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Lanikea B. King Date

Genetic Diversity in Oxytocin Receptor Sequence, Neural Expression, and Social Behavior

By

Lanikea B. King

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Neuroscience

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Genetic Diversity in Oxytocin Receptor Sequence, Neural Expression, and Social Behavior

By

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B.A., University of Hawai'i at Manoa, 2008

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ABSTRACT

Oxytocin (OXT) is a neuropeptide with conserved functions in reproductive physiology and social cognition through signalling with a single receptor, OXTR. OXTR expression occurs in diverse distributions within the brain and influences species-typical behavior. The socially monogamous prairie vole has high OXTR density in the nucleus accumbens (NAcc) compared to non-monogamous vole species. OXT and dopamine signalling converge in the prairie vole NAcc, enabling social attachments to distinct individuals. OXTR diversity in the NAcc also contributes to individual variation in prairie vole social behavior. Thus, regulatory elements (*cis*-REs) that influence transcription of the *oxtr* gene or synthesis of OXTR must be susceptible to variation. This dissertation examines the hypothesis that transcription of the prairie vole *oxtr* is modulated by polymorphic *cis*-REs. I found that a molecular signature of polymorphic *cis*-RE activity, allelic imbalance, occurs robustly and specifically in the NAcc. The single nucleotide polymorphism (SNP) marker used for allelic imbalance, NT204321, predicts OXTR density in the NAcc. NT204321 genotype groups also exhibit differences in mRNA levels in the NAcc and mRNA and OXTR binding are correlated in the NAcc. NT204321 also predicts individual differences in prairie vole social attachment. I next attempted to identify putative locations of *cis*-REs using next-generation sequencing and found approximately 3,000 SNPs within the *oxtr* locus. I discovered that SNPs in the prairie vole *oxtr* are in strong linkage disequilibrium (LD) with each other. One of these SNPs, which is located in the single intron, outperforms NT204321 as a marker of NAcc OXTR density, explaining 75% or more density variation in three separate replicates. Future studies using the intron SNP will investigate gene by environment interactions and probe more deeply into the mechanisms influence *oxtr* regulation.

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CHAPTER 1

General Introduction

Adapted from:

King, L. B. and Young, L. J. (2015) 'Oxytocin, Vasopressin and Diversity in Social Behavior', in Murphy, D. & Gainer, H. (eds.) Molecular Neuroendocrinology: "From Genome to Physiology"

Introduction

Neuroendocrine factors play a critical role in regulating many aspects of social behaviors, from individual recognition of conspecifics to the formation of social bonds. There is a remarkable diversity in social behavioral phenotype across vertebrate species, even among closely related species. Two neuropeptides, oxytocin (OXT) and arginine vasopressin (AVP), or their non-mammalian homologues (Figure 1.1), have been studied most intensively for their role in modulating social cognition and sociosexual behaviors. In this chapter I focus on OXT and AVP, collectively known as neurohypophyseal peptides, and their receptors (NHPRs), and examine how they modulate a rich diversity of social behavior in often a species-specific manner. Following a brief overview of the roles of OXT and AVP in regulating social behavior, I explore the evolution of the OXT and AVP family of neuropeptides and highlight the evolutionarily conserved role of these peptides in regulating social behavior. While some aspects of the OXT and AVP systems are evolutionarily very ancient and well conserved, there is a remarkable evolutionary plasticity in the receptors for these peptides in terms of their neural expression. I consider this plasticity in NHPR expression to be critical to diversity in social behaviors across and within species. Detailed knowledge of NHPR diversity comes from elegant research in monogamous prairie voles. A section on voles illustrates the neural mechanisms by which OXT and AVP regulate behaviors associated with monogamy. The chapter concludes with a highlight of recent research in humans demonstrating remarkable parallels between the roles of these peptides in animals and in our own species.

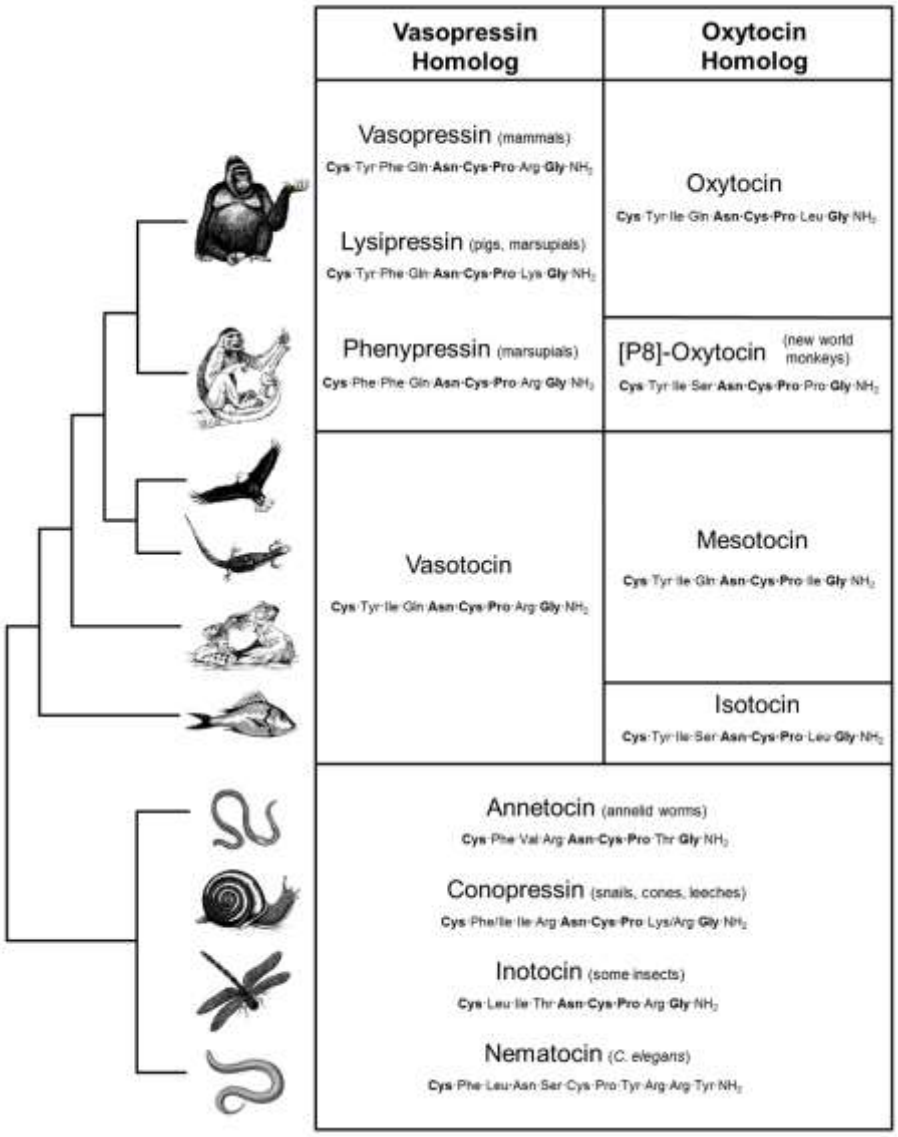

Figure 1.1. Oxytocin and neuropeptide homologs across the animal kingdom. The ancestral peptide gene was duplicated before the last common vertebrate ancestor; most extant vertebrates have two neuropeptides one oxytocin and one vasopressin homolog.

Oxytocin, vasopressin and social behavior

Oxytocin is best known for its role in initiating labor and delivery through regulating uterine contractions, and stimulating milk letdown during nursing. But mammalian mothers do more than deliver and provide sustenance to their offspring, they provide nurturing care, and in some cases develop mother-infant bonds. This caring in parturient mothers contrasts with virgin females in many

species, which ignore or attack infants. OXT guides this shift in behavior. Central OXT injections facilitate onset of maternal behavior while antagonists for the OXT receptor (OXTR) delay onset in rats (Numan and Young, 2015). Furthermore, *Oxtr* knockout (KO) mice are impaired in maternal retrieval and care (Takayanagi et al., 2005). Dams also aggressively defend offspring against intruders. OXT, as well as AVP, regulate maternal aggression in rodents (Bosch and Neumann, 2012).

Rats and mice are promiscuously maternal. In ungulates however, mothers live in herds and deliver offspring that are immediately mobile. Thus mothers not only have to become motivated to care for infants, but they must selectively care for their own young. To achieve this, ungulates, like sheep, develop a strong mother-infant bond. OXT is released during labor in the mother's brain, and infusions of OXT into the brain of steroid primed ewe lead to the development of a bond between the female and a novel lamb, through modulation of olfactory processing (Kendrick et al., 1988, Kendrick et al., 1986, Kendrick et al., 1987). These studies and many others in animal models demonstrate that the same molecule that regulates the peripheral physiology of reproduction also coordinates the onset of maternal responsiveness and bonding (Ross and Young, 2009).

Although best known for a role in female reproduction, OXT plays a more general role in social cognition, as revealed by more recent studies in knockout mice. Mice distinguish each other by smell, a process referred to as social recognition. Male *Oxt* KO mice fail to recognize mice that they have previously encountered (Ferguson et al., 2000, Ferguson et al., 2001, Choleris et al., 2007). However, a central infusion of OXT restores social recognition abilities. Thus, OXT plays a critical role in the neural processing of social information, which is

key to many aspects of social relationships, including mother-infant bonding and as we will see, pair bonding (Donaldson and Young, 2008, Ross and Young, 2009). Indeed, one of the fundamental processes by which OXT is thought to modulate social behavior is by enhancing the salience of social cues, which in rodents is primarily olfactory (Young, 2015). OXT also appears to mediate the rewarding aspects of social interactions though its interaction with the serotonin system in mice (Dolen et al., 2013). It is likely that these two fundamental processes, social information processing and social reward, contribute significantly to many of the more complex behavioral roles of OXT.

Vasopressin was named for its constricting effects of the vascular system. AVP is also referred to as antidiuretic hormone because of its role in regulating water retention in the kidney. Like OXT, AVP also modulates many aspects of social behavior, particularly male-typical social behaviors. For instance, two of the first behavioral effects of central AVP to be described were scent marking behavior and territorial aggression in hamsters (Ferris et al., 1984). Why did AVP evolve a role in regulating territorial behaviors? One intriguing possibility is that its role in regulating water balance, and hence urine production, became linked behaviorally and neurobiologically to scent marking of territory, which many species achieve through urination (Freeman and Young, 2013).

Neurohypophyseal peptide gene structure, evolution and conserved function

The genes encoding OXT and AVP share a common ancestral sequence, which underwent a duplication yielding the two new genes just after the emergence of the vertebrate lineage (Gimpl and Fahrenholz, 2001). This original

peptide is estimated to be at least 700 million years old (Donaldson and Young, 2008) and neuropeptide homologs of OXT and AVP have been discovered in distantly related invertebrates, including cephalopods, insects and nematodes (Gruber, 2014, Koehbach et al., 2013). All peptides in the OXT/AVP family share a cyclical structure and most are nine-amino acid peptides, with some examples of length variants, like nematocin (Venkatesh et al., 1997, Gilligan et al., 2003, Beets et al., 2012) (Figure 1.1). In vertebrates, the *Oxt* and *Avp* genes are adjacent to one another and transcribed towards each other. Each of the genes encode a prohormone that is cleaved by a protease to yield the OXT or AVP peptide, neurophysin, and other peptide products (Figure 1.2).

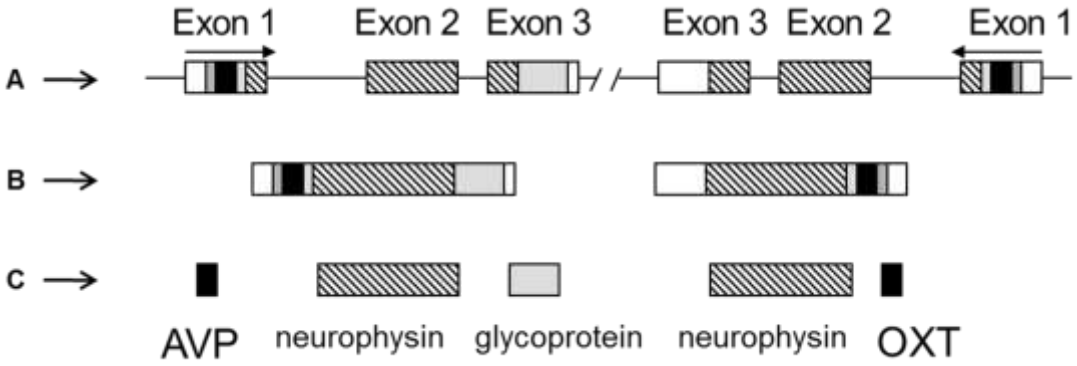

Figure 1.2. Oxytocin and vasopressin genes: structure and processing. (**A**) Gene structure in the DNA, (**B**) preprohormone mRNA, (**C**) final processed protein products including oxytocin and vasopressin. On line A, boxed regions indicate the locations of the exons. Shaded or hatched regions indicate coding regions. Vasopressin (AVP) and oxytocin (OXT) neurohypophyseal peptides are shown in black.

The general physiological functions of the OXT/AVP family are conserved from invertebrates to vertebrates. Invertebrate homologues stimulate egg laying behavior in leeches and earthworms, analogous to the regulation of parturition by OXT in mammals, and activate water balance reflexes in sea squirts, flour beetles, locusts and leeches, analogous to the antidiuretic properties of AVP homologs in vertebrates. The involvement in regulating sociosexual behaviors

emerged early in invertebrate evolution as well. Neurohypophyseal peptides activate stereotypical mating behaviors in leeches and snails (van Kesteren et al., 1995b, Van Kesteren et al., 1995a, Wagenaar et al., 2010). In *C. elegans*, nematocin plays a role in sensory processing involved in mating, suggesting that very early in evolution this peptide family became involved in regulating social interactions and social information processing (Gruber, 2014, Garrison et al., 2012).

Conservation in neural expression

In mammals, OXT and AVP are primarily synthesized by neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, although other populations can be found. OXT and AVP neurons project primarily, but not exclusively as we shall see, to the posterior pituitary, where they secrete peptide into the blood for distribution to peripheral organs. Across taxa of the animal kingdom there is an extraordinary conservation in the distribution or nature of neurohypophyseal peptide producing neurons (Knobloch and Grinevich, 2014).

The phylogenetic stability of the neuropeptide system appears to stem from a conservation of cell-type specific regulatory factors in the neurosecretory cells producing the peptide and of the *cis*-regulatory element s (*cis*-REs) surrounding the gene. In a detailed comparison between zebrafish (*Danio rerio*) and an annelid nereidid (*Platynereis dumerilii*), Tessmar-Raible and colleagues show that neurosecretory cells expressing AVP homologues in both species share common transcription factors, micro-RNA and embryonic migratory patterns (Tessmar-Raible et al., 2007). Even in the annelid, these neuroendocrine cells share anatomical features with the vertebrate hypothalamus such as ease of

access to the vasculature (de Velasco et al., 2007, Tessmar-Raible et al., 2007, Knobloch and Grinevich, 2014) and as a site of information integration (Goodson and Bass, 2000, Herman et al., 2003, Zhu et al., 2006).

In vertebrates, the transcriptional regulation of neurohypophyseal peptide genes is remarkably conserved across species. In a series of experiments, Venkatesh, Murphy and colleagues isolated the gene encoding the pufferfish (*Fugu rubripes*) homologue of OXT, isotocin, and generated transgenic rodents that expressed both the endogenous rodent and transgenic Fugu peptides (Venkatesh et al., 1997, Gilligan et al., 2003). In both rats and mice, the Fugu isotocin gene was expressed specifically within OXT containing neurons in the hypothalamus. In addition, the isotocin gene responded to increased salt concentrations at the same rate as OXT. This work remarkably demonstrates that a 5 kb region of the Fugu isotocin gene contains sufficient information for the gene to not only express in the correct secretory cells, but to respond to an environmental signal in an appropriate manner in a rodent as well. Similar results were seen for AVP and its fish homolog, vasotocin.

Oxytocin and vasopressin in the vertebrate brain

The best characterized secretory OXT neurons are the hypothalamic magnocellular neurons of the PVN, supraoptic nucleus SON and accessory nuclei (AN) that lies between PVN and SON (Knobloch and Grinevich, 2014, Ross and Young, 2009). These neurons project axons to the posterior pituitary, where they release OXT into circulation. The magnocellular neurons are also the sites of colocalization between rodent OXT and the fish isotocin discussed above and thus represent the most highly conserved population of OXT expressing cells. Parvocellular neurons in the PVN project primarily to the hind brain and brain

stem (Knobloch and Grinevich, 2014). Magnocellular neurons also provide OXT innervation to the forebrain. It is likely that these forebrain projections of magnocellular neurons are the major source of OXT regulating social behavior. These neurons release OXT both peripherally and centrally in response to vaginocervical stimulation during parturition and mating and to nipple stimulation during nursing. Oxytocin expression is not sexually dimorphic under normal physiological conditions, in contrast to AVP expression.

Like OXT, AVP is also synthesized in magnocellular neurons in the PVN and SON as well as parvocellular neurons in the PVN, although largely in separate neurons from OXT. The magnocellular neurons project to and release AVP from the posterior pituitary into the bloodstream in response to osmotic or blood pressure variation. In addition, AVP is expressed in the suprachiasmatic nucleus of the hypothalamus, where it may play a role in circadian rhythms. However, it is small parvocellular neurons in the medial amygdala and bed nucleus of the stria terminalis that project to forebrain regions and are likely to be the source of behaviorally relevant AVP in the vertebrate brain. The expression of AVP in these neurons is androgen dependent, and consequently sexually dimorphic. Males have more AVP producing neurons in these areas and more dense AVP fibers in projection sites, including the lateral septum and ventral pallidum (de Vries, 2008, Kelly and Goodson, 2014, Lim et al., 2004b).

OXT and AVP receptors

In mammals, there are four neurohypophyseal peptide receptors; a single OXT receptor (OXTR) and three subtypes of AVP receptor (AVPR1A, AVPR1B and AVPR2). OXTR is expressed in the brain and in the periphery. AVPR2 is expressed in the kidney and regulates the antidiuretic properties of AVP.

AVPR1B is expressed in the anterior pituitary and restricted brain areas. However, AVPR1A is widely expressed in the forebrain and is the receptor most often linked to the regulation of social behavior (Donaldson and Young, 2008). It should be noted that OXT can bind to AVPRs and AVP can bind to OXTR, as would be expected from their structural similarity.

In stark contrast to the conservation in peptide expression across vertebrates, the brain expression patterns of OXTR and AVPR1A vary remarkably across species. Figure 1.3 illustrates such a pattern for OXTR between rats, mice and prairie voles. Even closely related species within a genus exhibit this neural diversity and demonstrate that it may be linked to species-specific behaviors. Furthermore, individual variation in brain OXTR and AVPR1A distribution within a species has been associated with variation in social behavior. This diversity in neural expression patterns is a hallmark feature of OXTR and AVPR1A that clearly sets them apart from other receptor systems, including sex steroid receptors and neurotransmitter systems such as dopamine. In contrast to the receptor differences, there are few species differences in brain peptide projections. It appears that OXT or AVP can be released locally in brain regions and then diffuse to the nearby receptor expressing neurons. This paracrine type of transmission allows for the evolution of receptor distribution without necessitating coevolution of neuropeptide producing neurons.

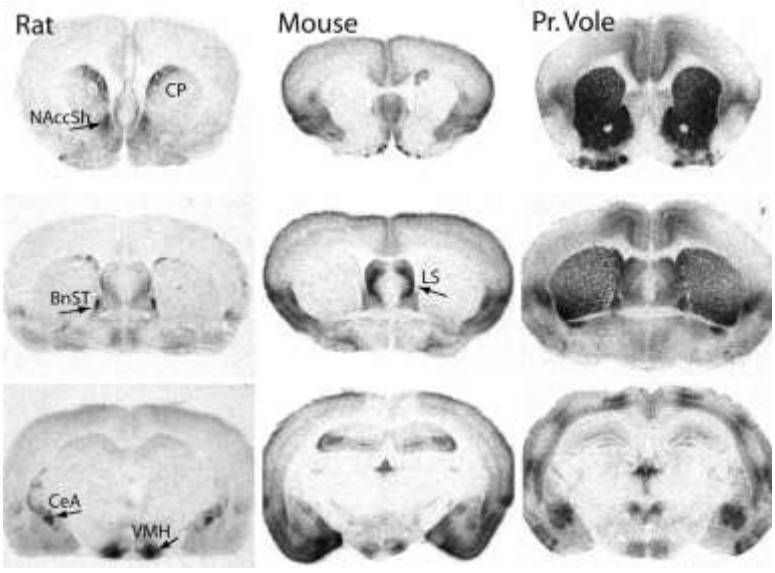

Figure 1.3. Receptor autoradiograms illustrating the distribution of OXT receptor binding sites in the forebrain of rat, mouse and prairie vole. Note that while thedistribution of receptors are conserved in some brain regions, such as the lateral septum (LS), bed nucleus of the stria terminalis (BnST), central nucleus of theamygdala (CeA) and the ventromedial nucleus of hypothalamus (VMH), several striking species differences are apparent. For example, in the rat striatum, OXT receptors are restricted to the dorsal caudate putamen (CP) and the shell of the nucleus accumbens (NAccSh), while OT receptors are abundant throughout the prairie vole striatum, and absent in the mouse striatum.

