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Neural Mechanisms of Variation in Pair Bond Formation in Prairie Voles

By

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Doctor of Philosophy

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ABSTRACT

The socially monogamous prairie vole (*Microtus ochrogaster*) forms long-term opposite-sex pair bonds, and provides a unique model to study the neural control of complex sociality. Much of the neural circuitry underlying these social bonds have been elucidated using comparative studies with non-monogamous, asocial *Microtus* species, and has been linked to the neuropeptides oxytocin and vasopressin, as well as dopaminergic reward systems. In addition to drastic species differences, a remarkable degree of individual variation in neuropeptide receptor expression and social behavior is observed within the prairie vole species. Here, we explored proximate mechanisms controlling individual variation in vole sociality. Using RNA interference to knockdown vasopressin V1a receptor expression, we overcome some past limitations of viral vector over-expression or pharmacological manipulations to show that subtle naturalistic-like variations in receptor expression drives within-species variation in pair bonding. Next, we show that although an individual's neuropeptide receptor expression is remarkably resilient to early adversity, variation in neuropeptide receptor expression may mediate behavioral responses to early life events. Early touch elicited immediate early gene activity in oxytocin neurons. Thus sensitivity to early social interactions may determine behavioral outcomes in adulthood. Finally, we show that neonatal treatment with melanocortin agonists, which promote adult attachment, prime oxytocin neurons, and stimulate dopamine release, lead to persistent changes in later play and bonding. Elucidation of the factors and mechanisms mediating normative social behavioral development can ultimately help to reveal potential targets for interventions for social deficits common among human psychiatric disorders.

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CHAPTER 1

Molecular neurobiology of social bonding and social cognition

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Abstract.

Many psychiatric illnesses, including autism spectrum disorders (ASD), schizophrenia, and depression, are characterized by impaired social cognition and a compromised ability to form social relationships. Although drugs are currently available to treat other symptoms of these disorders, none specifically target the social deficits. In order to develop pharmacotherapies to enhance social functioning, particularly for ASD where social impairment is a core symptom, we must first understand the basic neurobiology underlying complex social behaviors. The socially monogamous prairie vole (*Microtus ochrogaster*) has been a remarkably useful animal model for exploring the neural systems regulating complex social behaviors, including social bonding. Prairie voles form enduring social bonds between mated partners, or pair bonds, and display a biparental familial structure that is arguably very similar to that of humans. Here we discuss the neural systems underlying social bonding in prairie voles, including the neuropeptides oxytocin and vasopressin, opioids, dopaminergic reward and reinforcement, and stress-related circuitry, as well as the susceptibility of social functioning to early life experiences. We also highlight some of the remarkable parallels that have been discovered in humans.

Introduction

Monogamy in vertebrates

Monogamy is a rarely observed form of social organization among mammals, and arguably among the most complex behaviors exhibited in the animal kingdom (Kleiman, 1977). Although uncommon, phylogenetic analysis suggests that monogamy has repeatedly evolved across a variety of vertebrate taxa (Adkins-Regan, 2009), including, but not limited to, birds, canids, primates, and rodents. Monogamous mating systems are observed in less than 3% of mammals and, astonishingly, up to 90% of avian species (Kleiman, 1977; Mock and Fujioka, 1990). Understanding the characteristics unique to monogamous animals and the factors that lead to the evolution of these traits can reveal neurobiological and genetic variables that are susceptible to evolutionary pressures (Blumstein et al., 2010). These evolutionarily flexible pathways change in response to environmental demands, and are likely to reveal targets amenable to treatment for disorders characterized by deficits of the social brain. Furthermore, in order to choose an animal model suitable for studying complex sociality that may parallel the human condition, it is beneficial to consider the varied presentations and characteristics of this social system.

Accounts of monogamy across a wide variety of vertebrate taxa reveal varied, yet sometimes overlapping, factors in driving this mating system. Dispersion of females, high male to female sex ratios, necessity of paternal care, benefits of territorial defense, and a lack of the option for polygyny may lead to enhanced reproductive success when a male and female take on a monogamous social structure (Kleiman, 1977; Wittenberger and Tilson, 1980; Mock and Fujioka, 1990; Dunbar, 1995). The characteristics of monogamy

vary across the animal kingdom, but three dimensions of a monogamous mating system are widely shared: (1) an essentially exclusive mating relationship, (2) mutual parental care, and (3) preferential association with a specific opposite-sex partner (Wittenberger and Tilson, 1980; Dewsbury, 1987). Not all forms of monogamy necessarily meet each of these three features. Occasionally, males and females may take part in covert matings outside of the pair bond, but remain "socially monogamous" to their partner. Similarly, monogamous systems do not always include biparental care or frequent social or sexual interactions. As Dewsbury (1987) illustrated in his review of monogamy, the diverse presentation of monogamous pair bonds can be observed with examples from the rodent lineage (Dewsbury, 1987). Mongolian gerbil females seek out males, develop a preference for him and his home range, and defend the shared territory from curious females (Agren, 1984). However, upon estrus she leaves the territory in search of other mating partners, and the relationship is not restored until she comes out of estrus. Conversely, elephant shrews appear to mate exclusively and defend a common range (Kleiman, 1977; Rathbun, 1979), but "partners" rarely affiliate, do not show biparental care, and nest separately. Given the varied forms of monogamy across the animal kingdom, a model for the human condition must be wisely chosen and considered in terms of their naturalistic behaviors. Here, we argue that the prairie vole (Figure 1.1a, *Microtus ochrogaster*) provides a unique and valuable opportunity to investigate common underlying neurobiological systems regulating complex social behaviors. However, human and vole pair bonding likely evolved independently, and thus voles can not recapitulate the full complexity, including higher order cortical control, of human sociality.

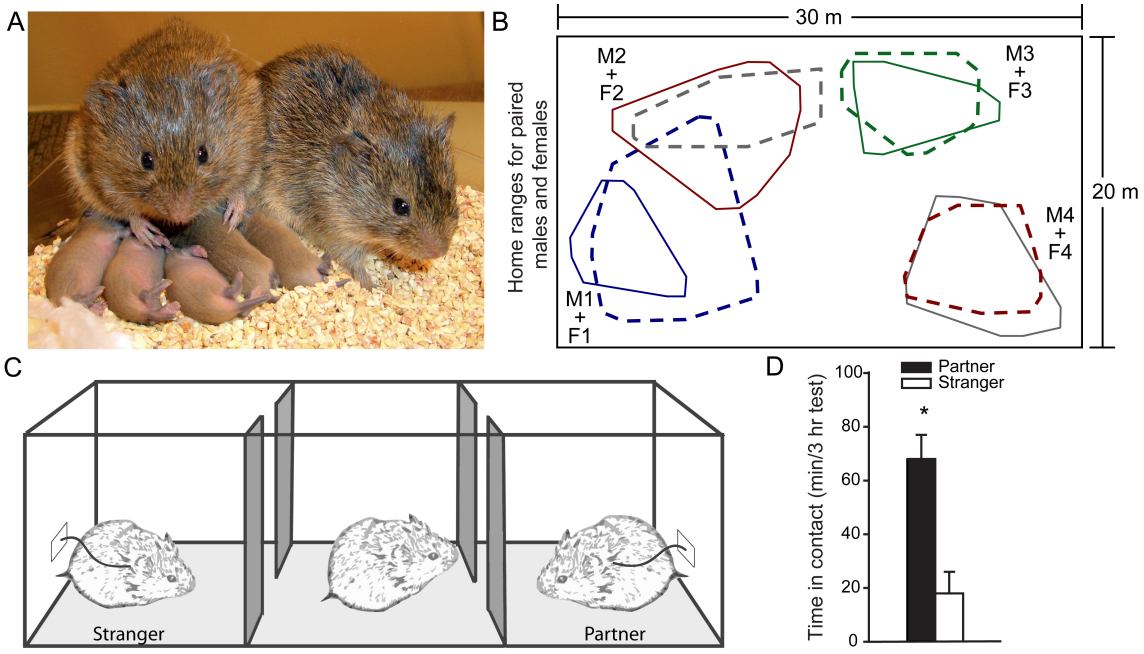

Figure 1.1. Prairie vole model.

(a) Typical prairie vole family units consist of a male-female biparental pair and their offspring. Prairie voles exhibit the 3 main characteristics of monogamy (essentially exclusive mating, biparental care, preferential association with partner). Photograph by Todd Ahern. (b) In the field, pair bonding can be measured with radiotelemetry and measuring the degree of home range overlap between partners (image from Ophir et al., 2008b). (c) Partner preference arena. Subject animal freely roams 3-chambered arena and time huddling with a opposite-sex "partner" or novel stimulus "stranger" is recorded over the 3-hr test. (d) In the partner preference test, prairie voles spend significantly more time with their familiar "partner" over a novel "stranger."

Prairie voles as an animal model of complex sociality.

Prairie voles display a rich social behavioral repertoire and meet all three of the criteria characteristic of monogamy and maintain these behaviors in a laboratory setting, making this species well suited as an animal model to understand the biology of complex social behavior. In contrast to the vast majority of mammalian species, including the more commonly researched laboratory mice and rats, prairie voles are among the 3-5% of mammals that form socially monogamous relationships (Kleiman, 1977; Getz et al.,

1981a). These pair bonds typically last for an entire lifetime and are characterized by biparental care, with the father contributing almost equally to caring for young (Carter et al., 1995). Voles display a preference for familiar partners over unfamiliar females and for novel unmated over novel mated animals of the opposite sex, behaviors that encourage both the formation and maintenance of a monogamous mating strategy (Ferguson et al., 1986; Shapiro et al., 1986; Dewsbury, 1987). Upon formation of the bond, partners defend a mutual territory, and males display mating-induced aggression towards both male and female intruders (Winslow et al., 1993; Gobrogge et al., 2009). Because pair bonding requires the integration of complex social abilities - including identification of socially salient cues, the long-term recognition of a familiar conspecific, and the motivation to preferentially associate with a partner (Lim et al., 2005a) - the ability to form a pair bond may serve as a general readout of social cognitive performance.

Prairie voles are small hamster-sized rodents with a wide-ranging distribution in the central United States. In the summer breeding months, pairs nest and burrow together, as was discovered from early field studies that repeatedly jointly captured male-female pairs (Getz et al., 1981a) and from radio-collared subjects (Figure 1.1b, (Hofmann et al., 1984; Getz and Hofmann, 1986; Ophir et al., 2008b). Communal groups consisting of a male-female pair and their juvenile offspring form in the late autumn-winter months (Carter et al., 1995; Getz and Carter, 1996). Alloparental behavior is commonly performed by these older offspring (Carter et al., 1995), a common monogamous social strategy in family groups with reproductively inhibited juvenile and sub-adult offspring (Kleiman, 1977), although cases of reproductively active prairie vole offspring are also

observed (Mcguire et al., 1993). Juveniles leave the nest and show high mortality rates in the summer, accounting for the absence of communal family groups during this season (Getz et al., 1993).

Prairie voles are well suited for laboratory studies in that they breed well, adapt to laboratory environments, and display pair bonding behavior in the lab, comparable to that observed in the field (Getz et al., 1981a; Shapiro et al., 1986; Dewsbury, 1987). The prairie vole pair bond is assessed in the laboratory using the 'partner preference test.' This reliable behavior assay of social bonding permits study of the neural mechanisms that control attachment and monogamy. In the partner preference test, the experimental subject is housed with an opposite-sex 'partner' for a defined length of time and subsequently tested for social preference for that 'partner' over a novel 'stranger' (Figure 1.1c; Williams et al., 1992). Following the cohabitation, the 'partner' and 'stranger' stimulus animals are restricted via tethering to either end of a 3-chambered arena and the subject has free access to all chambers. The amount of time the subject spends in close proximity or huddling with the partner is quantified over the three-hour test (Figure 1.1d). Using a computer coding system that has been validated against human scoring methods, high-throughput screening of up to 36 tests in a 24hr period can be performed (Ahern et al., 2009). In both sexes, mating facilitates the formation of a partner preference (operationally defined by spending twice as much time with the partner than the stranger), although lengthened cohabitation without mating can lead to a partner preference (Williams et al., 1992; DeVries and Carter, 1999). Pharmacological or other manipulations can be performed in the experimental subject during the cohabitation period in order to interrogate the neurobiological substrates of social bond formation.

Another valuable feature of the prairie vole model is the ability to perform comparative studies. Differences in sociality between closely related species have revealed valuable insight to the factors underlying and characterizing monogamy. Research into the neural control of complex behavior has taken advantage of the divergent social structures within the Microtus genus by comparing the highly social prairie voles to the minimally social meadow (*M. pennsylvanicus*) and montane (*M. montanus*) voles. These closely related species are promiscuous, uniparental, and relatively asocial, providing a unique evolutionary comparison to the highly social organization of the prairie vole. Males and females defend separate areas and nest alone during the breeding season (Jannett, 1982). Promiscuous male home ranges encompass the territories of multiple females, and many males converge to ranges of females in estrus (Madison, 1980; Jannett, 1982). These species do not form pair bonds in the laboratory, as montane voles show no preference for a familiar opposite sex mate (Shapiro et al., 1986). As will be discussed, comparative studies of these behaviorally divergent species have helped to reveal the neural characteristics that give prairie voles the ability to form pair bonds.

Neuropeptidergic control of the pair bond and proximate behaviors

Early studies of the neural control of attachment looked towards the neuropeptides oxytocin and vasopressin because of their implications in a number of the social and sexual behaviors related to the initiation and maintenance of the pair bond. Vasopressin (AVP) and oxytocin (OT) are nonapeptides, differing by only two amino acids, produced in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus where they are released into the periphery to coordinate a variety of

effects. Peripheral OT regulates the initiation of labor and stimulation of milk ejections, and peripheral AVP induces vasoconstriction and water reabsorbtion. Oxytocin is also released centrally from projections arising in the PVN and SON (Ross et al., 2009a), while central AVP arises from the hypothalamus in addition to sexually dimorphic neurons in the medial amygdala (MeA) and the bed nucleus of the stria terminalis (BNST) (Devries and Buijs, 1983). Before being implicated in monogamy, AVP was long known to play a role in facilitating social cognition (Dantzer et al., 1987; de Wied and van Ree, 1989), and territorial flank-marking displays in golden hamsters (Ferris et al., 1984).

Similarly, oxytocin was known to regulate social memory, promote affiliation (Van Wimersma Greidanus et al., 1990), and show pregnancy-induced increases in specific brain regions (Insel, 1990). Most importantly, it was linked to another important social relationship: the mother-infant bond. Central OT release coordinates peripheral events associated with birth with the behavioral changes required for the initiation of maternal care (Ross et al., 2009a). Virgin female rats will avoid or attack pups, but towards the end of pregnancy rats will instinctively become maternal. This pregnancy-induced change in the dam's motivation to nurture are due in part to OT release since infusion of OT in virgin female rats induces maternal responses to novel pups (Pedersen et al., 1982). In sheep, OT not only promotes maternal nurturing, but promotes a mother's bond specific to her own offspring (Kendrick et al., 1987). Using a variety of molecular techniques including pharmacological manipulations, immunochemical and histological mapping, viral vector technology, transgenic development, and genetic screens, the role of OT and AVP have been strongly related to pair bond formation and the behaviors

related to monogamy (Young and Wang, 2004b).

Species differences in neuropeptide circuitry

Comparative studies between Microtus species have uncovered drastic differences in the regulation and function of neuropeptidergic systems. Though both monogamous and promiscuous vole species display a conserved distribution of oxytocin peptide within the brain (Wang et al., 1996), the distribution of oxytocin receptors is strikingly different between prairie voles and their promiscuous relatives (Figure 1.2a,b). Prairie voles have high densities of OT receptor in the nucleus accumbens (NAcc), a brain region involved in reward, reinforcement, and addiction, as well as the BNST and medial prefrontal cortex (mPFC), whereas receptors are largely absent in NAcc in nonmonogamous species (Insel and Shapiro, 1992). In contrast, meadow and montane voles display elevated levels of OTR in the ventromedial hypothalamus and lateral septum (LS) compared to prairie voles (Insel and Shapiro, 1992).

In parallel to the OT circuitry among Microtus species, AVP projections are nearly identical between promiscuous and monogamous voles (Wang et al., 1996). Instead, divergent vasopressin V1a receptor (V1aR) distributions are thought to give rise to different behavioral phenotypes (Figure 1.2d,e). In prairie voles, V1aR binding is comparatively high in certain regions involved in socioemotional and reward processing, such as the anterior olfactory nuclei, cingulate cortex, amygdala, and ventral pallidum (Insel et al., 1994; Young et al., 1997; Lim et al., 2004a). Receptor binding in prairie and montane voles overlaps with *Avpr1a* mRNA, indicating that expression is regulated at a transcriptional level (Young et al., 1997). Some of this variation may be attributed to an upstream microsatellite region made of short repeat sequences more than 300 base pairs

longer in monogamous vole species (Young et al., 1999). While variation in the microsatellite may contribute to species differences in V1aR distribution, it does not explain species differences in social behavior among all vole species (Fink et al., 2006). Transgenic mice expressing the prairie vole vasopressin receptor gene (*Avpr1a*) neuroanatomical expression display a pattern similar to that of the prairie vole, and enhanced affiliation in response to AVP injections, whereas wild-type mice do not (Young et al., 1999).

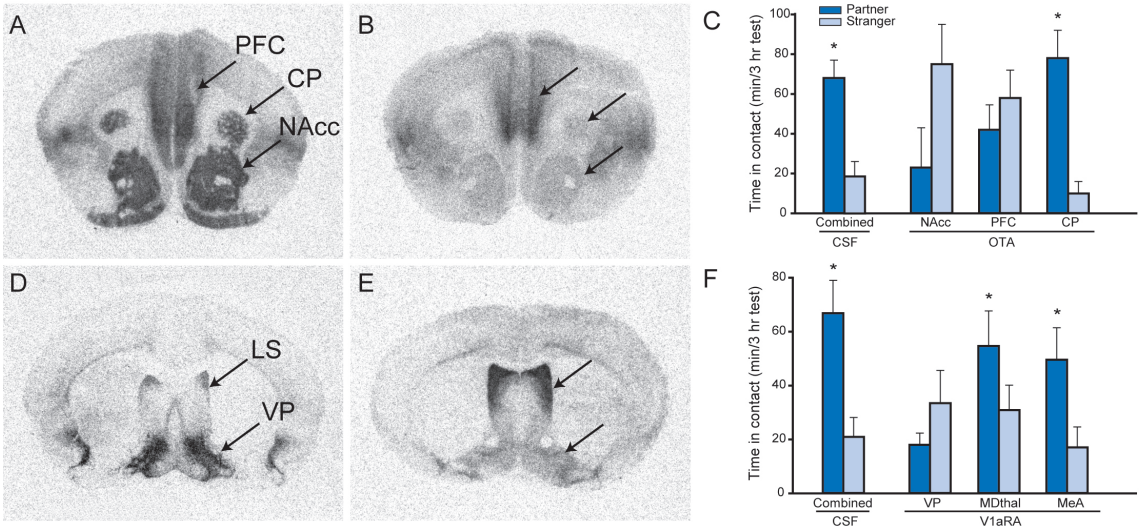

Figure 1.2. Comparative differences in neuropeptide receptor expression and functional effects on behavior.

(a,b) Monogamous prairie voles (a) have higher densities of OTR in the nucleus accumbens (NAcc) and caudate putamen (CP) than do nonmonogamous montane voles (b). Both species have OTR in the prefrontal cortex (PFC). (c) Infusion of a selective V1aR antagonist (V1aRA) into the VP, but not into the mediodorsal thalamus (MDthal) or medial amygdala (MeA), prevents mating-induced partner-preference formation in male prairie voles (Lim and Young, 2004). (d,e) Male prairie voles (d) have higher densities of V1aR in the ventral pallidum (VP) than do montane voles (e). (f) A selective OTR antagonist (OTA) infused bilaterally into the NAcc or PFC, but not the CP, blocks partner-preference formation in female prairie voles (Young et al., 2001). (modified from Young and Wang, 2004).

Similar differences in V1aR expression are found in comparisons of other groups of closely related, but behaviorally divergent species. Monogamous marmosets and California mice (Peromyscus californicus) display V1aR levels in the ventral pallidum that are absent in non-monogamous rhesus macaques and white-footed mice (P. leucopus) (Wang et al., 1997a; Bester-Meredith et al., 1999; Young, 1999). Thus selection of species with specific neural V1aR patterns may have led to the convergent evolution of a monogamous social strategy across multiple taxa (Lim and Young, 2004). However, V1aR distribution may not explain the evolution of monogamy in all other species (Turner et al., 2010).

After the formation of a pair bond, central AVP changes are observed in monogamous, but not promiscuous, male voles. In male prairie voles, but not females or meadow voles, vasopressin immunoreactive staining decreases in the lateral septum and lateral habenular nucleus after a mated cohabitation with a female and again after parturition (Bamshad et al., 1993; Bamshad et al., 1994). A decrease in immunoreactive fiber density may suggest a release of AVP peptide content from these terminals. Importantly, a concurrent increase in AVP mRNA in the BNST, a source of AVP to the LS and LH (Wang et al., 1994), suggests that AVP is released during pair bond formation, which may act to coordinate associated behaviors including partner preference, aggression, and paternal care. Activation of AVP circuitry though an injection of AVP into the brain has differing behavioral effects in monogamous and promiscuous species, as it enhances female-directed affiliation and resident-intruder aggression in prairie, but not meadow or montane vole males (Young et al., 1997; Young et al., 1999).

Partner preference

Originally inspired by the effects of oxytocin in maternal bonding, Sue Carter and colleagues explored the possibility that the same molecule might promote the formation of the pair bond between the female prairie vole and her male partner. In the first study to find functional effects of OT in voles, Witt (1990) reported enhanced grooming and huddling with a male partner after central OT administration to female prairie voles. They went on to investigate whether this enhancement of affiliation during cohabitation promotes bond formation. Pairing a female prairie vole with a male for six hours without mating is not sufficient for bond formation; however, infusion of OT into the brain during the shortened nonsexual cohabitation period pairing induces the female to bond with the male (Williams et al., 1994), and this effect is blocked by co-administration of an oxytocin antagonist (Cho et al., 1999). During the partner preference test, OT-treated females spend significantly more time with the familiar male partner than a novel male. Furthermore, pretreatment with an OT antagonist alone will prevent OT-or mating-induced partner preference formation (Williams et al., 1994; Insel and Hulihan, 1995), demonstrating that OT is necessary for the formation of the bond. In fact, OT levels in microdialysates are elevated in females during cohabitation and mating with a male (Ross et al., 2009a). Activation of the oxytocinergic system thus accelerates the formation of the pair bond and overcomes the absence of mating during a shortened cohabitation period. Thus the same neurobiological systems that promote maternal nurturing and mother-infant bonding play a critical role in regulating pair bonding in female prairie voles.

Although oxytocin may also contribute to pair bonding in male prairie voles (Cho

et al., 1999), a more convincing role of AVP has been demonstrated in the biology of male social bonding. Both OT and AVP injections accelerate pair bonding, even with only a 1hr cohabitation, in male prairie voles, and this enhancement is blocked by pretreatment with OT and AVP antagonists (Cho et al., 1999). However, only V1aR antagonists administered before mating block the formation of a pair bond in males (Winslow et al., 1993), indicating that AVP, but not OT, is required for male attachment formation.

Using site-specific pharmacological and viral manipulations, regions that show differences in receptor expression between Mictrotus species have also been identified as the critical sites of action for OT and AVP neurotransmission in pair bonding. OT antagonists administered directly to the nucleus accumbens (NAcc) or PFC, but not the caudate-putamen, prevent the formation of pair bonds in female prairie voles (Figure 1.2c; Young et al., 2001). . Pharmacological blockade of V1aR in the in the ventral pallidum or lateral septum, but not the medial amygdala or mediodorsal thalamus, inhibits the development of a partner preference in males (Figure 1.2f; Liu et al., 2001; Lim and Young, 2004). Increasing V1aR density in the ventral pallidum of male or OTR expression in the nucleus accumbens of female prairie voles using viral vector gene transfer promotes affiliation and accelerates pair bond formation (Pitkow et al., 2001; Ross et al., 2009b). Over-expression of V1aR in the ventral pallidum of male prairie voles enhances mating-induced Fos neurotransmission, and thus likely enhances social processing during cohabitation (Lim and Young, 2004). Astonishingly, performing this viral manipulation on V1aR expression in the ventral pallidum of male meadow voles transforms social behavior and causes this normally nonmonogamous species to form

partner preferences (Lim et al., 2004b), essentially recreating an evolutionary event in the lab.

Neuropeptide regulation of behaviors associated with pair bonding

In addition to the control of the pair bond itself, OT and AVP have been implicated in the behaviors associated with a monogamous mating strategy, including paternal care, alloparental care, spontaneous maternal behavior, and mating-induced aggression. During pregnancy and parturition, female rats exhibit plasticity in OT receptor (OTR) expression and are primed to respond to pups possibly through these changes in OT signaling (Insel, 1990). However, males do not experience pregnancy-related changes, and AVP signaling may instead have evolved to promote male parental care (De Vries and Villalba, 1997). A decrease in AVP fiber density in the lateral septum of prairie vole fathers is suggestive of vasopressin release, and is associated with a concurrent increase in the expression of paternal behaviors (Bamshad et al., 1993). However, as females enter postpartum estrus during this time, it is unknown whether this release is due to pup exposure or to the act of mating. In order to obtain a read-out of general neural activity controlling paternal care, Fos expression was mapped in response to pup exposure in male voles (Kirkpatrick et al., 1994c). The AOB, MeA, BNST, MPOA, and LS showed elevations in Fos activity, and all of these regions display V1aR expression. Additional work supports the notion that vasopressinergic activity in these areas controls paternal behavior. Site-specifically injecting AVP into the lateral septum of sexually-naïve male prairie voles promotes paternal responsiveness to novel pups, whereas a V1aR antagonist reduces it (Wang et al., 1994). Sexually naïve male prairie voles contacted and crouched over pups more than did saline-injected controls, and

antagonist-injected males groomed pups less. Though more evidence has pointed to a role of the lateral septum in mediating paternal care, males with bilateral lesions of the medial amygdala or olfactory bulbs display impairments in paternal behavior (Kirkpatrick et al., 1994a; Kirkpatrick et al., 1994c). AVP may also play a role in maternal behavior, maternal memory, and maternal aggression (Bosch and Neumann, 2008a; Nephew and Bridges, 2008; Bosch et al., 2010). Brattleboro rats are deficient in AVP and display inferior parental care (Engelmann and Landgraf, 1994). Similarly, injections of AVP promote maternal care in females (Pedersen et al., 1982).

Another social behavior in the monogamous vole repertoire, alloparenting by reproductively naïve juveniles in the communal nest, has also been linked to the OTR system. Within prairie vole juvenile females, levels of OTR expression in the NAcc correlate positively with alloparental behavior (Olazábal and Young, 2006a). Although virgin adult females also display spontaneous maternal care of pups, the display of this behavior is highly variable within the species with 40% attacking or ignoring pups (Roberts et al., 1998b). However, some of this variability may be accounted for by OT receptor distribution, since females with high OTR expression in the NAcc display spontaneous maternal care, and OTR blockade in this region prevents spontaneous maternal behavior (Olazábal and Young, 2006a). Interestingly, in adult female prairie voles, upregulation of OTR expression with viral vector gene transfer into the NAcc does not promote alloparental care (Ross et al., 2009b). However, over-expression in prepubertal juvenile females enhances adult female alloparenting, suggesting a role for long-term developmental OTR signaling (Keebaugh and Young, 2011a).

When prairie vole males form a pair bond, they display not only a social

preference for their partner, but also selective aggression toward novel male and female conspecifics (Winslow et al., 1993), another behavior under control of neuropeptides. Central AVP injections stimulate male-directed aggression in paired males even in the absence of mating, while blocking V1aR prevents mating-induced aggression (Winslow et al., 1993). V1aR antagonists do not block aggression in established breeders, and thus play a role in the initiation, but not the expression, of this behavior. More recently, anterior hypothalamic (AH) AVP has been implicated as necessary and sufficient in initiating selective aggression towards females (Gobrogge et al., 2009). AVP is released in the AH in pair bonded males displaying selective aggression towards intruders, and the amount of aggression correlated with AVP levels in the microdialysates from the AH during the social encounters. Furthermore, AVP injections or over-expression of V1aR using viral gene transfer into the AH promote selective aggression in sexually-naïve males towards a novel female. Given the role of AVP in promoting both territoriality, including mating-induced aggression in voles and flank marking behavior in golden hamsters (Ferris 1984), and bond formation primarily in males, it is possible that male pair bonding evolved through a system that supports territorial protection of his female partner and their offspring (Young and Flanagan-Cato, 2012).

Dopaminergic regulation of monogamy

OT and AVP appear to be tightly connected with dopaminergic signaling in prairie voles. Mesocorticolimbic dopamine pathways project from the ventral tegmental area to the NAcc, caudate putamen, and PFC (Wise, 2002). Oxytocin receptors are highly expressed in the NAcc of prairie voles, and this region projects to the ventral pallidum, the site of action of AVP. Mesolimbic dopamine is critical in processing the reward of

behaviors critical to life such as feeding and mating, and is also responsible for the addictive power of drugs of reward (Koob and Nestler, 1997; Di Chiara, 2002; Wise, 2002). In prairie voles, behavioral studies have shown that that dopaminergic activity in mesocorticolimbic regions modulates pair bond formation and maintenance, selective aggression, and paternal behavior (Wang et al., 1999; Lonstein, 2002; Aragona et al., 2003; Aragona et al., 2006) (Young et al., 2011). The dopamine system varies between species of Microtus, and may contribute to species differences in behavior. In addition to the ventral tegmentum, dopamine cell bodies are also found in the BNST and MeA of prairie voles, but nonmongamous meadow voles exhibit very little labeling in these regions (Northcutt et al., 2007). Furthermore, D2 dopamine receptor (D2R) levels are more densely expressed in the PFC of prairie vole brains (Smeltzer et al., 2006), whereas D1 receptor (D1R) levels are higher in the PFC and NAcc of promiscuous meadow voles (Aragona et al., 2006). High expression of D1R may inhibit the display of affiliative behaviors in meadow voles, as administration of D1R antagonists promotes social affiliation in these animals (Aragona et al., 2006).

Mating induces a release of dopamine in the NAcc of both sexes in prairie vole (Gingrich et al., 2000; Aragona et al., 2003), and this release is critical for pair bond formation. Central dopamine blockade before cohabitation using haloperidol inhibits partner preference in both sexes (Wang et al., 1999; Aragona et al., 2003). An enhancement of dopamine signaling with a low dose of the nonselective dopamine agonist apomorphine promotes pair bond formation after a shortened unmated cohabitation (Wang et al., 1999; Aragona et al., 2003). The facilitating effect of dopamine on pair bonding can be attributed to D2 receptor activation, as pharmacological

activation of D2R in the NAcc shell accelerates pair bond formation, whereas blockade prevents it in both prairie vole sexes (Gingrich et al., 2000; Aragona et al., 2006). Conversely, activation of D1R in the NAcc shell inhibits bond formation, further suggesting an inhibitory role of D1R in affiliative behaviors (Aragona et al., 2006).

Neuroplasticity and experience-induced changes in the dopamine system may account for the transformation in behavior after bond formation. After two weeks of cohabitation, D1R levels increase in the NAcc, but not caudate putamen, of male prairie voles (Aragona et al., 2006). This elevation of D1R expression may play a role in maintaining the pair bond by inhibiting a male's motivation to affiliate with a stranger female by decreasing the rewarding aspects of novel sexual encounters. Moreover, the temporal specificity in D1R changes may induce the behavioral transition to selective aggression towards stranger females. Twenty-four hours of cohabitation is not sufficient to alter D1R expression or induce selective aggression, indicating D1R plays a role in the maintenance of the pair bond and proximate behaviors, but not the early formation (Aragona et al., 2006). In support of a role in aggression, blockade of NAcc D1R signaling abolishes stranger female-directed aggression in paired male prairie voles. Similarly, inhibiting kappa-opioid receptor signaling in the NAcc shell prevents mating-induced aggression in both sexes, suggesting the opioid system is also an important regulating in the maintenance of the pair bond and associated behaviors (Resendez et al., 2012).

Social cognition, reward and the neural circuitry of social bonding

OT and AVP are essential for social information processing and recognition of conspecifics in mice and rats (Dantzer et al., 1987; Le Moal et al., 1987; Popik and van

Ree, 1991; Ferguson et al., 2000; Bielsky et al., 2004; Winslow and Insel, 2004). An important integrator of incoming social olfactory information is the medial amygdala (MeA), which receives direct input from the olfactory bulbs, and is among the first nodes in the forebrain to process conspecific odors. In prairie voles, the MeA is required for the social drive to affiliate with a partner and display paternal care (Kirkpatrick et al., 1994a). This region processes incoming information during sociosexual encounters, as cohabitation, mating, and pup exposure elicit MeA c-fos activity (Kirkpatrick et al., 1994c; Cushing et al., 2003; Lim and Young, 2004). OT activity in the MeA during an initial social encounter is necessary for the formation of a social memory (Ferguson et al., 2001).

The MeA is also a critical extrahypothalamic site of vasopressin cell bodies, which project out to forebrain regions including the ventral pallidum and lateral septum (LS) (Devries and Buijs, 1983). The LS is reciprocally connected to the hippocampus and receives information about social cues, thus priming it to stamp in the memory of a social encounter. This region also projects to hypothalamic areas, including the MPOA, and may thus regulate male sexual behavior (Lonstein, 2002). Blockade of V1aR in the LS, via application of antisense oligonucleotides or antagonists, blocks social recognition in rats and mice (Landgraf et al., 1995; Bielsky et al., 2005). Over-expression of V1aR in the LS of wildtype mice and rats enhances social recognition and the inter-exposure interval at which subjects can still recognize conspecifics (Landgraf et al., 1995; Bielsky et al., 2005). Further in support of a role in social memory, over-expression of V1aR in the LS restores social recognition in V1aR knockout mice (Bielsky et al., 2005).

In addition to acting in these social information processing regions, OT and AVP

converge upon areas involved in the mesolimbic dopaminergic reward pathway, and concurrent activation of the systems is necessary for bond formation. Pharmacological activation of D2-type dopamine receptors in the NAcc accelerates partner preference formation, an effect blocked by OT antagonist administration (Gingrich et al., 2000; Liu and Wang, 2003). Similarly, D2R blockade in the NAcc prevents oxytocinergic acceleration of bond formation (Liu 2003). Direct interactions between the two systems may occur as both dopamine and OT induce release of the other (Melis et al., 1989; Pfister and Muir, 1989). Co-activation of AVP and dopamine systems may also be necessary for pair bonding in males, as meadow voles with an over-expression of V1aR in the ventral pallidum are induced to pair bond, but D2R blockade prevents this enhanced bond formation (Lim 2004). In addition to dopaminergic reinforcement, new evidence has pointed to the necessity of opioid reward signaling in bond formation (Burkett et al., 2011b). Blockade of mu-opioid receptors before cohabitation with a male blocks the formation of a partner preference in female prairie voles. Thus, it appears the concurrent activation of OT, AVP, dopamine, and opioids are all required in the formation of a pair bond.

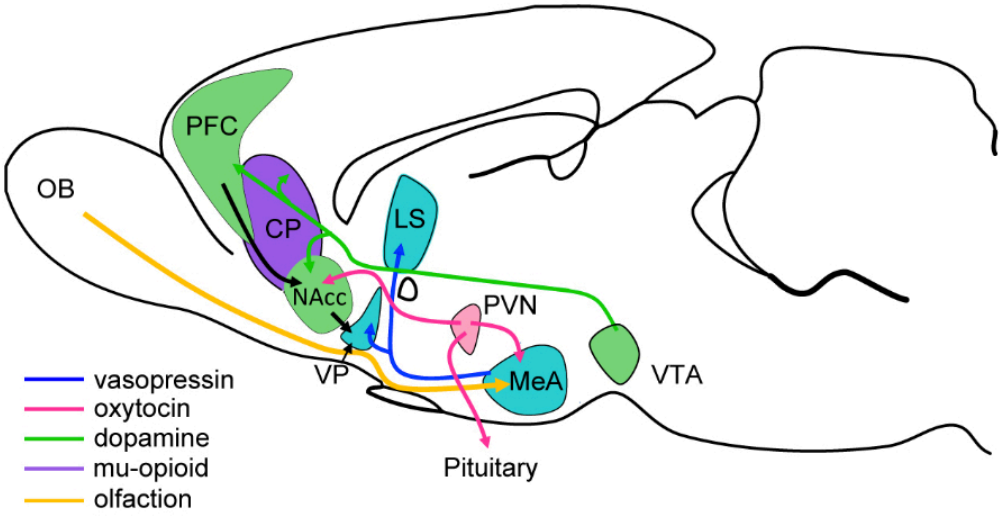

Figure 1.3. Neurocircuitry of pair bond formation.

A schematic of the proposed neural circuitry of pair bond formation in prairie voles. The neuropeptides OT and AVP carry important information on the olfactory signatures of a mate, enhance learning of socially relevant cues, and link information about the social cues of the partner with dopaminergic and opioid reward and reinforcement. Sociosexual interactions in female prairie voles stimulate the release of both OT from the paraventricular nucleus of the hypothalamus (PVN) and dopamine from the ventral tegmental area (VTA) into the NAcc. In males, AVP neurons in the extended amygdala release AVP into the ventral pallidum (VP) and the lateral septum (LS). Concurrently, olfactory signatures of the sexual partner are processed through the amygdala to ultimately lead to a condition partner preference (modified from Young et al., 2005).

OT and AVP are thought to promote bond formation by 1) increasing the saliency of incoming social information, 2) enhancing social motivation and approach, and 3) converging the olfactory signature of a mate upon dopaminergic reward pathways (Figure 1.3). High concentrations of OTR in the NAcc and V1aR in the ventral pallidum in prairie voles may serve to link up the olfactory signature of a mate with dopaminergic and opioid-mediated reward and reinforcement, in essence yielding a conditioned partner

preference (Young and Wang, 2004b; Young et al., 2005; Burkett et al., 2011b). Mating-induced mesolimbic dopamine release resulting from somatosensory information from the genitalia during sexual activity (unconditioned stimulus) may assign a rewarding value to the neuropeptide-enhanced social cues of the partner's specific olfactory signature (conditioned stimulus) (Young and Wang, 2004b). Opioid signalling in the striatum is also necessary for the development of a partner preference in female prairie voles and the maintenance of partner preference in both sexes (Burkett et al., 2011b; Resendez et al., 2012; Resendez et al., 2013). Although nonmonogamous mammals experience dopaminergic and opioid reward, the absence of the link with neuropeptidergic systems does not yield an enduring specific association with a partner. These neuroanatomical findings suggest that pair bonding is very similar to an addiction (Burkett et al., 2011b). Indeed, when separated from the partner, voles exhibit behaviors characteristic of withdrawal and depression (Pizzuto and Getz, 1998; Bosch et al., 2009).

The link between stress, anxiety, social loss, and pair bonding

The stress axis is another system that appears to regulate prairie vole sociality. The expression of monogamy may be regulated by variability in environmental pressures, and one of the main pathways by which the environment shapes behaviors is through stress. In fact, a demanding dry environment with limited food and water has also been implicated in the evolution of a mating strategy in which prairie vole pairs can combine efforts and share resources (Getz, 1978; Wang and Novak, 1992a; Mcguire et al., 1993; Carter et al., 1995). Conversely, *M. montanus* and *M. pennsylvanicus* live in thick grassy areas, and their diet consists of abundant and readily renewable grasses and sedges

(Ostfeld, 1990), an environment perhaps more amenable to promiscuity.

In response to a perceived or actual stressor, the hypothalamic-pituitary-adrenal (HPA) axis is activated beginning with hypothalamic corticotropin-releasing factor (CRF) stimulating the release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary and subsequently inducing the production of corticosterone from the adrenal cortex. Prairie voles display basal corticosterone levels that are 5-10 times greater than rats and promiscuous voles, and are considered glucocorticoid-resistant with 10-fold lower glucocorticoid receptor affinities (Carter et al., 1995; Taymans et al., 1997; Hastings et al., 1999), possibly suggestive of impaired negative feedback of the stress axis.

Stress axis in pair bond formation

Experimental activation of the HPA axis alters social behavior in male and female voles, although in divergent manner. In male prairie voles, stress activation, through either the psychological stress of forced swimming or exogenous corticosterone administration, accelerates the development of a partner preference (DeVries et al., 1996). Similarly, males recently exposed to a forced swim test display an elevation of alloparental behavior (Bales et al., 2006). Corticosterone not only facilitates bond formation, but also appears necessary for pair bonding to occur. Adrenalectomy prevents partner preference formation in male prairie voles, and replacement of corticosterone rescues the expression of this behavior (DeVries et al., 1996). However, prairie voles display sexual dimorphism in the effects of stress on sociality, as stress inhibits, rather than promotes, bond formation in females. Corticosterone injections or the stress of forced swimming, inhibit the development of partner preference in female prairie voles

(Devries et al., 1995). Conversely, adrenalectomized females are able to form a bond with only 1hr of cohabitation (Devries et al., 1995).

CRF innervates the NAcc, a region already extensively implicated in bond formation, and this accumbal CRF has been implicated in bond formation (Lim et al., 2007). Blockade of CRF 1 and 2 receptors prevent the development of partner preferences in males (DeVries et al., 2002). CRF administered centrally at doses insufficient to induce anxiety-like behaviors still accelerates bond formation in male prairie voles (DeVries et al., 2002). CRF administration directly into the NAcc before cohabitation with a female accelerates partner preference formation in male prairie voles, but not nonmonogamous meadow voles (Lim et al., 2007). CRF facilitation of bond formation was blocked by co-treatment with either CRF1 or CRF2 receptor antagonists.

