ABSTRACT

VOGEL, ANDREA RENEE. Neurobiology of Individual Social Behavior in a Monogamous Rodent, the Prairie Vole (*Microtus ochrogaster*). (Under the direction of Dr. Trudy Mackay.)

Across animal taxa, organisms vary tremendously in how much time they spend in close proximity to others of their own species, ranging from solitary species to animals that live in communal groups. Within a species, and even within a population, individuals vary in their propensity to engage in social interactions with others. In mammals, monogamy is a specialized social behavior, whereby less than 5% of mammals are considered monogamous. Within the definition of monogamy, there is both genetic monogamy, in which all the offspring are the product of the paired adults, and social monogamy, in which there is a high chance of extra-pair fertilizations. One monogamous mammalian species is the prairie vole, *Microtus ochrogaster*, which is a small, socially monogamous rodent that displays biparental care and partner preference. Socially monogamous means that a male and female prairie vole will mate, form a pair bond, and care for young; however, both male and female in the pair might stray outside the pair for copulations, which are termed extra-pair copulations. Prairie voles will display social behaviors, such as partner preference, as adults, which make them ideal model organisms for studying human social behaviors. My project examines the natural variation of partner preference and other social behaviors in a laboratory population of prairie voles. I explore the relationships between multiple social behaviors and mating, and calculate the heritability of these social behaviors. In order to figure out possible mechanisms of the partner preference, I examine vasopressin 1a receptor density in socially relevant brain regions. Furthermore, I attempt to show the effects of seminal vesicle proteins on partner preference and other social behaviors, as well as copulations, pregnancy, and offspring weight.

Neurobiology of Individual Social Behavior in a Monogamous Rodent, the Prairie Vole (*Microtus ochrogaster*)

by
Andrea Renee Vogel

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Genetics

Raleigh, North Carolina

2017

APPROVED BY:

_____	_____
Trudy Mackay	Lisa McGraw
Committee Chair	Committee Vice-Chair
_____	_____
Robert Anholt	Heather Patisaul
_____	
Brian Langerhans	

DEDICATION

To my parents

BIOGRAPHY

Andrea Vogel was born in West Bloomfield, Michigan in 1989, and grew up in Pottstown, Pennsylvania. Andrea spent many happy hours outside wandering the woods close to her family's home, which, among other things, inspired her to pursue studies in Biological Sciences with a Concentration in Cellular and Molecular Biology at Binghamton University. At Binghamton, she was an undergraduate researcher in Dr. Anne Clark's laboratory, which focused on social behavior in budgerigars. This work resulted in co-authorships on 3 publications. After graduation in 2011, Andrea joined Dr. Anthony Fiumera's laboratory at Binghamton as a Masters student. There she studied the environmental toxicology of atrazine in fruit fly reproduction, which earned her a first-authored manuscript. She graduated with a Masters of Science in Biological Sciences in 2013. Andrea then moved to North Carolina State University and joined the PhD program in Genetics. At North Carolina State University, she worked in Dr. Lisa McGraw's laboratory. This brought Andrea back to studies of social behavior, but this time with an emphasis on a mechanistic approach that encompassed both neurobiology and genetics using prairie voles. After graduation, Andrea plans to continue her studies of mechanisms of reproduction as a post-doctoral researcher.

ACKNOWLEDGMENTS

I would like to thank the many people who helped me over this incredible journey. I received support from so many people, in so many different ways. First off, I would like to thank my family, specifically my parents and my boyfriend. My parents always supported my interests. Without their guidance and help, I wouldn't be the person I am today. My boyfriend, Thomas, also needs a special acknowledgment because he stuck by me through all the struggles of the doctorate. He always encouraged me to do what was best for me, and supported me in all my endeavors. Without his support, I would not have finished my doctorate. I would also like to thank all the teachers and professors who helped me throughout this process. My advisory committee, including Drs. Lisa McGraw, Heather Patisaul, Trudy Mackay, Robert Anholt, and Brian Langerhans, were instrumental in helping me with all aspects of the graduate school process. I would also like to thank the many other professors who have helped me along the way, with a special thank you to Dr. John Meitzen and his lab. Of course, no one would be able to get through life without the help of friends. I have been honored to have many friends to laugh and commiserate with me, both within grad school and outside grad school. My office would be a lot quieter and lonelier without the voices of Sheryl Arambula, Megan Serr, and Cassie Rhodes. Genetics would not be the same without Jen Baltzegar, Tiffany Garbutt, and Shante Bryant. I would not have as much fun outside the office without Lura Kimbell, Katie Dunning, Victoria Shockley, and all the other wonderful unicorns from Aradia Fitness. Thank you to everyone who has helped me throughout these past years. I would not have made it without you.

TABLE OF CONTENTS

LIST OF TABLES vi
LIST OF FIGURES vii
CHAPTER 1 1
CHAPTER 2: ENVIRONMENTAL PLASTICITY OF PAIR BONDING IN THE SOCIALLY MONOGAMOUS PRAIRIE VOLE, *MICROTUS OCHROGASTER* 19
Abstract 19
Introduction 20
Results 24
Discussion 27
Materials and Methods 30
Acknowledgements 36
Contributions 36
CHAPTER 3: SEMINAL VESICLE PROTEINS DO NOT AFFECT FEMALE PAIR BONDING OR OFFSPRING BEHAVIOR IN PRAIRIE VOLES 48
Introduction 48
Materials and Methods 50
Results 54
Discussion 57
Acknowledgements 59
CHAPTER 4 64

LIST OF TABLES

Table 1.1 Overview of papers that examined the genetic basis of pair bonds in prairie voles 14

Table 2.1 Comparisons of time spent performing male behaviours before and after mating 42

Table S2.1 Narrow-sense heritability estimates 43

Table S2.2 Spearman's rank correlation tests between social behaviours and V1aR density in sections through the ventral pallidum 44

Table S2.3 Spearman's rank correlation tests between social behaviours and V1aR density in sections through the rostral part of the retrosplenial cortex 46

Table 3.1. Reproduction is not influenced by seminal vesicles proteins 61

Table 3.2 Female behaviors are not influenced by seminal vesicle proteins 62

Table 3.3 Offspring behaviors are not affected by seminal vesicle proteins 63

LIST OF FIGURES

Figure 2.1 Distribution histogram of pair bond formation in an outbred laboratory population of prairie voles 38

Figure 2.2 Association of variation in V1aR density in the ventral pallidum with affiliative behaviour 39

Figure 2.3 Association of variation in V1aR density in the RSC of male prairie voles with variation in affiliative behavior 40

Figure 2.4 Experimental animal crossing scheme 41

CHAPTER 1

Social behaviors in humans

Humans are social creatures and through partnerships (marriage) and friendships, mental and physical benefits manifest due to these strong social attachments. For example, people in marriages live longer and healthier lives than unmarried people; and people with strong social groups recover faster from illnesses (Robles and Kiecolt-Glaser, 2003; Barber, 2012). Social support can also buffer women against the effects of stress, regardless of the source of the stress (Stein and Smith, 2015). While social attachments can be influenced by environment, if a person is having difficulty in forming social attachments, the source of the impairment for social attachment may be a mental health disorder.

Mental health disorders in humans

Social deficits, where people are unable to or struggle in forming social bonds, are found in many mental health disorders such as autism, schizophrenia, and depression (Volkmar, 2001). A well-known mental health disorder that impairs the formation of social bonds is autism. Autism spectrum disorder is a mental health disorder that affects about 1 in 68 children, and is 4.5 times more common in boys than girls (Christensen et al., 2016). It is characterized as a heterogeneous neurodevelopmental disorder with characteristic behavioral abnormalities such as repetitive behaviors, deficits in language and communication, and lack of responsiveness in social interactions (American Psychiatric Association, 2013). People with autism spectrum disorder manifest symptoms of different severity, ranging from mild behavior abnormalities to severe impairments, which necessitates defining autism as a spectrum disorder (Persico and Bourgeron, 2006). Assessing autism spectrum disorder in

humans has increased awareness of the symptoms and allowed children to be diagnosed at an earlier age, which allows for behavioral therapy and other treatments to start earlier (Dawson, et al., 2010; Rogers et al., 2012; Eapen et al., 2013). By using human Genome-Wide Association Studies (GWAS), researchers have identified many genes that contribute to autism spectrum disorder (reviewed in Huguet et al., 2013). Schizophrenia research has benefited tremendously from GWAS (Schizophrenia Psychiatric Genome-Wide Association Study, 2011; Fanous et al., 2012; Levinson et al., 2012; Kanazawa et al., 2013; Ripke et al., 2013; Xu et al., 2014). GWAS has identified SNPs in loci that are important to neuronal development, neuronal calcium signaling, and in the major histocompatibility complex {Schizophrenia Psychiatric Genome-Wide Association Study, 2011; Irish Schizophrenia Genomics Consortium and the Wellcome Trust Case Control Consortium, 2012; Ripke et al, 2013}. Furthermore, through clustering SNPs by individual, researchers were able to create genetic networks with distinct clinical syndromes {Arnedo et al, 2015}. There are limitations when performing experiments with human subjects, such as the ability to control the environment, and large numbers of offspring for experimental testing, which is why a lot of research relies on animal models.

Animal models of human behavior

As helpful as studies done in humans have been to understand the neurobiological and genetic mechanisms of mental health disorders, research done with model organisms has also informed our understanding of mental health disorders and, more generally, human social behaviors. By examining social behaviors from a variety of angles, researchers can investigate if there are common neural or genetic pathways inherent to sociality. Traditional

mammalian laboratory animals such as mice and rats have been used to great success in understanding the mechanisms of social behavior and in identifying some underlying genes. Many of the neural pathways and genes implicated in social behavior are conserved between rodents and humans, such as the vasopressin and oxytocin pathways. Areas of the brain that are rich in vasopressin and oxytocin receptors, such as the amygdala (Caldwell et al., 2008; Lee et al., 2009), are also areas that are impacted in autism (Ha et al., 2015). It should be noted, however, that not all the brain regions implicated in autism are rich in vasopressin and oxytocin receptors {Ha et al, 2015}. In rats and mice, the vasopressin and oxytocin receptors are important for social memory recognition (Harony and Wagner, 2010). Vasopressin and oxytocin receptors show sex-specific locations in the brain, and it is thought to be the distribution of the receptors that elicit the behavioral responses (Dubois-Dauphin et al., 1996). By manipulating the receptors in rats and mice to reflect the impairment seen in humans with mental health disorders, researchers can determine which behaviors have been altered. Another way mice and rats have been used for learning about social behaviors impaired in mental health disorders, and non-disease state human social behaviors, is to identify strains of mice and rats that recapitulate behaviors associated with autism spectrum disorder or other mental disorders in humans. These animals can be examined to determine if there are genetic polymorphisms or changes in neurobiology that cause or correlate with the behavioral differences. Furthermore, most aspects of the environment can be controlled for animal models in laboratories, as opposed to human studies.

Mice and rats have also been useful to explore the relationship between the environment in early life and behavior. For example, mice have been used to understand how

behaviors are shaped by the social environment during early life (Veenema et al., 2007; Curley et al., 2009; Murgatroyd et al., 2009; Tsuda et al., 2011). The relationship between genes, environment during early life, and behavior in later life has been examined in rats, which showed epigenetic changes are responsible for maternal behavior in female rats (Weaver et al., 2004). Mice are also highly amenable to genetic manipulation, which allows for further understanding of how individual genes shape behaviors. However, an issue with using mice and rats to model human social behaviors is the lack of strong social attachment in adults in these rodents. Fortunately, a rodent model exists that shows partnership and social attachment as adults, in both males and females: the prairie vole.

