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Characterization of Oxytocin Receptors in the Nonhuman Primate Brain

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Characterization of Oxytocin Receptors in the Nonhuman Primate Brain

By

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B.S., University of Virginia, 2006

Advisor**:** Larry J. Young, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the
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Abstract

Characterization of Oxytocin Receptors in the Nonhuman Primate Brain
By Sara M. Freeman

Oxytocin (OT) and vasopressin (AVP) are two structurally related neuropeptides that act in the brain to modulate the expression of species-specific social behaviors. Based on decades of extensive work in rodent systems, research in this field has recently expanded to explore the effects of OT on social cognition in humans, including clinical populations. However, this expansion has proceeded without a fundamental understanding of the neurophysiology of the OT system in the primate brain. Studying the brains of nonhuman primates (NHP) provides an opportunity to elucidate the neural mechanisms by which OT and AVP modulate social cognition. However, the lack of highly selective radioligands for the primate oxytocin receptor (OXTR) and vasopressin 1a receptor (AVPR1a) has prevented the reliable mapping of central OXTR and AVPR1a distributions in NHP tissue. To ameliorate this issue, we developed a pharmacologically informed, competitive binding protocol for receptor autoradiography to selectively reveal OXTR and AVPR1a binding in NHP tissue. We then characterized OXTR binding in the brain of a common NHP model organism for biomedical research, the rhesus macaque (*Macaca mulatta*). We also characterized OXTR and AVPR1a binding in the brain of the coppery titi monkey (*Callicebus cupreus*), a socially monogamous New World monkey. Our results demonstrate that while there are species differences in OXTR distribution in the primate brain, OXTR is consistently found in brain regions that modulate visual attention and control orienting responses to visual stimuli, such as the nucleus basalis of Meynert and the superior colliculus. These results should inform future studies in NHP and ultimately facilitate the development of optimal pharmacological strategies to target the OT system for the improvement of social function in psychiatric disorders such as autism spectrum disorder.

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Chapter 1:

Introduction

Modified from the following reference:

Sara M. Freeman and Larry J. Young. Oxytocin, vasopressin, and the evolution of mating systems in mammals. (from) *Oxytocin, Vasopressin and Related Peptides in the Regulation of Behavior*, edited by Elena Choleris, Donald W. Pfaff and Martin Kavaliers.

GENERAL INTRODUCTION

"Safety in numbers." "Pack mentality." "All for one and one for all." The English language is full of phrases like these, expressing the almost instinctual human understanding of the benefit of being social. However, I would argue that the majority of the 6.7 billion people in our collective human society do not realize that the socially motivated nature of our behavior is not the default way of life for many other species. Our high degree of sociality seems so natural to us that we are unaware that it is quite rare in the animal world. We are surrounded by other individuals and depend on them for the goods and services we need for our survival. We cooperate with others and even exhibit altruistic behavior. Many of the choices we make everyday, from traffic decisions to those made in conversations or in business, are all made with a subconscious consideration for how that decision might affect other people in our lives, even if those people are complete strangers. Furthermore, a life of solitude would be an unimaginable horror for an individual of our gregarious species. However, for many animal species, a solitary life is the norm, and conspecifics only come into contact during aggressive interactions over territory or resources, or to mate.

"Survival of the fittest." "Every man for himself." These ideas have a selfish and negative connotation in our culture but are the behavioral rule for many mammalian species. This begs the question, if self-promoting rather than affiliative behaviors are so prevalent, then why, and how, would behaviors that facilitate social interactions beyond fighting and mating ever have evolved? In theory, the benefit of living in a social group is obvious; simply reread the first few words of this chapter. Group living, a level of social organization that is found in many species, requires that individuals at the very least

tolerate close contact between conspecifics in the group, and at the very most, behave in a way that promotes social contact and affiliation between individuals. In many cases of group living among mammals, the individuals are often related members of an extended family, so acting prosocially makes sense evolutionarily. One of the goals of this dissertation is to delve into social organization at a level beyond familial prosocial behavior on the spectrum of sociality: monogamy.

Monogamy can come in many different forms, ranging from strict genetic monogamy, in which a male and female pair to mate and procreate exclusively with each other, to social monogamy, in which a male and female share a territory, mate, and take care of offspring, but may also engage in extra-pair copulations on occasion. In either case, unrelated adult conspecifics will come to prefer to spend time together, to develop an attachment, to be pair bonded. Unlike the case in more than 90% of mammalian species, in animals that exhibit a "monogamous" mating strategy, a relationship develops between the mated pair that extends well beyond orgasm. This pair bond between a male and a female following mating can endure for a lifetime or merely for a breeding season (Barberis et al. 1992; Kleiman 1977; Jard 1998; Hibert et al. 1999). However, limiting one's mating opportunities to the reproductive status of a single individual for any length of time is counterintuitive in the context of evolutionary theory and reproductive fitness. So, what are the proximate and ultimate causes for monogamy in mammals?

Research in our laboratory over the last several years has focused on finding answers to this question from a neuroscientific perspective using the socially monogamous prairie vole. This introduction will review some of the main results from our lab and several other research groups regarding the theoretical and empirical basis for

the evolution of monogamy in mammals, with a focus on the two neuropeptides that have been shown to be particularly important in the regulation of pair bonding: oxytocin (OT) and vasopressin (AVP). The main research goal of investigating the neurochemistry of pair bonding in monogamous species is to better understand how the brain coordinates complex social behaviors more broadly. Understanding the neuroscientific basis for these types of behaviors, such as affiliation, cooperation, social memory, and selective attachment, will provide valuable insight into the mechanisms underlying social behavior in humans. This insight can then lead to translational research in clinical settings, for example in developing better treatments for humans with deficits in social functioning, such as individuals diagnosed with autism spectrum disorder or schizophrenia.

But before we are capable of fully translating the discoveries from research in highly affiliative rodents like the prairie vole, we need to expand the basic research being done in nonhuman primate models of complex social cognition. This chapter will end by outlining the specific aims of this dissertation, which focus on establishing the neuroanatomical and pharmacological foundations for future research on OT and AVP in nonhuman primates. In doing so, this chapter will set the stage for how the lessons from this earlier research on monogamy can contribute more broadly to the study of sociality in nonhuman primates in order to advance our understanding of social neuroscience and prepare us for translational research in the future.

Oxytocin, vasopressin, and the evolution of monogamy in mammals

Mammals are a unique class of vertebrates with a distinct set of features compared to other classes of animals. Five main characteristics of mammals set them

apart from other vertebrate organisms: fur/body hair, three middle ear bones, a neocortex in the brain, mammary glands, and (in all taxa except monotremes) live birth. The last two—giving birth to live young and nursing them with milk from mammary glands—are of particular interest here for two reasons. First, in the context of evolution, these two processes involve the reproductive system, a potent locus for natural selection. Second, these two mammalian characteristics are both modulated by OT, a neurohypophyseal peptide hormone intimately involved in female mammalian reproductive physiology. Specifically, circulating OT binds to receptors in the uterus, where it stimulates uterine contractions and facilitates labor, and in the mammary glands, where it stimulates the milk letdown reflex in response to tactile stimulation from nursing (Ferris et al. 1997; Burbach, Young, and Russell 2006; Young et al. 1997). This uniquely mammalian system also acts in the brain to initiate maternal nurturing behavior and mother-infant bonding after parturition (Wang et al. 1998; Pedersen et al. 1985; Kendrick et al. 1986; Kendrick, Keverne, Chapman, and Baldwin 1988a; Kendrick, Keverne, Chapman, and Baldwin 1988b; Pedersen et al. 1994). More recently, the oxytocinergic system has been examined for its role in modulating pair-bond formation in females of monogamous mammalian species (Ferris et al. 1984; Carter, DeVries, and Getz 1995; Albers et al. 1986; Young and Wang 2004). This relatively new direction in OT research suggests some interesting evolutionary relationships between the neural mechanisms of mother-infant bonding and pair bonding in monogamous species.

Only 3-5% of mammals are considered to be monogamous, whereas up to 90% of birds display some degree of monogamy (Albers 2012; Orians 1969; Kleiman 1977; Clutton-Brock 1989). Theories about the reasons for this disparity have been based on the

fact that, because only the mother lactates in mammals, only she can feed the offspring after birth, but in birds, both the mother and the father can contribute equally to feeding the young (Ferris et al. 1985; Orians 1969; Kleiman 1977). Due to this unshared parental investment in mammals, where a female is “physiologically capable of providing for her own offspring before and after birth", it is often a more adaptive strategy for the male to depart shortly after mating to find another female to impregnate (Ferris et al. 1984; Kleiman 1977). This strategy results in an increased prevalence of non-monogamous mating systems in mammals compared to birds and other classes of vertebrates that display parental care.

However, monogamy and biparental care of the young do exist in the mammalian phylogeny, usually in species where there is significantly increased offspring fitness when the father contributes as well, such as in harsh environmental conditions like low food availability and high predation rate, or in low population densities (Ferris et al. 1997; Getz and Hofmann 1986). Moreover, high levels of mate-guarding exhibited by the male after mating, in an effort to ensure paternity, have been hypothesized to be a corollary to monogamous mating systems in mammals as well (Ferris et al. 1984; Brotherton et al. 1997; Ferris et al. 1985; Schuiling 2003). Mate-guarding is a form of male-specific territorial behavior in which the male either physically or behaviorally prevents the female with whom he has just mated from subsequently mating with any other male after him. Male aggression and territorial behavior are strongly influenced by AVP, a peptide hormone that is phylogenetically and physiologically similar to OT. While most of the roles of OT have been elucidated in females, the behavioral functions of AVP have mainly been demonstrated in males. It is becoming increasingly clear,

however, that the concept that OT regulates female behaviors while AVP regulates male behaviors is a severe oversimplification and is simply incorrect. Nevertheless, AVP activates territorial scent marking behavior in male hamsters when injected directly into the brain (Gruder-Adams and Getz 1985; Ferris et al. 1984). Arginine vasotocin (AVT), a non-mammalian homolog of AVP, has also been shown to mediate male-specific territorial behavior in species other than mammals, such as vocalization in frogs and birds (Aragona and Wang 2004; Boyd 1994; Young and Wang 2004; Maney, Goode, and Wingfield 1997; McGraw and Young 2010; Goodson 1998). AVP can also induce aggression in hamsters and voles (Gruder-Adams and Getz 1985; Winslow et al. 1993; Ferris et al. 1997; Young et al. 1997). Based on the evidence that AVP is involved in territorial behavior, as well as recent work examining the role of AVP in mediating male pair-bond formation in monogamous voles, the AVP system is emerging as an integral player in the evolution of monogamy in mammals.

In this chapter, we will review the research exploring the role of AVP and OT in regulating pair-bond formation in monogamous prairie voles and speculate about how a complex social behavior such as monogamy could evolve *de novo.* We hypothesize that the evolutionarily ancient neurohypophyseal systems of AVP and OT, which carry out other essential functions in the body like regulating water balance and initiating parturition, respectively, have undergone modifications in the central nervous system of monogamous mammalian species to promote the formation of the pair bond following mating. We will explore the neuroanatomical specializations in the OT and AVP systems in monogamous species and discuss how interactions between these neuropeptide systems and reward systems in the brain can lead to the formation of a pair bond. We will

close with an outline of the aims of the current dissertation in moving the discoveries regarding OT and AVP into nonhuman primate species by laying the necessary neuroanatomical foundation for future behavioral pharmacological studies.

VASOPRESSIN

Antidiuretic hormone

Arginine vasopressin is a neurohypophyseal nonapeptide hormone that is closely related to OT, differing in only two positions in the nine amino acid sequence. The genes for these two peptides are believed to have arisen early in the vertebrate lineage from a duplication event; the genes are located on the same chromosome, separated by a small intergenic region, and transcribed in opposite directions (Young et al. 1997; Hara, Battey, and Gainer 1990; Burbach et al. 2001; Donaldson and Young 2008). The evolutionarily ancient functions of AVP are in the periphery, where it acts at the level of blood vessels via the vasopressin 1a receptor (AVPR1a) to control vasoconstriction, and at the level of the kidney via the V2 receptor to control water balance (Young et al. 1999; Barberis et al. 1992; Jard 1998; Hibert et al. 1999). Thus, AVP is also referred to by endocrinologists as antidiuretic hormone, or ADH. AVP has also been shown to act at AVPR1a in the brain to influence male-typical mammalian social behaviors such as aggression (Winslow et al. 1993; Ferris et al. 1997; Young et al. 1997), paternal behavior (Gobrogge et al. 2009; Wang et al. 1998), and territoriality (Williams, Carter, and Insel 1992; Ferris et al. 1984; Williams, Insel, and Harbaugh 1994; Albers et al. 1986).

At first, the peripheral and behavioral functions of AVP may seem entirely unrelated; how does maintaining proper blood osmolarity relate to male social behavior?

Urine concentration is a byproduct of water balance and blood osmolarity regulation, and scent marking with urine is one of the most common ways that male mammals establish and maintain a territory, within which all resources, food, and potential mates are his. Thus, it is logical that this system could have been "hijacked" by natural selection to develop a new function in territorial behavior: chemical communication (Wilson, Keuhn, and Beach 1963; Albers 2012).

Territoriality and aggression in hamsters

Territorial behavior is a common male-typical communication behavior that is used to delimit and defend the boundaries of an individual's territory. While territorial behavior can come in many forms, one that is often seen in mammals is scent marking, in which "excretions of urine, feces, sweat, or glandular secretions are disseminated in the environment" (Insel, Preston, and Winslow 1995; Ferris et al. 1985). In hamsters, scent marking is performed in a highly stereotyped manner called *flank marking* where the male rubs secretions of the flank glands on objects in his territory. This behavior is usually triggered by odors of other male hamsters, but flank marking can be stimulated in the absence of olfactory cues by injecting AVP into the medial preoptic area/anterior hypothalamus (MPOA-AH) of males (Insel, Preston, and Winslow 1995; Ferris et al. 1984). Injection of AVP into the hypothalamus also increases aggression in hamsters (Winslow et al. 1993; Ferris et al. 1997). The behavioral effects on both aggression and scent marking are mediated by the AVPR1a (Insel, Wang, and Ferris 1994; Ferris et al. 1984; Lim, Murphy, and Young 2004; Ferris et al. 1985).

From territoriality to pair bonding: vasopressin and the male prairie vole

Prairie voles are hamster-sized rodents that display high levels of sociality, including social monogamy and biparental care of the young (Liu, Curtis, and Wang 2001; Gruder-Adams and Getz 1985; Lim and Young 2004). These rodents provide an ideal system for the study of complex social behavior, especially the neural circuitry of bonding between adults in a monogamous pair (Liu, Curtis, and Wang 2001; Aragona and Wang 2004; Young and Wang 2004; McGraw and Young 2010). Research on this species has been strengthened by comparative work performed in two vole species closely related to the prairie vole, the meadow vole and the montane vole, which are both solitary, non-affiliative rodents that do not exhibit monogamy or paternal care (Liu, Curtis, and Wang 2001; Gruder-Adams and Getz 1985).

Vasopressin and social behavior in male voles

Comparative studies using these monogamous and non-monogamous vole species have demonstrated species-specific roles for AVP in social behavior. Infusions of AVP directly into the brain increase inter-male aggression in male prairie voles, but not in male montane voles (Lim et al. 2004; Young et al. 1997). Furthermore, central infusions of AVP enhance affiliative behavior toward a female in male prairie voles, but not in male montane voles (Young et al. 1999). Male prairie voles, but not male montane or meadow voles, develop long lasting partner preferences, or pair bonds, following cohabitation and mating with a female. Pair-bond formation is accompanied by the development of a selective aggression toward novel females they encounter, one component in a suite of social behaviors related to the monogamous mating strategy.

Mating-induced, territorial-like selective aggression can be blocked by injecting an AVPR1a antagonist either intracerebroventricularly (ICV) (Lim et al. 2004; Winslow et al. 1993) or into the anterior hypothalamus (Young et al. 1999; Gobrogge et al. 2009; Fink, Excoffier, and Heckel 2006).

The development of a pair bond in prairie voles can be tested in a laboratory using the partner preference test*,* in which the male, after a period of cohabitation with a female, is given a choice to either spend time with his familiar female partner, with a novel female, or in the neutral empty middle between the two (Figure 1.1) (Kashi and King 2006; Williams, Carter, and Insel 1992; Young and Hammock 2007; Williams, Insel, and Harbaugh 1994). The partner preference behavior exhibited by male prairie voles is in contrast to the "Coolidge Effect" in which after mating, male rodents generally display a preference for a novel female (Hammock and Young 2005; Wilson, Keuhn, and Beach 1963). In male prairie voles, mating or extended cohabitation time leads to the development of a partner preference, but cohabitation without mating or for less than 24 hours does not typically lead to partner preference formation (Ophir et al. 2008; Insel, Preston, and Winslow 1995). AVP mediates the formation of a partner preference in male prairie voles since infusion of an AVPR1a antagonist into the brain blocks the formation of a partner preference, and infusion of AVP induces a partner preference in the absence of mating (Solomon et al. 2009; Winslow et al. 1993). Thus it appears as though the neural systems that mediate territorial behavior, including scent marking and territorial aggression, have been co-opted to mediate the formation of a pair bond in the monogamous prairie vole.

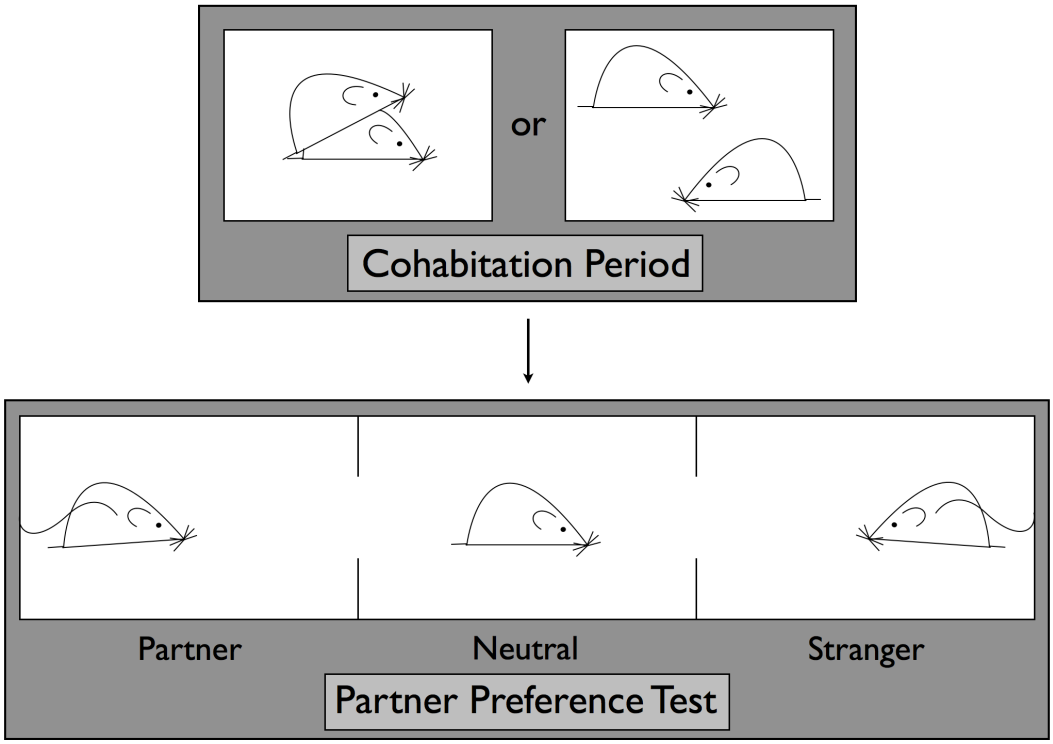

Figure 1.1. Partner preference paradigm.
First, the experimental animal, which can be a male or a female, is allowed to freely interact with an opposite sex conspecific for an extended period of time, called the cohabitation period. During this period, if there is mating or extended cohabitation time (18–24 h), the animals form a social bond; if mating is prevented or cohabitation duration is shortened (6 h), no pair bond will form (Hammock and Young 2005; Insel, Preston, and Winslow 1995). Next, the formation of a social bond is assayed in the laboratory using the partner preference test. In this test phase, the familiar opposite sex conspecific from the cohabitation phase ("partner") is tethered to one end of a three-chambered arena, and a novel opposite sex conspecific of equal stimulus value ("stranger") is tethered to the opposite end of the arena. The experimental animal is placed in the center of the arena and allowed to freely wander for 3 hours. The amount of time that the experimental animal spends in social proximity, or huddling, with either the partner or the stranger is recorded.

Neuroanatomy and pharmacology of vasopressin and pair-bond formation in male prairie voles

The first clues about the brain regions involved in AVP-mediated pair bonding came from comparative studies examining the distribution of AVPR1a in the brains of prairie, montane, and meadow voles. Receptor autoradiography for AVPR1a reveals striking species-specific differences in the pattern of binding. For example, prairie voles have higher densities of AVPR1a in several regions of the brain, including the ventral pallidum (VP), compared with montane and meadow voles (Figure 1.2C-D) (Fink, Excoffier, and Heckel 2006; Insel, Wang, and Ferris 1994; Lim, Murphy, and Young 2004). Furthermore, blocking specific populations of AVPR1a, including the VP and lateral septum (LS), prior to cohabitation and mating with a female blocks the development of a partner preference (Figure 1.2E-F) (Turner et al. 2010; Liu, Curtis, and Wang 2001; Lim and Young 2004). Infusion of AVP directly into the LS leads to a partner preference following cohabitation without mating (Figure 1.2F) (Ophir, Wolff, and Phelps 2008; Liu, Curtis, and Wang 2001). These results indicate that the LS and VP are critical sites for vasopressin's action in mediating pair-bond formation in male prairie voles.

The observation of species differences in AVPR1a distribution, particularly in the VP, led to investigations of the AVPR1a gene (*avpr1a)* in an effort to detect potential genetic mechanisms that could account for the species differences in brain expression and behavior. It has been suggested that changes in the distribution of AVPR1a in the brain evolutionarily, for example increases in expression of *avpr1a* in the VP, resulted in the capacity to form selective social bonds. This hypothesis was tested by recreating this

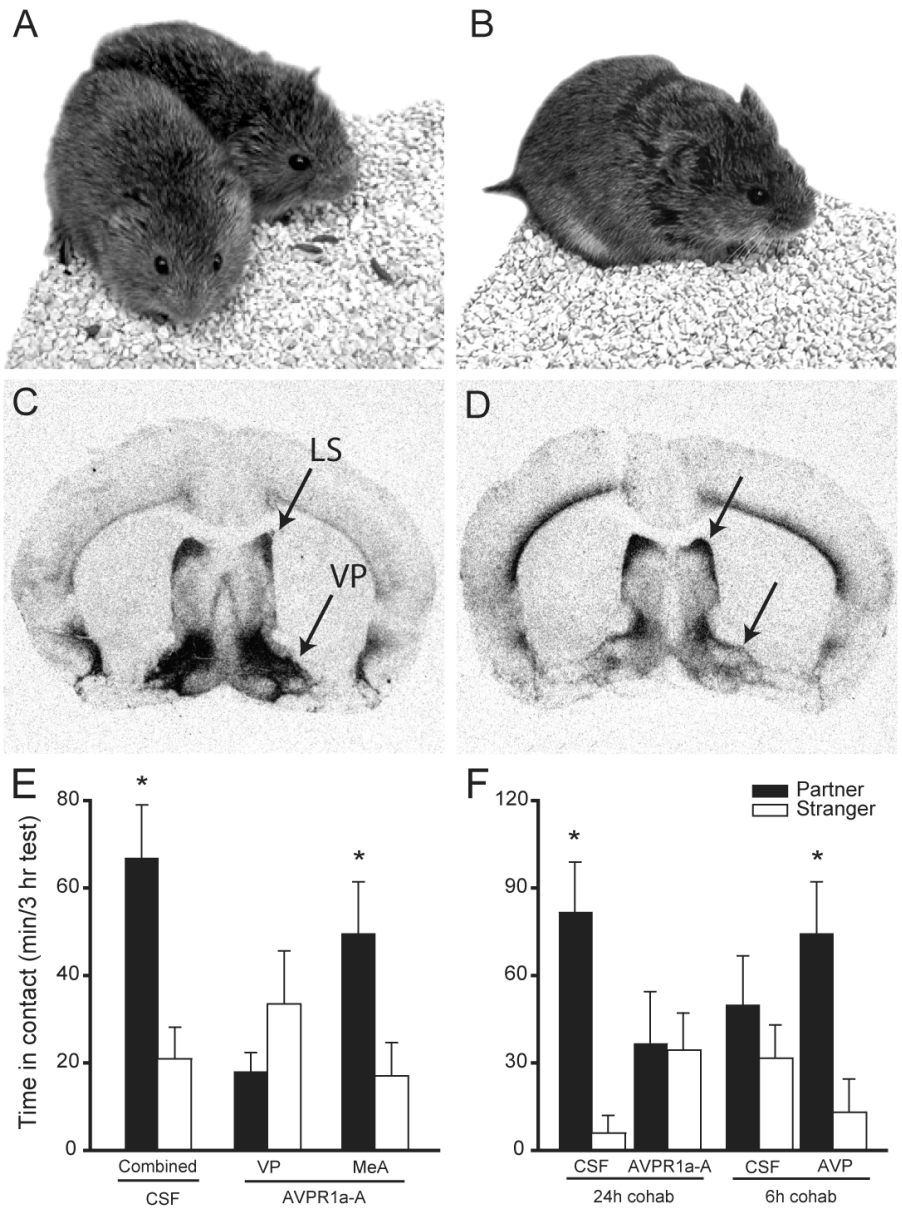

Figure 1.2. Vasopressin 1a receptor (AVPR1a) and social behavior in voles.
(A) Socially monogamous prairie voles. (B) Solitary and promiscuous meadow vole. (C and D) Autoradiograms of AVPR1a binding in the ventral pallidum (VP) in coronal sections through the forebrain of a prairie (C) or meadow (D) vole. (E) Infusion of an AVPR1a antagonist (AVPR1a-A) into the VP but not the medial amygdala (MeA) blocks partner preference in male prairie voles, but infusion of cerebrospinal fluid (CSF) in these regions does not affect partner preference behavior (Combined). (F) Infusion of a AVPR1aA into the lateral septum (LS) of male prairie voles disrupts partner preference after a cohabitation phase lasting 24 h, and infusion of vasopressin (AVP) into the LS during a shortened 6-h cohabitation induces a partner preference, which is not seen if CSF is infused (Ophir, Wolff, and Phelps 2008; Liu, Curtis, and Wang 2001). Figure adapted from Young and Wang, 2004 and Lim, Wang et al., 2004.

redistribution of the receptor expression in the lab using viral vector mediated gene transfer. Quite astoundingly, increasing *avpr1a* expression in the VP of non-monogamous meadow vole males results in the development of partner preference toward their mates (Figure 1.3) (Ophir, Wolff, and Phelps 2008; Lim et al. 2004). This result demonstrates that variation in the expression of a single gene in a single brain region can drastically affect the social behavior of a species, which suggests that the *avpr1a* is a potential locus for the evolution of monogamy in prairie voles from a previously non-monogamous mating system.

Genetic variation and pair bonding in male voles

To better elucidate the potential molecular mechanism for the species-specific differences in the distribution of AVPR1a in the brains of voles, the coding and regulatory regions of the *avpr1a* gene were examined in monogamous and non-monogamous species. The analysis indicated that the coding region of the *avpr1a* gene in prairie voles and montane voles are 99% homologous (Ophir, Wolff, and Phelps 2008; Young et al. 1999). However, the degree of homology is quite different in the 5' flanking region of the gene, a region that likely determines expression patterns. In prairie voles, there is a ~400 base pair (bp) polymorphism in the length of a repetitive DNA element, referred to as a microsatellite, approximately 700 bp upstream of the transcription start site, and in the non-monogamous meadow and montane voles, this microsatellite is rudimentary (~50 bp) (Landgraf and Neumann 2004; Young et al. 1999; Ludwig and Leng 2006; Fink, Excoffier, and Heckel 2006; Ross and Young 2009; Ross, Cole, et al. 2009). Repetitive microsatellite elements are evolutionarily unstable and prone to

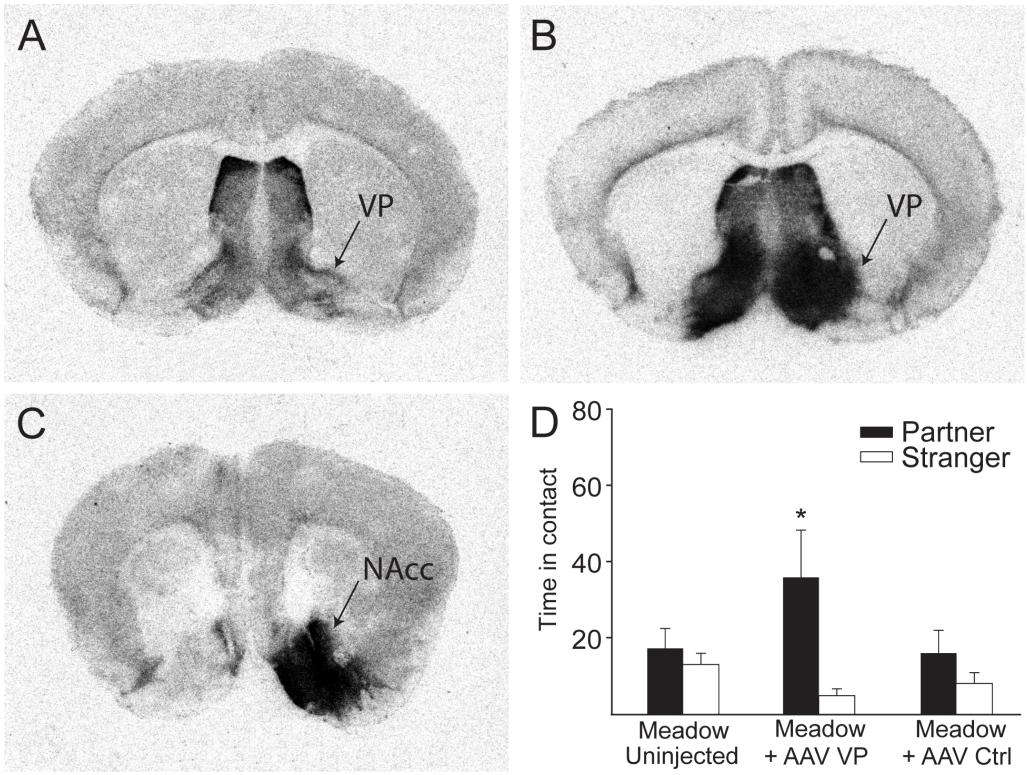

Figure 1.3. Manipulating vasopressin 1a receptor (AVPR1a) binding in meadow voles using viral vector mediated gene transfer.
(A) Autoradiogram showing AVPR1a binding in a typical meadow vole. (B) Overexpression of the AVPR1a gene in the ventral pallidum (VP) by adeno-associated viral vector (AAV)-mediated gene transfer (Meadow +AAV VP). (C) A stereotaxic injection inadvertently placed too rostral to the VP, in this case located just ventral to the nucleus accumbens, which serves as an anatomical control (Meadow +AAV Ctrl). (D) When AVPR1a levels are artificially increased within the VP using AAV gene transfer (Meadow+AAV VP), male meadow voles display a partner preference, preferring social contact with their partner than with a stranger. This behavior is not seen in uninjected meadow voles (Meadow Uninjected) or meadow voles receiving a viral vector injection that missed the VP (Meadow +AAV Ctrl) (Burbach, Young, and Russell 2006; Lim et al. 2004). Time in contact is given in minutes per 3-h test. Figure adapted from Lim, Wang et al., 2004.

mutation, which can in turn produce diversity in the expression of the gene (Pedersen and Prange 1979; Kashi and King 2006; Pedersen et al. 1982; Young and Hammock 2007). Therefore, this microsatellite polymorphism was hypothesized to be a potential causative polymorphism for the divergence in brain AVPR1a expression patterns and social behavior seen between species.

Several studies were conducted to acquire empirical evidence in support of this idea. First, individual variation in the length of this microsatellite in male prairie voles correlated with individual variation in levels of AVPR1a binding in some areas of the brain and also predicted social behavior in laboratory tests. Males homozygous for the long microsatellite allele had increased AVPR1a density in the LS and olfactory bulb, and they displayed a reduced approach latency and increased duration and frequency of olfactory investigation of a social odor (but not a non-social odor) as well as a reduced latency to social investigation of a novel animal (Hammock and Young 2005). In a partner preference test, the homozygous long males developed partner preferences more readily than the homozygous short males (Hammock and Young 2005). These neuroanatomical and behavioral results suggest that variation in the length of the microsatellite region in the *avpr1a* gene could be altering both global AVPR1a expression patterns in the brain and the repertoire of social behavior displayed in these animals. However, the relationship between microsatellite length and *avpr1a* expression and behavior is not likely to be as simple as originally hypothesized.

To further investigate the role that this microsatellite region might play in determining species-specific social behavior, prairie voles were taken into the field to examine the behavioral ecology of this system. However, the first study to evaluate the

relationship between microsatellite length, AVPR1a binding patterns in the brain, and male prairie vole behavior in mixed-sex, semi-natural field enclosures found results that are inconsistent with those in the laboratory experiments discussed above. Specifically, Ophir and colleagues reported that AVPR1a binding was associated with *avpr1a* microsatellite length, but the brain regions exhibiting the effect were different than those reported in Hammock et al. 2005. Furthermore, they found no association between *avpr1a* microsatellite length and social behavior in the field (Ophir et al. 2008). In another field study using semi-natural conditions, Solomon et al. evaluated the relationship between the length of the microsatellite and "indicators of social and genetic monogamy" in male prairie voles who were living in mixed-sex enclosures. These authors found that the length of the microsatellite did not correlate with social monogamy but did correlate with genetic monogamy (Solomon et al. 2009).

Thus, as is the case with many elegantly simple hypotheses, the data suggest that the mere expansion of the microsatellite is not responsible for the evolution of monogamy in prairie voles. A comparative analysis of *avpr1a* from 21 *Microtus* species revealed that the presence or absence of the microsatellite did not predict mating strategy within the genus (Fink, Excoffier, and Heckel 2006). Moreover, when eight species of deer mouse within the genus *Peromyscus*, which includes both monogamous and promiscuous species, were examined for *avpr1a* microsatellite length, for AVPR1a distribution, and for mating system, there were no significant correlations found between any of these features (Turner et al. 2010). Thus, while early work suggested that instability in the microsatellite drove the diversity in receptor distribution, more careful analysis suggests that simple variation in the length of the microsatellite is not the

primary cause of variation in expression patterns. Rather, individual alleles of the microsatellite may be linked to functional polymorphisms that contribute to variation in expression.

Vasopressin receptors & fitness in the wandering male prairie vole

Individual differences in AVPR1a binding levels in various brain structures contribute to both territorial and social behaviors in male prairie voles. Male prairie voles can adopt one of two different mating strategies. First, a "wanderer" is a male who does not reside with a single female but instead mates opportunistically in a larger home range that overlaps those of multiple females and males. Alternatively, a "resident" is a male who shares and defends a territory with a female with whom he is considered socially monogamous (Ophir, Wolff, and Phelps 2008). Ophir and colleagues hypothesized that in field enclosures occupied by males and females, the mating strategy and sexual behavior of the males would associate with individual variation in AVPR1a expression levels and patterns (Ophir, Wolff, and Phelps 2008). When measures of social and sexual fidelity in males were correlated with AVPR1a binding in various brain regions, the authors found that neither social nor sexual fidelity was associated with AVPR1a levels in regions that have been implicated in pair-bond formation or maintenance, namely the VP and LS (Ophir, Wolff, and Phelps 2008). However, they did find that AVPR1a binding in two brain regions involved in spatial memory, the posterior cingulate/retrosplenial cortex (PCing) and the laterodorsal thalamus, was inversely correlated with breeding success. Specifically, "wandering" males with low levels of binding in the PCing were more successful at siring offspring than those with high binding (Ophir, Wolff, and Phelps

2008). This result not only supports the notion that variation in receptor binding predicts some social behaviors, but it also links brain regions that are involved in spatial ability with a more flexible mating strategy and less stringent territorial boundaries.

OXYTOCIN

A maternal hormone

As mentioned in the introduction, OT is a hormone that is involved in the reproductive physiology of female mammals. Like AVP, this nine amino acid neuropeptide is synthesized in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. Cells from these regions send axon projections to the posterior pituitary, where they release OT in large amounts into the bloodstream at the neurohypophysis (Burbach, Young, and Russell 2006). While OT acts in the periphery to induce uterine contractions during labor and milk ejection during nursing, the OT-synthesizing neurons in the PVN and SON also release OT in the brain, where it acts centrally at oxytocin receptors (OXTR) to modulate social cognitive processes like social memory, maternal behavior, and affiliative behavior, to name a few (Landgraf and Neumann 2004; Ludwig and Leng 2006; Ross and Young 2009; Ross, Cole, et al. 2009).

