da Costa Araujo, Juliana

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Nonapeptide receptor expression in relation to mating system and social behavior in Richardson's ground squirrels

Department of Neuroscience

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NONAPEPTIDE RECEPTOR EXPRESSION IN RELATION TO MATING SYSTEM AND SOCIAL BEHAVIOR IN RICHARDSON’S GROUND SQUIRRELS

JULIANA DA COSTA ARAÚJO
Biological Science, Universidade Federal do Rio Grande do Norte, 2014

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NONAPEPTIDE RECEPTOR EXPRESSION IN RELATION TO MATING SYSTEM AND SOCIAL BEHAVIOR IN RICHARDSON’S GROUND SQUIRRELS

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ABSTRACT

Arginine vasopressin and oxytocin influence many aspects of behavior, including sociability, memory, social learning, reproduction, vocal signalling, and aggression. Many behavioral neuroendocrinology studies that address the behavioral effects of oxytocin and vasopressin have focused on male-female bonding among species/individuals that vary in their level of monogamy and polygamy. However, there are only a few studies of non-reproductive social interactions and the current study aims to provide more information about how vasopressin and oxytocin are regulating social relationships beyond pair-formation. Richardson’s ground squirrel (*Urocitellus richardsonii*) is an excellent choice for a study of this nature because the sexes differ in their social behavior, especially during breeding season; females are sociable with related females and males are agonistic, so sexual receptor differences are expected in the brains of males and females of this species. Using autoradiography, I identified the neuroanatomical distribution of arginine vasopressin receptors (V1aR) and oxytocin receptors (OTR) in the brains of male and female Richardson’s ground squirrels during the breeding season. V1aR expression was measured 28 times in 17 different brain regions and OTR had 23 measurements in 13 different brain regions. I also tested for sex differences because the sexes differ in their social bonding strategies. I found sex differences for V1aR in the mitral layer of the olfactory bulb and the bed nucleus of the stria terminalis. For OTR, I found sex differences in the medial amygdala and the molecular layer of the dentate gyrus. Based on previous studies, these sex differences seem to be related to social memory, aggression, exploratory behavior, and spatial performance.
ACKNOWLEDGEMENTS

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Finally, I thank Sean Warkentine for emotional support and helping to improve my English skills, and my amazing family, Juçara da Costa Araújo and Sebastião Araújo, and Alcelino L. da Costa Araújo and Maria M. L. de Oliveira, for understanding my long absence always supporting me in my goals.
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<tbody>
<tr>
<td>AMY</td>
<td>amygdala</td>
</tr>
<tr>
<td>AOB</td>
<td>accessory olfactory bulbs</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
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<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BSTdl</td>
<td>dorso-lateral part of the bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BSTdm</td>
<td>dorso-medial part of the bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BSTv</td>
<td>ventral part of the bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BSTov</td>
<td>oval nucleus of the bed nucleus of the stria terminalis</td>
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<tr>
<td>CB</td>
<td>cerebellum</td>
</tr>
<tr>
<td>CEA</td>
<td>central amygdala</td>
</tr>
<tr>
<td>CPu</td>
<td>caudoputamen</td>
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<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DGgr</td>
<td>granular layer of the dentate gyrus</td>
</tr>
<tr>
<td>DGmo</td>
<td>molecular layer of the dentate gyrus</td>
</tr>
<tr>
<td>DGpo</td>
<td>polymorph layer of the dentate gyrus</td>
</tr>
<tr>
<td>DGpo/gr</td>
<td>polymorph and granular layer of the dentate gyrus</td>
</tr>
<tr>
<td>EPI</td>
<td>external plexiform layer</td>
</tr>
<tr>
<td>FC</td>
<td>frontal cortex</td>
</tr>
<tr>
<td>GENd</td>
<td>geniculate group of the thalamus</td>
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<td>HN</td>
<td>hypoglossal nucleus</td>
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<td>HP</td>
<td>hippocampus</td>
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<td>HY</td>
<td>hypothalamus</td>
</tr>
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<td>HYmz</td>
<td>hypothalamic medial zone</td>
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<tr>
<td>IG</td>
<td>indusium griseum</td>
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<td>IPN</td>
<td>interpeduncular nucleus</td>
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<tr>
<td>LPO</td>
<td>lateral preoptic area</td>
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<td>LS</td>
<td>lateral septum</td>
</tr>
<tr>
<td>MEA</td>
<td>medial amygdala</td>
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<tr>
<td>MiL</td>
<td>mitral layer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MOs</td>
<td>secondary motor area</td>
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<td>MPO</td>
<td>medial preoptic area</td>
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<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>NAcC</td>
<td>nucleus accumbens core</td>
</tr>
<tr>
<td>NAcSh</td>
<td>nucleus accumbens shell</td>
</tr>
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<td>OB</td>
<td>olfactory bulbs</td>
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<tr>
<td>OT</td>
<td>oxytocin</td>
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<td>OTu</td>
<td>olfactory tubercle</td>
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<tr>
<td>OTR</td>
<td>oxytocin receptor</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>Pir</td>
<td>piriform cortex</td>
</tr>
<tr>
<td>PRP</td>
<td>nucleus prepositus</td>
</tr>
<tr>
<td>SC</td>
<td>superior colliculus</td>
</tr>
<tr>
<td>sptV</td>
<td>spinal tract of the trigeminal nerve</td>
</tr>
<tr>
<td>SUB</td>
<td>subiculum</td>
</tr>
<tr>
<td>TH</td>
<td>thalamus</td>
</tr>
<tr>
<td>VMH</td>
<td>ventromedial hypothalamic nucleus</td>
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<tr>
<td>VP</td>
<td>ventral pallidum</td>
</tr>
<tr>
<td>V1aR</td>
<td>arginine vasopressin 1a receptor</td>
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CHAPTER ONE: GENERAL INTRODUCTION

Neuropeptides constitute a distinctive group of neurotransmitters, which affect a variety of behaviors as well as different physiological functions in the body (Becker et al., 2002). Two specific neuropeptides are important for the expression of social behaviors in mammals, arginine vasopressin (AVP) and oxytocin (OT). AVP and OT are nonapeptides (i.e., comprised of nine amino acids) and differ from one another by only two amino acids (Dhakar et al., 2013; Gordon et al., 2011; Becker et al., 2002; Hara et al., 1990). AVP and OT originated from an ancestral peptide, vasotocin, around 700 million years ago (Acher and Chauvet, 1995). The expression of AVP, OT, and their respective receptors in the brain is associated with inter- and intraspecific variation in mating system and parental care (Dhakar et al., 2013). Because of the importance of AVP and OT in modulating these aspects of social behavior, they have become a major focus in behavioral neuroendocrinology research.

AVP and OT are involved in many functions in the periphery of the body and also in the central nervous system, so hormone release is systematic and can be triggered by numerous causes. Their activity is short-lived with around 20 minutes in cerebrospinal fluid (Ludwig and Leng, 2006) and release is dependent on environmental stimuli (Ludwig and Leng, 2006; Onaka et al., 1996; Onaka and Yagi, 1993). The levels of AVP and OT, therefore, fluctuate greatly within an individual throughout the day and in response to different stimuli. Consistent behavioural differences, such as parental care, mating behaviour, pair bonding and other aspects of sociality, are species-specific, less variable among individuals within species and, thus, reflect other aspects of the system. The
focus of most research into the evolution of species and sex differences in social behaviour in response to AVP and OT is, therefore, examining receptors. Receptor expression can change throughout an animal's lifetime (Nelson and Kriegsfeld, 2017), but has a longer time course and there is a wealth of data demonstrating that receptor expression is what is related to social behaviours. For those reasons, receptors were the focus of the current study in trying to understand how sociality is regulated.

Expression of AVP, OT and their receptors

AVP production occurs mainly in the hypothalamus, in the supraoptic nuclei and paraventricular nuclei, but in populations of cells distinct from that of OT (Vandesande and Dierickx, 1975). Small groups of AVP neurons are also found in the olfactory bulbs, the lateral septum medial septum, suprachiasmatic nucleus, medial amygdala, and bed nucleus of the stria terminalis (Rood et al., 2013; Rood and Vries, 2011; Wacker et al., 2010; Carter, 1998; Van Eerdenburg et al., 1992; Sofroniew, 1985; Sofroniew, 1983). AVP increases blood pressure and regulates fluid intake and is associated with memory, learning, stress-associated disorders, sexual behavior, pair bonding, modulating social recognition, and other types of social behavior (Gruber, 2014; Hofmann et al., 2014; Gordon et al., 2011; Becker et al., 2002; Dantzer et al., 1987; DeWied, 1971). In monogamous species, AVP also modulates mate recognition and promotes bonding after mating (Shapiro and Dewsbury, 1990; Wang et al., 1998). Unlike OT, AVP binds to several receptor subtypes, only two of which are found in the central nervous system: V1aR and V1bR (Dhakar et al., 2013). Of these
two receptors, the relationship between V1aR expression and behavior has been studied far more intensively than V1bR (Caldwell et al., 2008). V1aR is expressed in many parts of the brain, such as the olfactory bulbs (OB), prefrontal cortex (PFC), piriform cortex (Pir), indusium griseum (IG), nucleus accumbens (NAc), ventral pallidum (VP), bed nucleus of the stria terminalis (BST), lateral septum (LS), hippocampus (HP), arcuate nucleus (ARC), and hypothalamus (HY) (Nelson and Kriegsfeld, 2017; Dhakar et al., 2013; Beery et al., 2008; Becker et al., 2002).

Similar to AVP, OT neurons are found mainly in the hypothalamus, in the magnocellular and parvocellular cells of the paraventricular and supraoptic nuclei (Dhakar et al., 2013), but there are also reports of small groups of cells in places such as the medial preoptic area, mediobasal hypothalamus, periventricular complex, spinal cord, bed nucleus of the stria terminalis and anterior commissural nucleus (Kelly and Goodson, 2014; Dhakar et al., 2013; Kendrick, 2013; Becker et al., 2002; Moore and Lowry, 1998; Sofroniew, 1980). OT is responsible for the contractions of uterine muscles in childbirth, milk release during nursing, ejaculation, sexual behavior, maternal responsiveness, pair bonding, and a range of other complex social behaviors (Gruber, 2014; Gordon et al., 2011; Beery, et al., 2008; Insel, 1992). OT receptors (OTR), of which there is only one subtype, occur in many brain regions, including the OB, PFC, Pir, NAc, caudoputamen (CPu), LS, BST, amygdala (AMY), HP, thalamus (TH), HY, mid- and hindbrain, and spinal cord (Gruber, 2014; Beery et al., 2008; Insel, 1992; Sofroniew, 1983).
Complex social behaviors and social interactions are essential components of human behavior and evolution, and variations in the location and density of OTR can cause severe damage to social relationships (Hurlemann and Scheele, 2016). OT is even important for social bonding between humans and animals. Dogs are well known for being very cooperative and exogenous OT promotes social bonding between dogs as well as between dogs and humans (Romero et al., 2014). This and other evidence indicates that OT is a key component of modulating social relationships.