Prairie voles and pair bonding

Prairie voles (*Microtus ochrogaster*) are one of the 3-5% of mammal species that share a monogamous reproductive strategy (Carter and Getz, 1993, Kleiman, 1977) (Figure 1.4). In the wild, prairie voles form a robust pair bond, establishing an affiliative attachment that generally lasts over the lifetime of the animals (Carter and Getz, 1993). Prairie vole males and females sometimes engage in extra-pair copulations, making them socially rather than genetically monogamous (Ophir et al., 2008, Solomon et al., 2009). In addition to this bonding behavior, prairie voles live in philopatric family units where offspring delay dispersal from the nest (McGuire, 1993). Male and female prairie voles share parental care responsibilities and older pups alloparentally care for

younger siblings as well. Both sexes develop selective aggression towards unfamiliar conspecifics after bonding. These species-specific behaviors are generated by a brain hardwired for affiliation and attachment towards the family unit and aggression towards conspecifics outside of it.

Prairie voles offer a distinct advantage toward understanding neuroendocrine and other networks underlying their unique social behaviors because closely-related vole species are not monogamous, allowing comparative opportunities. Prairie voles belong to the rodent genus *Microtus*, which is highly differentiated taxonomically and has higher rates of genome evolution than other mammal genera (Triant and Dewoody, 2006). Despite these signs of diversity, vole species are often nearly indistinguishable by appearance alone. Social behavior is a phenotype where vole species do exhibit stronger diversity however. Monogamous behaviors evolved at least twice within the *Microtus* lineage and monogamous species are more closely related to non-monogamous species than to each other (Fink et al., 2006). Comparative genetic and neurobiological studies using the prairie, meadow (*Microtus pennsylvancius*) and montane voles (*Microtus montanus*) have provided insights into the neurobiology and evolution of social organization.

Figure 1.4. The monogamous prairie vole. Prairie voles form lifelong pair bonds after mating and develop an affiliative attachment with each other and selective aggression against unfamiliar voles. Prairie vole families can be philopatric, with multiple generations living in a single burrow, where males and older pups assist in caring for pups.

OXT and AVP influence pair bonding

In laboratory settings, the occurrence of a prairie vole pair bond is measured using the partner preference test and a test for selective aggression toward novel conspecifics. Before testing, adult subjects are cohabitated with members of the opposite sex. Cohabitation time can be varied depending on the needs of the experiment, with shorter cohabitations used when testing a hypothesis about enhancement of pair bond formation and longer cohabitations used when testing a hypothesis focused on blocking pair bond formation. Following the cohabitation period, subjects can be tested for pair bond formation.

The partner preference test uses a three-chamber apparatus, with two outer chambers containing a partner and a stranger stimulus vole, each tethered in opposite chambers to restrict their movement to a minimal area. The partner is the animal with which the subject was previously cohabitated while the stranger is a novel vole sharing the sex and reproductive experience of the

partner. The subject is allowed to freely roam the apparatus for three hours. The primary measure is immobile huddling next to either stimulus animal, and automated video analysis systems now allows for automatic scoring of partner preference test. Prairie voles of both sexes spend the majority of the test in social contact, while meadow voles spend most of the time in the empty chamber. A partner preference is considered to have formed when voles spend at least twice as much time huddling next to the partner compared to the stranger. Mating facilitates pair bonding, but partner preferences can develop in the absence of mating with extended cohabitation periods. A 24 hour cohabitation with mating is generally sufficient to stimulate a significant partner preference in males (Winslow et al., 1993). Females require shorter cohabitation before forming a preference (Williams et al., 1992). However, these parameters can vary and care should be taken to optimize them.

Pair bond formation also causes an increase in aggressive behaviors targeted at novel animals, particularly in males (Winslow et al., 1993), which is likely reflective of mate-guarding behavior. Thus pair bonding in males has an element reminiscent of territoriality. Both of these tests capture a unique aspect of the changes in prairie vole social behavior as a consequence of pair bonding. Many experiments using one or the other of these tests have revealed important biological factors for pair bonding in laboratory experiments.

Both OXT and AVP play critical roles in prairie vole pair bonding. Central injection of either peptides into the brain results in facilitated pair bonding during a brief cohabitation in the absence of mating (Williams et al., 1994, Winslow et al., 1993, Cho et al., 1999, Insel and Hulihan, 1995). Conversely, blocking endogenous OXT and AVP signalling using central infusions of antagonists for OXTR and AVRP1A prevent prairie vole pair bond formation.

Both peptides influence males and female bonding (Ophir et al., 2012, Zheng et al., 2013), although most studies suggest that OXT plays a more important role in females, while AVP plays a more important role in males.

Species differences in social behavior are patterned by receptor diversity

As mentioned, prairie voles are particularly useful as a model for studying social behavior due to the availability of non-monogamous *Microtus* species for comparative purposes. Indeed, when viewing anatomical data for OXTR and AVRP1A, the brains of different *Microtus* species appear very different. Relative to montane and meadow voles, prairie voles exhibit high OXTR density differences in several brain regions, particularly the nucleus accumbens (NAcc) (Figure 1.5A). OXTR signalling in both the NAcc and prefrontal cortex (PFC) is required for prairie vole pair bonding. Injections of an OXTR antagonist into either region in females prevent mating-induced partner preference formation (Figure 1.5C). Both of these regions are part of the mesolimbic reward pathway (MLR) and receive intensive dopaminergic projections from the ventral tegmental area (VTA) (Lim et al., 2004b). OXT signalling is also required in the prairie vole NAcc for alloparental behavior in adult virgin females, a behavior relevant to the unique prairie vole social structure (Ross and Young, 2009).

AVRP1A distributions also differ between vole species. Compared to montane and meadow voles, prairie voles express AVRP1A differentially in the ventral pallidum (VP) and other regions (Insel et al., 1994, Young et al., 1997b, Wang et al., 1997, Lim et al., 2004c) (Figure 1.5B). The VP is also a key region in the MLR pathway, serving as an output nucleus for the NAcc. Again, the species-specific expression of AVRP1A revealed a key region for AVP mediated

pair bonding. Injection of an AVRP1A antagonist into the VP, but not medial amygdala or thalamus, blocks partner preference in male prairie voles (Figure 1.5D). Remarkably, elevating AVRP1A density in the VP of meadow voles to levels resembling prairie voles using viral vector gene transfer allows the normally promiscuous species to form a partner preference (Lim et al., 2004c).

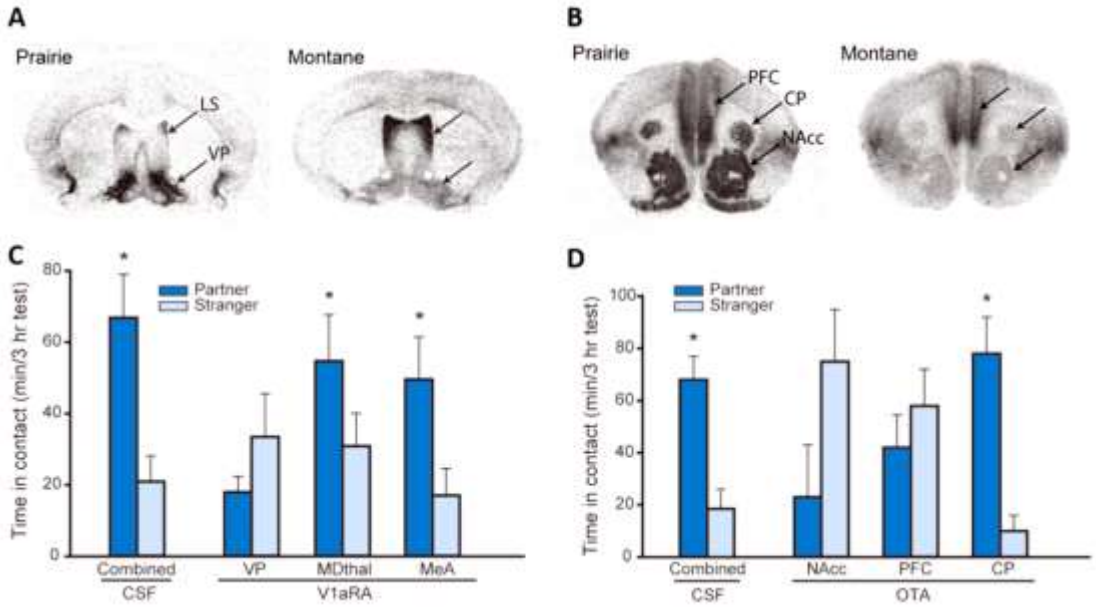

Figure 1.5. Receptor diversity reveals brain regions underpinning species specific social behavior. Autoradiograms of coronal brain sections reveal binding densities of oxytocin receptor (**A**) and vasopressin receptor 1a (**B**). Relative to montane voles, monogamous prairie voles express oxytocin receptor at higher density in the nucleus accumbens (NAcc) and caudate putamen (CP) (**A**) and vasopressin receptor 1a at higher density in the ventral pallidum (VP) (**B**). Infusions of oxytocin antagonist into the NAcc or prefrontal cortex (PFC) but not CP prevented partner preference in female prairie voles (**C**). Infusions of vasopressin 1a antagonist into the VP but not mediodorsal thalamus (MDThal) or medial amygdala (MeA) prevented blocked partner preference in male prairie voles (**D**). LS, lateral septum; CSF, cerebrospinal fluid; OTA, oxytocin receptor antagonist; V1aRA, vasopressin receptor 1a antagonist.

Primate species have a higher rate of social monogamy than other mammals (Lukas and Clutton-Brock, 2013). A general pattern first determined in voles is repeated in the primates: monogamous species express OXTR or AVRP1A in MLR regions. A monogamous titi monkey (*Callicebus cupreus*)

expresses AVRP1A but not OXTR in the NAcc (Freeman et al., 2014b). Another monogamous primate, the common marmoset (*Callithrix jaccus*), expresses OXTR and AVRP1A in the NAcc and VP, respectively (Schorscher-Petcu et al., 2009). The rhesus macaque (*Macaca mulatta*), which is not monogamous, does not express either receptor in the NAcc or VP. It is of interest to note that all primates analyzed so far express *OXTR* in brains regions involved in visual processing and attention such as the superior colliculus and nucleus basalis of Meynert, as well as auditory processing regions. Freeman et al. hypothesize this pattern may be adaptive for primates, allowing OXT to directly modulate brain regions involved in visual and auditory processing, sensory modalities clearly more important to primates than rodents (Freeman et al., 2014b).

Bird species also exhibit quantitative diversity in neuropeptide expression that has been linked to social behavior. After the suggestion of Kelly and Goodson to maintain simplicity in nomenclature, homologs of OXTR and AVRP1A, such as the closest homolog of OXTR in birds, the vasotocin 3 receptor, will be referred to using the mammalian nomenclature (Kelly and Goodson, 2014, Leung et al., 2011). Birds are more commonly monogamous than mammals but they also exhibit a diverse range of preferences for sociality, which leads to flocking in some species. A site of particular interest for bird sociality is the lateral septum (LS). In a survey of territorial and gregarious species, a consistent pattern emerges. Gregarious species, such as the zebra finch (*Taeniopygia guttata*), express *OXTR* in the dorsal portions of the LS. More isolated territorial species on the other hand lack dorsal LS OXTR but may express some receptor in the ventral LS. This socially segregated distribution is indeed functional. Injection of an OXTR antagonist into the LS of female zebra finches lowers sociality preference (Goodson, 2013, Goodson et al., 2012).

Although considerable diversity in density and regional distribution is found across species, the LS as a whole has a conserved presence of both OXTR and AVRP1A expression among vertebrates and researchers are now generating theories on general roles the region may play in various social behaviors (Kelly and Goodson, 2014).

Social behavior is also influenced by other neuromodulator and transmitter systems (Figure 1.6). For example, pair bonding requires dopamine (Aragona et al., 2006, Wang et al., 1999, Gingrich et al., 2000, Liu and Wang, 2003), endogenous opioids (Burkett et al., 2011, Resendez et al., 2013, Resendez et al., 2012) and other neuropeptides (Lim et al., 2007b, DeVries et al., 2002). In the case of dopamine, concurrent OXT and D2 receptor activation is required in the NAcc (Burkett and Young, 2012). This result enables a more elegant understanding of pair bonding. During copulation, dopamine and OXT are both released in females. D2 receptors mediate reinforcing properties of the experience while OXTR increases the salience of partner associated cues. AVP and dopamine interact in a similar fashion in males. This likely results in strengthening of connections between neural circuits encoding the olfactory cues of the partner with circuits encoding reward. Thus, pair bonding may occur in prairie voles as a more general conditioned partner preference through reliance on a species-specific OXTR or AVPR1A mechanism.

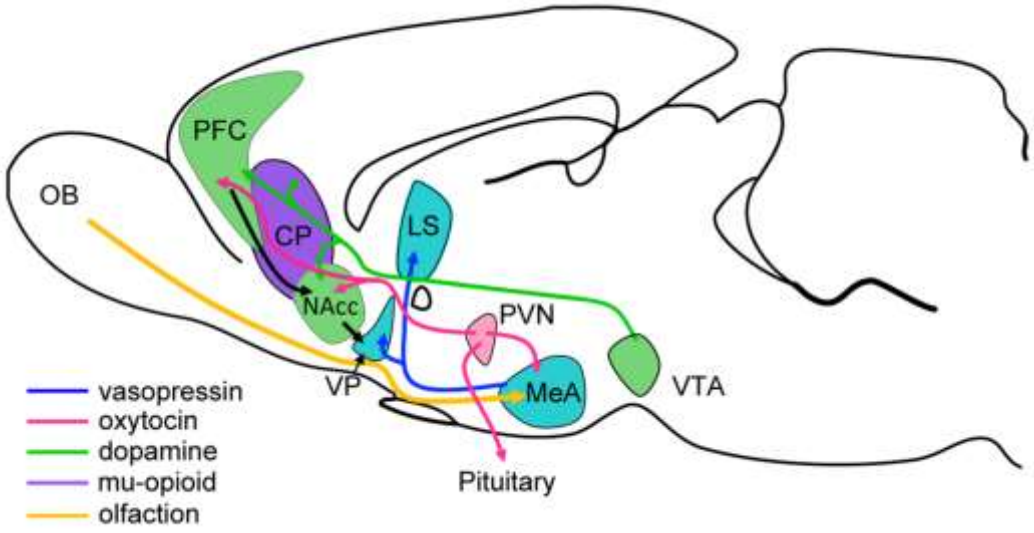

Figure 1.6. A model of a pair bonding network in the prairie vole brain. During mating, oxytocin and vasopressin are released from cells in the paraventricular nucleus (PVN) and dopamine is released from the ventral tegmental area (VTA). Neuropeptidergic innervation is widespread in the forebrain and receptors for theligands are localized in key regions involved in pair bonding. In females, concurrent oxytocin receptor and dopamine receptor D2 activation in the NAcc is required for partner preference. Additional oxytocin receptor activation in the prefrontal cortex (PFC) is also needed. Male prairie voles require vasopressin receptor 1a activation in the ventral pallidum (VP) and lateral septum (LS). All of these regions are part of the mesolimbic reward system which regulates learning and reinforcement in the brain. Finally, mu-opioid signalling in the caudate putamen (CP) signals reward. During the activation of these reinforcement pathways, social information about the sexual partner enters through the olfactory system into the medial amygdala (MeA), which relays this information to other areas. Oxytocin and vasopressin enhance the processing of this social information, allowing efficient dopamine-gated association with the rewarding properties of sex. Thus the prairie vole pair bond is a highly selective conditioned partner preference. OB, olfactory bulb.

Neuropeptide receptor expression contributes to individual differences in behavior

Neuropeptide receptors distributions are highly variable not only between species, but also between individuals within a species. In particular, brain regions that tend to show the highest inter-species variability are the same regions that show within-species variability. There is evidence that this

individual variation in receptor expression contributes to individual differences in social behavior. Prairie voles exhibit high levels of intraspecific variation in behavior and receptor expression across environmental context and as well as between individuals in a population (Cushing et al., 2001, Phelps and Young, 2003, Ross and Young, 2009). Experiments in the lab and field have yielded important information on the gene expression-behavior relationship between individuals.

In the laboratory, genetic tools and discrete behavioral analysis has allowed for an intimate dissection of the relationship between OXTR and AVPR1A densities in specific brain regions and prairie vole social behaviors. Viral vectors offer a powerful tool to manipulate the expression of a gene. Vectors containing a copy of a gene cause ectopic expression in cells within the vicinity of an injection site, leading to an upregulation of density in the case of OXTR and AVRP1A. Other vectors containing a silencing RNA (siRNA) allow knockdown, or reduction of endogenous expression, by exploiting RNA regulatory mechanisms. These tools, in conjunction with autoradiography to map densities, allow for behavioral variation to be associated with higher or lower levels of NHPRs.

Initial work with affiliative and parental behaviors sparked interest in a relationship with NHPR density. Natural variation in OXTR NAcc density is positively correlated with alloparental care in female prairie voles (Olazabal and Young, 2006a, Olazabal and Young, 2006b). Viral vector OXTR upregulation in the NAcc during the juvenile period, (Keebaugh and Young, 2011) but not during adulthood (Ross et al., 2009b) facilitated female alloparental behaviors. The differences in timing above suggest that some NHPR density effects occur by modulating development of social behavioral circuits. AVRP1A variation between

individuals is also associated with differences in affiliation and paternal care (Hammock et al., 2005). Upregulating AVRP1A density in the VP of male prairie voles increases VP neural responses to a social encounter (Lim and Young, 2004) and enhances affiliation (Lim et al., 2004a). Densities of both OXTR and AVRP1A in the LS differ between high and low investigating males (Ophir et al., 2009). Density variation in multiple regions can influence individual differences in affiliation.

NHPR density has been well investigated for its role in pair bonding. As mentioned above, AVRP1A upregulation in the meadow vole VP allows the normally promiscuous species to exhibit partner preferences (Lim et al., 2004c). AVRP1A density variation across the brain is associated with the propensity to form a partner preference in male prairie voles (Hammock and Young, 2005, Hammock and Young, 2002). More specifically, upregulation of AVRP1A in the prairie vole VP enhances individual propensity to form a partner preference (Lim et al., 2004a, Pitkow et al., 2001). Conversely, knockdown of AVRP1A expression in the VP prevents partner preference in males, demonstrating a direct function for endogenous AVRP1A density in pair bonding (Barrett et al., 2013). Viral vector injections ectopically increasing OXTR density in the NAcc facilitate partner preference formation in female prairie voles (Ross et al., 2009b, Keebaugh and Young, 2011). These studies on pair bonding have focused on NHPR density in the MLR pathway and reinforce the notion that NHPR involvement in prairie vole partner preference revolves around facilitating the association between individual social cues and reward signals (Young and Wang, 2004, Burkett and Young, 2012).(Young and Wang, 2004, Burkett and Young, 2012).

Outside of the laboratory, in a semi natural field setting, prairie voles can be tracked with radio telemetry collars to observe sociosexual behaviors in a complex spatial and social environment. As mentioned, prairie voles in the wild are socially monogamous and engage in extra-pair copulations. Telemetry allows individual territory formation to be analyzed in order to identify individual mating tactics. Individual males and females with overlapping territory can be considered pair bonded, or in this case residents - as opposed to wanderers that overlap with numerous other animals. Embryos can be collected from females following a field experiment, allowing parentage to be assessed with genetic techniques to determine mating success. Thus attachment and monogamy can be studied in complex environments, allowing insights into how circuits discovered in the laboratory operate in close to natural conditions (Phelps, 2010).

Field results do not follow perfectly from laboratory models. Male voles that adopt a resident mating tactic, and presumably have pair bonded with a female, have higher OXTR density in the NAcc (Ophir et al., 2012) but have no differences in AVRP1A in the VP as might be expected (Ophir et al., 2008). Females that have paired in the field have higher AVRP1A density in the VP than single females, but do not differ in OXTR density in the NAcc (Zheng et al., 2013). Interestingly, these results show that generally, NHPRs in the MLR pathway are still key, but in the field, males and females have a different sensitivity to the two peptides relative to the laboratory tests.

Field research allows analyses not possible in the laboratory. For example, there are complex relationships between NHPR density and reproductive success - whether a vole was able to mate and produce embryos. Mesolimbic reward pathway regions seem not to play a role in this measure of reproductive success. Instead, other regions that exhibit NHPR density variation

are associated. In males, AVRP1A density in the posterior cingulate and lateral dorsal thalamus is lower in un-paired wandering males that successfully mate, relative to wandering males that did not mate (Ophir et al., 2008). Mated wanderers also had less OXTR density in the hippocampus and septohippocampal nucleus (Ophir et al., 2012). These NHPR density-behavior relationships arise in a network of brain regions that are important for spatial cognition. Thus, OXTR and AVRP1A density change the sensitivity with which spatial reasoning networks receive information about social context. This allows voles with optimal individual NHPR levels to better navigate a particular sociospatial environment. Indeed, a common observation for successfully reproducing wanderer males is that their territory overlaps with more individual voles than unmated wanderers. Ophir et al. suggest that specific combinations of NHPR densities allow wandering animals to maximize the success of their mating strategy by increasing intrusions into other males' territory (Ophir et al., 2008, Ophir et al., 2012).

How diversity in receptor expression is achieved

So far, I have focused on NHPR densities and their influence on behavior. Receptor density is most often measured with autoradiography, which reveals receptor molecules in the cell membrane. Generally, a higher density of binding indicates a greater number of receptors; more receptors occur from increased expression of the gene, stability of mRNA or translation of mRNA to protein. In vole species, this appears to be the case: OXTR and AVRP1A density are significantly correlated with mRNA levels (Young et al., 1996, Young et al., 1997b). Both AVRP1A and OXTR density distributions have been related to transcriptional mechanisms.

