Carden & Hofer, 1990; Kalin et al., 1988). In further support, MOR agonists have been shown to reduce the secretion of the stress hormone cortisol in humans (Zis et al., 1984) and rhesus macaques (Broadbear et al., 2004) as well as in monogamous male titi-monkeys separated from their female partner (Ragen et al., 2013). It would be interesting to demonstrate in future research that changes to social context, such as social separation, reduce endogenous opioid release in the NAc and that, therefore, individuals seek out social interaction with a familiar conspecific to induce opioid release and alleviate distress.

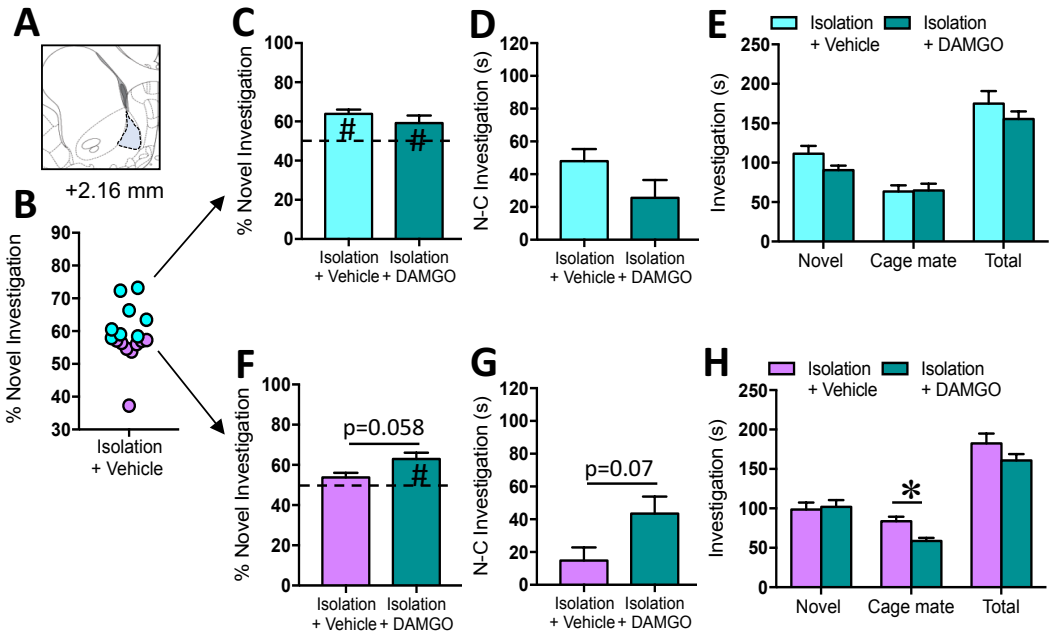

Figure 6.6. MOR agonism in the NAc following social isolation increases cage mate investigation following social isolation in susceptible male juvenile rats. Vehicle or the specific MOR agonist DAMGO were administered in the NAc (**A**). Under vehicle conditions, not all experimental subjects are "susceptible" to social isolation, i.e. display a reduction in social novelty preference following social isolation (**B**). In experimental rats not susceptible to social isolation, MOR activation locally in the NAc using the specific MOR agonist DAMGO, has no effect on social novelty preference (**C**), the difference between novel and cage mate investigation (**D**) or on novel, cage mate, or total social investigation (**E**). However, in experimental subjects susceptible to social isolation, DAMGO administration tends to increase social novelty preference, restoring a preference for social novelty (**F**). DAMGO administration also tended to increase the difference in novel and cage mate investigation (**G**) and significantly decreased cage mate investigation, but did not effect novel or total investigation (**H**). Subjects were divided into "not susceptible" and "susceptible" using a median split (57.5% social novelty preference). The percentage of novel investigation reflects the proportion of social investigation time spent investigating the novel stimulus. Dashed line indicates chance level (50 %). Bars indicate means + SEM. * $p<0.05$, paired-samples *t*-tests, # $p<0.05$ one-sample *t*-test against 50 %.

Finally, we found that some juvenile rats showed normal social novelty preference following social isolation and that MOR agonism did not alter their behavior. It is unclear why these rats were 'resilient' to social isolation, but the underlying mechanism could involve polymorphisms in the MOR gene. MOR gene variants have been associated with differences in social attachment and sensitivity to social isolation in

humans (Troisi et al., 2011), with differences in levels of maternal attachment in rhesus macaques (Barr et al., 2008), and with differences in the preference to interact with social versus object stimuli in mice (Briand et al., 2015). Therefore, it is plausible that individual differences in the MOR gene may be related to individual differences in social novelty preference and its disruption by social separation. This hypothesis remains to be tested.

Conclusion

Collectively, our results suggest that the MOR system, particularly within the NAc, mediates the motivation to engage in interaction with novel and familiar social stimuli, possibly by mediating the hedonic value of each. Social isolation (as well as social separation) were found to increase interaction with familiar conspecifics, an effect that was reduced by NAc-MOR activation in juvenile male rats "susceptible" to social isolation. Given the importance of the MOR system in the ventral striatum (which includes the NAc) to human social motivation (Hsu et al., 2013) and its involvement in impaired social motivation in individuals suffering from major depressive disorder (Hsu et al., 2015), future studies using this rat model may further our understanding of the MOR-mediated mechanisms underlying changes in social reward and motivation in neuropsychiatric disorders in humans.

Chapter Seven: General Discussion

The overarching aim of my dissertation work has been to better understand the distribution patterns of OTR, V1aR, and MOR in the juvenile brain, as well as the involvement of these receptors in the regulation of juvenile social novelty-seeking behavior. To this end, I first determined whether the expression of OTR, V1aR, and MOR in the rat brain depends on age and sex (Studies 1 and 2). I then aimed to explore the causal role of these receptors in the regulation of juvenile social novelty-seeking behavior using in-vivo pharmacological manipulations (Studies 3, 4, and 5). Finally, I examined the impact of social separation on social novelty-seeking behavior (Study 5).

Several major findings emerge from this body of research. First, my results demonstrate that there are numerous age differences between the juvenile period and adulthood in OTR, V1aR, and MOR binding density in the rat brain (Study 1 & 2). Second, I show that juvenile male and female rats robustly prefer to interact with a novel conspecific as compared to one which is familiar, i.e. they demonstrate a preference for social novelty (Study 3). Third, I find that while neither intracerebroventricular OTR nor V1aR blockade alters social novelty preference or social preference, intracerebroventricular MOR blockade reduces both (Study 3). Fourth, I show that blockade of either OTR or MOR signaling in the NAc reduces social novelty preference, suggesting a causal role for these receptors in the regulation of this behavior in juvenile animals (Studies 4 & 5). This effect appears to be brain region-specific as blockade of OTRs in the LS and BLA, and MORs in the BLA had no effect on social novelty-preference (Studies 4 & 5). Fifth, I demonstrate that an acute separation from familiar

conspecifics reduces social novelty preference (Study 5). Finally, I show that social novelty preference can subsequently be restored by MOR agonism, either centrally or in the NAc, in those juvenile male rats that showed no social novelty preference following separation (Study 5). Collectively, these findings suggest that both OXT and opioid systems regulate social novelty preference in brain region-specific ways during the juvenile period.