The neural circuitry of the CRF system differs between monogamous and promiscuous vole species and displays sexual dimorphism, further suggesting a role of this peptide hormone in regulating social behavior (Lim et al., 2005b). Monogamous prairie and pine voles display higher densities of CRFR2 and lower densities of CRFR1 in the NAcc than promiscuous meadow and montane voles. Within prairie voles, CRFR2 binding is greater in the BNST of males than that of females.

Stress axis in pair bond maintenance

In addition to influencing the willingness of an animal to form a pair bond, the systems controlling stress and anxiety may regulate the stability of a bond and are functionally altered after bond formation. Sexually-naïve female prairie voles respond to novel males with a decrease in corticosterone, which presumably facilitates bond formation, but pair-bonded females display an increase in serum corticosterone,

potentially functionally inhibiting bond formation (Devries et al., 1995). In male prairie voles, pairing with a female induces an increase in CRF mRNA in the BNST, and separation from the partner elevates circulating corticosterone and adrenal weight (Bosch et al., 2009). The increase in CRF mRNA may essentially prime the male to respond aversively to loss of the female partner. The CRF system, particularly that in the extended amygdala, displays parallel activation both in response to separation distress after partner removal and during withdrawal from drugs of reward (Burkett and Young, 2012b). After loss of the bonded partner, male voles display passive stress-coping in the forced swim and tail suspension tests, and this social-loss induced depression is prevented by blockade of CRF1 or 2 receptors using osmotic minipumps (Bosch et al., 2009). Loss of a bonded partner leads to a suite of traits associated with heightened stress and a depressive-like state, characteristics arguably similar to those of withdrawal (Burkett and Young, 2012b). Social isolation induces a depressive-like state in prairie voles and isolated animals display decreased sucrose intake, elevated plasma corticosterone, and increased heart rate (Grippo et al., 2007a). Meadow voles do not display an isolation-induced elevation in the stress response, suggesting this aversion to isolation is unique to animals preferring social contact (Stowe et al., 2005). A separation from their a female partner may induce proximity-seeking behavior in male voles toward their female partner, thus serve to stabilize the pair bond (Bosch et al., 2009).

Circuitry of OT and AVP within the stress axis

The circuitry by which stress affects pair bonding is largely unknown, but there may be overlap with neuropeptide systems. Along with CRF, vasopressin has long been known to stimulate ACTH release through the V1b receptor subtype (Whitnall, 1993).

Stressors, including social defeat, induce AVP release in rats as shown by an increase in microdialysates (Wotjak et al., 1996), and heightened stress levels have been associated with increased AVP mRNA in the MeA and BNST in rats (Linfoot et al., 2009; Ueta et al., 2011). Brattleboro rats deficient in AVP display reduced anxiety-like behaviors (Landgraf et al., 1995). The anxiety-provoking aspects of AVP may be mediated through the V1a receptor subtype, in addition to V1bR in the pituitary, as V1aR antisense oligonucleotides or antagonists into the LS reduce anxiety-like behavior in rats, and V1aR over-expression in the LS increases expression of these behaviors (Landgraf et al., 1995; Liebsch et al., 1996; Bielsky et al., 2005). In addition, overexpression of V1aR in the VP of male prairie voles increases anxiety-like behavior in the elevated-plus maze (Pitkow et al., 2001). The ventral pallidum is the main output of the limbic basal ganglia and also receives projects from the amygdala, and thus may be an important integrator of stress and reward (Napier and Mickiewicz, 2010). Stress axis and AVP systems may be reciprocally connected as corticosterone has been shown to increase V1aR expression in smooth muscle lines and centrally in the BNST and there is a glucocorticoid response element in the 5' region of the avpr1a gene (Colson et al., 1992; Watters et al., 1996).

In contrast to AVP, OT appears to be primarily anxiolytic and is associated with decreased levels of anxiety-like behaviors in rodents and lower corticosterone levels (Windle et al., 1997; Windle et al., 2004; Champagne and Meaney, 2007; Amico et al., 2008; Knobloch et al., 2012). It has been proposed that OT may act to disinhibit the approach to novelty and reduce the anxiety-provoking aspects of social stimuli, thus promoting the onset of social behaviors (Olazabol 2006). Recently, evidence for reciprocal regulation of OT and CRFR2 has been demonstrated in rats, with

hypothalamic OT neurons expressing CRFR2 and BNST CRF neurons expressing OTR (Dabrowska et al., 2011). Further in support of a functional relationship between CRFR2 activation and OT release, female prairie voles raised by a single mother display a positive relationship between OT mRNA in the anterior PVN and CRFR2 expression in the dorsal raphe (Ahern and Young, 2009).

The molecules controlling stress and anxiety may have co-evolved a role in social behavior, possibly through modulating an animal's motivation to form a social bond (Lim et al., 2007). It has been hypothesized that stressful conditions may provide an evolutionary advantage for pair bonding in male, but not female, prairie voles (DeVries et al., 1996). Females may benefit by staying in their natal nest and mating with non-family members (Getz et al., 1981a; McGuire and Getz, 1995; Getz and Carter, 1996), whereas sexually mature males may be too aggressive toward conspecifics other than the partner to stay in the nest (DeVries et al., 1996). Males would then obtain the most reproductive success by forming new partnerships, defending a new home range, and providing care to offspring. The divergent roles of OT and AVP in mediating anxiety parallel the opposing influence of stress on pair bond formation in males and females, and may, in part, account for sex differences in the influence of neuropeptide systems on bonding in prairie voles.

Within-species variability in social behavior

In addition to dramatic interspecies differences in vole social organization, significant natural variations in mating and parenting strategies also exist within the prairie vole species. In nature, breeding units vary between three types: male-female pairs, single-females, or communal groups composed of extended families (Getz and

Carter, 1996). Within the communal nest, the size of the unit can vary between 3 and a dozen or more individual adults and their offspring (Getz and Carter, 1996). Groups are larger in late autumn-winter with an average of 8 individuals as compared an average of 3 in spring-early autumn (Getz and Carter, 1996). Though most voles become "residents" and faithfully defend their partner and care for young, approximately 24% of females and 45% of males take on a "wandering" strategy, never forming a pair bond, (Getz and Carter, 1993). The percentage of wandering males in a population remains constant relative to population density (Getz and Carter, 1996). In the laboratory, voles vary significantly in their willingness to display spontaneous alloparental care, with up to 40% of females and 20% of male subjects attacking or ignoring pups (Roberts et al., 1998b).

Additionally, significant intraspecies variation in neuropeptide distribution has been reported in both wild and laboratory prairie vole populations. In a wild population of voles, V1aR densities can vary dramatically between individuals, with an approximately two-fold difference between upper and lower quartiles of expression (Phelps and Young, 2003). Diversity in V1aR expression is observed in regions such as the amygdala, thalamus, olfactory nuclei, and lateral septum and has been correlated to individual differences in behavior (see Figure 1.4a,b; Lim et al., 2004a, Hammock et al., 2005). In naturalistic field settings, regions involved in territoriality and spatial memory display lower V1aR levels in "wandering" males that sire offspring outside of their pair bond (Ophir et al., 2008c). A positive correlation in V1aR expression is observed between neural networks involved in reward processing and those involved in space use and territoriality, thus widespread V1aR expression may coordinate multiple systems relating to pair bonding (Phelps and Young, 2003). OTR expression also shows great

variability, particularly in the NAcc (Figure 1.4c,d). OTR binding variation in the NAcc correlates to alloparental and spontaneous maternal care in females in the lab (Olazábal and Young, 2006a), as well as mating tactics and social monogamy in the field (Ophir et al., 2012).

Thus it is possible that variation in neuropeptide systems is linked to behavioral diversity in prairie vole social behavior. Both proximate (neural) and ultimate (evolutionary) mechanisms are at play to shape behavioral and neural systems to be able to coordinate appropriate behaviors with given environmental demands (Crews and Moore, 1986; Blumstein et al., 2010). Understanding how this neural diversity is determined and regulated may lead to insights into the neurobiological basis of individual differences in social behavior, and may ultimately be more relevant to the human condition than are interspecies comparisons (Figure 1.5). Below we will describe the genetic variability, with a focus on the *Avpr1a* gene, and experience-induced changes, particularly those that occur early in life, that have been linked to variability in prairie vole social behavior and neuropeptide function.

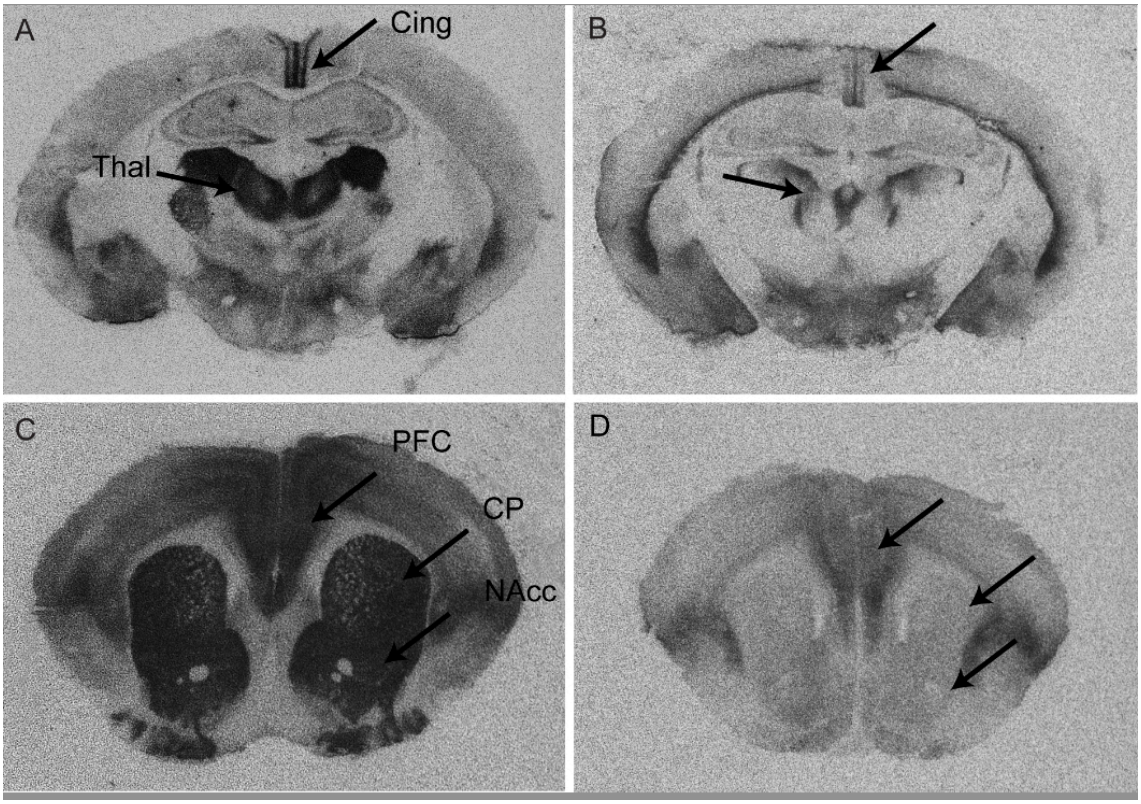

Figure 1.4. Intraspecies variation in neuropeptide receptor expression.

Dramatic individual variation in V1aR (a,b) and OTR (c,d) expression is observed within the prairie vole species, and may drive social behavioral diversity. Individual variation in V1aR expression in the cingulate (Cing) and thalamus (Thal) contributes to measures of male pair bonding in naturalistic field settings (Ophir et al., 2008c). In females, individual differences in OTR binding in the striatum (caudate putamen, CP; nucleus accumbens, NAcc), but not the prefrontal cortex (PFC), positively correlates with alloparenting (Olazabal and Young, 2006).

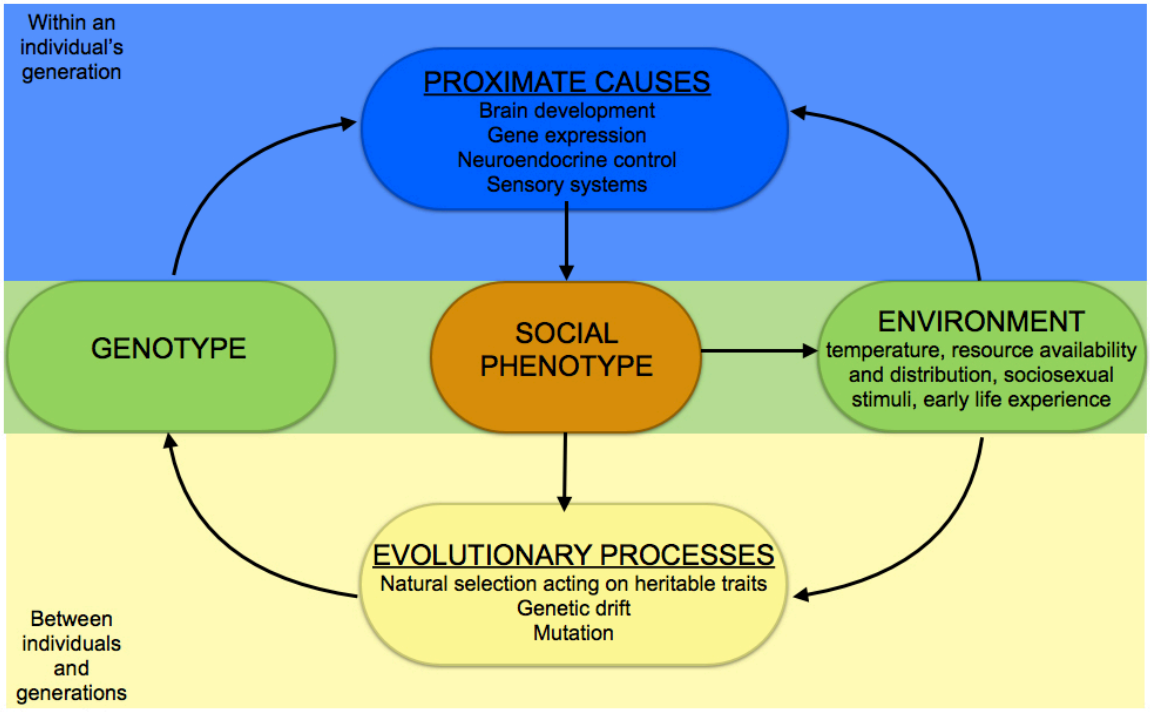

Figure 1.5. Proximate and evolutionary systems mediating plasticity in social behavior.

An organism's social phenotype is the result of a complex interplay between environmental conditions, genotype, evolutionary history, and development in shaping neuroanatomical and hormonal systems. For example, environmental influences both during development and later in life can impact the function of neuropeptides, neurotransmitters, and related molecules responsible for social motivation, reward and reinforcement, or stress and anxiety. Over time given environments may lead to socially monogamous mating systems by selecting for individuals that find it rewarding to be in social contact with a familiar mate or that are driven to maintain a bond because of the stress and withdrawal induced after separation. Understanding the neuroendocrine systems susceptible to environmental and experience-dependent mechanisms can help explain the causes of both individual variation in social behavior and of disease pathophysiology. Ultimately, an integrative study of social behavior may also offer insight to evolutionarily plastic targets amenable to pharmacotherapeutics. (Modified from a figure originally published in Blumstein et al. (2010) Toward an integrative understanding of social behavior: new models and new opportunities. *Front. Behav. Neurosci.* **4**: 34).

Genetic variation in *Avpr1a*

In addition to the species difference in the length of the microsatellite 5' of the *Avpr1a* gene between monogamous and promiscuous species, length and composition variation at this locus also varies among individual prairie voles and may account for differences in behavior and receptor binding that is also observed within this species (Young et al., 1999; Hammock and Young, 2002; Phelps and Young, 2003). The microsatellite is composed of di- and tetranucleotide sequences between 1,150-720 bp upstream of the *Avpr1a* transcriptional start site and microsatellite length varies by approximately 300 nucleotides among prairie voles (Hammock et al., 2005; Nair and Young, 2006). Microsatellite length may drive region-dependent variability, as brain V1aR distribution in monogamous vole species is enhanced in some but reduced in other regions in comparison with non-monogamous species (Insel et al., 1994; Young et al., 1997). However, the species differences in microsatellite is not an evolutionary "switch" leading to monogamy (Fink et al., 2006) but the instability of the microsatellite may function as an evolutionary tuning knob to produce subtle variation in V1aR distribution (Young and Hammock, 2007b). To provide evidence that individual differences in Avpr1a microsatellite structure might influence V1aR distribution and behavior *in vivo*, Hammock et al (2005) bred prairie vole lines to homozygosity for long or short microsatellite alleles. Long-allele males displayed higher levels of receptor binding in the olfactory bulb and lateral septum, regions previously attributed to pair-bond formation, but exhibited low levels of V1aR in the amygdala, hypothalamus, cingulate cortex and no difference in ventral pallidum binding as compared to short allele animals. These animals

also scored higher on measures of paternal care, social affiliation, and partner preferences. In a field population, long-long males display elevated V1aR expression in the ventral pallidum and medial amygdala, but this receptor pattern did not relate to monogamous behavior in terms of social or sexual fidelity (Ophir et al., 2008a). Further studies are needed to determine whether variation in the microsatellite structure are directly related to variation in V1aR distribution, or whether the microsatellite is simply linked to other functional elements.

Environmental variation

Intraspecies plasticity in behavior may be evolutionarily maintained because of the costs and benefits of certain social organizations under different ecological conditions (Kleiman, 1977; Dewsbury, 1987; Lott, 1991). Indeed, environmental pressures, including habitat quality, resource availability, and predation, appear to regulate mating strategies among prairie vole populations. Sociality varies according to geographical location, as Kansas voles are less social, more aggressive, less alloparental, are not found living in communal groups in the wild, and males overlap the territories of multiple females (Fitch, 1957; Gaines et al., 1985; Danielson and Gaines, 1987; Roberts et al., 1998c) as compared to Illinois voles. Both subpopulations exhibit partner preferences in the lab, but Kansas voles spend less time in heterosexual physical contact during preference testing (Roberts et al., 1998c; Cushing et al., 2001). These differences in behavioral strategies may reflect evolutionary adaptations to differing environments (Roberts et al., 1998b).

The habitat in central Illinois is generally more resource-rich than Kansas, and is moist, abundant with food sources, and can support year-round breeding and high

population densities (Cole and Batzli, 1979; Getz et al., 1981a; Getz and Carter, 1996; Roberts et al., 1998c). The environment in Kansas consists of dry grasslands, low resource availability, scattered food sources, and harsh, dry summers (Cushing and Kramer, 2005), yet paradoxically is thought to resemble the ancestral habitat of prairie voles that had led to monogamy (Cole and Batzli, 1979). However, the arid environment may have played a role in the evolution male pair bonding. Interestingly, it has been suggested that the role of AVP in conserving water in an arid environment may have co-evolved to support monogamy in male prairie voles (Cushing et al., 2001; Cushing and Kramer, 2005). Dry conditions stimulate peripheral AVP release to aid in water reabsorbtion and maintain water balance, and this elevated AVP production may concurrently promote pair bond formation in males (Cushing and Kramer, 2005). In support of this theory, Kansas prairie vole males are more sensitive to AVP injections than are Illinois males, and exhibit partner preferences after only a 1hr cohabitation if administered AVP, whereas Illinois males do not (Cushing et al., 2001). It is also possible that variations in early parental care can translate environmental conditions to offspring, and Kansas offspring are more susceptible to loss of a parent than are Illinois subjects (Roberts et al., 1998c).

Early life experience

Although prairie voles may exhibit flexibility in mating strategy into adulthood, individual differences in experience during critical periods of the development of social neural circuitry may have more drastic effects on adult expression of social behavior. The brain is particularly susceptible to perturbations in the environment during the malleable period of neural development early in life, and quality of parental care in early postnatal

life is a salient predictor of adult socioemotional behavior, stress reactivity and physiology. Much of the knowledge on the mechanistic effects of early life experience comes from studies done in more traditional laboratory rodents (for review, see Meaney, 2001; Veenema, 2012). For rat pups in the wild, the early environment in an isolated burrow does not offer many stimuli other than the care they receive from their mother (Francis et al., 1999). Tactile stimulation from dams, in terms of licking, grooming, and nursing, diminishes HPA axis activity in rat pups (Meaney, 2001). Mothers that display higher levels of maternal behavior in general produce offspring that have attenuated responses to stress and lower levels of anxiety.

Evidence suggests that social experiences early in development are also necessary for the formation of neural circuits controlling complex social behaviors, and that some of these effects may be mediated by neuropeptide systems. Indeed, female rat offspring display the maternal nurturing style of their mothers, and this effect is transmitted non-genomically (Francis et al., 1999). In the offspring of high licking and grooming rat mothers, females display elevated levels of OTR in the CeA and BNST and males display elevated V1aR expression in the CeA (Francis et al., 2002). The CeA in prairie voles may also be uniquely regulated, as it is the only region expressing V1aR where expression does not correlate with that seen in any other brain region (Phelps and Young, 2003).

In prairie voles, offspring receive biparental care from both parents, as well as alloparenting from older siblings, and this heightened level of postnatal social interactions may play a role in the development of the systems controlling adult bond formation. Interestingly, when meadow vole males, which normally do not display paternal care, are cross-fostered to prairie vole parents, they exhibit some paternal

behaviors (McGuire, 1988). Unfortunately, the reverse experiment is not possible as problems with milk delivery prevent montane or meadow vole mothers from feeding prairie vole pups, due to the presence of teeth in prairie voles (Shapiro and Insel, 1990). In comparison to asocial montane voles, prairie voles may be uniquely susceptible to early life manipulations, as they show heightened signs of distress when separated from parents (Shapiro and Insel, 1990). Isolated prairie vole pups make ultrasonic vocalizations, whereas montane voles do not, and show heightened corticosterone levels upon separation.

Even subtle manipulations early in life can have profound effects on later-life social behavior in prairie voles (Bales and Perkeybile, 2012). Bales and colleagues find that handling pups with a gloved hand ("MAN1") on the first day of life leads to enhancements in juvenile male alloparental behavior and pair bonding in both sexes as compared to pups transferred only with a cup ("MAN0") (Bales et al., 2007a), and that some of these behavioral effects are transmitted intergenerationally (Stone and Bales, 2010). Early handling resulted in enhanced OT peptide content in the SON, and diminished OTR in the BNST and LS of male MAN1 offspring, and NAcc, BNST, and LS of female MAN1 offspring (Bales et al., 2007a; Stone and Bales, 2010). Upon transferring to a new cage, MAN1 pups received a heightened amount of maternal attention, possibly accounting for their behavioral and physiological profiles into adulthood (Carter et al., 2009).

The presence of the prairie vole father in the natal nest also has significant effects on social development. Females raised to adulthood with their fathers display elevated alloparental behavior (Lonstein and De Vries, 2001). Prairie vole juveniles spend more

time with offspring from subsequent litters and spend less time alone in family groups where the father is present, over single-mother reared groups (Wang and Novak, 1994). Paternal deprivation in monogamous mandarin voles results in heightened anxiety-like behavior and lower levels of social interactions in offspring (Jia et al., 2009). Single-mother reared prairie voles receive less licking and grooming during the postnatal period and, as adults, females are less willing to care for novel pups and both sexes require a longer cohabitation to form partner preferences (Ahern and Young, 2009). Family structure also impacts how the next generation cares for their own offspring (Ahern et al., 2011). Female offspring of single mother family units also displayed increased OT peptide content in the hypothalamus and a greater dorsal raphe CRF2R density. However, this manipulation did not affect neuropeptide receptor distributions, suggesting these receptors are perhaps not always susceptible to environmental perturbations in prairie voles. Thus, deficits in normal social interactions, or even subtle variations in care during this critical period of development can significantly impact expression of adult social behavior.

Early activation of neuropeptide systems in regulating social bond development

One mechanism by which early experience may be functionally translated into behavior is through neuropeptide activation. A unique developmental role of OT in the natal nest is supported by the appearance of active OT peptide immunostaining only on the day of birth, but not in the prenatal period in rats (Buijs et al., 1980; Whitnall et al., 1985; Tribollet et al., 1989). Social contact induces peripheral and central OT release, as well as decreases blood pressure and heart rate and elevates anabolic metabolism in adult rats (Uvnas-Moberg et al., 1993; Uvnas-Moberg, 1997). As grooming is associated with

OT release in adults (Uvnas-Moberg, 1998), licking and grooming received from the parents in postnatal life may stimulate OT release in pups and be a neural transducer of early social contact (Insel, 1991), a phenomenon termed “chemical imprinting” (Francis et al., 2002). Indeed, both tactile stimulation to the anogential region of 7d old rabbit pups and suckling elicit immediate early gene activation in OT neurons in the PVN, suggestive of OT release in response to maternal licking and grooming and milk intake (Caba et al., 2003). The degree to which early social stimulation activates OT systems in neonatal prairie voles remains to be explored.

Based on the knowledge of the role of OT in adulthood, it is possible that early OT signaling may be necessary to assign a rewarding value to social stimulation from the mother and stimulate the neural circuitry involved in the development of social bonding. Central administration of OT, designed to mimic the experience of social contact, to isolated 6-8d old rat pups decreases separation distress calls (Insel and Winslow, 1991). OT release during mother-infant interactions may be necessary to develop a drive for social contact. OT and OTR knockout (OTKO and OTRKO, respectively) mice emit fewer ultrasonic vocalizations when separated from their mothers and OTKO pups take longer to reunite with their mothers compared to wildtype littermates (Winslow et al., 2000; Takayanagi et al., 2005; Ross and Young, 2009). This decreased distress to separation may reflect a weakened mother-infant bond, suggestive of disrupted development of social motivation during the neonatal period. Rat pups normally display a preference for a maternally-associated odor after conditioning sessions, but this social learning is blocked by central administration of an OT antagonist (Nelson and Panksepp, 1996). Preference for a maternal odor placed on the ventrum of the mother leads to filial

huddling, which is similarly blocked by OT antagonist treatment during conditioning (Kojima and Alberts, 2011).

In prairie voles, peripheral injections of OT on the day of birth, which are thought to penetrate the still underdeveloped neonate blood brain barrier, lead to increased pair bond formation in males as adults, elevated intruder aggression in females, altered sociosexual behavior, and reductions in male alloparental behavior as compared to vehicle injected animals (Bales and Carter, 2003b; Kramer et al., 2003; Cushing et al., 2005; Bales and Perkeybile, 2012). Early OT activation also has mechanistic effects on OT and AVP expression in the PVN and BNST (Yamamoto et al., 2004) and V1aR expression (Bales et al., 2007b). Although most findings of peripheral OT or OTA administration have reported effects on male pair bonding (Bales and Carter, 2003b), alloparenting (Bales et al., 2004), and neuropeptide receptor expression (Bales et al., 2007b), subsequent studies have reported a dose-dependent effect of OT neonatally on female pair bonding in adulthood (Bales et al., 2007c).

Given that V1aR regulates social cognition in adulthood and high expression levels are observed during the postnatal period in which significant familial interactions occur, V1aR is also a potential candidate for the transduction of early environmental influences (Carter et al., 2009). In some brain regions that control social behavior, such as the ventral pallidum, ventromedial and laterodorsal hypothalamus, and cingulate cortex, V1aR levels peak in the second postnatal week in prairie voles and do not reach adult levels until weaning (Wang et al., 1997b). These receptors are indeed functionally active as postnatal AVP injections alter neonatal vocalization responses to social isolation in neonatal prairie voles (Winslow and Insel, 1993). As expression in neonates is markedly

different from that of adult animals, V1aR may have a different role during development by organizing neural networks compared to regulating expression of social behavior in adults. In support of this hypothesis, neonatal exposure to AVP enhances aggression in adult prairie voles (Stribley and Carter, 1999), although other social behaviors were not examined. As described above, times of drought can stimulate AVP release and, subsequently, induce aggressive behavioral responses that are well suited to harsh environmental conditions (Stribley and Carter, 1999). Taken together, studies of early life manipulations of neuropeptide systems can potentially inform the development of early intervention strategies for human disorders characterized by deficits in social function.

Parallels with human social cognition

Do the same mechanisms regulating prairie vole bonding also influence human social relationships? Although human social behavior involves modulation from higher-order brain structures absent in rodents, comparative studies in voles may help elucidate the basic genetic and physiological underpinnings of human social behavior. During sexual stimulation in males, AVP levels measures in blood plasma are increased (Murphy et al., 1987). Similarly, vaginocervical and nipple stimulation in females induces the release of OT, which, during pregnancy and lactation functions to bring on uterine contractions and milk letdown. Originally evolutionarily important for maternal bonding, the OT system may have been exapted to play a role in bonding to a sexual partner to form a monogamous relationship (Ross and Young, 2009). Intriguingly, recent studies have discovered a link between variation in the OT receptor (OXTR) and AVPR1A genes and human pair bonding behaviors. Genetic association studies have linked a specific variant of a microsatellite in the AVPR1A gene to an increased risk of marital crisis in

males (Walum et al., 2008) and a single nucleotide polymorphism (SNP) in the OXTR gene (rs7632287) with traits associated with pair bonding in women (Walum et al., 2012). These commonalities between the vole and human peptide systems suggest that indeed, vole and human social behaviors may share some neurophysiological substrates (Meyer-Lindenberg et al., 2011).

In humans, neuropeptides partially bypass the blood brain barrier through intranasal delivery (Born et al., 2002), providing a noninvasive means to study the effects of OT on human behavior and neural responses. Intranasal approaches have demonstrated a role for OT in social learning, social anxiety, and the processing of social cues (for review, see Meyer-Lindenberg et al., 2011). In the first study to link intranasal OT to behavior in humans, OT enhanced the ability of social interactions to buffer stress responses (Heinrichs et al., 2003), suggesting that OT may diminish perceived social threats. OT dampens fMRI amygdala responses to threatening social images, more so than to nonsocial scenes, and may uncouple the amygdala from brainstem autonomic fear responses (Kirsch et al., 2005). In addition to diminishing negative associations of social contact, OT enhances prosocial behavior, which is defined as voluntary behavior intended to benefit another individual (Eisenberg et al., 2007), such as trust (Kosfeld et al., 2005), generosity (Zak et al., 2007), parental care (Naber et al., 2010; Weisman et al., 2012) and positive communication (Ditzen et al., 2009). OT infusions increase the accuracy of inferring the emotions of others through subtle facial cues, in essence increasing affective “mind reading” (Domes et al., 2007). Intranasal OT infusions enhanced trust of an “investor” for a “trustee” in an investment scenario (Kosfeld et al., 2005). OT enhances the amount of time individuals spend looking into the eye region of

human faces, suggesting that it may enhance interest in socially-relevant sensory information (Guastella et al., 2008a). In fact, direct eye gaze to attractive individuals activates brain regions involved in dopaminergic reward (Kampe et al., 2001). Perhaps mediated by an intensified reward for social stimuli, OT reinforces encoding of positive social cues and improves performance on a memory task for happy, but not angry or neutral, human faces (Guastella et al., 2008b). In another social learning paradigm in which human faces with positive or negative valence are displayed in response to correct or incorrect responses, respectively, oxytocin infusions enhanced task performance (Hurlemann et al., 2010). Intranasal OT also enhances cooperation in modified Prisoner's Dilemma game, which associated with enhanced striatal and amygdala activation (Rilling et al., 2012). More recently, OT has been linked to bond maintenance in men, as it increases the distance they keep from an attractive female (Scheele et al., 2012). Additionally, in men in relationships, OT increases the perceived attractiveness of female partners and increases fMRI responses in the ventral tegmental area and nucleus accumbens, regions mediating bonding in voles, in response to images of their partner (Scheele et al., 2013). These and other intranasal OT studies suggest that OT modulates the processing of social cues, socially reinforced learning, social anxiety, as well as other prosocial processes, including social bonding. The remarkable parallels in neuropeptide function in man and voles suggest that prairie voles can provide valuable insights into human social cognition.

Several studies over the last few years have applied research done in animals, and prairie voles in particular, to human clinical studies, demonstrating that the OT system may be a viable target for social cognitive enhancement in autistic individuals (Modi and

Young, 2012). Intravenous administration of OT to males diagnosed with Autism Spectrum Disorder(s) or Asperger Syndrome reduces repetitive stereotyped behaviors (Hollander et al., 2003) and enhances comprehension of affective speech (Hollander et al., 2007), both of which are core phenotypes of the disorders. In two recent studies, intranasal OT enhanced social cognitive function in male autistic individuals. Upon presentation of images of eyes in various affective states, the ability to read emotions from subtle facial cues was enhanced after OT infusion (Guastella et al., 2010). Similarly, intranasal OT enhanced gaze to the eye region in an autistic population (Andari et al., 2010). In the same study, socially reinforced learning improved with oxytocin infusion in a simulated socially interactive ball game. Participants were better able to discriminate between players that included them in the game over those that ignored them. Thus OT, OTR agonists, or drugs that stimulate OT release may enhance social functioning. Social cognitive enhancers could act synergistically with applied behavioral therapies that use socially reinforced learning techniques to teach autistic individuals social rules, language, and social cues (Young, 2011; Modi and Young, 2012). Pair bonding in monogamous voles may be a useful behavioral paradigm to test for drugs that, if given to an autistic individual just prior to a social learning session, may enhance the effectiveness of social behavioral therapies. Thus in addition to being useful for understanding the neural and genetic mechanisms underlying social behavior, pair bonding in voles may help with drug discovery to identify drugs that enhance social function.

Specific Aims of this Dissertation

The objective of this dissertation is to examine the proximate neural and experience-dependent mechanisms that drive within-species variation in social behavior.

There is significant inter- and intraspecies variation in neuropeptide receptors in the brain. Previously, Lim et al., (2004) demonstrated using viral vector mediated gene transfer that species differences in V1aR expression in the ventral pallidum likely mediate species differences in male pair bonding ability in voles. Several studies have demonstrated correlations between OTR or V1aR density and social behavior in voles (Olazabal et al., 2006, Ophir et al., 2008, Hammock et al., 2005), and over-expression of V1aR in the ventral pallidum or OTR in the NAcc accelerates pair bonding in prairie voles (Pitkow et al. 2001, Ross et al. 2009). While informative, the viral vectors used in the over-expression studies ectopically expressed the receptors in all neurons around the injection site, whether or not they endogenously express the receptors. Therefore, the studies do not precisely recapitulate natural variation in endogenous receptor expression within a given brain region. In chapter 2, we use shRNA viral vectors to selectively down-regulate V1aR expression in the ventral pallidum to more precisely model natural variation in expression gene expression. Vasopressin signaling in this region is necessary and sufficient to mediate pair bonding in males, thus we tested the hypothesis that subtle variation in neuropeptide receptor gene expression, within the range that is normally observed, can contribute to variation in social bonding.

In chapter 3, we switch our focus from the contribution from diversity in gene expression to the contribution of variation in early life experiences on sociality. We find that early-life social deprivation can significantly impact variation in later-life social behaviors in a sex-specific way. More importantly, natural variation in OTR density in the NAcc moderates the impact of early-life social experiences. These results suggest that neuropeptide signaling early in life may be mediating responses to early adversity and

nurturing behaviors. The long-term impact of parental nurturing behavior are likely to be mediated, in part, by the modulatory effects of the oxytocin and dopamine systems.

In chapter 4, we examined the impact of stimulating the melanocortin receptor, which is known to stimulate central OT release and interacts with the dopamine system. We reasoned that targeting the MCR might pharmacologically mimic the neurochemical changes associated with early-life licking and grooming. Indeed neonatal MCR agonists activated hypothalamic neuropeptides system and enhanced partner preference formation in adulthood. Given these prosocial effects of early-life MCR activation, we then tested whether neonatal MCR agonists could rescue the deficits in pair bonding behavior produced by early-life social isolation. The results from these studies are consistent with the hypothesis that the endogenous MCR system may play a role in transducing parental nurturing into prosocial behavior, and the MC4R could be explored as a target for developmentally treating social deficits in psychiatric conditions.

Therefore, in this dissertation I explore the role of variation in gene expression, early-life social experience, and interaction between brain receptor density and early-life social experience on later life social behaviors. Based on these findings we explored a pharmacological intervention that may mimic some aspects of early-life nurturing, highlighting the translational potential of the exploration of natural variation in social behavior.

CHAPTER 2

Variation in vasopressin receptor (*Avpr1a*) expression creates diversity in behaviors related to monogamy in prairie voles.

Modified from the following reference:

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Abstract

Polymorphisms in noncoding regions of the vasopressin 1a receptor gene (*Avpr1a*) are associated with a variety of socioemotional characteristics in humans, chimpanzees, and voles, and may impact behavior through site-specific variation in gene expression. The socially monogamous prairie vole offers a unique opportunity to study such neurobiological control of individual differences in complex behavior. Vasopressin 1a receptor (V1aR) signaling is necessary for the formation of the pair bond in males, and prairie voles exhibit greater V1aR binding in the reward-processing ventral pallidum than do asocial voles of the same genus. Diversity in social behavior within prairie voles has been correlated to natural variation in neuropeptide receptor expression in specific brain regions. Here we use RNA interference to examine the causal relationship between intraspecific variation in V1aR and behavioral outcomes, by approximating the degree of naturalistic variation in V1aR expression. Juvenile male prairie voles were injected with viral vectors expressing shRNA sequences targeting *Avpr1a* mRNA into the ventral pallidum. Down-regulation of pallidal V1aR density resulted in a significant impairment in the preference for a mated female partner and a reduction in anxiety-like behavior in adulthood. No effect on alloparenting was detected. These data demonstrate that within-species naturalistic-like variation in *Avpr1a* expression has a profound effect on individual differences in social attachment and emotionality. RNA interference may prove a useful technique to unite the fields of behavioral ecology and neurogenetics to perform ethologically relevant studies of the control of individual variation and offer insight into the evolutionary mechanisms leading to behavioral diversity.

Introduction

Central vasopressin 1a receptors (V1aR) regulate a variety of socioemotional behaviors including conspecific recognition and memory (Landgraf et al., 1995; Bielsky et al., 2004), territoriality (Ferris et al., 1984; Albers, 2012), aggression (Winslow et al., 1993; Gobrogge et al., 2009), paternal care (Wang et al., 1994), and anxiety-related behaviors (Wigger et al., 2004). Prairie voles (*Microtus ochrogaster*) provide an excellent opportunity to study the neural mechanisms underlying complex social behavior as they form life-long socially monogamous relationships with their mates. In male prairie voles, V1aR activation is necessary and sufficient for pair bonding and other behaviors associated with monogamy (Winslow and Insel, 1993; Wang et al., 1994; Liu et al., 2001a; Lim and Young, 2004; Donaldson et al., 2010).

Comparative studies with closely related, asocial *Microtus* species have suggested that variation in V1aR distribution in the brain is associated with diversity in social behavioral phenotypes (Insel et al., 1994; Young et al., 1997). Non-monogamous vole species have lower densities of V1aR in the ventral pallidum (VP) than do prairie voles and infusion of a V1aR antagonist into the VP of male prairie voles prevents mating-induced partner preferences, a laboratory index of pair bonding (Lim and Young, 2004). In addition, over-expressing the V1aR gene (*Avpr1a*) in the VP of male meadow voles confers the ability to develop partner preferences in this promiscuous species (Lim et al., 2004b).

Variation in length and composition of a microsatellite upstream of the *Avpr1a* gene between monogamous and promiscuous vole species and has been hypothesized to impact gene expression and behavior (Young et al., 1999; Hammock and Young, 2004;

Young and Hammock, 2007a). Similar allelic variation in microsatellites upstream of the *AVPR1A* gene in both humans and chimpanzees has been linked to relationship quality (Walum et al., 2008), personality traits (Ebstein, 2006; Meyer-Lindenberg et al., 2009; Ebstein et al., 2012; Hopkins et al., 2012), and autism spectrum disorders (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006). Together, these observations suggest that natural variation in neural expression patterns, not protein structure, significantly contributes to diversity in sociobehavioral traits.

Significant natural variation in mating and parenting strategies also exist within prairie voles, with some males taking on a life of "wandering" from mate to mate while others become "residents" and faithfully defend their partner (Getz and Carter, 1993; Roberts et al., 1998b). Individual variation in V1aR expression has been correlated with behavioral variation and *Avpr1a* microsatellite composition in males (Phelps and Young, 2003; Hammock et al., 2005; Hammock and Young, 2005; Ophir et al., 2008a). However, the exact contributions of the microsatellite to both inter and intra-specific regional V1aR binding and behavior remains controversial (Hammock et al., 2005; Hammock and Young, 2005; Fink et al., 2006; Young and Hammock, 2007a; Ophir et al., 2008a). While previous studies demonstrated that ectopically expressing V1aR alters social behaviors (Young et al., 1999; Pitkow et al., 2001; Lim et al., 2004b; Gobrogge et al., 2009), there has been no direct, causal demonstration that endogenous variation in *Avpr1a* expression is behaviorally relevant. Prairie voles typically display an approximately 30-40% difference in ventral pallidal V1aR density between upper and lower quartiles in both laboratory and wild-caught populations (Barrett and Young, unpublished observations; Hammock et al., 2005, Phelps and Young, 2003). Here, we use

RNA interference (RNAi) to manipulate endogenous *Avpr1a* expression and examined social behavior in male prairie voles. We hypothesized that a naturalistic degree of variation in *Avpr1a* expression *causally* generates behavioral diversity within a species.

Materials and Methods

Development of short hairpin RNA sequences.