Mechanisms that control gene transcription can be thought of in terms of two broad classes: transcription factors and non-coding *cis*-regulatory elements (cis-REs). Transcription factors are specialized proteins that bind to cis-REs in DNA in order to modulate gene expression. These cis-REs occur commonly in promoters near genes, and at other sites, such as enhancers that can be further from protein coding regions. Mutations in cis-REs are the most common cause of phenotypic differences between species (Wittkopp and Kalay, 2012), and thus are likely contribute to the evolutionary diversity exhibited by NHPRs.

The prairie vole NHPR distributions help differentiate them from other *Microtus* species. The promoter regions of both *oxtr* and *avpr1a* contain cis-REs that generate expression in brain regions where prairie vole receptor distributions occur. Mice transgenic for 5kb of prairie vole *oxtr* promoter expressed an ectopic label in many brain areas that contain *oxtr* mRNA in prairie voles (Young et al., 1997a, Young et al., 1997c). This powerful genetic technique revealed that a minimal portion of promoter does indeed contain cis-REs that drive gene expression in certain brain regions. The *avpr1a* promoter also contains important cis-REs. Mice similarly transgenic for minimal components of the prairie vole AVRP1A gene, including 2.2 kb of promoter sequence, exhibited a unique AVPR1A distribution (Young et al., 1999a). The transgenic mice expressed AVPR1A in regions where wild-type mice normally lack expression of the gene (Young et al., 1999a). The cis-REs contained in the transgenic construct were so potent that the mutant mice exhibited increased affiliative behavior, which wild-type mice did not, in response to an AVP injection.

Some cis-RE sequences are conserved across species and these motifs can be analyzed in genomic areas suspected of harboring cis-REs. Sequence analysis

of the *oxtr* promoter between prairie and montane voles identified few differences in putative cis-REs. The *avpr1a* promoter on the other hand contains a stark difference between meadow and prairie voles. There is an approximately 600 bp complex microsatellite in the prairie vole *avpr1a* promoter. Microsatellites are highly unstable and polymorphic genetic features. Montane voles have a 200 bp microsatellite. The prairie vole microsatellite demonstrates direct functional properties in *in vitro* luciferase assays either when deleted or when compared to the meadow vole microsatellite. The *avpr1a* promoter microsatellite contains cis-REs that contribute to differences in gene regulation between species.

Interestingly, the prairie vole *avpr1a* microsatellite is also polymorphic in length between individuals (Hammock and Young, 2005). These length variants are also functional in a luciferase assay. Genotype-based breeding to generate F1 prairie voles that were homozygous for long or short alleles revealed that the length of the microsatellite was associated with complex AVRP1a density differences throughout the brain (Figure 1.7). The microsatellite alleles were also associated with differences in behavior, including variation in partner preference formation. Males with long alleles formed a partner preference after a brief cohabitation, while males with short alleles did not. The prairie vole *avpr1a* promoter microsatellite therefore exerts a robust influence on gene expression, such that it can also predict differences in social behavior.

A detailed analysis of the specific contributions of cis-REs in the vole microsatellite was performed using transgenic mice. Knock-in mice were created using homologous recombination, replacing 3.4kb of mouse DNA with prairie vole *avpr1a* promoter sequence (Donaldson and Young, 2013). In addition, three separate lines also had unique microsatellite content: short prairie vole, long prairie vole or meadow vole microsatellites. The long prairie vole microsatellite

increases AVRP1A density in three brain regions relative to both wild-type background and the meadow vole microsatellite and, the long prairie vole microsatellite increases dentate gyrus AVPR1A density relative to the short. This experiment conclusively demonstrates that the *Microtus* microsatellite contains cis-REs, and that instability in the microsatellite is likely a mechanism that creates diversity in receptor expression patterns and likely social behavior.

These increasingly detailed genetic studies have dissected one particular cis-RE that contributes to prairie vole social diversity through regulation of *avpr1a* in the brain. Vole species provide natural diversity while proving tractable to a combination of advanced behavioral and genetic techniques. Vole genetic resources are now catching up with more traditional models like the mouse, including sequence information and transgenic techniques (McGraw and Young, 2010). The experiments described above suggest a strategy to identify sources of diversity in social behavior and then to narrow down influences to the level of brain regions and/or DNA sequences. For example, mechanisms regulating *oxtr* expression in the brain have proven difficult to explain, whether as a result of cis-REs or otherwise. Prairie voles exhibit high individual variation in OXTR in the NAcc, which may be subject to as of yet unknown cis-REs.

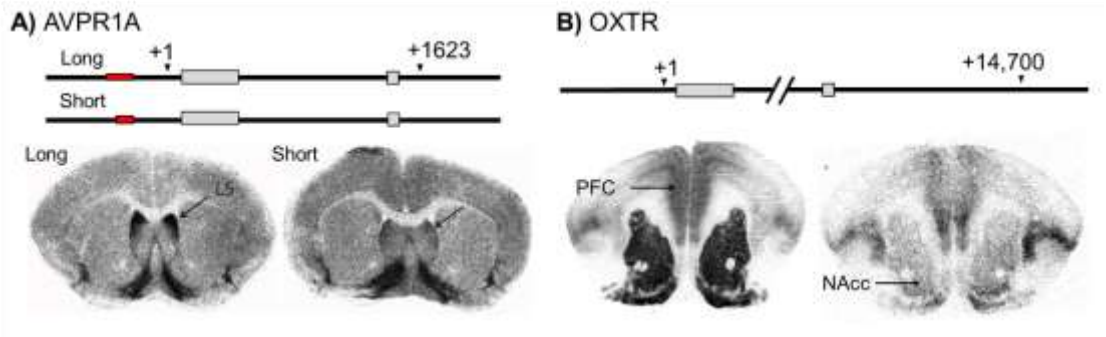

Figure 1.7. Individual diversity in OXTR and AVPR1A. Autoradiograms of coronal brain sections from individual prairie voles. The *Avpr1a* promoter microsatellite (in red) predicts individual differences in AVPR1A in many regions including the lateral septum (LS) (A). OXTR also exhibits high individual variation in expression, particular in the nucleus accumbens (NAcc), while other regions such as the prefrontal cortex (PFC) are more stable between individuals (B). The *oxtr* gene differs in structure from *avpr1a*: it lacks a microsatellite but has larger non-coding regions, leading to a longer total gene sequence.

Translational implications for OXTR and AVRP1A

The biomedical community has substantial interest in uncovering biological mechanisms that influence human social cognition. This is in part due to a growing concern with psychiatric disorders with deficits in social cognition, namely autism spectrum disorder (ASD) (Young et al., 2002, Modi and Young, 2012, Insel, 2010). Work with animal models has provided a strong conceptual base on which to base hypotheses about human social behavior (Guastella and MacLeod, 2012). Of course, human research necessarily allows far less intrusive methods, which is a problem for OXT and AVP research, since large molecules such as OXT cannot pass from the periphery across the blood brain barrier. Intranasal OXT (IN-OXT) delivery was proposed as a solution, as the nasal epithelium has a weak barrier.

Human IN-OXT research has progressed rapidly over the past decade since the first effects were seen. Two major research themes have emerged: social salience and prosociality (Bartz et al., 2011), although other categories have been proposed (Kemp and Guastella, 2011). Prosocial effects include increases in trust

and generosity to name just two (Kosfeld et al., 2005, Zak et al., 2007). Enhancements in social saliency include increased gaze to the eyes (Guastella et al., 2008, Andari et al., 2010) and leads to improved abilities to detect emotion in faces and a selective increase in social recognition (Rimmele et al., 2009, Domes et al., 2007, Prehn et al., 2013). The effects parallel nicely the work reviewed above in rodents and suggest that the role of OXT in regulating the processing of social information and the salience of social information is evolutionarily conserved from rodent to man, even across sensory modalities. Brain networks underlying these behavioral effects are now being elucidated. Increased eye gaze, as stimulated by IN-OXT, is associated with activation of the superior colliculus and its connectivity with the amygdala, suggesting a modulation of a circuit for directing visual saccades to salient features (Gamer et al., 2010). This finding is particularly interesting when considering that the superior colliculus is a site of conserved *OXTR* expression in primates, potentially including humans (Freeman et al., 2014b).

Basic processes like these may lead to significant effects on human social behavior. In an experiment on human attachment, IN-OXT caused male subjects, if they were in a monogamous relationship, to maintain a longer distance from a novel female (Scheele et al., 2012). The treatment also selectively increases the attractiveness of a partner's photograph and increased NAcc and VTA activation, suggesting IN-OXT stimulates MLR pathway signaling in humans (Scheele et al., 2013).

IN-OXT has a robust effect on human social cognition and behavior. Researchers are now developing strategies to combine IN-OXT or drugs that stimulate endogenous central OXT release with social behavioral training to improve social symptoms in ASD (Modi and Young, 2012, Modi and Young, 2011).

Early results suggest that IN-OXT can indeed improve social cognitive processing in ASD. The ability of IN-OXT to increase eye gaze, emotion detection and social recognition holds in ASD patients (Andari et al., 2010, Bartz et al., 2010, Guastella et al., 2010a, Domes et al., 2013). IN-OT also enhances social reciprocity in high functioning autistic subjects (Andari et al., 2010). Within some tests, ASD subjects exhibit individual variability in their response to IN-OXT (Andari et al., 2010, Bartz et al., 2010). This variation in response could likely result from individual differences in OXTR expression in the brain.

Central *OXTR* gene expression has not been characterized in humans. However, social behavior has been associated with single nucleotide polymorphisms (SNPs) in the *OXTR* gene. Studies focused on ASD etiology found numerous SNPs in *OXTR* that are associated with ASD diagnosis in patients or endophenotypes relating to social interaction or communication in both typically developing subjects or patients (Campbell et al., 2011, Jacob et al., 2007, Liu et al., 2010, Walum et al., 2008, Wu et al., 2005, Ylisaukko-oja et al., 2006, Yrigollen et al., 2008, Lerer et al., 2008, Parker et al., 2014, LoParo and Waldman, 2014) . I have postulated that a general function of OXTR is to increase salience of social information, deficits in that process could help explain the genetic associations with ASD mentioned above (Young, 2015). Along those lines, studies with infants that have or would later be diagnosed with ASD found deficient gaze to socially relevant features such as eyes or biological motion (Jones and Klin, 2013, Klin et al., 2009). It has been suggested that this early loss of attention towards social information denies the brain opportunities to learn about the social environment, potentially leading to further deficits. Interestingly then, *OXTR* variation may contribute to early gaze deficits, as SNPs in the gene are associated with individual variation in social cognition at

18 months (Wade et al., 2014). *OXTR* SNPs may further interact with basic neural development. OXTR expression in mice undergoes temporary increases during a developmental window that may play a significant role in the development of cortical contributions to cognition (Hammock, 2014, Hammock and Levitt, 2013, Zheng et al., 2014).

Variation in *OXTR* is related to human social cognition in adulthood. SNPs in the gene are associated with complex human behaviors such as prosocial dispositions, trait empathy and even pair bonding (Kogan et al., 2011, Walum et al., 2012, Wu et al., 2012). These effects may stem in part from a role for OXTR in basic processing of visual and auditory information (Lucht et al., 2013, Tops et al., 2011). Skuse et al. revealed a profound association with *OXTR* after testing ASD patients, their parents and siblings in a facial recognition memory task (Skuse et al., 2014). The recognition ability of all subjects was modulated by the genotype of *OXTR* SNP rs237887. Within each group, i.e. proband or parents, subjects with the A/A genotype of the SNP were significantly more competent in the recognition task than those with the G/G genotype while the A/G genotype responded intermediately. This study revealed a clear association between *OXTR* and social cognition, with variation in the gene explaining 10% of individual behavioral variation. Finally, *OXTR* SNPs interact with social factors to mediate responses to stressors. Kim et al. found that *OXTR* interacted with cultural background in stress-induced social support seeking (Kim et al., 2010). A similar study showed that OXTR genotype modulated the ability of social support to dampen physiological and subjective responses to stress (Chen et al., 2011).

All of the *OXTR* SNPs discussed in the above section are in non-coding regions such as introns. Thus, genetic variation near the human *OXTR* is more likely to have functional consequences on gene expression than on amino acid

composition of the OXTR protein. A cis-RE has been identified in a human *OXTR* intron (Mizumoto et al., 1997). *OXTR* expression is reduced in the cortex of ASD patients, who also have increased *OXTR* methylation, an epigenetic mark associated with transcriptional suppression (Gregory et al., 2009). Another *OXTR* SNP exhibits allelic expression imbalance in human brain tissue (Tansey et al., 2010). Such imbalance is a specific effect of heterozygosity in cis-REs actively involved in expression of a gene. Thus, this result provides direct evidence of association between *OXTR* SNPs and expression of the receptor. It is tempting to speculate that such allelic effects on expression could lead to associations between SNPs and OXTR density in brain regions responsible for visual processing and lead to robust modulation of social cognition like those reported by Skuse and colleagues.

The abundance of findings regarding the influence of *OXTR* SNPs on social cognition would benefit from animal models providing more detailed information on mechanisms by which OXTR density is regulated in the brain. This information has so far proven elusive. In the case of AVRP1A however, work with prairie voles has provided detailed insights into mechanisms by which a promoter microsatellite containing cis-REs generates diversity of the receptor in the brain (Donaldson and Young, 2013, Hammock and Young, 2005). Intranasal AVP enhances recognition of emotional faces and sexual cues, suggesting that AVRP1A activation modulates social salience in the human brain similarly to OXTR (Guastella et al., 2010b, Guastella et al., 2011). The primate *AVRP1A* promoter also contains unstable microsatellites that vary in length between individuals (Donaldson et al., 2008, Hammock and Young, 2005). Using these variants as markers, *AVPR1a* has been associated with altruism, pair bonding and autism (Kim et al., 2002, Knafo et al., 2008, Walum et al., 2008, Wassink et

al., 2004, Yirmiya et al., 2006). Long alleles of the RS3 microsatellite are associated with elevated *AVPR1a* expression in the brain and cell culture (Knafo et al., 2008, Tansey et al., 2011).

As noted, humans share some *AVRP1A* microsatellites in common with other primates. The RS3 microsatellite has received much of the focus in human studies and its length polymorphism seems to add diversity the influence of AVRP1A on human social cognition (Donaldson et al., 2008). The chimpanzee (*Pan troglodytes*) is polymorphic for a deletion of RS3. Most chimpanzees are therefore either homozygous for the RS3 deletion, or heterozygous, meaning they have only a single genomic copy of the RS3 microsatellite. Research in voles suggests that rich genetic diversity will lead to interesting individual differences in behavior. Indeed, the RS3 deletion polymorphism associates with a number of chimpanzee social behavioral measures, including personality traits such as dominance and conscientiousness and receptive joint attention (Hopkins et al., 2012, Hopkins et al., 2014, Latzman et al., 2014).

Opportunities for discovery

I hypothesize that NHPR diversity underlies complex behavioral differences between species. The genes for these receptors may be prone to containing genetic variation in regulatory elements controlling expression throughout important neural networks. Additionally, similar variation may contribute to individual differences in humans and other animals. The variation inherent in these systems is important to our understanding of complex biological systems and also offers tractability for detailed study.

The goal of the present dissertation is to explore potential genetic influences contributing to individual variation in OXTR distribution in the brain

with the ultimate goal of identifying potential cis-REs governing variation in expression. The identification of genetic factors leading to variation in OXTR expression and behavior in prairie voles would make prairie voles a premier model organism to understand how genetic variation in human NHPR genes contribute to variation in social cognition as well as psychopathology.

CHAPTER 2

Genetic variation in the oxytocin receptor contributes to individual diversity in brain receptor distribution and social attachment in monogamous prairie voles

ABSTRACT

Social behavior is a complex evolutionarily plastic phenotype that varies markedly even between closely related species. The OXT system is ideally suited to underpin such a diverse phenotype, as the OXTR plays an evolutionarily conserved role in regulating sociosexual behaviors, yet exhibits highly diverse distributions in the brain between species as well. The diversity of OXTR binding allows OXT to modulate neural networks differentially between species while maintaining a conserved role in peripheral reproductive physiology. A homologous neuropeptide to OXT, AVP and its receptor AVPR1A have similar properties. A variable microsatellite serves as a mutable *cis*-RE in the promoter of the *avpr1a* gene that confers phylogenetic flexibility to AVPR1A binding. OXTR likely relies on a similar *cis*-RE mechanism to produce its inherent diversity in distribution. The socially monogamous prairie vole is an ideal model of complex social behavior. A key feature of the prairie vole brain is high OXTR density in the NAcc, which aids the development of social attachments. OXTR density variation in the NAcc additionally leads to individual differences in prairie vole social behavior. In this chapter, I hypothesized that diversity in OXTR density in the prairie vole NAcc is caused by *cis*-RE activity. I discovered that *oxtr* mRNA exhibits allelic imbalance in the NAcc but no other brain regions where OXTR binding is observed. The single nucleotide polymorphism (SNP) marker, NT204321, used for allelic imbalance is in LD with one or more *cis*-REs and predicts OXTR density in the NAcc. Voles with the high-OXTR associated T/T genotype also had a greater propensity to form a partner preference. The

current study that prairie vole OXTR density is under a strong genetic influence that extends to the level of individual differences in social attachment.

INTRODUCTION

Social behavior is a complex and malleable phenotype, requiring flexibility in underlying neural systems in order to achieve diversity between species and among individuals to maximize fitness based on natural selection. Neuromodulators facilitate flexibility in neural networks by coordinating distributed cellular activity to suit internal states and external demands (Bargmann, 2012, Marder, 2012, Louis and Tomchik, 2014, Kelly and Goodson, 2014). Modulatory transmitters generally have restricted sites of production but widespread dispersal in the brain (Ludwig and Leng, 2006, Ross et al., 2009a, Woolf and Butcher, 2011). Target cells and brain nuclei express unique compliments of receptors and intracellular signalling cascades, allowing discrete responses to a dispersed signal (Boto et al., 2014, Gordus et al., 2015, Katz and Lillvis, 2014, Macosko et al., 2009). Genetic mechanisms that introduce variation to these receptor systems in targeted nuclei can introduce behavioral diversity.

The neuropeptide oxytocin (OXT) is a neuromodulator that selectively influences reproductive and social behavior through signalling via a single oxytocin receptor (OXTR). In vertebrates, the *Oxt* gene expression in the brain is remarkably conserved as are projections of OXT axons (Ross et al., 2009a, Venkatesh et al., 1997, Gilligan et al., 2003). In contrast, *oxtr* gene expression in the brain is strikingly different across vertebrates, even among closely related species (Goodson et al., 2012, Anacker and Beery, 2013, King and Young, 2015, Freeman et al., 2014a, Freeman et al., 2014b). Disruption of *Oxt* or *Oxtr* in mice impairs social recognition, suggesting a role for this system in social information

processing (Ferguson et al., 2000, Takayanagi et al., 2005, Lee et al., 2008). Similarly, loss or blockade of *Oxtr* reduces olfactory investigation in mice and rats, and OXTR protein density is associated with olfactory investigation in prairie voles (Ophir et al., 2009, Nakajima et al., 2014, Lukas et al., 2011, Pobbe et al., 2012). OXT system signalling is involved in both processing social information as well as behavioral attending to social cues. These findings are paralleled by work with humans and non-human primates for whom intranasal delivery of OXT modulates visual attention to social stimuli (Andari et al., 2010, Guastella et al., 2008, Ebitz et al., 2013, Modi et al., 2014). Furthermore, variants in the human *OXTR* gene are associated with face recognition skills (Skuse et al., 2014). Thus, OXT signalling has an evolutionarily conserved role in modulating social cognition by enhancing the salience of social cues in mammals, regardless of the sensory modality used (Freeman et al., 2014a, Young, 2015).

OXT signalling is also important for the normal development of social behavior. OXTR density varies through development (Hammock and Levitt, 2013, Hammock, 2015, Wang and Young, 1997). Oxytocin and *Oxtr* KO mice have deficiencies in isolation-induced ultrasonic vocalizations (USVs) (Takayanagi et al., 2005, Ferguson et al., 2000). Differential rates of USV calling can influence maternal care of pups (Bowers et al., 2013). Variation in early life care affects adult social behavior (Ahern and Young, 2009). Such effects may in part be moderated by OXT signalling (Bales et al., 2011), and direct manipulation of OXT signalling during development changes the expression of social behavior later in life (Keebaugh and Young, 2011, Barrett et al., 2014, Bales et al., 2014, Bales et al., 2013, Carter et al., 2009, Bales et al., 2007).

In a socially monogamous rodent, the prairie vole, brain OXTR distribution reveals important regions within a neural network for pair bonding

(Young and Wang, 2004, Johnson and Young, 2015). The nucleus accumbens (NAcc), a critical node of the mesolimbic dopamine reward system, is one such region. OXTR density is much higher in the NAcc of prairie voles than in promiscuous vole species (Olazabal and Young, 2006b, Insel and Shapiro, 1992), and OXTR activation in the NAcc but not caudate putamen is necessary for mating-induced partner preference formation (Young et al., 2001, Keebaugh et al., 2015).

Many regions in the prairie vole brain express *oxtr* variably between individuals, especially the NAcc and caudate (Ophir et al., 2009, Olazabal and Young, 2006b, Young, 1999). Increasing OXTR density in the NAcc using viral-vector mediated gene transfer facilitates, while decreasing OXTR density in the same region using RNAi inhibits pair bonding (Ross et al., 2009b, Keebaugh and Young, 2011, Keebaugh et al., 2015). Furthermore, variation in NAcc OXTR density is correlated with individual differences in monogamy-related behavior (Ophir et al., 2012). Thus, OXTR diversity in the prairie vole NAcc is an intriguing neural phenotype intermediate between behavior and genomic regulation.