Oxytocin and maternal behavior

OT not only facilitates parturition and nursing, but it also mediates the onset of maternal nurturing by transforming the female's behavior after giving birth. Central infusion of OT in virgin rats results in the rapid onset of full maternal behavior (Pedersen and Prange 1979; Pedersen et al. 1982), while blocking OXTR with an antagonist delays

the onset of maternal responsiveness and diminishes the expression of species-specific maternal behavior (Burbach, Young, and Russell 2006; Pedersen et al. 1985; Pedersen et al. 1994).

In sheep, elevated levels of OT in the brain at parturition trigger not only the onset of maternal nurturing behavior, but also the selective mother-infant bond (Pedersen et al. 1985; Kendrick et al. 1986; Kendrick, Keverne, and Baldwin 1987; Kendrick, Keverne, Chapman, and Baldwin 1988a; Kendrick, Keverne, Chapman, and Baldwin 1988b; Pedersen et al. 1994). Sheep live in herds and many females give birth at the same time during the breeding season. Lambs are ambulatory soon after birth so ewes must quickly form a selective bond to their lamb in order to distinguish it from all other lambs. After bonding to their lamb, ewes will show aggression toward foreign lambs but nurture their own. However, ICV infusions of OT in estrogen-primed ewes increases maternal behaviors and decreases aggressive behaviors toward novel lambs (Carter, DeVries, and Getz 1995; Kendrick, Keverne, and Baldwin 1987; Young and Wang 2004). Furthermore, both infusion of OT and vaginocervical stimulation (VCS), which stimulates release of OT in the brain, results in a ewe bonding to a novel lamb (Orians 1969; Kendrick, Keverne, Chapman, and Baldwin 1988b; Kleiman 1977; Clutton-Brock 1989).

To further support this evidence from sheep and rats, two mutant mouse strains have been generated to analyze the oxytocinergic system: an oxytocin peptide knock out mouse (OTKO), and an oxytocin receptor knock out mouse (OXTRKO). Both of these mutant strains exhibit deficits in various social parameters, including aggression, social recognition, parental care, and reproductive behavior. Specifically, OXTRKO postpartum

mothers and virgins show disruptions in maternal behavior as assayed by latency to retrieve pups, latency to crouch over pups, and time spent crouching over pups (Orians 1969; Takayanagi et al. 2005; Kleiman 1977). This deficit is not as robust in OT peptide knock out animals, perhaps because the OXTR is being activated by structurally and functionally related AVP (Kleiman 1977; Nishimori et al. 1996; Young, Shepard, and Amico 1996; Winslow 2002; Pedersen et al. 2006).

Although the role of OXTR expression in maternal behavior has yet to be explored in prairie voles, there is evidence that OXTR plays a role in regulating alloparental behavior in this highly social species. Alloparental behavior is defined as parental-like behavior expressed toward infants that are not the offspring of that animal; in prairie voles, it is often used to describe the behavior of juveniles, which will often take care of their parents' next litter if they do not leave the nest after weaning (Getz and Hofmann 1986; Carter and Getz 1993). Individual variation in the density of OXTR binding in the nucleus accumbens (NAcc) of both juvenile and adult female prairie voles is positively correlated with levels of alloparental behavior (Brotherton et al. 1997; Olazábal and Young 2006b; Schuiling 2003; Olazábal and Young 2006a). Furthermore, infusion of an OXTR antagonist into the NAcc of adult sexually naïve females reduces the percentage of animals that display alloparental behavior toward novel pups (Olazábal and Young 2006b). Thus in rats, mice, sheep and prairie voles, there is evidence that the OT system plays a role in the regulation of maternal nurturing behavior and, at least in sheep, in the development of the maternal bond.

Oxytocin and pair bonding in female voles

Based on this evidence that OT is required for mother-infant bonding in sheep and that OXTR activation is necessary for appropriate maternal nurturing behavior in rodents, this social attachment system became a focus for research into the neural basis of pair bonding between the female and male prairie vole. The first study to examine the role of OT in prairie vole pair bonding found that in the absence of mating, central infusion of OT was sufficient to stimulate the formation of a partner preference in female prairie voles (Williams, Insel, and Harbaugh 1994). Furthermore, infusion of an OXTR antagonist ICV was able to block mating-induced partner preferences in the female prairie vole (Insel, Preston, and Winslow 1995). These studies suggest that common neuroendocrine mechanisms to some degree regulate maternal bonding and bonding between the female and the male partner.

In an effort to begin to understand the neural circuitry underlying OT-dependent partner preference formation, and to explore the nature of the variation in mating strategies among vole species, the neuroanatomical distribution of OXTR binding was examined in prairie voles and non-monogamous montane voles. As previously discussed for the AVPR1a, there are also remarkable species differences in OXTR distribution in the brain, which may account in part for the species differences in pair bonding between monogamous and non-monogamous voles. There is a high density of OXTR expression in the NAcc in the monogamous prairie vole that is absent in the promiscuous montane vole (Figure 1.4) (Insel and Shapiro 1992; Lim, Murphy, and Young 2004). OXTRs are present in both species in the prefrontal cortex (PFC), which, along with NAcc, is part of the brain's reward and reinforcement pathway (Kelley 2004; Wallace et al. 2008). Blocking OXTR in the PFC or the NAcc by infusing an OXTR antagonist site-

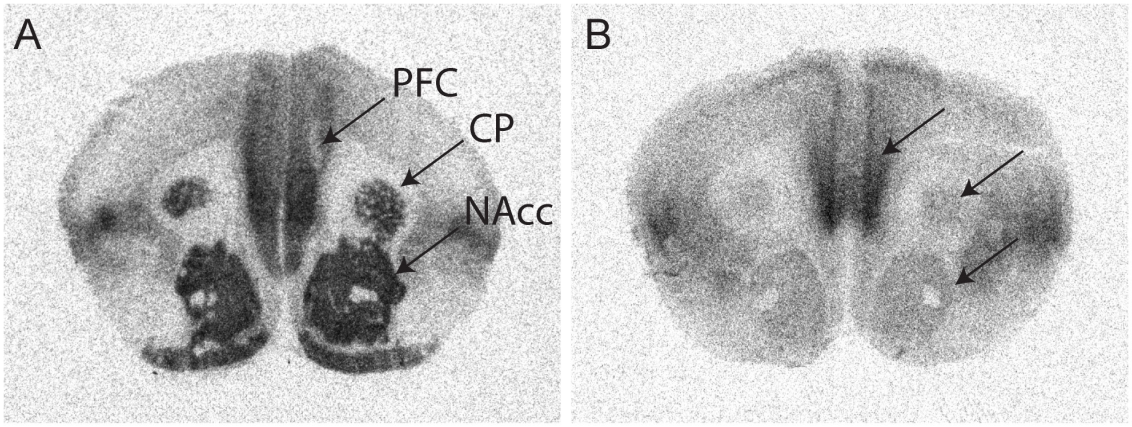

Figure 1.4. Oxytocin receptor distribution in voles.
(A and B) Autoradiograms of oxytocin receptor binding densities in coronal sections through the forebrain of a prairie vole (A) and a meadow vole (B), showing the prefrontal cortex (PFC), caudate putamen (CP), and nucleus accumbens (NAcc). Figure adapted from Young and Wang, 2004.

specifically into the brains of female prairie vole prevents partner preference formation, indicating that OXTR activation in these regions is necessary for development of partner preferences in female prairie voles (Young et al. 2001). Furthermore, increasing OXTR density in the NAcc by site-specific viral vector mediated gene transfer facilitates the formation of a partner preference in female prairie voles as well (Ross, Freeman, et al. 2009).

In vivo microdialysis to monitor OT release in the NAcc in female prairie voles during cohabitation with males showed that an increase in extracellular OT during mating (Ross, Cole, et al. 2009). Mating in prairie voles involves multiple intromissions, which likely results in significant VCS, a potent OT-releasing stimulus. Thus, it appears that while VCS during labor initiates OT release leading to the formation of a maternal bond in mother sheep, VCS during mating initiates OT release leading to the formation of a pair bond in the female prairie vole. Based on the data described above, the female's bond to a male is mediated by similar neural underpinnings as the bond of a mother to her offspring. Therefore, social monogamy as a mating strategy for females may result from an adaptation of the maternal circuitry in mammals.

SOCIAL RECOGNITION AND REWARD: THE PATHWAY TO BONDING

We have discussed that both AVP and OT acting within specific brain regions stimulate the formation of pair bonds in male and female prairie voles. But how does activation of these neuropeptide receptors lead to a social preference for the partner, and hence, to a pair bond? Two components of social cognition that are critical to the formation of a pair bond are reinforcement of social interactions via the reward systems

in the brain, and individual recognition based on social memory circuits. In rodents, social memory is tested in the laboratory by quantifying the duration of social investigation after repeated exposures to the same individual. Rodents will show decreased olfactory investigation time after each trial, and investigators interpret this habituation to indicate that the rodent has formed a memory of that individual. Exposure to a novel animal after this habituation process results in a return to the original high levels of investigation seen during the first presentation.

Paradigms such as this one have been commonly used to assess social memory in various rodent species. Experiments using knockout mice have been instrumental in the investigation of the role of AVP and OT in social memory. Transgenic mice lacking the *avpr1a* gene, called AVPR1a knock out mice, exhibit social amnesia but have normal spatial memory and normal memory for non-social odors, indicating that both learning ability and olfactory function in these animals are still intact (Bielsky et al. 2004). Recovery of social memory in these animals can be attained by experimentally replacing *avpr1a* using viral vector mediated gene transfer in the LS (Bielsky et al. 2005). Also, studies in rats have examined the role of AVP in social memory. In male rats, AVPR1a activation in the LS has been shown to be involved in retention of social memory; injecting AVP into the LS increases social memory, while injection of an AVPR1a antagonist impairs it (Dantzer et al. 1987; Dantzer et al. 1988). This result is important in the context of social memory and pair bonding, because LS is one of the brain regions that has been shown to be essential for the AVP-mediated formation of a partner preference in male prairie voles. Selective amnesia for social information is also seen in OTKO mice and can be induced in wildtype mice by treatment with an OT antagonist

(Ferguson et al. 2000). This deficit in knockout mice can be rescued by infusions of OT ICV as well as into the medial amygdala (MeA) (Ferguson et al. 2000; Ferguson et al. 2001). Social memory deficits have also been documented in OXTRKO male mice (Takayanagi et al. 2005). These experiments support the role of OT and AVP in individual recognition, a process that partner preference formation depends on.

The other essential component to the formation of a pair bond besides individual recognition is the reinforcement of social interactions by activation of the brain's reward circuits. In female prairie voles, the formation of a partner preference relies on the expression of OXTR in the NAcc, a region in the brain's mesolimbic reward pathway (Young et al. 2001). Another region in this pathway is the VP, an area critical for partner preference in male prairie voles. The VP has also been shown to be important for the reinforcing properties of drugs of abuse. When injected stereotaxically into the VP of a rat, psychostimulants like cocaine and amphetamine induce a conditioned place preference (CPP) for the area of the test arena where the rat received the dose, and this cocaine-induced CPP can be prevented if dopamine (DA) is depleted from the VP (Gong, Neill, and Justice 1996; Gong, Neill, and Justice 1997). In a CPP task, animals receive a reinforcing stimulus only when they cross into a certain area of a place preference arena; after a few conditioning trials, the animal will prefer to spend time in this part of the arena. This process is analogous to the partner preference exhibited by pair bonded prairie voles: the partner is the conditioned stimulus reinforced by the rewarding aspects of mating.

This reward is believed to be mediated by DA. In female prairie voles, microinjection of a dopamine D2-like receptor antagonist into the NAcc, but not the PFC,

blocked the development of a partner preference, and a dopamine D2-like receptor agonist injected in the NAcc caused females that had cohabitated with a male in the absence of mating to form a partner preference (Gingrich et al. 2000). This result implies that perhaps the rewarding aspect of mating, a behavior that increases DA levels in the NAcc, could be combining with activation of OXTR in that region to cause a positive association between the social stimuli from that specific partner and the reward of mating (Young and Wang 2004). Thus, it seems that in female prairie voles, the mating-induced increases in OT and DA converging on the NAcc may be causing natural reward learning and reinforcing the characteristics of the individual partner after mating to result in a partner preference.

Therefore, we suggest that with the convergence of reward systems, learning and memory circuits, and the activation of OT and AVP inputs in response to social stimuli could result in an increased desire to spend time with an opposite sex conspecific with which an individual has previously mated, or a "conditioned partner preference." This proposed neural circuitry of pair bonding is illustrated in Figure 1.5.

BEYOND MONOGAMY: COMPARATIVE WORK IN OTHER SPECIES

Oxytocin and African mole rats

The OT system has also been examined in African mole rats, a taxonomic group of rodents that exhibit a wide variety in their social organization (Kalamatianos et al. 2010). Despite being in the same phylogenetic family, the eusocial naked mole rat and the solitary Cape mole rat represent opposite ends of the spectrum of sociality. The eusocial naked mole rat, *Heterocephalus glaber*, lives in colonies that can contain

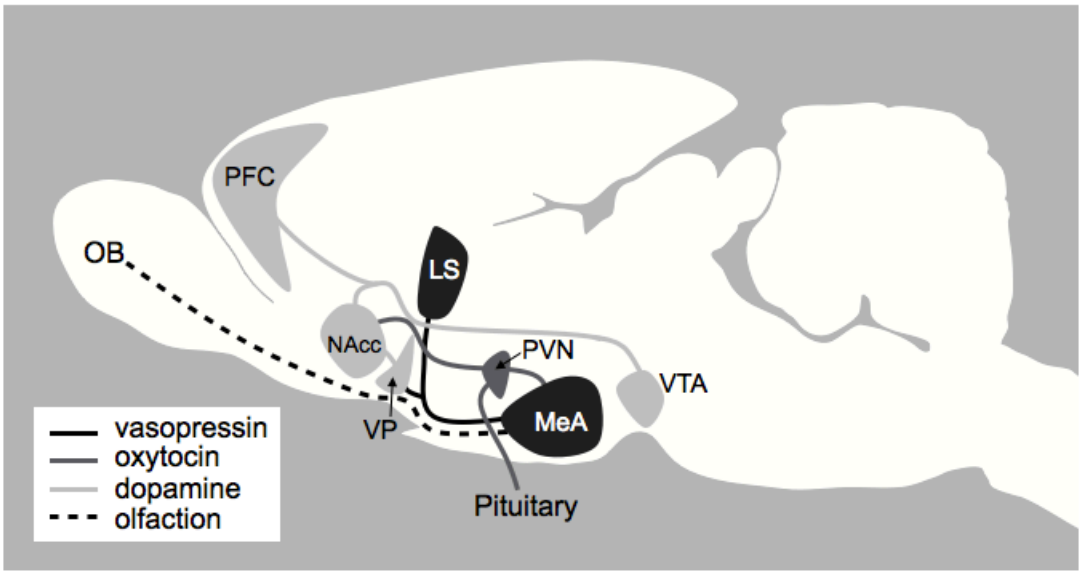

Figure 1.5. Sagittal view of a prairie vole brain illustrating a proposed neural circuit model for pair bonding.
In this model, mating activates the ventral tegmental area (VTA), resulting in increased dopamine activity in the prefrontal cortex (PFC) and nucleus accumbens (NAcc). Concurrently, olfactory signals from the mate are transmitted via the olfactory bulb (OB) to the medial amygdala (MeA). Oxytocin (OT) acts in the MeA, and vasopressin (AVP) acts in the lateral septum (LS) to facilitate olfactory learning and memory. Mating also stimulates increased extracellular concentrations of OT in the PFC and NAcc of females, and of AVP in the ventral pallidum (VP) of males. AVP fibers in the LS and VP originate from cell bodies in the MeA. The source of OT projections to the NAcc, MeA, and PFC most likely originate from a population of cell bodies in the paraventricular nucleus of the hypothalamus (PVN). The concurrent activation of the dopaminergic system and the OT or AVP systems in the NAcc or VP, respectively, potentially results in the development of a conditioned partner preference. Figure adapted from Young and Wang, 2004.

hundreds of individuals, and like other eusocial species, have a unique system of reproduction that includes one reproductively active queen and her one or few male mates. The other male and female individuals in the colony are reproductively suppressed, provide cooperative care of the young, and work together to burrow, forage, and protect the colony (Brett 1991; Jarvis 1991). On the other side of the spectrum, the Cape mole rat, *Georychus capensis*, is a solitary species with interaction between conspecifics limited to mating and contact between the mother, pups, and littermates (Bennett and Jarvis 1988). In Cape mole rats, even the interaction between the mother and pups is limited; the mother becomes aggressive toward her offspring and drives them out of the burrow shortly after weaning (Bennett and Jarvis 1988).

This difference in sociality was theorized by Kalamatianos and colleagues to be mediated in part by neurochemical alterations in the OT system (Kalamatianos et al. 2010). When these authors performed receptor autoradiography for the OXTR in both species, they found that the naked mole rats have higher levels of expression than the caped mole rats in multiple brain regions that also have high OXTR binding in the prairie vole, most notably the NAcc (Kalamatianos et al. 2010). Drawing a parallel between monogamous prairie voles and eusocial naked mole rats is not as direct as comparing two solitary rodent species, like the caped mole rat and the meadow vole, but the eusocial organization of naked mole rats involves similar prosocial behaviors as seen in the monogamous prairie vole, such as extended periods of time in close side-by-side contact huddling together (Jarvis 1991; Lacey and Sherman 1991). Thus, authors hypothesize that the high levels of OXTR seen in the NAcc of naked mole rats might mediate the high

levels of prosocial contact and alloparental behavior required to maintain such an extended colonial social structure in this species.

Vasopressin and monogamy in deer mice

Another rodent species that has been examined in the context of social bonding and neurohypophyseal hormones is the monogamous California mouse, *Peromyscus californicus*. This species is an excellent model for the study of paternal behavior and male aggression, especially in comparison with the closely related promiscuous white-footed mouse, *Peromyscus leucopus*, which exhibits much less paternal care and lower levels of aggression than the California mouse (Bester-Meredith, Young, and Marler 1999). It was hypothesized that there would be high levels of AVPR1a binding in the California mouse in the brain regions that have high AVPR1a binding in the prairie vole. While the authors found many unexpected differences in the AVPR1a pattern of binding between the monogamous California mouse and the prairie vole, there was dense AVPR1a binding in the LS and VP, two notable areas also high in AVPR1a in the prairie vole (Insel, Gelhard, and Shapiro 1991; Bester-Meredith, Young, and Marler 1999). Both of these brain areas were also found to have much lower or even undetectable levels of AVPR1a binding in the promiscuous white-footed mouse as compared to the California mouse (Bester-Meredith, Young, and Marler 1999). These results suggest that there are similarities in the underlying neurological systems for social behaviors such as monogamy, paternal care, and aggression, and the role that these systems play more generally in the mammalian brain may be conserved across phylogenetic groups.

Oxytocin, vasopressin, and primate neuroanatomy and social behavior

Variation in oxytocin and social behavior in macaques

While the comparative study of OT and AVP in different mammalian social systems has expanded greatly since the first rodent studies were conducted, the physiology and neuroendocrinology of the OT system in primate social behavior has only recently begun to be investigated. In 2003, it was shown in rhesus macaques that OT levels in cerebrospinal fluid (CSF) correlate significantly with the level of expressed affiliative behavior (Winslow et al. 2003). Rosenblum and colleagues took advantage of the natural variation in social behavior within the macaque genus and compared CSF OT between the affiliative bonnet macaque and the aggressive pigtail macaque (Rosenblum et al. 2002). Bonnets spend much of their time in prosocial contact, such as grooming and huddling; they are more likely than pigtails to engage in social interaction with conspecific strangers; and they do not have a strong social hierarchy. Pigtails on the other hand have a strict hierarchy, maintain large individual distances, and are not likely to interact positively with strangers. As hypothesized, the affiliative bonnets were found to have higher CSF OT and lower CSF levels of corticotropin releasing factor (CRF; mediates response to stress) than the more aggressive pigtails (Rosenblum et al. 2002).

In a recent study, Parr and colleagues demonstrate that OT can affect the attentional bias of rhesus macaques to visual social stimuli (Parr et al. 2013). In this study, rhesus macaques performed a computerized attention task following administration of an intranasal dose of OT (IN-OT) or placebo. Treatment with OT reduced the subjects' attention to images of negative facial expressions (open mouth threats and bared-teeth displays) but did not affect attention to neutral faces or non-social images (clip art).

Furthermore, IN-OT treatment caused macaques to increase their attention to images of monkey faces with direct gaze and reverse their attentional bias toward faces with averted gaze (Parr et al. 2013). It is believed that monkeys with direct gaze are threatening for viewer monkeys, so if IN-OT increases attention to images of faces with direct gaze, then this study supports conclusions from some recent human studies, which show that IN-OT can attenuate the arousing or aversive nature of emotional images (Norman et al. 2011; Ellenbogen et al. 2012). While future studies in primates are needed to establish a mechanism for these macaque results, one explanation may be that IN-OT is decreasing the amygdala's typical increase in activity in response to seeing emotional faces or fearful images (Domes, Heinrichs, Gläscher, et al. 2007; Kirsch et al. 2005).

There have been two other studies examining the effects of IN-OT in rhesus macaques. First, Chang and colleagues investigated whether IN-OT would change the behavior of rhesus macaques in a task where the subjects could choose to give juice rewards to themselves, to a nearby monkey, or to neither. The authors found that IN-OT increases both selfish and altruistic choices to dole out juice rewards, and it also enhanced "other-oriented attention" to the recipient monkey selectively after an altruistic choice was made (Chang et al. 2012). Furthermore, this paper was the first to show that IN-OT causes increases in OT concentration in the CSF, indicating that IN delivery can result in changes in central levels of OT, although the mechanisms for this change are still unknown. Second, Ebitz and colleagues found that IN-OT increased gaze to the eyes when monkeys viewed images of monkey faces (Ebitz, Watson, and Platt 2013), which supports evidence from human studies that IN-OT increases gaze to the eye region of human faces (Gamer, Zurowski, and Büchel 2010; Andari et al. 2010; Guastella,

Mitchell, and Dadds 2008). Further, this study reports that IN-OT reduces the expected species-typical social vigilance toward images of unfamiliar, emotional, or dominant male faces (Ebitz, Watson, and Platt 2013). These results partly agree with the results of Parr et al. (2013) discussed above, with both studies showing that IN-OT reduces attention to pictures of open mouth threat and bared-teeth displays. While future studies are needed to investigate the neural mechanisms underlying these effects, these recent studies suggest that OT is capable of modulating social visual attention, social orienting, and social decision-making in the rhesus macaque.

Monogamous primates: marmosets, titi monkeys, & tamarins

Although monogamy is rare in primates, a few socially monogamous species of New World monkey, including the common marmoset (*Callithrix jacchus*), the black-pencilled marmoset (*Callitrhix pencillata*), the coppery titi monkey (*Callicebus cupreus*), and the cotton-top tamarin (*Saguinus Oedipus*) have become new animal models for the neuroendocrine basis of social bonding and parental care. In marmosets, there is preliminary evidence that OT may play a role in regulating some aspects of pair bonding. Daily administration of IN-OT in black-penciled marmosets during a three-week period in which males and females were paired with opposite sex conspecifics resulted in increased huddling with their partner (Smith et al. 2010). Furthermore, daily delivery of an OXTR antagonist caused decreased proximity to their partner as well as decreased food sharing, which is a highly social and cooperative behavior in marmosets (Smith et al. 2010). Although there are some neuropharmalogical issues in reliably identifying the location of OXTR and AVPR1a in primate brain tissue (discussed further below), the

neuroanatomical map of OXTR and AVPR1a in the socially monogamous common marmoset has been published (Schorscher-Petcu, Dupré, and Tribollet 2009; Wang et al. 1997). Briefly, common marmosets have high levels of OXTR binding in the NAcc and high levels of AVPR1a binding in the VP, which is consistent with the findings in voles. However, these results need to be corroborated by future studies in order to ensure their reliability.

Coppery titi monkeys, which have also been used to study pair-bonding behavior in primates, have recently been studied for the role of AVP on pair bonding in males. In one experiment, males that have been paired with a female for at least one year were given either saline, a low dose of IN-AVP, or a high dose of IN-AVP before being presented in turn with either its female partner or an unfamiliar female stranger. When given saline, males contacted the unfamiliar female more frequently than their partner, which is expected because most mammalian males are attracted to novel females. But when they were given the high dose of AVP, males contacted their partner more frequently than the unfamiliar female (Jarcho et al. 2011). This result supports the idea that AVP underlies affiliative behavior in highly social primates, as it does in social rodent species.

The relationship of OT in pair-bonding behavior in cotton-top tamarins has also begun to be examined. Urinary levels of OT and various affiliative behaviors were measured in male-female pairs over the course of three weeks. OT levels within each pair was directly related to sexual behavior and the frequency of affiliative behaviors between male-female pairs, specifically initiation of huddling by males and initiation of sex by females (Snowdon et al. 2010). Although peripheral measures of OT, like urinary and

salivary OT, still need further verification regarding their connection to central levels, this preliminary evidence in tamarins supports the hypothesis that OT may be released during sociosexual encounters in primates.

CONCLUSIONS AND FUTURE DIRECTIONS

General Conclusions

In this chapter, we have reviewed the evidence in support of one possible mechanism by which the physiologically ancient functions of OT and AVP in mammals may have changed under evolutionary pressure to result in social bonding from a previously solitary system of social organization. Our proposed theory for the evolutionary basis for monogamy in voles is not necessarily generalizable to all monogamous mammalian species, but does provide some insight into the neural basis for attachment and bonding in these species. It seems that over evolutionary time the neural and genetic correlates of AVP-dependent male territorial behavior have been modified to give rise to the behavioral patterns associated with social monogamy in prairie voles. We hypothesize that in prairie voles, when this system interacts with those involved in social recognition, spatial memory, and reward, previously generalized territorial behaviors can become focused on the sensory signature of the female mate, resulting in the partner becoming a valued aspect of his territory. Thus, the male's pair bond to the female can be viewed in the context of an AVP-mediated territorial behavior, especially considering the selective aggression that the male displays toward novel adult conspecifics after becoming pair bonded to a female. In females, the circuits that mediate the onset of maternal nurturing and infant attachment after parturition and during nursing have been

exapted to give rise to the pair bond. These circuits converge via activation of OXTRs with those involved in social recognition and reward, resulting in pair bonding to a male after mating.

While work in marmosets, macaques, and tamarins has begun to move this field further toward more investigations in primate social behavior, it is still unknown whether the same neural circuits so eloquently mapped in rodents also apply to highly social primates, such as humans. The work described in this chapter has paved the way for investigations into how the human brain functions socially, but there is much left to be discovered here. What are the implications for the neural basis of social bonding in our species? While this chapter will not discuss whether humans should be considered monogamous or not, it is undeniable that men and women develop a relationship in our species that goes beyond orgasm.

There have been a growing number of studies examining both the AVP and OT systems in humans. A recently published study examined whether polymorphisms that exist in the human AVPR1a gene (*AVPR1A*) correlate with pair-bonding behavior among couples. Among men in long-term, live-in relationships lasting at least 5 years, those with one specific variant at a repeat polymorphism, called RS3, were more likely to be uncommitted in their relationship by remaining unmarried; if they were married, they were more likely to report a crisis in their marriage (Walum et al. 2008). Also, the partners of males with this variant were more likely to report dissatisfaction in their relationship (Walum et al. 2008). Studies delivering OT intranasally in humans have shown that OT can affect some aspects of interpersonal relationships, including face perception, emotion recognition, trust, and even perhaps fidelity (Kirsch et al. 2005;

Kosfeld et al. 2005; Domes, Heinrichs, Gläscher, et al. 2007; Domes, Heinrichs, Michel, et al. 2007; Guastella, Mitchell, and Dadds 2008; Scheele et al. 2012). While some recent evidence may suggest that OT has context-specific effects on human social behavior and may not always promote positive social responses (Bartz et al. 2011; Guastella, Graustella, and MacLeod 2012; Churchland and Winkielman 2012), investigations into the neurophysiology of the OT (and AVP) system in humans are promising for the advancement of our understanding of complex social cognition and for the potential development of therapeutics for human conditions characterized by deficits in social function.

Thus, it is apparent that understanding the roles of AVP and OT in regulating monogamy-related behaviors in animal models can provide important insights into the evolution of our own social structure.

Challenges in identifying OXTR and AVPR1a in primate brain tissue

Although some the aforementioned studies highlight recent work examining the role that OT and AVP may be playing in primate social behavior, there is still a considerable lack of knowledge regarding basic neurophysiology of these systems in primate species. This is largely due to limitations not only in access to primate brain tissue but, more importantly, to the lack of reliable detection and localization methods for OT and AVP receptors in the primate brain. Currently, there are no reliable antibodies for immunohistochemistry (IHC) in brain tissue for OXTR; six different antibodies were used for IHC in brain tissue from wildtype mice and OXTRKO mice, and the pattern of staining produced by each of the antibodies was the same for both genotypes, which

demonstrates that IHC is not a reliable method for localizing OXTR in brain tissue (Yoshida et al. 2009). Furthermore, despite recent efforts from our own lab (Smith et al. 2012; Smith, Freeman, Voll, Young, and Goodman 2013a; Smith, Freeman, Voll, Young, and Goodman 2013b), there are currently no *in vivo* neuroimaging agents for OXTR for PET imaging, a technique that would be invaluable in research using humans and nonhuman primates. Early stage development of an AVPR1a tracer for PET neuroimaging is currently underway.

The remaining technique that has been used for decades to localize OXTR and AVPR1a in rodent brain tissue is receptor autoradiography using radiolabeled ligands, but recent work from our own lab, which will be discussed further in **Chapter 2**, confirms previous evidence that these radioligands have mixed affinities for OXTR and AVPR1a in primates (Manning et al. 2008; Manning et al. 2012; Freeman *in prep*). Thus, this technique needs further pharmacological optimization before it can be used reliably to determine the distribution of these receptors in primate brain tissue. The work described in **Chapter 2** provides this pharmacological optimization, and **Chapters 3 and 4** describe how this technique has been used in brain tissue from both a non-monogamous and a monogamous species of primate to elucidate the central distribution of OXTR and AVPR1a. These studies help to establish the necessary neuroanatomical background for future studies of the role of OT and AVP in primate social cognition.

Specific Aims of this Dissertation

The aims of the current study were designed to ameliorate some of the technological limitations of research on OT in primates and to establish a

pharmacological and neuroanatomical foundation of the OT system for future work in primate species.

In **Chapter 2**, I describe the pharmacological characterization of several different OXTR and AVPR1a ligands for binding to the human OXTR and AVPR1a in order to develop an optimized and pharmacologically informed protocol for competitive binding receptor autoradiography for primate brain tissue sections. The design of this protocol will allow for the first time reliable and specific detection of OXTR and AVPR1a in human and nonhuman primate tissue. First, I validated and optimized the general ligand binding protocols then performed *in vitro* ligand binding assays with a variety of OXTR and AVPR1a ligands. Specifically, I performed saturation binding experiments with the commercially available iodinated radioligands for OXTR and AVPR1a in order to determine their binding affinities to the human OXTR and AVPR1a. These binding affinity values will determine what concentration of each radioligand should be used in receptor autoradiography to target OXTR or AVPR1a. Next, I performed competitive binding assays on four ligands (two peptide ligands and two non-peptide ligands) to determine their selectivity between human OXTR and AVPR1a. These experiments determined which ligands are most selective to occupy either OXTR or AVPR1a and also determined the optimal concentration for these unlabeled competitor ligands to target the receptors in a competitive binding protocol. The experiments in Chapter 2 informed the design of a pharmacologically based competitive binding protocol for receptor autoradiography for the reliable localization of OXTR and AVPR1a in primate brain tissue.

The experiments described in **Chapter 3** use this protocol, as well as *in situ* hybridization, to detect OXTR binding patterns in brain tissue sections from the rhesus macaque (*Macaca mulatta*). The rhesus macaque is a model organism that is becoming more and more popular for the study of OT and complex social cognition, yet the central distribution of OXTR has not been elucidated. The AVPR1a map has been published previously by my advisor (Young, Toloczko, and Insel 1999), although those results should be confirmed using the new and more reliable autoradiography protocol outlined in Chapter 2. Therefore, I performed competitive binding receptor autoradiography in brain tissue sections from 9 animals using the iodinated radioligands characterized in Chapter 2. Lastly, adjacent sections of tissue from 9 animals were used for *in situ* hybridization to localize the mRNA for OXTR. Sections were later counterstained for acetycholinesterase to precisely establish anatomical landmarks for accurate interpretation of our receptor autoradiography and *in situ* hybridization results. These experiments characterize for the first time the distribution of OXTR expression in the rhesus macaque brain.

In **Chapter 4**, I extend these neuroanatomical investigations while returning to our lab's focus on monogamy and examine the brain of the socially monogamous coppery titi monkey (*Callicebus cupreus*). This species has been studied for several years for its social behavior, but only recently have investigations involving OT and AVP begun to be conducted (Jarcho et al. 2011; KL Bales, 1R01HD071998-01A1). In the experiments described in Chapter 4, I use the protocol from Chapter 2 to perform receptor autoradiography for OXTR and AVPR1a in five individuals. The sections were counterstained for acetylcholinesterase to determine anatomical landmarks for accurate

description of the results. This study characterizes the central distribution of OXTR and AVPR1a for the first time in this species.

The work of this dissertation builds upon previous studies on the neurophysiology of OT and AVP and the roles these systems play in modulating social behavior in rodents by providing neuroanatomical foundations for future research in primate species. However, we generally still know very little about the neurophysiology of the OT system in primates. Thus, in **Chapter 5**, I review how sophisticated experimental techniques have been used to elucidate the neurophysiology of the OT and AVP systems in rodents, and I identify several specific ways that these established technologies can be used to answer basic questions in nonhuman primate models. I highlight areas of future research in nonhuman primates that are experimentally poised to yield critical insights into the anatomy and physiology of the OT system and apply these perspectives to human research goals in order to advance our understanding of the OT system into the 21st century.

In summary, the work put forth in this dissertation helps to inform the design of future studies of the OT system in humans and nonhuman primates, which can ultimately advance the development of therapeutics for human social dysfunction.

Chapter 2:

Pharmacological Characterization of the Oxytocin Receptor and Vasopressin 1a Receptor in Primates: Design of a Competitive Binding Receptor Autoradiography Protocol

This chapter presents work to be published within:

Sara M. Freeman, Kiyoshi Inoue, Aaron L. Smith, and Larry J. Young. Localization of oxytocin receptors in the brain of the rhesus macaque (*Macaca mulatta*).

ABSTRACT

Oxytocin (OT) and vasopressin (AVP) are two evolutionarily ancient peptides that are similar in their structure and function. They differ in only two amino acid positions out of nine and are capable of binding to each other's receptors. As a consequence, many of the agonists and antagonists that have been developed to probe this system also exhibit mixed affinities, especially in human and nonhuman primate species. Therefore, we have pharmacologically characterized the binding properties of commercially available radioligands and unlabeled ligands using the human oxytocin receptor (hOXTR) and human vasopressin 1a receptor (hAVPR1a). Ligand binding assays were performed using membrane preparations from CHO cell lines that constitutively express either the gene for hOXTR or for hAVPR1a. Saturation binding assays using the iodinated OXTR radioligand (^{125}I-OVTA) or the iodinated AVPR1a radioligand (^{125}I-LVA) determined for the first time their binding affinities for the human receptors. ^{125}I-OVTA shows a high affinity for both the hOXTR (K_d = 90 ± 17 pM) and the hAVPR1a (K_d = 360 ± 51 pM). Similarly, ^{125}I-LVA also shows a high affinity for the hAVPR1a (K_d = 30 ± 3.0 pM) and the hOXTR (K_d = 590 ± 450 pM). Competitive binding assays were performed on hOXTR and hAVPR1a using a novel, nonpeptide small molecule OXTR ligand (ALS-II-69) and a nonpeptide small molecule reported to be selective for hAVPR1a (SR49059). In competition with ^{125}I-LVA, ALS-II-69 has a K_i of 3.41 nM for the hOXTR and a very low affinity for the hAVPR1a, with a K_i > $2.47x10^3$ nM. In competition with ^{125}I-OVTA, SR49059 has a K_i of 19.7 nM for the hOXTR and a K_i of 1.68 nM for hAVPR1a. Analysis of the competition curves suggests that performing OXTR autoradiography with ^{125}I-OVTA in combination with 10 nM

SR4905 would reveal OXTR binding by displacing ~85% of the radioligand binding to hAVPR1a without drastically reducing binding to hOXTR. Due to its high selectivity, ALS-II-69 can be co-incubated with ^{125}I-LVA at concentrations up to 1 μM to selectively displace radioligand binding to OXTR and reveal AVPR1a. The parameters identified in the ligand binding assays in the current study have resulted in a pharmacologically informed protocol for competitive binding receptor autoradiography that will be useful for accurately mapping these receptors in the brains of humans and nonhuman primates.