In terms of social behavior, the effects of AVP and OT and their receptors have been researched extensively over the past 10-15 years. One of the conclusions of these studies is that there are consistent patterns of AVP and OT receptor expression in relation to sociality. In mammals, the expression levels of AVP and OT and their receptors are associated with social recognition, affiliation, parental and alloparental care, trust, and aggression (Dhakar et al., 2013; Insel, 2010; Olazabal and Young, 2006; Kosfeld, et al., 2005; Lim and Young, 2004; Shapiro and Dewsbury, 1990; Wang et al., 1998). For example, OTR density in the medial preoptic area (Pedersen et al., 1994), NAc and CPu (Olazabal and Young, 2006) is positively correlated with parental care within and across mammal species. That is, individuals and species that express more parental care have a higher density of OTR in these three brain regions. Conversely, OTR density in the LS is negatively correlated with parental care (Olazabal and Young, 2006). Species with higher expression of AVP in the BST and higher density of V1aR in the LS also exhibit more parental care (Bester-Meredith et al., 1999). Apart from parental care, levels of AVP, OT, V1aR and OTR also covary with
mating system. More specifically, monogamous species differ from polygamous species in the density and distribution of AVP and OT receptors throughout the brain (Insel et al., 1994; Insel and Hulihan, 1995; Insel et al., 1995; Bester-Meredith et al., 1999; Cho et al., 1999; Smith et al., 2010). The majority of studies have tended to focus on vole species: the prairie voles (*Microtus ochrogaster*), montane voles (*M. montanus*), meadow voles (*M. pennsylvanicus*) and pine voles (*M. pinetorum*). Elevated levels of V1aR and OTR in the NAc and the VP occur in the monogamous prairie vole, but not in the other two polygamous species leading some to suggest that increases in V1aR and OTR expression are associated with pair bonding (Lim et al., 2004). However, some patterns of AVP and OT receptor distribution are not consistent with this dichotomy and the expression of both receptors can vary significantly among other species (Chappell et al., 2016; Freeman et al., 2014; Kalamatianos et al., 2010; Campbell et al., 2009; Beery et al., 2008; Insel et al., 1991). For example, Beery et al. (2008) conducted a study comparing two closely related rodents with contrasting social behavior from South America, the social colonial tuco-tuco (*Ctenomys sociabilis*) and the solitary Patagonian tuco-tuco (*Ctenomys haigi*), and found clear differences in receptor distribution in the two species. The social species (*C. sociabilis*) had high levels of OTR in the Pir and TH and V1aR in the OB. However, in the solitary species (*C. haigi*), OTR was denser in the LS and HP. OTR was not expressed in NAc in either species. Thus, the tuco-tucos have a different expression pattern to that of the voles. In yet another two species comparison, eusocial naked mole-rats (*Heterocephalus glaber*) have a different neuroanatomical distribution of OTR compared with the asocial Cape mole-rats.
(Georychus capensis) as well as differing from the voles and tuco-tucos (Kalamatianos et al., 2010). Naked mole-rats have much higher levels of OTR in NAc, amygdaloid nuclei, HP and BST. In contrast, the Cape mole-rat has strong OTR expression in the Pir, but OTR is undetectable in naked mole-rats. These are only two examples of how mammal species can vary in their OTR and V1aR expression patterns from that of voles, but it is clear from cross-species comparisons that V1aR and OTR expression patterns vary greatly among and within species and differ from the ‘classic’ vole comparison (Chappell et al., 2016; Freeman et al., 2014; Anacker and Beery, 2013; Turner et al., 2010; Campbell et al., 2009). Although species-specific distribution of V1aR and OTR in the brain is attributed primarily to social behavior (Young, 1999; Insel and Shapiro, 1992a), the variability across a wider range of species calls into question the specific role that receptor expression has on the evolution of sociality.

One approach that can aid in resolving the putative relationship between V1aR, OTR, and social behavior is to examine species that differ in their social behavior from previously studied species. Mammals display an immense variety of social systems that can differ in organization, structure and mating system (Kappeler et al., 2013). By focusing primarily on the false dichotomy of monogamy-polygamy, the majority of previous studies on OTR and V1aR and social behaviors are not taking into account the diversity of social behaviors that have evolved. There are, however, species that can fill this knowledge gap and potentially yield significant insight into how social behavior, mating systems, AVP, OT, and receptor expression evolve together.
Ground squirrels

Ground squirrels (Marmotini, Rodentia) are a group of closely related species that vary greatly in their degree of social cohesion, which makes them an ideal group for comparative analyses (Blumstein and Armitage, 1998). The first comparative studies on this group were by David Barash in 1973 and 1974 (Hare and Murie, 2007). Barash identified a latitudinal relationship with sociality in the genus *Marmota*, with species in high elevations (e.g., hoary marmots, *Marmota caligata*) being more social than those in low elevations (e.g., woodchucks, *Marmota monax*). Following those observations, hundreds of subsequent studies were conducted in this group to determine how different selection pressures resulted in the diverse array of social systems and social behavior that occur in ground squirrels. Social systems in ground squirrels were divided into five categories or ‘grades’ by Armitage (1981) and Michener (1983a). Michener used similar definitions for the grades to Armitage, but highlighted kinship as the main factor differentiating the grades. The grades in increasing degree of sociality are: 1 – asocial, 2 – single family female kin cluster, 3 – female kin cluster with territoriality, 4 – female kin cluster with male dominance, and 5 – egalitarian, polygynous harems (Hare and Murie, 2007). It is important to note that in almost all ground squirrels, the mating system is polygamous, but they vary in terms of the amount of social interactions between and within sexes and the size and density of colonies. Although Mateju et al. (2016) found no correlation between relative brain size and sociality in ground squirrels, the role of V1aR and OTR expression in the evolution of ground squirrel sociality has not been investigated. Given the range of social systems within this closely related group, ground
squirrels could provide insight into the molecular basis of social system evolution in a more refined manner than previous rodent studies.

Richardson’s ground squirrel (*Urocitellus richardsonii*) is a polygamous species in which males and females vary tremendously in their degree of sociality and the composition of the social group during breeding season (Michener, 1998). There is a considerable amount of behavioral data on sex differences in their social system, reproductive efforts, and timing of reproduction. Males are solitary and emerge earlier from hibernation to start competing for territories and females, while females live close to related females, express low aggressive behavior and raise the litters alone (Michener, 1998; Michener and McLean, 1996; Michener, 1979). However, the neural structures responsible for expressing these sex differences has not been examined.

The goal of this thesis was to describe for the first time the neuroanatomical regions expressing receptors for AVP and OT in Richardson’s ground squirrel during the breeding season using autoradiography. As detailed in Chapter 2, I compared the densities of V1aR and OTR across brain regions between males and females and compared my results to previously studied species. This thesis is a starting point for comparative studies of ground squirrels and it will bring valuable neuroanatomical information about Richardson’s ground squirrels, and adds a new perspective on the putative role of nonapeptide receptors in the expression of sociality and sex differences in behavior.
CHAPTER 2: DISTRIBUTION OF VASOPRESSIN 1A AND OXYTOCIN RECEPTORS IN MALE AND FEMALE RICHARDSON’S GROUND SQUIRRELS

Introduction

The nonapeptides arginine vasopressin (AVP) and oxytocin (OT) influence many aspects of behavior (Hurlemann and Scheele, 2016; Gruber, 2014; Gordon et al., 2011; Becker et al., 2002; Insel, 1992). Perhaps one of the most intensively studied effects of both AVP and OT in recent years is social behavior. AVP and OT modulate fear, anxiety, maternal care, aggression, memory, social bonds, and other complex social behaviors in humans and in other animals (Litvin and Pfaff, 2013; McCarth and Altemus, 1997). In the context of social behavior, voles (Arvicolinae, Rodentia) have been important animal models for understanding the roles of AVP and OT in the formation and maintenance of pair bonds, as well as parental care (Kelly and Ophir, 2015). In particular, AVP and OT modulate mate recognition and promote pair bonding after mating and therefore the actions of both nonapeptides are intimately related to monogamy (Young and Wang, 2004; Wang et al., 1998; Carter et al., 1995; Shapiro and Dewsbury, 1990). Further, the evolution of a monogamous or polygamous mating system in voles appears to be mediated by differential expression of the receptors for AVP and OT and has even been manipulated experimentally in the lab (Young et al., 1999). For example, the level of expression of AVP receptors (V1aR) in the ventral pallidum (VP) and oxytocin receptors (OTR) in the nucleus accumbens (NAc) and prefrontal cortex (PFC) appear to reflect the strength of the pair bond both within and across vole species (Chappell et al., 2016; Ophir et al., 2012; Lim & Young,
The large number of studies on pair bonding and other social behaviors in voles in relation to V1aR and OTR expression, have been key to identifying putative neurogenetic mechanisms underlying the evolution of sociality and the identification of the social behavior network (McGraw and Young, 2010) and mesolimbic reward system (Young et al., 2011) as key neural pathways that modulate social behavior (Kelly and Ophir, 2015).

The neural circuits that modulate monogamy and sociality share many of the same brain regions and pathways as parental care. From an evolutionary perspective, parental behavior likely was the first expression of social interactions and the other forms of positive social interactions evolved from it (Nelson and Kriegsfeld, 2017). In voles, NAc is important for monogamous relationships, but also plays a crucial role in parental behavior, with higher expression of receptors enhancing parental care (Keebaugh et al., 2015). V1aR and OTR density in the lateral septum (LS), another structure important for pair bonding (Liu et al., 2001), are positively correlated with nursing and allogrooming (Curley et al., 2012; Ophir et al. 2008). Aggressive behaviors that support the protection of offspring are also regulated by OT in the amygdala, paraventricular nucleus (Bosch et al, 2005; Francis et al., 2000), bed nucleus of the stria terminalis, and LS (Caughey et al., 2011).

Although inter- and intraspecific studies of voles have been immensely informative in terms of the neuroendocrine control of parental care, pair bonding and other social behaviors, they may be limited in terms of explaining the evolution of different mating and social systems in other species. Although two species comparisons of monogamous versus polygamous voles revealed
consistent differences in V1aR and OTR expression (Insel et al., 1994; Insel and Shapiro, 1992a), studies of other rodent species suggest that the relationship between V1aR and OTR expression in specific brain regions in relation to mating system is more complicated (Turner et al., 2010). For example, in a comparative study across eight species of mice (Peromyscus spp.), V1aR receptor densities did not have any consistent difference between monogamous and polygamous species in brain regions that regulate mating system in voles (Turner et al., 2010). Thus, despite the differences in V1aR and OTR expression found between monogamous and polygamous voles, similar differences are not found in deer mice, casting doubt on whether there are consistent patterns in V1aR and OTR expression that relate to mating system.