Genetic variants that affect *cis*-regulatory elements (*cis*-REs) generate variation in expression of target genes and this process plays a prominent role in evolutionary change (Wittkopp and Kalay, 2012, King and Wilson, 1975, Bendesky and Bargmann, 2011, Young and Hammock, 2007, Bendesky et al., 2012, Linnen et al., 2013). Indeed, a class of hyper-variable *cis*-RE, microsatellites, has functional influence over species and individual diversity in neuropeptide receptor expression and social behavior (Hammock and Young, 2005, Donaldson and Young, 2013, Hammock et al., 2005). While variation in OXTR density in the NAcc has likewise been associated with variation in

behavior, the genetic mechanisms leading to the behaviourally relevant OXTR diversity has not been explored.

I hypothesized that prairie vole *oxtr* gene expression is influenced by polymorphic *cis*-REs. To test this hypothesis, I analyzed brain-derived mRNA for allelic imbalance, a molecular signature of *cis*-RE activity. I further hypothesized that allelic imbalance occurs with higher magnitude in regions with higher variation in OXTR density. I assessed allelic imbalance by pyrosequencing mRNA derived from target brain nuclei of individuals that were heterozygous for common single nucleotide polymorphisms (SNPs) in the *oxtr* transcribed region. I found evidence of strong allelic imbalance in NAcc, but not in other brain regions. Furthermore, for one SNP marker, NT204321 (C/T), the T allele was consistently overexpressed relative to the C allele and this effect was selective to the NAcc and caudate putamen (CP). I therefore hypothesized that NT204321 was in substantial LD with one or more polymorphisms of functional consequence. To test this hypothesis, I selected heterozygous NT204321 breeders from our outbred colony to produce offspring off all three NT204321 genotypes: C/C, C/T, and T/T and quantified OXTR in the brains of these animals. The NT204321 T-allele was associated with higher OXTR density compared to C/C homozygotes. Given prior findings that OXTR density variation in NAcc influences individual differences in behavior, I tested siblings homozygous for NT204321 genotypes for isolation-induced vocalizations as pups and as adults in the partner preference test. While no difference in ability to form a partner preference was observed in females, male prairie voles with the high-OXTR predicting T/T genotype formed a partner preference following a brief cohabitation with a female while their C/C siblings did not. These data strongly suggest that *cis*-REs for the prairie vole *oxtr*

generate diversity in neural molecular and behavioral phenotypes related to social attachment.

RESULTS

Variation in OXTR density across brain regions

Previous experiments reported exceptional diversity in OXTR binding between individual prairie voles, particularly within the NAcc (Ophir et al., 2009, Young, 1999), however a systematic comparison of individual variation across brain regions has not been reported. I performed OXTR autoradiography to quantify differences in OXTR binding between individual prairie voles (n=12). The individual variation in density was prominent within the NAcc relative to other brain regions such as the amygdala and prefrontal cortex (PFC), which express OXTR with similar mean densities (Figure 2.1).

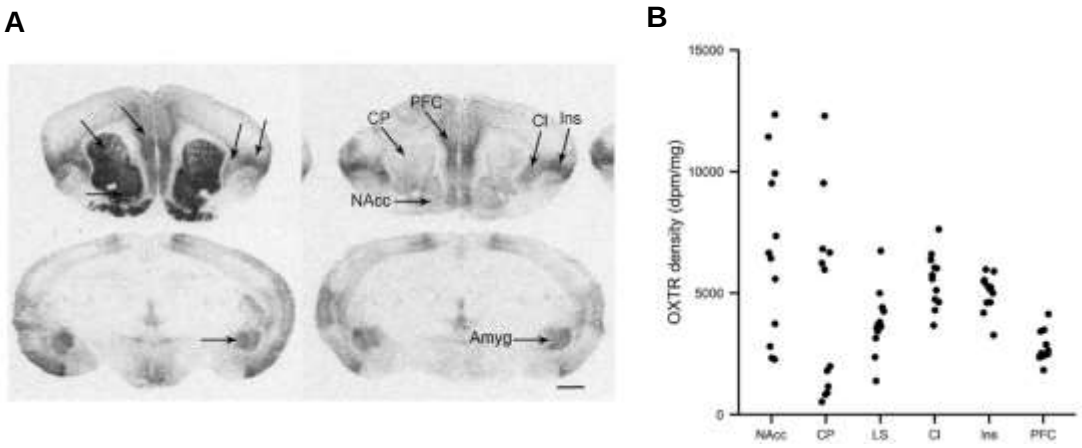

Figure 2.1. Individual variation in OXTR density in the prairie vole forebrain. (**A**) Representative audioradiograms of two individual prairie voles illustrating the range of expression exhibited by OXTR. The NAcc has striking differences between individual prairie voles. (**B**) Individual OXTR density for twelve individual male prairie voles across five brain regions. NAcc, nucleus accumbens; LS, dorsal lateral septum; PFC, prefrontal cortex including prelimbic and infralimbic cortex (not shown in **A**); Cl, claustrum; Ins, insular cotex; Amyg, amygdala nuclei, not quantified here. Black scale bar represents 100 µm.

In order to compare OXTR density correlated with *oxtr* mRNA levels I performed *in situ* hybridization on adjacent sections from these same brains (Figure 2.2). I observed that mRNA and OXTR density are quite similar within individuals, suggesting that the regulatory mechanisms generating the individual differences act on either transcription or stability of the *oxtr* mRNA.

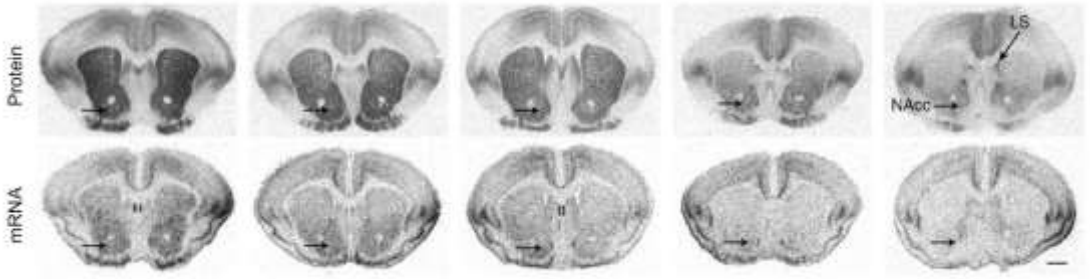

Figure 2.2. OXTR protein and *oxtr* mRNA distributions are similar within individuals. Five individual prairie voles with a range of NAcc OXTR binding densities also exhibit high variation in mRNA levels in the NAcc, as measured by *in situ* hybridization. NAcc, nucleus accumbens; LS, dorsal lateral septum. Black scale bar represents 100 µm.

Allelic imbalance occurs selectively in the dorsal and ventral striatum

To test the hypothesis that variability in *oxtr* expression within the striatum (NAcc and caudate putamen) was due to the influence of *cis*-REs, I assayed for allelic imbalance, a molecular phenomenon that occurs when two alleles of the same gene are transcribed at differential rates (Wittkopp, 2011). The simplest explanation for allelic imbalance is that one or more linked polymorphic *cis*-REs are modulating expression of the target gene differentially between alleles.

In order to assess allelic imbalance, I identified SNPs in the transcribed region of the prairie vole *oxtr*, as the technique requires a measurement of mRNA. I designed five sets of primers for PCR amplification of approximately 500 bp amplicons spanning *oxtr* exons including untranslated regions (UTRs) based on sequence from a prairie vole BAC clone, DP001215.2 (McGraw et al., 2012). I sequenced the five amplicons for 19 voles from which I collected brains. I identified 26 SNPs and initially chose three to use as markers because multiple individuals were heterozygous at all three. It was important to have animals heterozygous at more than one SNP to verify that any allelic imbalance was not due to technical bias from the primers used at any particular marker. The PCR reaction for one SNP, NT2043215 was unreliable so I proceeded using two SNPs as markers for allelic imbalance: NT204321 and SNP18 (Table 2.1).

I used pyrosequencing of cDNA from heterozygotes at the two SNPs to measure relative abundance of mRNA isolated from the PFC (here, prelimbic and infralimbic cortex combined), NAcc, CP, dorsal lateral septum (LS) and amygdala. Genomic DNA (gDNA) and mRNA were isolated and cDNA was generated from mRNA. The gDNA served as an internal control, since genomic alleles should be present at a 1:1 ratio. The cDNA is the target molecule and here I observe that cDNA from striatal tissues, both NAcc and CP, exhibit allelic imbalance (Figure 2.3, A&C).

For NT204321 in the 3' UTR, cDNA allelic ratios in NAcc are significantly greater than gDNA in those regions (Student's *t*-test, $p < 0.05$). This result is also obtained when measuring a second SNP, also in the 3' UTR (Figure 2.3B). Interestingly, in other regions that are less variable than striatal tissues, the cDNA allelic ratios do not differ from those of gDNA (Figure 2.3). The one

exception to this pattern is seen in the Amyg data shown in Figure 2.3C, and in this case the allelic imbalance is of a much smaller magnitude than the NAcc. These data suggest the hypothesis that *cis*-REs that are uniquely active within striatal tissue generate individual differences in expression of *oxtr*.

SNP ID	BAC position	Nucleotides	Number of hets	maf
SNP26	21232	G/T	7	0.5
SNP15	8489	C/T	11	0.47
SNP7	8289	G/T	0	0.45
SNP18	8878	C/T	7	0.44
SNP16	8502	C/T	7	0.39
SNP5	6310	G/C	9	0.36
SNP2	6152/**204321**	C/T	8	0.33
SNP22	9093	G/T	6	0.31
SNP21	9087	A/G	4	0.25
SNP25	20638	C/T	9	0.24
SNP19	8944	A/G	3	0.19
SNP17	8508	A/C	6	0.16
SNP9	8355	C/T	3	0.14
SNP11	8417	C/T	5	0.13
SNP20	9043	A/G	4	0.11
SNP12	8424	C/T	4	0.11

Table 2.1. Initial polymorphism discovery in 19 individual prairie voles. SNPs are numbered in the order they appear along BAC clone AC238822.2. SNPs are arranged from highest to lowest by minor allele frequency (maf). Three SNPs were chosen as potential markers for allelic imbalance because a number of individuals were heterozygotic at all three markers. SNP25 PCRs failed and so allelic imbalance assays were completed with SNP2 and SNP18. The location of SNP2, nucleotide (NT) 204321 on another prairie vole BAC clone DP001215.2 is also shown in bold.

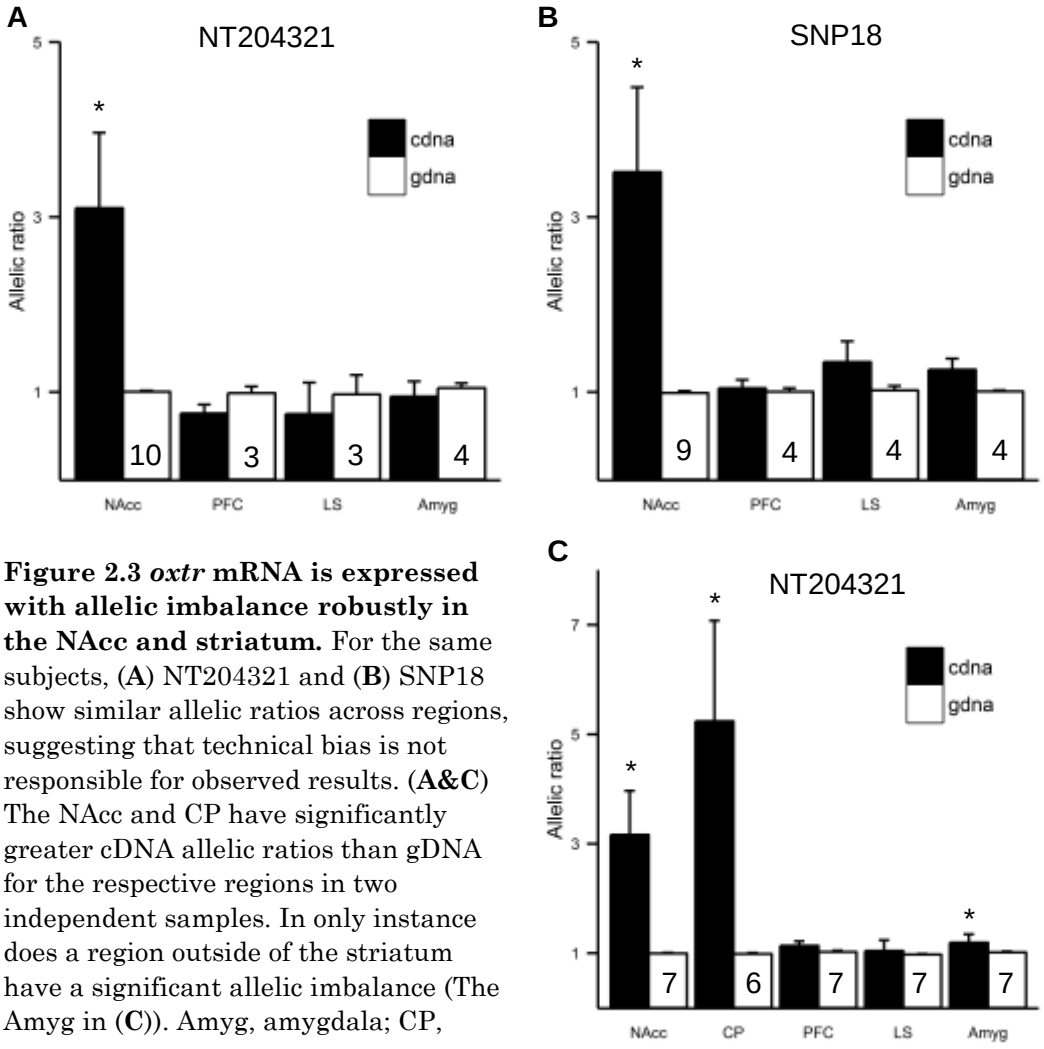

Figure 2.3 *oxtr* mRNA is expressed with allelic imbalance robustly in the NAcc and striatum. For the same subjects, (**A**) NT204321 and (**B**) SNP18 show similar allelic ratios across regions, suggesting that technical bias is not responsible for observed results. (**A&C**) The NAcc and CP have significantly greater cDNA allelic ratios than gDNA for the respective regions in two independent samples. In only instance does a region outside of the striatum have a significant allelic imbalance (The Amyg in (**C**)). Amyg, amygdala; CP, caudate putamen; LS, lateral septum; NAcc, nucleus accumbens; PFC, prefrontal cortex. * cDNA allelic ratio is significantly greater than a threshold calculated by the mean of the gDNA allelic ratio + 3 gDNA standard deviations. Sample size (n) is shown for each region in the gDNA column.

NT204321 is associated with OXTR density

I found that that the T-allele of NT204321 conferred sensitivity to a restriction enzyme, SSP1. I therefore were able to genotype new individuals for NT204321 genotype using PCR and a restriction digest, followed by resolution on an agarose gel. I was able to use this method to quickly screen individuals for NT204321 genotype for the remaining experiments described in this chapter.

In the allelic expression imbalance data, I noted that the T-allele of NT204321 was consistently more highly expressed than the C-allele. This led us

to hypothesize that NT204321 might serve as a marker to predict higher overall OXTR density in the NAcc. To test this hypothesis, I bred heterozygous (C/T) males and females, establishing 33 breeder pairs to generate offspring of all possible genotypes within the same litter. Data on OXTR expression was collected for 12 brain regions using autoradiography. OXTR density did not differ between males and females in any region (Three-way ANOVA, genotype x region x sex, no main effect of sex).

I expected OXTR binding to be correlated across regions. To test this hypothesis, I used exploratory factor analysis to determine the correlation structure between OXTR binding data from all 12 brain regions investigated (See Methods for details). I hypothesized that correlations between OXTR data from different brain regions can be explained by unobserved variables reflecting transcriptional processes giving rise to the patterns of correlation across brain regions. Factor analysis is a method to identify such unobserved, latent variables. Our analysis revealed two factors together explaining the majority of variance (58%) in the twelve brain regions in which I measured OXTR density. As can be seen in Table 2.2A, Factor 1 strongly reflects co-variability in MLR regions (NAcc, CP, olfactory tubercle (Tu)). The second factor reflects covariation between cortical and subcortical regions that have relatively uniform levels of OXTR density (Table 2.2A). Interestingly, the correlation between Factor 1 and NAcc is very close to 1, indicating that this factor is almost perfectly predicted by NAcc expression. Similarly, the claustrum (Cl) and insular cortex (Ins) load strongly on the second factor. I identified similar patterns when I investigated the associations between NT204321 and the OXTR expression in the 12 brain regions, with regions loading into Factor 1 being more related to genotype than those loading into Factor 2. Only in some MLR regions (NAcc, CP)

A	Factor 1	Factor 2
NAcc	0.99	0.14
Tu	0.82	0.09
CP	0.82	0.18
CeA	0.69	0.3
Bulb	0.56	0.5
LS	0.56	0.15
Cl	0.17	0.88
Ins	0.12	0.85
BLA	0.04	0.71
PFC	0.5	0.51
VMH	0.3	0.47
AON	0.28	0.41

B	Factor 1	Factor 2
NAcc	0.99	0.04
CP	0.92	0.1
Tu	0.91	-0.01
LS	0.56	0.14
Ins	-0.06	0.99
Cl	0.07	0.75
PFC	0.51	0.6

Table 2.2. Two factors encompass the covariation in OXTR binding amongst brain regions in two independent samples. Values in the table represent factor correlations. NAcc, nucleus accumbens; Tu, olfactory tubercle; CP, caudate putamen; CeA, central amygdala; OB, olfactory bulb; LS, dorsal lateral septum. Cl, claustrum; Ins, insular cortex; BLA, basal lateral amygdala; PFC, prefrontal cortex; VMH, ventral medial hypothalamus; AON, anterior olfactory nucleus.

and olfactory processing regions (olfactory bulb (OB)) did I observe a significant association between OXTR density and NT204321 genotype in this cohort of animals with a relatively small sample size (Figure 2.4). These finding are significant given the hypothesis that OXTR influences social behavior by modulating the salience and reinforcing value of social cues, and the importance of olfaction in social communication in rodents. These data suggest that the NT204321 marker of *cis*-RE activity is a significant predictor of OXTR protein density, but in a highly brain region-specific manner. I performed a second factor analysis in an additional sample (N=85) from which data was collected for

behavioral analyses described below (Table 2.2B). In this sample I assessed fewer regions in order to process more subjects and tissue in a single autoradiography experiment. The second factor analysis, like the first one, revealed two factors explaining most of the variance (74%). NAcc was again almost perfectly correlated with the first factor. Here the Ins was close to perfectly correlated with Factor 2. The factors revealed in our analysis suggest a divergence in oxtr transcriptional regulation machinery across factors, but may reflect commonalities in transcriptional regulation between brain regions within a factor. Further, our results suggest that most of the variability in OXTR expression across brain regions is explained by the variability in NAcc and the Ins. Thus further analyses including brain data focused on these two regions as representatives of Factor 1 and Factor 2 brain regions.

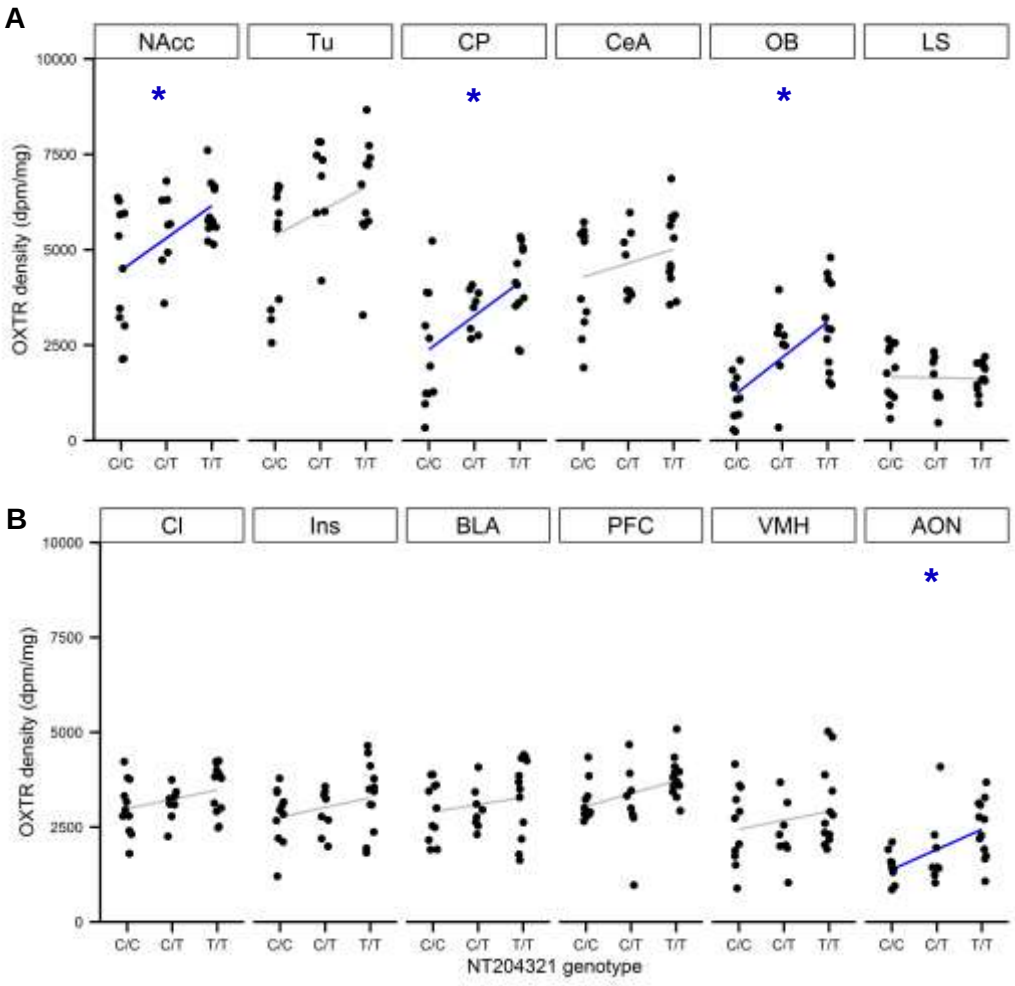

Figure 2.4. OXTR binding density in striatal and olfactory regions is associated with NT204321. Samples sizes for each genotype are C/C=11, C/T=8 and T/T=12. (**A**) Brain regions are sorted based on the how strongly the regions loaded on Factor 1. Within Factor 1 regions, OXTR binding is significantly related to genotype in NAcc, CP and OB. (**B**) Brain regions are sorted based on the how strongly the regions loaded on Factor 2. In the Factor 2 grouping, only the AON was significantly associated with genotype. Associations were investigated using simple linear regression. * $P < 0.004$ (α corrected for 12 comparisons). NAcc, nucleus accumbens; Tu, olfactory tubercle; CP, caudate putamen; CeA, central amygdala; OB, olfactory bulb; LS, dorsal lateral septum; Cl, claustrum; Ins, insular cortex; BLA, basal lateral amygdala; PFC, prefrontal cortex; VMH, ventral medial hypothalamus; AON, anterior olfactory nucleus. Data is shown as individual OXTR density (dpm/mg) with trend line for the linear regression.