Prairie vole behavior

Prairie voles (*Microtus ochrogaster*) are small, microtine rodents that show biparental care of offspring, aggression towards same-sex intruders, and form long-lasting partner preferences. Both males and females will show a preference for their partner after six hours if mating occurs (Williams et al., 1992). Prairie voles were first identified as socially monogamous by field work in which the same male and female were trapped together several times, thus indicating a pair bond (Getz et al., 1981; Getz and Hofmann, 1986; Getz et al., 1993). A pair bond is defined as when two mated animals show an enduring preference for each other, usually accompanied by increased aggression towards intruders (Winslow et al., 1993). The pair bond was confirmed in laboratory research by both males and females displaying a partner preference using an assay called the partner preference test (Williams et al., 1992; Getz et al., 1993; Winslow et al., 1993). The partner preference test uses a three-chambered apparatus, with one member of the pair tethered at one end of the apparatus, and a

stranger animal that is not related to the pair tethered at the other end, while the experimental animal is free to spend time with either the partner or the stranger, or can be alone. The use of the partner preference test established the uniqueness of the prairie vole as a mammalian species that displays social monogamy, because the experimental animals typically spend more time with their partner than with the stranger or alone. This is in contrast to promiscuous species, such as the meadow (*Microtus pennsylvanicus*) or montane vole (*Microtus montanus*), which spend more time alone than with a partner or stranger during the partner preference test (Shapiro and Dewsbury, 1990). Field research shows that not all prairie voles form a pair bond, as ~50% of prairie voles are found to be in a monogamous state, but ~25% are found in communal groups, and ~25% are found as "wanderers", which are solitary animals that have only opportunistic copulations (Solomon and Jacquot, 2002; Ophir et al., 2007). Additionally, these percentages can vary between populations, and there are usually more male wanderers than females (Ophir et al., 2007). The percentage of "resident" (pair-bonded and territory-defending) males differs between populations in Kansas and Illinois (Danielson and Gaines, 1987; Swihart and Slade, 1989; Roberts et al., 1998; Cushing et al., 2001). Other researchers have noted differences between Illinois and Tennessee populations, whereby female prairie voles in Tennessee would mate with multiple males, even when showing a pair bond with a male (Wolff and Dunlap, 2002; Wolff et al., 2002). However, no differences between populations were seen when prairie voles from Tennessee and Illinois were tested in a semi-naturalistic arena (Ophir et al., 2007). The variation among populations shows that pair bonding, and partner preference, is a plastic

behavior. The variation in pair bonding seen in field research should be mirrored in laboratory testing using the partner preference test.

In addition to the partner preference test, other commonly used behavioral assays to measure social behavior in laboratory research include the alloparental care test, the open field test, the elevated plus maze, and the resident intruder test. The alloparental care test examines care for pups, and in prairie voles both males and females will care for young. Juvenile and adult prairie voles of both sexes will care for young, even with no prior exposure to young pups, although nulliparous females are less likely to care for young than others (Ross and Young, 2009). Both the open field test and the elevated plus maze investigate anxiety-like behaviors, by providing an area thought to be more anxiety-inducing (the center of the open field test and the open arms of the elevated plus maze) and an area that is less anxiety-inducing (the edge of the open field test and the closed arms of the elevated plus maze). Finally, the resident intruder test looks at aggression against same-sex intruders in a quantifiable fashion. Both laboratory and field research has shown that after mating, aggression against same-sex intruders becomes more common, and it is thought that this aggression is used to guard the mate from opportunistic copulations. Both field and laboratory research has informed us about the underlying mechanisms of social behaviors, such as partner preference.

Neurobiology of social behavior

Several neuropeptides and their receptors have been identified as integral to partner preference formation and maintenance. Those that have been well studied include oxytocin, vasopressin, and dopamine. Oxytocin and vasopressin are similar in structure and have

demonstrated roles in social behavior (Carter et al., 2009: Lukas et al., 2011). Oxytocin plays a larger role in females than in males, while vasopressin is more involved in male behavior than female behavior (Winslow et al., 1993; Williams et al., 1994; Lim and Young, 2004; Insel, 2010). Dopamine is an important neuropeptide in both sexes and works alongside oxytocin in the nucleus accumbens to form the partner preference (Wang et al., 1999; Gingrich et al., 2000; Aragona et al., 2003). Together, these neuropeptides have demonstrated effects on partner preference formation and maintenance, along with parental care and mate guarding, in their respective sexes.

Many studies have examined V1aR density in regions across the brain to try to elucidate the associations between brain regions and social behaviors. The vasopressin 1a receptor (V1aR) pathway (which will be discussed in detail below) influences both partner preference and anxiety in prairie voles (Pitkow et al., 2001). One laboratory study, which used V1aR receptor autoradiography to determine the V1aR densities in 31 brain regions, found correlations between anxiety-related behavior and V1aR binding in thalamic areas, social behaviors and V1aR binding in olfactory nuclei, and partner preference behavior and V1aR binding in amygdala, thalamic, and ventral pallidum (Hammock et al., 2005). The link between the ventral pallidum and partner preference formation has been investigated at length in the laboratory. Strikingly, when monogamous male prairie vole brains are compared to non-monogamous male montane vole brains, the prairie vole brains show a greater density of V1aR in the ventral pallidum (Insel et al., 1994). Additionally, when V1aR density was increased through viral vector-mediated gene transfer in the promiscuous

meadow vole, the meadow vole showed more time spent with the partner during a partner preference test, similar to the behavior shown by the prairie vole (Lim et al., 2004).

Several studies have examined V1aR density in several brain regions and its association with monogamy over a longer period of time, such as weeks or a breeding season. By allowing pairs to coexist in semi-naturalistic enclosures for several weeks so that pairs have time to breed, and then using paternity testing to determine which males and females had extra-pair fertilizations, researchers compared the density of V1aR in both the group that had extra-pair fertilizations, and those that did not. One such study found that mating success was correlated with V1aR binding in the posterior cingulate/retrosplenial cortex and laterodorsal thalamus, such that males with successful extra-pair fertilizations have significantly lower binding in these regions (Ophir et al., 2008). Another study confirmed this finding, showing that low levels of V1aR in areas of the brain associated with spatial memory, such as the laterodorsal thalamus and retrosplenial cortex, are predictive of high intrusion rates and poor mate-guarding (Okhovat et al., 2015). These studies did not manipulate the density of V1aR, but measured the individual variation inherent in the population. The amount of natural variation in V1aR density is considerable, as Phelps and Young discovered in their study of wild prairie voles (Phelps and Young, 2003).

The *avpr1a* locus and its relationship to neurobiology and social behaviors

A compelling argument for the natural variation seen in pair bond and other social behaviors is the presence of a polymorphic microsatellite region upstream of the *avpr1a* locus. This locus is responsible for the vasopressin receptor in the brain. V1aR density in the brain is highly variable, and the density is correlated with social structure between vole

species. Thus, it stands to reason that the diversity at the *avpr1a* locus is generating diversity in the V1aR in regions of the brain. Investigations of polymorphisms at the avpr1a locus can not explain the variation in V1aR density in some brain regions, which implies that more genes than just *avpr1a* influence the individual variation seen in V1aR density. As shown in Table 1.1, the results from laboratory studies show that long (greater than median length in the study) alleles of *avpr1a* correlate with higher densities of V1aR binding as observed by receptor autoradiography in some brain regions, although the regions differ between studies (Hammock et al., 2005; Hammock and Young, 2005). A study done in a semi-naturalistic setting also found that the long *avpr1a* alleles were associated with higher densities of V1aR in the ventral pallidum, medial amygdala, ventromedial hypothalamus, and average of the whole brain, evidenced using receptor autoradiography (Ophir et al., 2008). The semi-naturalistic study only found significant correlations between the summed total length of *avpr1a* microsatellites and V1aR expression in the ventral pallidum, medial amygdala, and central amygdala (Ophir et al., 2008). One study, which examined single nucleotide polymorphisms (SNPs) in and around the *avpr1a* locus, found four tightly linked SNPs that predicted V1aR expression level in the retrosplenial cortex and one SNP that predicted V1aR expression in the laterodorsal thalamus (Okhovat et al., 2015). This same study associated *avpr1a* mRNA levels with differences in V1aR protein levels in these brain regions using qPCR, and found that the retrosplenial cortex-associated SNPs coincide with markers of transcriptional regulation. The study's major finding was that one SNP was a G/T polymorphism that altered CpG sites which led to less enhancer methylation in animals with

high V1aR expression (Okhovat et al., 2015). Overall, these studies show how polymorphisms in a single gene can contribute to variation in neural expression patterns.

When studies attempt to associate *avpr1a* microsatellite length with other social behaviors, no clear patterns emerge. One laboratory study of male prairie voles found that males with short *avpr1a* microsatellites had significantly more anogenital sniffing than males with long *avpr1a* microsatellites, but only during the light photoperiod on the first two days of testing (Graham et al., 2016). The overall conclusion of this study was that microsatellite length had no effect on courtship in male prairie voles (Graham et al., 2016). A different laboratory study of breeder pairs where both animals in the pair had either short or long *avpr1a* microsatellites found that short allele breeder pairs had more pup mortality in the first litter and short allele males in the breeder pairs had less frequent grooming of pups (Hammock and Young, 2005). When these pairs were tested with a novel social odor, males with long *avpr1a* microsatellite alleles had a shorter latency to approach the social odor and a higher frequency and longer duration of investigation of the social odor (Hammock and Young, 2005). These same males with long *avpr1a* microsatellite alleles also spent more time with their partner and were more likely to display partner preference than males with short *avpr1a* microsatellite alleles during the partner preference test (Hammock and Young, 2005). This study would suggest that the length of the microsatellite is associated with pair bond formation, sociality, and paternal care. A study done in the wild also supports this association between the length of the microsatellite and paternity, as it found that males with longer total *avpr1a* microsatellite length were more likely to sire offspring with one or more females, and to sire more offspring than males with shorter total *avpr1a* microsatellite length

(Keane et al., 2014). This association between longer microsatellite length and fertility is also shown by females, as females with longer total *avpr1a* microsatellite length produced almost double the amount of offspring, by having double the amount of litters with twice as many males as females with shorter total *avpr1a* microsatellite length (Harris et al., 2014). In contrast, one semi-naturalistic study found the opposite effect, whereby males with short *avpr1a* alleles sired offspring with significantly more females and had more offspring than males with long *avpr1a* alleles. However, other studies find no association between microsatellite length and behavior. The study that had the largest number of animals genotyped was done in the wild, and examined social and genetic monogamy and reproductive success. It found no relationship between the *avpr1a* microsatellite and any behavior measured (Mabry et al., 2011). (For complete review of the studies, see Table 1.1).

One striking difference between the laboratory studies, the semi-naturalistic studies, and the wild-caught studies, beyond their different conclusions, is the number of animals included in the study. Most laboratory studies use smaller numbers of animals than the other studies do. Also, the laboratory studies use animals from a maintained colony of prairie voles. Over time, genetic diversity can be lost in a colony, and genetic drift can occur, driving differences between laboratory and wild prairie voles. The studies discussed in chapters 2 and 3 used a laboratory population of prairie voles that was maintained to keep genetic diversity and we used, for a laboratory experiment, a large number of animals.

I attempt to bring clarity to the relationships between suites of behaviors, and how pair bonding changes behaviors. I sought to determine how V1aR expression is associated with suites of behaviors, which others have done before but not with the number of animals

that we included in this study. Furthermore, I determine the heritability of socially relevant behaviors, which has not been shown before.

Seminal vesicle proteins

One area of interest that has not been explored in prairie voles is the contribution of seminal fluid proteins (SFPs), and particularly seminal vesicle proteins (SVPs). SFPs are transferred from the male to the female in the ejaculate during mating. Studies done in insects, and notably in Drosophila, have shown physiological and behavioral changes in the mated female due to SFPs (Avila et al., 2011; Hopkins et al., 2017). These include decreasing a female's need to eat and sleep, her probability of re-mating, and her lifespan, while increasing her rate of ovulation (Avila et al., 2011; Hopkins et al., 2017). SFPs are also necessary for sperm health and formation of the mating plug in *Drosophila melanogaster* (Neubaum and Wolfner, 1999; Tram and Wolfner, 1999; Lung and Wolfner, 2001).