Amicable social interactions can occur in social groups independent of mating systems and treating mating system as dichotomous (i.e., monogamous or polygamous) ignores the diversity of mating and social systems that have evolved (Tang-Martinez, 2003). One of the few exceptions to this was a study by Beery et al. (2008) in which V1aR and OTR expression were compared between the social Colonial tuco-tuco (Ctenomys sociabilis) and the solitary Patagonian tuco-tuco (Ctenomys haigi). In contrast to the monogamous prairie voles (McGraw and Young, 2010), C. sociabilis forms social groups comprised of a female kin group and one male in a polygamous mating system with the male defending the group territory. The second species, C. haigi, is also polygamous, but asocial with each individual living alone in a separate burrow system. Comparing these two tuco-tuco species therefore provides a test of whether a difference in sociality is reflected in OTR and V1aR expression under a common
mating system (polygamy). The social species had OTR in the piriform cortex and thalamus and lower expression of OTR across many brain regions compared with the asocial species. In contrast, the asocial species had OTR expression in the hippocampus whereas the social species did not. The two species also differed in V1aR expression; the social species had lower expression in the NAc and ventral pallidum and expressed V1aR in the olfactory bulbs. In contrast, the asocial species lacked V1aR in the olfactory bulbs, but did have receptors in the prefrontal cortex. These two species did differ in terms of their V1aR and OTR expression, but not in all of the same brain regions shown to be different in the monogamous-polygamous vole comparisons.

In a second study that did not focus on a strict monogamous-polygamous species comparison, Kalamatianos et al. (2010) examined OTR expression in two African mole-rats, a family of subterranean rodents. Like Beery et al. (2008), they compared two species that differ markedly in social behavior: naked mole-rats (*Heterocephalus glaber*) and Cape mole-rats (*Georychus capensis*). The Cape mole-rat is similar to the asocial tuco-tuco and polygamous voles; it lives a largely solitary existence and expresses minimum parental care. In contrast, naked mole-rats live in large social colonies of around 80 to nearly 300 individuals (Kalamatianos et al., 2010; Faulkes and Bennett, 2007). They express alloparental care and monogamy/polyandry, in which usually one female breeds with 1-3 males (Kalamatianos et al., 2010). They are eusocial, a feature rare in mammals and usually described in insects, in which the colony is formed mostly by nonbreeding helpers that help taking care of the queen, offspring and the duties of the colony (Kalamatianos et al., 2010; Faulkes and Bennett, 2007). As
with the tuco-tuco study, there were numerous differences in OTR expression between the two mole-rat species. Cape mole-rats lacked expression in several brain regions compared to naked mole-rats: NAc, indusium griseum, medial amygdaloid nucleus, cortical amygdaloid nucleus and anterior pole of bed nucleus of the stria terminalis (BST) (Kalamatianos et al., 2010). However, Cape mole-rats had higher expression in the olfactory tubercle and expression in regions that is not found naked mole-rats, such as piriform cortex (Pir), cingulate cortex and dentate gyrus (DG) (Kalamatianos et al., 2010). Thus, the mole-rats have yet another pattern of differences in OTR expression across brain regions in relation to species differences in sociality.

The consensus that is emerging from these comparative studies is that V1aR and OTR expression differs between social and asocial species, but that the specific brain regions that have differential receptor expression can vary from one pair of species to the next. Whether there is an overall pattern of expression that is typical of a more social lifestyle is difficult to assess at this stage because there are too few comparisons outside of the monogamy/polygamy dichotomy. To address whether specific patterns of V1aR and OTR expression are indeed associated with the evolution of a more or less social lifestyle, a broader range of species that vary in social behavior should be examined. This is especially true of understanding the roles that AVP and OT play in social relationships beyond the pair bond, such as social relationships within rather than between sexes. Some species even express sex differences in social behavior such that one sex is markedly more social than the other. This kind of sex difference in social behavior characterizes the social system of Richardson’s ground squirrel (Urocitellus
*richardsonii*. Their mating strategy is quite common among rodents; males try to copulate with as many females as possible, and females have multiple mates (Waterman 2007; Michener and McLean, 1996), in what has been described as defense/non-defense polygyny (Davis and Murie, 1985). That is, males can choose to actively defend a territory with many females, or move to another area to try to copulate with other females (Michener, 1998). Within this polygynous mating system, there are marked sex differences in many different behaviors (Michener, 1998), one of which is social behavior. Adult males are largely asocial, only interacting with conspecifics during the breeding season and those interactions are either agonistic or mating attempts (Michener, 1998, 1990). In contrast, female Richardson’s ground squirrels form lasting bonds with closely related females throughout the year. This sex difference in social behavior provides yet another perspective on the relationship between nonapeptide receptor expression and sociality and could yield more insights into the mechanisms underlying the evolution of sociality.

Here, I examined the distribution and intensity of V1aR and OTR expression in the brains of Richardson’s ground squirrels captured during the breeding season. Despite the aforementioned variability across rodent studies in what brain regions express V1aR and OTR, I can make some predictions about what brain regions should have sexually dimorphic receptor expression based upon the neural control of agonistic and amicable social behaviors. For example, I expect a sex difference in BST because this structure plays a key role in modulating aggression (Nelson and Trainor, 2007). NAc plays an important role in pair bonding and often differs between monogamous and polygamous species.
(Ross et al., 2009; Liu and Wang, 2003), but males and female Richardson’s ground squirrels do not form pair bonds, so I do not expect sex differences within this brain region. For most other brain regions, however, it is difficult to predict whether a sex difference will be present or not as sex differences in OTR and V1aR are so variable across species (Dumais and Veenema, 2016).

**Material and Methods**

**Animals**

I collected 11 male and 14 female wild Richardson’s ground squirrels for analysis. Animals were adults and trapped during breeding season, between February and March of 2015 and 2016, in several areas within the City of Lethbridge. Animals were captured using wire traps (Tomahawk Model 103, Hazelhurst, WI, USA) and food placed outside of their burrow entrances. Traps were monitored continuously from a distance of 50-100m using binoculars. After they were captured, the squirrels were removed with the aid of a cone-shaped, zippered bag and sex was determined visually. The squirrels were then weighed with a Pesola spring scale (±5g), euthanized with an intra-peritoneal injection of sodium pentobarbital (450 mg/kg), and brains were quickly removed. All protocols adhered to the standards of the Canadian Council of Animal Care, were approved by the University of Lethbridge Animal Welfare Committee (protocol # 1427) and all research conducted under permits issued by the Alberta Department of Environment and Parks (55980, 55981, 53998, 53999).

*Tissue collection and autoradiography*
Once removed, the brains were immediately frozen on pulverized dry ice in the field. They were then stored in a -80°C freezer until sectioning. All brains were sectioned coronally at a thickness of 20 µm on a cryostat set at -20°C, and thaw-mounted onto electrostatic slides (Fisher Superfrost-Plus). Sections were collected throughout the rostro-caudal extent of the brain from the olfactory bulbs to the medulla and are 140µm apart from each other in alternate 1:8 series. Once the sections were mounted onto slides, they were stored at -80°C until processing.

 Autoradiography is a widely used technique for studies of nonapeptide receptors, including comparative studies (Freeman and Young, 2016). Following the protocols outlined in previous studies (Ophir et al., 2013), two alternate series of sections were processed with $^{125}\text{I}$ radioligands to visualize either V1aR (Vasopressin (linear), V1A antagonist (Phenylacetyl1-0-Me-D-Tyr2) Arg6-[125I]) (NEX254, PerkinElmer) or OTR (ornithine vasotocin analog ([$^{125}\text{I}$]-OVTA), NEX 310 PerkinElmer). Before the autoradiography procedures, slides were removed from the -80°C freezer and exposed to room temperature to dry for approximately 1 hour. Sections were then submerged in cold (4-5°C) 0.1% paraformaldehyde (pH 7.6) for 2 min, followed by two washes of 10 min in a buffering solution (Tris-HCl, 50 mM, pH = 7.4). Then, slides were incubated for 1 hour in a solution with either $^{125}\text{I}$-linear-vasopressin (V1aR) or $^{125}\text{I}$-ornithine vasotocin (OTR) with tracer buffer (tracer = 50 mM Tris (7.4), 10mM MgCl, 0.1% BSA, 0.05% bacitracin), followed by four washes of 5 min each in Tris +Mg (50mM) and one wash of 30 min. Trays were water dipped and blow dried before being placed with $^{125}$I standards for autoradiography on glass slide (American Radiolabeled Chemicals
Inc., St Louis, MO, US, lot number 140627, batch number I140611) in a film cartridge with film (Carestream Kodak Biomax-MS autoradiography film) in a dark room. Films for OTR were developed 72 hours later and films for V1aR were developed 48 hours after being placed in the cartridge. An additional series of sections from the same individuals were then stained with cresyl violet to aid in the identification of brain regions in the developed film.

*Optical density measurements*

The developed autoradiography film was digitally scanned on a Microtek ScanMaker 5900 (Microtek International, Inc., Hsinchu, Taiwan) at a resolution of 1200 dpi and saved as Tagged Image File Format (TIFF) files. Regions of the brain that were radiolabeled for OTR or V1aR were identified with reference to rodent brain atlases (Paxinos and Watson, 2015, http://atlas.brain-map.org/). Optical density measurements of the scanned film were then made in ImageJ (NIH, http://imagej.nih.gov/ij/) following the same procedures as in Ophir et al. (2012). The measurements were taken at three different sections of the brain in both right and left hemispheres and from fibre tracts in the same sections. The raw data was then converted to disintegrations per minute/milligram of tissue estimated from rat brain (dpm/mg) using a power function for OTR and exponential function for V1aR to fit the curves generated in relation to the $^{125}$I standards. The final values were calculated by subtracting the density of each receptor fiber tracts (measures were taken from fiber tracts on the same sections as the region of interest) from receptor density of each region of interest. These
values were then averaged across sections to yield an estimate of the receptor density of each brain region for each individual.

Only structures with apparent labeling were measured and I used the same rostro-caudal location across individuals. Measurements in the majority of brain regions were taken by highlighting the entire brain region, but for some brain regions I measured subregions separately as well or focused only layers in which expression was evident. For example, in the nucleus accumbens, the core (NAcC) and shell (NAcSh) were measured separately as well as receptor density for the entire nucleus accumbens (i.e., NAcc + NAcSh). I also divided the BST into dorso-medial, dorso-lateral and ventral divisions following Paxinos and Watson (2005) and Campbell et al. (2009). Within the olfactory bulbs (OB), the layers could be identified and were therefore measured separately. Similarly, I measured different parts of the hippocampus separately, including the subiculum, CA regions and layers within the dentate gyrus. Receptor density measurements in the cerebral cortex were restricted to layers 5 and 6 and measured in two different regions. The first was around the anterior forceps of the corpus callosum trying to include the primary somatosensory, anterior cingulate, prelimbic and infralimbic areas of the layer 6 and 5, which I refer to as frontal cortex (FC). The second was more caudal in the secondary motor area (MOs).