NT204321 genotype predicts individual differences in social behaviors

OXT and OXTR are important for the development of social behavior (Bales et al., 2013, Bales and Carter, 2003, Barrett et al., 2014, Hammock and Levitt, 2013, Hammock, 2015, Keebaugh and Young, 2011). OXT and OXTR knockout mice display a robust reduction in ultrasonic vocalizations (USVs) following isolation from the natal nest compared to wild-type mice (Takayanagi et al., 2005, Ferguson et al., 2000). At postnatal day 7, I measured isolation-induced USVs in offspring from our heterozygous breeders. I observed 73 males: 24 T/Ts, 17 C/Ts and 32 C/Cs and 75 females: 16 T/Ts, 24 C/Ts and 35 C/Cs. USV calls were not normally distributed (Shapiro-Wilk test, $p = 2.93\text{x}10^{-7}$. In males, I found a significant effect of genotype on the number of USV calls emitted (Kruskal-Wallis rank sum test, $X^2(2)=6.34$, $p = 0.042$; Figure 2.5). In females, there was not a significant effect of genotype on the number of USVs (Kruskal-Wallis rank sum test, $X^2(2)=2.06$, $p = 0.36$).

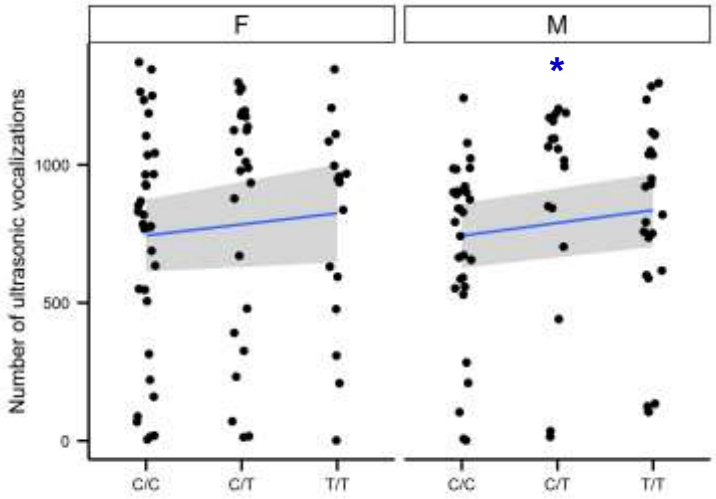

Figure 2.5. NT204321 predicts isolation induced ultrasonic vocalizations in males. Males (M) had a significant association between numbers of USVs and NT204321 genotypes. Females (F) USVs were not associated with genotypes. * $P < 0.04$, Kruskal-Wallis test. Data is shown as individual number of USV calls with trend line ± standard error of the mean.

Previous experiments found that artificially elevating NAcc OXTR density using viral vector gene transfer enhanced the propensity of subjects to form a partner preference. Since T/T voles have high NAcc OXTR density I reasoned the T/T group would have enhanced ability to develop a social attachment following a brief cohabitation with a partner. I paired male subjects with a sexually naïve, unreceptive female for only 6 hours and female subjects were ovariectomized and paired with sexually naïve males for 6 hours.

Males and females were run through partner preference tests and analyzed independently. I evaluated the effects of genotype on behavior by analyzing huddling duration in a two-way ANOVA with genotype and stimulus (partner or stranger) animal as factors. In males, there was a main effect of stimulus ($F_{1,136}$=5.77, p = 0.0177) but not of genotype ($F_{1,136}$=0.34, p = 0.56) and no significant interaction ($F_{1,136}$=2.68, p = 0.10). Based on Tukey's HSD pair-wise comparisons, I found that male T/T voles spent significantly more time huddling with his partner than the stranger (Mean difference: 21.3 min, p = 0.0248) while C/C males did not (Figure 2.6A). Females of both genotypes formed a partner preference (Student's t-test, $p < 0.05$; Figure 2.6B). Males with the T/T show an enhanced ability to form a partner preference compared to their C/C siblings.

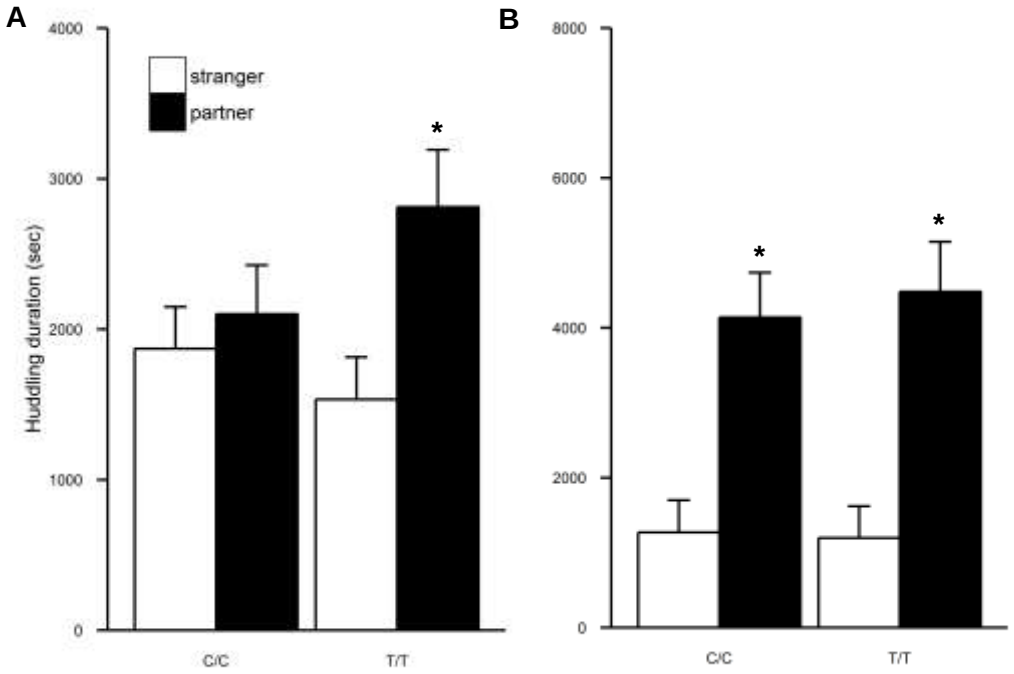

Figure 2.6. The effect of NT204321 genotype on partner preference formation in male and female prairie voles. For both sexes experimental subjects were cohabitated with an opposite sex stimulus animal for 6 hours and immediately tested in the partner preference test. (**A**) The effect of genotype on behavior was investigated using two-way ANOVA. The interaction of genotype x stimulus on huddling duration did not reach significance ($F_{(1,136)}$=2.68, P=0.10). However, males with a T/T NT204321 genotype (n=36) spent significantly more time with the partner than the stranger, while males with the C/C NT204321 genotype (n=34) did not show a partner preference. (**B**) Females of both T/T (n=16) and C/C (n=14) genotypes showed a partner preference under these conditions. * indicates a partner preference, mean partner huddling time is significantly greater than mean stranger huddling duration (t-test, $P < 0.01$).

Since male voles of differing NT204321 genotype demonstrated a varying propensity to pair bond, I aimed to verify that the *oxtr* gene was indeed differentially regulated in the NAcc of the two groups. I performed OXTR autoradiography on the 70 males that underwent partner preference testing. A two-way ANOVA showed a significant main effect of genotype ($F_{1,330}$=169.7, $p < 10^{-15}$) and region ($F_{4,330}$=226.8, $p < 10^{-15}$) and a significant interaction between genotype and region ($F_{4,330}$=67.7, $p < 10^{-15}$). A Tukey's HSD test for pair-wise comparisons revealed that T/T voles had substantially greater OXTR density in

the NAcc than C/C siblings (133% greater, $p < 10^{-6}$). In this cohort, the T/T voles also had higher OXTR density in the PFC (33% greater; $p = 0.000017$). T/T voles did not differ from C/C voles in the Ins, Cl or LS.

To verify that the differences in OXTR density I observed in the previous experiments are indeed due to regulation of *oxtr* mRNA, I performed *in situ* hybridization for *oxtr* mRNA in a randomly selected subset of the males used in the partner preference study (n=31), approximately half were C/C and half T/T. T/T voles had significantly higher *oxtr* mRNA density than C/C voles (linear regression, $p = 8.63 \times 10^{-8}$; Figure 2.7A). Further, I found that *oxtr* mRNA is strongly and significantly correlated with OXTR density ($r = 0.80$, $p = 5.64 \times 10^{-8}$; Figure 2.7B).

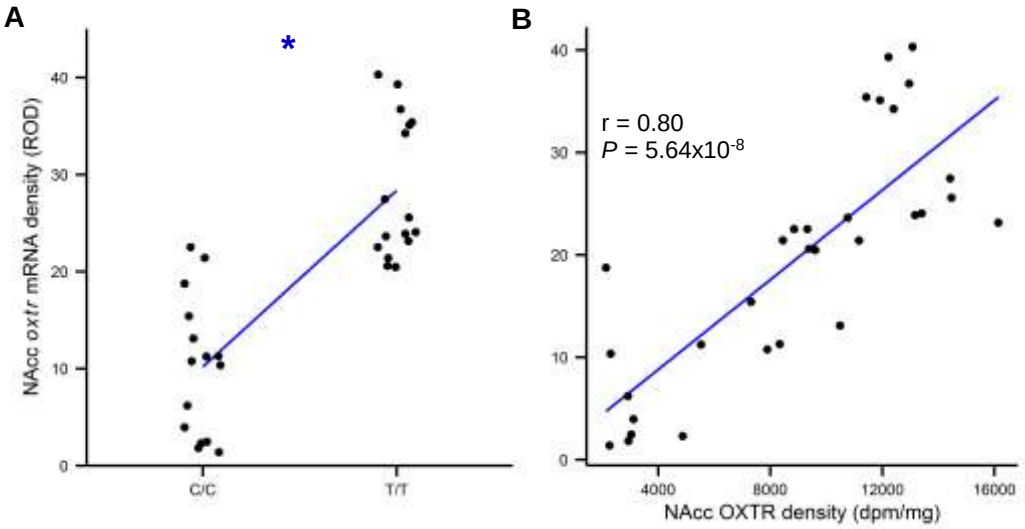

Figure 2.7. OXTR density diversity in the NAcc results from variation in *oxtr* mRNA transcription. Linear regressions were run for *oxtr* mRNA density vs. NT204321 genotype or vs. OXTR density (**A**) *oxtr* mRNA levels in the NAcc are associated NT204321 genotypes. (**B**) NAcc OXTR binding density is significantly correlated with *oxtr* mRNA levels. * $P < 1 \times 10^{-7}$.

DISCUSSION

Diversity in OXTR distribution in the brain, particularly within behaviourally relevant regions like the NAcc, underlies inter- and intra-species

variation in social behavior. The genetic mechanisms leading to brain region specific variation in OXTR have not been explored until now. Genetic variation in functional regulatory elements is very important for the emergence of phenotypic diversity. I here demonstrate that genetic variation in the o*xtr* associates with individual diversity in *oxtr* expression in the prairie vole brain. I found evidence for allelic imbalance of *oxtr* expression in the striatum that implies differential regulation of mRNA production or stability. Additionally, I found that OXTR density and mRNA levels are highly correlated, providing independent evidence that diversity in OXTR expression is controlled at the level of mRNA regulation. Based on these results, I propose that the prairie vole *oxtr* gene has genetic variation in *cis*-REs in LD with SNPs in allelic imbalance that exert substantial influence over expression of the gene.

I note that the data reported here relied on a marker SNP that was initially selected for heterozygosity to be leveraged in the allelic imbalance assay. I do not anticipate that NT204321 is functional, rather it is in LD with one or more other polymorphisms that disrupt *cis*-RE function. Previous studies have identified putative sites for functional interaction with transcription factors or DNA methylation in the human *OXTR* promoter and intron (Mizumoto et al., 1997, Tansey et al., 2010, Gregory et al., 2009, Kusui et al., 2001, Puglia et al., 2015). The prairie vole NAcc may serve as a model of brain region specific control OXTR variation to guide future molecular studies in humans.

In *avpr1a*, a close homolog of *oxtr*, the process of identifying a putative *cis*-RE was facilitated by the presence of a strong signature of variation in the promoter sequence. The *avpr1a* gene resembles *oxtr* in exhibiting diversity in expression between and within species, also with influence over behavioral differences (Phelps and Young, 2003, Hammock and Young, 2005, Young et al.,

1999a, Lim et al., 2004c, Donaldson et al., 2008, Walum et al., 2008, Barrett et al., 2013). In contrast to *oxtr*, *avpr1a* has a large, hypervariable microsatellite in its proximal 5' promoter. This microsatellite contains functional *cis*-REs that drive some of the *avpr1a* diversity (Hammock and Young, 2005, Hammock and Young, 2004). Candidate *cis*-REs can be tested definitively for function *in vivo* using knock-in genetic strategies (Cretekos et al., 2008). Importantly, the prairie vole *avpr1a* microsatellite was shown to drive species and individual differences in such a genetic model (Donaldson and Young, 2013). Such a focused effect of any given *cis*-RE is expected as each functional sequence interacts with factors that can occur selectively in distinct cell types (Linnen et al., 2013).

I found that the genetic influence on *oxtr* expression occurs in a region-specific manner. Robust allelic imbalance is specific to the striatum, and genotype-OXTR density associations are strongest in this region. In this manner, *cis*-REs appear to contribute modular control over OXTR diversity in prairie voles. Given the variety of OXTR distributions across mammalian species, further examination of the regulation of prairie vole striatal *oxtr* expression may provide general insights into the tremendous species diversity in OXTR brain distribution across mammals. The neuropeptide receptors such as *oxtr* and *avpr1a* seem particularly prone to *cis*-RE influence and may thus be uniquely suited for evolutionarily rapid, targeted adjustments to neural networks that are otherwise stable between species.

One such important network with a high degree of conservation is the mesolimbic reward (MLR) network (O'Connell and Hofmann, 2011). In a key region of the MLR, the NAcc, OXTR activation is necessary for partner preference and OXTR variation mediates individual differences. Here, I confirm a role for naturally occurring OXTR density differences in individual variation in

social attachment (Ophir et al., 2012). Males with different genotypes had differential propensity to form a partner preference. While I did not see an effect in females, OXTR activation in NAcc is absolutely necessary for female partner preference (Young, 1999, Young et al., 2001) and previous work with viral vector gene transfer demonstrated effects on female behavioral diversity (Keebaugh and Young, 2011, Ross et al., 2009b). I saw that both C/C and T/T females formed a partner preference in the conditions I tested in this experiment and may indicate a higher female sensitivity to OXT signalling.

Indeed, a previous study from our lab found that high NAcc OXTR density individual females conferred resilience against early life stress (Barrett and Young, submitted). OXTR density is dynamic through development, offering many opportunities for interaction between OXTR variation and environmental influences on sociocognitive development (Wang and Young, 1997, Hammock, 2015). Further work on gene by environment interactions on social behavior will need to understand how *oxtr* variation contributes to diversity throughout development.

The role of OXTR variation as a contributor to individual behavioral differences in humans has become an intensely researched topic, with calls to better understand molecular mechanisms regulating OXTR variation (Kumsta and Heinrichs, 2013). Intranasal delivery of OXT influences basic social cognition in humans (Andari et al., 2010, Guastella et al., 2008, Bartz et al., 2010, Marsh et al., 2010). Individuals have heterogeneous responses to OXT (Bartz et al., 2011, Olff et al., 2013) that sometimes involve interaction with *OXTR* variation (Marsh et al., 2012, Montag et al., 2013). Similar to our findings of gene by environment interactions in voles, early life stress can interact with *OXTR* variation to influence adult social behavior and emotional regulation (Loth et al.,

2014, Lucas-Thompson and Holman, 2013, Smearman et al., 2015, Thompson et al., 2011). Additionally, *OXTR* variation influences whether individuals seek out or benefit from social support during stress (Chen et al., 2011, Kim et al., 2010), demonstrating that, in humans, *OXTR* variation mediates sensitivity to social context and information to influence individual behavior. More work is needed to understand how *OXTR* variation provides this influence to human neural networks.

I have shown that OXTR and AVPR1A exhibit high diversity between and within species. This fact necessitates caution when generalizing findings about these systems between species. However, I would like to suggest here that the distributional diversity of OXTR may, somewhat paradoxically, confer functional stability to OXT across alternative social strategies. I and others hypothesize that a conserved function of OXT is to increase the attentional and motivational salience of social information (Modi and Young, 2012, Young, 2015). Within the diversity in OXTR distributions, conserved sites of OXTR binding emerge within members of a given clade. Rodents generally share binding of OXTR, AVPR1A or both in olfactory processing regions such as the olfactory bulb and anterior olfactory nucleus, as reviewed by Beery et al. (Beery et al., 2008). Primates, on the other hand, share OXTR binding in attentional, visual and auditory processing nuclei (Freeman et al., 2014a, Freeman et al., 2014b, Schorscher-Petcu et al., 2009, Young et al., 1999b). In both cases, primary sensory systems are modulated by OXTR. Intriguingly, Bendesky and Bargmann identify sensory processing systems as a target system on which genetic polymorphisms may act to generate individual variation in behavior (Bendesky and Bargmann, 2011). The susceptibility of OXTR to *cis*-RE generated variation may allow evolutionarily rapid placement of OXTR within brain regions involved in

processing of species-favored modalities. Future studies may focus on OXTR density variation in brain regions involved in sensory integration and attention as possible loci of individual behavioral variation.

I have identified a genetic marker in the *oxtr* gene that robustly predicts OXTR binding in a brain-region specific manner. These findings provide an opportunity to explore genetic mechanisms underlying individual variation in social behavior with implications for human social cognition and even psychiatric disorders. Further studies to identify putative functional elements may provide exciting insights into the precise genetic mechanism generating social diversity.

METHODS

Animals

Prairie voles were housed in same-sex groups with 2–3 voles/cage from the time of weaning at 21 days of age. Housing consisted of a ventilated 36×18×19 cm Plexigas cage filled with Bed-o-Cob Laboratory Animal Bedding under a 14:10 h light/dark cycle (lights on 7:00 AM–9:00 PM) at 22 °C with access to food (rabbit LabDiet) and water ad libitum. The prairie voles were obtained from our laboratory breeding colony that was originally derived from field captured voles in Illinois. Subjects were weaned at 21 days. Voles were either toe clipped at post-natal day 7 (PND7) or ear clipped at PND21 to provide individual identification and collect tissue for genotyping. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Sanger sequencing and polymorphism discovery

I collected brain and liver tissue from 19 male prairies voles that were euthanized with CO_2. Tissue was immediately frozen in crushed dry ice. DNA

was isolated using a Qiagen DNeasy kit (Germantown, MD). PCR primers targeting five targets presumed to be transcribed into *oxtr* mRNA were designed using sequence from a BAC clone including prairie vole *oxtr* sequence, DP001215.2. The putative prairie vole transcript was identified by BLAST alignment of the mouse *Oxtr* transcript (NM_001081147.1) to the prairie vole BAC clone. PCR primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The five loci covered the two coding sequence exons plus the 5' UTR and parts of the 3' UTR. The thermocycler program used for all five reactions was, initial denature: 94˚ C – 5m 30 s, 35x cycles: 1) denature: 94˚ C – 30 s, 2) anneal: 51˚ C – 30 s, 3) elongation: 72˚ C – 30 s; final elongation: 72˚ C – 10 min. The primers for the loci, in order and beginning at the 5' end of the gene were, locus 1, forward: 5'-ATAAGCAGCAGCAGGTAGG-3', reverse: 5'-ACACGCGTCCTAAGAGAAG-3'; locus 2, forward: 5'-TAGCAGAACTAGGGCCTACC-3', reverse: 5'-AGGTGCACATTTTCTCACTG-3'; locus 3, forward: 5'-CTGAAGATGGTTGAGAGCAG-3', reverse: 5'-AGTTCAGAGAAAGGGTGAGG-3', locus 4, forward: 5'-CAAGCATCAGCTTCTTCAAC-3', reverse: 5'-GTGCTTCTACTACCCCAACC-3'; locus 5, forward: 5'-GTGGAGTCTGAGGAAGGAAG-3', reverse: 5'-TGCTGTTCACTGAGTATTTGC-3'.

