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Heather E. Ross	Date

Oxytocin and affiliative behavior in prairie voles

By

Heather E. Ross
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Neuroscience

_____

Larry Young, PhD
Advisor

_____

Aras Petrulis, PhD
Committee Member

_____

Kerry Ressler, PhD, MD
Committee Member

_____

Yoland Smith, PhD
Committee Member

_____

David Weinshenker, PhD
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D. Dean of the Graduate School

_____ Date

Oxytocin and affiliative behavior in prairie voles

By

Heather E. Ross
B.S. Michigan State University, 2001

Advisor: Dr. Larry Young

An abstract of
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Abstract

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By Heather E. Ross

Oxytocin is produced in the hypothalamus and released into the circulation through the neurohypophyseal system. Peripherally released oxytocin facilitates parturition and lactation. Centrally released oxytocin plays a role in maternal nurturing behavior and affiliative behavior in adults. Oxytocin receptors in the nucleus accumbens have been implicated in the regulation of alloparental behavior and pair bond formation in the socially monogamous prairie vole. There are both interspecies differences in oxytocin receptor density in the nucleus accumbens of monogamous and non-monogamous prairie voles, as well as, individual differences within prairie voles, that are functionally related to differences in affiliative behaviors. Here we show that the distribution of oxytocin-immunoreactive fibers in the nucleus accumbens is conserved in prairie voles, mice and rats, despite remarkable species differences in oxytocin receptor expression in the region. However, the origin of these accumbal OT fibers is unknown. Using a combination of site-specific and peripheral infusions of the retrograde tracer, Fluorogold, we demonstrate that oxytocin-immunoreactive fibers in the nucleus accumbens likely originate from paraventricular and supraoptic hypothalamic neurons. This distribution of retrogradely labeled neurons is consistent with the hypothesis that striatal oxytocin fibers arise from collaterals of magnocellular neurons of the neurohypophysial system. If correct, this may serve to coordinate peripheral and central release of oxytocin with appropriate behavioral responses associated with reproduction, including pair bonding after mating, and

maternal responsiveness following parturition and during lactation. In addition, we used adeno-associated viral vector gene transfer to examine the functional relationship between accumbal oxytocin receptor density and social behavior in prairie and meadow voles. Adult female prairie voles that over-express oxytocin receptor in the nucleus accumbens displayed accelerated partner preference formation after cohabitation with a male, but did not display enhanced alloparental behavior. However, partner preference was not facilitated in non-monogamous meadow voles by introducing oxytocin receptor into the nucleus accumbens. These data are the first to demonstrate a direct relationship between oxytocin receptor density in the nucleus accumbens and variation in social attachment behaviors. Thus, individual variation in oxytocin receptor expression in the striatum may contribute to natural diversity in social behaviors.

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TABLE OF CONTENTS

Appendix A

Y. Takayanagi, M. Yoshida, I. F. Bielsky, **H. E. Ross**, M. Kawamata, T. Onaka, T. Yanagisawa, T. Kimura, M. M. Matzuk, L. J. Young and K. Nishimori, Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice, Proc Natl Acad Sci U S A. 102 (2005) 16096-101.

Appendix B

Ross HE and LJYoung. Genetic regulation of complex social behavior in a monogamous rodent. 2006. In Beyond Nature & Nurture in Psychiatry: Genes, Environment and Their Interplay. p57-65.

LIST OF FIGURES

CHAPTER 1:

CHAPTER 2:

CHAPTER 3:

CHAPTER 1

General Introduction

This chapter presents work to be published as: Ross HE and LJ Young. Oxytocin and the Neural Mechanisms Regulating Social Cognition and Affiliative Behavior. Front Neuroendocrinol, Submitted.

1.1 INTRODUCTION

Oxytocin (OT), derived from Greek meaning "rapid birth," is a nine amino acid, cyclic neuropeptide produced in the brain that has well characterized functions both on peripheral reproductive tissues and in the central nervous system [for a review see: (Burbach, Young et al. 2006)]. This molecule has an impressive history in the biological sciences. In 1906, Sir Henry Dale found that extracts of the posterior lobe of the pituitary contained substances that promoted contractile activity of the uterus (Dale 1906). OT was the first peptide to have its structure chemically identified and synthesized in the laboratory, leading to a Nobel Prize for Vincent du Vigneaud in 1955. Later neuroanatomical studies revealed that in the brain, OT is synthesized primarily in magnocellular neurons of the hypothalamus which project directly to the posterior pituitary where it is released into the bloodstream. This neurosecretory component of the OT system is known as the neurohypophyseal OT system, and it plays a critical role in the onset of parturition and milk ejection during lactation. For the past seventy years, the neurohypophyseal OT system has been a quintessential neuronal model for understanding the regulation of neurosecretion and more recently of dynamic neuronal-glial interactions (Scharrer and Scharrer 1940; Theodosis and Poulain 1984). Beginning in the late 1970's, pharmacological studies began to reveal a role for OT not only in birth and lactation, but in coordinating a suite of behavioral changes in the mother necessary for the survival of the offspring, i.e. maternal behavior. Since that time, it has become apparent that OT has many functions within the brain, modulating a constellation of behaviors associated with sociality. While many of the early studies have focused on the role of OT in regulating female behavior with a focus on reproduction, it is now clear that OT modulates social

cognition and affiliative behavior in both sexes. In contrast to the well understood neurosecretory OT system, the neuroanatomical details of central OT release have been woefully understudied. In this review, we will focus on OT's role in regulating a subset of its central nervous system affects; social cognition and affiliative behaviors. We will first review the literature pertaining to its role in initiating maternal nurturing behavior and the mother-infant bond. We will then briefly describe studies demonstrating a role for OT in the formation of social memories. Then we will review in detail the growing literature suggesting that OT is involved in social bond formation in monogamous rodents. Finally, we will discuss recent neuroanatomical studies of the OT neuronal systems that likely regulate affiliative behaviors in rodents.

1.2 COORDINATING BIRTH AND PARENTAL CARE

During pregnancy and parturition, a series of hormonal changes occur to prepare the womb for nurturing the developing fetus and ultimate birth and provisioning nutrients for the offspring. These include a rise and fall in progesterone, a steady increase in estrogen, and release of pituitary hormones, including OT and prolactin (for a review see (Mann and Bridges 2001)). These hormones prepare the uterus for supporting the placenta, are involved in initiating birth, and are critical for the lactation process. However, it is clear that each of these signaling molecules are also critical for the change in behaviors of the mother that are necessary for the survival of her offspring. Thus, nature uses conserved biochemical pathways to coordinate peripheral physiology with behavioral motivation. Here we will focus on the role of OT in regulating the maternal brain, with an emphasis on the induction of maternal nurturing behavior and the mother-infant bond.

1.3 MATERNAL CARE IN RODENTS

In most rodents, virgin females are either indifferent to, or have an aversion to, conspecific pups. However, around the time of birth, there is a dramatic shift in behavior of the dam towards maternal nurturing behavior. In rodents, maternal behavior includes nest building, retrieving pups to the nest, licking and grooming, hovering over, and nursing pups. Adult nulliparous female rats are neophobic and avoid or attack pups presented to them. This fear of pups diminishes at the time of parturition and pup odors become attractive. However, a virgin female will eventually begin caring for pups if exposed to them over multiple days (Rosenblatt 1967). The first suggestion that OT plays a role in regulating the onset of maternal behavior came from a study showing that OT injected into the cerebral ventricles (ICV) of a virgin rat could produce nurturing behavior towards pups within two hours (Pedersen and Prange 1979). Since this initial study, there have been conflicting reports possibly due to strain differences and testing protocol (Bolwerk and Swanson 1984; Fahrbach, Morrell et al. 1986). It has been determined that along with OT, estrogen priming, as well as anosmia, will increase the probability of spontaneous maternal care in virgin rats (Fahrbach, Morrell et al. 1984; Wamboldt and Insel 1987). Support for the role of endogenous OT in regulating maternal behavior has been derived from studies showing that an OT antagonist or OT antisera infused into the brain blocks the onset of maternal behavior in rats that have just given birth (van Leengoed, Kerker et al. 1987) (Fahrbach, Morrell et al. 1985). OT appears to play a more important role in regulating the onset of maternal behavior than its maintenance since OT antagonists fail to inhibit maternal behavior once nurturing behavior has been established (Fahrbach, Morrell et al. 1985).

Genetic experiments using mice with a deletion in either the OT or oxytocin receptor (OTR) gene have supported these initial pharmacological studies in rats. In addition, they have challenged the long held belief that OT is critical for the onset of parturition. Surprisingly, OT knock-out (OTKO) and OTR knock-out (OTRKO) mice give birth on time and without incident (Nishimori, Young et al. 1996; Takayanagi, Yoshida et al. 2005) (Young, Shepard et al. 1996). Thus, while OT may play an important role in initiating parturition, there appears to be redundant mechanisms whereby prostaglandin activation is able to compensate for the loss of OT to promote labor (Russell and Leng 1998). Although these genetically altered mice were able to give birth, they were unable to lactate, confirming an essential role for OT in lactation. As a result, their pups perish within 24 hours of birth.

Initial results in two independently generated OTKO lines of mice produced paradoxical results with regard to the role of OT in regulating maternal behavior (Nishimori, Young et al. 1996; Young, Shepard et al. 1996). In both lines, maternal motivation appeared grossly normal. Indeed, even virgin mice of these strains displayed pup retrieval and licking and grooming behavior (Young, Winslow et al. 1997). However, more detailed studies of these mice revealed that nulliparous OTKO mice displayed decreased levels of retrieval and licking and grooming of pups compared to wild-types (Pedersen, Vadlamudi et al. 2006). In a separate study, OTKO mice housed in a social semi-natural environment were significantly more likely to display infanticidal behavior than wild-type mice in the same environment (Ragnauth, Devidze et al. 2005).

Compared to peptide knock-out mice, receptor deficient females display a more robust deficit in maternal behavior. Both wild-type and OTRKO post partum females built nests

and crouch over their pups. However, pups of knock-out females are often observed scattered throughout the cage. When tested for pup retrieval, OTRKO dams display longer latencies to retrieve the pups and spend less time crouching over their pups (Takayanagi, Yoshida et al. 2005). The discrepancy between the results of maternal behavior testing in OTKO and OTRKO mice suggest that in OTKO mice another ligand, perhaps vasopressin, partially compensates for the lack of OT, allowing for the expression of maternal behavior.

Other knock-out mouse studies provide additional evidence for a role of OT involvement in the regulation of maternal behavior. Mice lacking CD38, a transmembrane receptor involved in the immune response and mobilizing of Ca^{++} from intracellular stores, display maternal nurturing deficits almost identical to the retrieving and crouching deficits seen in OTRKO dams. Furthermore, studies revealed that these mice display deficits in OT release. Interestingly, a subcutaneous infusion of OT rescued the deficits in maternal nurturing (Jin, Liu et al. 2007). A second line of mice provided circumstantial evidence consistent with a role for OTR in regulating maternal behavior, although alternative explanations are equally plausible. The OTR is a seven transmembrane G-protein coupled receptor that signals through the G(q/11) family (Kimura and Ivell 1999). Mice have been generated that lack the alpha-subunits of the two main members of the G(q/11) family, Galpha(q) and Galpha(11), selectively in the forebrain. These forebrain Galpha(q/11)-deficient females have a profound deficit in maternal behaviors. They do not display nest building, pup retrieving, crouching, or nursing (Wettschureck, Moers et al. 2004). Taken together, these genetic manipulation studies provide convincing evidence that OT plays a role in some aspects of maternal nurturing behaviors in mice.

There is some evidence that natural variation in maternal nurturing received by pups alters brain OTR expression levels which in turn may alter the quality of maternal behavior they provide to their own offspring when they reach adulthood. Post partum rats dams can be classified as high licking and grooming (LG) or low LG. Pups reared by high LG dams display high LG when they become mothers, while pups reared by low LG mothers display low LG when they become mothers, regardless of the maternal behavior of their biological mother (Francis, Diorio et al. 1999). High LG moms have increased OTR density in the medial preoptic area, bed nucleus of the stria terminalis, lateral septum, amygdala and the paraventricular nucleus of the hypothalamus (PVN) compared to low LG moms (Francis, Champagne et al. 2000). Furthermore, infusion of an OTR antagonist reduces LG levels in high LG dams (Champagne, Diorio et al. 2001). The maternal nurturing received as pups has a long term impact on OTR expression in the brain as female pups reared by high LG dams have increased OTR binding in the central amygdala when they reach adulthood (Francis, Young et al. 2002). Post-weaning social and environmental enrichment also affects both maternal behavior and adult OTR densities in the brain. Pups from low LG dams weaned into socially and environmentally enriched cages display higher LG and elevated OTR density than LG dams weaned into impoverished isolated housing conditions (Champagne and Meaney 2007). Thus it appears as though the transgenerational transmission of maternal behavior may be mediated by changes in the expression of OTR.

1.4 MATERNAL BONDING IN SHEEP

Rodents typically display promiscuous maternal behavior, and maternal dams will nurture any pup placed in her nest. There is no particular bond between the mother and

the pups, since the likelihood of a foreign pup being in the dams nest is rather low. However, ungulates such as sheep live in large herds and give birth to precocial young during a defined breeding season. Mothers therefore need to discriminate between their own lamb and foreign lambs, and have subsequently evolved mechanisms to produce selective maternal-infant bonds. Vaginocervial stimulation during labor initiates a cascade of neurochemical events that ultimately leads to the development of the selective bond between the mother and the lamb (Kendrick, Levy et al. 1991). The selective bond appears to be mediated in part by the development of an olfactory memory of the lamb which occurs in the main olfactory bulb (Levy, Locatelli et al. 1995). Once the odor is learned, the mother actively rejects any other lamb from nursing. Vaginocervical stimulation is a potent releaser of OT in the ewe (Kendrick, Keverne et al. 1986), and is capable of promoting adoption of a foreign lamb in a steroid primed ewes (Keverne, Levy et al. 1983). Furthermore, OT administered intracerebroventricularly can induce full maternal behavior in less than a minute in oestrogen-primed nonpregnant ewes (Kendrick, Keverne et al. 1987). In addition to initiating maternal nurturing behavior, OT also facilitates the formation of olfactory memories by modulating noradrenaline and influencing the release of neurotransmitters in the olfactory bulb. After giving birth, there is a strengthening of the mitral to granule and periglomerular cell synapses to bias the network to respond to lamb odors, with a higher number of output mitral cells responding to the ewes own lamb odors (Kendrick, Levy et al. 1992; Levy, Kendrick et al. 1995; Kendrick, Da Costa et al. 1997). As a consequence of this olfactory learning, the lamb accepts her own lamb, but rejects foreign lambs, indicative of a selective mother-infant bond.

1.5 ALLOPARENTAL BEHAVIOR IN VOLES

Monogamous prairie voles (*Microtus ochrogaster*) have been instrumental in understanding the role of OT in the regulation of affiliative behaviors. Prairie voles display biparental care and often nest in communal dens comprised of a breeding pair and multiple litters of their offspring (Getz and Carter 1996). Perhaps as an adaptation to this communal living, prairie vole juveniles and some adult females display nurturing behavior toward pups who are not their own. This "baby sitting" behavior is referred to as alloparental care, and provides an opportunity to investigate the regulation of maternal-like behavior in the absence of the physiological changes associated with pregnancy and parturition. The majority of juvenile prairie voles (< 20 days of age) display high levels of alloparental behavior when exposed to novel pups (Olazabal and Young 2005). However, as females reach puberty, only about 50% of the females will spontaneously retrieve, lick/groom, and hover over pups presented to them, with the remainder of the females either ignoring or attacking pups (Lonstein and De Vries 1999; Bales and Carter 2003; Olazabal and Young 2005). The transition from juvenile to adult patterns of alloparental behavior in females does not appear to be related to changes in sex hormones (Lonstein and De Vries 1999). In prairie voles, the rearing environment is able to influence the amount of affiliative behaviors exhibited as an adult. For example, females are more likely to be alloparental as adults if they remain in the natal nest after weaning, with continued exposure to parents being a more critical factor than previous pup exposure (Lonstein and De Vries 2001). The presence of the father in the natal nest also increases the amount of time juveniles spend in alloparental behavior towards their younger siblings (Wang and Novak 1994).

While gonadal steroids do not appear to influence the expression of alloparental behavior, there is evidence that OT may play an important role, at least in female prairie voles. There is a remarkable amount of individual variation in OTR density in the nucleus accumbens (NAcc) in prairie voles, and OTR density in the NAcc is significantly correlated with the display of alloparental behavior in both juvenile and adult virgin females (Olazabal and Young 2006; Olazabal and Young 2006). Indeed, adult females that display alloparental behavior have higher densities of OTR in the NAcc than those that either ignore or attack pups (Olazabal and Young 2006) (Figure 1.1A, B). Administration of an OT antagonist into the NAcc, but not into the adjacent caudate, is able to block all expression of maternal-like behavior towards pups in adult females (Figure 1.1C). This relationship between OTR density in the NAcc and the natural variation in alloparental behavior in adult female prairie voles led us to hypothesize that differences in OTR density in the NAcc could directly contributes to individual differences in behavior.

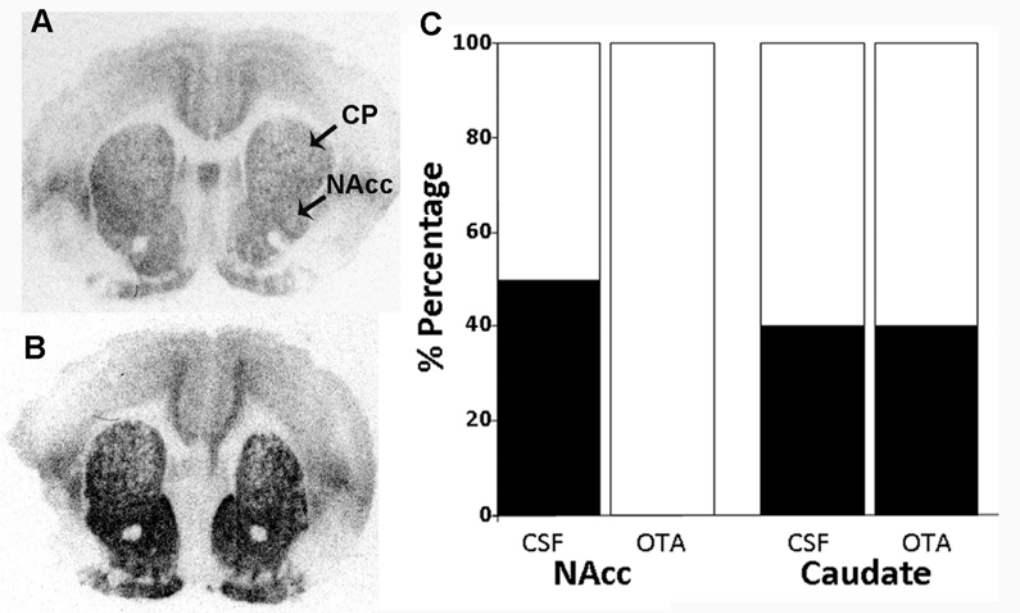

Figure 1.1. OTR and alloparental behavior in female prairie voles. Autoradiography showing oxytocin receptor density in the nucleus accumbens (NAcc) and caudate putamen (CP) in spontaneously maternal (A), and non-maternal (B) female prairie voles. Females that have a high density of OTR in the NAcc are more likely to exhibit alloparental care than those with a low level of accumbal OTR. C) Graph showing the effect of administering oxytocin antagonist (OTA) or cerebrospinal fluid (CSF) into the NAcc or CP on alloparental behavior of female prairie voles. Nulliparous females injected with CSF in the NAcc or CP, or OTA in the CP, showed the normal variation in propensity for alloparental behavior. However, injecting OTA in NAcc inhibited alloparental behavior in all the females, suggesting that endogenous oxytocin is necessary for the expression of alloparental behavior in female prairie voles. Adapted from Olazabal and Young, Neuroscience, 2006.

1.6 PUP-MOTHER INTERACTIONS

There is some evidence that OT may also modulate the infant's response to the mother. For example, when mouse pups are separated from their mother, they emit ultrasonic vocalizations in protest of the separation. OTKO and OTRKO pups emit significantly fewer vocalization following separation from their mother than do wild-type littermates (Winslow, Hearn et al. 2000; Takayanagi, Yoshida et al. 2005). This difference could reflect differences in emotionality (e.g. anxiety-like behavior), but could also reflect a decreased motivation to be in contact with the mother. To support this latter interpretation, tests were performed in OTKO pups to assess motivation to reunite with their mother. In the first test, 10 day old pups were placed in one chamber of a two chambered testing arena in which the mother was restricted to the other chamber. After an initial training trial, OTKO pups exhibited a significantly longer latency than wild-type pups to cross into the mother's chamber in the second and third trials (Young, unpublished data) (Figure 1.2) .The second test used a three-chambered arena to determine if 15 day old pups exhibited a preference for their mother over a novel lactating female. Wild-type pups showed a strong preference for their mother, spending significantly more time in the mother's chamber than with the novel female. By contrast, OTKO pups show no preference (Young, unpublished data). OT also has an influence on pups attraction to their mother in that OT antagonist is able to block a preference for maternal odors in 15 day old rat pups (Nelson and Panksepp 1996). In this experiment, pups were conditioned to a lemon odor associated with maternal reunion. This was done by removing the pups from the maternal nest for three hours and then returning them to their now lemon-scented mother or to a control lemon-scented cotton ball. Pups were

given OT-antagonist or saline ICV before placement with the lemon odor. When tested the next day, pups that had received OT-antagonist did not show a preference for the maternal-associated odor, while those receiving saline did.

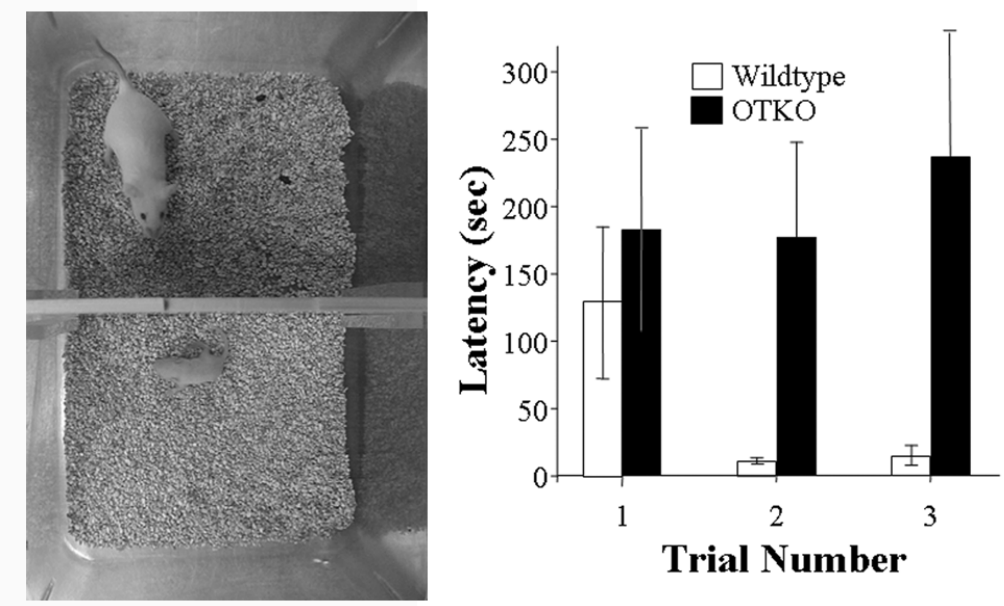

Figure 1.2. Infant-mother interactions of oxytocin knock-out (OTKO) pups. Ten day old pups were placed in one chamber of a testing arena in which the mother was restricted to a second chamber. The divider contained small holes that were large enough for the pup to pass, but that prevented the mother from passing. The latency for the pup to enter the mother's chamber was recorded on three successive trials. There was no difference between wildtype and and OTKO pups in the initial training trial. However, in the subsequent trials OTKO pups exhibited a significantly longer latency than wild-type pups to cross into the mother's chamber ($p < 0.05$). Thus OT may be involved in motivating pups to seek contact with their caregiver.

1.7 SOCIAL BONDING IN ADULTS

Monogamous prairie voles have become an important model for understanding the neurochemical basis of social bond formation between mating pairs, known as the pair bond. In nature, a large percentage of mating pairs nest together for extended periods of time and display biparental care (Getz, Carter et al. 1981). In the laboratory, the formation of a pair bond has been investigated by using a partner preference test (Williams, Catania et al. 1992). In this test, a male and female are paired and allowed to co-habitate, during which time mating may or may not occur. At the time of testing, the co-habitation partner is tethered to one side chamber of a three-chamber apparatus. A novel animal of equal stimulus value, termed the 'stranger', is tethered to the opposite side chamber. The test animal is placed in the center chamber and allowed to explore all three chambers freely. The amount of time this test animal spends in close proximity to, or huddling with either the partner or the stranger is recorded over a three hour testing period. The experimental animal is said to have a partner preference if it spends at least twice as much time in contact with the partner compared to the stranger (Insel and Hulihan 1995).

Females who have cohabitated with a male typically display a partner preference if mating occurs. However, longer cohabitations without mating can also result in the development of a pair bond. Because of the role that OT plays in mother-infant attachment, and since mating results in vaginocervical stimulation, which is known to release OT in the brain, OT was a prime candidate for regulating the formation of the pair bond. Indeed, there is now convincing evidence that OT plays a critical role in the development of partner preferences in female prairie voles. Intracerebroventricular

infusion of OT during a six hour cohabitation period with a male is able to induce a partner preference in unmated female prairie voles (Williams, Insel et al. 1994). Likewise, an OT antagonist blocks mating-induced pair bond formation after a 24 hr cohabitation (Insel and Hulihan 1995). The role of OT in partner bonding in male prairie voles is less clear. The original study in males showed that ICV infusion of OT for 24 hours was not enough to induce a pair bond, nor did OT-antagonist inhibit partner preference formation (Winslow, Hastings et al. 1993). However, another group found that a central infusion of a high dose of OT followed by a one hour cohabitation was enough for males to prefer a partner over a stranger. Conversely, giving an OT-antagonist or vasopressin antagonist, a closely related peptide, with OT was able to block OT induced partner preferences (Cho, DeVries et al. 1999). These data suggest that exogenous OT may stimulate partner preference formation in males through interactions with the OTR and vasopressin receptors, but it is not clear whether endogenous OT is released in males during mating, or whether it contributes to partner preference formation in males. Rather, the related peptide, vasopressin appears to play a more important role in partner preference formation in male prairie voles (Winslow, Hastings et al. 1993; Young and Wang 2004; Donaldson and Young 2008).

Comparative studies in voles with different social organizations have been useful for identifying the neuroanatomical substrate on which OT acts to promote partner preference formation. For example, monogamous and non-monogamous species of voles have remarkably different distribution of OTR within the brain. Prairie voles have significantly higher densities of OTR in the NAcc than non-monogamous meadow and montane voles (Insel and Shapiro 1992) (Figure 1.3). To test whether these areas are

involved in partner preference formation, OT antagonist or vehicle was injected site specifically into the NAcc, prefrontal cortex, or caudate putamen of female prairie voles prior to cohabitation with a male. Following pairing, the females were tested for a partner preference. Females receiving vehicle into any brain region or OT antagonist into the caudate putamen formed a partner preference, while those receiving the antagonist into the NAcc or prefrontal cortex failed to display a partner preference (Young, Lim et al. 2001) (Figure 1.3). OT release in the NAcc has not been directly measured during the pair bonding process.

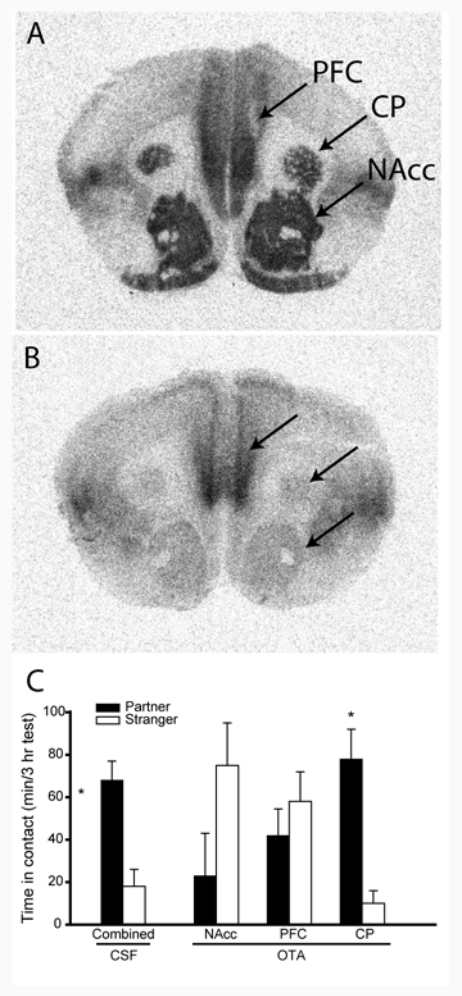

Figure 1.3. **Species differences in oxytocin receptor (OTR) expression in prairie and montane voles**. Notice the higher level of OTR binding in the caudate (CP), and nucleus accumbens (NAcc) of the prairie vole (A) than the montane vole (B). Both species have OTR binding in the prefrontal cortex (PFC) C) Graph illustrating the effects of administering oxytocin antagonist (OTA) or cerebral spinal fluid (CSF) into the PFC, CP, or NAcc on pair bonding behavior in female prairie voles. Administering OTA into the CP or CSF during a 24-hour cohabitation with mating does not effect the formation of a partner preference. However, injecting OTA into the PFC or NAcc blocked females from bonding with their mating partner, showing that oxytocin in these areas is important for affiliative behavior in a monogamous vole. Adapted from Young et al., Horm Behav, 2001 and Young and Wang, Nat Neurosci, 2004.

