VASOPRESSIN AND SOCIAL BEHAVIOR IN RICHARDSON’S GROUND SQUIRRELS

A dissertation submitted
to Kent State University in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

By

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December, 2016

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LIST OF ABBREVIATIONS

Avp – arginine vasopressin
Avp-ir – arginine vasopressin immunoreactivity
Avpr1a – arginine vasopressin 1a receptor
Avpr – arginine vasopressin receptor
Avpr1b – arginine vasopressin 1b receptor
Avt – arginine vasotocin
ACTH – adrenocorticotropic hormone
AH – anterior hypothalamus
APZ – Assiniboine Park Zoo
ARF – Angela Rose Freeman
BNST – bed nucleus of the stria terminalis
CeB – basolateral nuclei of the amygdala
DG – dentate gyrus
FGM – fecal glucocorticoid metabolite
HPA – hypothalamic pituitary adrenal
i.c.v. – intracerebroventricular
i.p. – intraperitoneal
ir – immunoreactivity
LS – lateral septum
MeA – medial amygdala
NAcc – nucleus accumbens
PBS – phosphate-buffered saline
POA – preoptic area
PVH – paraventricular nucleus of the hypothalamus
PVN – paraventricular nucleus of the thalamus
Oxt – oxytocin
Oxtr – oxytocin receptor
SCN – suprachiasmatic nucleus
SON – supraoptic nucleus
RGS – Richardson’s ground squirrel
RIA – radioimmunoassay
USV – ultrasonic separation vocalization
VMH – ventromedial hypothalamus
VP – ventral pallidum
ACKNOWLEDGMENTS

This work was ultimately made possible with the support of amazing scientists, friends and family and loved ones.

I was especially lucky to have two amazing supervisors to help this incredible project and PhD come to fruition. To Dr. Heather Caldwell, an amazing PI and PhD supervisor, thank you for pushing me to accomplish more than I thought I could and working with this crazy Canuck to merge the lab and the field. To Dr. Jim Hare, the often behind-the-scenes mentor and supervisor, your efforts to make everything run smoothly were not unnoticed. I wish every Friday was a ‘patio’ day. There aren’t enough words to express my gratitude to both of you. At times I thought we might all be crazy for taking this on, and I’m still unsure that we aren’t. I would not have gotten here without both your guidance and insight (and funding too). I often talk about being ‘lucky’ with great mentors, but more honestly, good people attract good people, I felt at home in both your labs.

To Katherine May Feser: I love you. You’re the best confidante/comrade/partner-in-crime/best friend a gal could have. We have been through so much and I can’t wait to see where we go next. Thank you for feeding me, housing me, supporting me, and teaching me to climb!

To my sweethearts and friends and colleagues: thank you all for your support, also feeding me, housing me, and caring for me. Squirrel wranglin’ ain’t easy, and you made it better. Kyle Eros: you came along at just the right time. I’m so glad we found each other. Jordon Busch: you remind me every day to live in the present. Matt Kliot: your couch is the best place to relax after a hard science day. Liz
Aulino: I think it will be odd to try to find a new local BFF as sassy as you who can find the most ridiculous papers. Megan Rich, Shannah Witchey, Karla Rodriguez: thank you for helping me find my way to the bar and back. Katie, Rodrick, Lisa, Brittany, James, and other undergrads: thank you for helping in all the little things. To past lab members Travis Miller, Steven Tamborski, Erica Stevenson, and Julio César Morales-Medina thank you for teaching me, helping me, reminding me why certain things don’t work. To my field assistants and the Manitoba crew (Ellen, Levi, Thomas, Justin, Jillian, Taylor, Kaitlin, Melanie), especially Kevin-Bairos Novak and Calen Ryan: you made the hard work fun, when I think of the field, I think of the zoo ninja and the silly accents. A special thank you to Drs. Charlene Berkvens and Dr. Chris Enright who were in my corner every step of the way. Without the amazing vet staff this project wouldn’t exist (Drs. Laurence LaBrecque, Amelie Mathieu, Kelleigh Waters, and Shannon McCarthy, Brandi Nesplak, Desiree Proulx, Megan Desai, Brittany Semeniuk). You taught me everything I know about sterile surgical technique. Everyone at the APZ – you’ve been so helpful and supportive, thank you (Dr. Steven Peterson, C-Jae Breiter, and a nearly endless list of keepers, curators, directors and volunteers).

To my committee and collaborators: thank you so much for all your efforts to see this dissertation and project completed. Dr. Eric Mintz, Dr. Sean Veney, and earlier, Dr. Chi-hua Chiu: thank you for your advice, recommendations, and general good nature. You made the process painless.

Finally: thanks to my family for their love: Mike, Netta, Josh, Mr. Nibs. And to mom and dad for always being there for anything and everything.
Chapter 1

Introduction

Introduction

Arginine vasopressin (Avp) is a well-known modulator of social behavior (Caldwell et al., 2008; Caldwell and Albers, 2015). This peptide can act in the brain to influence behavior in mammals, including aggression (Albers, 2012), affiliation (Caldwell et al., 2008), social recognition and memory (Albers, 2012), predator responses (Bowen and McGregor, 2014) and vocalizations (Scattoni et al., 2008). The majority of work investigating the role of Avp in mammals has been conducted on traditional laboratory models, voles, and other rodents in laboratory environments (Caldwell and Young, 2009; Phelps et al., 2010; Young et al., 1999a), or in a few cases, outdoor enclosures (Ophir et al., 2008). Given its prevalence across taxa, and its implications for a variety of social behaviors, Avp is an ideal neuropeptide for comparative behavioral research. This introduction outlines the role Avp plays in modulating behavior through central mechanisms, focusing on social behavior and vocalizations.

1. Vasopressin

Avp, a nine-amino acid peptide, is made as a preprohormone in magnocellular neurons of the hypothalamic paraventricular (PVH) and supraoptic (SON) nuclei and released into circulation from the posterior pituitary (Brownstein
et al., 1980). While traditionally known for its importance in blood pressure and water balance, AVP is also important for the neuromodulation of many social behaviors (reviewed in Caldwell et al., 2008). Since AVP and its sister peptide oxytocin (OXT) exist in nearly every vertebrate taxa, and show conservation of structure and function, using these peptides for comparative work on social behavior can elucidate which functions are shared among closely and distantly related species.

1.1 Structure

AVP in most mammals is a nine amino acid protein (Cys-Tyr-Phe-Gln-Ast-Cys-Pro-Arg-Gly-NH$_2$) (Donaldson and Young, 2008). Its sister peptide, OXT, differs at position 3 and 8, with an isoleucine and leucine substitution respectively (Donaldson and Young, 2008). The AVP gene (in humans and mice) contains three exons and two introns located on the same chromosome (chromosome 20 and 2 respectively), and is adjacent to the OXT gene, but sits in the opposite transcriptional direction (Caldwell et al., 2008). Based on the similarities in structure among taxa (Figure 1.), it has been suggested that a gene duplication event prior to the divergence of vertebrates from a common ancestor led to the AVP and OXT genes we observe in rodents such as mice, rats, voles, and the subject of this dissertation, Richardson’s ground squirrels (Donaldson and Young, 2008).

1.2 Conservation of structure and function

Because of the similar structure between OXT and AVP (or their homologs in other taxa) and among taxa, the functions of these peptides are often conserved. One
Figure 1: Oxytocin and vasopressin homologs. From Donaldson and Young, 2008. Reprinted with permission from AAAS.
example of this conservation is the sexual dimorphism of Avp or arginine vasotocin (Avt)-producing neurons in the bed nucleus of the stria terminalis (BNST), in the vast majority of taxa, with males having more Avp-immunoreactive (Avp-ir) cells (Kelly and Goodson, 2013). Furthermore, Avp and Avt innervation to the BNST is also functionally conserved, and influences courtship, aggression, and affiliation (Goodson and Bass, 2001; Kelly and Goodson, 2013; Veenema et al., 2010). Avp and Avt have been implicated in a wider range of social behavior via exogenous peptide administration, including sexual and parental behavior, pair bonding, social recognition, aggression, mate choice, and vocalizations (reviewed in Goodson and Bass, 2001).

1.3 Overview of vasopressin’s effects on physiology and behavior

In mammals, Avp may influence social behavior through its interactions with the two centrally-expressed receptors: Avp 1a receptor (Avpr1a) and the Avp 1b receptor (Avpr1b). The Avpr1a is widely expressed in the rodent brain (Dubois-Dauphin et al., 1996), while the Avpr1b is predominantly found in the CA2 regions of the hippocampus (Hernando et al., 2001; Young et al., 2006). Furthermore, the expression levels of these receptors are often species and sex-specific (Hernando et al., 2001; Insel, 2010; Ostrowski et al., 1994; Vaccari et al., 1998). Avp projections throughout the brain originate in numerous brain areas and release Avp into the hypothalamo-hypophyseal portal system where it acts on Avpr1b in the anterior pituitary to control adrenocorticotropic hormone (ACTH) release and ultimately modulate the hypothalamic-pituitary adrenal (HPA) axis (Antoni, 1993; Herman and Cullinan, 1997). This activation of the HPA axis through stimulation of ACTH release
can in turn affect behavior (Caldwell et al., 2008; Gibbs, 1986). Thus, Avp can influence behavior directly by acting on central receptors, or indirectly by activating the HPA axis.

Central Avp influences affiliation, including grooming and sniffing (Young et al., 1999a), social bond and memory formation (Lim and Young, 2006), aggression (Keverne and Curley, 2004), and maternal behavior (Wang et al., 2000). Furthermore, Avp can act centrally to affect social communication across species. Specifically, Avp and its homologues influence vocalizations in: plainfin midshipman fish (*Porichthys notatus*; Goodson and Bass, 2000), birds (*Serinus canaria*; Voorhuis et al., 1991), *Spizella pusilla*; Goodson, 1998; *Zonotrichia leucophrys gambelii*: Maney et al., 1997), amphibians (reviewed in Goodson and Bass, 2001), rats (*Rattus spp.*, Sprague Dawley Rat; Iijima and Chaki, 2005; Winslow and Insel, 1993), squirrel monkeys (*Saimiri sciureus*; Winslow and Insel, 1991), and mice (*Mus musculus*, C57BL/6J and 129/SvJ strains, Scattoni et al., 2008, *Scotinomys spp.*: Campbell et al., 2009).

2. Vasopressin and social behavior

Avp can influence a variety of social behaviors. Of particular importance to this dissertation is how Avp may affect affiliation, social recognition, aggression, and antipredator responses.

2.1 Affiliation

Most of our knowledge of Avp and affiliation comes from comparative studies on *Microtus* voles. Specifically, differences in the distribution of the Avpr1a in the brain contribute to whether or not a vole is socially monogamous (prairie
voles, *Microtus ochrogaster*) or socially promiscuous (montane, *Microtus montanus* and meadow, *Microtus pennsylvanicus* voles) (Insel et al., 1993; Young et al., 1999a). In the landmark study by Young and colleagues (1999), mice were genetically modified to express the prairie vole *Avpr1a* (including the promoter). This resulted in *Avpr1a* expression patterns similar to those observed in prairie voles as well as increases in social affiliation, as measured by the grooming and sniffing of conspecifics. In promiscuous montane voles central Avp injections increase autogrooming but not social affiliation (Young et al., 1999a; Young et al., 1997). Additionally, Avp administration in the prairie vole, but not the montane vole, increases social interactions (Young et al., 1999a).

Avp may also induce and reinforce pair-bonding behavior in both sexes of prairie voles, with *Avpr1a* antagonists blocking the formation of pair bonds without reducing mating behavior (Cho et al., 1999; Wang et al., 1994; Winslow and Insel, 1993). In prairie voles in particular, injection of an *Avpr1a* antagonist into the ventral pallidum (VP; a brain region important in reward; Smith et al., 2009) can block pair bonding in males (Lim and Young, 2004). Similarly, overexpression of the *Avpr1a* in the VP can facilitate pair bonding without mating in prairie voles (Pitkow et al., 2001), and expressing the *Avpr1a* in the VP of montane voles via gene transfer through a viral vector can induce pair bonding similar to prairie voles (Lim et al., 2004; Young and Wang, 2004).

Avp may also influence maternal behavior and grooming. An *Avpr1a* antagonist injected into the medial amygdala (MeA) of female rats increases the latency to display maternal behavior to pups after a 10-day separation, while both
Avp and a Avpr1a antagonist reduce overall maternal behavior via unknown mechanisms (Nephew and Bridges, 2008a; Nephew and Bridges, 2008b). In mice, Avp stimulates autogrooming in wild type but not transgenic mice lacking the Avpr1a (Avpr1a⁻/⁻), though expression of the Avp mRNA does not differ between groups (Bielsky et al., 2004).

Avp can also impact play-specific affiliation in rodents. In juvenile male and female rats, an Avpr1a antagonist given intracerebroventricularly (i.c.v.) reduced play behaviors in males, but increased these behaviors in females (Veenema et al., 2013). Similar results were found in Syrian hamsters (also called ‘golden hamsters’, Mesocricetus auratus); injecting Avp into the AH induced play fighting in juvenile males while an Avpr1a antagonist inhibited this behavior (Cheng and Delville, 2009). Interestingly, injection into the lateral septum (LS) increased these same social play behaviors in male rats but decreased the behavior in females (Veenema et al., 2013), but only when tested in a familiar environment (Bredewold et al., 2014), further suggesting that the location of action by Avp, and the social context is integral to its influence on behavior.

2.2 Social recognition

Social recognition is the ability to remember conspecifics, and is often tested via novel-familiar paradigms where recognition of a familiar conspecific is inferred by decreasing social investigation (Ferguson et al., 2002). A common version of this test involves observing social interactions between the individual of interest and a conspecific in repeated trials, then substituting the known conspecific for an unfamiliar conspecific. If the individual has intact social recognition, one predicts
that in repeated trials with the familiar individual, investigation will decrease. Once a new conspecific is introduced, investigation should increase to the same level as the first trial. Individuals with impaired social recognition show no decreases in investigation over time, acting in each trial as if the conspecific is novel. Many studies have investigated how Avp may influence social recognition, especially in rodents (for an extensive review see Albers, 2012). Indeed, the Brattleboro rat is an excellent model as it lacks endogenous Avp, while transgenic mice lacking either of the central Avp receptors (Avpr) have also improved our understanding of Avp’s influence on social recognition.

Early studies of rats found that Avp agonists could facilitate social memory by improving recognition of a conspecific over longer time periods (Dantzer et al., 1987; Le Moal et al., 1987). The administration of Avp was not required during the initial encounter, suggesting that Avp may be particularly important in social memory consolidation (Dantzer et al., 1987). In Long Evans rats, but not Brattleboro rats, an Avpr1a antagonist impairs social recognition, though endogenous Avp restores function (Engelmann and Landgraf, 1994). Further work with Avpr1a antagonists found that they impaired social recognition in rats, but did not affect object recognition (Everts and Koolhaas, 1997; Everts and Koolhaas, 1999; Ferguson et al., 2002; van Wimersma Greidanus and Maigret, 1996).

Research then turned to genetic and molecular tools to examine Avp’s further influence on social recognition. One line of Avpr1a −/− males appears to have impaired social recognition, as tested through olfactory investigation of a stimulus female, while maintaining normal non-social olfaction (Bielsky et al., 2004). A
follow-up study from the same lab determined that the LS and not the MeA was important for this behavior in these mice, as re-expressing the Avpr1a receptor in the LS of these Avpr1a −/− mice completely rescued social recognition (Bielsky et al., 2005). However, in another line of Avpr1a −/− mice there is no evidence of impaired social recognition memory (Wersinger et al., 2007a).

In rats, viral vector gene transfer of the Avpr1a prairie vole gene into the LS of rats improves social recognition (Landgraf et al., 2003). Similarly, knockdown of the Avpr1a via antisense oligodeoxynucleotide administration into the LS of rats reduces social recognition and prevents males from responding to exogenous Avp (Landgraf et al., 1995). There is recent evidence that in Syrian hamsters, Avp given i.c.v. can influence social recognition via action at the oxytocin receptor (Oxtr) (Song et al., 2016b), which may explain some of the behavioral variation seen in previous work in mice (Bielsky et al., 2004; Wersinger et al., 2007a,b). Indeed, future work examining receptor ‘cross-talk’ in other species might elucidate mechanisms of some conflicting findings in the Avp and Oxt behavioral research.

The Avpr1b is also important in social recognition; Avpr1b −/− mice have mildly impaired social recognition (and reduced aggression, discussed below), but display other social behaviors normally (Wersinger et al., 2002). Female, Avpr1b −/− mice also have impaired social recognition, as measured by the Bruce effect, while Avpr1a −/− females do not have this impairment (Wersinger et al., 2008).

2.3 Aggression

Avp has also been implicated in aggression in rodents, though its effects differ based on species and sex. In Syrian hamsters, Avp injected into the AH
increases offensive aggression during long-photoperiods (Caldwell and Albers, 2004; Ferris et al., 1997). Likewise, injection of an Avpr1a antagonist in the same brain region reduces aggression (Caldwell and Albers, 2004; Ferris and Potegal, 1988). In socially isolated males, the AH as well as the PVH tend to have higher densities of Avpr1a; potentially increasing the probability of aggressive behaviors (Albers et al., 2006). In contrast, socially experienced male hamsters tend to have higher densities of the Avpr1a in the central amygdala (Albers et al., 2006).

In female hamsters, however, antagonism of the Avpr1a in the AH stimulates offensive aggression, while Avp administration reduces aggression dose dependently in the resident-intruder test (Gutzler et al., 2010). With respect to maternal aggression, Avp (i.c.v.) also decreases -while an Avpr1a antagonist increases- aggression in lactating female rats (Nephew and Bridges, 2008b; Nephew et al., 2010). In mice, Avpr1a −/− dams’ maternal aggression do not differ from wild type dams (reviewed in Bosch, 2013, but see Avpr1b below). This emphasizes that Avp may act differentially based on an animal’s sex and species.