Short hairpin RNA sequences (shRNAs) targeting the prairie vole *Avpr1a* coding sequence were designed using Invitrogen's BLOCK-iT™ RNAi Designer software (Invitrogen, Carlsbad, CA). To minimize off-target effects, a BLAST search against other *Microtus* sequences was performed to verify specificity. We subsequently confirmed using a BLAST search of the recently available *M. ochrogaster* genome (taxid:79684) that the shRNA sequences do not target any gene other than the *Avpr1a*. Three sequences targeting exon 1 and two targeting exon 2 were chosen based on the efficacy predicted by the algorithm (Figure 2.1A,B). One scrambled control sequence, composed of the same nucleotide makeup as sh4141 but in a different order, was also designed and does not target any known mammalian gene sequence. Numbers in the shRNA descriptors indicate the distance from the beginning of the *M. ochrogaster Avpr1a* locus as numerated in the Genbank entry Accession number AF069304.2. Sequences were cloned into a pENTR™/U6 vector, with polymerase III-dependent U6 driven expression, sequenced to identify clones with proper insertion, and tested for knockdown efficacy *in vitro* before *in vivo* use. ShRNAs were cloned into adeno-associated viral vectors also expressing GFP for *in vivo* testing (Figure 2.1C). Similar shRNA technology has been used before to successfully knockdown target gene expression in other rodents (Musatov et al., 2006; Tiscornia et al., 2006; Garza et al., 2008).

(A)	sense sequence (passenger)	hairpin loop	antisense sequence (guide)
scrambled	GCTCCGTGACTTCACTACTCA	CGAA	TGAGTAGTGAAGTCACGGAGC
sh3854	GCACCTCTTCATCCGACATCT	CGAA	AGATGTCGGATGAAGAGGTGC
sh4141	GCACGCCTCAGTACTTCATCT	CGAA	AGATGAAGTACTGAGGCGTGC
sh4312	GCTTCATCTGCTACCACATCT	CGAA	AGATGTGGTAGCAGATGAAGC
sh7010	GCAAGACTGTGTCCAAAGTTT	CGAA	AAACTTTGGACACAGTCTTGC
sh7087	GCAGAAGACATACTTCTTACT	CGAA	AGTAAGAAGTATGTCTTCTGC

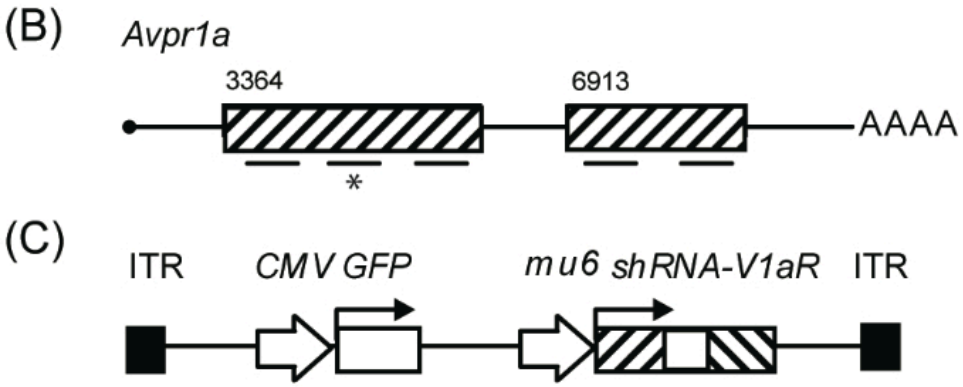

Figure 2.1. Design of short hairpin RNA sequences

Candidate shRNA sequences consisted of a 21nt sense sequence followed by a 4nt hairpin loop and a 21nt antisense guide strand that is complementary to the target *Avpr1a* sequence (A). Three shRNA sequences against exon 1 and two against exon 2 of the prairie vole *Avpr1a* gene were designed (B) and ultimately inserted into an adenoassociated viral vector driving shRNA expression with murine U6 and co-expressing GFP under control of a uniquitous CMV promoter (C). The asterisk indicates sh4141, the sequence used to generate virus for behavioral testing.

Cell culture testing of shRNA sequences.

To identify the most effective shRNA sequence, an *in vitro* reporter assay tested the ability of 5 shRNA plasmids to knockdown a prairie vole *Avpr1a-GFP* fusion protein in comparison to a scrambled control sequence. As specific antibodies against the V1aR are not available, the amount of GFP immunoreactivity relative to scrambled transfected

controls was used to assess knockdown of the V1aR. The day before transfection, HEK 293T cells were plated with Dulbecco Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and incubated at 37˚C in 6-well plates (5ml media and 2E6 cells per well). Once at 70-80% confluence, 3ml media was removed and cells were cotransfected with 6μg of RNAi entry vector, 2μg of a target *Avpr1a -GFP* fusion protein vector, and 10μl Lipofectamine-2000 Invitrogen transfection reagent brought up to 500μl with Opti-MEM (Invitrogen, Carlsbad, CA). After 4-6hr, 2.5ml DMEM was added back. Knockdown was assessed 48hr after co-transfection in three experiments, each with duplicate wells. Half of each sample was saved for analysis by either western blot or quantitative real-time PCR.

Western blot analysis.

Cells were washed with cold phosphate-buffered saline (PBS) and homogenized in Ripa Lysis Buffer (sc-24948, Santa Cruz Biotechnology). After determining protein concentration by BCA assay (Pierce, Rockford, IL), 5mg of denatured protein extract was loaded into NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were separated by gel electrophoresis and transferred to a nitrocellulose membrane. The blot was blocked with 2% milk and incubated in 1:1000 rabbit anti-GFP (A6455, Invitrogen, Carlsbad, CA) for 80min. Primary antibody reactivity was detected by incubation 1:10,000 anti-rabbit fractionate and detected using Pierce SuperSignal West Pico Chemiluminescence followed by 20s film exposure. Blots were stripped for 15 minutes with Pierce ReStore Plus stripping solution and incubated in primary antibody specific for GAPDH (10R-G1099, Fitzgerald, Acton, MA) for 80 minutes at a dilution of 1:5000. Primary antibody reactivity was detected by incubation in secondary antibody and

detected using Pierce SuperSignal West Pico Chemiluminescence followed by 15s film exposure. Images were taken with Alpha Innotech imager, and optical density measuremets were analyzed using NIH Image J Software. *In vitro* knockdown efficiencies were determined relative to the scrambled control.

Quantitative real time PCR (qRT PCR).

RT PCR was performed on transfected cells using the POWER SYBR Green Cells-to CT Kit according the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, cells were washed with cold PBS, counted, and mixed with cell lysis buffer. Genomic DNA was degraded by treatment with DNAse. Complementary DNA (cDNA) was synthesized by reverse transcription under the following cycling conditions: 60 min at 37°C; 5 min at 95°C; hold at 4°C. Quantitative PCR was performed with 4ul cDNA, 10mM forward and reverse primers, and 12.5ul Power SYBR Green PCR master mix on an AB 7500 (Applied Biosystems, Carlsbad, CA) under the following cycling conditions: 10 min at 95°C, 40 cycles of 15s at 95°C and 1min at 60°C. Both *Avpr1a* (FWD 5-ACTGAGCACGCCTCAGTACTTCAT-3, RVS 5-TGAAGCCATAGCAGGTACCCAAGA-3) and human GAPDH control (FWD 5-AGCCTCAAGATCATCAGCAATGCC-3 and RVS 5-TGTGGTCATGAGTCCTTCCACGAT-3) primer sets were used. Relative quantification to cDNA from scrambled transfected cells using the ΔΔCT method was used to compare expression across samples. The reference gene GAPDH was used as an internal control for each individual, and samples from three replicate experiments were run in duplicate. Fold changes in expression were calculated as $2^{(-\Delta\Delta CT)}$.

Animal handling and care.

Animals were laboratory-bred male prairie voles, derived from a field-caught stock originating from Illinois. The colony is systematically outbred to maintain genetic diversity. Animals were maintained on a 14:10 h light:dark cycle with lights on at 07:00 at 22˚C with access to food (Purina high-fiber rabbit chow) and water ad libitum. Breeder housing consisted of large ventilated cages (34 × 30 × 19 cm) lined with bedding (bed-o-cob, Maumee, OH, USA). At 21 days of age, male subjects were injected with either scrambled (n=13) or shRNA-*Avpr1a* (n=14) virus, and weaned into same-sex same-treatment pairs or trios in smaller (30 × 18 × 19 cm) cages. No animals were exposed to subsequent litters in their natal group. Subjects remained undisturbed until adulthood, at which point they underwent testing for partner preference, alloparental behavior, and anxiety-like behavior in the elevated plus maze (EPM), with a two-week interval between each test (PND60-100). Stimulus animals were estrogen-primed sexually-experienced ovariectomized adult female voles, and served as either "partner" or "stranger" in the partner preference test (see below). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Viral vector production.

Sequences verified to knockdown *in vitro* were made into adeno-associated viral (AAV) vectors as previously described (Ross et al., 2009). The constructs consisted of a cytomegalovirus (CMV) promoter driving GFP expression, followed by a high-output murine U6 Pol III element driving expression of the shRNA sequence. The AAV vectors used in this study are of the AAVrh10 serotype, which is highly neurotropic and exhibits

particularly strong gene transfer into brain neurons. AAV vector stock titters were 10^{13} infectious particles per ml as determined by real-time PCR. To verify their knockdown ability *in vivo*, 1μl of either AAV-sh4141 and AAV-sh7087 were injected unilaterally into the mediodorsal thalamus (MD Thal; coordinates -0.14 anteroposterior (A/P), -0.11 mediolateral (M/L), -0.35 dorsoventral (D/V)) in 3 animals each with methods as described below. Brains were harvested at least 2 months after injection.

Viral vector injection.

In order to knockdown V1aR expression long-term, males were injected at the time of weaning with shRNA-*pvAvpr1a* and expression lasted through adulthood. Juvenile male prairie voles were bilaterally injected into the ventral pallidum with 1μl of sh4141, the AAV virus found to most efficiently produce *Avpr1a* knockdown *in vitro* and *in vivo*, from here on simply referred to as shRNA-*pvAvpr1a* (n=14) or the scrambled AAV control (n=13). AAV infusions were performed under isoflurane anesthesia in a Kopf stereotax fitted with an Ultra Micro Pump II (World Precision Instruments, Sarasota, FL) apparatus and a 26 gauge 5ml Hamilton syringe. Ventral pallidum coordinates were modified from (Lim et al., 2004b) for juveniles and verified using dye injections. Coordinates relative to bregma in the anterior-posterior and medial-lateral planes and the top of the skill at the injection site in the dorsal-ventral plane were +0.14 A/P, ±0.08 M/L, and –0.54 D/V. Virus was infused at a rate of 3nl/s, and the needle was left in place for 3 min after the injection to prevent capillary action. The incision was closed with absorbable sutures (Vicryl 5-0 Ethicon, Piscataway, NJ). Animals were group housed until adulthood when they underwent behavioral testing. Subjects were paired with a female for a total of 3d for partner preference testing. As males display mating-

induced aggression, animals were singly housed for the remainder of behavioral testing. After all of the behavioral studies, brains were harvested and analyzed for V1aR knockdown using autoradiography and injection site accuracy using GFP fluorescence. Preliminary studies indicated that expression was stable after 14d.

Behavioral testing

Partner preference test.

After 1.5-2 months of viral expression, adult males (range 64-78 days of age) were paired with age-matched, sexually-experienced stimulus females in a clean cage. Females were ovariectomized and induced into estrus with 2μg estradiol benzoate (Sigma-Aldrich BP958) daily for 3 days prior to pairing as previously described (Modi and Young, 2011). The cohabitation period was video-recorded for 3 hr to verify mating. Four animals that did not mate were excluded from the analysis (three from the control group and one from the shRNA-*pvAvpr1a* group). Partner preference was performed 24hr after pairing using a paradigm previously described (Williams et al., 1992). After 24hr of cohabitation, the male subjects were put in the center of a 3-chambered arena with "partner" females tethered to one end and novel "stranger" females tethered to the other. "Stranger" females underwent the same sociosexual experience as the "partners." The experimental animal was allowed to freely move throughout the arena. Time spent in immobile social contact (huddling) with each female during this 3hr session is scored with an automated system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA) as previously described (Ahern et al., 2009). After testing, males were placed back in the cohabitation cages alone, and females were returned to the colony.

Alloparental behavior.

Because exposure to pups has been shown to alter subsequent social behavior (Stone et al., 2010), alloparental care was assessed after partner preference testing. Two weeks after partner preference testing, males (age range 75-95d) were tested for their willingness to care for novel, genetically unrelated stimulus pups. Subject animals were habituated to the testing room for at least 1 hr. They were then placed in a large clean cage (37 × 31 × 19cm) lined with bedding (bed-o-cob) and allowed to acclimate for 10min. The test began when 2 novel pups (PND2-5) were placed in the opposite corner. For 10min the latency, frequency, and duration of behaviors including pup licking, carrying, and crouching over pups were scored live by an observer blind to the experimental condition (Stopwatch+, Center for Behavioral Neuroscience, GA; http://www.cbn-atl.org/ research/stopwatch.shtml). Testing immediately stopped if pups were attacked. A latency of 600s was assigned if subjects did not approach the pups for the duration of the test. Males were categorized as "alloparental" if they spent a total of at least 5s licking and 30s crouching over the pups without attacking (Lonstein and De Vries, 1999; Olazábal and Young, 2005).

Elevated Plus Maze (EPM).

At least two weeks after alloparental testing, males (age range 100-109) were tested for anxiety-like behavior in an EPM as previously described (Ahern and Young, 2009). Males had been isolated for at least 4 wks prior to EPM testing. Animals were habituated in a room adjacent to the behavioral testing room for at least 1 hr prior to testing, which occurred between 09:00 and 12:00 h. The apparatus was raised 80 cm above the floor, and consisted of 2 closed arms (50 × 6 × 15.5 cm), 2 open arms (50 × 6 ×

0.6 cm), and a center platform (6 × 6 cm). The subjects were placed in the center, and behavior was scored from above using an automated scoring system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA) for 5 min. The arena was cleaned with 5% EtOH between animals. Arms were further divided into distal and proximal subsections, as willingness to enter the distal portion of the open arm may be a more potent measure of anxiety (Wright et al., 1992; Fernandes and File, 1996; Garcia-Cairasco et al., 1998). Raw measurements included duration, frequency, and distance in each subsection (open, distal open, closed, distal closed, center) and total distance moved in the entire arena. Measures relevant to anxiety and exploration were then calculated as follows: percent entries in the distal open arms [entries into distal open arms/ (entries into distal open arms + entries into distal closed arms) X 100], percent duration in the distal open arm [time in distal open arms/ (time in distal open arms + time in distal closed arms) X 100]. The same calculations were performed for percent time and entries into the entire open arms.

Brain harvest and processing.

At the conclusion of behavioral testing, subject males were euthanized with CO2 inhalation in their home cage. Brains were rapidly removed from the skull, frozen in methylbutane chilled with dry ice, and stored at -80˚C until sectioning. The brains were sectioned through the ventral pallidum in 4 series at 20μM onto super-frost plus slides (Fisher Scientifi c, 12-550-15), and maintained at −80°C until assayed.

GFP visualization.

One series of slides was coverslipped with Krystalon (EMD Chemicals) to visualize GFP expression at the injection site. Subjects were excluded if GFP expression

was not observed in the ventral pallidum. Four shRNA and one scrambled-injected animals were excluded from the autoradiographic and behavioral analyses due to injection misses. One shRNA-injected animal was only successfully targeted unilaterally, but was kept in the analysis. This left samples sizes of n=9 s shRNA-*pvAvpr1a* and n=9 scrambled for brain and behavioral analyses.

Receptor autoradiography.

Receptor binding for V1aR to determine whether group differences in expression was achieved, and for oxytocin receptor (OTR) as a control, was localized by autoradiography as described previously (Young et al., 1997; Ahern et al., 2009; Ross et al., 2009b). Slides were removed from -80˚C storage, air dried, dipped in 0.1% paraformaldehyde-PBS (pH 7.4), and washed twice in 50mM Tris buffer (pH 7.4) to remove endogenous OT and AVP. Sections were then incubated for 1 hr in tracer buffer (pH 7.4) containing 0.05nM of either ^{125}I- linier V1a antagonist for V1aR (NEX310050UC Perkin Elmer) or ^{125}I-OVTA for OTR (NEX 254050UC PerkinElmer). Slides were washed four times in 4˚C 50mM Tris-MgCl (pH 7.4), followed by a 30min room temperature stirring rinse to remove unbound radioligand. After a dip in dH2O and air drying, slides were exposed to BioMaxMRfilm (Kodak, Rochester, NY) for 72 hr for quantification and a subsequent 8d for higher resolution images. For quantification, ^{125}I autoradiographic standards (ARI 0133A, American Radiolabeled Chemicals) were included in the cassette.

Autoradiograms were quantified as previously described (Phelps and Young 2003, Lim et al., 2005, Ahern 2009). Films were digitized (MTI CCD72 camera) and quantified using AIS software version 6.0 (Imaging Research Inc., Ontario, Canada).

Optical density measures of V1aR binding were taken for each brain region bilaterally and averaged across 6-12 sections encompassing the entire ventral pallidum (VP), and 3-4 sections for the olfactory bulb (OB), lateral septum (LS), and S1 region of the cortex by an experimenter blind to the experimental treatment. A total of 9 animals from each group were analyzed for V1aR expression. To assess the specificity of knockdown, OTR binding the in nucleus accumbens (NAcc) shell and core was quantified across 6-12 sections. Tissue was damaged from one shRNA-injected male, thus leaving 8 shRNA and 9 scrambled subjects for OTR analysis. For initial *in vivo* testing of the virus, 5-10 bilateral sections in the MD Thal were measured for V1aR binding. Optical densities were converted to decompositions per minute (DPM)/mg tissue equivalents using ^{125}I microscale standards on each film. Specific binding was calculated by subtracting non-specific background binding in the S1 region of the cortex from total binding in each region. This region was chosen over the corpus callosum because it contains neuronal cell bodies rather than just axon tracks.

Perfusion and immunohistochemistry.

An additional 5 adult animals were injected bilaterally into the ventral pallidum with 1μl of either shRNA-*pvAvpr1a* or scrambled virus for post-processing with immunohistochemistry for GFP to assess viral spread and neuronal health. At least 2 months after injection, an approximately equivalent length of viral expression in the experimental subjects, animals were sacrificed by isoflurane. Animals were perfused transcardially with 4% paraformaldehyde. Brains were then removed, stored in 4˚C sucrose, and sectioned coronally at 40μM on a freezing microtome. Free-floating sections were stored in cryoprotectant in a 24 well tissue culture plate until assayed for GFP

expression. Every other section (every 80μM) was run in one assay. Sections were rinsed thoroughly in PBS (pH 7.4) to remove cryoprotectant, pre-blocked in PBS containing 0.3% Triton X-100 and 5% normal goat serum (NGS), and incubated for 44 hr at 4˚C with 1:2000 chicken polyclonal anti-GFP (ab13970, Abcam, Cambridge, MA) in PBST with 1%NGS. Tissue was then rinsed with PBST, and incubated in 1:2000 Alexa fluor anti-chicken secondary antibody (A-11039, Invitrogen, Carlsbad, CA) in PBST for 3 hr at 4˚C. After washing in PBST followed by PBS, sections were mounted onto superfrost-plus slides, air dried, and coverslipped using vectashield-mounting medium (Vector Labs, Burlingame, CA).

Statistical analyses.

Data are presented as mean ± SEM, unless otherwise noted. The normality of behavioral data was tested with a one-sample Kolmogorov-Smirnov test. In the partner preference test, time spent huddling with the partner and stranger animals was analyzed with repeated measures (RM) ANOVA with viral treatment as a between subjects factor and stimulus animal as a within-subjects repeated measure. Planned *post-hoc* paired t-tests were performed on huddling time with partner versus stranger if a significant interaction effect was detected. A Fisher exact test was used to compare the number of animals that formed a partner preference in each treatment group, with preference defined as spending twice as much time in social contact with the partner over the stranger animal. In addition to group differences, behavioral parameters in the partner preference test after 24hr of cohabitation were correlated with V1aR binding using linear regression. In the alloparental test, one-way ANOVAs were performed after excluding one scrambled injected animal that attacked the pups. A Fisher exact test was used to

compare the proportion of animals defined as alloparental between groups. One-way ANOVAs were used to compare behavior in the EPM between groups, and linear regression was used to correlate behavior with V1aR expression. To determine whether group differences in V1aR expression was achieved, independent t-tests were used to compare mean V1aR in the VP, LS, and OB. Similarly, OTR expression in the NAcc core and shell was analyzed with independent t-tests. Statistics were performed with SPSS Statistics 17.0 with significance set at $p<0.05$ and two-tailed tests unless otherwise noted.

Results

In vitro and *in vivo* efficacy.

The 5 candidate shRNA sequences were tested for knockdown of a target V1aR-GFP fusion protein by examination of immunofluorescence, qRT-PCR, and western blot. V1aR mRNA was suppressed 83-93% and protein levels were reduced 71-88% as compared to scrambled transfected controls (Fig 2.2A-C). Neurotropic AAV vectors were constructed from the shRNA sequences that consistently and efficiently knocked down expression *in vitro* (sh4141 and sh7087) and tested for knockdown ability *in vivo*. When injected unilaterally into the MD Thal, AAV-sh4141 and AAV-sh7087 were found to knockdown 41% and 32% of *Avpr1a* expression in comparison to the non-injected site (Fig 2.2D). Thus, we proceeded with AAV-sh4141 in the behavioral experiments.

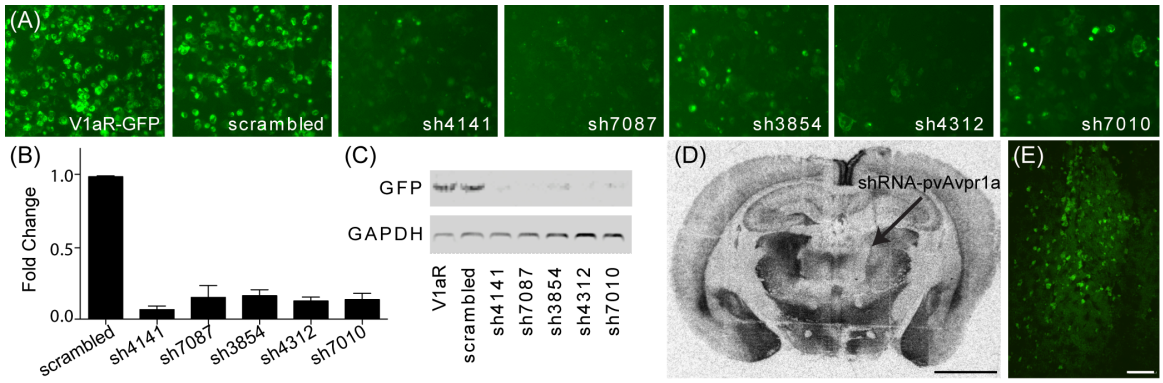

Figure 2.2. Characterization of shRNA knockdown in vitro and in vivo.

ShRNA plasmids targeting different regions of the prairie vole *Avpr1a* gene, but not a scrambled control, knocked-down expression of a V1aR-GFP fusion protein as assessed by GFP visualization in HEK 293T cells (A), quantitative RT PCR (B), and western blot (C; V1aR-GFP, 74kDa, GAPDH, 37kDa) Unilateral injection of shRNA(sh4141)-*pvAvpr1a* into the mediodorsal thalamus resulted in 41% average knockdown as compared to contralateral expression (D, scale bar 1mm). Viral injection sites were verified with GFP visualization (E, scale bar 50μm).

Partner preference testing results.

Males injected with shRNA-*pvAvpr1a* showed significant impairments in partner preference after 24hr of cohabitation with a sexually receptive female (Figure 2.3A-B). RM-ANOVA revealed that there was a significant main effect of virus ($F_{(1,16)}$=6.36, p=0.023) between groups, indicating the scrambled males spent more overall time in social contact than did the shRNA males. A significant interaction between virus and time spent with either stimulus animal was detected ($F_{(1,16)}$= 48.84, p<0.001), with shRNA males spending less time in social contact overall. There was no main effect of stimulus animal across both groups ($F_{(1,16)}$=3.25, p=0.09). Planned *posthoc* pairwise t-tests revealed significantly more time spent huddling with that partner over the stranger in the scrambled group (p=0.001), but significantly more time spent with the stranger over the partner in the shRNA group (p=0.002; Figure 2.3A). Additionally, significantly

more scrambled animals formed a partner preference ($p=0.0004$, Fisher's test, Figure 2.3B). As a measure of activity, distance travelled in the center arena was compared between groups and there was no difference ($p=0.336$), suggesting differences in testing were not due to overall changes in locomotion. Finally, to compare the degree of *Avpr1a* knockdown to partner preference behavior, VP V1aR densities were compared to time spent huddling with the partner or stranger during the test. Across both treatments, V1aR densities significantly predicted time spent with the stranger ($R=-0.523$, $R^2=0.273$, $p=0.026$), with high expressing animals spending less time in stranger contact, however no correlation was detected between V1aR and partner contact.

Alloparental behavior.

One scrambled injected animal was removed from the analyses of mean duration, frequency and latency of paternal care because of attacking the pups. No significant group differences in any paternal behavior measured were observed ($p>0.05$, one-way ANOVA). Likewise, there was no significant difference between scrambled (n=6/9) and shRNA (n=9/9) in the number of animals that displayed alloparental care ($p=1.00$, Fisher's test).

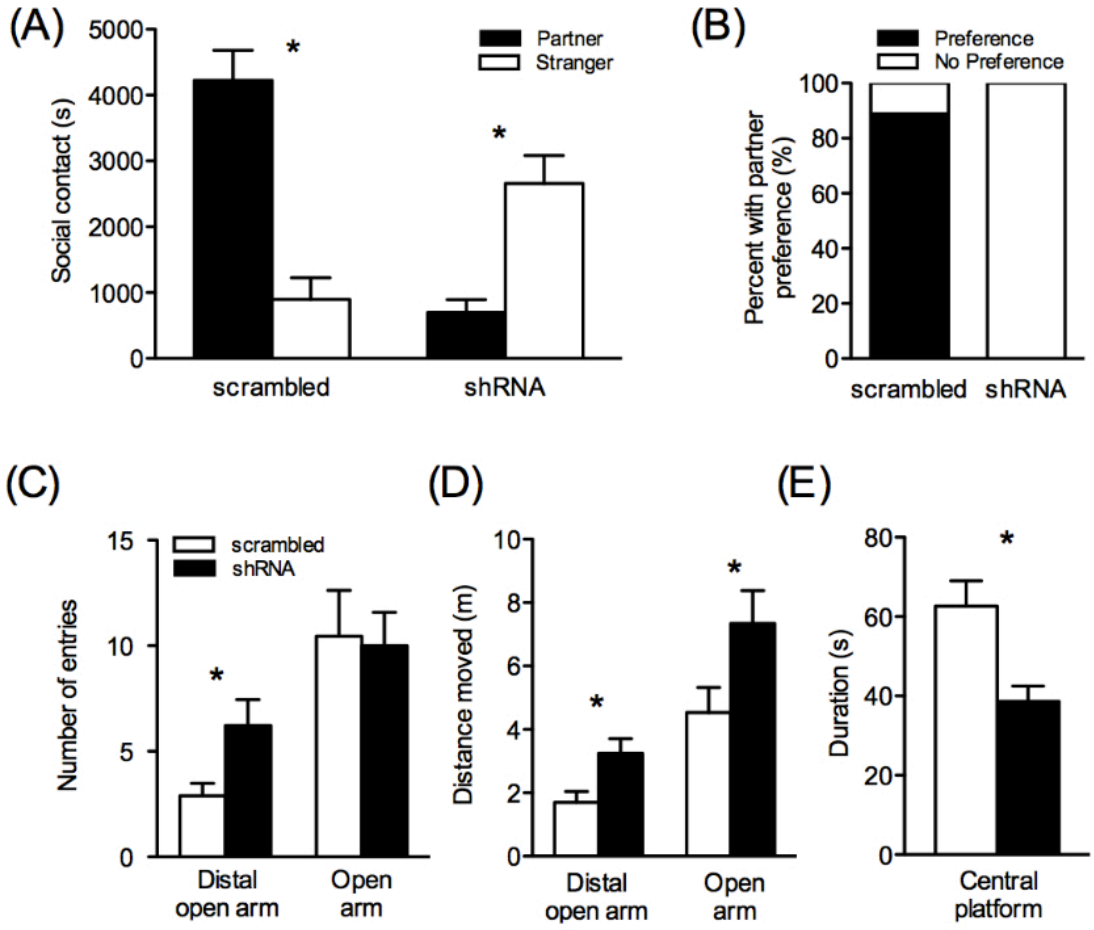

Figure 2.3. Partner preference and elevated-plus maze behavior in shRNA-*pvAvpr1a* and scrambled injected male prairie voles.

After 24hr of mated cohabitation with a female, scrambled injected males spent significantly more time in social contact with the partner than the stranger, whereas shRNA injected males spent significantly more time with the stranger (A). The percent of animals forming a partner preference, defined as spending more than twice as much time with the partner than the stranger, differed significantly between groups, with no shRNA injected males forming a preference after 24hr (B). shRNA injected males displayed decreased measures of anxiety-like behaviors, with a higher frequency of distal, but not entire, open arm entries (C), a greater distance moved in the distal and entire open arms (D), and less time spent in the central platform (E). Asterisks indicates p-values <0.05.

Elevated plus maze.

No difference in the time spent in the entire open ($F_{(1,16)}$=0.116, p=0.737, one-way ANOVA) or closed ($F_{(1,16)}$=0.895, p=0.358, one-way ANOVA) arms was detected between shRNA and scrambled males. Groups also did not differ in total number of entries into the entire open ($F_{(1,16)}$=0.027, p=0.871, one-way ANOVA, Figure 2.3C) or closed ($F_{(1,16)}$=0.636, p=0.437, one-way ANOVA). However, the shRNA males entered the distal portion of the open arm significantly more than did the scrambled injected males ($F_{(1,16)}$=6.04, p=0.026, one-way ANOVA, Figure 2.3C), indicative of decreased anxiety. Similarly, the shRNA group moved a greater distance in both the distal open arm ($F_{(1,16)}$=7.25, p=0.016, one-way ANOVA, Figure 2.3D) and the entire open arm ($F_{(1,16)}$=4.70, p=0.046, one-way ANOVA, Figure 2.3D). Time spent in the central platform also differed between groups, with scrambled males spending significantly more time in this region ($F_{(1,16)}$=10.17, p=0.006, one-way ANOVA, Figure 2.3E). No difference in overall activity in the EPM as measured by total distance moved was detected ($F_{(1,16)}$=3.44, p=0.082, one-way ANOVA). Duration in the central platform correlated both with V1aR binding in the VP (R=0.495, R^2=0.245, p=0.037) and with time spent huddling with the partner after 24hr cohabitation (R=0.512, R^2=0.262, p=0.030) across both treatment groups. Only within the shRNA injected group, percent entries into the open arm correlated with pallidal V1aR (R=-0.667, R^2=0.444, p=0.045).

V1aR, OTR, and GFP expression.

Autoradiography was performed to assess the degree of knockdown in the ventral pallidum. Figure 2.4 shows representative images of V1aR in the ventral pallidum (A,B),

and OTR binding in the NAcc (E,F) between shRNA and scrambled males. Injection site was verified by visualization of GFP expression, and a total of 5 animals were excluded from all analyses (1 scrambled, 4 shRNA). Injection of the shRNA-*pvAvpr1a* vector led to a 30% reduction in V1aR binding in the ventral pallidum as compared to scrambled controls ($p=0.008$, t-test, Figure 2.4D). V1aR binding in the LS ($p=0.13$) or OB ($p=0.477$) and OTR binding in the nucleus accumbens (NAcc) core ($p=0.28$) or shell ($p=0.26$) was not significantly different between groups (t-tests). Additional animals were injected with either virus to visualize viral spread with immunohistochemistry on perfused tissue. Representative images of GFP expression as visualized by immunohistochemistry shows clear neuronal processes and health of cells, confirming that neurons were successfully targeted and alive (Figure 2.4G,H). Thus, the observed knockdown of V1aR binding was not due to neuronal death.

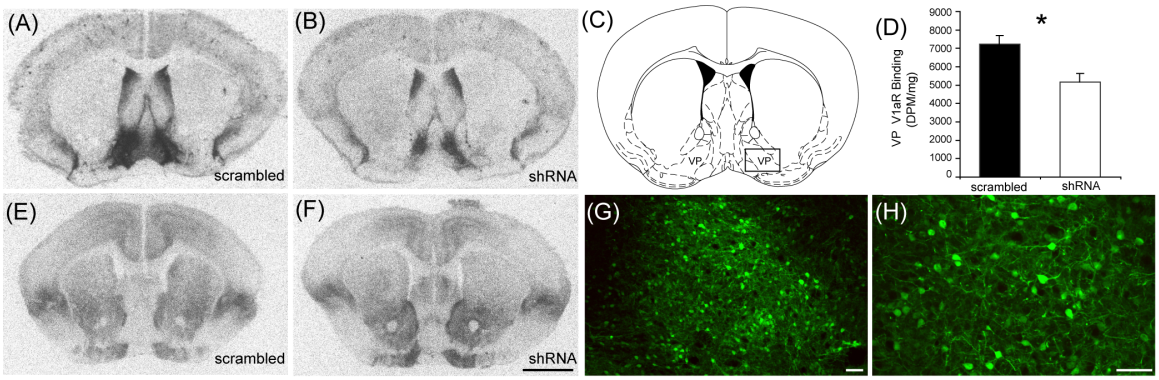

Figure 2.4. Analysis of V1aR knockdown, OTR and GFP expression in shRNA-*pvAvpr1a* and scrambled injected male prairie voles.

Representative V1aR autoradiograms display a reduction in binding in shRNA-injected animals as compared to control, scramble injected animals (A, B). The square surrounding the ventral pallidum (VP) represents the location of injection sites (reprinted from Paxinos and Watson, 1998) (C). V1aR expression was reduced by 30% in shRNA-injected subjects as compared to controls (D). OTR expression in the nucleus accumbens was unchanged, as seen in representative autoradiograms (E, F). GFP immunoreactivity at the injection site displays typical viral spread (10X, G) and clear expression in neuronal cell bodies and processes (20X, H). Scale bars are 1mm in F and 50μm in G, H. Asterisk indicates p-value <0.05.

Discussion

These results support the hypothesis that variation in *Avrp1a* expression directly contributes to natural variation in pair bonding and anxiety-related behaviors and are consistent with previous work demonstrating the role of V1aR in promoting partner preferences (Donaldson et al., 2010) and increasing anxiety (Pitkow et al., 2001). We achieved approximately 30% knockdown of V1aR binding in the ventral pallidum of male prairie voles, comparable to the degree of natural variation seen in this brain region (Phelps and Young, 2003; Hammock et al., 2005; Ophir et al., 2008a). This chronic, but only partial, down-regulation of expression profoundly impaired partner preference formation after one day of cohabitation, with shRNA males displaying significantly more

social contact with a novel stranger female. V1aR density in the VP correlated with time huddling with the stranger during testing, indicating that the degree of intraspecific variation in expression is behaviorally relevant. However, there was no difference in alloparental responsiveness between virally injected males. Vasopressin-mediated pair bond formation and paternal care may be controlled by disparate neural circuitry, as enhancing VP V1aR density in meadow voles also promotes partner preference but not alloparenting (Lim et al., 2004b). In the EPM, shRNA males displayed a reduction in anxiety-like behaviors. Those with low V1aR binding entered and explored distal ends of the open arms more willingly, and spent less time in the central platform. Duration in the central platform has previously been associated with anxiety, approach/avoidance conflict and reduced decision making, and is reduced after treatment with anxiolytics (Cruz et al., 1994; Rodgers and Johnson, 1995; Ohl et al., 2001; Olazábal and Young, 2005). Thus, natural variation in *Avpr1a* expression contributes to variation in both behavioral responsivity to a stressor and sociality.

Pair bonding is believed to arise from neuropeptide-mediated coding of socially-relevant cues of a partner (e.g. olfactory signatures) converging with dopaminergic and opioid mediated reward and reinforcement pathways activated during mating or social interaction (Young and Wang, 2004b; Burkett and Young, 2012a). This convergence essentially leads to a conditioned partner preference. The VP receives direct GABAergic input from the NAcc and dopamanergic input form the ventral tegmental area, and is associated with natural and drug-related reward(Childress et al., 2008). The VP can be further subdivided into medial and lateral divisions, which are functionally and anatomically distinct. The medial VP receives input from the NAcc shell, projects to the

VTA and MDthal, and is thought to be involved in limbic motivation and reward (Lim et al., 2004a). In contrast, the lateral VP receives input from the NAcc core, projects to the substantia nigra, subthalamic nucleus, and globus pallidus, and is thought to be important for reinforcement driven motor behavior. V1aR cell bodies are located within the medial VP (Lim et al., 2004a). Blockade of VP V1aR inhibits the formation of a pair bond (Lim and Young, 2004) and over-expression accelerates partner preference formation in male voles (Pitkow et al., 2001; Lim et al., 2004b). Thus, V1aR in the VP is thought to strengthen the neural connections encoding social cues and reward, and this process appears directly related to V1aR density in the VP. A naturalistic-degree of V1aR knockdown in this region significantly impaired male partner preference formation, possibly by reducing the strength of the connection between the neuropeptide's and reward circuitries. More generally, the VP is an important integrator of limbic and motivational processing. Indeed, we saw a reduction in overall time spent in social contact in shRNA injected males, suggestive of a decrease in social motivation. Whether a long-term reduction in V1aR expression has consequences on the expression or function of other receptor systems remains to be explored, although we did not detect a difference in OTR NAcc expression. Interestingly, in kidney cell lines, knockdown of *Avpr1a* expression resulted in an impairment of aldosterone function by reducing transport of the mineralcorticoid receptor (Izumi et al., 2011; Hori et al., 2012). Thus, the use of RNAi may help reveal such downstream molecular effects of V1aR in the brain.

In addition to linking to social reward circuitry, the V1aR may also impact social attachment through its regulation of anxiety. Evolutionarily, it has been hypothesized that demanding environments with sparse resources were one of the driving forces behind

prairie vole mating systems; and indeed activation of the stress axis promotes male pair bonding (Getz, 1978; DeVries et al., 1996). Infusion of V1aR antisense oligonucleotides or antagonists into the LS or complete knockout of the V1aR reduce anxiety-like behaviors, whereas V1aR over-expression in the LS or VP increases these behaviors (Landgraf et al., 1995; Liebsch et al., 1996; Pitkow et al., 2001; Bielsky et al., 2005). AVP originating in the MeA and BSNT projects directly to the VP, thus the VP may integrate stress and anxiety states with striatopallidal pathways mediating reward and reinforcement. We observed reduced anxiety-like behavior in the males with partial V1aR knockdown. Time spent in the central platform correlated with partner huddling in the partner preference test, suggesting a possible interaction between the V1aR in linking social cues with the reward system, and in regulating emotionality. Diversity in VP V1aR, both between and within species, may be a means to behaviorally adapt to evolutionary pressures. It is possible that in times of stress, VP V1aR density may increase, in turn promoting a male's willingness to pair bond and enhancing behavioral responsivity to stressors.

More broadly, the VP is an important integrator of emotional states and affect. Reduction in VP mu-opioid signaling are associated with sadness in humans (Zubieta et al., 2003). Opioid signaling in the VP is also thought to modify mesolimbic dopamine signaling from the VTA and is necessary and sufficient for the development of behavioral sensitization to morphine (Mickiewicz et al., 2009). Striatal mu-opioid stimulation promotes partner preference formation in female prairie voles, although the role in males has not been explored (Burkett et al., 2011b; Resendez et al., 2012; Resendez et al., 2013). Perhaps, high-anxiety males with presumably reduced opioid signaling would be

benefit more from positive effects of mating-induced opioid release on emotionality, and thus be more willing to form a partner preference.

Although even a 30% reduction in pallidal V1aR density has behavioral relevance, V1aR densities in certain other socioemotional brain regions can vary even more dramatically between prairie voles, with up to two-fold differences in expression between individuals (Phelps and Young, 2003). Variation in V1aR density in the laterodorsal thalamus, posterior cingulate, LS, and medial amygdala has also been correlated with social behavior in prairie voles (Hammock et al., 2005; Hammock and Young, 2005). V1aR binding in the laterodorsal thalamus and posterior cingulate predicts pair bonding measures in the field and may be important in space use and territoriality in wild populations (Ophir et al., 2008c). Within-species variation in the LS of prairie voles may be behaviorally relevant, as males with high levels V1aR exhibit enhanced social motivation to investigate females (Ophir et al., 2009). Examination of region-specific expression both in laboratory and naturalistic contexts can offer insight to the neural mechanisms controlling individual variation in behavior.

RNAi may be a particularly useful tool to study the causal effects of diversity in receptor expression in an ethologically significant context. Antisense oligonucleotides have been used to temporarily knockdown neural *Avpr1a* expression previously (Landgraf et al., 1995; Bosch and Neumann, 2008b; Kelly et al., 2011). However, to our knowledge, this is the first report to use viral vector mediated RNAi to manipulate endogenous gene expression in the brain in a nontraditional mammalian species. RNAi has several advantages to other approaches used to investigate the role of neuropeptide receptors in regulating behavior. Viral vector mediated over expression, which has been

used in voles (Pitkow et al., 2001; Lim et al., 2004b), mice (Bielsky et al., 2005) and rat (Landgraf et al., 2003), drives expression of transgenes indiscriminately and ectopically in the infused region, making circuits not involved in the natural regulation of the behavior sensitive to the peptide. Additionally, commonly used pharmacological manipulations are transient and use agents that often diffuse far beyond the site of infusion and bind to related receptor subtypes (Manning et al., 2008), and are optimally designed for studying acute effects, rather than long-term investigations into the role of social neuropeptides. Conventional knockout mouse approaches often involve developmental compensation (Landgraf, 2006). RNAi utilizes an endogenous regulatory pathway to selectively degrade double stranded RNA and results in a reduction, not absolute loss, of the targeted transcript. Consequently, in our study, the reduction in V1aR expression was limited to the region near the injection site, was within the range of what is naturally observed, and only affected cells that endogenously express the *Avpr1a*.