The NAcc is a key component of the mesolimbic dopamine reward/reinforcement pathway and has been implicated in mediating the effects of drugs of abuse as well as addiction (Kelley 2004) (Carelli 2002). In addition to a high density of OTR, the NAcc of prairie voles also contains dopamine projections from the ventral tegmental area, and dopamine receptors. Blocking D2-like dopamine receptors in the rostral shell of the NAcc prevents partner preference formation, and D2 agonists induce partner preferences in the absence of mating (Gingrich, Liu et al. 2000). Activation of D1-like DA receptors inhibits partner preference formation induced by either mating or by D2 activation (Aragona, Liu et al. 2003; Aragona, Liu et al. 2006). Aragona and colleagues have begun to elucidate the second messengers involved in DA receptor mediated partner preference formation. They found that reducing cAMP signaling in the shell of the NAcc also facilitates partner preference formation. Conversely increasing cAMP signaling, by decreasing PKA activity, blocks mating-induced partner preference (Aragona and Wang 2007). These results are consistent with the fact that D2 receptor activation decreases cAMP while D1 receptor activation stimulates cAMP production. The dopamine and OT system interact in the NAcc of female prairie voles to promote partner preference formation. A seminal study showed that OTR and D2 receptor activation must occur concurrently for partner preference to develop in female prairie voles (Liu and Wang 2003). Blockade of D2 receptors in the NAcc shell prevented OT-mediated partner preferences, while blockade of OTR in the NAcc shell prevented D2 receptor mediated partner preferences in female prairie voles. Future studies are needed to determine whether and how the second messenger systems of OT and DA interact to produce a partner preference.

The fact that OT is involved in mediating maternal nurturing, mother-infant bonding, alloparental behavior and pair bonding raises an intriguing hypothesis about the evolution of pair bonding in monogamous species. Maternal behavior is present in all mammalian species, while pair bonding has evolved multiple times independently in unrelated monogamous species, and is quite rare. We propose that the ability a female to form an attachment with her male partner arose from a modification of the cellular machinery and circuitry involved in regulating maternal behavior. Indeed, OTR in the NAcc is involved in both alloparental nurturing behavior and partner preference formation. Vaginocervical stimulation during parturition stimulates central OT release, while similar stimulation during mating bouts also promotes OT release. We predict that similar exaptations of maternal behavior circuitry may have occurred in the evolution of monogamy in other species as well. It will be important to determine if OT is involved in pair bonding in other monogamous species as well.

1.8 SOCIAL RECOGNITION IN RODENTS

We hypothesize that pair bond formation is the result of an association between the rewarding mating experience and the olfactory signature of the partner (Young and Wang 2004). The ability to distinguish familiar conspecifics from strangers and to remember individuals previously encountered is critical for successful group living and survival many species. This process is called social recognition, and OT has been shown to be important for this memory ability. The modulation of social recognition in rodents was first investigated in rats (Dantzer, Bluthe et al. 1987), and central injection of low doses of OT enhanced the amount of time a male remembers a conspecific (Popik, Vetulani et al. 1992; Benelli, Bertolini et al. 1995). There have been many sites of action

implicated for OT in rat social recognition, including the ventral hippocampus, septum, medial preoptic area, and olfactory bulb (Popik and van Ree 1991; Popik, Vos et al. 1992; van Wimersma Greidanus and Maigret 1996; Dluzen, Muraoka et al. 1998). It should be noted that although OT in these areas have been shown to enhance memory, antagonist to the OTR have not been able to block memory performance. Thus the mechanism by which OT acts in these regions to enhance social recognition is unclear. It is known, however, that OTs effects in the olfactory bulb on social recognition are mediated by norepinephrine (Dluzen, Muraoka et al. 1998; Dluzen, Muraoka et al. 2000).

Studies of OTKO and OTRKO mice have been instrumental in further investigating the mechanisms by which OT enhances social recognition (Ferguson, Young et al. 2000; Choleris, Gustafsson et al. 2003; Kavaliers, Colwell et al. 2003; Takayanagi, Yoshida et al. 2005; Crawley, Chen et al. 2007; Lee, Caldwell et al. 2008). OTKO mice have been studied most extensively with regard to the neuroanatomical localization of OT actions in mediating social recognition. Social recognition is assessed by quantifying the duration of olfactory investigation upon repeated exposure of the experimental mouse to a stimulus mouse. Wild-type mice habituate to familiar mice as reflected by a decreased investigation time over subsequent exposures. However, OTKO males fail to habituate after repeated exposures to the same mouse (Ferguson, Young et al. 2000). This is not due to deficits in general learning and memory or olfaction since they do habituate to non-social odors (Ferguson, Young et al. 2000). A single intracerebroventricular infusion of OT before, but not after, the initial exposure completely rescues the deficit in social recognition (Ferguson, Aldag et al. 2001). This suggests that the deficit in OTKO mice lies in the processing of the olfactory signals or encoding the memory. Analysis of neural

activation patterns using Fos immunohistochemistry revealed that following a social encounter, Fos activation is normal in the olfactory bulb, but is markedly impaired in the medial amydala and downstream projection sites of the amygdala (Ferguson, Aldag et al. 2001). Site-specific infusion of OT into the medial amygdala, but not into the olfactory bulb rescued the social recognition deficits (Ferguson, Aldag et al. 2001). Likewise, infusion of OTR antisense oligonucleotides into the medial amygdala of wild-type females impairs social recognition abilities (Choleris, Little et al. 2007). The medial amygdala receives olfactory information directly from the olfactory bulb and is therefore in an exquisite position to process social odors. OTKO female mice also are unable to discriminate parasite load on potential mates, which would increase their risk of infection (Kavaliers, Colwell et al. 2003). OTRKO mice had a milder deficit in social recognition in that they were unable to distinguish inbred mouse strains (C57BL/6) but could differentiate females of an outbred strain (CD-1) (Takayanagi, Yoshida et al. 2005).

A further line of evidence suggesting that OT is required for social recognition comes from studies in the CD38-KO mouse mentioned above. This mutant has a deficit in OT secretion and is also impaired in its ability to recognize familiar conspecifics. Expressing CD-38 in the hypothalamus using a lenti-viral vector or by infusing OT was able to rescue this deficit (Jin, Liu et al. 2007).

These studies suggest that OT is involved in enhancing the processing of social information. Precisely how OT enhances social information processing is unknown, but it may serve to mark the social signal with saliency or alter the valence of the signal. It is unknown whether OT is involved in the processing of social information of other modalities (e.g. visual, auditory), or whether it is specific for olfactory information.

However, we hypothesize that OT may be acting in multiple brain regions to enhance the saliency of social stimuli and to encode social memories, and that this may play a pivotal role in mother-infant interactions, pair bond formation, as well as in the formation of other social relationships.

1.9 SOCIAL COGNITION IN HUMANS

In recent years, pharmacological studies have suggested that OT is also able to enhance human social cognition. When administered intranasally to human subjects, OT stimulates behaviors consistent with an enhancement of interpersonal trust during economic games (Kosfeld, Heinrichs et al. 2005). In fact, when given intranasal OT, human subjects continue to trust others even after having been betrayed by another (Baumgartner, Heinrichs et al. 2008). This increase in trust, and lack of feedback following betrayal may be due to a decrease in activation of circuits involved in fear processing, such as the amygdala (Kirsch, Esslinger et al. 2005) (Baumgartner, Heinrichs et al. 2008). Intranasal OT also improves identity recognition memory for neutral and angry faces, independently of participant's gender (Savaskan, Ehrhardt et al. 2008), perhaps by increasing gaze to the eye region of human faces (Guastella, Mitchell et al. 2008). A more recent study revealed that OT increased the accuracy of judging a face that the subject previously viewed as familiar (Rimmele, Hediger et al. 2009). This finding is remarkably consistent with the well documented role of OT on social recognition in rodents, and suggests that OT enhances social information processes from multiple modalities, including visual. Intranasal OT also improves the ability to infer the emotional state of others based on subtle facial stimuli, a phenomenon referred to as

"mind reading" (Domes, Heinrichs et al. 2007); which could have profound effects on the maintenance of social groups.

The effects of OT on animal social behavior and the impact on social cognition in the human studies has important implications for psychiatric disorders associated with social deficits such as autism spectrum disorders and schizophrenia. A single study has reported that plasma OT is decreased in autistic subjects compared to typical control subjects (Modahl, Green et al. 1998), but this finding has not been replicated. Subjects with autism spectrum disorder or schizophrenia often do not attend to the appropriate facial cues of others (Klin, Jones et al. 2002; Pelphrey, Sasson et al. 2002). These observations have led to the hypothesis that OT may be a viable pharmacotherapy to enhance social cognitive abilities in subjects with autism spectrum disorder (Bartz and Hollander 2006). In fact, a single study has shown that OT can increase autistic individuals' ability to recall emotion in a voice intonation task. A more important finding of this study was that prior exposure to OT increased the learning ability to subsequent emotional tasks (Hollander, Bartz et al. 2007). Further studies are needed to determine whether targeting the OT system may be a viable treatment strategy for enhancing social cognition in psychiatric disorders.

1.10 MEDIATION OF THE POSITIVE EFFECTS OF SOCIAL SUPPORT:

Stress responses and immune function.

In social species, social support provides beneficial effects on immune function and stress reactivity. There is some evidence that OT may play a role in mediating these effects but more research is needed in this area, particularly with respect to immune function.

Functioning of the immune system has become of interest in the context of autism spectrum disorders since these disorders have been associated with a vulnerability to toxins and excessive food allergies (Burger and Warren 1998; Fatemi, Earle et al. 2002; Ashwood, Wills et al. 2006; Bello 2007). Social support accelerates healing time and can be beneficial to those dealing with potentially fatal diseases (DeVries, Craft et al. 2007). When stressed, wounds are bigger in isolated than paired hamsters (Detillion, Craft et al. 2004). However wound size was similar in groups that were pair housed and stressed, pair housed with no-stress, and isolated with no-stress. Cortisol levels post-stress are higher in isolated than paired individuals and wound size is larger in stressed than adrenalectomized individuals with stress or no-stress. In other words, stress-induced cortisol release inhibits wound healing and social housing buffers against stress-induced activation of the HPA. To test whether OT was facilitating the positive effects of pair housing, OT was given intraperitoneally to isolated animals. Treatment with OT decreased wound size and lowered the release of stress-induced cortisol; while OT-antagonist centrally administered into the ventricles impaired wound healing in paired hamsters (Detillion, Craft et al. 2004). The physical contact during pair housing may stimulate the release of OT to promote these healing effects. Innocuous somatosensory stimulation such as touch, warm temperature, vibration, and electroacupuncture increase oxytocin levels in plasma and cerebrospinal fluid (Stock and Uvnas-Moberg 1988; Uvnas-Moberg, Bruzelius et al. 1993).

Although these experiments suggest that OT affects wound healing by modulating the hypothalamic, pituitary adrenal axis, there is a possibility that it can directly interact with the immune system. OT and OTR are present in the thymus, the organ where

lymphocytes mature into T cells that are involved in the adaptive immune system (Gimpl and Fahrenholz 2001). The human OTR gene has binding sited for the inflammatory cytokine nucleofactor interleukin-6 (NF IL-6) and for acute-phase response elements, which are induced by infection or inflammation [(Inoue, Kimura et al. 1994) for review see (Gimpl and Fahrenholz 2001)]. Binding to these sites on the OTR gene may explain why immune elements have the ability to induce labor [for review (Russell and Leng 1998; Gimpl and Fahrenholz 2001)]. In fact, cytokines like interferon-γ, can affect OTR mRNA levels in a manner not matched by the sex hormones progesterone and estradial (Ivell, Bathgate et al. 1997). In the brain, interleukin-1b directly stimulates intranuclear release of OT from the supraoptic nucleus (SON) (Landgraf, Neumann et al. 1995). Furthermore, infusion of interleukin-6 increases OTR expression in the brain of female rats (Young, Muns et al. 1997). Further studies are needed to understand the relationship between OT, social buffering, and the immune system.

1.11 HUMAN SOCIAL RELATIONSHIPS

Animal studies have implicated a role for OT in mediating maternal behavior, mother-infant bonding, and pair bonding and beg the question of whether OT might modulate human social relationships. Data addressing this issue is scarce and inconclusive. However, there are reports that plasma OT concentrations are correlated with emotional responses of mothers to their infant. In particular, active maternal behavior, which comprises mother's gaze at infant's face, positive affect, affectionate touch, and motherese vocalizations and cognitive maternal representations, which includes feelings of attachment and frequent checking behavior, are correlated with high OT levels during the first trimester and first postpartum month (Feldman, Weller et al. 2007).

Another interesting possibility worthy of speculation is whether OT may play a role in human pair bonding. It has been shown that OT is increased in the plasma during sexual arousal and ejaculation or orgasm in humans (Carmichael, Humbert et al. 1987) similar to the release seen in prairie voles during mating (Ross, Cole et al. 2009) and rats and sheep following vaginocervical stimulation (Kendrick, Keverne et al. 1986; Sansone, Gerdes et al. 2002; Waldherr and Neumann 2007). Human sexuality may have evolved to promote pair bonding by the incorporation of behaviors that maximize the frequency and extent of OT release during intimacy. For example, in most species female sexual receptivity is tightly coupled to the reproductive cycle. However, in human females sexual desire has become uncoupled from fertility, resulting in more frequent copulations. Humans copulate face to face, maximizing exposure to visual stimuli of the partners face. In addition, humans are the only species where the female breast has become a secondary sexual characteristic. Indeed, nipple stimulation can be sexually arousing to both men and women (Levin and Meston 2006), and both vaginocervical and nipple stimulation increases OT release in plasma (Christensson, Nilsson et al. 1989). Thus, in humans, sexual intimacy recapitulates the physiological stimuli of delivery and nursing, maximizing the release of OT, which may serve to strengthen the bond between the female and the male. If this speculation is correct, it would be consistent with our hypothesis that the mechanisms underlying pair bonding emerged through alterations in the mechanisms underlying the mother-infant bond.

There is evidence that early life social relationships can alter the adult OT system. In rhesus macaques, adolescents raised in a nursery by human caregivers have lower OT concentrations in their cerebrospinal fluid compared to those raised by their mother

(Winslow, Noble et al. 2003). A recent study suggests a similar phenomenon in humans. Women who experienced early childhood abuse or neglect had significantly lower levels of OT in their CSF compared to women who did not experience early abuse or neglect (Heim, Young et al. 2008). This provides evidence that, as mentioned above in rats, early parent-infant interactions can have enduring consequences on the OT system, which may impact adult social cognition and the ability to form social relationships.

1.12 NEURAL CIRCUITRY OF THE OXYTOCIN SYSTEM

With the emerging interest in the central effects of OT on affiliative behavior, attention should be directed in understanding the distribution and modes of release of OT within brain regions regulating affiliative behavior. While numerous studies have documented the release of OT within the PVN and SON reviewed in (Landgraf and Neumann 2004; Ludwig and Leng 2006), little attention has been given to the source and mode of release of OT within structures that modulate social behavior, such as the NAcc. Here we briefly describe the brain OT system, including forebrain OT projections, and speculate on the origin of behaviorally relevant OT. We will focus on the prairie vole system where appropriate.

1.13 CHARACTERIZATION OF OXYTOCIN CELLS

The PVN and SON of the hypothalamus are the main sites of OT production in the brain. In rats, there are two types of OT neurons present in the PVN, large magnocellular neurons and smaller parvocellular neurons, which differ not only with respect to size, but also with regard to their projections (Swanson and Sawchenko 1983). The SON contains only magnocellular OT cells (Sofroniew 1983; Swanson and Sawchenko 1983). In

prairie voles, OT neurons can also be seen in the medial preotic nucleus, median preoptic nucleus, and preoptic paraventricular nucleus; all which are continuous with the PVN population. Additionally, individual OT labeled neurons can be found in the bed nucleus of the stria terminalis and the lateral hypothalamic area (Wang, Zhou et al. 1996). Although the soma of OT neurons are mainly restricted to the hypothalamus, OT fibers are spread throughout the entire brain. Sparse fibers can be found in the NAcc, amygdala, lateral septum and hippocampus (Figure 1.4). In the rat, one of the densest central OT projections is to the brainstem and spinal cord (Sofroniew 1983).

These dense OT fibers in caudal brain areas caught the attention of early researchers. Tracer experiments were done to determine if individual PVN cells project to the brainstem and spinal cord, to the pituitary, or both. It was found that magnocellular cells of the PVN project to the posterior pituitary; while the smaller parvocellular cells project to the hind brain or spinal cord, with 0.2% projecting to both the pituitary and brainstem (Ono, Nishino et al. 1978; Hosoya and Matsushita 1979; Swanson and Kuypers 1980; Kozlowski 1986). Since these studies, it has been assumed by many in the field that all the central OT fibers originate from parvocellular cells of the PVN, creating a dissociation between the centrally projecting OT system and the neurohypophyseal system. This separation of projection targets is supported by microdialysis studies showing that peripheral and central release are dissociated under certain circumstances (Neumann, Ludwig et al. 1993; Engelmann, Landgraf et al. 2004; Neumann 2007).

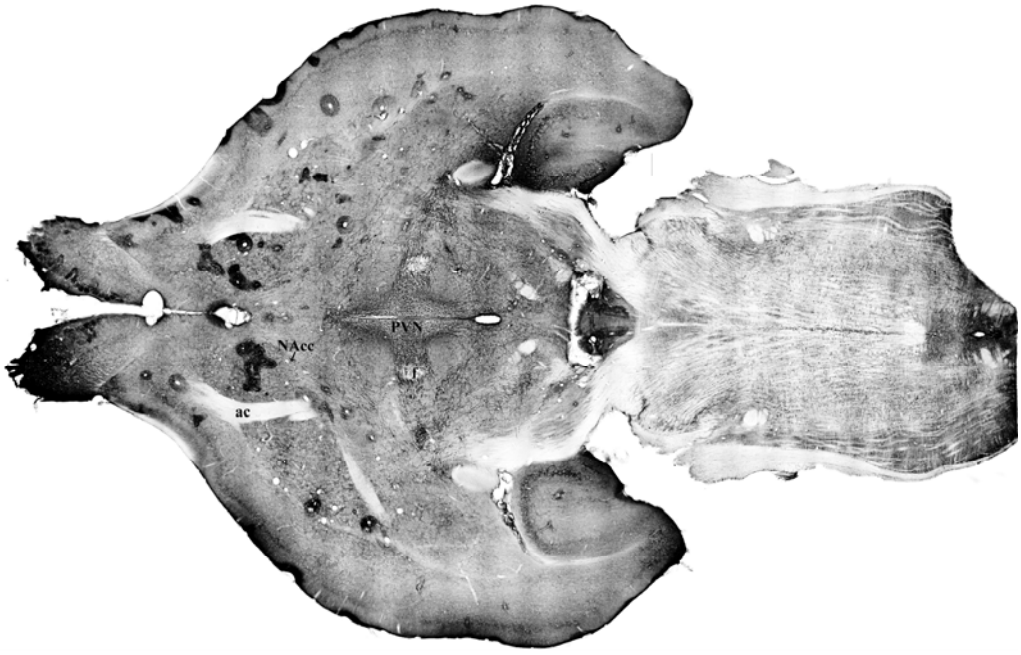

Figure 1.4. Light micrograph of oxytocin-immunoreactive fibers in the prairie vole from a horizontal section. PVN = paraventricular nucleus of the hypothalamus. ac = anterior commisure, f = fornix. NAcc = nucleus accumbens.

Unlike classical neurotransmitters, which are released primarily at the synaptic cleft, neuropeptide neurons can release peptide from its entire surface area (Pow and Morris 1989) and can diffuse through the extracellular space due to long half-lives. Peptide specificity is achieved through a high binding affinity for the receptors, about 1000x higher than classical neurotransmitters (Landgraf and Neumann 2004; Salio, Lossi et al. 2006). Dendritic release of OT has been well characterized and is independent of neuronal firing [for review see (Ludwig and Leng 2006)]. Ca^{++} released from intracellular stores favors dendritic release, while Ca^{++} influx from ion channels favors axonal release. Recent studies have begun to elucidate the mechanism by which dendritic release occurs. First, glutamate can trigger dendritic release of OT through the activation of NMDA receptors without producing action potentials (Ludwig and Leng 2006). Alpha melanocyte stimulating hormone (αMSH) evokes dendritic release of OT and inhibits axonal release in the SON. OT neurons in the PVN and SON express the MC4 receptor that binds αMSH and triggers intracellular calcium release to evoke dendritic OT release. At the same time, αMSH inhibits electrical activity and reduces OT release from axons by releasing endocannabinoids to presynaptically inhibit afferent glutamate release [(Sabatier, Caquineau et al. 2003; Sabatier and Leng 2006), for review see (Ludwig and Leng 2006; Sabatier 2006)]. It has been speculated that dendritically released OT from the SON or PVN diffuses to distant brain regions where it activates OTRs mediating social behavior (Landgraf and Neumann 2004; Ludwig and Leng 2006). However, little attention has been given to the release of OT from the sparse network of OT fibers coursing through forebrain limbic regions.

1.14 OXYTOCIN FIBERS OF THE NUCLEUS ACCUMBENS

The density of OTR binding in the NAcc is highly species specific, with prairie voles having high densities of receptors throughout the striatum, rats having intermediate receptor binding, and mice and meadow voles having little or no OTR binding in the Nacc (Figure 1.5). This variability in OTR localization may provide a mechanism by which evolution acts to change social systems from one species to another, as seen between the monogamous and polygamous voles. As OTR in the NAcc plays a critical role in regulating alloparental behavior and partner preference formation in prairie voles, detailed characterization of OT immunoreactive fibers in the NAcc is needed. To date, there have been no studies that have investigated the origin on these fibers in any species.

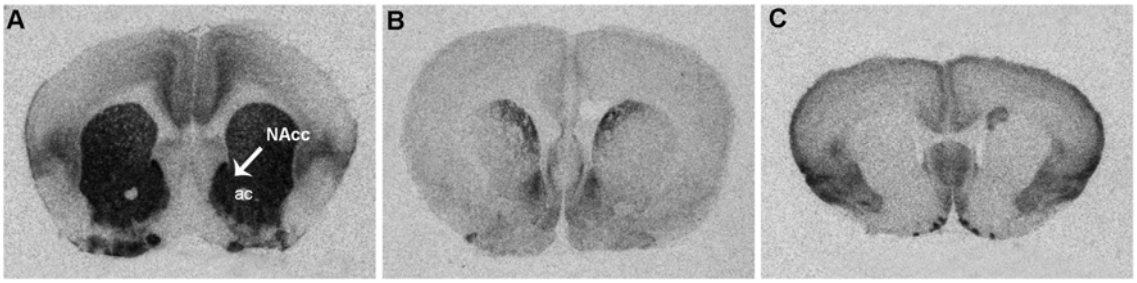

Figure 1.5. Oxytocin receptor (OTR) in species with varying levels of social behavior (from high to low, respectively). Autoradiography for OTR in the nucleus accumbens (NAcc) showing A) high density in the prairie vole, B) low levels in the rat, and C) no expression in the mouse. ac = anterior commisure.

1.15 CONCLUSIONS

Historically, the neurohypophyseal OT system has served as a model of neurosecretion. Decades of research have demonstrated the role of this neuropeptide in the regulation of reproductive physiology, including parturition and lactation. OT is an excellent example of how the same hormone can coordinate peripheral physiology with behavior as can be seen in its role in initiation of maternal nurturing behavior and mother-infant bonding following parturition. More detailed studies have now revealed that OT also plays a role in focusing the brain's attention to social stimuli in both males and females. Evolutionarily, the behavioral effects of OT are shaped by the plasticity in the neural expression of OTR. At least in prairie voles, this maternal behavioral circuitry has been co-opted to promote social bond formation between mates. Elegant human pharmacological studies are revealing an intriguing constellation of effects of this neuropeptide on social information processing and interpersonal relationships. Despite these discoveries, major gaps remain in the presynaptic side of OT function. Further study is needed to examine the relationship between peripheral and central release within the structures where OT modulates behavior, so that we may gain a more complete understanding of the circuitry regulating the social brain.

1.16 DISSERTATION GOALS

This dissertation will begin to fill in gaps in the understanding of oxytocin circuitry and how it is involved in affiliative behaviors in female prairie voles. In chapter 2, I investigate the hypothesis that differences in OTR density in the NAcc directly contributes to individual differences in affiliative behaviors. By using an adeno-

associated viral vector carrying the prairie vole OTR gene, the levels of OTR in the NAcc of adult female prairie voles was increased. These females were then tested for alloparental and partner preference behaviors. Accumbal OTR was also increased in female meadow voles to determine if OTR in this reward area can influence affiliative behavior in a non-monagoumous species. In chapter 3, we characterized the oxytocin system that influences these affiliative behaviors by using retrograde tract tracing, immunohistochemistry, and electron microscopy. I conclude this thesis by discussing how the central OT system may be organized to promote the coordination of peripheral physiology and behavioral changes associated with reproduction.

CHAPTER 2

Variation in Oxytocin Receptor Density in the Nucleus Accumbens has Differential Effects on Affiliative Behaviors in Monogamous and Polygamous Voles

This chapter presents work published as: Ross HE, Freeman SM, Spiegel LL, Ren X, Terwilliger E, and LJ Young. 2009. Variation in oxytocin receptor density in the nucleus accumbens has differential effects on affiliative behaviors in monogamous and polygamous voles. J Neurosci. Feb 4;29(5):3112-3119.

2.1 ABSTRACT

Oxytocin receptors in the nucleus accumbens have been implicated in the regulation of alloparental behavior and pair bond formation in the socially monogamous prairie vole. Oxytocin receptor density in the nucleus accumbens is positively correlated with alloparenting in juvenile and adult female prairie voles, and oxytocin receptor antagonist infused into the nucleus accumbens blocks this behavior. Furthermore, prairie voles have higher densities of oxytocin receptors in the accumbens than non-monogamous rodent species, and blocking accumbal oxytocin receptors prevents mating-induced partner preference formation. Here we used adeno-associated viral vector gene transfer to examine the functional relationship between accumbal oxytocin receptor density and social behavior in prairie and meadow voles. Adult female prairie voles that over-express oxytocin receptor in the nucleus accumbens displayed accelerated partner preference formation after cohabitation with a male, but did not display enhanced alloparental behavior. However, partner preference was not facilitated in non-monogamous meadow voles by introducing oxytocin receptor into the nucleus accumbens. These data confirm a role for oxytocin receptor in the accumbens in the regulation of partner preferences in female prairie voles, and suggest that oxytocin receptor expression in the accumbens is not sufficient to promote partner preferences in non-monogamous species. These data are the first to demonstrate a direct relationship between oxytocin receptor density in the nucleus accumbens and variation in social attachment behaviors. Thus, individual variation in oxytocin receptor expression in the striatum may contribute to natural diversity in social behaviors.

2.2 **INTRODUCTION**

Microtine rodents display a remarkable diversity in social behaviors, ranging from highly affiliative and socially monogamous, to relatively asocial and promiscuous mating strategies (Gruderadams and Getz 1985). The socially monogamous prairie vole (*Microtus ochrogaster*) forms enduring social attachments, or pair bonds, to an opposite-sex partner following cohabitation and mating, while non-monogamous meadow voles (*Microtus pennsylvanicus*) typically do not. Adult sexually naïve female prairie voles display considerable diversity in their spontaneous nurturing behavior, or alloparental behavior, with approximately half showing maternal-like behavior toward pups while the remainder either ignore or attack pups (Lonstein and De Vries 1999; Bales and Carter 2003; Olazabal and Young 2005) . Thus voles provide an excellent opportunity to examine the neurobiological mechanisms underlying social attachment and alloparental care, as well as the mechanisms leading to diversity in these behaviors both across species and between individuals.

The nonapeptide oxytocin (OT) has been implicated in the regulation of both partner preference formation and alloparental behavior in prairie voles. Infusion of an oxytocin receptor (OTR) antagonist into the nucleus accumbens (NAcc), but not into the adjacent caudate putamen blocks mating-induced partner preference formation, a laboratory proxy of pair bond formation (Young, Lim et al. 2001). Similar infusions of OTR antagonist into the NAcc also block alloparental behavior in virgin females (Olazabal and Young 2006). Thus activation of OTRs in the NAcc facilitates both partner preference formation and alloparental behavior in female prairie voles.