The copulatory plug in mammals is also comprised of SFPs (Cukierski et al., 1991). However, changes in the physiology and behavior of mated mammalian females have not been as well studied as in insects. In both insects and mammalian species, SFPs impact ovulation. In induced ovulators such as the alpaca (*Vicugna pacos*) and the llama (*Lama glama*), an SFP is responsible for inducing ovulation through a surge of luteinizing hormone (LH) from the pituitary gland (Paolicchi et al., 1999; Adams et al., 2005; Bogle et al., 2012). This SFP, called the ovulation inducing factor (OIF), was later found to be beta-nerve growth factor (NGFβ), a highly conserved protein found in the ejaculate of both spontaneous and induced ovulator species (Ratto et al., 2012). The induction of ovulation is the most striking example of an SFP having a physiological effect in mammalian females.

SFPs have also been shown to impact pregnancy in mammalian females. In mice, females mated to males without seminal vesicles (SVs) had a harder time getting pregnant and more instances of reabsorbed embryos (Bromfield et al., 2014). Males without SVs not only impacted their mates and pregnancy, but also their offspring. In the same mouse (*Mus musculus*) study, male offspring of sires without SVs weighed more than male offspring of sires who retained their SVs (Bromfield et al., 2014). A different study in golden hamsters (*Mesocricetus auratus*) found that offspring of sires without accessory sex glands weighed less at birth and had behavioral abnormalities, such as quicker habituation to acoustic stimuli (Wong et al., 2007). This study implies that SFPs could have effects on behavior.

I predicted that SFPs would have physiological and behavioral effects in prairie voles based on the above studies. Prairie vole females are induced ovulators, so while it has not been shown that SFPs have any effects in prairie voles, I expect ovulation to be affected due to the studies in alpacas and llamas. Furthermore, the mouse and golden hamster studies imply that SFPs might have behavioral effects. Prairie voles undergo several behavioral changes after mating, such as the formation of partner preference and aggression towards same sex intruders. I hypothesize that SFPs play a role in the rapid behavioral changes seen in prairie voles.

Table 1.1 Overview of papers that examined the genetic basis of pair bonds in prairie voles

Authors	Year	Habitat	N	Genetics	Autoradiography	Behavior	Genetic results	Other results	Conclusion
Hammock, EA; Lim, MM; Nair, HP; and Young, LJ	2005	laboratory, single sex (2-3 males per cage) then individually housed, 14/10 h light/dark cycle	20	V1aR microsatellite (40 alleles) PCR length: 742 ± 9 bp (723-760 range)	V1aR autoradiography of 31 brain regions from the olfactory bulb to the level of the superior colliculus (Table 1)	Elevated plus maze, open field test, paternal care, juvenile affiliation, partner preference, and resident-intruder (1 test per day)	V1aR binding strongly correlated with microsatellite in main and accessory olfactory bulbs, the amygdala, and the thalamus	Correlations between V1aR binding in thalamic areas and anxiety-related behavior, V1aR bindingin olfactory nuclei and social behaviors, and V1aR binding in amygdala, thalamus, and VP and partner preference	V1aR microsatellite correlated with V1aR binding in brain regions that are correlated with behavior
Hammock, EA and Young, LJ	2005	laboratory	25 breeding pairs, cross foster 2/3 litters 6-8 hrs after birth. Autorad N = 45 short-allele maled, 49 long-allele males	homozygous *avpr1a* microsatellites for long or short	V1aR autoradiography of 23 brain sections from the olfactory bulbs to the VTA	Huddling, nursing posture, licking/grooming, social and non-social odor investigation, social approach and investigation, partner preference	Short-allele breeder pairs had higher rates of pup mortality in 1st litter. Higher grooming frequency of pups of long-allele males compared to short-allele males. Long-allele males had shorter latency to first approach, higher frequency and longer duration of investigation to social odor. Long-allele males spent more time with partner, displayed partner preference. V1aR binding associated with genotype differences in 16 regions (unadjusted p-value) or 9 regions (Bonferroni cut-off)		Genotype differences in V1aR binding in the lateral septum and olfactory bulb are likely neural substrates underlying genotype differences in social behavior.

Table 1.1 continued

Barrett, CE; Keebaugh, AC; Ahern, TH; Bass, CE; Terwilliger, EF; and Young, LJ	2013	laboratory, 14/10 hr light:dark cycle	9 scrambled, 9 shRNA-*Avpr1a* virus males	shRNAs for *Avpr1a* coding sequence	V1aR autoradiography through ventral pallidum	Partner preference, alloparental care, elevated plus maze	Males injected with shRNA-*pvAvpr1a* showed significant impairment in partner preference. shRNA males entered the distal portion of open arm in EPM and moved a greater distance in distal open arm and entire open arm, and spend less time in the central platform.	VP V1aR densities significantly predicted time spent with the stranger, with high expression spending less time in stranger contact. Duration in central platform of EPM correlated with VP V1aR expression and time spent huddling with the partner. Within the shRNA injected group, % entries into open arm correlated with VP V1aR.	*Avpr1a* expression directly contributes to natural variation in pair bonding and anxiety-related beahviors.
Graham, BM; Solomon, NG; Noe, DA; Keane, B	2016	laboratory, Illinois and Kansas separate, 14/10 h light/dark cycle	16 of each population	V1aR microsatellite (Ilinois mean 724.7 ± 1.9 bp for short length, 765.7 ± 1.8 bp for long; Kansas mean 719.7 ± 1.4 bp for short, 767.4 ± 2.4 bp for long)		Partner preference (1 male long allele, 1 male short); naso-nasal sniffing and anogenital sniffing by males; side-by-side contact, allogrooming, active exploratory contact by male and male recipient	Males with short *avpr1a* microsatellite allele lengths did significantly more anogenital sniffing during light period of days 1 and 2 than males with long	No differences between voles from Kansas vs Illinois in any behavior measured	No difference in courtship behavior due to microsatellite length
Okhovat, M; Berrio, A; Wallace, G; Ophir, AG; Phelps, SM	2015	1) semi-natural; 2) laboratory		151 SNPSs at *avpr1a* locus (4 linked).	V1aR autoradiography in Vpall, LS, RSC, LDThal	Space use, IPF/EPF	4 SNPs predicted RSC-V1aR, 1 SNP predicted LDThal-V1aR. qPCR associated *avpr1a* mRNA differences with RSC-V1aR protein.	IPF males have more RSC-V1aR than EPF males. Low levels of RSC-V1aR associated with high intrusion rates and poor mate-guarding. LDThal-V1aR also predicted sexual fidelity and spaces use.	Association between HI-RSC allele and high RSC-V1aR expression

Table 1.1 continued

Ophir, AG; Wolff, JO; and Phelps, SM	2008	4 field enclosures (20 x 30 m)	6 males and 6 females in each enclosure, 8 groups total (48 male, 48 female total). Recovered 43 males and 38 females, excluded 2 more females		V1aR autoradiography in Vpall, LS, Pcing, LDThal	space use, RER, paternity		Resident male home ranges overlapped fewer male home ranges than wandering male ranges. Successful males overlapped fewer males than unsuccessful. Successful wanderers overlapped more than unsuccessful wanderers and successful residents. Successful residents overlapped few females than unsuccessful, but successful wanderers overlapped more females than successful residents. EPF males overlapped more home ranges and opposite-sex overlaps than IPF. Mating success correlated with PCing and LDThal. Successful wanderers and EPF males have significantly lower binding than unsuccessful in PCing and LDThal.	Regions implicated in spatial navigation predict space use and extra-pair paternity.
Ophir, AG; Campbell, P; Hanna, K; and Phelps, SM	2008	8 semi-natural (20 x 30 m) enclosures	48 male and 48 female (6 of each sex in each group)	*avpr1a* microsatellite of males (505-585 bp per allele)(1026-1170 bp summed allele lengths)	V1aR autoradiography of 15 brain areas from the olfactory bulbs to the anterior hypothalamus(Table 1)	# of offspring, embryo size, home range size, male-male encounter rate, male-male overlap, male-female encounter rate, male-female overlap, relative encounter rate	VPall, MeA, VMH and average V1aR expression higher in 'long' males. Vpall, MeA, and CeA V1aR expression significantly correlated with summed allele length.	No influence of allele length on behavior	*avpr1a* allele length and V1aR expression in several brain regions are positively correlated

Table 1.1 continued

Solomon, NG; Richmond, AR; Harding, PA; Fries, A; Jacquemin, S; Schaefer, RL; Lucia, KE; and Keane, B	2009	20 semi-natural (32 x 32 m) enclosures	either 8 voles (4 male; 4 female) or 24 (12 male; 12 female) per enclosure	*avpr1a* microsatellite of males (2004: 733 bp (703-778 bp); 2005: 743 bp (713-798 bp) each allele) Longer allele per male was 738 bp (738-778 bp; 2004) and 753 bp (728-798 bp; 2005)			Males with short *avpr1a* alleles sired offspring with significantly more females and more offspring than males with longer alleles		Genetic monogamy correlated with *avpr1a* allele length in males
Harris, MN; Alvarez, RM; Keane, B; Talib, AD; Eiswerth, MJ; Solomon, NG	2014	20 semi-natural (32 x 32 m) enclosures	either 8 voles (4 male; 4 female) or 24 (12 male; 12 female) per enclosure	*avpr1a* microsatellite (Males: 1476 bp (1441-1616); Females: 1461 bp (1426-1566 bp))			Females with >median allele length (long) produced almost 2x offspring and 2x litter with 2x as many males than females with short alleles		Females *avpr1a* genotype associated with significantly greater reproductive success
Mabry, KE; Streatfeild, CA; Keane, B; and Solomon, NG	2011	wild	589 males trapped, 470 genotyped	summed length of *avpr1a* microsatellite (KS: 1398-1574; IN: 1392-1579)		# females associated with a male, genetic monogamy, reproductive success, # of genetic mates, # of offspring	No relationship between *avpr1a* genotype of males and any variable measured		*avpr1a* length polymorphism is not strongly associated with behavior under ecologically relevant conditions

Table 1.1 continued

Keane, B; Parsons, S; Smucker, BJ; and Solomon, NG	2014	wild	238 adult and subadult males, 168 genotyped	*avpr1a* microsatellite (1470 bp (1390-1570 bp) summed)		home range (Mean Square Distance), # females a male overlapped,	*avpr1a* microsatellite length associated with # of mated females and offspring. Males with long *avpr1a* length were more likely to sire offspring with 1 or more females and have more offspring than males with short *avpr1a* length.	No influence of allele length on behavior	Males with long *avpr1a* microsatellites were more likely to sire offspring with more than 1 female and to sire more offspring than males with short microsatellites.

CHAPTER 2: ENVIRONMENTAL PLASTICITY OF PAIR BONDING IN THE SOCIALLY MONOGAMOUS PRAIRIE VOLE, *MICROTUS OCHROGASTER*

Andrea R. Vogel[1,2,3,*], Heather B. Patisaul[1,2], Sheryl E. Arambula[1,2], Francesco Tiezzi[3,4], and Lisa A. McGraw[1,2,3]

[1] Department of Biological Sciences, [2] W. M. Keck Center for Behavioral Biology, [3]Program in Genetics, and [4]Department of Animal Science

* Address correspondence to Andrea Vogel, email: arvogel@ncsu.edu

Abstract

The genetic and environmental factors that contribute to pair bonding behaviour remain poorly understood. Prairie voles (*Microtus ochrogaster*) often, but not always, form stable pair bonds and present an ideal model species for investigating the genetic and environmental factors that contribute to variation in monogamy. Here, we assessed variation in partner preference, a measure of pair bonding, and related social behaviours in a population of laboratory reared prairie voles under controlled environmental conditions. We estimated the heritability of these behaviours, and evaluated to what extent variation in these behaviors correlate with vasopressin 1a receptor (V1aR) expression in the ventral pallidum (VP) and retrosplenial cortex (RSC). We found substantial variation in partner preference and measures of aggression, paternal care, and anxiety-like behaviours, but no correlation between these traits. We also found variation in V1aR density in the VP and RSC can

account for behavioural components of paternal care and aggression, but not in partner preference. Heritability estimates of variation in partner preference were low, indicating that the extensive variation in partner preference observed within this population is due largely to environmental plasticity.