RNAi also has limitations in terms of potential off-target effects (Grimm et al., 2006; Manjunath et al., 2009). Indeed, there is a possibility that the shRNA sequences used here could target other genes, which could in turn impact behavior. It is important to note that there was no impact on the expression of OTR, which shares high homology with V1aR, in the adjacent NAcc, suggesting that the knockdown was specific to the *Avpr1a*. Furthermore, a BLAST search of the available *M. ochrogaster genome* did not reveal any other sequences within the genome matching our shRNA.

Viral spread may have reached surrounding regions including the NAcc and LS as has been reported before (Pitkow et al., 2001), but only significant knockdown in the VP was achieved, and regions without V1aR were not affected. Thus, a viral vector mediated

shRNA approach provides an ideal means to examine the impact of natural variation in neuropeptide receptor on behavioral diversity in an ethologically relevant manner, and subjects can be examined over multiple behavioral tests.

Social and mating systems in *Microtus* are evolutionarily labile and thus must evolve rapidly under pressure of natural selection forces. Even within the prairie vole species, there is substantial variation across geographic locations, environmental conditions, and within shared enclosures (Thomas and Birney, 1979; Roberts et al., 1998c). It has been proposed that the polymorphic microsatellite in the 5' flanking region of the prairie vole *Avpr1a*, due to inherent instability of the repetitive DNA, may generate diversity in *Avpr1a* gene expression and therefore behavior. Microsatellite length has been associated with V1aR expression in the ventral pallidum, with long allele males displaying approximately 20% higher levels of binding (Ophir et al., 2008a). However, the same report did not find a link between microsatellite length and reproductive success or mating tactics in field enclosures. Although there are conflicting reports on the specific relationship between microsatellite lengths, site-specific V1aR binding, and laboratory and field behavior (Hammock et al., 2005; Hammock and Young, 2005; Ophir et al., 2008a; Solomon et al., 2009; Mabry et al., 2011), these discrepancies may be in part be due to microsatellite length being an imperfect indicator of the functional polymorphism, which may be better assessed by analyzing microsatellite sequence. Further, not all studies control for parental genotype and the possibility of epigenetic effects on *Avpr1a* expression, as early postnatal experiences can impact adult V1aR (Francis et al., 2002; Bales et al., 2007b; Lukas et al., 2010). Our results suggest that if the microsatellite does influence expression in the ventral pallidum, it represents a genomic mechanism for

generating diversity in social behaviors. Whether a causal down-regulation of V1aR density impairs field measures of monogamy warrants further investigation.

Microsatellite sequences in the human *AVPR1a* 5' flanking region have been associated with variation in human social behaviors. Specifically the $(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$ complex repeat (RS3) has been related to autism spectrum disorder, increased amygdala activation, and lower relationship quality in males (Kim et al., 2002; Walum et al., 2008; Meyer-Lindenberg et al., 2009). Interestingly, chimpanzees (*Pan troglodytes)* are polymorphic for a 5' deletion flanking the *AVPR1A* gene that encompasses RS3 (Donaldson et al., 2008), and individuals homozygous for the deletion display sex differences in dominance and conscientiousness not present in heterozygous animals (Hopkins et al., 2012). Thus, microsatellite evidence from humans, chimpanzees, and voles alike suggest that polymorphisms in noncoding regions may mediate rapid evolution in social behavior.

These results indicate that the degree of *Avpr1a* expression in the ventral pallidum is a means to control individual differences in socioemotional behaviors, and this diversity may maintain adaptability of prairie vole social structures to changing socioecological conditions. The prairie vole mating system was once purely a focus in field ecology, but has since proven invaluable in the study of the neurogenetic control of sociality. Whether this degree of variation has relevance in field measures of sociality remains to be explored. The advancements in our ability to manipulate the genome of this nontraditional, behaviorally rich species with innovative technologies, including viral vector mediated shRNA and transgenesis (Donaldson et al., 2009a; Keebaugh et al., 2012), will advance our ability to explore the mechanistic underpinnings of social

behavior in an ecologically relevant context.

Conflict of interest

The authors declare no competing financial interests.

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CHAPTER 3

Interaction of early-life social experience and brain oxytocin receptor expression on pair bonding behavior.

A modified version of this chapter will be submitted for publication as:

Barrett, C.E., Arambula, S., Young, L.J. (In prep) Striatal oxytocin receptor density predicts resilience to early adversity in female prairie voles (*Microtus ochrogaster*).

Abstract

The highly social, monogamous prairie vole displays a remarkable individual variation in neuropeptide receptor expression and sociality. Some of this variation may explained by genetic diversity, however individual differences in social experience and in interactions between genes and the environment are likely to be a major contributor to variation in social behavior. Here, we examine the interaction of early-life nurturing and brain neuropeptide receptor density on later-life social behaviors. We found (1) that daily 3hr isolations from parents and siblings from postnatal day 1-14 impaired adult partner preference formation in females, but did not impact oxytocin receptor (OTR), vasopressin 1a receptor, or corticotropin-releasing factor (CRFR) 1 or 2 binding, (2) that OTR density in the nucleus accumbens (NAcc) moderated the effect of early life isolation such that females with low OTR binding were most susceptible to early isolation, while females with high OTR binding were resilient to early isolation, and (3) a 5 minute tactile stimulation mimicking parental licking and grooming induced significant immediate early gene activity in OT neurons in the paraventricular nucleus of the hypothalamus (PVN) of postnatal day 6-7 neonates. These results suggest that NAcc OTR signaling may transduce parental nurturing behaviors to produce long-term changes in the neural circuitry involved in the formation of social relationships. We hypothesize that females with higher density of OTR in the NAcc experience greater modulation of NAcc OTR expressing neurons when in the natal nest, thus mitigating the impact of transient social isolation.

Introduction

Experience during critical periods of development impact adult expression of social behavior and the underlying neural circuitry. The postnatal brain is particularly susceptible to perturbations in the environment during this malleable period of neural development. In particular, the quality of parental care in early postnatal life is a salient predictor of adult socioemotional behavior, stress reactivity, and physiology. Disruptions in mother-infant interactions can lead to increased fear responsiveness, hyperactive stress physiology, impaired social competence, and, in humans, increase vulnerability for mood and anxiety, addiction, and personality disorders (Frank and Paris, 1981; Heim and Nemeroff, 1999; Penza et al., 2003; Heim et al., 2004; Sanchez, 2006). Much of the understanding of the mechanistic effects of early life experience comes from work in rodents (for review see Meaney, 2001; Veenema, 2012). High levels of maternal behavior produce offspring with attenuated responses to stress, lower levels of anxiety, and heightened maternal care (Liu et al., 1997; Caldji et al., 1998; Champagne et al., 2001). Offspring subjected to daily maternal separations over the first two weeks of life display hyperactive corticotropin-releasing factor (CRF) responses to stress, impaired glucocorticoid feedback, and heightened stress and anxiety-like responses in behavioral tests (Plotsky and Meaney, 1993; Liu et al., 1997; Caldji et al., 2000; Ladd et al., 2000).

Tactile stimulation from the mother is one of the salient factors affecting offspring responses to early care, and mediates adult behavioral, neurobiological, and physiological outcomes. Stroking neonates with a paint brush to simulate licking and grooming attenuates stress responses (Pauk et al., 1986; Suchecki et al., 1993; Levine, 1994) and drops in growth hormone secretion (Schanberg et al., 1984) that result from maternal

separation. Repeated stroking also alleviates some of the long-term negative effects of rearing without a mother (Gonzalez et al., 2001). Interestingly, humans display a similar phenomenon. Preterm birth interferes with normal parent-infant interactions while the child is in an incubator. Interventions that apply supplemental tactile stimulation improve the child's emotional self-regulation, growth rate, and social reciprocity throughout development (Field et al., 1986; Field, 1995; Feldman et al., 2003). One candidate mechanism by which early tactile experience may be functionally translated into behavior is through neuropeptide activation (Carter et al., 2009). In adult rats, oxytocin (OT) is released after social interactions and touch (Uvnas-Moberg, 1998; Ross et al., 2009a). There is some evidence that OT may be released in infants after tactile or maternal contact in mammals (Caba et al., 2003; Lenz and Sengelaub, 2010; Kojima et al., 2012), including humans (Feldman et al., 2010). A unique developmental role of postnatal OT is supported by the appearance of active OT peptide immunostaining only on the day of birth in rats (Buijs et al., 1980; Whitnall et al., 1985; Tribollet et al., 1989). OT released at parturition plays a role in the shift of GABA neurotransmission from being excitatory to inhibitory, a process which is impaired in animal models of autism (Tyzio et al., 2014). Early licking and grooming shapes OT pathways into adulthood, and offspring exhibit an oxytocin receptor (OTR) profile characteristic of that of their rearing mother (Champagne et al., 2001; Francis et al., 2002). Elevated licking and grooming, or peripheral OT injections in neonatal rat pups enhances adult maternal behavior (Francis et al., 1999; Pedersen and Boccia, 2002). Together these findings suggest that early OT signaling mediates some of the behavioral changes induced by heightened early social interactions.

The prairie vole (*Microtus ochrogaster*) is a socially monogamous rodent that

provides an excellent means to assess the impact of early care on offspring social, neurobiological, and behavioral development. Monogamy is characterized by preference for the partner, maintenance of a joint territory, and mutual care for offspring, and a pair bond can be assessed in the lab using the partner preference test, where time with the partner over a novel opposite-sex stranger indicates a social preference (Williams et al., 1992). Oxytocinergic and vasopressinergic neurons are thought to enhance processing of incoming social information and link up circuits encoding social cues of the partner with dopaminergic and opioid mediated reward and reinforcement systems (Young and Wang, 2004b; Burkett and Young, 2012a). In adult female prairie voles, heightening OT signaling in the nucleus accumbens (NAcc), either through central injections of OT agonists or viral vector-mediated over expression of OTR, facilitates pair bond formation (Williams et al., 1994; Young et al., 2001; Ross et al., 2009b). Early experience impacts the same neuropeptide circuitry that has been linked to social attachment (Ahern and Young, 2009; Veenema and Neumann, 2009; Lukas et al., 2010; Bales et al., 2011; Cao et al., 2014). Previously, our lab found that rearing pups without a father impairs adult pair bonding and alloparental care (Ahern and Young, 2009). In the monogamous mandarin vole, early social deprivation also impairs adult social bonding, reduces social interactions, and enhances anxiety-like behavior, and some of these effects are reversed with supplemental tactile stimulation (Jia et al., 2009; Wei et al., 2013; Yu et al., 2013).

Prairie voles are outbred and display considerable behavioral and genetic diversity, providing a unique system in which to study the neural and genetic mechanisms underlying individual variation in sociality. There is a remarkable individual variation in OTR and vasopressin 1a receptor (V1aR) expression in the brain that has been associated

with variation in alloparental and pair bonding behaviors (Pitkow et al., 2001; Phelps and Young, 2003; Hammock and Young, 2005; Ophir et al., 2008a; Ross et al., 2009b; Keebaugh and Young, 2011b; Barrett et al., 2013). Manipulations of accumbal OTR or pallidal V1aR beginning at weaning alters adult alloparental bonding in prairie voles (Keebaugh and Young, 2011b; Barrett et al., 2013), suggesting developmental differences in neuropeptide signaling can have a long-term impact on behavior. Differences in sensitivity to OT or arginine vasopressin (AVP) could differentially affect encoding of social information over development.

Based on these observations, we hypothesized that variation in early-life social experiences would produce diversity in adult social and anxiety-like behavior in prairie voles. We further hypothesized that the responses to early-life experience (e.g. nurturing or neglect) are mediated by early neuropeptide signaling. We then examined the interaction of early-life experience and individual variation in brain neuropeptide receptor expression on adult social behavior. Our results suggest that OT signaling in the NAcc in response to parental tactile stimulation mitigates the negative impact of bouts of social isolation and are consistent with the observation that genetic variation in the human *OXTR* gene is associated with differential susceptibility to the negative outcomes of early adversity in humans (Bradley et al., 2011; Thompson et al., 2011; McQuaid et al., 2013) as well as endocrine studies showing that parental engagement leads to increase OT release in infants (Feldman et al., 2010; Weisman et al., 2012).

Materials and Methods

Animal care and handling.

Prairie voles were from a laboratory-bred colony originally derived from a field-

caught stock from Illinois. The colony is systematically outbred to maintain genetic diversity. Animals were maintained on a 14:10h light:dark cycle with lights on at 07:00 at 22˚C with access to food (Purina high-fiber rabbit chow) and water ad libitum. Breeder housing consisted of large ventilated cages (34×30×19cm) lined with bedding (bed-o-cob, Maumee, OH, USA). Cages were not changed for the first 3 postnatal days in accordance with normal husbandy procedures. In Experiments 1 and 4, subjects were socially isolated from PND1-14, with PND0 defined as the day of birth, and were weaned into same-sex same-treatment pairs or trios in smaller (30×18×19 cm) cages at PND21. In Experiment 3, pups were euthanized at postnatal day (PND) 6-7 and brains were removed for analysis. No animals were exposed to subsequent litters in their natal group. No more than 2 animals per sex and experimental group were used from the same litter within each experiment. Experimental timelines and animal numbers are outlined in Figure 3.1. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

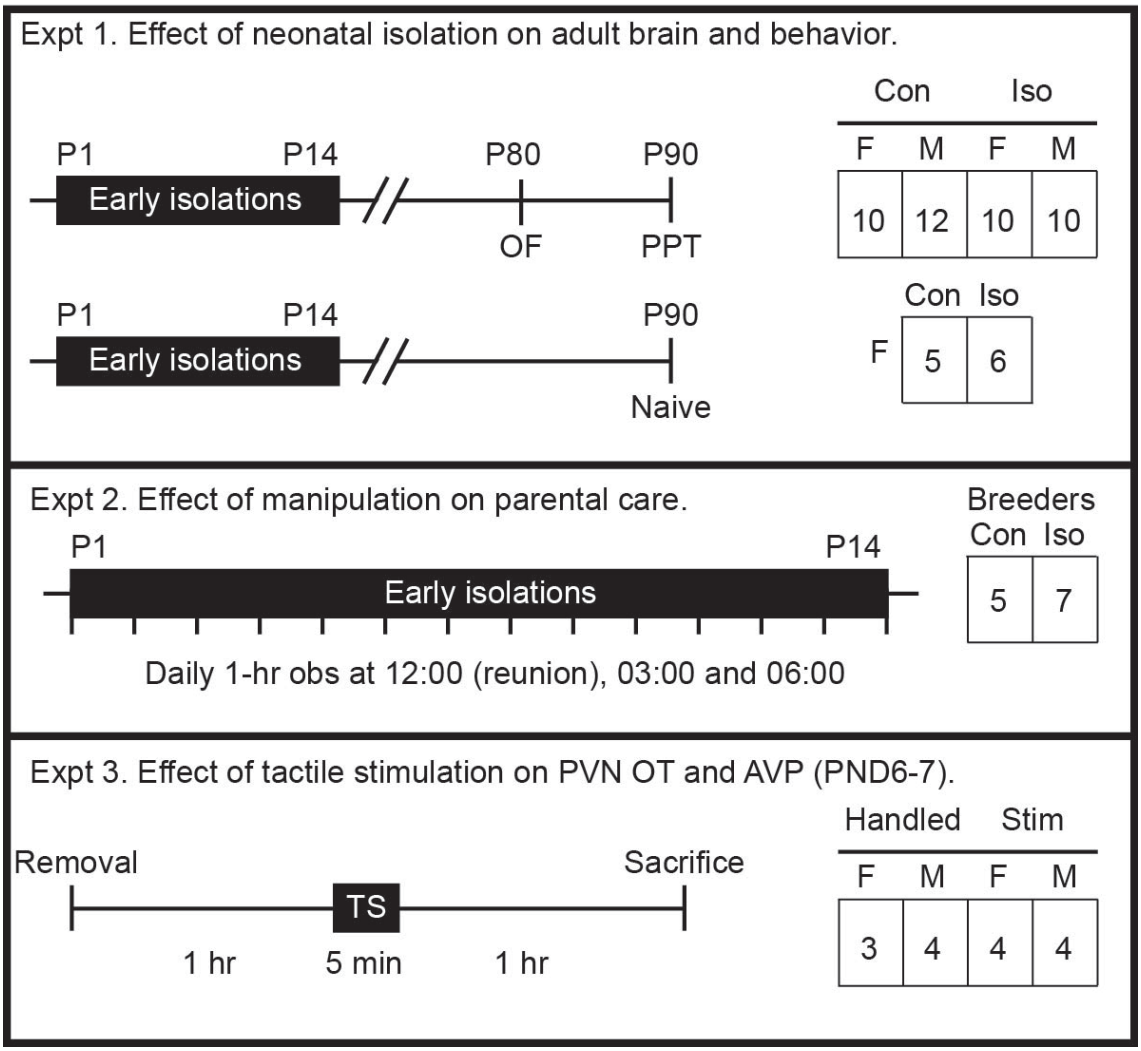

Figure 3.1. Experimental design.

In experiment 1, entire litters were isolated from both parents and siblings in a temperature controlled incubator for 3-hr per day from PND1-14, with PND0 as the day of birth. Control litters were left undisturbed, apart from being weighed every 3 days. Anxiety-like behavior was tested in the open field (OF), social attachment in the partner preference test (PPT), and autoradiography for OTR, V1aR and CRFR1/2 was performed on brains at sacrifice. Brains from a subset of females who were naïve to behavioral testing also underwent autoradiography. In experiment 2, the effect of the isolations on parental care was tested at 3 time points from PND1-14. In experiment 3, the impact of a 5-min body and anogenital paint-brush stimulation on hypothalamic oxytocin (OT) and vasopressin (AVP) was tested in PND6-7 pups. For 1 hr before and after stimulation, pups were kept in a temperature-controlled incubator. Animal numbers are outlined in tables to the right. F, female. M, male.

Experiment 1. Effect of neonatal social isolation on adult behavior and neuropeptide systems.

Procedure.

Between PND1-14, whole litters were removed from the cage, weighed, and isolated in single holding units in a temperature-controlled incubator (30-32˚C) from 0900-1200. Control litters were removed from parents every 3d to measure weights. To assess the impact of early isolation on weight gain, animals were weighed on PND1, 4, 7, 10, 13 and on weaning at PND21. From PND1-13, weights were averaged within each litter and sexes were not determined until weaning. After PND14, pups were left undisturbed until PND21, at which time offspring were weaned into same sex and treatment duos or trios. To avoid litter effects, no more than 1 animal per sex per litter underwent behavioral testing and brain analysis. Additionally, no more than 1 animal per sex per litter was left undisturbed until sacrifice for naïve brain analysis.

Open field.

Anxiety-like behavior in adulthood was assessed using the open field test. At approximately 80d of age, subjects were placed in a corner of an open field arena (40 × 40 × 40cm) and the duration and distance moved within the center or periphery was recorded using an automated system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA) as previously described (Ahern and Young, 2009).

Partner preference.

To assess the impact of early social isolation on adult bond formation, subjects were tested for their preference for an opposite-sex conspecific. At approximately 90d of age, female subjects (n=10 control, 10 isolated) were paired in a clean cage with a

sexually-experienced vasectomized male partner to prevent pregnancy-induced neural changes. Males (n=12 control, 10 isolated) were paired with a sexually-experienced ovariectomized female partner that was primed with estradiol 3 days prior to testing to induce sexual receptivity, as previously described (Barrett et al., 2013). After 24hr, subjects were placed in a partner preference test in which the partner and a novel stranger male were tethered to either end of a 3-chambered arena. At the end of the test, subjects and partners were put back into their home cage and retested the following day (48hr from pairing). An overhead camera recorded the test for 3hr, and time spent huddling with either stimulus animal was scored with an automated system (SocialScan 2.0, Clever Sys.) as previously described (Ahern and Young, 2009). Female prairie voles are induced ovulators and take approximately 2 days to go into estrus and mate (Carter et al., 1988), thus females likely began mating toward the end of the 48hr cohabitation.

Tissue collection and receptor autoradiography.

Four days following partner preference testing, animals were sacrificed with CO2 and brains were collected in isopentane on dry ice. In addition to behaviorally-experienced female brains, autoradiography was performed on naïve females (n= 5 control, 6 isolated).

Autoradiography for OTR, V1aR, corticotropin-releaseing factor (CRFR) subtypes 1 and 2 was performed as previously described (Lim et al., 2005b; Ahern and Young, 2009). For OTR and V1aR, sections were incubated for 1 hr in 0.05 nM of either ^{125}I- linier V1a antagonist for V1aR (NEX310050UC Perkin Elmer) or ^{125}I-OVTA for OTR (NEX 254050UC PerkinElmer). For CRFR1 and CRFR2, sections were incubated for 2hr in 0.2nM ^{125}I-sauvagine (NEX306010UC PerkinElmer). As ^{125}I-sauvagine binds

to both CRFR subtypes, the CRFR2 antagonist (500 nM Astressin-2B; Tocris Biosciences, Ellisville, MO; Cat #2391) and CRFR1 anatonigist (500 nM CP-154,526; Tocris Biosciences; Cat #2779) were added to the tracer to reveal CRFR1 and CRFR2 binding, respectively. Slides were exposed to BioMaxMRfilm (Kodak, Rochester, NY) for 72 hr for densitometry and a subsequent 8d for photographic quality images. For quantification, ^{125}I autoradiographic standards (ARI 0133A, American Radiolabeled Chemicals) were included in the cassette. Films were digitized (MTI CCD72 camera) and quantified using AIS software version 6.0 (Imaging Research Inc., Ontario, Canada). Optical densities were converted to decompositions per minute (DPM)/mg tissue equivalents using ^{125}I microscale standards. Specific binding was calculated by subtracting non-specific background binding in the S1 region of the cortex (OTR, V1aR) or the corpus callocsum (CRFR1,2) from total binding in each region. For OTR quantification, optical density measures were taken across 3 sections in the olfactory bulb (OB), prefrontal cortex (PFC), nucleus accumbens (NAcc) shell and core, lateral septum (LS), bed nucleus of the stria terminalis (BNST), and the central (CeA) and basolateral amygdala (BLA). For V1aR, the OB, ventral pallidum (VP), LS, BNST, central-lateral (CeL) and central-medial (CeM) amygdala, mediodorsal thalamus (MDThal), and posterior cingulate (PCing). For CRFR1, density measures were taken in the dentate gyrus (DG), LS, medial PFC, and NAcc core and shell. For CRFR2, density was quantified in NAcc-septal pole (NAcc-sp), dorsal raphe (DR), PFC, CA1 region of the hippocampus, and LS.

Corticosterone assay.

Basal corticosterone concentration was assessed using ELISA. Approximately

500 ml trunk blood was collected with heparinized capillary tubes into EDTA-coated tubes with 10 ul aprotinin (Trysol, Fisher Scientific) to prevent protease activity. Blood was spun at 5000rpm for 5min and plasma was collected and stored at -20˚C until assayed. Plasma samples were diluted 1:10 and assayed with a commercially available kit (Siemens, Los Angeles, CA) in duplicate (inter-assay CV, 10.36%, intra-assay CV, 1.31%). Assay services were provided by the Biomarkers Core Laboratory at the Yerkes National Primate Research Center. This facility is supported by the Yerkes National Primate Research Center base grant 2P51RR000165-51.

Experiment 2. Effect of manipulation on parental care

To examine the impact of the 3 hr isolation on parental care received by the pups upon returning to the natal nests, home cage observations of parental care were taken between PND1-14 during three 1-hr blocks (12:00-01:00, immediately after reunion; 03:00-04:00; 06:00-07:00) similar to previously described protocols in the lab (Ahern and Young, 2009). Twelve breeder pairs were observed (5 control, 7 early isolated), and early manipulations were performed as described above. Within each 1-hr block, 15 point observations were taken every 4min. Male and female voles were scored for nest occupancy and type of activity: licking and grooming pups; carrying pups; self-grooming; grooming mate; resting; nest building; wandering around cage; eating; drinking; digging; climbing. Females were also monitored for active (arched-back or standing crouch) or passive (blanket or side) nursing status. The frequency of each behavior was calculated by dividing by the total number of observations (630 over PND1-14, 210 per each observation block over PND1-14).

Experiment 3. Effect of Tactile Stimulation on immediate-early gene expression in PVN Oxytocin and Vasopressin neurons.

Procedure and tissue collection.

Prairie vole pups (age PND6-7) were removed from the postnatal nest and put in individual holding units in a 30˚C incubator. After 1hr, pups were removed by a gloved investigator and stroked with a damp stiff-haired paint brush (McCarthy et al., 1997) or held for 5 min. A total of 15 pups from 5 different litters were used in the experiment (n=3 handled females, 4 handled males, 4 stroked females, 4 stroked males). Tactile stimulation consisted of 3 min of body stimulation, and 2 min of anogenital stimulation, alternating every 1 min. After tactile stimulation, subjects were returned to the incubator. Pups were deeply anesthetized with isofluorane and euthanized by rapid decapitation 1hr after the end of the stimulation. The presence of testes was examined to determine sex. Brains were removed and post-fixed in 10ml of 5% acrolein diluted in phosphate buffered saline (PBS, pH 7.4) for 3hr, followed by two 10 min washes in PBS. Brains were transferred to 30% sucrose at 4˚C for at least 24hr and sectioned on a microtome at 40mM. Sections were stored in cryoprotectant at 4˚C until immunohistochemical processing.

Immunohistochemistry.

Every third section was processed for the immediate early gene (IEG) protein Early Growth Response Protein-1 (EGR-1) and either OT or AVP as described previously (Modi et al., Submitted; Barrett et al., Under revision). EGR-1 was chosen as a measure of neural activity as it has been reported to be a more sensitive marker of

activity in the hypothalamus than c-Fos (Polston 1995). Briefly, sections were rinsed for 15 min in 0.1% sodium borohydride, pre-blocked for 1hr with normal goat serum, and then incubated for 47hr at 4˚C in primary antibody in PBS (pH 7.4) with 0.3% triton-X and 2% NGS (1:8,000 rabbit polyclonal anti-EGR1, sc-189, Santa Cruz Biotechnology, Santa Cruz, CA; 1:10,000 mouse monoclonal anti-OT, mAb5296, Millipore, Billerica, MA). For AVP-EGR1 double labeling, sections were incubated in primary for 46hr (1:1,000 mouse monoclonal anti-VP, PS41, generously donated by Dr. H. Gainer, NIH, USA (Ben-Barak et al., 1985)). Sections were washed in PBS and incubated in secondary Alexa Fluor conjugated antibodies for 3hr at 4˚C (1:1,000 Alexa Fluor 568 for EGR-1 and Alexa Fluor 488 for OT or AVP, Life Technologies, Grand Island, NY). Tissue was mounted and cover slipped using Vectashield mounting medium with Dapi (Vector, Burlingame, CA), and Z-stack images were taken on a Leica confocal microscope at 40X magnification. The total number of OT or AVP cells and EGR-1 double labeled neurons in the PVN were counted in five to six bilateral sections for OT and two to five for AVP from each subject by an experimenter blind to the sex and treatment condition. The total percentage of EGR-1 positive OT or AVP cells was calculated across all sections for each animal.

Statistical analyses.

Data are presented as mean ± SEM, unless otherwise noted. In experiments 1 and 4, weights were analyzed with repeated measures (RM) ANOVA with day as a repeated measure and rearing condition and sex (only in Expt 4) as a between subjects factor and *posthoc* Student's t-tests. Weanling weights were analyzed with Student's t-tests between groups within each sex. Behavioral data was analyzed for normality with the

Kolmogorov-Smirnov test. Open field data (Duration in center, periphery; Latency and frequency to enter center; Distance travelled in center, periphery, total arena) from experiment 1 were analyzed with multivariate (M) ANOVA. In the partner preference test in experiments 1 and 4, time spent huddling with the partner and stranger animals was analyzed with repeated measures (RM) ANOVA with isolation (Expt 1) or drug (Expt 4) and sex (Expt 1) as between subjects factors and stimulus animal (partner or stranger) and time as within-subjects repeated measures. Planned *posthoc* paired t-tests were performed on huddling time with partner versus stranger to assess partner preference within each group, and corrected for multiple comparisons using the Bonferonni-Holm test. Autoradiographic data were analyzed with MANOVA across brain regions. Receptor binding was correlated to the percent time huddling with the partner (partner huddling / total huddling time with the stranger and partner). A median split was performed on all NAcc OTR densities, and RMANOVA was performed with rearing condition and NAcc OTR (hi vs lo) as a between subject factor and stimulus animal (partner v stranger) as a within subjects factor. In Experiment 3, immunohistochemistry results were analyzed with planned Student's t-tests between groups. Statistics were performed with SPSS Statistics 17.0 with significance set at $p<0.05$ and two-tailed tests.

Results

Experiment 1. Effects of neonatal social isolation on weight gain, adult behavior and receptor density.

Weight.

Early isolated animals displayed slowed weight gain from PND1-13 in

comparison to controls (time X rearing condition; $F_{4,72}$=4.78, p=0.004). Early isolated animals weighed significantly less than controls only on PND13 (p=0.017; Figure 3.2A). Weights were normalized by weaning (p>0.05).

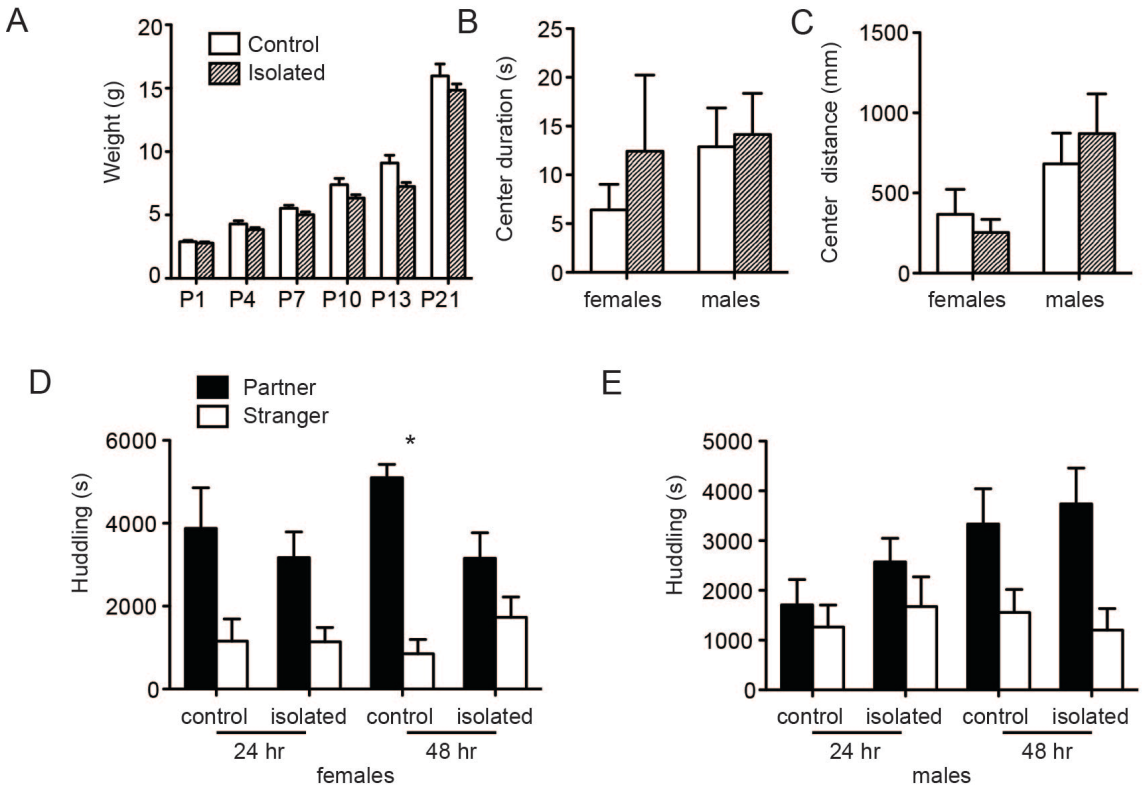

Figure 3.2. Impact of early isolation on pup weight and adult behavior.

Prairie vole litters were exposed to daily 3-hr separations from both parents and siblings, or left undisturbed aside from weighing every 3d. Isolated litters displayed slowed weight gain, and weighed significantly less only on PND13 (A). As adults, no differences were observed between control and isolated litters in the open field (time in center, B; distance moved in center, C). In females, only the control group displayed a significant partner preference after 48hr of cohabitation (D). No differences in partner preference were detected in males (E). Asterisk indicates *post-hoc* t-test, p<0.05.

Open field.

Early isolation did not impact any measure of anxiety-like behavior in the open field (Figure 3.2B,C).

Partner preference.

In females after a 24hr cohabitation, a RMANOVA revealed a main effect of time spent with the partner over the stranger stimulus ($F_{1,18}$=8.70, p=0.009), but no significant interaction effect in females. Neither rearing group formed a significant partner preference after 24hr (p>0.05, Figure 3.2D). After 48hr from the time of pairing, there was a main effect of rearing condition ($F_{1,18}$=6.24, p=0.022), stimulus ($F_{1,18}$=21.56, p<0.001), and an interaction effect between condition and stimulus animal ($F_{1,18}$=5.33, p=0.033). Control females spent significantly more time with the partner over the stranger (p<0.001), but females socially isolated as neonates did not (p=0.219; Figure 2D). No impact of the early isolation on male partner preference was detected after either 24- or 48hr of cohabitation (Figure 3.2E). No difference in distance moved in the center arena between rearing groups was detected at 48hr, indicating that partner preference results were not due to differences in general locomotion.

Corticosterone.

Early isolation did not significantly impact adult basal corticosterone levels in either males (control, 2317±229 ng/ml; isolated, 2305±283) or females (control, 3756±370 ng/ml; isolated, 3969±312 ng/ml). There was no difference in corticosterone between behaviorally experienced and naïve groups, thus values were averaged within each rearing group.

Autoradiography.

The impact of early isolation on adult neuropeptide receptor binding was assessed in behaviorally naïve and experienced females. No changes in OTR, CRFR1, or CRFR2

were detected in any region examined. Naïve females who had experienced early isolation, however, displayed reduced V1aR binding in the CeM ($F_{1,9}$=5.15, p=0.049) and BNST ($F_{1,9}$=8.10, p=0.019). Similarly, in behaviorally experienced females, a reduction in V1aR binding was detected in the CeM ($F_{1,18}$=4.63, p=0.045) and CeL ($F_{1,18}$=5.32, p=0.033). However, V1aR effects do not hold up to multiple corrections testing.

NAcc OTR and partner preference.

Multiple linear regression revealed a significant interaction effect between rearing condition and NAcc (core and shell combined) OTR binding (ANOVA, $F_{3,16}$=7.50, p=0.002, Condition X NAcc OTR, p=0.006; Figure 3.3A), but not with neuropeptide receptor binding in other regions. The percent of time spent huddling with the partner after 48hr of cohabitation was significantly positively correlated with OTR binding in the NAcc core (R=0.743, R^2=0.552, p=0.014) and shell (R=0.798, R^2=0.637, p=0.006) in the early isolated group. Females were then divided into either "high" or "low" OTR NAcc groups using a median split. RMANOVA revealed a significant interaction between stimulus animal and NAcc OTR ($F_{1,16}$=5.87, p=0.028), and between stimulus animal, rearing condition, and NAcc OTR ($F_{1,16}$=8.11, p=0.012), suggesting that NAcc OTR binding was related to adult social responses to early isolation. Both low and high OTR expressing control females spent significantly more time with the partner than the stranger (lo, p=0.015; hi, p=0.002). Only early isolated females in the high accumbal OTR group spent more time with the partner than the stranger (lo, p=0.417; hi, p=0.010; Figure 3.3B). Thus, females with high accumbal OTR (Figure 3.3C,D) were resilient to impairments in partner preference that resulted from early isolation.

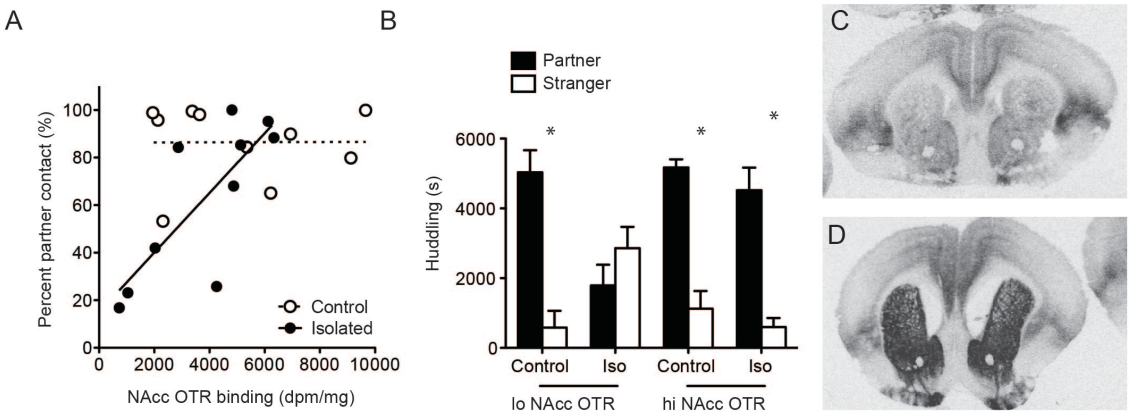

Figure 3.3. Females with low NAcc OTR susceptible to early adversity.

After 48hr of cohabitation, the percent of time females spent huddling with their partner over total huddling significantly correlated with NAcc OTR binding in the early isolated ($R=0.779$, $R^2=0.607$, $p=0.008$), but not control ($R=0.006$, $R^2<0.0001$, $p>0.05$) females (A). Only females with low OTR binding exposed to early isolation did not form a partner preference (B). Representative autoradiographs of low (C) and high (D) OTR NAcc females. Asterisk indicates *post-hoc* t-test, $p<0.05$. NAcc, nucleus accumbens. OTR, oxytocin receptor.

Experiment 2. Effect of separations on parental care.

To determine how the transient social isolation impacted parental licking and grooming, we quantified parental care over the 2-week isolation. Licking and grooming from both parents was enhanced during the 1-hr observation upon reunion of isolated pups to the nest (12pm, dam $p=0.006$, sire, $p=0.030$; Figure 3.4A), but not at later time points in the day ($p>0.05$). Across all time points from PND1-14, licking and grooming from the mothers of early isolated litters was greater than that from controls ($p=0.047$). No significant differences in the frequencies of nursing, nest occupancy, or other parental activities were detected. Likewise, no significant differences in measures of parental care were detected over time from PND1-14.

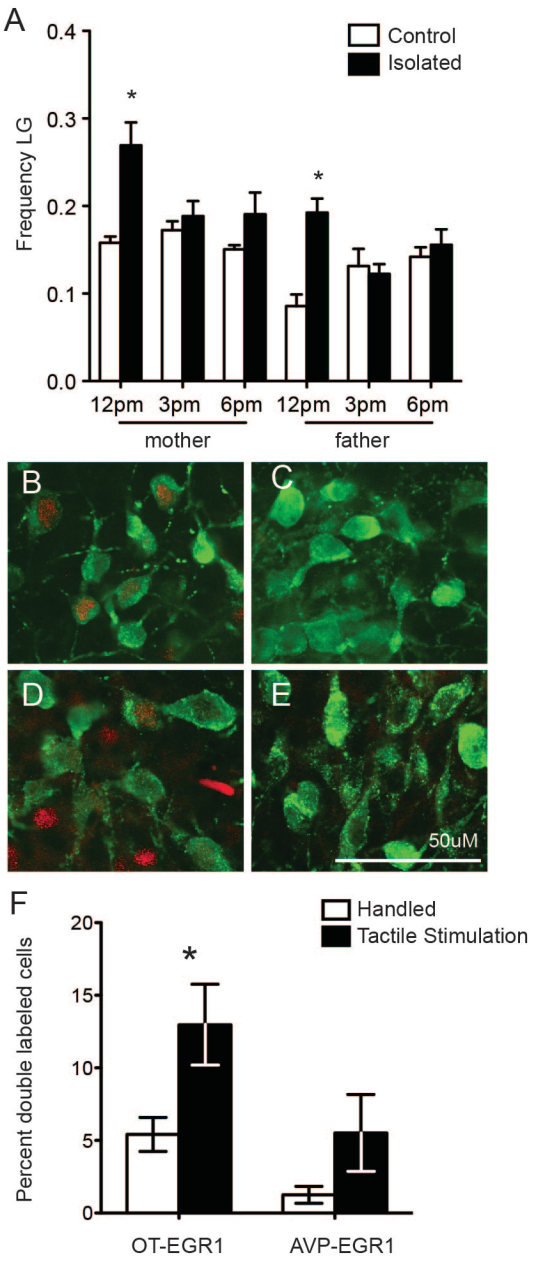

Figure 3.4. Tactile stimulation activates OT neurons in the PVN of females.

For 1-hr after reunion of the litters, both parents displayed heightened licking and grooming in comparison to controls (A). PND6-7 neonates were brushed for 5-minute to the anogenital and body region or only handled and sacrificed after 1-hr. Representative oxytocin (A, C) and vasopressin (D,E) sections in tactile stimulated (left) and handled (right) animals. Oxytocin cells are green and EGR-1 nuclei are labeled red. Tactile stimulation induced significant EGR-1 activity in oxytocin, but not vasopressin, neurons (F). Asterisk indicates Student's t-test, $p<0.05$. EGR-1, Early growth response factor-1.

Experiment 3. Effect of Tactile Stimulation on immediate-early gene expression in PVN Oxytocin and Vasopressin neurons.

To determine if tactile stimulation activates hypothalamic OT and AVP neurons in neonatal prairie voles, we examined immediate early gene activity in the PVN one hour after 5 min anogenital and body stimulation with a paintbrush. Tactile stimulation significantly increased the percentage of PVN OT neurons double labeled for EGR-1 ($p=0.035$), but no significant differences in AVP-EGR1 immunostaining were detected ($p=0.165$; Figure 3.4B-F). Thus, simulated licking and grooming of a week-old prairie vole significantly activated OT, but not AVP hypothalamic neurons.