Overall, I find that, in the majority of brain regions analyzed, OTR and MOR binding density is higher in juveniles than adults. In contrast, V1aR binding density is lower in juveniles than adults. Furthermore, I find few brain regions that exhibit sex differences in binding density for either the OTR, V1aR or MOR. Therefore, below, I will discuss how these patterns of age and sex differences in receptor binding density compare and contrast across the three receptor types, as well as what the functional significance of these patterns might be. Next, I will discuss how my findings herein enhance our knowledge of the broader neural circuitry within which OXT and opioids act to facilitate juvenile social behavior, with the NAc as a key node in this network. Finally, I will discuss the ways in which these findings relate to and inform our understanding of the potential roles of OXT and opioids in the etiology and treatment of neurodevelopmental disorders such as autism spectrum disorders (ASD). I will argue that opioids may have promising therapeutic potential in the treatment of disorders such as ASD, and that further research is needed, particularly into the roles of both OXT and opioids in the regulation of social behavior during development.

Comparisons of age and sex differences in OTR, V1aR, and MOR binding density

Determining OTR, V1aR, and MOR binding densities within the brains of the same cohort of juvenile and adult male and female rats gave me the unique opportunity to compare age and sex differences in binding patterns across receptor types and to consider, as a whole, what these patterns might suggest about the neural regulation of juvenile social behavior. Interestingly, I observed that there were very few brain regions in which there was measurable binding of all three receptor types. For example, of the 35 regions analyzed for OTR binding and 33 regions analyzed for MOR binding, in only 11 regions were both OTR and MOR measurable. Similarly, OTR and V1aR binding (measured in 29 regions) overlapped in only 6 regions and MOR and V1aR in only 7 (Fig. 7.1). While OTR and V1aR are expressed in many sub-regions of the hypothalamus, MOR binding was not detectable in any nuclei within the hypothalamus. Conversely, MOR binding was densely expressed throughout sub-regions of the thalamus and midbrain, while OTR and V1aR binding were localized to only a few sub-regions in these areas. Furthermore, I observed contrasting age and sex differences in OTR and MOR binding densities. For instance, age differences are seen for both OTR and MOR binding densities in the posterior BNST, but OTR binding is denser in adults as compared to juveniles, while MOR binding is denser in juveniles as compared to adults. Moreover, in the same brain region, a sex difference is seen in OTR binding density (denser in males), but there is no sex difference in MOR binding density. This example, among others,

demonstrates the specificity of age and sex differences in receptor binding and highlights the complexity inherent to discerning the functional implications of these differences.

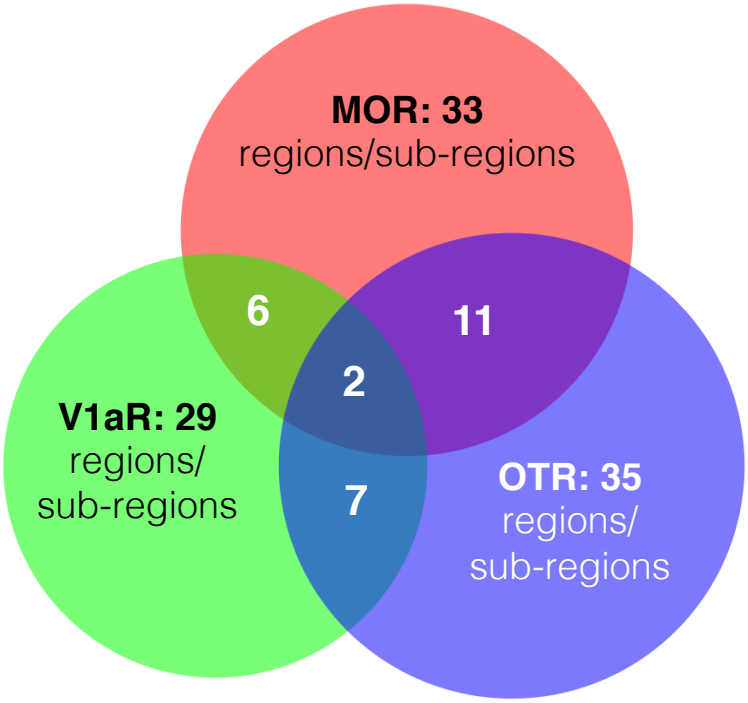

Figure 7.1. Overview of OTR, V1aR, and MOR binding analyzed in various regions or sub-regions of the rat brain shown as a Venn diagram to illustrate the overlap in receptor binding in any given region. Note that OTR, V1aR, and MOR binding overlap in only two regions; OTR and MOR binding overlap in 13 regions, OTR and V1aR overlap in 9 regions, and V1aR and MOR overlap in 8 regions. Thus, OTR, V1aR, and MOR are uniquely expressed in the majority of regions analyzed in the rat brain.

Despite the variation observed between receptor types, over-arching patterns of age and sex differences emerge in OTR, V1aR, and MOR binding. For instance, the majority of brain regions with age differences in OTR and MOR binding density show higher densities in juveniles as compared to adults (Fig. 7.2a). In contrast, the majority of brain regions with age differences in V1aR binding densities exhibit higher densities in adults as compared to juveniles (Fig. 7.2a). These age differences may be best understood in the context of age differences in brain development more broadly. During the juvenile

period, the striatal reward-seeking neural circuitry is more developed than cortical brain regions (Giedd et al., 1999; Huttenlocher et al., 1990; Sowell et al., 2003; Sturman & Moghaddam, 2011; 2012). In adulthood, these cortical brain regions exert a regulatory influence over striatal reward systems (Kravitz et al., 2015). Thus, the uninhibited, hyperactive reward circuitry in the juvenile brain has been suggested to underlie the increased propensity of juveniles to engage in risk-taking and novelty-seeking behaviors (Somerville & Casey, 2010; Doremus-Fitzwater, 2016). It is possible that the heightened binding density of OTR and MOR in the juvenile brain may contribute to this reward-seeking behavioral phenotype. In Studies 4 & 5, I show that both OTR and MOR activation in the NAc (a striatal brain region) facilitate juvenile social novelty-seeking behavior, likely by modulating the reward value of these social stimuli. Furthermore, I find that social novelty-seeking behavior is more robustly displayed by juveniles than adults (discussed further in the next section). These findings support the possibility that higher OTR and MOR activation in the juvenile brain enhance reward-seeking behavior during the juvenile period.

On the other hand, less is known regarding the importance of age differences in V1aR binding density. However, V1aR activation plays an important role in the regulation of adult behaviors such as pair-bond formation in prairie voles (Lim & Young, 2004; Lim et al., 2004) and opposite sex preference in rats (DiBenedictis et al., unpublished results), suggesting that higher V1aR in the adult brain may sub-serve these behaviors. Alternatively, it is possible that lower V1aR binding in the juvenile brain may be permissive of juvenile-specific risk-taking and novelty-seeking behaviors. A better

understanding of the role of age differences in the AVP system in the regulation of age-specific social behaviors should be the focus of future studies.