In Microtus spp. voles, Avp has different effects depending on the species. Avp increases aggression in prairie voles, with an antagonist decreasing aggression, however, Avp does not increase aggression in the closely-related montane vole (Winslow et al., 1993; Young et al., 1997). Again, this is likely attributable to variation in Avpr distributions.

The Avpr1b also plays a role in modulating aggression, as male Avpr1b −/− mice show reduced aggression compared to wildtype and heterozygous littermates (Wersinger et al., 2002), and this reduced aggression persists even when the Avpr1b
lab strain is crossed with a more 'wild' subspecies (Caldwell and Young, 2009). Through viral replacement of this receptor, specifically in the CA2 region of the hippocampus, aggressive behavior can be restored (Pagani et al., 2015). Furthermore, the reduction in aggression is specifically associated with conspecific aggression, as Avpr1b −/− mice show regular prey aggression (Wersinger et al., 2007a). In contrast to the Avpr1a studies, female Avpr1b −/− mice are similar to males, and have reduced maternal aggression towards a male conspecific (Wersinger et al., 2007a). Antagonism of the Avpr1b also results in reduced offensive aggression in male Syrian hamsters (Blanchard et al., 2005), and reduced biting during defensive aggression in mice (Griebel et al., 2002). Thus, both the Avpr1a and Avpr1b may influence aggressive behaviors in rodents.

2.4 Predator responses

In recent work, there have been some data illustrating that Avp may also play a role in antipredator behavior. Specifically, Avpr1a antagonism i.c.v. reduced neuronal activity as measured with fMRI to predator odor in rats (Reed et al., 2013). The authors noted that the Avpr1a in the amygdala are modulating these neural responses to predator odor. In a different rat study, an Avpr1a antagonist (SR49059) given intraperitoneal (i.p.) reduced huddling behavior in rats exposed to cat fur (Bowen and McGregor, 2014, Figure 2). Similarly, Avp increased huddling behavior in response to the cat fur odor; this response with Avp administration was blocked by an Avpr1b antagonist, but not an Avpr1a antagonist (Bowen and McGregor, 2014). Hypothalamic Avp and Avpr1a mRNA after predator odor in rats
Figure 2: The effects of the Avpr1a antagonist on huddling induced by cat fur and the number of contacts with the cat fur stimulus. (a) Rats in a VEH quad engaging in the characteristic huddling response. (b): Rats in a quad pre-treated with 3 mg/kg SR49059 displaying avoidance of physical contact with other rats instead of the usual huddling response. Compared to VEH, 3 mg/kg SR49059, but not 1 mg/kg, inhibited huddling (c) and neither dose of SR49059 had any significant effect on the number of contacts with the cat odour stimulus (d). ***p < 0.001. From Bowen and McGregor 2014. Reprinted with permission from Oxford University Press.
is unchanged, though Oxt mRNA is reduced (Muroy et al., 2016). However, the predator odor reduced huddling behavior, supporting an undetected change in Avpr1b signaling due to prior immobilization stress. In response to a predator (rat) CD1 mice typically give vocalizations and will bite the predator, and these behaviors are reduced when given an Avpr1b antagonist (Griebel et al., 2002).

Avp might also influence antipredator responses via its effects on anxiety in general. In the majority of studies on rodents, Avp is reported to have an anxiogenic effect (Neumann and Landgraf, 2012). Male Avpr1a+/− mice are reported as having reduced anxiety as measured in the elevated plus, open field, and light/darkbox tests (Bielsky et al., 2004; Egashira et al., 2007). However, in a different line of mice, which also lack this receptor, no changes in anxiety were observed (Wersinger et al., 2007b). In rats, there are similar, somewhat contradictory findings with regards to anxiety, as knockdown of the Avpr1a in the septum reduced anxiety-like behavior in the elevated plus test (Landgraf et al., 1995), as did administration of the Avpr antagonist (Liebsch et al., 1996), though antagonism of both the Avpr1a and Avpr1b in the LS increased anxiety (Everts and Koolhaas, 1999). These differences in observed anxiety modulation are likely due to the neural substrates where these peptides are binding, as well as potential confounds associated with the testing location and how these experiments are performed.

3. The central vasopressin system

The LS, amygdala, POA, midbrain, limbic structures, and hypothalamic nuclei, which are connected, are sometimes referred to as the ‘social behavior neural
network’ (Albers, 2012). These areas and their relation to Avp function are discussed below.

3.1 Vasopressin receptor distributions

As mentioned above, the Avpr1a is widely distributed throughout the brain, while the Avpr1b is more limited and localized to the CA2 region of the hippocampus (inferred from in situ; Young et al., 2006). Even though Avpr1a is widely distributed, its expression varies by species. The Avpr1a typically shows high densities in the LS (Hernando et al., 2001; Ostrowski et al., 1994; Vaccari et al., 1998). However, in monogamous, social prairie voles the Avpr1a is found in high densities in the ventral pallidum (VP; often identified in early research as the diagonal band), an area associated with reward and reinforcement (Insel and Young, 2001; Young et al., 1999a). In the non-social promiscuous montane and meadow voles, Avpr1a is most dense in the LS and amygdala (Insel et al., 1993; Young et al., 1999a).

Other socially monogamous species such as California mice (Peromyscus californicus; Bester-Meredith et al., 1999; Insel et al., 1991) and the common marmoset (Callithrix jacchus; Schorscher-Petcu et al., 2009) have dense Avpr1a expression in both the VP and LS, contrasting to the prairie voles’ limited expression in the LS (Wang et al., 1997). In contrast, the non-monogamous rhesus macaque (Macaca mulatta) has Avpr1a binding in some areas similar to rodents, including the BNST, LS, hypothalamus and amygdala (Young et al., 1999b). Variation in the expression in Avpr1a in the VP leads to changes in partner preference and anxiety, demonstrating its role in social monogamy (Barrett et al., 2013). Generally,
monogamous and social species are thought to have dense receptor binding in the VP, while non-social species are described as having expression in the LS.

Unsurprisingly, gonadal hormones can also affect Avpr1a expression in some species (Reviewed in Caldwell et al., 2008). For example, castrated male hamsters have reduced Avpr binding in the hypothalamus (Johnson et al., 1995), compared to intact males. Social experience (often related to gonadal hormones) can also influence Avp and Avpr1a distribution. Dominant hamsters have more Avpr1a binding than subordinates in the lateral ventromedial hypothalamus (VMH) (Cooper et al., 2005), and subordinate Long-Evans rats have high levels of binding in the LS (Askew et al., 2006). Furthermore, socially isolated Syrian hamsters and rats show increased Avpr1a binding in the AH, PVH and lateral hypothalamus, LS, BNST, and basolateral nuclei of the amygdala (CeB) respectively, over socially experienced conspecifics (Albers et al., 2006; Askew et al., 2006).

In prairie and montane voles, however, species but not sex-differences were reported (Insel et al., 1994), though a more recent study with a larger sample size showed males having more Avpr1a binding in the medial prefrontal cortex in montane, but not prairie voles (Smeltzer et al., 2006). Furthermore, early life experience may also have sex-dependent effects on Avpr distributions, as male rats that received high levels of maternal care had higher densities of Avpr1a in the amygdala, but there were no changes in female receptor densities (Francis et al., 2002).

In addition to receptor expression, the regulatory elements of the receptor genes can vary among species. In monogamous voles, a variable repeat microsatellite sequence lies upstream of the Avpr1a coding region in its promoter
(Hammock and Young, 2002; Young et al., 1999a). This variation has been a focus of recent human studies, showing that the *Avpr1a* gene microsatellite sequences are associated with pair-bonding or ‘marital quality’ in males (Walum et al., 2008) though not in females (Cherkas et al., 2004), as well as social adjustment and communication impairments (Kim et al., 2002; Yirmiya et al., 2006), altruism (Knafo et al., 2008), empathy (Uzefovsky et al., 2015), maternal behavior (Avinun et al., 2012), and even musical memory and aptitude (Granot et al., 2007; Ukkola-Vuoti et al., 2011). It is thought that this promoter (Hammock et al., 2005) region may show differences between closely-related species and may impact both receptor distribution and social behavior (Donaldson and Young, 2013; Hammock et al., 2005).

### 3.2 Vasopressin distribution

The presence of Avp-ir in cell bodies and fibers also varies among species and sexes. As expected, Avp-ir is often found in brains areas involved in the neural regulation of social behavior. In rats, early work established the primary locations of Avp neurons including the SON, PVH, suprachiasmatic nucleus (SCN), amygdala, BNST, LS, VP, as well as a few other places in the hypothalamus (Buijs et al., 1978; DeVries et al., 1985; Sofroniew, 1985). These Avp-neurons are in similar locations in lab (Castel and Morris, 1988; Mayes et al., 1988) and California mice (Bester-Meredith et al., 1999). In a study of both monogamous and non-monogamous voles, Avp-ir was found in cells in the SON, PVH, and SCN (Wang et al., 1996). Male voles have connections between the LS, BNST, and medial POA (Wang et al., 1996). In the monogamous pine vole (*Microtus pinetorum*), most Avp-ir is found in the LS, though
some differences in fiber distribution are detected between vole species (Wang et al., 1996). In Syrian hamsters, Avp is detected in both neurons and fibers of the SON, PVH, and SCN, and some in the medial POA-AH (Albers, 2012). However, the Syrian hamster lacks Avp-ir in the fibers of the LS, which differs from most other rodents (Albers et al., 1991; Dubois-Dauphin et al., 1990).

Avp-ir patterns can also be affected by gonadal hormones, especially in rats (De Vries and Al-Shamma, 1990), European hamsters (Cricetus cricetus; Buijs et al., 1986) mice (Mayes et al., 1988), Mongolian gerbils (Meriones unguiculatus; Wang et al., 2013), Chinese striped hamsters (Cricetulus barabensis; Wang et al., 2013), prairie and meadow voles (Bamshad et al., 1993; Wang and De Vries, 1993), Syrian hamsters (Delville and Ferris, 1995), and others (reviewed in De Vries and Panzica, 2006), and also by social experience. Generally, Avp in males is more abundant than females, and seasonal changes in Avp are thought to be modulated by testosterone in males (Insel and Young, 2000). The dimorphism between males and females is particularly well conserved in the medial BNST, and is related to aggression and courtship (Kelly and Goodson, 2013). In females, estrogens such as estradiol may modulate Avp in specific brain regions: in the SON of female rats Avp and estrogen receptors co-localize (Alves et al., 1998), while in hamsters, estradiol may modulate the effects of Avp in the medial POA and AH (Huhman and Albers, 1993).

Social status can also modulate Avp-ir (Ferris et al., 1989). Subjugation in hamsters decreases Avp in the AH (Delville et al., 1998), but increases Avp (co-localized with Fos protein) in the PVH in mice (Litvin et al., 2011). Furthermore, Avp-ir in brain regions important to social behaviors is altered through cross-
fostering in *Peromyscus* spp. (Bester-Meredith and Marler, 2001). The modulation of Avp with social status may be related to anxiety as well, as Avp may promote passive coping to social defeat in these experiments (Neumann and Landgraf, 2012). Thus, the distribution of Avp and its receptors is modulated by a number of factors, and can be both sex- and species-specific.

4. Vasopressin and vocalizations

Recent research alludes to Avp’s potential influence on social vocalizations. Similar to social behavior and Avp and Avpr distribution, Avp’s influence on vocalizations is also often species- and sex-specific. Furthermore, Avp and Avt modulate vocalizations in a number of vertebrate taxa, and Avp is commonly detected in brain regions important for vocalization (Goodson and Bass, 2001).

4.1 Fish

Avt (a non-mammalian Avp homologue) modulates vocalizations from the forebrain in plainfin midshipman fish through the POA and AH (Goodson and Bass, 2000). These fish have two morphs which exhibit differences in sexual behavior – Type I males are ‘traditional males’ that use courtship to attract females and exhibit parental care, while Type II males are ‘sneaker males’ which are smaller and similar in phenotype to females (Goodson and Bass, 2000). Type II males attempt to fertilize eggs by covertly approaching nests. In this species, fictive (forebrain-evoked) vocalization is inhibited by Avt in type I males (traditional males), and facilitated by an Avpr1a antagonist (Goodson and Bass, 2000). Isotocin, but not Avt or the Avpr1a antagonist, influenced Type II males and females, inhibiting vocalizations in both
groups (Goodson and Bass, 2000). This suggests that vocalizations are modulated by Avp and its homologs in both a sex-specific and status-specific manner.

4.2 Amphibians

In amphibians, Avt can also influence vocalization behavior. Avt administration typically increases frequency and duration of calling behavior (male cricket frogs, Acris crepitans (Chu et al., 1998); gray treefrog, Hyla versicolor (Tito et al., 1999); bullfrogs, Rana catesbeiana (Boyd, 1994)), but can also alter the types of calls given (Túngara frog males, Physalaemus pustulosus (Kime et al., 2007)).

Furthermore, Avt impacts call perception, as female bullfrogs (Boyd, 1994), and female American toads (Bufo americanus, Schmidt, 1985) are more likely to respond to advertisement calls when given Avt.

4.3 Birds

The impact of Avt on bird song and calls has been relatively well-studied, likely due to the variety of vocalizations that birds exhibit, and the relative ease of eliciting these calls. Birds have the Avp homologue Avt, similar to amphibians, and likewise, Avt also influences avian vocalizations. While by no means exhaustive, this section highlights some of the variation of responses among avian species when given Avt.

Generally, Avt’s effects are thought to be linked to circulating steroid levels (usually, testosterone) (Panzica et al., 2001), though there are a few studies that show the separation of song and gonadal hormones. Central administration of Avt (i.c.v) increases the number of territorial songs given by female white-crowned sparrows (Zonotrichia leucophrys gambelii) in the absence of a reproductive state.
(Maney et al., 1997). Furthermore, in field sparrows (*Spizella pusilla*) Avt only influences complex song, not courtship song (LS injection, Goodson, 1998).

Interestingly, in Lincoln’s sparrows (*Melospiza lincolnii*), there is evidence that song perception and environment can feedback on the Avt system, influencing Avt-ir in the forebrain (Sewall et al., 2010). This indicates that at least in some species, gonadal steroids are not required for the interaction of Avt and song.

In young male canaries (*Serinus canaria*), Avt given subcutaneously increased song in the early autumn, but decreased song in the winter (Voorhuis et al., 1991), though this effect may be influenced by circulating steroids. In zebra finches (*Taeniopygia guttata*), however, neither Avt nor its receptor antagonist influences courtship calling (Goodson et al., 2004), indicating that while Avt’s effects on vocalizations may be widespread, its impacts may differ among species, sex, and context.

**4.4 Mammals, including rodents**

While there is extensive literature on bird vocalizations and Avt, there are far fewer studies regarding the vocalizations of mammals and Avp. The work that has been conducted focuses mainly on anxiety-induced vocalizations such as isolation calls. When offspring are separated from their parents they typically give calls to elicit reuniting with their parent. In mice and rats, these calls typically elicit retrieval by the dam, and she brings the pup to the nest. These ultrasonic separation vocalizations (USVs) are modulated by Avp acting on both Avpr1a and Avpr1b (Bleckardt et al., 2009; Scattoni et al., 2008). While most evidence is from work with traditional lab models (i.e. mice, rats), in male squirrel monkeys (*Saimiri*...
*sciureus*), both Avp and Oxt reduce ‘isolation-poop’ calls in a social separation test (Winslow and Insel, 1991). Early work showed that Avp administration (i.c.v) reduces rat pup USVs, which is hypothesized to be via its action on the Avpr1a (Winslow and Insel, 1993), though more recently, an antagonist given i.p. also reduced pup USVs (Bleickardt et al., 2009). An Avpr1b antagonist reduces USVs from rat pups, potentially by altering stress hormone levels (Iijima and Chaki, 2005). Thus, while Avp’s central receptors are involved in the modulation of USVs in rodents, it is still unclear what neural substrates (and potential peripheral mechanisms) might be involved.

Adult female Avpr1b −/− mice emit fewer ultrasonic vocalizations than wild type controls when faced with an intruder female (Scattoni et al., 2008), supporting the hypothesis that the Avpr1b is important for stress-induced vocalizations. Recent research has revealed that in juvenile male rats, an Avpr1a antagonist (i.c.v) reduces pro-social 50-kHz ultrasonic vocalizations and play behavior, however Avp had no effect (Lukas and Wöhr, 2015). Thus, there is some evidence that Avp and Avpr have the ability to influence social vocalizations in addition to anxiety-related vocalizations.

The rest of the work regarding rodents, Avp, and vocalizations have examined Avp and Avpr binding and areas important for vocalizations. For example, in the mustached bat (*Pteronotus parnelli*), Avp-containing fibers were localized in the periaqueductal gray which is an area important for vocal communication, and potentially kin recognition (Prasada Rao and Kanwal, 2004). Highly-vocal singing mice (*Scotinomys xerampelinus*) show dense binding of the Avpr1a in the anterior
and laterodorsal thalamus which are important for social and spatial memory (Campbell et al., 2009). Thus, in order to understand what Avp’s mechanisms for influencing vocalizations are, and to determine if these mechanisms are conserved, more comparative studies need to be conducted.

5. Richardson’s ground squirrel: A model for social behavior and vocalizations

Avp has been established as a modulator of social behavior, including vocalizations in some species (Albers, 2012; Caldwell et al., 2008; Caldwell and Albers, 2015; Insel, 2010). However, there is limited work on how Avp might influence social vocalizations (Lukas and Wöhr, 2015; Scattoni et al., 2008). Furthermore, while we know Avp influences social behavior in a variety of rodents, few studies have extended these manipulations of the central Avp system in free-living rodents in an ecologically-relevant environment (Caldwell and Young, 2009; Campbell et al., 2009; Ophir et al., 2008). Thus, I selected RGS as a model for social behavior and vocalizations, as this species exhibits alarm calling behavior in social contexts.