Variation in OTR density in the NAcc has been hypothesized to contribute to species differences in social organization and alloparental behavior. Prairie voles have high densities of OTR in the NAcc, while non-monogamous meadow voles, mice and rats do not (Insel and Shapiro 1992; Olazabal and Young 2006). Prairie voles also display higher levels of alloparental behavior than do meadow voles, mice or rats (Olazabal and Young 2006). Paralleling this inter-species relationship between OTR density in the NAcc and parental behavior, OTR density in the NAcc is positively correlated with alloparental behavior in both juvenile and adult female prairie voles (Olazabal and Young 2006; Olazabal and Young 2006). In this study, we used adeno-associated viral vector (AAV) gene transfer to directly test the hypothesis that variation in NAcc OTR density can contribute to variation in social attachment and alloparental behavior. Adult female prairie voles were bilaterally infused into the NAcc with an AAV encoding the prairie vole OTR gene, resulting in a significant elevation in OTR binding. Animals were then tested for alloparental behavior and partner preference formation. We then tested the hypothesis that OTR expression in the NAcc was sufficient to facilitate partner preference formation by infusing female meadow voles with the same vector. We predicted that compared to controls, OTR over-expressing female prairie voles would show increased alloparental behavior and accelerated partner preference formation. Furthermore, we predicted that meadow vole females expressing OTR in the NAcc would develop partner preferences toward male partners.

2.3 MATERIALS AND METHODS

Animals: Prairie and meadow voles were housed in same sex groups with 2-3 voles/cage from the time of weaning at 21-23 days of age. Housing consisted of a ventilated

36x18x19cm Plexiglass cage filled with Bed-ocobbs Laboratory Animal Bedding under a 14:10 hr light/dark cycle at 22ºC with access to food (rabbit LabDiet, Richmond, IN) and water ad libitum. The prairie voles were obtained from our laboratory breeding colony that originally derived from field-captured voles in Illinois. Meadow voles were derived from stock obtained from a breeding colony at Florida State University. Subjects were 2-5 month old intact sexually naïve female voles. Stimulus animals were sexually experienced adult male voles. Each male served as a "partner" and a "stranger" during the partner preference test (see below). Littermates were assigned to different treatment groups to control for variability within litters and within cages. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Adeno-Associated Viral Vectors: The OTR coding sequence was created by splicing the first exon of a prairie vole OTR genomic clone encoding the first five transmembrane domains (Genbank accession number AF079980) and the 3' end of the OTR amplified from prairie vole uterus cDNA. The coding sequence was re-framed using PCR to eliminate the UTRs and provide new flanking restriction sites to facilitate cloning into the AAV vector plasmid. The modified gene was then cloned into an AAV2 vector plasmid between a 0.6 kb cytomegalovirus (CMV) promoter and a SV40 DNA fragment containing the SV40 small t intron and polyA signal. The AAV2-OTR was cross-packaged in AAV9 by a triple plasmid transfection into AAV-293 cells (Stratagene, La Jolla, CA) using a standard calcium phosphate precipitation method. No helper virus was employed. An AAV2-eGFP plasmid was packaged in parallel in AAV9 as a negative control vector. Briefly, each AAV vector plasmid, an AAV2/9 rep/cap plasmid providing AAV2 replicase and AAV9 capsid functions, and a 3rd plasmid encoding Adenovirus

helper functions, pHelper (Stratagene), were co-transfected into 293 cells at a molar ratio of 1:1:1. Cells were harvested 48 hrs post-transfection. The cell pellets were then re-suspended in DMEM, and the intracellular virus particles released by three consecutive rounds of freeze-thaw, followed by centrifugation at 13,000 rpm for 10 min on a table-top centrifuge to remove particulates. The vector stocks were stored at –80 C, and titered by real-time PCR using an ABI Prism 7700 Sequence Detection System from Perkin-Elmer Applied Biosystems (Foster City, CA). Titers were on the order of 10^{12} DRP/ml. (DRP = Dnase Resistant Particles).

Viral Vector Infusion: Stereotaxic infusions were performed under isoflurane anesthesia in a Kopf stereotax fitted with an Ultra Micro Pump II (World Precision Instruments, Sarasota, FL) and a 26-gauge Hamilton syringe. Females were injected bilaterally into the shell of the NAcc (prairie voles: AP +1.7mm, ML .9mm, DV -4.5mm, meadow voles: AP +1.6mm, ML .9mm, DV -4.3mm) with 750nl of either an AAV containing the vole oxytocin receptor (CMV-OTR, N=12), or a control eGFP expressing vector (CMV-GFP, N=16). Virus was infused at a rate of 93.8nl/min. The syringe was left in place for 5 min following infusion to minimize diffusion of vector up the needle track. Sham operated animals (N =9) were anesthetized and had their scalps incised and sutured. Following surgery, animals were group housed until time of partner preference behavior testing. Preliminary studies indicated that OTR expression at the site of injection was stable after 10 days.

Alloparental Behavior Testing: One month after injection, the prairie voles were tested for alloparental behavior. Testing occurred between 0800hr and 1800hr. Test animals were placed in a large clean cage (45.5x24x20cm) and allowed to acclimate for 15min.

Two pups (2-5 days of age) were placed on one end of the cage. The latency to approach the pups, number of animals that attacked pups, and the amount of time spent grooming, hovering, and retrieving the pups was recorded. A latency of 900 seconds was assigned to animals that did not approach the pups during the 15 minute test for the purpose of statistical analysis. Testing was immediately stopped if the female attacked the pups. Animals were categorized as alloparental if they spent >30 sec licking the pups without attacking. Based on the results from the experiment with prairie voles, meadow voles were not tested for alloparental behavior.

Partner Preference Testing (Prairie Vole): One month following the alloparental behavior testing, and two months following AAV infusion, females were given 4 μg of estradiol benzoate (EB; Fisher, Pittsburgh, PA) dissolved in 0.1ml of sesame oil IP daily for 3 days prior to mating to induce sexual receptivity. 16 hours following the last EB injection animals were placed in a clean cage (28x17x12cm) with a sexually experienced adult male for 6 hours and then returned to group housing. Mating behavior was recorded during the initial 6 hour co-habitation. The latency to first intromission and the number of mating bouts in the first hour were scored. A latency of 3600 seconds was assigned to animals that did not mate during the 1 hour period for the purpose of statistical analysis. The following morning (14 hrs after the cohabitation), the animals were tested for partner preference. In a partner preference test, the experimental female is placed in a neutral center chamber of a three-chambered apparatus, in which the partner male is tethered in one side chamber and a novel "stranger" male is tethered in the other (Williams, Catania et al. 1992). The experimental animal is free to move throughout the chambers and the

time spent in close proximity to each male is recorded using an automated beam break system (Curtis and Wang 2005; Curtis and Wang 2005; Lim, Liu et al. 2007).

A day later, the females were re-paired with the same partner for an additional 12 hours of cohabitation. The females were then tested again for a partner preference (18 hour total contact time). This two stage paradigm was used to maximize our detection of a facilitation of partner preference test, since there is variability in the threshold time needed to form a partner preference. Animals were considered to have a partner preference if they spent twice as much time in close proximity with the partner compared to with the stranger.

Partner Preference Testing (Meadow vole): Typically meadow voles from our colony will not form a pair bond after 24hrs of mate exposure. Therefore for this experiment, partner preference tests were performed after a cohabitation of 24 and an additional 48 hrs (72 hrs total). All other treatment and testing methods are the same as above.

Tissue collection and processing: Following the behavioral experiments animals were decapitated following deep anesthetization with isofluorane. Brains were then collected and frozen on powdered dry ice. The brains were sectioned through the NAcc in 6 series at 20μm, on a cryostat, onto Fisher Frost-plus slides. Slides were stored at -80C until used in autoradiography.

OTR Autoradiography: OTR receptor autoradiography was used to assess OTR binding in AAV injected animals. Autoradiography was performed as described previously with slight modifications (Insel, Gelhard et al. 1991; Wang and Young 1997). Sections were removed from -80C storage, allowed to air, dipped in 0.1% paraformaldehyde in

phosphate-buffered saline (pH 7.4), and rinsed twice in 50 mM Tris buffer (pH 7.4) to remove endogenous OT. Next the tissue was incubated in 50 pM ^{125}I-OVTA (NEX 254050UC PerkinElmer, Waltham, MA) for one hour. Unbound radioligand was removed by four washes in 50mM Tris plus 2% $MgCl_2$ (pH 7.4) and then dipped into dH_20 and air dried under a stream of cool air. Once dry, the slides were exposed to BioMax MR film (Kodak, Rochester, NY) for 72 hours. Two CMV-OTR and one CMV-GFP animals were excluded from the behavioral analysis due to injection misses.

GFP Immunohistochemistry: A subset of the animals (N=5) injected with CMV-GFP, was perfused transcardially with 50 ml of PBS, followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer containing 2.5% acrolein (Polysciences, Warrington, PA). Immediately following perfusion, the brains were removed and stored at 4°C in 30% sucrose solution until sectioning. The brains were cut into 25µm coronal sections with a freezing microtome and stored free-floating in cryoprotectant solution at -20°C until immunohistochemical processing.

A 1:6 series through the rostrocaudal axis of each brain was processed for GFP. Briefly, sections were removed from the cryoprotectant solution, rinsed extensively in potassium phosphate-buffered saline (pH 7.4), and then reacted for 15 minutes in 1% sodium borohydride to remove excess aldehydes. Sections were then incubated in primary antibody solution directed against GFP in potassium phosphate-buffered saline (KPBS) containing 0.1% Triton-X for 1 hour at room temperature followed by 48 hours at 4°C. Cells containing GFP were identified by using a polyclonal rabbit anti-GFP antibody (Cat. No. A6455, Invitrogen, Carlsbad, CA) at a concentration of 1:100,000. After primary antibody incubation, the tissue was rinsed in KPBS, incubated for 1 hour in

biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) at a concentration of 1:600, and rinsed in KPBS, followed by a 1-hour incubation in avidin-biotin peroxidase complex (ABC Elite Kit PK-6100 Vector, Burlingame, CA) at a concentration of 1:200. After rinsing in KPBS and Tris buffer (pH 7.2), GFP was visualized as a brown reaction product by using 3,3′-diaminobenzidine containing 0.08% hydrogen peroxide in Tris buffer. The reaction product was terminated after approximately 20 minutes by rinsing in Tris buffer. Sections were mounted out of saline onto gelatin-subbed slides, air-dried, dehydrated in a series of graded alcohols, cleared in Histoclear (National Diagnostics, Atlanta, GA), and coverslipped using Krystalon (EMD Chemicals, Gibbstown, NJ).

Statistical Analysis: Data are presented as mean ± standard error of the mean (SEM). A two way RM ANOVA was run using time spent with each stimulus animal as the dependent variable, the within-subjects factor being partner or stranger, and treatment group as the between subject factor. The Holm-Sidak Test was used for post hoc pairwise comparisons when a significant interaction effect was detected. One way ANOVAs were run on alloparental and mating behaviors for prairie voles. Those behaviors that did not meet the criteria for normality were analyzed using the Kruskal-Wallis One Way Analysis of Variance on Ranks. A Fisher's Exact Test was used to determine group differences in the proportion of animals displaying alloparental behavior. Meadow vole mating behavior was analyzed using a t-Test and Mann-Whitney Rank Sum Test when the normality test failed.

2.4 RESULTS

Alloparental Behavior in Prairie Voles: The proportion of CMV-OTR injected female prairie voles displaying alloparental behavior (3/9) did not differ from the control (4/15) or sham voles (3/10) ($p > 0.5$; Figure 2.1A). Two CMV-OTR, four CMV-GFP control, and two sham females, which were categorized as non-alloparental, attacked the pups. The latency to approach pups (Figure 2.1B) and time spent licking/grooming also did not differ significantly between the groups ($H= 0.31$, $P > 0.8$ for latency, $H= 0.40$, $P > 0.8$ for grooming). When only the animals that reached the criteria of being alloparental were compared, the latency to approach pups for CMV-OTR females (140.2±65.2 seconds) was not different than CMV-GFP (62.5±39.3 seconds) or sham (169.4±51.7 seconds) females ($F(2,7)= 1.27$, $p > 0.3$). There was also no difference in the amount of time that the alloparental CMV-OTR females spent licking pups compared to the alloparental CMV-GFP or sham females ($F(2,7)= 1.94$, $p > 0.2$; Figure 2.1C). The total amount of time each group spent licking/grooming, hovering, and carrying the pups was also not different between the alloparental CMV-OTR (695.2±77.1 seconds) CMV-GFP (387.5±132.7 seconds) or sham (503.8±82.8 seconds) animals ($F(2,7)= 1.98$, $p > 0.2$).

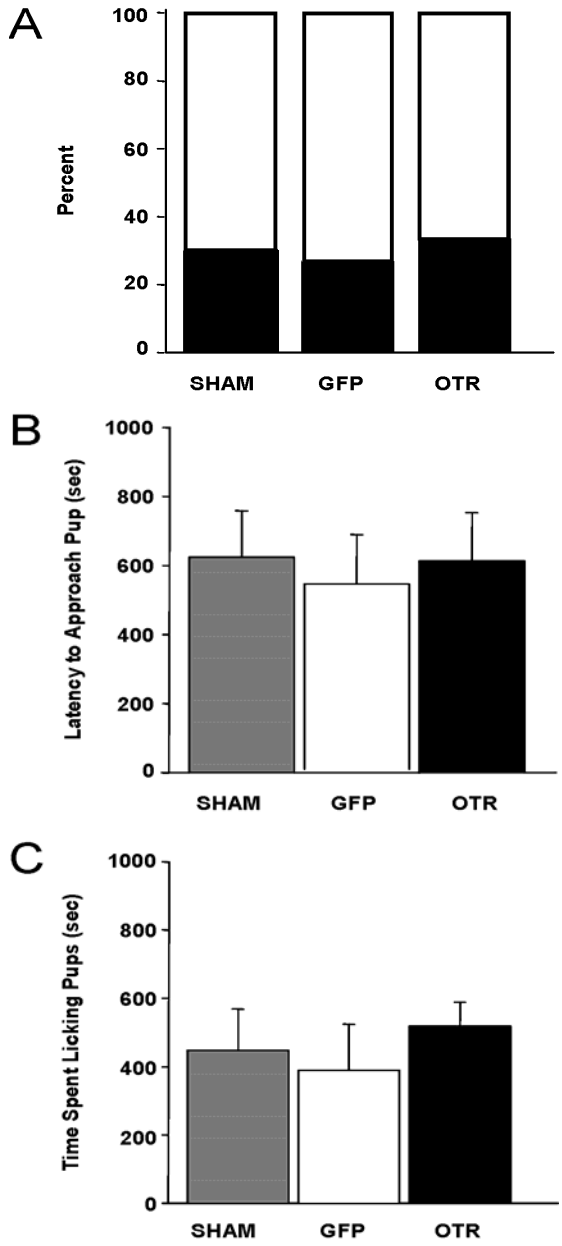

Figure 2.1. Alloparental behavior in sham, CMV-GFP and CMV-OTR female prairie voles. A) There was no effect of treatment on the proportion of females in each treatment group that displayed alloparental behavior. The dark bars represent the percent of animals that were categorized as alloparental, while the light bars depict the number of females that attacked or ignored the pups. B) The latency to approach pups was not different between groups. C) There was no difference between groups in the amount of time the females spent licking pups, when only the females in each group that displayed alloparental behavior towards the pups were compared. Data are presented as mean ± SEM.

Mating Behavior in Prairie Voles: Mating behavior was not significantly affected by the females' prior surgical treatment. The latency to first intromission was not significantly different in males paired with CMV-OTR females compared to males mated to CMV-GFP or sham females ($H= 5.043$, $p = .08$; Figure 2.2A). Although the CMV-OTR group tended to mate sooner than the other groups, they did not mate more often. The number of mating bouts in the first hour did not differ in pairs containing CMV-OTR females from pairs containing CMV-GFP or sham females ($F(2,31) = 0.46$, $p > 0.6$; Figure 2.2B).

Partner Preference Behavior in Prairie Voles: After the 6 hr cohabitation period, none of the groups displayed a significant partner preference (Figure 2.2C). There was no main effect of treatment ($F(2,31) = 0.56$, $p > 0.5$) or time spent with the partner versus the stranger ($F(1,31)= 0.46$, $p > 0.5$). After an additional 12 hours of cohabitation, there was no main effect of treatment ($F(2,29) =.78$, $p = 0.5$) or the amount of time spent with the partner versus stranger ($F(1,29)= 3.71$, $p = 0.06$). However, there was a significant interaction effect ($F(2,29)=5.56$, $p = 0.009$). The post-hoc test revealed that CMV-OTR females spent significantly more time in close proximity to the partner than to the stranger ($p < 0.001$; Figure 2.2D). However, the CMV-GFP and sham females failed to show a partner preference after this period of time ($p > 0.05$; Figure 2.2D). In addition, 80% of CMV-OTR injected voles reached the criteria of having a partner preference, i.e. spending twice as much time with the partner than the stranger, compared to only 31% of CMV-GFP injected and 44% of sham animals (Figure 2.2E,F).

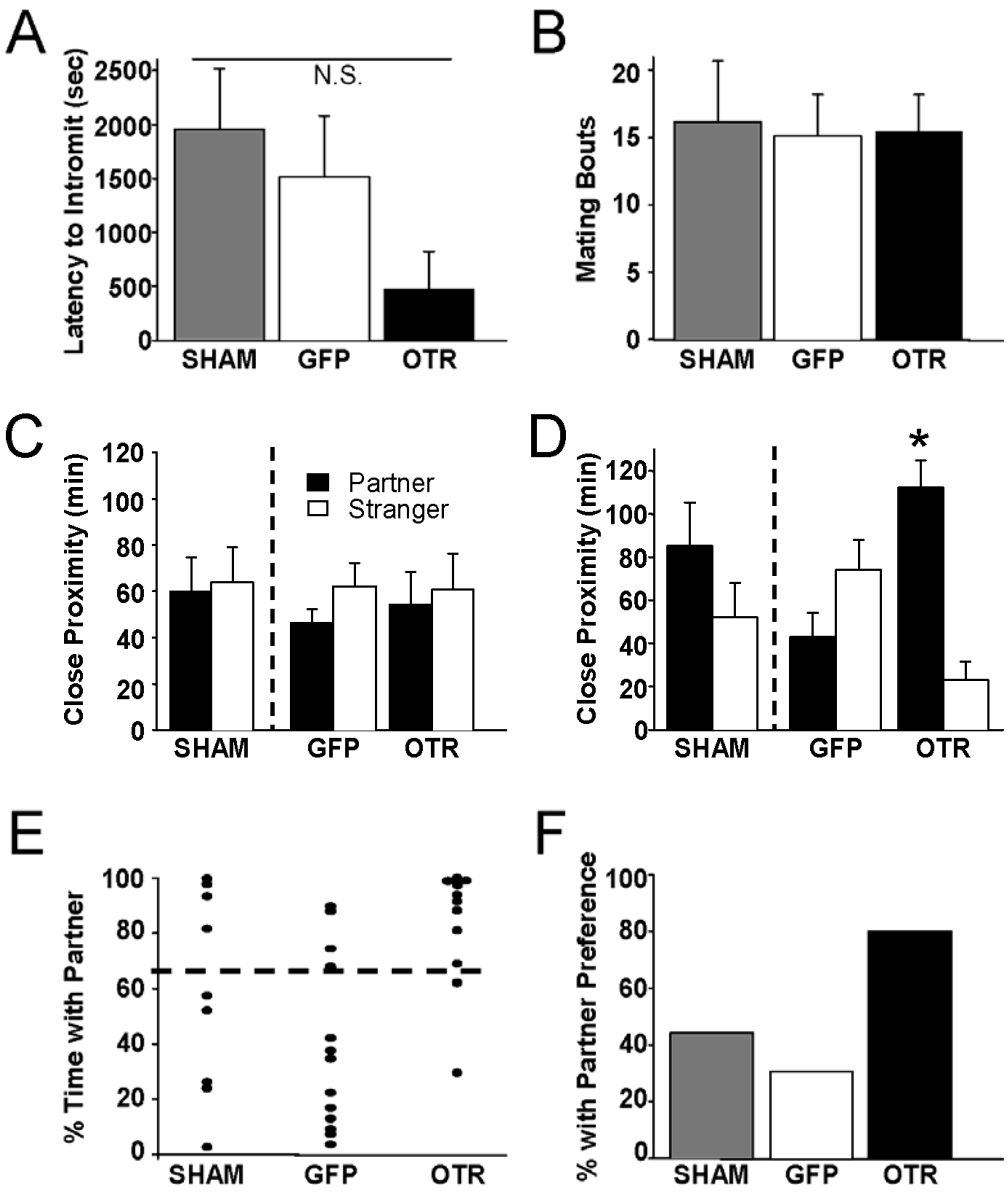

Figure 2.2. Mating and partner preference behavior in sham, CMV-GFP, and CMV-OTR female prairie voles. The latency to first intromission (A) and the number of mating bouts (B) were not significantly different between the groups. C) After a 6 hour cohabitation period, none of the groups displayed a significant preference for the partner over the stranger. D) After a cumulative 18 hour cohabitation, CMV-OTR injected females spent significantly more time with the partner than the stranger ($p<.001$). The sham and CMV-GFP injected females did not spend significantly more time with either male at either time point. E) Scatter plot illustrating the ratio of the time spent with partner/stranger for each individual in the groups. Females spending greater than 67% of their total social contact time with the partner (above the dashed line) are considered to have displayed a partner preference. F) The overall percentage of animals in each group that displayed a partner preference. Data are presented as mean ± SEM.

OTR and GFP Expression in the NAcc of Prairie Voles: Autoradiography was done to determine placement of AAV injection and to verify that the CMV-OTR vector resulted in increased OTR binding compared to controls. As previously reported, there was significant individual variation in OTR binding in the NAcc of control CMV-GFP prairie voles (Figure 2.3A,B). Distinct elevations in OTR binding were detected in the NAcc of CMV-OTR injected prairie voles (Figure 2.3C). In addition, GFP immunohistochemistry was performed on brain sections of CMV-GFP animals to determine the extent of expression in neurons of the NAcc. Clear labeling of cells with neuronal characteristics was detected, confirming that the CMV AAV vectors drove expression in neurons (Figure 2.3E,F).

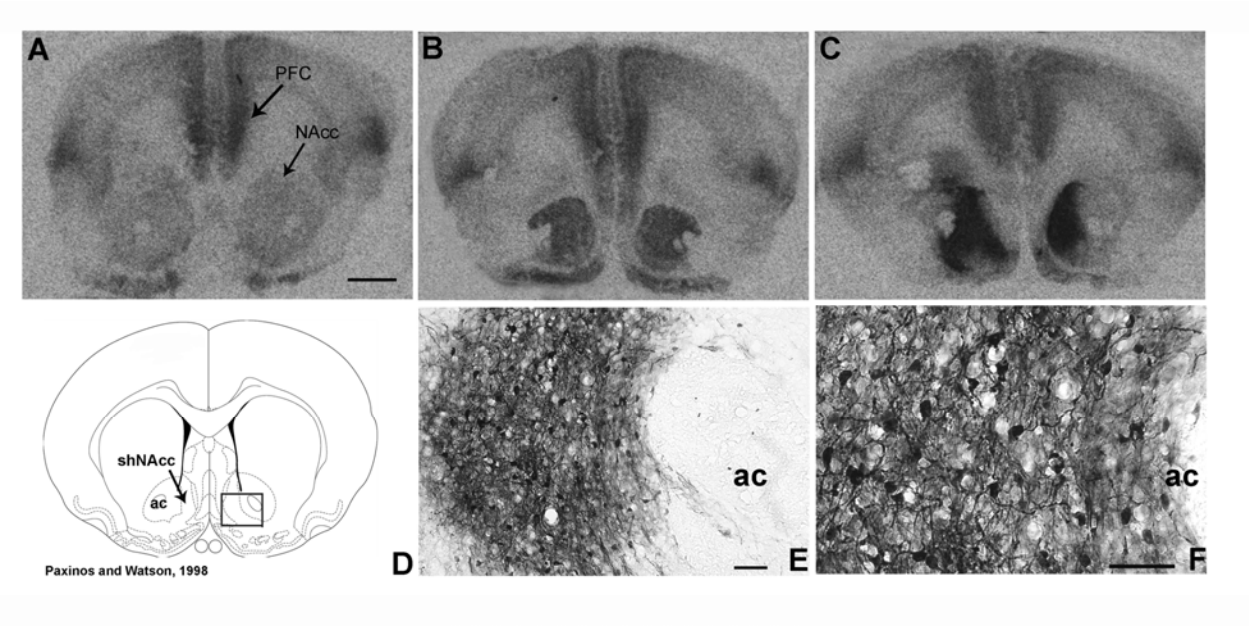

Figure 2.3. Analysis of OTR and GFP expression in CMV-OTR and CMV-GFP female prairie voles. OTR density was determined using receptor autoradiography. A, B) There was significant variation in the density of OTR binding in the NAcc of CMV-GFP females. This is in contrast to the prefrontal cortex (PFC), where there is little individual variation in OTR binding density. Bar = 1mm. C) CMV-OTR injected females had elevated OTR binding in the NAcc relative to controls. D) The dark square surrounding the NAcc depicts the position of the photomicrograph in E, shNAcc = shell of the NAcc, ac = anterior commissure. E) Photomicrograph taken using a 20x objective of GFP-immunoreactivity in the shell of the NAcc of a CMV-GFP female. Immunoreactivity was distributed widely in soma as well as fiber processes. F) Higher magnification photomicrograph of the image in E illustrating the clear neuronal characteristics of the GFP-immunoreactive cells. Bars in E and F = 50 μm.

Mating Behavior in Meadow Voles: No significant difference in mating behavior was observed between the treatment groups in meadow voles. The latency to first intromission was not different in males paired with CMV-OTR (1245.1±1465.9 seconds) females compared to males mated to control CMV-GFP (566.6±989.8 seconds) females ($T= 128.0$, $p > 0.2$). The number of mating bouts in the first hour also did not differ in pairs containing CMV-OTR (6.8±5.1) females from pairs containing CMV-GFP (9.5±4.4) females ($t= 1.42$, $p > 0.1$).

Partner Preference in Meadow Voles: After a 24hr cohabitation period, none of the groups displayed a significant partner preference (Figure 2.4A). There was no main effect of treatment ($F(1,20) = 0.19$, $p > 0.6$) or time spent with partner versus stranger ($F(1,20) = 0.09$, $p > 0.7$). After an additional 48 hours of cohabitation, there was again no significant main effect of treatment ($F(1,19)=.65$, $p > 0.4$) or time spent with either female ($F(1,19) = 0.62$, $p>.4$; Figure 2.4B).

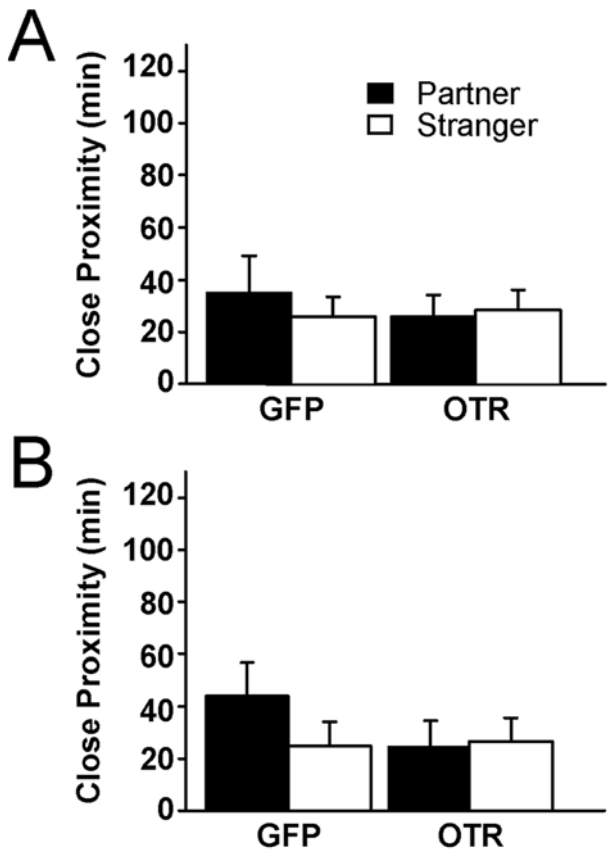

Figure 2.4. Partner preference behavior of female meadow voles after a 24 hr co-habitation (A) and after a culmulative 72 hour co-habitation (B). CMV-OTR and CMV-GFP injected females did not show a partner preference. They spent equal amounts of time with the partner and the stranger at both time points. Data are presented as mean ± SEM.