Introduction

Monogamy, defined as a mated pair that stays together through several breeding seasons is rare among mammals, and when it occurs, monogamy is typically accompanied by the formation of pair bonds – strong, lasting social bonds between mates (Kleiman, 1977). Prairie voles (*Microtus ochrogaster*) are socially monogamous rodents that often form pair bonds but, in their natural habitats, display enormous variation in their level of social monogamy (Getz et al., 1981; Getz et al., 1993; Solomon and Jacquot, 2002; McGuire and Getz, 2010; Young et al., 2011). For example, male prairie voles vary considerably in their mating strategies, ranging from "resident" strategies, where they defend a territory with their respective paired female; to "wanderer" strategies, where they do not have defined territories and gain paternity through extra-pair matings (Getz et al., 1981; Getz et al., 1993; Solomon and Jacquot, 2002; Ophir et al., 2008; McGuire and Getz, 2010; Okhovat et al., 2015). In addition to decades of field research describing mating system variation in their natural habitats, prairie voles have also become an invaluable laboratory species for understanding the neurobiological and genetic basis of pair bonding and related social behaviours. While these laboratory studies have increased our understanding of the biological basis of the pair bond, our understanding of why individuals show incredible variation in mating strategies,

including whether or not they form pair bonds, is still poorly understood. In this study, we set out to further characterise natural genetic variation in male pair bonding and related social behaviours, its neurobiological biological correlates, and the heritability of these traits in a controlled laboratory environment.

Pair bonding and related social behaviours in prairie voles have been studied in the laboratory using standard rodent behavioural assays The pair bond is commonly measured using the partner preference test, which measures the amount of time a focal animal spends with its mate (partner) versus an unrelated, unfamiliar animal of the same sex (stranger). In prairie voles, males typically form a "partner preference" whereby they spend more than twice the amount of time with their "partner" than the "stranger" female following a brief cohabitation and mating (Williams et al., 1992; Young and Wang, 2004; Ahern et al., 2009). Pair bonding also alters related social behaviours in prairie vole males that can also be easily measured in the laboratory. Increased aggression towards a same-sex intruder is perhaps the most well-characterised post-pair bonding alteration of male behaviour and is thought to be an adaptation to territorial defense and mate guarding (reviewed in Gobrogge and Wang, 2011). In addition, paternal behaviour, measured indirectly as alloparental care; and anxiety-like behaviours, measured with the open field test, are also sometimes altered following the formation of a pair bond (Insel et al., 1995; Terleph et al., 2004). The close association of pair bonding and related social behaviours is partially explained by the extensive overlap of the neural circuitry encoding these behaviours, and presumably, a common genetic basis (reviewed in Gobrogge and Wang, 2015).

Several neurobiological systems underlying male pair bond formation and maintenance have implicated multiple neurotransmitters and their respective receptors as an important mediator of male social behaviours (reviewed in Carter et al., 1995; Young and Wang, 2004; Aragona et al., 2006; Young et al., 2011). Most relevant to this study is the role of arginine vasopressin and its receptor (V1aR) within the ventral pallidum (VP) and the retrosplenial cortex (RSC) in mediating male social behaviours including pair bond formation, aggression, and anxiety (Winslow et al., 1993; Pitkow et al., 2001; Lim and Young, 2004; Barrett et al., 2013; Okhovat et al., 2015). V1aR expression within the VP shows considerable natural variation, and manipulation of the density of V1aR is strongly correlated with male social behaviour (Phelps and Young, 2003; Lim and Young, 2004; Hammock and Young, 2005; Barrett et al., 2013). For example, antagonists against V1aR in the VP reduce a male's propensity for pair bonding (Lim and Young, 2004) and anxiety (Barrett et al., 2013), whereas increasing V1aR binding in this brain region increases partner preference formation and alloparental care (Pitkow et al., 2001). In prairie voles studies in semi-natural environments, V1aR expression in the RSC predicts male behaviours associated with pair bonding including sexual fidelity and intrusion rate, whereby male voles who had higher levels of V1aR were more likely to only have offspring with their partner and less likely to intrude on another male's territory (Okhovat et al., 2015). Expression of V1aR in both the VP and RSC exhibits considerable natural variation, yet whether or not this variation predicts pair bonding behaviours or whether or not this expression is heritable is poorly understood (Phelps and Young, 2003; Hammock et al., 2005).

Although pair bonding, related social behaviours, and V1aR expression in the brain are complex traits presumably encoded by multiple genes, polymorphisms in and near *avpr1a*, the gene encoding V1aR, appear to play a significant role in modulating male social behaviour. A polymorphic microsatellite region upstream of the *avpr1a* locus has repeatedly been shown to influence several male social behaviours, including partner preference, along with paternal care and anxiety (Hammock et al., 2005; Hammock and Young, 2005; Solomon et al., 2009). Congruence of these results, however, has been inconsistent and other studies did not observe correlations between *avpr1a* microsatellites and social behaviours, showing that other mechanisms are involved in complex social behaviours (Ophir et al., 2008; Mabry et al., 2011; Graham et al., 2016). Even so, V1aR levels in the RSC were associated with single nucleotide polymorphisms (SNPs) in *avpr1a*, which points to a genetic basis for spatial memory and sexual fidelity (Okhovat et al., 2015). Although the role of polymorphisms at the *avpr1a* locus undoubtedly influence both social behaviour and brain V1aR expression, studies of these polymorphisms in complex, natural or semi-natural environments attribute a smaller role (Ophir et al., 2008; Mabry et al., 2011; Graham et al., 2016). We speculate that genetic background effects or genotype x environment interactions could be the basis for phenotypic variation in prairie voles.

Based on decades of previous research on social behaviours in these rodents, we have discovered extensive variation in social behaviours, variation in V1aR density in socially-relevant areas of the brain, and potentially functional allelic variation in *avpr1a*. Social behaviours, in general, are complex traits that arise from the influences of many genes. These behaviours have been assumed to be heritable in prairie voles, since work in other organisms

has shown measurable heritability for most behavioural traits (Anholt and Mackay, 2010). In order to test the hypothesis that genetic variation in part accounts for variation in social behaviour observed in field and laboratory populations of prairie voles, we assessed variation in male partner preference behaviour and other social behaviours in a population of laboratory reared prairie voles under carefully controlled environmental conditions. We estimated the heritability of partner preference and social behaviours, including anxiety, alloparental care (care for young that are not offspring of the experimental male), and aggression towards a same-sex intruder (a proxy for mate guarding). We also evaluated the extent to which variation in V1aR expression in the VP and RSC is correlated with behavioural variation. We discovered that heritability of the social behaviours, including partner preference, anxiety-like behaviours, alloparental care, and aggression towards a same-sex intruder, is not significantly different from zero; but that variation in vasopressin receptor expression in the VP and RSC is significantly correlated with affiliative behaviours.

Results

Males vary in their partner preference. We used a standard partner preference test to assess partner preference (Williams et al., 1992). Briefly, the focal male's partner was tethered at one end of a three chambered apparatus, while a stranger female neither the male nor female partner had met before was tethered at the other end of the apparatus. The male was free to move around the chamber and spend time with his partner, the stranger, or by himself. We measured the time each of 180 males spent with his partner for 180 minutes. The

average time (± SE) spent with the partner was 59 ± 3 min. However, we observed extensive variation in partner preference, ranging from 0 to 147 min (Fig. 2.1).

Partner preference is not associated with other social behaviours. Because behaviours including aggression toward conspecifics, paternal behaviour, and anxiety-like behaviours are often influenced by pair bonding, we measured these behaviours both before males were paired and after the partner preference test. Aggression was measured using the resident intruder test where we measured latency to approach a strange male, time spent away from the strange male, affiliative behaviour towards the strange male, defensive behaviour, and aggressive behaviour. Aggressive behaviour in the resident intruder test increased dramatically after mating (Table 2.1). Alloparental behaviour was assessed by introducing a male to unfamiliar pups. We measured latency to approach pups, time away from the pups, licking and grooming, cuddling with the pups, carrying the pups, and aggression towards pups. Most aspects of alloparental care were unaffected, except for licking and grooming of the pups, which decreased after mating (Table 2.1). Further, we did not observe changes in anxiety-like behaviours as measured using the open field test. Interestingly, we did not observe a significant correlation between any of these male behaviours and partner preference (Supplementary Fig. S1), demonstrating that there is substantial individual variation in the responses of males to mating.

V1aR density correlates with some pair bonding related behaviours. To evaluate the correlation between V1aR abundance with variation in partner preference, aggression,

alloparental care, and anxiety-like behaviours, we quantified the V1aR density in the VP and RSC of male prairie voles. We collected brain tissue for V1aR assessment in a subset of tested males. Autoradiography was performed immediately after the final behavioural test. We used rank-order Spearman correlation tests to assess variation in each behavioural component with V1aR density in each of eight rostral to caudal sections through the VP and seven sections through the rostral RSC. We observed significant variation in V1aR densities across the males both in the VP (Fig. 2.2A and B) and the RSC (Fig. 2.3A and B). In contrast to previous observations, we did not observe a correlation with partner preference and V1aR density in either the VP or RSC (Supplementary Table S2.2 and S2.3). Licking and grooming of pups, cuddling, carrying, and defensive behaviour toward a stranger were correlated with V1aR density in the VP (Fig. 2.2C; Supplementary Table S2.2;). Similarly, we found that variation in V1aR density in the RSC was associated with time away from the pups, licking and grooming, cuddling, carrying, time away from a strange male, and affiliative behaviour toward a stranger (Fig. 2.3C; Supplementary Table S2.3). Thus, our results show that variation in V1aR density in the VP and RSC correlates with the behavioural components of alloparental care and affiliative behaviour, but not in partner preference.

Heritability is not significantly different from zero. The males tested for variation in partner preference and related social behaviours were derived from a crossing scheme to minimize genetic background variation. We crossed two full-sib males with two unrelated full-sib females. The male offspring of these pairings were mated to two unrelated full-sib females (Fig. 4) to generate two generations of test subjects. We measured behaviours of

parents and offspring before and after mating. We were able to utilize this design to estimate the heritability of these traits. Although the laboratory population was outbred, individuals were related due to maintenance of a finite population. Therefore, we used pedigree data of all the animals in the study to derive a relationship matrix for heritability estimation. Heritability estimates of variation in the amount of time spent with the partner were not significantly different from zero (Supplementary Table S2.1), indicating that the extensive variation in partner preference observed within this population is due largely to environmental variation.

Discussion

We found substantial variation in partner preference and other social behaviours in 180 male prairie voles from a laboratory-reared population but no evidence of genetic variation for these traits. Of particular interest is the considerable variation observed in partner preference, whereby some male prairie voles spent over two hours in contact with their partner, while other males spent less than twenty minutes with their partner, during a three-hour long test. Although there was individual variation in the other behaviours we examined, there were no significant correlations between suites of behaviours.

Our inability to detect heritability of partner preference and related behaviours may potentially be resolved by increasing the sample size to reduce standard errors and resolve a contribution of genetic variation to the phenotypic variance. Social behaviours, including partner preference, are complex behaviours that are most likely influenced by multiple genes, not all of which have been identified (Mackay et al., 2009). Although our population has

been outbred since its arrival at North Carolina State University and DNA sequencing of a subset of BAC clones derived from this population revealed genetic variation among individuals (McGraw et al., 2012), we do not know about the historical inbreeding that may have occurred prior to establishing the population, thus weakening our ability to detect contributions of genetic variance to the social behaviours. Since we did not characterise polymorphisms at the *avpr1a* locus in this population, we cannot exclude a minor contribution to the observed variation in pair bonding by variation at the microsatellite region in this gene. Although absence of polymorphic variation at the *avpr1a* locus is unlikely, it cannot be excluded.