Statistical analyses

To test for significant differences between the sexes for each brain region, I used a generalized linear model (GLM), as implemented in JMP (v. 12, SAS Institute). Analyses for V1aR and OTR were performed separately. For each
model, sex, brain region and their interaction term were included as effects and treated as a normal distribution. Pairwise t-tests were then used to determine if there were significant sex differences within individual brain regions.

**Results**

V1aR is expressed in all regions of the social behavior network (SBN), in regions that overlap between the SBN and the reward system, such as lateral septum (LS) and bed nucleus of the stria terminalis (BST) and in regions of the mesolimbic reward network (striatum, ventral tegmental area, nucleus accumbens, hippocampus, ventral pallidum). Also, V1aR was expressed in many brain regions with expression appearing outside of these networks, including in the cerebellum, medulla and superior colliculus (Table 1). Overall, in comparison to V1aR, the number of structures with OTR expression was relatively low, with strong expression in the dentate gyrus, amygdala, thalamus and LS (Table 1).

**V1aR**

**Olfactory bulbs**

Radiolabeling was present and uniform throughout the mitral and external plexiform layers of the olfactory bulbs and was more intense in the mitral cell layer than in the external plexiform layer (Figure 1a). The olfactory nerve, glomerular, inner plexiform and granule layers did not appear to have any labeling. V1aR expression was also moderate to strong in the accessory olfactory bulb (Figure 1a), but determining what layer or layers within the accessory olfactory bulb had radiolabeling was not possible.
Cerebral cortex

Labeling was present in layer 6 of the frontal cortex, with weak labeling in layer 5 and no labeling in layers 1-4 (Figure 1b). The labeling in layer 6 of the cortex varied from weak to moderate among individuals. Going from rostral to caudal, the labeling is concentrated in the center of the hemisphere, at the beginning of the frontal cortex, and separates when the corpus callosum appears. The labeled areas always contacted the dorsal and medial parts of the corpus callosum. The radiolabeling is strongest in the secondary motor area. It also occurs in the primary somatosensory, anterior cingulate, prelimbic and infralimbic areas, but the radiolabeling intensity was lower than in the primary motor area. More caudally, at the level of the lateral septum, the labeling becomes concentrated dorsally in the primary and secondary motor areas only. The labeling is slightly stronger on the dorsal and medial part of the cortex around the anterior forceps of the corpus callosum, and dorsally when the fibers of both hemispheres connect.

Striatum

Nucleus accumbens (Figure 1c) labeling varied from moderate to strong across individuals (Table 2). In the nucleus accumbens, receptor binding occurred at the ventral edge of the structure, forming a consistent curve outlining nucleus accumbens shell (Figure 1c). The nucleus accumbens core (Figure 1c) also had labeling, but it was diffuse and punctate and not uniformly labeled. Binding within both nucleus accumbens core and shell appear to be slightly
stronger around the location where the corpus callosum unites both hemispheres. The punctate labeling in the nucleus accumbens core increases in number and size moving caudally through the brain until it becomes more ventral and then dissipates, just before the lateral septum appears.

The olfactory tubercle labeling (Figure 1d), like in the nucleus accumbens core, is punctate and varies from weak to moderate in different individuals. Unlike the ‘dotted’ labeling in the nucleus accumbens core, the olfactory tubercle appears to have ‘stripes’ of labeling in a dorso-ventral orientation. The labeling is also expressed in a rostro-caudal gradient that begins rostrally with weak labeling that intensifies and then weakens until it disappears shortly after at the caudal pole of the nucleus accumbens.

The labeling in the lateral septum (Figure 1e) is strong in all individuals. At its rostral pole, the labeling is weak, but becomes very strong after the nucleus accumbens ends. Moving caudally, the labeling remains very strong until it disappears at the level of the anterior commissure.

**Amygdala**

The central amygdala has no V1aR expression, but the labeling is strong in the medial amygdala (Figure 1g and Figure 1h). The labeling in the most medial part of the medial amygdala has a well-defined, rounded shaped whereas the most lateral part of the medial amygdala has more diffuse and variable labeling. The labeling becomes darker and the shape of the medial amygdala changes to a parabola, with the two ends of the U-shape losing distinction near their ends. The amygdala expression starts to fade slowly until disappearing
shortly after the medial amygdala turns into this U-like shape. The labeling is not uniform throughout the medial amygdala and suggests that subregions within it differentially express V1aR.

**Hypothalamus**

Three hypothalamic nuclei could be identified by V1aR expression: the lateral preoptic area (Figure 1e and Figure 1f), medial preoptic area (Figure 1e) and ventromedial hypothalamic nucleus (Figure 1h). When expression is first observed at the rostral end of the hypothalamus, it is possible to identify the lateral preoptic area (LPO), which had a moderate amount of labeling. Initially, the LPO is localized medially, forming a V-like shape across the hemispheres. More dorsally, the LPO moves laterally and ventrally and until the expression disappears. Shortly after the LPO expression appears, what seems to be the medial preoptic area (MPO) becomes visible with weak labeling. Further dorsally, some parts of the hypothalamic medial zone show weak labeling, including the ventromedial hypothalamic nucleus. Defining the exact regions of HY expressing receptors is quite challenging because the HY has many nuclei and the ones showing expression lack well-defined cytoarchitectonic boundaries.

**Thalamus**

V1aR expression was very strong in rostral (Figure 1g) and caudal thalamus (Figure 1i and Figure 1j) and restricted to specific regions. Although defining precisely what regions are expressing V1aR is difficult, several thalamic regions do not express any V1aR: ventral medial and lateral groups of the dorsal
thalamus and the reticular nucleus of the thalamus. The strongest expression is in the rostral thalamus along the midline, which likely represents the anterior nuclei and nucleus reuniens. The lateral geniculate nucleus of the thalamus also expressed V1aR and the intensity of expression increases towards the caudal pole.

**Bed nucleus of the stria terminalis**

The labeling in the bed nucleus of the stria terminalis overall is moderate/strong (Figure 1f). Three parts could be distinguished: dorso-medial (BSTdm), dorso-lateral (BSTdl) and ventral (BSTv) (Figure 3). The labeling varies slightly between these parts, with the dorso-medial being the darkest. The receptor expression starts at the same rostro-caudal level as the anterior commissure and is strongest when the anterior commissure connects the brain hemispheres, separating the bed nucleus of the stria terminalis into dorsal and ventral parts (Figure 3).

**Hippocampus**

Several parts of the hippocampus express V1aR. The strongest labeling is in the polymorphic layer of the dentate gyrus (Figure 1g, Figure 1h, Figure I and Figure 1j) and appears to be equally strong in rostro-caudal and dorso-ventral aspects. The molecular layer of the dentate gyrus also had radiolabeling that was uniform throughout all sections (Figure 1i and Figure 1j), but the intensity was much weaker than that of the polymorphic layer. In contrast, I detected no labeling in the granule layer. Measurements were taken in the rostral and caudal
hippocampus. The subiculum (Figure 1i and 1j) had weak labeling that was uniform throughout rostro-caudal and dorso-ventral axes as well. In the subiculum, the molecular and pyramidal layer have a slightly stronger receptor expression than the stratum radiatum (Figure 1i and 1j).

**Midbrain/Mesencephalon**

The superior colliculus has strong labeling in the superficial, primarily sensory layers: the zonal, superficial grey and optic layers, with the labeling slightly stronger in the optic layer (Figure 1i and Figure 1j). For all layers, the labeling is uniform throughout the extent of the superior colliculus. In contrast, the deeper layers did not have any labeling. The periaqueductal gray (PAG) (Figure 1i and 1j) has weak and diffuse labeling.

**Brainstem**

Two regions within the brainstem had V1aR labeling: the spinal tract of the trigeminal nerve (Figure 1m) and the hypoglossal nucleus (Figure 1l). The labeling in the spinal tract of the trigeminal nerve is strong and uniform throughout the medulla. The labeling in the hypoglossal nucleus is moderate.

**Cerebellum**

Moderate labeling in the cerebellum appeared to be localized to the granule cell layer of both the vermis and hemispheres (Figure 1k). The labeling is slightly weaker towards the rostral end of the cerebellum, but otherwise uniform.
with no obvious differentiation among lobules or between the vermis and hemispheres.

**OTR**

*Olfactory bulb*

Moderate labeling was present and uniform throughout the external plexiform layer of the olfactory bulb (Figure 2a), with no labeling in the other layers. Labeling was also present in the accessory olfactory bulb (Figure 2a), but as with V1aR, the resolution was insufficient to determine whether this occurred in a specific layer or throughout the accessory olfactory bulb.

**Striatum**

The nucleus accumbens core labeling was weak in general (Figure 2b), but was moderate in a few individuals. The nucleus accumbens shell was moderate in the caudal pole (Figure 2c). As with V1aR, OTR binding occurred on the ventral edge of the structure, but does not form a consistent curve along nucleus accumbens shell. The curve in the dorsal part of NAcc shell was also punctate like in the nucleus accumbens core. The dotting of the nucleus accumbens core and the half circle line of the nucleus accumbens shell appear to be slightly stronger around the location where the corpus callosum unites both hemispheres. The punctate labeling in the nucleus accumbens core was uniform and extends to the caudal pole, increasing in number and size until the punctate structures becomes more ventral and less distinct.
The labeling in the lateral septum (LS) (Figure 2c and Figure 2d) was strong in all individuals, but not uniform and its shape was very similar to that of V1aR. LS became visible in the medial brain when the nucleus accumbens appeared. It was more visible, but still weak, around the level that the nucleus accumbens disappeared, and it becomes very strong until it vanishes near the anterior commissure connection.

**Bed nucleus of the stria terminalis**

The labeling in the BST was strong in the oval nucleus (Figure 2e) and moderate in the rest of the structure (Figure 2d and Figure 2e). Like in V1aR (Figure 3), the structure was divided into three parts, dorso-medial, dorso-lateral and ventral and all three regions had a moderate level of labeling. Even though they all express moderate labeling relative to other brain regions, the labeling varied slightly among these parts, with the dorso-medial and ventral being the densest. The receptor expression started at the level of the anterior commissure and was strongest when the anterior commissure connected, separating the BST into dorsal and ventral regions. The oval nucleus expression was not present in V1aR and it was the region with strong labelling in OTR.

**Amygdala**

Both the central and medial amygdala express OTR. Within the central amygdala, the labeling was strong and uniform throughout (Figure 2f and Figure 2g). As with V1aR, OTR labeling in the most medial part of the medial amygdala was well-defined, but the labeling in the lateral part was diffuse (Figure 2f, Figure
2g and Figure 2h). The amygdala expression started to fade caudally. However, one part of the medial amygdala did not fade and maintains strong labeling until the ventral part of the lateral ventricle was observed (Figure 2h).

**Thalamus**

The thalamic labeling was very similar to the V1aR expression (Figure 2f), with very strong labeling in the dorsal thalamus, nucleus reuniens and caudal thalamus and no apparent labeling in the ventral, medial or lateral groups or the reticular nucleus. The strongest expression was in the rostral thalamus in what is likely the anterior group of the dorsal thalamus and the nucleus of reuniens, which merge together along the midline. The expression in the geniculate nucleus of the thalamus was localized laterally and continues to the caudal pole of the thalamus (Figure 2i and 2j). Although it was difficult to determine exactly in which nuclei this caudal labeling occurs, the most likely candidate was the medial geniculate complex.