OTR Expression in the NAcc of Meadow Voles: CMV-GFP injected meadow voles had little or no OTR binding in the NAcc (Figure 2.5A). In contrast, CMV-OTR injected meadow voles had significant OTR binding in the NAcc, comparable to that of the prairie voles (Figure 2.5B).

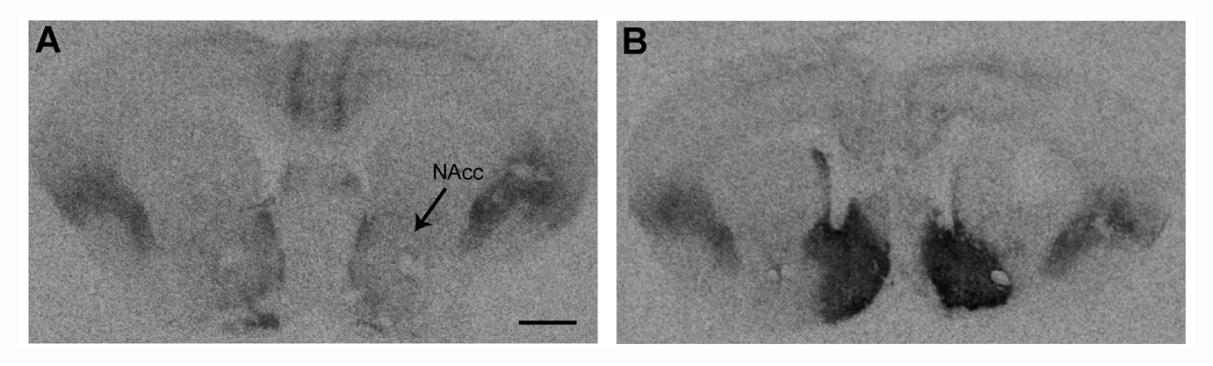

Figure **2.5. Receptor autoradiography illustrating OTR binding density in the NAcc** of CMV-GFP (A) and CMV-OTR (B) meadow voles. Note that CMV-GFP female meadow voles had little or no OTR binding in the NAcc. However, OTR binding in the NAcc was dramatically elevated in CMV-OTR female meadow voles. Bar = 1mm.

2.5 DISCUSSION

Species differences in OTR density in the NAcc have been associated with species differences in mating strategy (social monogamy vs polygamy) and alloparental behavior, suggesting that variation in OTR receptor expression may underlie species differences in social organization and behavior (Insel, Gelhard et al. 1991; Lim, Murphy et al. 2004; Young and Wang 2004). Socially monogamous prairie voles have higher densities of OTR in the NAcc than non-monogamous meadow and montane voles, and pharmacological blockade of those receptors prevents mating-induced partner preference formation (Young, Lim et al. 2001; Olazabal and Young 2006). There is also significant individual variation in OTR density in the NAcc within prairie voles (Figure 3A,B). OTR density in this region is positively correlated with individual variation in alloparental behavior of adult sexually naïve female prairie voles (Young, 1999; Olazabal 2006) and OTR antagonist administration into the NAcc eliminates this behavior (Olazabal and Young 2006). Therefore, in this study we sought to directly test the relationship between receptor density in the NAcc and affiliative behavior in voles, by using AAV gene transfer.

Our results show that, as predicted, female prairie voles with elevated levels of OTR in the NAcc display accelerated partner preference formation compared to females with lower OTR density (CMV-GFP or shams). Contrary to our prediction, there was no difference in alloparental behavior between groups, suggesting differential mechanisms by which accumbal OTR regulates partner preference formation and alloparental behavior. Our results support the hypothesis that individual differences in OTR expression contribute to intra-species variation in some aspects of affiliative behavior.

However, increasing OTR expression in the NAcc was not sufficient to induce partner preference formation in meadow voles, even after 72 hours of cohabitation, suggesting that the species differences in accumbal OTR alone are not sufficient to explain the species differences in the ability to form partner preferences. It is important to note that there were no group differences in mating behavior in any of parameters examined. Therefore, the enhanced partner preference in the experimental prairie voles cannot be attributed to increased sexual activity during the initial cohabitation period.

Adult female prairie voles show remarkable individual variation in their display of alloparental care. About 50% of sexually naïve females will spontaneously retrieve, lick/groom, and hover over pups presented to them (Lonstein and De Vries 1999; Bales and Carter 2003; Olazabal and Young 2005). There is also significant individual variation in OTR density in the NAcc among prairie voles (Young 1999). Females with higher densities of OTR in the NAcc are more likely to display alloparental responsiveness than animals with lower OTR in this area. Thus it was surprising that elevating OTR density in the NAcc of prairie voles did not enhance alloparental behavior. The fact that these animals did show accelerated partner preference formation suggest that the OTR derived from the viral vector transgene was functionally coupled to signal transduction pathways in the NAcc.

We hypothesize that individual variation in OTR activation in the NAcc during development may play a more important role in producing intra-species variation in alloparental behavior. The positive correlation in OTR density in the NAcc and alloparental behavior has been shown in juvenile prairie voles as well as adults (Olazabal and Young 2006; Olazabal and Young 2006). Alloparental behavior in prairie voles

appears to be particularly sensitive to perturbations during development. For example, perinatal exposure to OT alters alloparental behavior in female prairie voles (Bales, van Westerhuyzen et al. 2007). Therefore, individuals with higher densities of OTR during development may experience increased OTR signaling in the NAcc, resulting in long-lasting neurochemical changes that increase the probability of displaying alloparental behavior as they become adults. If this hypothesis is correct, we would predict that increasing OTR expression in the NAcc neonatally would increase the frequency of alloparental behavior.

Another explanation for the failure of enhancing OTR expression in the NAcc to increase alloparental behavior is that variation in OTR expression in multiple brain regions may be necessary to produce the expected diversity in behavior. For example, OTR expression in the lateral septum is negatively correlated with alloparental behavior (Olazabal and Young 2006). Finally, it is also possible that variation in hormone exposure or social experience influences OTR density in the NAcc in addition to altering alloparental behavior. For example, the presence or absence of the father during development or perinatal manipulation of steroid hormones can influence the display of alloparental responsiveness in female prairie voles (Roberts, Zullo et al. 1996; Roberts, Williams et al. 1998; Lonstein and De Vries 2000).

In male prairie voles, vasopressin plays a critical role in the display of paternal behavior as well as partner preference formation, paralleling the role of oxytocin in females (Winslow, Hastings et al. 1993; Wang, Ferris et al. 1994). Site-specific pharmacological studies demonstrate that V1aR in the ventral pallidum, a major output of the NAcc, are critical for pair bond formation (Lim and Young 2004). Increasing V1aR in the ventral

pallidum using viral vector mediated gene transfer accelerates partner preference formation of male prairie voles (Pitkow, Sharer et al. 2001), a finding that parallels the current study. Although over-expressing V1aR in the ventral pallidum of male meadow voles was not sufficient to induce paternal behavior, it did promote partner preference (Lim, Wang et al. 2004); in contrast, in the present study overexpression of OTR in the NAcc did not stimulate partner preference formation in female meadow voles.

There are several potential explanations for the failure of female meadow voles with elevated OTR in the NAcc to form partner preferences. First, species differences in OT release within the NAcc during mating may differ between female prairie voles and meadow voles. Although OTR localization differs between the species, both prairie voles and meadow voles have OT-immunoreactive fibers in the NAcc (Ross and Young, unpublished data). *In vivo* microdialysis experiments have shown that mating stimulates OT release in the NAcc of female prairie voles, but parallel studies have not been performed in meadow voles (C.D. Cole and Young, unpublished data). It is possible that infusion of OT into CMV-OTR treated animals would facilitate partner preference formation in meadow voles. However, it should be noted that in rats and sheep, vaginocervical stimulation increases central OT release (Kendrick, Keverne et al. 1986; Sansone, Gerdes et al. 2002). Therefore it is likely that meadow voles are already experiencing a rise in accumbal OT with mating. Another explanation for the failure of NAcc OTR expression to stimulate partner preference formation in female meadow voles is that species differences in OTR expression in other brain regions are also necessary for pair bonding. For example, OTR density is also higher in the prefrontal cortex and lateral amygdala in prairie voles compared to nonmonogamous vole species (Insel, Gelhard et

al. 1991; Young, Huot et al. 1996; Smeltzer, Curtis et al. 2006). Therefore, elevating OTR expression in multiple sites may be necessary for stimulating mating-induced partner preferences in meadow voles. Finally, it is possible that multiple neurochemical differences between the species are responsible for species differences in behavior (e.g. dopamine, corticotropin releasing factor)(Gingrich, Liu et al. 2000; Liu and Wang 2003; Smeltzer, Curtis et al. 2006; Lim, Liu et al. 2007).

There are several important caveats of our experimental approach that warrant discussion. First, the CMV-OTR likely increased OTR expression in all cell types in the injected area, including neuronal populations that normally do not respond to OT. However, it is possible that the transgenic OTR resulted in greater signaling in neurons that normally express endogenous OTR because of enhanced sensitization to OT released onto those neurons, or the presence of the appropriate downstream signaling molecules. Secondly, the area of transgene expression produced by viral vector infusion was not uniformly expressed over the entire rostral-caudal or medio-lateral extent of the NAcc. In the CMV-OTR females, OTR expression was consistently elevated in the shell and adjacent core regions of the NAcc. However, it should be noted that shell of the NAcc has been most implicated in the regulation of partner preference formation and OTR density in this same region is more highly correlated with alloparental behavior than the core region.

This is the first study to demonstrate conclusively that variation in OTR expression in the brain can contribute to variation in social behavior. OT has been widely implicated in the regulation of several behaviors, including social information processing and memory, mate choice, maternal nurturing and attachment (Dantzer, Bluthe et al. 1987; Kendrick 2000; Ferguson, Aldag et al. 2001; Kavaliers, Colwell et al. 2003). There is now clear

evidence that OT modulates human social cognition as well, including interpersonal trust, eye gaze, facial memory, and emotion perception (Kosfeld, Heinrichs et al. 2005; Domes, Heinrichs et al. 2007; Guastella, Mitchell et al. 2008; Savaskan, Ehrhardt et al. 2008). OT administration increases the retention of social cognition in a voice intonation task in autistic subjects (Hollander, Bartz et al. 2007), and several genetic studies have reported modest associations between non-coding polymorphisms of the OTR gene and autism spectrum disorder (Wu, Jia et al. 2005; Jacob, Brune et al. 2007; Lerer, Levi et al. 2007; Yrigollen, Han et al. 2008). There is limited information on the distribution of OTR, and nothing is known regarding individual variation in OTR density in the human brain. Our results suggest that variation in OTR density in specific brain regions may contribute to individual differences in social cognitive function in humans.

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CHAPTER 3

Characterization of the Oxytocin System Regulating Affiliative Behavior in Female Prairie Voles

This chapter presents work to be published as: Ross HE, Cole C, Smith Y, Neumann I, Landgraf R, and LJ Young. Characterization of the oxytocin system that regulates affiliative behavior in female prairie voles. Neurosci, Submitted. Charlene Cole performed and analyzed the microdialysis work included in this paper (Figure 3.1.).

3.1 ABSTRACT

Oxytocin regulates partner preference formation and alloparental behavior in the socially monogamous prairie vole (*Microtus ochrogaster*) by activating oxytocin receptors in the nucleus accumbens of females. Mating facilitates partner preference formation, and oxytocin-immunoreactive fibers in the nucleus accumbens have been described in prairie voles. However, there has been no direct evidence of oxytocin release in the nucleus accumbens as a function of mating, and the origin of the oxytocin fibers is unknown. Here we show for the first time that extracellular concentrations of oxytocin are increased in the nucleus accumbens of female prairie vole during mating. We further show that the distribution of oxytocin-immunoreactive fibers in the nucleus accumbens is conserved in prairie voles, mice and rats, despite remarkable species differences in oxytocin receptor expression in the region. Using a combination of site-specific and peripheral infusions of the retrograde tracer, Fluorogold, we demonstrate that the nucleus accumbens oxytocin-immunoreactive fibers likely originate from paraventricular and supraoptic hypothalamic neurons. This distribution of retrogradely labeled neurons is consistent with the hypothesis that striatal oxytocin fibers arise from collaterals of magnocellular neurons of the neurohypophysial system. If correct, this may serve to coordinate peripheral and central release of oxytocin with appropriate behavioral responses associated with reproduction, including pair bonding after mating, and maternal responsiveness following parturition and during lactation.

3.2 INTRODUCTION

Oxytocin (OT) released from the neurohypophysial system has been implicated in the regulation of reproductive physiology in mammals, including uterine contractions during parturition and milk ejection during lactation (Burbach, Young et al. 2006). In addition, OT released within the brain coordinates the onset of maternal responsiveness and maternal bonding at the time of parturition (Pedersen and Prange 1979; Kendrick, Keverne et al. 1987). Recent studies in humans have also suggested that central OT modulates social cognition, including increasing interpersonal trust, eye gaze, face recognition, and the ability to infer the emotions of others based on facial cues (Kosfeld, Heinrichs et al. 2005; Domes, Heinrichs et al. 2007; Donaldson and Young 2008; Guastella, Mitchell et al. 2008; Savaskan, Ehrhardt et al. 2008).

Prairie voles (*Microtus ochrogaster*) have become an important animal model for elucidating the behavioral roles of OT and the neurobiology of affiliative behavior (Carter, DeVries et al. 1995; Young and Wang 2004). Prairie voles are a highly affiliative rodent species characterized by a socially monogamous mating strategy and high levels of alloparental care. In the laboratory, the formation of selective pair bonds between mates can be assessed using a partner preference test in which the time spent with the partner versus a novel stimulus animal is quantified. Mating facilitates the formation of partner preferences in female prairie voles (Williams, Carter et al. 1992). Pharmacological and genetic manipulation studies have demonstrated that oxytocin receptors (OTR) in the nucleus accumbens (NAcc) play a significant role in the regulation of behaviors associated with social monogamy and alloparental care. Infusion of an OTR antagonist into the NAcc prevents mating-induced partner preferences in

female prairie voles (Young, Lim et al. 2001). Conversely, increasing OTR density in the NAcc using viral vector gene transfer can accelerate the formation of a partner preference (Ross et al., in press). Furthermore, OTR binding density in the NAcc is positively associated with alloparental behavior, and infusion of an OTR antagonist into the NAcc inhibits spontaneous alloparental behavior in sexually naïve female prairie voles (Domes, Heinrichs et al. 2007). Interestingly, non-monogamous rodent species, including meadow voles, mice and rats have very low levels of OTR binding in the NAcc, which may contribute to the species differences in social behavior (Insel and Young 2001; Burbach, Young et al. 2006).

Despite the evidence that OT signaling in the NAcc plays a critical role in regulating affiliative behavior in prairie voles, the presynaptic OT system in this region has not been characterized. Specifically, mating-induced OT release has not been directly demonstrated, and the morphology and source of the OT-immunoreactive fibers projecting to the NAcc has not been determined. OT is produced primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. Large diameter magnocellular neurons in these regions form the neurohypophysial system and are thought to project primarily to the posterior pituitary (Bargmann 1949). Early tract tracing studies demonstrated that OT-immunoreactive fibers of the brainstem originate from the smaller diameter parvocellular OT neurons in the PVN, leading to the assumption that these neurons also project to the forebrain regions regulating behavior, providing a dissociation between the neurohypophysial and central OT systems (for review see (Landgraf and Neumann 2004).

In this study we examined the NAcc OT system in detail. Using microdialysis in awake, behaving female prairie voles, we measured OT release within the NAcc as a function of exposure to a male and mating. We then compared the distribution of OT-immunoreactive fibers in the NAcc of prairie voles, meadow voles, rats and mice to determine whether the OT innervation of the NAcc is conserved across species with diverse social behavior and OTR distributions in this area. The ultrastructural features of the OT-immunoreactive processes in the NAcc were then examined using electron microscopy. Finally, Fluorogold tract tracing was used to identify the neuronal origin of the OT projections to the NAcc. The results of these studies add significantly to our understanding of the circuitry involved in regulating affiliative behavior in female prairie voles, and provide a potential mechanism for coordinating central OT release with reproductive physiology in all mammals.

3.3 MATERIALS AND METHODS

Animals: Prairie and meadow voles were housed in same sex groups with 2-3 voles/cage from the time of weaning at 21-23 days of age. Housing consisted of a ventilated 36x18x19cm Plexiglass cage filled with Bed-o-cobbs Laboratory Animal Bedding under a 14:10 hr light/dark cycle at 22ºC with access to food (rabbit LabDiet, Richmond, IN) and water *ad libitum*. The prairie voles were obtained from our laboratory breeding colony that originally derived from field-captured voles in Illinois. Meadow voles originated from a colony at Florida State University. For the microdialysis experiment, subjects were sexually naïve female prairie voles 70-90 days of age (30-45g).

For anatomical studies, subjects were adult (>60days old) female prairie voles. In addition, two female meadow voles from our breeding colony, two mice (C57BL/6J) (one male and one female), and two Sprague Dawley (Charles River) female rats were used for a species comparison. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Hormone Treatment and Microdialysis Probe Implantation: Subjects were ovariectomized two weeks prior to probe implantation and microdialysis, and administered 1μg estradiol benzoate in peanut oil, intraperitoneally (IP), for four consecutive days prior to testing to induce receptivity. Subjects were anesthetized with isoflurane and U-shaped microdialysis probes were stereotaxically implanted unilaterally into the NAcc (nose bar -2.5mm, AP +1.8mm, ML -0.9mm, DV -4.5mm). After a one day recovery, the probe was connected to two lengths of PE20 tubing (Polymicro Tech, Phoenix, AZ). The microdialysis probes were self made as previously described, and had a molecular cut-off of 18 kDa (for details see Neumann et al., 1993; Bosch et al., 2005). The inlet of the probe was connected to a 10-ml Hamilton syringe controlled by a CMA/100 microinfusion pump (Bioanalytical Systems, West Lafayette, IN). The outlet fed into a refrigerated collector (SciPro, Inc, Sanborn, NY) housing polyethylene tubes (Fisher Scientific, Houston, TX). A single-channel swivel and counter-balanced lever arm allowed the animals to move freely during mating. Ringer's solution was perfused through the probe via the inlet into the NAcc during the experiment at a flow rate of 1.0μl/min.

Oxytocin Collection: To determine the effects of mating on OT release, eight consecutive 30-minute microdialysates were collected from the NAcc of estrogen-primed female

prairie voles (n=26). Sample collections were divided into three phases: basal (1-4), restricted exposure (5-8), and free exposure (9-16). Basal dialysates were collected from individually housed females and served as the baseline for extracellular concentration of OT. After the basal phase, a sexually experienced male prairie vole of similar age and weight, housed in a wire mesh cage (restricted exposure phase), was introduced into the test cage. After 2 hrs the male was removed from the mesh cage and allowed to physically interact with the female (free exposure phase) for 4 h. This portion of the test was videotaped. Female subjects that did not mate during this period because they were not sexually receptive were categorized as non-mated.

Following microdialysis, brains were rapidly removed, frozen on dry ice, and stored at -80C until use. Brains were later sectioned on a cryostat into 20-µm slices mounted on Superfrost plus microscope slides (Fisher, Pittsburgh, PA). Slides were stored at -80ºC. Proper placement of the probes was confirmed by cresyl violet staining.

Quantification of OT: Dialysates collected during microdialysis were stored at -80ºC until analyzed for content. Samples were lyophilized and the concentration of OT in each dialysate was determined by radioimmunoassay as described previously (Neumann et al., 1993). Cross-reactivity of the polyclonal antiserum with arginine-vasopressin and other related peptides was <0.7%. Intra- and inter-assay coefficients of variation were in the 5-9% and 8-12% ranges, respectively; all dialysates to be compared were assayed in the same run. 25 µl of each dialysate was assayed and the level of detectability of the assay was 0.05pg/dialysate.

Fluorogold Infusions: Female prairie voles (n=18) were anesthetized with isoflurane and placed in a Kopf stereotaxic apparatus. Fluorogold (FG) was iontophoretically injected unilaterally into the NAcc (AP +1.7mm, ML -0.9mm, DV -4.5mm) using a glass micropipette (tip diameter 10–20 μM) filled with the retrograde tracer Fluorogold (2-4% soln. w/v in dH_20; Fluorochrome LLC; Denver, CO) for 15 min at 1-7 μA (50/50 duty cycle). The syringe was left in place for 5 min following infusion to minimize diffusion of tracer up the needle track. 7-14 days after injection, the animals were perfused as described below. Animals with injections that did not reach the NAcc, core or shell, contaminated the lateral ventricle, or showed no FG staining at the injection site were not included in the analysis. To control for the possibility that leakage of FG into capillaries near the injection site or into the ventricles resulted in labeling of hypothalamic neurons, we also performed control infusions consisting of an injection of 0.01 μg of FG in 500nl dH_2O into the lateral ventricle, or 0.5 μg of FG in 50 μl dH_2O injected IP.

In order to label neurons of the neurohypophysial OT that send projections to the pituitary, a region in the CNS that lack blood-brain barrier (Merchenthaler 1991), 600 μg of FG (Horvath 1998) in 75 μl dH_2O was injected IP into female voles. Five days later, the tissue was collected as described below.

Tissue Collection for Immunohistochemistry: Animals were euthanized and perfused transcardially with 50 ml of PBS, followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer containing either 0.1% glutaraldehyde for EM or 2.5% acrolein (Polysciences, Warrington, PA) for all other anatomical studies. Immediately following perfusion, the brains were removed and stored at 4°C in 30% sucrose solution until

sectioned. The brains were cut into 25 µm coronal or 60 µm horizontal sections with a freezing microtome, or 60 µm coronal sections with a vibratome, and stored free-floating in cryoprotectant solution at -20°C until immunocytochemical processing.

Single labeling immunohistochemistry for OT or FG: A 1:6 series through each brain was processed for FG and/or OT for either electron (EM), light (LM), or fluorescent (FM) microscopy. Briefly, sections were removed from the cryoprotectant solution, rinsed extensively in potassium phosphate-buffered saline (KPBS; pH 7.4), and then reacted for 15 minutes in 1% sodium borohydride to remove excess aldehydes. Sections were then incubated in primary antibody solution directed against either FG or OT in KPBS containing 0.1% Triton-X for 48 hours at 4°C. Cells containing OT were identified by using a polyclonal rabbit anti-OT antibody (20068; Immunostar, Hudson, WI) (Lim, Murphy et al. 2004) at a concentration of 1:1000 for EM, 1:70,000 for LM and 1:10,000 for FM. Cells containing FG were identified by using the polyclonal rabbit anti-Fluoro-Gold antibody (AB153;Chemicon, Temecula, CA) at a concentration of 1:5000. After primary antibody incubation, the tissue was rinsed in KPBS, incubated for 1 hour in biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) at a concentration of 1:600 for LM and 1:5000 for FM, and rinsed in KPBS, followed by a 1-hour incubation in avidin-biotin peroxidase complex (Vector, Burlingame, CA, ABC Elite Kit PK-6100) at a concentration of 1:222 for LM and 1:888 for FM. At this point, single labeled tissue for LM was rinsed in either 1) KPBS and sodium acetate (0.175 M; pH 6.5), and visualized as a black reaction product by using nickel sulfate-intensified 3,3′-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer, or 2) KPBS and Tris buffer (pH 7.2), and visualized as a brown reaction product

by using 3,3′-diaminobenzidine (DAB) containing 0.08% hydrogen peroxide in Tris buffer (pH 7.2). The reaction product was terminated after approximately 20 minutes by rinsing in sodium acetate buffer or Tris buffer, respectively. Double labeled tissue for FM continued to be processed as described below.

Double Label Tyramine Amplification Protocol: After rinsing FG-labeled tissue out of the ABC solution (see above), the tissue was incubated in biotin-tyramine (BT) solution (5ul BT/ml KPBS) for 20 minutes, rinsed in KPBS, incubated with Texas Red-streptavidin (T02,Biomeda, Foster City,CA) or stretavidin Alexa Fluor 350 (S11249; Invitrogen) in heated KPBS containing 0.4% Triton-X-100 for 3 hours at 37ºC at a concentration of 1:200. Slides were then rinsed in KPBS, incubated overnight in anti-OT antibody solution in KPBS containing 0.1% Triton-X-100 and normal goat serum (Jackson Immunoresearch, West Grove, PA) (1:100) at room temperature, rinsed in KPBS, incubated in Cy2 conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) (1:50) and normal goat serum (1:100) for 2.5 hours, and then rinsed in KPBS. All sections were mounted out of saline onto gelatin-subbed slides, air-dried overnight, dehydrated in a series of graded alcohols, cleared in Xylene, and coverslipped using Krystalon (EMD Chemicals, Gibbstown, NJ). The tyramine amplification method allows for the use of primary antibodies raised in the same species to be used for double labeling immunohistochemistry without cross-reactivity (Shindler and Roth 1996; Guidetti, Hoffman et al. 2007).

Single Pre-embedding Immunoperoxidase Labeling for EM: Following sodium borohydride treatment (see above), sections were placed in a cryoprotectant solution (phostphate buffer (PB) 0.05M, pH 7.4, 25% sucrose, and 10% glycerol) for 20 minutes,

frozen at -80ºC for 20 minutes, returned to a decreasing gradient of cryoprotectant solutions, and rinsed in PBS. Sections were then incubated in primary and secondary antibody solutions identical to those used for LM (see above), except for the omission of Triton X-100.

After the DAB reaction, the tissue was rinsed in PB (0.1M, pH 7.4) and treated with 1% OsO_4 for 20 minutes. It was then returned to PB and dehydrated with increasing concentrations of ethanol. When exposed to 70% ETOH, 1% uranyl acetate was added to the solution for 35 minutes to increase the contrast of the tissue at the electron microscope. Following dehydration, sections were treated with propylene oxide and embedded in epoxy resin for 12 hours (Durcupan ACM, Fluka, Buchs, Switzerland), mounted onto slides and placed in a 60ºC oven for 48 hours. Separate samples of the NAcc were cut out of the larger sections, mounted onto resin blocks and cut into 60 nm sections using an ultramicrotome (Leica Ultracut T2). The 60 nm sections were collected on Pioloform-coated copper grids, stained with lead citrate for 5 minutes to enhance tissue contrast and examined on the Zeiss EM-10C electron microscope. Electron micrographs were taken with a CCD camera (DualView 300W; Gatan, Inc., Pleasanton, CA) controlled by DigitalMicrograph software (Gatan, Inc.).

Cell Size Measurements in the PVN: Sections that contained both FG-positive and FG-negative OT cells were sampled throughout the rostrocaudal extent of the PVN of three animals. The long-axis diameter of 107 (63 FG- and 44 FG+) OT cells with visible processes were measured using SPOT software calibrated with a micrometer (Diagnostics Instruments, Sterling Heights, MI).

Statistical Analysis: The level of detectability for OT was 0.5pg/dialysate. As most samples were below the level of detectability for OT we used a binary categorization—detectable or non-detectable—for each dialysate. McNemar's test was used to compare the frequency of detectable OT between phases and groups. This is a non-parametric test used on nominal data to determine if row and column marginal frequencies are equal and generally used when the data consist of paired observations. A t-Test was used to determine if there was a size difference between OT cells labeled with FG and those not labeled. Data are presented as mean ± standard error of the mean (SEM).

3.4 RESULTS

OT Release During Mating: *In vivo* microdialysis was performed in female prairie voles at baseline, during a restricted exposure when the male was confined to a wire cage, and during free exposure when mating could occur. OT concentrations in the dialysates were below the level of detectability in the majority of samples. Therefore, samples were categorized as being detectable (> 0.05 pg/25 μl sample) or non-detectable (< 0.05 pg/25 μl sample) and analyzed using the non-parametric McNemar's test. The frequency of detectable OT was greater during the free exposure phase than the restricted exposure phase (Figure 3.1). In addition, the frequency of detectable levels of OT was higher in females that mated during the free exposure phase than females that failed to mate. Five of the 13 (38.5%) animals that mated produced samples with detectable OT during the free exposure phase, while not showing detectable OT during the basal or restricted exposure sessions (McNemar's test, restricted exposure vs free exposure: $p < 0.035$). By contrast, only two of the 13 animals, that were unreceptive and failed to mate, displayed

detectable OT in the free exposure phase (McNemar's test, restricted exposure vs free exposure: $p > 0.05$) (Figure 3.1). It should be noted that animals that mated received multiple intromissions throughout the free exposure phase, while the animals that did not mate received attempted mounts from the males.