As in previous studies, we saw considerable variation in V1aR density in the VP and RSC (Phelps and Young, 2003; Hammock et al., 2005). We predicted that V1aR density in these regions would correlate with social behaviours. However, variation in V1aR density did not correlate with partner preference. Our observations contrast previous studies, in which partner preference is associated with V1aR density in the VP, although Hammock et al. (2005) reported a strong negative correlation between V1aR density and partner preference (reviewed in Young et al., 2011). In contrast, affiliative behaviours, such as cuddling, licking/grooming, and carrying the pup, and affiliative and defensive behaviour towards a strange male, showed consistent correlations with V1aR density across the VP and RSC. However, our data show that there is no correlation between these social behaviours and partner preference based on differences in V1aR expression.

Despite the controlled environment under which these studies were performed, the substantial variation in social behaviours that we observed, as well as our inability to detect

heritability of these traits suggests a strong environmental component to pair bond formation. Genetic and neurobiological studies of prairie voles in naturalistic environments support our conclusions that environmental variance may play a larger role in expression of social behaviours than genetic variance. For example, space use, such as territory size and whether or not a male wanders off territory, was a greater correlate of *avpr1a* microsatellite polymorphisms and SNPs at the *avpr1a* locus and V1aR density at the RSC than pair bonding (Ophir et al., 2008; Okhovat et al., 2015). Although we were able to carefully control the laboratory environment in which these experiments were performed, we are unable to exclude the role of the social environment in influencing pair bonding-related behaviours in this study. For example, our study confined animals to standard laboratory caging, limiting the male's choice of mating strategy by only providing him with one possible mate. It is widely accepted that females play an equally important role in mating, yet this study did not account for female choice of mates or other male x female genotypic or phenotypic interactions.

In summary, we found substantial variation and non-significant heritability of behaviours related to monogamy among prairie voles, suggestive of a strong role for environmental plasticity of pair bonding-related behaviours and its neurobiological correlates. Future studies of prairie vole behaviour both in the laboratory and in natural populations will continue to resolve the complexity of social behaviours and help resolve the role of the environment in influencing the genetic and neurobiological underpinnings of these traits.

Materials and Methods

Animals. All animals were reared in house and housed in single-sex 0.3 x 0.3 x 0.2 m cages containing two to four individuals at the Biological Resources Facility at North Carolina State University (72°F, 30% average relative humidity). Food and water were provided ad libitum, with corncob bedding (Anderson Bed-o-cob, Granville Milling Co., Creedmoor, NC) and paper strips for nesting material. All rooms were on a 12-hour light/dark schedule (6AM-6PM lights on). Experimental animals were eight to twelve weeks old. Experimental protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC) and the resident veterinarian.

Mating. We injected female prairie voles with 0.1mL of 20μg/mL estradiol benzoate (Fisher BioReagents) once a day for the two days prior to mating in order to induce ovulation and receptivity towards males. We then paired the females with a sexually naïve male for 18 hours. After mating, paired males and females were housed together.

Behavioural assays. We subjected sexually naïve male prairie voles to three subsequent behavioural tests (open field test, alloparental care test, and resident intruder test), described in detail below. These tests were performed on the same day. Following the behavioural assays, we paired the males with an unrelated, sexually naïve female. After an 18-hour cohabitation period, males were tested in the partner preference test. The open field test, alloparental care test, and the resident intruder tests were repeated on the next day. The total

sample size consisted of 180 males. If an animal or its mate died during the procedure, both sibling experimental males were removed from analyses.

Partner preference test. The partner preference test measures social preferences (Slob et al., 1987; Williams et al., 1992; Ahern et al., 2009). In this test, the partner female and a stranger female were tethered at opposite ends of a 0.6 x 0.15 x 0.3 m box. The male was introduced in the middle of the cage and interactions were recorded for 3 hours. Testing was done in the morning and afternoon, at 8:00 AM and 12:00 PM, respectively. We used the same females for both sessions, and each male was tested in a single session. Between testing sessions, the corncob bedding was removed and the arena cleaned with 70% isopropyl alcohol. All tests were video recorded and scored using TopScan (version 3.00), as previously described (Ahern et al., 2009). We recorded the time each male spent alone, in social contact with his partner, or in social contact with the stranger female. At the end of the test, each male was returned to his cage with his partner female.

Open field test. The open field test evaluates anxiety-like behaviour (Archer, 1973). A single male vole was placed in an empty 0.6 x 0.6 x 0.6 m box. The vole was allowed to move naturally and was videotaped for 15 min, starting immediately when it was placed into the testing arena. All testing was done under overhead illumination, between the hours of 8:30 and 11:30 AM. We analysed the videos using TopScan (Clever Sys Inc., version 3.00, Reston, VA, 2011). Aggregated times spent in the centre or the edge were used for statistical

analyses. The edge was defined as the area 0.1 m from the edge of the box on all sides, and the centre was defined as the remaining inner area.

Alloparental care assay. The alloparental care assay evaluates parental behaviour towards an unfamiliar pup (Roberts et al., 1996). A single male vole was exposed to two 1-4 day old pups in a 0.3 x 0.3 x 0.2 m box with corncob bedding. The experimental vole had 5 min to interact with the pup unless the adult displayed aggressive behaviour, at which point the test ended. All testing was done under overhead illumination between the hours of 8:30 and 11:30 AM. Behaviour was videotaped and we used Stopwatch+ (version 1.5.1, 2003) to quantify the latency of time the animal took to approach the pup and the amount of time spent away from the pup, huddling/hovering over the pup, licking/grooming the pup, and carrying the pup (Kenkel et al., 2012). Aggressive behaviour was analysed in a binary format: 0 for no aggression, 1 for aggression.

Resident intruder test. The resident intruder assay measures aggressive behaviours toward an unfamiliar, unrelated male (Koolhaas et al., 2013). The experimental male was placed in a 0.5 x 0.15 x 0.2 m box with corncob bedding. After 1 min the "intruder", a sexually naive animal of the same sex, was placed into the box and interactions were video recorded for 5 min. The weights of both the experimental and "intruder" animals were recorded before testing and did not influence the outcome of the behavioural assays. After testing both animals were returned to their respective home cages. All testing was done under overhead illumination and between the hours of 8:30 and 11:30 AM. We analysed the videos using

Stopwatch (version 1.5.1, 2003) to determine the latency of time the experimental animal took to approach the intruder, the amount of time spent alone, and the amount of time the experimental animal displayed affiliative, defensive, or aggressive behaviour towards the intruder.

Autoradiography. We chose 60 males from across the partner preference distribution to examine V1aR density in the VP and RSC through autoradiography. Brains were removed from males at the end of the behavioural assays and flash-frozen on dry ice, then stored at -80°C. Six sets of 20µm thick coronal slices from the medial geniculate to the front of the ventral pallidum at 120µm intervals were mounted on Superfrost Plus slides (Fisher Scientific) and stored at -80°C. To visualize and quantify V1aR binding we used standard protocols for receptor autoradiography by using 125I-labeled linear vasopressin V1a receptor ligand ([125I]-Phenylacetyl-D-Tyr(M)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH2, PerkinElmer Scientific [NEX310]; (Insel et al., 1994; Young et al., 1997). Briefly, sections were lightly fixed in 0.1% paraformaldehyde in phosphate-buffered saline (pH 7.2) for 2 min at room temperature, then washed twice for 10 min in 50 mM Tris-HCl (pH 7.4). Slides were incubated at room temperature for 60 min in 50 pM 125I-antagonist in 50 mM Tris-HCl (pH 7.4) with 10 mM MgCl2 and 0.1% bovine serum albumin (radioimmunoassay grade, Sigma, St. Louis, MO). All the slices for a specific region were incubated for autoradiography using the same batch of buffer solution. Unbound ligand was removed by four washes in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2 buffer, and sections were air-dried. Sections were exposed to BioMax MR film (Kodak, Rochester, NY) for either 11 (VP) or 14 (RSC) days alongside

radioactive standards (American Radiolabeled Chemicals, Inc.). Neuroanatomic boundaries were defined using two rat brain atlases (Paxinos and Watson, 1998; Paxinos and Watson, 2006) and a mouse brain atlas (Paxinos and Franklin, 2013). High expression of V1aR results in high binding of radioactive ligand and can be measured by the optical density of film exposed to the tissue sections. We investigated VP and RSC V1aR by digitizing films and quantifying the standardized scans using MCID software. We examined 8 slices of the VP, spanning the entire region, and 7 slices of the RSC, spanning its rostral area. We estimated nonspecific binding from background levels of binding in the caudate putamen for the VP slices, and the stria terminalis for the RSC slices.

Statistical analyses. A paired t-test was used to determine if male open field behaviour, alloparental care, or aggression in the resident intruder assay changed after mating. To assess correlations between partner preference and male behaviours, we used the duration of each behaviour before and after mating (from the open field, alloparental care, and resident intruder assays) and the difference between the two and plotted these values against the time an experimental male spent with his partner during the partner preference test. Adjusted R2 and P-values were calculated for each scatterplot. All statistical analyses were performed in R (version 3.0.1, 2012).

Estimates of correlations between V1aR densities and behaviours. We used a Spearman's rank correlation test to determine significant correlations between V1aR densities and the duration of behaviours. V1aR density in the focal region of each brain section was correlated

separately with all behaviours. A P-value and a correlation value (R) were calculated for each correlation in R (version 3.0.1, 2012). A P-value of < 0.05 was considered significant.

Estimates of heritability. Heritabilities for all the traits were analysed using univariate linear analyses according to the following models:

$$y = 1\beta + \mathbf{Z}_m m + \mathbf{Z}_p p + \mathbf{Z}_s s + e \qquad (1)$$

where y is the vector of observations; β is the vector of systematic effects (intercept); m is the vector of random additive genetic effect of the male; p is the vector of random additive genetic effect of the partner; s is the vector of random additive genetic effect of the stranger; e is the vector of random residuals; 1 is a vector containing '1' entries and length equal to the number of records, and $\mathbf{Z}_m$, $\mathbf{Z}_p$, and $\mathbf{Z}_s$ are incidence matrices relating the corresponding effects to the dependent variable. Vector of solutions random additive genetic effects were assumed normally distributed with null mean and variance equal to the effect estimated variance, respectively $m \sim N(0, \mathbf{A}\sigma^2_m)$, $p \sim N(0, \mathbf{A}\sigma^2_p)$ and $s \sim N(0, \mathbf{A}\sigma^2_s)$, where **A** is a pedigree-derived relationship matrix (Henderson, 1984). Residuals were assumed uncorrelated $e \sim N(0, \mathbf{I}\sigma^2_e)$, where **I** is an identity matrix and σ2e is the residual variance.

Variance components σ^2_m, σ^2_p, σ^2_s and σ^2_e were estimated in a Bayesian framework using the Gibbs sampling algorithm as implemented in the MCMCglmm R package (Hadfield, 2010). Chains of 280,000 iterations were run, removing the first 30,000 iterations as burn-in and storing 1 sample every 25 iterations, leaving 10,000 samples for inference. Convergence was assessed by visual inspection of trace plots, number of effective samples and autocorrelation among subsequent samples, computed using functions that are built into

the package MCMCglmm. The posterior mean of the saved samples was used as estimate of the parameter, standard deviation was computed and used as standard error of the estimates.

Total phenotypic variance was computed as $\sigma^2_m + \sigma^2_p + \sigma^2_s + \sigma^2_e$, heritability for each additive genetic effect (male, partner and stranger) was computed as the ratio of the estimate for that variance on total phenotypic variance.

Data Availability Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

The authors would like to thank Caitlin Clement, Megan Serr, Caroline Leitschuh, Bryson Deanhardt, Elizabeth Dustin, Nicole Wehbie, Shannon Baksa, and Brian Horman for assistance with the behavioural experiments, and David Dorris and Drs. Robert Anholt, Trudy Mackay, John Meitzen, Jinyan Cao and Wen Huang for helpful discussions.

Contributions

ARV and LAM designed the experiments. ARV performed the experiments and compiled the data. SEA assisted with aspects of behavioural testing. FT performed analyses, ARV wrote the manuscript with editorial and technical input from all authors. HBP and LAM supervised the research.

Competing interests

The authors declare no competing financial interests.

Figure 2.1 Distribution histogram of pair bond formation in an outbred laboratory population of prairie voles.