PCR amplicons were sequenced at Beckman Coulter Genomics (Danvers, MA). Reads were analyzed using Lasergene software (DNASTAR, Madison, WI). NT204321, located in the 3'UTR at bp 204321, was polymorphic (minor allele frequency: 0.33). Due to the prevalence of NT204321 in our colony, it was chosen as a marker for allelic expression imbalance.

Allelic imbalance

Adult prairie voles used for this experiment were identified as heterozygous at NT204321. Subjects were euthanized with CO_2. Brains were frozen in crushed dry ice and stored at -80˚ C. Brains were sectioned in a cryostat at 300 μm. Sections were placed onto chilled glass slides. A microdissecting tool was used to collect tissue. Tissue was transferred to lysis buffer from the Qiagen mRNA/DNA Micro Kit. The mRNA was isolated immediately then stored at -20˚ C. DNA fractions were stored at 4˚ C and isolated later. I made cDNA from mRNA using poly-d(T) primers (Promega, Madison, WI), reverse transcription was performed with the Qiagen Omniscript Kit.

Sequences containing NT204321 PCR amplified from cDNA and genomic DNA (gDNA). PCRs on brain-derived nucleic acid samples were performed using the FailSafe PCR kit master mix plus Buffer E (Epicentre, Madison, WI). All PCRs used the following touch-down PCR protocol: initial denature: 98˚ C – 5 min; 30x cycles: 1) denature: 94˚ C – 30 s, 2) anneal: *variable see below – 30 s, 3) elongation: 72˚ C – 45 s; final elongation: 72˚ C – 10 min. Annealing temperatures were variable, for each set of cycles, given temperatures were used: 1-2: 65˚ C, 3-4: 63˚ C, 5-6: 61˚ C, 7-8: 59˚ C, 9-10: 57˚ C, 11-12: 55˚ C, 13-14: 53˚ C, 15-16: 51˚ C, 17-30: 50˚ C. The primers used were thus (note that reverse primers were biotin labelled at the 5' end), NT204321: forward, 5'-CTAGGCTTTGGTTGGGGAAATAAC-3',reverse: biotinylation-5'-TTGGGTCTTGTTATGGTCCTGAC-3', sequencing primer: 5'-GGAAATAACAAGAAATGG-3'.

PCR amplicons were pyrosequenced at the University of Alabama, Heflin Center for Genomic Sciences Genomics Core (Birmingham, AL). For each individual-brain region combination, two cDNA reactions were performed. Three replicate PCRs were performed per nucleic acid sample; for each individual brain

region, I pyrosequenced 6 cDNA replicates and 3 gDNA replicates. A sample was included in analysis if 66% of these reads passed a quality check.

Allelic ratios were calculated for cDNA (representing mRNA) and gDNA by dividing the T-allele allelic quotient by the C-allele quotient. A mean ratio for cDNA and gDNA was calculated for each individual-brain region combination. An average allelic ratio was calculated for all gDNA samples, and this mean gDNA ratio was used to normalize individual allelic ratios. The cDNA allelic ratio was considered to have significant allelic expression imbalance if it was greater than the mean gDNA allelic ratio plus three gDNA standard deviations for that region (Barrie et al., 2014).

Genotyping

DNA was isolated using a Qiagen DNeasy Kit. A 140 bp amplicon including NT204321 was amplified by PCR, using a Qiagen Taq PCR Master Mix Kit. The thermocycler program used was, initial denature: 94˚ C – 5 min 30s; 35x cycles: 1) denature: 94˚ C – 30 s, 2) anneal: 53˚ C – 30 s, 3) elongation: 72˚ C – 30 s; final elongation: 72˚ C – 10 min. Amplicons were digested for 1.5 hours at 37˚ C with the SSP1 restriction enzyme (New England Biolabs, Ipswich, MA). SSP1 cuts the T-allele of NT204321 but not the C-allele. Thus, resultant banding patterns were used to identify genotypes: a single band 140 bp band for C/Cs, three bands for C/Ts, two smaller bands for T/Ts. The primers used for this reaction were, forward: 5'-CTAGGCTTTGGTTGGGGAAATAAC-3', reverse: 5'-TTGGGTCTTGTTATGGTCCTGAC-3'.

Ultrasonic vocalizations

At PND7, breeder cages were transported to a behavioral testing room and allowed to habituate for at least 15 minutes before pups were disturbed. On a cage by cage basis, the entire litter was removed and placed in a plexiglass cage with fresh bedding at room temperature. Each pup was transported into an adjacent room, where it was placed into another plexiglass cage, recorded by video for locomotion and recorded for ultrasonic vocalizations (USVs) over 5 minutes. After recording, the pup was transported back to the staging room, toe clipped for ID and tissue and replaced to the breeder cage.

USVs were recorded using a Sonotrack microphone and recording apparatus (Metris, Netherlands). Using default parameters, the Sonotrack calculated the total number of USV calls emitted by each subject.

Partner preference test

The partner preference was performed as previously described[47]. Each subject was first conditioned to a stimulus animal, the partner. The duration of cohabitation is an important parameter, longer durations increase the probability that individuals will form a partner preference formation. For these experiments, all cohabitations were 6 h and took place during the morning preceding testing. Two partner preference tests were run each day, with all handling taking place during light hours. Following cohabitation, the subject and partner were isolated. Partners as well as strangers unfamiliar to the subject but with the same gender and sociosexual experience as the partner were tethered to opposing ends of a three-chamber Plexiglass arena, total dimensions: 75 cm x 20 cm x 30 cm. The subject was placed in the centre of the arena and allowed to freely roam and interact with stimulus animals for 3 h. Experimenters were blind to subject genotype during this setup period. Subjects were counterbalanced across

genotype for day and order of testing. Behavior was recorded from a digital camera above the arenas and side-by-side huddling duration between the subject and each stimulus is scored automatically with SocialScan 2.0 (Clever Sys Inc, Reston, VA).

Males

Male subjects were toe-clipped at PND7 for genotyping then cohoused with same-sex siblings or age matched cage mates until adulthood. Subjects were grouped by NT204321 genotype, 36 voles with a T/T genotype and 34 voles with C/C genotype were tested between PND60 and PND190, I confirmed genotypes for all of these subjects. Stimulus animals for the partner preference tests were sexually naïve and intact females. Female stimulus voles were cohoused in cages of 2-3 until the time of pairing with each subject. Male subjects were cohoused with sexually naive females (partner) for 6 hours. Sexually naïve females require olfactory stimulation from the urine of a novel male to become sexually active, this process requires approximately 24 hours[48] so I presume that no mating occurred during the brief cohabitation. After cohabitation, females were removed from the cage and tethered to serve as stimuli for the partner preference test. Each partner was used as a stimulus in two subsequent tests during the same day. Subjects were counter-balanced between genotype groups for order of test during a day.

Females

Females were toe-clipped at PND7 then cohoused with same-sex siblings or age matched cagemates until PND 60 - 120, when they were ovariectomized under isoflurane anesthesia. Subjects were grouped by NT204321 genotype, 14 voles with a T/T genotype and 16 voles with C/C genotype were tested and tissue was

not collected from these subjects. After a minimum of two weeks recovery, subjects were placed into a fresh cage with a novel male, which was also naïve to opposite sex encounters. After 6 hours of cohabitation. Male partners were removed from the cage and tethered to serve as a stimulus during a 3 h partner preference test. Each partner was used as a stimulus in two subsequent tests during the same day. Subjects were counter-balanced between genotype groups for order of test during a day.

Oxytocin receptor autoradiography

All animals analyzed for genotype-phenotype associations were confirmed for *oxtr* genotypes as above through additional genotyping of DNA isolated from caudal brain tissue. Freshly frozen brains were stored at -80˚ C. Coronal sections were cut in a cryostat at temperatures between -16˚ C and 18˚ C. 20 µm sections were collected and then stored at -80C until use in autoradiography or *in situ* hybridization. Oxytocin receptor autoradiography was performed as previously reported[19]. Tissue was removed from -80˚ C storage and air dried, then dipped into 0.1% paraformaldehyde in 7.4 pH PBS. Next, tissue was rinsed in 50 mM Tris buffer before a 1 h incubation in 50 pM ^{125}I-OVTA (NEX 254050UC, PerkinElmer). Finally, sections were dipped through four washes 50 mM Tris with 2% MgCl and underwent a rinse in ddH_20 and then air dried. Dry sections were exposed to BioMax MR film (Kodak) for 72 h. Processed film was imaged on a light box. Images were taken and analyzed with MCID software. Digital images were obtained with a light box and a 12-bit QICAM camera (QImaging, Surrey, BC, Canada). OXTR binding density was calculated by capturing raw optical density (ROD) values from regions of interest. Evaluators were blind to genotype during scoring. A semi-quantitative measure of OXTR binding density was

calculated: disintegrations per minute per milligram of tissue (dpm/mg) was estimated by comparing ROD values to a ^{125}I standard. Background binding was captured from regions of the brain with consistent lack of signal such as the corpus callosum. Specific OXTR binding density was finally calculated by subtracting mean background binding from the regions quantified for each section of tissue. Brightness and contrast of representative images were equally adjusted for all autoradiography images within a panel using Adobe Photoshop CS3.

In situ hybridization

Sense and antisense ^{35}S-UTP-labeled RNA probes for prairie vole *oxtr* mRNA were generated as described previously[33]. The antisense RNA probe was complementary to the prairie vole sequence corresponding to base pairs 101–1272 of predicted *oxtr* mRNA (RefSeq accession number XM005364985). Twenty μm cryosections adjacent to the slices used for OVTA autoradiography were hybridized with the probes, and were exposed to BAS-TR2025 phosphoimaging plate (Fujifilm, Tokyo, Japan) for a month and then, to Kodak BioMax MR films for 7 months. For quantitative analysis, phoshoimaging plates were scanned with BAS-5000 and analyzed using Multi Gauge V3.1 software. ROD was captured for NAcc and a region of each section with background, such as the corpus callosum Evaluators were blind to genotype during scoring. Specific NAcc ROD was calculated by subtracting mean background from mean NAcc for each section. Representative images were taken from film autoradiograms, digital images were obtained using a light box and a QICAM camera connected to a computer. Brightness and contrast of representative images were equally adjusted for all *in situ* images within a panel using Adobe Photoshop CS3.

Statistical analysis

All statistical analyses were performed in the R statistical software package version 3.1.1, unless stated otherwise. No *a priori* power calculations were performed to select group sizes, however our samples are similar in size to a previous report from our lab investigating phenotype – genotype relationships (Carter et al., 1987). Analyses for partner preference data were performed using 2-way ANOVAS, with time spent huddling as the outcome measure and the interaction between genotype and stimulus animal as the main predictor. The ultrasonic vocalization data was not normally distributed and I analyzed those data with a Kruskal-Wallis test. All partner preference data was normally distributed. The ANOVAS were followed up by planned post hoc analyses using the Welch two sample t-test, with the Bonferroni correction for multiple comparisons. Allelic expression imbalance data were analyzed as stated above: cDNA allelic ratio was considered to have significant allelic expression imbalance if it was greater than the mean gDNA allelic ratio plus three gDNA standard deviations for that region (Barrie et al., 2014). Associations between genetic information and brain data were examined using linear regression with Bonferroni corrections for multiple comparisons, the common method for analysing genotype-phenotype associations. For linear regression, genotypes were modelled as 0, 1 and 2, with the number representing the number of high OXTR-associated alleles: the T-allele in the case of NT204321 and C-allele in the case of NT213739. For all reported associations using linear regression I investigated the residuals and found they were normally distributed. Regarding the factor analyses, to determine how many factors to extract I used the "nFactors" package in R and the factor extraction decisions were based on the eigenvalues-greater-than-one rule, parallel analysis, the optimal coordinates

method and acceleration factor. Exploratory factor analyses were performed with the factanal() function, using “varimax” as the rotation method.

CHAPTER 3

Dissection of the prairie vole oxytocin receptor gene to identify robust markers of brain region specific transcriptional variation

ABSTRACT

OXT signalling is critical in social cognition. OXT achieves a conserved function across diverse species by interacting with a single receptor, OXTR. OXTR binding occurs in unique distributions across species and confers unique network responses to widespread OXT secretion. Understanding OXTR diversity is therefore critical to understand the evolution of social behaviors between and within species. In Chapter 2 I identified a 3' UTR SNP, NT204321, I believe to be in LD with *cis*-REs that stimulate OXTR diversity. NT204321 genotype predicts NAcc OXTR density and social attachment behavior in males. Here, I performed targeted, next-generation sequencing of the *oxtr* gene in 45 prairie voles with the aim of identifying copious polymorphism information to be used in a detailed association study. I identified close to 3,000 SNPs, of which 967 were selected for further study after conservative filtering. Microsatellites and insertion/deletions were not analyzed. The 967 SNPs were enriched for strong associations with OXTR density in the NAcc and this inflation was driven by strong LD (LD) structure amongst SNPs. After pruning based on LD, only 15 SNPs remained, including NT213739, an intronic SNP that explains at least 75% of the variation in NAcc OXTR density. None of the other 14 SNPs proved additionally informative. The strong LD structure prevents further localization of putative *cis*-REs with this sample. Future studies will investigate a small set of SNPs in 100% LD with NT213739. NT213739 will serve as robust marker of NAcc OXTR density for future studies on individual variation in neural and behavioral phenotypes.

INTRODUCTION

Oxytocin (OXT) and its single receptor (OXTR) constitute a critical neuromodulatory system underpinning social cognition and behavior. Furthermore, the OXT system contributes to inter- and intra-species behavioral diversity through variable expression of the *oxtr* gene. Vertebrate species exhibit unique distributions of OXTR binding throughout the brain. In the socially monogamous prairie vole, OXTR binding is highly variable in the nucleus accumbens (NAcc), which has implications for individual differences in behavior. In Chapter 2, I identified a marker for NAcc OXTR binding density, NT204321, and provided evidence that OXTR density variation stems from transcriptional regulation of the prairie vole *oxtr*.

The prairie vole offers a tractable model system to understand how OXTR diversity contributes to neural organization of individual differences in social behavior. Such a model will be useful in understanding the basic biology of the OXT system, which may have implications for human health. Genetic variants in the human *OXTR* are associated with autism spectrum diagnosis (Di Napoli et al., 2014, Egawa et al., 2015, Egawa et al., 2013, Campbell et al., 2011, Wu et al., 2005, Liu et al., 2010, Lerer et al., 2008, Jacob et al., 2007, Wermter et al., 2010). While some studies have failed to observe associations, or found inconsistent results for certain variants, a recent meta-analysis of these studies concludes that *OXTR* is indeed involved with the development of autism (LoParo and Waldman, 2014). There is a clear role of *OXTR* in human social cognition that could contribute to the deficits in social interaction seen in autism. Variants in *OXTR* have a general role in in social information processing, with effects on attention to social cues (Tops et al., 2011, Wade et al., 2014), social recognition (Skuse et al., 2014), and ability to infer emotions (Uzefovsky et al., 2015, Lucht et

al., 2013, Slane et al., 2014, Parker et al., 2014, Massey et al., 2015). All of the variants discussed above are non-coding, suggesting a role for differential regulation of *OXTR* in the individual differences and diseases risk presented by the gene. Indeed, the expression of the human *OXTR* gene is under *cis*-RE influence in the human brain (Tansey et al., 2010, Gregory et al., 2009).

Diversity in complex phenotypes arises largely due to mutations in non-coding regulatory elements (*cis*-REs) such as promoters and enhancers (Wittkopp and Kalay, 2012). These *cis*-REs interact with cell specific transcription factors and changes *cis*-RE sequence leads to variation in gene expression that result in discrete phenotypic changes, which are much less likely to be deleterious than protein coding mutations (Wittkopp et al., 2009, Linnen et al., 2013, Andzelm et al., 2015). Given the relatively subtle effects of *cis*-REs, functional evidence can be difficult to amass. *In vitro* reporter expression assays can be useful but require testing a sequence outside of the endogenous nuclear context, which offers limited validity for cell-specific expression phenotypes. Another strategy involves the fine-scale mapping of variants to identify haplotypes, or predictable sets of genetic variants in LD, and map them to native expression phenotypes (Bendesky et al., 2012, Barrie et al., 2014, Wittkopp et al., 2009, Lim et al., 2007a, Wang et al., 2013). After establishing strong evidence for a *cis*-RE, an *in vivo* test of function can be performed with knock-in genetic models (Donaldson and Young, 2013, Cretekos et al., 2008).

While identifying *cis*-REs in prairie voles is unlikely to reveal conserved *cis*-REs in humans, understanding the kinds of transcription factors that interact with *oxtr* in neural tissues may reveal general patterns of regulation. For example, monogamous mammals have convergent OXTR or homologous AVPR1A binding in mesolimbic reward regions (Schorscher-Petcu et al., 2009, Insel and

Shapiro, 1992, Freeman et al., 2014b). Perhaps these peptide receptor genes have a propensity to evolve *cis*-REs sensitive to transcription factors that are unique to striatal tissues, such as the dopamine receptor related factor (DRRF), which competes with a ubiquitous activator to modulate expression in a brain region specific manner (Hwang et al., 2001).

In Chapter 2, using a marker of NAcc OXTR density – NT204321, I established C/T breeding pairs and produced C/C and T/T offspring for anatomical and behavioral comparisons. In a group of 70 animals that were analyzed for partner preference behavior, I found that NT204321 predicted OXTR density robustly in the NAcc and significantly but less robustly in the PFC. Using exploratory factor analysis, I identified patterns of covariation for OXTR density between brain regions.

Some patterns of covariation in OXTR binding have previously been observed. One study previously found that NAcc and LS were negatively correlated (Olazabal and Young, 2006b). Using an unsupervised hierarchical clustering method, Ophir et al. observed that OXTR binding in NAcc and PFC are related (Ophir et al., 2009). In addition, a colleague in the Young lab noted a positive correlation between NAcc and PFC (Inoue and Young, unpublished data). Further, in an OXTR autoradiography experiment where *oxtr* mRNA-targeting siRNA was injected unilaterally into the NAcc through viral vector mediated gene transfer, the ipsilateral PFC appeared to have diminished OXTR density scaling with reduced NAcc OXTR binding (Burkett, Keebaugh and Young, unpublished data). This observation could be explained if OXTR is transported to axonal terminals of projections from NAcc or CP or both to the PFC. Dolen et al found that OXTR originally synthesized in dorsal raphe neurons is found in axon terminals in the NAcc, where OXTR modulates 5HT1b receptors

to influence social reward (Dolen et al., 2013). Some instances of OXTR density covariation between brain regions may be due in part to OXTR stored in presynaptic compartments.

With the goal of better understanding the genetic structure of the prairie vole *oxtr* gene and a long term goal of aiding discovery of *cis*-REs for the gene, I finely mapped genetic variants across 70kb of sequence including the gene and surrounding non-coding regions using next-generation sequencing. I selected subjects based on extreme OXTR density in the NAcc in combination with homozygotic genotypes for NT204321 in the 3' untranslated region of the gene. Amongst these voles that were selected from an outbred prairie vole colony, I discovered close to 3,000 single nucleotide polymorphisms (SNPs) as well as insertion/deletion and repeat elements. I used a conservative filtering regime to isolate 967 high confidence SNPs. I found an enrichment of strong associations between most of these SNPs and NAcc OXTR density. One intronic SNP (NT213739, so named because of its location in the reference sequence) emerged as the most strongly associated, NT213739 in the intron, so named because of its location in the reference sequence. Unexpectedly, most SNPs exhibited strong LD across the entire region. Because LD severely limited my ability to use this data to isolate putative locations of *cis*-REs, I removed SNPs in LD with NT213739 that exceeded $R^2 = 0.25$. After further filtering, NT213739 remained the best predictor of NAcc OXTR density. After genotyping more individuals for NT213739, I found that this intronic SNP explains 75% or more of the variation in NAcc OXTR density in three independent replicates. This represented an improvement in predictive power over NT204321, which explained between 30 – 70% of the variation in the same samples. I conclude that NT213739 is a

predictive marker of a haplotype containing an allele of a *cis*-RE with a robust influence over *oxtr* expression in the prairie vole striatum.

RESULTS

A genetic marker of *cis*-RE activity selectively predicts OXTR density variation in the NAcc

I here revisit autoradiography data presented in Chapter 2. In addition to those 70 subjects, I analyzed an additional 8 male voles in the same autoradiography exposure. For NAcc and six additional brain regions, I ran a linear regression for OXTR density against NT204321 genotype (C/C or T/T). For all regions except NAcc, I added NAcc as a covariate, thus controlling for the possibility that OXTR synthesized in that region influence patterns of variation elsewhere.

As illustrated in Figure 3.1, the striatal subregions and olfactory tubercle (Tu), all mesolimbic reward regions, exhibited a high range of variation in OXTR density, while other brain regions had more constrained OXTR variation. As previously shown, NT204321 genotype significantly predicted NAcc OXTR binding density in the NAcc, $b = 3240$, $t(76) = 11.73$, $p < 2.2 \times 10^{-16}$. In addition, NT204321 genotype explained a significant portion of variance in OXTR density, $R^2 = 0.64$. NT204321 genotype also significantly predicted OXTR binding density in the CP, $b = 1035$, $t(75) = 5.05$, $p = 3.07 \times 10^{-6}$. NT204321 genotype did not significantly predict OXTR binding density in the Tu, prefrontal cortex (PFC), insular cortex (Ins), claustrum (Cl) or dorsal lateral septum (LS) ($p > 0.05$).