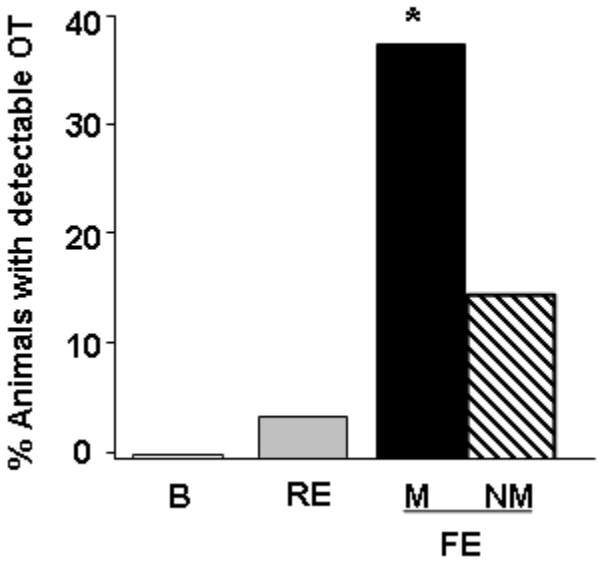

Figure 3.1. *In vivo* microdialysis to detect extracellular oxytocin (OT) as a function of social exposure and mating. Four 30-min samples were collected and analyzed for each phase. The graph illustrates the percentage of animals yielding microdialysates in each phase with detectable OT concentrations. Under basal conditions (B) OT concentrations were below the level of detectability (<0.05 pg/sample) in all samples. Detectable OT was observed significantly more frequently during the free exposure (FE) phase in females that mated (M) compared to during the restricted exposure (RE) phase when the male was housed in a wire cage (* = McNemar's test, $P = 0.035$). In the group of females that failed to mate (NM) during the free exposure phase, the percentage with detectable OT during that phase was not significantly different from the restricted exposure phase (McNemar's test, $P > 0.05$).

Species Comparisons of OT Immunoreactivity in the NAcc: The expression of OTR in the NAcc is highly species specific, with prairie voles having high densities of OTR, rats having intermediate densities of OTR, and mice and meadow voles having little or no OTR (Burbach, Young et al. 2006). By contrast, OT peptide expression is highly conserved across these species. We examined OT fiber-immunoreactivity in prairie voles, meadow voles, mice and rats. The distribution of OT-immunoreactive fibers was qualitatively similar across species, with sparse fibers in the anterior NAcc, primarily in the shell, which in more caudal regions become denser at the ventral pallidum and diagonal band (Figure 3.2).

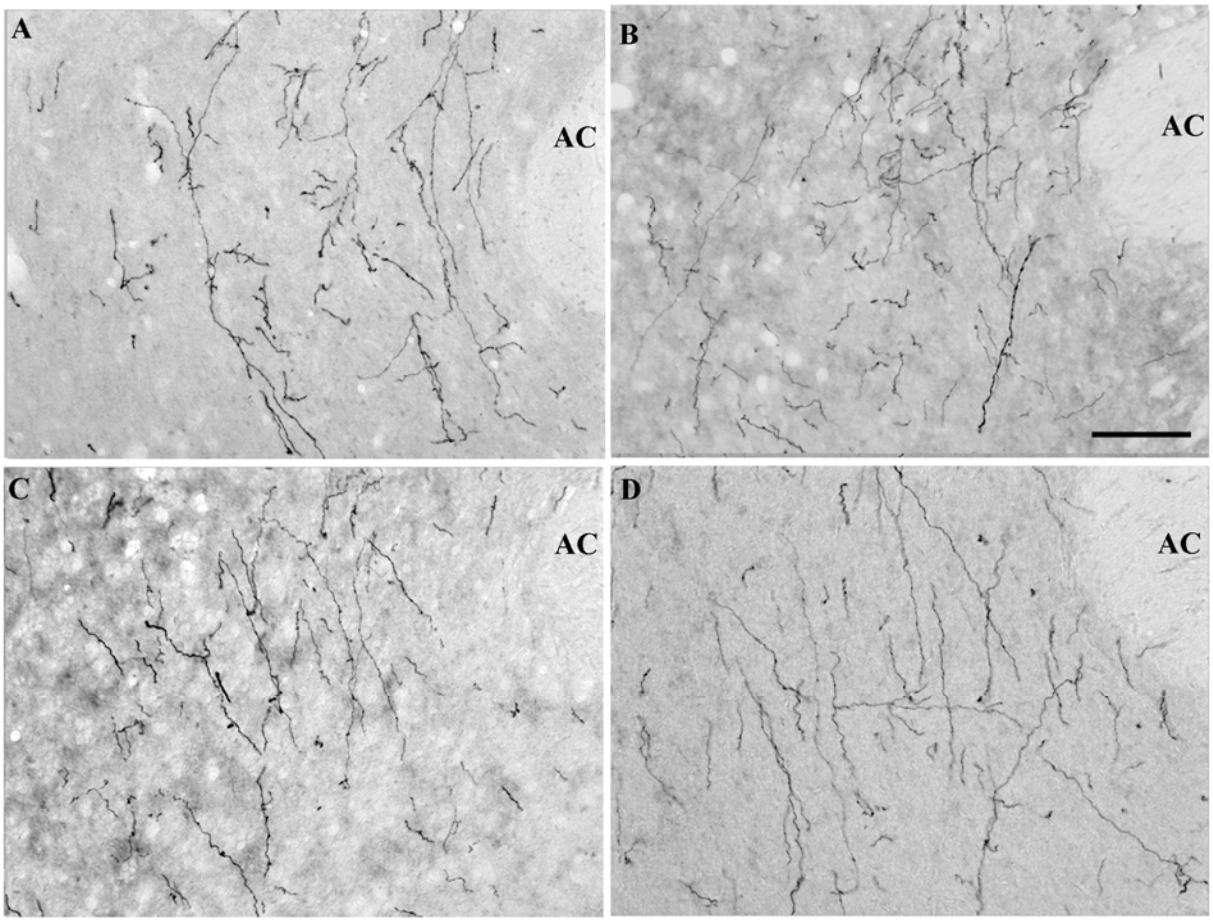

Figure 3.2. Species comparison of OT immunoreactive fibers in coronal sections of the nucleus accumbens (NAcc). Prairie voles (A), meadow voles (B), mice (C) and rats (D) all displayed a comparable pattern of distribution and relative density of OT-immunoreactive fibers in the NAcc. Scale bar = 500 μm (valid for A-D). ac = anterior commisure, PVN = paraventricular nucleus.

Morphological Characteristics of NAcc OT Processes: Electron microscopy was used to characterize the ultrastructure of the OT-immunoreactive processes in the NAcc. Two major types of OT-immunoreactive elements were found in NAcc: (1) Large caliber unmyelinated structures filled with OT-immunoreactive dense core vesicles that do not form clear synaptic contacts but are often apposed to dendrites of striatal neurons (Figure 3.3A,B). (2) Vesicle-filled synaptic terminals that form clear asymmetric synapses most commonly with spine heads and less frequently with dendritic shafts of striatal neurons (Figure 3.3C,D). The latter type of terminals displayed ultrastructural features of glutamatergic boutons and were most often devoid of OT-immunoreactive dense core vesicles. Random analysis of all labeled structures seen in this material revealed that the non-synaptic dense core vesicle filled structures accounted for 73% (32 out of 44) of total labeled elements examined, whereas OT-positive terminals represented only 23% (12 out of 44) of total labeled structures. Of these 12 OT-positive terminals, 83% (10 out of 12) formed asymmetric synaptic contact with spines, while 17% (2 out of 12) were in contact with dendritic shafts.

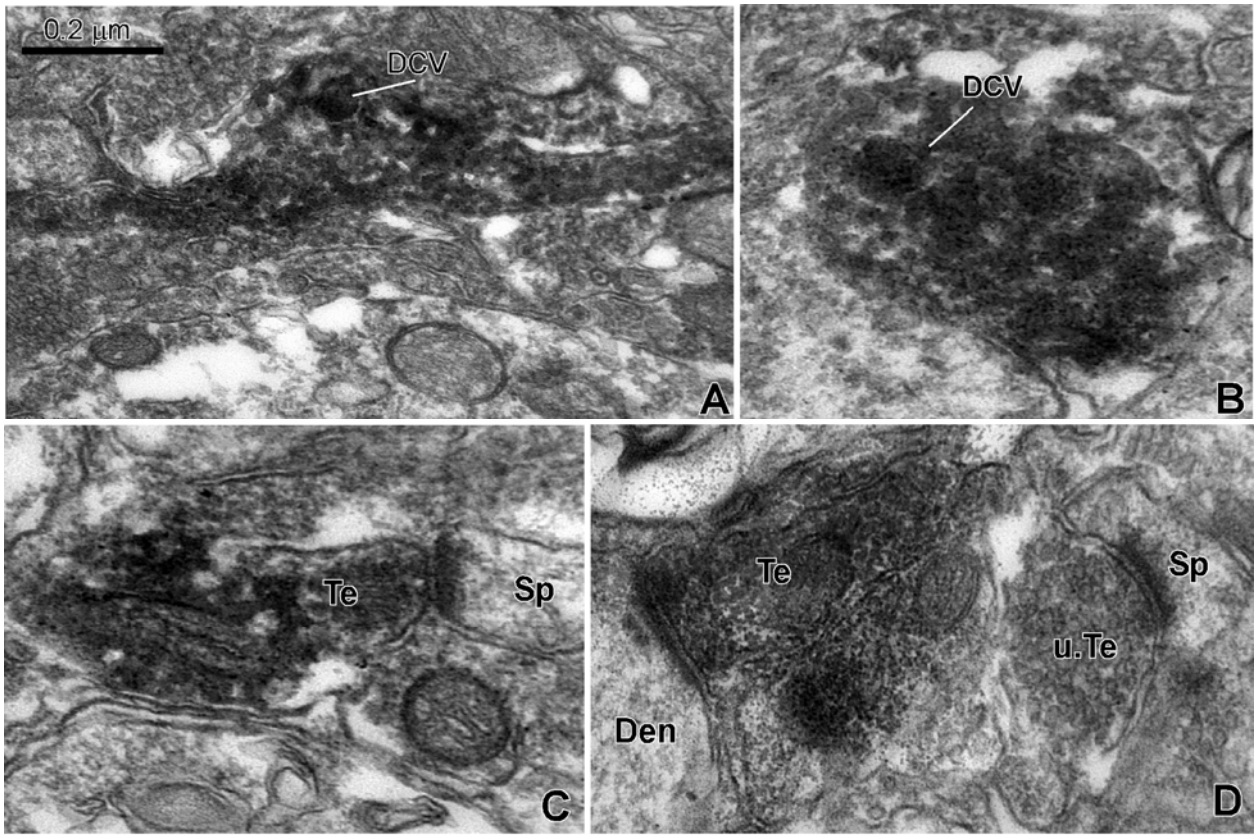

Figure 3.3. Electron micrographs of OT-immunoreactive elements on the nucleus accumbens. (A and B) show strongly labeled profiles that contains darkly stained dense core vesicles (DCV). These elements did not form clear synaptic contact in the planes of sections examined. (C and D) illustrate examples of OT-immunoreactive terminal boutons (Te) forming asymmetric synapses with a spine (sp) and a dendritic shaft (Den). An unlabeled terminal forming an asymmetric axo-spinous synapse is shown in D. Scale bar in A valid for B-D.

Visualization of OT Fiber Projections in Horizontal Sections: To delineate the course of OT-immunoreative fibers to the NAcc, horizontal sections through the NAcc and PVN were stained for OT. Figure 3.4 illustrates that the OT-immunoreactive fibers traversing toward the NAcc often emerge from, and are indistinguishable from, the dense hypothalamic fiber tracts of the neurohypophysial system. Some of these fibers seem to deviate from the other fibers and travel rostrally towards the striatum. This view also illustrates the diffuse pattern of OT-immunoreactive throughout the forebrain and striatum.

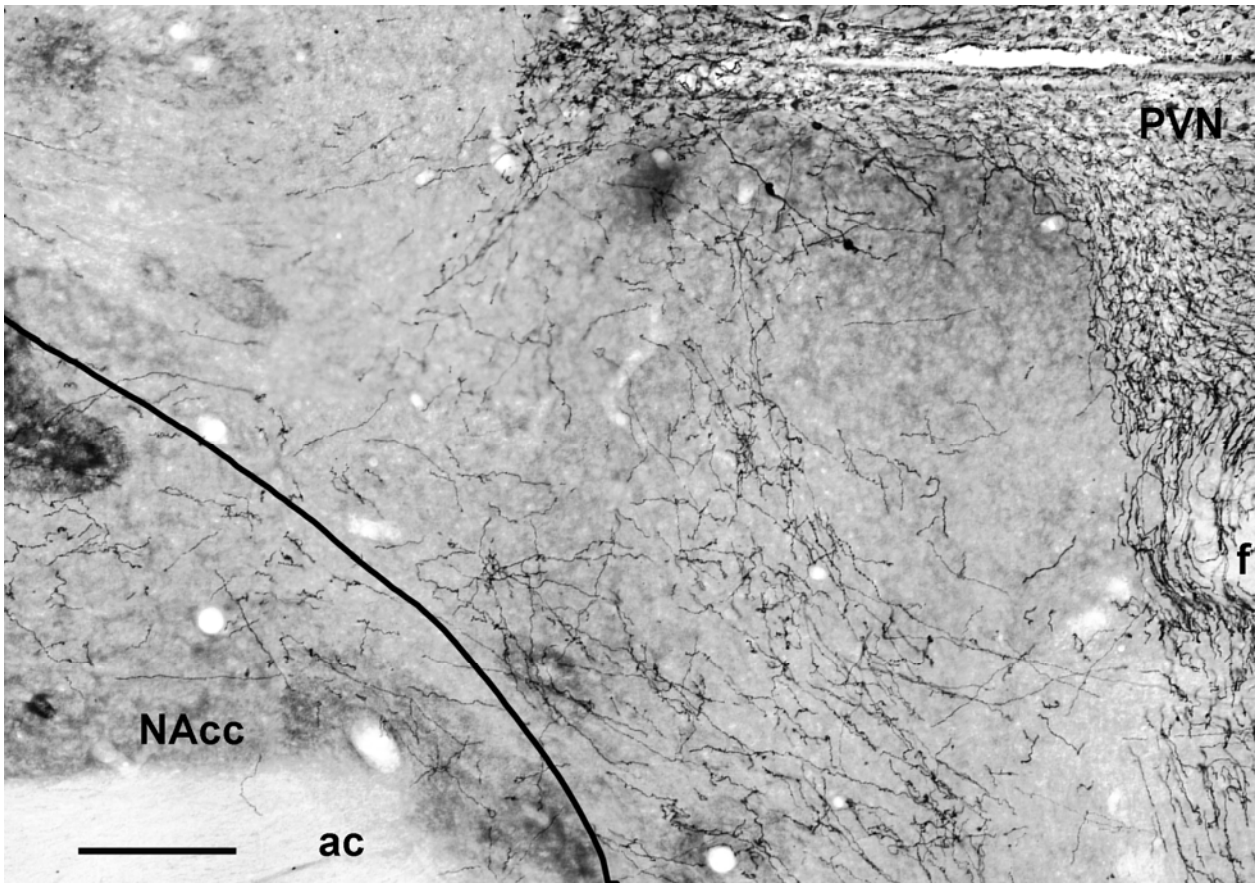

Figure 3.4. Light micrograph of oxytocin-immunoreactive fibers in the paraventricular nucleus of the hypothalamus (PVN) and nucleus accumbens (NAcc) of the prairie vole from a horizontal section. Black line delineates the boundary of the striatum as determined by a marker for KChIP2. Note the diffuse pattern of immunoreactive fibers coursing toward the striatum. A few fibers deviate from the neurohypophysial pathway of the PVN and project toward the striatum. Scale bar = 100 μm. ac = anterior commisure, f = fornix.

Identification of the OT Neurons Projecting to the NAcc: The retrograde tracer FG was used to determine the origin of OT neurons that project to the NAcc. The FG infusions targeted the shell of the NAcc; the same region of the microdialysis probe placement, and the location of the OT-immunoreactive fibers. Examination of the FG injection halo by immunohistochemistry revealed that 11 of the 18 animals had FG halos in the shell and included portions of the medial core of the NAcc. The FG halo of two of these animals also included the medial and dorsal core, and one included the shell only. An additional animal had an injection that included the shell and portions of the lateral septum and was therefore removed from analysis. Thus 10 animals had FG injection sites that met the inclusion criteria (Figure 3.5A). One of the strongest projections to the NAcc originated from the midline thalamic cell groups, including the reuniens nucleus, xiphoid nucleus, parataenial nucleus, and medial dorsal nucleus (Figure 3.5B). Other FG-labeled areas included the prelimbic cortex, ventral pallidum, lateral septum, anterior amygdala, and ventral tegmental area. FG labeling in these areas was consistent across all of the animals. In addition, double labeled (FG-positive and OT-positive) cells were present in the PVN (Figure 3.5C,D) and, to a lesser extent, in the SON (Figure 3.5E,F). A 1:6 series yielded 3-5 double labeled cells in the PVN of 6 of the 10 females, while two animals had a single SON double-labeled soma. Since projections from the PVN and SON to the NAcc have not been previously reported, control injections into the lateral ventricles and periphery were performed that used similar amounts of FG to what was injected into the NAcc. The peripheral IP infusion (0.5 μg/vole) was performed because FG in the bloodstream is taken up by hypothalamic terminals in the pituitary. No FG-labeled neurons were detected in the PVN or SON of any of the control animals. This suggests

that the double-labeled neurons in the NAcc-infused brains represent *bona fide* projections from these nuclei to the NAcc.

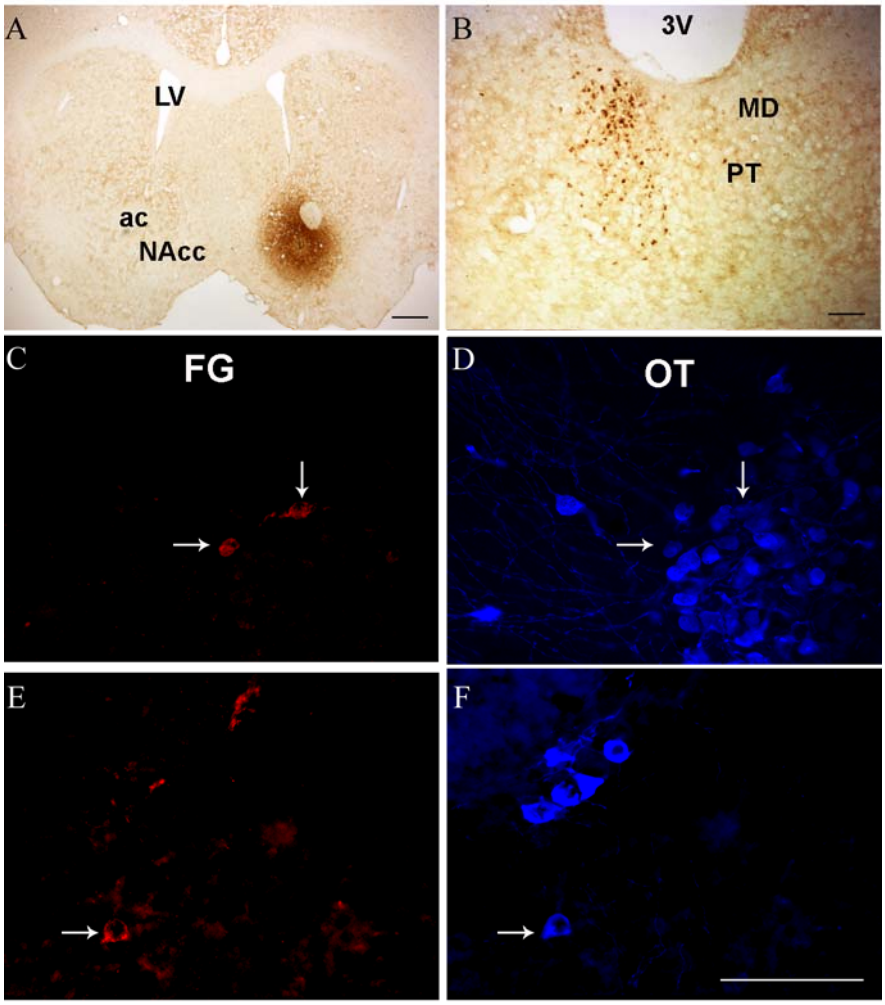

Figure 3.5. Retrograde labeling from the nucleus accumbens (NAcc) of female prairie voles. A) Representative injection site of Fluorogold (FG) in the NAcc. B) FG+ cells in the medial dorsal (MD) and paratenial (PT) thalamic nuclei, one of the most heavily stained regions after these NAcc injections. C-D) Arrows indicate double labeled neurons for FG (C) and OT (D) in the paraventricular nucleus of the hypothalamus. E-F) The arrows indicates a double labeled neurons for FG (E) and OT (F) in the supraoptic nucleus of the hypothalamus. Scale bars: A = 200 µm; B = 100 µm; F = 100 µm (valid for C-E). LV = lateral ventricle, ac = anterior commisure, 3V = third ventricle.

Organization of the Pituitary Projecting OT System: Peripherally injected retrograde tracer can be taken up by cells that have terminals outside the blood brain barrier, including the magnocellular neurons projecting to the posterior pituitary and the parvocellular neurons projecting to the median eminence. Thus, OT cells labeled with FG following a peripheral injection represent magnocellular neurons of the neurohypophysial system as well as potentially parvocellular neurons that project to the median eminence (Silverman, Witkin et al. 1990). Following an IP injection of 600 μg of FG, all SON OT neurons were FG-immunoreactive. Most (~96%) of the OT neurons in the anterior and middle extent of PVN were also FG positive (Figure 3.6 A-K). However, a cluster of non-FG labeled OT neurons was observed in the dorsal part of the posterior PVN (Figure 3.6C,F,I,L), and isolated non-FG neurons were scattered throughout the extent of PVN. Since magnocellular neurons of the neurohypophyseal system of the rat are significantly larger than the parvocellular neurons that do not project to the pituitary [(30-50 μm versus 15-25 μm, respectively) (Sofroniew 1983)], we measured the neuronal diameter along the long axis of FG/OT double labeled neurons, corresponding to pituitary projecting neurons in the rat, and FG-negative OT neurons, corresponding to parvocellular neurons. The soma of double-labeled neurons (42.7μm ± .72 diameter) were slightly larger than the single labeled OT neurons (37.0μm ±.82). While this difference in diameter is statically significant ($p < 0.001$), the size distinction between parvocellular and magnocellular neurons in the prairie vole is not as robust as reported in rats but is similar to the size difference in mice (Castel and Morris 1988).

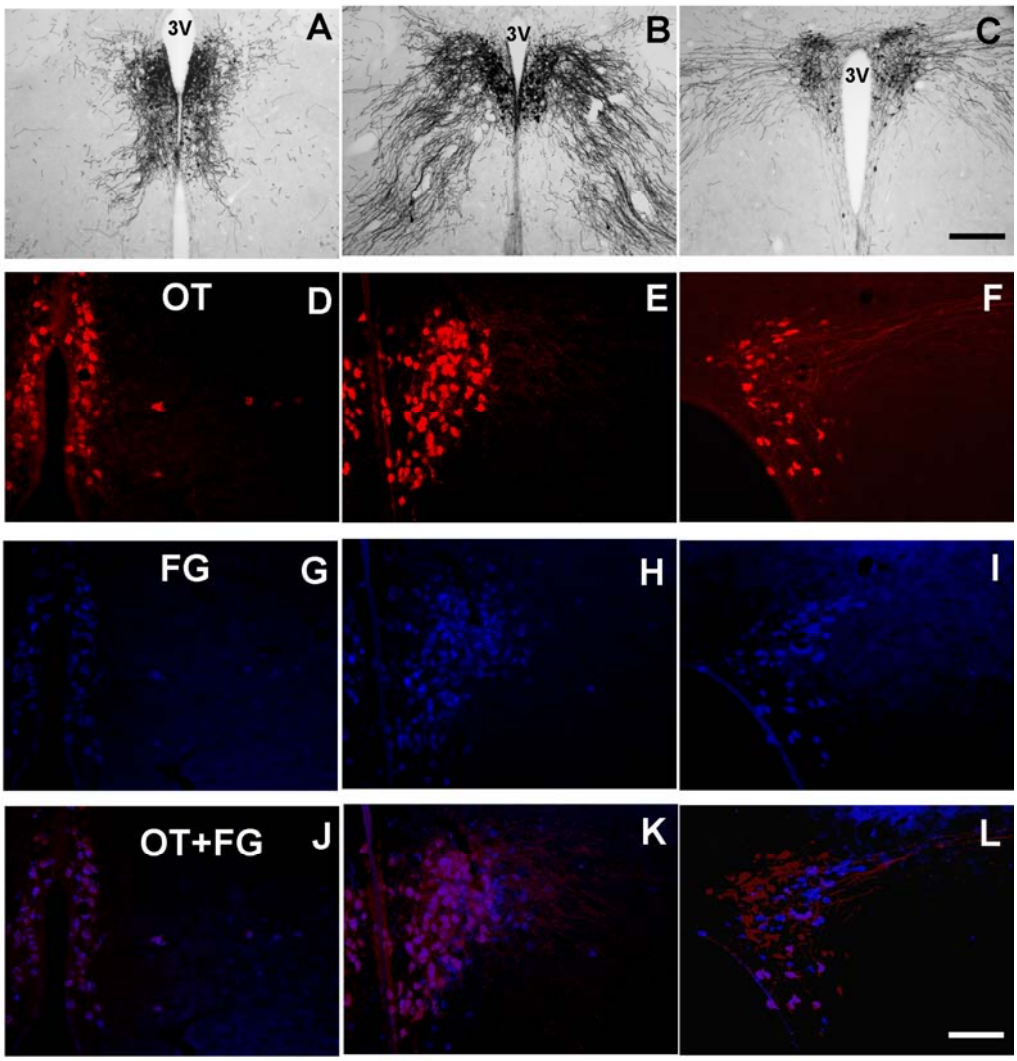

Figure 3.6. Organization of the neurohypophysial system of the paraventricular nucleus of the hypothalamus (PVN). Prairie voles were injected intraperitoneally with FG and brain sections were processed for FG and OT immunoreactivity. OT immunoreactivity in the anterior (A) middle (B) and posterior (C) regions of the PVN as revealed with the immunoperoxidase method. D-F) Micrographs of immunofluorescent (Texas Red-labeled) OT-containing neurons in sections of the hypothalamus representative of those shown in A-C. G-I) FG-labeled cells labeled with Alexa Fluor 350 in the same sections shown as in D-F. J-I) Overlay of OT and FG labeling in D-I. Notice that the majority of cells in the anterior and middle levels of the PVN are labeled for both OT and FG, suggesting that they are magnocellular neurohypophysial neurons. Notice the two populations of cells in F, a small dorsal and a larger ventral group. The dorsal cluster does not have FG immunoreactivity suggesting that these are the parvocellular OT cells that project to the hindbrain and spinal cord. Scale bar in C = 200 μm (valid for A-B); bar in L = 100 μm (valid for D-K). 3V = third ventricle.

3.5 DISCUSSION

OT modulates a wide range of social behaviors, including maternal nurturing and bonding, sexual behavior, and social attachment. In humans, intranasal OT enhances interpersonal trust, eye gaze, recognition of familiar faces, and the ability to infer the emotions of others (Kosfeld, Heinrichs et al. 2005; Domes, Heinrichs et al. 2007; Guastella, Mitchell et al. 2008; Savaskan, Ehrhardt et al. 2008). Yet remarkably little is known about the origins of neurons that modulate these behaviors. In prairie voles, pharmacological and genetic manipulation studies have suggested that OTRs in the NAcc are involved in regulating spontaneous maternal behavior and partner preference formation (Young, Lim et al. 2001; Olazabal and Young 2006; Ross, Freeman et al. 2009). Here we provide the first demonstration that extracellular OT concentrations in the NAcc increase during mating. In this study, despite using the highest sensitivity of radioimmunoassay, basal levels of OT were undetectable due to the low recovery rate of OT through the microdialysis tubing (< 5%) (Neumann, Ludwig et al. 1993) and the diffuse pattern of OT fibers within the NAcc Therefore, it is important to note that we cannot conclude that the NAcc is devoid of extracellular OT under basal conditions or following exposure to a male without mating. However, the fact that 38.5% of the females that mated produced microdialysates with measurable OT provides strong evidence that OT is released during mating from the OT-immunoreactive processes coursing through the NAcc. We also cannot conclude that OT is not released within the NAcc during social exposure with a male in the absence of mating. Indeed 2 of the 13 females that did not receive an intromission because they were not sexually receptive produced microdialysis samples with measurable OT, but this failed to be statistically

different from baseline. It is plausible that social interaction, including mounting attempts, also results in OT release, albeit to a lesser extent than what occurrs with mating. This would be consistent with the observation that female prairie voles can form partner preferences following cohabitation with a male without mating, although this requires longer time periods (Williams, Carter et al. 1992). Indeed, OT release within the PVN has been reported in male rats during restricted exposure to a female (Waldherr and Neumann 2007).