**Hypothalamus**

In contrast to V1aR, the hypothalamus labeling was weak and diffuse whenever it was visible, and individual nuclei could not be reliably identified based on labeling alone. The hypothalamus region with expression was in the middle of the structure, and has therefore been tentatively identified it as medial zone of the hypothalamus (Figure 2f and 2g).

**Hippocampus**
The hippocampus had OTR labeling throughout all layers of the dentate gyrus (DG) and CA. The strongest labeling in the hippocampus was in the molecular layer of the dentate gyrus (Figure 2f, Figure 2g, Figure 2h, Figure 1i, and Figure 1j) but it was not uniform. Expression in all layers of DG were stronger (particularly in the molecular layer) caudally at the point where the dorsal and ventral DG approach each other and the sections stretch further dorsally until they connect forming a crescent shape (Figure 8 – 2:14, 2:15 and 2:16). It was this crescent shape structure, formed by all the DG layers that had the strongest expression in the DG. The polymorphic and granule cell layers (Figures 2g, 2h, 1i, and 1j) had moderate and weak expression and in contrast to the molecular layer. Measurements were taken in the rostral and caudal hippocampus (Figure 2h).

Midbrain/Mesencephalon

The superior colliculus (Figure 2h, 2i, and 2j) had moderate to strong labeling in the zonal layer and weak labeling in the optic and superficial gray layers. Like in V1aR, there was expression in all three layers, but the labeling was stronger in the zonal layer rather than in the optic layer. The labeling was uniform throughout its progress towards the caudal part. Also, like the V1aR expression, the deeper layers of the superior colliculus did not express any OTR. The periaqueductal gray had weak and diffuse labeling (Figures 2h, 2i, and 2j).)

Brainstem
Two regions in the brainstem had OTR expression: the spinal tract of the trigeminal nerve (Figure 2m) and nucleus prepositus (Figure 2l). The labeling in both regions was moderate to strong and uniform throughout their rostro-caudal axes.

Cerebellum

Weak labeling was present in the granule cell layer of both the vermis and hemispheres (Figure 2k), but it was inconsistent within individuals. The inconsistency did not occur along medio-lateral or rostro-caudal gradients and there were no consistent differences among lobules or between the vermis and hemispheres.

Sex differences in V1aR and OTR binding

Due to tissue quality and radiolabeling intensity, I could only test for sex differences in 18/19 individuals that were processed for both V1aR and OTR. The GLM of V1aR labeling revealed significant differences among brain regions ($\chi^2 = 826.02$, df = 30, 491, $p < 0.0001$), but no overall sex difference ($\chi^2 = 0.23$, df = 1, 491, $p = 0.63$) and no significant interaction term ($\chi^2 = 36.51$, df = 30, 491, $p = 0.19$). However, pairwise comparisons yielded significant sex differences in the mitral cell layer of the olfactory bulbs ($p = 0.01$), BSTdm ($p = 0.001$) and a trend for a sex difference in BSTv ($p = 0.051$). As shown in the boxplots (Figure 4a), males had significantly higher expression than females in MiL (+19%). In contrast, females had higher V1aR expression in both dorsomedial (+84%) and
ventral (41%) parts of BST (Figure 4b and 4d). There was no significant sex difference within the dorsolateral part of BST (p = 0.47, Figure 4c).

The GLM of OTR labeling revealed significant main effects of brain region ($\chi^2 = 963.12$, df = 24, 407, p < 0.001) and sex ($\chi^2 = 8.90$, df = 1, 407, p = 0.003), but the interaction term was not significant ($\chi^2 = 25.14$, df = 24, 407, p = 0.40). Males had significantly higher overall OTR expression than females, although this amounted to only an 11% difference based on means. Pairwise comparisons between the sexes within each brain region yielded significant sex differences in two brain regions: dentate gyrus and the amygdala. Within the dentate gyrus, males had significantly higher expression than females in the molecular layer (p = 0.002, +20%, Figure 5a), but not in the granule/polymorphic layer (p = 0.80, Figure 5b). Males also had significantly higher OTR expression in the medial amygdala (p = 0.006, +28%, Figure 5c) than females, but there was no sex difference in the central amygdala (p = 0.62, Figure 5d).

**Discussion**

**V1aR expression**

V1aR expression was present in at least 17 different brain regions (not counting variable expression within some brain regions) in Richardson’s ground squirrels; more than those that expressed OTR (see below). Overall, the number of brain regions reported to express V1aR varies greatly across rodent species. *Peromyscus* mice, tuco-tucos and the guinea pig appear to express V1aR in relatively few brain regions (Tribollet et al., 1992a; Insel et al., 1991; Beery et al.,
but singing mice, rats, voles and hamsters express V1aR in a similar range and number of brain regions to the ground squirrel (Campbell et al., 2009; Johnson et al., 1993; Kremarik et al., 1993, Wang et al., 1997). However, directly comparing the number of brain regions that express V1aR across species is difficult due to variations in how the neuroanatomical distribution of receptor expression is reported. First, not all studies examine expression throughout the entire brain, so finding more or fewer brain regions expressing V1aR in one species versus another is often the product of what was processed and reported. Second, many brain regions have heterogeneous expression such that some parts express V1aR whereas other parts do not, or expression varies in intensity within a brain region. Both of these patterns were clearly apparent in Richardson’s ground squirrels in the olfactory bulbs and BST (Figures 1a, 3 and 6). Not all studies report this kind of variation or measure receptor density separately for subregions, which makes comparing the number of brain regions expressing V1aR difficult across species.

Despite these two caveats in comparing number and overall distribution of V1aR across species, it was expressed in all of the brain regions in which it is typically found in rodents in Richardson’s ground squirrels (Campbell et al., 2009; Beery et al., 2008; Wang et al., 1997; Johnson et al., 1993; Kremarik et al., 1993; Tribollet et al., 1992a). Thus, V1aR was expressed in all brain regions within the social behavior network (lateral septum, preoptic area, ventromedial hypothalamus, anterior hypothalamus, periaqueductal gray, medial amygdala, and bed nucleus of the stria terminalis), most brain regions within the mesolimbic reward network (ventral tegmental area, nucleus accumbens, basolateral
amygdala, ventral pallidum, lateral septum and bed nucleus of the stria terminalis), as well as the olfactory and accessory olfactory bulbs (table 1). The social behavior network is a strongly connected network that regulates numerous social behaviors (O’Connel and Hofmann, 2011). All the structures in this network also have the presence of gonadal hormones (Wood and Newman, 1995; Simerly et al., 1990; Commins and Yahr, 1985). The most well understood behaviors regulated by the social behavior network are sexual, maternal and reproductive behavior (Newman, 1999). The mesolimbic reward system also regulates behavior, but is mainly controlled by the reward answer caused by certain behavior. It works as a measurement of motivation based on reward stimuli (O’Connel and Hofmann, 2011). The brainstem and cerebellum are rarely discussed or reported in studies of nonapeptide receptor expression, presumably because they are not examined, not sectioned or are otherwise damaged. It is therefore difficult to interpret much from V1aR expression of the brainstem and cerebellum until a broader comparative dataset becomes available.

Apart from the social behavior and mesolimbic reward networks, I found strong V1aR expression in the superior colliculus and olfactory bulbs (table 1). V1aR is expressed in the superior colliculus of some other rodents (Insel et al., 1994; Kremarik et al., 1993; Insel et al., 1991; Dubois-Dauphin et al., 1990; Tribollet et al., 1988), but not all of them (Campbell et al., 2009; Beery et al., 2008). Comparisons of receptor density across studies are not possible due to variations in autoradiographic techniques, but the labeling intensity appears to be relatively higher in the ground squirrels compared with other species. In terms of function, it is well established that the superior colliculus plays a critical role in
processing visual and multisensory information (May, 2006; Krauzlis et al., 2013). The dorsal layers primarily receive visual inputs and are critical for generating responses to visual stimuli (Gandhi & Katnani 2011). However, the intermediate and deep layers are multisensory and respond to visual, auditory and tactile stimuli (Castro-Alamancos and Favero, 2016; May, 2006; Wise and Irvine, 1983). Although there did not appear to be any differentiation in V1aR expression between dorsal, intermediate and deep layers, it is possible that the very strong expression I observed through the superior colliculus of ground squirrels reflects some aspects of vocal communication. The other rodent species in which V1aR is expressed in the SC have relatively large vocal repertoires (e.g., Campbell et al., 2009) and vocal communication, especially alarm calling, is a key component of the social system of Richardson's ground squirrels (Hare, 1998). Individual Richardson's ground squirrels quickly orient towards alarm calls and can discriminate between neighbours and non-neighbours as well as reliable and non-reliable callers (Hare and Atkins, 2001; Hare, 1998). The superior colliculus modulates pinna movements towards sounds and integrates visual and auditory inputs in sound source localization (May, 2006) and is likely playing a major role in detecting and localizing alarm calls. Whether the superior colliculus plays a role in discriminating callers or not and what function(s) AVP might have in that process are, however, unknown.

Social communication is also likely a factor that explains the strong expression of V1aR in the olfactory bulb. AVP and OT are both important in social recognition based on olfaction (Wacker and Ludwig, 2012). In rats, the olfactory bulb has a large population of AVP expressing neurons as well as
expression of V1aR. Infusion of AVP into the bulbs enhances social recognition and blocking V1aR within the OB significantly impairs social recognition (Dluzen et al., 1998; Tobin et al., 2010). Similarly, infusion of AVP and OT increases how long hamsters (*Mesocricetus auratus*) can remember the scent of a previously encountered individual (Song et al., 2016). Activation of V1aR can even increase scent-marking behaviors (Song et al., 2014). The strong expression of V1aR observed in the mitral cell layer of the olfactory bulbs suggests that AVP could also mediate olfactory-based social recognition in Richardson’s ground squirrels.

Olfactory communication is important for all species of ground squirrels (Mateo, 2009, 2003). Ground squirrels have relatively large numbers of scent glands along the cheeks (oral glands) and the back and sides (dorsal glands) in addition to extrusible anal glands (Kivett et al., 1976). These glands are used to scent mark and are contacted directly during ‘greeting’, an amicable behavior in which both squirrels sniff one another’s oral gland regions (Kivett et al., 1976). Other ground squirrel species can discriminate familiar from unfamiliar individuals and kin and non-kin based on scent alone (Mateo, 2009, 2006, 2003; Harris and Murie, 1982). Like other ground squirrels, Richardson’s ground squirrels differentiate kin from non-kin (Hare, 1998; Davis, 1982; Michener, 1974) and although it has not been tested directly, it is likely that this recognition is at least partially based on olfactory cues. High V1aR expression in the olfactory bulbs would then enhance individual discrimination in Richardson’s ground squirrels, enabling members of a colony to recognize siblings as well as potentially identify reproductive status and/or age, assist dispersal and avoid inbreeding (Mateo, 2006, 2003).
**OTR expression**

OTR was expressed in fewer brain regions than V1aR (tables 1,3). As with V1aR, the number of brain regions reported to express OTR varies greatly across rodent species; rats express OTR across many brain regions (Tribollet et al., 1992b; Freund-Mercier et al., 1987; De Kloet et al., 1985), but most other rodents express OTR in a similar number of regions to the ground squirrel (Kalamatianos et al., 2010; Campbell et al., 2009; Dubois-Dauphin et al., 1992; Insel and Shapiro, 1992a; Tribollet et al., 1992a). Again, direct comparisons across species and studies are difficult due to variations in what parts of the brain are analyzed and even reported upon.