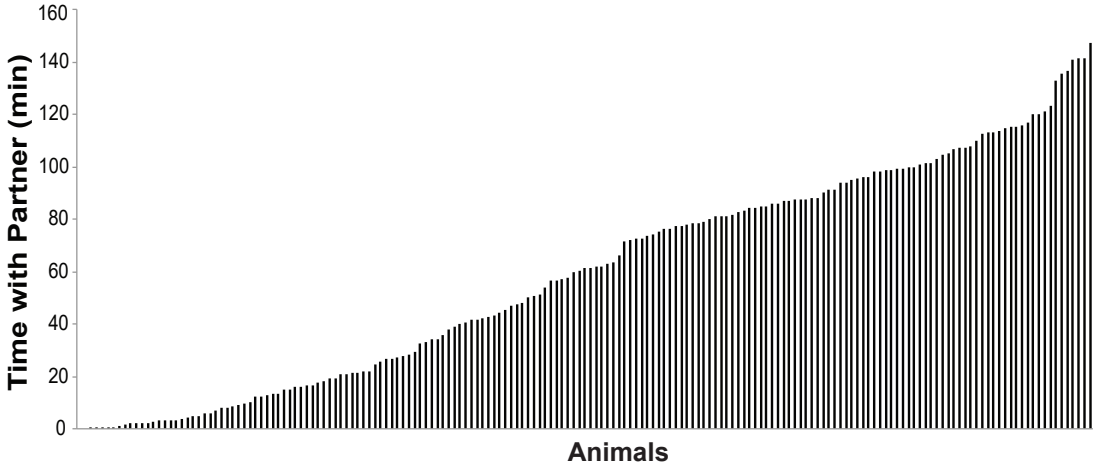

Figure 2.2 Association of variation in V1aR density in the ventral pallidum with affiliative behaviour. (A) A representative section showing low V1aR density in the VP (B) A representative section showing high V1aR density in the VP (C) A representative graph of the Spearman's rank correlation in section 1 of the VP against 2 significantly correlated behaviours. VP, indicated in the circled region, designates ventral pallidum, AC for anterior commissure, CP for caudate putamen, LS for lateral septum. Scale bar = 100 μm.

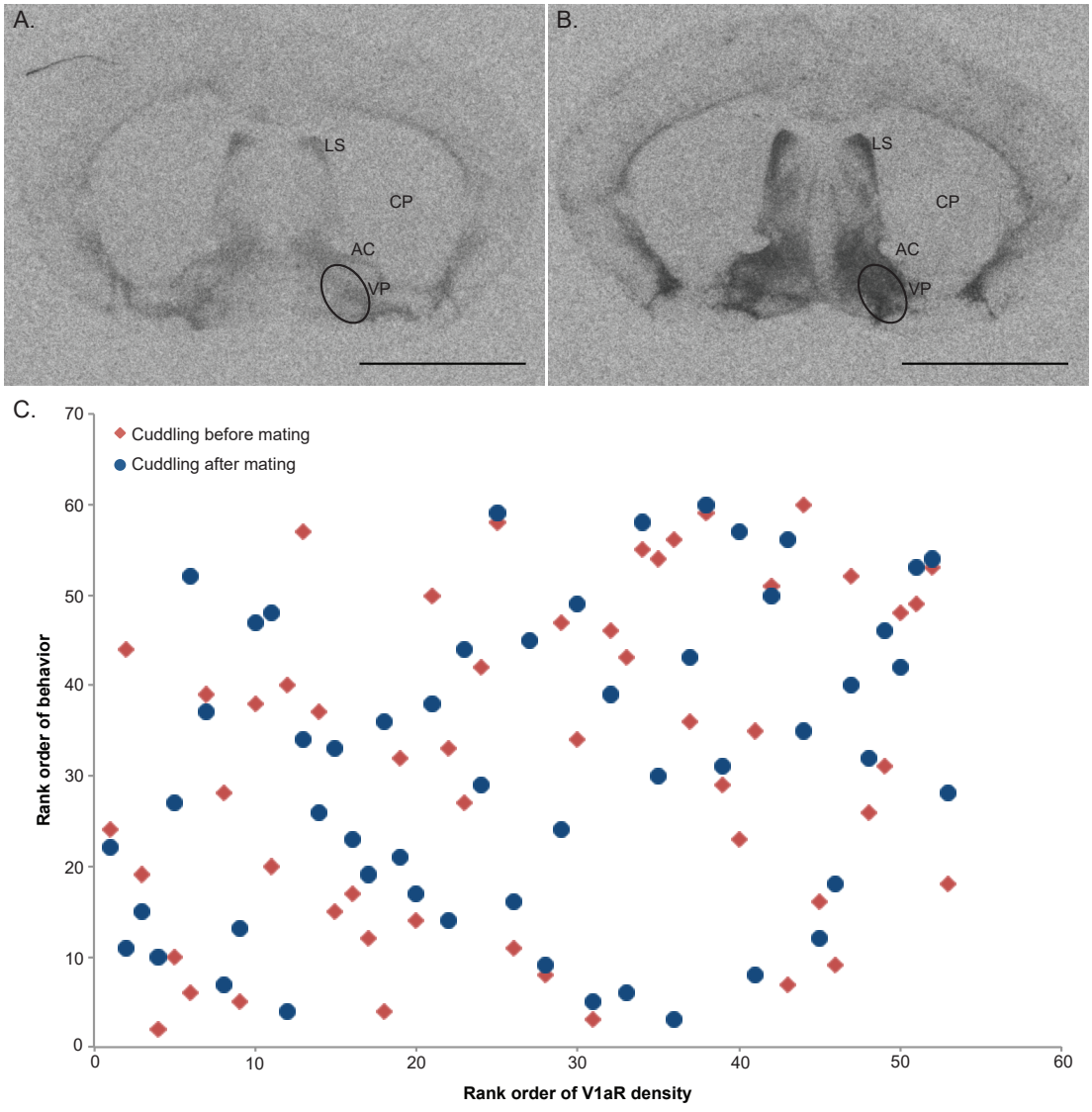

Figure 2.3 Association of variation in V1aR density in the RSC of male prairie voles with variation in affiliative behaviour. (A) A representative section showing low V1aR density in the RSC (B) A representative section showing high V1aR density in the RSC (C) A representative graph of the Spearman rank correlation for section 1 of the RSC against 2 significantly correlated behaviours. RSC, indicated by the circled region, designates retrosplenial cortex, HP for hippocampus. Scale bar = 100 µm.

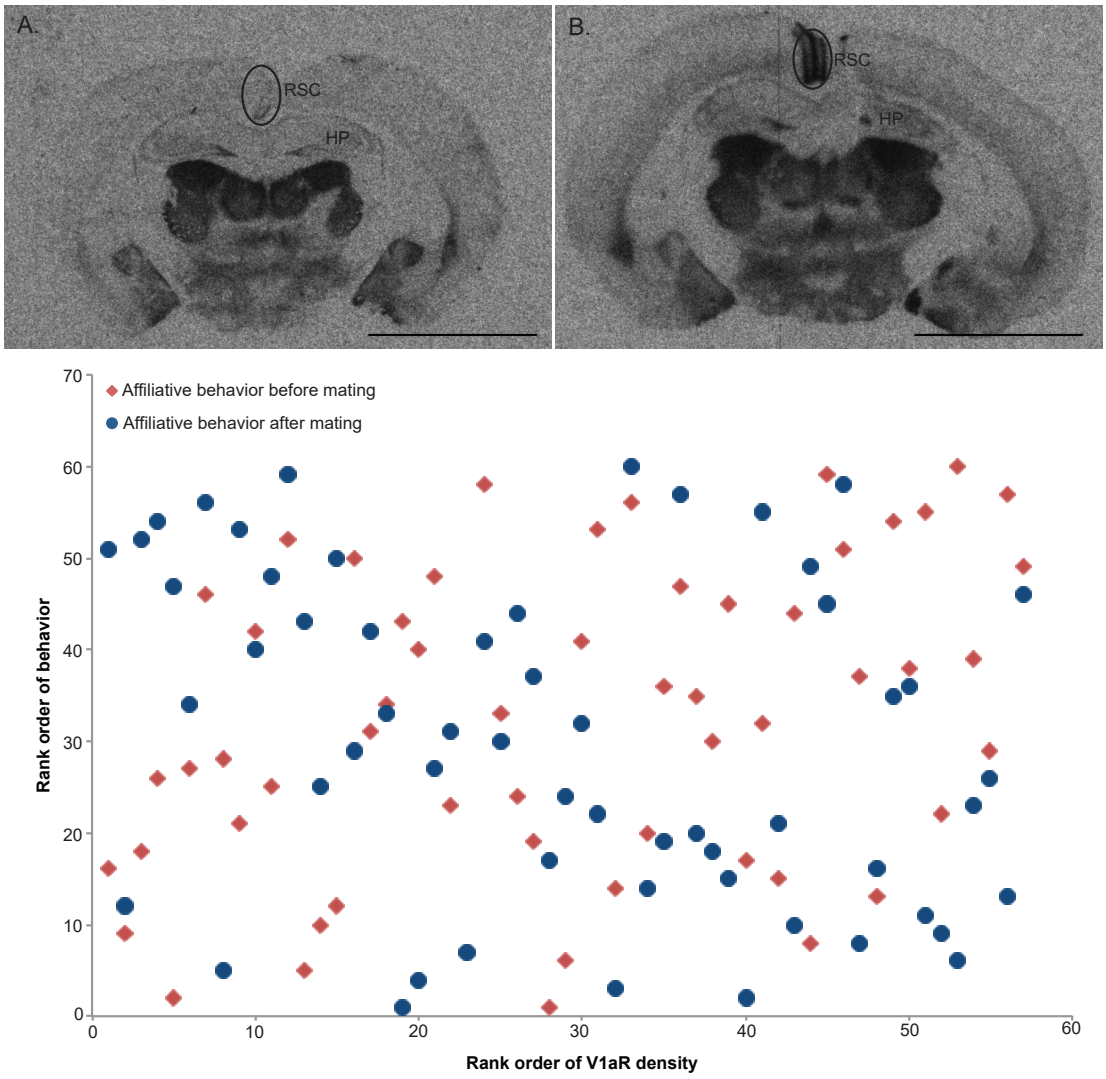

Figure 2.4 Experimental animal crossing scheme. Animals in the black boxes are the experimental males. Males were mated to unrelated full-sib females. Following behavioural testing of the parents (red male symbols), one male and one female were sacrificed and brains harvested. The remaining male and female were mated to produce the next generation offspring (purple male symbols).