5.1 RGS and alarm calling

RGS - and specifically the population at the APZ - are an excellent field model for studying social behavior as they are abundant, easy to observe, and highly social. In addition, known relatedness and ages of individuals of the population at the APZ allows for incorporation of these factors when conducting social research. Thus, instead of using a laboratory model, which can be highly inbred, by using a wild population we can observe how kinship may influence behavior in addition to Avp administration.
With regards to alarm communication, RGS are most responsive to calls of neighbors relative to non-neighbors (Hare, 1998b; Thompson and Hare, 2010). While the age of the caller does not affect the responses of adults or juveniles (Swan and Hare, 2008), juveniles do respond differently than adults to alarm calls. Specifically, adults may perceive the level of risk from the number of callers, while juveniles do not (Sloan and Hare, 2008). Juveniles adjust responses based on call rate and length, while adults do not (Sloan and Hare, 2006; Warkentin et al., 2001). Finally, adults and juveniles respond differently to an approaching predator, with adults increasing vigilance coincident with decreasing distance to the predator model (Sloan and Hare, 2004). Furthermore, the sex of the animal can affect their response; females call more than males, and this is true for all age classes (Hare, 1998a).

The alarm calls themselves contain information about the predator, including whether it is avian or terrestrial (Davis, 1984; Sloan et al., 2005), whether it is approaching (Thompson and Hare, 2010), and distance to the presumptive predator (Warkentin et al., 2001).

5.2 Alarm Calling

Alarm calling behavior was originally thought of as altruistic (Trivers, 1971), but has been established as nepotistic in ground squirrels (Sherman, 1977); as social animals benefit from ‘altruistic’ behavior towards kin (i.e. kin-selection) (Hamilton, 1964). Further supporting a nepotistic interpretation, RGS are more likely to call when kin are near, and adult females with young are more likely to alarm call than others (Blumstein, 2007; Davis, 1984). However, reciprocal altruism
is also a possible factor in social alarm call behavior, as RGS are able to discriminate amongst individuals (Davis, 1982; Hare, 1998a), and weight responses to alarm calls based on a caller’s past performance (Hare and Atkins, 2001).

Whether alarm calling in RGS is nepotistic, reciprocally altruistic, or a combination of both; alarm calling behavior is a social behavior in this species. Indeed, previous behavioral research from the Hare lab at the University of Manitoba has established through a long-term study of RGS at the Assiniboine Park Zoo (APZ) that these animals’ behaviors are greatly influenced by kinship and sociality (Hare, 1998b; Hare et al., 2007).

6. Scope of the dissertation

The aim of the work described in this dissertation was to investigate the neurobiological basis of social alarm calling behavior in a free-living rodent. I employed both field-based and lab-based experiments to determine whether central Avp influenced social behavior in RGS, what brain regions are the probable locations for Avp’s action, and finally, which brain regions are integral to alarm call perception. Our central hypothesis was that central Avp modulates alarm calling and social behavior in RGS.

Chapter 2: Sex-specific effects of arginine vasopressin on Richardson’s ground squirrel social behavior and communication

Chapter 2 focuses on the impact of central Avp on the social behavior of RGS. I hypothesized that supplementation of Avp would alter social behavior and alarm
calling, by acting at central Avp receptors. This chapter consists of two major parts: the impact of Avp on behavior, and the contributions of cortisol to these results.

I further hypothesized that Avp acting mainly through central mechanisms would not change cortisol peripherally. I predicted that Avp would increase cortisol by acting through the Avpr1b and the HPA axis, but that feedback mechanisms would then bring cortisol back to a baseline level (Antoni, 1993; Herman and Cullinan, 1997). I predicted that Avp would promote affiliation and modulate vocalizations, based on findings in other rodents.

Chapter 3: Central distribution of oxytocin and vasopressin 1a receptors in Richardson's ground squirrel

Chapter 3 focuses on the species-specific binding patterns of Oxtr and Avpr1a in RGS. I hypothesized that both sociality and reproductive tactics would influence receptor binding patterns. Further, I predicted that based on the lack of pair bonding and biparental care in this species I would see binding patterns similar to the montane vole (high in the LS and amygdala and low in the VP) (Insel et al., 1994; Wang et al., 1997), and given their keen ability to remember and recognize each other, I further predicted dense binding in the LS (Landgraf et al., 1995; Landgraf et al., 2003). Since RGS exhibit alarm calling behavior, receptor binding in areas important for auditory processing is predicted. Similar to Avpr, I predicted that Oxtr binding patterns would mirror those seen in other promiscuous rodents, such as the montane vole. Thus, I expected dense binding in the LS, VMH and amygdala.
Chapter 4: Perception of alarm calls and neuronal activation in Richardson’s ground squirrel

In Chapter 4 I examined perception of alarm calls and associated neuronal activation in RGS. I hypothesized that alarm call perception would result in neuronal activation in specific brain regions. I predicted that the type of call (i.e. chirp vs. whistle) would result in differential activation in the brain. Specifically, I predicted that the high threat vocalization (chirp; Sloan et al., 2005) would cause activation in areas such as the amygdala, or other areas important in anxiety and emotion, while the whistle vocalization would show activation in the social behavior neural network (i.e. POA, hypothalamic nuclei, BNST, amygdala, nucleus accumbens). Both types of vocalizations are expected to activate auditory cortex areas.

In conclusion, by examining the impacts of central Avp, Avpr, Oxtr, and neuronal activation in RGS social behavior and communication, I will add to a growing body of comparative work regarding sociality and nonapeptide modulation of behavior. Specifically, the work outlined in this dissertation will help determine whether Avp influences social behavior in this species, where alarm calling behavior might be centrally modulated, and whether Avp binding is correlated with sociality and/or reproductive strategies.
Chapter 2

Sex-specific effects of arginine vasopressin on Richardson’s ground squirrel social behavior and vocalizations
(Submitted to Hormones and Behavior)

Introduction

Avp is a nine-amino acid neuropeptide that is important for regulating social behavior in a variety of animals (Caldwell and Albers, 2015; Goodson and Bass, 2001; Stevenson and Caldwell, 2012). This peptide works through two central receptors: Avpr1a and Avpr1b, to influence behavioral changes (Caldwell et al., 2008; Young et al., 1999b). Recent reviews have highlighted both sex-specific and species-specific effects on behavior following central administration of Avp, and thus highlight the importance of comparative research (Dumais and Veenema, 2015; Phelps et al., 2010; Stoesz et al., 2013). Central administration of Avp influences affiliation, including grooming and sniffing (Young et al., 1999a), social bond and memory formation (Caldwell and Albers, 2015; Lim and Young, 2006), aggression (Keverne and Curley, 2004; Nephew and Bridges, 2008b), maternal behavior (Wang et al., 2000), vocal communication (Goodson and Bass, 2001), and anxiety (Neumann and Landgraf, 2012). Avp is also known to influence behavior through the HPA axis via binding to Avpr1b in the anterior pituitary, which leads to release of ACTH. ACTH then acts on the adrenal glands to promote glucocorticoid release.
(Antoni, 1993). These circulating glucocorticoids are essential for the 'stress response' in animals, facilitating both physiological and behavioral responses to 'stressors' (Romero, 2004).

The majority of work on Avp and behavior has focused on traditional model species (i.e. rats, mice (Mus spp.) (McGraw and Young, 2010), and Syrian hamsters (Albers et al., 2006; Caldwell et al., 2008; Cooper et al., 2005; Delville et al., 1998; Ferris et al., 1997), but also includes comparative work using Microtus voles (McGraw and Young, 2010), which revealed that life history is correlated with Avpr distribution patterns (Insel et al., 1993; Young et al., 1999a). Indeed, more recent work has shown that Avpr distributions may be species-specific and can vary among individuals (Caldwell and Albers, 2003; Campbell et al., 2009; Everts et al., 1997; Phelps et al., 2010). Furthermore, researchers have started to examine the roles of Avp in more complex social environments, such as outdoor enclosures (Ophir et al., 2008), and beyond “traditional” laboratory models, such as in laboratory populations derived from wild-caught animals (reviewed in Caldwell and Young, 2009; Phelps et al., 2010; Young et al., 1999a). These studies are particularly important because rodents bred in captivity and without appropriate environmental enrichment are known to develop stereotyped behavior (Würbel, 2001). Further complicating matters, the location of the testing laboratory may produce differences in behaviors (Crabbe et al., 1999), and laboratory and wild strains (or laboratory and field studies) of rodents can differ in both behavior (Barnett and Stoddart, 1969; Blanchard et al., 2003) and physiology (Calisi and Bentley, 2009). Thus, rigorous scientific testing of different animal species under a
variety of behavioral conditions likely represents the best approach to elucidate the conserved functions of Avp in modulating social behavior.

RGS are a social, colonial, semi-fossorial rodent that are native to the prairies of central Canada and the United States (Michener and Koeppl, 1985). Like other ground squirrels, this species uses alarm calls to alert nearby conspecifics to potential predators (Davis, 1984; Hare, 1998a). In ground squirrels, this calling behavior can be nepotistic, as these social animals benefit from their ‘altruistic’ behavior towards kin (i.e. kin-selection, Hamilton, 1964; Sherman, 1977). Consistent with the kin-selection hypothesis, RGS are more likely to call when kin are near, and adult females with young are more likely to call than others (Davis, 1984). The alarm calls themselves contain a wealth of information that is communicated to receivers (Thompson and Hare, 2010). RGS are an especially salient model for social communication as they are able to discriminate amongst individuals and weight responses based on a caller’s past reliability (Hare, 1998a; Hare and Atkins, 2001). Similar to other squirrels, RGS also produce other vocalizations in the presence of conspecifics – including tooth chatters and squeals during agonistic encounters (Balph and Balph, 1966; Holmes and Sherman, 1982; Owings and Leger, 1980), and varied squeaks during other social interactions.

Previous work regarding Avp and vocalizations in rodents has mostly focused on USVs from pups. In rat pups, i.c.v. Avp reduces USV production, while an Avpr1a antagonist blocks this effect; suggesting the Avpr1a as the site of action (Winslow and Insel, 1993). However, these effects were not seen in later peripheral injection experiments. Furthermore, the Avpr1b may also be important in these
vocalizations, as an Avpr1b antagonist (given i.p.) reduces USVs in rat pups (Iijima and Chaki, 2005; Varga et al., 2015). It is possible that the peripheral versus central administration of these antagonists is the reason for this behavioral difference. Avp-deficient Brattleboro rat pups also emit fewer USVs when separated from their dam compared to controls, suggesting endogenous Avp is important for these behaviors (Varga et al., 2015). However, USVs tend to be considered an indicator of anxiety as they are suppressed by anxiolytic drugs (Gardner, 1985), and thus, may be modulated differently than other social vocalizations.

There are a few studies that have investigated Avp’s influence on other vocalizations in rodents. In juvenile male rats, i.c.v. Avp has no effect on prosocial play vocalizations, though an Avpr1a antagonist reduces the number of these vocalizations (Lukas and Wöhr, 2015). In highly-vocal singing mice (*Scotinomys teguina* and *Scotinomys xerampelinus*), high densities of Avpr1a are seen in areas important in vocal communication (i.e. AH, periaqueductal gray, and medial geniculate), and these densities correlate with the likelihood of vocal behavior (Campbell et al., 2009). Adult female mice that lack the Avpr1b emit fewer vocalizations during territorial intrusions; these calls are lower in frequency than those emitted by wild-type controls (Scattoni et al., 2008). Given Avp’s modulation of vocalizations in other rodents, we hypothesized that central Avp would modulate alarm calling behavior in RGS.

Our study population has been tagged and monitored since 2003, providing a long-term record of matrilineal relatedness. Furthermore, we have validated a non-invasive method for measuring fecal glucocorticoid metabolites (FGMs) in this
species, allowing the examination of stress effects on behavior (Hare et al., 2014).

This particular population is habituated to human presence, making it easy to observe. It is also a free-ranging population that is genetically diverse and lives in a complex environment characterized by environmental change, predation, and social challenges. Thus, it is ideal for comparative work on Avp's behavioral effects on wild species in an ecologically-relevant setting. Given Avp’s varied effects on social behavior in rodents, we hypothesized that central Avp administration in RGS would alter social behaviors, including vocalizations. Here we tested how central Avp administration in this free-living ground squirrel species influenced behavior and vocal communication in a sex-dependent manner. We predicted that Avp would alter alarm call vocalizations and promote affiliation, similar to effects seen in other rodent species. We further predicted that Avp would alter female territorial vocalizations based on work conducted on transgenic mice (Scattoni et al., 2008), and that Avp would alter aggression differently in males and females as seen in Syrian hamsters (Caldwell and Albers, 2004; Gutzler et al., 2010). In male hamsters, Avp injected into the AH increases aggression, but the same injection in females reduces aggression compared to controls (Caldwell and Albers, 2004; Gutzler et al., 2010). Thus, we expect that Avp will have sex-dependent effects.

**Methods**

From March through July 2014 we studied 11 male and 6 female RGS, and from May through July 2015 we studied 13 female RGS. All were free-living RGS at the APZ in Winnipeg, MB (49°52’N, 97°14’W). This population has been studied since 2003, and is annually trapped using Tomahawk live traps (Tomahawk Live
Trap Co., Tomahawk, WI, USA) baited with peanut butter (No Name™ Smooth Peanut Butter; Loblaw Inc, Toronto, ON, Canada). Squirrels were given individual marks on the dorsal pelage with human hair dye (Clairol Hydrience 52S, Pearl Black, Stamford, CT, USA), and were permanently marked with numbered metal ear tags (National Band and Tag Company, Monel no. 1, Newport, KY, USA) to allow differentiation from other individuals in the field. Individuals were weighed at capture with a Pesola spring scale (Pesola AG, Baar, CH) to the nearest 5g. All research with animals was conducted in accordance with the Canadian Council on Animal Care guidelines and was approved by the University of Manitoba Fort Garry Campus Animal Care Committee (F13-014/1), APZ Research Ethics and Review Committee (2014-A003), and Manitoba Conservation (Wildlife Scientific Permit WB14952).

**Surgeries**

**Surgeries – selection and transportation**

Individuals were selected for surgery based on location, so that squirrels in the same area (<30m away) would not be tested during the same time frame. Treatment (Avp or saline) was selected randomly and females were only selected for surgery after their young had been weaned. Individuals were live trapped as described above and transported in a pillowcase-covered trap to the APZ veterinary hospital (on-site). Surgeries were conducted on afternoons between 13:00 and 16:00 CST. Fresh scat was collected from subjects before surgery for FGMs radioimmunoassays (RIAs) to measure concentration of FGMs, and then frozen at -20°C. Squirrels were weighed immediately prior to surgery; individuals weighing
more than 400g or less than 250g were not used in this study as those larger than 400g were about to begin hibernation, and those under 250g were unlikely to survive.

**Surgeries – pump implantation**

Squirrels were anesthetized using isoflurane gas (5% for induction, and then maintained at 2-4% and confirmed with testing blink and pedal reflex), and heart rate monitoring via a Doppler heart rate monitor. The head of the squirrel was shaved and prepared for surgery using an ethanol and a chlorohexidine wash. Squirrels were given meloxicam (0.3mg/kg) and bupivacaine (1mg/kg) subcutaneously in the body or at the incision site, respectively. An ocular lubricant was used on squirrels’ eyes to prevent discomfort.

All the following surgical procedures were completed under sterile conditions. A midline incision was made from the eyes caudal to the ears, to allow for visualization of the skull and skull sutures. A subcutaneous pocket for the pump (Alzet model 1007D, Durect Corp., Cupertino, CA, USA) was then created between the shoulder blades via blunt dissection, and a vinyl catheter (ALZET Brain Infusion Kit 2, Durect Corp., Cupertino, CA, USA) was trimmed to remove excess tubing under the skin. A 0.45mm hole was then made in the skull caudal to the eyes and approximately 1-2mm left of the midline using a sterile dental drill to allow cannulation of the lateral ventricle, as verified in pilot work. The area around the drilled hole was rinsed of bone fragments and dust using warm sterile saline. A low-profile cannula (Alzet Brain Infusion Kit 2, DURECT Corp., Cupertino, CA, USA) was modified to penetrate 4mm below the skull, and was implanted into the prepared
area. The cannula was attached to the catheter and the pre-filled pump; both the catheter and pump were positioned under the skin between the shoulder blades. Pumps were prepared in accordance with the manufacturer’s guidelines to deliver 0.05 µg Avp h⁻¹ in 0.5 µl of sterile saline or the equivalent volume of sterile saline alone as a control, based on pharmacological doses in other rodents which induced self-grooming behavior. The cannula was affixed to the skull using Vetbond (3M, St. Paul, MN, USA) or Glustitch (Glustitch Inc, Delta, BC, Canada) cyanoacrylate adhesive. The incision was then closed using 4.0 monocryl sutures, and the animal given subcutaneous saline (10 ml/kg/hour of anaesthesia) to rehydrate.

Surgeries – recovery and collection

Animals recovered within 30 minutes to 2 hours, and once ambulatory, were released at the point of capture. All animals were monitored daily after surgery to ensure the incision was healing and did not show sign of infection. Behavioral testing was completed between 3-6 days post-surgery. We selected a 3-day waiting period to ensure that animals would have similar levels of Avp-related FGMs in their fecal samples, as FGMs post stressful events peaks at day four in RGS (Hare et al., 2014). Once behavioral testing was complete, a second fresh fecal sample was collected as previously described, and subjects were humanely euthanized using isoflurane gas (induction at 5%) and intracardiac sodium pentobarbital injection. Up to 0.5 ml diluted India ink was then injected into the cannula’s catheter and the brain was removed to verify cannula placement. Brains were immediately post fixed in 4% paraformaldehyde prior to sectioning at 100 µm on a vibratome.
Behavioral experiments

To determine how Avp influenced behaviors of subjects, three tests, both before and after surgery, were conducted: a general survey, and a predator model experiment, and a social challenge experiment.