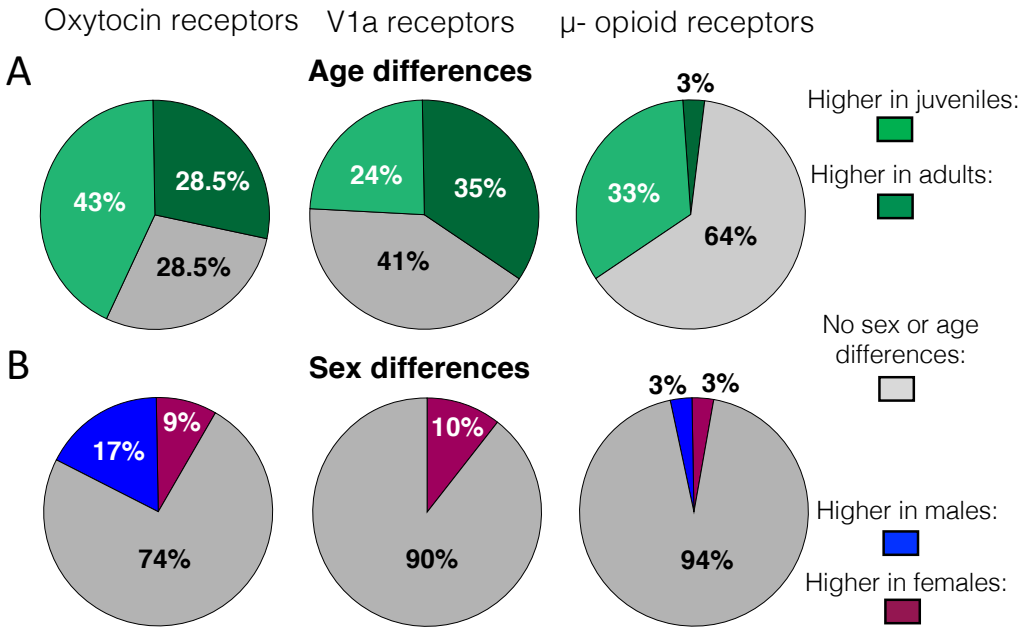

Figure 7.2. Pie charts representing the proportion of regions in which OTR, V1aR, and MOR binding were measured in the rat brain that differed by age (**A**) and sex (**B**).

In comparison with age differences in OTR, V1aR, and MOR binding, sex differences were far less prevalent. Specifically, OTR binding densities differed with age in almost 71.5% of the analyzed regions, but differed by sex in only 25% (Fig. 7.2b). Age and sex differences for the V1aR were present in 59% and 10% of analyzed regions, respectively and for the MOR in 36% and 6%, respectively (Fig. 7.b). Moreover, the effect sizes of most sex differences in OTR, V1aR, and MOR binding were smaller than the effect sizes of age differences. These findings lend powerful support to the suggestion that age may be a more robust determinant of binding density for these three receptors than sex. These findings also highlight the importance of age as a biological variable in

studies of the role of these systems in the regulation of social behavior. This is especially the case given the current investigation of OXT, AVP and opioid systems as therapeutic targets in the treatment of neurodevelopmental disorders that display an early age of onset (Guastella & Hickie, 2016; Scifo et al., 1996; Umbricht et al., 2016). Interestingly, in the majority of instances where sex differences were found, they were already present in juveniles, prior to the onset of puberty. This is also of importance given that many neurodevelopmental disorders exhibit a sex difference in prevalence (Fombonne et al., 2003) and it suggests that both males and females should be included in studies during development.

Comparison with age and sex differences in AVP and OXT fiber density

In order to understand the functional relevance of age and sex differences in OTR, V1aR and MOR receptor binding density, it is helpful to consider whether there are corresponding age and sex differences in the expression of their endogenous ligands. Therefore, in collaboration with Brett DiBenedictis, I have begun to compare patterns of age and sex differences in OTR and V1aR binding density with patterns of age and sex differences in OXT and AVP fiber immunoreactivity in the rat brain (Smith et al., 2016; DiBenedictis et al., 2016, unpublished results). We chose to focus on component brain regions of the social behavior neural network (SBNN). The SBNN was first proposed by Sara Newman (Newman, 1999) as a reciprocally interconnected set of brain regions involved in the regulation of diverse social behaviors e.g. aggression, sexual behavior, and parental behavior. The brain regions included in this network are the posterior aspect of the medial amygdala, posterior BNST, LS, medial preoptic area, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal grey. It has been

proposed that activation across these interconnected nodes of the SBNN leads to the expression of social behavior (Newman, 1999; O'Connell & Hofmann, 2012) and that differences in the relative activation across this network lead to the display of distinct forms of social behavior i.e. aggression vs. parental behavior (Newman, 1999; Goodson et al., 2005; Crews et al., 2006). Importantly, OXT and AVP have been shown to regulate social behaviors by acting in nodes of the SBNN in species ranging from teleost fish to mammals (For review see: Goodson & Bass, 2001; Godwin & Thompson, 2012; Albers, 2015).

Our comparison of age and sex differences in OTR and V1aR binding and OXT and AVP fiber density across the nodes of the SBNN has revealed two intriguing results. First, there is often a considerable lack of overlap between receptors and fibers within a system. For example, in the medial amygdala, posterior BNST, medial preoptic area, anterior hypothalamus, and ventromedial hypothalamus, AVP fibers are abundant, but there is no detectable V1aR binding (DiBenedicitis et al., 2016, unpublished results; Smith et al., 2016). Similarly, in the medial amygdala, medial preoptic area, and ventromedial hypothalamus, we find high OTR binding densities, but very sparse OXT fiber densities (DiBenedicitis et al., 2016, unpublished results; Smith et al., 2016). Secondly, we find that in the medial amygdala, posterior BNST and LS, age and sex differences in OTR but not V1aR binding density co-occur with age and sex differences in AVP but not OXT fiber density, especially in adulthood (DiBenedicitis et al., 2016, unpublished results; Smith et al., 2016; Fig. 7.3). These findings may suggest the possibility that, in these brain regions, signaling is occurring across OXT and AVP systems. In support, there is high sequence homology (80%) between the OTR and V1aR

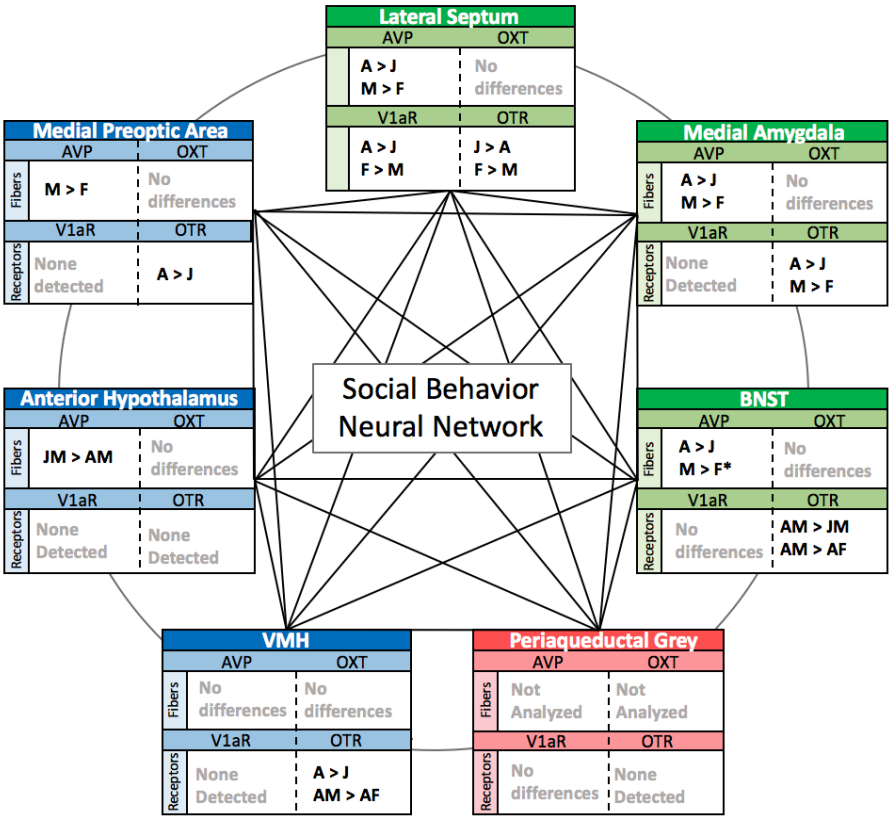

Figure 7.3. Summary of sex and age differences in AVP immunoreactive and OXT immunoreactive cells/fiber density (measured as cell number/pixels: DiBenedictis et al., 2016, unpublished results), as well as OTR and V1aR binding density (measured as disintegrations per minute/milligram tissue: Smith et al., 2016) throughout the regions of the social behavior neural network (Newman, 1999). M: males, F: females, J: juveniles, A: adults; <,> indicate significantly less than or more than, respectively.