INTRODUCTION

Oxytocin (OT) and vasopressin (AVP) are two structurally related peptide hormones that have a range of physiological effects in both the central nervous system as well as in the periphery. The effects of OT in the periphery on stimulating uterine contractions to initiate labor and mediating lactation at the mammary gland have been well established (Soloff, Alexandrova, and Fernstrom 1979; Fuchs et al. 1982). Several pharmaceutical companies and independent researchers have been working for decades to develop OT receptor (OXTR) antagonists for the prevention of preterm labor (Manning et al. 2012; Barberis et al. 1999; Williams et al. 1998; Chan et al. 1996; Akerlund, Melin, and Maggi 1995; Pettibone et al. 1995) in human, although the high sequence homology between the OXTR and the vasopressin 1a receptor (AVPR1a) have made the development of selective ligands for these receptors quite challenging. Similarly, the actions of AVP in the periphery at the AVPR1a to control vasoconstriction of the blood vessels and at the vasopressin 2 receptor (AVPR2) in the kidney to maintain water balance have also been targets for the development of pharmacotherapies for the regulation of blood pressure and for treating diabetes insipidus, respectively (Thibonnier et al. 2002; Manning et al. 2008; Manning et al. 2012). Due to the high therapeutic value of developing selective ligands for the treatment of these fairly common human conditions, these peripheral actions of OT and AVP have been the focus of decades of research, often beginning with rodent models. Unfortunately, as researchers in this field are aware, many of the agonists and antagonists that were developed in rodent models and shown to exhibit reliable receptor subtype selectivity, fail to demonstrate selectivity in humans or nonhuman primates (Manning et al. 2012). For example, ligands that are

highly specific for AVPR1a in rodents will exhibit a mixed affinity for both OXTR and AVPR1a in humans, or for multiple receptor subtypes of AVP receptors. This cross reactivity of OXTR and AVP receptor ligands presents a significant obstacle in translating rodent research into human pharmacotherapies.

This issue extends into research on the central mechanisms of these peptides as well. In the brain, decades of research has shown that OT and AVP can act as potent neuromodulators in a variety of species to influence complex social behaviors including, but not limited to: maternal behavior (Kendrick et al. 1986; Kendrick, Keverne, and Baldwin 1987; Kendrick, Keverne, Chapman, and Baldwin 1988a; Olazábal and Young 2006b; Pedersen and Prange 1979; Pedersen et al. 1982; Pedersen et al. 1994; Takayanagi et al. 2005; Pedersen et al. 2006), aggression (Winslow et al. 1993; Ferris et al. 1997; Young et al. 1997; Gobrogge et al. 2009), affiliation (Rosenblum et al. 2002; Winslow et al. 2003; Snowdon et al. 2010), territoriality (Ferris et al. 1984; Albers et al. 1986), social monogamy (Young and Wang 2004; Smith et al. 2010; Jarcho et al. 2011), and social memory (Dantzer et al. 1987; Dantzer et al. 1988; Ferguson et al. 2000; Ferguson et al. 2001; Bielsky et al. 2004; Bielsky et al. 2005). Similar to the research on peripheral mechanisms of these hormones, many of the studies that established these central effects also used rodent models, such as mice, rats, hamsters, and voles. Thus, as research in behavioral and social neuroscience builds upon the foundational work conducted in rodents and expands to include research in nonhuman primates as well as humans, it is becoming crucial to understand how these pharmacological tools are acting when they are used to probe the OT and AVP systems in primates. Without a solid understanding of how these ligands function in humans and nonhuman primates, researchers working on

these systems risk misinterpreting their findings due to the off-target effects of these compounds.

Furthermore, due to this issue of mixed affinities of the available ligands, research progress on the basic physiology of OXTR and AVPR1a in primates has been markedly hindered, especially fundamental neuroanatomical research efforts to localize the distributions of these receptors in brain tissue. Characterizing the central distributions of receptors will identify the brain regions that respond to these peptides and furthermore, will inform the design of future experiments to target these brain areas in order to begin to elucidate the neurophysiology of these systems. However, the OXTR radioligand ^{125}I-ornithine vasotocin analog (^{125}I-OVTA) and the AVPR1a radioligand ^{125}I-linear vasopressin-1a antagonist (^{125}I-LVA), which have been used extensively in rodent species for receptor autoradiography to identify the location of OXTR and AVPR1a in brain tissue, have been used only minimally and with caution in brain tissue from primates. For example, in an early effort to map the distribution of OXTR in the rhesus macaque brain, Tolockzo and colleagues concluded that the binding pattern produced by ^{125}I-OVTA in this species is due to binding to the AVPR1a, and subsequently asks the question, “Are there oxytocin receptors in the primate brain?” (Toloczko, Young, and Insel 1997). There have been two reports of OXTR and/or AVPR1a binding in the marmoset brain (Wang et al. 1997; Schorscher-Petcu, Dupré, and Tribollet 2009), although these studies did not take into account the fact that these radioligands have not been pharmacologically characterized for their binding properties in primates. The more recent paper of the two was the first to use a competitive binding protocol for receptor autoradiography, and the authors performed a more extensive competition experiment

than previous publications, in an effort to show selectivity of the radioligands and to help inform the concentration of the unlabeled competitors (Schorscher-Petcu, Dupré, and Tribollet 2009). However, the concentrations they chose were arbitrary and not based on the pharmacological profile of the specific molecules in use.

Therefore, the specific aims of the present study include: (i) to characterize the binding affinity and selectivity for the human OXTR and AVPR1a of the two commercially available radioligands: the OXTR radioligand ^{125}I-OVTA and the AVPR1a radioligand ^{125}I-LVA; (ii) to characterize the binding affinity and selectivity for the human OXTR and AVPR1a for two purported human-selective, small molecule ligands: SR49059, an AVPR1a antagonist (Gal, Wagnon, and Garcia 1993) and ALS-II-69, a novel OXTR antagonist (Smith, Freeman, Voll, Young, and Goodman 2013b) in comparison with two commonly used peptide ligands; and (iii) to identify a pharmacologically-informed, competitive-binding protocol for receptor autoradiography which should selectively reveal the OXTR and AVPR1a distributions in primate brain tissue. In doing so, the present study seeks to begin to ameliorate the pharmacological and neuroanatomical limitations currently facing research on OT and AVP physiology in primate species.

METHODS

Cell culture

Two stably transfected Chinese hamster ovary (CHO) cell lines were used: one expressing the gene for the human oxytocin receptor (hOXTR) and one expressing the gene for the human vasopressin 1a receptor (hAVPR1a). These cell lines were gifts of the

Dr. Brian Roth at UNC-Chapel Hill, a part of the NIH Psychoactive Drug Screening Program (PDSP). We used cell lines that constitutively express the human receptors as a proxy for the rhesus receptors because cell lines expressing the rhesus OXTR or AVPR1a gene are not readily available. Due to the high homology between the rhesus OXTR and human OXTR (97% identical amino acid sequence), we feel that using the human receptors is an appropriate screening tool for pharmacology (Salvatore et al. 1998). Cells were thawed and put into culture in sterile, filtered Ham's F-12 media (GIBCO; Grand Island, NY) with 10% fetal bovine serum (Hyclone; Waltham, MA), 1% Pen/Strep (GIBCO; Grand Island, NY), 15 mM 1X HEPES buffer solution (GIBCO; Grand Island, NY), and 400 μg/mL of the selection agent G418 sulfate (cellgro; Manassas, VA), which ensures growth of only the cells expressing the vector of interest. Cells were grown in 75 cm^2 culture flasks (Corning; Manassas, VA) in an incubator at 37°C, 100% humidity, 5% CO_2, and were passaged with trypsin when they reached ~80% confluency. For control experiments, a line of untransfected control CHO cells (CHO K1) were cultured in the same way as described above, except that the culture medium did not include the selection agent G418 sulfate. These cells were a gift of Dr. Randy Hall at Emory University.

Membrane Preparation & Protein Quantification

Cells were plated in 6-well plates and allowed to grow until they reached confluency. The media was vacuumed off, and 1X sterile PBS (pH 7.4) was added to each well. The cells were harvested into the PBS using a cell scraper, pipetted into 1 mL centrifuge tubes, and placed on ice. Cells were centrifuged for 5 min at 2,000 rpm at 4°C.

The supernatent was vacuumed off, and 500 mL of ice cold Buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) was added. Cells were homogenized (Cole-Parmer LabGEN 125 homogenizer) on medium speed for approximately 10 sec and then ultracentrifuged for 10 min at 44,000 rpm at 4°C. The supernatent was vacuumed off, 500 mL of ice cold Buffer A was added, and cells were homogenized and ultracentrifuged again at the same settings as before. After this second ultracentrifugation, the supernatent was vacuumed off, and 355 mL of Buffer A was added. Protein in the membrane preparation samples was quantified using the Pierce BCA Protein Assay Kit according to the kit's instructions (Thermo Scientific 23227). Diluted BSA standards were made according to the kit's preparation instructions using Buffer A as the diluent. The BCA protein quantification assay was performed as described below using 5 mL of each sample, and the remaining 350 mL of cell membranes in Buffer A were prepared for freezing: 50 mL of a 10X protease inhibitor was added to prevent protein degradation in the samples, and then 100 mL of a 5 mg/mL BSA, 25 mM $MgCl_2$ solution was added, which resulted in a final volume of 500 mL and a final buffer concentration of 1 mg/mL BSA, 5 mM $MgCl_2$ for freezing. For each of the three groups of samples (hOXTR, hAVPR1a, and CHO K1 controls), after protein quantification was complete, samples were mixed together, protein concentration results were averaged, and cell membrane samples were aliquoted prior to freezing. Membrane preparations remained frozen at -20°C until use for ligand binding assays.

Ligand Binding Assay Optimization

Determination of best pretreatment of filter paper

Based on the pretreatment options outlined in Vergote et al. (2009), we decided to test the following soaking conditions for the filter paper: 4-hour soak in 10 mg/ml BSA, 4-hour soak 0.5% PEI, or 4-hour soak 0.5% PEI followed by a rinse in 10 mg/ml BSA (Vergote et al. 2009). We tested both radioligands (^{125}I-OVTA and ^{125}I-LVA) to determine the filter pretreatment's effect on non-specific binding. Reaction mixtures were prepared with 0.5 nM of ^{125}I-OVTA or ^{125}I-LVA and 18 µg of membrane protein from the untransfected CHO K1 cell controls in a total volume of 200 mL of Buffer B (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mg/ml BSA, pH 7.4). One set of triplicate reactions was performed. After a 1-hour incubation at room temperature (RT), reactions were rapidly vacuum-filtered onto Whatman GF/C filter paper using a Brandel Cell Harvester and washed three times with cold Buffer A (4°C). The radioactivity of the filter paper was then read using a gamma counter. Unpaired, two tailed t-tests were used to compare groups; multiple comparisons were corrected by setting the alpha level to 0.01.

Determination of non-specific binding to untransfected CHO cells

In order to establish that our radioligands were not binding to any non-specific sites on the CHO cells themselves, we measured the total binding of 1 nM ^{125}I-OVTA or ^{125}I-LVA in three different reaction preparations (final volume 200 uL in Buffer B): 20 µg membrane protein from untransfected CHO K1 cells, 20 µg membrane protein from stably transfected CHO cells (hOXTR preparations for ^{125}I-OVTA; hAVPR1a preparations for ^{125}I-LVA), or no protein added. Specificity of binding was determined in the presence of 1 µM purportedly primate-selective unlabeled competitors: SR49059 for hAVPR1a and ALS-I-41 for hOXTR. After a 1-hour incubation at room temperature

(RT), reactions were rapidly vacuum filtered using a Brandel Cell Harvester onto Whatman GF/C filter paper (soaked in the optimal pretreatment determined above) and washed three times with cold Buffer A (4°C). The radioactivity of the filter paper was quantified using a gamma counter. One set of triplicate reactions was performed. Unpaired, two tailed t-tests were used to compare groups; multiple comparisons were corrected by setting the alpha level to 0.01.

Determination of optimal amount (µg) of protein to prevent ligand depletion

In order to determine the optimal number of micrograms of membrane protein to add to each reaction mixture to prevent ligand depletion, increasing amounts of membrane protein (hOXTR or hAVPR1a) were added to constant concentrations of radioligand (50 pM of ^{125}I-OVTA or ^{125}I-LVA). The amounts of membrane protein included: 0, 2, 4, 6, 8, 10, 15, and 20 µg of protein. Non-specific binding was measured in the presence of 1 µM OT (for hOXTR) or AVP (for hAVPR1a). The final volume of all reactions was 200 mL in Buffer B. One set of triplicate reactions was performed. After a 1-hour incubation at room temperature (RT), reactions were rapidly vacuum filtered using a Brandel Cell Harvester onto Whatman GF/C filter paper (soaked in the optimal pretreatment determined above) and washed three times with cold Buffer A (4°C). The radioactivity of the filter paper was quantified using a gamma counter. Measurements of the percent of total activity for total binding and non-specific binding were calculated, and only those reactions in which total and nonspecific binding were below 10% were considered as optimal amounts of membrane protein.

Saturation Binding Assay

In order to determine the binding affinity (measured by the dissociation constant, K_d) of two available ^{125}I-labeled radioligands for hOXTR and hAVPR1a, saturation binding assays were performed. Eleven increasing concentrations of each radioligand were tested: 0.008, 0.016, 0.024, 0.031, 0.046, 0.062, 0.093, 0.125, 0.187, 0.250, and 0.500 nM. Reactions were initiated by adding 3 μg of protein (optimal amount determined above) from the membrane preparations to each reaction tube. Both radioligands (^{125}I-OVTA and ^{125}I-LVA) were tested for their ability to saturate both receptors (hOXTR and hAVPR1a), resulting in four different combinations of radioligand binding to receptor. All reactions took place in 200 mL Buffer B. Reactions were incubated for 1 hr at RT and then rapidly vacuum filtered using a Brandel Cell Harvester onto Whatman GF/C filter paper (soaked in the optimal pretreatment determined above) and washed three times with cold Buffer A (4°C). The radioactivity of the filter paper was read using a gamma counter. All reactions in this assay were performed in triplicate, and each set of triplicate values was averaged. Outliers were removed using the Grubbs test (critical Z = 1.15; two-sided alpha set to 0.01). Two independent replications of this assay were performed. Non-specific binding averages were subtracted from total binding averages to give a measure of specific binding of the radioligand to its receptor. Nonlinear regression analyses for one-site saturation binding were performed on the resulting specific binding data using GraphPad Prism 5.0 (San Diego, CA) in order to determine K_d and B_{max}.

Competitive Binding Assay

We tested four compounds: two OXTR ligands and two AVPR1a ligands. Two of the ligands were developed for use in rodent and have been shown to be selective for their respective receptors in rodent tissue: the OXTR agonist [Thr^4,Gly^7]OT (T4G7OT) and the AVPR1a antagonist d$(CH_2)_5$[$Try(Me)^2$]AVP (Manning compound). The other two ligands we tested are both small molecule, non-peptide ligands and were developed specifically for use in human tissue. They have been reported to be highly selective between hOXTR and hAVPR1a: a novel OXTR antagonist developed by Dr. Aaron Smith in our lab (ALS-II-69) and an AVPR1a antagonist developed by Sanofi Recherche (SR49059). The original synthesis reactions of these molecules have been published elsewhere (Smith, Freeman, Voll, Young, and Goodman 2013b; Gal, Wagnon, and Garcia 1993). Twelve different increasing concentrations (on a log and semilog scale) of each of the four competitor ligands were tested: 10^{-13}, 10^{-11}, 10^{-10}, $10^{-9.5}$, 10^{-9}, $10^{-8.5}$, 10^{-8}, $10^{-7.5}$, 10^{-7}, $10^{-6.5}$, 10^{-6}, and 10^{-5}. AVPR1a compounds were tested for their ability to compete ^{125}I-OVTA off of hOXTR and hAVPR1a. OXTR compounds were tested for their ability to compete ^{125}I-LVA off of hOXTR and hAVPR1a. The concentrations of the radioligands were held constant at the radioligand's K_d (determined by the saturation experiments above) for the receptor in use. All reactions took place in 200 µL Buffer B. Reactions were incubated for 1 hr at RT and then rapidly vacuum filtered using a Brandel Cell Harvester onto Whatman GF/C filter paper (soaked in the optimal pretreatment determined above) and washed three times with cold Buffer A (4°C). The radioactivity of the filter paper was read using a gamma counter. All reactions in this assay were performed in triplicate, and each set of triplicate values was averaged. Two independent replications of this assay were performed. Nonlinear regression analyses for one-site

competition binding were performed on the resulting specific binding data using GraphPad Prism 5.0 (San Diego, CA) in order to determine K_i.

RESULTS

Ligand Binding Assay Optimization

Determination of best pretreatment of filter paper

In order to reduce the non-specific binding of positively charged or peptide radioligands to the filter paper in ligand binding assays, the filter paper needs to be pre-treated in a solution of either polyethylenimine (PEI) or bovine serum albumin (BSA). PEI is a cation and therefore repels positively charged radioligands from the filter paper, and BSA prevents adhesion of ligands to non-specific binding sites in a variety of *in vitro* assays, including ligand binding assays. Based on the pretreatment options outlined in Vergote et al. (2009), we decided to test the following soaking conditions for the filter paper: 4-hour soak in 10 mg/ml BSA, 4-hour soak 0.5% PEI, or 4-hour soak 0.5% PEI followed by a rinse in 10 mg/ml BSA (Vergote et al. 2009).

The 0.05% PEI and 0.05% PEI plus dip in BSA were not significantly different from each other for either radioligand (for ^{125}I-LVA, $p=0.492$, Figure 2.1A; for ^{125}I-OVTA, $p=0.077$, Figure 2.1B). We found that the pretreatment that reduced background levels of binding the most for both radioligands is a 4-hour soak in 10 mg/ml BSA ($p<0.0001$ for each radioligand; Figure 2.1). All subsequent pharmacology experiments were performed using a 4-hour pretreatment of the filter paper in 10 mg/ml BSA.

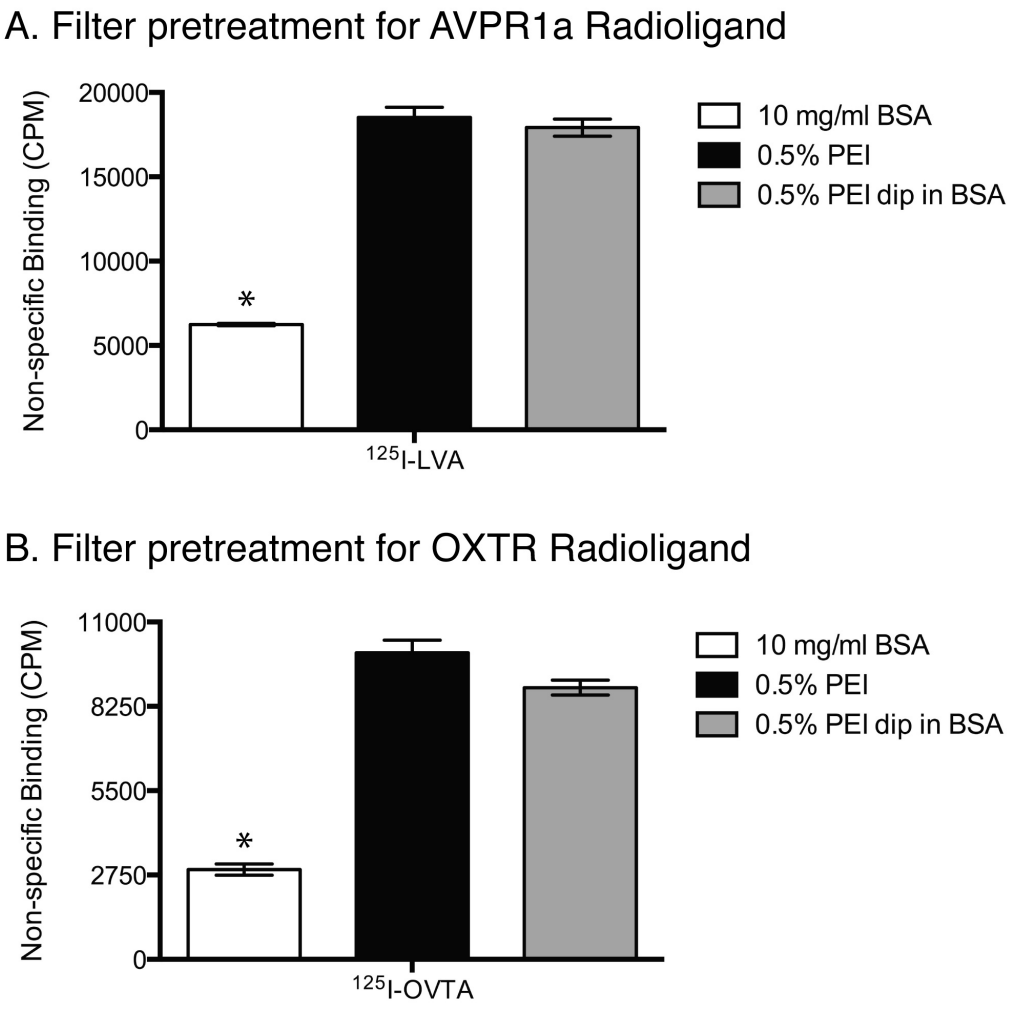

Figure 2.1. Determination of optimal pretreatment for filter paper in ligand binding assays.
A 4-hour soak in 10 mg/ml BSA significantly reduced background binding of both radioligands to control CHO K1 cell membranes compared to the other two pretreatment conditions ($p<0.0001$). n=3 replicates per group.

Determination of non-specific binding to untransfected CHO cells

Because the receptors of interest are expressed in CHO cells, it is necessary to determine whether the radioligands that are being characterized are capable of binding to sites on the CHO cell membranes other than the receptors of interest. These sites could be other receptors that are endogenously expressed by the CHO cells, including hamster OXTR and AVPR1a, or non-specific interactions with the membranes. Binding to these sites could affect our analysis of the binding properties of the radioligands in question. In order to establish that our radioligands are not binding to any of these non-specific sites on the CHO cell membranes themselves, we measured the total binding of 1 nM ^{125}I-OVTA or ^{125}I-LVA to three different reaction preparations: untransfected CHO K1 cells, stably transfected CHO cells (hAVPR1a preparations for ^{125}I-LVA; hOXTR preparations for ^{125}I-OVTA), or no protein added. Specificity of binding was determined in the presence of the reportedly primate-selective unlabeled competitors: SR49059 for hAVPR1a and ALS-I-41 (a slight variant of ALS-II-69, only differing in molecular weight by 80 daltons) for hOXTR.

We found that ^{125}I-LVA binds to hAVPR1a membranes significantly more than to untransfected CHO cell membranes ($p=0.0014$), and its binding to untransfected CHO cells does not differ from the radioactivity levels when no protein was added ($p=0.6249$; Figure 2.2A). Furthermore, SR49059 reduces binding of ^{125}I-LVA to hAVPR1a membranes down to the levels of background binding observed with untransfected CHO membranes and no protein added (Figure 2.2A). Similarly, we found that ^{125}I-OVTA binds to hOXTR membranes significantly more than to untransfected CHO cell membranes ($p<0.0001$), and its level of binding to untransfected CHO cells does not

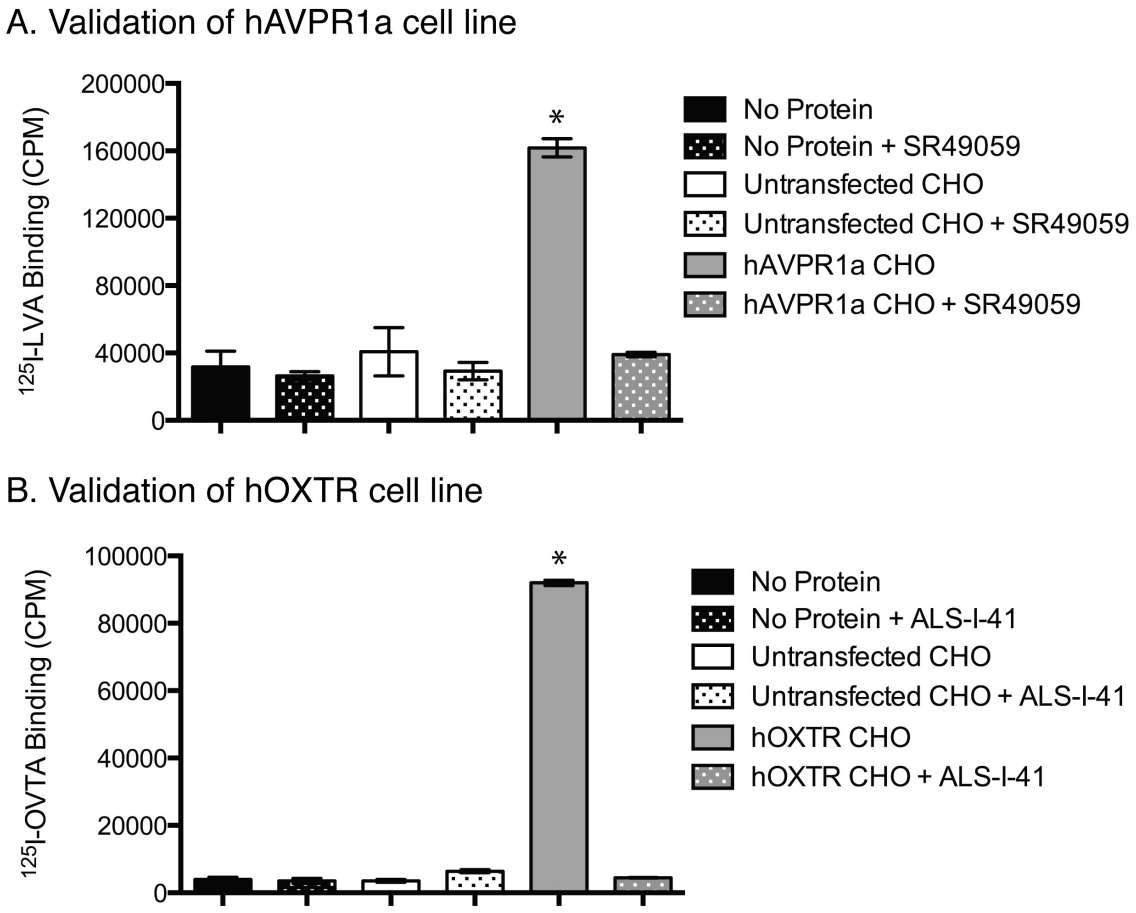

Figure 2.2. Determination of binding specificity to transfected CHO cells. Membrane preparations from the hAVPR1a (A) and hOXTR (B) cell lines used in the current study do not have endogenous binding sites for the radioligands of interest in subsequent experiments. Selective competitors are capable of displacing radioligand binding to the level detected in untransfected CHO cells and no protein conditions. *$p<0.001$. n=3 replicates per group.

differ from the binding levels when no protein was added (p=0.5878; Figure 2.2B). Furthermore, ALS-I-41 is capable of reducing the binding of ^{125}I-OVTA to hOXTR membranes down to the levels of background binding that is seen in the presence of untransfected CHO membranes or no protein at all (Figure 2.2B). Therefore, our hOXTR and hAVPR1a CHO cell lines are reliable and will not contaminate our ligand binding assays with nonspecific binding sites intrinsic to the cells themselves.

Determination of optimal amount (µg) of protein to prevent ligand depletion

Ligand binding assays are analyzed under the assumption that only a very small fraction of the available ligand binds to the available receptors (or to non-specific sites) in the reaction mixture. Based on this assumption, one can also assume that the concentration of free ligand in the reaction mixture is the same as the concentration of the ligand that was added. Standard analyses of ligand binding assays such as the ones described in this paper mathematically require that the concentration of free ligand is in effect equal to the concentration added at the start of the binding assay. Therefore, if the radioligand binding is larger than 10% of the amount of radioligand added to the reaction mixture, one can assume that the free ligand concentration in the reaction mixture was depleted by binding to both specific and non-specific sites and is now less than the concentration added. Because it is experimentally impossible to determine this new concentration of free ligand when using the vacuum filtration method used in the present experiment, the occurrence of ligand depletion during ligand binding assays indicates that optimization of the reaction is needed. The most common cause of ligand depletion is the presence of too large an amount of available receptors; therefore, we ran optimization

experiments to determine the ideal amount of membrane protein to add to the reaction mixtures in order to prevent ligand depletion in all subsequent assays.

In order to determine if ligand depletion is present in an assay with radioactive ligands, one must measure the total radioactivity of the concentration of radioligand added to the reaction mixture before filtration, the total binding of the radioligand, and the non-specific binding of the radioligand in the presence of 1 μM of a competitor ligand. From this information, the percent of the total activity added can be calculated for both total and nonspecific binding. We found that the ideal amount of protein to prevent ligand depletion (defined as less than 10% of total binding) when ^{125}I-LVA is incubated with hAVPR1a is 2 μg (Figure 2.3A); for ^{125}I-OVTA with hOXTR, the ideal amount is 4 μg (Figure 2.3B). Therefore, we continued all subsequent experiments using an average value of 3 μg of protein per reaction tube.

Saturation Binding Assay

In order to determine the binding affinity (measured by the dissociation constant, K_d) of two available ^{125}I-labeled radioligands for hOXTR and hAVPR1a, saturation binding assays were performed. In these assays, a constant amount of protein (3 μg, as determined from optimization experiments) is added to increasing concentrations of a radioligand. Because the amount of available receptors remains constant as the concentration of free radioligand increases in each reaction, the specific binding of the radioligand will eventually level off as the receptors become saturated with bound radioligand. This resulting saturation binding curve can be analyzed in order to yield the K_d and the B_{max} of the receptors in the sample.

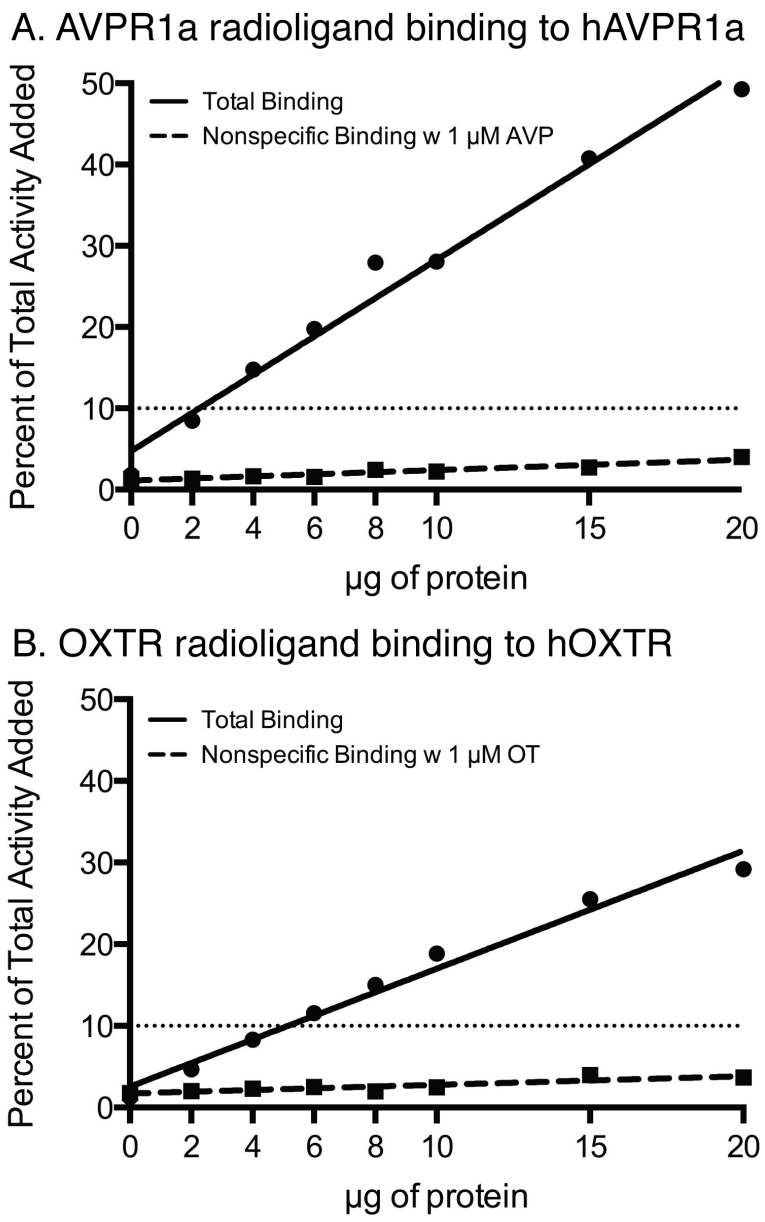

Figure 2.3. Determination of optimal µg of protein to add to test tubes to prevent ligand depletion.
Dotted line at 10 percent indicates cut-off margin for ligand depletion; only values below this criteria will be considered safe to prevent ligand depletion. (A) Total and nonspecific AVPR1a radioligand binding to hAVPR1a membranes as a percent of total activity added, showing that 2 µg is the ideal amount of protein to prevent ligand depletion. (B) Total and nonspecific OXTR radioligand binding to hOXTR membranes as a percent of total activity added, showing that 2-4 µg is the ideal amount of protein to prevent ligand depletion. n=3 replicates per group.

We found that both radioligands have a high (subnanomolar) affinity in the pM range for both of the human receptors (Figure 2.4; Table 2.1). ^{125}I-LVA binds to hAVPR1a with a higher affinity (30 pM) than it binds to hOXTR (~590 pM). Similarly, ^{125}I-OVTA binds to hOXTR with a higher affinity (92 pM) than it binds to hAVPR1a (360 pM). The binding affinity value of 92 pM for ^{125}I-OVTA agrees with the only other previous evaluation of this molecule on hOXTR, which reports its K_d as 0.095 nM (Chini et al. 1995). These data support the conclusion that when assessed in human, these radioligands still retain a slightly higher affinity for their own receptor, but they lose their overall selectivity and exhibit a high affinity for the opposite receptor. Therefore, these radioligands are capable of cross-reacting between hOXTR and hAVPR1a when used in tissue from humans or non-human primates.

Despite the fact that both of these available radioligands can bind to both of the receptors, they can still be used for primate receptor autoradiography if they are used in the presence of an unlabeled displacer ligand. This experimental design is called competitive binding autoradiography and takes advantage of the fact that the unlabeled competitor can occupy the receptors that are not of interest. In this way, when the radioligand is co-incubated along with a selective competitor, the radioligand's only available binding sites are those remaining from the receptor of interest. Thus, we have determined the optimal concentration at which these radioligands should be used in order to promote binding to their receptors: 30 pM for ^{125}I-LVA and 90 pM for ^{125}I-OVTA.

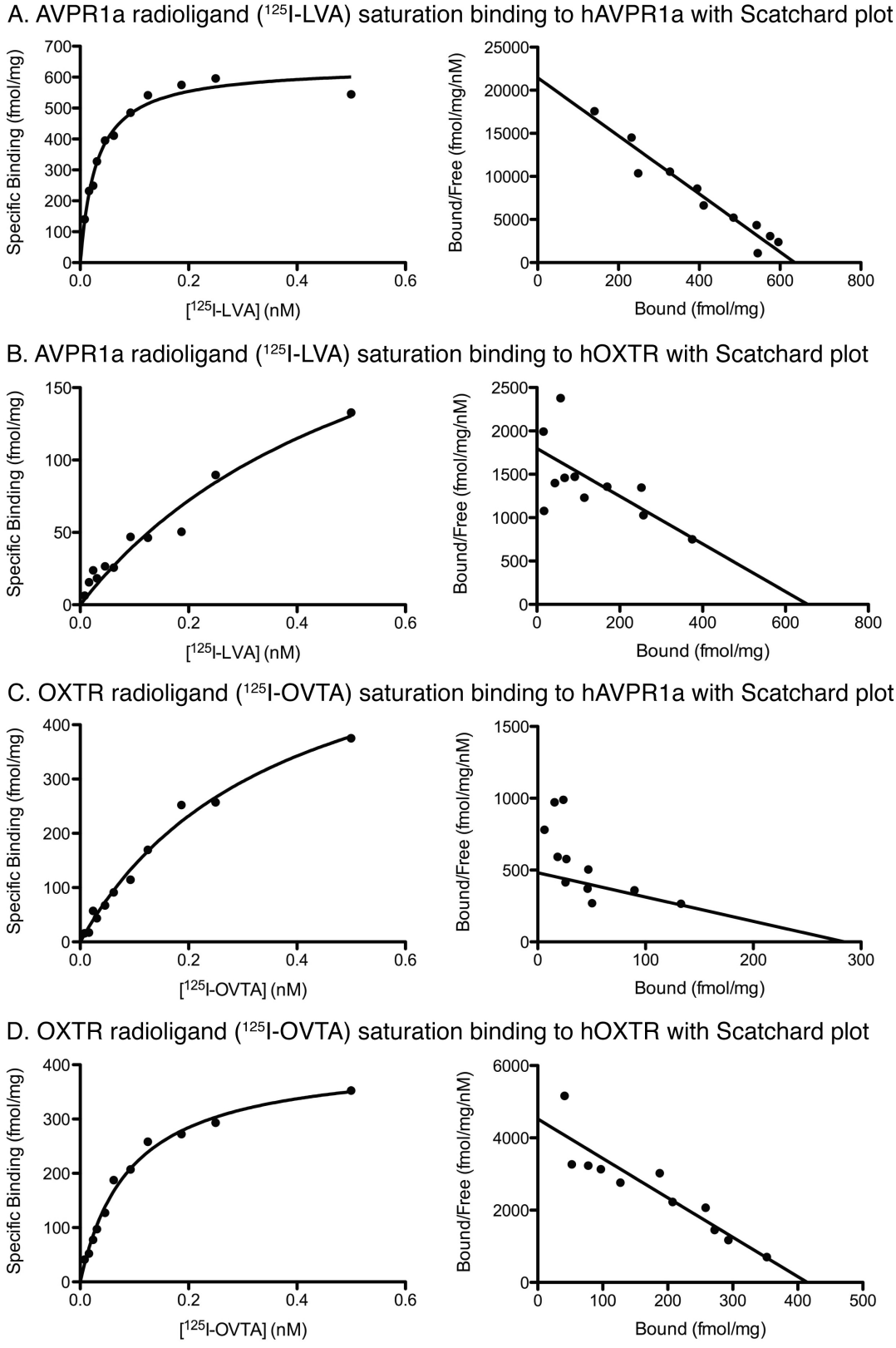

Figure 2.4. Saturation binding curves and Scatchard plot transformations for OXTR and AVPR1a radioligand binding to human receptors. Details on next page.