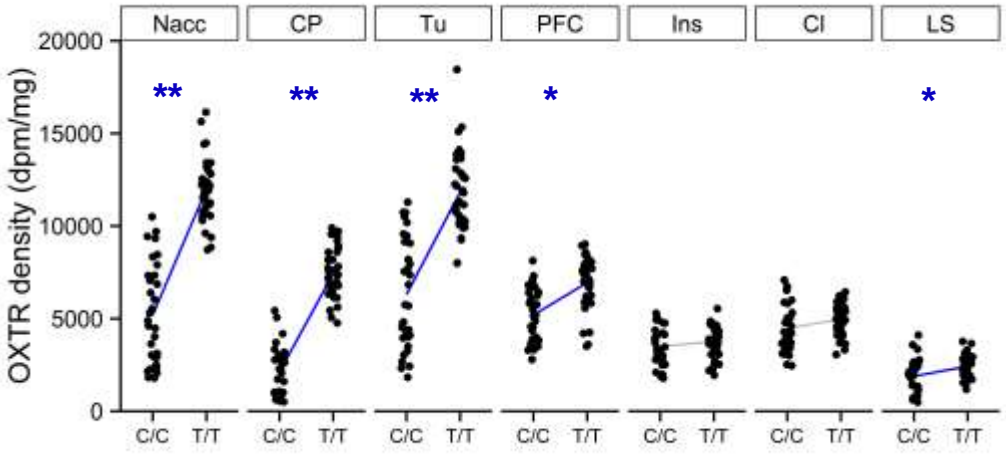

Figure 3.1. NT204321 is strongly associated with OXTR density in striatal subregions. Individual OXTR densities for 78 prairie voles are plotted for each region. Of interest is the large variation in the NAcc, CP and Tu, all regions in the mesolimbic reward network. Linear regressions were performed to analyze associations between OXTR density and NT204321 genotype. NAcc, nucleus accumbens; CP, caudate putamen; Tu, olfactory tubercle; PFC, prefrontal cortex (including prelimbic and infralimbic cortex); Ins, insular cortex; Cl, claustrum; LS, dorsal lateral septum. $^{**}P < 10^{-12}$, $^{*}P < 0.007$.

As indicated by the factor analyses in Chapter 2, there were some patterns of covariation for OXTR density between NAcc and other brain regions. NAcc OXTR binding was associated with OXTR density in the CP, $t(75) = 9.28$, $p = 4.32 \times 10^{-14}$; Tu, $t(75) = 11.36$, $p = 2.2 \times 10^{-16}$; and LS, $t(75) = 4.63$, $p = 1.52 \times 10^{-5}$. The NAcc and CP are both striatal subregions and Tu OXTR density is noticeably correlated with striatal OXTR in autoradiography film images. The Tu is both a region in the mesolimbic reward network and an olfactory processing region that stores odor cue valence following reinforcement training (Gadziola et al., 2015). Given the hypothesis that OXTR plays a general role in processing socially relevant information, the Tu may be an important mediator of the role of OXT on social recognition and reward in prairie voles.

Sample selection and sequencing for polymorphism discovery in the prairie vole *oxtr* gene

As reported in the previous chapter and illustrated above, striatal sub-regions have an expanded range of variation in OXTR density relative to other brain regions exhibit OXTR binding. Additionally, *oxtr* mRNA in the NAcc uniquely exhibits allelic imbalance, a signature of *cis*-RE activity. A marker of allelic imbalance, NT204321, further predicts OXTR density only the NAcc, suggesting that *cis*-RE activity uniquely stimulates variation in *oxtr* expression. Identifying these functional sequences could yield important information about how the OXT system contributes to individual variation in social behavior in the prairie vole model and potentially in other mammals such as humans.

Given the strong genetic influence on NAcc OXTR binding, I reasoned that a detailed expression quantitative trait locus (eQTL) mapping approach could reveal locations of putative functional significance in non-coding sequence near the *oxtr* gene. The eQTL approach has recently yielded much success through the use of next-generation sequencing technologies (Albert and Kruglyak, 2015). Here, I planned to enrich and sequence the regions surrounding and including the prairie vole *oxtr* gene in a number of individuals to characterize the pattern of genetic polymorphisms.

I chose 45 individual prairie voles for sequencing with the Illumina platform. These voles were selected from the pool of 78 that I had previously phenotyped. As seen in Figure 3.1, individuals within the NT204321 C/C and T/T genotype groups, while having significant mean differences, still overlap to a certain degree. I selected 10-13 individuals from each of four categories, intersecting on either end of the range: high (HI) or low (LO) and by NT204321 genotype T/T (T) or C/C (C) (Figure 3.2A). The HI and LO categories approximately consisted of the upper and lower quartile of NAcc OXTR density from each NT204321 genotype. The HI-C and LO-T individuals have very similar

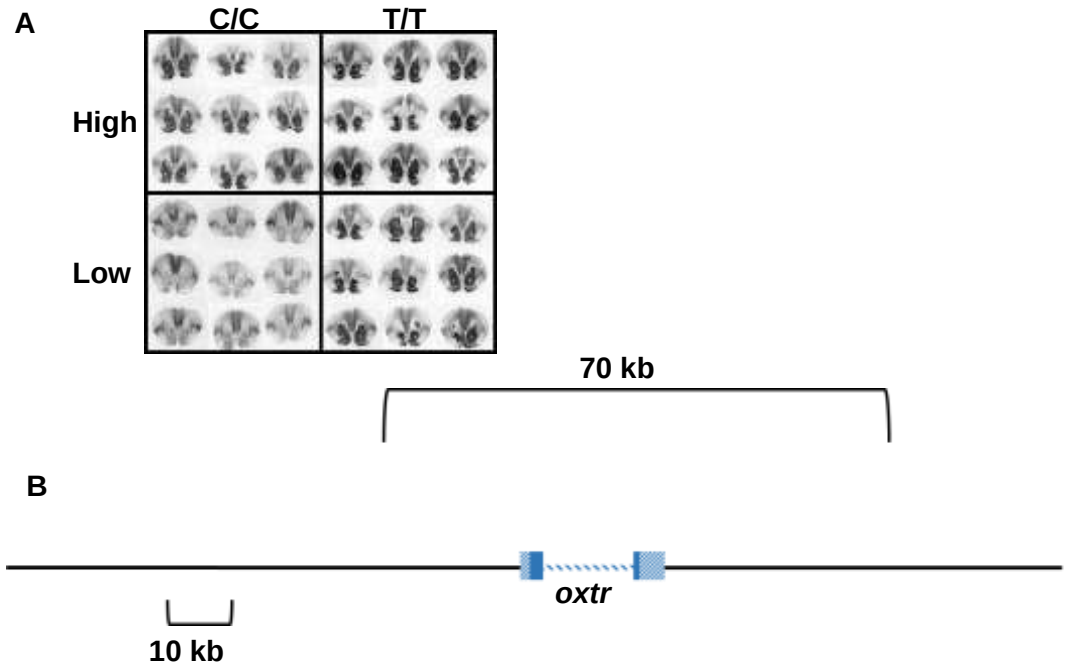

Figure 3.2. Subject selection and sequencing strategy. (**A**) Representative images from the 45 subjects selected for OXTR binding density. I identified the highest and lowest OXTR density animals from each NT204321 genotype (**B**) I targeted approximately 70kb of DNA for sequencing.

mean densities, as given in disintegrations per minute per milligram of tissue (dpm/mg) (11086 vs. 12222 dpm/mg respectively). I hypothesized that NT204321 was either an imperfect marker of the actual functional *cis*-RE or that NT204321 was a high-fidelity marker for an element that interacts with secondary elements to generate the full range of OXTR variation. In the first case, I expected that an eQTL approach might identify another SNP or set of SNPs in LD (as a haplotype) with an even stronger relationship with NAcc OXTR density than NT204321. In such a case, I expected HI-C and LO-T individuals would tend to be heterozygous for the newly discovered polymorphism, which could explain their intermediate phenotypes. In the second case, LO-C and LO-T individuals might share allele identities at secondary markers relative to the HI-C and HI-T individuals. Such secondary markers would represent one or more *cis*-REs in epistasis with the NT204321 associated elemenAfter verifying NT204321 genotype for each of the

45 individuals from DNA isolated from brain tissue, I amplified approximately 70kb of DNA sequence from each individual with 10 long-range PCR amplicons (6-10 kb each). The *oxtr* gene is approximately 22 kb long in prairie voles, so I enriched close to 30 kb of downstream and upstream sequence in addition to the gene itself, where *cis*-REs could potentially be found (Figure 3.2B). Individuals were pooled and given multiplex IDs before sequencing on an Illumina HiSeq1000 machine. Reads were processed and mapped to a prairie vole BAC clone, DP001215, which consists of two smaller BAC clone sequences spliced together. The smaller BAC clone used in Chapter 2 was used to create the DP001215 sequence so the sequence composition for overlapping regions is exactly the same between the two. Read and mapping quality were high, yielding high confidence variant information.

Association analyses

I initially identified approximately 3,000 SNPs within the 70kb window of sequencing. Other polymorphic features such as insertion/deletions and repetitive sequences are present in abundance but more difficult to systematically characterize. The SNPs offer a substantial amount of variant information to work with for a first pass analysis. Using VCFtools to filter out low quality, less informative SNPs, I pruned SNPs with a minor allele frequency less than 0.01 (to exclude markers without variability in my sample), mean depth below 100 reads, mean quality score below 1000 and markers with missing values for any individual. This process left 967 SNPs for further analysis.

Linear regression models were used to investigate the associations between the 967 remaining SNPS and NAcc OXTR density using PLINK. Figure 3.3 shows a QQ-plot of *p*-values from those regressions. The plot shows an

enrichment of unexpectedly small *p*-values given the number of associations performed. Many of the 967 SNPS have strong associations with NAcc OXTR density. There is strong LD between many of these SNPs, which results in horizontal organization of some *P*-values, indicating groups of SNPs in perfect LD thus giving the same *P*-value when regressed against OXTR density. Since this strong LD is not conducive to the goal of isolating regions with the strong correlations, I aimed to further reduce the number of SNPs to be tested by pruning for LD.

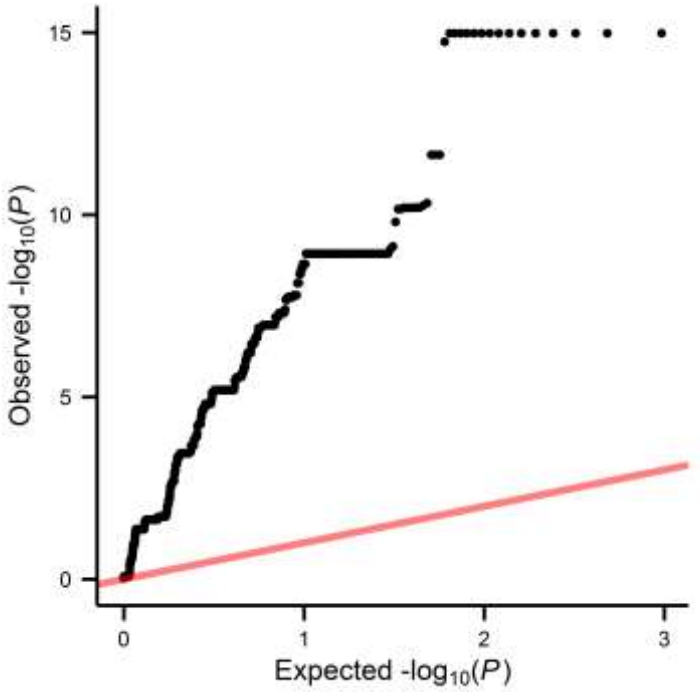

Figure 3.3. SNPs near *oxtr* are strongly associated with OXTR density in the NAcc. Quantile-quantile plot of the associations between the 967 oxtr markers and NAcc OXTR density. The plot shows that there are many more significant *P*-values than expected by chance for the number of association tests performed.

I was primarily interested in identifying variants that could potentially be functional and therefore focused on the SNPs with the lowest *P*-values (and largest effect sizes). Due to linkage disequilibrium, 15 SNPs spanning a 30 kb

region were associated with NAcc OXTR density with the same, minimal *P*-value (*P*=1.06x10-15, adjusted R^2=0.78).

In order to assess whether any of the 15 most associated SNPs are more likely than others to lie in a putative *cis*-RE, I investigated their homology with regions of the mouse *Oxtr* gene that overlap with signatures of functional activity occurring within neural tissues where OXTR is expressed in the mouse (Mouse et al., 2012). I mapped vole sequence containing these SNPs and surrounding sequence of approximately 500 bp per SNP to the mouse Oxtr gene (NCBI37/mm9 build). I compared our vole sequences to ENCODE tracks for markers of general transcriptional activity such as DNase hypersensitivity, H3K4me1, and H3K27a as well as binding of the transcription factor CTCF, which can act as a canonical transcription factor or as an organizer of genomic architecture (Ong and Corces, 2014). Only one SNP overlapped with strong signatures of transcriptional function, a SNP occurring at nucleotide 213739 (NT213739, minor allele frequency: 0.32) (Figure 3.4). The sequence containing NT213739 overlapped peaks of DNase hypersensitivity and CTCF binding within the large intron that is proposed to contain cis-REs in mammals. Based on this evidence I chose to further investigate the predictive power of NT213739 in two additional samples of voles to determine how effective that SNP is at predicting OXTR NAcc.

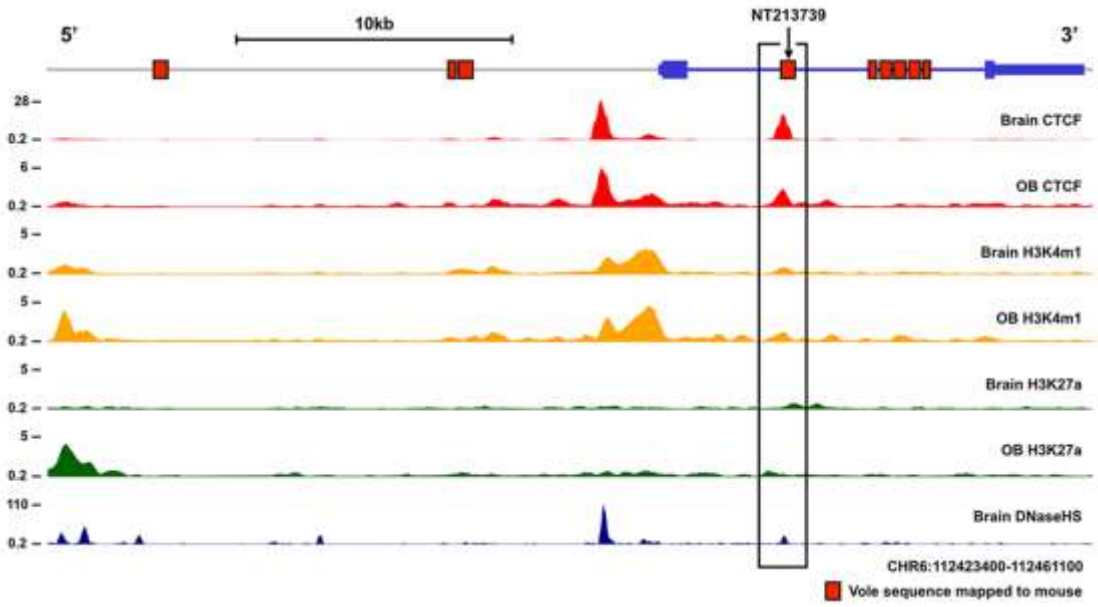

Figure 3.4. Identification of NT213739 as a marker of NAcc OXTR density. A schematic of the mouse *Oxtr* gene with accompanying functional data from the ENCODE project. The prairie vole sequences containing the SNPs showing the strongest association with NAcc OXTR density that also map to mouse *Oxtr* sequence. Approximately 500 bp per SNP of prairie vole sequence surrounding each SNP was aligned to the mouse genome as indicated by red rectangles in the figure. ChIP-seq signal or DNaseHS signal is shown. OB, olfactory bulb; Brain, whole brain; CTCF, CCCTC-binding factor (red) ; H3K4m1, a single methyl modification of lysine 4 of the H3 histone (orange); H3K27a, acetylation modification of lysine 27 on the H3 histone (green); DNaseHS, deoxyribonuclease I hypersensitive sites (dark blue).

NT213739 is an improved marker for striatum OXTR density

The strong LD structure described above was not expected. The *Microtus* genus that prairie voles belong to has the highest rate of molecular evolution of all mammalian species analyzed to date (Triant and Dewoody, 2006). While this phenomenon may prove informative to the natural history of the prairie vole *oxtr* gene in future studies, here it hindered my project goals. The strong LD structure prevented the use of associations between OXTR density and independent SNPs to isolate regions of interest that could contain putative *cis*-REs; none of the SNPs are independent from one another.

Nonetheless, I identified an improved marker of OXTR density, NT213739 (C/T). NT213739 is in the middle of the large 10kb intron of *oxtr*. Indeed this SNP has improved power to predict OXTR density in the NAcc compared to the initial marker, NT204321 (Figure 3.5). I performed linear regressions for three independent OXTR autoradiography data sets, containing more than 30 individuals each.

As reported above, in the sample of 45 sequenced voles (Figure 3.5A), NT213739 genotype significantly predicted OXTR binding density in the NAcc and explained a significant portion of the variation, $R^2 = 0.75$. In comparison, the NT204321 genotype explained a significant, but smaller portion of variance, $R^2 = 0.59$, $F(1,43) = 60.5$, $p = 9.79 \times 10^{-10}$.

In the remaining 33 voles from the same autoradiography exposure that were not selected for sequencing, NT213739 genotype again significantly predicted OXTR binding density in the NAcc. The T/T genotype group had significantly less OXTR density in the NAcc than both the heterozygotic genotype C/T, $b = 1868$ dpm/mg, $t(30) = 3.58$, $p = 0.001$, and C/C genotype, $b = 7461$ dpm/mg, $t(30) = 19.23$, $p < 2 \times 10^{-16}$. In addition, NT213739 genotype explained a significant portion of variance in NAcc OXTR density, $R^2 = 0.94$. In comparison, the NT204321 genotype explained a significant, but smaller portion of variance, $R^2 = 0.77$, $F(2,30) = 104.4$ $p = 1.91 \times 10^{-11}$. Since the extreme values of OXTR density were removed from this group for the sequencing, the variance explained is likely inflated by the non-random nature of the group composition. Nonetheless, this set of subjects is at least independent from the other two samples and serves to confirm that NT213739 genotype is a robust marker across samples.

In the sample of 31 mixed sex voles, NT213739 genotype significantly predicted OXTR binding density in the NAcc. The T/T genotype has significantly less OXTR density in the NAcc than both the heterozygotic genotype C/T, b = 2217 dpm/mg, $t(28) = 5.55$, $p = 6.\ 13 \times 10^{-6}$, and C/C genotype, b = 3331 dpm/mg, $t(28)$ = 9.718, $p = 1.80 \times 10^{-10}$. In addition, NT213739 genotype explained a significant portion of variance in NAcc OXTR density, $R^2 = 0.77$. In comparison, the NT204321 genotype explained a significant, but smaller portion of variance, R^2 = 0.28, $F(2,28) = 3.35$, $p = 0.008$.

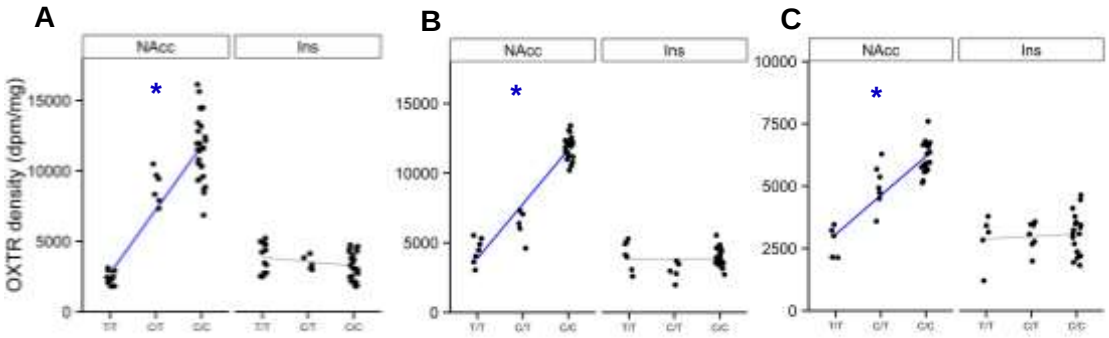

Figure 3.5. NT213739 genotype robustly predicts *oxtr* expression across independent replicates. NAcc OXTR density was associated with NT213739 genotype across three independent samples (**A**) adjusted R^2=0.81, (**B**) adjusted R^2=0.90 and (**C**) adjusted R^2=0.74. OXTR density in the insula (Ins) was not associated with NT213739. Data is shown as individual OXTR density (dpm/mg) or individual mRNA density (ROD) with trend line for the linear regressions. *$P < 1\text{x}10^{-8}$.

DISCUSSION

I found that an intronic SNP, NT213739, is a robust marker of OXTR density in the NAcc. In three independent samples, this marker showed a marked improvement to explain variance of NAcc OXTR density compared to the 3' UTR NT204321. I anticipate that NT213739 will be used to generate groups for future experiments exploring the role of OXTR diversity in neural behavioral phenotypes.