(Koehbach et al., 2013) and, in rats and mice, AVP and OXT have similar affinities for the OTR and V1aR (Manning et al., 2008; 2012). Furthermore, an emerging body of evidence suggests that in some circumstances AVP and OXT may regulate behavior by acting at the receptors of the other system, i.e. AVP at OTR and OXT at V1aR (Song et al., 2016a,b; Schorscher-Petcu et al., 2010; Sala et al., 2011; Anacker et al., 2016), albeit not specifically within nodes of the SBNN. However, these behavioral studies have only been conducted in adult animals. Moreover, age differences in which receptor either OXT

or AVP interacts with during any given behavior have not been compared. Future studies should aim to determine whether or not age and sex differences in the behavioral function of neuropeptide systems involve age and sex differences in signaling via the OTR or the V1aR.

The social novelty preference test: A new paradigm in which to examine juvenile social behavior

In order to investigate the neural mechanisms underlying juvenile social novelty-seeking behavior, I developed the social novelty preference paradigm. The need to develop such a test was precipitated by the fact that despite our knowledge of juvenile novelty-seeking and exploratory behavior in the context of novel objects and environments (Bronstein & Spear, 1972; Philpot & Wecker, 2008; Douglas et al., 2003; Cyrenne et al., 2011), very little was known regarding novelty-seeking behavior within a social context. Thus far, social behavior tests such as the social discrimination test (Engelmann et al., 1995) and the Crawley's 3-chambered social preference test (Crawley, 2004; Moy et al., 2004; Nadler et al., 2004) have been utilized to determine differences between novel and familiar conspecific investigation. However, these tests differ from the social novelty preference test in crucial ways. First, in the social discrimination test, subject animals are exposed to freely-moving stimulus conspecifics, allowing for the possibility that the behavior of the stimulus animal could influence measures of social investigation. In contrast, in the social novelty preference test, stimulus animals are confined to smaller Plexiglas chambers which restrict their movement while still allowing

for social investigation. Secondly, in both the social discrimination test and Crawley's 3-chambered social preference test, the familiar conspecific is one to which the subject has previously been exposed for approximately 4-5 minutes. In the social novelty preference test, the familiar stimulus animal is the subjects' cage mate, with whom they have been co-housed for at least 5 days. Therefore, while these previously established tests are excellent for assessing social memory, this new test allowed me, for the first time, to assess the motivation of juvenile rats to interact with either a novel or a familiar individual.

Having demonstrated that social novelty preference is present at the juvenile age, the question remained as to whether social novelty preference was indeed higher in juveniles as compared to adults, as is the case for non-social novelty-seeking behavior (Bronstein & Spear, 1972; Spear, 2000; Philpot & Wecker, 2008; Douglas et al., 2003). To address this question, I exposed juvenile and adult male and female rats to the social novelty preference test and compared behavior between the ages (Fig. 7.4; according to the methods used for social novelty preference testing in Chapter 4). I hypothesized that social novelty-seeking behavior would be higher in juvenile as compared to adult rats of both sexes. My results suggest that this is indeed the case (for complete statistics see Table 7.1). Specifically, juvenile male and female rats spend more time interacting with a novel conspecific as compared to their adult counterparts (Fig. 7.4a). This also translates into a larger difference between novel and familiar investigation times in juveniles as compared to adults (Fig 7.4b). Notably, both juveniles and adults showed a preference for social novelty, as indicated by a significantly higher percentage of time spent interacting with the novel individual than would be predicted by chance (Fig. 7.4c). Finally, total

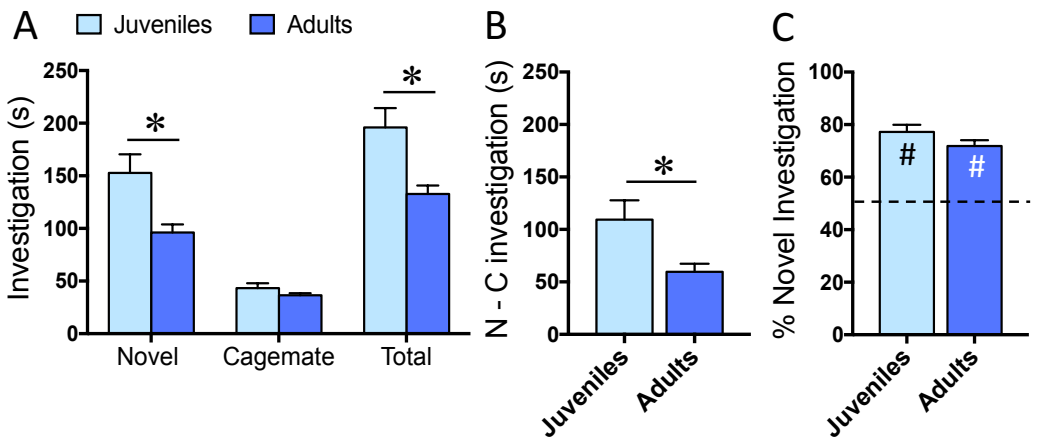

Figure 7.4. Age differences in social novelty-seeking behavior. Juvenile (5 week old) and adult (12 week old) rats of both sexes were exposed to the social novelty preference test, in which they have the choice to interact with a novel conspecific or with their cage mate (both age- and sex- matched. (**A)** Juveniles spent significantly more time investigating a novel conspecific than adults, resulting in more time spent in total social investigation**.** (**B**).The difference score between novel (N) and cage mate (C) investigation time was higher in juveniles as compared to adults. (**C**) Both juveniles and adults displayed a preference for social novelty, as indicated by a significantly higher percentage of novel investigation (which reflects the proportion of total social investigation time spent investigating the novel conspecific) than would be predicted by chance. No sex differences were observed and, thus, data was collapsed across sexes. Bars represent mean + SEM: results of three-way ANOVA (age x sex x social context) followed by Bonferroni posthoc tests. ∗ : $p<0.05$; # : significantly different from 50%.

social interaction time is higher in juveniles as compared to adults, while time spent in the central (empty) chamber of the social novelty preference test is higher in adults as compared to juveniles (Fig. 7.4a). These last findings suggest that the motivation to engage in social interaction is higher in juveniles as compared to adults. In support, juvenile rats have previously been shown to be more likely than adults to develop a conditioned place preference for environments in which they had previously engaged in social interaction (Douglas et al., 2004), suggesting that social interaction is more rewarding during the juvenile period. No main effects of sex were found on any

parameters of the social novelty preference test (Table 7.1). The presence of age differences in social novelty-seeking behavior suggests that there may be age differences in the neural mechanisms underlying this behavior. My research suggests that both OTR and MOR activation in the NAc facilitate social novelty preference in juveniles (Chapters 5 & 6). It would therefore be interesting to determine the involvement of these receptors in social novelty-seeking behavior in adult rats. Interestingly, I found that OTR binding density in the NAc is higher in juveniles as compared to adults, but that MOR binding density in this brain region did not differ with age. These findings may suggest that the age-specific regulation of social novelty-seeking behavior may be mediated by activation of the OTR. Future studies should aim to test this hypothesis.