Figure 2.4 (*previous page*). Saturation binding curves and scatchard plot transformations for OXTR and AVPR1a radioligand binding to human receptors. Saturation curves (left) and scatchard plots (right) for radioligand binding to hOXTR and hAVPR1a. (A) AVPR1a radioligand binding to hAVPR1a. (B) AVPR1a radioligand binding to hOXTR. (C) OXTR radioligand binding to hAVPR1a. (D) OXTR radioligand binding to hOXTR.

	K_d (pM)		B_{max} (fmol/mg)	
	hOXTR	hAVPR1a	hOXTR	hAVPR1a
^{125}I-LVA	590 ± 450	30 ± 3.1	290 ± 150	640 ± 19
^{125}I-OVTA	92 ± 17	360 ± 51	410 ± 31	650 ± 54

Table 2.1. Binding affinities (K_d) and B_{max} values from saturation binding analyses.

Competition Binding Assay

The next step in pharmacologically optimizing a competitive binding autoradiography protocol is to determine the binding affinities and selectivity profile of the competitors for the two receptors of interest. In order to determine the binding affinity (measured by the dissociation constant, K_i) of two unlabeled OXTR ligands and of two unlabeled AVPR1a ligands, competitive binding assays were performed. In these assays, a constant amount of protein (3 μg, as determined from optimization experiments described above) and a constant concentration of radioligand (at the radioligand's K_d, determined by the saturation experiments above) are added to each of twelve different, increasing concentrations of the unlabeled competitor ligand. Because the amount of available receptors and the amount of radioligand is held constant in each reaction, the binding of the radioligand to receptor is consistent in each reaction tube when no competitor is present. With increasing concentrations of added competitor, the radioligand binding will eventually drop off as the competitor ligand outcompetes the radioligand for binding to the available receptors. The resulting competitive binding curve can be analyzed to yield the K_i for the competitor compound, and when the two curves are compared, the ideal concentration to use in competitive binding receptor autoradiography can be determined. This ideal concentration is one at which a majority of binding to one receptor is displaced but the binding to the other receptor is unaffected. For example, for an AVPR1a competitor, the ideal concentration is a concentration that displaces almost all of the binding to the AVPR1a, but leaves the majority of the binding to OXTR intact.

The resulting competition binding curves and binding affinity values for the two purported human-selective, non-peptide ligands are shown in Figure 2.5 and Table 2.2, respectively. We determined that the best concentration for SR49059 to bind to hAVPR1a without binding to hOXTR is 10 nM (Figure 2.5A). As the curves in Figure 2.5B show, the OXTR antagonist ALS-II-69 is extremely selective for the human OXTR, and it only barely begins to bind to hAVPR1a at concentrations higher than 1 μM. Therefore, concentrations of ALS-II-60 up to 1 μM could be used to displace binding to OXTR without affecting the binding to AVPR1a.

Furthermore, we also characterized two ligands that have been used for decades in the rodent literature. Here, we show that the most frequently used AVPR1a antagonist, the Manning compound, is as selective for the human receptors as SR49059 (Table 2.2). This result is somewhat expected, given that SR49059 was reported originally to have a potency similar to that of the Manning compound, despite its structural differences (Gal, Wagnon, and Garcia 1993). However, previous reports of the binding affinities of the Manning compound for the human OXTR and AVPR1a show that it may be even less selective that our data indicate here, with a K_i for hOXTR at 3 nM and a K_i for hAVPR1a at 1.6 nM (Manning et al. 2012). Regardless of this slight discrepancy, the Manning compound has been used in at least one previous experiment in primate tissue in order to show specificity of radioligand binding (Young, Toloczko, and Insel 1999), and it was used at a concentration of 1 uM, which is a high enough concentration for this ligand to bind fully to both AVPR1a and OXTR in primate tissue. Thus, although our data indicate that the Manning compound may be as selective as SR49059 for the human receptors, there have been several reports cautioning the use of the Manning compound in

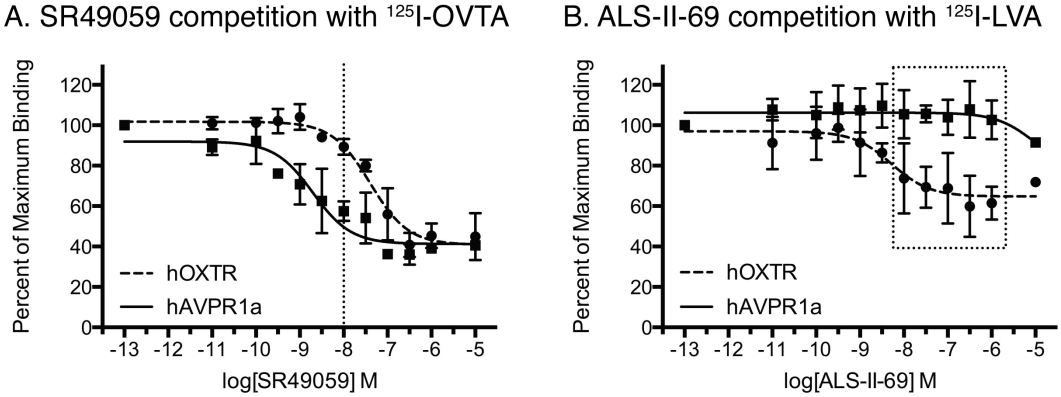

Figure 2.5. Competition binding curves and binding affinity determination for competitor ligands.
A. Competition curves for the AVPR1a ligand SR49059 showing that 10^{-8} M, or 10 nM, is the optimal concentration to occupy hAVPR1a without binding significantly to hOXTR (dotted line). B. Competition curves for the OXTR ligand ALS-II-69 showing that this ligand does not begin to occupy hAVPR1a until 10^{-5} M, or 10 μM. Thus, this ligand can be used at concentrations ranging to 1 μM (box).

K_i (nM)	SR49059	ALS-II-69	Manning	T4G7OT
hAVPR1a	1.68	≥ 2.47x10^3	0.66	85.5
hOXTR	19.7	3.41	11.2	72.5

Table 2.2. Binding affinities (K_i) from competition binding analyses (nM).

human or nonhuman primate systems (Manning et al. 2012; Manning et al. 2008). Therefore, we feel that SR49059 is a better candidate for competitive binding receptor autoradiography in primate tissue.

We also report the binding affinities for the OXTR agonist T4G7OT and show that this compound has effectively equivalent affinities for hOXTR and hAVPR1a (Table 2.2). However, these values do not agree with previously published affinities for hOXTR and hAVPR1a for this compound, which report the K_i for hOXTR as 6.6 nM and the K_i for hAVPR1a as 305 nM (Manning et al. 2012). These authors indicate that T4G7OT does still retain enough selectivity in human to be considered a reliable pharmacological tool (Manning et al. 2012). Even though our affinity values for the human receptors are inconsistent with this previous report, which may be due to slightly different experimental conditions, T4G7OT is still much more selective in rodent (K_i for rat OXTR = 0.8 nM; K_i for rat AVPR1a = >10,000 nM) than in human, regardless of which human receptor affinity values are used for comparison (Manning et al. 2012). Thus, these results indicate that although this compound is a reliable and selective pharmacological agent in rodents, it should be considered cautiously if it will be used in humans or nonhuman primates. For our purposes, this compound is not a good option for competitive binding receptor autoradiography in primate tissue, because we have identified a novel and much more highly selective hOXTR ligand, ALS-II-69. We feel that ALS-II-69 should be used for future studies targeting OXTR in primate systems.

DISCUSSION

The purpose of the current study was to pharmacologically characterize several radiolabeled and unlabeled OXTR and AVPR1a ligands for use in primate tissue, for the extended purpose of pharmacologically informing a modified protocol for competitive binding receptor autoradiography. By developing this protocol, it is now possible to identify and localize OXTR and AVPR1a binding sites reliably in primate brain tissue, a task that has until now been generally neglected due to the previously described issues in ligand selectivity.

Until now, the OXTR radioligand ^{125}I-OVTA and the AVPR1a radioligand ^{125}I-LVA had not been evaluated for their binding to human OXTR and AVPR1a, aside from one previous report of the binding affinity (K_d) of ^{125}I-OVTA to hOXTR (Chini et al. 1995). But in this previous study, the binding affinity of ^{125}I-OVTA to hAVPR1a was not determined. Furthermore, the published K_d for ^{125}I-OVTA to hOXTR (0.095 ± 0.033 nM) is consistent with the K_d that we determined in the current study (0.092 ± 0.018 nM), which gives our methods external validity. We found that these radioligands are capable of binding to both hOXTR and hAVPR1a with a high, subnanomolar affinity, with each radioligand having a slightly higher affinity for its own receptor (Table 2.1). Furthermore, these experiments have determined the optimal concentration at which these radioligands should be used in order to promote selective binding in competitive binding approaches in primate tissue: 30 pM for ^{125}I-LVA and 90 pM for ^{125}I-OVTA.

To complete the competitive binding design of receptor autoradiography, we also generated competitive binding curves for two non-peptide ligands and two peptide ligands of OXTR and AVPR1a. We found that the nonpeptide ligand ALS-II-69 is extremely selective for hOXTR over hAVPR1a and have identified this molecule as the

ideal competitor for a competitive binding receptor autoradiography approach. This molecule represents a much better option than the peptide agonist T4G7OT, despite conflicting reports that T4G7OT may be selective enough to be used in human or nonhuman primate preparations (Manning et al. 2012). We have also characterized the nonpeptide AVPR1a ligand SR49059 and determined it to be mildly selective for hAVPR1a. Interestingly, its selectivity profile based on the current study does not differ much from that of the Manning compound, a peptide ligand for AVPR1a that is commonly used in rodent. However, because our best candidate for OXTR competition, ALS-II-69, is also a nonpeptide ligand and because previous reports have cautioned use of Manning in primate systems, we have identified SR49059 as the best AVPR1a competitor for a competitive binding receptor autoradiography approach when it is used at a 10 nM concentration.

Based on these results, our pharmacologically informed, competitive binding receptor autoradiography protocol is shown in Table 2.3, below. This approach should be the most reliable for future studies seeking to identify OXTR and AVPR1a binding sites in primate brain tissue.

	Radioligand alone	To reveal OXTR (by occupying AVPR1a)	To reveal AVPR1a (by occupying OXTR)
30 pM ^{125}I-LVA	none	+ 10 nM SR49059	+ 20 nM ALS-II-69
90 pM ^{125}I-OVTA	none	+ 10 nM SR49059	+ 20 nM ALS-II-69

Table 2.3. Experimental design using pharmacologically informed competitive binding protocol for receptor autoradiography.

Chapter 3:

Characterization of the Distribution of the Oxytocin Receptor in the Brain of the Rhesus Macaque (*Macaca mulatta*)

This chapter presents work to be published within:

Sara M. Freeman, Kiyoshi Inoue, Aaron L. Smith, and Larry J. Young. Localization of oxytocin receptors in the brain of the rhesus macaque (*Macaca mulatta*).

ABSTRACT

While the neuroanatomical distributions of oxytocin (OT) and vasopressin (AVP) receptors have been well described in rodents using highly selective radioligands, there are few reports of central OT receptor (OXTR) or vasopressin 1a receptor (AVPR1a) distribution in primates. The rhesus macaque (*Macaca mulatta*) is an important primate model for social behavior, and while OT has been shown to modulate social behavior in this species, the pharmacological tools used to map OXTR in rodents have failed to yield convincing results in rhesus macaque brain tissue. This is due to a lack of reliable OXTR antibodies for immunohistochemistry and to non-selective radioligands, which exhibit mixed affinities for OXTR and AVPR1a in primates. We have previously developed a pharmacologically-informed modification for receptor autoradiography to selectively reveal OXTR and AVPR1a binding distributions in primate brain sections, and here we report the resulting distribution of OXTR in the rhesus macaque brain from this competitive binding receptor autoradiography protocol, as well as from *in situ* hybridization. Our results demonstrate that OXTR expression in the macaque brain is much more restricted compared to AVPR1a. Specifically, the signal for OXTR appears to be limited to: the ventromedial hypothalamus, which is an area rich in OXTR in several other species and is important for sexual motivation; the nucleus basalis of Meynert, which is critical for allocating attention to incoming visual stimuli; and the superficial gray layer of the superior colliculus, which is important in multimodal sensory processing, such as saccadic eye movements and head orientation responses to visual stimuli. OXTR was also detected in three brainstem nuclei: the trapezoid body, which receives auditory input directly from the anterior cochlear nucleus; the pedunculopontine

tegmental nucleus, which has been implicated in a variety of functions, from attention and arousal to consciousness and reinforcement learning; and the oculomotor nucleus, which contributes to the motor control of eye movements, such as gaze-holding, saccades, and smooth pursuit. While this distribution of OXTR likely has a variety of functions, OXTR expression in the rhesus macaque, a species which uses vision and audition as the primary modalities for social communication, is found in areas of the brain that modulate visual attention, process auditory and multimodal sensory stimuli, and control orienting responses to visual stimuli—functions that are critical during social interactions for the appropriate interpretation of and response to species-typical social behavior.

INTRODUCTION

Over the last several decades, oxytocin (OT) has been shown to mediate several aspects of social behavior in mammals. Seminal work from the 1980s and '90s showed that OT is capable of acting centrally to trigger the onset of mother-infant bonding and maternal nurturing behavior in several species, including sheep and rats (Kendrick, Keverne, and Baldwin 1987; Pedersen and Prange 1979; Pedersen et al. 1994). In mice, the OT system is important in maternal responsiveness as well, and it has also been shown to be required for social recognition and social memory in this species (Takayanagi et al. 2005; Ferguson et al. 2000; Ferguson et al. 2001). Research from our lab, as well as several others, has shown that OT also mediates species-specific social behaviors like pair bonding and parental care in the socially monogamous prairie vole (Young and Wang 2004; Insel, Preston, and Winslow 1995; Williams, Insel, and Harbaugh 1994). More recent research efforts have now begun to investigate the OT system in various nonhuman primate species, including macaques, marmosets, and tamarins, and these studies have shown that OT is also related to complex social behaviors such as affiliation, cooperation, and sexual behavior (Rosenblum et al. 2002; Winslow et al. 2003; Smith et al. 2010; Snowdon et al. 2010). Intranasally administered OT (IN-OT) to rhesus macaques has been shown to increase central OT concentrations (Chang et al. 2012) and affect a variety of social behaviors including increasing gaze to the eye region of monkey faces (Ebitz, Watson, and Platt 2013), increasing both selfish and altruistic choices to dole out juice rewards (Chang et al. 2012), and changing social visual attentional bias in a variety of ways (Ebitz, Watson, and Platt 2013; Parr et al.

2013). The details of these studies have been discussed previously in Chapter 1 (see pages 32-34).

Based on these studies showing that OT is capable of modulating complex social behaviors in animals, there has been a recent boom in the number of studies examining the effects of administering exogenous OT to humans (Young and Flanagan-Cato 2012). In these studies, IN-OT is shown to increase several aspects of social function, including but not limited to: gaze to the eyes (Guastella, Mitchell, and Dadds 2008; Gamer, Zurowski, and Büchel 2010; Andari et al. 2010), the ability to identify emotions in pictures of the eye region of human faces (Domes, Heinrichs, Michel, et al. 2007), and trust (Kosfeld et al. 2005). The various behavioral effects of IN-OT in humans have been recently reviewed elsewhere (Guastella, Graustella, and MacLeod 2012). Based on this evidence that IN-OT can enhance several aspects of social functioning in humans, researchers hypothesized that IN-OT could be a promising treatment for psychiatric conditions that are characterized by social deficits, such as autism spectrum disorders (ASD), social anxiety, and schizophrenia (Modi and Young 2012). Indeed, in the last few years, several seminal papers have been published showing that IN-OT can improve aspects of social functioning in clinical populations of humans compared to healthy controls (Andari et al. 2010; Hollander et al. 2003; Hollander et al. 2007; Tachibana et al. 2013; Domes et al. 2013). However, there have also been conflicting reports of the effect of IN-OT in clinical and healthy human populations, suggesting that its effects are context-specific, can be highly variable from person to person, and in some cases, have no effect (Dadds 2013; for reviews and critiques of IN-OT studies, see Bartz et al. 2011;

Guastella, Graustella, and MacLeod 2012; Guastella et al. 2013; Churchland and Winkielman 2012; Olff et al. 2013; Macdonald and Feifel 2013).

Thus, while this body of work in humans represents a great advancement, it is apparent that our understanding of the OT system in humans (and non-human primates alike) is still incredibly limited. Research in this field has plowed ahead to investigate the effects of OT in humans without a fundamental understanding of the neurophysiology of the OT system in humans and nonhuman primates alike (Churchland and Winkielman 2012). Furthermore, basic research on the OT system in a primate model organism, such as the rhesus macaque, has also been mostly left by the wayside, despite the very recent work examining the effects of IN-OT on face processing and prosocial behavior (Chang et al. 2012; Parr et al. 2013; Ebitz, Watson, and Platt 2013). One of the basic and most critical research goals that remains undetermined is the site of action of OT—the distribution of the oxytocin receptor (OXTR)—in the rhesus macaque brain.

There are several reasons why the distribution of OXTR in primate brain tissue has remained elusive. First, the techniques available to localize these receptors are limited. There aren't any reliable antibodies available for immunohistochemistry for OXTR in brain tissue (Yoshida et al. 2009), and, despite recent work to develop an OXTR tracer for *in vivo* PET neuroimaging, there are currently no widely available PET ligands for these receptors (Smith et al. 2012; Smith, Freeman, Voll, Young, and Goodman 2013a; Smith, Freeman, Voll, Young, and Goodman 2013b). Thus, the primary technique that is used to identify OXTR densities in tissue sections is receptor autoradiography. This method has been used for decades in various rodent species to successfully determine where OXTR and the closely related vasopressin 1a receptor

(AVPR1a) are expressed in brain tissue in rodents (Insel et al. 1993; Tribollet et al. 1988; Tribollet et al. 1990; Elands, Beetsma, et al. 1988; Dubois-Dauphin et al. 1990; Dubois-Dauphin et al. 1992; Young et al. 2000; Tribollet, Barberis, et al. 1992; Insel, Gelhard, and Shapiro 1991; Beery, Lacey, and Francis 2008; P. Campbell, Ophir, and Phelps 2009; Kalamatianos et al. 2010; Insel and Shapiro 1992; Lim, Murphy, and Young 2004). Second, many of the pharmacological tools that are available to probe the OT system are not selective for OXTR when used in primate species and also exhibit a high affinity for AVPR1a (Manning et al. 2012; Freeman *in prep*). This issue is especially relevant for receptor autoradiography, because the radioligands that are used for binding to OXTR or AVPR1a are not selective when used in primate brain sections (Chapter 2; Manning et al. 2012; Freeman et al. *in prep*). This mixed affinity issue is not surprising given that the endogenous hormones OT and vasopressin (AVP) themselves are highly homologous peptide hormones differing in only 2 out of 9 amino acid positions, and it has been well known that OT and AVP are capable of binding to and activating each others' receptors. Therefore, it is crucial that alternative strategies are used when studying these systems in primates.

As an example, previous attempts to map OXTR resulted in the conclusion that the commercially available OXTR radioligand (^{125}I-OVTA) binds only to AVPR1a in this species, but this conclusion was based on misinformed results from the selectivity determination of the radioligand (Toloczko, Young, and Insel 1997). These authors even questioned whether the primate brain expresses OXTR at all (Toloczko, Young, and Insel 1997). In Chapter 2, I demonstrated that ^{125}I-OVTA has a high affinity for both the human OXTR and AVPR1a, suggesting that the binding pattern it produces in rhesus

macaque tissue is likely a combination of binding to both of these receptors. Furthermore, in the early attempts to map the OXTR distribution, the only unlabeled competitor that was successful in competing off the binding signal from ^{125}I-OVTA was the commonly used AVPR1a ligand referred to as the Manning compound. This result lead to the conclusion that ^{125}I-OVTA binds to AVPR1a in rhesus macaque (Toloczko, Young, and Insel 1997). However, more careful pharmacological characterization demonstrated that the Manning compound also has a high affinity for both OXTR and AVPR1a when used in primate (Manning et al. 2008; Manning et al. 2012), and because this compound was used at a very high concentration (1 μM) in the selectivity determination experiment by Tolockzo et al. (1997), the conclusion that ^{125}I-OVTA is binding solely to AVPR1a in this tissue is no longer an accurate interpretation of the results.

The goal of the current study is to identify the neuroanatomical distribution of OXTR in brain tissue of the rhesus macaque using more sophisticated techniques. Specifically, this study uses a pharmacologically-informed, competitive-binding receptor autoradiography protocol combined with *in situ* hybridization, to reliably characterize for the first time the OXTR distribution in the rhesus macaque brain. This study helps to establish the neuroanatomical foundation for a nonhuman primate model of complex social cognition that will aid in our understanding of OT's role in mediating social functioning in primates more broadly.

METHODS

Tissue preparation and sectioning

Rhesus macaque brains were removed promptly after death, rinsed with PBS, and blocked into 3 or 4 coronal blocks, which were then allowed to freeze completely while laying flat on top of aluminum foil on a slab of dry ice, wrapped tightly in foil, and placed at -80ºC until sectioning. Brain blocks were removed from -80ºC and brought up to -20ºC for sectioning. The brains were sectioned in ten series at 20 µm, on a cryostat, and mounted on Fisher Frost-plus slides. Slides were stored in a sealed slide box with desiccant and kept at -80ºC until use for receptor autoradiography. Sections from nine animals (males and females) were used for this study, with sections ranging from frontal cortex through the hypothalamus and temporal lobe and to the cerebellum and brainstem.

Receptor autoradiography

Rhesus macaque brain sections were removed from -80ºC and allowed to thaw in the sealed slide box containing a desiccant packet for 1 hour at 4ºC followed by 1 hour at RT in a vacuum desiccator. The slides were then dipped in 0.1% paraformaldehyde in PBS, pH7.4, and rinsed twice in 50 mM Tris buffer, pH 7.4, to remove endogenous ligand. Then, sections were incubated for 1 hr in one of two different radioligands, at concentrations matching their K_d for the receptor of interest: 30 pM ^{125}I-linear vasopressin-1a antagonist (^{125}I-LVA) to target AVPR1a or 90 pM ^{125}I-ornithine vasotocin analog (^{125}I-OVTA) to target OXTR (Perkin Elmer, Waltham, MA). Sets of three adjacent sections were incubated in three different conditions: radioligand alone, radioligand plus an unlabeled, human-selective AVPR1a ligand (SR49059; Gal, Wagnon, and Garcia 1993), or radioligand plus an unlabeled, human-selective OXTR ligand (ALS-II-69; Smith, Freeman, Voll, Young, and Goodman 2013b). SR49059 was purchased

from Tocris (Minneapolis, MN). ALS-II-69 was synthesized in our lab by Dr. Aaron Smith. These unlabeled competitors were incubated at concentrations that were determined by competitive binding pharmacology experiments (described in Chapter 2) to be ideal for selective binding to the receptor of interest: 10 nM SR49059 to target AVPR1a and 20 nM ALS-II-69 to target OXTR. The use of SR49059 to selectively occupy AVPR1a for a competitive binding approach has been suggested previously (Ala et al. 1997). Next, unbound radioligand was removed by four washes in 50 mM Tris buffer plus 2% $MgCl_2$, pH 7.4, and then dipped into ddH_2O and air dried. Once dry, the slides were exposed to BioMax MR film (Kodak, Rochester, NY) for 8 days. Digital images were obtained from the films using a light box and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) connected to a computer. Brightness and contrast of the images were equally adjusted for all the sections using Adobe Photoshop (San Jose, CA).

In situ hybridization

Rhesus macaque brain sections were removed from -80ºC and allowed to thaw as described above for autoradiography. Probes derived from cDNAs encoding the human OXTR and AVPR1a were used for *in situ* hybridization for the rhesus monkey brains due to the high homology between the two species (97% identical amino acid sequence between human and rhesus OXTR; Salvatore et al. 1998). The human OXTR fragment, corresponding to nucleotide 631 to 1751 of human OXTR mRNA (NM_000916.3), and the human AVPR1a fragment, corresponding to nucleotide 2014 to 3198 of human AVPR1a (NM_000706.4), were amplified from human OXTR and AVPR1a plasmids

(gift from Dr. Bice Chini), respectively, using polymerase chain reaction (PCR) and a forward primer (5'-GGAAGATTTAGGCGAGTCCTTCCACA-3') and reverse primer (5'-CCGTACTGTTTGTTGGGCTTCGATTG-3') for AVPR1a and a forward primer (5'-CGCGCTCGCAGCCAACTGGA-3') and reverse primer (5'-TGCGATGGCTCAGGACAAAGGA-3') for OXTR. PCR products were inserted to pCRII vector (Invitrogen, Grand Island, NY). ^{35}S-UTP labeled sense and antisense probes were generated from linearized plasmids using T7 or SP6 RNA polymerases.

Rhesus monkey brain sections were hybridized with the probes and washed as described previously with minor modifications (Inoue et al. 2004; Inoue, Burkett, and Young 2013). Specifically, the length of the proteinase K treatment was increased from 2 min to 6 min, and the parameters for the high stringency wash step were changed as follows: 5% beta-mercaptoethanal was added to the 50% formamide/2x standard sodium citrate solution, and the temperature for this wash step was decreased from 65ºC to 62.5ºC.

The sections were then exposed to BAS-IP TR 2025 E phosphorimaging screens (FujiFilm, Tokyo) for 28 days and then to BioMax MR film (Kodak, Rochester, NY) for 100 days. Phosphorimaging screens were analyzed using a BAS5000 phosphorimager (FujiFilm, Tokyo). After film development, digital images were obtained from the films using a light box and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) connected to a computer. Brightness and contrast of the images are equally adjusted for all the sections using Adobe Photoshop (San Jose, CA).

Acetylcholinesterase staining

Following receptor autoradiography and film development, slides were counterstained for acetylcholinesterase (AChE) to delineate the brain regions for anatomical analysis. Staining for AChE was performed as previously described using a modified protocol for the traditional AChE protocol that has been shown to amplify AChE signal in tissue previously used for receptor autoradiography (Lim, Hammock, and Young 2004). Images of the resulting counterstained sections were compared with images from a rhesus macaque brain atlas (Paxinos, Huang, and Toga 1999) to determine neuroanatomical landmarks and identify regions.

RESULTS

Radioligands alone

When the radioligands were incubated in the absence of competitors, ^{125}I-OVTA and ^{125}I-LVA produced grossly similar binding patterns, although the intensity of binding was greater for ^{125}I-LVA than for ^{125}I-OVTA (Figure 3.1). Furthermore, the binding pattern of both radioligands was consistent with what was previously published for the binding of ^{125}I-LVA on rhesus macaque brain tissue (Young, Toloczko, and Insel 1999). In the three anatomical levels shown in Figure 3.1, dense binding was detected in the anterior cingulated cortex (ACC), bed nucleus of stria terminalis (BNST), central amygdala (CeA), claustrum (Cl), entorhinal cortex (Ent), infundibulum (Inf), insular cortex (Ins), median eminence (ME), parasubiculum (PASB), and subiculum (SB). There are some areas with very low levels of ^{125}I-OVTA binding that do not seem to overlap with ^{125}I-LVA binding, for example in the nucleus basalis of Meynert (NBM) and ventromedial hypothalamus (VMH) (Figure 3.1, white arrows), but it is difficult to assess

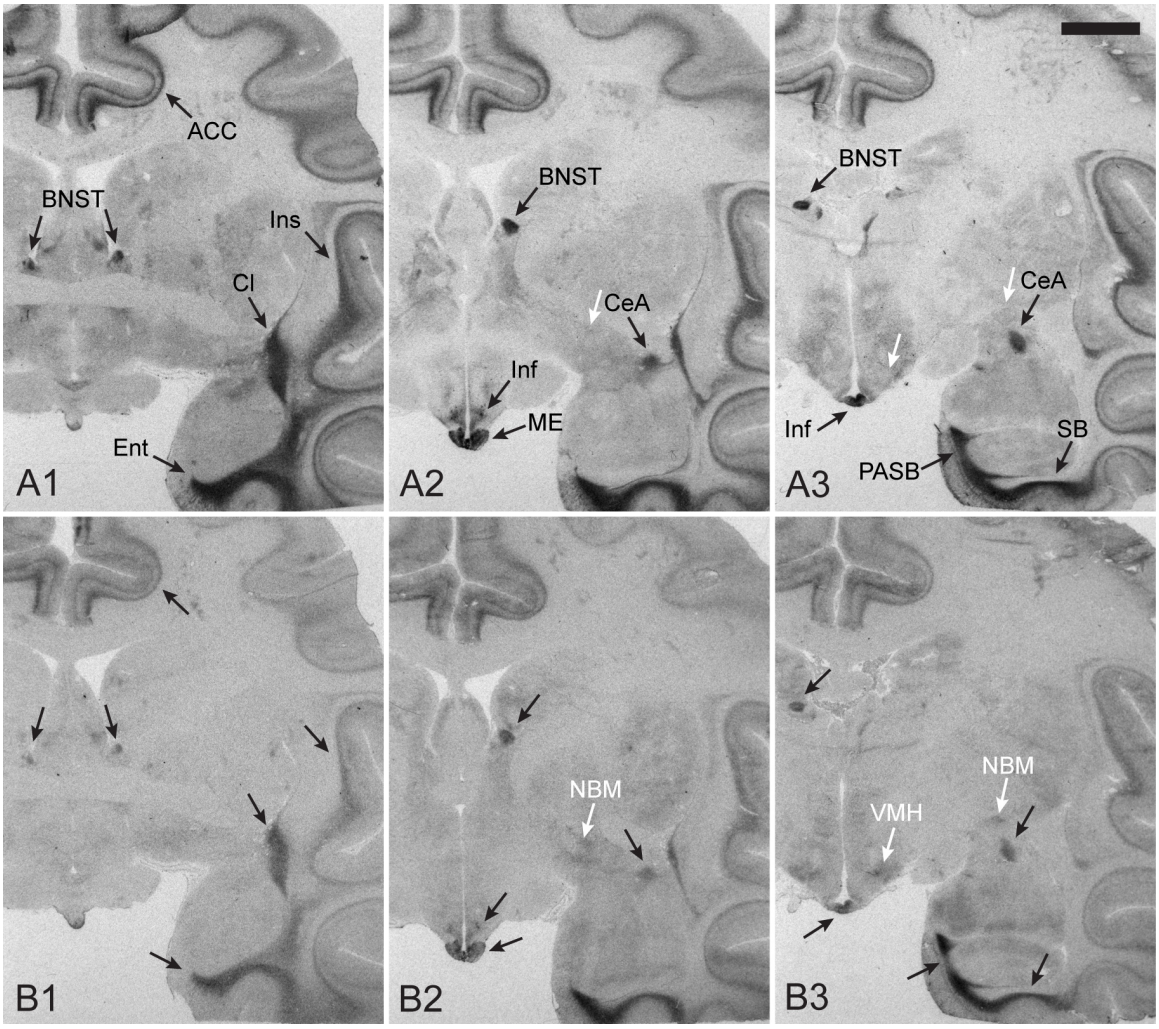

Figure 3.1. Radioligand binding to the rhesus macaque brain.
The binding patterns produced by the AVPR1a radioligand, ^{125}I-LVA (A), and the OXTR radioligand, ^{125}I-OVTA (B), in rhesus monkey brain tissue sections at three anatomical levels (1, 2, 3), showing the overlap in binding by these two radioligands across brain regions. Black arrows indicate areas with overlapping binding between the two radioligands. White arrows highlight an example of one region, the nucleus basalis of Meynert, which has low levels of binding to ^{125}I-OVTA but not ^{125}I-LVA. Scale bar = 5mm. Abbreviations: ACC, anterior cingulate cortex; BNST, bed nucleus of stria terminalis; CeA, central amygdala; Cl, claustrum; Ent, entorhinal cortex; Inf, infundibulum; Ins, insular cortex; ME, median eminence; NBM, nucleus basalis of Meynert; PASB, parasubiculum; SB, subiculum; VMH, ventromedial hypothalamus

with certainty if this is specific OXTR binding by only analyzing the radioligand binding in the absence of selective competitors.

Taken together, these results can be interpreted in one of two ways. Either these receptors are expressed mostly in overlapping areas of the brain, or the resulting binding patterns reflect a combination of AVPR1a and OXTR densities in the tissue. Given what we know about the pharmacological properties and mixed affinities of these two radioligands, the latter explanation is much more likely.

Radioligand binding in the presence of unlabeled competitors

In order to more selectively reveal binding to OXTR, we used a competitive binding approach (Figure 3.2). Broadly, when the selective OXTR competitor ALS-II-69 was co-incubated on the tissue, it did not reduce the overall levels of binding for either radioligand (Figure 3.2E,F) compared to the radioligand alone condition (Figure 3.2A,B). Therefore, the majority of dense binding from ^{125}I-OVTA is apparently not due to binding to the OXTR. In contrast, the selective AVPR1a competitor SR49059 drastically reduced binding of both ^{125}I-LVA and ^{125}I-OVTA in most brain regions, especially in the two representative brain regions indicated by arrows, the BNST and the CeA (Figure 3.2C,D). Therefore, the general binding pattern from both radioligands in the alone condition is likely due to binding to the AVPR1a. Furthermore, this competitive approach revealed a few brain regions with modest levels of ^{125}I-OVTA binding and no binding from ^{125}I-LVA, including the nucleus basalis of Meynert (NBM) shown in Figure 3.2 by the white circles in panels B and D. This region was also noted above as a possible area of specific OXTR binding, and this competitive binding approach supports the conclusion

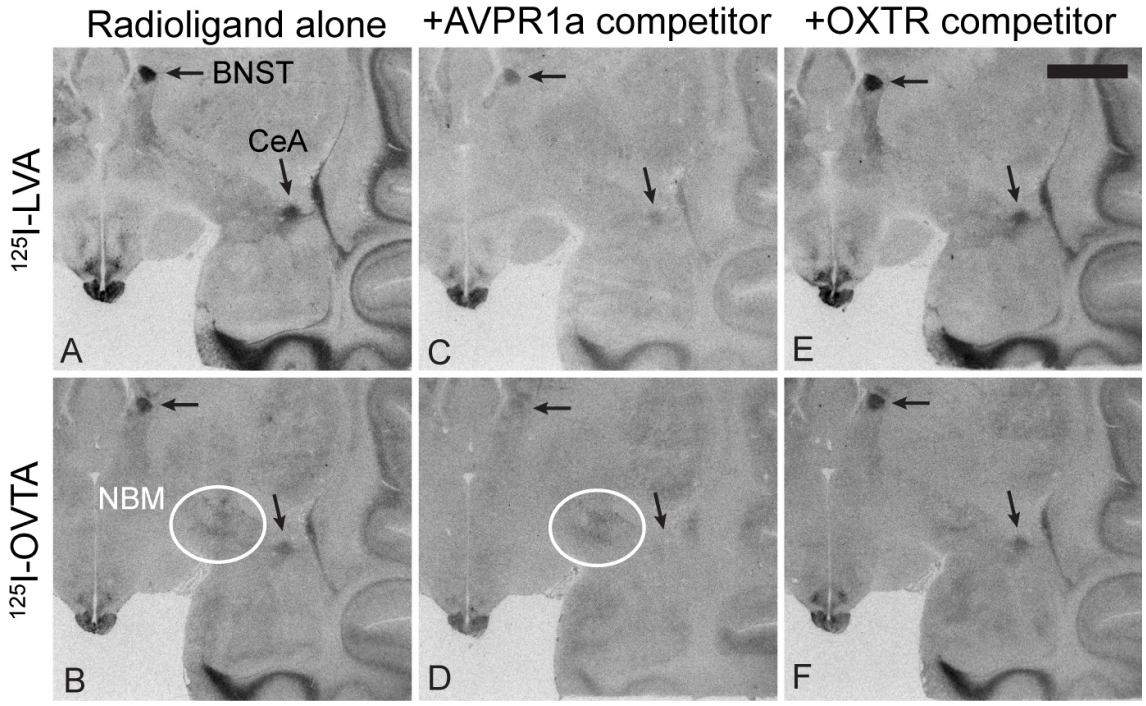

Figure 3.2. Representative results from competitive binding receptor autoradiography using ^{125}I-LVA and ^{125}I-OVTA.
(A, B) Radioligand alone. (C, D) Radioligand coincubated with 10 nM of the AVPR1a competitor SR49059, showing that this compound is capable of competing off most of the signal from both radioligands, especially in the areas highlighted with arrows, the bed nucleus of stria terminalis (BNST) and the central amygdala (CeA). (E, F) Radioligand coincubated with 20 nM of the OXTR competitor ALS-II-69, showing little knockdown of ^{125}I-OVTA or ^{125}I-LVA compared to the radioligand alone condition. White circles in panels B and D indicate modest binding of ^{125}I-OVTA to the nucleus basalis of Meynert (NBM), an area which does not show binding to ^{125}I-LVA and does shows reduced binding in the presence of the OXTR competitor in panel F. Scale bar = 5mm.

that this area expresses OXTR and not AVPR1a. However, the overall signal that remains for OXTR in the presence of SR49059 (Figure 3.2D) is very light and difficult to interpret in general.