Discussion

Early isolation impaired female bonding

A large body of literature has demonstrated the importance of early maternal care, specifically licking and grooming, in shaping offspring physiology and behavior. Rat dams that exhibit high levels of licking and grooming raise offspring that display low behavioral indices of anxiety, reduced HPA responses to stressors, enhanced glucocorticoid negative feedback, reductions in central CRF mRNA, and high levels of maternal care themselves (for review, see Meaney, 2001; Kaffman and Meaney, 2007). In rats and mice, early maternal separation impairs social recognition (Lukas et al., 2011), alters juvenile and adult aggressive behavior (Veenema et al., 2006; Veenema and Neumann, 2009), and enhances maternal aggression (Veenema et al., 2007). Prairie voles may be particularly susceptible to disruptions in early care as pups mount corticosterone responses and display ultrasonic vocalizations in response to separations from the parents whereas the nonmonogamous meadow vole (*Microtus pennsylvanicus*)

do not (Shapiro and Insel, 1990). Here, we found that repeated daily isolations from both siblings and parents over the first two weeks of life impaired adult pair bonding in females, but not in males. Although we did not empirically quantify mating, it is possible that early isolation impacted latency to mating or mating frequency during cohabitation, thus impacting mating-induced bond formation. Sex-specific effects of early manipulations on later vole social attachment have previously been reported, with models of reduced postnatal care impairing female but not male pair bonding (Bales et al., 2007a; Yu et al., 2012). While rearing without a father impaired only female bond formation in mandarin voles (Yu et al., 2012), our lab previously found that single-mother reared prairie voles of both sexes display delayed pair bonding and reduced alloparental care (Ahern and Young, 2009). In the monogamous mandarin vole, daily social isolations lead to greater anxiety-like behavior in the open field, reduced social interactions, and decreased bond formation in both sexes (Jia et al., 2009; Yu et al., 2013). As stress and CRF administration promotes pair bonding in male prairie voles (DeVries et al., 1996; Lim et al., 2007), enhanced stress reactivity in adulthood may override negative effects of reduction in social interactions early in life on male sociality. Furthermore, maternal separations in rats have been reported to enhance adult AVP expression (Veenema et al., 2006; Murgatroyd et al., 2009; Veenema and Neumann, 2009), which would also serve to promote male bonding. The mechanisms by which male vole sociality is more resilient to early social isolations remain to be determined.

No differences in baseline anxiety-like behavior in the open field or plasma corticosterone levels were detected in the current study. The absence of anxiety differences as a result of early parental separations in the present study is somewhat

surprising, but consistent with some previous studies (Plotsky and Meaney, 1993; Caldji et al., 2000; Ahern and Young, 2009). It is possible that behavioral and hormonal stress reactivity measures, rather than basal anxiety states, are impacted from early separations, as occurs in other rodent species (Ladd et al., 1996; Kalinichev et al., 2002). However, the rise in licking and grooming upon reunion of pups to the nest may negate negative impacts of isolations on stress behavior and physiology.

Neuropeptide receptor expression and early isolation

Early social isolation did not impact adult OTR, CRFR1, or CRFR2 expression, but did lead to decreases in V1aR expression in the centromedial amygdala and BNST, areas involved in processing stress and anxiety. However, previous studies have reported an impact of early maternal care or maternal separations on later OTR and V1aR binding in rats (Francis et al., 2002; Lukas et al., 2010). Females were nulliparous at the time of sacrifice, and it is possible that differences in OTR expression from early adversity may not be detectable until lactation. Early licking and grooming impacts adult estrogen receptor alpha expression, and estrogen injections enhance OTR in the medial preoptic area and LS only in high LG offspring (Champagne et al., 2001). It is also possible that differences in OTR expression may be detectable at different points in development, as rats exposed to early separations display transient reductions in hippocampal OTR neonatally (Noonan et al., 1994). We previously reported no change in neuropeptide receptor levels in single mother reared prairie voles, except for an enhancement of dorsal raphe CRFR2 and a modest reduction of OTR in the BNST (Ahern and Young, 2009). However, early handling decreases OTR in the NAcc, LS, and BNST in prairie voles (Bales et al., 2011) and paternal deprivation decreases OTR mRNA in the NAcc and

MeA of mandarin voles (Cao et al., 2014), although these effects are not in the same expected direction. Nonetheless, the results of this and previous studies suggest that the wide variation in accumbal OTR is not entirely attributable to variation in early experience.

Interestingly, diversity in OTR expression in the nucleus accumbens was related to behavioral outcomes of early isolation. Specifically, OTR binding significantly correlated with the percent of time females spent huddling with their partner in the early isolated group. Non-isolated females displayed no relationship between accumbal OTR and partner huddling. Thus, it appears that low levels of NAcc OTR binding is sufficient to form a partner preference after a sufficient length of cohabitation (48hr). Given the remarkable behavioral and genetic heterogeneity in prairie voles, it is possible that a subset of individuals display heightened susceptibility to early adversity. Dividing females into "low" or "high" OTR NAcc expressers revealed that only females with low accumbal OTR expression were impacted by the social isolation and did not display a partner preference. This interaction between receptor expression and early experience suggests that differential OTR signaling in the NAcc over development may mediate differences in vulnerability or resilience to adverse life events.

In adult prairie voles, OT release during social interactions is thought to converge upon dopaminergic and opioid mediated reward and reinforcement pathways, thereby creating an association between a conditioned reward and exposure to their partner (Young and Wang, 2004b; Burkett and Young, 2012a). Oxytocin signaling over development may assign a rewarding value to social stimulation and strengthen the circuitry involved in the development of social bonding. For example, oxytocin may alter

dopaminergic transmission in medium spiny neurons in the NAcc or may lead to changes in synaptic plasticity (Dolen et al., 2013; Zheng et al., 2014). In prairie voles, long-term developmental elevation of NAcc OTR from weaning age but not acutely in adulthood recapitulates the relationship between OTR density and alloparental care (Ross et al., 2009b; Keebaugh and Young, 2011b). Interestingly, the mature oxytocin peptide is not expressed until birth in rats (Alstein et al., 1988), suggesting oxytocin is not functionally active until birth. Oxytocin receptor expression is also present in the neonatal prairie vole brains (Shapiro and Insel, 1989). Neonatal OT regulates maternal-infant interactions, as central OT administration decreases separation distress calls in infant rats (Insel and Winslow, 1991), isolated OTKO and OTRKO neonatal mice do not emit ultrasonic vocalizations (Winslow et al., 2000; Takayanagi et al., 2005), and OT is necessary for developing a preference for maternal odors (Nelson and Panksepp, 1996; Kojima and Alberts, 2011). Postnatal OT is also important for neuronal development and necessary for the GABA switch from excitatory to inhibitory, an effect disrupted in mouse models of autism (Tyzio et al., 2006; Tyzio et al., 2014).

Mediators of early touch

OT neurons in the PVN displayed significant immediate early gene activity in response to a five-minute tactile stimulation in week old prairie vole neonates. This effect appears to be selective hypothalamic activation since AVP neurons did not display a significant activation compared with handled-controls. We also found that after the 3hr separation, parental care is enhanced upon reunion with the litter, as has been reported previously in rats (Pryce et al., 2001). It is conceivable that females with high OTR sensitivity benefited more from this burst in care upon reunion. Similar to offspring of

high-licking mothers, pups that receive supplemental tactile stimulation over the first week of life display elevated hippocampal glucocorticoid receptors in adulthood (Jutapakdeegul et al., 2003). Repeatedly stroking pups with a paintbrush alone to mimic licking and grooming reduces separation distress (Guzzetta et al., 2009) and alleviates some of the behavioral deficits associated with early deprivation in both mice (Gonzalez et al., 2001) and voles (Wei et al., 2013). Rat pups reared in the absence of a mother show less impairments in maternal care and emotionality in the open field if stroked daily with a paint brush (Gonzalez et al., 2001). Supplemental tactile stimulation to neonatally isolated mandarin voles reverses the reduction in PVN OT and AVP immunoreactivity, sociability, body weight and the increase in neonatal and adult corticosterone . The neonatal period in prairie voles is characterized by high levels of licking and grooming from both parents and older siblings in the neonatal nest, and touch-induced oxytocin release may be an important integrator of early care. If adult OTR is reflective of postnatal OTR expression, low-expressing females may have been less sensitive to nurturing-induced oxytocin release neonatally, whereas those with high accumbal OTR sensitivity could benefit from the heightened licking and grooming upon return to the nest. Interestingly, a recent paper found that postnatal OT is necessary for sensory experience-dependent cortical plasticity (Zheng et al., 2014). Whisker deprivation reduces PVN OT expression and release and impairs excitatory transmission in the S1 cortex, an effect rescued with local OT injection to neonates. Whether OT can mediate plasticity in regions outside of the cortex, such as forebrain limbic regions underlying sociality, is an exciting area of future research.

Some of the downstream effectors of maternal licking and grooming have been

elucidated. Tactile stimulation or licking and grooming from the mother activates a pathway by which elevated plasma T3 thyroid hormone stimulates hippocampal serotonin (5-HT) which subsequently enhances hippocampal glucocorticoid receptor expression, an effect which persists into adulthood (Champagne et al., 2003; Hellstrom et al., 2012). In adults, serotonin stimulates the release of OT (Jorgensen et al., 2003) and vice versa (Yoshida et al., 2009), thus oxytocin may indeed fit within this molecular framework. The exact pathways by which licking and grooming elicit activation of central OT systems in neonates still remain to be determined.

Human relevance

Early adverse experiences have also been associated with lowered OT levels in the CSF in both humans and rhesus macaques (Winslow et al., 2003; Heim et al., 2009) as well as lowered OT in urine of children that have experienced parental abuse (Wismer Fries et al., 2005). Single nucleotide polymorphisms (SNPs) of the human *OXTR* have also been associated with OT signaling with socioemotional behavior in humans, including variability in parental sensitivity in adults (Bakermans-Kranenburg and van Ijzendoorn, 2008), empathy and stress reactivity (Rodrigues et al., 2009), affect and self-esteem (Lucht et al., 2009; Saphire-Bernstein et al., 2011), and social recognition (Skuse et al., 2014). These SNPs have also been linked to psychopathologies including depression and anxiety (Costa et al., 2009) and the occurrence of autism (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2008; Liu et al., 2010).

Recently, it has been suggested that oxytocin receptor polymorphisms interact with early life adversity, including maternal depression or childhood maltreatment, to enhance the risk for exhibiting emotional dysregulation, social anxiety, and depressive

symptoms (Bradley et al., 2011; Thompson et al., 2011; McQuaid et al., 2013). Interestingly, Thompson et al (2011) found this effect in female adolescents, which parallels our finding that female voles were more responsive to early isolations. OXTR polymorphisms have also been linked to resilient functioning in response to positive early environments (Cicchetti and Rogosch, 2012; Bradley et al., 2013). Taken together, this suggests that differences in the expression or sequence of the OTR gene can interact with early experience to impact adult sociality. Oxytocin release is coordinated between parents and infants during social interactions (Feldman et al., 2010) and *OXTR* polymorphisms have been linked to the security of infant attachment in humans (Chen et al., 2011), suggesting that oxytocin early in life may regulate early social interactions in humans in a similar manner to voles.

Conclusion

To our knowledge, these results are the first to report that variation in accumbal OTR expression is associated with responses to early adversity, and that hypothalamic OT may mediate responses to early touch in prairie voles. This is the first to establish a relationship between individual variation in neuropeptide receptor expression in the brain and susceptibility/resilience to early-life social experience/neglect. Child abuse, parental neglect, and preterm infancy all involve disruptions in socioemotional development and health, thus development of interventions or therapeutics is critical. It has been proposed that drugs that stimulate OT release are viable candidates for improving social cognition in psychiatric conditions associated with abnormal social functioning (Modi and Young, 2012). Future studies should investigate whether pharmacological potentiation of the OT system augments social experiences and potential beneficial effects on the development

of neural pathways involved in social engagement. However, it should be noted that other studies in prairie voles have found that daily intranasal OT in adolescence actually reduced partner preference formation in adult male prairie voles. Therefore, more research is needed to understand the potential beneficial, as well as detrimental, impacts of manipulating the OT system developmentally (Bales et al., 2012; Young, 2013).

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CHAPTER 4

Neonatal melanocortin receptor agonist treatment activates hypothalamic peptide systems, promotes adult attachment, and reduces play fighting in prairie voles.

Modified from the following reference:

Barrett, C.E., Modi, M.E., Zhang, B.Z., Inoue, K., Young, L.J. (Under revision) Neonatal melanocortin receptor agonist treatment promotes adult attachment and reduces play in prairie voles in a sex dependent manner. Neuropharmacology.

Abstract

The melanocortin receptor (MCR) system has been studied extensively for its effects on feeding and sexual behavior, but its effects on social behavior have received little attention. Alpha-MSH interacts with the dopamine system and stimulates oxytocin (OT) release. Acute melanotan-II (MTII), an MC3/4R agonist, potentiates brain oxytocin (OT) release and facilitates OT-dependent partner preference formation in socially monogamous prairie voles. Here we examined the long-term impact of early-life MCR stimulation on hypothalamic neuronal activity and social development in prairie voles. Finally, we tested the hypothesis that MTII can buffer against early isolations. Male and female voles were given daily subcutaneous injections of 10 mg/kg MTII or saline between postnatal days (PND)1-7. Neonatally-treated males displayed a reduction in initiated play fighting bouts as juveniles. Neonatal exposure to MTII facilitated partner preference formation in adult females, but not males, after a brief cohabitation with an opposite-sex partner. Acute MTII injection elicited a significant burst of the immediate early gene EGR-1 immunoreactivity in hypothalamic OT, vasopressin, and corticotrophin releasing factor neurons, when tested in PND 6-7 animals. Daily neonatal treatment with 1 mg/kg of a more selective, brain penetrant MC4R agonist, PF44687, promoted adult partner preferences in both females and males compared with vehicle controls. Furthermore, neonatal exposure to MTII rescued the deficits in partner preference formation induced by early life social isolation described in Chapter 3. Thus, developmental exposure to MC4R agonists lead to a persistent change in social behavior, suggestive of structural or functional changes in the neural circuits involved in the formation of social relationships, and may mimic or enhance the effects of maternal

nurturing in models of parental neglect.

Introduction

The melanocortin (MC) system has been studied extensively for it role in coordinating feeding (Poggioli et al., 1986), stress and anxiety (De Barioglio et al., 1991; Lu et al., 2003; Chaki and Okuyama, 2005) and sexual behavior (Argiolas et al., 2000; Rossler et al., 2006), among other physiological and behavioral processes (for review, see Wikberg et al., 2000; Mountjoy, 2010; Tao, 2010). However, the effects of melanocortin receptor activation on social behavior have received little attention. Alpha-melanocyte stimulating hormone (α–MSH) stimulates central OT release from rat hypothalamic slices, an effect that is blocked by an MC4 receptor (MC4R) antagonist (Sabatier et al., 2003). The MC4R also interacts with additional systems involved in the regulation of social behaviors, including dopamine (Lindblom et al., 2001), opioids (Alvaro et al., 1997), and corticotropin-releasing factor (CRF; Lu et al., 2003).

Recently, we have found that melanocortin signaling promotes social attachment in prairie voles (Modi et al., Submitted). The socially monogamous, biparental prairie vole (*Microtus ochrogaster*) exhibits a complex repertoire of social behaviors that have been associated with oxytocin, dopamine, opioid, and CRF signaling (Young and Wang, 2004b; Aragona et al., 2006; Lim et al., 2007; Bosch et al., 2009; Burkett et al., 2011b), and provides a excellent model to assess the neural underpinnings of social behavior (McGraw and Young, 2010). Pair bond formation is assessed using the partner preference test, in which a subject animal's preference for a cohabitated partner or novel stranger animal is tested (Williams et al., 1992). In prairie voles, the partially brain penetrant MC3/4 agonist, melanotan-II (MTII), activates oxytocin (OT) neurons in the

paraventricular nucleus of the hypothalamus (PVN), potentiates central OT release in response to a physiological stimulus (hypertonic saline), and facilitates partner preference formation. These effects are thought to be mediated via MC4R as the selective, brain penetrant MC4R agonist, PF446687, also promotes partner preference formation (Modi et al., Submitted).

Adult prairie vole sociality is sensitive to early-life manipulations of parental care (Ahern and Young, 2009). One mechanism by which early social experience, in particular parent-infant interactions, may be translated into long-term behavioral alterations is through long-term organizational effects of neuropeptide activation through restructuring neural circuitry (Carter et al., 2009). For example, neonatal treatment with OT at birth impacts later socioemotional behaviors in prairie voles (Bales and Carter, 2003b; Kramer et al., 2003; Cushing et al., 2005; Bales et al., 2007c). Interestingly, neonatal administration of α-MSH and ACTH-like peptides to rats leads to enhancements in adult attention to relevant stimuli (Champney et al., 1976), learning (Beckwith et al., 1977b; Acker et al., 1985), social contact in an open field (Beckwith et al., 1977a), and reductions in adult anxiety (Felszeghy et al., 1993). Early α-MSH treatment also impacts hypothalamic cytoskeletal proteins (Wu et al., 2006), hypothalamic dopamine neuron development (Egles et al., 1998), and neurite outgrowth (Joosten et al., 1996), thus leading to functional and structural changes in neural development. As MC agonists impact a variety of neural systems involved in sociality and acute MC4R stimulation promotes adult pair bonding, early MC stimulation may also stimulate encoding of early social information and lead to long-term organizational changes in social behavior.

Here, we investigated the hypothesis that daily MTII and the more selective, small

molecule MC4R agonist, PF446687 (Lansdell et al., 2010) injections during the first week of life would alter juvenile play and adult pair bonding in males and female prairie voles. We also assessed the impact of peripheral MTII on hypothalamic neuropeptide systems using the immediate early gene, early growth response factor-1, as a marker of neuronal activity. Daily neonatal treatment with MTII altered juvenile play behavior and both agonists enhanced adult social bonding, suggesting central MC4R stimulation has long-lasting, organizational effects on social neural circuitry. We further demonstrate that neonatal MT II rescues the deficits in social bonding in females who experienced early-life social isolations during the first two weeks of life. Thus MT II may mimic or enhance the beneficial effects of parental nurturing on the development of social behavior.

Materials and Methods

Animal care and handling.

Subjects were laboratory-bred prairie voles, derived from a field-caught Illinois stock. The olony was maintained at 22˚C and on a 14:10 h light:dark cycle with access to food (Purina high-fiber rabbit chow) and water *ad libitum*. Breeder housing consisted of large ventilated cages (34 × 30 × 19 cm) lined with bedding (bed-o-cob, Maumee, OH, USA). Subjects were not exposed to subsequent litters. Pups were weaned into same-sex same-treatment pairs or trios in smaller (30 × 18 × 19 cm) cages at PND21. From each litter, no more than 2 animals of the same sex and treatment group were used for each experiment. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee. All efforts were made

to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

Experimental design.

The timelines of experiments and animal numbers can be found in Figure 4.1 and the legend. Experiments 1 and 3 investigated the behavioral consequences of daily nonselective MC agonist MTII or selective MC4R agonist PF446687 in males and females. Experiment 2 examined the impact of acute MTII injection on hypothalamic systems and plasma corticosterone.

Experiment 1. Effect of neonatal MTII on social behavior.

Drug Administration.

To determine the developmental effects of early-life MTII administration, neonatal prairie vole pups were subcutaneously injected daily with MTII acetate (Alpha diagnostics Intl, San Antonio, TX) or 0.9% saline vehicle (sal: Hospira, Lake Forest, IL) from PND1-7, with PND0 as the day of birth. Pups were toe clipped to identify treatment groups. Animals were injected with a sufficient volume of drug solution at a 0.08 mg/ml concentration to deliver 10 mg/kg using a 250 μl syringe (Hamilton, Reno, NV) with a 30 gauge needle. Volumes were adjusted for growth rate and ranged from 35 μl on day 1 to 65 μl on day 7, yielding doses roughly equivalent to the 10mg/kg previously shown to facilitate partner preference formation in adults (Modi et al., Submitted). Weights were taken from PND1-7 and at weaning.

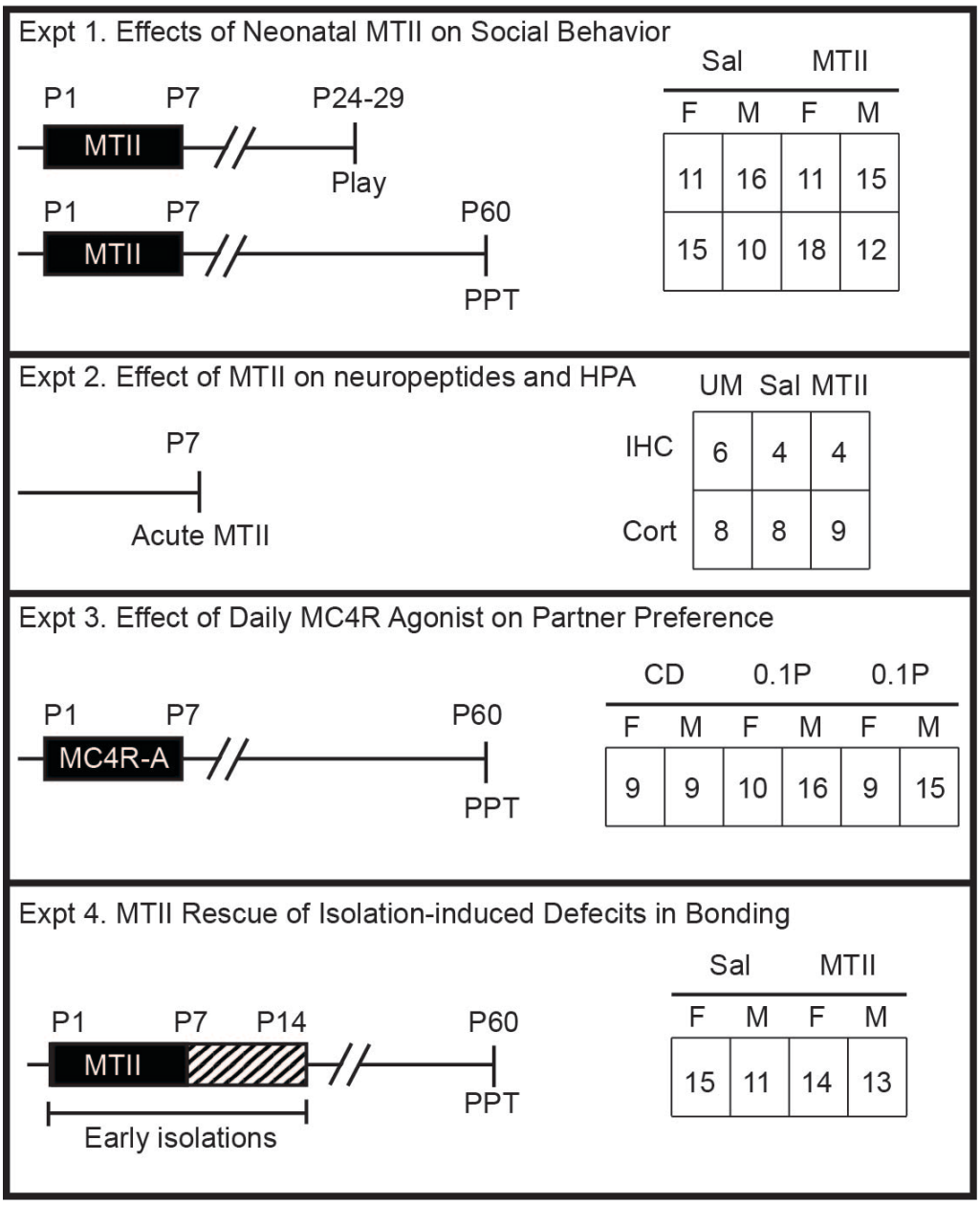

Figure 4.1. Experimental design.

In experiment 1, neonates were injected daily from PND1-7 with 10mg/kg of melanotan-II (MTII) and either tested for juvenile play behavior or adult partner preference (partner preference test, PPT). In experiment 2, PND6-7 neonates were sacrificed 1-hr after an acute injection of 10mg/kg MTII or saline, or no injection (UM, unmanipulated). Either immunohistochemistry (IHC) for hypothalamic neuropeptide activation was performed or plasma corticosterone was assayed. In experiment 3, neonates were injected daily with a melanocortin-4 agonist (MC4R-A, PF-446687, Pfizer) and tested for partner preference in adulthood. In experiment 4, neonates were isolated from parents for 3hr per day from PND1-14 (Chapter 3), and treated with either saline or MTII for the first week of the two-week isolation. Animal numbers are outlined in tables on the right. F, female; M, male.

Juvenile play.

One cohort of animals was treated with MTII (n=11 females, 15 males) or saline (n=11 females, 16 males) and tested for juvenile social play between ages 24-29. As isolation increases play behavior in rodents (Panksepp and Beatty, 1980), subjects were singly housed and tested daily for 3 d after the first 24 hr isolation. Between 0700 and 1200 each day, subjects were paired with an age, weight, and sex-matched colony stimulus animal in a clean arena (37 × 31 × 19cm) and video-taped for play behavior for 10 min. The frequencies of juvenile play and affiliative behaviors were coded by a blind observer using Noldus Observer (Noldus, Sterling, VA). Coat color variations between treatment groups were not distinguishable in the video recordings. Behaviors were scored based on ethograms previously described (Gavish et al., 1983; Chau et al., 2008; Parent and Meaney, 2008; Veenema and Neumann, 2009; Wang et al., 2012; Branchi et al., 2013). Working definitions of the play and social exploratory/affiliative behaviors can be found in Table 1.

Category	Behavior	Definition
Play	Aggressive grooming (AG)	one animal violently grabs at the fur of another and grooms, usually in the back region
	Boxing (B)	both animals stand on the hind legs and use only their forepaws to attack repeatedly
	Chasing (C)	active following
	Pinning (Pi)	the action of one animal holding another down overhead and the other is typically on its back or supine
	Pouncing (Po)	jumping on another animal making contact with forepaws and a common play initiation behavior
	Pulling/biting (PB)	mouth to body contact
	Supine (Su)	subject on its back, commonly a result of physical attack or pinning or can be independently induced
	Wrestling/tackling (WT)	ventrum-to-ventrum embracing with biting without inflicting wounds
Social Affiliative/Exploratory	Allogrooming (Allo)	received or directed grooming to another subject
	Genital investigation (GI)	sniffing or grooming directed towards other animal's genital region
	Huddling (H)	immobilized and in close contact
	Sniffing (Sn)	general sniffing the stimulus animal
	Social Contact (SC)	passive bodily contact

Table 1. Juvenile play ethogram.

The frequency of juvenile play and affiliation with a novel conspecific was coded using the above working definitions of behaviors. Behaviors initiated by the subject animal over a 10min period for 3d were coded by a blind observer.

Partner preference test.

A second cohort of males (n=12 MTII, 10 sal) and females (n=18 MTII, 15 sal) were tested for partner preference (PP). PP is a laboratory proxy for pair bond formation in which subjects spend significantly more time in immobile social contact with a previously cohabitated partner than with a novel stranger stimulus animal over a 3 hr

testing period (Williams et al., 1992). Subjects (~65 d of age) were paired with sexually-experienced, vasectomized or ovariectomized opposite-sex stimulus animals in a clean cage. Females were videotaped throughout the first 6hr cohabitation to verify that no mating occurred. In contrast, males were paired with an ovariectomized female who was not primed with estradiol to prevent mating. The use of vasectomized males ensured a prevention of pregnancy in experimental females, eliminating any potential confounds of hormonal changes on neural neuropeptide or receptor expression.

For females 6 hr of cohabitation without mating is not typically sufficient to stimulate PP (Williams et al., 1992). However, 24 hrs of cohabitation even without mating can lead to PP. In contrast, in our laboratory, males require longer cohabitation times without mating in order to develop PP. For this reason, females and males were tested on different schedules at two different time points (6-, 24-hr for females; 24-, 48-hr for males) to maximize the likelihood of detecting either enhanced or impaired PP. Subjects were placed in a 3-chambered arena with "partner" and novel "stranger" stimuli tethered to either end. Time spent in immobile social contact (huddling) with each female during this 3 hr session was scored with an automated system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA) as previously described (Ahern et al., 2009). After behavior testing, brains were collected for analysis of OT receptor, vasopressin V1a receptor and OT mRNA. As no significant differences were detected in any of these variables, these data are not presented.

Experiment 2. Effect of neonatal MTII on immediate early gene expression (IEG) in peptidergic PVN neurons and corticosterone release.

Procedure and tissue collection.

As MTII stimulates IEG activity in the PVN of neonates (Glavas et al., 2007), we examined the neuropeptide phenotype of activated neurons of the PVN 60 min after MTII administration. We also assayed plasma corticosterone to examine the impact of MC activation of the HPA axis of neonates, as MTII induces corticosterone release in adults (Lu et al., 2003). At PND6-7, entire mixed-sex litters were removed from the nest and immediately subcutaneously injected with 65 μl of either saline (sal; n=4) or 10 mg/kg MTII (n=4) or un-injected and only removed from the nest (unmanipulated, UM; n=6). After injection, pups were left isolated in a 30˚C incubator (Water-jacketed warmer base, Thermocare, Incline Village, NV) for 1hr. Pups were deeply anesthetized with isoflourane, euthanized by rapid decapitation, and brains were removed and acrolein fixed. A second cohort of pups (n= 8 sal, 9 MTII, 8 UM) was similarly injected and blood was collected for analysis of corticosterone response 1 hr after injection. Approximately equal numbers of each sex were used and combined in the analyses.

Brain processing and immunohistochemistry.

Brains were removed and immersion fixed in 10 ml of 5% acrolein diluted in PBS for 3hr, followed by two 10min washes in phosphate buffered saline (PBS, pH 7.4). Brains were transferred to 30% sucrose at 4 ˚C for at least 24 hr and sectioned on a microtome at 40 μM. Sections were stored in cryoprotectant at 4˚C until immunohistochemical processing. Every third section was processed for Early Growth Response Protein-1 (EGR-1) and either OT, vasopressin (AVP) as described previously (Modi et al., Submitted) or corticotropin releasing factor (CRF). EGR-1 was chosen as a measure of neural activity as it has been reported to be a more sensitive marker of activity in the hypothalamus than Fos (Polston and Erskine, 1995). For immunofluorescence,

sections were rinsed for 15 min in 0.1% sodium borohydride, pre-blocked for 1hr with normal goat serum, and then incubated for 47 hr at 4˚C in primary antibody in PBS (pH 7.4) with 0.3% triton-X and 2% NGS (1:8,000 rabbit polyclonal anti-EGR1, sc-189, Santa Cruz Biotechnology, Santa Cruz, CA; 1:10,000 mouse monoclonal anti-OT, mAb5296, Millipore, Billerica, MA). For AVP-EGR1 double labeling, sections were incubated in primary for 46 hr (1:1,000 mouse monoclocal anti-VP, PS41, generously donated by Dr. H. Gainer, NIH, USA, (Ben-Barak et al., 1985)). Sections were washed in PBS and incubated in secondary Alexa Fluor conjugated antibodies for 3hr at 4˚C (1:1,000 Alexa Fluor 568 for EGR-1 and Alexa Fluor 488 for OT or AVP, Life Technologies, Grand Island, NY). Tissue was mounted and cover slipped with Vectashield mounting medium with Dapi (Vector, Burlingame, CA), and Z-stack images were taken on a Leica confocal microscope at 40X magnification. Images were processed in Image J (NIH). EGR-1 positive nuclei were distinguished by red immunofluorescence, and OT or AVP cell bodies were labeled with green immunofluorescence.

CRF-EGR1 was processed with diaminobenzidine (DAB) immunostaining as the primary antibodies were produced in the same host. Sections were rinsed in 0.1% sodium borohydride for 15min, washed in PBS, rinsed in 0.05% hydrogen peroxide for 30min, washed in PBS-0.3% triton-X (PBST), preblocked in 5% NGS for 1hr, and incubated in primary antibody in PBST-2% NGS at 4˚C for 45 hr (1:20,000 rabbit polyclonal anti-CRF, c-5348, Sigma-Aldrich, St. Louis, MO). Sections were then washed in PBST-2% NGS and incubated in biotinylated secondary in PBST-2% NGS (goat anti-rabbit, Vector, Burlingame, CA). An ABC kit (Vector, Burlingame, CA) was used to detect secondary binding, and a DAB kit was used for visualization (Vector, Burlingame, CA). Sections

were washed and then incubated in anti-EGR-1 antibody for 42 hr at 4˚C. EGR-1 was visualized with Nickel-DAB using the same kit. Sections were mounted, ethanol dehydrated, and coverslipped with Krystalon mounting media (Thermo Fisher Scientific, Waltham, MA). EGR1 nuclei were distinguished by purple nickel DAB staining, and CRF cell bodies by brown DAB staining.

The total number of EGR-1 double-labeled neurons in the PVN were counted in four to six bilateral sections for OT, two to four for AVP, and two to eleven for CRF from each subject. The total percentage of EGR-1 positive OT, AVP, or CRF cells was calculated across all PVN sections for each animal.

Blood collection and corticosterone assay.

Plasma was assayed for corticosterone response to drug injection as previously described (Ahern et al., 2009). Approximately 100 μl trunk blood was collected with heparinized capillary tubes into EDTA-coated tubes with 5 μl aprotinin (Trysol, Fisher Scientifi) to prevent protease activity. Blood was spun at 5000rpm for 5min and plasma was collected and stored at -20˚C until assayed. Plasma samples were diluted 1:100 and assayed with a commercially available kit (MP Biomedicals, Orangeburg, NY) in triplicate (inter-assay CV, 7.2%, intra-assay CV, 10.3%). Assay services were provided by the Biomarkers Core Laboratory at the Yerkes National Primate Research Center. This facility is supported by the Yerkes National Primate Research Center base grant 2P51RR000165-51.

Experiment 3. Effect of Neonatal MC4R agonist PF446687 on Social Behavior.

Procedure.

To determine if behavioral effects of MTII are attributable to MC4R activation, pups were injected subcutaneously daily from PND 1-7 with the selective, highly brain penetrant MC4R agonist PF446687 (Lansdell et al., 2010, supplied by Pfizer Global Research and Development, Cambridge MA) as described for MTII. Neonates received vehicle (10% hydroxypropyl-cyclodextrin; Sigma-Aldrich, St. Louis, MO), 0.1mg/kg, or 1mg/kg PF446687. Animals were injected with a sufficient volume of drug solution at a 0.087 mg/ml concentration to deliver 1 mg/kg or 0.0087 mg/ml to deliver 0.1 mg/kg. Volumes ranged from 32 μl on day 1 to 60 μl on day 7. These doses are roughly equivalent to the 0.1 mg/kg and 1 mg/kg doses given to adults (Modi et al., Submitted).

Partner preference test.

PP testing was performed as described above in males (n=9 veh, 16 0.1 mg/kg, 15 1 mg/kg PF44687) and females (n=9 veh, 10 0.1 mg.kg, 9 1 mg/kg PF446687). However, subjects (~70 d of age) were paired with intact partners rather than gonadectomized partners as we did not intend to analyze receptor expression levels following the procedure as in Experiment 1. Female prairie voles are induced ovulators and take approximately 2 days to go into estrus and mate (Carter et al., 1988). Thus, female subjects likely did not mate for the entire 24hr while males paired with intact stimuli likely began mating toward the end of the 48hr cohabitation.

Experiment 4: MT II rescue of social deficits induced by early-life social isolation.

Procedure.

Whole litters were exposed to early isolation from PND1-14 as described in Chapter 3. Within a litter, individual pups were injected with either melanotan-II (MTII)

or saline. Pups were toe clipped on the left or right forepaw to identify drug or saline treatment groups. Neonates received 28μg MTII in volume of 35μl 0.1% sterile saline for the first 2 days of life, 40μg MTII in volume of 50μl for days 3-5 and 52μg MTII in volume of 65μl for days 6-7 or an equivalent volume of saline. These doses are roughly equivalent to the 10mg/kg doses given to prairie voles in previous studies (Modi et al., Submitted; Barrett et al., Under revision). Weights were measured daily between PND1-14 to determine the effect of MTII treatment on weight gain and at weaning on PND21. Each pup within a litter was uniquely toe clipped and thus weights were tracked for each individual animal and not averaged across a litter. After PND14, pups were left undisturbed until PND21, at which time offspring were weaned into same sex and treatment duos or trios. No more than 2 females of the same treatment were used from each litter.

Partner preference.

To determine if daily MTII treatment enhanced adult bonding, subjects were tested for their preference for an opposite-sex conspecific after a shortened non-mated cohabitation. At approximately 60d of age, subjects were paired with a sexually-naïve, intact opposite-sex partner. Females (n=15 saline, 14 MTII) were paired for 6hr and video-taped to ensure no mating occurred. One female that mated within the MTII group was excluded from behavioral analyses. Males (n=11 saline, 13 MTII) were paired for a longer 24hr cohabitation period, as they typically take longer to form a partner preference in our lab. Female stimulus animals were not primed, and thus mating likely did not take place as voles are induced-ovulators and take 2-3d to go into estrus after exposure to male scent (Witt et al., 1988).

Corticosterone assay.

At approximately 70 days of age, animals were sacrificed with CO2 and trunk blood was collected and assayed for corticosterone as described above (inter-assay CV, 14.9%, intra-assay CV, 12.2%).

Statistical analyses.

Data are presented as mean ± SEM, unless otherwise noted. Weights were analyzed with repeated measures (RM) ANOVA with day as a within-subjects factor and treatment as a between-subjects factor, followed by *post-hoc* Student's t-tests. Litter effects were tested using multivariate ANOVA within each experiment, but no significant main effects of breeder pair were detected. For play data, the frequency of each individual behavioral event initiated by the subject animal was summed across each 10 min period into either a "play" or "social exploratory/affiliative" category and a RM-ANOVA with sex and treatment as between-subjects factors and time as a within-subjects factor was performed. Planned Student's t-tests were performed between treatment groups on the last day of testing, as pilot studies showed that play is enhanced over time. Individual play and social exploratory/affiliative behaviors were analyzed using a non-parametric Mann-Whitney U test, as data failed to reach normality using the Kolomogoroc-Smirnov test. Partner preference data were analyzed with planned Student's t-tests comparing huddling with the partner over the stranger. A Bonferonni correction was applied for multiple analyses within each sex and PP test (alpha = 0.025 for Exp 1; alpha = 0.017 for Exp 3). Immunohistochemistry results were analyzed with univariate ANOVA's with drug treatment as a factor and post-hoc Bonferonni t-tests between the three treatments. Statistics were performed with SPSS Statistics 17.0 with

significance set at $p<0.05$ and two-tailed tests.

Results

Experiment 1. Effect of neonatal MTII on social behavior.

Weight and coloration.

MTII treated animals weighed significantly less than vehicle controls from PND 2-7 (Figure 4.2A; $F_{1,31}=17.25$, $p<0.001$). Similar results were obtained from Cohort 1. MTII treatment impaired weight gain over time (time by treatment $F_{6,186}=14.92$, $p<0.001$). Weights did not differ at weaning ($p > 0.05$; Figure 4.2B). Although not empirically quantified, MTII treated animals displayed darkened pigmentation as neonates (Figure 4.2C) and weanlings (Figure 4.2D), which was normalized in adults.

Juvenile play and affiliation.

Initiated play bouts increased over subsequent testing days ($F_{2,98}=6.04$, $p=0.003$). A significant interaction between sex, treatment and time on initiated play bouts was detected ($F_{2,98}=3.19$, $p=0.045$; Figure 4.3A). A significant increase in play from D1 to D3 was seen in saline-injected males ($p=0.005$), but not in MTII males. In contrast, MTII-treated ($p=0.032$), but not saline-treated, females displayed increased play from D1 to D3. On the last day of testing, there was a main effect of drug ($F_{1,49}=4.76$,$p=0.034$) and a sex by drug interaction ($F_{1,49}=4.22$, $p=0.045$; Figure 4.3B). Neonatally MTII-treated males, but not females, initiated less play bouts compared with saline controls ($p=0.008$; Figure 4.3B). This effect was driven by a decrease in aggression-like wrestling/tackling ($U=65.5$, exact $p=0.030$) and boxing ($U=54.5$, exact $p=0.008$) in MTII males (Figure 4.3C). Thus, early treatment with MTII reduced male, but not female, juvenile play, but

only those components involving aggressive-like behaviors. Contrastingly, MTII females initiated significantly more bouts of social exploratory/affiliative behaviors than did saline treated females (sex X treatment, $F_{1,49}$=9.66, p=0.003; female MTII vs Sal, p=0.021), which was specifically driven by an increase in sniffing (U=18.5, exact p=0.004; Figure 3D). There was no effect of MTII treatment on social exploratory/affiliative behavior in males.

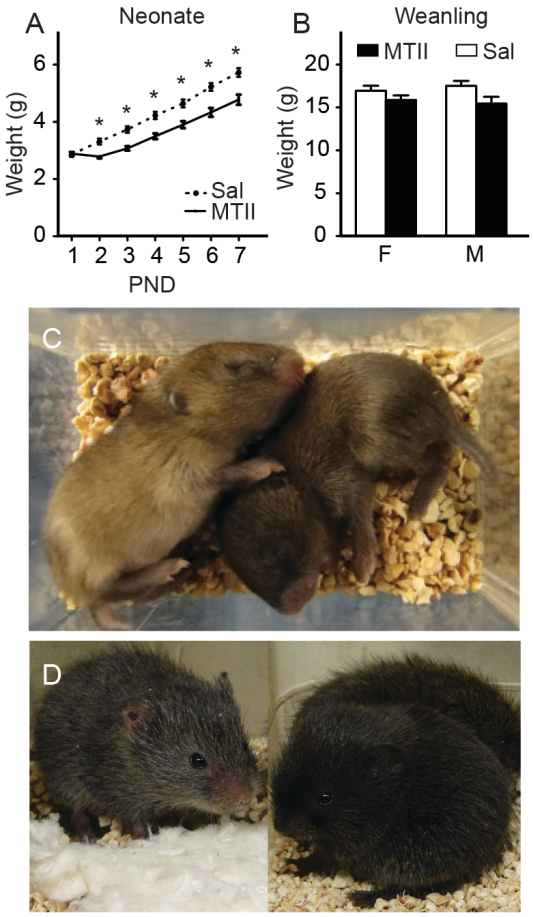

Figure 4.2. Daily neonatal MTII treatment reduced weight gain and induced darkened pigmentation.