Survey

Subjects were observed for one hour during the day (0700 to 1600, CST) both before (range 23 to 0 days, average 4.3 days) and after (range 3 to 5 days, average 3.5 days) surgery, using all occurrences sampling for aggression (chases, ball fights, lateral displays, tooth chatters, piloerection; a full list of behaviors and definitions are presented in appendix A), and affiliation (greeting, play, allogrooming, following) and fixed-interval point sampling every 30s for all other behaviors (standing, eating, running, walking, calling, self-grooming, digging, in the burrow, out of sight, and other). Behaviors were coded as previously defined in Hare (1998a) and Holmes and Sherman (1982).

Predator model experiment

To determine the effects of Avp on alarm calling behavior a call-eliciting stimulus was used, following the paradigm used by Hare (Hare, 1998a; Sloan et al., 2005). Once a subject was located, a Sony HDR-XR100 video camera on a tripod (SONY, Tokyo, Japan) and hand-held shotgun microphone (Audio-Technica AT835B, Audio-Technica, Machida, Japan) were set up approximately 10m away from the subject. All model presentations were done by ARF wearing the same outer clothing each day to minimize confounding effects of the observer. Once the subject was at a low level of vigilance and activity (eating or standing with the head down (s4hd))
for approximately 2 minutes, the trial was started. For this test, we coded all behaviors continuously starting 30s before and ending 30s after presentation of the stimulus. Also recorded were: the number of individuals nearby (approximately 20m from subject), and whether those individuals were kin, when known. Behaviors were thus calculated as a change from the 30s prior to the 30s immediately after the stimulus using video recordings. Additionally, pre-vigilant latency, defined as the amount of time to reduce vigilance after the first vigilant response after the presentation of the stimulus, was calculated (behavioral coding/definition details in appendix A).

*Social challenge experiment*

For male subjects, the subject was trapped and video recorded for 10 minutes in the location where he was trapped. For female subjects, there were two versions of the test conducted for each subject. In the first version, the female subject was trapped in her core area, and video recorded for ten minutes in the location where she was trapped. In the second version, an unrelated adult female with an adjacent (<15 m) territory was trapped and moved into the subject’s core area, and the behavior of the subject interacting with the ‘intruder’ was recorded for ten minutes. This simulated intrusion was only conducted when the subject female was above ground and within 10m at the start of the trial. Only females defend a core area outside of the breeding season. Thus, only females were tested using the ‘intruder’ test.

All behaviors (see appendix A) of the experimental animals were scored including the duration of: vocalizations (chirps, whistles, social ‘squeaks’, aggressive
chatters), hitting and biting the trap, self-grooming, social behaviors (greetings, close contact with conspecifics without aggression), and aggressive/agonistic behaviors (lunging, biting conspecific, rolling onto back, lateral displays, and piloerection of tail hair). Close contact was defined when the squirrel outside the trap was within 5 cm of the trap. For simulated intrusions, behaviors of the subject that happened within the frame of the video (approximately 1m square with trap in the center of the frame) were scored. All other behaviors were defined as in Hare (1998b) and Holmes and Sherman (1982). Latency for social behavior, latency for aggression, and the number of call syllables issued in the context of social behavior or aggressive behavior were also recorded. This experiment was conducted when conspecifics were near (<10m), though in some cases conspecifics avoided the trap (Table 1).

Table 1: Avoidance in social challenge tests

<table>
<thead>
<tr>
<th>Sex</th>
<th>Timepoint</th>
<th>Number Avoiding</th>
<th>Number Avoiding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Saline)</td>
<td>(Avp)</td>
</tr>
<tr>
<td>Male</td>
<td>PRE</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Male</td>
<td>POST</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>PRE</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>POST</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Female - Intrusion</td>
<td>PRE</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Female - Intrusion</td>
<td>POST</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

_Fecal glucocorticoid analysis – collection_

Fecal samples were collected at the time of surgery and euthanasia to examine FGMs. Fecal samples were frozen and stored at -20°C, and served as a time-integrated measure of circulating FGMs over the previous four days (Hare et al., 2014).
**Fecal glucocorticoid analysis – fecal extraction**

Fecal samples were dried overnight at 60°C, then approximately 0.1g of dried, ground fecal matter was added to 1.5ml 95% ethanol. Samples were mixed then centrifuged at 4°C for 10 minutes at 13,000 x g. The supernatant was removed and 200μl was transferred to a tube and the ethanol was evaporated off using a sample concentrator (2014 samples: Savant, Thermo-Fisher), or at room temperature in a fume hood (2015 samples). FGM concentrations were equivalent in pooled samples regardless of drying method. The dried pellet was then re-suspended in 200μl of RIA buffer (0.1M phosphate buffer, 0.9% NaCl (w/v) and 0.5% bovine serum albumin (w/v)), and separated into duplicate subsamples immediately prior to RIA.

**Fecal glucocorticoid analysis – radioimmunoassay**

The protocol described in Ryan et al. (2012) and validated by Hare et al. (2014) was followed with minor adjustments. Briefly, 100μl of resuspended sample was combined with 100μl of cortisol-specific antibody (1:9,000 dilution, Fitzgerald Industries, North Acton, MA, US), and 100μl (5,000 ± 250 dpm) of radioactively-labeled (tritium, ³H) free cortisol (GE Healthcare, Piscataway, NJ, USA). Assay tubes were incubated at room temperature for 1h and then overnight at 4°C. To terminate the assay, 100μl of dextran-coated charcoal (0.5% w/v dextran and 5% w/v charcoal) was added to all assay tubes and incubated on ice for 15 minutes. Tubes were then centrifuged at 4°C for 30 minutes at 2,500 x g and the supernatant was decanted into 7ml scintillation vials with 4ml of liquid scintillation cocktail (Ultima Gold AB, Perkin Elmer, MA, USA). Vials were briefly mixed, and radioactivity was
measured using a liquid scintillation counter (LS6500, Beckman Coulter). FGM concentration was extrapolated from standard curves using known concentrations of cold cortisol (Steraloids, Newport RI, USA). All samples were processed in duplicate and standards in triplicate. For fecal samples, male samples had an inter-assay variation of 7%, and intra-assay variation of 6.5%, while females had an inter-assay variation of 15.1% and intra-assay variation of 13-17.8%. These variances, minimum detectable levels, and extraction efficiencies were comparable to values previously reported for RGS (Hare et al., 2014; Ryan et al., 2012). Duplicate or triplicates with a standard deviation of > 125 dpm were re-assayed or rejected.

Statistical analysis

ANCOVAs, using type III sums of squares, were used to determine if Avp-treated and saline-treated animals differed in behavior, using each individual’s baseline scores (prior to surgery) as a covariate. FGM data were log-transformed (fecal ng/g measurements), and normality was examined using Q-Q plots and Shapiro-Wilk tests. Since data were normal (P>0.05 for Shapiro-Wilk test), FGM measurements within saline and Avp treatment groups were compared using paired t-tests. All statistics were conducted using R (version 3.2.3) and considered statistically significant where \( P < 0.05 \).

Results

Males – survey

There was a significant effect of Avp on combined aggression (chasing, lateral displays, tooth chatters, and ball fights) during the behavior survey after controlling for the effect of baseline (“PRE” scores) (Figure 3. \( F_{2, 8} = 5.735, p = 0.0285 \)).
Figure 3: Male aggression bouts from survey experiments. Red lines indicate changes between the means for grouped PRE and POST scores within saline (solid) and Avp (dashed) treatment groups. Inset indicates mean aggressive bout changes (POST-PRE) + SE for saline (black) and Avp (grey). Avp treated animals had significantly (p <0.05) fewer aggressive bouts than saline-treated controls.
were no statistically significant effects of Avp treatment on any other behaviors after controlling for the effect of baseline scores (Table 2). While the differences between Avp and saline groups seem large prior to random assignment into treatment and surgery, these differences before treatment were non-significant (Wilcoxon-Mann-Whitney test, Saline: n = 6, mean ± SE = 0.667 ± 0.494; Avp: n = 5, mean ± SE = 2.20 ± 0.735; W = 5.735, p = 0.1450).

Table 2: Male behaviors during survey experiments. Significant differences between saline and Avp-treated animals indicated in italics.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Treatment</th>
<th>PRE mean counts ± SE</th>
<th>POST mean counts ± SE</th>
<th>$F_{2,8}$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined Aggression</td>
<td>Saline (n = 6)</td>
<td>0.667 ± 0.494</td>
<td>0.167 ± 0.167</td>
<td>5.735</td>
<td>*0.0285</td>
</tr>
<tr>
<td></td>
<td>Avp (n = 5)</td>
<td>2.200 ± 0.735</td>
<td>0.600 ± 0.400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled Vigilance (Slouch, s4p, Alert)</td>
<td>Saline</td>
<td>6.917 ± 2.016</td>
<td>4.667 ± 1.840</td>
<td>0.7884</td>
<td>0.4869</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>4.450 ± 1.271</td>
<td>3.450 ± 1.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Social</td>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vocalizations</td>
<td>Saline</td>
<td>0.333 ± 0.333</td>
<td>1.167 ± 0.749</td>
<td>0.7268</td>
<td>0.5128</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>3.20 ± 2.010</td>
<td>1.800 ± 1.800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self-groom</td>
<td>Saline</td>
<td>1.5 ± 0.563</td>
<td>1.5 ± 0.847</td>
<td>1.417</td>
<td>0.2973</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>2.8 ± 0.735</td>
<td>1.2 ± 0.735</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Non-social</td>
<td>Saline</td>
<td>109 ± 3.786</td>
<td>111.5 ± 2.405</td>
<td>2.282</td>
<td>0.1644</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>106.2 ± 2.745</td>
<td>112.8 ± 2.634</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Males – predator model experiment*

There was a significant effect of Avp on escape behavior and pooled vigilance scores (Figure 8. $F_{2,8} = 4.47$, p = 0.0497) and (Fig 9. $F_{2,8} = 5.78$, p = 0.0280)
respectively, but no effect on other measures (Figure 4: non-vigilance, Figure 5: low-vigilance, Figure 6: slouch, Figure 7: alert) after controlling for the effect of baseline during the predator model experiment. Pooled vigilance/escape was calculated as 

\[
(1 \times \text{run/at burrow/in burrow}) + (0.75 \times \text{s2p}) + (0.5 \times \text{slouch}) + (0.25 \times \text{s4phu}) / \text{total time scored (based on Wilson and Hare, 2003).}
\]

There were no effects of Avp on any vocalization measures (call, call latency, call rate, syllables), previgilant latency, or nonvigilance behavior during the predator model experiment (Table 3).

Table 3: Male behaviors during predator model experiments. Significant differences between saline and Avp-treated animals indicated in italics.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Treatment</th>
<th>PRE mean difference score ± SE</th>
<th>POST mean difference score ± SE</th>
<th>F_{2,8}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Vigilance</td>
<td>Saline (n=6)</td>
<td>-0.306 ± 0.145</td>
<td>-0.478 ± 0.155</td>
<td>0.3329</td>
<td>0.7263</td>
</tr>
<tr>
<td></td>
<td>Avp (n=5)</td>
<td>-0.060 ± 0.110</td>
<td>-0.647 ± 0.152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Vigilance</td>
<td>Saline</td>
<td>0.211 ± 0.101</td>
<td>0.339 ± 0.091</td>
<td>0.4379</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>-0.053 ± 0.056</td>
<td>0.247 ± 0.106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slouch</td>
<td>Saline</td>
<td>-0.056 ± 0.056</td>
<td>-0.017 ± 0.017</td>
<td>0.5455</td>
<td>0.5997</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alert</td>
<td>Saline</td>
<td>0.022 ± 0.022</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.060 ± 0.037</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escape</td>
<td>Saline</td>
<td>0.022 ± 0.011</td>
<td>0.028 ± 0.026</td>
<td>4.47</td>
<td>*0.0497</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.013 ± 0.053</td>
<td>0.286 ± 0.166</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled Vigilance</td>
<td>Saline</td>
<td>0.002 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td>5.78</td>
<td>*0.0280</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.002 ± 0.003</td>
<td>0.012 ± 0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previgilant Latency</td>
<td>Saline</td>
<td>0.367 ± 0.139</td>
<td>0.139 ± 0.061</td>
<td>1.481</td>
<td>0.2837</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.087 ± 0.079</td>
<td>0.367 ± 0.139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call</td>
<td>Saline</td>
<td>0.106 ± 0.162</td>
<td>0.133 ± 0.080</td>
<td>0.522</td>
<td>0.6122</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.033 ± 0.033</td>
<td>0.047 ± 0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call Latency</td>
<td>Saline</td>
<td>18.50 ± 5.162</td>
<td>20.67 ± 4.674</td>
<td>1.3</td>
<td>0.3244</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>25.2 ± 4.8</td>
<td>26.4 ± 3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syllable</td>
<td>Saline</td>
<td>2.5 ± 1.455</td>
<td>1.5 ± 0.806</td>
<td>0.6882</td>
<td>0.5299</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>2.2 ± 2.2</td>
<td>0.4 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call Rate</td>
<td>Saline</td>
<td>0.392 ± 0.192</td>
<td>0.257 ± 0.158</td>
<td>1.8</td>
<td>0.2262</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.096 ± 0.096</td>
<td>0.076 ± 0.076</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4: Changes in male non-vigilance behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 5: Changes in male low-vigilance behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 6: Changes in male slouch behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 7: Changes in male alert behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 8: Changes in male escape behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 9: Changes in male pooled vigilance behavior scores during predator model experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes ± SE.
Males – social challenge experiment

There was a significant effect of Avp on social and non-social behavior duration ($F_{2, 8} = 5.928, p = 0.0264$) and ($F_{2, 8} = 6.428, p = 0.0217$) respectively, after controlling for the effect of baseline during the social challenge experiment. Social behavior decreased in Avp-treated males, while the duration of non-social behavior increased. Avp treatment also altered aggressive behavior during the social challenge experiment, as Avp treatment significantly decreased the duration of offensive aggression (biting, lunging, lateral displays; Figure 10. $F_{2, 8} = 10.45, p = 0.0059$), and decreased the duration of all aggressive encounters (including submissive behavior), though this effect was not statistically significant.

Avp treatment also altered vocalizations during the social challenge experiment in males. The total duration of all vocalizations was significantly affected by Avp treatment (Figure 11. $F_{2, 8} = 16.22, p = 0.0015$) after controlling for the effect of baseline scores. Avp treatment did not significantly affect social vocalizations (Figure 12) or whistles (Figure 13), though males treated with Avp had a greater incidence of chirps (Figure 14. $F_{2, 8} = 5.289, p = 0.0344$), and number of total syllables (Figure 15. $F_{2, 8} = 5.321, p = 0.0339$) given during the social challenge experiment compared to saline treatment, after controlling for baseline scores. Males treated with Avp did not differ from controls with aggressive vocalizations (Figure 16), grooming behavior, anxiety-like behavior (biting or hitting the cage repeatedly), or ‘other’ behaviors (sitting, standing, eating; Table 4).
Figure 10: Male offensive aggression duration from social challenge experiments. Red lines indicate changes between the means for grouped PRE and POST scores within saline (solid) and Avp (dashed) treatment groups. Inset indicates mean changes (POST-PRE) + SE for saline (black) and Avp (grey). Avp treated animals had a significant (p < 0.05) decrease in offensive aggression duration compared to saline-treated controls.
Figure 11: Changes in male vocalization duration during social challenge experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 12: Changes in male social vocalization duration during social challenge experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 13: Changes in male whistle bouts during social challenge experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 14: Changes in male chirp vocalizations during social challenge experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 15: Changes in male call syllables during social challenge experiments. *Saline*-treated animals are indicated with solid lines, *vasopressin*-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 16: Changes in male aggressive vocalization duration during social challenge experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Table 4: Male behaviors during social challenge experiment. Significant differences between saline and Avp-treated animals indicated in italics.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Treatment</th>
<th>PRE mean proportion or count ± SE</th>
<th>POST mean proportion or count ± SE</th>
<th>F_{2,8}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social</td>
<td>Saline (n = 6)</td>
<td>0.005 ± 0.005</td>
<td>0.004 ± 0.002</td>
<td>5.928</td>
<td>*0.0264</td>
</tr>
<tr>
<td></td>
<td>Avp (n = 5)</td>
<td>0.065 ± 0.027</td>
<td>0.016 ± 0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Social</td>
<td>Saline</td>
<td>0.983 ± 0.014</td>
<td>0.996 ± 0.002</td>
<td>6.428</td>
<td>*0.0217</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.805 ± 0.096</td>
<td>0.921 ± 0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>Saline</td>
<td>0.011 ± 0.009</td>
<td>0</td>
<td>2.977</td>
<td>0.1081</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.130 ± 0.083</td>
<td>0.063 ± 0.045</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chirp Syllable Count</td>
<td>Saline</td>
<td>3.83 ± 3.64</td>
<td>0.83 ± 0.65</td>
<td>5.289</td>
<td>*0.0344</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>17.2 ± 11.9</td>
<td>29.2 ± 11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whistle Bout Count</td>
<td>Saline</td>
<td>2.83 ± 2.83</td>
<td>1.17 ± 0.83</td>
<td>0.8413</td>
<td>0.466</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>9.2 ± 8.7</td>
<td>5.4 ± 3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Call Syllables Count</td>
<td>Saline</td>
<td>6.67 ± 6.47</td>
<td>2.17 ± 1.22</td>
<td>5.321</td>
<td>*0.0339</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>34.0 ± 17.24</td>
<td>39.4 ± 16.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggressive Vocalization</td>
<td>Saline</td>
<td>0.015 ± 0.009</td>
<td>0.007 ± 0.003</td>
<td>2.035</td>
<td>0.1930</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.052 ± 0.029</td>
<td>0.027 ± 0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social Vocalization</td>
<td>Saline</td>
<td>0.001 ± 0.001</td>
<td>0.001 ± 0.0004</td>
<td>1.01</td>
<td>0.4063</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.003 ± 0.001</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Vocalization</td>
<td>Saline</td>
<td>0.026 ± 0.014</td>
<td>0.011 ± 0.003</td>
<td>16.22</td>
<td>*0.0015</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.111 ± 0.049</td>
<td>0.093 ± 0.031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Offensive Aggression</td>
<td>Saline</td>
<td>0.012 ± 0.010</td>
<td>0</td>
<td>10.45</td>
<td>*0.0059</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.075 ± 0.045</td>
<td>0.023 ± 0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self Groom</td>
<td>Saline</td>
<td>0.015 ± 0.007</td>
<td>0.014 ± 0.002</td>
<td>0.3141</td>
<td>0.739</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.028 ± 0.013</td>
<td>0.017 ± 0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite/hit Cage</td>
<td>Saline</td>
<td>0.125 ± 0.077</td>
<td>0.181 ± 0.029</td>
<td>0.1487</td>
<td>0.8641</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.020 ± 0.014</td>
<td>0.152 ± 0.086</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Saline</td>
<td>0.847 ± 0.073</td>
<td>0.803 ± 0.028</td>
<td>0.0357</td>
<td>0.965</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.704 ± 0.154</td>
<td>0.797 ± 0.075</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Females – survey**

There was an effect of Avp on female vocalizations during the behavior survey (F_{2,16} = 3.51, p = 0.0545) after controlling for the effect of baseline, with Avp-treated females producing more vocalizations. Avp treatment in females also significantly influenced foraging behavior ('eat') in females, (F_{2,16} = 3.819, p =
0.0441). Avp treatment did not significantly affect any other behaviors measured during the survey experiment after correcting for baseline scores (Table 5; aggression, vigilance, social duration, self grooming, non-social duration, time in burrow).