In contrast to V1aR, OTR was not expressed in all brain regions within the social behavior network. More specifically, I did not find expression of OTR in the preoptic area. Other species, Patagonian tuco-tuco and social tuco-tuco, have OTR expression in the preoptic area (Beery et al., 2008). I did see weak OTR expression in the periaqueductal grey (PAG), which has been reported in rats only (Yang et al., 2011). Singing mice express V1aR in the PAG, but not OTR (Campbell et al., 2009). The PAG is one of the regions involved in coordinating fear responses (Watson et al., 2016) as well as pain modulation (Yang et al., 2011). In rats, the pain threshold is increased with injection of oxytocin in the PAG (Yang et al., 2011). The regulation of pain threshold might be especially important for male Richardson’s ground squirrels. During breeding season, they engage in strong agonistic behavior with other males competing for access to females and extensive injuries are relatively common (Michener, 1983b).
In relation to the brain regions of the mesolimbic reward network, OTR was present in some, but not all regions (Tables 1 and 3. Figures 2a, 2b, 2f, 2g, 2h, 2i, and 2j). Although OTR was expressed in the nucleus accumbens, there was no expression in the ventral tegmental area, ventral pallidum or basolateral amygdala. Instead, the ground squirrels expressed OTR in the central and medial nuclei of the amygdala (Table 3. Figures 2f, 2g, and 2h) and there was a significant difference in receptor density between these two regions. The central amygdala had the strongest expression for OTR, whereas the medial amygdala had more moderate labeling (Table 3. Figures 2f, 2g, and 2h). The central amygdala is known for regulating fear (Ciocchi et al., 2010) and its activity can be modulated by oxytocin (Huber et al., 2005). It is likely performing the same function in ground squirrels, which is supported by a lack of sex difference in the central amygdala (in contrast to the medial amygdala, see below).

The dentate gyrus (DG) also has very strong OTR expression, but the strength of expression varied across its layers. Expression in OTR was strongest in the molecular layer (Table 3. Figures 2f, 2g, 2h, 2i, and 2j), which is comprised primarily of dendrites from the granule layer and fibers from the entorhinal cortex (Treves et al., 2008; Amaral et al., 2007). In contrast, OTR within the granule and polymorphic layers was significantly lower (Table 3. Figures 2g, 2h, 2i, and 2j). Adult neurogenesis in the DG is known for its relation to glucocorticoids, anxiety and stress (Snyder et al., 2011). However, a recent study in rats demonstrated that oxytocin also regulates neurogenesis in the DG (Opendak et al., 2016). OT, during periods of social instability and recovery, were able to restore normal levels of neurogenesis in stressed rats (Opendak et al., 2016). So, OT in the DG
is responsible for resilience in period of stress. In the rat study, the stress was caused by the disruption of the hierarchy that is present in the rats’ social system. Although male Richardson’s ground squirrels are not known to have a dominance hierarchy, they are strongly territorial (Michener, 1979) and stress levels are likely very high during intense periods of male-male competition for females (Michener, 1998). Under these high stress conditions, OT could be acting on OTR to help offset the effects of high corticosteroid levels on DG neurogenesis in an analogous fashion to that observed in rats (Opendak et al., 2016).

**Sex differences**

Despite profound behavioral sex differences in Richardson’s ground squirrels, including social behaviors (Michener, 1998), I detected relatively few sex differences in V1aR or OTR expression. Although this might be unexpected given the social behavior of Richardson’s ground squirrels, reports of sex differences in the expression of V1aR and OTR are uncommon in the literature and are highly specific to both species and brain region (reviewed in Dumais and Veenema 2016). For example, sex differences in V1aR were found in two regions, medial preoptic area and mammillary nuclei, in C57b6 mice (Dubois-Dauphin et al., 1996), but none were found in ICR mice (Tribollet et al., 2002). In prairie and montane voles, only V1aR in the medial prefrontal cortex is significantly different between the sexes (Smeltzer et al., 2006) and singing mice (Campbell et al., 2009) and tuco-tucos (Beery et al., 2008) have no sex differences in V1aR. Similar patterns are reported in OTR expression; sex differences are present across many brain regions in *Peromyscus* mice (Insel et
al. 1991) and rats (Dumais et al. 2013), but only one or two brain regions in other species (Cao et al., 2014; Campbell et al., 2009; Beery et al., 2008; Smeltzer et al., 2006; Tribollet et al., 2002). Some species even lack sex differences in OTR entirely (Hammock and Levitt, 2013; Dubois-Dauphin et al., 1992). Thus, our results are similar to those of previous studies in that sex differences only occurred in a subset of the brain regions that showed nonapeptide receptor expression.

As shown in figures 4a and 4b, V1aR was significantly different between the sexes in two brain regions: the mitral cell layer of the olfactory bulbs and the dorsomedial part of BST. The sex difference in the olfactory bulbs was, however, relatively small and there was clearly a lot of overlap in V1aRdensity between males and females (Figure 4a). Nevertheless, this could reflect a difference in the action of vasopressin on olfactory-mediated behaviors. Vasopressin appears to play an important role in memory for male rodents and oxytocin for females (Engelmann et al., 1998; Bluthe and Dantzer, 1990). In Richardson’s ground squirrel, the higher expression of V1aR in the mitral layer in males might aid in recognizing and remembering other individuals. This would enable males to avoid inbreeding with related females and optimize reproductive success by identifying the females that they had already copulated with.

The largest sex difference in V1aR was in BST. More specifically, the dorsomedial part of BST had a significantly higher optical density in females than males (Figure 4b). A similar trend was observed in the ventral region (Figure 4c), but no difference was present in dorsolateral BST (Figure 4d). Other studies subdivided BST based on receptor expression (Dumais and Veenema, 2016;
Campbell et al., 2009), but there are relatively few reports of sex differences in BST for either V1aR or OTR. In all cases that reported sex differences, males, not females, had higher receptor expression. Only one study reported higher V1aR in the BST in male rats (Dumais et al., 2016). In the other cases, male rats and male mice had higher expression for OTR, and not V1aR (Dumais et al., 2013; Insel et al., 1991). BST is responsible for aggression, parental behavior, avoidance behavior and copulatory behavior (Nelson and Kriegsfeld, 2017) and injection of AVP in the BST reduces aggressive behavior in male rats (Veenema et al., 2010). The synthesis of vasopressin in the BST is regulated by gonadal hormones (Nelson and Kriegsfeld, 2017), which also affect the V1aR density within the BST (Caldwell and Albers, 2004; Young et al., 2000). Many studies have shown that the sex difference in AVP production in the BST is affected by testosterone (Rasri et al., 2008; De Vries et al., 1994; Wang et al., 1994), but it remains unknown if testosterone can also affect V1aR expression. Although it is unclear what the higher expression of V1aR in female Richardson’s ground squirrels means in terms of behavior, one possible explanation is that the higher expression of V1aR in females is modulating their aggression and territorial behavior towards each other (Michener, 1979). Although there are no reports of a dominance hierarchy among females, they do vary in their behavior towards one another with more aggression directed towards non-kin than kin (Michener, 1979). Similar to how Veenema et al. (2010) showed that AVP injections reduce aggression, having more V1aR in BST could enable females to have finer control over their aggressive behavior and cause an overall reduction in aggression.
In contrast to the large sex difference in BST, I found moderate sex differences in OTR expression in the medial amygdala (MEA, Figure 5c) and molecular layer of the DG (Figure 5a). The MEA receives direct projections from the AOB and sends information to the hypothalamus (Keshavarzi et al., 2014) and it is responsible for fear responses, defense, copulatory behavior, social interest and parental care (Cao et al., 2014; Phelps and LeDoux, 2005). Two previous studies reported sex differences in MEA for OTR: singing mice (Campbell et al., 2009) and rats (Dumais et al., 2013). In both studies, males had higher expression and the males compete for dominance or territories (Pasch et al., 2013; Blanchard et al., 1984). Cao et al. (2014) conducted a study in mandarin voles (Microtus mandarinus) in which behavioral changes were linked to OTR expression. OTR in MEA was reduced due to paternal deprivation, and reduced time of social investigation during contact with new individual and time of exploratory behavior (Cao et al., 2014). If OTR in Richardson’s ground squirrels is regulating social interest, it could be because males are more likely to explore the environment when trying to find receptive females. If this is correct, then after the breeding season, when males stop interacting with other individuals and concentrate on gaining weight, males would have lower OTR in the MEA. OTR could also, like the experiments of aggressive behavior and AVP (Koolhaas et al., 1990), be regulating aggressive behavior in Richardson’s ground squirrel. In the Koolhaas et al. (1990) study, injection of AVP in the medial amygdala increased aggression in males, but it is possible that in Richardson's ground squirrels, OTR would be regulating aggression instead. The regulation of the same behavior in the same structure by different nonapeptides is not uncommon and supports the
idea that the expression of specific nonapeptides and receptors are species-specific (Dumais and Veenema, 2016; Goodson and Thompson, 2010).