The increase in central extracellular OT following mating is consistent with studies in sheep and rats, that demonstrate that vaginocervical stimulation results in central OT release (Kendrick, Keverne et al. 1986; Sansone, Gerdes et al. 2002). Vaginocervical stimulation occurs naturally during parturition and mating. The OT released during parturition likely plays a role in the onset of maternal behavior (Pedersen and Prange 1979; Kendrick, Keverne et al. 1991), while OT released during mating, at least in prairie voles, facilitates the formation of a pair bond. A recent study also reported that OT is released in the hypothalamus of male rats following mating (Waldherr and Neumann 2007). The role of OT in facilitating partner preference in male prairie voles is not clear, as one study found that OT or OT antagonist administered ICV did not effect partner preference formation (Winslow, Hastings et al. 1993); while another lab found central OT infusion in males did facilitate partner preference formation (Cho, DeVries et al. 1999). In humans, OT has been reported to increase in the plasma during sexual arousal and ejaculation or orgasm, although changes in central OT concentrations have not been examined (Carmichael, Humbert et al. 1987).

Our results also illustrate the remarkable evolutionary conservation of OT innervation in the ventral striatum despite marked species differences in receptor distribution. Prairie voles have high densities of OTR in the NAcc, while meadow voles and mice are virtually devoid of receptors in this region (Insel and Shapiro 1992; Olazabal and Young 2006). We expected to find similar species differences in OT fibers in the NAcc. However, at least qualitatively, each species displayed a similar diffuse pattern of OT-immunoreactive fibers. A recent report described similar OT-immunoreactive fibers in the NAcc of the eusocial naked-mole rat (Rosen, de Vries et al. 2008). The contrast between the conservation of distribution of peptide and receptor suggests that diversity in receptor distribution, and not OT projections, drives the evolution of central OT function. As can be seen in horizontal slice, OT fibers course diffusely through the forebrain rather than traveling in discrete tracts targeting specific regions. This is consistent with the observation that OT released from fibers can diffuse to target regions, in a paracrine fashion (Ludwig and Leng 2006).

The electron microscopic analysis revealed that the majority of OT-immunoreactive elements in the NAcc are large diameter (~200nm), unmyelinated processes packed with dense core vesicles. OT-immunoreactive elements containing asymmetric synapses were also clearly present. Interestingly, these synapses were largely devoid of OT-containing dense core vesicles, but instead contained smaller synaptic vesicles. Hypothalamic OT neurons exhibit somatic and dendritic release of dense core vesicles (Pow and Morris 1989; Landgraf and Neumann 2004; Ludwig and Leng 2006). Based on these ultrastructural observations, we hypothesize that OT may be released from these *en passant* processes in a mechanism similar to somato-dendritic release in the

hypothalamus (Pow and Morris 1989). Magnocellular OT neurons express vesicular glutamate transporter, suggesting that they are glutamatergic (Ponzio, Ni et al. 2006), and have unmyelinated axons (Swanson and Sawchenko 1983). Likewise, the asymmetric specialization of the synapses seen on OT-immunoreactive terminals in the NAcc of prairie voles is indicative of glutamatergic neurotransmission. It should be noted that we could not definitively demonstrate that the OT-containing elements were axons or extended dendrites. However, the presence of synapses associated with some terminals suggests that at least some fibers are axonal projections.

The results from the retrograde tract tracing studies are most consistent with the hypothesis that NAcc OT fibers originate from the PVN and SON. This result was surprising since neither of these regions have been reported as being labeled following injection of retrograde tracers into the rat NAcc (Phillipson and Griffiths 1985; Brog, Salyapongse et al. 1993). Initially we hypothesized that FG leaking into the capillary system or into the ventricles may have been producing false positive FG-labeled cells. However, our control infusions of similar amounts of FG directly into the lateral ventricle or IP did not result in detectable FG labeling in the PVN or SON. The double-labeled neurons that were identified were strongly labeled while surrounding PVN and SON neurons were not labeled, which also argues against leakage of the FG into the bloodstream. We suspect that the diffuse nature of the OT projections in the NAcc, and the sparseness of OT-containing axon terminals, the main sites of FG uptake (Ju, Han et al. 1989), limited considerably the amount of tracer being taken up by the OT projection system. Consequently, only a few retrogradely labeled PVN or SON neurons were detected in 6 out of 10 animals with FG injections into the NAcc. This phenomenon has

also been seen on other neuronal systems which led to controversy regarding the degree of axonal collateralization of the striatofugal system to both segments of the globus pallidus (see (Parent, Sato et al. 2000) for review). However, although the number of double-labeled OT neurons was small, they were only seen in the hypothalamic brain areas of the PVN and SON. This finding is consistent with lesion studies that found a loss of OT-immunoreactive fibers after PVN or SON ablation (De Vries and Buijs 1983; Hawthorn, Ang et al. 1985).

Early retrograde tracing studies examining the OT projections of the hindbrain and brainstem indicated that those projections arise from parvocellular OT neurons in the PVN (Ono, Nishino et al. 1978; Swanson and Sawchenko 1983). This has led to the extrapolation by many in the field that forebrain OT projections arise from the parvocellular PVN neurons, while the magnocellular neurons of the PVN and SON project exclusively to the posterior pituitary. Our results bring that assumption into question. First, all SON neurons are magnocellular (Swanson and Sawchenko 1983), yet a few SON neurons were clearly FG-positive following infusion into the NAcc. In addition, within the PVN of prairie voles, non-pituitary projecting OT neurons were clustered in the dorsal posterior region. These neurons may be giving rise to the hindbrain and brainstem OT system. However, FG-positive OT neurons were not found in this population, but were most often found in the anterior PVN close to the third ventricle, an area predominated by neurohyophyseal neurons. In prairie voles, OT soma are also found in the medial preoptic area, median preoptic nucleus, bed nucleus of the stria terminalis, and lateral hypothalamic area (Wang, Zhou et al. 1996). However, we did not detect any FG-labeled OT neurons in these regions. The large caliber, and

unmyelinated nature of the NAcc OT fibers is also consistent with magnocellular origin (Harris, Manabe et al. 1969). In the horizontal slices, the OT fibers coursing toward the NAcc appear to emerge from the axonal projections of the neurohypophyseal system. Given the low detection rate of double-labeled OT neurons, future studies using alternative techniques, are needed before definitive conclusions are drawn regarding the origin of the OT fibers in the NAcc.

While not examined in the present study, we hypothesize that the NAcc OT fibers may be from collaterals of the magnocellular neurohypophysial OT neurons. In the mouse, a minority of PVN magnocellular neurons produce collaterals around the level of the fornix that turn anteriorly and become perpendicular to the section, exactly as would be expected if going to the NAcc (Hatton, Cobbett et al. 1985). If the prairie accumbal OT fibers are indeed collaterals of magnocellular hypothalamic neurons, this would provide a direct mechanism for coordination of central release deep in the forebrain with peripheral release under the appropriate physiological conditions, such as vaginocervical stimulation during mating or parturition, or sensory stimulation during suckling. Indeed, it is possible that extracelluar OT in the NAcc reflects a combination of somatodendritic release within both the PVN and SON that diffuses to the NAcc, and *en passant* or terminal release from OT fibers located in the NAcc and originating from PVN and SON. These multiple modes of central release of OT may explain high temporal and spatial resolution and a theoretically unlimited variability in OT signaling modes (Landgraf and Neumann 2004). While central and peripheral release patterns may be coordinated to trigger synergistic effects, local release independent of peripheral secretion may also be possible (Neumann, Ludwig et al. 1993; Landgraf and Neumann 2004; Ludwig and Leng 2006).

The present study provides the most complete characterization of the OT system involved in regulating affiliative behavior to date. We demonstrate that extracellular OT concentrations in the NAcc increase with mating in the female prairie vole. OTR antagonist infused in this region block mating-induced partner preference formation as well as alloparental behavior (Young, Lim et al. 2001; Olazabal and Young 2006). This OT projection pattern is not an oddity of the monogamous prairie vole, but represents an evolutionary conserved forebrain projection. The ultrastructure and the diffuse nature of the projections are consistent with a paracrine, neuromodulatory function of forebrain OT. Finally, if these projections are indeed collaterals of magnocellular OT neurons of the neurohypophysial system, they provide a mechanism of coordination of peripheral physiology and behavior necessary for reproductive success.

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CHAPTER 4

Conclusions and Future Directions

4.1 SUMMARY OF KEY FINDINGS

Oxytocin's (OT) involvement in reproduction has been well established. It is essential for lactation and OT release in the brain encourages care of offspring. In addition, its release in social species promotes attachment and partner bonding. OT action in the nucleus accumbens (NAcc), an area critical for reward processing, is found to be involved in all bonding behaviors studied thus far in female prairie voles. However, the breadth of its role in individual differences in affiliative behaviors and social cognition, especially in humans, is still under active investigation. I found in female prairie voles with increased oxytocin receptor (OTR) in the NAcc accelerated partner preference formation after cohabitation with a male, but did not enhance alloparental behavior. However, partner preference was not facilitated in non-monogamous meadow voles by introducing oxytocin receptor into this same area. These data are the first to demonstrate a direct relationship between oxytocin receptor density in the NAcc and variation in social attachment behavior. Thus, individual variation in oxytocin receptor expression in the striatum may contribute to natural diversity in social behaviors.

Although OT is one of the oldest studied systems, there are still fundamental unknowns about fiber origin and function of magnocellular cells. Until now, the source of OT to the NAcc had been unidentified. I found that the paraventricular nucleus of the hypothalamus and the supraoptic nucleus send fibers towards forebrain reward areas. This data then brought into question long held beliefs about the characteristics of central OT projections; mainly that they are separate from the neurohypophyseal system that releases peptide from the pituitary. The distribution of retrogradely labeled neurons from the accumbens to hypothalamic nuclei is consistent with the hypothesis that striatal oxytocin fibers arise

from collaterals of magnocellular neurons of the neurohypophysial system. This may serve to coordinate peripheral and central release of oxytocin with appropriate behavioral responses associated with reproduction, including pair bonding after mating, and maternal responsiveness following parturition and during lactation.

4.2 VIRAL CONCLUSIONS

Like humans, prairie voles show natural variation in their propensity for social attachment. In our vole model, activation of OTR promotes nurturing behavior and selective preferences for mating partners. The level of OTR in reward areas is found to positively correlate with the expression of one of these affiliative behaviors, alloparental care. One goal of this thesis was to directly test the relationship between receptor density in the NAcc and affiliative behavior in voles. To this end, I used an adeno-associated virus expressing the prairie vole OTR to increase receptor expression in the NAcc of females and tested whether there was an increased propensity to exhibit alloparental care and partner bonding. I found that OTR level did not affect affiliative behaviors equally. While there was no difference in nurturing behavior towards pups between groups, I found that high OTR levels in the NAcc decreased the time needed to produce a selective partner bond. These results suggest that variation in OTR density in the adult NAcc directly modulates the ability to form social attachments to a mating partner but not nurturing behavior towards pups.

Next we investigated whether expression of OTR in a non-monogamous species would induce affiliative behavior. Meadow voles have very low levels of endogenous OTR in the NAcc and do not normally show a pair bond. Using the OTR-containing adenovirus,

we increased the density of OTR in the NAcc of female meadow voles. When these females were tested, they failed to show a preference for their mating partner. This suggests that OTR expression in the NAcc is not sufficient to produce the species differences in bonding behavior.

4.3 ALLOPARENTAL IMPLICATIONS AND FUTURE DIRECTIONS

Contrary to our prediction, there was no difference in alloparental behavior between groups, suggesting differential mechanisms by which accumbal OTR regulates partner preference formation and alloparental behavior. Previously, our lab has shown that the level of OTR in the NAcc highly correlates with the likelihood of being alloparental. We now hypothesize that individual variation in OTR activation in the NAcc during development may play a more important role in producing intra-species variation in alloparental behavior than adult levels. The positive correlation in OTR density in the NAcc and alloparental behavior has been shown in juvenile prairie voles, in addition to adults (Olazabal and Young 2006; Olazabal and Young 2006). If this hypothesis is correct, individuals with higher densities of OTR during development may experience increased OTR signaling in the NAcc, resulting in long-lasting neurochemical changes that increase the probability of displaying alloparental behavior as they become adults. This is similar to epigenetic effects seen in OTR density in female rats. High licking/grooming (LG) dams have increased OTR density in specific brain areas compared to low LG moms (Francis, Champagne et al. 2000). In this way, the quality of maternal care received influences the amount of OTR expressed in certain brain areas and determines the amount of care the next generation will experience. However, in prairie voles no developmental manipulation has been shown to change OTR levels. To directly

test whether OTR levels during development are crucial for the expression of alloparental behavior in prairie voles, one could use viral vectors to enhance OTR density in the NAcc of juveniles and test for alloparental behavior as adults. It would also be possible to decrease the amount of receptors using siRNA.

Furthermore, alloparental behavior in prairie voles appears to be particularly sensitive to social experiences. For example, the presence or absence of the father during development influences the display of alloparental responsiveness in adult female prairie voles. In fact, preliminary data from our laboratory (Ahern and Young, unpublished) showed that OT mRNA in the anterior PVN is affected by rearing condition. The individual difference in nurturing behavior is associated with endogenous OT release in both rats and voles; since ICV administration of OTR-antagonist was able to reduce LG levels in high LG dams (Champagne, Diorio et al. 2001) and diminishes alloparental behavior in female prairie voles (Olazabal and Young 2006). It may be that non-alloparental animals have a reduction in OT release during this affiliative behavior. Microdialysis could be used to determine if there is a difference in extracellular OT release following pup presentation.

Another explanation for the failure of enhancing OTR expression in the NAcc to increase alloparental behavior is that variation in OTR expression in multiple brain regions may be necessary to produce the expected diversity in behavior. For example, OTR expression in the lateral septum is negatively correlated with alloparental behavior in juveniles (Olazabal and Young 2006). Juveniles are more likely to be alloparental, less anxious, and more affiliative towards same-sex conspecifics than adults. In addition, adults that were less anxious and more affiliative, towards a same-sex partner, tended to

be more alloparental. However, OTR density in other brain areas did not correlate with alloparental behavior as adults. Again this is evidence for a role of OTR, in different brain areas, during development being most important for the expression of adult behavior. Therefore increasing OTR in the NAcc and decreasing it simultaneously in LS may produce a more reliable outcome.

4.4 PARTNER PREFERENCE IMPLICATIONS AND FUTURE DIRECTIONS

Meadow Vole PP

Increasing OTR expression in the NAcc was not sufficient to induce partner preference formation in meadow voles, suggesting that the species differences in OTR in the NAcc alone are not sufficient to explain the species differences in the ability to form partner preferences. This finding is in contrast to previous studies from the lab that showed increased affiliative behavior in male meadow voles with elevated levels of vasopressin receptor in the ventral pallidum, another reward area (Lim, Wang et al. 2004). There are a few reasons for the difference in the behavioral response to upregulated neuropeptide receptors between the sexes. First, while the ventral pallidum is the only brain area shown to affect pair bonding behavior in males, there are multiple brain areas in females. For example, OTR density is also higher in the prefrontal cortex and lateral amygdala in prairie voles compared to nonmonogamous vole species (Insel, Gelhard et al. 1991; Young, Huot et al. 1996; Smeltzer, Curtis et al. 2006). Therefore, elevating OTR expression in multiple sites may be necessary for stimulating mating-induced partner preferences in meadow voles. Finally, it is possible that multiple neurochemical differences between the species are responsible for species differences in behavior (e.g.

dopamine, corticotropin releasing factor)(Gingrich, Liu et al. 2000; Liu and Wang 2003; Smeltzer, Curtis et al. 2006; Lim, Liu et al. 2007).

Prairie Vole PP

Increasing OTR in the NAcc of adult female prairie voles produced an accelerated partner preference. Females injected with AAV-OTR showed a significant partner preference after 18 hours of co-habitation; while AAV-GFP and sham animals did not. This difference was not due to differences in mating behavior. In this study, I tested how long it took to form a partner preference as a consequence of OTR density in the NAcc. There are two possible mechanisms that could produce this outcome. One is by learning the association between the partner's olfactory signature and the reward of mating thereby forming a specific attachment sooner. Partner preference is thought to use many of the same mechanisms as other forms of associative learning, changing synaptic strength with repeated input pairings. Our lab has shown that many synaptic plasticity proteins are upregulated after mating in female prairie voles. This learning mechanism is the simplest explanation and best tested with the partner preference test, which is an all or none event. However, increasing OTR in reward areas may also produce an accelerated bond by increasing the amount attraction felt towards the mate. Although these are subtle differences, it may be a question of motivation. It would be interesting to see if with increasing co-habitation time there is an increase in motivation to be with the partner. A way to test this would be to see if a female would work for access to the partner or cross an electrified grid.

The finding that variation in receptor levels can influence partner bonding is very exciting because it has implications for human behavior and social cognition. Perhaps a mechanism similar to what we found in prairie voles is acting to produce some of the individual differences in human behaviors, especially as it pertains to social relationships and attachment to partners. It is also possible that subtle variation in receptor levels also impacts general sociability and the ease in which we interact in groups. The development of a PET ligand for the OTR would greatly enhance our understanding of human receptor localization and density; since the ligands used for autoradiography in rodents do not bind well to primate tissue.

Our lab has investigated the promoter region of OTR and the closely related neuropeptide, vasopressin receptor, towards controlling the species-specific pattern of receptor location. Creating a transgenic mouse expressing the prairie vole OTR gene and promoter caused a vole-type pattern of receptor expression (Young, Winslow et al. 1997). Although studies have not identified a mechanism for brain area variability in receptor density, differences in microsatellite length in the vasopressin receptor gene (avpr1a) have been shown to correlate with receptor level expression within prairie voles. Recently one polymorphism in the human avpr1a has been shown to correlate with partner bonding, marital status, and perceived marital problems. In addition, the genotype of this polymorphism in males not only influences their bonding behavior but affects marital quality perceived by their spouse (Walum, Westberg et al. 2008). Therefore, variability within promoter regions of neuropeptides are able to influence perceived partner attachment and perhaps account for some of the individuality people exhibit in their social behaviors.

4.5 ANATOMY CONCLUSIONS

The location of OTR, particularly in the NAcc, is species-specific and ranges from no expression in mice to high expression in prairie voles, with rat and meadow vole levels falling in between. A feature of neuropeptide systems is that very often there is a mis-match between the presence of receptors and peptide for a given location. Therefore we were interested in knowing whether rodents with known OTR expression patterns also had OT fibers present in the NAcc. Using immunohistochemistry, we saw that rats, mice, meadow, and prairie voles had OT-immunoreactive fibers in the NAcc regardless of OTR levels. A recent study in the naked mole rat has shown that OT fibers are present in the NAcc of this eusocial species as well (Rosen, de Vries et al. 2008). Thus it appears that in contrast to the localization of OTR, the distribution of OT fibers is remarkably conserved across species.

Previous research has shown that accumbal OTR is critical for the expression of affiliative behaviors in female prairie voles. In addition, Cole has shown using microdialysis that OT is released in this reward area during mating. However, the origin of the fibers that are presumably releasing this OT has not been investigated. To determine the location of OT soma that project to the NAcc, retrograde tracer was injected into this area. I found the accumbal afferents of prairie voles to be very similar to those found in the rat, except that none of these brain regions produce OT. The only brain region where OT and retrograde tracer were found to co-localize was in the paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON). Although these hypothalamic nuclei are the main sites of OT production, the SON is thought to contain only magnocellular cells that release neuropeptides into peripheral

blood circulation (Sofroniew 1983; Swanson and Sawchenko 1983). However, there is some evidence in the literature to the contrary. Lesions of the SON affect different populations of OT fibers, using RIA (Hawthorn, Ang et al. 1985). Important for our investigation, both types of lesions produced a loss in caudate-putamen OT content. In addition, a study injecting 3H-leucine into the SON found multiple extrahypothalamic projections, including olfactory bulb, amygdala, cingulum, and locus coeruleus (Alonso, Szafarczyk et al. 1986).

The PVN contains these posterior pituitary projecting magnocellular cells as well as smaller cells that project to the hindbrain and spinal cord. These smaller parvocellular cells are assumed to be controlling all central neuropeptide release. Since the distribution of magno and parvocellular cells is unknown in the prairie vole PVN, I used a peripherally administered retrograde tracer to distinguish pituitary-projecting soma from centrally-projecting soma. In the prairie voles, the majority (~96%) of OT cells in the anterior and medial PVN contained tracer. However, in the posterior PVN there was a dorsal group of OT cells that remained unlabelled, implying that these are the parvocellular cells that project to the hindbrain. Interestingly, the accumbal projecting OT somas were not located in this "parvocellular" area, again suggesting that magnocellular cells send fibers to forebrain nuclei, like the NAcc.

The ultrastructure of these accumbal OT fibers has revealed that they are thick unmyelinated processes filled with dense core vesicles. A minority of these fibers form synapses (23%) and terminate mainly on spines (83%) and to a lesser extent on dendritic shafts (17%). This is in accordance with OT synapses in other areas of the brain which showed a preference for dendritic over axonal contacts (Buijs and Swaab 1979).

Interestingly, the synaptic boutons of the OT-immunoreactive fibers were devoid of dense core vesicles. These synapses make asymmetric contacts and therefore appear to be glutamatergic. However, the majority of OT-immunoreactive elements in the NAcc are packed with OT-immunoreactive vesicles and do not form clear synapses, even in cases where the axonal segments were examined through serial ultrathin sections (Ross, Cole et al. 2009). This suggests that OT may be released from the fiber surface in an *en passant* manner. From our studies, it is not possible to determine conclusively whether these processes are axonal or dendritic in nature. The presence of synapses associated with some terminals, however, suggests that at least some fibers are axonal projections.

4.6 ANATOMY IMPLICATIONS AND FUTURE DIRECTIONS

The initial experimental design was to inject cholera toxin (CT) into the NAcc and FG into the periphery in order to determine whether the cells that project to the accumbens also project to the posterior pituitary using triple-label immunohistochemistry. Since CT did not produce reliable staining in the PVN or SON, it would be possible to reverse the tracers and inject CT into the periphery. However, CT has not been extensively tested as a peripheral tracer and therefore many controls would need to be included (Alisky, van de Wetering et al. 2002). A more involved experiment, but one that could produce clearer results, would be to inject a retrograde tracer directly into the posterior pituitary. This would have the advantage of getting rid of the ambiguity of peripheral tracers labeling the anterior pituitary as well. An anatomical method that could visualize the collaterals of magnocellular neurons is biocytin injection into individual cells in the PVN or SON (Usrey, Muly et al. 1992). This tracer will fill the processes in the NAcc and pituitary and

then serial reconstruction would determine the type of neurons that project to the forebrain.

An area of increasing interest for our lab is the link between glutamate and OT in the NAcc of female prairie voles. Cole has shown that both extracellular glutamate and OT are increased in dialysates collected during mating, compared to non-contact exposure. In addition, when an OT-antagonist is retro-dialysized into the NAcc it blocks the mating induced glutamate release. Although the source of this extracellular glutamate cannot be verified, the finding that glutamate release is functionally coupled to OTR activation is very interesting when considering data presented in this dissertation. We showed that some OT terminals make asymmetric synapses in the NAcc and that small synaptic vesicles are present in terminals, suggesting that glutamate release is possible from OT neurons. Yet, the number of OT axons found to make synapses in the NAcc was very low. This leads to the question of what type of information is being conveyed to this selected population of neurons. Presumably OT is released in abundance from *en passant* terminals and therefore able to bind OTR in a broad area, but only a few of these neurons will also be getting an excitatory signal concurrently. One possibility is that glutamate is involved in the associative learning required for pair bond formation, perhaps through traditional long-term depression mechanisms already well known for learning in the NAcc. Our lab is beginning to address the role of glutamate in enhancing learning. Modi has shown that giving an allosteric modulator of NMDA receptors alone can enhance partner preference formation. Future experiments will need to address the interaction between glutamate and OTR signaling cascades.

4.7 MODEL FOR BEHAVIORALLY RELEVANT OXYTOCIN RELEASE IN THE FOREBRAIN

Based on our observations in the prairie vole system, and notions prevalent in the literature, we can propose three cellular models with respect to the origin of the OT immunoreactive processes in NAcc. First, the prevailing assumption is that separate neuronal populations comprise the neurohyphyseal OT system and the centrally projecting system (Fig 4.1A). Alternatively, centrally projecting OT fibers may be axon collaterals of neurohyphyseal OT neurons that are diverted from their path toward the pituitary (Fig 4.1B). Finally, given the well documented dendritic release of OT, the OT filled processes in the NAcc may actually be long dentrites of magnocellular neurons (Fig 4.1C).

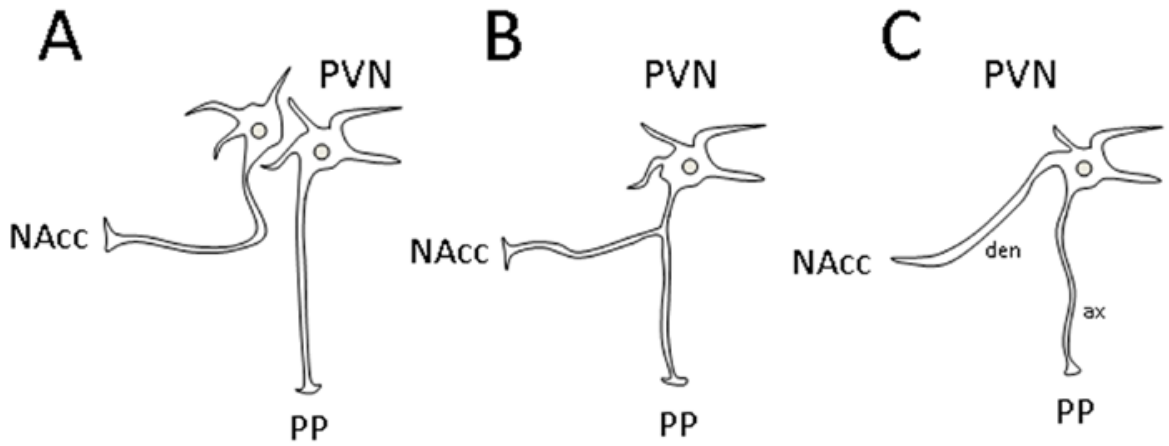

Figure 4.1. Three models illustrating the possible origin of the oxytocin (OT) immunoreactive processes in the nucleus accumbens (NAcc). A) Separate neuronal populations comprise the neurohyphyseal OT system and the centrally projecting system. Many investigators favor this model and presume that the centrally projecting neurons are parvocellular neurons. B) Centrally projecting OT fibers may be axon collaterals of neurohyphyseal OT neurons that are projecting towards the pituitary. Based on our work in voles, we predict that this may actually reflect the organization of the OT system in the NAcc C) Given the well documented dendritic release of OT, the OT filled processes in the NAcc may actually be long dentrites of magnocellular neurons. The presents of oxytocin immunoreactive structures containing synapses in the NAcc suggests that this model may not be correct. Note that in each model, bulk diffusion of somatodendritically released oxytocin from the supraoptic or paraventricular nucleus of the hypothalamus may be influencing extracellular OT concentration in the Nacc. PVN= paraventricular nucleus of the hypothalamus. PP= posterior pituitary

Based on the tract tracing studies discussed above, we hypothesize that many of the OT projections coursing through the forebrain are projections from magnocellular neurons, or collateral of magnocellular axons projecting to the posterior pituitary. In the mouse, a minority of PVN magnocellular axons produce collaterals around the level of the fornix that turn anteriorly and become perpendicular to the section, exactly as would be expected if going to the NAcc (Hatton, Cobbett et al. 1985). In addition, axon collaterals from the magnocellular SON has also been reported (Mason, Ho et al. 1984). In fact, it was noted thirty years ago that the multipolarity of neurosecretory cells, containing more than one axon, would allow for simultaneous release from the pituitary and extrahypothalamic sites (Buijs and Swaab 1979). This view, however, has not gained wide acceptance in the field. If the prairie accumbal OT fibers are indeed collaterals of magnocellular hypothalamic neurons, this would provide a direct mechanism for coordination of central release deep in the forebrain with peripheral release under the appropriate physiological conditions, such as vaginocervical stimulation during mating or parturition, or sensory stimulation during suckling. Coordinated release has been suggested for other neuropeptides to aid in body temperature regulation (Buijs, De Vries et al. 1983). Indeed, it is possible that extracelluar OT in the NAcc reflects a combination of somatodendritic release within both the PVN and SON that diffuses to the NAcc, and *en passant* or terminal release from OT fibers located in the NAcc and originating from PVN and SON. These multiple modes of central release of OT may explain high temporal and spatial resolution and a theoretically unlimited variability in OT signaling modes (Landgraf and Neumann 2004). While central and peripheral release patterns may

be coordinated to trigger synergistic effects, local release independent of peripheral secretion may also be possible (Neumann, Ludwig et al. 1993; Landgraf and Neumann 2004; Ludwig and Leng 2006).