In situ hybridization reveals OXTR expression

Thus, while our competitive binding approach reveals promising results for selective OXTR binding in the rhesus macaque brain, the specific signal remaining for OXTR is generally difficult to interpret with confidence. Therefore, we used adjacent sections from the same individual for *in situ* hybridization for OXTR and AVPR1a mRNA and compared the resulting signals for AVPR1a mRNA and OXTR mRNA to the pattern of binding produced by ^{125}I-OVTA alone (Figure 3.3). In contrast to radioligand binding patterns, the distribution of AVPR1a and OXTR mRNAs were non-overlapping (Figure 3.3B,C). Furthermore, the binding pattern produced by the AVPR1a antisense probe matched the binding of ^{125}I-OVTA in the BNST, CeA, Ent, Inf, PASB, and presubiculum (PSB) (black arrows, Figure 3.3A,B). In contrast, the binding pattern produced by the OXTR mRNA antisense probe resulted in a signal in the NBM and VMH, which matched areas of modest binding from ^{125}I-OVTA alone (white arrows, Figure 3.3A,C). OXTR sense strand probes were used to distinguish the mRNA signal from nonspecific binding of the probes, which was apparent in the hippocampus (Hipp) (gray arrows, Figure 3.3C,D).

Comparison of OXTR mRNA with ^{125}I-OVTA binding in the presence of SR49059

In order to confidently determine the brain areas where OXTR is expressed in the

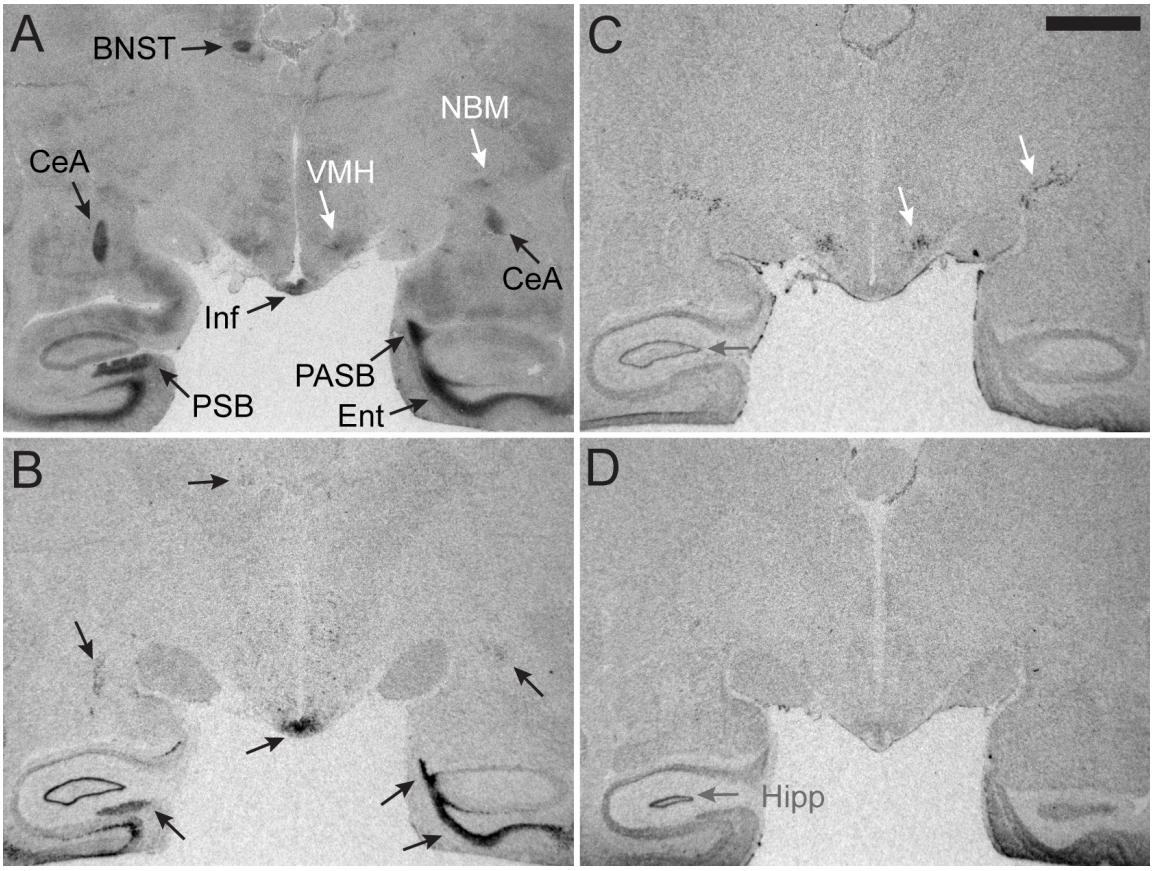

Figure 3.3. Comparison of ^{125}I-OVTA radioligand binding alone to *in situ* hybridization results.
The binding pattern produced by ^{125}I-OVTA alone (A) includes binding to both AVPR1a and OXTR, which can be parsed apart by comparing the radioligand binding densities to the *in situ* hybridization results for mRNA for these receptors. (B) An antisense probe for AVPR1a mRNA yields a binding pattern that matches areas of radioligand binding (black arrows, A,B). (C) An antisense probe for OXTR mRNA yields a binding pattern which allows the remaining lighter gray densities produced by ^{125}I-OVTA to be interpreted as distinctly OXTR binding and not background (white arrows, A,C). (D) A sense probe designed as a negative control for *in situ* hybridization shows that the mRNA signal seen in the hippocampus and surrounding areas is background and not specific mRNA for OXTR (gray arrows, C,D). Scale bar = 5mm. Abbreviations: BNST, bed nucleus of stria terminalis; CeA, central amygdala; Ent, entorhinal cortex; Inf, infundibulum; NBM, nucleus basalis of Meynert; PASB, parasubiculum; PSB, presubiculum; VMH, ventromedial hypothalamus

brain of the rhesus macaque, we a) performed competitive binding receptor autoradiography to enhance the selectivity of ^{125}I-OVTA binding to OXTR, b) aligned those results with *in situ* hybridization for OXTR mRNA in adjacent sections, and finally c) compared the resulting signal to sections that have been counterstained for acetylcholinesterase (AChE). By comparing the binding signals from OXTR protein and OXTR mRNA to the counterstained sections and a published rhesus macaque brain atlas (Paxinos, Huang, and Toga 1999), we were able to confidently identify areas of OXTR expression in the rhesus macaque brain (Figure 3.4 and 3.5).

First, we used sections from one individual in order to optimize and confirm this competitive approach in combination with *in situ* hybridization. The results from two anatomical levels in the forebrain are shown in Figure 3.4. This figure highlights OXTR expression in the VMH and NBM, shown in white and black arrows, respectively.

Detailed neuroanatomical distribution of OXTR in the rhesus macaque brain

This comparison demonstrated that it was sufficient to perform the competitive binding receptor autoradiography using only two conditions: OXTR radioligand alone and OXTR radioligand with the AVPR1a competitor, which enhances the selectivity of the radioligand binding. Therefore, in subsequent tissue sections from 8 individuals, in order to map the distribution of OXTR in detail throughout the rhesus macaque brain (Figure 3.5), we used three adjacent sections from each animal in the following manner: 1) ^{125}I-OVTA alone, 2) ^{125}I-OVTA with 10 nM SR49059, and 3) *in situ* hybridization for *OXTR* mRNA.

The overall expression of OXTR in the rhesus brain is significantly more

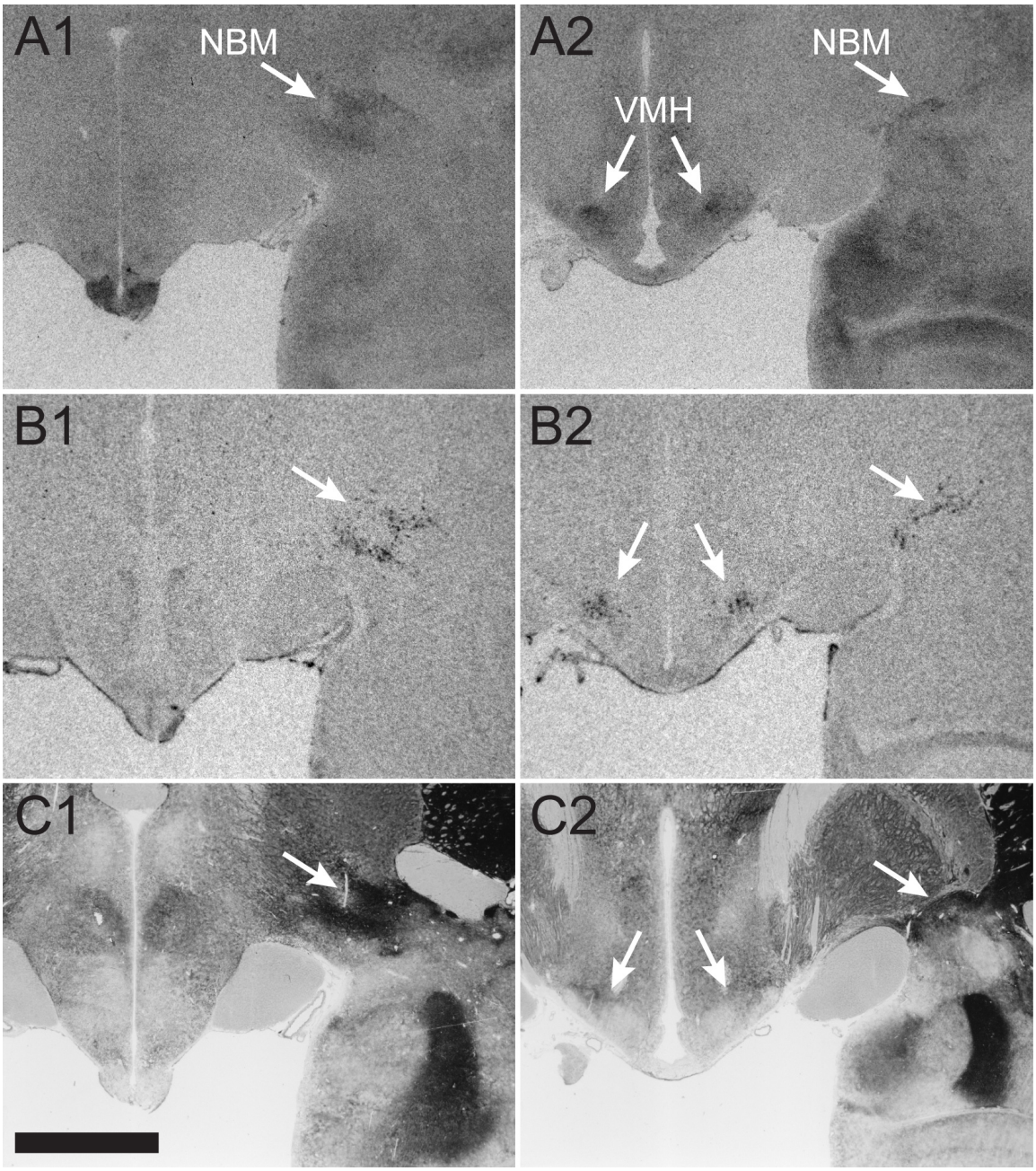

Figure 3.4. Binding to OXTR protein and OXTR mRNA, aligned to an acetylcholinesterase counterstain, in two anatomical levels of the forebrain of one representative rhesus macaque.
By comparing OXTR radioligand binding in the presence of a selective AVPR1a competitor (A) to the results from *in situ* hybridization for OXTR mRNA (B), it is possible to determine the brain areas expressing OXTR in the rhesus macaque brain. Here, when these areas are aligned to an acetycholinesterase counterstain (C), we see OXTR expression in the nucleus basalis of Meynert (NBM) and the ventromedial hypothalamus (VMH). Column 1 is more anterior to column 2. Scale bar = 5mm.

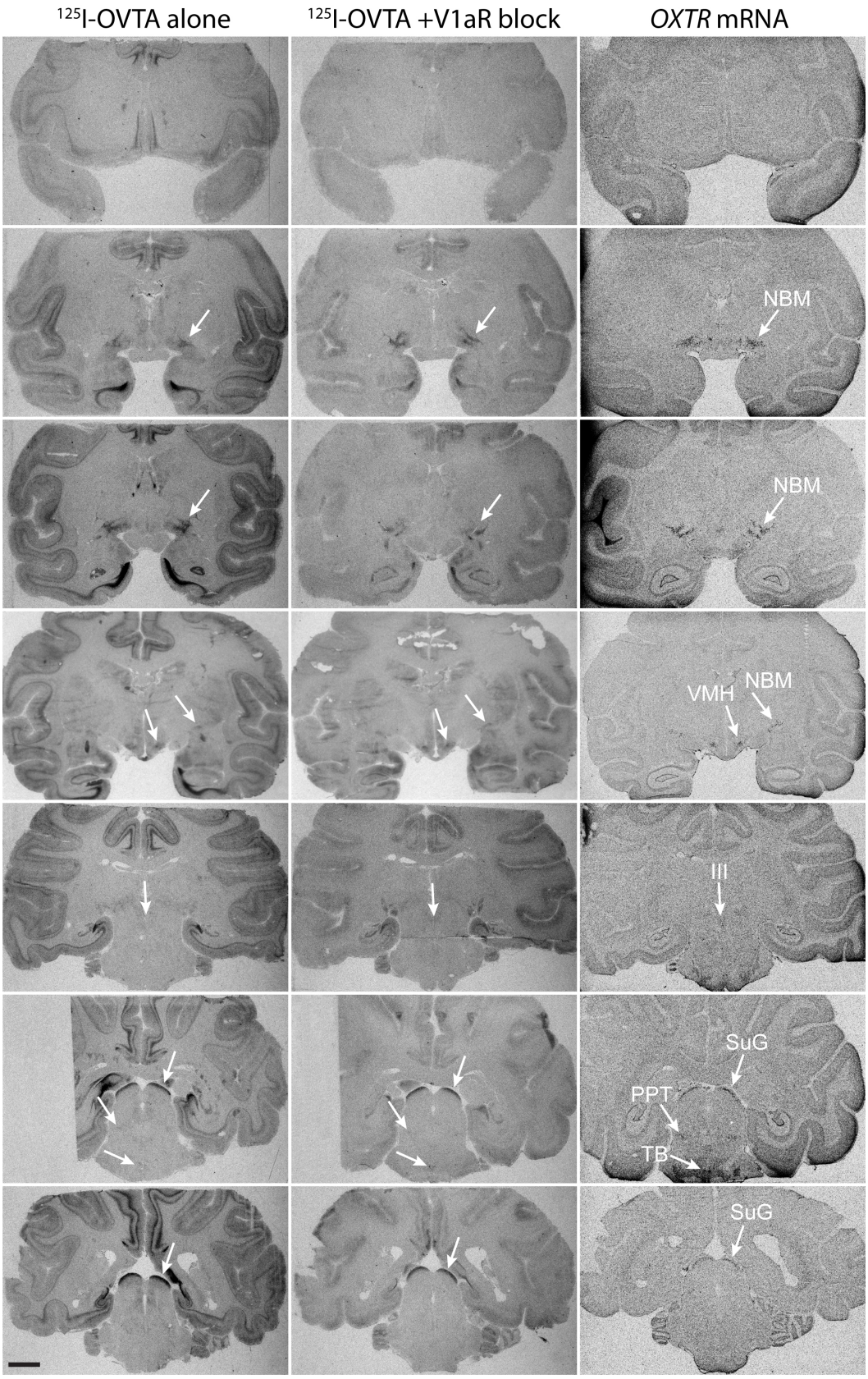

Figure 3.5. OXTR distribution in the rhesus macaque brain. Details on next page.

restricted than AVPR1a, with a clear, consistent signal detected in three main areas of the brain—VMH, NBM, and the superficial gray layer of the superior colliculus (SuG)—as well as three areas of the brainstem that have lower levels of expression: the oculomotor nucleus (III), the peduncopontine tegmental nucleus (PPT), and the trapezoid body (TB). While it is not likely that these are the only regions that express OXTR, these are the areas with densities high enough to be detectable by the current methods.

DISCUSSION

Summary of findings and potential caveats

These results indicate that the overall expression of OXTR in the rhesus macaque brain is sparse and difficult to detect reliably with receptor autoradiography alone, due to mixed affinities of the available radioligands. This supports previous efforts to map OXTR in this species (Toloczko, Young, and Insel 1997). However, we demonstrate that it is possible, by using a combination of competitive binding autoradiography with selective ligands and *in situ* hybridization to establish the distribution of OXTR expression in primate brain tissue. In the rhesus macaque brain, OXTR was detected in six regions: VMH, NBM, SuG, TB, III, and PPT. While this study is the first to

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Figure 3.5 (*previous page*). OXTR distribution in the rhesus macaque brain. Receptor autoradiography results using ^{125}I-OVTA alone (A) and ^{125}I-OVTA plus 10 nM SR49059 (B) aligned with *in situ* hybridization results for *OXTR* mRNA (C) in 6 representative individuals at 7 different levels (rows). White arrows indicate areas of specific OXTR expression based on a visual comparison of the results from *in situ* hybridization (C) and competitive binding (B). Scale bar = 5mm. Abbreviations: III, oculomotor nucleus; NBM, nucleus basalis of Meynert; PPT, pedunculopontine tegmental nucleus; SuG, superficial gray layer of the superior colliculus; TB, trapezoid body; VMH, ventromedial hypothalamus

successfully detect OXTR in rhesus macaque brain tissue, there are some possible caveats worth discussing.

First, some radioligand binding remains in the presence of the AVPR1a competitor. This result complicates the interpretation of the specific OXTR binding pattern seen in the presence of the competitor. Is this signal simply from small amounts of radioligand that cannot be outcompeted by the competitor? Or is this an indication of true expression of both OXTR and AVPR1a in those brain regions? The radioligands used in this study are peptide ligands that mimic the structure of the endogenous neuropeptides and are designed to bind tightly to the receptors. Thus, it is highly probable that some of the radioligand may not be completely prevented from binding by the small, nonpeptide competitors. Furthermore, it is likely that small molecule ligands, such as the competitors used in the current study, could be binding to sites on the receptor other than the orthosteric site. For example, nonpeptide compounds can act as allosteric modulators, which affect the affinity of the orthosteric ligand (in our case, the peptide radioligand) without directly competing it off of the putative ligand-binding pocket (Gruber, Muttenthaler, and Freissmuth 2010). Thus, it is not unexpected that some binding will remain in the presence of the competitor in the current study. By comparing the resulting binding signal to the distribution of OXTR mRNA, we can overcome this small issue and identify regions of specific OXTR expression.

There may be other areas of the rhesus macaque brain with OXTR expression in densities that are below the detectable limit of our methods. It may be possible to detect these low levels of expression by using variations of *in situ* hybridization techniques. For example, using fluorescently labeled probes in combination with confocal microscopy or

using radioactive probes with emulsion dipped slides and longer incubation times may reveal more discrete areas of OXTR expression that escaped detection in the present study. It is also possible to quantify gene expression in tissue homogenates (rather than tissue sections) by using reverse transcription PCR either qualitatively to determine the presence or absence of OXTR mRNA, or quantitatively to determine relative or absolute levels of expression.

Tissue from nine animals was used in this study, with the anatomical range of sections varying from brain to brain. Thus, while some regions were represented in all or almost all of the individuals, many regions were only present in a subset of the total. Although we were able to acquire sections from the frontal cortex through the brainstem, we acknowledge the limitations in the sampling of tissue used in this study. Despite these issues, we provide the first convincing evidence of OXTR distribution in the rhesus macaque brain, and we invite follow-up studies to confirm and expand these results.

Functions of OXTR-expressing regions of the macaque brain

Ventromedial hypothalamus: sexual behavior

OXTR expression in the VMH has been shown to be important in the control of sexual behavior and appetite, and activation of OXTR in this region has been proposed to be the regulator of the motivational switching between the suppression of feeding and appetite and the initiation of seeking a mate (Leng et al. 2008). OXTR binding has been found in VMH in mice (Insel et al. 1993), rats (Johnson 1992), guinea pigs (Tribollet, Barberis, et al. 1992), prairie voles and montane voles (Insel and Shapiro 1992), two species of Central America singing mice (Campbell, Ophir, and Phelps 2009), and two

species of the South American rodent the tuco-tuco (Beery, Lacey, and Francis 2008). Thus, the VMH may represent one of the most evolutionarily conserved regions of OXTR expression across mammals.

There are several lines of evidence from rodent research suggesting OXTR in the VMH mediates steroid-hormone dependent changes in sexual receptivity. First, in male and female rats, 17-beta estradiol (E2) increases OXTR binding in the VMH (de Kloet, Voorhuis, and Elands 1985; Coirini et al. 1992). In female rats, levels of OXTR binding in the VMH increase within 24h after treatment with E2 implants and decrease within 24h after implant removal, and these changes are accompanied by paralleled changes in uterine weight (Johnson et al. 1989). Furthermore, OXTR mRNA increases during the estrous cycle as concentrations of E2 increase (Bale, Dorsa, and Johnston 1995). In E2-primed female rats, site-specific infusions of OT into the VMH increase sexual receptivity (Bale et al. 2001), and infusion of antisense oligodeoxynucleotides to the OXTR into the VMH (which decreases OXTR binding) decreases female sexual receptivity (McCarthy et al. 1994). In primates, it has been shown that the frequency of female rhesus macaque sexual behavior is highest during the peak in secreted E2 during estrous (Wallen et al. 1984), and furthermore, female sexual proceptivity is increased after treatment with E2 in ovariectomized rhesus macaques (Zehr, Maestripieri, and Wallen 1998). Although it is not known whether levels of OXTR in the primate brain increase in response to increasing concentrations of circulating E2, it is possible that E2-dependent increases in sexual motivation in monkeys may be due to increases in OXTR expression in the VMH, in an evolutionarily conserved mechanism as the one so elegantly elucidated in rodents.

Trapezoid body: auditory input from the cochlea

OXTR was detected in the trapezoid body of the brainstem. Auditory nerves carrying signals from the hair cells in the cochlea synapse on neurons in the cochlear nucleus, which in turn synapse on the contralateral principal neurons of the medial nucleus of the trapezoid body (TB) in the auditory brainstem (Borst and Soria van Hoeve 2012). The TB is organized in a tonotopic fashion, maintaining the frequency map in the cochlea, and the principal neurons from the medial nucleus of the TB project ipsilaterally to the superior olivary complex and to the lateral lemniscus, two downstream regions involved in early auditory processing (Borst and Soria van Hoeve 2012). The characteristics of the connectivity between the TB and other regions in the auditory pathway are considered to underlie the ability to localize sound sources in space (Schneggenburger and Forsythe 2006).

It is speculative at best to prescribe a functional outcome of OXTR expression in this fast-acting auditory relay nucleus, but given the macaque's use of vocal communication by both infants and adults in a variety of social settings, it seems logical that brain regions involved in the earliest stages of auditory processing may be sensitive to OT in this species. In humans, OT has begun to be studied for its effects on social auditory processing, and preliminary reports suggest that it can affect reaction to infant cries and infant laughter (Riem, Bakermans-Kranenburg, and Pieper 2011; Riem et al. 2012; Bakermans-Kranenburg et al. 2012) and may even play a role in navigating the "cocktail party effect" in a crowded room (Tops et al. 2011). In the brain of the mustached bat (*Pteronotus parnellii*), OT-fibers have been found in several auditory

nuclei, including the medial and ventral nuclei of the trapezoid body (Kanwal and Rao 2002) although the receptor distribution for this species is not yet known.

Superior colliculus and oculomotor nucleus: visual processing and eye movement

The involvement of the superior colliculus in the control of eye movements and visual attention in monkeys was first established in the early 1970s (Wurtz and Goldberg 1971; Wurtz and Goldberg 1972). The superior colliculus is made up of seven layers, with the three most superficial layers responding almost exclusively to visual input. The intermediate and deep layers are important in the premotor preparation and motor control of saccadic eye movements and changes in head direction in response to visual and other sensory cues (Gandhi and Katnani 2011). While this multimodal sensory and motor processing is carried out by the deep layers of the SC to produce motor responses, it is the superficial gray layer of the SC (SuG)—which receives input from the retina and is organized to contain a map of visual space (Goldberg and Wurtz 1972a; Goldberg and Wurtz 1972b; Schiller and Stryker 1972)—that provides synaptic input to the deeper layers in order to mediate orienting movements in response to specific visual cues (Helms, Ozen, and Hall 2004).

The results of the current study show that high levels of OXTR mRNA and protein are expressed in the SuG, possibly providing a role for OT to influence visually guided orienting responses to socially relevant stimuli detected in visual space. Indirect support for this idea comes from a study in humans that reported increased functional coupling between the SC and the amygdala during following treatment with IN-OT during a face viewing task (Gamer, Zurowski, and Büchel 2010). Furthermore, this study

also reported that IN-OT increased the likelihood that subjects would change their gaze toward the eyes, a socially salient visual target, which further supports a role for OT in the SC in visually guided changes in gaze direction (Gamer, Zurowski, and Büchel 2010). Furthermore, in a recent study that examined the effects of IN-OT on altruistic reward allocation in macaques, treatment with OT selectively increased the donor monkey's attention to the passive recipient monkey only after the altruistic reward delivery, and the authors suggest that perhaps this is due to OT's influence on "neural circuits mediating orienting behavior, including... [the] superior colliculus" (Chang et al. 2012).

Another visual area of the brain where we detected modest OXTR signal is the oculomotor nucleus (III). This nucleus contains motoneurons that innervate the eye muscles (along with motoneurons from the trochlear nucleus and abducens nucleus) and receive input from premotor networks to generate various types of eye movements, including saccades, smooth pursuit, and gaze holding, among others (Büttner-Ennever 2007). OXTR expression in this region may be playing an analogous role to the one described above for the SuG, but in the behavioral motor output rather than visual integration of incoming stimuli.

Nucleus basalis of Meynert and pedunculopontine tegmental nucleus: visual attention, reinforcement learning, and cholinergic innervation of the brain

The NBM is a highly interconnected area of the basal forebrain that provides cholinergic innervation into the entire neocortex (Mesulam et al. 1983; Jones et al. 1976). Due to early evidence that the cholinergic neurons in this region (as well as their projections to the cortex) degenerate in patients with Alzheimer's disease (Whitehouse et

al. 1981), much of the early research on the function of the NBM revolved around the hypothesis that this region must be critical in learning and memory—two of the hallmark cognitive deficits of Alzheimer's disease. After decades of research in both rodents and monkeys using lesion and inactivation studies in combination with various learning and memory tasks, it is now understood that the main function of NBM is in selective attention, attentional effort, and motivation, specifically with respect to visual attention (Muir et al. 1993; Sarter, Givens, and Bruno 2001; Power, Vazdarjanova, and McGaugh 2003; Balducci et al. 2003; Sarter, Gehring, and Kozak 2006; Demeter and Sarter 2013). OXTR expression in this region of the rhesus macaque brain likely influences visual attention to social cues. A recent study reported that IN-OT in rhesus macaques changes the visual social attentional bias toward emotional faces, and the authors suggest a role for OT in "the earliest stages of social information processing" (Ebitz, Watson, and Platt 2013). Furthermore, the study by Chang and colleagues (2012) discussed above also lends credence to this idea of OT modulating selective attention; the authors say that their findings "invite the possibility that OT gates the activity of attention circuits in the brain specifically during active interaction with others", perhaps via action at OXTR in the NBM (Chang et al. 2012).

We also detected modest OXTR mRNA in the PPT, which a highly interconnected region of the brain that has been implicated in a variety of functions, including sleep, locomotion, reaction time, reinforcement learning, sensation, attention, and even consciousness (Winn 2006; Garcia-Rill 1991). This nucleus is made up of two subdivisions, a cholinergic and non-cholinergic region. The cholinergic subdivision, where we see OXTR expression, is the major source of cholinergic input to the midbrain

and one of the major ascending arousal pathways in the brainstem (Garcia-Rill 1991; Kobayashi et al. 2002). The PPT is essential for integrating the sensory and motor signals necessary for complex processes like perception and conscious awareness (Kobayashi et al. 2002; Steckler et al. 1994), and the localization of OXTR in this area is reminiscent of its expression in the NBM, the major source of cholinergic input to the neocortex and forebrain. Both of these regions have been suggested to mediate aspects of selective attention, an undoubtedly important function for navigating social environments. Furthermore, cholinergic input from the PPT strongly innervates the macaque SC (Ma, Graybiel, and Wurtz 1991), a region important in controlling eye movements, as discussed above, and also a region where OXTR expression was detected. Neurons in the PPT have also been shown to be active in monkeys during saccade tasks (Okada and Kobayashi 2009) and to directly stimulate SC neurons and facilitate the initiation of saccadic eye movements (Kobayashi, Saito, and Isa 2001). Thus, it is possible that these OT-sensitive cholinergic areas could be mediating some aspects of the changes in eye movements and shifts in visual attention in response to changing social cues in the environment.

The PPT is also the primary excitatory input to dopaminergic neurons in the midbrain (Matsumura 2005), and this aspect of its connectivity has been has been studied for its involvement in reinforcement learning. Support for this concept comes from electrophysiological studies in macaques where animals were trained to saccade to and fixate on a target in order to receive a juice reward (Kobayashi et al. 2002). Recordings from the PPT showed increased activity in neurons during saccade execution as well as during reward delivery, and the authors interpret this multimodal responding of the PPT

as an indication that this region integrates a variety of signals and "may help create attentional/motivational states and reinforce behaviors" (Kobayashi et al. 2002). In rats, bilateral lesions of the PPT disrupts operant responding for natural rewards and conditioned reinforcers and also affects self-administration of various drugs of abuse (reviewed in Winn 2006). However, it was found that this disruption was not present during tasks where prior learning or training was involved, so researchers now believe that this area is actually important in learning rather than reward, specifically in the acquisition of "action-outcome associations" (the Alderson hypothesis; reviewed in Winn 2006). Although speculative, it is possible that OXTR in this region in macaques may underlie some aspects of socially reinforced learning. For example, IN-OT in healthy humans has been shown to improve performance on a learning task, but only when social reinforcers (happy face for a correct trial vs. angry face for an incorrect trial) were used and not when nonsocial reinforcers (green circle vs. red circle) were used (Hurlemann et al. 2010).

While the distribution of OXTR in the rhesus macaque likely contributes to a variety of functions, such as sexual behavior and auditory processing discussed above, it is noteworthy that the majority of OXTR expression in this species is primarily found in areas of the brain that modulate visual attention, eye movements, and orienting responses to visual stimuli—functions that are critical in a species that uses vision as the primary modality for social communication in order to appropriately interpret and subsequent respond to species-typical social behavior.

Comparison to OXTR in other primates

To our knowledge, there are only two other primate species that have had the distribution of OXTR mapped in the brain: the common marmoset, *Callithrix jacchus* (Schorscher-Petcu, Dupré, and Tribollet 2009) and the human, *Homo sapiens* (Loup et al. 1991; Loup et al. 1989). However, neither of these studies used *in situ* hybridization; both relied only on receptor autoradiography. Both studies attempted to show radioligand specificity with displacement experiments designed to the best of their knowledge at the time of publication. For example, both studies used at least three different concentrations of various unlabeled competitors to establish specificity of ^{125}I-OVTA binding; however, these concentrations were arbitrary. Thus, while the results of these studies are now slightly tenuous due to the aforementioned issues of radioligand specificity and displacer selectivity, the results are still worth evaluating in light of the current study. Future studies using a pharmacologically optimized competitive binding protocol are needed to confirm the results of these previous attempts to map OXTR in primate tissue.

In the brain of the socially monogamous and highly affiliative common marmoset, the distribution of OXTR is generally very restricted compared to AVPR1a, which is a pattern that also emerges in the rhesus macaque brain (Young, Toloczko, and Insel 1999). The most notable region of OXTR binding in the marmoset is the nucleus accumbens (NAcc), an area of the brain highly important to addiction and reward. Another socially monogamous species with dense OXTR expression in the NAcc is the socially monogamous prairie vole (Lim, Murphy, and Young 2004), and the closely related but solitary montane vole has no OXTR expression in this area (Insel and Shapiro 1992). Because of these species differences and experimental evidence showing that OT acting in the NAcc is necessary for the formation of the pair bond in female prairie voles

(Young et al. 2001), it has been hypothesized that OXTR density in the NAcc may be consistent across species with social monogamy or otherwise high levels of social affiliation. Although rhesus macaques are group living primates with tight familial bonds, these monkeys maintain strict dominance hierarchies and many of the interactions between unrelated conspecifics are aggressive, not affiliative. Thus, we did not expect to see OXTR binding in the NAcc of this species, and our results confirmed this expectation.

There were only a few other areas with OXTR binding in the marmoset brain: the diagonal band of Broca, nucleus limitans, superficial gray layer of the superior colliculus, olivary nucleus, motor trigeminal nucleus, and spinal trigeminal nucleus (Schorscher-Petcu, Dupré, and Tribollet 2009). Nucleus limitans lies just rostral to the superior colliculus, from which it also receives most of its input (Burton and Jones 1976). While we did detect OXTR in the SuG in the rhesus macaque, we did not detect a signal in nucleus limitans. OXTR is found in the olivary nucleus of marmosets, and although it first appeared to us as though there is a signal for OXTR in the same area of the rhesus macaque, this region lies close to the edge of the tissue and seems to be darkened due to an edge effect (Figure 5, OXTR mRNA column, row 5, ventral surface).

As described above, rhesus macaques and most species other species that have been investigated for OXTR binding have OXTR expression in the VMH, with the exception of rabbits (Tribollet, Dubois-Dauphin, et al. 1992). Quite unexpectedly, marmosets do not express OXTR in the VMH, but they do have AVPR1a binding in this region (Schorscher-Petcu, Dupré, and Tribollet 2009; Tribollet, Dubois-Dauphin, et al. 1992). In the human brain, no OXTR or AVPR1a binding was reported for VMH (Loup

et al. 1991). In marmosets, there is dense OXTR binding in the spinal trigeminal nucleus, which is an area of OXTR binding in the human as well (Loup et al. 1991; Loup et al. 1989). However, we did not have sections from the rhesus macaque brain that were posterior enough to capture this nucleus for the current study.

In the human brain, intense putative OXTR binding was observed in the NBM, the ventral lateral septum, and the diagonal band of Broca, with moderate binding in the preoptic hypothalamus, globus pallidus, and ventral pallidum, and inconstant low levels of binding in a few other areas (Loup et al. 1991). Of these, the only area that is consistent with the rhesus macaque is the NBM. In marmosets, the authors labeled an area of OXTR expression in the basal forebrain as the diagonal band of Broca (DB). In marmoset brain tissue, the DB is very near to the NBM, and based on a replication experiment performed in our lab, we feel that this region is in fact the NBM and not the DB (Freeman, unpublished). Thus, it is possible that the NBM represents a conserved region of OT-mediated visual attention across primate species.

Conclusion

The current study finally establishes the much-needed neuroanatomical foundation to inform future research on the effects of OT in a nonhuman primate model of social cognition. Research examining OT and human behavior will undoubtedly continue to march forward, and it is our hope that the results of this study will help researchers design and interpret future studies and ultimately contribute to a more complete understanding of the neurophysiology of the OT system.