Effects of social isolation and social separation on social novelty preference

Social isolation has long been known to have both acute and enduring effects on social behavior (Terranova, et al., 1999; Niesink & van Ree, 1982; Van den berg, et al., 1999; Hol et al., 1998; Gerall, et al., 1967; Gruendel & Arnold, 1969; Toth et al., 2011). Moreover, when juvenile rats were reunited with a cage mate from which they had been separated for 24 hours, they played more and engaged more in social interaction than if they were united with a novel individual (Terranova et al., 1999). The social novelty preference test allowed me to assess the impact of social environment on choice of social partner in juvenile male and female rats. I find that a brief period (3 h) social isolation or a brief 3-h separation from a familiar peer decreased social novelty preference (Chapter 6). This effect is driven by an increase in time spent interacting with the familiar cage mate from which subjects had been separated. The fact that social separation, even in the absence of social isolation, is enough to reduce social novelty preference suggests that

Table 7.1. Effects of age, sex, and social context on investigation behavior in the social novelty preference test. Results represent three-way ANOVAs (age x sex x context) followed by Bonferroni post-hoc tests for those behaviors showing significant interaction effects. There were no significant age x sex x context comparisons. Significant ($p<0.05$) differences are bolded.

Main Effects	**Age Effect**	**Sex Effect**	**Context Effect**
Social Novelty Preference	$F_{(1,54)}$=0.72; p=0.40	$F_{(1,54)}$=1.57; p=0.22	**$F_{(1,54)}$=30.6; p<0.001**
Novel-Cage mate Investigation	**$F_{(1,54)}$=8.98; p=0.004**	$F_{(1,54)}$=0.01; p=0.93	**$F_{(1,54)}$=28.8; p<0.001**
Novel Investigation	**$F_{(1,54)}$=48.3; p<0.001**	$F_{(1,54)}$=2.92; p=0.09	**$F_{(1,54)}$=17.7; p<0.001**
Cage mate Investigation	**$F_{(1,54)}$=31.5; p<0.001**	$F_{(1,54)}$=11.3; p=0.09	**$F_{(1,54)}$=26.1; p<0.001**
Total Investigation	**$F_{(1,54)}$=110; p<0.001**	**$F_{(1,54)}$=13.8; p<0.001**	$F_{(1,54)}$=2.27; p=0.14
Time in Center Chamber	**$F_{(1,54)}$=85.9; p<0.001**	$F_{(1,54)}$=3.70; p=0.06	$F_{(1,54)}$=2.20; p=0.14

Interaction Effects	**Age x Sex**	**Age x Context**	**Sex x Context**
Social Novelty Preference	$F_{(2,54)}$=1.34; p=0.25	$F_{(2,54)}$=0.62; p=0.43	$F_{(2,54)}$=1.14; p=0.29
Novel-Cage mate Investigation	$F_{(2,54)}$=0.63; p=0.43	$F_{(2,54)}$=1.47; p=0.23	$F_{(2,54)}$=0.93; p=0.34
Novel Investigation	$F_{(2,54)}$=0.54; p=0.47	$F_{(2,54)}$=0.02; p=0.89	$F_{(2,54)}$=1.80; p=0.19
Cage mate Investigation	$F_{(2,54)}$=0.30; p=0.59	**$F_{(2,54)}$=6.99; p=0.01**	$F_{(2,54)}$=0.03; p=0.88
Total Investigation	$F_{(2,54)}$=0.21; p=0.65	$F_{(2,54)}$=1.88; p=0.18	$F_{(2,54)}$=2.25; p=0.77
Time in Center Chamber	$F_{(2,54)}$=0.12; p=0.73	$F_{(2,54)}$=0.05; p=0.83	$F_{(2,54)}$=1.23; p=0.27

Post-hoc Comparisons:

Age: Juvenile vs. Adult	**Social Males**	**Isolated Males**	**Social Females**	**Isolated Females**
Novel-Cage mate Investigation	**p<0.05**	p=0.83	p=0.07	**p<0.05**
Novel Investigation	**p<0.001**	**p<0.01**	**p<0.001**	**p<0.001**
Cage mate Investigation	p=0.46	**p<0.001**	**p=0.03**	**p<0.005**
Total Investigation	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**
Time in Center Chamber	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**

Sex: Male vs. Female	**Social Juveniles**	**Isolated Juveniles**	**Social Adults**	**Isolated Adults**
Total Investigation	p=0.17	**p<0.01**	p=0.46	**p<0.012**

Context: Social vs. Isolated	**Juvenile Males**	**Juvenile Females**	**Adult Males**	**Adult Females**
Social Novelty Preference	**p<0.001**	p=0.07	**p<0.05**	**p<0.005**
Novel-Cage mate Investigation	**p<0.001**	**p<0.05**	p=0.06	**p<0.05**
Novel Investigation	**p<0.005**	p=0.26	**p<0.02**	p=0.08
Cage mate Investigation	**p<0.001**	**p<0.004**	p=0.86	**p<0.02**
Total Investigation	p=0.53	p=0.59	**p<0.02**	p=0.58

M, male; F, female. J, Juvenile; A, Adult. Social novelty preference was calculated in percentage investigation time; all other parameters are in seconds.

shifts in social motivation can be induced by a change to social context in juveniles. Given that social interactions are of heightened salience to juvenile as compared to adult animals (Doremus-Fitzwater et al., 2016, Spear et al., 2000), I hypothesized that social isolation would have less of an effect on social novelty preference in adults as compared to juveniles. To test this, adult and juvenile rats of both sexes were exposed to 3 hours of social isolation before exposure to the social novelty preference test (Fig. 7.5; methods according to those used to test the effects of social isolation on social novelty preference in Chapter 6). Contrary to my hypothesis, social isolation had a robust effect on social novelty preference in adult rats (for complete statistical details see Table 7.1). This was reflected by a significant decrease in novel interaction following social isolation, as well a decrease in the difference between time spent in novel and cage mate interaction (Fig. 7.5a,b). No sex differences in the effects of social isolation on social novelty preference were observed (Table 7.1). Of note, however, these results suggest that while social isolation decreases social novelty preference in both sexes and at both ages, the behavioral changes that underlie this decrease may be different. Specifically, while juvenile rats of both sexes and adult female rats spent more time interacting with a familiar cage mate following social isolation, adult male rats did not (Fig. 7.5b). Instead, adult males spent less time in total social interaction following social isolation as compared to all other groups (Fig. 7.5a). Taken together, these findings demonstrate that social isolation influences social preferences, across ages. In Chapter 6, I began to address the neural mechanisms underlying these effects by showing that MOR activation modulates social preferences following social isolation.