4.8 FINAL CONCLUSIONS

OT has a variety of roles in affiliation and cognition in males and females of both rodents and human. Voles are unique in that they are rodents with complex social behavior. They continue to be a prove themselves as a valuable tool for investigating the role of OT in promoting affiliation and are even able to inform us on the mechanism by which OT enhances social cognition in the human brain. Results from chapter 2 show that it is possible for subtle differences in receptor level to translate into individuality in behavior, in voles and therefore perhaps humans as well. This dissertation highlights the need for further study in the basic neuroanatomical connections thought to be understood. Chapter 3 showed evidence that magnocellular neurons can contribute to the central release of OT from axons, in addition to the established mode of dendritic release. This opens a new era in how OT controls maternal behaviors with lactation, and partner bonding behavior with mating, by synchronizing systemic and peripheral release.

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APPENDIX A

Takayanagi Y, Yoshida M, Bielsky IF, Ross HE, Kawamata M, OnakaT, Yanagisawa T, Kimura T, Matzuk MM, Young LJ, and K Nishimori. 2005. Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. Proc Natl Acad Sci U S A. Nov 1;102(44):16096-101. Heather E. Ross contributed the pup ultrasonic vocalization and social discrimination data (Figure 4).

A.1 ABSTRACT

The oxytocin receptor (OXTR) and its ligand, oxytocin (OXT), regulate reproductive physiology (i.e., parturition and lactation) and sociosexual behaviors. To further define the function of OXTR, we generated mice with a null mutation in the *Oxtr* gene (*Oxtr*$^{-/-}$). *Oxtr*$^{-/-}$ mice were viable and had no obvious deficits in fertility or reproductive behavior. *Oxtr*$^{-/-}$ dams exhibited normal parturition, but demonstrated defects in lactation and maternal nurturing. Infant *Oxtr*$^{-/-}$ males emitted fewer ultrasonic vocalizations than wild-type littermates in response to social isolation. Adult *Oxtr*$^{-/-}$ males also showed deficits in social discrimination and elevated aggressive behavior. OXT-deficient (*Oxt*$^{-/-}$) males from *Oxt*$^{-/-}$ dams, but not from *Oxt*$^{+/-}$ dams, showed similar high levels of aggression. These data suggest a developmental role for OXTR in shaping adult aggressive behavior. Our study demonstrates that OXTR plays a critical role in regulating several aspects of social behavior, and may have important implications for developmental psychiatric disorders characterized by deficits in social behavior.

A.2 INTRODUCTION

Oxytocin (OXT), a nonapeptide hormone, was the first peptide hormone to have its structure determined and the first to be chemically synthesized in a biologically active form (1, 2). OXT is produced primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (3) and secreted mainly from the posterior pituitary gland. In addition, OXT fibers project to various brain regions (4) where OXT functions as a neurotransmitter or neuromodulator. Besides its classical functions, i.e., induction of labor and milk ejection, OXT plays an important role in social behavior (i.e., sexual behavior, maternal behavior, affiliation and social memory), the estrous cycle, penile erection, and ejaculation (4-7).

The actions of OXT are mediated via binding to the oxytocin receptor (OXTR). OXTR contains seven transmembrane domains and belongs to the class 1 family of G protein-coupled receptors. In response to ligand binding, OXTR mainly leads to stimulation of phospholipase C by interacting $G\square_{q/11}$. OXTR is widely expressed in the reproductive tract (i.e., uterus, mammary gland, ovary, testis, and prostate), brain, and kidney in mammals (4).

OXT-deficient ($Oxt^{-/-}$) mice displayed impairments in milk ejection (8, 9) and social recognition (10), but no obvious defects in parturition (8). Although OXT is a strong uterotonin and is considered to drive parturition, our observations unexpectedly and clearly prove that OXT is not essential for labour in mice. In contrast, *Oxtr* mRNA expression in the uterus is upregulated dramatically at term (11), uterine sensitivity to

OXT increases just before parturition (12, 13), and OXTR antagonists delay parturition in mice (14), suggesting an indispensable role for OXTR in mouse parturition. We therefore suspected that a functional redundancy supporting the OXT system might compensate for the defective *Oxt* gene in *Oxt*$^{-/-}$ mice. Furthermore, growing evidence suggests a role for OXTR in modulation of social behaviors. To further study the function of OXT/OXTR system, we generated mice lacking OXTR (*Oxtr*$^{-/-}$) and evaluated the reproductive functions including parturition and sociosexual behaviours. In addition, we further compared their maternal and male aggressive behaviors with that of previously generated ligand *Oxt*$^{-/-}$ mice.

A.3 MATERIALS AND METHODS

Generation of *Oxtr*$^{-/-}$ Mutant Mice and Genotyping**.** To construct the targeting vector, mouse 129/Sv strain-derived genomic clones (11) were used. The targeting vector was designed to substitute exons 2 and 3, containing most of the *Oxtr* coding region with the same sequence and phosphoglycerate kinase promoter-neomycin resistance cassette (PGK-Neo) flanked by three *lox*P sites (Fig. 1*A*). A 6.1 kb *Xho*I-*Bam*HI fragment was used as the 5' homology region; a 2.2 kb *Bam*HI-*Sph*I fragment containing exons 2 and 3 was inserted between two *lox*P sites; and a 2.8 kb *Sph*I-*Sph*I fragment was used as the 3' homology region. An MC1 promoter-herpes simplex virus-thymidine kinase cassette (MC1-TK) was used for negative selection. We linearized this construct with *Sal*I and electroporated it into E14TG2a embryonic stem (ES) cells. G418 and FIAU (Moravek Biochemicals) doubly resistant clones were screened by Southern blot analysis. We generated chimeric mice by microinjection of heterozygous ES clones into C57BL/6J blastocysts. We mated

chimeric males to CAG-*cre* transgenic female mice (15) to yield *Oxtr*$^{+/-}$ mice. Offspring from intercrosses of heterozygous littermates were genotyped by Southern blot analysis (Fig. 1*B*). The care and use of mice in this study was approved by the Institutional Animal Care and Use Committee of Tohoku University.

Mice. In addition to *Oxtr*$^{-/-}$ mice generated in the present study, we also used *Oxt*$^{-/-}$ mice generated previously (8), in order to distinguish between the role of the ligand, OXT, and that of the receptor, OXTR, in the regulation of maternal and aggressive behaviors. *Oxtr*$^{-/-}$, *Oxtr*$^{+/-}$ and *Oxtr*$^{+/+}$ mice were maintained on a mixed 129 x C57BL/6J background. *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice used in this study were descended from a mixed 129 x C57BL/6J strain as previously described (8). In the maternal behavior and aggressive behavior tests, we used *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ mice, and *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice from heterozygous intercrosses. For analysis of the potential effects of maternal OXT, we also used intercrosses of homozygous *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice to generate *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice from homozygous parents followed by cross-fostering with C57BL/6J females.

Southern Blot and Northern Blot Analysis**.** We isolated genomic DNA from mouse tail and poly(A)$^{+}$ RNA from tissues using TRIzol Reagent (Invitrogen) and oligotex-dT30/super (Takara). For Southern blot analysis, about 3 μg of DNA, digested with *Sac*I, was loaded on 1% agarose gels. For Northern blot analysis, equal amounts of RNA (uterus, 2 μg; brain, 5 μg and 20 μg per lane) were loaded on formaldehyde agarose gels. These were subjected to electrophoresis, and transferred to Byodyne B nylon membranes (Pall). The membranes were hybridized to ^{32}P-labeled probes. Probes for Southern blots were obtained by digestion with restriction enzyme, and probes for Northern blots were obtained by reverse transcriptase-mediated polymerase chain reaction (RT-PCR)

[*Oxtr* probe A and B, spanning from 170 nucleotides (nt) to 429 nt (259 base pairs (bp)) and from 500 nt to 787 nt (288 bp) in the mouse *Oxtr* mRNA (GenBank accession number D86599) coding region, respectively; glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) probe, spanning from –8 nt to +1065 nt (1073 bp) of the mouse *Gapd* mRNA (GenBank accession number NM_008084) coding region] and labeled with Megaprime DNA labeling systems (Amersham Biosciences) with [^{32}P]-dCTP. Membranes were stripped and reprobed for *Gapd* to ensure equal loading.

Maternal Behavior Test**.** Maternal behavior was tested on both postpartum and virgin females. All postpartum females (10-15 weeks old) were individually housed once pregnant. Nest material was provided 1 day prior to testing. Births were recorded each morning. Each new mother was observed for 20 minutes with minimal disturbance. 1 hour after the removal of her pups, each female was exposed to three 1-3-day-old foster pups from an *Oxtr*$^{+/+}$ dam, which were placed in each corner of the cage distant from her nest. During the next 30 minutes, each female was continuously observed, and the following data points were recorded: latency to sniff and retrieve each pup, and latency and duration of crouching over all three pups in the nest. Different pups were used in each test. All virgin females (7-9 weeks old) were individually housed for 2 days prior to testing. For 2 consecutive days, each female was exposed to three 1-3-day-old foster pups for 30 minutes as described above. Only the second test was scored.

Ultrasonic Vocalization Test and Measurement of Locomotor Activity in Pups**.** 7-day-old male pups from 7 *Oxtr*$^{+/-}$ breeding pairs were tested between 1-4 hours before the dark phase. The parents were removed from the home cage 20 minutes prior to testing and the cage was placed on a heated surface at 35°C until testing was completed. Each

pup was placed into a Plexiglass recording chamber (40 x 40 cm) for 3 minutes. Vocalizations were recorded using an ultrasonic detector and analyzed as WAV files (16). The number of 4.5 x 4.5 cm grids crossed during the test was also noted.

Social Discrimination Test**.** Prior to testing, males (4-7 months old) were individually housed and exposed to ovariectomized females for varying periods of time (15-40 minutes) for 2 days in order to reduce mating bouts during test sessions. The social discrimination test (17) consisted of placing an ovariectomized C57BL/6J stimulus female into the home cage of the experimental male for 5 minutes. After a 30 minute interexposure interval, the female (SAME) was then placed back into the cage along with another C57BL/6J ovariectomized stimulus female (NOVEL) for 5 minutes. The amount of time spent investigating each females anogenital or perioral area was then scored from the video-recorded session. Interactions including sexual behaviour were excluded from the analysis. Females were only exposed to one male per day to reduce male odour contamination.

Resident-Intruder Aggression Test**.** 10-week-old resident males were individually housed for about 4 weeks before testing. 10-week-old C57BL/6J mice, housed in groups, were used as intruders. Two tests of 5 minutes each with a 5 minute interval were performed. New intruder mice were used in each test. The following data points were recorded: attack duration, frequency and latency.

For further details, see *Supporting Text*, which is published as supporting information on the PNAS web site.

A.4 RESULTS

Generation of *Oxtr*$^{-/-}$ Mutant Mice**.** To analyze the roles of OXTR in the reproductive and central nervous systems, we generated OXTR-deficient mice by gene targeting (Fig.1*A*). The disruption of the *Oxtr* gene locus (Fig. 1*B*), the absence of *Oxtr* transcripts (Fig. 1*C*), and the absence of OXTR binding (Fig. 1*D*) in *Oxtr*$^{-/-}$ mice confirmed that the recombined allele is null. A 1:2:1 Mendelian distribution of the progeny was observed [(*Oxtr*$^{+/+}$:*Oxtr*$^{+/-}$:*Oxtr*$^{-/-}$), 65:133:69 (males); 79:133:78 (females)].

Reproductive Functions in *Oxtr*$^{-/-}$ Mice**.** In *Oxtr*$^{-/-}$ mice, OXT-induced contractions in pregnant myometrium were not evident (Fig. 2*A*). Since arginine vasopressin (AVP), another nonapeptide hormone synthesized in the PVN and SON and secreted from the posterior pituitary, acts as a partial agonist of OXTR (18, 19) and also stimulates uterine contractions (20, 21), we also examined AVP-induced myometrium contractions in *Oxtr*$^{-/-}$ mice. The myometrium of *Oxtr*$^{-/-}$ mice did not respond to AVP (Fig. 2*B*). Receptor autoradiography confirmed the absence of OXTR binding binding in pregnant myometrium of *Oxtr*$^{-/-}$ mice (Fig. 1*D* and *Supporting Text* which are published as supporting information on the PNAS web site). We previously reported the inability to detect *Avpr1a* mRNA in the uterus of wild-type mouse using RT-PCR (21). Taken together, these findings prove that AVP-induced uterine contractions in pregnant wild-type mice are mediated solely by OXTR, consistent with our previous report (21).

To examine reproductive function, *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ mice were mated in all possible combinations. Contrary to our prediction, the onset and the duration of labor were normal in *Oxtr*$^{-/-}$ females (Fig. 2*C*). Furthermore, *Oxtr*$^{-/-}$ mice exhibited normal rates of

mating and pregnancy, and litter sizes, demonstrating that OXTR is not essential for either male or female reproductive function. However, all offspring from *Oxtr*$^{-/-}$ dams died within 24hr after birth, regardless of the genotype of the offspring (Fig. 2*C*). This mortality was likely due to defects in lactation since milk was not observed in the digestive tract of pups from *Oxtr*$^{-/-}$ dams. All offspring from *Oxtr*$^{-/-}$ mice were successfully fostered to *Oxtr*$^{+/+}$ mice, and thus the survival defect in the pups lies entirely with the *Oxtr*$^{-/-}$ dams. Histological analysis of mammary glands in *Oxtr*$^{-/-}$ females indicated that the development of mammary tissues during gestation and milk production were normal, except accumulation of milk in ducts of the postpartum mammary glands (data not shown). Thus, *Oxtr*$^{-/-}$ females failed to lactate, similar to *Oxt*$^{-/-}$ females (8).

Oxtr$^{-/-}$ Female Mice Display Defects in Maternal Nurturing**.** Since OXT plays a role in maternal behavior (7), we examined maternal behavior in *Oxtr*$^{-/-}$ mice. Initially, the behavior of postpartum females was observed for 20 minutes in their home cages. Both *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ females built nests and spent the majority of this period crouching over their pups ($P > 0.05$), but pups of *Oxtr*$^{-/-}$ females were often found scattered around the cage ($P < 0.01$) (Fig. 3*A*). Following this initial observation, we monitored the dams' responses to three pups placed in different corners of the cage for 30 minutes. *Oxtr*$^{-/-}$ dams displayed a significantly longer latency to retrieve the pups (first retrieval, $P > 0.05$; second retrieval, $P < 0.05$; and complete retrieval, $P < 0.01$) or to crouch over the pups ($P < 0.01$), and spent less time crouching over the pups ($P < 0.05$), than *Oxtr*$^{+/+}$ females (Fig. 3*B*). The impairment of retrieval was not due to the failure of the mother to detect the pups, since latency to approach and sniff their offspring was similar to wild-type mothers (11.7 □ 5.0 s compared with 9.9 □ 4.2 s; $P > 0.05$). Additionally, virgin *Oxtr*$^{-/-}$ females

displayed a similar phenotype (Fig. 3*C*), suggesting that OXTR is required for nurturing responses to pups outside the physiological context of pregnancy and parturition. Interestingly, we also demonstrated that both postpartum and virgin *Oxt*$^{-/-}$ females displayed normal maternal behavior (data not shown), consistent with an earlier study (22). This could be explained by other ligands, such as AVP, activating this receptor.

The decrease in maternal behavior of postpartum *Oxtr*$^{-/-}$ females could be explained as reflection of the inability to lactate. However, our data showed that virgin *Oxtr*$^{-/-}$ females that have not experienced lactation, displayed an impairment of maternal behavior (Fig. 3*C*), and *Oxt*$^{-/-}$ females showed normal maternal behavior despite their inability to lactate (data not shown). These results strongly suggest that any deficits in maternal behavior in these mice would be a clear indication of a specific behavioral deficit.

Decreased Ultrasonic Vocalizations and Increased Locomotor Activity in Infant *Oxtr*$^{-/-}$ Males**.** Next, we examined isolation-induced ultrasonic vocalizations and locomotor activity of infant *Oxtr*$^{-/-}$ males. *Oxtr*$^{-/-}$ males, like *Oxt*$^{-/-}$ males (16), emitted significantly less calls than did wild-type littermates ($P < 0.05$) (Fig. 4*A*), while displaying significantly higher levels of locomotor activity during the test ($P < 0.05$) (Fig. 4*A*). These results suggest that perhaps *Oxtr*$^{-/-}$ males are less distressed by social isolation and shift their behavior toward more exploratory activity than do wild-type littermates.

Social Amnesia in *Oxtr*$^{-/-}$ Mice**.** Since *Oxt*$^{-/-}$ mice display social amnesia (10), we examined social discrimination in adult *Oxtr*$^{-/-}$ males. Males were initially exposed to an ovariectomized C57BL/6J female. After a 30 minute separation, the male was simultaneously exposed to this same female and a novel female of the same strain. As

expected, *Oxtr*$^{+/+}$ males spent significantly more time investigating the novel compared to the familiar female ($P < 0.05$) (Fig. 4*B*), and therefore were able to discriminate between the two. However, *Oxtr*$^{-/-}$ males spent a similar amount of time investigating both females ($P > 0.05$) (Fig. 4*B*) suggesting an impairment of social discrimination. There was no difference between the two genotypes in the amount of time spent investigating the initial female ($P > 0.05$), indicating that the *Oxtr*$^{-/-}$ males' deficit was not due to a difference in exposure time or motivation to investigate a female (data not shown). However, *Oxtr*$^{-/-}$ males were able to discriminate outbred CD-1 stimulus females (data not shown), suggesting that the deficit represents an impairment rather than a complete disruption in social recognition.

Oxtr$^{-/-}$ Males Display High Levels of Aggression Due To the Lack of OXTR Activation During Prenatal Development**.** Since we observed more wounded mice in group-housed males from cages containing *Oxtr*$^{-/-}$ mice than in cages containing only *Oxtr*$^{+/+}$ mice (Fig. 5*A*), we assessed aggressive behavior using the resident-intruder test. *Oxtr*$^{-/-}$ resident males attacked the intruder with shorter latency ($P < 0.05$), for longer duration (test1, $P < 0.05$; and test2, $P > 0.05$), and with higher frequency (test1, $P < 0.05$; and test2, $P < 0.05$) compared to *Oxtr*$^{+/+}$ residents (Fig. 5*B*). In contrast, in adjacent cages containing male *Oxt*$^{-/-}$ mice, the rate of wounded mice was similar to that in *Oxt*$^{+/+}$ mice (Fig. 5*A*). Furthermore, aggressive behavior of *Oxt*$^{-/-}$ mice in the resident-intruder test was indistinguishable from *Oxt*$^{+/+}$ males (Fig. 5*C*).

To investigate this discrepancy in aggression phenotypes between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice, we examined possible compensatory effects of the *Oxtr* mutation. OXTR and AVPR1a autoradiography in the brain showed no OXTR binding in *Oxtr*$^{-/-}$ mice and the density of

AVPR1a -binding was similar between *Oxtr*$^{+/+}$ (5826 ± 602.3 dpm/mg) and *Oxtr*$^{-/-}$ (4787 ± 463 dpm/mg) mice (Fig. 6*A* and *B*). Northern blot analysis failed to detect any RNA products with sequence similarity to *Oxtr* in the brain (Fig. 6*C*). In addition, *Oxtr*$^{-/-}$ mice showed no differences in *Oxt* and *Avp* mRNA expression in the hypothalamus (data not shown), pituitary OXT (*Oxtr*$^{+/+}$, 202.6 ± 10.7 ng/pituitary, n=9; *Oxtr*$^{-/-}$, 222.8 ± 9.3 ng/pituitary, n=7) and AVP (*Oxtr*$^{+/+}$, 471.6 ± 70.9 ng/pituitary, n=9; *Oxtr*$^{-/-}$, 453.3 ± 28.3 ng/pituitary, n=7), plasma OXT (*Oxtr*$^{+/+}$, 36.3 ± 5.2 pg/ml, n=5; *Oxtr*$^{-/-}$, 39.3 ± 6.1 pg/ml, n=5), or plasma testosterone levels (*Oxtr*$^{+/+}$, 1329.4 ± 277.7 pg/ml, n=10; *Oxtr*$^{-/-}$, 1148.7 ± 225.5 pg/ml; n=10). These results indicated that there were no apparent dysregulations of AVP, AVPR1a or testosterone, each of which are known to influence aggression.

OXT in the dam can transfer through the placental barrier (23-25), and *Oxtr* mRNA is present in the mouse brain during embryonic development (Fig. 6*D*). Furthermore, perinatal injections of OXT in prairie voles have an impact on adult behavior (26). Therefore, we hypothesized that *in utero* exposure to OXT might have rescued the aggression phenotype in *Oxt*$^{-/-}$ mice derived from heterozygous matings. Therefore, we analyzed aggression in *Oxt*$^{-/-}$ mice generated from homozygous matings (Fig. 6*E*), creating an OXT-free developmental environment. Like *Oxtr*$^{-/-}$ males, *Oxt*$^{-/-}$ males generated from homozygous matings exhibited highly aggressive behavior, as previously reported (16). Our findings indicate that embryonic exposure to OXT affects the development of aggression in adulthood, although other intrauterine factors in *Oxt*$^{-/-}$ dams could also influence aggression. However, since increased aggression is seen in *Oxtr*$^{-/-}$ mice from *Oxtr*$^{+/-}$ mothers, it suggests that the defect lies in the pup.

A.5 DISCUSSION

This study provides a comprehensive analysis of mice lacking the *Oxtr* gene. Although OXT has been used to induce or augment labor in humans, and the OXT antagonist delays labor in wild-type outbred mice (14), parturition is initiated and proceeds normally in $Oxtr^{-/-}$, similar to $Oxt^{-/-}$ mice (8). Although this unexpected phenotype may be due to functional redundancy in the OXT signaling system or a compensatory effect resulting from the absence of *Oxtr* throughout development, it is clear that the OXT signaling pathway is not essential for normal parturition in mice.

The impairment in social discrimination in $Oxtr^{-/-}$ in our study is consistent with previous results from ligand knockout mice. $Oxt^{-/-}$ females as well as $Oxt^{-/-}$ males also show significant deficit in social recognition (10, 27). The comparison of social discrimination in $Oxtr^{-/-}$ mice between genders would be important for understanding the regulation of social discrimination that is related to estrogen, gonadal steroid (27).

In contrast to our results, it was reported by another other group that $Oxt^{-/-}$ males from $Oxt^{+/-}$ dams displayed reduced aggression (28). This contradiction could be caused by differences in the targeting construct used, which did not result in the complete loss of Oxt peptide, or in the testing paradigm used (9, 28). The study reporting decreased male aggression performed the tests in a neutral arena, while our study and others reporting increased aggression used a resident intruder paradigm. Therefore the changes in aggression due to disruption of the OXT system may be context dependent"

In both aggressive behavior and maternal behavior, phenotypes were different between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice. *Oxtr*$^{-/-}$ males from *Oxtr*$^{+/-}$ dams displayed elevated aggressive behavior (Fig. 5*B*). *Oxt*$^{-/-}$ males from *Oxt*$^{-/-}$ dams, but not from *Oxt*$^{+/-}$ dams, displayed similar high levels of aggression (Fig. 5*C* and 6*E*). This indicates that *in utero* exposure to maternal OXT may affect adult aggressive behavior. In addition, maternal behavior in *Oxtr*$^{-/-}$ females from *Oxtr*$^{+/-}$ dams was impaired (Fig. 3*A*-*C*), but *Oxt*$^{-/-}$ females from *Oxt*$^{+/-}$ or *Oxt*$^{-/-}$ dams showed normal maternal behavior (data not shown). These results suggest that although prenatal activation of OXT/OXTR system may significantly affect adult aggressive behavior in males, it is not sufficient for the establishment of maternal behavior. Thus, these causative mechanisms seem to be different between aggressive behavior and maternal behavior. We speculate that the difference in phenotypes of maternal behavior between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice can be explained by a possibility that other ligands than OXT that activate OXTR can compensate for the defect of the *Oxt* gene.

Semi-natural environment-housed *Oxt*$^{-/-}$ females from *Oxt*$^{+/-}$ dams showed high levels of aggression and infanticidal behaviors, unlike cage-housed *Oxt*$^{-/-}$ females from *Oxt*$^{+/-}$ dams (29). These results indicate that postnatal environment also affects the behavior via OXT/OXTR system. In semi-natural environment, phenotypes of *Oxtr*$^{-/-}$ mice, such as impaired maternal behavior or elevated aggressive behavior might be altered, and *Oxt*$^{-/-}$ males from *Oxt*$^{+/-}$ dams might be aggressive. These studies suggest an important interaction between environment and the OXT/OXTR system in regulating social behavior.

Our observations demonstrated that the OXT/OXTR system plays an important role in regulating social behavior, and might have important implications for human behavioral disruptions. Further comprehensive investigation of *Oxtr*$^{-/-}$ mice may provide new insight into the neurobiological mechanisms resulting in psychiatric disorders associated with disruptions in social behavior, including autism.

Acknowledgments

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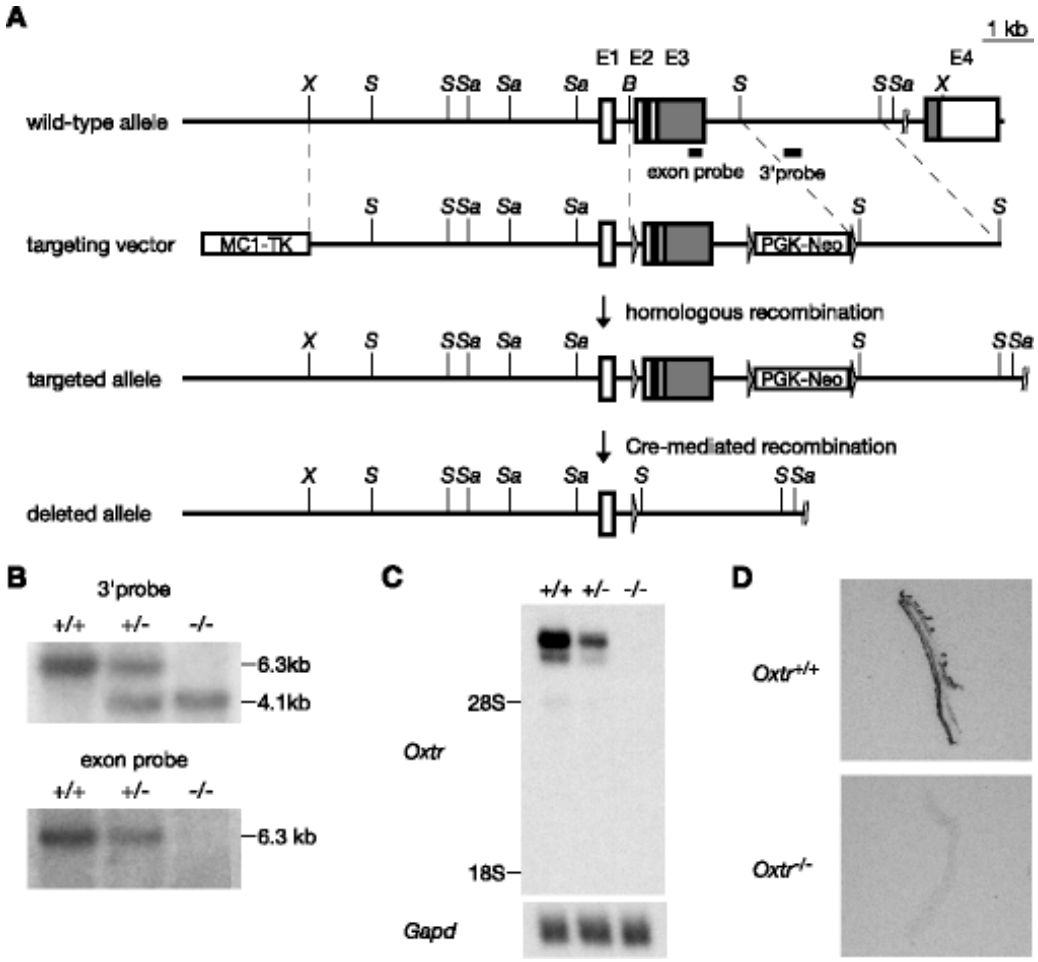
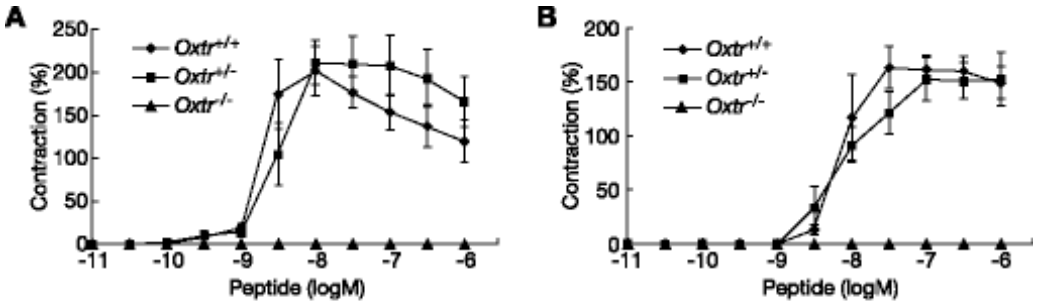


Fig. 1. Generation of *Oxtr* $^{-/-}$ mice. (*A*) The *Oxtr* locus and gene targeting constructs. Exons (E) are indicated by boxes (white boxes, 5' and 3' UTRs; gray boxes, coding regions). Positions of restriction enzyme sites and the probes used for Southern blot analysis are shown (*X*, *Xho*I; *S*, *Sph*I; *Sa*, *Sac*I; *B*, *Bam*HI). The *lox*P sites are represented by arrowheads (not to scale). PGK-Neo, phosphoglycerate kinase promoter-neomycin resistance cassette; MC1-TK, thymidine kinase cassette. (*B*) Southern blot analysis of genomic DNA from littermate progeny from *Oxtr* heterozygote crosses. *Sac*I-digested tail DNA was hybridized with the radiolabeled probes indicated in (*A*). (*C*) Northern blot analysis of poly(A)$^+$ RNA from the pregnant uteri (day 19 of gestation) of *Oxtr*$^{+/+}$, *Oxtr*$^{+/-}$ and *Oxtr*$^{-/-}$ mice. The blot was sequentially hybridized with *Oxtr* probe A and a *Gapd* probe. (*D*) OXTR-binding autoradiograms in the pregnant uteri (day 19 of gestation) of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice.