Prairie voles injected with MTII from PND1-7 weighed less than saline controls on PND2-7 (A), which was normalized by weaning at PND21 (B). Although not empirically quantified, daily MTII led to fur darkened pigmentation in neonates (C, left Sal, right MT II). Darkened pigmentation from early MTII injections was still observed at weaning (D, left Sal, right MT II). Asterisks in A indicate *post-hoc* Student's t-tests with p<0.05

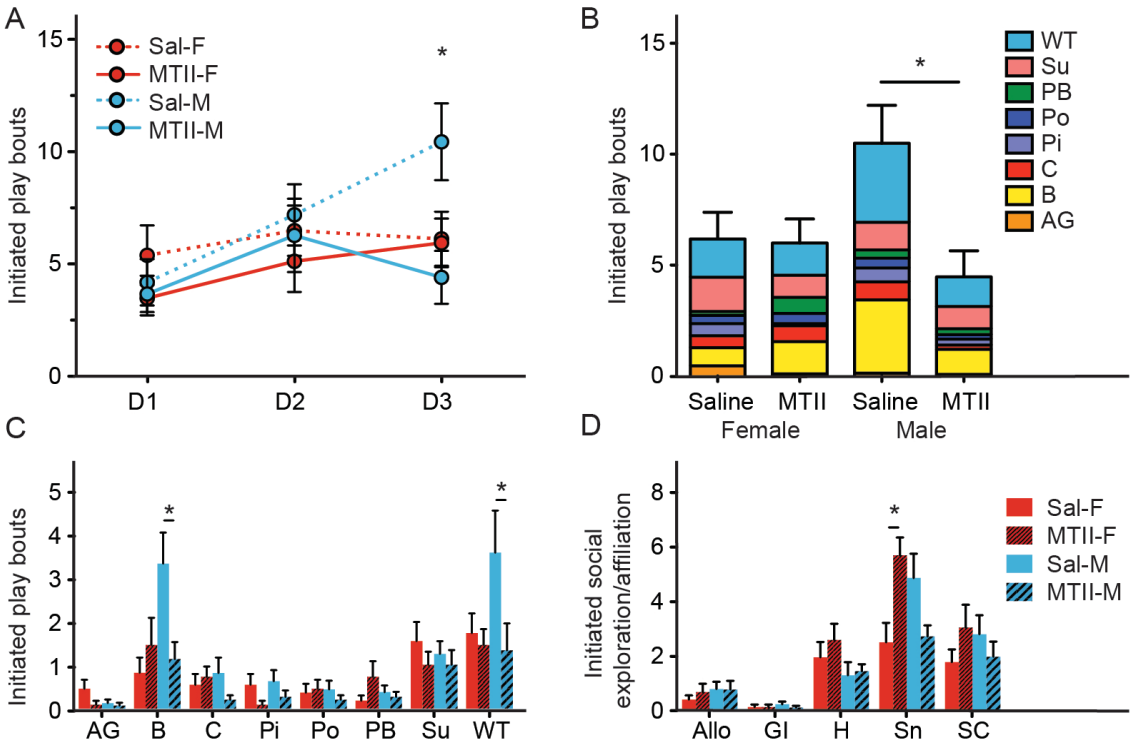

Figure 4.3. Daily neonatal MTII treatment reduced male juvenile play bouts.

Between PND24-29, juvenile prairie voles injected with MTII or saline from PND1-7 were singly housed and tested for play behaviors for 10min with a novel untreated stimulus animal over a 3d period. Bouts of initiated play increased over time in saline males, but remained stable in MTII males (A). On the third testing day, MTII male play was significantly lower than saline male play (B), which was driven by a decrease in boxing and wrestling/tackling (C). MTII treated females displayed greater levels of sniffing than saline females on the third day of testing (D). Asterisks in A-B indicate *post-hoc* Student's t-tests with $p<0.05$ and in C-D indicate Mann-Whitney U tests with $p<0.05$. AG, aggressive grooming; B, boxing; C, chasing; Pi, Pinning; Po, pouncing; PB, pulling/biting; Su, supine; WT, wrestling/tackling; Allo, allogrooming; GI, genital investigation; H, huddling; Sn, sniffing; SC, social contact.

Partner preference test.

Females that received neonatal MTII, but not saline, spent significantly more time with the partner than the stranger male after a 6hr non-mated cohabitation (Figure 4.4A; MTII, p=0.007, sal, p=0.793). After the full 24hr cohabitation, both experimental and control groups displayed a preference for the partner (Figure 4.4A; MTII p<0.001, Sal p=0.001). MTII treatment did not impact male partner preference, and neither experimental nor control groups spent significantly more time with the partner over the stranger at either point (Figure 4.4B).

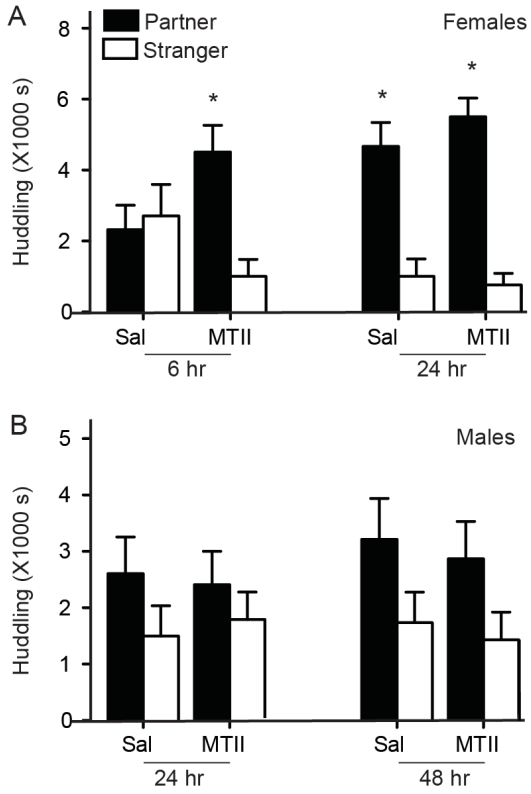

Figure 4.4. Daily neonatal MTII facilitated adult female partner preference.

In females, early MTII treatment facilitated adult partner preference after an abbreviated 6 hr cohabitation without mating (A). MTII treatment had no effect on male partner preference after 24- or 48-hr of cohabitation with an unprimed female (B). Asterisk indicates planned Student's t-tests with p<0.025.

Experiment 2. Effect of neonatal MTII on IEG expression in peptidergic neurons in PVN and corticosterone release.

Activation of OT, AVP, and CRF neurons.

The percent of peptidergic neurons immunopositive for EGR1 differed significantly between un-manipulated, saline, and MTII groups (OT, $F_{2,\ 11}$=51.51, p<0.001; AVP, $F_{2,\ 11}$=16.63, p<0.01; CRF, $F_{2,\ 11}$=605.34, p<0.01; Figure 4.5A-J). A significant increase in EGR1 positive neurons for all neuropeptidergic populations was observed following an MTII injection, in comparison to either saline or no injection (p<0.005). Saline-injected neonates displayed a greater percentage of CRF-EGR1 cells (p<0.001), but not OT-EGR1 or AVP-EGR1, in comparison to unmanipulated controls.

Corticosterone.

MTII injected neonates displayed a significant increase in plasma corticosterone 1hr after the injection in both sexes (Figure 4.5K; $F_{2,22}$=25.73, p<0.001; MTII vs UM, p<0.001; MTII vs Sal, p=0.001, Sal vs UM, p=0.201). There was no significant sex by treatment effect.

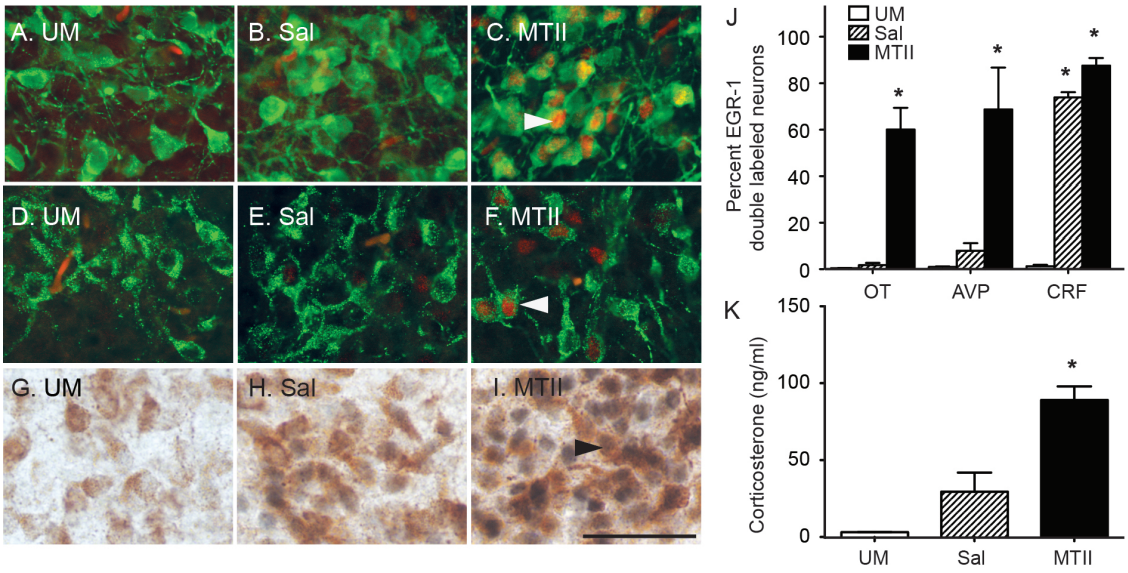

Figure 4.5. MTII-induced neuropeptide activation in the PVN.

PND6-7 neonates were euthanized 1hr after an acute peripheral injection of 10mg/kg MTII and representative images of brains processed for immunohistochemistry for OT-EGR1 (A,B,C), AVP-EGR1 (D,E,F), and CRF-EGR1 (G,H,I) are shown. The left panel was uninjected (A,D,G), the middle was injected with saline (B,E,H), and the right received MTII (E,F,I). In fluorescent images, OT and AVP positive cell bodies are green and EGR1-labeled nuclei are red. In DAB processed slices, CRF cell bodies are brown, and EGR1 nuclei are black. MTII elicited significant activation in OT, AVP, and CRF neurons, whereas saline only activated CRF cells in comparison to uninjected controls (J). MTII injected neonates mounted a corticosterone response (K). UM=unmanipulated. Color channels were adjusted in Image J (NIH) for fluorescent images. Asterisks indicate Student's t-tests with $p < 0.05$. Scale bar represents 50μM.

Experiment 3. Effect of neonatal selective MC4R agonist PF446687 on social behavior.

Weight and coloration.

PF446687 treated neonates had slowed weight gain over the first week of life ($F_{6,312}$=2.88, p=0.001) but did not weigh significantly less than vehicle treated controls at any age (Figure 4.6A,B). Although we did not quantify pigmentation objectively, there was no obvious difference in pigmentation following daily PF446687 treatment.

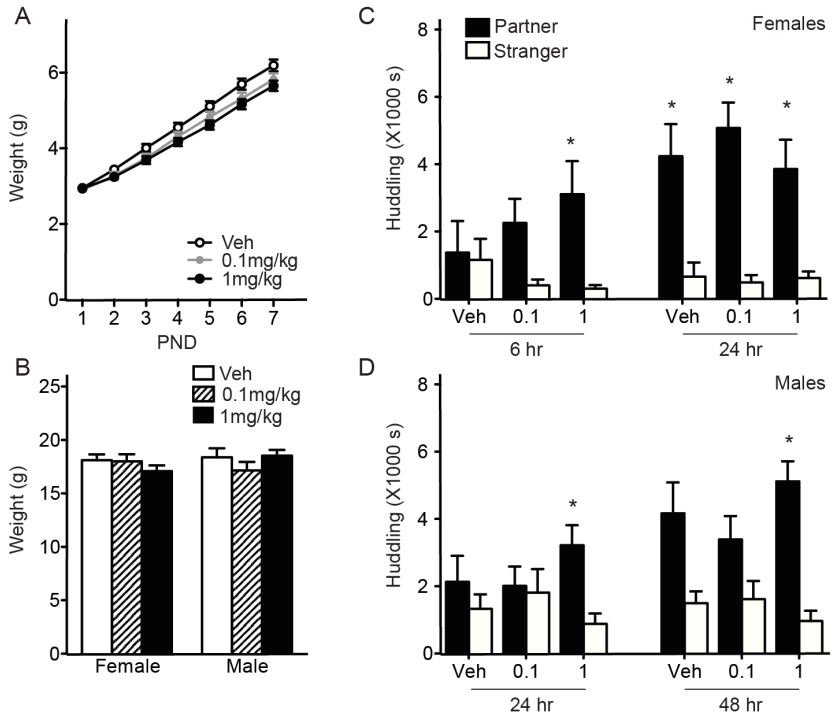

Figure 4.6. Daily neonatal treatment with the specific MC4R agonist PF446687 facilitated partner preference in both sexes.
Daily injection with either 0.1mg/kg or 1mg/kg PF446687 did not lead to a significant weight loss in neonates (A) or juveniles (B) in comparison to vehicle-injected controls. Prairie vole females injected with the either dose of PF446687 from PND1-7 spent significantly more time huddling with the partner over the stranger after an abbreviated 6hr cohabitation (C). Only those males injected with the high dose of PF446687 spent more time with the partner at both 24 and 48hr of cohabitation with an unprimed female (D). Asterisks indicate planned Student's t-tests with $p < 0.017$.

Partner preference.

Adult females neonatally treated with either the low (0.1 mg/kg) or high (1 mg/kg) dose of PF446687, but not the vehicle, spent significantly more time huddling with the partner over the stranger following a 6hr-cohabitation (Figure 4.6C; vehicle, p=0.855, 0.1mg/kg, p=0.025; 1mg/kg, p=0.015). Only the high dose remained significant after Bonferonni correction (alpha = 0.017). All groups displayed a preference after 24hr of cohabitation (vehicle, p=0.015; 0.1mg/kg, p<0.001; 1mg/kg, p=0.009). In males, 1mg/kg PF446687 treatment resulted in significantly more time spent huddling with the partner over the stranger at both 24- and 48-hour time points (Figure 4.6D; 24hr, p=0.006; 48hr, p<0.001). Vehicle and 0.1mg/kg PF446687 treated males did not show a significant preference at either time point.

Experiment 4: MT II rescue of social deficits induced by early-life social isolation.

Weight.

A RMANOVA within each sex revealed a significant interaction between drug and PND ($F_{13,354}$=2.77, p=0.001) in females, but not males (Figure 4.7A,B). In females, MTII treatment significantly reduced weights on PND2 (p=0.012), 3 (p=0.032), and 5 (p=0.036). Weights were reduced in males on PND2 (p=0.021), 4 (p=0.028), 7 (p=0.038), and 8 (p=0.032). Weights were no different between treatment groups between PND6-14 and at weaning (p>0.05).

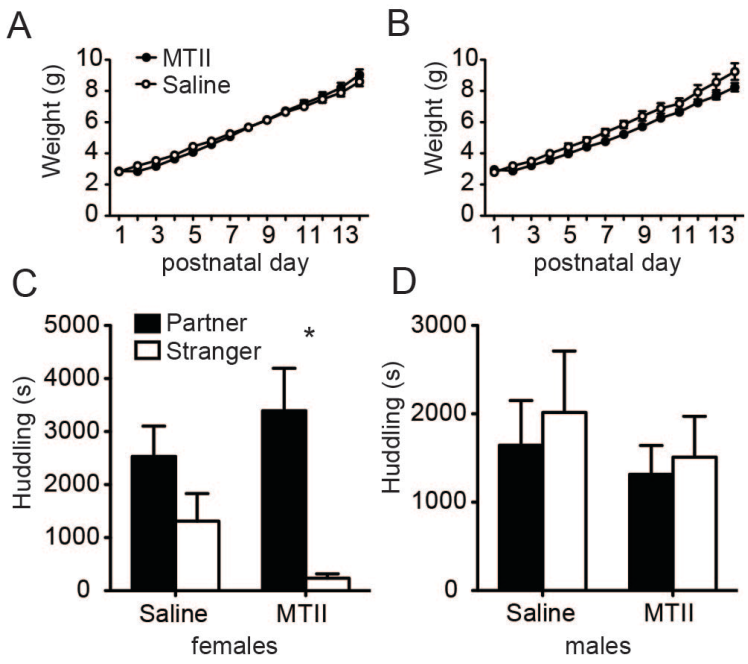

Figure 4.7 Females exposed to early isolation form PP when given MT2.
Voles were treated with the melanocortin agonist MTII for the first week of the two-week isolation. Females displayed slowed weight gain for the first week of life, but accelerated weight gain when the drug was off-board (A). Males displayed slowed weight gain during the entire two-weeks (B). MTII treatment significantly reduced female weights on PND2, 3, 5 and male weights on PND2, 4, 7, 8. Neonatal MTII treatment facilitated female pair bonding after a 6-hr nonmated cohabitation (C), but had no effect in males (D). Asterisk indicates *post-hoc* t-test, $p<0.05$.

Partner preference.

To determine if promoting early OT release with the melanocortin receptor agonist MTII would buffer against early isolation described in Chapter 3, we tested adult partner preference in early isolated females that received either MTII or saline daily for the first week of life. Neonatal MTII treatment has previously been shown to facilitate adult partner preference after a shortened 6hr cohabitation period (Barrett et al., Under revision). After an abbreviated 6hr non-mated cohabitation, a RMANOVA (stimulus animal X treatment) revealed a main effect of partner ($F_{1,28}=16.00$, $p<0.001$), but a nonsignificant interaction effect ($F_{1,27}=2.74$, $p=0.109$). Planned pairwise t-tests revealed

that the isolated females treated with chronic MTII spent significantly more time huddling with the partner over the stranger (p=0.002; Figure 4.7C) whereas the saline injected controls did not (p=0.166). No impact of early MTII treatment was detected in males (Figure 4.7D).

Corticosterone.

Adult basal corticosterone levels were decreased in MTII treated males (p=0.032; Sal, 1701±192 ng/ml, MTII, 2611±366 ng/ml). Female corticosterone levels did not differ (Sal, 3915.5±367.2; MTII, 3869.7±475.7).

Discussion

Developmental MC agonist administration impacted later juvenile and adult social behaviors. Daily peripheral injection (PND1-7) with the nonselective MC agonist MTII reduced later male, but not female, juvenile play behaviors (Figure 3A-D). We previously found that an acute injection of MTII robustly facilitates partner preference, and this effect is blocked by pre-treatment with an OT antagonist (Modi et al., Submitted). Here, we found this same compound to lead to persistent changes in social bonding into adulthood (Figure 4A). Females, but not males, given neonatal MTII displayed an enhancement in partner preference after an abbreviated non-mated cohabitation with an opposite-sex partner. PND6-7 neonates injected acutely with MTII mounted a corticosterone response and displayed robust hypothalamic EGR-1 activity in OT, AVP, and CRF neurons (Figure 4). When given the more selective, highly brain penetrant MCR4 agonist PF446687, both sexes exhibited enhanced adult partner

preference (Figure 6C,D), suggesting that perhaps females are more sensitive to the MC manipulation. Sex-specific behavioral effects of neonatal alpha-MSH administration have previously been reported (Beckwith et al., 1977a; Beckwith et al., 1977b). Activation of MC receptor subtypes other than MC4R may mask the effects of MC4R activation on partner preference in MTII-treated males. Finally, early MTII treatment prevented the impairments in adult female bonding that we observed in Chapter 3 (Figure 7C,D). Overall, the presence of persistent behavioral effects in adults resulting from neonatal MTII or PF446687 administration suggests that MC4R activation in neonates developmentally alters the social brain circuitry, leading to an enhancement of adult social attachment.

MTII and PF446687 treatment also had differential effects on weight and pigmentation. MTII induced neonatal weight loss, likely via MC3 and MC4R (Huszar et al., 1997; Chen et al., 2000a), and darkened pigmentation, via MC1R (Thody, 1999; Figure 1). PF446687 treated neonates did not weight less than vehicle controls, but displayed slowed weight gain (Figure 5A). Although the MC4R system is critically involved in feeding, MC agonists do not always lead to weight loss (Wu et al., 2006; Muceniece et al., 2007). Additionally, multiple second messenger pathways activated from the same receptor or from different MCR4 agonists (Konda et al., 1994; Nickolls et al., 2005) or additional activation of the MC3R (Chen et al., 2000a) may account for this discrepancy.

Saline-treated males displayed a trajectory of increased initiated play bouts over subsequent testing days, but saline-treated females displayed consistent levels throughout testing. Neonatal MTII-treated males fail to show this trajectory, and display significantly

reduced play on the last day of testing compared to saline-treated males. A similar phenomenon is found in male rats with altered early care, as high levels of licking and grooming or a simulated tactile stimulation regime reduce juvenile play (Moore and Power, 1992; Parent and Meaney, 2008; Edelmann et al., 2013). In contrast, maternal separation increases rough and tumble play (Veenema and Neumann, 2009). Thus MTII treatment mimicked the effects of high levels of maternal nurturing. Social play during the juvenile period is part of the normal development of aggressive and sexual behaviors in males (Taylor, 1980; Spear and Brake, 1983) and is often higher in males than females (Poole and Fish, 1976; Thor and Holloway JR, 1983). MTII-treated females did not play more than saline females, although MTII treatment increased female play over time. Thus, neonatal MTII treatment differentially impacted male and female play. MTII treated females also displayed enhanced sniffing during the social play test.

It should be noted that isolation was necessary to achieve a significant amount of play in voles, as has previously been shown in mandarin voles (Wang et al., 2012) and rats (Panksepp and Beatty, 1980). As isolation in voles increases basal corticosterone, PVN CRF levels, and AVP levels in the supraoptic nuclei, (Ruscio et al., 2007), it is possible that these systems act to promote play fighting. Indeed, play fighting in rats increases PVN CRF mRNA, and maternally separated rats display increased play fighting and increased PVN AVP mRNA (Veenema and Neumann, 2009). However, whether play is endogenously important for the social development of voles remains to be determined.

Developmental effects of neonatal alpha-MSH and ACTH have previously been reported. Daily (PND2-7) peripheral administration of alpha-MSH to rat pups improves adult male learning and visual discrimination (Beckwith et al., 1977b) and increases time

spent in social contact in both sexes (Beckwith et al., 1977a). Melanocortins promote neuroplasticity and neurotrophin activity (Joosten et al., 1996; Xu et al., 2003; Shen et al., 2013), which may lead to persistent changes in neural architecture into adulthood. Indeed, alpha-MSH treatment for the first two weeks of life alters hypothalamic expression of cytoskeletal proteins involved in synaptic plasticity (Wu et al., 2006). MC4R stimulation also induces hypothalamic brain derived neurotropic factor release, which exerts effects on feeding and cardiovascular function, but may also position the system to regulate synaptic plasticity (Nicholson et al., 2007; Gomez-Pinilla et al., 2008). As pair bond formation requires social learning, attention to relevant social cues, and the drive to preferentially associate with a partner (Young and Wang, 2004b), these changes in adult behavior would serve to promote social attachment.

An acute peripheral MTII injection in adult prairie voles results in IEG expression in OT neurons but not AVP neurons, suggesting a selective response (Modi et al., Submitted). Here, we found an acute injection of MTII robustly activated EGR-1 immunostaining in OT, AVP and CRF neurons in the PVN in prairie vole pups. Thus, neonatal MC stimulation has a more robust and general activation of hypothalamic neuropeptide systems, perhaps due to increased permeability of the blood brain barrier (Glavas et al., 2007). In adult mice, subsets of MC4R neurons in the PVN contain OT, CRF, and TRH mRNA, but no co-labeling with AVP mRNA was detected (Liu et al., 2003). However, alpha-MSH increases AVP release in hypothalamic explants (Dhillo et al., 2002). MTII has higher affinity for MC4R (Oosterom et al., 1999) and may not be getting into the brain of adults at a level high enough to activate the subtype of MCRs on AVP neurons. It is also possible that neonates display a different developmental pattern

of expression of MC receptors, suggestive of a functional difference in melanocortin systems during development.

One possible mechanism by which MC agonists are impacting socioemotional behavior is through the potentiation of the central OT system. Oxytocin decreases feeding (Arletti et al., 1989), promotes sexual behavior (Gorzalka and Lester, 1987), and induces the stretching, yawning, grooming response (Melis et al., 1986), thus may mediate some of the downstream anorexigenic and behavioral effects of MC stimulation (Sabatier, 2006). Activation of the OT system during early life, either through endogenous mechanisms or pharmacological interventions, can have a life-long impact on social behavior and neuroendocrine function (Carter et al., 2009; Keebaugh and Young, 2011b). An injection of OT on the first day of life promotes adult partner preference in prairie voles (Bales and Carter, 2003a; Bales et al., 2007c), thus our results are in line with an OT-dependent mechanism of MTII. The closely related neuropeptide AVP has also act early in life to mediate adult sociality, as daily postnatal treatment with AVP enhances later aggression in males prairie voles (Stribley and Carter, 1999). However, the long-term effects on partner preference formation are unknown. Neuropeptide signaling during infancy, specifically during parent-infant interactions (Caba et al., 2003), may assign a rewarding value to social stimulation and shape the neural networks that shape social bonding. The possibility that α-MSH may endogenously be released during nursing in neonatal animals and acts to prime OT neurons and promote early social attachment warrants investigation. We did not detect any effect of MTII treatment on later OT mRNA in the PVN or OTR or vasopressin 1a receptor binding in any brain region examined (data not shown), suggesting early neuropeptide treatment may have led to

changes in other neurotransmitter systems.

Prairie vole neonates mounted a corticosterone response and showed CRF neuron activation after MTII injection. However, saline injection alone induced significant CRF neuron activation, but not corticosterone release, as described previously in rats (Smith et al., 1997). Neonatal saline injection has been shown to have behavioral consequences in male prairie voles (Stribley and Carter, 1999; Bales and Carter, 2003a). It is possible that early CRF activation impaired control male pair bonding, and MTII did not rescue this behavior. Although in adulthood MCR activation may promote stress and anxiety (Lu et al., 2003; Liu et al., 2013), developmental effects may be act in the opposite direction. Neonatal POMC ablation leads to an anxiogenic phenotype in adulthood (Greenman et al., 2013), whereas neonate ACTH4-9 administration reduces later anxiety and depressive-like behavior (Felszeghy et al., 1993). In a phenomenon known as stress inoculation, a certain amount of activation of the stress axis early in development can have beneficial effects on emotionality later in life (Parker and Maestripieri, 2011). However, MTII acting directly on adrenal MC2R likely increases corticosterone release in comparison to the selective MC4R agonist PF446687, and the heightened stress activation may counteract effects on adult prosociality in MTII-treated male voles. Additionally, MC4R stimulation in the central amygdala leads to reductions in CRF mRNA (Brunson et al., 2001). It is therefore possible that the modulation of stress axis by MC4R underlies the observed changes in adult sociality.

MC4R are located in multiple limbic brain areas outside of the hypothalamus (see Mountjoy, 2010), including regions mediating sociality in prairie voles including the amygdala and prefrontal cortex (Modi et al., Submitted). In addition to impacting

hypothalamic neuropeptide systems, MC4R signaling has been linked to dopaminergic and opioid-mediating reward and reinforcement systems, which are critically involved in pair bond formation (Burkett and Young, 2012a). Alpha-MSH or MTII stimulates dopamine release in the ventral tegmental area through MC4R activation (Lindblom et al., 2001), alters dopamine receptor expression in the nucleus accumbens and tegmentum (Lindblom et al., 2002), modifies accumbal D1R signaling in response to stress (Lim et al., 2012), and influences hypothalamic dopamine neuron differentiation (Egles et al., 1998). The rewarding and addictive effects of cocaine and opiates are thought to be mediated by MC4R signaling (Alvaro et al., 1996; Alvaro et al., 1997; Hsu et al., 2005). The MC4R mediates a pleitropy of behavioral and neurobiological effects, and future studies will address the mechanism by which MC4R activation impacts sociality.

Melanocortin enhancement of adult bonding in isolated females

Given the enhancement of later social bonding, we assessed the ability of MCR agonists to buffer against early life disruptions in parental care and rescue behavioral deficits in social attachment. Indeed, melanocortin agonist treatment for the first week of life buffered against early isolation. Female voles that experienced early isolation and were given MTII, but not saline, formed a partner preference after an abbreviated 6hr cohabitation. Interestingly, MTII lead to a reduction in male basal corticosterone levels compared to isolated-only males. A reduction in basal anxiety states may in part explain the absence of pair bond facilitation in males. MTII activates central OT neurons and primes them for subsequent stimulation (Sabatier et al., 2003; Modi et al., Submitted), thus promoting endogenous OT release in response to physiologically relevant stimuli. As we saw in chapter 3, tactile stimulation activated OT neurons in prairie vole neonates.

Thus, is it possible that heighted OT release early in life increased encoding of incoming social information, and overcame the negative impact of social isolation. Although our results are in line with an OT-dependent mechanism, the MC4R mediates a variety of other effects and, thus, our study cannot be construed as proof that MC4R effects on behavior are due to effects on the OT system. The precise mechanism by which daily MC4R activation is promoting adult social bonding warrants further investigation.

Conclusions and future directions

Our findings provide the first evidence that daily neonatal modulation of MC4R enhances later social relationships. Early activation of the MC system led to persistent changes in juvenile and adult sociality, indicating that MC4R stimulation has long-term effects on the development of social behavior. The effects of MC activation on both play and partner preference formation are in the opposite direction of early social deprivation, suggesting that perhaps MC activation mimics the neural impact of parental nurturing. MC4R activation coordinates a variety of neurobiological and behavioral responses that are linked to social bond formation in prairie voles. Whether the MC system plays a role in the transduction of parental nurturing to prosocial neural development warrants further investigation. Elucidation of the factors and mechanisms mediating normative social behavioral development can ultimately help to shed light on preventative strategies in the treatment of human disorders of the social domain.

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CHAPTER 5

General Conclusions and Future Directions

Partly modified from the following reference:

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Summary

Deficits in social communication and formation of stable interpersonal attachments are characteristic of mental illnesses such as borderline personality disorder, social anxiety, schizophrenia and autism spectrum disorders. Understanding the neural circuitry underlying complex social behaviors is key to developing effective preventative interventions or pharmacological treatments for psychiatric disorders. The goal of this dissertation was to examine within-species diversity in neuropeptide receptors and sociality in prairie voles. We examined proximate mechanisms of variation in sociality by examining both the impact of receptor variation and early experience on social behavior, including adult bonding. In chapter 2, we found that subtle variation in V1aR expression in the ventral pallidum mediates diversity in pair bonding in male prairie voles, demonstrating a direct impact of variation in gene expression on behavior In chapter 3, we found that early life adversity reduces adult bonding, but only in females with low accumbal OTR expression. This effect may be mediated by reduced sensitivity to touch-induced OT release in neonates. In chapter 4, we found that early MC4R treatment, which primes and activates OT neurons, impacts later sociality in a sex-dependent manner. Below, we briefly summarize our main findings and discuss future directions.

Chapter 2. Future directions.

In this chapter we found that long-term down-regulation of V1aR expression in the ventral pallidum beginning shortly after weaning impairs pair bond formation and reduces exploratory behavior in the EPM in male prairie voles. In line with previous studies on V1aR in the VP, we did not detect an impact on alloparental care, suggesting distinct circuitries mediating these two behaviors. Two next logical questions are: (1)

would knockdown in adulthood induce the same behavioral responses, or is reduced signaling during a particular developmental window critical for the impact on behavior? and (2) how does V1aR variation in other brain regions mediate social behavior?

Previous work from the lab has found that over-expression of OTR in the NAcc promotes alloparental care in females, but only if injected at a juvenile age, not in adulthood (Keebaugh and Young, 2011; Ross et al., 2009). These results suggest that long-term developmental knockdown has greater consequences on later sociality. It is possible that knockdown in adult males would not have an impact on pair bonding. In the wild, prairie vole weanlings typically stay in the natal nest to take part in rearing the next litter and these complex multi-generational, biparental nests are characterized by high levels of social interactions. Could pallidal vasopressin signaling be activated during these social interactions and impact development of the circuitry that mediates adult bonding and anxiety? Little is known about the downstream mediators of vasopressin signaling in voles, or even the cell types on which pallidal V1aR is expressed (mainly due to the lack of available antibodies for the V1aR). It would be interesting to test whether synaptic plasticity and function is impacted by juvenile experience, and whether V1aR expression mediates responses to social experience.

Vasopressin 1a receptor signaling during the neonatal period may also be important for the development of individual differences in behavior adulthood. As V1aR expression peaks in key social brain regions in neonates during this postnatal period (Wang et al., 1997b), vasopressin transmission during this critical period of development may play a role in translating early social experiences. Although much is known about the functional role of V1aR in adult animals, very little is known about vasopressin

transmission early in development. In some brain regions that control social behavior, such as the ventral pallidum, ventromedial and laterodorsal hypothalamus, and cingulate cortex, V1aR levels peak in the second postnatal week in prairie voles (Wang et al., 1997b). Transient postnatal peak levels of expression are also observed in rats (Tribollet et al., 1991). These receptors are indeed functionally active as AVP injections alter neonatal vocalization responses to social isolation in neonatal prairie voles (Winslow and Insel, 1993). As expression in neonates is markedly different from that of adult animals, V1aR may have a different role during development by organizing neural networks compared to regulating expression of social behavior in adults. In support of this hypothesis, neonatal exposure to vasopressin enhances aggression in adult prairie voles (Stribley and Carter, 1999), although other social behaviors were not examined.

To address the second question posed above, AAV-*avpr1a*-shRNA could be used to investigate the role natural variation in V1aR in other regions implicated in bonding. For example, V1aR variation in the lateral septum (LS) and medial amygdala (MeA) have been linked to variation in bonding in the lab and a high degree of variation in V1aR expression is observed in these regions. In male prairie voles, LS AVP has been linked to paternal care (Bamshad et al., 1994) (Wang et al., 1994), and promotes partner preference formation in males (Liu et al., 2001b). Male prairie voles with high levels of LS V1aR exhibit enhanced motivation to investigate females (Ophir et al., 2009). LS V1aR is reciprocally connected to the hippocampus, receives vasopressinergic information about social cues, and thus may mediate the development of social memory for the partner (Bielsky et al., 2005). The LS may also be important for the anxiety-provoking aspects of V1aR (Landgraf et al., 1995; Liebsch et al., 1996). Variation in V1aR binding in the

medial amygdala correlates with partner preference formation within male prairie voles (Hammock et al., 2005). The MeA receives direct projections from the olfactory bulb and is a major source of forebrain vasopressin, thus making it an important integrator of social olfactory information in rodents. In male prairie voles, this region is significantly activated in response to mating and pup exposure (Kirkpatrick et al., 1994b; Lim and Young, 2004), and axon-sparing lesions of the MeA decrease paternal care in voles (Kirkpatrick et al., 1994a). The medial amygdala may be particularly relevant for translational studies, as high levels of AVPR1A expression within this region are observed in humans (Loup et al., 1991) and abnormalities in amygdala activity in response to socially-relevant stimuli has been linked to the human AVPR1A gene (Meyer-Lindenberg et al., 2008). Finally, in the wild, variation in the laterodorsal thalamus and posterior cingulate has been linked to variation in pair bonding, and is hypothesized to play a role in mediating the formation of spatial memory of a rewarding social interaction (Ophir et al., 2008c) However, we have to consider is that our laboratory colony is genetically heterogeneous and a wide degree of V1aR variation is observed before injection of shRNA viruses. If this technique is to be used in regions with a high degree of natural variation in V1aR, such as in the mediodorsal thamalus or posterior cingulate, it would be beneficial to know whether these animals are “high” or “low” expressors to begin with.

Technically, there are some limitations to the use of shRNA for brain injections. After bilateral injection of the AAV-*avpr1a*-shRNA, we achieved 30% knockdown in comparison to scrambled injection control males. Although we achieved 80% knockdown in cell culture, decreased *in vivo* infection rate, differences in transcription machinery

between HEK cells and vole neurons may account for the reduced knockdown in vole brains. Furthermore, V1aR mRNA in the ventral pallidum is limited to thin linear strips and achieving accurate hits is technically difficult. Using the same methodology, Dr. Alaine Keebaugh has found that bilateral injection of a large volume (2ul) of OTR shRNA to the NAcc leads to a 40-50% knockdown of OTR. Accumbal OTR mRNA is widely distributed throughout the accumbens. Interestingly, this knockdown lead to impaired partner preference formation and alloparental care in female voles (Fig 5.1)

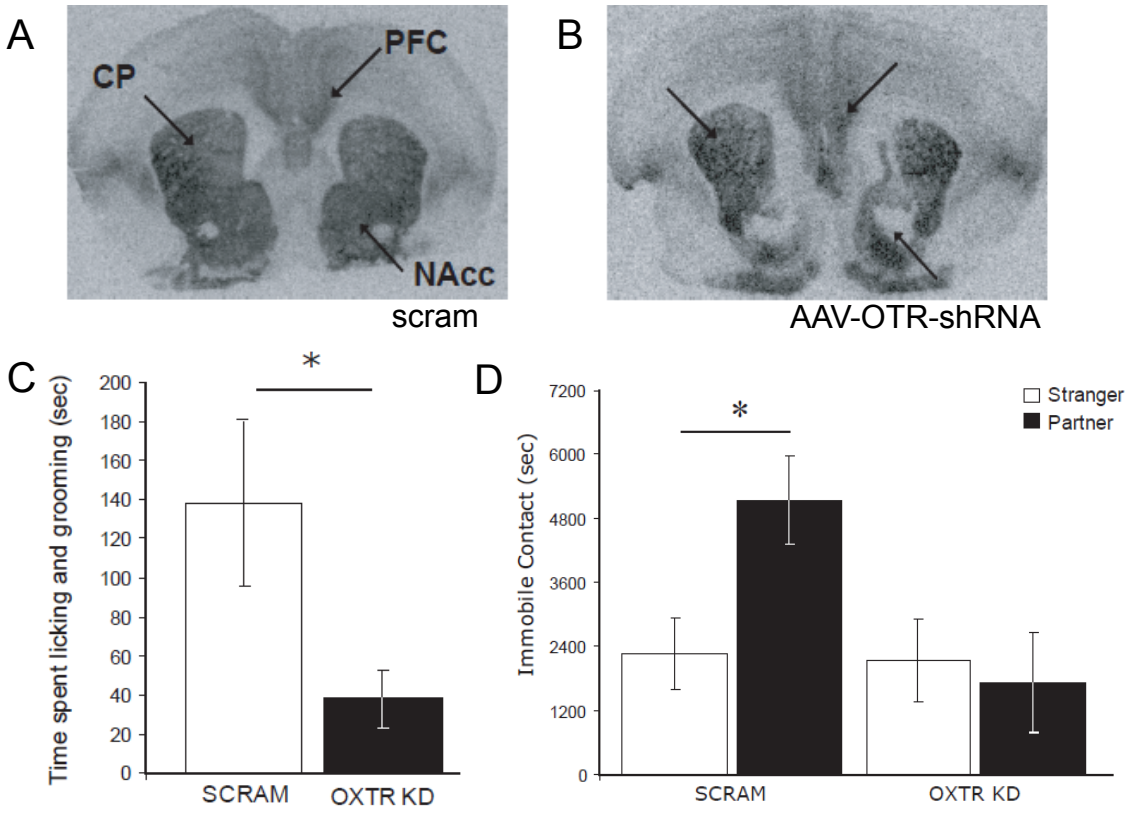

Figure 5.1 NAcc OTR knockdown in females.

Female prairie voles were bilaterally injected with 2ul of AAV-OTR-shRNA or AAV-scrambled at weaning (PND21). Representative autoradiograms of scrambled (A) and OTR-shRNA (B) brains. Approximately 40% knockdown was achieved in the NAcc. OTR knockdown reduced the amount of time spent licking and grooming novel pups (C) and impaired partner preference after a 24-hr mated cohabitation (D).

Currently, we are working towards using these shRNA sequences to generate transgenic prairie voles with life-long knockdown of V1aR or OTR. This line of transgenic voles would have permanently altered expression of V1aR throughout the brain, allowing us to investigate the role of the V1aR in development, intra-species differences, and naturalistic behavior as they will not experience invasive pharmacological manipulations. Our progress towards developing transgenic technologies by developing superovulation techniques that work in voles is outlined in Appendix 1. Prior to this, Dr. Zoe Donaldson, in collaboration with Dr. Anthony Chan and colleagues, generated the first transgenic prairie vole, which displayed germline, heritable *GFP* expression (Donaldson et al., 2009b). Our attempts up to this point to generate transgenic voles with targeted neuropeptide receptor knockdown through the injection of lentivirus into single-cell embryos have been unsuccessful. We have recently employed a new technique in which the DNA construct (CMV-GFP-U6-shRNA) was electroporated into the testes of males. If offspring display insertion of the DNA construct as verified by genotyping and southern blot, we will characterize neuropeptide receptor expression and the impact on socioemotional behavior (elevated-plus maze, open field, social odor investigation, social interest, spontaneous paternal behavior, sexual behavior, partner preference formation, and paternal behavior).