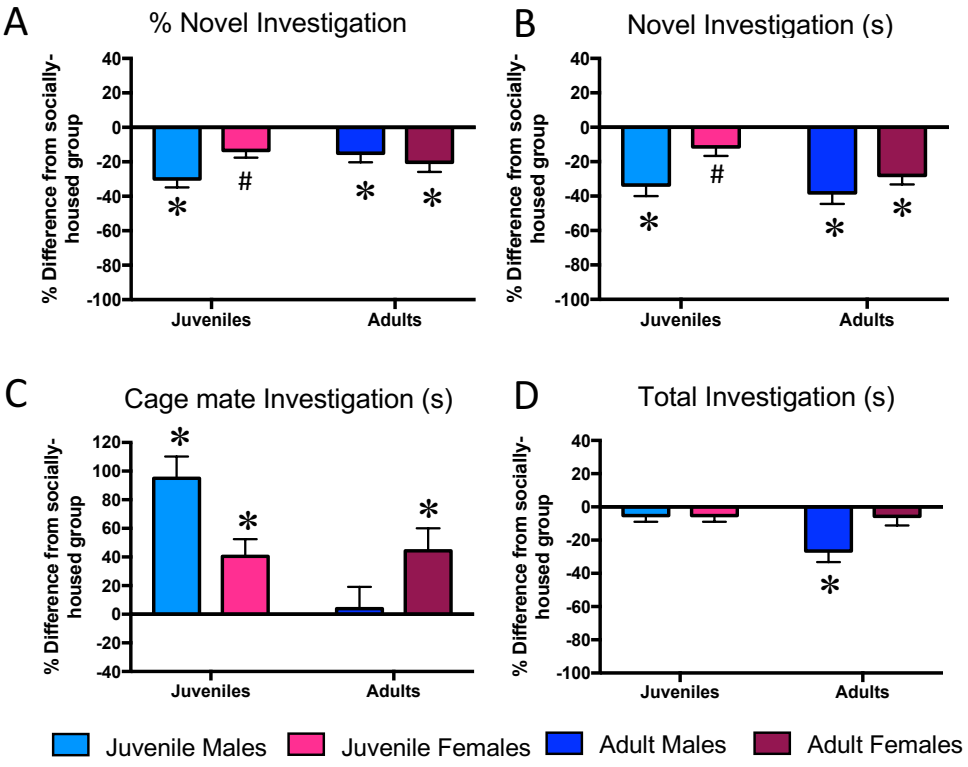

Figure 7.5. Effects of social isolation on social novelty-seeking behavior in juvenile and adult, male and female rats. Data represent percentage difference in behavior following social isolation as compared to socially housed controls. In both juveniles and adults, social novelty preference (**A**) and novel investigation (**B**) decrease following social isolation. In contrast, cage mate investigation increases following social isolation in all groups except adult males (**C**). Total social interaction decreases following social isolation only in adult males (**D**). Bars represent mean + SEM: results of three-way ANOVA (age x sex x social context) followed by Bonferroni posthoc tests. ∗ : $p<0.05$; # : $p=0.08$.

Novel Insights in the neural mechanisms underlying juvenile social novelty preference

My results demonstrate that either OTR or MOR blockade in the NAc impairs juvenile social novelty-seeking behavior. These effects appear to be brain region-specific,

as neither OTR blockade in the LS, nor OTR or MOR blockade in the BLA altered social novelty preference. These findings expand previous work showing that both OTRs and MORs in the NAc mediate social behavior. For example, during the juvenile period, OTR blockade or OTR downregulation in the NAc reduces spontaneous alloparental behavior in female prairie voles (Keebaugh et al., 2015) and conditioned place preference for a conspecific in male mice (Dolen et al., 2013). MOR blockade in the NAc reduces social play behavior as well as play-conditioned place preference in juvenile rats (Trezza et al., 2011). In adulthood, viral vector-induced over-expression of OTRs in the NAc facilitates pair-bond formation (Liu & Wang, 2003; Ross et al., 2009), while viral vector-induced OTR downregulation or OTR antagonist administration into the NAc impairs pair-bond formation (Liu & Wang, 2003; Keebaugh et al., 2015) in female prairie voles. Furthermore, MOR blockade in the NAc disrupts pair-bond formation in adult female prairie voles (Resendez et al., 2013). Together, these and my current findings demonstrate that the NAc is a critical node in the neural networks in which OTR and MOR modulate social behavior.

Importantly, the mechanisms by which the OTR and the MOR in the NAc facilitate social behavior may be different. Activation of the OTR may induce social learning by altering the signaling of other neurotransmitter systems in the nucleus accumbens. For example, in adult female prairie voles, administration of a dopamine D2 receptor agonist can induce the formation of a pair bond and this effect is blocked by co-administration of an OTR antagonist. Conversely, blockade of D2 receptors inhibits OTR-agonist induced partner preferences (Liu & Wang, 2003). This suggests that both OTR and D2 activation in the nucleus accumbens are required for the expression of

partner preferences. In another example, OXT system activation increases anandamide availability in the NAc, and this is attenuated by central OTR blockade. (Wei et al., 2015). Anandamide signaling then regulates socially-conditioned place preference in juvenile male mice (Wei et al., 2015), suggesting that OTR activation may enhance the regulation of social behavior by the endocannabinoid system. Finally, in juvenile mice, the formation of socially-conditioned place preferences has been shown to depend on the coordinated activity of OXT and serotonin in the NAc (Dolen et al., 2013). Specifically, OTR antagonist administration in the NAc blocked socially-conditioned place preference formation in male mice. Moreover, in the NAc, OTRs were found to be pre-synaptically located on the axon terminals of serotonin neurons projecting to the NAc from the dorsal raphe. Interestingly, OXT administration was found to induce long term depression (LTD) of medium spiny neuron synapses in the NAc and this effect could be blockaded by antagonism of serotonin 1B receptors (Dolen et al., 2013). Together, these findings support a model in which the OTR acts in concert with various neurotransmitter systems in the NAc, including the dopamine, endocannabinoid, and serotonin systems to support social reward, and induces social learning through long term changes in synaptic strength within the NAc.

In contrast to the role of NAc-OTRs, NAc-MORs have been more closely linked with setting the pleasurable or hedonic value of social interactions. Indeed, in Chapter 6, I find that MOR blockade in the NAc decreases time spent interacting with a novel conspecific, but also increases time spent interacting with a familiar cage mate. This may suggest that MOR activation in the NAc is important for assigning relative reward value between novel and familiar social stimuli. In support, opioid action in the NAc has been

associated with the regulation of the 'hedonic' or pleasurable nature of a wide array of rewarding stimuli, including food, drugs, and sex (Berridge & Kringelbach, 2015; Le Merrer et al., 2009). Within the social domain, NAc-MOR blockade disrupts pair bond formation without reducing mating behavior, on which the formation of the pair bond depends. Therefore, it has been suggested that NAc-MOR blockade reduces the hedonic value associated with the mating experience (Resendez et al., 2013). The fact that MOR blockade in the NAc disrupts play-conditioned place preference further supports the suggestion that MORs in the NAc code the hedonic value of these interactions (Trezza et al., 2011). Collectively, these findings suggest that the hedonic reward value of social stimuli is mediated by the NAc-MOR system. Notably, however, how the OXT and MOR systems might interact within the NAc to facilitate juvenile social behavior remains unknown and should be the focus of future investigations.