C

Effect of genotype on reproductive functions

Fertility and sexual behavior in male and female mice

Pairing		No. of mating trials	Mating rate (%)	Pregnancy rate (%)	Average litter size
Male	Female				
$Oxtr^{+/+}$ ×	$Oxtr^{+/+}$	79	35	71	7.5±0.7
$Oxtr^{-/-}$ ×	$Oxtr^{+/+}$	63	41	65	8.2±0.9
$Oxtr^{+/+}$ ×	$Oxtr^{-/-}$	43	42	67	8.6±0.5
$Oxtr^{-/-}$ ×	$Oxtr^{-/-}$	82	33	59	8.4±0.8

Parturition and maternal behavior in female mice

Pairing		No. of pregnant females	Onset of labor (day)	Duration of labor (hour)	Postnatal survivors per total births (%)
Male	Female				
$Oxtr^{+/+}$ ×	$Oxtr^{+/+}$	11	19.1±0.1	3.8±0.4	96
$Oxtr^{-/-}$ ×	$Oxtr^{+/+}$	10	19.2±0.1	4.8±0.9	92
$Oxtr^{+/+}$ ×	$Oxtr^{-/-}$	10	19.2±0.1	4.8±0.9	0
$Oxtr^{-/-}$ ×	$Oxtr^{-/-}$	13	19.3±0.1	3.2±0.2	0

Fig. 2. Reproductive functions in *Oxtr* $^{-/-}$ mice. (*A* and *B*) The amplitude of OXT (*A*)- or AVP (*B*)-stimulated contractions of myometrial strips isolated from pregnant mice (day 19 of gestation) of each genotype. These investigations were performed as previously described (21). (*C*) The profile of reproductive functions in $Oxtr^{+/+}$ and $Oxtr^{-/-}$ mice (male, 10-25 weeks old; female, 10-15 weeks old). Each genotype was mated and females were selected without reference to ovulatory cycle. Mating rate denotes the ratio of plugged females to matings and pregnancy rate denotes the ratio of pregnant females to plugged females. The morning of finding the copulation plug was designated as day 0.5 of gestation. The data represents mean ± SEM.

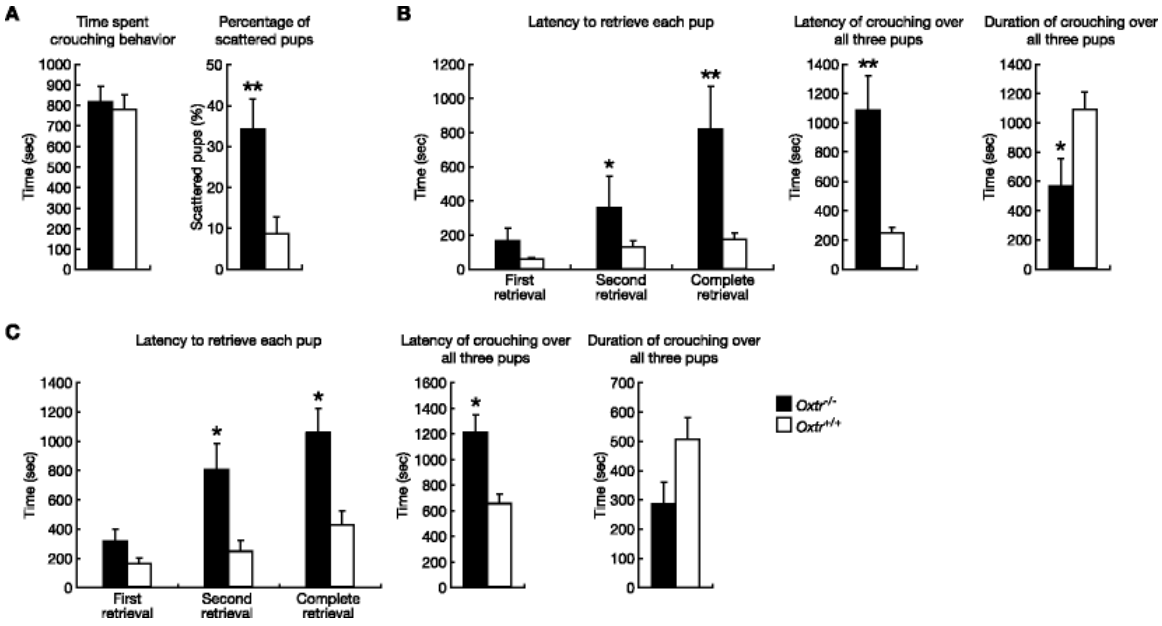


Fig. 3. Maternal nurturing in female *Oxtr* $^{-/-}$ mice. (*A*) Observation of newly postpartum *Oxtr*$^{-/-}$ (n=9) and *Oxtr*$^{+/+}$ (n=10) females before tests for maternal behavior. Time crouching over pups and percentage of newborns scattered was recorded. (*B* and *C*) Tests for maternal behavior. Latency to retrieve each pup, and latency and duration of crouching over all three pups of *Oxtr*$^{-/-}$ (n=9) and *Oxtr*$^{+/+}$ (n=10) postpartum females (*B*), and *Oxtr*$^{-/-}$ (n=15) and *Oxtr*$^{+/+}$ (n=7) virgin females (*C*) from heterozygous intercrosses. Failure to retrieve or crouch was assigned as 30 minutes, the length of observation period. **P* < 0.05 and ***P* < 0.01 (Mann-Whitney *U*-test). Error bars, standard error.

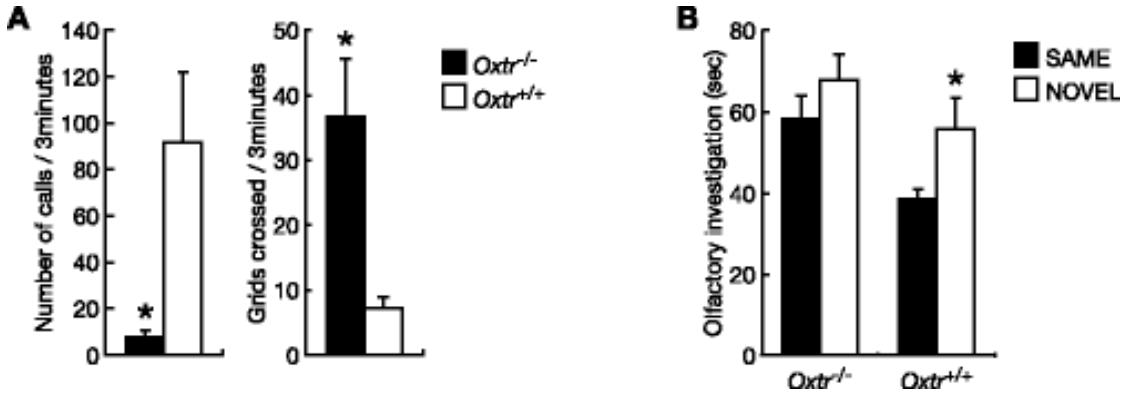


Fig. 4. Infant ultrasonic vocalization and adult social discrimination. (*A*) Measurements of social isolation-induced ultrasonic vocalizations (left) and locomotor activity (right) in *Oxtr*$^{-/-}$ (n=8) and *Oxtr*$^{+/+}$ (n=10) male pups from heterozygous intercrosses. (*B*) Test for social discrimination test. After the first exposure to a female and an interexposure interval, this female (SAME) was placed back along with another female (NOVEL). *Oxtr*$^{-/-}$ (n=14) and *Oxtr*$^{+/+}$ (n=10) males were examined for investigation times directed to the SAME or NOVEL females. *$P < 0.05$ (Mann-Whitney *U*-test). Error bars, standard error.

A

Wounded mice					
Genotypes of members in the same cage	No. of observed mice	Wounded mice	No-wounded mice	Wounded / total (%)	Mice / cage
$Oxtr^{+/+}$	37	1	36	2.7	4.1
$Oxtr^{+/+}$, $Oxtr^{+/-}$, $Oxtr^{-/-}$	54	18	36	33.3	4.0
$Oxt^{+/+}$	21	0	21	0	3.5
$Oxt^{+/+}$, $Oxt^{+/-}$, $Oxt^{-/-}$	62	2	60	3.2	4.1

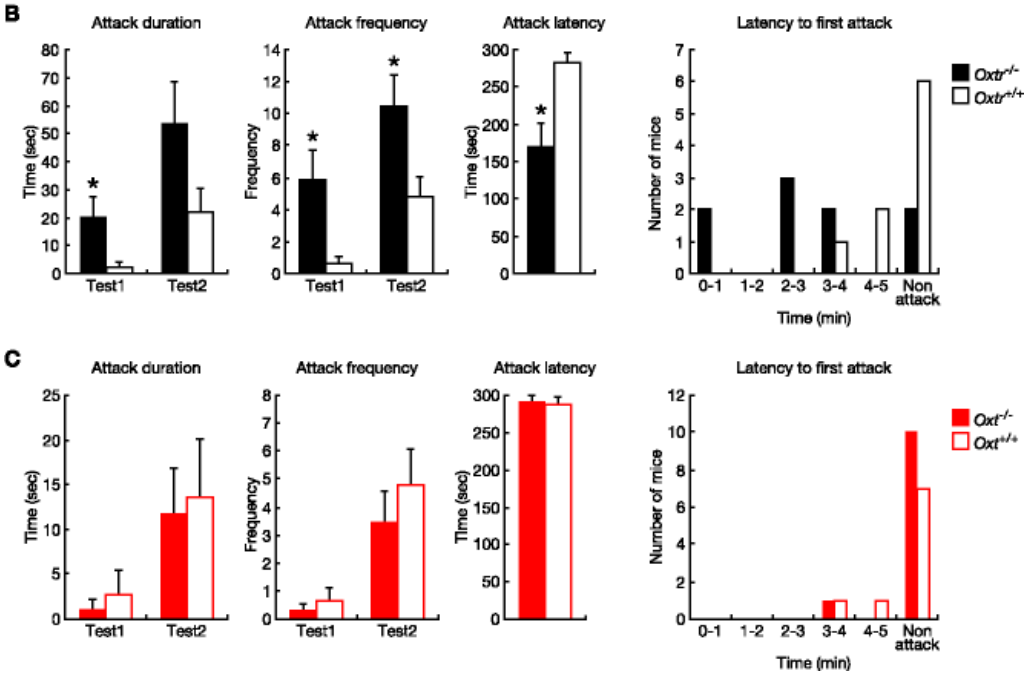


Fig. 5. Aggressive behavior as measured by the resident-intruder test. (*A*) The number of wounded mice (3-9 months old) in cages including each genotype. (*B* and *C*) Aggressive behavior of $Oxtr^{-/-}$ (n=9) and $Oxtr^{+/+}$ (n=9) mice (*B*), and $Oxt^{-/-}$ (n=11) and $Oxt^{+/+}$ (n=9) mice (*C*) from heterozygous intercrosses in the resident-intruder test. Attack duration, frequency and latency, and latency to first attack were recorded. $*P < 0.05$ (Mann-Whitney *U*-test). Error bars, standard error.

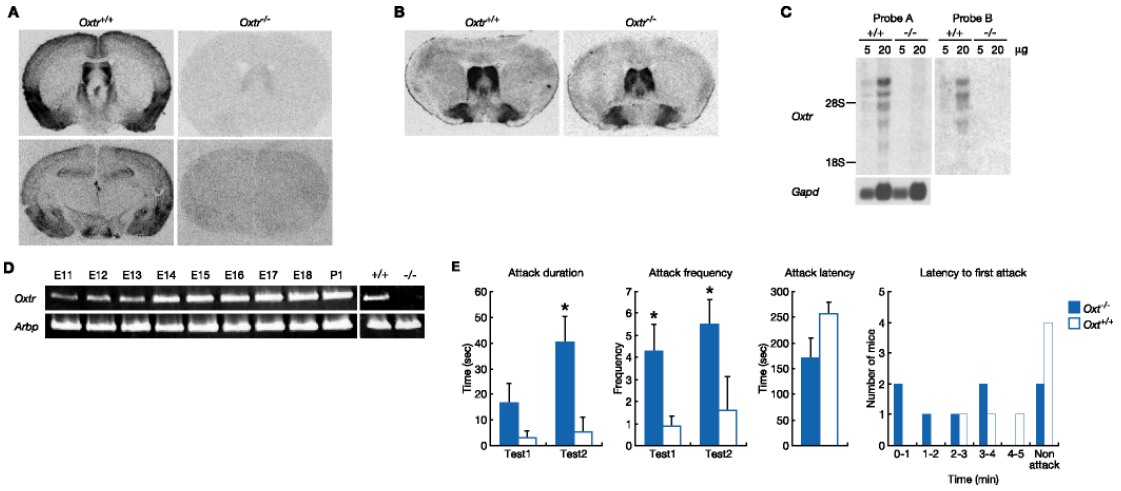


Fig. 6. Causal analysis of aggressiveness between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice. (*A* and *B*) OXTR (*A*) and AVPR1a (*B*)-binding autoradiograms in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ brain. (*C*) Northern blot analysis of *Oxtr* mRNA expression in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ brain using *Oxtr* probe A and B. The same membrane was rehybridized with a *Gapd* probe. Indicated amounts of poly(A)$^{+}$ RNA were used. (*D*) RT-PCR analysis for *Oxtr* and *Arbp*, in the brain of male fetus (C57BL/6J) and adult *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ males. (*E*) Aggressive behavior of *Oxt*$^{-/-}$ (n=8) and *Oxt*$^{+/+}$ (n=7) mice, from intercrosses of each of *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice and fostering by C57BL/6J females, in the resident-intruder test. **P* < 0.05 (Mann-Whitney *U*-test). Error bars, standard error.

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APPENDIX B

Ross HE and LJYoung. Genetic regulation of complex social behavior in a monogamous rodent. 2006. In Beyond Nature & Nurture in Psychiatry: Genes, Environment and Their Interplay. p57-65.

B.1 INTRODUCTION

Over the past decade, great strides have been made in understanding role of genes and environment in the regulation of complex behaviours. Animal models have been particularly useful for gaining insights into the neurobiological mechanisms by which genes and environment interact to influence behaviour. These insights have important implications for understanding the biological basis for individual differences in human behaviour as well as psychiatric disorders. Our research on the molecular basis of social behaviour and pair bonding in prairie voles provides an example of the power of animal models in understanding the relationship between genes, the brain, and complex behaviours.

B.2 PRAIRIE VOLES

Voles are hamster-sized rodents that are ideally suited for mechanistic studies of social behavior. Prairie voles are highly social, biparental and form life-long pair bonds between mates (Getz et al., 1981; Carter et al., 1995). This social organization is unusual among mammals since only 5% of mammalian species are monogamous (Kleiman, 1977). In contrast, montane and meadow voles, which are genetically closely related to prairie voles, are relatively asocial, do not form social bonds between mates, and the females raise their offspring alone (Young and Wang, 2004). Thus, comparative studies using vole species with different social structures provides an opportunity to investigate

the molecular, cellular, and neurobiological mechanisms regulating affiliative behaviour and social attachment. The insights gained by these studies may have important implications for psychiatric disorders characterized by deficits in social behaviour, such as autism.

In the laboratory, pair bonding is assessed using a partner preference test. In this test, a male and female prairie are housed together, during which the length of cohabitation and the presence or absence of mating can be varied. Pharmacological manipulations can also be administered during this cohabitation period. The pair is then separated and placed in a three-chambered testing apparatus in which the partner is tethered to one side chamber and a novel animal, of equal stimulus value as the partner, is tethered to the other side. The tethered animals can move around their respective cages only, while the test animal can freely explore the entire apparatus. During this three-hour test, the amount of time spent in each chamber and in contact with each partner is recorded. Generally a mated prairie vole will spend most of their time with the partner and very little time in the center or stranger's chamber. In contrast, montane and meadow voles will spend equal amounts of time with their partner and stranger; actually spending most of their time isolated in the middle (Young and Wang, 2004).

B.3 ROLE OF VASOPRESSIN IN PAIR BOND FORMATION

Pair bonds develop in both male and female prairie voles after an overnight cohabitation, and this process if facilitated by mating. Pharmacological studies have demonstrated that in the male prairie vole, the neuropeptide vasopressin stimulates the formation of the pair bond (Winslow et al., 1993). Infusion of a selective vasopressin V1a receptor

(V1aR) antagonist into the brain prevents pair bond formation after extended mating, while infusion of vasopressin facilitates pair bond formation in the absence of mating. This peptide also facilitates other behaviours associated with monogamy, including mate guarding and paternal care (Winslow et al., 1993; Wang and Insel, 1996). Vasopressin, also known as antidiuretic hormone, is produced in the paraventricular nucleus of the hypothalamus, which projects to the posterior pituitary, as well as in extrahypothalamic regions such as the medial amygdala and bed nucleus of the stria terminalis (De Vries and Buijs, 1983), which project throughout the brain. It is these extrahypothalamic projections, which are more extensive in males than in females, which are thought to regulate social behaviours.

To begin to understand the divergence in pair bonding behaviour between monogamous and nonmonogamous vole species, we performed comparative studies of the brain vasopressin system in these species. Immunocytochemical studies revealed few if any differences in the distribution of vasopressin-producing cells or their projections (Wang et al., 1996). In contrast, there are significant species-differences in the neuroanatomical distribution of V1aR. For example, the ventral pallidum, medial amygdala, and mediodorsal thalamus have a high density of receptors in the monogamous species but virtually none in the promiscuous species (Insel et al., 1994). The begin to identify which brain regions might be involved in pair bonding, we examined the distribution of V1aR in several other monogamous and non-monogamous species which are not related to voles. The monogamous *Peromyscus californicus*, or California mouse, also has high levels of V1aR in the ventral pallidum, while the related P. *luecopus* does not. Additionally, the monogamous common marmoset has a high density of receptors in this region, while the

rhesus macaque does not (Young, 1999). Thus at least among these species, there appears to be an association between V1aR binding in the ventral pallidum and pair bonding.

To directly identify which brain are critical for the pair bonding process, we performed site specific infusions of V1aR antagonist and examined its effects on partner preference formation in male prairie voles. A dose of antagonist which was ineffective when given ICV, was targeted to the ventral pallidum, medial amygdala and the mediodorsal thalamus. Only antagonist injections into the ventral pallidum were able to completely block pair bond formation (Lim and Young, 2004), demonstrating the critical role of this region in pair bonding. The ventral pallidum is a major output of the nucleus accumbens, and both of these areas are critical components of the mesolimbic dopamine reward pathway. *In vivo* microdialysis studies have also demonstrated that there is an increase in extracellular vasopressin in the ventral pallidum in samples collected after ejaculation (Morales et al., 2004).

In order to understand how vasopressin may facilitate pair bonding, we turn to behavioural studies in knockout mice, which suggest that vasopressin and the V1aR is critical for the processing social olfactory cues. V1aR knockout mice mouse have social amnesia and do not recognize individuals that they have previously encountered (Bielsky et al., 2004). This deficit is selective to social recognition, since these mice easily learn to recognize non-social odours and perform normally in other cognitive tasks. Furthermore, increasing V1aR in a wildtype mouse using viral vector gene transfer enhances social recognition abilities (Bielsky et al., 2005).

B.4 ROLE OF DOPAMINE IN PAIR BOND FORMATION

In addition to vasopressin, dopamine also plays a critical role in pair bond formation. Blocking the dopamine D2 receptor in the nucleus accumbens prevents pair bond formation (Gingrich et al., 2000; Aragona et al., 2003). In fact, both dopamine D2 and V1aR must be simultaneously activated in order for a pair bond to form in a male prairie vole.

B.5 NEUROCHEMICAL MODEL OF PAIR BOND FORMATION

Based on this information, we have developed a working model of pair bond formation that incorporates all of these elements (Young and Wang, 2004). During courtship and mating, dopamine from ventral tagmental area neurons projecting to nucleus accumbens is released, resulting in an activation of the reward circuitry. Thus mating is rewarding. During the mating bout, the male is also processing olfactory stimuli from the female. Olfactory information is transmitted from the olfactory bulbs to the medial amygdala, an area where vasopressin is synthesized. The vasopressinergic neurons of the medial amygdale project to the ventral pallidum and release vasopressin at the time of ejaculation. The ventral pallidum is the major output of the nucleus accumbens, and is an area involved in reward and conditioning. Since vasopressin is involved in processing olfactory signatures used for individual recognition, we hypothesize that the concurrent activation of the dopamine reward system of the accumbens, and the activation of V1aR in the ventral pallidum results in an association between the reward of sex and the olfactory signatures of the partner, culminating in a conditioned partner preference. Since non-monogamous animal do not have V1aR in the ventral pallidum, this specific

association does not develop. Thus while sex is likely rewarding in both monogamous and non-monogamous species, only in species with high levels of V1aR in the reward circuit does the specific olfactory signature of the partner become associated with the partner.

B.6 GENETIC CONTROL OF PAIR BONDING

If this hypothesis is correct, it suggests a relatively simple evolutionary mechanism where a mutation in a single gene can have a profound effect on complex behaviour. To test this hypothesis directly, we used an AAV viral vector to increase V1aR expression in the ventral pallidum of the nonmonogamous meadow vole. Meadow voles injected into the ventral pallidum with an AAV vector containing the prairie vole V1aR under the control of the neuron-specific enolase promoter expressed high levels of V1aR binding in this region and from pair bonds with their partner after a 24 hour cohabitation . In contrast, control animals in which the injections fell outside of the ventral pallidum, or that received a control vector, did not form pair bonds. This remarkable study demonstrates that altering the expression of a single gene in a single brain region can have a profound effect on complex behaviour. This is likely because the circuitry involved in pair bonding, ie the reward circuit, is present in all species. Expressing the V1aR gene in this regions simply links this pre-existing circuitry to a system involved in processing of social cues.

The species differences in V1aR binding appear to be due to differences in the regulation of gene expression, since the distribution of V1aR mRNA is similar to the distribution of V1aR protein in both species (Young et al., 1997). To identify the potential mechanisms

resulting in this differential gene regulation, we compared the sequence of the prairie and montane vole V1aR gene, including the 5' flanking region. Although the coding regions and most of the 5' flanking region are nearly identical, there an insertion of a 500 bp highly repetitive element, or microsatellite, approximately 700 base pairs upstream of the transcription start site in the prairie vole gene which is nearly absent in the montane vole and meadow vole (Young et al., 1999). It is clear that the sequences surround the coding region determine region-specific expression, since a transgenic mouse harbouring the prairie vole V1aR gene displayed a pattern of V1aR binding that was similar to that of the prairie vole (Young et al., 1999). Therefore, we hypothesised that the expansion of the microsatellite could be enough to alter the V1aR expression pattern of the brain, resulting in the differences in behaviour (Young et al., 1999). In fact, when we compared the V1aR promoter in two other vole species we found that the monogamous pine vole, like the prairie vole, also has the microsatellite insertion, while the nonmonogamous meadow does not. Therefore, at least among these four vole species there seems to be an association between social structure and the presence of the microsatellite.

To directly test the hypothesis that the microsatellite itself might alter gene expression, we used a cell transcription reporter assay. Specifically, the sequences before and after the prairie vole microsatellite were amplified using PCR and spliced together incorporating a restriction enzyme site where the microsatellite would naturally be located. This entire construct, which is essentially the prairie vole promoter without a microsatellite, was placed upstream of a luciferase reporter. With this design, any microsatellite can be added back into the construct to study the effect of this inserted sequence on expression. This technique showed that the microsatellite sequence alone

could effect expression. Deleting the microsatellite resulted in an increase in activity of the reporter gene in some cell lines but not others (Hammock and Young, 2004). We then examined the expression driven by the prairie vole V1aR promoter containing either the prairie vole microsatellite or the montane vole microsatellite sequence. The results demonstrate that in some cell lines, but not others, the two constructs resulted in differential expression. This cell-type specific effect of the microsatellite on gene expression provides a mechanism by which V1aR expression differs in some brain regions but not others in these two vole species. Taken together, these results demonstrate that this microsatellite is indeed functional and the expansion of this repetitive sequence may be the mechanism resulting in emergence of the species differences in receptor expression and the evolution of monogamous behaviour in prairie voles.

B.7 INDIVIDUAL DIFFERENCES

Comparing brain receptor distribution and gene regulation has been very fruitful in highlighting potential mechanisms that produced the diverse behavioural repertoires seen in nature across species. However, within a species there is also individual variability in behaviour. These individual differences become even more important when dealing with psychiatric disorders in our own species. Voles, like humans, exhibit a range of individual differences in both behaviour and physiology. For example, the distribution of vasopressin receptors throughout the prairie vole brain varies significantly from one individual to another. Some prairie vole males have very high densities of V1aR binding in areas like the thalamus, posterior cingulate cortex, and medial amygdala while other have virtually no receptors in these regions (Phelps and Young, 2003). Given the

hypothesised role of the microsatellite in the evolution of prairie vole social behaviour, we hypothesized that individual differences in microsatellite lengh may underlie these individual differences in brain expression pattern and potentially behaviour.

The highly repetitive nature of microsatellites makes them inherently unstable in nature and we see a tremendous amount of heterogeneity in the length of the microsatellite among our laboratory voles, although not to the extent found across species. To test whether this variation in length might result in differences in expression, we examined at whether individual variations in prairie vole microsatellites can cause differences in gene transcription. Again using a cell transcription reporter assay, we showed that there was a difference in the ability to induce transcription depending on the length of the prairie voles microsatellite tested (Hammock and Young, 2005). Next we genotyped our entire colony to create breeding pairs that were homozygous long or homozygous short for their microsatellite length. The offspring of these pairs were cross fostered to control for environmental factors. About half the newborns fostered to short microsatellite parents did not survive, perhaps suggesting a difference in the quality of paternal care given based on the genotype of this one gene.

We then examined able the behaviour and receptor binding of voles from these two lines, selected for microsatellite length only. Cross-fostered animals with a long microsatellite have a much greater density of vasopressin receptors than animals with shorter microsatellite lengths in several brain areas, including the olfactory bulb and lateral septum (Hammock and Young, 2005). Males of these two strains also differed in their social behaviour. Males with a long microsatellite, and hence higher levels of V1aR in the lateral septum and olfactory bulb, spent more time licking and grooming their pups

than males with short microsatellites. Long allele males also spent more time investigating a social odour like female bedding than did short allele males, but the strains did not differ in their investigation of a non-social odour (Hammock and Young, 2005). Finally, long allele males were more likely to form a partner preference after an abbreviated cohabitation with a female, than were short allele males. These results demonstrate that in the brain, individual variation in a microsatellite located in the regulatory region of a gene can have a profound effect on not only protein expression but also behaviour.

B.8 POSSIBLE HUMAN IMPLICATIONS

Humans also have microsatellites in their V1aR gene that are almost as a variable between individuals as our voles. One microsatellite found in the 5' flanking region varies in the number of GT's, ranging from 18 to 50 repeats (Thibonnier, 2004). In fact, there are now two independent studies that have found a modest association between autism and this same microsatellite allele (Kim et al., 2002; Wassink et al., 2004). These results suggest that while the V1aR gene may not be a major determinant of autism, variations in the microsatillite may interact with other genes and environmental insults to influence specific aspects of social cognition in autistic individuals.

Interestingly, this microsatellite which has been associated with autism is absent in the common chimpanzee, although the remainder of the promoter is very highly conserved between these two highly related species (Hammock and Young, 2005). However, this microsatellite is present in the bonobo chimpanzee, which is known for its high level of sociosexual reciprocity. It is interesting to speculate that the incorporation of this

microsatellite into the bonobo and human V1aR may have contributed the evolution of their complex social behaviours.

Together, these studies demonstrate the utility of using animal models to elucidate the roles of specific genes in the regulation of complex behaviours. They further demonstrate that simple genetic differences in a single gene can have profound impact on these behaviours. This has important implications for the evolution of social behaviours as well as for individual differences in human behaviour, including psychiatric disorders such as autism.

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