As an alternative approach, we are working towards developing zinc finger nuclease pairs to generate lines of voles lacking V1aR expression by creating a targeted mutation in the *avpr1a* gene. In this technique, a pair of zinc fingers conjugated to endonucleases bind specifically to either strand of target DNA to induce a double-stranded break, which is subsequently stitched back by an inherently imperfect repair

mechanism that leads to mutation, which can be either an insertion or deletion, in a portion of the repaired DNA. This technology has recently been used in rats (Geurts et al., 2009) and zebrafish (Doyon et al., 2008). ZFNs would be transiently expressed, resulting in a double-stranded break in a portion of inject embryos. Offspring will be assessed for mutation using a PCR mismatch genotyping assay and positive animals will be sequenced to assess the degree of mutation. This technology can be used to create complete knockout of a gene, in addition to partial knockout in heterozygotes or animals in which mutation resulted in a frame shift deletion that impairs transcription or translation of the endogenous gene.

One particularly exciting avenue of research with these animals would be to study the impact of V1aR diversity on monogamy-related behaviors in naturalistic field settings. As these animals will not experience any invasive pharmacological manipulation and the induced variation in V1aR expression will last for their lifetime, we plan to perform field studies examining their undisturbed naturalistic social behavior. In collaboration with Dr. Steve Phelps at the University of Florida, transgenic males and wild-type female voles would be released into 20X30m field enclosures. Radio-tracking will be used to measure home range overlap as a proxy for pair bonding and male fidelity and reproductive success will be determined via parentage analysis (Carter and Getz, 1993; Ophir et al., 2008c).

Countless potential applications of this technology will elucidate our understanding of the vasopressinergic social circuit, and can easily be extended to manipulations of other neural regulators of social behavior, such as OT and dopamine receptors. For example, region and temporal specific gene alteration using Cre

recombinase or Tet on/off expression systems will permit more precise examinations of the social behavior circuit. In addition, reporter gene constructs using gene-specific promoters will allow easy fluorescent identification of specific neuronal populations within the social behavioral network for functional and electrophysiological characterization. These technologies have the potential to greatly accelerate our understanding of the interaction of genes in well-defined brain circuits in producing complex social behaviors and will dramatically transform how we study the neurobiology of social behavior.

Chapter 3. Future directions.

In this chapter, we found that early social isolations impaired adult bonding in female prairie voles and that this effect was driven by females with low accumbal OTR. We also found that early tactile stimulation activated hypothalamic OT neurons, suggesting OT is a potential integrator of early social information.

Male pair bonding was not impaired as a result of the early isolation. Although not statistically significant, early isolated males formed a stronger partner preference that controls after 48hr. In adults, stress has sexually dimorphic effects on pair bonding. An acute swim stress or injection of corticosterone or central CRF promotes pair bonding in males, while inhibiting it in females (DeVries et al., 1996; Lim et al., 2007). Perhaps stress reactivity is increased in males exposed to early adversity, and this either promotes pair bonding or masks negative effects mediated by other neurobiological systems.

Of note, we originally chose to run the early separation experiment to test whether early experience impacts adult V1aR expression. In chapter 2, we saw that a subtle difference in ventral pallidal V1aR has a drastic impact on behavior. But whether the

variation in neuropeptide receptors is driven by genetic differences or by the environment is largely unknown. In a previous study by Todd Ahern in the lab, single mother rearing did not impact adult OTR, V1aR, CRFR1, or CRF2R expression across a wide variety of regions, apart from an increase in CRFR2 in the dorsal raphe and a modest decrease in OTR in the BNST. Here, we opted to perform an arguably more drastic early insult, yet again did not detect any large differences, apart form V1aR in the BSNT and CeA. Although studies in other species have found differences in OTR and V1aR from the level of early licking and grooming (rats, mice) or from paternal deprivation (mandarin voles, mRNA measured), we believe that the large variation in receptor expression observed between vole individuals is likely driven primarily by genetic differences. Indeed, microsatellite length variation upstream of the V1aR accounts for some inter- and intraspecific variation in binding. Recently, Lani King discovered a SNP in the 3' UTR of the OTR gene that is highly predictive of OTR binding in the NAcc, but not other brain regions. Whether this SNP is linked to regulatory regions in other brain regions or is functional itself (miRNA regulation occurs in 3' UTR) remains to be determined.

The OTR SNP provides a very useful model to ask the question: does genetic variation in OTR confer susceptibility or resilience to early adversity? We found that NAcc OTR binding correlated to the percent time huddling with the partner in the early isolated females, but not controls. To test whether this is truly a gene by environment interaction, litters from heterozygous breeder pairs would be exposed to early isolation and tested for adult bonding. We would expect females carrying the allele that predicts low OTR NAcc binding would be more impacted by the early insult. If this is the case, it would be interesting to track behavioral and neurobiological development in these

females and what goes awry in susceptible females exposed to the early isolation. Conversely, there could be active processes occurring in resilient females to buffer against early adversity. These active processes may be most relevant for the development of interventions or therapeutics in susceptible populations. Of relevance to humans, Hasse Walum has data suggesting that OTR polymorphisms interact with self-reports of early childhood parental care to predict marital satisfaction. However, the functional relevance of human OTR polymorphisms is yet unknown. The same SNPs that conferred susceptibility to early adversity in humans have also been linked to enhancements in prosociality, suggesting that individuals more attuned to social cues early in life may be more impacted by maltreatment, a theory known as "differential susceptibility" (Brune, 2012). However, human SNPs are likely in linkage disequilibrium with other functional elements, and the same SNP can be associated with opposite behaviors depending on the population sampled.

One potential resilient process that occurs in high-NAcc OTR females is enhanced responding to touch-induced OT release. We found that oxytocin, but not vasopressin, neurons were significantly activated in response to a 5-min tactile stimulation of the anogenital and body regions. This finding begs the question: what is downstream of neonatal oxytocin release? Is it impacting functional plasticity of neural circuitry involved in social bonding? In rats, early tactile stimulation induces thyroid hormone release, subsequence 5-HT release, and de-methylation of the glucocorticoid receptor (Hellstrom et al., 2012). What are the downstream molecular targets of oxytocin and does oxytocin impact epigenetic changes in these genes? A recent paper found that neonatal OT in mice mediates S1 cortical plasticity in response to sensory deprivation

(Zheng et al., 2014). Whether neonatal OT mediates plasticity in the NAcc or other limbic brain regions would be an exciting area of future research.

Resilient females displayed high levels of adult OTR, but we still don't know whether OTR in adulthood correlates with OTR in the neonatal period. Oxytocin receptor expression is present in the neonatal rodent brains, but this early pattern of expression is distinct from that in adults (Shapiro and Insel, 1989). Do high NAcc OTR females also have high levels of OTR in the accumbens as neonates? Understanding the natural progression of gene and protein expression is essential to draw conclusions about the functional importance of given neural factors during development (Nelson and Winslow, 2009). In terms of the oxytocin peptide, only the OT precursor (OT-neurophysin) and C-terminal extended forms of OT are detectable embryonically in rats (Alstein et al., 1988), suggesting OT is not functionally active around the time of birth. Interestingly, we found that the cleaved form of OT is already present in neonatal prairie voles as early as E16 (Fig 5.2a). We did detect OT in mouse E18 brains, but the pattern and level of expression was distinct from prairie voles at this same time (FIG 5.2b). Oxytocin expression was more caudal in the mouse brain at E18, and more resembled prairie vole E16 expression. This early species-specific expression may suggest that neonatal oxytocin signaling plays a distinct role in prairie voles.

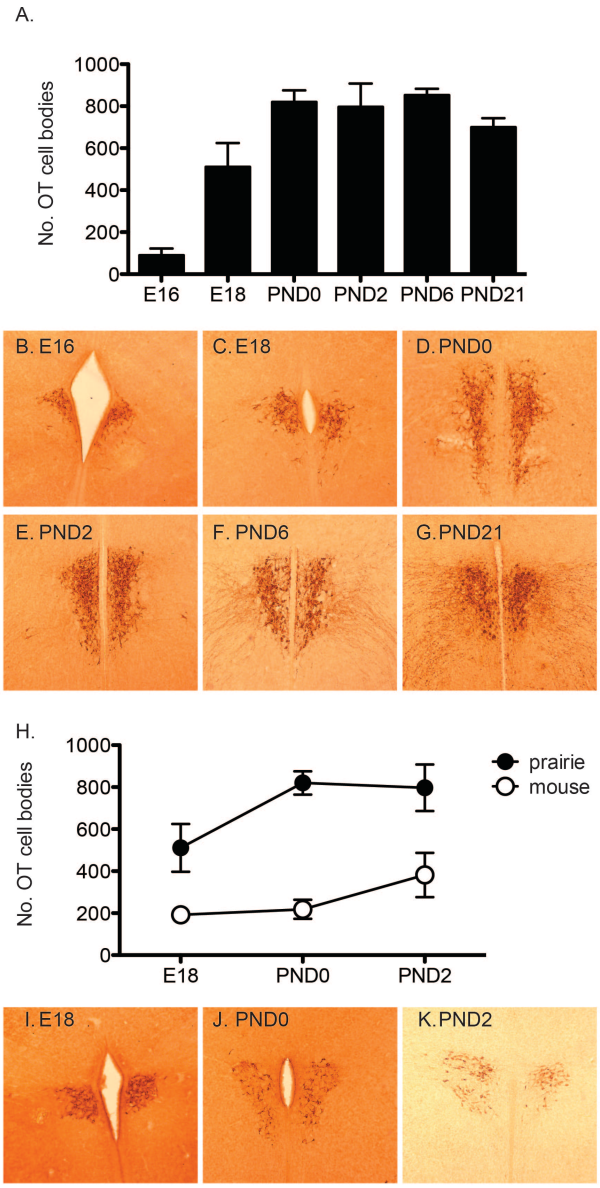

Figure 5.2 Ontogeny of oxytocin in prairie voles and mice

Immunohistochemistry on prairie vole and mouse tissue was performed using an antibody targeting the cleaved OT peptide (VA10, generously donated by H. Gainer, NIH). The number of OT-positive neurons in the PVN increased from E16 to PND0 (birth) and decreased to a final count at PND21 (weaning; A, representative images B-G). OT was detectable in both mouse and prairie vole embryonically, but prairie voles displayed significantly more OT neurons in the PVN between E18-PND2 (H, representative images J-K). N=3 brains per time point per species.

Chapter 4. Future directions.

In this chapter, we found that early melanocortin agonists induce general hypothalamic activation, reduce later male play fighting, and enhance female pair bonding. Neonatal MC4R agonists enhanced adult pair bonding in both sexes. Finally we saw that early treatment with a melanocortin agonist induced bond formation in females that had experience the early isolation. We originally chose to target the MC4R because of recent findings that MC4R stimulation induces OT neuron priming and enhances adult OT-dependent partner preference in prairie voles. However, in addition to oxytocin, MC4R activation interacts with additional neurocircuitry involved in social bond formation (Aragona et al., 2006; Lim et al., 2007; Burkett et al., 2011b), including dopamine (Lindblom et al., 2001), opioids (Alvaro et al., 1997), and CRF (Lu et al., 2003) signaling.

To give a broader overview of the melanocortin system: it is comprised of the neuropeptide ligands, α, β, γ melanocyte stimulating hormone (MSH) and ACTH, derived from the cleavage of proopiomelanocortin (POMC; Smith and Funder, 1988; Bertagna, 1994). POMC neurons in the arcuate nucleus of the hypothalamus project throughout the brain (see Mountjoy, 2010; Coupe and Bouret, 2013) and coordinate a large array of physiological and behavioral processes, including feeding (Poggioli et al., 1986), thermoregulation (Chen et al., 1997), cardiovascular function (Ni et al., 2006), pigmentation (Thody, 1999), brain development (Strand et al., 1991), synaptic plasticity (Shen et al., 2013), stress and anxiety (De Barioglio et al., 1991; Lu et al., 2003; Chaki and Okuyama, 2005), learning and memory (De Wied and Jolles, 1982), addiction (Hsu et al., 2005), and sexual behavior (Argiolas et al., 2000; Rossler et al., 2006). Five

receptor subtypes (MC1-5) are distributed throughout the periphery and the brain. MC3 and MC4 receptors are expressed primarily in the central nervous system and display the highest neural expression pattern (Lindblom et al., 1998; Kishi et al., 2003). Both neural receptors have been linked to feeding and energy homeostasis (Fan et al., 1997; Huszar et al., 1997; Chen et al., 2000a; Sutton et al., 2008), but the MC4R has been linked to much of the central action of MSH (for review, see Wikberg et al., 2000; Mountjoy, 2010; Tao, 2010).

Dr. Meera Modi recently showed that acute MC4R stimulation induces a robust partner preference after an abbreviated cohabitation (Appendix 2). Here, we found that MC4R stimulation also has long-term developmental consequences. It is intriguing to consider that MC4R stimulation is endogenously involved in social bond formation in adulthood and early life through coordinating multiple neural systems involved in sociality. Interestingly, leptin is found in milk and is transferred to the suckling offspring (Casabiell et al., 1997), providing a potential mechanism by which early alpha-MSH is stimulated. Other potential stimuli are the pup's endogenous leptin release after feeding, stress (Liu et al., 2007) and dopamine release (Tong and Pelletier, 1992). Would reduction of MC4R signaling, perhaps with shRNA-mediated knockdown, impair adult pair bonding?

We also developed a novel behavioral test, juvenile play, to assess sociality at an earlier developmental time point in prairie voles. MTII reduced play fighting in males on the third day of testing, essentially making the males behave more similarly to females. In females, MTII treatment enhanced affiliative behavior during this test. It is possible that MTII treatment reduced males' drive to engage in aggressive-like behavior. Whether the

display of play during the juvenile period is important for later pair bonding warrants further study. Very little is known about juvenile play in voles or the neural systems underlying development of this behavior. Monogamous mandarin vole males raised without a father display decreased play fighting, which the authors hypothesized may be a result of by reduced AVP levels in the anterior hypothalamus (Wang et al., 2012). In the mandarin vole study, voles were isolated 6hr per day before play testing. Here, voles were isolated for 3d prior to testing, and tested with an un-treated partner for 10min each day. In our pilot studies, we found baseline levels of play to be very low, both as assessed by home cage observations and by pairing with novel subjects. We found it necessary to isolate voles for multiple days to detect significant levels of play. Thus, the ethological relevance of this behavior is questionable. Perhaps rats are a more suitable model for studies of juvenile play, as they display very high levels of this behavior.

Nonetheless, it would be beneficial to develop behavioral assays to assess social behavior in the vole throughout development. Toward this end, we worked towards developing a test of pup-motivated social interactions. Previous work in the lab has shown that OTR knockout mice do not learn to cross a barrier to reach their mother. We sought to extend this work to voles, and found that pups treated with MC4R agonist (PF446687) for the first week of life display shorter latencies to cross the barrier on the second trial (Fig 5.3). This result is suggestive of enhanced social learning in MC4R-A treated pups, which may be mediated by increased motivation to reunite with the mother.

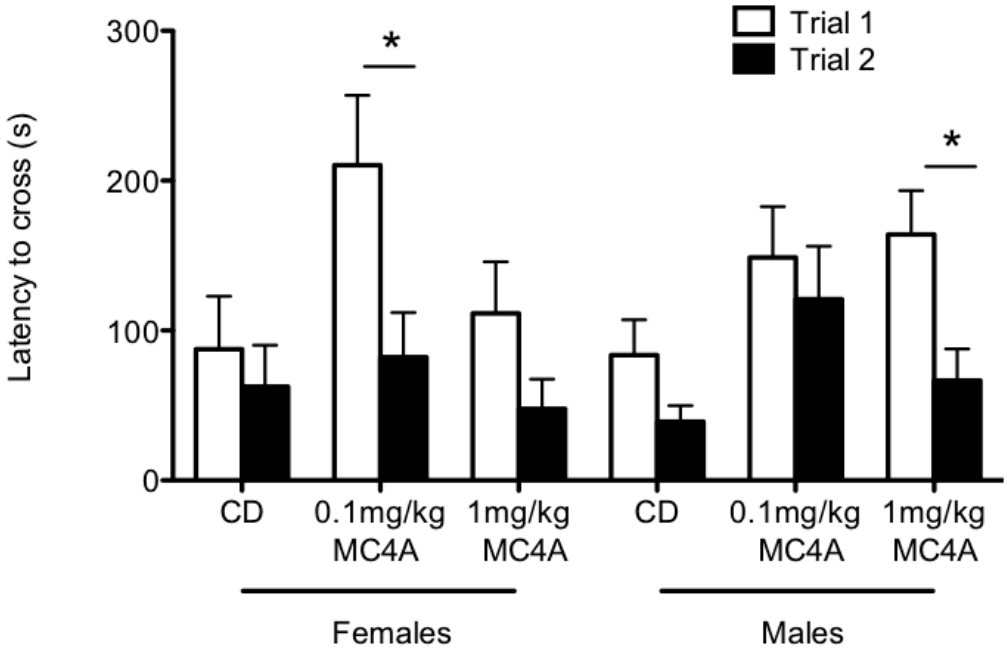

Figure 5.3 MC4R agonist promote pup motivated reunion with the mother

Prairie vole neonates were treated with the MC4R agonist (PF-446687, Pfizer) from PND1-7 and tested for latency to reunite with the mother over two successive trials (30min inter-trial interval). Females treated with the low (0.1mg/kg) dose and males treated with the high (1mg/kg) dose of MC4R displayed significant decreased in latencies to cross the barrier. Asterisks indicate planned Student's t-tests, $p<0.05$. CD, vehicle hydroxypropyl cyclodextrin. MC4A, melanocortin 4 receptor agonist.

But what are the downstream mediators of MC4R effects on sociality? Although our results are in line with an OT-dependent mechanism, the MC4R mediates a variety of other effects and, thus, our study cannot be construed as proof that MC4R effects on behavior are due to effects on the OT system. We saw that an acute injection of MTII stimulated OT, AVP, and CRF neurons in the hypothalamus of PND6-7 voles. There is conflicting evidence on the effects of MC agonists on AVP release. MTII does not activate AVP neurons in the PVN of adult prairie voles (Modi et al., Submitted). Similarly, MC4R agonists and alpha-MSH fail to stimulate AVP neurons in the SON of rats (Sabatier et al., 2003). Future research should address whether MC4R enhancements

in partner preference are mediated by OT, AVP, CRF, or dopamine, or, more interestingly, a combination of these neural systems.

Finally, we found the administration of the melacocortin agonist MTII for the first week of the two-week isolation induced a partner preference after an abbreviated non-mated cohabitation. MC4R agonists facilitate somatodendritic OT release and prime OT neurons so that a later stimulation induces a greater OT response (Sabatier et al., 2003; Ludwig and Leng, 2006; Sabatier, 2006; Modi et al., Submitted). It is possible that MTII given at the beginning of the 3hr isolation activated and primed OT neurons. In this model, when neonates were reunited with the parents and experienced the heightened hour of licking and grooming, a heightened level of OT would have been released from endogenous. Future experiments should directly test this OT-dependent hypothesis, perhaps by giving OTA prior to reunion period. We performed an experiment where PND6-7 prairie voles that had been given MTII 3hr prior were tactilely stimulated and sacrificed 1hr later for immediate early gene immunohistochemistry. However, we did not detect a difference in IEG activity in OT neurons between MTII treated alone or MTII+tactile stimulation. Electrophysiological measures on neonate slice preps previously dosed with MTII may provide a useful means to test this hypothesis.

Conclusion and Future Directions

Humans have a unique drive to engage in social interactions and display intricate social bonding, behaviors which until recently have been considered too complex to link to any neurobiological bases. The past few decades of study into the highly social prairie vole, once only a model for behavioral ecology and mammalogy, has helped to uncover the neural circuits driving sociality, and has generated hypotheses into our own behavior

and physiology. In particular, the nonapeptides OT and AVP have been strongly linked to pair bonding and other behaviors associated with a monogamous social structure. These neuropeptides carry important information on the olfactory signatures of a mate, enhance learning of socially relevant cues, and link up information about a conspecific with dopaminergic reward and reinforcement. The stress axis and related neurotransmitter systems are important for regulating monogamous strategies in variable environments and for maintaining the expression of a pair bond. The systems controlling social attachment are also susceptible to experience early in life, pinpointing the neonatal period as a critical time in the development of pair bonding. OT and AVP signaling may both translate the experience of early social interactions and regulate the development of neural circuits controlling sociality throughout development.

The continued progress is making the prairie voles a premier model organism for social behavior may help reveal yet unanswered questions in the field such as: What are the underlying mechanisms behind individual variation in social attachment and functioning of the social brain? How do neural circuits controlling social information processing, social motivation, learning, reward, and stress and anxiety interact at cellular, developmental, and context-specific levels? What is the impact of neuropeptide systems on a temporal and region specific scale? The field of behavioral neuroendocrinology is also lacking in studies that bridge the wide gap between well-controlled laboratory-based neuroscience and evolutionarily relevant field studies. The integration of modern molecular and pharmacological approaches with behavioral ecology would help to uncover mechanistic underpinnings of social behavior to explore the origin of diversity in an ecologically relevant context.

The processes underlying social bond formation in prairie voles and social cognition in humans display remarkable similarities. A burst in studies using intranasal neuropeptide administration, genetic association, and neuroimaging approaches in recent years has revealed striking human parallels to vole research and promising therapeutic strategies. Thus insights from this highly social species have the potential to generate hypotheses and drive drug discovery for treating deficits in social ability in humans. As there are presently no approved treatments that target the deficits in social reciprocity and social cognition seen in all cases of ASDs, there is a dire need to understand the pathophysiology of this disease and to identify treatable neurobiological targets. We are beginning to expand the genetic toolbox available for the prairie vole and thus its usefulness as an animal model (Donaldson and Young, 2008; McGraw et al., 2010; McGraw and Young, 2010; Keebaugh et al., 2012). The prairie vole genome was recently sequenced, thus paving the way for more sophisticated temporal and target specific transgenic development using prairie vole specific gene sequences (McGraw and Young, 2010). Lentiviral-mediated gene transfer has been successfully used to create transgenic prairie voles expressing green-fluorescent protein (Donaldson 2009). Future transgenic approaches to generate prairie voles with reduced or abolished neuropeptide receptors and impaired sociality may be viable systems with which to test drugs that target social learning in a socially compromised individual (Donaldson et al., 2009a).

APPENDIX 1

Identification of variables contributing to superovulation efficiency for production of transgenic prairie voles (Microtus ochrogaster).

Modified from the following reference:

Keebaugh, A.C., Modi, M.E., Barrett, C.E., Jin, C., Young, L.J. (2012) Identification of variables contributing to superovulation efficiency for production of transgenic prairie voles (*Microtus ochrogaster*). 10(1):54.

ABSTRACT

The prairie vole is an emerging animal model for biomedical research because of its rich sociobehavioral repertoire. Recently, lentiviral transgenic technology has been used to introduce the gene encoding the green fluorescent protein (GFP) into the prairie vole germline. However, the efficiency of transgenesis in this species is limited by the inability to reliably produce large numbers of fertilized embryos. Here we examined several factors that may contribute to variability in superovulation success including, age and parentage of the female, and latency to mating after being placed with the male. Females produced from 5 genetically distinct breeder lines were treated with 100 IU of pregnant mare serum gonadotrophin (PMSG) and immediately housed with a male separated by a perforated Plexiglas divider. Ovulation was induced 72 hr later with 30IU of human chorionic gonadotropin (hCG) and 2 hrs later mating was allowed. Superovulation was most efficient in young females. For example, females aged 6-11 weeks produced more embryos (14 +/- 1.4 embryos) as compared to females aged 12-20 weeks (4 +/- 1.6 embryos). Females aged 4-5 weeks did not produce embryos. Further, females that mated within 15 min of male exposure produced significantly more embryos than those that did not. Interestingly, there was a significant effect of parentage. The results of this work suggest that age and genetic background of the female are the most important factors contributing to superovulation success and that latency to mating is a good predictor of the number of embryos to be recovered. Surprisingly we found that cohabitation with the male prior to mating is not necessary for the recovery of embryos but is necessary to recover oocytes. This information will dramatically reduce the number of females required to generate embryos for transgenesis in this species.

Introduction

The socially monogamous prairie vole (*Microtus ochragaster*) is an excellent model organism for understanding the genetics and neurobiology regulating social bonding and other behaviors associated with monogamy (McGraw and Young, 2010), which are not exhibited by polygamous laboratory mouse and rat species. Because prairie voles can be systematically outbred they are ideal for the study of individual variation in neurochemistry and sociobehavioral traits (Hammock and Young, 2005; Olazábal and Young, 2006b). Over two decades of research in this species has provided insights into the neurobiological basis of social attachment (Insel and Shapiro, 1992; Insel et al., 1994) and nurturing behavior (Olazábal and Young, 2006a; Olazábal and Young, 2006b; Ross et al., 2009b; Keebaugh and Young, 2011b), and voles have served as a model of how social experience affects adult social behavior (Ahern and Young, 2009), depression (Grippo et al., 2007a; Bosch et al., 2009) and cardiac function (Grippo et al., 2007b). Discoveries in prairie voles are beginning to inform novel treatment strategies for psychiatric disorders with impairments in social behavior (Modi and Young, 2011; Modi and Young, 2012). The prairie vole is of great interest for biomedical research and the ability to genetically manipulate this non-traditional animal model would allow for the study of diseases associated with social deficits in a more behaviorally relevant species.

There is an ongoing effort within the prairie vole research community to develop comprehensive genomic resources to facilitate biomedical research in this model organism, including a 10X BAC library (McGraw et al., 2010; McGraw et al., 2012), a cytogenetic and genetic linkage map (McGraw et al., 2011), and the genome is

forthcoming. Recently, lentiviral mediated transgenic technology was used to introduce the green fluorescent protein (GFP) gene into the prairie vole germline, as a proof of principle (Donaldson et al., 2009a). Progress is being made combining this approach with RNAi technology to silence gene expression, but the inefficiency of superovulation and embryo transfer has been a significant impediment to further use of this technology to explore genetic mechanisms of behavior. Further, the powerful technology of gene targeting using homologous recombination has not yet been applied to the prairie vole. The future success of these transgenic technologies in this species requires reliable methods of superovulation that yield large numbers of viable oocytes and embryos with normal developmental potential.

Superovulation is a procedure used to produce a large number of developmentally synchronized embryos and protocols based on administration of gonadotrophic hormones have been standardized in species such as mouse (Eistetter, 1989), rat (Hirabayashi et al., 2001; Cornejo-Cortes et al., 2006), pig (Cuello et al., 2004), cow (Donaldson and Ward, 1986), rabbit (Treloar et al., 1997) and goat (Graff et al., 2000). However, the responsiveness of each species to superovulation treatment varies and must be optimized to account for species differences (Vanderhyden and Armstrong, 1989; Goh et al., 1992). Within the laboratory mouse, optimal age, hormone dose, and other factors vary between strains (Hogan, 1994). Further, among rats there is also considerable variation between laboratories with respect to the choice of strain, optimal age, hormone and hormone dose (Popova et al., 2002; Popova et al., 2005).

In addition to the prairie vole's unusual social system (characterized by social monogamy, formation of extended families, and cooperative breeding) (Getz et al.,

1981b; Solomon, 1991), their reproductive physiology differs significantly from traditional laboratory rodents (Richmond and Conaway, 1969; Sawrey and Dewsbry, 1985). Prairie voles are unusual in that they are more responsive to social factors rather than environmental cues to reach estrus (Richmond and Conaway, 1969; Sawrey and Dewsbry, 1985; Carter-Su and Roberts, 1997). Behavioral estrus in the prairie vole occurs 1-3 days after the female is introduced to a novel male or male urine and ovulation is typically induced following mating (Richmond and Conaway, 1969; Carter et al., 1987; Roberts et al., 1998a). Prairie vole's mate repeatedly for 24h (3-31 bouts) (Roberts et al., 1999) and successful reproduction in this species requires prolonged contact with a male (McGuire et al., 1992; Dewsbury, 1995). The social cues initiating the ovarian development are olfactory (Richmond and Stehn, 1976; Dluzen et al., 1981). Estrus synchronization, superovulation and fertilization represent a significant challenge for the efficient production of transgenic prairie voles. Since prairie voles are not currently commercially available, meaning that donor females are often limiting, it is essential to understand the variables that can contribute to successful embryo harvesting. In our own experience, inconsistency in the success of superovulation and fertilization has been a significant barrier to efficient transgenesis. Thus, the main goal of this study was to explore some of the variables that contribute to superovulation success in the prairie vole. We examine several factors that are known to contribute to variability in superovulatory success in other species including age of female, latency to mate following exposure to a male, and parental lineage of the female. Further, the single published study inducing superovulation in the prairie vole incorporates separated cohabitation with a male combined with hormone administration (Donaldson et al., 2009a); however, the need for

this extra step (i.e. separated cohabitation) is just a hypothesis. Thus, we test the importance of including this extra step. We describe in detail the method for inducing synchronized ovulation via hormonal manipulation (pregnant mare serum gonadotrophin / human chorionic gonadotropin) *without* the need for sociosexual manipulation, a time intensive procedure that requires specialized housing.

Methods

Subjects

Subjects were sexually naive female prairie voles 4-20 weeks old, and stud males were adult (90–365 days of age), sexually experienced prairie voles. All prairie voles were generated from an in-house breeding colony originally derived from Illinois prairie voles. After weaning at 21 days of age, subjects were housed in same sex pairs or trios with water and Purina rabbit chow provided *ad libitum* under a 14:10 light:dark cycle with lights on at 7am. All experiments were done in accordance to the Institutional Animal Care and Use Committee at Emory University.

Superovulation Protocol: Separated cohabitation & hormone treatment

Prairie voles do not display spontaneous ovarian activity or ovulation. Exposure to olfactory scents from a male is necessary to induce sexual receptivity and follicle development under nonhormonally-primed conditions (Richmond and Stehn, 1976; Sawrey and Dewsbry, 1985; Carter et al., 1987). Once receptive, ovulation occurs only after 10 or more hours of pairing after mating takes place (Roberts et al., 1999). To induce receptivity and synchronize ovulation in multiple animals, each naive female was either housed in a cage with a sexually experienced stud male but separated from him by a Plexiglass divider or returned to her home cage. Prior to pairing, to increase the

number of mature follicles, females were administered 100IU of PMSG intraperitoneally (Adachi et al., 1993) at 3PM immediately before being placed into a separated cohabitation with the stud male. Seventy-two hours later females were administered 30IU IP of hCG at 3PM to induce ovulation (Adachi et al., 1993). Two hours later at 5PM the divider was removed to allow mating. In experiment 1, control animals were injected with sterile saline on the same time schedule as PMSG and hCG as described above. As previously shown for the polygamous Japanese field vole, *Microtus montebelli* (Adachi et al., 1993), Experiment 1 demonstrates that this hormone regimen is effective at inducing superovulation in the prairie vole in the absence of pre-exposure to a male (e.g. separated cohabitation). Much of this data, however, was collected retrospectively; thus, in experiments 2 and 3 the hormone and separated cohabitation was held constant in order to explore the contribution of other variables. It should be noted that this high hormone dose was chosen based on pilot studies testing various PMSG/hCG doses (5IU/5IU as is done in mice and rats, 25IU/25IU, 50IU/25IU and 75IU/25IU) which failed to reliably induce superovulation (Keebaugh and Young, unpublished data).

Oocyte and Embryo harvesting

Seventeen hours following administrations of hCG females were sacrificed using CO_2 asphyxiation (approximately 8AM), their oviducts removed and placed into M2 media (Millipore, Billerica, MA). Under a stereoscope, a 32 gauge needle was placed into the infundibulum and oviducts were flushed with ~0.3ml M2 media. Harvested embryos were stored in M16 media (Millipore) microdrops under mineral oil at 37ºC and 5% CO_2.

Experiment 1. Effectiveness of hormone administration and pre-exposure to a male on oocyte and embryo production

Two studies were conducted to determine the importance of hormone administration and pre-exposure to a male on oocyte (no mating) and embryo (mating) production. For each experiment, females 9-10 weeks of age i) were given either saline/saline or PMSG/hCG and ii) underwent either separated cohabitation or were singly housed prior to saline or hCG treatment (n=15/treatment). Superovulation and oocyte/embryo harvesting protocols were done as described above. Females producing no oocytes/embryos were considered not to have ovulated; females producing 1-6 oocytes/embryos were classified as having ovulated; females producing more than 7 oocytes/embryos were classified as having superovulated since the typical litter sizes are 3-5 pups.

In experiment 1a we examined oocyte production; thus, females did not mate. Treatment group 1.1 (G1.1) served as the control. They received saline, did not mate, and were singly housed. To determine if hormone administration and/or pre-exposure to a male would lead to differences in the number of oocytes produced, treatment group 1.2 (G 1.2) and treatment group 1.3 (G 1.3) females both received hormone but G1.2 females were singly housed while G1.3 females were pre-exposed to a male via separated cohabitation.

In experiment 1b we looked at embryo production; thus, females did mate. Females in treatment group 2.1 (G2.1) received saline and were pre-exposed to a male. Treatment group 2.2 (G2.2) and treatment group 2.3 (G2.3) females both received hormone but G2.2 females were singly housed while G2.3 females were pre-exposed to a male.

Experiment 2. Importance of female age and occurrence of mating as indicators of

superovulatory success

Females ranging in age from 4-20 weeks old were given PMSG/hCG according to the superovulation protocol described. Mating was scored as occurring or not occurring during the first 15 minutes after removing the divider and embryos were collected the following morning beginning at 8AM.

Experiment 3. Role of female parentage in superovulatory efficiency

Previous studies have identified variation in response to superovulation (i.e. high versus low responders) across substrains of mice. Among Illinois derived laboratory prairie vole colonies it has been reported that about half of females exposed to males for 2-3 days will fail to show lordosis (Carter et al., 1989). Our prairie vole colony is derived from Illinois and maintained as an outbred population; thus we are interested in identifying if genetic background contributes to variation in superovulatory response and ultimately genetic lines optimal for inducing superovulation. Females 7-11 weeks of age from five breeder pairs (N=36; BP1=7, BP2=7, BP3=7, BP4=9, BP5=6) were given PMSG/hCG according to the superovulation protocol described above.

Statistical analysis

For experiment 1 the Freeman-Halton extension of Fisher's exact test was used to compute two-tailed probabilities of obtaining a distribution of values in a 3x3 contingency table for each experimental group. For oocyte and embryo production, statistical analysis was used to determine 1) is hormone priming critical for superovulation and 2) is pre-exposure to a male important. For experiment 2 regression analysis was used to determine the predictive value of female age (n=85) on the number of embryos recovered. A chi-square test was used to determine if the occurrence of

mating within the first 15 minutes of male access (n=85) contributed to superovulatory response. For experiment 3 one-way ANOVAs were run to compare the number of embryos produced between breeder pairs. Hochberg's GT2 test was used for post hoc analysis when significant effects were detected.

Results

Experiment 1. Effectiveness of sociosexual manipulation and hormone administration on ovulatory response and embryo production.

In studies where it is desired to collect oocytes, for example in IVF, some exposure to a male is necessary; however, for studies where embryos are desired, for example lentiviral mediated transgensis, pre-exposure to a male is not necessary. For oocyte collection, pre-exposure to a male and hormone treatment are necessary for superovulation success ($p=0.03$, Fisher's exact test) (Figure A1.1a). However, for embryo collection, only hormone administration ($p=0.012$, Fisher's exact test) is necessary for superovulation success (Figure A1.1b).

Experiment 2. Importance of female age and occurrence of mating as indicators of superovulatory success

Regression analysis indicated that female age ($p=0.001$, $r^2= 0.357$) is a significant factor contributing to variation in superovulatory response (Figure A1.2a). Further, the females that mated within 15 minutes of exposure to the male superovulated more than those that did not ($\chi^2=20.82$, df=2, $p=0.00003$, Figure A1.2b).

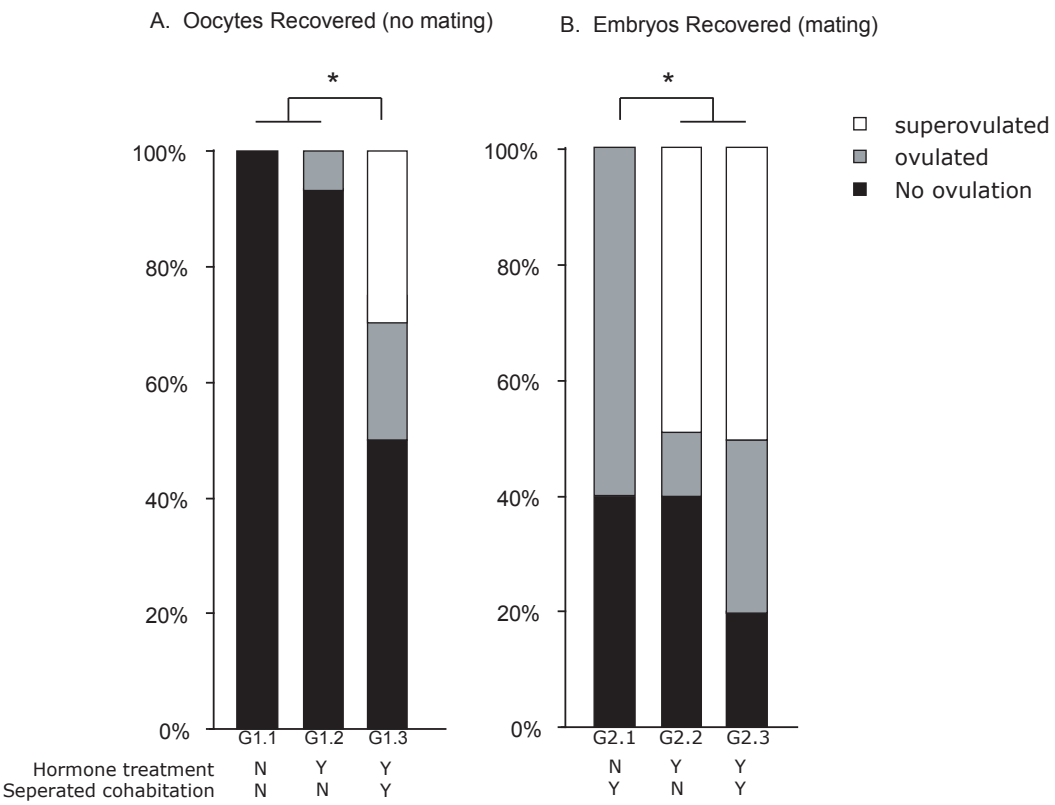

Figure A1.1. Impact of hormone administration and separated cohabitation on superovulatory response in the presence and absence of mating.

The percentage of female's not ovulating (black bar), ovulating (gray bar) and superovulating (white bar) following hormone treatment and separated cohabitation in (A) the absence of mating and (B) the presence of mating. These data suggest that to recover large numbers of oocytes hormone treatment and separated cohabitation are needed for superovulation to occur in the absence of mating. However, for the recovery of embryos hormone treatment and mating alone are sufficient. Treatment group 1.1 (G1.1) did not receive hormone treatment or separated cohabitation. Treatment group 1.2 (G1.2) did receive hormone treatment but did not undergo separated cohabitation. Treatment group 1.3 (G1.3) received hormone treatment and underwent separated cohabitation. Treatment group 2.1 (G2.1) did not receive hormone treatment but did undergo separated cohabitation while Treatment group 2.2 (G2.2) received hormone treatment but did not undergo separated cohabitation. Treatment group 2.3 (G2.3) received hormone treatment and underwent separated cohabitation. Asterisk represents a significant p-value ($p<0.05$).

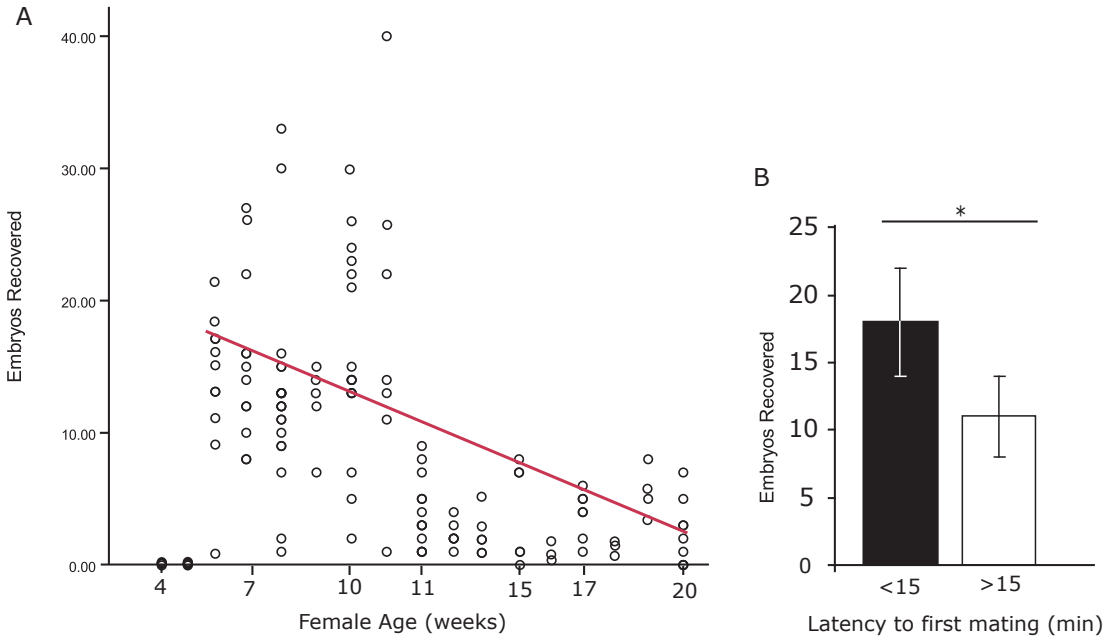

Figure A1.2. Female age and occurrence of mating are indicators of superovulation efficiency.

(A) The number of embryos collected per female aged 6-20 weeks significantly decreased with female age. (B) Frequency of ovulatory response in females that mated within the first 15 minutes of male access (<15) compared to those that mated after the first 15 minutes of male access. Females that mated within the first 15 minutes of male access were more likely to superovulate than those that did not immediately mate. Error bars are represented as + SEM. Asterisk represents a significant p-value (<0.05).