While the neural circuitry within which the NAc acts to regulate social novelty preference has yet to be fully elucidated, I found that temporary inactivation of the BLA alters social novelty-seeking behavior. Specifically, local bilateral administration of the $GABA_A$ receptor agonist muscimol (1µg/µl, 0.3 µl/side) 20 min. prior to exposure to the social novelty preference test reduced novel interaction time, while leaving cage mate interaction unaffected (see Fig.7.6 legend for statistical details, methods according to those used for local pharmacological manipulations in Chapters 5 & 6). This is in line with previous findings in juvenile mice showing that exposure to a social stimulus induces an increase in Fos expression (an indication of increased neuronal activation) in the BLA (Ferri et al., 2016). It is possible that the BLA participates in the regulation of social novelty preference via direct interactions with the NAc. In support, the BLA has

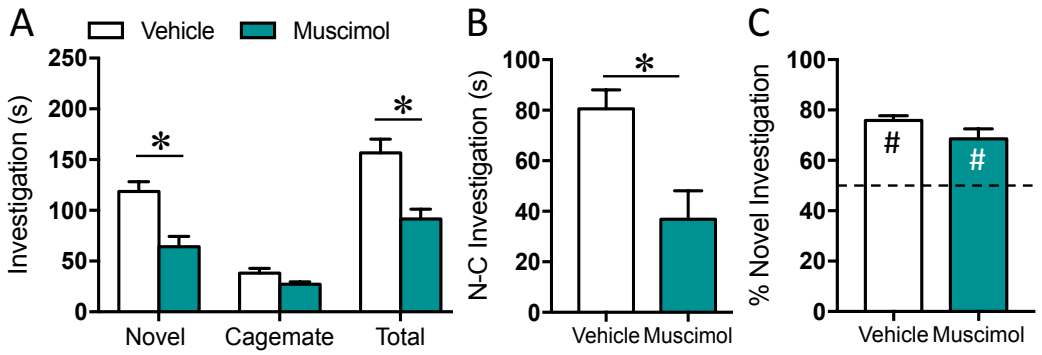

Figure 7.6. Effect of temporary inhibition of the BLA on juvenile social novelty preference**. (A)** Local, bilateral administration of the $GABA_A$ receptor agonist muscimol (1µg/µl, 0.3 µl/side) 20 min. prior to exposure to the social novelty preference test reduced novel interaction time ($t_{(1, 5)}$=4.64, p<0.01), as well as total interaction time ($t_{(1, 5)}$=4.3, p<0.01), while leaving cage mate interaction unaffected ($t_{(1, 5)}$=1.9, p=0.11). (B) Muscimol administration significantly decreased the difference between novel (N) and cage mate (C) investigation times ($t_{(1, 5)}$=4.8, p<0.01). (C) Muscimol administration did not alter the percentage novel investigation ($t_{(1, 5)}$=1.8, p=0.13). The percentage novel investigation reflects the proportion of total social investigation time spent investigating the novel conspecific. Bars represent mean + SEM: results of paired-samples t-tests. ∗ : p<0.05, #: significantly different from 50%.

been shown to send glutamatergic projections to the NAc (Kelley et al., 1982; Russchen & Price, 1984; Petrovich et al., 1996; McDonald, 1991; Britt et al., 2012). Furthermore, pharmacological inhibition of the BLA has been shown to decrease the firing of NAc neurons during a cued-instrumental task (Jones et al., 2010) and photo-inhibition of glutamatergic projections from the BLA to the NAc decreases cue-induced sucrose consumption and prevents cue-induced reinstatement of drug seeking behavior (Stuber et al., 2011; Stefanik & Kalivas, 2013), suggesting that this pathway may be involved in non-social reward seeking. Moreover, the BLA plays an important role in assigning meaning to sensory stimuli based on contextual factors and learned associations (Bordi & LeDoux, 1992; Holland & Gallagher, 1999; Maren & Fanselow, 1995; Janak & Tye, 2005; Beyeler et al., 2016). While this has largely been studied in the context of

associative learning, it is possible that the BLA is also involved in the process of assigning value to innately motivating stimuli – such as novel or familiar conspecifics.

There are also other brain regions that are interconnected with the NAc and may therefore be part of the NAc-mediated neural circuitry regulating social novelty preference. For example, the NAc receives excitatory input from the cerebral cortex and sends both direct and indirect (via the ventral pallidum) inhibitory descending projections to the motor systems of the brainstem and spinal cord (Swanson, 2000; 2005). The NAc is largely composed of medium spiny neurons (MSNs) and their participation in either the direct or indirect projection pathways can be distinguished based on expression of dopamine D1 (direct) or D2 (indirect) receptors (Gerfen & Young, 1988; Gerfen, 1992; Lobo & Nestler, 2011; although see Kupchik & Kalivas, 2017 for exceptions). As a part of this descending pathway from the cerebral hemispheres to the motor pattern generators of the brainstem, the nucleus accumbens participates in the generation of motivated behaviors (Swanson, 2000). Furthermore, inputs from several other brain regions give the nucleus accumbens access to information regarding context and motivational state, which can then influence its output. For instance, the nucleus accumbens receives input from the ventral tegmental area (VTA) (Beckstead, 1979; Swanson et al., 1982), hippocampus, dorsal raphe nucleus, and paraventricular nucleus of the hypothalamus (Kelley & Domesick, 1982; Dolen et al., 2013; Vertes & Hoover, 2008). It would be interesting to determine in future studies which of these connections with the NAc are involved in the regulation of social novelty-seeking behavior.

Oxytocin and opioids as potential therapeutic targets in the treatment of neurodevelopmental disorders

Here, I provide evidence supporting the involvement of both OXT and opioid systems in the regulation of juvenile social novelty-seeking behavior. Importantly, I find that OTR blockade in the NAc, but not ICV, impairs social novelty preference while MOR blockade, either ICV or in the NAc, impairs this behavior. Both OXT and opioids have been suggested to play a role in the etiology of ASD, and have been investigated as potential therapeutic targets in its treatment. The term ASD encompasses a range of neurodevelopmental disorders that are characterized by impairments in social interaction, language delays, and repetitive behavioral repertoires (American Psychiatric Association, 2013). The prevalence of ASD has almost doubled over the past two decades (Centers for Disease Control and Prevention, 2012). An ASD diagnosis is a significant emotional and psychosocial burden to the individual and their family. In addition, the cost burden to society of an ASD diagnosis, due largely to special education needs and loss of parental care, is approximately 1-2 million dollars per individual in the US (Buescher et al., 2014). Therefore, it is critical that we better understand and develop better treatments for ASD. Below, I will discuss the evidence for each system (OXT and opioid) in the treatment of ASD, and how it relates to my current findings.

Oxytocin

A role for OXT in the etiology and treatment of social deficits in ASD was first investigated because of the well-known importance of OXT to the regulation of social behavior across the animal kingdom (Lim et al., 2005; Carter et al., 2008). Of specific

relevance to ASD, OXT knock-out mice have been shown to display impairments in social interaction and social memory (Ferguson et al., 2001) and OTR knock-out mice exhibit impairments in social interaction and social memory, as well as repetitive behaviors and seizures (commonly co-morbid with ASD; Sala et al., 2011). In humans, genetic polymorphisms in the OTR gene have been associated with ASD risk in a number of cohorts (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2008; Cambell et al., 2011; Francis et al., 2016). Moreover, rare variants of the OTR gene are more common in individuals with ASD than in healthy controls (Liu et al., 2015). Additionally, male autistic children have been shown to have lower plasma OXT levels as compared to healthy controls and higher levels of the extended OXT precursor protein, suggesting the possibility of OXT processing impairments in individuals with ASD (Green et al., 2001). These findings suggest that changes in the OXT system may, at least in part, be involved in the etiology of ASD.