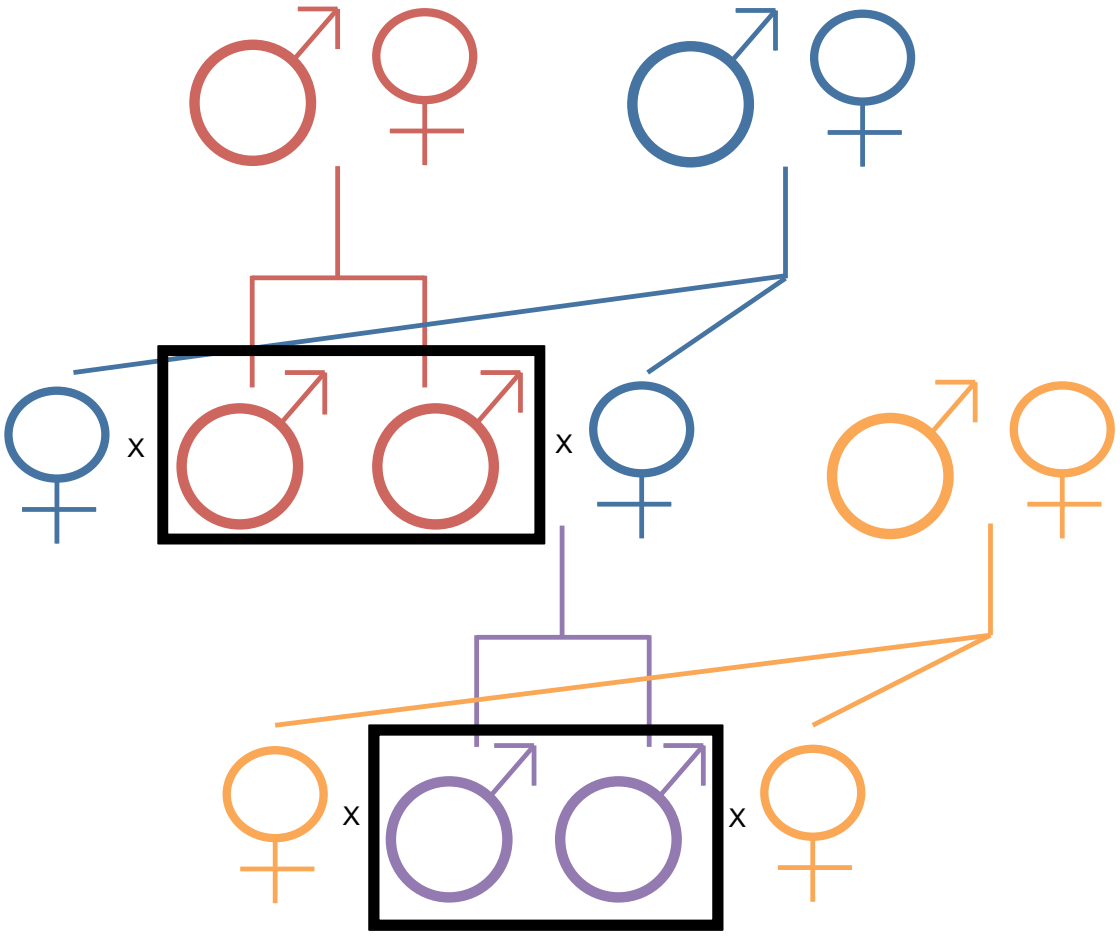

Table 2.1 Comparisons of time spent performing male behaviours before and after mating.

Behaviour	Before Mating (s, Mean ± SE)	After Mating (s, Mean ± SE)	P
Alloparental Care			
Latency to Approach	29 ± 4	21 ± 3	0.0753
Move Away	80 ± 5	80 ± 6	0.9555
Licking/Grooming	38 ± 3	30 ± 2	0.0048
Huddling/Hovering	142 ± 6	148 ± 7	0.3402
Carry	4 ± 1	3 ± 1	0.2442
Aggression	0.02 ± 0.01	0.07 ± 0.01	< 0.01
Resident Intruder			
Latency to Approach	19 ± 2	13 ± 1	< 0.01
Alone	205 ± 4	229 ± 3	< 0.0001
Non-aggressive	63 ± 3	27 ± 3	< 0.00001
Defensive	4 ± 1	3 ± 0.5	0.0204
Aggressive	8 ± 1	28 ± 2	< 0.00001
Open Field			
Centre	124 ± 6	113 ± 6	0.0713
Edge	779 ± 6	790 ± 6	0.0718

Supplementary Table S2.1 Narrow-sense heritability estimates.

Behavioral test	Male h^2 ± se	Partner h^2 ± se	Strange h^2 ± se
Time spent with partner	0.09 ± 0.10	0.16 ± 0.22	0.26 ± 0.27
Alloparental care before mating	0.08 ± 0.12	0.32 ± 0.26	0.13 ± 0.19
Alloparental care after mating	0.14 ± 0.17	0.31 ± 0.26	0.16 ± 0.23
Aggression towards a strange male before mating	0.03 ± 0.04	0.05 ± 0.09	0.19 ± 0.24
Aggression towards a strange male after mating	0.06 ± 0.11	0.07 ± 0.13	0.22 ± 0.28
Time in center before mating	0.30 ± 0.26	0.05 ± 0.10	0.26 ± 0.27
Time in center after mating	0.37 ± 0.18	0.04 ± 0.07	0.12 ± 0.16

Supplementary Table S2.2 Spearman's rank correlation tests between social behaviours and V1aR density in sections through the ventral pallidum. Highlighted squares are those with a significant p-value (<0.05).

	VP Sect 1		VP Sect 2		VP Sect 3		VP Sect 4		VP Sect 5		VP Sect 6		VP Sect 7		VP Sect 8	
Behaviors	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R
Time spent with partner in partner preference test	0.117	0.22	0.072	0.24	0.845	-0.03	0.822	0.03	0.082	0.23	0.136	0.20	0.060	0.24	0.597	0.07
Latency to approach pup before mating	0.875	0.02	0.783	0.04	0.177	0.18	0.258	0.15	0.912	-0.01	0.996	0.00	0.349	0.12	0.208	0.16
Latency to approach pup after mating	0.099	-0.23	0.182	-0.18	0.554	-0.08	0.705	0.05	0.109	-0.21	0.346	-0.12	0.861	0.02	0.926	-0.01
Latency to approach pup difference	0.202	-0.18	0.210	-0.17	0.144	-0.19	0.452	-0.10	0.207	-0.17	0.347	-0.12	0.351	-0.12	0.293	-0.14
Time away from pup before mating	0.256	-0.16	0.120	-0.21	0.407	-0.11	0.544	-0.08	0.867	-0.02	0.745	0.04	0.802	-0.03	0.367	0.12
Time away from pup after mating	0.489	-0.10	0.206	-0.17	0.822	-0.03	0.539	-0.08	0.364	-0.12	0.969	0.01	0.773	-0.04	0.748	0.04
Time away from pup difference	0.717	0.05	0.992	0.00	0.619	0.07	0.913	0.01	0.476	-0.09	0.745	-0.04	0.628	-0.06	0.702	-0.05
Licking/grooming pup before mating	0.185	0.18	0.121	0.21	0.770	-0.04	0.615	0.07	0.025	0.29	0.117	0.21	0.280	0.14	0.389	0.11
Licking/grooming pup after mating	0.342	0.13	0.038	0.27	0.152	0.19	0.839	0.03	0.179	0.18	0.069	0.24	0.129	0.20	0.560	0.08
Licking/grooming pup difference	0.586	-0.08	0.741	-0.04	0.211	0.17	0.820	-0.03	0.457	-0.10	0.748	-0.04	0.989	0.00	0.648	-0.06
Cuddling pups before mating	0.029	0.30	0.160	0.19	0.504	0.09	0.514	0.09	0.025	0.29	0.565	0.08	0.690	0.05	0.911	0.01
Cuddling pups after mating	0.024	0.31	0.002	0.40	0.348	0.13	0.547	0.08	0.007	0.35	0.047	0.26	0.128	0.20	0.799	0.03
Cuddling pups difference	0.790	0.04	0.069	0.24	0.518	0.09	0.689	0.05	0.205	0.17	0.055	0.25	0.077	0.23	0.422	0.11
Carrying pups before mating	0.723	-0.05	0.539	0.08	0.331	-0.13	0.348	-0.13	0.734	-0.05	0.276	0.14	0.963	0.01	0.080	-0.23
Carrying pups after mating	0.668	0.06	0.923	0.01	0.154	0.19	0.445	-0.10	0.336	-0.13	0.827	0.03	0.379	0.12	0.384	-0.11
Carrying pups difference	0.597	0.07	0.626	-0.07	0.029	0.29	0.515	0.09	0.530	-0.08	0.375	-0.12	0.602	0.07	0.339	0.13
Aggression towards pups before mating	0.477	-0.10	0.837	-0.03	0.976	0.00	0.791	0.04	0.322	-0.13	0.954	0.01	0.977	0.00	0.887	0.02
Aggression towards pups after mating	0.125	-0.21	0.179	-0.18	0.504	-0.09	0.928	-0.01	0.531	-0.08	0.282	-0.14	0.251	-0.15	0.815	-0.03
Latency to approach strange male before mating	0.416	-0.11	0.854	-0.02	0.141	-0.20	0.883	-0.02	0.269	-0.15	0.339	-0.13	0.566	-0.08	0.955	-0.01
Latency to approach strange male after mating	0.182	-0.19	0.495	-0.09	0.734	-0.05	0.773	0.04	0.533	-0.08	0.501	-0.09	0.256	-0.15	0.544	-0.08
Latency to approach strange male difference	0.706	-0.05	0.667	-0.06	0.420	0.11	0.869	-0.02	0.839	0.03	0.735	0.05	0.507	-0.09	0.338	-0.13

Table S2.2 continued

Time away from strange male before mating	0.736	-0.05	0.812	0.03	0.116	0.21	0.421	-0.11	0.446	-0.10	0.913	-0.01	0.817	0.03	0.386	-0.11
Time away from strange male after mating	0.697	-0.05	0.664	0.06	0.220	0.16	0.651	-0.06	0.194	-0.17	0.742	-0.04	0.538	0.08	0.886	-0.02
Time away from strange male difference	0.966	0.01	0.995	0.00	0.955	0.01	0.542	0.08	0.576	-0.07	0.629	-0.06	0.972	0.00	0.548	0.08
Affiliative behavior towards strange male before mating	0.859	0.02	0.945	-0.01	0.228	-0.16	0.580	0.07	0.211	0.17	0.419	0.11	0.898	-0.02	0.689	0.05
Affiliative behavior towards strange male after mating	0.485	0.10	0.921	-0.01	0.832	-0.03	0.474	0.10	0.656	0.06	0.640	-0.06	0.987	0.00	0.845	0.03
Affiliative behavior towards strange male difference	0.370	0.13	0.695	0.05	0.337	0.13	0.989	0.00	0.930	0.01	0.671	-0.06	0.609	0.07	0.807	-0.03
Defensive behavior towards strange male before mating	0.365	0.13	0.260	0.15	0.893	-0.02	0.539	0.08	0.389	0.11	0.473	0.10	0.996	0.00	0.032	0.28
Defensive behavior towards strange male after mating	0.802	-0.04	0.751	0.04	0.243	-0.16	0.377	-0.12	0.955	-0.01	0.368	0.12	0.289	-0.14	0.752	-0.04
Defensive behavior towards strange male difference	0.580	-0.08	0.312	-0.13	0.395	-0.11	0.620	-0.07	0.729	-0.05	0.776	-0.04	0.337	-0.13	0.228	-0.16
Aggression towards strange male before mating	0.401	0.12	0.382	0.12	0.639	0.06	0.741	0.04	0.204	0.17	0.185	0.17	0.610	0.07	0.710	0.05
Aggression towards strange male after mating	0.638	-0.07	0.961	-0.01	0.351	-0.12	0.829	-0.03	0.621	0.07	0.275	0.14	0.723	-0.05	0.280	-0.14
Aggression towards strange male difference	0.418	-0.11	0.697	-0.05	0.321	-0.13	0.843	-0.03	0.882	0.02	0.472	0.10	0.891	-0.02	0.566	-0.08
Time spent in center before mating	0.354	0.13	0.980	0.00	0.289	-0.14	0.883	0.02	0.717	0.05	0.500	0.09	0.980	0.00	0.199	0.17
Time spent in center after mating	0.257	0.16	0.682	-0.06	0.806	-0.03	0.987	0.00	0.833	0.03	0.670	0.06	0.755	0.04	0.251	0.15
Time spent in center difference	0.789	0.04	0.328	-0.13	0.793	0.04	0.745	-0.04	0.607	0.07	0.884	0.02	0.985	0.00	0.893	0.02
Time spent in edge before mating	0.348	-0.13	0.974	0.00	0.313	0.13	0.874	-0.02	0.730	-0.05	0.500	-0.09	0.946	-0.01	0.196	-0.17
Time spent in edge after mating	0.273	-0.16	0.641	0.06	0.772	0.04	0.994	0.00	0.899	-0.02	0.722	-0.05	0.756	-0.04	0.258	-0.15
Time spent in edge difference	0.775	-0.04	0.309	0.14	0.792	-0.04	0.739	0.05	0.642	-0.06	0.891	-0.02	0.930	-0.01	0.912	-0.01

Supplementary Table S2.3 Spearman's rank correlation tests between social behaviours and V1aR density in sections through the rostral part of the retrosplenial cortex.