Last, I found a significant sex difference in OTR expression in the molecular layer of the dentate gyrus (DG), with males having higher expression than females (Table 3). This external layer of the DG has very few cells, and is composed mostly of dendrites of the granule cells from the granular layers of the DG, and some axons that are mostly from the entorhinal cortex (Scharfman, 2016). During the process of neurogenesis, new granule cells send the dendrites to the molecular layers (Zhao et al., 2006) and these new neurons play a role in learning and memory (Winocur et al., 2006). Sex differences in spatial learning and memory have yet to be tested in Richardson’s ground squirrels, but males do have much larger home ranges and travel further than females during the breeding season (Michener and McLean, 1996). This sex difference in spatial behavior is not associated with sex differences in DG size or number of granule cells in the breeding season (Burger et al., 2014, 2013). Neurogenesis rates in male Richardson’s ground squirrels does not differ between breeding and non-breeding seasons, but it does differ markedly in females (Burger et al. 2014). This might occur because females are more susceptible to changes caused by stress or the synergistic effects of estrogens and stress hormones on hippocampal neurogenesis (Galea, 2008; Mirescu and Gould, 2006; Ormerod et al., 2004; Galea and McEwen, 1999). Although not a functional explanation, the higher OTR expression could help to preserve neurogenesis in males and support their larger movements during the breeding season.
Conclusions

Overall, the expression of vasopressin receptors (V1aR) was more prevalent than oxytocin receptors (OTR) in Richardson's ground squirrel. V1aR is also more prevalent in structures of importance to sociality, such as all of the regions of the social behavior network, most brain regions within the mesolimbic reward network, and the olfactory and accessory olfactory bulbs. Based on available information of anatomy and function of these structures, all the regions with sex differences are contributing directly or indirectly to sex differences in behavior during breeding season. For V1aR, the sex differences in the mitral cell layer of the olfactory bulb and dorsomedial part of the BST might be associated, respectively, with memory mediated by olfactory clues and aggressive behavior. For OTR, the sex differences in the MEA are most likely related to exploratory or aggressive behavior, and for the molecular layer of the dentate gyrus to spatial performance differences. An important future direction will be comparing the pattern of nonapeptide receptors expression between and within sexes in different seasons. Freeman (2016) provides preliminary data from male juvenile Richardson's ground squirrels that appears to have expression differences in a few structures from the autoradiography figures in the current study, in the paraventricular nucleus and the thalamus for OTR and in the bed nucleus of the stria terminalis and the hippocampus for V1aR. While studies with Richardson's ground squirrels provide a starting point to understand how nonapeptide receptor expression is related to sociality in ground squirrels, other species within the group will need to be studied to find patterns typical of this group and compare across social systems.
Table 1. A summary of the brain regions that had radiolabeling for V1aR and OTR in Richardson’s ground squirrels. For each region of interest, a score is provided for radiolabeling intensity as follows: - none, + weak, ++ moderate, +++ strong, and ++++ very strong.

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>V1aR</th>
<th>OTR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Social behavior network</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial amygdala</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Preoptic area</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Anterior hypothalamus</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Ventromedial hypothalamus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Mesolimbic reward network</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Basolateral amygdala</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><strong>Social behaviour and mesolimbic reward networks</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bed nucleus of the stria terminalis</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Lateral septum</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>CA field</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subiculum</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Other brain regions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral layer</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>External plexiform layer</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Accessory olfactory bulb</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Secondary motor area</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thalamus</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Dorsal thalamus</td>
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<td>+++</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Brainstem nuclei</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>
Table 2. The mean receptor density and standard deviation (SD) of each sex for each of the 28 brain regions that had radiolabeling for V1aR.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Females (n = 11)</th>
<th>Males (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Olfactory bulb - mitral Layer</td>
<td>2592.88</td>
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<td>Olfactory bulb - external plexiform layer</td>
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<tr>
<td>Frontal cortex</td>
<td>591.26</td>
<td>289.17</td>
</tr>
<tr>
<td>Nucleus accumbens shell</td>
<td>1349.95</td>
<td>559.45</td>
</tr>
<tr>
<td>Nucleus accumbens core</td>
<td>717.29</td>
<td>239.35</td>
</tr>
<tr>
<td>Nucleus accumbens core – Full ^1</td>
<td>648.30</td>
<td>260.57</td>
</tr>
<tr>
<td>Olfactory tubercle</td>
<td>728.46</td>
<td>354.66</td>
</tr>
<tr>
<td>Olfactory tubercle – Full ^2</td>
<td>344.58</td>
<td>79.81</td>
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<tr>
<td>Secondary motor area</td>
<td>451.96</td>
<td>157.13</td>
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<td>Lateral septum</td>
<td>2776.14</td>
<td>655.68</td>
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<td>Lateral preoptic area</td>
<td>1717.87</td>
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<tr>
<td>Medial preoptic area</td>
<td>1142.63</td>
<td>360.41</td>
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<td>Ventromedial hypothalamic nucleus</td>
<td>1303.34</td>
<td>349.90</td>
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<td>Bed nucleus of the stria terminalis (dorso-medial)</td>
<td>1427.39</td>
<td>309.86</td>
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<tr>
<td>Bed nucleus of the stria terminalis (dorso-lateral)</td>
<td>954.11</td>
<td>202.99</td>
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<tr>
<td>Bed nucleus of the stria terminalis (ventral)</td>
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<td>338.67</td>
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<td>Anterior thalamus</td>
<td>3374.23</td>
<td>503.19</td>
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<tr>
<td>Geniculate group – posterior thalamus</td>
<td>2831.08</td>
<td>574.16</td>
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<td>Amygdala</td>
<td>1612.23</td>
<td>433.20</td>
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<tr>
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<td>823.55</td>
</tr>
<tr>
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<td>555.41</td>
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<td>Dentate gyrus – molecular layer</td>
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<tr>
<td>Cerebellum</td>
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<td>73.01</td>
</tr>
<tr>
<td>Spinal tract of the trigeminal nerve</td>
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<td>400.42</td>
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<tr>
<td>Hypoglossal nucleus</td>
<td>2238.71</td>
<td>1084.59</td>
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</tbody>
</table>

^1 Only the dots with binding expression were selected and measured. The number and size of the dots vary among individuals.

^2 An area that includes the dots with binding expression and parts without expression were selected in all sections that were measured. All sections were measured trying to keep the shape and size consistent.
Table 3. The mean receptor density and standard deviation (SD) of each sex for each of the 24 brain regions that had radiolabeling for OTR.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Females (n = 10)</th>
<th>Males (n = 8)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>External plexiform layer</td>
<td>278.01</td>
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<td>Accessory olfactory bulb</td>
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<td>Nucleus accumbens core</td>
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<td>Nucleus accumbens core – Full</td>
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<td>Lateral septum</td>
<td>322.24</td>
<td>50.74</td>
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<td>56.46</td>
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<td>Bed nucleus of the stria terminalis (dorso-lateral)</td>
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<tr>
<td>Bed nucleus of the stria terminalis (ventral)</td>
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<td>55.56</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminalis (oval nucleus)</td>
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<td>55.56</td>
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<td>Central amygdala</td>
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<td>Medial amygdala</td>
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<td>Hypothalamic medial zone</td>
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<td>Periaqueductal grey</td>
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<td>Dentate gyrus – molecular layer (rostral)</td>
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<td>Dentate gyrus – granular and polymorphic layers (rostral)</td>
<td>369.23</td>
<td>95.98</td>
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<tr>
<td>Dentate Gyrus – molecular layer (caudal)</td>
<td>1145.25</td>
<td>137.77</td>
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<tr>
<td>Dentate Gyrus – granular and polymorphic layers (caudal)</td>
<td>657.17</td>
<td>127.15</td>
</tr>
<tr>
<td>Hippocampus proper</td>
<td>227.34</td>
<td>73.33</td>
</tr>
<tr>
<td>Superior colliculus zonal layer</td>
<td>226.22</td>
<td>66.17</td>
</tr>
<tr>
<td>Geniculate group – posterior thalamus</td>
<td>307.34</td>
<td>52.97</td>
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<td>Cerebellum</td>
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<td>Nucleus prepositus</td>
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<tr>
<td>Spinal tract of the trigeminal nerve</td>
<td>233.67</td>
<td>74.72</td>
</tr>
</tbody>
</table>

1 Only the dots with binding expression were selected and measured. The number and size of the dots vary among individuals.

2 An area that includes the dots with binding expression and parts without expression were selected in all sections that were measured. All sections were measured trying to keep the shape and size consistent.
Figure 1: Brain sections from a Richardson’s ground squirrel showing Nissl stain (left) and autoradiography of V1aR binding (right). Arrows indicate binding in a: accessory olfactory bulb (AOB), mitral layer (MiL), and external plexiform layer, (EPI); b: frontal cortex (FC); c: secondary motor area (MOs), nucleus accumbens core (NAcC), and nucleus accumbens shell (NAcSh); d: lateral septum (LS) and olfactory tubercles (OTu); e: LS, lateral preoptic area (LPO), and medial preoptic area (MPO); f: bed nucleus of the stria terminalis (BST) and LPO. Scale bar = 1mm.
Figure 1 (cont’d): Brain sections from a Richardson’s ground squirrel showing Nissl stain (left) and autoradiography of V1aR binding (right). Arrows indicate binding in: g - polymorph layer of the dentate gyrus (DGpo), thalamus (TH), and amygdala (AMY); h - subiculum (SUB), DGpo, TH, AMY, and ventromedial hypothalamic nucleus (VMH); i - superior colliculus (SC), SUB, geniculate nucleus of the thalamus (GENd), DGpo, molecular layer of the dentate gyrus (DGmo), periaqueductal grey (PAG); j - SC, SUB, GENd, DGpo, DGmo, PAG, and interpeduncular nucleus (IPN); k - cerebellum; l - hypoglossal nucleus (HN); m - spinal tract of the trigeminal nerve (sptV).
Figure 2: Brain sections from a Richardson’s ground squirrel showing Nissl stain (left) and autoradiography of OTR binding (right). Arrows indicate binding in: a - accessory olfactory bulb (AOB) and external plexiform layer (EPI); b - nucleus accumbens core (NAcC), and nucleus accumbens shell (NAcSh); c - lateral septum (LS) and NAcSh; d - LS and bed nucleus of the stria terminalis (BST); e - oval nucleus of the bed nucleus of the stria terminalis (BSTov) and BST; f - molecular layer of the dentate gyrus (DGmo), thalamus (TH), central amygdala (CEA), and medial amygdala (MEA). Scale bar = 1mm.
Figure 2 (cont’d): Brain sections from a Richardson’s ground squirrel showing Nissl stain (left) and autoradiography of OTR binding (right). Arrows indicate binding in: g - molecular layer of the dentate gyrus (DGmo), polymorph and granular layer of the dentate gyrus (DGpo/gr), central amygdala (CEA), medial amygdala (MEA), and hypothalamic medial zone (HYmz); h - superior colliculus (SC), DGmo, DGpo/gr, hippocampus proper (CA), MEA, and periaqueductal grey (PAG); i - SC, geniculate group of the thalamus (GENd), DGmo, DGpo/gr, and PAG; j - SC, GENd, DGmo, DGpo/gr, and PAG; k - cerebellum (CB); l - nucleus prepositus (PRP); m - spinal tract of the trigeminal nerve (sptV). Scale bar = 1 mm.
**Figure 3:** Subregions of the bed nucleus of the stria terminalis (BST); arrows indicate the dorso-medial (BSTdm), dorso-lateral (BSTdl) and ventral (BSTv) parts of the BST.
Figure 4: Boxplots of the receptor density values for V1aR in the mitral cell layer of the olfactory bulb (MiL) and the bed nucleus of the stria terminalis (BST). The boxplots indicate the mean and minimum and maximum values within each sex. a – male (M) Richardson’s ground squirrels had statistically significantly higher receptor density values in the MiL than females (F); b – male Richardson’s ground squirrels had statistically significantly lower receptor density values in the BSTdm than females; c – males tended to have a lower receptor density values in the dorso-lateral part of the bed nucleus of the stria terminalis (BSTdl) than females, but this was not statistically significant (p = 0.051). d – males and females had no statistically significant difference in the receptor density values of the ventral part of the bed nucleus of the stria terminalis (BSTv).
Figure 5: Boxplots of the receptor density values for OTR in the dentate gyrus (DG) and medial (MEA) and central (CEA) amygdala. The boxplots indicate the mean and minimum and maximum values within each sex. a – male (M) Richardson’s ground squirrels had statistically significantly higher receptor density values in the molecular layer of DG than females (F); b – males and females had no statistically significant difference in the receptor density values of the granular and polymorph layer of the DG; c – male Richardson’s ground squirrels had statistically significantly higher receptor density values in the MEA than females. d – males and females had no statistically significant difference in the receptor density values of the CEA.
CHAPTER 3: GENERAL DISCUSSION