A large body of work also supports the possibility that acute intranasal OXT may improve deficits in social functioning in individuals with ASD. In healthy humans, intranasal OXT administration has been shown to enhance many measures of social cognition, including eye gaze, emotion recognition, trust, and social synchrony (see Guastella & LacLeod for review). Numerous studies of acute intranasal OXT administration in autistic individuals have now been conducted, with largely promising findings. For example, intranasal OXT has been shown to improve gaze to the eye region of the face in both adults and youth with autism (Andari et al., 2010; Guastella et al., 2010). This is particularly relevant given that diminished eye gaze is one of the earliest predictors of ASD (Spezio et al., 2007, Dawson et al., 2005; Dalton et al., 2005).

Intranasal OXT has also been shown to improve social reciprocity in children with certain types of autism (Andari et al., 2010) and to alter brain activity and functional connectivity in response to social cues and conditions (Gordon et al., 2013; Domes et al., 2013; Gordon et al., 2016; Guastella & Hickie, 2015).

Despite the promise of the above studies, questions still remain as to whether OXT will prove to be a clinically effective treatment for ASD. In comparison to acute administration studies, chronic OXT administration studies have been less conclusive (Guastella & Hickie, 2016; Anagnostou et al., 2012; Guastella et al., 2015; Dadds et al., 2014). For example, Guastella et al. (2015) administered 18-24 International Units (IU) of OXT intranasally to 8-12 year olds with ASD for a period of 8 weeks. They found no effect of OXT on social cognition (Guastella et al., 2015). In contrast, a second study from the same group found that a 5-week course of the same dose of OXT lead to significant improvements in social cognition in younger children (3-8 year olds). These studies suggest that length of administration, as well as exact developmental stage may modulate the effects of OXT. This is in line with studies in male prairie voles demonstrating that while acute neonatal OXT administration enhances partner preference formation in adulthood (Bales et al., 2003), chronic intranasal administration during the juvenile period leads to impairments in partner preference formation in adulthood (Bales et al., 2013).

These later studies clearly indicate that the impact of chronic OXT administration on social functioning in children with ASD depends on the developmental phase in during which children receive OXT administration. Given our findings herein of age differences in central OTR expression in the rat brain, it is possible that age differences in

susceptibility to intranasal OXT administration in children with ASD may be due to age differences in central OTR expression. However, whether age differences in central OTR expression exist in humans, or are altered in ASD, remains to be determined. Moreover, our findings of higher OTR binding in the NAc in juveniles as compared to adults and a role for NAc-OTRs in the regulation of social novelty preference in juvenile males point to the NAc as an importance locus of OXT action to be studied further in children with ASD.

Opioids

In contrast to OXT, far less research has been conducted on the potential role of opioids in the etiology and treatment of ASD, despite the fact that such a role has long been postulated. In the late 1970's Panksepp (1979) proposed "A neurochemical theory of autism", suggesting that ASD was related to dysregulation in the brain opioid system. This hypothesis arose from the observation that opiate treatment in animal models mimicked many of the behavioral symptoms seen in children with ASD (Panksepp et al., 1979). For example, opiate treated pups showed reduced crying upon separation from their mothers, reduced clinging behaviors, and less desire for social bonds. Furthermore, these animals also displayed motor stereotypies and increased risk of seizures (Panksepp et al., 1979; Sahley & Panksepp, 1987).

Based on these findings, Panksepp and colleagues suggested that symptoms of ASD might be caused by a hyperactivity of the opioid system due to elevated levels of opioid peptides, such as endorphins (Sahley & Panksepp, 1987). Several studies have since tested this hypothesis, with mixed results. The first, in 1984, found that autistic children had lower blood levels of humoral endorphin as compared to healthy controls

(Weizman et al., 1984). However, since then, both central (as measured in cerebrospinal fluid; Gillberg et al., 1985) and plasma (Sandman et al., 1990; Tordjman et al., 2009) endorphin levels have been shown to be higher in children with ASD. Several studies have also investigated the effects of opioid antagonists such as naloxone and naltrexone, on the symptoms of ASD. The earliest studies found that peripheral opioid blockade reduced self-injurious behaviors in autistic patients (Davidson et al., 1983; Richardson & Zaleski, 1983; Sandman et al., 1983; 1987, Bernstein et al., 1987, Herman et al., 1987; Campbell et al., 1988; Leboyer et al., 1998). Moreover, peripheral naltrexone in autistic girls was found to increase smiling, social interaction and social play (Scifo et al., 1996).

More recently, a number of studies have reported that MOR knock-out mice exhibit behavioral characteristics that are very similar to symptoms seen in children with ASD. In detail, MOR knock out mouse pups vocalize less in response to separation from their mother, and show no preference for mother-associated odors. Importantly, they still vocalize in response to a fear-inducing cue (male odor), suggesting that this reduction in vocalization is specific to the maternal separation (Moles et al., 2004). Similarly, both juvenile and adult MOR knock out mice show deficits in social behavior, such as a reduced preference for social over non-social stimuli and a lack of socially conditioned place preference (Cinque et al., 2012; Becker et al., 2014). These effects were also observed in juvenile wild-type mice treated neonatally with peripheral naloxone (Cinque et al., 2012). My findings here add substantially to this body of evidence. First, I find that the expression of MORs is higher in the juvenile as compared to the adult brain in many reward related brain regions (Study 2). Second, I find that central MOR activatioin facilitates the preference of juvenile rats to interact with a novel conspecific as compared

to either an object or a familiar conspecific (Study 3). Finally, I find that modulation of social novelty preference by the MOR occurs in the NAc and extends to the regulation of changes to social motivation following social isolation (Study 5). Together, these findings strongly support the notion that the MOR system plays an important role in the regulation of juvenile social behavior and may, therefore, be part of the etiology of social deficits in ASD. Furthermore, this suggests that the MOR system could be considered as a promising therapeutic target for the treatment of ASD symptoms that deserves more attention in future research.

Future directions & Conclusions

This body of dissertation work substantially adds to our understanding of OTR, V1aR, and MOR expression in the juvenile brain, as well as to our knowledge regarding the functional roles of these receptors in the regulation of juvenile social behavior. However, as much as these findings provide answers to the questions at hand, they also open up new avenues for future research. For instance, these studies reveal dozens of novel age differences in OTR, V1aR, and MOR binding density throughout the rat brain. Here, I only begin to explore the functional relevance of these myriad age differences in receptor binding density to age differences in behavior. Future studies should aim to provide a deeper understanding of the importance of these diverse patterns to the regulation of juvenile behavior. Furthermore, I demonstrate that both OTR and MOR activation in the NAc facilitate juvenile social novelty preference. However, how activation of these two receptors interacts within the NAc to regulate this behavior

remains unknown, as does the larger neural circuitry within which the NAc functions to produce social novelty preference. Future studies addressing these questions will provide us with a more comprehensive picture of the complex neural systems that produce the juvenile propensity to engage in social behavior. Finally, I find that social isolation or social separation can robustly change social preferences, possibly by altering the hedonic value of novel and familiar social stimuli. While we demonstrate that activation of the MOR in the NAc is involved in these changes in social preference following social isolation, much more work is needed to fully understand the neural changes that occur upon social isolation and how this leads to changes in behavior. For example, it would be highly interesting to use microdialysis to measure local opioid release within the NAc and determine whether this is altered by changes to social context. In its entirety, this body of work significantly advances our understanding of the neural systems underlying juvenile social novelty preference, and suggests that both oxytocin and opioid systems in the brain may be potential clinical targets for restoring social novelty-seeking behavior in neurodevelopmental disorders, such as autism.

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