Chapter 4:

Distribution of Oxytocin and Vasopressin 1a Receptors in the Brain of the Socially Monogamous Coppery Titi Monkey (*Callicebus cupreus*)

This chapter presents work to be published within:

Sara M. Freeman, Larry J. Young, and Karen L. Bales. Distribution of vasopressin 1a receptors in the brain of the socially monogamous titi monkey (*Callicebus cupreus*).

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ABSTRACT

The coppery titi monkey is a socially monogamous New World primate that has been studied in the field and the laboratory to investigate the behavioral neuroendocrinology of primate pair bonding and parental care. The neuropeptide vasopressin has been shown to influence male titi monkey pair-bonding behavior, and studies are currently underway to examine the effects of oxytocin on titi monkey behavior and physiology. Here, we use receptor autoradiography to identify the distribution of vasopressin 1a (AVPR1a) and oxytocin receptors (OXTR) in hemispheres of titi monkey brain (n=5). Because the available radioligands for AVPR1a and OXTR have modest affinities for both of these receptors in primate tissue, we used a pharmacologically-informed, competitive-binding autoradioagraphy protocol to selectively reveal AVPR1a and OXTR. In this procedure, either the AVPR1a radioligand (^{125}I-LVA) or the OXTR radioligand (^{125}I-OVTA) was incubated on tissue in one of three conditions: 50 pM radioligand alone, or in the presence of either 10 nM SR49059 (a selective human AVPR1a ligand) or 20 nM ALS-II-69 (a selective human OXTR ligand). The AVPR1a distribution is widespread throughout the brain, but the OXTR distribution is much more limited, with the most abundant binding in the hippocampal formation (dentate gyrus, CA1 field, presubiculum layers I and III). Moderate binding was detected in the nucleus basalis of meynert, pulvinar, superior colliculus, layer 4C of primary visual cortex, periaqueductal gray, pontine gray, nucleus prepositus, and spinal trigeminal nucleus. AVPR1a binding exists throughout the cortex (especially cingulate, insular, and occipital cortices), as well as in the caudate, putamen, nucleus accumbens, central amygdala, endopiriform nucleus, hippocampus (CA4 field), globus pallidus, lateral

geniculate nucleus, infundibulum, habenula, periaqueductal gray, substantia nigra, olivary nucleus, hypoglossal nucleus, and cerebellum. Furthermore, we show that the OXTR antagonist ALS-II-69 reduces ^{125}I-OVTA binding by 43% without affecting binding of ^{125}I-LVA, and that the AVPR1a antagonist SR49059 is capable of reducing ^{125}I-LVA binding by 74% or more, without significantly affecting binding of ^{125}I-OVTA. Based on these results and the fact that both ALS-II-69 and SR49059 are non-peptide, small-molecule antagonists that should be capable of crossing the blood brain barrier, these two compounds emerge as excellent candidates for the pharmacological manipulation of OXTR and AVPR1a in future behavioral experiments in titi monkeys and other primate species.

INTRODUCTION

The neuropeptide hormones oxytocin (OT) and vasopressin (AVP) have been demonstrated in the last several decades to be important modulators of social behavior in mammalian species ranging from rodents to sheep to humans. These molecules are capable of influencing a range of social behaviors including, but not limited to, maternal behavior, territoriality, sexual behavior, and affiliation. These neuropeptides have also been extensively studied in the socially monogamous prairie vole and shown to be critical in the formation of the pair bond that occurs between opposite sex adult conspecifics after mating (Young and Wang 2004).

Socially monogamous primate species have also begun to be studied to determine if OT and AVP play similar roles in modulating pair bonding and social attachment, as they do in the prairie vole. In the black-penciled marmoset (*Callithrix pencillata*), OT delivered intranasally (IN-OT) increased the frequency of huddling with the subject's pair-mate, and treatment with an OT receptor (OXTR) antagonist decreased proximity and huddling (Smith et al. 2010). Treatment with an OXTR antagonist also eliminated food sharing between partners (Smith et al. 2010). In another socially monogamous primate species, the coppery titi monkey (*Callicebus cupreus*), males treated intranasally with AVP increased contact time with their partner compared to a stranger female (Jarcho et al. 2011). The relationship of OT in pair-bonding behavior has also been studied in cotton-top tamarins (*Saguinus oedipus*), and urinary levels of OT in male–female pairs measured over the course of 3 weeks were directly related to sexual behavior and the frequency of affiliative behaviors, specifically initiation of huddling by males and initiation of sex by females (Snowdon et al. 2010). These studies indicate that OT and

AVP can influence species-specific, pair-bond-related behaviors in socially monogamous primates.

While studies using behavioral pharmacology and endocrine measurements have been useful in demonstrating that OT and AVP can modulate pair bonding and related behaviors in primates, it is important to establish which brain regions are sensitive to extracellular concentrations of these neuropeptides. Identifying the locations of the receptors for OT and AVP in the brain can better determine how these circuits function to produce species-specific social behaviors and can inform future studies investigating their neural basis. Reliable antibodies for immunohistochemistry for the OXTR or the vasopressin 1a receptor (AVPR1a) are not available, so the most common technique available to localize these proteins in brain tissue sections is receptor autoradiography. This method has been used for decades to successfully determine where OXTR and AVPR1a are expressed in brain tissue of several species of rodent, including laboratory mice (Insel et al. 1993) and rats (Tribollet et al. 1988; Tribollet et al. 1990; Elands, Beetsma, et al. 1988), Syrian hamsters (Young et al. 2000; Dubois-Dauphin et al. 1992; Dubois-Dauphin et al. 1990), guinea pigs (Tribollet, Barberis, et al. 1992), the socially monogamous California mouse and the promiscuous deer mouse (Insel, Gelhard, and Shapiro 1991), a social and a solitary species of tuco-tuco (Beery, Lacey, and Francis 2008), two species of Central American singing mice (P. Campbell, Ophir, and Phelps 2009), the eusocial naked mole-rat and the solitary Cape mole-rat (Kalamatianos et al. 2010), and the socially monogamous prairie and pine voles and the non-monogamous montane and meadow voles (Insel and Shapiro 1992; Lim, Murphy, and Young 2004).

Based on this extensive collection of mapping data, there is a significant amount of species variation in the distributions of OXTR and AVPR1a. In prairie voles, OXTR is highly expressed in the nucleus accumbens (NAcc), which is part of the mesolimbic reward system in the brain, but in montane and meadow voles, this brain area lacks OXTR (Insel and Shapiro 1992; Lim, Murphy, and Young 2004). Similarly AVPR1a is also expressed in an area in the reward circuit in prairie voles, the ventral pallidum (VP), and this area lacks AVPR1a in the montane and meadow voles (Insel and Shapiro 1992; Lim, Murphy, and Young 2004). It has been hypothesized that the sensitivity of these reward regions to OT and AVP may provide a mechanism for linking the neural encoding of the olfactory cues used for social communication to the reinforcing aspects of mating and affiliative behavior, which promotes the establishment a conditioned partner preference in socially monogamous species. Furthermore, high OXTR density in the NAcc is also seen in the eusocial naked mole-rat (Kalamatianos et al. 2010) and the socially monogamous common marmoset (Schorscher-Petcu, Dupré, and Tribollet 2009), two other highly social species which spend extended periods of time in close proximity with other individuals. This cross-species similarity supports the idea that the distributions of these receptors in reward regions of the brain could be important in mediating affiliative and prosocial behaviors in these species. While neural and genetic variations in AVPR1a have not been consistently associated with mating strategies across rodents (Turner et al. 2010), the action of neuropeptides in reward regions in the brain could indicate one possible evolutionary mechanism for the development of prosociality from more solitary lifestyles over time.

Despite the extensive work done in rodents to map these receptors, the distributions of OXTR and AVPR1a in the brains of primate species are still relatively unknown. This is due to the pharmacological profiles of the OXTR radioligand ^{125}I-ornithine vasotocin analog (^{125}I-OVTA) and the AVPR1a radioligand ^{125}I-linear vasopressin-1a antagonist (^{125}I-LVA), which have been used extensively for receptor autoradiography to identify the location of OXTR and AVPR1a in rodent brain tissue. As described in Chapters 2 and 3, while these two radioligands are reliable in rodent tissue, when they are used in human or nonhuman primate tissue, they lose their selectivity and exhibit a mixed affinity for OXTR and AVPR1a (Freeman et al. *in prep*; Manning et al. 2012). This issue results in radioligand binding to both receptor types in primate tissue and thus an inability to reliably identify the locations of one receptor over the other.

Attempts to overcome this issue have used a competitive binding approach that involves co-incubating the tissue with the radioligand and an unlabeled competitor (an agonist or antagonist) for the receptor that is not of interest (Schorscher-Petcu, Dupré, and Tribollet 2009; Freeman et al. *in prep*). This method blocks radioligand binding to the nonspecific receptor site so that the signal produced by radioligand binding is only due to binding to the receptor of interest. This approach was used in brain tissue sections from the common marmoset (*Callithrix jacchus*), however, the competitor ligands were used at somewhat arbitrary concentrations, rather than those informed by rigorous pharmacological assessments (Schorscher-Petcu, Dupré, and Tribollet 2009). While this does not necessarily mean that the results are inaccurate, it does point to the need for a pharmacologically informed competitive binding approach.

Furthermore, because several of the unlabeled OXTR and AVPR1a ligands that could be used as competitors also exhibit mixed affinities for both of these receptors in primates, it is necessary to use highly selective competitor ligands when working in primate tissue sections. Therefore, we have chosen in the current study to use the pharmacologically optimized, competitive binding autoradiography protocol described in Chapter 2, which uses two competitor ligands that have been shown to be highly selective in primates: an OXTR antagonist ALS-II-69 and an AVPR1a antagonist SR49059.

The goal of the current study was to map the OXTR and AVPR1a distributions in the brain of the socially monogamous coppery titi monkey. We hypothesized that there would be high densities of OXTR in the NAcc and AVPR1a in the ventral pallidum (VP) based on the data from other socially monogamous mammals. Based on results from the common marmoset (Schorscher-Petcu, Dupré, and Tribollet 2009) and rhesus macaque (Chapter 3; Young, Toloczko, and Insel 1999), we also hypothesized that OXTR binding would generally be much lower and more sparse than AVPR1a binding.

METHODS

Animals

Animals were housed at the California National Primate Research Center in cages (1.2 m × 1.2 m × 2.1 m) and were on a 12:12 light:dark cycle with lights on at 0600 hr and lights off at 1800 hr. Temperature was maintained at 21ºC. Housing conditions are identical to that in (Valeggia and Mendoza 1999). Animals were fed a diet of monkey chow, banana, marmoset jelly, cottage cheese, apple, and carrot at 0800 hr and 1300 hr. Animals were euthanized on veterinary advice due to health reasons, none of which

included a neurological component, and brains were harvested opportunistically. Two males (aged 6.97 and 5.21 years) and three females (ages: 4.28, 4.33, and 18.81 years) were used for the study. All animals were in stable, long-term pair bonds, and the females had all previously had infants; the males had not previously reproduced.

Tissue preparation

Titi monkey brains were removed promptly after death, rinsed with PBS, and cut into two hemispheres. The hemispheres were blocked coronally, allowed to freeze completely on dry ice, and placed at -80ºC until sectioning. Hemisphere blocks were removed from -80ºC and brought up to -20ºC for sectioning. The hemispheres were sectioned at 20 μm on a cryostat and mounted on Fisher Frost-plus slides. Slides were stored in a sealed slide box with desiccant and kept at -80ºC until use.

Receptor autoradiography

Sections of titi monkey brain hemispheres were removed from -80ºC and allowed to thaw in sealed slide boxes containing desiccant packets for 1 hour at 4ºC followed by 1 hour at RT. The slides were then dipped in 0.1% paraformaldehyde in PBS, pH 7.4, and rinsed twice in 50 mM Tris buffer, pH 7.4, to remove endogenous ligand. Then, sections were incubated for 1 hr with one of two different radioligands: 50 pM ^{125}I-linear vasopressin-1a antagonist (^{125}I-LVA) to target AVPR1a or 50 pM ^{125}I-ornithine vasotocin analog (^{125}I-OVTA) to target OXTR. To achieve selective binding of these radioligands, sets of three adjacent sections were co-incubated in three different competitive binding treatments: radioligand alone, radioligand plus SR49059, which is a human-selective

AVPR1a ligand (Tocris, Minneapolis, MN; Gal, Wagnon, and Garcia 1993), or radioligand plus ALS-II-69, which is a human-selective OXTR ligand synthesized by our own lab (Smith, Freeman, Voll, Young, and Goodman 2013b). These unlabeled competitors were incubated at concentrations that were determined by previous competitive binding pharmacology experiments to be ideal for selective binding to the receptor of interest: 10nM of SR49059 to target AVPR1a and 20nM of ALS-II-69 to target OXTR (Chapter 2; Freeman et al. *in prep*). Next, unbound radioligand was removed by four washes in 50 mM Tris buffer plus 2% $MgCl_2$, pH 7.4, and then dipped into ddH_2O and air dried. Once dry, the slides were exposed to BioMax MR film (Kodak, Rochester, NY) for 3 days with a set of ten ^{125}I standards (American Radiolabeled Chemicals, Inc., St. Louis, MO). Radioligand tracers ^{125}I-LVA and ^{125}I-OVTA were obtained from Perkin Elmer (Waltham, MA). Digital images were obtained from the films using a light box and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) connected to a computer. Brightness and contrast of the images are equally adjusted for all the sections using Adobe Photoshop (San Jose, CA).

Quantification and Statistical Analysis

Quantification of the optical binding density (OBD) was conducted on the resulting autoradiogram images in the following manner using AIS software (Imaging Research, Inc.). After determining a flat field correction for luminosity levels, optical binding values from the set of standards (American Radiolabeled Chemicals, St. Louis, MO) were loaded into the software and used to generate a standard curve, from which binding density values from brain regions of interest would be extrapolated. In each

animal, two separate measurements were made per brain region per treatment and averaged. OBD averages were calculated for six regions of interest (ROI): three for ^{125}I-LVA binding and three for ^{125}I-OVTA binding. Two-tailed, paired t-tests (alpha = 0.05) were performed to compare results from each of the competitor conditions to the radioligand alone condition. Finally, to generate a measurement of the overall efficacy of the competitors in reducing radioligand binding in this species, average percentages were calculated for the two competitor conditions in each ROI; the OBD values were transformed to reflect the percentage of binding in the radioligand alone condition. Then, for each competitor, these percentages were then averaged across the three ROIs to yield a global picture of radioligand binding reduction. Due to the variation in the location of the midline bisection in each animal and to some slight issues with tissue integrity at the edges, some brain regions, especially hypothalamic areas, were either not present or non-quantifiable and were therefore excluded from analyses.

Acetylcholinesterase staining

Following receptor autoradiography and film development, slides were counterstained for acetylcholinesterase (AChE) to delineate the brain regions for image analysis as previously described using a modified protocol for the traditional AChE protocol that has been shown to amplify AChE signal in tissue previously used for receptor autoradiography (Lim, Murphy, and Young 2004). Images of the resulting counterstained sections were compared with images from a red-bellied titi monkey brain atlas (www.brainmuseum.org), a rhesus macaque brain atlas (Paxinos, Huang, and Toga 1999), a common marmoset brain atlas (Newman et al. 2009), and a tufted capuchin brain

atlas (Manocha, Shantha, and Bourne 1968) to determine neuroanatomical landmarks and identify regions.

RESULTS

Selectivity of Radioligands

The radioligands ^{125}I-LVA and ^{125}I-OVTA produce distinct and mostly non-overlapping patterns of binding in the titi monkey brain (Figure 4.1A,B). This result is strikingly different from what is seen when these compounds are used in rhesus macaque brain tissue, where they produce overlapping patterns of binding (Chapter 3, Figure 3.1). In order to confirm selective binding of the radioligands in this species, radioligands were co-incubated on adjacent sections in one of three treatments: radioligand alone, radioligand with the AVPR1a antagonist SR49059 ("+AVPR1a Block"), or radioligand with the OXTR antagonist ALS-II-69 ("+OXTR Block"). Results of this competitive binding approach in one individual are shown in Figure 4.1, with representative binding highlighted in the lateral geniculate nucleus (LG) for AVPR1a binding and in the presubiculum (PSB) for OXTR binding.

Quantification and analysis of the raw optical binding density (OBD) show that SR49059 significantly reduces binding of the AVPR1a radioligand ^{125}I-LVA in the LG ($p=0.0009$; Figure 4.2A, Figure 4.1A,C), and this result was consistent in the other two representative brain regions that were quantified: the primary visual cortex and central amygdala (Table 4.1). The binding of ^{125}I-LVA in the LG in the presence of the OXTR antagonist ALS-II-69 was not significantly different ^{125}I-LVA alone ($p=0.5564$; Figure 4.2A; Figure 4.1A, E), and this result was also consistent across regions (Table 4.1).

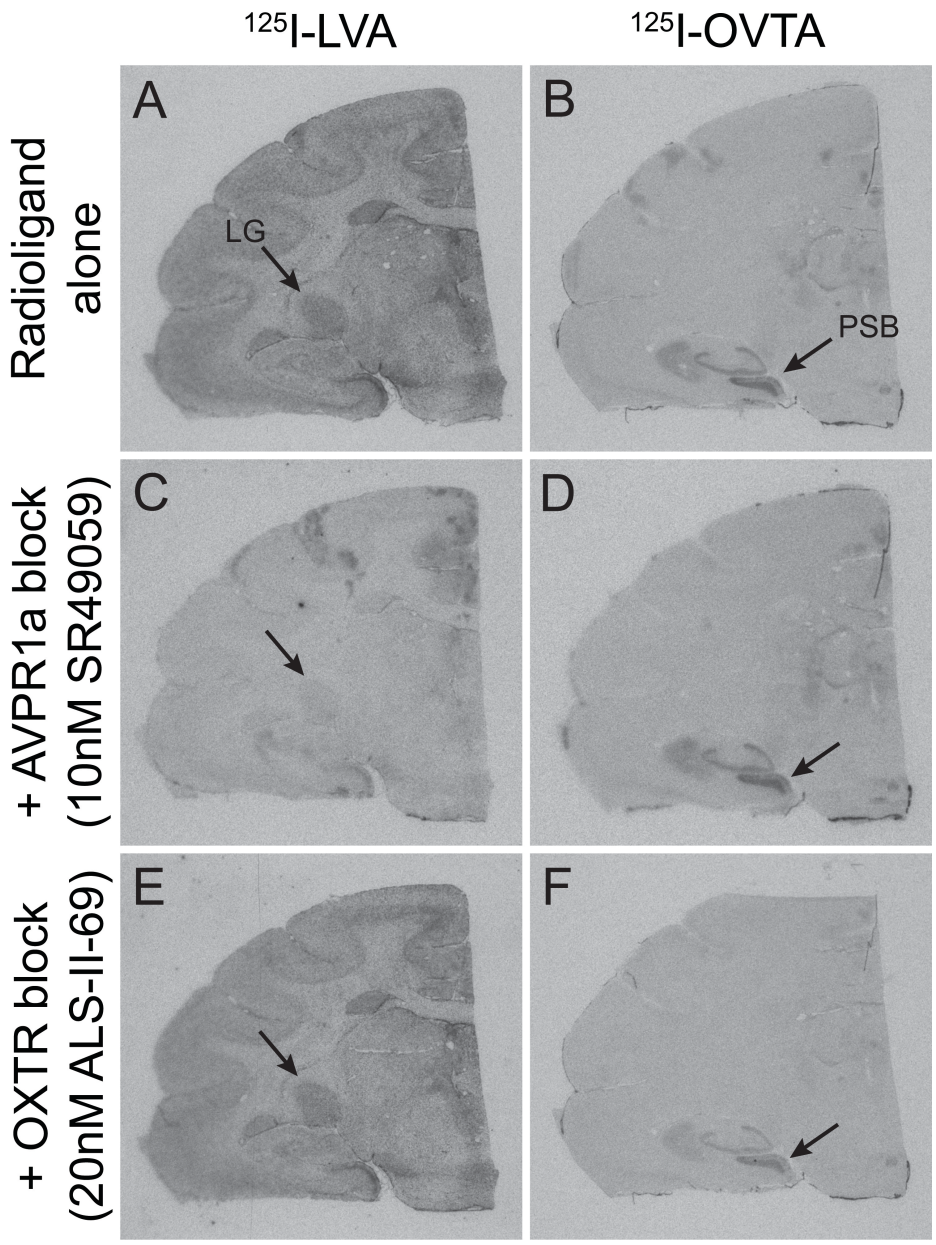

Figure 4.1. Selectivity of radioligand binding in titi monkey brain tissue.
Binding of the AVPR1a radioligand ^{125}I-LVA (A, C, E) and the OXTR radioligand ^{125}I-OVTA (B, D, F) in adjacent titi monkey brain sections. Arrows highlight two representative regions with specific AVPR1a binding (lateral geniculate, LG) and OXTR binding (presubiculum, PSB). (A, B) Radioligand binding alone. (C, D) Radioligand binding in the presence of the AVPR1a antagonist SR49059. (E, F) Radioligand binding in the presence of the OXTR antagonist ALS-II-69.

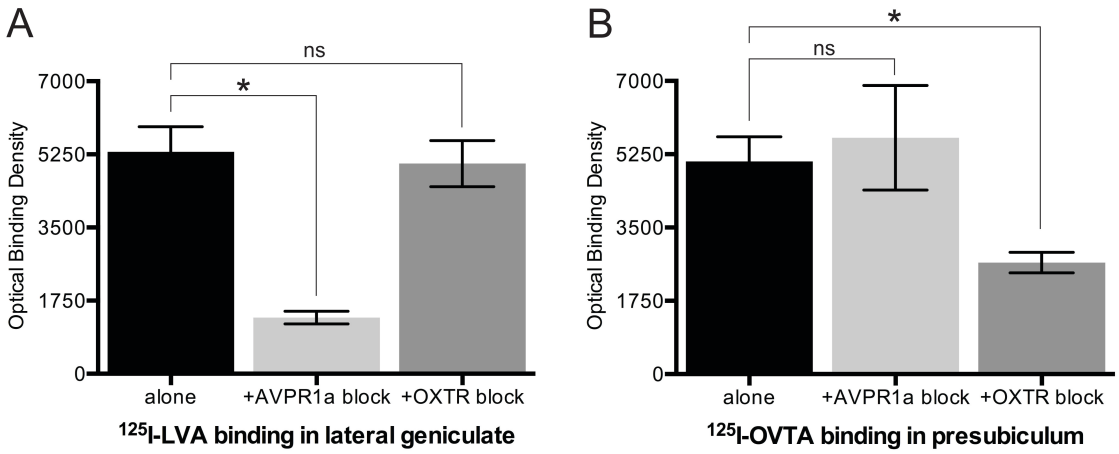

Figure 4.2. Representative quantification of competitive binding.
(A) ^{125}I-LVA binding in the lateral geniculate nucleus was significantly reduced by the AVPR1a block with SR49059 (p=0.0009) but not by the OXTR block (p=0.5564). (B) ^{125}I-OVTA binding in the presubiculum was significantly reduced by the OXTR block with ALS-II-69 (p=0.0055) but not by the AVPR1a block (p=0.6761).

	^{125}I-LVA Alone	^{125}I-LVA + AVPR1a Block	^{125}I-LVA + OXTR Block	Alone vs. AVPR1a Block	Alone vs. OXTR Block
CeA	6712 ± 357.1	1530 ± 141.1	6169 ± 417.0	p=0.0002*	p=0.1057
LG	5310 ± 600.0	1344 ± 150.1	5029 ± 550.1	p=0.0009*	p=0.5564
V1	5244 ± 758.9	1464 ± 323.3	5291 ± 609.3	p=0.0013*	p=0.8939

Table 4.1. Quantification of competition binding for the AVPR1a radioligand.
Optical binding densities (mean±SEM) in three representative brain regions for each binding condition (n=5 animals). Paired t-tests (alpha=0.05) reveal a significant reduction in binding by the AVPR1a block compared to radioligand alone. The OXTR block did not significantly change binding compared to radioligand alone. CeA, central amygdala; LG, lateral geniculate; V1, primary visual cortex

	^{125}I-OVTA Alone	^{125}I-OVTA + AVPR1a Block	^{125}I-OVTA + OXTR Block	Alone vs. AVPR1a Block	Alone vs. OXTR Block
DG	3267 ± 675.0	3413 ± 301.3	1793 ± 230.5	p=0.8764	p=0.0463*
PSB	5079 ± 585.6	5644 ± 1246	2665 ± 244.7	p=0.6761	p=0.0055*
CA1	2808 ± 487.8	2418 ± 165.8	1719 ± 188.5	p=0.4220	p=0.0541

Table 4.2. Quantification of competition binding for the OXTR radioligand.
Optical binding densities (mean±SEM) in three representative brain regions for each binding condition (n=5 animals). Paired t-tests (alpha=0.05) reveal a significant reduction in binding by the OXTR block compared to radioligand alone in two out of the three regions. The AVPR1a block did not significantly change binding compared to radioligand alone. DG, dentate gyrus; CA1, CA1 field of hippocampus; PSB, presubiculum

These results indicate ^{125}I-LVA binds selectively to AVPR1a in this species.

In competition with ^{125}I-OVTA, ALS-II-69 significantly reduces ^{125}I-OVTA binding in the PSB (p=0.0055; Figure 4.2B; Figure 4.1B,F) but SR49059 does not (0.6761; Figure 4.2B; Figure 4.1B,D). In the other two regions that were quantified (Table 4.2), ALS-II-69 significantly reduced binding of ^{125}I-OVTA in the dentate gyrus (DG) of the hippocampus (p=0.0463) but not in the CA1 field of the hippocampus, although it approached significance (p=0.0541). In both of these regions, however, competition with SR49059 did not significantly reduce binding of ^{125}I-OVTA (Table 4.2), indicating that ^{125}I-OVTA binds selectively to OXTR in titi monkey tissue.

Efficacy of Competitors

Our results indicate that the competitors used in the current study are effective at selectively displacing radioligand binding in the titi monkey brain. Overall, SR49059 reduced binding of ^{125}I-LVA by an average of 74.0±1.7%, without affecting the binding of ^{125}I-OVTA (Figure 4.2, dark gray bars). Similarly, ALS-II-69 is capable of reducing binding of ^{125}I-OVTA by an average of 43.0±5.5%, without affecting the binding of ^{125}I-LVA (Figure 4.2, light gray bars). These results provide preliminary evidence that these two small-molecule, nonpeptide antagonists are effective and selective ligands for use in titi monkey tissue.

Vasopressin 1a Receptor Distribution

The distribution of AVPR1a in the titi monkey brain is generally diffuse and widespread (Figure 4.4A). AVPR1a binding exists throughout the cortex, especially in

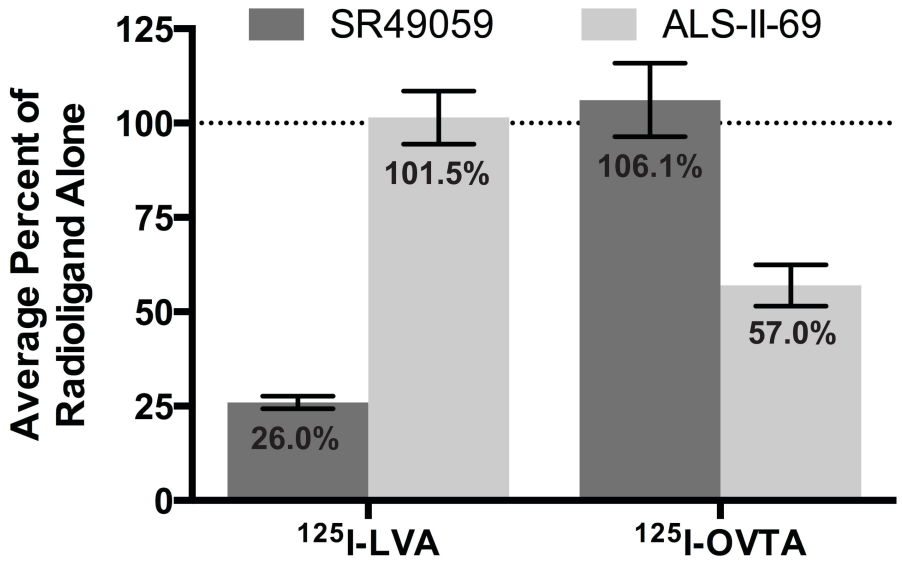

Figure 4.3. Overall efficacy of competitors.
Percent reduction in radioligand binding by SR49059 and ALS-II-69, averaged across three brain regions in five animals for each radioligand. SR49059 competes off 74.0±1.7% (average±SD) of binding of ^{125}I-LVA without significantly reducing binding of ^{125}I-OVTA (106.1±9.7%). ALS-II-69 competes off 43.0±5.5% of binding of ^{125}I-OVTA without significantly reducing binding of ^{125}I-LVA (101.5±7.1%).

the insular (Ins), cingulate (Cg), and occipital cortices. In the occipital cortex, it is most dense in primary visual cortex (V1) in layers 5-6 and is also present in secondary visual cortex (V2). It was also observed in the claustrum (Cl).

AVPR1a binding is also prominent in several regions of the basal ganglia. In the dorsal striatum, it is expressed throughout the caudate nucleus (Cd), and putamen (Pu). In the ventral striatum, it is expressed in the rostral nucleus accumbens (NAcc). AVPR1a binding also appears in and the globus pallidus (GP), in both the internal (GPi) and external (GPe) segments, although to a lesser degree than in the caudate and putamen. The substantia nigra (SN) is a major input nucleus for the basal ganglia, and we also detected AVPR1a binding in this region.

In the temporal lobe, AVPR1a can be detected in several areas. Rostrally, very dense binding is seen in what we believe to be the endopiriform nucleus (EN), although there is very little known about this nucleus in monkeys. We also observed dense binding in the central amygdala (CeA). In the hippocampus, AVPR1a is expressed in the CA4 pathway of the hippocampus (CA4), also commonly referred to as the hilus of the dentate

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Figure 4.4. Distribution of AVPR1a (A) and OXTR (B) in adjacent sections from one representative titi monkey brain, aligned with AChE counterstain (C). Panels 1-3. Abbreviations: CA1, CA1 fild of the hippocampus; CA4, CA4 field of the hippocampus; Cb, cerebellar cortex; Cd, caudate; CeA, central amygdala; Cg, cingulate cortex; Cl, claustrum; cp, choroid plexus; EN, endopiriform nucleus; DG, dentate gyrus; GP, globus pallidus; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; Hb, habenula; HN, hypoglossal nucleus; Hipp, hippocampus formation; Inf, infundibulum; Ins, insular cortex; LG, lateral geniculate; LS, lateral septum; mT, medial thalamus; NAcc, nucleus accumbens; NBM, nucleus basalis; NP, nucleus prepositus; Occ, occipital cortex; ON, olivary nucleus; PAG, periaqueductal gray; PG, pontine gray; PSB,I, PSB,II, or PSB,III, presubiculum layer 1, 2, or 3; Pt, pretectum; Pu, putamen; Pv, pulvinar; SC, superior colliculus; SN, substantia nigra; Sp5, trigeminal nucleus of the spinal tract; SuG, superficial gray later of the superior colliculus; V1,4C, primary visual cortex layer 4C; V1,5-6, primary visual cortex, layers 5-6

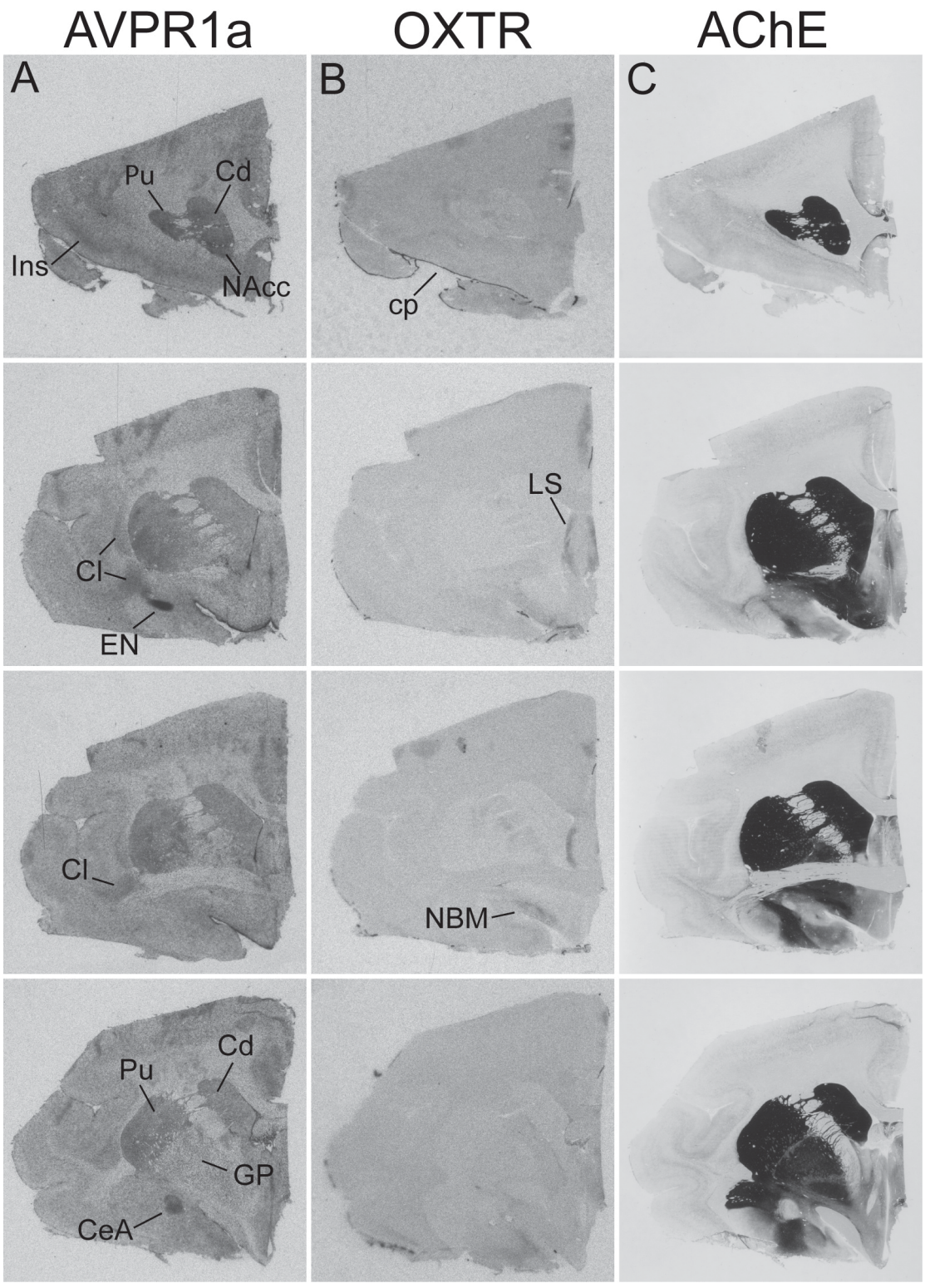

Figure 4.4. Distribution of AVPR1a (A) and OXTR (B) in adjacent sections from one representative titi monkey brain, aligned with AChE counterstain (C). Panel 1. Abbreviations on the previous page