Table 5: Female behaviors during survey experiments. Significant differences between saline and Avp-treated animals indicated in italics.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Treatment</th>
<th>PRE mean proportion of time scored ± SE</th>
<th>POST mean proportion of time scored ± SE</th>
<th>F&lt;sub&gt;2,16&lt;/sub&gt;</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined Aggression</td>
<td>Saline (n = 10)</td>
<td>0.010 ± 0.004</td>
<td>0.007 ± 0.003</td>
<td>0.2484</td>
<td>0.783</td>
</tr>
<tr>
<td></td>
<td>Avp (n = 9)</td>
<td>0.006 ± 0.003</td>
<td>0.011 ± 0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled Vigilance</td>
<td>Saline</td>
<td>0.100 ± 0.024</td>
<td>0.091 ± 0.031</td>
<td>1.185</td>
<td>0.3313</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>8.15 ± 2.35</td>
<td>4.63 ± 1.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Social</td>
<td>Saline</td>
<td>0.002 ± 0.002</td>
<td>0.002 ± 0.001</td>
<td>0.7019</td>
<td>0.5103</td>
</tr>
<tr>
<td>(Greeting,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allogroom, sniff,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>play, follow)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.003 ± 0.002</td>
<td>0.004 ± 0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vocalization</td>
<td>Saline</td>
<td>0.013 ± 0.009</td>
<td>0.020 ± 0.011</td>
<td>3.51</td>
<td>*0.0545</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.028 ± 0.012</td>
<td>0.020 ± 0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self Groom</td>
<td>Saline</td>
<td>0.014 ± 0.004</td>
<td>0.020 ± 0.007</td>
<td>1.197</td>
<td>0.3277</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.021 ± 0.008</td>
<td>0.011 ± 0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Non</td>
<td>Saline</td>
<td>0.222 ± 0.034</td>
<td>0.153 ± 0.025</td>
<td>1.375</td>
<td>0.281</td>
</tr>
<tr>
<td>Social</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.186 ± 0.039</td>
<td>0.207 ± 0.049</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In Burrow</td>
<td>Saline</td>
<td>0.276 ± 0.061</td>
<td>0.318 ± 0.088</td>
<td>0.7724</td>
<td>0.4784</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.291 ± 0.063</td>
<td>0.336 ± 0.089</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eat</td>
<td>Saline</td>
<td>0.340 ± 0.069</td>
<td>0.363 ± 0.077</td>
<td>3.819</td>
<td>*0.0441</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.321 ± 0.055</td>
<td>0.330 ± 0.062</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Females – predator model experiment

There was a significant effect of Avp on female slouching vigilance (Figure 19. F<sub>2,16</sub> = 4.005, p = 0.0389), but not pooled vigilance/escape (Figure 20. F<sub>2,16</sub> = 0.4674, p = 0.6349), after controlling for the effect of baseline during the predator model experiment. Females did not differ for any other measures when treated with
Avp compared to those treated with saline after controlling for baseline (non-vigilance (Figure 17), low vigilance (Figure 18), alert (s2p) (Figure 21), escape (Figure 22), previgilant latency, calls, call latency, call rate, number of syllables).

Females - social challenge experiment

Avp treatment did not influence total vocal duration (Figure 23), or social vocal duration (Figure 24) during the social challenge experiment in females, however Avp treatment did alter other vocalization measures. Females treated with Avp produced fewer whistle bouts than did saline-treated females (Figure 25, $F_{2,16} = 3.836, p = 0.0436$) after controlling for the effect of baseline scores. Avp-treated females also exhibited a reduction in aggressive vocalizations compared to saline controls, though this trend was not statistically significant (Figure 28, $F_{2,16} = 2.332, p = 0.1292$). Females treated with Avp had a marked decrease in anxiety-like behavior (repeatedly hitting and biting the cage) compared to saline treated females, and this effect was significant after controlling for baseline scores ($F_{2,16} = 5.129, p = 0.019$). We also detected an effect of Avp treatment on females as measured in ‘other’ behaviors (eating, s4p), due to these females conducting other non-social and social behaviors ($F_{2,16} = 4.161, p = 0.0351$). Avp treatment did not significantly affect durations of social, non-social, or aggressive behaviors (Figure 28. Unlike with males, female chirp vocalizations (Figure 26), total alarm call syllables (Figure 27), and offensive aggression were not affected by Avp treatment (Table 6). The number of calls given per the amount of time interacting with conspecifics (Call notes per social duration and call notes per aggressive duration) was calculated; this was also unaffected by Avp treatment (Table 6).
Figure 17: Changes in female non-vigilance behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 18: Changes in female low-vigilance behavior proportions during predator model experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 19: Changes in female slouch behavior proportions during predator model experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 20: Changes in female alert behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 21: Changes in female escape behavior proportions during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 22: Changes in female pooled vigilance scores during predator model experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 23: Changes in female total vocalization duration during social challenge experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 24: Changes in female social vocalization duration during social challenge experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 25: Changes in female whistle bouts during social challenge experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 26: Changes in female chirp vocalizations during social challenge experiments. *Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.*
Figure 27: Changes in female call syllables during social challenge experiments. 
Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Figure 28: Changes in female aggressive vocalization duration during social challenge experiments. Saline-treated animals are indicated with solid lines, vasopressin-treated animals are indicated with dashed lines. Mean changes are indicated in red. Inset indicates mean changes + SE.
Table 6: Female behaviors during social challenge experiment. Significant differences between saline and Avp-treated animals indicated in italics.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Treatment</th>
<th>PRE mean proportion or count ± SE</th>
<th>POST mean proportion or count ± SE</th>
<th>F_{2,16}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social</td>
<td>Saline (n = 10)</td>
<td>0.170 ± 0.049</td>
<td>0.170 ± 0.038</td>
<td>2.477</td>
<td>0.1155</td>
</tr>
<tr>
<td></td>
<td>Avp (n = 9)</td>
<td>0.081 ± 0.021</td>
<td>0.095 ± 0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Social</td>
<td>Saline</td>
<td>0.828 ± 0.049</td>
<td>0.827 ± 0.039</td>
<td>2.038</td>
<td>0.1628</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.885 ± 0.041</td>
<td>0.903 ± 0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>Saline</td>
<td>0.001 ± 0.009</td>
<td>0.003 ± 0.002</td>
<td>0.38</td>
<td>0.6899</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.033 ± 0.031</td>
<td>0.001 ± 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chirp Syllable Count</td>
<td>Saline</td>
<td>1.9 ± 0.781</td>
<td>13.5 ± 8.741</td>
<td>0.8299</td>
<td>0.454</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>4.667 ± 4.052</td>
<td>1.333 ± 0.986</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whistle Bout Count</td>
<td>Saline</td>
<td>1.5 ± 1.185</td>
<td>1.8 ± 1.389</td>
<td>3.836</td>
<td>*0.0436</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>2.667 ± 1.772</td>
<td>1.389 ± 0.564</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Call Syllables</td>
<td>Saline</td>
<td>4.3 ± 1.550</td>
<td>17.5 ± 8.916</td>
<td>1.169</td>
<td>0.336</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>8.778 ± 6.957</td>
<td>2.556 ± 1.107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggressive Vocalization</td>
<td>Saline</td>
<td>0.009 ± 0.007</td>
<td>0.009 ± 0.003</td>
<td>2.332</td>
<td>0.1292</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.017 ± 0.007</td>
<td>0.002 ± 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social Vocalization</td>
<td>Saline</td>
<td>0.004 ± 0.002</td>
<td>0.003 ± 0.002</td>
<td>0.7064</td>
<td>0.5082</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.001 ± 0.001</td>
<td>0.006 ± 0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Vocalization</td>
<td>Saline</td>
<td>0.012 ± 0.007</td>
<td>0.013 ± 0.004</td>
<td>1.278</td>
<td>0.3056</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.018 ± 0.007</td>
<td>0.008 ± 0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Offensive Aggression</td>
<td>Saline</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.002</td>
<td>0.4061</td>
<td>0.6729</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.012 ± 0.011</td>
<td>0.001 ± 0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call Notes per Social Duration</td>
<td>Saline</td>
<td>4031.9 ± 3996.5</td>
<td>30.17 ± 29.98</td>
<td>0.4061</td>
<td>0.6729</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>62.57 ± 61.81</td>
<td>21.50 ± 14.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call Notes per Aggressive Duration</td>
<td>Saline</td>
<td>1.698 ± 1.207</td>
<td>0.047 ± 0.035</td>
<td>0.1109</td>
<td>0.8957</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>31.56 ± 25.65</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self Groom</td>
<td>Saline</td>
<td>0.073 ± 0.018</td>
<td>0.041 ± 0.006</td>
<td>1.284</td>
<td>0.3041</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.031 ± 0.018</td>
<td>0.027 ± 0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite/hit Cage</td>
<td>Saline</td>
<td>0.059 ± 0.028</td>
<td>0.042 ± 0.017</td>
<td>5.129</td>
<td>*0.019</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.187 ± 0.068</td>
<td>0.232 ± 0.062</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Saline</td>
<td>0.842 ± 0.036</td>
<td>0.915 ± 0.019</td>
<td>4.161</td>
<td>*0.0351</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.758 ± 0.060</td>
<td>0.739 ± 0.059</td>
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</tr>
</tbody>
</table>
Females - social challenge experiment: simulated intrusions

There were no significant effects of Avp on any of the measured behaviors in females during the simulated intrusions social challenge experiment after controlling for the effect of baseline (“PRE” scores) (Table 7). Avp-treated females tended to have a longer duration of non-social behavior compared to saline-treated animals, though the effect fell short of statistical significance (F_{2,16} = 3.248, p = 0.0655).
Table 7: Female behaviors during simulated intrusions social challenge experiment.

Significant differences between saline and Avp-treated animals indicated in italics.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Treatment</th>
<th>PRE mean proportion or count ± SE</th>
<th>POST mean proportion or count ± SE</th>
<th>F2,16</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social</td>
<td>Saline (n=10)</td>
<td>0.028 ± 0.011</td>
<td>0.028 ± 0.011</td>
<td>2.091</td>
<td>0.1561</td>
</tr>
<tr>
<td></td>
<td>Avp (n=9)</td>
<td>0.094 ± 0.052</td>
<td>0.024 ± 0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Social</td>
<td>Saline</td>
<td>0.860 ± 0.067</td>
<td>0.929 ± 0.030</td>
<td>3.248</td>
<td>0.0655</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.711 ± 0.083</td>
<td>0.855 ± 0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>Saline</td>
<td>0.112 ± 0.063</td>
<td>0.043 ± 0.024</td>
<td>1.769</td>
<td>0.2022</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.249 ± 0.076</td>
<td>0.121 ± 0.067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chirp syllable Count</td>
<td>Saline</td>
<td>0.2 ± 0.2</td>
<td>0.8 ± 0.8</td>
<td>0.316</td>
<td>0.7335</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>1.444 ± 1.444</td>
<td>0.111 ± 0.111</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whistle Bout Count</td>
<td>Saline</td>
<td>0.6 ± 0.499</td>
<td>1.3 ± 0.895</td>
<td>1.501</td>
<td>0.2526</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>4.556 ± 1.980</td>
<td>1.222 ± 0.547</td>
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</tr>
<tr>
<td>Total Call Syllables</td>
<td>Saline</td>
<td>1.1 ± 0.706</td>
<td>6.9 ± 5.527</td>
<td>0.4537</td>
<td>0.6432</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>9.556 ± 4.568</td>
<td>2.667 ± 1.344</td>
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<td></td>
</tr>
<tr>
<td>Aggressive Vocalization</td>
<td>Saline</td>
<td>0.069 ± 0.037</td>
<td>0.027 ± 0.018</td>
<td>1.853</td>
<td>0.1888</td>
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<tr>
<td></td>
<td>Avp</td>
<td>0.161 ± 0.057</td>
<td>0.084 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social Vocalization</td>
<td>Saline</td>
<td>0.001 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>0.3718</td>
<td>0.6953</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.001 ± 0.001</td>
<td>0.001 ± 0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Vocalization</td>
<td>Saline</td>
<td>0.069 ± 0.037</td>
<td>0.028 ± 0.018</td>
<td>1.883</td>
<td>0.1843</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.161 ± 0.057</td>
<td>0.084 ± 0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Offensive Aggression</td>
<td>Saline</td>
<td>0.063 ± 0.033</td>
<td>0.030 ± 0.018</td>
<td>2.468</td>
<td>0.1164</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.172 ± 0.061</td>
<td>0.079 ± 0.046</td>
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</tr>
<tr>
<td>Call notes per social duration</td>
<td>Saline</td>
<td>0.084 ± 0.072</td>
<td>30.54 ± 30.30</td>
<td>1.559</td>
<td>0.2407</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>208.49 ± 141.77</td>
<td>53.81 ± 53.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Call notes per aggression duration</td>
<td>Saline</td>
<td>0.003 ± 0.002</td>
<td>0.098 ± 0.091</td>
<td>1.679</td>
<td>0.2178</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>16.9 ± 11.9</td>
<td>33.34 ± 33.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SelfGroom</td>
<td>Saline</td>
<td>0.002 ± 0.002</td>
<td>0.011 ± 0.007</td>
<td>1.73</td>
<td>0.1089</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.002 ± 0.002</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Saline</td>
<td>0.881 ± 0.938</td>
<td>0.938 ± 0.031</td>
<td>1.49</td>
<td>0.2549</td>
</tr>
<tr>
<td></td>
<td>Avp</td>
<td>0.768 ± 0.067</td>
<td>0.782 ± 0.116</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FGM Results

Male FGMs

FGM difference values for saline-treated and Avp-treated animals were normally distributed after log-transformation (Shapiro-Wilk test, W = 0.9663, p = 0.8548). One Avp-treated animal did not have a post-surgery sample and was excluded from analysis (Avp n = 4, Saline n = 6). Paired t-tests were used to determine if treatment affected FGM measurements in either group. FGM levels were not affected by saline treatment ($t_5 = 0.701, p = 0.5145$) or Avp treatment ($t_3 = 2.000, p=0.1393$). Change in FGMs was not dependent on number of days from surgery for Avp-treated males ($n = 4, R^2 = 0.1013, p = 0.6817$).

Female FGMs

Females treated with Avp and saline had FGM difference values that were normally distributed after log-transformation (Shapiro-Wilk test, W = 0.9635, p = 0.7256). Three individuals had insufficient samples, and/or had standard deviations $> 125$ DPM in duplicates, and were thus excluded from analysis (Saline n=8, Avp n=8). Saline treatment ($t_7 = 0.288, p = 0.7817$) and Avp treatment ($t_7 = 0.579, p = 0.581$) did not significantly influence FGM levels in females. Change in FGMs was not dependent on the number of days from surgery for Avp-treated females ($n = 8, R^2 = 0.015, p = 0.7746$).

Discussion

Avp has a wide-range of behavioral effects and is well-suited for comparative studies because of its modulatory role in the physiology of various taxa. We hypothesized that Avp would alter social behavior including vocalization production
since Avp affects these behaviors in other species. While our specific hypothesis that Avp might increase affiliative behavior was unsupported; the prediction that Avp would influence calling behavior was supported, but only in some contexts. This work expands our current understanding of Avp’s central effects on behavior to a free-living species in its natural environment by documenting effects on social behavior, aggression, antipredator vigilance, and vocal communication.

Predator vigilance and anxiety

Our findings suggest that central Avp administration can increase antipredator vigilance and escape responses in males without significantly altering FGMs. Male RGS increased escape and overall vigilance behaviors in response to the predator model when treated with Avp, and this response occurred without a concomitant increase in FGMs. Further, males treated with Avp did not differ in ‘anxiety-like’ behavior from saline-treated animals, suggesting this change in vigilance is not related to enhanced anxiety in general. In rats, peripheral treatment with Avp induces huddling and hiding behavior in response to predator odor compared to vehicle injections (Bowen and McGregor, 2014). The authors suggest that this effect is likely due to action at the Avpr1a to induce a social response, while they suggest action through the Avpr1b might increase anxiety, and thus, hiding behavior. However, an unknown site of action (i.e. peripheral or central), along with the absence of data on the stress response of their subjects preclude definitive inferences from being drawn (Bowen and McGregor, 2014). These rats were exposed to cat-fur odor only, and this odor was sufficient to elicit a response in male
rats. In our work, we show that central Avp can influence predator responses through the visual domain in the absence of a predator-specific olfactory stimulus.