The primary goal of my thesis was to describe the neuroanatomical distribution of arginine vasopressin (V1aR) and oxytocin (OTR) receptors in the brains of male and female Richardson’s ground squirrels during their breeding period, when males display very aggressive behaviors when competing for access to females. Based on studies of V1aR and OTR in other rodent species and what is known about the behavior of Richardson’s ground squirrels, I expected that V1aR and OTR expression would differ between male and female ground squirrels because of the aforementioned sex differences in social behavior. More specifically, I expected to find sex differences in the expression patterns in the hippocampus, medial amygdala and laterodorsal thalamus (Campbell et al., 2009) because of sex differences in both spatial and social behavior (Michener, 1998). I also expected that females would have higher expression of V1aR in the bed nucleus of the stria terminalis and lateral septum, because they have fewer aggressive interactions (Veenema et. al. 2010). I did confirm sex differences in the DG of the hippocampal formation, medial amygdala, and bed nucleus of the stria terminalis but not in the laterodorsal thalamus and lateral septum. However, I did find a sex difference in the mitral layer of the olfactory bulbs, which was not predicted.

Overall, V1aR was expressed throughout the social behavior and mesolimbic reward networks as well as a number of other brain regions. OTR was also expressed in many of the same brain regions, but generally at lower levels. Despite the large number of structures expressing nonapeptide receptors, I only found sex differences in four regions. Determining why these brain regions
were sexually dimorphic, whereas others were not, is complicated because sex differences in OTR and V1aR are so variable across species (Campbell et al., 2009; Kalamatianos et al., 2010; Beery et al., 2008; Wang et al., 1997; Insel et al., 1994; Johnson et al., 1993; Kremarik et al., 1993; Dubois-Dauphin et al., 1992; Insel and Shapiro, 1992a; Tribollet et al., 1992a; Tribollet et al., 1992b; Insel et al., 1991; Dubois-Dauphin et al., 1990; Freund-Mercier et al., 1987; De Kloet et al., 1985). As I discussed in Chapter 2, sex differences in behavior are likely associated with sexual dimorphic receptor expression, but the precise roles of AVP and OT in these behaviors are unclear. Further studies with Richardson’s ground squirrels will be necessary to better understand the role of nonapeptides in their social interactions. For example, comparing receptor expression patterns across different seasons could be very beneficial for further understanding the role of nonapeptides in this group. A recently completed PhD thesis by Freeman (2016) on juvenile Richardson’s ground squirrels appears to show different receptor densities in some brain regions compared with my results. Specifically, there appear to be differences in the paraventricular nucleus and thalamus for OTR and in the bed nucleus of the stria terminalis and hippocampus for V1aR, but an insufficient number of sections were provided to determine this conclusively. Although these differences could be attributed to methodological differences, it is also possible that this represents a seasonal difference. Seasonal receptor variation is related to seasonal variation of grouping size in two wild sparrows (Wilson et al., 2016). It is also known that parental behaviors are regulated by nonapeptide hormones (Perea-Rodriguez et al., 2015), so differences in receptor densities of female Richardson’s ground squirrels would
very likely be found. Also, in voles, space used seems to be related to the laterodorsal thalamus, with wandering males, those with large home ranges that overlap the home ranges of many other males and females, having lower V1aR density than resident males, those with small home ranges (Ophir et al., 2008). According to this, male Richardson’s ground squirrels could have some seasonal differences due to their changes in space use too. Especially in males, differences found between the breeding and non-breeding periods would be valuable in order to understand the changes in aggressive behavior. Males stop aggressive interactions with other males remain solitary in the post-breeding season (Michener, 1979). In this case, the sex differences would be more closely related to sociality itself, but not with aggression.

As mentioned in Chapter 2, I came across several problems when comparing the nonapeptide receptor expression of ground squirrels with other studies. Often studies are missing information in regions like the brainstem, cerebellum and medulla. Many studies do not section the entire brain or fail to provide images throughout the brain and information about regions beyond the social behavior or mesolimbic reward networks (Beery et al., 2008). In fact, the studies on mole-rats (Kalamatianos et al., 2010; Mooney et al., 2015) did not even examine regions outside of the telencephalon. Providing at least one complete series of images throughout the entire brain allows them to be consulted in future studies and a better comparison analysis to be done. In addition, studies that fail to notice weak expression in structures and fail to report it will still be able to be consulted and used in future studies. In the accompanying appendix, I therefore provided an entire set of scanned film from olfactory bulbs
through to the medulla for each of the two receptors that I examined and I hope for future studies to engage in a similar degree of data transparency.

Second, heterogeneity of receptor expression in many brain structures also poses significant problems. The pattern of expression within structures like the thalamus, bed nucleus of the stria terminalis or hypothalamus, can vary a great deal, but when reported, are mentioned only as expression without any qualifying statements. For example, many species can express receptors in the thalamus, but the expression might be in different parts within it (for example, figure 1b-g and Campbell (2009) for V1aR expression). Stating only expression in the thalamus might therefore obscure the fact that the receptors are expressed in totally different parts of the thalamus in different species. This further emphasizes the importance of providing at least one entire set of images throughout the brain for each species being examined. An example of how important this can be is provided by examining the pattern of expression in the olfactory bulbs in Richardson’s ground squirrels and the colonial tuco-tuco (Beery et al, 2008). Both have V1aR expression in the olfactory bulbs, but when comparing the figures, the pattern of expression is different (Figure 6). From looking at what is provided in Beery et al. (2008) compared to my data, it appears that the expression occurs in different layers. Richardson’s ground squirrels do not have expression in the inner-most layer of granule cells, while it seems to be present in that of the colonial tuco-tuco. If I had access to the whole brain section sequence for the tuco-tuco, I would be able to make precise comparisons. Authors should therefore be encouraged to share their entire dataset more effectively in a similar way to other fields in neuroscience (Poldrack and Gorgolewski, 2014).
Lastly, not all studies report the variations present within structures, measurement units vary across studies and the measurements are usually made across the entire region and not their subregions. For example, I was able to identify and separate the different layers of the dentate gyrus, but most studies simply measure the entire hippocampal region. The importance of examining expression at finer levels of resolution (e.g., subregions within BST or DG) is potentially huge. In the case of my study, I would not have found sex differences if I had measured the entire BST or DG. Refining the specific parts of brain regions that express nonapeptide receptors enables a better postulation of the role of nonapeptides in modulating brain region function.

The autoradiography technique itself makes it difficult to compare values made in different runs and often the degree of resolution provided by scanned film is lower than that of brightfield or fluorescent histology. Add to this, the lack of consistency in how autoradiography results are presented and comparisons across studies become even more challenging. Thus, providing the films of the entire brain can make comparative studies easier, so I suggest that autoradiography studies provide entire films as supplementary material (appendices). A similar recommendation was made by Karten et al. (2013) for neuronal tract tracing and should be applied more broadly across neuroanatomical studies.

Conclusions

Vasopressin, oxytocin and their receptors are immensely important in various forms of social behavior. With the increasing amount of data in new
species, it appears clearer that the paths regulating specific types of behavior are distinct in each species, which is very likely linked to independent evolutionary paths of some social behaviors in distinct groups. This study of male and female Richardson’s ground squirrels was the first one to describe the neuroanatomical position and density of vasopressin and oxytocin receptors during breeding season. It helps to better frame a wider understanding of the evolution of social behavior since the nonapeptides can be considered the most important group in creating behavior diversity (Goodson, 2008). These hormones and their receptors have been conserved and are considered ancients in structural terms (Dhakar et al., 2013), but are very diverse in the species-specific way they regulate behavior (Goodson, 2008). To better support comparative studies of nonapeptide receptor expression, I suggest that future studies (and even past ones) release a complete set of sections. Often the reports do not include all the brain regions expressing receptors, for not being important to the study or because the expression of a region was missing. Such information could be valuable for other studies, and will help future comparative studies be more complete.
**Figure 6:** Different V1aR pattern expression in the olfactory bulbs of a Richardson’s ground squirrel and Colonial tuco-tuco (Beery et al., 2008).
REFERENCES


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Figure 7: Complete V1aR autoradiography film with brain sections of a female Richardson’s ground squirrel (part 1 of 2). Slides are labeled from slide number 4:1 to 4:25. Sequence start on top right slide with 4:1; continuous on film part 2 of 2 (following page) on the top right (4:7); goes to top left of part 1 of 2 (4:14) and continuous with top left of part 2 of 2 (4:20) until the last slides (4:25). Animal number 24. Collected on March 9th, 2016. Animal number 24 (identified as “JA24”). Collected on March 9th, 2016 and weighing 293g.
Figure 7 (cont’d): Complete V1aR autoradiography film with brain sections of a female Richardson’s ground squirrel (part 2 of 2). Slides are labeled from slide number 4:1 to 4:25. Sequence start on top right slide with 4:1 of part 1 of 2 (previous page), continuous on film part 2 of 2 on the top right (4:7), then goes to top left of part 1 of 2 (4:14) and continuous with top left of part 2 of 2 (4:20) until the last slides (4:25). Animal number 24. Collected on March 9th, 2016. Animal number 24 (identified as “JA24”). Collected on March 9th, 2016 and weighing 293g.
Figure 8: Complete OTR autoradiography film with brain sections of a female Richardson’s ground squirrel (part 1 of 2). Slides are labeled from slide number 4:1 to 4:25. Sequence start on top right slide with 4:1, continuous on film part 2 of 2 (following page) on the top right (4:7), then goes to top left of part 1 of 2 (4:14) and continuous with top left of part 2 of 2 (4:20) until the last slides (4:25). Animal number 24 (identified as “JA24”). Collected on March 9th, 2016 and weighing 293g.
Figure 8 (cont’d): Complete OTR autoradiography film with brain sections of a female Richardson’s ground squirrel (part 2 of 2). Slides are labeled from slide number 4:1 to 4:25. Sequence start on top right slide with 4:1 of part 1 of 2 (previous page), continuous on film part 2 of 2 on the top right (4:7), then goes to top left of part 1 of 2 (4:14) and continuous with top left of part 2 of 2 (4:20) until the last slides (4:25). Animal number 24. Collected on March 9th, 2016. Animal number 24 (identified as “JA24”). Collected on March 9th, 2016 and weighing 293g.