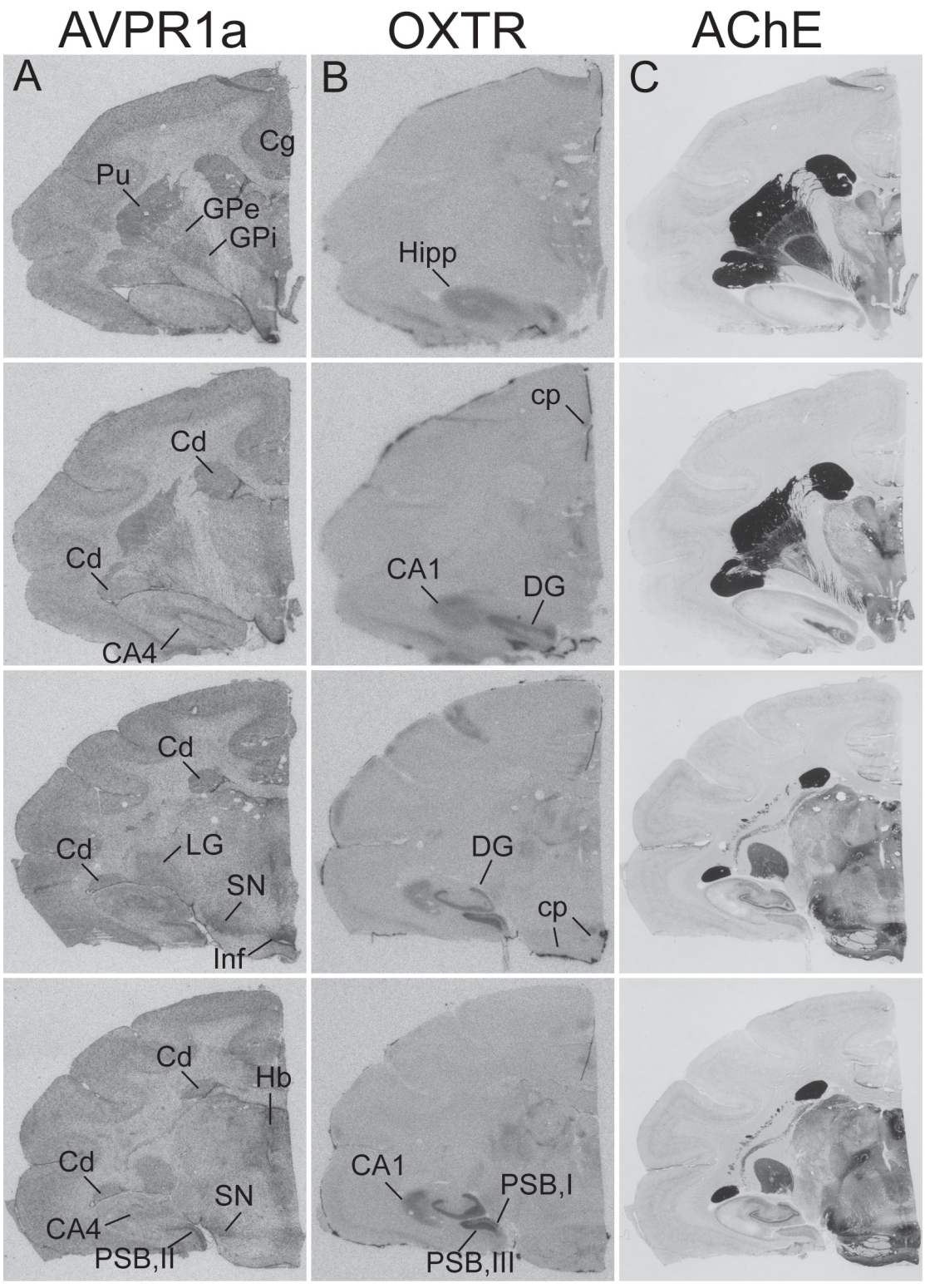

Figure 4.4. ***Continued*****. Panel 2**

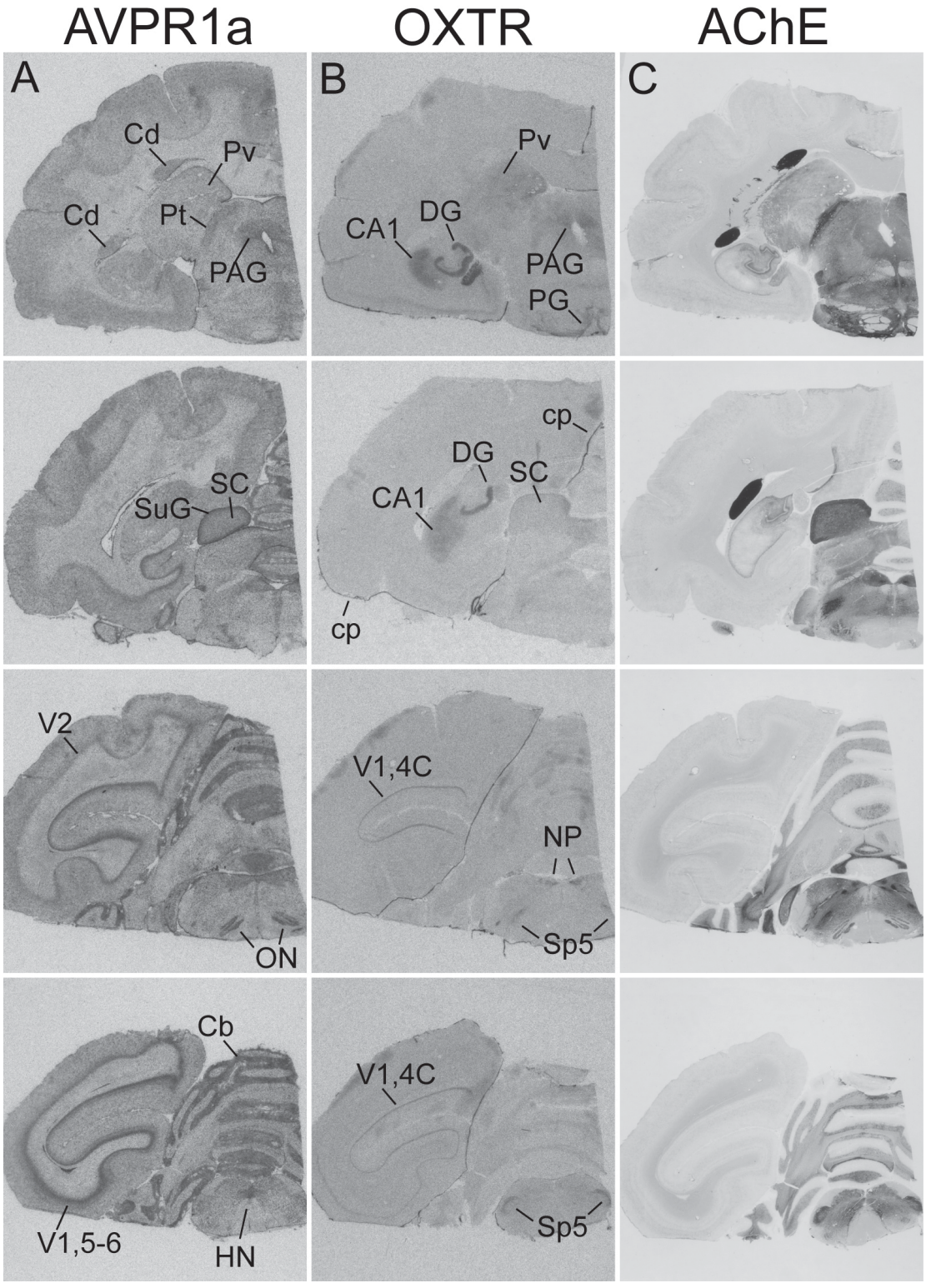

Figure 4.4. ***Continued.*** **Panel 3.**

gyrus. There is also modest binding for AVPR1a in layer II of the presubiculum (PSB, II).

The distribution of AVPR1a also includes several subcortical nuclei involved in the processing of visual information. The lateral geniculate nucleus of the thalamus (LG), which receives input from the retina, has moderate AVPR1a binding (Hubel, *Eye, Brain, and Vision*. 1995). The superficial gray layer of the superior colliculus (SuG), which also receives visual information from the retina, has dense AVPR1a binding, and there also is binding in the deeper layers of the superior colliculus (SC), which mediate the premotor and motor control of eye movements (Gandhi and Katnani 2011). The primary visual cortex, as previously mentioned above, has dense AVPR1a binding in layers 5 and 6, which project back to the LG and SC, respectively (Hubel 1995).

We also detected AVPR1a binding in several structures at the level of the midbrain and hindbrain. These areas include the habenula (Hb), periaqueductal gray (PAG), the infundibulum (Inf), olivary nuclei (ON), and the hypoglossal nucleus (HN). AVPR1a binding was also seen throughout the cerebellum (Cb).

Oxytocin Receptor Distribution

The distribution of OXTR in the brain of the titi monkey is generally quite sparse and much more restricted than AVPR1a (Figure 4.4B). The areas where the binding was most dense were in the hippocampal formation (Hipp), particularly the CA1 field (CA1), the dentate gyrus of the hippocampus (DG), and layers I and III of the presubiculum (PSB,I and PSB,III, respectively). These regions are all found in the temporal lobe and are important in various aspects of learning and memory (Squire and Zola-Morgan 1991).

There were several other structures with modest levels of OXTR binding. These areas are the nucleus basalis of Meynert (NBM), periaqueductal gray (PAG), pulvinar (Pv), layer 4C of the primary visual cortex (V1,4C) in the occipital lobe, the deeper layers of the superior colliculus (SC), and some hindbrain regions, including the nucleus prepositus (NP), pontine gray (PG), and trigeminal nucleus of the spinal tract (Sp5).

Because hemispheres of brain tissue were used for this study, areas along the midline were either absent, bisected, or damaged in a few subjects, but in at least one animal we were able to detect specific OXTR binding in the lateral septum (LS).

DISCUSSION

Comparison to OXTR in other primates

This is the first study to characterize the OXTR and AVPR1a distribution in the socially monogamous coppery titi monkey. The results support our hypothesis that the OXTR binding would be much more limited than AVPR1a binding, as it is in the common marmoset (*Callithrix jacchus*) and rhesus macaque (*Macaca mulatta*) (Schorscher-Petcu, Dupré, and Tribollet 2009; Young, Toloczko, and Insel 1999; Chapter 3; Freeman et al. *in prep*). In the rhesus macaque, OXTR distribution is also incredibly restricted, with low levels of expression in only a few small areas of the brain: NBM, VMH, SuG, III, trapezoid body (TB), and the pedunculopontine tegmental nucleus (PPT) (Chapter 3; Freeman et al. *in prep*). In titi monkeys, the distribution of OXTR binding is also in the NBM, but mostly in areas that are not seen in the rhesus macaque: Hipp, DG, PSB, PAG, and a few hindbrain regions not identified in rhesus tissue. In the common marmoset, which is the only other socially monogamous primate to have its OXTR and

AVPR1a distributions characterized, the OXTR distribution is also very limited compared to AVPR1a. Both marmosets and titi monkeys have dense OXTR in spinal trigeminal nucleus (Sp5), as well as humans (Loup et al. 1991; Loup et al. 1989).

Interestingly, marmosets have dense OXTR binding in the NAcc, as would be expected (Schorscher-Petcu, Dupré, and Tribollet 2009). While we expected to see OXTR in the NAcc in the titi monkey, another socially monogamous New World monkey like the marmoset, our results do not support this hypothesis. While at first this lack of NAcc OXTR in titi monkeys seems unusual, especially in light of the fact that OXTR is found in the NAcc in monogamous and highly social rodents as well (Lim, Murphy, and Young 2004; Kalamatianos et al. 2010), it is common to see vast species differences in OXTR binding both within the rodent clade as well as across mammalian clades. Furthermore, due to the high levels of homology between OT and AVP as well as between their receptors, and due to the mixed affinities of OT and AVP for OXTR and AVPR1a in primates, it is quite possible that the dense AVPR1a binding in the NAcc in titi monkeys may be serving an analogous function as OXTR in this same area in other monogamous species. Furthermore, the widespread, low levels of expression of AVPR1a in titi monkey brain may be an indication that expression of this receptor is less tightly regulated in this species. Additional studies are needed in primates to better elucidate the similarities, differences, and evolutionary origins of the physiology of OXTR and AVPR1a and their neuroanatomical locations across species.

Taken together, these comparative primate results confirm the observation from rodents that both OXTR and AVPR1a display remarkable phylogenetic plasticity in terms of central distributions. Thus, while it is tempting, it is important to remember that the

receptor distribution in the brain of one primate species cannot be a surrogate for the receptor distribution in another, including human.

Visual processing centers of the brains of primates

One striking commonality between the OXTR and AVPR1a distributions of the titi monkey and the binding patterns seen in other primates is the expression of this class of receptors in areas that are important in visual processing (and also multimodal sensory information), and the allocation of attention to sensory stimuli. The titi monkey, human, and rhesus macaque all have OXTR expression in the NBM (Chapter 3; Loup et al. 1991), an area that is critical for mediating attention to visual stimuli (Sarter, Givens, and Bruno 2001; Sarter, Gehring, and Kozak 2006; Muir et al. 1993; Balducci et al. 2003). The common marmoset also has OXTR (and AVPR1a) binding in the NBM (Freeman, unpublished data), although in the published map (Schorscher-Petcu, Dupré, and Tribollet 2009), OXTR binding in this area was labeled as the nearby and closely related structure, the diagonal band of Broca.

OXTR and AVPR1a binding is also seen in two visual areas of the thalamus. First, AVPR1a binding is dense in the LG, which is the primary thalamic relay nucleus for visual input from the retina to downstream regions (Hubel 1995). The LG sends visual input from the retina to the SC and also projects to (and receives projections from) the primary visual cortex (Hubel 1995). In titi monkeys, both OXTR and AVPR1a are observed in V1, with OXTR in layer 4C and AVPR1a in layers 5 and 6. AVPR1a binding was also detected in the secondary visual cortex (V2) and generally throughout the occipital cortex (Occ). Second, both AVPR1a and OXTR binding were present in the

pulvinar nucleus of the thalamus (Pv), which also receives input from the retina, as well as from the SC (Berman and Wurtz 2011). Inhibiting neurotransmission by injection of GABA-related drugs into the Pv of behaving monkeys disrupts performance on a visual attention task (Petersen, Robinson, and Morris 1987). Furthermore, electrophysiological evidence suggests that neuronal activity in the Pv is related to the salience of visually presented objects (Robinson and Petersen 1992). These OT- and AVP-sensitive thalamic areas are early processing areas for visual input and are capable of mediating visual attention and salience.

Two areas that are important for the processing of visual information and especially for the control of eye direction and gaze are the SC and the nucleus prepositus (NP). The dense AVPR1a binding in the SuG layer of SC in the titi monkey closely resembles the dense OXTR binding in the SuG of the rhesus macaque (Chapter 3) and the marmoset (Schorscher-Petcu, Dupré, and Tribollet 2009). In the deeper layers of the SC in the titi monkey, there is also modest OXTR binding as well as dense AVPR1a binding. As explained in Chapter 3, the layers of the SC play distinct and important roles in stabilizing gaze direction, issuing motor commands to produce saccadic eye movements, and incorporating cognitive information to orient to (or away from) a stimulus (Gandhi and Katnani 2011). Furthermore, OXTR was detected in NP, a brainstem nucleus that is part of the horizontal gaze holding system and is considered an important neural integrator for the oculomotor system (McCrea and Horn 2006). The NP also has OXTR binding in the human brain (Loup et al. 1989) and seems to have low levels of OXTR binding in the marmoset brain, which was overlooked (Schorscher-Petcu, Dupré, and

Tribollet 2009). OT and AVP acting in these areas may be important regulators of social visual attention, gaze shifting, and gaze stabilization.

The AVPR1a binding in the pretectum (Pt) of the titi monkey closely resembles the OXTR binding seen in the area labeled as nucleus limitans (NL) in the marmoset paper (Schorscher-Petcu, Dupré, and Tribollet 2009). While the accuracy of the identification and labeling of the Pt vs. the NL may be arguable in the current study vs. the published marmoset map, it is important to note that these regions: 1) are anatomically immediately adjacent to each other, 2) are located between the Pv and SC and receive input from these areas (Burton and Jones 1976), and therefore 3) likely contribute to visual processing, attention, and gaze control as well.

We observed dense AVPR1a binding in the CeA in titi monkeys, which is an area of dense AVPR1a expression in rhesus macaques (Chapter 3; Young, Toloczko, and Insel 1999). In macaques, neurons in the amygdala respond to the head direction and gaze direction (Tazumi et al. 2010) and to the identity and facial expression (Gothard et al. 2007) of images of monkey faces. In an fMRI study in awake macaques, there was a stronger BOLD signal in the CeA in response to images of faces with averted gaze than with direct gaze, and the behavioral responses of the monkeys indicated that averted gaze faces were more arousing (Hoffman et al. 2007). This paper also showed the same effect for neurons in the BNST, which is an area with dense AVPR1a binding in rhesus macaques. Although there as been relatively little research on the effect of AVP on behavior and amygdala activity in humans or nonhuman primates (but see Rilling et al. 2012 and Zink et al. 2010), several studies have begun to investigate the effects of IN-OT on these outcomes. In fMRI studies, IN-OT reduces the increased BOLD signal in the

amygdala detected in response to viewing emotional faces (Domes, Heinrichs, Gläscher, et al. 2007) or fear-inducing visual stimuli (Kirsch et al. 2005), and in individuals with social anxiety disorder, OT specifically reduces the heightened amygdala response to fearful faces (Labuschagne et al. 2010). This growing literature in humans is closely aligned to evidence from rodents as well. In rats, OXTR and AVPR1a are expressed in distinct subpopulations of the CeA (Veinante and Freund-Mercier 1997), and OT and AVP each activate these different subdivisions to differentially regulate fear behavior (Huber, Veinante, and Stoop 2005; Viviani et al. 2011). Specifically, OT acting in the CeA decreases the freezing response to conditioned fear in rats (Viviani et al. 2011; Knobloch et al. 2012). While future studies in primates are needed to investigate the effect of AVP (and OT) in the amygdala on neuronal activity and behavioral measures, this preliminary evidence suggests that the CeA and other aspects of the extended amygdala are important for face processing as well as fear behavior.

While there may be species differences between OXTR and AVPR1a distributions in primates, one main commonality across species is that these neurohypophyseal receptors are expressed in areas which process visual stimuli, allocate attention, and control gaze direction and eye movement. These functions are highly relevant to the processing social stimuli in primates, which are species that use vision as the primary modality for social interactions.

OXTR, AVPR1a, and brain regions important for reinforcement learning

While we did not detect OXTR in the NAcc or AVPR1a in the VP, two regions of the mesolimbic dopamine reward pathway that have been shown in prairie voles to be

important for pair-bond formation (Young and Wang 2004), these receptors were located in several areas of the brain that mediate various aspects of learning and memory, as well as the processing of rewarding or novel stimuli. For example, the three main forebrain regions in the titi monkey brain where we detected strong OXTR binding—CA1, DG, and PSB—are highly interconnected temporal lobe structures critical for learning and memory (Squire and Zola-Morgan 1991; Squire 1992; Demeter, Rosene, and Van Hoesen 1985). We also detected modest AVPR1a binding in the hippocampus. Knockout mice lacking either OXTR or AVPR1a show deficits in social memory (Ferguson et al. 2000; Ferguson et al. 2001; Bielsky et al. 2004), and studies are currently ongoing to assess the effects of IN-OT on primate social cognition (in titi monkeys: KL Bales 1R01HD071998-01A1) and social memory for faces (in rhesus macaques: LJ Young 1P50MH100023-01). Thus, it is possible that OT and AVP acting in these parts of the brain may contribute to the acquisition and/or recall of social memories in primates. Interestingly, head direction cells have been found in the PSB of the rhesus macaque (Robertson et al. 1999), and "spatial view" cells that respond to viewing specific aspects of an environment have been detected in CA1 (Robertson, Rolls, and Georges-Francois 1998), two areas where we detected dense OXTR expression. This suggests an exciting (yet speculative) OT-sensitive visuo-motor link between these temporal lobe structures and the brain regions controlling eye movements, gaze/head direction, and shifts in attention in response to visual stimuli, discussed above (and in Chapter 3).

AVPR1a binding in the titi monkey brain was also detected in the Cd, Pu, GP, and SN. The striatum (Cd, Pu, GP) is highly important in reinforcement learning (Schultz et al. 1992; Schultz, Tremblay, and Hollerman 2003), and SN is a main source of

dopaminergic input into the striatum. Dopaminergic neurons in the substantia nigra pars compacta (SNc) have been shown to be involved in learned responses to stimuli and decrease their firing after repeated presentations of a stimulus (Ljungberg, Apicella, and Schultz 1992). However, behaviorally relevant stimuli, especially those stimuli that are reinforced during classical conditioning paradigms, continue to activate the dopaminergic neurons in the SNc, a response that has been theorized to underlie the addictive properties of drugs of abuse. It has been proposed that many of the neural circuits involved in addiction are also key regions mediating social attachment (Burkett and Young 2012). Thus, in the context of primate social behavior and pair bonding, its possible that AVPR1a in the areas like SN and striatum may be integral to the formation of the pair bond, or to social memory of familiar individuals more broadly.

Assessment of primate-selective antagonists

Finally, this is the first study to assess the efficacy of two small molecule, non-peptide antagonists as competitors for the iodinated OXTR and AVPR1a radioligands in a New World primate species. The AVPR1a antagonist SR49059 significantly and selectively reduces binding of the AVPR1a radioligand ^{125}I-LVA. Similarly, the OXTR antagonist ALS-II-69 significantly and selectively reduces binding of the OXTR radioligand ^{125}I-OVTA. The iodinated radioligands used in this study have significantly higher affinities for their respective receptors than the endogenous neuropeptides (nanomolar affinities for OT and AVP vs. picomolar affinities for the radioligands) and have also been designed to bind to the receptors longer than the endogenous ligands. Therefore, if the two nonpeptide ligands used in this study can effectively displace

binding of the radioligands, then it is likely that they could be even more effective at displacing endogenous OT and AVP *in vivo*. While future studies are needed to assess the efficacy of ALS-II-69 when given peripherally, SR49059 has been reported to have good oral bioavailability (Gal, Wagnon, and Garcia 1993). Thus, these results provide preliminary evidence that these two small-molecule, nonpeptide antagonists are effective and selective ligands for use in titi monkeys and are also excellent candidate antagonists for future behavioral pharmacological studies in primates.

Chapter 5:

Discussion

This chapter presents work to be published within:

Sara M. Freeman, Hasse Walum, and Larry J. Young. Diversity in the oxytocin receptor system in primates: perspectives on past and future research. Invited review; to be published in a special issue 'Oxytocin's routes in social behavior: into the 21st century' in Frontiers in Neuroscience. 2013.

ABSTRACT

In the last several decades, sophisticated experimental techniques have been used to elucidate the neurophysiology of the oxytocin and vasopressin systems in rodents. We have been able to take advantage of a suite of methodologies including but not limited to: intracerebroventricular and site-specific infusions of selective agonists and antagonists into the brain, receptor autoradiography, microdialysis, conditional genetic knock out animals, site-specific viral vector-mediated gene transfer, and elegantly designed behavioral paradigms. Based on the plethora of results from these rodent studies, we have now begun to create new hypotheses about how the oxytocin system could be acting in our own species. However, despite the recent inundation of publications using intranasal oxytocin in humans, we still know very little about the neurophysiology of the oxytocin system in primates. Furthermore, the design and analysis of these human studies have generally remained experimentally ignorant to the potential mechanisms underlying their published results. While the methods available to study the oxytocin system in humans are incredibly limited due to practical and ethical considerations, there is great potential to remedy the gaps in our knowledge by working to develop better nonhuman primate models of social functioning. At present, a subset of the aforementioned technologies, as well as several others, such as neuroimaging, have been used in a very limited way to study the oxytocin and vasopressin systems in nonhuman primates, but there is great potential to broaden our understanding of the neurophysiology of these systems. Therefore, in my concluding chapter, I (i) identify several specific ways that the established technologies can be used to answer basic research questions in a primate model, (ii) highlight areas of future research in nonhuman primates that are

experimentally poised to yield critical insights into the anatomy and physiology of the oxytocin system, and (iii) apply an evolutionary perspective to the results of this dissertation in order to more soundly develop our hypotheses and refine our methods so that we are advancing our understanding of the primate oxytocin system.

Introduction

Research on the oxytocin (OT) system in rodents has provided a wealth of information about the role this peptide plays in the expression of species-specific behaviors. One reason for these advancements is the availability of sophisticated and validated methods that can be used across rodent species, as we saw in Chapter 1. These methods include histological, neurological, pharmacological, genetic, and behavioral techniques that have been used alone and in combination to provide the field with reliable and replicable data. Without this extensive data set from the rodent literature, it is unlikely that the growth rate in the number of studies examining OT in humans and nonhuman primates (NHP) would be as high as it is currently (Young and Flanagan-Cato 2012). However, this rapid expansion of research into primate OT neurophysiology has resulted in several weaknesses, including: inconsistent methodologies used across studies, a lack of validated behavioral paradigms, and a significant gap in the histological and neurological understanding of these circuits in the primate brain. In the following section, I review some of the elegant rodent work that paved the way for the current advancements in OT research and link these methods to potential primate research aims. I will also review what has been done in my dissertation, as well as what could be done in future experiments, to advance our understanding of the function of OT in NHP models of social cognition.

Methodological comparisons between rodent and primate OT research

Intracerebroventricular injections of drugs into the brain

A common starting place for neuropharmalogical experiments is by intracerebroventricular (ICV) administration of drugs. Delivering agonists and/or antagonists directly into the cerebrospinal fluid (CSF) of the ventricular system bypasses several biological obstacles: the blood brain barrier (BBB), the metabolism of compounds by enzymes and proteases in blood, and any off-target effects of drugs acting at peripheral receptors throughout the body. One example for how ICV injections have been used in rodents to further our understanding of the function of OT in social behavior comes from studies of the socially monogamous prairie vole, a species whose capacity to form selective pair bonds has resulted in their use a model organism for the neurochemical basis of social attachment. In female prairie voles, ICV administration of OT was shown to promote pair-bond formation, and ICV delivery of an OTR antagonist blocked it (Williams, Insel, and Harbaugh 1994). Invasive methods like ICV injections are much less common in studies of social function in NHP and require a more thorough ethical evaluation than in rodents. Consequently, there is only a single study that used ICV injections of OT in NHP, in male squirrel monkeys, and the authors found that it increased aggression in dominant individuals and also increased social contact in subordinates (Winslow and Insel 1991).

Receptor autoradiography

By performing histological experiments to characterize the oxytocin receptor (OXTR) distribution in the brain, investigators become able to move forward from ICV injections toward the design of experiments that involve site-specific manipulations of and measurements within the brain. As early as the 1980s, researchers were working to

identify the location of OXTR binding sites in the brains of rodents. This work was made possible by the development of radiolabeled OXTR ligands for receptor autoradiography. The earliest radioligand available for this purpose was a tritiated version of OT (Brinton et al. 1984; Freund-Mercier et al. 1987; Van Leeuwen et al. 1985), but the cross reactivity of this ligand with the closely related vasopressin 1a receptor (AVPR1a), combined with long exposure times needed on film before results can be attained, resulted in it being an unfavorable and generally inconvenient tool for autoradiographic analysis of the brain.

Later, highly selective radiolabeled peptide analogs for OXTR and AVPR1a were developed for use in rodent brain tissue (Elands, Barberis, Jard, Tribollet, et al. 1988; Elands, Barberis, Jard, Lammek, et al. 1988). The iodinated OXTR radioligand, ^{125}I-ornithine vasotocin analog (^{125}I-OVTA), and V1aR radioligand, ^{125}I-linear vasopressin-1a antagonist (^{125}I-LVA), allowed researchers to quickly and reliably map the central distributions of both OXTR and AVPR1a (Perkin Elmer, Waltham MA, USA). To our knowledge, rodent species with known OXTR and AVPR1a distributions include: laboratory mice (Insel et al. 1993) and rats (Tribollet et al. 1988; Tribollet et al. 1990; Elands, Beetsma, et al. 1988), hamsters (Young et al. 2000; Dubois-Dauphin et al. 1990; Dubois-Dauphin et al. 1992), guinea pigs (Tribollet, Barberis, et al. 1992), the socially monogamous California mouse and the promiscuous deer mouse (Insel, Gelhard, and Shapiro 1991), a social and a solitary species of tuco-tuco (Beery, Lacey, and Francis 2008), two species of Central American singing mice (P. Campbell, Ophir, and Phelps 2009), the eusocial naked mole-rat and the solitary Cape mole-rat (Kalamatianos et al. 2010), and the socially monogamous prairie and pine voles and the non-monogamous montane and meadow voles (Insel and Shapiro 1992; Lim, Murphy, and Young 2004).

However, there has been a significant lag in the mapping of OXTR in the brains of primate species. This knowledge gap can be attributed mainly to the loss of radioligand selectivity when ^{125}I-OVTA and ^{125}I-LVA are used in primate tissue. While these compounds are selective for their respective receptors in rodents, they have now been shown to exhibit high affinities for both OXTR and AVPR1a in primates (Chapter 2; Freeman et al. *in prep*; Manning et al. 2012). Nevertheless, before these pharmacological limitations were fully understood, these radioligands (as well as the older tritiated versions of the endogenous neuropeptides themselves) were used for receptor autoradiography in three primate species: in the common marmoset for OXTR and AVPR1a (Wang et al. 1997; Schorscher-Petcu, Dupré, and Tribollet 2009), in the rhesus macaque for AVPR1a (L. J. Young, Toloczko, and Insel 1999), and in human for OXTR and AVPR1a (Loup et al. 1991; Loup et al. 1989). While these efforts give us a preliminary view of the localization of OXTR and AVPR1a across a few primate species, the results need to be reevaluated, confirmed, and expanded, given the new knowledge of the lack of selectivity exhibited by the radioligands that were used.

In chapter 2, I reported the pharmacological characterization of the iodinated radioligands as well as several unlabeled ligands for OXTR and AVPR1a and then used this pharmacological information to design an optimized method for competitive binding receptor autoradiography that can selectively and reliably reveal the distributions of OXTR and AVPR1a in primate tissue (Chapter 2; Freeman *in prep*). One of the OXTR competitor ligands I characterized was a novel, nonpeptide antagonist developed in our own lab (Smith, Freeman, Voll, Young, and Goodman 2013b), and I have also contributed to the characterization of several other candidate OXTR antagonist

compounds (Smith et al. 2012; Smith, Freeman, Voll, Young, and Goodman 2013a). We have now successfully mapped OXTR in the brain of a common NHP model organism for biomedical research, the rhesus macaque (*Macaca mulatta*) (Chapter 3); this mapping was attempted in the 1990s but abandoned due to the apparent non-selectivity of ^{125}I-OVTA (Toloczko, Young, and Insel 1997). Additionally, we have also mapped OXTR and AVPR1a in the brain of the socially monogamous coppery titi monkey (*Callicebus cupreus*) (Chapter 4). We have independently confirmed the findings for OXTR and AVPR1a in the forebrain of the common marmoset (*Callithrix jacchus*) (Freeman, unpublished). A comparative analysis across primate species and the possible evolutionary significance of these receptor distributions will be discussed further below. This neuroanatomical information about receptor distributions is critical for informing the design and analysis of future experiments in primates and should be a renewed focus for NHP research on OT.

Measuring concentrations of OT in the brain and periphery

Site-specific measurements of neuropeptide release using implantation of a microdialysis probe have allowed concentrations of OT within specific brain regions to be assessed during specific behaviors, like stress (Ebner et al. 2005), mating (Ross, Cole, et al. 2009), maternal behavior (Bosch et al. 2004), and parturition and nursing (Neumann and Landgraf 1989; Landgraf, Neumann, and Pittman 1991; Neumann, Russell, and Landgraf 1993; Neumann et al. 1993). In female prairie voles, dialysates collected from the NAcc—an area with dense OXTR expression in this species—during mating showed increased levels of OT when compared to interactions with a male without mating (Ross,

Cole, et al. 2009). Furthermore, microdialysis experiments in female rats have shown that OT is elevated during parturition and nursing within brain regions that synthesize OT, specifically the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus (Neumann, Russell, and Landgraf 1993; Neumann et al. 1993). Assessments such as these have helped to provide important information regarding the neurocircuitry of OT in the rodent brain.

Due to the overall difficulty in directly probing the brains of NHP, researchers in the primate field have substituted methods like microdialysis with measurements from CSF of the spinal column, as well as with peripheral measures of OT from urine, plasma, and saliva. While studies using this design tend to be purely correlational, it is start. For example, in a species comparison between pigtail (*Macaca nemestrina*) and bonnet macaques (*Macaca radiata*), Rosenblum and colleagues hypothesized that CSF levels of OT would be higher in the more gregarious bonnet macaques while CSF levels of corticotropin releasing factor (CRF) would be higher in the more aggressive pigtails, and they found that this was indeed the case (Rosenblum et al. 2002). In comparisons between nursery-reared and mother-reared rhesus macaques, CSF OT was lower in nursery-reared animals, but across both groups, CSF OT was positively correlated with levels of affiliative behavior (Winslow et al. 2003). In male-female pairs of socially monogamous cotton-top tamarins (*Saguinus Oedipus*), urinary OT was highly correlated to the initiation of huddling by the male pair-mate and by the initiation of sex by the female (Snowdon et al. 2010). A recent study of wild chimpanzees (*Pan troglodytes*) reports increases in urinary OT after single grooming bouts between bonded individuals

(kin or not) but not between non-bonded individuals, indicating a possible effect of social relationship strength (Crockford et al. 2013).

While using peripheral measures of hormones is common in studies using NHP, but the use of urinary, salivary, or plasma OT as a biological measure of central OT activity is tenuous. Neither urinary, salivary, nor plasma concentrations of OT have yet been directly linked to levels of OT release in the brain, although this is an active area of ongoing research. Some recent evidence from rodents even suggests that CSF concentrations of OT do reflect the dynamic changes in extracellular fluid within specific brain regions (Neumann et al. 2013). Therefore, microdialysis in the brains of NHP for direct measurement of OT release should be used site-specifically within brain regions. The work of my dissertation has now made it possible to use the receptor distributions in three NHP species to design experiments using site-specific measurements of OT in relevant brain regions are sensitive to extracellular OT concentrations.

Furthermore, by using a combination of measurements from site-specific samples, CSF, and peripheral fluids, it would become possible to evaluate the temporal connection between OT release in the brain and increases in OT concentrations in CSF, urine, saliva, and/or plasma, thus answering one of the long-standing questions in primate OT research.

Site-specific injections of OT-related compounds into the brain

In rodents, OXTR distributions have been used to inform the design of more specific behavioral pharmacology experiments that involve targeted infusions of OT or OXTR agonists or antagonists into the brain regions that have high OXTR densities. For example, in voles, local infusion of an OXTR antagonist into the nucleus accumbens

(NAcc) or the prefrontal cortex (PFC) of the prairie vole is sufficient to block the formation of a partner preference (Young et al. 2001). Furthermore, infusions of an OXTR antagonist into the medial amygdala (MeA) of wildtype mice produce deficits in social memory (Ferguson et al. 2001). Because the central OXTR distribution in the most common primate species used for biomedical research, such as the rhesus macaque, has not been evaluated until now, it has been impossible to design experiments like the ones performed in rodents, where neurological information about the locations of OXTR is taken into account.

But now that we are equipped with this knowledge, what sorts of future experiments are possible? In the socially monogamous marmoset, which has dense OXTR in the NAcc like the prairie vole, it would be interesting to perform an analogous experiment to the one referenced above in voles in order to determine whether OT acting in NAcc is necessary for pair-bond formation (or pair-bond-related behaviors) in marmosets. For example, site-specific infusion of a selective OXTR antagonist during the period of pair-bond formation followed by a behavioral assessment of pair-bond strength would be one way to assess the function of OT in the marmoset brain during social bonding. Similar experiments could be done in marmosets with respect to their high levels of parental (especially paternal) care. By combining these antagonist studies with site-specific microdialysis experiments as described above, it now becomes possible to assess where OT is released in the brain as well as where it is acting to produce behavior.

In rhesus macaques, a similar experimental design targeting the dense OXTR in the nucleus basalis of Meynert (NBM) or the superficial gray layer of the superior colliculus (SuG), followed by a social visual attention task, would yield critical insights

into OT's regulation of social attention. The importance of OT in early stages of visual processing as well as in "other-oriented attention" have been alluded to recently in behavioral studies administering OT intranasally to macaques (Chang et al. 2012; Ebitz, Watson, and Platt 2013). We now have the ability to perform targeted manipulations of the macaque brain to better elucidate the function of OT in primate social cognition.

Peripheral administration of OT

While these sophisticated neurobiological manipulations are now available for NHP as they have been in rodents, the best available method until now has been to deliver OT to primates is peripherally via an intranasal spray (IN-OT). In squirrel monkeys (*Samiri sciureus*), IN-OT was shown to reduce the ACTH response to stress (Parker et al. 2005). In black-penciled marmosets (*Callitrhix pencillata*), daily IN-OT administration during a three-week period in which males and females were paired with opposite sex conspecifics resulted in increased huddling with their partner (Smith et al. 2010). Furthermore, daily treatment with an OXTR antagonist caused decreased proximity to their partner, as well as decreased food sharing, which is a highly social and cooperative behavior in marmosets (Smith et al. 2010).

There have been three papers examining the effect of IN-OT in rhesus macaque social cognition, the first of which showed that rhesus monkeys increased both selfish and altruistic allocation of juice rewards after administration of OT (Chang et al. 2012). Further, this study also showed that treatment with OT selectively increased the donor monkey's attention to the passive recipient monkey only after the altruistic reward delivery (Chang et al. 2012). In another recent study, IN-OT reduced the subjects'

attention to images of negative facial expressions (monkey threats and bared teeth displays), but did not affect attention to neutral faces or non-social images (Parr et al. 2013). Furthermore, IN-OT treatment caused these macaques to increase their attention to images of monkey faces with direct gaze, thereby reversing their typical attentional bias toward faces with averted gaze (Parr et al. 2013). These findings are somewhat corroborated by results from a study by Ebitz and colleagues, who found that IN-OT increased attention to the eyes of pictures of rhesus faces in one task, but the same study found that IN-OT reduced the frequency that the subjects chose to view pictures of dominant male faces in a different task (Ebitz, Watson, and Platt 2013). Thus, it seems that IN-OT modulates the visual social attention of macaques, which agrees with the receptor distribution (Chapter 3) in areas of the brain that are important for gaze direction, eye movements, and attention. Future studies in macaques should use the locations of OXTR in the macaque brain to further elucidate the neural mechanism underlying the behavioral changes observed in these recent studies.