Unlike males, female RGS did not alter antipredator vigilance and escape behavior when treated with Avp. Females showed an increase in ‘slouch-type’ vigilance, but overall vigilance was not altered by Avp administration. The time of year may have influenced female responses, as females were tested immediately after lactation (See Time of year/breeding state). It may be that female vigilance was already at a physiological ceiling as females are more likely to alarm call (associated with vigilance) when young have been weaned (Davis, 1984). Avp treatment reduced ‘anxiety-like’ responses in female RGS without impacting FGMs. Alarm calling in ground squirrels is often nepotistic, as it serves to alert kin to potential predator threats. Females in particular are more likely to become vigilant and alarm call, due to the potential fitness benefits of surviving kin (Sherman, 1977). For females, the fitness benefit gained from alerting kin to predatory threats may override the influence of Avp on hiding and vigilance behavior. In the survey, there was a significant increase in overall vocalizations. However, we did not attempt to record the context in which these calls were given. In predator experiments, alarm calling was not altered with Avp treatment in either females or males. Since vocalizations were altered by Avp administration in social but not predator-prey situations, the context of these calls may be important for Avp’s action (See Vocalizations below).

We used stereotyped biting and hitting of the cage during the social challenge experiment as a measure of ‘anxiety-like’ behavior. In males, Avp had no impact on
this behavior, however, in females, administration of Avp decreased the duration of this behavior. This effect could be due to the difference in administration of Avp (i.c.v versus other site-specific injections), or due to species-specific effects. Past work on Avp and anxiety in rodents typically shows Avp as having an anxiogenic effect. Male mice that lack the Avpr1a had reduced anxiety-like behavior in the elevated plus, open field, and light/dark box tests (Bielsky et al., 2004; Egashira et al., 2007), and rats with knockdown of the Avpr1a in the septum have reduced anxiety-like behavior in the elevated plus test (Landgraf et al., 1995). Consistent with the proposed link between anxiety and Avp, high-anxiety male and female rats have increased Avp expression in the PVH (Murgatroyd et al., 2004). However, antagonism of the Avpr1a and Avpr1b in the LS of rats increases anxiety (Everts and Koolhaas, 1999), and a different line of Avpr1a knockout mice had no measured deficits in anxiety (Wersinger et al., 2007b). These differences among studies could be due to differences in testing location or in the methods used. More work on Avp and anxiety in RGS will help to determine where Avp might be acting in the brain, and which receptor(s) are important for the Avp-induced behavioral changes.

**Vocalizations**

Vocalizations during the social challenge experiments were affected by Avp treatment. In both male and female RGS, treatment with Avp altered call syllables and duration of overall vocalizations. In males, Avp increased the numbers of chirps, call syllables, and reduced the duration of other vocalizations overall. In Avp-treated females, the total duration of vocalizations was not significantly reduced, however, females emitted significantly fewer whistles, and tended to emit fewer chirps and
aggressive chatters. Chirp vocalizations are a ‘high-threat’ vocalization, given typically in response to avian predators (Davis, 1984; Sloan et al., 2005). In this context, trapped male squirrels emitted chirps in response to approaching conspecifics. It is possible that these vocalizations could be anxiety-related, in addition to having a social component, as approaching females often showed aggression towards the trapped male subjects. The reduction of whistles in females was unexpected, though these changes are probably not associated with anxiogenesis from Avp given the reduction in stereotyped cage-biting in Avp-treated females. Furthermore, both males and females did not show significant increases in FGMs, thus, changes in vocalizations are likely independent of associated HPA axis activation and glucocorticoid release.

It is possible that central Avp administration working through the Avpr1a may reduce specific types of vocalizations in RGS, but not others. Further, peripheral Avp might not have discernable effects on these varied types of rodent vocalizations, especially if the peripheral Avp dose is low, since peripheral and central peptide administration have differential effects (Williams et al., 1994). Future work on female rodents should investigate central Avp mechanisms for influencing these varied social vocalizations.

Aggression

In most male rodents, central Avp administration increases aggressive behaviors (male prairie voles: (Winslow et al., 1993); Syrian hamsters, AH: (Caldwell and Albers, 2004; Ferris et al., 1997)). In RGS males, central administration of Avp reduced aggression in the survey and the social challenge
experiments. While male aggression measures prior to supplementation appear different between randomly assigned saline and Avp groups, these differences are not statistically significant, and attributable to the issues inherent with small sample sizes from invasive and ambitious manipulative field work. Overall, these responses are similar to female Syrian hamsters (AH injection; Gutzler et al., 2010) and lactating rats (i.c.v.; Nephew and Bridges, 2008b). It is likely that Avpr distributions are responsible for this difference in aggression, though time of year may also play a role, as males are generally less aggressive during the post-weaning period (Michener and McLean, 1996; Yeaton, 1972). Additionally, site-specific injections limit Avp’s action to a particular neural substrate, and thus, differences in behavioral responses from this work may be due to the area where Avp is acting via one of its receptors. In female RGS subjects, central Avp administration did not significantly affect aggression in any of the behavioral measures, potentially due to females already being in a highly aggressive state in the post-weaning period (Michener and McLean, 1996; Yeaton, 1972).

Affiliation

Despite the wealth of literature for other rodents, particularly socially monogamous voles, highlighting the importance of Avp on affiliative and pro-social behaviors, we did not detect any effects of Avp administration on male or female affiliation for RGS. In males, there was an effect of Avp on social behavior during the social challenge – males spent more time being non-social and less time being social overall. There were no differences in any grooming, sniffing, or play behaviors in any of the experiments. In some species, such as montane voles, Avp administration
does not increase social affiliation, but does increase self-grooming (Young et al., 1999a; Young et al., 1997). It is hypothesized that the differences in Avpr distribution between montane voles and prairie voles leads to the differential effects of Avp on behavior. In addition to potential differences in Avpr distributions, there have been suggestions that sociality in addition to or instead of social monogamy may be related to nonapeptide-influenced behavioral changes (Bales et al., 2004; Goodson, 2008; Insel et al., 1994). Since in this social species, affiliation was not influenced by Avp, it may be that affiliation due to socially monogamous mating strategies (i.e. within-pair huddling and grooming, or biparental care, which are not known for RGS) are influenced by Avp while more general affiliation (i.e. between siblings or closely-related individuals outside of mating contexts) is not. Future investigation into the distribution of Avpr in the brain of RGS will help elucidate where Avp acts centrally in this species, and comparing other promiscuous but social species will aid in determining the range of Avp's effects. Given the lack of effect on affiliation, we predict that this species will have receptor distribution patterns similar to the montane vole.

**Time of year/breeding state**

RGS breed in mid to late March in Manitoba, with males fighting to establish access to females (Michener and McLean, 1996), after which, males become relatively non-aggressive and females peak in aggression at the time of juvenile emergence (Yeaton, 1972). In this study, males were supplemented with Avp in late April and May, when males are relatively non-aggressive, while females were supplemented right after juvenile emergence in June and early July, when females
are most aggressive. In Syrian hamsters, breeding state is an important modulator of the effects of Avp, as injecting Avp into the AH increased aggression in breeding conditions (long photoperiod), but did not influence hamsters in a short, winter-like, photoperiod (Caldwell and Albers, 2004). In the male RGS, Avp administration reduced aggression, which was unexpected based on previous rodent work. However, since these males were in a non-breeding, low-aggressive state, Avp may have influenced these males by promoting season-appropriate behavioral responses. In females, which were already relatively aggressive (ranging 4.3 to 25% of time during simulated intrusions spent being aggressive), Avp treatment did not change aggression. Since females were already at a physiologically demanding time (immediately after lactation) and were typically aggressive, their season-appropriate aggressive behaviors may have reached a physiological limit, and thus a ceiling effect may have precluded the detection of any treatment effect. Future research should investigate whether Avp treatment in breeding males also alters aggression, and whether Avp changes aggression in females when they are in a typically less-aggressive state, and hence address season-dependent effects of Avp on aggressive behavior.

Social recognition

For social recognition in rodents, many studies have focused on olfactory cues and the influence of Avp (Bielsky and Young, 2004). Previous work has shown that animals lacking the Avpr1a gene show a disruption in social recognition, but habituate to non-social odors (Bielsky et al., 2004; and thus are not simply anosmic, though see Wersinger et al., 2007b). RGS are able to recognize individuals without
olfactory cues, as they habituate to alarm vocalizations from the same caller, but not from different callers (Hare, 1998a), and discriminate kin from non-kin and familiar neighbors from unfamiliar individuals in the context of behavioral interactions (Hare, 1998b). Future work should investigate whether Avp influences social recognition in this species. Since predator responses were altered in the absence of predator olfactory cues, it is possible that Avp could also alter social recognition in this species in the absence of social odor cues.

**Summary**

Our findings for RGS demonstrate that Avp can have sex-specific effects on vocalizations, anxiety, aggression, and predator vigilance in an ecologically-appropriate setting. Of particular interest is our finding that olfactory cues associated with a predator are not required for Avp to influence antipredator vigilance. Our discovery that Avp affects aggression in a sex-dependent manner requires further investigation to determine whether breeding state is driving this variation. Furthermore, context is important for Avp to influence vocalizations, as social vocalizations, but not predator-elicited vocalizations, were influenced by Avp administration. While Avp typically increases affiliation in social rodents, affiliation was not affected in this social species. Future work should investigate which neural substrates are important for Avp’s effects in this species. Investigating Avp’s conserved functions across species will advance our understanding of the neurobiology of social behavior.
Chapter 3

Central distribution of oxytocin and vasopressin 1a receptors in Richardson’s ground squirrel

Introduction

The distribution of both AoVr and Oxtr is often sex- and species-specific, and these distributions are often linked to the ecology of the animal (Insel et al., 1991; Insel et al., 1994; Ophir et al., 2008; Phelps et al., 2010, Figure 29). There is evidence that social monogamy vs promiscuity can lead to changes in Avpr1a distributions in voles (Insel and Young, 2001; Insel et al., 1994; Ophir et al., 2008; Young et al., 1999a), but it is not clear whether general sociality will mirror distribution patterns based on social living, or on reproductive promiscuity in RGS.

I hypothesized that both sociality and reproductive tactics would influence receptor binding patterns. Further, I predicted that based on the lack of pair bonding and biparental care in this species I would see binding patterns similar to the montane vole (high in the LS and amygdala and low in the VP) (Insel et al., 1994; Wang et al., 1997), and given their keen ability to remember and recognize each other, I further predicted dense binding in the LS (Landgraf et al., 1995; Landgraf et al., 2003). While not a rodent, the ecology of the rhesus macaque for which the Avpr1a and Oxtr distributions are known is similar to RGS – females form
Figure 29: Oxytocin and vasopressin signaling in brain areas important to social motivation. Oxytocin (Oxtr) and vasopressin receptors (Avprs) are found throughout the structures of the social behavior neural network (SBNN) and the mesocorticolimbic dopamine (DA) system. Their localization in these nuclei is critical for oxytocin’s and vasopressin’s modulation of socially motivated behaviors and may serve as the functional connection between the SBNN and DA systems, particularly by their action in the lateral septum (LS) and extended amygdala, including the bed nucleus of the stria terminalis (BNST). Other abbreviations: AH anterior hypothalamus; BLA basolateral amygdala; HIPP hippocampus; MeA medial amygdala; NAcc nucleus accumbens; OB olfactory bulb; PAG periaqueductal gray; PFC prefrontal cortex; POA preoptic area; VP ventral pallidum; VTA ventral tegmental area; VMH ventromedial hypothalamus. “Current topics in behavioral neuroscience, Oxytocin, vasopressin, and the motivational forces that drive social behaviors, vol 27, 2016, pgs 51-103, Caldwell, HK and Albers, H.E. © Springer International Publishing Switzerland 2015, With permission of Springer”
matrilineal hierarchies, females are philopatric while males disperse, the animals live in groups, and males and females both mate multiply (Bercovitch and Berard, 1993; Young et al., 1999b). The rhesus macaque shows dense binding of Avpr1a in the cortex, the LS, BNST, and amygdala and slightly less in the POA and SCN; surprisingly, no binding was seen in the hippocampus or dentate gyrus (Young et al., 1999b). The Oxtr in this species is limited to the superior colliculus, trapezoid body, nucleus basalis of Meynert, pedunculopontine tegmental nucleus and VMH– areas important for visual and auditory perception and processing (Freeman et al., 2014). Since RGS exhibit alarm calling behavior, receptor binding in areas important for auditory processing is predicted.

While Oxt is not the focus of this dissertation, determining the location of both Oxtr and Avpr1a can aid in determining where receptor crosstalk might occur when data are contradictory among studies. In the prairie vole, Oxtr binding is dense in the prelimbic cortex, BNST, nucleus accumbens (NAcc), thalamic nuclei, and lateral amygdala (Insel and Shapiro, 1992). In the montane vole, however, Oxtr is found in the LS, VMH, and cortical amygdala (Insel and Shapiro, 1992). These differences were replicated in the related pine vole and meadow vole respectively (Insel and Shapiro, 1992). In mice, Oxtr is expressed in many brain regions including the olfactory bulbs, BNST, hypothalamic nuclei, amygdala, and hippocampus (Gould and Zingg, 2003; Insel et al., 1991). Similar regions in the rat have Oxtr binding, and also include the preoptic and supraoptic nuclei and several cortical regions (Elands et al., 1988; Yoshimura et al., 1993).
Similar to Avpr, I predicted that Oxtr binding patterns would mirror those seen in other promiscuous rodents, such as the montane vole. Thus, I expected dense binding in the LS, VMH and amygdala.

**Methods**

8 RGS (5 females, 3 males) were humanely euthanized using an intracardiac injection of pentobarbital as part of a population control program. Brains were quickly removed, and frozen on powdered dry ice, and then stored at -80°C until sectioning. The brains were sectioned at 20 µm in six serial sets on a cryostat set at -20°C. After a section was thaw mounted onto Superfrost™ slides for each set, the next section within a set was mounted 240 µm after. Slides were temporarily stored at -20, then stored long term at -80. All brains were sectioned and then processed together within a 3-month period. All research with animals was conducted in accordance with the Canadian Council on Animal Care guidelines and was approved by the University of Manitoba Fort Garry Campus Animal Care Committee (F13-014/1), APZ Research Ethics and Review Committee (2014-A003), and Manitoba Conservation (Wildlife Scientific Permit WB14952).

Oxtr and Avpr1a autoradiography procedures were as described in previous work with minor changes (Caldwell and Albers, 2003; Lee et al., 2008). Briefly, sections were allowed to thaw to room temperature and dry for approximately one hour. Then, slides with mounted sections were fixed for 2 min in 0.1% paraformaldehyde (pH 7.4), and then washed in 50mM Tris buffer (pH 7.4) twice for 10 min each time. Sections were then incubated in a tracer buffer containing 50mM Tris with 10 mM MgCl₂ (pH 7.4), 0.1% bovine serum albumin, 0.05% bacitracin, and
50pM tracer for 1 h at room temperature. Forebrain mouse sections served as positive controls. Controls for competitive binding assays had Oxt (Oxt acetate salt, LOT SLBG9296V, Sigma-Aldrich, St. Louis, MO, USA) or Avp ([Arg^8]-vasopressin acetate salt, LOT 113M4751V, Sigma-Aldrich, St. Louis, MO, USA) dissolved in sterile H$_2$O added to the tracer buffer for a final concentration of 20nM. Radioligands used were specific for Avpr1a (Linear vasopressin V$_{1a}$ receptor antagonist, [$^{125}$I]-Phenylacetyl-D-Tyr(Me)-Pre-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$, LOT GS81260, PerkinElmer, Boston, MA, USA) or Oxtr ([$^{125}$I]-OVTA: Ornithine vasotocin analog, Vasotocin, d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr$^4$,Orn$^8$,[$^{125}$I]Tyr$^9$-NH$_2$], LOT EP91660, PerkinElmer, Boston, MA, USA). Following incubation, all sections were washed in 50mM Tris with 10 mM MgCl$_2$ (pH 7.4) twice for 10 minutes each time, and finally for 35 min while stirring. Sections were then washed in cold dH$_2$O, and blown dry with cool air. Slides with mounted sections, and a microscale for $^{125}$I (2.8 to 1500 nCi/mg, LOT 160512, American Radiolabeled Chemicals Inc, St. Louis, MO) were laid in a cassette with Kodak Bio-Max MR Film (Kodak, Rochester, NY), and left to process for three weeks. Film was then developed.

Quantification

Standard Curves for binding density were established using [$^{125}$I] microscales. We used an adjacent set to the autoradiography sets which had cresyl violet stained sections to identify binding in the following anatomical areas: Medial POA, LS, VP, Amygdala, AH, BNST, VMH, PVN, PVH, NAcc, Medial Geniculate and Dentate Gyrus (DG).
We used a set box size on 3 sections within a set (240um apart; Table 8). The box was placed into the center of the anatomical area using neuroanatomical landmarks. Optical densities were determined using ImageJ.