The *cis*-RE variation that I isolated by selecting NT204321 alleles for breeding likely interacts with a transcription factor or factors specific to the striatum. The allelic imbalance phenotype reported in Chapter 2 was unique to the NAcc. In addition, OXTR density by genotype associations show specificity for NAcc and CP as well. The mechanisms of cell-specific transcription are just beginning to be understood. Two examples that I am aware of involve tissue-specific factors that compete with ubiquitous activators to enact tissue specific regulation (Hwang et al., 2001, Andzelm et al., 2015). What is clear is that *cis*-REs confer exquisite control over which cells express an mRNA and how much mRNA is expressed.

Interestingly, after controlling for NAcc density as a covariate, the NT204321 genotype was shown to predict OXTR density exclusively in the striatum, even in a large, well-powered sample. One study has demonstrated that OXTR signalling in presynaptic compartments can have a profound impact on behavior (Dolen et al., 2013). It is currently unknown to what degree OXTR distributions, as visualized with OXTR autoradiography, are due to presynaptic densities. Future characterization of OXTR diversity should take account into account his added layer of complexity.

One goal of this experiment was to leverage the high variation in OXTR density to identify an isolated region or set of regions with exceptional correlation with the phenotype. Such regions could be considered to contain putative *cis*-REs and offer targets for future functional studies. I discovered that in this sample of sequenced individuals, the *oxtr* gene has very strong LD structure. Given that I isolated NT204321 heterozygotes from our outbred laboratory colony, I may have to some degree introduced artificial LD structure into my breeder pairs. Alternatively, a subset of the wild prairie voles Ibred into our colony may have

been carrying this T-associated haplotype from the wild. If this is the case, it may indicate the prairie vole *oxtr* locus is under strong selection or some kind of demographic pressure in the wild. Any such conclusions cannot be made without further analysis of a large number of randomly sampled individuals from a wild population however.

The most highly associated marker, NT213739, is in perfect LD with 12 other SNPs, at least amongst the 45 voles I sequenced. These 13 SNPs will be the target of upcoming investigations aiming to further dissect the prairie vole *oxtr* gene. For example, these SNPs could be sequenced or genotyped in more individuals to track whether any of them are in less LD than currently assumed. Given the growing interest in understanding the role of the OXT system in human social behavior, the prairie vole NAcc may become an important model to understand how diversity in neural and behavioral phenotypes is generated at the genetic level.

METHODS

Long-range PCRs for target enrichment of 70kb surrounding *oxtr*

DNA was isolated from previously sectioned brains stored at -80˚ C with the Qiagen DNeasy Kit. All PCRs were performed using the Qiagen LongRange PCR Kit. Ten loci between 6.6 – 10 kb were amplified. Loci 2 through 10 were run with the same thermocycler program, initial denature: 93˚ C – 3 min; 35x cycles: 1) denature: 93˚ C – 15 s, 2) anneal: 62˚ C – 30 s, 3) elongation: 68˚ C – 10 min 20 s. Loci 2 and 4 through 10 were amplified with a single reaction. Locus 1 and 3 required an initial amplification followed by a second amplification using internally nested primers. Locus 3 was run with the same above program for initial and nested reactions. Locus 1 required six replicates run on the central

wells of the thermocycler with a 5˚ C gradient. All initial amplicons were then run with nested primers on the same program without a gradient.

Primers for each loci are listed below: Locus 1 – initial, forward: 5'-TTTGGACACTGTGACTTGGCATTG-3', reverse: 5'-ACGTCCACCTTGGGTATCGTTTTG-3', Locus 1 – nested, forward: 5'-GGTGGGTCATCTGTCTATCTGTTGC-3', reverse: 5'-GGCTCCTGATTTTCCCAGGTACAAG-3', Locus 2, forward: 5'-CGAAGGTCAGGGGAGAAAAGTGAC-3', reverse: 5'-ACTCCAGTCCTTGTGGAACTAATGTGG-3', Locus 3 – initial, forward: 5'-CAATAAGCAGCTAGACAGGGCCCA-3', reverse: 5'-CCCTGGATCTACATGCTGTTCACG-3', Locus 3 – nested, forward: 5'-CGCTGCAGTAGTGGGAAGACATTG-3', reverse: 5'-ACGAACTTGTGCAGCGCTTTCTC-3', Locus 4, forward: 5'-GACCCTCTGATGGCTGAGTGACTG-3', reverse: 5'-CCCAGAGGGAACTGCATCTGAGTC-3', Locus 5, forward: 5'-TCAGCCCTCAGAAACTTTTTCAAACAC-3', reverse: 5'-GAAGGGTGCCTGTCTTCTTTGGTC-3', Locus 6, forward: 5'-AAGGGGAGTGACTTTCAGGGGAAG-3', reverse: 5'-AGTGTGTGACAGCATTGGGACTTTG-3', Locus 7, forward: 5'-CCAAGGGATGACACAGCTTTGAGAG-3', reverse: 5'-CCAGCTTTGCTACAGAGGATCAGC-3', Locus 8, forward: 5'-CCAGGGCAGCTTTATTCATGTGTG-3', reverse: 5'-TGCTGCTACCAGTCATGTCTCTGC-3', Locus 9, forward: 5'-AAATCCTGGATGGTGATATTGTCTGC-3', reverse: 5'-AGTAACATGCCTGCTCCTGTGTGTG-3', Locus 10, forward: 5'-GGCGAAACTACTTTCCACGTTTGC-3', reverse: 5'-TGTGCTAGCCAGTTCACCATCAGC-3'

Amplicon library preparation

Sequencing library preparation and sequencing analyses were performed by the Yerkes Nonhuman Primate Genomics Core (Atlanta, GA). PCR amplicons from each animal were pooled and cleaned using Solid Phase Reversible Immobilization (SPRI) beads (Beckman Coulter). Libraries were generated using

the Illumina Nextera XT DNA Library Prep Kit (San Diego, CA), dual barcoding and sequencing primers were added according to the manufacturer protocol. Libraries were validated by microelectrophoresis, quantified, pooled and clustered on Illumina TruSeq v3 flowcell. Clustered flowcell was sequenced on an Illumina HiSeq 1000 in 100-base single-read reactions.

Amplicon sequencing analysis

Sequencing reads were mapped to *Microtus ochrogaster* target 220 haplotype 2 genomic scaffold (DP001215.2) using the Burrows-Wheeler Aligner (bwa version 0.7.10). The aligned reads were processed with the DNAseq Variant Analysis workflow of the Genome Analysis toolkit (GATK v3.2.2)[46], including marking duplicate reads and local realignment around insertion/deletions. Variants were called on a per-sample basis and then combined to produce a joint variant call file.

Genotyping

Tissue used in this experiment was collected from previously sectioned brains stored at -80C. DNA was isolated using a Qiagen DNeasy kit. For NT204321 genotyping, a 140 bp amplicon including NT204321 was amplified by PCR. The PCR protocol did not require the touch-down procedure when using high concentration genomic DNA. Amplicons were digested for 1.5 hours at 37 deg C with SSP1 restriction enzyme (New England Bio). SSP1 cuts the T-allele of NT204321 but not the C-allele. Thus, resultant banding patterns were used to identify genotypes: a single band 140 bp band for C/Cs, three bands for C/Ts, two smaller bands for T/Ts.

For NT213739, a 117 bp amplicon including was amplified by PCR, using a Qiagen Taq PCR Master Mix Kit. The thermocycler program used was, initial denature: 94˚ C – 5 min 30s; 35x cycles: 1) denature: 94˚ C – 30 s, 2) anneal: 55˚ C

– 30 s, 3) elongation: 72˚ C – 30 s; final elongation: 72˚ C – 10 min. Amplicons were digested for 1.5 hours at 65˚ C with the BsiHKAI restriction enzyme (New England Biolabs). BsiHKAI cuts the C-allele of NT213739 but not the T-allele. Thus, resultant banding patterns were used to identify genotypes: a single band 117 bp band for T/Ts, three bands for C/Ts, two smaller bands for C/Cs. The primers used for this reaction were, forward: 5'- GGGACGTTCACGTTACATGG -3', reverse: 5'- AGACGGGACAGAGTCTCCAG -3'.

Statistical analysis

Statistical analyses focused on associations with NT204321 and NT213739 were performed in R. Associations between genetic information and brain data were examined using linear regression with Bonferroni corrections for multiple comparisons, the common method for analysing genotype-phenotype associations. For linear regression, genotypes were modelled as 0, 1 and 2, with the number representing the number of high OXTR-associated alleles: the T-allele in the case of NT204321 and C-allele in the case of NT213739. For all reported associations using linear regression I investigated the residuals and found they were normally distributed. Processing of next-generation sequencing data was performed in VCFtools, a program package designed for working with VCF files from sequencing projects[50]. In particular used the --maf, --min-meanDP, --minQ and --max-missing functions for filtering and quality control of the sequence data. For associations between individual markers in the *oxtr* sequence and autoradiography expression data, linear regressions were performed using PLINK/SEQ, an open-source library for working with genetic variation data (URL: https://atgu.mgh.harvard.edu/plinkseq/). All figures were generated using the data visualization package ggplot2 in R.

CHAPTER 4

Concluding Remarks

SUMMARY OF FINDINGS

The OXT system is an evolutionarily conserved hormonal and neuromodulatory system with important functions in reproductive physiology and social cognition and behavior. OXTR exhibits diversity in brain distributions that drive differences in social behavior between species. These patterns suggest *cis*-RE sequences regulating the *oxtr* gene are a critical substrate of selection during the evolution of social behavior. Recent work has revealed the NAcc to be a site where individual OXTR density variation drives individual diversity in social attachment behavior. These developments are exciting as they present a model for understanding how subtle adjustments at specific nuclei in a neural network can lead, relatively straightforwardly, to individual variation in behaviors underpinned by the network. For my thesis, I set out to test the long-standing hypothesis that transcriptional mechanisms give rise to individual OXTR diversity in the NAcc.

In Chapter 1, I reviewed the evidence that the neuropeptides OXT and AVP have a selective function in the brain, which is to modulate neural networks that underpin social cognition and behavior. A prevailing hypothesis in the field is that a fundamental and general function of OXT is to increase the salience, or signal to noise ratio, of sensory information relevant to the social environment. These peptides are able to achieve the general effect of social behavior modulation in diverse species because the receptors are flexibly distributed. Functional genetic elements, *cis*-REs, undergo adaptive evolution to achieve modular and rapid changes in gene expression. Transcriptional flexibility extends to individual members of species, where gene expression variation generates individual behavioral differences. Individual diversity in social

behavior is relevant to psychiatric disorders such as autism spectrum disorder, which often involves disruption of social interaction.

In Chapter 2, I examined the role polymorphisms in the *oxtr* gene play in generating individual diversity in neural and behavioral phenotypes. I began by quantifying OXTR density in forebrain regions and found that the striatum, including the NAcc, exhibits higher variation than other brain regions. This unique feature of the NAcc suggests cells in this region regulate the *oxtr* gene in a unique manner, with one possible mechanism of regulation being *cis*-REs. Following up on this insight, I next observed that *oxtr* is indeed under the influence of *cis*-REs in the prairie vole brain, as indicated by allelic imbalance. Interestingly, allelic imbalance is specific to striatal sub-regions: NAcc and CP. Further, this initial experiment revealed a SNP marker, NT204321 (C/T) that specifically predicts OXTR density in NAcc and CP. T/T voles have higher densities than C/C voles. These genotypes also have differential propensity to form social attachments, with the T/T genotype facilitating male partner preference formation after a brief cohabitation. I independently confirmed the effect of NT204321 on NAcc OXTR density in the behavioral cohort. Finally, I showed that *oxtr* mRNA in the NAcc differs between T/T and C/C genotypes and correlates strongly with OXTR density in that region.

The demonstration that *oxtr* expression is strongly influenced by *cis*-REs is an important contribution to our understanding of neurohypophyseal peptide receptors. Previous studies have provided important evidence that a homolog with similar properties, *avpr1a*, responds to *cis*-REs in a microsatellite. Microsatellites alleles were shown to affect AVPR1A distribution in a modular fashion, and to influence individual behavioral diversity. I revealed that genetic variation also strongly influences diversity in OXTR distribution and OXTR-

dependent behaviors. Thus, neuropeptide receptor genes may have a general property of acquiring variation in *cis*-REs that drives significant diversity in expression.

OXTR binding occurs in neural networks for sensory and reward processing as well as a social decision network. These networks are conserved between vertebrates. Major disruptions of these networks would result in loss of fitness. The susceptibility of *oxtr* cis-REs to variation allows relatively subtle adjustments to subsets of regions instead of entire networks. In this manner, a small mutation can influence a network's output in an evolutionarily incremental fashion. I discussed specific examples of evolutionary patterns of OXTR distribution adjustments. Monogamous mammals gain OXTR or AVPR1A binding convergently in mesolimbic reward network nuclei and I hypothesize this enables a gain of function to associate discrete social cues with hedonic reward, and thus attachments form with specific individuals. Importantly, I identified genetic variation that seems to respond to transcription factors unique to these reward regions, thus the *cis*-RE generating the variation I studied in this thesis may potentially respond to a transcription factor involved in gain and loss of monogamy behavior.

Regarding sensory processing, rodents gain OXTR binding in olfactory processing regions while primates gain OXTR binding in visual and auditory processing regions. Thus, OXT modulates sensory processing of whichever modality a species uses to communicate critical social information. I confirmed that a simple mechanism, variation in *cis*-REs, can explain the process by which these adjustments to networks occur.

Prairie voles are an excellent model species for the study of complex social behavior. Unfortunately, vole researchers currently lack advanced tools to

manipulate genes in a region specific manner, as is possible in mice. I identified a genetic marker that predicts OXTR density differences in a region specific manner. The NT204321 marker is likely in linkage with the *cis*-RE, rather than conferring function itself. I demonstrated the possibility of using this marker as a tool for behavioral experiments. However, the marker is less feasible for more complex behavioral experiments, such as those that might detect gene by environment interactions, which would require numerous groups.

The model system would benefit greatly from knowledge of the specific *cis*-RE generating the NAcc phenotype, or as a first step forward, a better marker of individual OXTR density. In Chapter 3, I analyzed the prairie vole *oxtr* gene in detail with the goal of identifying a more powerful marker and generating information that will aid discovery of the *cis*-RE. To this end, I sequenced 70kb of sequence including the prairie vole *oxtr* gene and surrounding non-coding regions. I enriched the target sequence with long-range PCRs and sequenced these amplicons using the Illumina next-generation sequencing platform. I selected 45 voles that were either C/C or T/T at the NT204321 marker and that in addition had either the highest or lowest NAcc OXTR density for their respective genotype. Moderate OXTR individuals were high C/C and low T/T and had similar mean OXTR density. I hypothesized that such individuals have *cis*-RE alleles that were de-correlated from NT204321 that sequencing could reveal.

The sequencing process produced usable data, with high quality and mapping measures. I filtered the variant call data to remove microsatellites, insertion/deletions and low frequency SNPs. Future studies may seek to perform more sophisticated analyses with those data. This filtering was conservative, however close to 1,000 SNPs remained, which was enough to proceed with my current goals. Unexpectedly, in this sample, I found strong LD for SNPs

throughout the *oxtr* gene. The NT204321 T-allele occurs on a haplotype with almost no sequence variation across the entire 70kb locus. The C-allele haplotypes are more diverse. Nonetheless, I proceeded on a first pass to perform association tests between the 967 SNPs and OXTR density. There was significant enrichment of significant associations. Because of strong LD, many of these SNPs were not informative. I further filtered out SNPs in strong LD with the top, most highly associated SNP, only 12 remained. Of these, only two were significantly correlated with OXTR density following multiple test corrections. The second SNP does not explain additional variance. The top SNP is a C/T substitution in the intron of *oxtr*. The intron SNP explains an astounding 75% or more of the NAcc OXTR density variation in three independent analyses.

I discovered markers linked to a robust, brain region specific *cis*-RE. More work is needed to identify the functional site. Nonetheless, I identified an improved marker of NAcc OXTR density. This marker will enable experiments focused on behavior and network properties that may depend on OXTR diversity.

FUTURE DIRECTIONS

OXTR in neural networks

The most immediate impacts of the work presented in this dissertation will be the use of the SNP markers I identified to generate phenotypically distinct groups for use in behavioral experiments. The behavioral analyses I presented here relied on the NT204321 marker, which has weaker predictive power than NT213739. In figure 3.1, it is apparent that some homozygotes for each of the NT204321 alleles still exhibit overlapping NAcc OXTR density. The behavioural analyses I performed required large group sizes to achieve the power

required to discern group differences, close to 40 animals in a given group for both partner preference and USVs. The one other study that analysed *cis*-RE contributions to behaviour in prairie voles also reported similar group sizes (Hammock and Young, 2005). Such large group sizes could be prohibitive for future experiments that would focus on gene by environment interactions. For example, groups with high NAcc OXTR density are resilient to inhibitory influences of prenatal or early post-natal stressors on adult social function. NT213739 is a better predictor of this particular neural phenotype, the homozygotes groups in the three samples I analyzed had no overlap in the NAcc (Figure 3.5). Future studies should characterize the power of this marker to predict behavioural differences.

The genotype associations with OXTR density I have observed are more or less specific to the NAcc and perhaps one or two other regions with functions at the intersection of mesolimbic reward network (MLR) processing and olfactory sensory processing. The CP, or dorsal striatum, is essentially the same tissue as NAcc at least as far as developmental origins are concerned. However, OXTR activation in CP is not required for prairie vole partner preference (Young et al., 2001). OXTR density in the olfactory tubercle (Tu) is also very strongly correlated with NAcc OXTR density. The Tu was recently shown to encode odor valence (Gadziola et al., 2015). It may be difficult to differentiate the contribution of OXT to Tu and NAcc and perhaps the two regions should be considered as a module of the MLR that OXTR has influence over in prairie voles.

Future behavioural experiments could leverage the relative specificity of my genetic markers to predict MLR sensitivity to OXT to help dissect the specific role OXT signalling plays in the formation of prairie vole social attachments. OXT and dopaminergic signalling must occur concurrently for prairie voles to

form a partner preference (Young and Wang, 2004, Liu and Wang, 2003). But does OXT merely act in synergy with dopamine, helping to "boost" a general reward signal? OXTR appears to act in this manner in the NAcc of mice, where OXTR occurs on axonal terminations from dorsal raphe serotonergic inputs (Dolen et al., 2013). The effects of OXT are dependent upon subsequent 5HT1B activation, suggesting that OXTR enhances the effect of serotonin, rather than playing a complimentary role. In the prairie vole however, *oxtr* is expressed natively in the NAcc. It is unknown whether OXTR occurs in medium spiny neurons, interneurons or both. Either way, native OXTR binding suggests that OXT signalling could play a complimentary rather than synergistic role. For example, if OXTRs are present in dendritic spines of NAcc medium spiny neurons, OXT secretion could act to prime synapses to respond to incoming olfactory cue information in a discrete manner. In this way, OXT would boost cue signal rather than reward signal.

Future experiments could test such hypotheses by determining whether OXT can serve as a rewarding signal in a non-social context, such as an operant conditioning chamber. Would a prairie vole lever press to receive an intracerebroventricular OXT injection? High NAcc OXTR voles might be expected to perform this task in response to lower concentrations of OXT. Alternatively, perhaps OXT would instead be required to discriminate a rewarding signal from a noisy mix of neutral cues. Tu neurons are able to discriminate a rewarding olfactory cue from a complex mixture (Gadziola et al., 2015).

Experiments such as these could help resolve whether OXTR diversity has multiple functional roles across species or whether there is a single or limited set of coherent functions across species. OXT is hypothesized to play a general role in social information processing by increasing the salience of discrete cues (Young,

2015). In monogamous species such as prairie voles and the common marmoset that express *oxtr* in MLR regions, does OXTR expand its role in information processing to a larger set of networks, or instead gain a new reward-boosting function unique to certain species?

Throughout this thesis I have ignored a major debate about the general function of OXT, namely that it also appears to be potently anxiolytic. Indeed, some researchers have made the strong proposition that all observed effects of OXT could be due to its anxiolytic properties (Churchland and Winkielman, 2012). More likely, OXT has a role in both stress and social cognition. Some studies have shown a dissociation between central and peripheral roles of OXT, with peripheral injections having specifically anxiolytic effects (Ayers et al., 2011). However, other groups have shown central anxiolytic effects (Smith and Wang, 2014). I have not produced any novel data that could contribute greatly to an attempt to dissociate a social vs. anxiety role. Nonetheless I would like to share one speculation I developed while considering theories about the role of OXT in information processing. Could some portion of the anxiolytic role of OXT be due its general role in increasing the salience of discrete sensory information for the rest of the brain? The amygdala is an extremely important brain region for stress and anxiety (Shekhar et al., 2003, LeDoux, 2007). The amygdala receives relatively raw sensory information of multiple modalities. The amygdala is also one of the most consistent brain regions in which to observe OXTR density. As I have discussed previously, primary sensory processing brain regions also commonly express *oxtr*, giving OXT an early role in determining what information arrives at the amygdala in the first place. In bats, BLA neurons respond discretely to conspecific auditory calls containing emotional and individual-specific information (Peterson and Wenstrup, 2012). Perhaps OXT

reduces the proportion of amygdala neurons active at a given time by increasing the signal to noise ratio of discrete cues, thereby reducing anxiogenic amygdala signalling.

More immediately, groups of prairie voles organized by their NT213739 genotype can be used to ask questions about the role of OXTR density variation in molding larger networks responses. For example, I have begun a collaboration with a colleague in the Young lab to test the hypothesis that high NAcc OXTR density voles will exhibit increased functional coordination across the brain, as measured with correlations of c-*fos* activation throughout brain regions in the MLR and social decision making network. Future experiments are also planned to test the hypothesis that OXTR density differences might influence functional connectivity between the PFC and NAcc, as measured with multi-electrode recording arrays in the NAcc.

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