While these results highlight the recent efforts in investigating OT's impact on primate social cognition, they are also an example of the difficulty in comparing the overall implication of results when different behavioral tasks are used across studies (an issue we will discuss below). Another issue regarding IN-OT studies is that the efficacy of IN-OT to reach receptors in the brains of NHP has not yet been evaluated. Ongoing research efforts have begun to evaluate whether IN-OT is capable of increasing central concentrations of OT in CSF, and recent work from our own lab has shown that CSF concentrations of OT do in fact increase after intranasal delivery, as well as plasma concentrations (Modi et al. *submitted*).

However, this demonstration still leaves many questions unanswered. Is the subsequent increase in CSF OT after intranasal delivery due to an accumulation of the delivered dose or due to triggered release of endogenous stores of OT? If IN-OT is accumulating in CSF, does this increase indicate that OT concentrations also increase in specific brain regions? If the increase in CSF OT after IN-OT is not related to concentrations in extracellular fluid within the brain (Neumann et al. 2013), but IN-OT is capable of changing behavior, is it acting via a peripheral mechanism? What is this peripheral mechanism; does the increased OT in plasma after IN-OT result in feedback to the brain that then releases endogenous central stores of OT? If so, is this increase in endogenous OT what is being detected in CSF rather than OT from exogenous IN delivery?

In my future studies, I hope to answer some of these questions by taking advantage of an interesting new discovery in New World monkeys. In placental mammals and most marsupials, the nine amino acid sequence of OT has a leucine in the 8^{th} position (Acher, Chauvet, and Chauvet 1995). It has now been shown that almost all genera of New World monkeys have a single nucleotide substitution in the OT gene which results in a novel version of OT that has a proline in the 8^{th} position ("Pro^8-OT") rather than a leucine (Lee et al. 2011; Wallis 2012). Interestingly, however, the coppery titi monkey (*Callicebus cupreus*) does not have this mutation and therefore synthesizes the conserved mammalian form of the peptide ("Leu^8-OT"). By administering Pro^8-OT intranasally to titi monkeys, which synthesize Leu^8-OT, and then sampling CSF and plasma for concentrations of each the two peptides (using mass spectrometry), it becomes

possible to disentangle the mechanisms by which IN-OT affects behavior and to finally answer many of the lingering questions described above.

Genetic studies of the OT system in rodents and primates

There have been several lines of genetic knock out mice developed that aid in the investigation of the OT system in rodents. First, mice lacking the gene for OT (OTKO) fail to develop a memory of a familiar conspecific after repeated presentations (Ferguson et al. 2000), and infusions of OT into the MeA of OTKO mice rescued this behavior (Ferguson et al. 2001). Mice lacking the oxytocin receptor gene (OXTRKO) have also been studied, and it was found that these mice have several deficits in social behavior (Takayanagi et al. 2005). Furthermore, conditional knock out mouse lines for OXTR, such as mice selectively lacking OXTR expression in the forebrain (Lee, Caldwell, Macbeth, Tolu, et al. 2008; Lee, Caldwell, Macbeth, and Young 2008) and mice lacking the ability to release OT from intracellular stores (Jin et al. 2007; Liu et al. 2008) have been generated to further aid in the investigation of the contribution of OT and OXTR to normal mouse social behavior. These genetic resources have enabled comparisons not only to wildtype but across several knock out lines (Higashida et al. 2010).

While knock out technologies are valuable resources for the investigation of gene function, they are not available for NHP (however, see Chan et al. 2001). Therefore, studies of genetic influences on behavior in these species are limited to association studies looking at naturally occurring genetic variation. There are no published studies examining potential genetic variation in OT-related genes in NHP to our knowledge. However, genetic variation in the AVPR1a gene has been studied for its possible

association in NHP sociality in chimps (Hopkins, Donaldson, and Young 2012) and owl monkeys (Babb, Fernandez-Duque, and Schurr 2010). While this dearth severely limits our understanding of the potential genetic contribution of OT-related genes to NHP behavior, there have been some studies of genes related to the OT system in humans. These studies have focused on variation in the human oxytocin receptor gene (*OXTR*), which has primarily been shown to be linked to autism (Wu et al. 2005; Jacob et al. 2007; Lerer et al. 2008; Yrigollen et al. 2008; Tansey et al. 2010; Campbell et al. 2011; Liu et al. 2010; Wermter et al. 2010), but also to other measures of social behavior, such as empathy (Rodrigues et al. 2009), attachment style in patients with depression (Costa et al. 2009), social cognition in ADHD (Park et al. 2010), emotional support seeking (Kim et al. 2010), prosocial temperament (Tost et al. 2010), maternal sensitivity (Bakermans-Kranenburg and van IJzendoorn 2008), prosocial decision making (Israel et al. 2009), and pair-bonding behavior (Walum et al. 2012). While studies of this kind are limited due to the lack of functional evidence and the small effects sizes of studied markers, they benefit from the fact that variation the human genome is relatively well defined and that genotyping is becoming increasingly cheap, which enables the use of large population sizes. Information about functional variation in genes involved in the OT system can be useful for pharmacological studies in humans and NHP, for example to investigate whether specific genetic markers related to OT signaling affects response to drug treatment, and we would like to highlight this area for future research in NHP.

Viral vector-mediated gene transfer is another important technology that has permitted the evaluation of the function isolated gene products, with the benefit of manipulating specific brain areas. In the study of the vole OT system, Ross and

colleagues were able to experimentally increase OXTR density in the NAcc by injecting a viral vector carrying the prairie vole OXTR gene bilaterally into this area of adult females (Ross, Freeman, et al. 2009). There is a great degree of individual variation in the density of OXTR in the NAcc of prairie voles, and this density positively correlates with the level of expressed alloparental behavior (Olazábal and Young 2006b). By comparing experimental animals to control animals, the authors were able to establish whether there is a causal relationship between the density of NAcc OXTR and prairie vole alloparental behavior (Ross, Freeman, et al. 2009). Surprisingly, this manipulation did not affect the expression of alloparental care (but did accelerate pair bond formation), but a recent follow up to this study showed that elevating OXTR in the NAcc in juvenile females did increase alloparental behavior when these individuals were adults (Keebaugh and Young 2011). Thus, the inclusion of genetic manipulations to alter protein expression in the brain via viral vector mediated gene transfer has promoted a deeper understanding of the potential activational and organizational roles that OT plays in social behavior. While long lasting alterations to the genome of any NHP using viral vector mediated gene transfer may never become commonplace, it may be possible (perhaps in small bodied primates with relatively short lifespans?) to use this technology for the assessment of OXTR expression in NHP. In cases where this does become a reality, knowing the existing receptor distributions in the brain is key to designing experiments that manipulate levels of expression, and this receptor map is now available for three commonly studied NHP species.

Behavioral paradigms

The last advantage of rodent research that can be translated to strengthen NHP work is the suite of validated and reliable behavioral paradigms. The aforementioned techniques that can genetically or pharmacologically manipulate the brain would be of little use without established behavioral tests. These established paradigms have been used for decades to investigate the potential influence of any candidate hormone on phenotypes of interest. In the context of social neuroscience, there are several well-accepted paradigms for the assessment of social behavior in rodents, as well as for other potentially confounding behaviors, like anxiety. Specific assessments include the open field (Prut and Belzung 2003), elevated plus maze (Pellow et al. 1985), social defeat (Martinez and Torrent 1998), social learning (Ferguson et al. 2000), and partner preference (Williams, Carter, and Insel 1992). This list is not exhaustive, and of course, there are several classic paradigms used across laboratories to assess other behavioral phenotypes, such as depressive-like behaviors, reward/reinforcement, and pain. The great benefit of using established behavioral paradigms, such as the ones available for rodent research, is the replicability of results and the consistency of methods used across studies. These are two critical components of behavioral research that the primate OT field is lacking.

While the design of behavioral studies of OT in primates is clearly inspired by previous rodent research, these investigations are often not informed by the breadth and depth of knowledge of the OT system that resulted from rodent research. For example, OT seems to be viewed within the field of primate behavior as purely a prosocial peptide that can potentially affect any behavioral phenotype related social interaction, regardless of whether there is a neurobiological hypothesis supporting the idea. Rather than simply

using the fact that OT has an effect in rodents to justify the investigation of OT in primates, we must use the design of rodent studies to inform the design of behavioral paradigms in primates. For example, most of the rodent literature suggests that OT influences the process of social learning rather than the maintenance of social memories, yet primate research often assesses the influences of OT on maintenance of social memory or the facilitation of social behavior more broadly.

In the future, the design of NHP behavioral paradigms for the assessment of OT should take into account lessons from the rodent literature and be informed by hypotheses of functional receptor distributions, in order to move the field forward, and should also complement the studies of IN-OT in humans in order to better inform the potential mechanisms of those effects. Furthermore, rodent studies that show influences of OT on different social behaviors have been extensively replicated, but in the human and NHP literature, replications are rarely found. Thus, two important goals for the future of behavioral neuroendocrinology in primates should be to rigorously define behavioral phenotypes and to design behavioral paradigms that have translational relevance from the established rodent tasks. In addition, future work within this field would benefit from a small set of replicable behavioral tasks that can repeatedly be shown to be influenced by manipulations of neuropeptidergic systems.

Future directions: selective non-peptide ligands for behavioral neuropharmacology in primates

One obvious issue in translating rodent research to NHP is that many of the techniques described above involve invasive manipulations of the brain in large cohorts

of animals. While we are aware that ethical, cost, and animal welfare considerations may be prohibitive to the widespread use of invasive manipulations of the brains of NHP, we also feel that there is still great potential to gain important insights into the neurocircuitry of the OT system in circumstances when these neurosurgical techniques are available and appropriate. To close our discussion of the methodological possibilities in investigations of OT in NHP, we would like to highlight a few of the emerging candidate drugs for behavioral pharmacological manipulation of OXTR after peripheral delivery.

As we discussed above, there is still a critical need for the confirmation that IN-OT crosses the BBB in high enough volumes to accumulate in brain tissue and bind to available receptors. One paper has shown a significant elevation in CSF OT in two rhesus macaques after IN-OT administration compared to individuals given placebo (Chang et al. 2012), but further studies are needed to confirm the efficacy of varying doses of IN-OT. However, this issue is beginning to be somewhat superseded by the availability of several new compounds that can manipulate the central OT system after peripheral administration. The first are two compounds that cross the BBB and cause release of endogenous stores of OT in the brain: melanotan-II and PT141. These compounds are currently being evaluated in our lab and others for use in humans and NHP.

There are a few small-molecule, nonpeptide OXTR antagonists for OTR available that putatively cross the BBB after peripheral delivery. One of these compounds called L-368,899 even has excellent bioavailability after oral administration (Thompson et al. 1997) and has been also reported to accumulate in limbic structures of the brains of rhesus macaques (Boccia et al. 2007). However, after radiolabeling this ligand and performing *in vivo* PET imaging in macaques, there is minimal uptake in the brain, which

seems to be only in the ventricles and not in neural tissue (Smith, Freeman, Voll, Young, and Goodman 2013a). It is still possible that this drug may be capable to binding to OXTR in the brain after peripheral delivery, but in concentrations that are below the detectable limit of PET imaging. Our lab has also developed other novel, small-molecule, highly selective OXTR antagonists as tools for behavioral pharmacology in NHP as well as for potential tracers for *in vivo* PET neuroimaging (Smith et al. 2012; Smith, Freeman, Voll, Young, and Goodman 2013a; Smith, Freeman, Voll, Young, and Goodman 2013b). One of these compounds, ALS-II-69, was evaluated in this dissertation and shown to effectively block OXTR radioligand binding to primate tissue (Chapter 4). Behavioral pharmacology experiments are ongoing to assess the effectiveness of this ligand and others *in vivo* in altering social behavior in a rhesus macaque model (1P50MH100023-01 to LJ Young).

Thus, as investigations of OT in NHP continues in the future, the best way to advance our knowledge of OT neurophysiology is by using a combination of 1) behavioral pharmacology experiments with selective, small-molecule compounds that cross the BBB, 2) replicable, validated, and biologically informed behavioral paradigms, and 3) site-specific manipulations and measurements informed by central receptor distributions.

Mixed affinities of OT and AVP: relevance to future research

This dissertation has highlighted the importance of using selective ligands when probing the OXTR and AVPR1a systems in primates due to the high levels of homology between OT and AVP themselves, as well as their receptors. This issue brings to light

several important considerations for future work in these systems in both humans and NHP more broadly.

First, the mixed selectivity issue within the study of OT and AVP in primates suggests that many of the effects of OT could be mediated by AVPR1a, and vice versa. This concept is not new; several previous publications suggest that the effects of OT and AVP are due to activation of the "opposite" receptor (Ragnauth et al. 2004; Gupta et al. 2008; Schorscher-Petcu et al. 2010; Bales et al. 2007; Takayanagi et al. 2005; Song et al. 2013). Thus, with the growing number of IN-OT studies in humans, rhesus, and other primates, we need to consider the idea that the effects of intranasal OT may be mediated in part by AVPR1a. Now that several highly selective ligands have become available, such as SR49059 and ALS-II-69 highlighted by this dissertation, it is possible to use these ligands in combination with IN-OT in order to parse apart the contribution of OXTR and AVPR1a to the behavioral effects observed. By using selective antagonists in conjunction with IN-OT treatment, it becomes possible to determine the relative contribution of each of these receptors to OT-dependent changes in behavior. This issue is especially relevant with respect to the current drug development efforts to treat ASD with drugs targeting the OT system. If some of the beneficial prosocial effects of IN-OT are due to activation of AVPR1a, then drug development efforts should also include selective targets for AVPR1a.

Second, given the aforementioned mixed affinity issues, it is critical for researchers in this field to know the pharmacological properties of the ligands in their specific species of interest in order to conclusively determine the contribution of either of these receptors to behavior and physiology. Species differences in receptor binding

characteristics vary widely; simply comparing binding characteristics of many of the most common ligands used in this field for the human and rat OT and AVP receptors reveals considerable variation in pharmacological properties across species (Table 5.1).

Finally, we suggest that the nomenclature for the OT and AVP receptors should be augmented to reflect the mixed affinities of the endogenous hormones. By using the current nomenclature of "oxytocin receptor" and "vasopressin receptors", researchers are automatically biased to think that AVPR1a is activated only by AVP and that OXTR is activated only by OT. These biased perspectives about the function of these receptors may limit the design and interpretation of future experiments. Therefore, we recommend that OXTR, AVPR1a, and the other vasopressin receptors (vasopressin 1b receptor and vasopressin 2 receptor) should be referred to as neurohypophyseal peptide receptors, subtypes 1-4 (NHPR1-4).

Evolutionary perspectives on central OXTR distributions

OXTR in rodents is concentrated in brain regions involved in olfactory processing

Most of the elegant behavioral studies in rodents that were reviewed above and in Chapter 1 showed OT's involvement in various aspects of social interactions between individuals. In rodents, social interactions are driven mainly by olfactory investigations and chemical communication, such as by scent marking and anogenital sniffing. Identification of individuals as either kin, a familiar conspecific, or an unrelated potential mate of the opposite sex is based on olfactory processing of major urinary proteins (Brennan 2004). In laboratory experiments, duration of olfactory investigation is a common measurement in social interaction tests. For example, in the studies in mice that

Binding Affinities (nM) of various compounds for human and rat hypophyseal hormone receptors (K_i reported; if K_d reported, values are noted with an *; ND = not determined)

	hOTR	hV1aR	hV1bR	hV2R	rOTR	rV1aR	rV1bR	rV2R
AVP	1.7±0.5[a,o] 1.65±0.5[d] 1.6[h] 7.0±1.6[p] (native) 2.4±1.0[p] (cloned) 48±20[w]	1.1±0.1[a,o] 1.7[d,n] 1.73±0.08[m] 1.40±0.28[w]	0.68±0.01[a,o] 1.1[d] 1.1±0.05[m] 3.2[n] 0.8±0.25[w]	1.2±0.2[a,o] 1.1[d] 1.1±0.1[m] 0.4[n] 4.2±0.5[w]	1.7±0.4[a,h] 1.17±0.11[b]	2.6±0.1[a,h] 1.54±0.97[b]	3.3±0.5[a] 0.29±0.05[b,h]	0.45±0.03[a,h] 0.45±0.1[b,h]
[^{3}H]-AVP	3.4[w] *	0.27±0.06[k] *	0.43±0.06[k] *		1.7[c] *	0.6-3[c] *	1-3[c] *	0.4[c] *
OT	0.8[l,u] 0.21 ±0.08[e] 1.9±0.11[n,p] (native) 0.34±0.12[p] (cloned) 6.8±1.9[w]	120[l,u] 1.18±0.38[e] 64±12[m] 56[n] 34.9±7.7[w]	241±56[e] 1789±79[m] 251[n] 1782[u] 1016±322[w]	3500[l] 303±142[e] 167±12[m] 59[n] 1544[u] 6747±2298[w]	1.45* 0.44±0.23[e] 1.0±0.1[u]	56-78* 49±4[e] 845±99[u]	ND[e]	37±9[e]
[^{3}H]-OT	2[r] *				1.0-2.5[c,h] *	78[c] *	250[c] *	370[c] *
LVA	1.1[d]	0.8±0.08[d,m]	9.4±0.2[d,m]	282±2[d,m]				
[^{125}I]-LVA	0.59±0.45*	0.03±0.003*	ND[l]	ND[l]	1.4[c,v] *	0.18[c] * 0.06[v] *	92[c,v] *	62[c,v] *
OVTA	0.28±0.30[e] 0.59[g] 0.18[d,n]	1.39±2.36[e] 5.26[g] 3.9±0.5[d,m,n]	>1000[e] 10229±1439[d,m,n]	657±269[e] 929±19[d,m]	0.02[e] 0.17±0.03[f] 0.031±0.028[r] (iodinated OVTA)	27±9[e] 25.6±10.6[f] 13.6±5.6[r] (iodinated OVTA)	ND[e,f]	44±21[e] 15.1±1.1[f] 10.2±1.2[r] (iodinated OVTA)
[^{125}I]-OVTA	0.09±0.018[l] * 0.095±0.033[a] * 0.1[w] *	0.36±0.51[l] *	ND[l]	ND[l]	0.03[c,v] * 0.069±0.009[r] * (uterus) 0.073±0.038[r] * (hippocampus)	13.6[c,v] *	ND[c]	10.2[c,v] *
SR49059	130[l] (pregnant uterus) 340±96[w] 19.7[l]	6.3±0.6[l] (platelets) 1.1±0.2[l] (adrenals) 1.5±0.4[l] (nonpregnant uterus) 0.89±0.21[k] 1.06±0.06[m] 7.2±2.8[w] 1.68[l]	220±30[l] 218±91[k] 129±17[m] 238±8[w]	275±50[l] 119±8[m] 1216±146[w]	1080±115[j]	2.2±0.4[j]	ND[j]	ND[j]
Manning	24[n] 11.2[l]	1.59±0.03[m,n] 0.66[l]	359±14[m,n]	82±10[m,n]		0.28±0.02[t]		65.4±16.6[t]
[Thr^4,Gly^7]OT	6.62[n,u] 72.5[l]	78.8[n] 305±85.1[u] 85.5[l]	>10,000[u]	>10,000[u]	0.8±0.2[u]	>10,000[u]		
[Phe^2,Orn^8]VT	21.9[n]	2.1[n]	ND[n]	ND[n]				
dDAVP	203[u]	62.4[u]	5.8[u]	23.3[u]		100[u]	9.3[u]	0.3[u]
ALS-I-41	16[s]	8,800[s]	>10,000[s]	1,800[s]	2.9[s] (prairie vole brain)	9.4[s] (prairie vole brain)	ND[s]	ND[s]
ALS-II-69	3.41[l]	>2,400[l]	ND[l]	ND[l]				

[a] Derick et al. 2002
[b] Pena et al. 2007
[c] Schmidt et al. 1991
[d] Durroux et al. 1999
[e] Lemaire et al. 2002
[f] Manning et al. 1995a
[g] Manning et al. 2005
[h] Manning et al. 2008
[i] Terrillon et al. 2002
[j] Serradeil-Le Gal 2002
[k] Rodrigo et al. 2007
[l] Freeman unpublished
[m] Thibonnier et al. 1997
[n] Barberis et al. 1999
[o] Cheng et al. 2004
[p] Salvatore et al. 1998
[q] Chini et al. 1995
[r] Elands et al. 1988
[s] Smith et al. 2012
[t] Howl et al. 1993
[u] Chini et al. 2007
[v] Manning et al. 1993
[w] Akerlund et al. 1999

Table 5.1. Binding affinities (nM) of various OXTR and AVPR1a ligands for human and rat OT and AVP receptor subtypes. K_d reported; * indicates K_i

demonstrated the requirement of OT in the MeA for social memory of a familiar conspecific, social memory was measured by the habituation of time spend in olfactory exploration of the stimulus animal after repeated presentations (Ferguson et al. 2000; Ferguson et al. 2001).

Accordingly, the sites of action of OT in the rodent brain (i.e. the OXTR distribution) are most frequently found in areas of the olfactory pathway (Figure 5.1A,B). OXTR expression across rodent species is observed in the olfactory bulb (OB) and accessory olfactory nucleus (AOB), as well as downstream regions that receive projections from this primary sensory area and are involved in olfactory processing circuits, including the MeA, central amygdala (CeA), bed nucleus of stria terminalsi (BNST), the piriform cortex (Pir), the lateral septum (LS), and the hippocampus (Hipp). There are areas involved in the olfactory path that do not express OXTR in rodents, including the medial preoptic nucleus of the hypothalamus (MPOA), just as there are areas of OXTR expression that are not part of the olfactory pathway, like the NAcc and prefrontal cortex (PFC). Taken together, it is clear that areas that are involved in the processing of olfactory input and areas that are sensitive to OT are highly co-expressed in the rodent brain, providing an ecologically relevant neural circuit for the processing of social information.

OXTR in primates is concentrated in brain regions involved in visual processing

Across the animal kingdom, eyes are arguably one of the most salient stimuli in an animal's visual environment. Detecting and recognizing eyes immediately gives an individual a great deal of information, which in most cases needs to be interpreted and

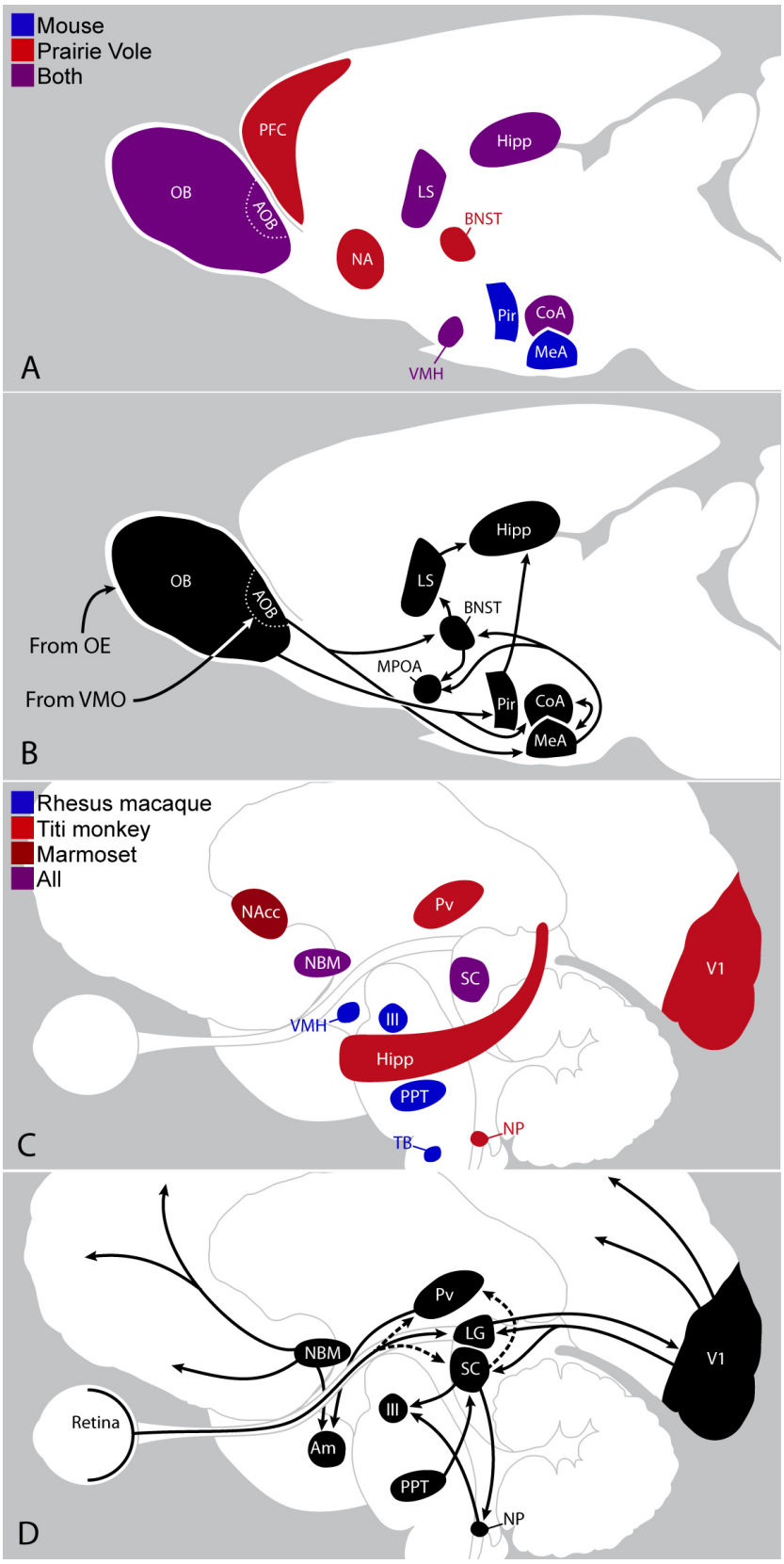

Figure 5.1. OXTR expression in rodent and primate brain areas that modulate attention to relevant social stimuli. Details on following page.

acted on quickly in order for an animal to survive. In primates, the eyes, along with the other components of the face, have developed important functions in social communication (Emery 2000). The location and orientation of the eyes in the face provides information about individual identity, and an individual's gaze direction can give clues to nearby conspecifics about potentially relevant stimuli in the environment, such as an incoming predator. Primates need to be able to quickly detect subtle shifts in gaze by nearby individuals—like a flash of eye contact from the dominant male—in order to adequately navigate social environments. Not only do primates need to be able to detect subtle changes in the eyes of others in order to gain important socially relevant information, but they also need to adjust their own gaze direction in response. This reciprocal relationship between the gaze of two individuals is arguably one of the most important aspects of primate social communication.

In this context, it is not surprising that there is considerable overlap in OXTR expression across primate species in areas that are important in visual processing, shifts in gaze direction, and the allocation of attention to visual stimuli (Figure 5.1C,D; Table

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Figure 5.3. OXTR expression in rodent and primate brain areas that modulate attention to relevant social stimuli.
(A) OXTR expression in select areas of the rodent brain (Insel et al. 1993; Beery, Lacey, and Francis 2008)
(B) The olfactory information pathway in the rodent brain, including regions involved in social recognition (Richter, Wolf, and Engelmann 2005; Cooke et al. 1998).
(C) OXTR expression in the nonhuman primate brain (Schorscher-Petcu, Dupré, and Tribollet 2009; Chapter 3, Chapter 4).
(D) The pathways in the primate brain that process visual input, modulate visual attention, and control eye movements (Hubel 1995; J. L. Labandeira-Garcia, Guerra-Seijas, and Labandeira-Garcia 1990; Lopez-Barneo, Ribas, and Delgado-Garcia 1981; Gandhi and Katnani 2011; Mesulam et al. 1983; Büttner-Ennever 2007; Ma, Graybiel, and Wurtz 1991; Balducci et al. 2003; Kobayashi et al. 2002; Okada and Kobayashi 2009; E. G. Jones et al. 1976)

5.2). In the primary visual path from the retina, OXTR has been detected in the superior colliculus (SC), pulvinar (Pv), and primary visual cortex. Interesting support for the involvement of these subcortical structures in the detection of another's gaze direction comes from evidence from cortically blind patients with damage to the visual cortex damage (Morris et al. 2001; Burra et al. 2013). Activity in the right amygdala was increased these patients in response to presentation of images of faces with direct gaze compared to averted gaze (Morris et al. 2001; Burra et al. 2013), and this activity co-varied in a condition-dependent way with activity in the SC and Pv (Morris et al. 2001). The Pv has also been implicated in the salience of visual stimuli (Robinson and Petersen 1992). In areas involved in saccadic eye movements in response to visual stimuli, OXTR is expressed in the superficial layer of the superior colliculus (SuG) as well as deeper layers, and in the oculomotor nucleus (III) and the nucleus prepositus (NP), which are two brainstem motor nuclei that control the muscles of the eye and participate in the horizontal gaze stabilization system, respectively (Büttner-Ennever 2007; McCrea and Horn 2006; Gandhi and Katnani 2011).

OXTR expression across primate species has also been found in two major cholinergic regions of the brain: the nucleus basalis of Meynert (NBM) and the pedunculopontine tegmental nucleus (PPT). These nuclei are major sources of cholinergic input to the rest of the brain (Mesulam et al. 1983; Garcia-Rill 1991; Jones et al. 1976) and are important regulators of selective attention and motivation (Demeter and Sarter 2013; Sarter, Gehring, and Kozak 2006; Balducci et al. 2003; Sarter, Givens, and Bruno 2001; Muir et al. 1993; Kobayashi et al. 2002). Furthermore, cholinergic input from the PPT strongly innervates the macaque SC (Ma, Graybiel, and Wurtz 1991), and

		Common Marmoset		Rhesus Macaque		Titi Monkey		Human	
		OXTR[1]	AVPR1a[1]	OXTR[2]	AVPR1a[3]	OXTR[4]	AVPR1a[4]	OXTR[5,6]	AVPR1a[5,6]
Thalamic	LG	-	-	-	-	-	Y	-	-
	Pv	-	-	-	-	Y	Y	-	-
Subcortical	SC	-	?	-	-	Y	Y	Y	-
	SuG	Y	?	Y	-	-	Y	-	-
	Pt/NL	Y	-	-	-	-	Y	ND	ND
Cortical	V1	ND	ND	-	Y	Y	Y	ND	ND
	V2	ND	ND	-	Y	-	Y	ND	ND
	Occ	-	Y	-	Y	-	Y	ND	ND
Visuomotor	III	-	-	Y	ND	-	-	ND	ND
	NP	?	-	ND	ND	Y	-	Y	-
	PSB	-	-	-	Y	Y	Y	-	-
Attentional	NBM	Y	Y	Y	-	Y	-	Y	-
	PPT	-	?	Y	ND	-	-	ND	ND
	CeA	-	-	-	Y	-	Y	-	-

Table 5.2. Comparison of OXTR and AVPR1a binding sites across primate species, only in areas of the brain that process visual input, control visuomotor responses (eye/head direction), and/or mediate visual attention.

Y binding detected.
– no binding detected.
ND binding not determined in this region.
?, binding detected in that region, but the authors did not label or discuss it.

[1] Schorscher-Petcu et al. 2009.
[2] Chapter 3.
[3] Young et al. 1999.
[4] Chapter 4.
[5] Loup et al. 1989.
[6] Loup et al. 1991.

Abbreviations: LG, lateral geniculate nucleus; Pv, pulivinar; SC, superior colliculus; SuG, superficial gray layer of the superior colliculus; Pt, pretectum; NL, nucleus limitans, V1, primary visual cortex; V2, secondary visual cortex; Occ, occipital lobe; III, oculomotor nucleus; NP, nucleus prepositus; PSB, presubiculum; NBM, nucleus basalis of Meynert; PPT, pedunculopontine tegmental nucleus; CeA, central amygdala

neurons in the PPT have also been shown to be active in monkeys during saccade tasks (Okada and Kobayashi 2009) and to directly stimulate SC neurons and facilitate the initiation of saccadic eye movements (Kobayashi, Saito, and Isa 2001). Furthermore, NBM projects to the amygdala and is the primary source of cholinergic input to the basolateral amygdala (Mesulam et al. 1983; Jones et al. 1976; Woolf and Butcher 1982; Nagai et al. 1982). This cholinergic input is required for memory consolidation (Power, Vazdarjanova, and McGaugh 2003; Power and McGaugh 2002), possibly promoting the encoding of memory during sustained attention to visual stimuli. Thus, it is possible that these OT-sensitive cholinergic areas could be mediating some aspects of the changes in eye movements and shifts in visual attention in response to changing social cues in the environment.

Conclusions and future directions

Informing the design of experiments in NHP

The existence of OXTR in brain regions involved in visual attention and eye movements has several implications for future studies. First, the behavioral paradigms used to investigate the effects of OT in NHP should be designed to test for attention to social stimuli. We recommend that researchers in this field review the established paradigms from the literature on the neurobiology of attention and modify those existing paradigms to incorporate social elements. By preserving the overall parameters of these tasks, future research on the effects of OT will also benefit from consistency and replicability with studies from the well-established field of the neurobiology of attention. Similarly, we suggest that future studies of the OT system in NHP borrow techniques

from the field of visual neuroscience, such as eye tracking, in order to incorporate fine measurements of subtle changes in eye movements and/or head direction in social viewing tasks. Furthermore, implementing electrophysiological recordings in relevant brain regions during behavioral tasks can contribute to a more complete understanding of the circuitry underlying complex social behavior.

The expression of OXTR in two of the main cholinergic areas of the brain indicates a potential involvement of acetylcholine in the regulation of social behavior in primates. Manipulations of the muscarinic cholinergic receptor or nicotinic cholinergic receptor have been suggested as a way to enhance attention (Demeter and Sarter 2013). Even the authors of the human OXTR mapping paper from 1991, who first detected OXTR binding in the NBM, suggested that OT binding sites on cholinergic neurons in this area may manipulate cholinergic transmission to downstream regions like the cerebral cortex or hippocampus (Loup et al. 1991). Investigations into the possible functional connection between OT and acetylcholine would be an exciting new direction for the study of social behavior and neurophysiology more broadly.

Clinical relevance of this dissertation

It has been shown that infants, toddlers, and adolescents with ASD all show disrupted patterns of eye movements in response to images/videos of human faces or naturalistic social scenes, including avoidance of the eye region of faces, atypical fixation targets to the nonsocial aspects of social scenes, and a lack of attentional bias toward faces, as measured by faster saccadic disengagement from images of faces (Rice et al. 2012; Chawarska, Volkmar, and Klin 2010; Jones, Carr, and Klin 2008; Klin et al. 2002).

More severe disruptions in eye movements and social visual attention in ASD patients correlate with intensity of social dysfunction (Jones, Carr, and Klin 2008; Klin et al. 2002). Thus it has been hypothesized that intervention at an early age in these patients to increase gaze to faces may be able to ameliorate later life social outcomes. Indeed, IN-OT has been shown to increase gaze to the eye region of pictures of human faces in autistic populations (Andari et al. 2010), although long term studies are still needed. The distribution of OXTR in the brains of ASD patients is not known, and although the central OXTR distribution in macaques may or may not be predictive of that of humans, evidence is building from behavioral studies in both humans and nonhuman primates that IN-OT is capable of altering social visual attention. Perhaps this action of OT is due to modulation of areas such as the nucleus basalis of Meynert or the superior colliculus. Basic and clinical research is needed (and currently ongoing, 1P50MH100023-01 to LJ Young) to unwind the neural underpinnings of OT's effects in humans and nonhuman primates alike.

The research put forth in this dissertation is situated in an ongoing, decades-long body of work on the neurochemical basis of complex social behavior. The results of this work should inform future studies in NHP and ultimately facilitate the development of optimal pharmacological strategies targeting the OT system for improving social function in psychiatric disorders such as autism spectrum disorder.

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APPENDIX: LIST OF CO-AUTHORED PUBLICATIONS

Aaron L. Smith, **Sara M. Freeman**, Ronald J. Voll, Larry J. Young, Mark M. Goodman**.** Investigation of an F-18 Oxytocin Receptor Selective Ligand via PET Imaging. *Bioorganic and Medicinal Chemstry Letters*. doi: http://dx.doi.org/10.1016/j.bmcl.2013.07.045.

Aaron L. Smith, **Sara M. Freeman**, Ronald J. Voll, Larry J. Young, Mark M. Goodman. Carbon-11 *N*-methyl alkylation of L-368,899 and *in vivo* PET imaging investigations for neural oxytocin receptors. *Bioorganic and Medicinal Chemistry*. 2012 Oct 29. doi: http://dx.doi.org/10.1016/j.bmcl.2012.10.116.

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