Table 8: Neuroanatomical areas and associated box sizes

<table>
<thead>
<tr>
<th>Area measured</th>
<th>Box size (pixels)</th>
<th>Box size, Width by Height (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPOA</td>
<td>22 by 22</td>
<td>0.382 X 0.382</td>
</tr>
<tr>
<td>LS</td>
<td>30 by 25</td>
<td>0.521 X 0.434</td>
</tr>
<tr>
<td>VP</td>
<td>25 by 25</td>
<td>0.434 X 0.434</td>
</tr>
<tr>
<td>Medial Amygdala</td>
<td>25 by 30</td>
<td>0.434 X 0.521</td>
</tr>
<tr>
<td>Central Amygdala</td>
<td>30 by 30</td>
<td>0.521 X 0.521</td>
</tr>
<tr>
<td>Basolateral Amygdala</td>
<td>20 by 20</td>
<td>0.347 X 0.347</td>
</tr>
<tr>
<td>AH</td>
<td>15 by 15</td>
<td>0.260 X 0.260</td>
</tr>
<tr>
<td>Anterior BNST</td>
<td>20 by 20</td>
<td>0.347 X 0.347</td>
</tr>
<tr>
<td>Posterior BNST</td>
<td>20 by 30</td>
<td>0.347 X 0.521</td>
</tr>
<tr>
<td>VMH</td>
<td>25 by 25</td>
<td>0.434 X 0.434</td>
</tr>
<tr>
<td>PVN</td>
<td>12 by 20</td>
<td>0.208 X 0.347</td>
</tr>
<tr>
<td>PVH</td>
<td>5 by 40</td>
<td>0.087 X 0.694</td>
</tr>
<tr>
<td>NAcc</td>
<td>15 by 15</td>
<td>0.260 X 0.260</td>
</tr>
<tr>
<td>DG</td>
<td>40 by 120</td>
<td>0.694 X 2.083</td>
</tr>
<tr>
<td>Medial Geniculate</td>
<td>40 by 45</td>
<td>0.694 X 0.781</td>
</tr>
</tbody>
</table>
For analysis, the dpm/mg measurements from the three adjacent sections within an animal for each neuroanatomical area were averaged. These averages were then compared between sexes using t-tests.

Results

RGS did not differ in Oxtr binding density based on sex in any of the regions of interest (Table 9). RGS also did not differ in Avpr1a binding density based on sex in any the brain regions of interest (Table 10).
Table 9: t-test of mean density values for Oxtr in selected brain areas between male and female RGS

<table>
<thead>
<tr>
<th>Sex</th>
<th>Mean (dpm/mg)</th>
<th>df</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPOA M</td>
<td>486</td>
<td>6</td>
<td>1.13</td>
<td>0.3135</td>
</tr>
<tr>
<td>MPOA F</td>
<td>682</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS M</td>
<td>1597</td>
<td>6</td>
<td>1.16</td>
<td>0.2984</td>
</tr>
<tr>
<td>LS F</td>
<td>2205</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP M</td>
<td>697</td>
<td>6</td>
<td>0.937</td>
<td>0.3851</td>
</tr>
<tr>
<td>VP F</td>
<td>950</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial Amygdala M</td>
<td>2555</td>
<td>6</td>
<td>0.037564</td>
<td>0.9726</td>
</tr>
<tr>
<td>Medial Amygdala F</td>
<td>5293</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central Amygdala M</td>
<td>2876</td>
<td>6</td>
<td>0.92126</td>
<td>0.4265</td>
</tr>
<tr>
<td>Central Amygdala F</td>
<td>4941</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basolateral Amygdala M</td>
<td>311</td>
<td>6</td>
<td>-0.67339</td>
<td>0.5421</td>
</tr>
<tr>
<td>Basolateral Amygdala F</td>
<td>191</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AH M</td>
<td>664</td>
<td>6</td>
<td>0.769</td>
<td>0.4724</td>
</tr>
<tr>
<td>AH F</td>
<td>849</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior BNST M</td>
<td>1628</td>
<td>6</td>
<td>1.0103</td>
<td>0.3665</td>
</tr>
<tr>
<td>Anterior BNST F</td>
<td>2327</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior BNST M</td>
<td>1893</td>
<td>6</td>
<td>-1.5873</td>
<td>0.2247</td>
</tr>
<tr>
<td>Posterior BNST F</td>
<td>1117</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMH M</td>
<td>572</td>
<td>6</td>
<td>1.14</td>
<td>0.3009</td>
</tr>
<tr>
<td>VMH F</td>
<td>684</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVN M</td>
<td>1866</td>
<td>6</td>
<td>0.231</td>
<td>0.8316</td>
</tr>
<tr>
<td>PVN F</td>
<td>1978</td>
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</tr>
<tr>
<td>PVH M</td>
<td>1997</td>
<td>6</td>
<td>-0.80015</td>
<td>0.4948</td>
</tr>
<tr>
<td>PVH F</td>
<td>1547</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NAcc M</td>
<td>291</td>
<td>6</td>
<td>2.43</td>
<td>0.06101</td>
</tr>
<tr>
<td>NAcc F</td>
<td>489</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DG M</td>
<td>8449</td>
<td>5</td>
<td>-1.04</td>
<td>0.3787</td>
</tr>
<tr>
<td>DG F</td>
<td>6715</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10: t-test of mean density values for Avpr1a in selected brain areas between male and female RGS

<table>
<thead>
<tr>
<th>Sex</th>
<th>Area</th>
<th>Mean (dpm/mg)</th>
<th>df</th>
<th>t</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>MPOA</td>
<td>M</td>
<td>585</td>
<td>6</td>
<td>0.19911</td>
<td>0.8522</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>686</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS</td>
<td>M</td>
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<td>6</td>
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<td>0.9813</td>
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<tr>
<td></td>
<td>F</td>
<td>2554</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP</td>
<td>M</td>
<td>955</td>
<td>6</td>
<td>1.5109</td>
<td>0.186</td>
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<td></td>
<td>F</td>
<td>734</td>
<td></td>
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<tr>
<td>Medial Amygdala</td>
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<td>1404</td>
<td>6</td>
<td>-2.2899</td>
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<tr>
<td></td>
<td>F</td>
<td>598</td>
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<tr>
<td>Central Amygdala</td>
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<td>6</td>
<td>0.65035</td>
<td>0.5495</td>
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<tr>
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<td>F</td>
<td>500</td>
<td></td>
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<tr>
<td>Basolateral Amygdala</td>
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<td>6</td>
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<td></td>
<td>F</td>
<td>339</td>
<td></td>
<td></td>
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<tr>
<td>AH</td>
<td>M</td>
<td>679</td>
<td>6</td>
<td>-0.87643</td>
<td>0.4191</td>
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<tr>
<td></td>
<td>F</td>
<td>492</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior BNST</td>
<td>M</td>
<td>874</td>
<td>6</td>
<td>1.3255</td>
<td>0.2592</td>
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<tr>
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<td>F</td>
<td>1522</td>
<td></td>
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<tr>
<td>Posterior BNST</td>
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<td>0.8754</td>
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<tr>
<td></td>
<td>F</td>
<td>1548</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMH</td>
<td>M</td>
<td>390</td>
<td>6</td>
<td>-0.92253</td>
<td>0.4253</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>76</td>
<td></td>
<td></td>
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<tr>
<td>PVN</td>
<td>M</td>
<td>6458</td>
<td>6</td>
<td>0.44303</td>
<td>0.6855</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7716</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PVH</td>
<td>M</td>
<td>282</td>
<td>6</td>
<td>-0.17925</td>
<td>0.8728</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>212</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAcc</td>
<td>M</td>
<td>1322</td>
<td>6</td>
<td>-0.9258</td>
<td>0.3923</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1711</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>M</td>
<td>873</td>
<td>6</td>
<td>-1.0386</td>
<td>0.3398</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>672</td>
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<td></td>
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<tr>
<td>Medial Geniculate</td>
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<td>2960</td>
<td>6</td>
<td>0.028532</td>
<td>0.9782</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2974</td>
<td></td>
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</tr>
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</table>
Thus, Avpr and Oxtr binding pooled across sexes is summarized in Table 11, including areas where binding was not quantified, but examined for general presence (+) or absence (-). Oxtr was present in expected regions including the areas within the social behavior neural network. Oxtr binding was most dense in the LS, DG, and Amygdala. Avpr1a was also present in areas within the social behavior neural network, including the LS, Amygdala, and BNST.

Table 11: Oxtr and Avpr1a binding in RGS brain regions. +++ indicates strong binding, ++ moderate binding, + presence, and – absence absence

<table>
<thead>
<tr>
<th>Area</th>
<th>Relative Oxtr Binding</th>
<th>Relative Avpr1a Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPOA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LS</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amygdala</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>AH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BNST</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>VMH</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PVN</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>PVH</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NAcc</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>DG</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Ca3 region of Hippocampus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Islands of Calleja</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Prelimbic area</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ca1 region</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Superior Colliculus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inferior Colliculus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Olfactory tubercle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Medial pretectal area</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Medial Geniculate</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cortex</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Competition with Oxt but not Avp in conjunction with the Oxtr radioligand reduced binding to undetectable levels in the LS, BNST and other thalamic nuclei (Figure 30). Competition with Avp but not Oxt in conjunction with the Avpr1a radioligand also reduced binding.
Figure 30: Representative oxytocin receptor autoradiograph of brain regions (L) with associated cresyl violet (R). DG: dentate gyrus; VMH: ventromedial nucleus of the hypothalamus; PVN: paraventricular nucleus; AH: anterior hypothalamus; Amy: amygdala. Scale bar: 5 mm.
Discussion

Oxytocin receptor binding

Our predictions for oxytocin binding suggested that we would see Oxtr in the LS, VMH, and amygdala. Dense binding of Oxtr was observed in both the amygdala, and LS, though only a presence-level of binding was observed in the VMH (Figure 31). Similar to mice, Oxtr in RGS are localized to the olfactory bulbs, thalamic nuclei, BNST, and hippocampus (Figure 32). Unlike rats, the RGS does not appear to have strong Oxtr binding in the PVH and SON. The greatest density of receptors in these juvenile RGS is in the amygdala, the DG, and the LS. The CeB is known for its importance in fear and anxiety (Gale et al., 2004), specifically infusion of Oxt into the CeB can increase freezing behavior in rats (Lahoud and Maroun, 2013). The medial amygdala is also involved in aggressive behaviors, and Oxt and Avp in the amygdala overall work in an opposing fashion to modulate fear responses in rats (and likely other rodents; Huber et al., 2005). It is possible that Oxtr in the amygdala is important for the regulation of fear and anxiety in RGS, and Avp in our previous work (Chapter 2) could be potentially working through Oxtr in the amygdala to promote vigilance behaviors. Site-specific infusions of Avp, Oxt, and associated antagonists might elucidate what regions are important for the effects seen in Chapter 2, though based on binding, the amygdala seems like a potential candidate.

The DG of RGS also has dense Oxtr binding. The DG is a main site of adult neurogenesis (Gage et al., 1998; Kuhn et al., 1996), and is thus, implicated in the formation of memories (Shors et al., 2001). There is evidence that Oxt in the DG is important for promoting proliferation of new cells, as well as buffering against
Figure 31: Representative oxytocin receptor autoradiograph (L) with associated cresyl violet (R). **LS: lateral septum; BNST: bed nucleus of the stria terminalis; VP: ventral pallidum. Scale bar: 5 mm.**
Figure 32: Representative oxytocin receptor autoradiograph (L) with associated cresyl violet (R). *NAcc: nucleus accumbens. Scale bar: 5 mm.*
stressful situations (Leuner et al., 2012). The hippocampus (specifically the DG, CA1 and CA3 regions) in RGS males tends to be larger than females, and larger in the non-breeding season (Burger et al., 2013). This difference in size is likely attributable to the breeding system (i.e. promiscuity) of RGS, and the necessity for juvenile males to find food in the non-breeding season (Burger et al., 2013). The Oxtr in these animals might promote spatial memories of food sources in these animals by promoting neurogenesis. Further research will need to be conducted to determine whether this is the case.

*Vasopressin 1a receptor binding*

In some areas, such as the LS, BNST, thalamic nuclei, and amygdala, Avpr1a binding was observed in addition to Oxtr binding (Figure 33). The LS in RGS had high densities of Oxtr and Avpr1a, which was predicted. This binding is similar to other promiscuous rodents (Montane and meadow voles: Insel and Shapiro, 1992; Insel et al., 1994). Recently, Oxtr and Avpr1a were found to be correlated with anxiety and maternal behavior in mice (Curley et al., 2012). Likewise, in rats, the Avpr1a in the LS is essential in social memory and anxiety (Landgraf et al., 1995). It is likely that in RGS the septum is also important in anxiety and other social behavior. Future work should focus on how the receptors in this area might modulate these behaviors.

The BNST also showed Avpr1a binding, and is an area important in the social behavior neural network. In socially isolated rats and hamsters, the BNST has increased Avpr1a binding (Albers et al., 2006; Askew et al., 2006), suggesting a role
Figure 33: Representative vasopressin 1a receptor autoradiograph of brain regions (L) with associated cresyl violet (R). *LS: lateral septum; BNST: bed nucleus of the stria terminalis. Scale bar: 5 mm.*
for Avp in the social environment. It is not known what role the BNST might play in this species.

In contrast to the Oxtr results, our RGS had moderate binding in both the medial geniculate and the NAcc. The binding of Oxtr and Avpr1a in the NAcc was mostly restricted to the NAcc shell, while small pockets of binding were seen throughout the NAcc core for Avpr1a, leading to an increase in overall density estimates (Figure 34). It is unclear why binding was sporadic in the NAcc core, and this pattern of binding in the NAcc has not been described in other species for Oxtr or Avpr1a binding. It is possible that groups of neurons in the NAcc core are involved in Avp-related behavior, including reward, and future work should investigate what makes these groups of neurons different from others in the NAcc core (in addition to Avpr1a binding).

Avpr1a binding was also seen in the medial geniculate in moderate densities (Figure 35). Oxtr binding is also present here, but was not quantified for the sexes. The medial geniculate is part of the auditory circuit, linking the auditory cortex, and the inferior and superior colliculus. The medial geniculate also has connections to the amygdala, and is important for fear conditioning using acoustic stimuli (LeDoux et al., 1984). In RGS, we hypothesized that social communication via alarm calling might be modulated by Avp and Oxt, and the presence of receptors in this region suggests a potential route of action.
Figure 34: Representative vasopressin 1a receptor autoradiograph of brain regions (L) with associated cresyl violet (R). NAcc: nucleus accumbens. Scale bar: 5 mm.
Figure 35: Representative vasopressin 1a receptor autoradiograph of brain regions (L) with associated cresyl violet (R). MeG: medial geniculate; Hipp: Hippocampus. Scale bar: 5 mm.
Chapter 4

Perception of alarm calls and neuronal activation in Richardson’s ground squirrel

Introduction

While there is some evidence regarding the interactions between Avp and the propensity for an animal to vocalize (Lukas and Wöhr, 2015; Scattoni et al., 2008), there are far fewer studies which examine an animal’s perception of vocalizations and the associated neural activation (Sadananda et al., 2008). In rats, the 50 kHz vocalization indicates a positive affective state, while the 22 kHz typically indicates aversion or anxiety (Sadananda et al., 2008). When given playback of 50kHz vocalizations, rats show Fos reactivity in auditory areas, as well as the frontal cortex, the NAcc, the thalamic parafascicular and PVH. The 22kHz playback, however, activates the amygdala, hippocampus and periaqueductal gray (Ouda et al., 2016; Sadananda et al., 2008). c-Fos is typically used as a proximate measure of neuronal activation, as it is in the class of immediate early genes – genes which are not typically transcribed until the cell receives external stimuli, and which are transcribed usually within minutes of this stimulus (Sheng and Greenberg,
Thus, Fos protein, typically detectable at 60-90 minutes post-stimulus is used to indicate neuronal activation.

I hypothesized that alarm call perception would result in neuronal activation in specific brain regions. I predicted that the type of call (i.e. chirp vs. whistle) would result in differential activation in the brain. Specifically, I predicted that the high threat vocalization (chirp; (Sloan et al., 2005)) would cause activation in areas such as the amygdala, or other areas important in anxiety and emotion, while the whistle vocalization would show activation in the social behavior neural network (i.e. POA, hypothalamic nuclei, BNST, NAcc). Both types of vocalizations are expected to activate auditory cortex areas.

Methods

29 female juvenile RGS were trapped at the APZ in Winnipeg, MB ((49°52’N, 97°14’W) using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) baited with peanut butter (No Name™ Smooth Peanut Butter; Loblaws Inc, Toronto, ON, Canada). All trials were completed between 9 July 2015 and 3 August 2015.

Once trapped, RGS were transported in the live trap covered in a pillowcase to a building onsite which housed acoustic isolation chambers (Figure 36). These chambers were approximately 1m X .75m X .75m, and prevented the conduction of ambient sound outside the chamber under 80dB SPL. The pillowcases were then removed and the squirrels were placed in the chamber, while still in the trap to habituate to the chamber. The chamber light and fan were switched on when the squirrel was placed in the chamber. After two hours of habituation time, squirrels
Figure 36: Acoustic isolation chamber for call perception experiments.
were given either a whistle call, a chirp call, or no stimulus as a control. The playback tracks were adjusted to play for 2 min and contained 49 syllables (0.426 syllable/s call rate) (Warkentin et al., 2001). All calls were recorded in 2010 and came from a set of 112 total syllables from 3 individuals. The playback unit (Honeytone amplifier (Danelectro Co., Camarillo, CA, USA) connected to a SONY Minidisc player (Sony MZ-N707, SONY, Tokyo, Japan)) was adjusted to play calls at ecologically appropriate sound pressure levels (85dB ± 5dB).