Experiment 3. Role of parentage in superovulatory efficiency

Given the variation in ovulatory response seen in prairie voles upon exposure to a male as well as the significant strain variation in mice (see discussion), we were interested in the influence of parentage on superovulatory success in the prairie vole. There was a significant effect of parentage on superovulatory response ($F(4,31)=9.373$, $p=0.001$, one-way ANOVA). Post hoc analysis revealed that breeder pair 5 responded more efficiently than all other breeder pairs ($p=0.039$, Figure A1.3).

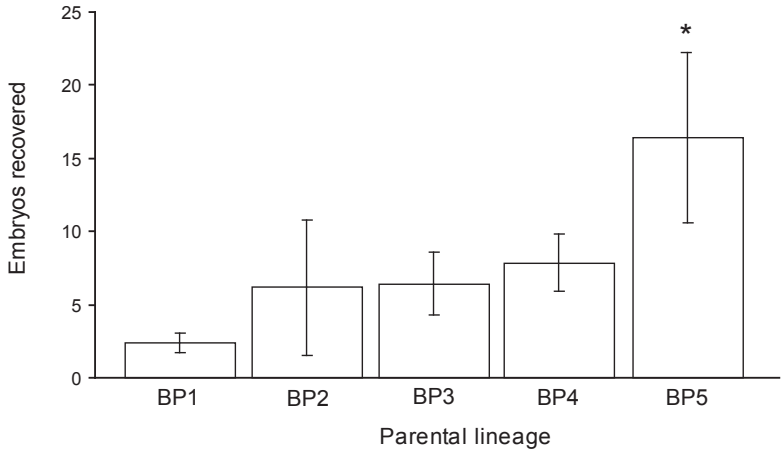

Figure A1.3. Impact of parentage on superovulation efficiency.

Mean number of embryos recovered per superovulated female derived from distinct breeder pairs. The numbers of females examined per breeder pair (BP) are as follows: 7 for BP1, 7 for pair BP2, 7 for pair BP3, 9 for pair BP4, and 6 for pair BP5. Posthoc analysis indicates that BP 5 is significantly more sensitive to the superovulation paradigm than the other parental lineages. This data demonstrates that genetic background influences superovulation efficiency in the prairie vole. Error bars are represented as + SEM. Asterisk represents a significant p-value.

Discussion

Here we demonstrate that this superovulation method is a viable and effective technique for generating germline transgenic prairie voles. Specifically, we show that pre-exposure to a male in combination with PMSG/hCG is necessary to induce superovulation in the prairie vole for the collection of oocytes (in the absence of mating); however, pre-exposure to a male is not necessary for the collection of embryos in this speices (presence of mating) (Figure A1.1). The implications of this finding suggest that when fertilized embryos are needed for transgenesis the extra time and resources needed for separated cohabitation are not necessary as reported previously (Donaldson et al., 2009a); however, to generate large numbers of oocytes, for example with in vitro fertilization, separated cohabitation is necessary. Thus, some exposure to a male cue is

required to achieve ovulation even in the presence of PMSG/hCG. The unusual reproductive physiology of the prairie vole leads to subtle differences that need to be considered when using different types of transgenic technologies.

In addition to social manipulation, other variables known to differ among species, as well as substrains of mice, were examined. The efficiency of superovulation within the prairie vole varies with female age, parentage and latency to first mating bout. Superovulation was most efficient in young females. Notably females aged 6-11 weeks produced a mean of 14 +/- 1.4 embryos while females aged 12-20 weeks produced only a mean of 4 +/- 1.6 embryos. Females that mated within 15 min of access to the male produced significantly more embryos than those that did not. Thus, the latency to mate is a good indicator of oviduction status. Further, there was a significant effect of parentage. For example, 9 out of 9 females from breeder pair 5 superovulated (defined as producing 7 or more embryos), while only 1 out of 6 females superovulated from breeder pair 1. This suggests that some parental lineages of prairie voles are more sensitive to this superovulation paradigm than others. The experiments here suggest that age and genetic background of the female are important factors contributing to superovulatory success, and that occurrence of mating is a good predictor of the number of embryos to be recovered. Further, this work cannot distinguish between the effects of environmental factors as discussed below. This information will dramatically reduce the number of females required, as well as the time and the equipment investment needed to generate a large number of embryos for future gene manipulation studies in this species.

Why is there variability in ovulatory response?

Previous studies using Illinois derived prairie vole populations have shown that

approximately 50% of female prairie voles exposed to males for 2-3 days fail to display lordosis (Carter et al., 1989) and therefore did not mate or ovulate. In the present study 60% of females exposed to males for 3 days (separated cohabitation only, group 2.1) ovulated. When females underwent the superovulation paradigm 80% of the females showed an ovulatory response, with 52.5% of those females superovulating (group 2.3). This suggests that the superovulation protocol decreases the variability previously seen in mating and ovulatory response; however, some of the variation remains unaccounted for. Such variation could be explained by environmental and/or genetic differences among individual females.

It is possible that the social environment in the natal nest (i.e. parental nurturing behavior, juvenile-juvenile interactions) could be factors that result in long-lasting neurochemical changes that increase reproductive potential as adults. For example females are more likely to engage in affiliative behavior as adults if they remain in the natal nest after weaning (Lonstein and De Vries, 1999). The presence of the father in the natal nest also increases their propensity to form a partner preference as adults (Wang and Novak, 1992b; Ahern et al., 2011). These studies suggest that the social environment during development, perhaps when the pups are interacting with their parents and siblings, is critical for shaping factors that result in long-lasting neurochemical changes that impact reproductive potential as adults.

In addition to environmental factors, genetic background could also be playing a role in the variation seen in ovulatory response among female prairie voles. In laboratory *Mus* species, the average number of embryos induced by superovulation is highly strain-dependent. Females of the strains B6, BALB/cByJ, 129/SvJ, CBA/CaJ, SJL/J, C58/J are

considered high responders to superovulation and can be induced to ovulate 40-60 embryos while females of the strains A/J, C3H/HeJ, BALB/cJ, 129/J 129/ReJ, DBA/2J, C57L/J are considered low responders and produce at most 15 embryos per mouse (Hogan, 1994). This suggests that subtle differences in genotype can have dramatic consequences on the expression of this particular reproductive trait. Studies in female prairie voles have shown that there is a graded ovulatory response to prolonged social stimulation and that some females show exceptional ovulatory sensitivity following mating with only 6-12 hours of male exposure (Roberts et al., 1999). In the present study 100% of females from breeder pair 5 superovulated while only 16% of females from breeder pair 1 superovulated, demonstrating that some lineages of prairie voles are more sensitive to this superovulation paradigm than others. Prairie voles are maintained as an outbred population; however, as transgenesis in this species matures it may become necessary to establish optimal breeding lines for superovulation and embryo production as has been done in the mouse.

Conclusion

In conclusion, the present study has identified a superovulation and mating paradigm that allows for the recovery of a large number of developmentally synchronized oocytes and embryos that can be used for the production of transgenic prairie voles. However, this paradigm is not perfect and variation in ovulatory response still exists. Several variables warrant further studies. First, the diet provided to a prairie vole breeding colony may have important consequences on the reproductive responsiveness of offspring. Second, increasing the hours of light per day (for example from 14:10 to 18:6) may increase the ovulatory response of hormone treated females. And, finally it may

prove necessary to establish optimal donor and recipient strains as prairie vole transgenesis matures.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

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APPENDIX 2.

Melanocortin receptor agonists facilitate oxytocin-dependent social behavior

Modified from the following reference:

Modi, M.E., Inoue, K., Barrett, C.E., Kittleberger, K., Smith, D., Landgraf, R., Young, L.J. (Submitted) Melanocortin receptor agonists facilitate oxytocin-dependent partner preference formation in the prairie vole. Biol Psych.

Abstract

Oxytocin (OT) enhances prosocial behavior in animal models and intranasal OT (IN-OT) improves aspects of social cognition in both the normative and psychiatric human populations. The therapeutic potential of IN-OT is limited, though, by poor penetration of the peptide through the blood-brain barrier. Drugs that stimulate endogenous OT release represent an alternative approach for therapeutically enhancing social cognition. Here we report that two melanocortin receptor (MCR) agonists, Melanotan II (MTII) and PF-446687, enhance OT-dependent social behavior in the monogamous prairie vole. Peripherally administered MTII increased activity in OT neurons, potentiated central OT release, and enhanced partner preference formation in female prairie voles. This effect was blocked by an infusion of OT receptor antagonist into the nucleus accumbens. MTII-induced partner preferences were maintained at least one week after drug manipulation, indicating enduring effects on social learning. Collectively, these results indicate that MC4R agonists may be a viable alternative strategy to enhance OT signaling in the brain for the treatment of social deficits in psychiatric disorders.

Introduction

Oxytocin (OT) is a neuropeptide that facilitates social behavior, including social recognition, parental nurturing and social attachment, in animals (Lim and Young, 2006; Modi and Young, 2012; Neumann and Landgraf, 2012). The prosocial effects of OT are thought to be mediated in part by increasing the salience of social stimuli and enhancing social information processing, thereby facilitating social learning and social motivation (Ross and Young, 2009). In humans, intranasal OT (IN-OT) increases attention to the eye region of faces, enhances socially reinforced learning, and improves attention to and comprehension of emotional expression (Domes et al., 2010; Hurlemann et al., 2010; Guastella and Macleod, 2012). The effects of OT on social behavior and cognition in healthy human subjects suggests that the OT system may be a viable pharmacological target for improving social functioning in psychiatric disorders characterized by social deficits, including autism spectrum disorders (ASD) and schizophrenia (Modi and Young, 2012). IN-OT increases social reciprocity, attention to social stimuli, emotion recognition and decreases social anxiety in individuals with ASD (Andari et al., 2010; Guastella et al., 2010; Hall et al., 2011). In schizophrenia, IN-OT reduces symptom severity for positive and negative symptoms including those related to social cognition not typically affected by antipsychotics (Feifel et al., 2010; Pedersen et al., 2011). As ASD and schizophrenia are heterogeneous disorders with perhaps hundreds of underlying causes, improving social cognition through enhancing OT signaling is particularly exciting as it may be effective regardless of the primary neurological etiology.

The clinical efficacy of IN-OT, though, is likely limited by its poor penetration of the blood-brain barrier (BBB) (Landgraf and Neumann, 2004; Churchland and

Winkielman, 2012; Young and Flanagan-Cato, 2012). Neuropharmacological studies have suggested that peptides gains better access to the brain through IN administration compared to other peripheral routes of administration (Born et al., 2002), however, it is questionable as whether maximal receptor occupancy in behaviorally relevant brain regions regulating social behavior can be achieved through this method. An alternative approach to increase central OT signaling is to pharmacologically stimulate endogenous OT release (Modi and Young, 2012). The neuropeptide α-melanocyte stimulating hormone (α-MSH) promotes the release of central but not peripheral OT through the melanocortin 4 receptor (MC4R) (Sabatier et al., 2003). Therefore, we hypothesize that MC4R agonists may be an effective pharmacological approach to induce OT-mediated improvements in social cognition, providing a novel therapeutic approach to improve social functioning in disorders such as ASD.

To determine if stimulation of the MC4R can recapitulate the behavioral response of central OT administration, we examined the effects of three structurally-distinct melanocortin receptor (MCR) agonists on partner preference formation in the prairie vole (*Microtus ochrogaster)*. Prairie voles are highly affiliative, socially monogamous rodents that form enduring pair bonds, which can be assessed in the laboratory using a partner preference test. Partner preference formation engages the cognitive domains of social information processing, social reward and learning/memory, and can therefore be used as a behavioral assessment of the effects of drugs on improving social cognition (Modi and Young, 2011; Millan et al., 2012). In female prairie voles, OT facilitates partner preference formation by activating OT receptors (OTR) in the nucleus accumbens (NAcc) (Young et al., 2001; Young and Wang, 2004a). Correspondingly, mating-

induced partner preferences are inhibited by infusion of OTR antagonist into the NAcc (Young et al., 2001; Liu and Wang, 2003).

In this study, we assess the hypothesis that MCR agonists would recapitulate the central effects of OT in the vole model. Our results suggest that MC4R agonists enhance social learning, in part through an OTR dependent mechanism, and that the social information learned during drug treatment is enduring. These findings have important implications for the development of novel pharmacotherapies for the treatment of social deficits in psychiatric disorders.

Methods

Subjects

Subjects were adult, sexually naïve female prairie voles (60-120 days of age) and meadow voles (see Supplementary Information) from our colony maintained at the Yerkes National Primate Research Center at Emory University. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Emory University.

Partner Preference Test

Female prairie voles were cohabited with a male prairie vole for six hours with mating prevented by ovariectomy. Subjects were then tested in a 3-hour partner preference test which were scored using TopScan Behavioral Analysis System (CleverSys, Inc. Reston, VA) as previously reported (Ahern et al., 2009). Partner preference data were analyzed with planned paired Student's t-test comparing time spent with partner versus stranger in each treatment group. Groups were considered to have a

partner preference if they spent significantly more time in social contact with partner than the stranger. Individual animals were considered to have formed a partner preference if they spent twice as much time with the partner than with the stranger.

Peripheral Administration of Melanotan I and II

The effect of Melanotan I (MTI) and Melanotan II (MTII) on partner preference formation was tested in female (N=10/group) prairie voles. Subjects were injected intraperitoneally (i.p.) with either MTI, MTII (1,10 mg/kg in 0.1 ml 0.9% saline); Alpha Diagnostics, San Antonio, TX), or vehicle. Doses were based on those given to elicit behavioral responses in other rodent models (Rossler et al., 2006; Klenerova et al., 2008). The experimental animals were then cohabitated and tested in the partner preference test.

Long-term Effect of MTII Administration in Female Prairie Voles

Adult female prairie voles were injected with MTII (10mg/kg) or a saline vehicle (n=9-11/group) and paired with a stimulus male. After cohabitation, the animals were singly housed for seven days and then tested in the partner preference test without additional interaction.

In vivo *Microdialysis Sampling of Oxytocin Release*

Adult female prairie voles (n=10 saline; n=13 MTII) were unilaterally implanted with microdialysis probe into the NAcc as described previously (Ross et al., 2009a). After two days of recovery, the animals were anesthetized with urethane (1.6g/kg i.p.) and maintained under urethane for the duration of the experiment. Isotonic Ringer's solution was perfused through the probe at a flow rate of 3µl per minute. After a 90-minute equilibration period, samples were collected in 30-minute bins. Two 30-minute

baseline samples were collected. The animals were then injected i.p. with either 10mg/kg of MTII or isotonic saline. Three 30-minute post drug samples were then collected. The perfusion solution was then switched to a hypertonic Ringer's solution containing 1M NaCl. Two final 30-minute samples were collected under the hypertonic conditions. Animals whose probes did not remain patent for the duration of the sampling or were mistargeted were removed from analysis.

OT concentration was determined using a highly sensitive radioimmunoassay (RIAgnosis, Munich, Germany) (Neumann et al., 1993; Neumann et al., 2013). Cross-reactivity of the polyclonal antiserum with arginine-vasopressin (AVP) and other related peptides was <0.7%. Intra- and inter-assay coefficients of variation were in the 5–9% and 8–12% ranges, respectively. The level of detectability of the assay was in the 0.3-0.5pg/dialysate ranges.

Co-administration of Melanotan II and Oxytocin Receptor Antagonist

Females were anesthetized and implanted with 26 gauge bilateral guide cannulas (Plastics One, Roanoke, VA) aimed at the NAcc as described previously (Modi and Young, 2011). After 3 days of recovery, subjects received microinjections of Ringer's Solution or 10 μg of the OTR antagonist (OTA; $d(CH_2)_5^1$,Tyr(Me)^2,Thr^4,Orn^8,des-Gly-NH_2^9)-Vasotocin (H-2908 Bachem, Torrance, CA) dissolved in 500nl of Ringer's Solution (Fisher Scientific, Pittsburg, PA) per side (N=11-12/treatment). Subjects were with clogged or loose cannulae were excluded. Simultaneously the females received either MTII (10mg/kg) or saline i.p.. Subjects were then cohabitated with males for six hours and tested in the partner preference test.

Activation of OT-Positive Neurons after MTII Administration

Females were injected with 10mg/kg of MTII or saline (n=6, MTII; n=5, Sal) and returned to their home cage. Ninety minutes after injection, subjects were anesthetized and perfused transcardially with 4% paraformaldehyde and sectioned on a microtome at 35 μm coronally. Every sixth section underwent immunohistochemical processing to detect EGR1 (Early Growth Response protein 1) and OT as previously described (Maney et al., 2003). Sections were incubated in primary antibody against EGR1 (rabbit polyclonal, sc-189, Santa Cruz Biotechnology, Santa Cruz, CA; 1:8,000 dilution) and OT (mouse monoclonal, mAb5296, Millipore, Billerica, MA; 1:10,000 dilution), washed and incubated in secondary Alexa Flour conjugated antibodies (Alexa Flour 568 for EGR1 and Alexa Flour 488 for OT, 1:1,000 dilution, Life Technologies, Grand Island, NY). EGR1 activity in AVP positive cells was also inspected. Every sixth section was incubated in primary antibody for AVP (mouse monoclonal antibody PS41, generously donated by Dr. H. Gainer, NIH, USA; 1:1,000 dilution) and EGR1. Four to six bilateral sections from each subject containing the PVN were quantified at 20X magnification. The ratio of EGR1 positive OT to total OT positive cells was quantified in each section and then averaged to yield a total ratio for each animal.

Localization of Melanocortin 4 Receptor (MC4R) mRNA

RNA probes for the MC4R were generated from a 920 bp prairie vole MC4R cDNA using S^{35}UTP. *In situ* hybridization using sense and antisense probes was carried out in brains from female prairie voles as described previously (Burkett et al., 2011a). MC4R mRNA signal was visualized using the BAS-5000 Phosphoimager (FujiFilm, Valhalla, NY). For more detail see Supplemental Information.

Peripheral Administration of PF-446687

Adult female prairie voles (n=10/group) were injected i.p. with PF-446687 (1mg/kg or 10mg/kg; Pfizer Inc. New York, NY), dissolved in a volume of 0.1ml of 10% β-cyclodextrin in 0.9% sterile saline or a vehicle control. The females were then cohabitated and then tested in the partner preference test.

Results

Melanocortin Receptor Agonist Facilitates Partner Preferences

Peripheral administration of the linear, non-brain penetrant melanocortin agonist, Melanotan I (MTI) did not induce a partner preference at either 1 or 10 mg/kg (Figure 1a). In contrast, females receiving 10 mg/kg, but not 1 mg/kg of the cyclized agonist Melanotan II (MTII), spent significantly more time in social contact with the partner than with the stranger, indicative of the formation of a partner preference (Figure A2.1b; paired Student's t-test, $p=0.008$). This demonstrates that peripheral administration of melanocortin agonist can recapitulate the behavioral effects of central OT administration in prairie voles. Correspondingly, MTII failed to facilitate partner preferences in female meadow voles, which do not express OTR in the NAcc, after a 24-hour cohabitation (see Supplementary Information).

MTII-induced Partner Preference is Enduring

To determine if the social learning that occurred during MTII exposure was retained after the drug was cleared, we tested for partner preference one week after cohabitation and MTII administration. The half-life of MTII in plasma is between 1-2 hours (Hruby et al., 2011). A single dose of MTII (10mg/kg) followed by a 6 hour cohabitation period is sufficient to induce the expression partner preference in the

experimental animals after a 7 day period of isolation (Figure A2.1c; paired Student's T-test p=0.05) with 9 out of 11 animals showing a preference for the partner, but not in saline treated animals, with only 6 out of 12 showing a preference for the partner.

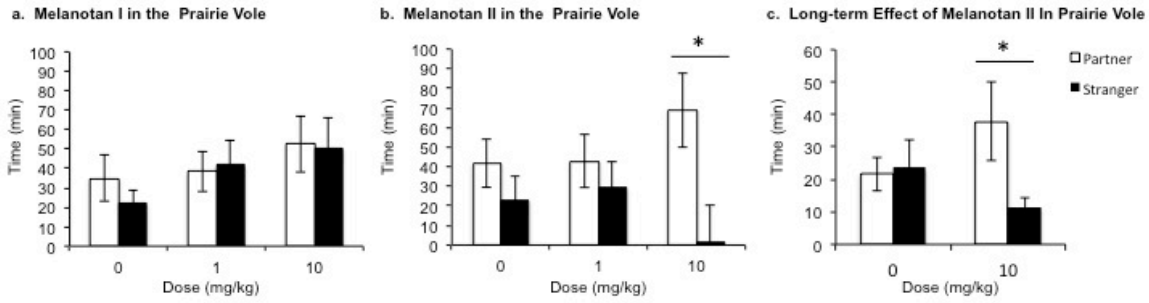

Figure A2.1. MTII facilitates immediate and long-term partner preference in female prairie voles.

A) Female prairie voles given either a low (partner vs. stranger p= 0.85) or high dose of MTI (partner vs. stranger p=0.923), performing as the vehicle control group did, failed to spend significantly more time with either stimulus animal and therefore did not form a "partner preference". B) Female prairie voles receiving a high dose (10mg/kg) of MTII (partner vs. stranger p=0.003) spent significantly more time with the partner male vs. the stranger male, indicative of a partner preference. C) The effect of MTII (10mg/kg) is maintained in the experimental animal after a 7 day period of separation. Comparison of time spent with the partner vs. the stranger after MTII ($p<0.05$) but not saline ($p=0.85$) results in a significant difference between groups, with an average of thrice more time spent with the partner than the stranger. * Indicates a statistically significant partner preference.

MTII Enhances the Release of OT in the NAcc in Response to Hypertonic Saline.

We used *in vivo* microdialysis to determine whether MTII stimulates OT release in the NAcc. Peripheral MTII administration alone did not increase OT concentrations in the microdialysate samples in the anesthetized subjects. However, MTII results in a significantly greater OT release following the hypertonic osmotic challenge (Figure A2.2a). Comparison of OT concentration using a two-way mixed model ANOVA

(sample phase X treatment) revealed a significant difference in OT levels across sample phase ($F(6,16)=10.04$, $p<0.001$), reflecting the significant increase in OT concentrations stimulated by hypertonic saline administration. There was no significant interaction between treatment and sample phase ($F(6, 16)=0.85$, $p=0.55$). However, the MTII treated animals had significantly elevated OT in the NAcc microdialysates following hypertonic saline (in an average of the two samples) compared to baseline condition (in an average of the two samples) as demonstrated in a paired Student's T-test ($p=0.02$). Animals receiving the control treatment showed no significant difference in OT levels between averaged hypertonic saline samples and baseline samples in a paired Student's T-test ($p=0.98$).

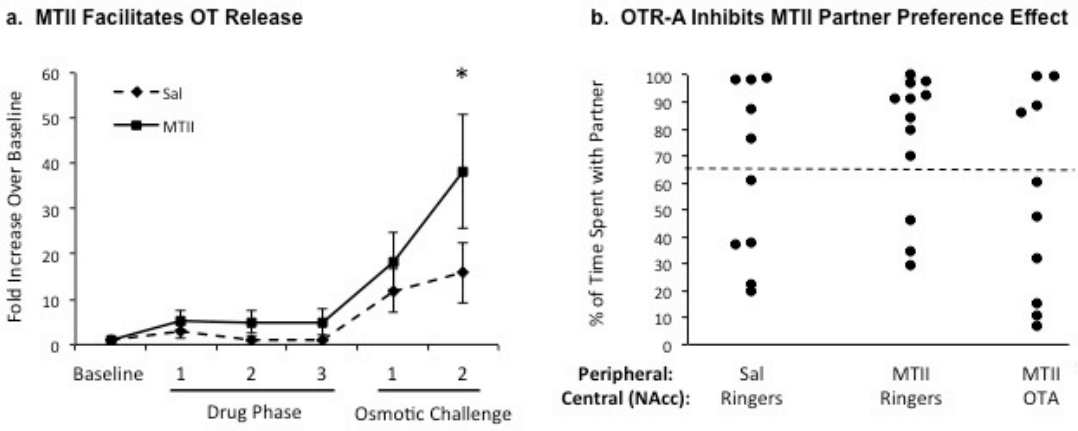

Figure A2.2. MTII interacts with the OT system.

A) Administration of MTII alone does not increase OT release relative to a vehicle control (Sal) in the drug phase. However, MTII does increase OT release in the NAcc of female prairie voles in response to a hypertonic osmotic challenge, indicating a potentiating effect. The average OT levels after osmotic challenge are significantly higher than the average baseline OT levels in MTII but not saline treated animals ($p=0.02$) * Indicates a statistically significant increase in post hoc comparisons. B) The facilitatory effect of MTII administration on partner preference was inhibited by administration of an OT receptor antagonist into the NA (OTA). Animals receiving the vehicle control (Sal+Ringers) showed no preference to spend time with the partner (partner vs. stranger $p=0.22$), whereas animals receiving MTII (MTII+Ringers) spent significantly more time with the partner than the stranger (partner vs. stranger $p=0.008$). OTA blocked the MTII

mediated preference for the partner (partner vs. stranger p=0.18). The dashed line is indicative of the "twice as much time spent with the partner than the stranger" definition of binary partner preference.

The Effect of MTII on Partner Preference is blocked by an OT Receptor Antagonist in the NAcc

Female prairie voles receiving control injections of both saline peripherally and Ringer's centrally did not show a group preference in a paired Student's T-test (p=0.32), with 5 of the 10 animals showing a partner preference. Females receiving MTII peripherally and vehicle centrally did show a group preference in a paired Student's T-test (p=0.02), with 9 of the 12 animals showing a partner preference (Figure A2.2b). Females that received both MTII peripherally and the OT receptor antagonist centrally did not show a partner preference in a paired Student's T-test (p=0.27) with 4 of the 10 animals showing a partner preference.

Melanocortin 4 Receptor mRNA Expression in the PVN of Prairie Voles

In situ hybridization confirmed the presence of intense MC4R mRNA signal in the PVN that overlaps with OT mRNA signal. MC4R mRNA signal was also distributed in a number of other brain regions that could be involved the regulation of social behavior, including the medial amygdala and nucleus accumbens (Figure A2.3, see Supplementary Information for more detail). No signal was detected in slices incubated with the corresponding sense probe.

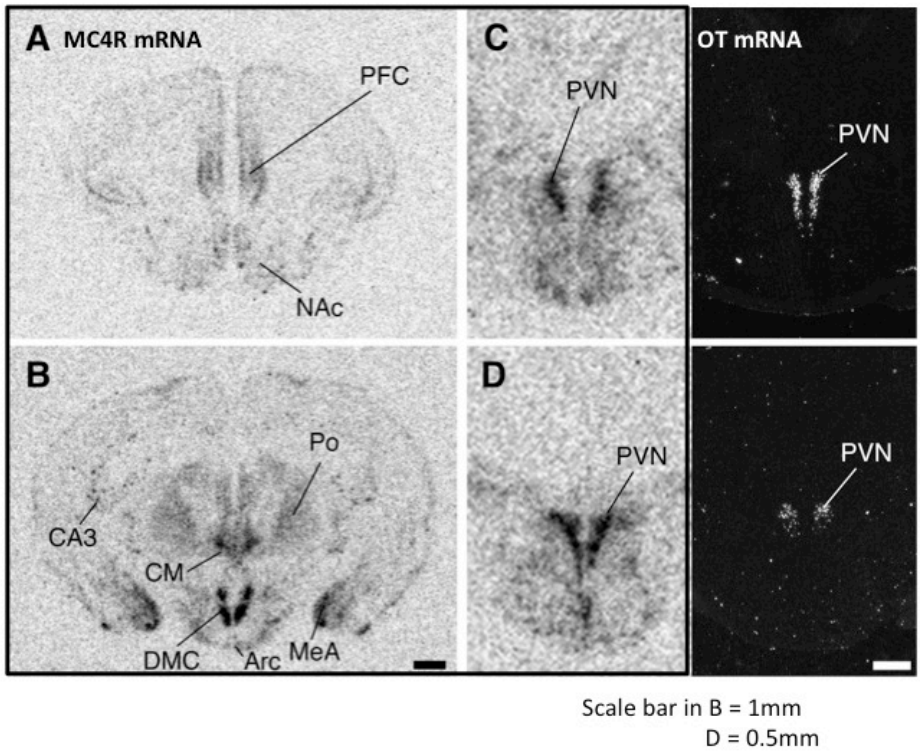

Figure A2.3. MC4R mRNA is present in the PVN of the prairie vole brain.

A, B) MC4R mRNA was detected via in situ hybridization in numerous regions of the prairie vole brain involved the regulation of social behavior, including: the medial amygdala (MeA), nucleus accumbens (NaC), prefrontal cortex (PFC), CA3, the dorsal medial (DMC) and arcuate (Arc) nuclei of the hypothalamus, and the posterior (Po) and central medial (CM) thalamic nuclei. C, D) Adjacent sections were used for MC4R (left) and for OT in situ hybridization (right.) Relevantly, MC4R mRNA was present in the paraventricular nucleus of the hypothalamus (PVN), one of the primary hypothalamic sites of OT synthesis of the prairie vole brains. The distribution of MC4R mRNA overlaps with the PVN regions expressing OT mRNA (dark field micrographs) in the caudal region (D) but not in the rostral region (C.)

Activation of OT-Positive Neurons after MTII Administration

There was a significant increase in EGR1-immunoreactive OT neurons following MTII administration (Figure A2.4; Student's T-test $p<0.005$). EGR1 was present in 14% of OT positive cells following MTII treatment compared (Figure A2.4c) to 1% of OT positive cells in saline treated animals (Figure A2.4b). Contrastingly, MTII induced

EGR1 expression in less than 1% of AVP positive cells, which was a small but significant increase over 0% activation in the saline treated animals (Student's T-test $p<0.05$).

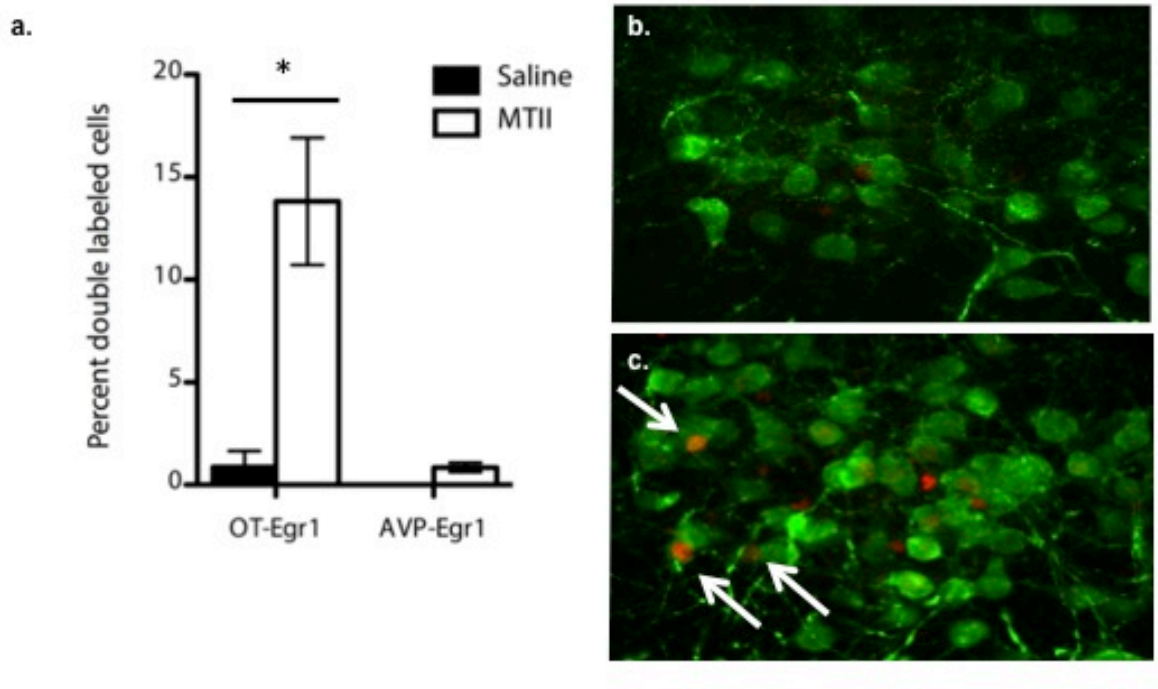

Figure A2.4. MTII activates OT-positive neurons in the paraventricular nucleus.

A) In the paraventricular nucleus of the hypothalamus, 13.8% of OT positive cells were activated by *in vivo* MTII administration, as indicated by EGR1 expression, compared to less than 1% of cells under control conditions (b; Student's T-test p=0.0049). Less than 1% of vasopressin positive cell were activated by MTII administration. B) OT positive paraventricular cells after saline administration (green=OT or AVP, red=EGR1). C) OT positive cells after MTII administration. Arrows indicate co-localization of OT and EGR1. * indicates a significant difference in proportion of double-labeled neurons.

Pf-446687, a Brain Penetrant and Selective MC4 Receptor Agonist, Induces Partner Preference

Using LS-MS to analyze distribution of MC4R agonists in plasma and brain, we found that MTII had a brain to plasma concentration ratio of 0.02. The behaviorally

effective 10mg/kg dose resulted in a brain concentration of 370.54nM. By contrast, Pf-446687, a highly selective, small molecule MC4R agonist (Lansdell et al., 2010), is highly brain penetrant with a brain to plasma concentration ratio of 0.68 and a 23.25nM brain concentration at the 1mg/kg behaviorally effective dose (see Supplementary Information).

Peripheral administration of PF-446687 resulted in a significant increase in time spent with the partner compared to the stranger at 1mg/kg (paired Student's T-test, $p=0.007$; Figure A2.5), but not at 10 mg/kg (paired Student's T-test, $p > 0.05$). At 1mg/kg, 9 out of 10 animals formed a preference, compared to 7 out of 10 at 10mg/kg and 5 out of 10 for controls.

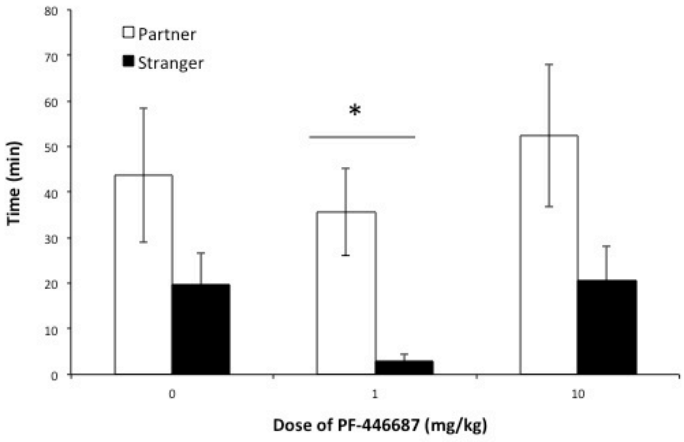

Figure A2.5. Pf-446687 facilitates partner preference in prairie voles.

Administration of PF-446687 resulted in significant partner preference. Female prairie voles receiving a low dose (1mg/kg) of MTII (partner vs. stranger p=0.007) spent significantly more time with the partner male vs. the stranger male. The 10mg/kg doses trended towards the formation of a preference but did not reach significance (p=0.08). * Indicates a statistically significant partner preference.

Discussion

Effect of Melanocortin Agonists on the OT system and Partner Preference Formation

Peripheral administration of brain penetrant MCR agonists recapitulates the behavioral effects of central OT administration in the partner preference paradigm. Both MTII and Pf-446687 induced partner preferences after 6 hour cohabitation without mating, a stimulus insufficient for natural preference formation. Moreover, the effects of MTII on partner preference persisted and were observable 7 days post-injection, well beyond its reported half-life. The effects of MTII on partner preference were inhibited by blockade of OTR within the NAcc, suggesting that the behavioral effects of MC4R agonists are mediated, in part, by central OTR signaling.

Stimulation of MCR engages the central OT system. Intracerebroventricular injection of α-MSH, the endogenous ligand of the melanocortin receptors, induces the expression of c-fos, a neuronal marker of excitation, in OT containing neurons of SON (Sabatier et al., 2003). In vitro α-MSH promotes the somatodendritic release of OT in rat hypothalamic brain slices through activation of the MC4R (Sabatier et al., 2003). Consistent with these data, we found that MC4R mRNA is concentrated in the PVN and that peripheral infusion of MTII in our model robustly increases EGR1 immunoreactivity in PVN OT neurons, albeit in a subset of neurons. Additionally, MTII potentiates OT release in the NAcc following a physiological stimulus (hypertonic saline) that causes OT release. Thus we hypothesize that MTII and Pf-446687 stimulate somatodendritic release of OT from a subset of OT neurons in the PVN, which increases local concentrations of OT in the PVN but not distant brain regions (Neumann et al., 1994). This serves to prime the entire population of OT neurons, leading to enhanced OT release from terminals in the NAcc during social interactions with the partner and accelerated partner preference

formation in the same way as a central infusion of OT.

The efficacy of an MCR agonist in facilitating social behavior, though, is dependent on the pharmacological profile of the compound, as MTII and PF-446687 but not MTI enhanced partner preference. Both MTI and MTII are agonists for MC1,3,4 and 5R , though MTII has a 10-fold greater affinity for MC4R. MTII also has enhanced *in vivo* stability and BBB permeability, compared to MTI (Sawyer et al., 1980; Al-Obeidi et al., 1989; Oosterom et al., 1999; Hruby et al., 2011). PF-446687 is highly selective for MC4R and is both brain penetrant and stable *in vivo* (Lansdell et al., 2010). Thus, the differential effects of MTII and PF-44687 compared to MTI may be due to either the increased stability and/or access to central MC4Rs of these compounds.

Pharmacokinetic analysis indicated both MTII and PF-446687 were detected in the brain after peripheral administration, though the level of penetrance differed. MTII concentrations in the brain were 2% of that found in plasma, a sufficient concentration to occupy central MC4Rs and elicit MC4R-mediated behavioral responses. The EC_{50} of MTII at the MC4R is 2.87nM and the measured concentration in the brain in our study was 370nM, including both bound and free fractions (see Supplemental Information)(Grieco et al., 2007). Contrastingly, Pf-446687 was thirty times more brain penetrant, though the total concentration in the brain was only twice as high at 10 mg/kg due to the tissue permeability of the compound. At 1mg/kg Pf-446687, the more behaviorally efficacious dose, a brain concentration of 23nM sufficient to meet the EC_{50} of 12nM of the compound at MC4R was achieved(Lansdell et al., 2010). It is possible that at the very high brain concentrations achieved with the 10mg/kg dose Pf-446687 has off-target effects or activates alternative intracellular signaling pathways diminishing the

behavioral response(Cai et al., 2004). The fact that this compound had a similar effect as MTII at the lower more pharmacological relevant dose, though, supports the role of MC4R specifically in the facilitation of partner preferences.

While our data support the hypothesis that the effects of MC4R agonists are mediated by a potentiation of OT release, our data do not prove this mechanism of action. The behavioral effects of MC4R agonists could be mediated by alternative mechanisms. MC4R mRNA is localized in several brain regions implicated in social and emotional behavior that could affect partner preference formation, including the medial amygdala and prefrontal cortex. Furthermore, MTII induces dopamine release through the activation the ventral tegmental area (VTA) (Lindblom et al., 2001). Dopamine D2 receptor agonists infused into the NAcc facilitate partner preference formation and blocking OTR in the NAcc prevents this facilitation (Liu and Wang, 2003). Thus, MC4R agonists may promote social learning through activation of NAcc dopamine circuits. Site-specific infusion of MTII into the PVN, VTA and other brain regions would be informative for further elucidating the mechanism of action for the behavioral results found in our study.

Therapeutic Implications

Several clinical trials involving intranasal OT in psychiatric populations are currently underway (Young and Flanagan-Cato, 2012). Intranasal OT, however, may not achieve maximal receptor occupancy in the brain due to poor brain penetrance, limiting its therapeutic potential. Pharmacological stimulation of central OT release is a viable alternative approach for enhancing social cognition. Stimulating endogenous OT release from terminals in the brain in close proximity to its receptor targets could achieve

significantly greater OTR signaling. Our results suggest that MC4R agonists stimulate OT release only following appropriate physiological stimuli. Thus MC4R agonists would enhance OTR signaling only under conditions when brain OT is endogenously released, e.g. upon positive social stimuli (Neumann and Landgraf, 2012), which would maximize the therapeutic potential while minimizing off target effects.

If our hypothesis is correct, MC4R agonists should enhance social learning by increasing attention to social stimuli and the perception of socially relevant information (Andari et al., 2010; Guastella et al., 2010). Our data suggest the social information learned during MC4R stimulation is enduring, as the partner preference formed under the influence of MTII persisted for at least one week after treatment. We propose that pharmacologically enhanced OT release specifically paired with behavioral therapies involving social reinforcement to improve social skills could have enduring effects. That is, MC4R agonists represent a potential adjunct to behavioral therapies and have the potential to enhance the efficacy of those therapies for generating long-term improvements in social functioning.

There are important caveats to MC4R agonist-based therapies. Stimulation of MC4R suppresses eating, a significant side effect that may be particularly problematic in ASD populations (Chen et al., 2000b). Unwanted sexual effects are also likely to be a limiting factor in the clinical use of MC4R agonists (Wessells et al., 2000). It is possible, however, that low doses of MC4R agonists given in controlled clinical settings would result improved social cognition with limited side-effects. Our data suggest that clinical investigations of the effects of MC4R agonists on social cognition are warranted.

Conflicts of Interest

LJY and MEM have applied for a patent for the use of MC4R agonists in the treatment of social cognitive deficits**.** MEM is currently employed by Pfizer Inc., but was not so during the collection of the data presented. DGS was employed by Pfizer Inc. during the course of these experiments. KI, CEB and RL have no conflicts of interest to disclose.

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