One hour after receiving the stimulus, squirrels were humanely euthanized and had brains swiftly removed and post-fixed in 4% paraformaldehyde before slicing at 50um on a vibratome in 5 serial sets. Tissue was stored once sliced in cyroprotectant (50% v/v 0.1M potassium phosphate buffer, 30% w/v sucrose, 1% w/v polyvinyl pyrrolidione, 30% v/v ethylene glycol) at -20°C until immunohistochemistry. All research with animals was conducted in accordance with the Canadian Council on Animal Care guidelines and was approved by the University of Manitoba Fort Garry Campus Animal Care Committee (F13-014/1), APZ Research Ethics and Review Committee (2014-A003), and Manitoba Conservation (Wildlife Scientific Permit WB14952).

c-Fos immunohistochemistry

Tissue was washed six times in 1 X phosphate-buffered saline (PBS) for 10 minutes at room temperature to remove cryoprotectant. Tissue was processed as previously described (Dhakar et al., 2012). Sections were washed at room temperature in 1X PBS twice for 5 min each time, then incubated in 1.5% hydrogen peroxide for 5 min, and again washed twice in 1X PBS for 5 min. I then incubated
sections for 30 minutes in 1X Power Block Universal Blocking Reagent (BioGenex, San Ramon, CA). Sections were then incubated overnight at 4°C in primary c-fos antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA, rabbit anti-c-fos, sc-52) at 1:5000 in antisera diluent (PBS + 1% v/v normal goat serum + 0.3% v/v Triton-X-100). Following overnight incubation, sections were washed three times in 1 X PBS for 5 minutes at room temperature, then incubated in a biotinylated secondary antibody (goat anti-rabbit, Vectastain kit, Vector Laboratories, Burlingame, CA, USA) as indicated in kit instructions. Sections were again washed three times for 5 min each in 1 X PBS, then incubated in the Vectastain avidin-biotin-complex solution per kit instructions (Vector Laboratories, Burlingame, CA, USA). Finally, sections were washed again for 5 minutes each three times in 1X PBS prior to incubation with diaminobenzidine for 2-10 min (Vector Laboratories, Burlingame, CA, USA). I then washed sections twice for 5 min in 1X PBS, mounted sections onto microscope slides, which, once dry were coverslipped using DPX mounting medium.

I am still processing tissue at this time, but will be counting cells in neuroanatomical areas of interest with box-sizes outlined in Chapter 3. In particular, I am interested in potential activation in the POA, LS, BNST, periaqueductal grey, PVH, and amygdala.

**Results**

I am still processing the data for c-Fos expression in this species. Currently, it seems that a higher than usual (1:5000, Santa Cruz rabbit anti-c-Fos, in PBS + 1% normal goat serum + 0.3% Triton-X 100) concentration of primary antibody will be necessary, as the protocol used for mice only produced some faint staining of cells.
After trial and error, a primary antibody concentration of 1:1000, and incubating at room temperature instead of 4°C has resulted in the staining of cells in RGS tissue (Figure 37). It is expected that completion of the staining of this tissue and counting of immunopositive cells will be complete by end of November, 2016.

Discussion

We have recently determined that a higher concentration of primary antibody is necessary for staining cFos immoreactive cells. This experiment has taken longer than expected due to the lack of established protocols and molecular tools in this species. Nonetheless, given the presence of Oxtr and Avpr in auditory circuits in the brain of RGS (Chapter 3), and the effect of central Avp on RGS vocal behavior (Chapter 2), examining neuronal activation during call perception will aid in our understanding of how these sounds are processed and perceived. We expect to see staining in areas important in auditory processing; activation in the medial geniculate, or inferior and superior colliculus would indicate areas where Avp might act to influence alarm call perception. Furthermore, potential differences in activation in animals receiving whistle vs chirp vocalization stimuli might reveal more about these different types of alarm calls in general.
Figure 37: Micrographs of cFos stained tissue in mouse (L) and RGS (R), using primary antibody at 1:1000 concentration.
Chapter 5

General Discussion

This dissertation adds to the ongoing comparative research concerning central Avp and social behavior in rodents. Specifically, this research demonstrates that Avp is important for influencing predator vigilance, for social vocalizations, and for aggression. Furthermore, the distribution of Oxtr and Avpr1a are similar to other promiscuous species of rodents, with high densities of binding in the LS, BNST, amygdala and DG. This dissertation thus reveals how Avp (and in some cases, Oxt) may modulate species-specific behavior in a free-living social rodent.

Vasopressin and social behavior

Avp central administration in free-living RGS did not influence affiliation, but did affect aggression, vigilance, and vocalizations. While administration of Avp was via the lateral ventricle in Chapter 2, in Chapter 3 receptor autoradiography was employed to determine where Avp could act to influence these behaviors. In social, monogamous species, Avpr and Oxtr tend to be localized to the NAcc, VP, BNST and hippocampus, while promiscuous species tend to have Avpr and Oxtr in the LS, VMH, amygdala and BNST. These generalizations suggest that social behavior might be innately rewarding in animals that form tight social bonds and pair bonds, as the VP and NAcc show dense binding in these animals (Young and Wang, 2004).
contrast, RGS do not show dense Oxtr or Avpr in these reward areas (Figure 38), suggesting that perhaps, sexually-motivated social bonding but not general social living is modulated by receptors in these regions (see Promiscuity versus sociality below for further discussion). In other areas of the social behavior neural network (Figure 29), Avpr and Oxtr are present: the BNST, LS and amygdala (Albers, 2012). However, it is not clear whether these regions are modulating the behaviors that were impacted by Avp administration in Chapter 2.

I expected based on the influence on aggression, that Avpr and Oxtr might be dense in the AH and amygdala (Ferris and Potegal, 1988; Ferris et al., 1997; Koolhaas et al., 1990). Oxtr (and Avpr) were localized to the amygdala, however, very faint binding was found in the AH. Additionally, the PVH may play a role in aggression, based on some findings in hamsters (Albers et al., 2006). While there were no sex differences in the receptor distributions in juvenile RGS, male RGS had reduced overall aggression when given central Avp. Originally, I hypothesized that the AH might be the location of action based on previous work completed in Syrian hamsters. Given the distributions of Oxtr and Avpr, it seems more likely that aggression might be modulated through the PVH and amygdala. Indeed, our male RGS did show slight increases in Avpr binding in the MeA over females, and if this divergence continued into adulthood, significant impacts on sex-specific behavior could occur via these receptors. While the AH might be involved through connections to the LS and PVH, direct action via Avpr or Oxtr in the AH is unlikely.
Figure 38: Representative oxytocin receptor autoradiograph of brain regions (L) with associated cresyl violet (R) after oxytocin competition. LS: lateral septum; BNST: bed nucleus of the stria terminalis; 3V: third ventricle; LV: lateral ventricle. Scale bar: 5 mm.
Additionally, Avp and Oxt in the amygdala are modulators of fear and anxiety, and may play a role in vigilance behavior in RGS, while receptors in the septum may regulate more general anxiety. In RGS, administration of Avp increased predator vigilance and escape behavior in males and decreased anxiety-like behavior in females. In other rodents, Avp is generally anxiogenic, and is thought to act through Avpr1b receptors. While a lack of molecular tools makes the localization of Avpr1b difficult, based on previous research it is likely that the distribution Avpr1b does have an effect on anxiety behavior in RGS. Nonetheless, The Oxtr and Avpr1a also can influence anxiety and fear. In particular, the Avpr1a in the septum (Bielsky et al., 2004; Egashira et al., 2007; Landgraf et al., 1995; Liebsch et al., 1996) may modulate anxiety, and in RGS, dense binding of Oxtr and Avpr1a in this region may be the location for changing anxiety. In the amygdala, Oxt and Avp are thought to have opposite effects, altering anxiety and fear (Neumann and Landgraf, 2012), and thus, might be involved in the effects seen in male RGS with respect to predator vigilance. Further work investigating predator-related responses and Avp should investigate if these changes are mediated through the amygdala exclusively, or the septum, or both.

In addition to modulating anxiety, the LS plays a role in social recognition in rodents (Bielsky et al., 2005; Landgraf et al., 2003). The preliminary autoradiography data from Chapter 3 supports that Oxtr and Avpr1a are both found in the LS. RGS have the ability to recognize individuals on the basis of their alarm calls (Hare, 1998a), which might be mediated through Avp. Future work should
focus on the Avp system in this species and whether Avp in the LS is essential for recognition of individuals based on alarm calls.

Promiscuity versus sociality

The majority of work on social behavior and nonapeptides (i.e. Avp and Oxt), has focused on pair bonding behavior in voles (Barrett et al., 2013; Gobrogge et al., 2009; Lim and Young, 2004; Lim et al., 2004; Pitkow et al., 2001). However, non-reproductive sociality (e.g. between siblings) is less frequently studied. This has led to the question of: do these nonapeptides influence sociality in general, or just pair-bonding behavior and affiliation in the context of reproduction? In Chapter 2, I briefly discuss that Avp did not influence affiliation in RGS adults, and that nonapeptides might affect reproductive affiliation (within-pair huddling, partner preference, etc.), but not general sociality. Indeed, the autoradiography data presented in Chapter 3 suggests that this social species does not have the Avpr and Oxtr in the reward regions typically seen in pair-bonding rodents.

Previous work showed that expressing the Avpr1a in the reward region can induce pair bonding in a typically non-affiliative species (Young et al., 1999a), which suggests that it is the receptor distribution driving reproductive affiliation. The Avpr and Oxtr distribution in RGS seem to mirror those found in promiscuous voles. Therefore, the affiliation seen in RGS is probably facilitated through pathways other than Avp, though what that mechanism is remains to be seen. Another possibility is that receptor density might change over the season, with higher densities of Avpr and Oxtr in the reward areas during the breeding season, as the RGS brain already shows seasonal and sex differences and is plastic (Burger et al., 2013) (Figure 39).
Furthermore, behavior in RGS is influenced by season, and may have influenced the behavior seen in Chapter 2 (i.e. physiological ceiling effects). Thus, examination of other seasonal changes in these neuropeptide systems will be a logical future avenue for research in this species.

*Oxytocin and vasopressin: working together*

This dissertation focuses on Avp and its influence on behavior in RGS. While these data demonstrate that central Avp does influence a number of behaviors, Avp’s sister peptide Oxt is likely also playing a role. Recent work has indicated that crosstalk between Oxt and the Avpr and Avp and the Oxtr can influence behavior (Bowen and McGregor, 2014; Song et al., 2014; Song et al., 2016a; Song et al., 2016b). Oxt and Avp, however, are thought to regulate different behaviors and occasionally the same behavior in separate fashions (i.e. Oxt is anxiolytic, while Avp is anxiogenic; Neumann and Landgraf, 2012). In this dissertation, we examined both Oxtr and Avpr1a distributions, as Avp may act through either receptor due to its similarities in structure to Oxt (Table 12).
Figure 39: Model of oxytocin and vasopressin influence on behavior. Lack of oxytocin and vasopressin receptors in the VP (ventral pallidum) and NAcc (nucleus accumbens) suggest that reproductive affiliation but not social affiliation is modulated through this mechanism. Receptors in the DG (dentate gyrus), LS (lateral septum), and amygdala influence fear (predator responses and vigilance), and aggression behavior. However, season also influences both the expression of behavior through both nonapeptide-dependent and independent mechanisms.
Table 12: Evidence of oxytocin and vasopressin crosstalk

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Peptide</th>
<th>Receptor</th>
<th>Effect</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social recognition</td>
<td>Avp and Oxt</td>
<td>Avpr1a</td>
<td>Restored social exploration and recognition in Oxtr -/- mice</td>
<td>Sala et al. 2011</td>
</tr>
<tr>
<td>Social communication</td>
<td>Avp and Oxt</td>
<td>Avpr1a</td>
<td>Induced flank marking in Syrian hamster</td>
<td>Song et al. 2014</td>
</tr>
<tr>
<td>Social recognition</td>
<td>Avp and Oxt</td>
<td>Oxtr</td>
<td>Enhance social odor recognition in Syrian Hamster</td>
<td>Song et al. 2016</td>
</tr>
<tr>
<td>Social reward</td>
<td>Avp and Oxt</td>
<td>Oxtr</td>
<td>Activation in the ventral tegmental area reduced place avoidance</td>
<td>Song et al. 2016b</td>
</tr>
<tr>
<td>Social response to predator threat</td>
<td>Oxt</td>
<td>Avpr1a</td>
<td>Huddling by rats in response to predator odor</td>
<td>Bowen and McGregor 2014</td>
</tr>
</tbody>
</table>

Thus, future work should focus on both Avp and Oxt as both may influence social behavior.

Summary

This body of work illustrates the importance of Avp in modulating social behavior and vocalizations in a free-living rodent. The data in this dissertation highlight the extensive influence of Avp on social behavior, and have laid groundwork for future work investigating neural regulation of social behavior in the field. Indeed, the sex differences in behavior after supplementation indicate that season, breeding state, and sex in general might interact with nonapeptide systems to modulate season-appropriate behaviors.

My work on receptor binding shows that RGS are more similar to promiscuous voles and mice than monogamous rodents, suggesting that reproductive tactics and not sociality are driven by Avpr and Oxtr distributions. Future work will need to examine how more general sociality is regulated in the
brain. This work also adds to the comparative body of research on nonapeptides, rodents, and social behavior, and helps to elucidate which behaviors are influenced by Avp, and how life history influences receptor distributions.
Appendix A: Behavioral and Scoring Definitions

**Alert/S2p**: the subject stands erect on its hindpaws; its body position perpendicular to the ground

**Allogroom**: the subject grooms a conspecific

**Self-groom**: the subject licks, chews, or scratches its pelage

**Anxiety-like behavior**: the subject repeatedly bites or presses its nose against the cage for > 1s

**Ball fight**: the subject attaches to another squirrel during an aggressive encounter, and the two squirrels form a ‘ball shape’

**Belly-up**: the subject rolls onto its back exposing its belly towards another squirrel

**Bury**: the subject uses its forepaws to create a forward sweeping motion towards a conspecific

**Calling**: the squirrel vocalizes a temporally distinct syllable, includes chirps, whistles, and ultrasonic vocalizations

**Chase**: the subject runs in apparent pursuit of a conspecific for > 1s

**Chased**: the subject is pursued by a running conspecific for > 1s

**Dig**: the subject uses its forelimbs and legs to move dirt or soil

**Eating/Foraging**: the subject consumes edible material

**Escape**: the subject locomotes away from a potential threat

**Follow**: the subject moves in close proximity (<30cm) behind a locomoting conspecific slower than 0.5m/s

**Greet**: the subject’s nose in contact with the nose or oral angle of a conspecific

**In burrow**: the subject inside a ground squirrel burrow
Lateral display: the subject arches its body, forming a ‘c’ shape while facing a conspecific.

Lunge/Bite: the subject moves with its forepaws in the air towards a conspecific, sometimes contacting the conspecific with its mouth.

Out of sight: the subject was in a location other than a burrow where it could not be seen (e.g. in bushes, or under a building).

Piloerection: the pelage on the tail of the squirrel becomes erect or bristled.

Play: subject interacts with a juvenile with mounting behavior aimed at the side or head of the conspecific.

Pre-vigilant Latency: time from first vigilant posture increase after predator model presentation to a decrease in vigilance.

Run: locomotion faster than 0.5m/s.

S4d: subject stands with all four paws in contact with the ground.

S4hd: subject stands with four paws in contact with ground, and head is not raised above the horizontal plane of the body.

S4hu: subject stands with four paws in contact with ground, and head is raised above the horizontal plane of the body.

Slouch: subject stands on hindpaws, back has a curved posture, and body is not fully erect.

Tooth chatter: a mechanical sound produced by the subject by rattling its teeth together.

Walk: the subject locomotes slower than 0.5m/s.
Survey coding definitions:

Combined aggression: All chases, ball fights, lunges, tooth chatters, piloerection and lateral displays

Pooled vigilance/Escape: a weighted score of $1 \times \text{escape} + 0.75 \times \text{alert/s2p} + 0.5 \times \text{slouch} + 0.25 \times \text{s4hu}$

Combined social: all follows, greetings, allogrooming, play

Vocalizations: chirp, whistle and ultrasonic calls

Self groom: as prev. defined

Combined non-social: all other behaviors

Predator model coding definitions:

Non-vigilance: amount of time spent foraging or s4hd in the 30 seconds after model presentation - amount of time spent foraging or s4hd in the 30 seconds before model presentation

Low Vigilance: amount of time spent in s4hu posture 30 seconds after model presentation - amount of time spent in s4hu posture 30 seconds after model presentation

Slouch: amount of time spent in a slouch posture 30 seconds after model presentation - amount of time spent in a slouch posture 30 seconds after model presentation

Alert: amount of time spent in an alert/s2p posture 30 seconds after model presentation - amount of time spent in an alert/s2p posture 30 seconds after model presentation
Escape: amount of time spent walking, running, or hiding in burrow 30 seconds after model presentation - amount of time spent walking, running, or hiding in burrow 30 seconds after model presentation

Pooled vigilance/Escape: a weighted score of 1 * escape [as defined in this test] + 0.75 * alert/s2p + 0.5 * slouch + 0.25 * low-vigilance

Previgilant latency: time from first vigilant response after model presentation to the first decrease in vigilance posture

Call: duration of vocalizations 30 seconds after model presentation – duration of vocalizations 30 seconds after model presentation

Call latency: time to first vocalization after model presentation (a score of 30 is maximum latency/no call)

Syllable: number of call notes after the model presentation

Call rate: number of call notes after presentation of hat / total duration of vocalization bout

Social challenge coding definitions:

Social: total amount of time in close proximity (<5cm) to conspecific (includes ‘greet’ behavior), without aggressive behavior

Non-social: amount of time that is neither social nor aggressive. For simulated intrusions, this includes when the subject is not in the video frame

Aggression: total amount of time engaged in ‘aggressive behaviors’, including lunging/biting, piloerection, lateral displays, belly-up, or responding to a conspecific
conducting these behaviors towards the trapped subject. For simulated intrusions, this also included bury, standing on the trap, and rolling the trap.

Chirp bouts: chirps were short, highly frequency-modulated vocalizations, that typically were not repeated. However, if chirps were separated by 3s or less they were considered one ‘bout’.

Whistle bout: whistles were vocalizations of relatively longer duration of chirps, and do not have noticeable frequency modulation. These vocalizations are typically repeated and if separated by 3s or less are considered one ‘bout’.

Call syllables: total number of chirps and whistles given by the subject.

Aggressive vocalization: any other vocalization other than a chirp, whistle given during aggressive behavior.

Social vocalization: any other vocalization other than a chirp or whistle given during social behavior.

Total vocalization: time spent vocalizing (aggressive and social).

Total offensive aggression: time spent engaged in lunging/biting, piloerection, lateral display, standing on the trap, bury.

Self groom: any licking or scratching of the pelage.

Bite/hit cage: repeated (>1s) biting or hitting trap with nose, also called ‘anxiety-like’ behavior.

Other: standing, eating, or digging.
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