Highlighted squares are those with a significant p-value (<0.05).

	RSC Sect 1		RSC Sect 2		RSC Sect 3		RSC Sect 4		RSC Sect 5		RSC Sect 6		RSC Sect 7	
Behaviors	P	R	P	R	P	R	P	R	P	R	P	R	P	R
Time spent with partner in partner preference test	0.399	0.11	0.972	0.00	0.978	0.00	0.486	0.10	0.193	0.17	0.383	0.12	0.279	0.15
Latency to approach pup before mating	0.987	0.00	0.708	0.05	0.943	-0.01	0.681	-0.06	0.645	-0.06	0.951	-0.01	0.642	-0.06
Latency to approach pup after mating	0.762	0.04	0.920	-0.01	0.826	-0.03	0.747	0.04	0.923	-0.01	0.894	0.02	0.665	-0.06
Latency to approach pup difference	0.562	0.08	0.980	0.00	0.920	0.01	0.441	0.11	0.607	0.07	0.721	0.05	0.785	0.04
Time away from pup before mating	0.115	0.21	0.590	0.07	0.710	0.05	0.286	0.15	0.074	0.24	0.080	0.23	0.043	0.27
Time away from pup after mating	0.225	-0.16	0.271	-0.15	0.170	-0.19	0.110	-0.22	0.126	-0.20	0.110	-0.21	0.510	-0.09
Time away from pup difference	0.002	-0.40	0.084	-0.23	0.038	-0.28	0.006	-0.36	0.001	-0.41	0.001	-0.42	0.007	-0.35
Licking/grooming pup before mating	0.007	0.36	0.001	0.42	0.008	0.35	0.028	0.30	0.020	0.30	0.010	0.33	0.032	0.28
Licking/grooming pup after mating	0.415	0.11	0.071	0.24	0.063	0.25	0.602	0.07	0.184	0.18	0.180	0.18	0.043	0.27
Licking/grooming pup difference	0.205	-0.17	0.317	-0.14	0.479	-0.10	0.303	-0.14	0.422	-0.11	0.386	-0.12	0.695	-0.05
Cuddling pups before mating	0.636	-0.06	0.708	-0.05	0.939	-0.01	0.888	-0.02	0.502	-0.09	0.507	-0.09	0.369	-0.12
Cuddling pups after mating	0.445	0.10	0.399	0.11	0.323	0.13	0.173	0.19	0.080	0.23	0.265	0.15	0.122	0.21
Cuddling pups difference	0.176	0.18	0.168	0.19	0.211	0.17	0.168	0.19	0.055	0.25	0.103	0.22	0.025	0.30
Carrying pups before mating	0.337	0.13	0.035	0.28	0.105	0.22	0.363	0.13	0.079	0.23	0.026	0.29	0.142	0.20
Carrying pups after mating	0.777	0.04	0.583	0.07	0.864	0.02	0.777	-0.04	0.948	0.01	0.780	0.04	0.853	0.03
Carrying pups difference	0.646	-0.06	0.184	-0.18	0.131	-0.20	0.179	-0.18	0.104	-0.22	0.147	-0.19	0.318	-0.13
Aggression towards pups before mating	0.810	-0.03	0.784	0.04	0.879	-0.02	0.663	-0.06	0.535	-0.08	0.658	-0.06	0.400	-0.11
Aggression towards pups after mating	0.975	0.00	0.826	0.03	0.975	0.00	1.000	0.00	0.832	-0.03	1.000	0.00	0.192	-0.18
Latency to approach strange male before mating	0.632	0.06	0.847	0.03	0.233	0.16	0.340	0.13	0.722	0.05	0.662	0.06	0.267	0.15
Latency to approach strange male after mating	0.801	0.03	0.875	0.02	0.246	0.16	0.471	0.10	0.383	0.12	0.477	0.10	0.109	0.21
Latency to approach strange male difference	0.756	-0.04	0.936	0.01	0.876	-0.02	0.577	-0.08	0.733	0.05	0.826	0.03	0.945	0.01
Time away from strange male before mating	0.119	-0.21	0.458	-0.10	0.320	-0.14	0.198	-0.18	0.154	-0.19	0.129	-0.20	0.030	-0.29

Table S2.3 continued

Time away from strange male after mating	0.223	0.16	0.501	0.09	0.357	0.13	0.715	0.05	0.449	0.10	0.427	0.11	0.781	0.04
Time away from strange male difference	0.112	0.21	0.419	0.11	0.345	0.13	0.448	0.10	0.195	0.17	0.198	0.17	0.199	0.17
Affiliative behavior towards strange male before mating	0.007	0.35	0.109	0.22	0.111	0.22	0.029	0.29	0.018	0.31	0.024	0.30	0.009	0.34
Affiliative behavior towards strange male after mating	0.016	-0.32	0.101	-0.22	0.029	-0.29	0.077	-0.24	0.019	-0.31	0.062	-0.25	0.168	-0.19
Affiliative behavior towards strange male difference	0.001	-0.43	0.027	-0.29	0.015	-0.32	0.008	-0.36	0.002	-0.39	0.007	-0.35	0.005	-0.36
Defensive behavior towards strange male before mating	0.761	-0.04	0.552	0.08	0.838	0.03	0.416	-0.11	0.619	-0.07	0.964	0.01	0.828	0.03
Defensive behavior towards strange male after mating	0.664	0.06	0.344	0.13	0.788	0.04	0.910	-0.02	0.945	0.01	0.761	0.04	0.801	0.03
Defensive behavior towards strange male difference	0.834	0.03	0.943	-0.01	0.907	-0.02	0.644	0.06	0.935	0.01	0.832	-0.03	0.798	-0.03
Aggression towards strange male before mating	0.128	-0.20	0.477	-0.10	0.321	-0.14	0.050	-0.27	0.155	-0.19	0.714	-0.05	0.805	-0.03
Aggression towards strange male after mating	0.716	0.05	0.328	0.13	0.249	0.16	0.379	0.12	0.254	0.15	0.589	0.07	0.421	0.11
Aggression towards strange male difference	0.528	0.09	0.355	0.13	0.269	0.15	0.161	0.19	0.168	0.18	0.527	0.08	0.369	0.12
Time spent in center before mating	0.520	0.09	0.410	0.11	0.454	0.10	0.496	0.09	0.541	0.08	0.526	0.08	0.526	0.09
Time spent in center after mating	0.852	0.03	0.537	-0.09	0.751	-0.04	0.595	-0.08	0.740	-0.05	0.489	-0.10	0.531	-0.09
Time spent in center difference	0.673	-0.06	0.307	-0.14	0.368	-0.13	0.307	-0.14	0.382	-0.12	0.411	-0.11	0.386	-0.12
Time spent in edge before mating	0.535	-0.08	0.435	-0.11	0.485	-0.10	0.512	-0.09	0.536	-0.08	0.543	-0.08	0.546	-0.08
Time spent in edge after mating	0.838	-0.03	0.546	0.08	0.751	0.04	0.589	0.08	0.755	0.04	0.496	0.09	0.546	0.08
Time spent in edge difference	0.690	0.06	0.321	0.14	0.382	0.12	0.299	0.15	0.396	0.12	0.429	0.11	0.416	0.11

CHAPTER 3: NO EVIDENCE THAT SEMINAL VESICLE PROTEINS AFFECT PAIR BONDING AND RELATED BEHAVIORS IN PRAIRIE VOLE (*MICROTUS OCHROGASTER*) FEMALES OR THEIR OFFSPRING

Andrea Vogel*, Caitlin Clement, Lisa McGraw

Department of Biological Sciences, Program in Genetics, and W.M. Keck Center for Behavioral Biology

North Carolina State University, Raleigh, NC 27695

* Corresponding author: arvogel@ncsu.edu

Introduction

Seminal fluid proteins (SFPs), a component of seminal fluid, are produced in male reproductive tract secretory tissues, transferred from the male to the female during mating, and include many classes of proteins (reviewed in Poiani, 2006; Avila et al., 2011; Hopkins et al., 2017). While SFPs have been studied in a wide variety of organisms, studies in insects, particularly *Drosophila melanogaster*, have indicated that SFPs, independent of other components of the ejaculate or mating, are important not only for contributing to sperm health, sperm viability, and fertilization, but also affect female physiology and behavior. For example, in *D. melanogaster*, SFPs decrease a female's probability of re-mating, increase

ovulation, decrease her need to sleep and eat, and even decrease her lifespan (reviewed in Avila et al., 2011; Hopkins et al., 2017).

Like insects, male mammals also transfer SFPs to their mates. Whether or not these proteins affect the behavior and physiology of the female has been largely unexplored, yet a few intriguing studies have suggested that SFPs are more than just a passive medium for sperm transfer. For example, in species that have induced ovulation, such as llamas (*Lama glama*) and alpacas (*Vicugna pacos*), a SFP, ovulation-inducing factor (OIF), has been demonstrated to produce a surge of luteinizing hormone (LH) from the pituitary gland in llamas that stimulates ovulation (Paolicchi et al., 1999; Adams et al., 2005; Bogle et al., 2012). OIF was found to be beta-nerve growth factor, which is a highly conserved protein found in both spontaneous and induced ovulators (Ratto et al., 2012). In a second example of SFPs playing a role in more than sperm support, Bromfield et al. (2014a) demonstrated transgenerational effects of seminal vesicle proteins (SVPs) in mice (*Mus musculus*). Male mice whose sires had their seminal vesicles (SVs) surgically removed at adulthood, and subsequently did not transfer SVPs, weighed more and showed symptoms of metabolic syndrome compared to male conspecifics born to fathers with intact SVs (Bromfield et al., 2014a). SFP include cytokines, which interact with uterine epithelial cells and induce an inflammatory response, which has been hypothesized to mediate downstream effects in the female reproductive tract {Bromfield, 2014}. As pregnancy requires a change in immune tolerance to the embryo, it may be that the cytokines present in seminal fluid direct the quality of the future immune response {Bromfield, 2014}. These studies provide convincing evidence that some mammalian SFPs are capable of providing more than sperm support;

however, these potential functions have been largely unexplored (McGraw et al., 2015, but see Bromfield 2014b)).

Prairie voles (*Microtus ochrogaster*) are socially monogamous rodents that form long-lasting social attachments, or pair bonds. Following mating, pair bonded males not only contribute to offspring care, but also defend their nests and territories by displaying increased aggression towards same-sex intruders. In rats, vaginocervical stimulation during coitus produces a surge of oxytocin (OT) (Sansone et al., 2002). In prairie voles the release of OT facilitates pair bonding, but whether SFPs also influence pair bonding or affect copulation, pregnancy, female post-mating behavior, offspring body weight, or offspring behavior (such as described in the studies above) in prairie voles was unexplored (Williams et al., 1994). However, we found no evidence that SVPs affected the behavioral traits we measured in the male's mate and his offspring, nor did we observe a transgenerational effect of SVPs on male offspring weight as was observed in mice (Bromfield et al., 2014).

Materials and Methods

Animals and Housing. Experimental protocols were approved by and performed in accordance with the North Carolina State University Institutional Animal Care and Use Committee (IACUC) and in consultation with the resident veterinarian. Animals were housed in single-sex 0.3 x 0.3 x 0.2 m cages containing two to four adult individuals at the Biological Resources Facility at North Carolina State University (72°F, 30% average relative humidity). Food and water were provided ad libitum, with corncob bedding (Anderson Bed-o-cob, Granville Milling Co., Creedmoor, NC) and paper strips for nesting material, with

extra nesting material provided to breeders. All rooms were on a 12-hour light/dark schedule. Experimental animals were eight to twelve weeks old and sexually mature.

Surgical manipulation of seminal vesicles. All cages had at least two animals that underwent surgery. Experimental males were anesthetized with isoflurane in oxygen gas. Because in rodents, SVs are the main contributor of SFPs (Druart et al., 2013), we removed the SVs by placing a small vertical incision (~0.5 cm) on the lower abdomen of the male prairie vole and the SVs were exposed and brought out of the incision. Males undergoing the experimental treatment (SVX males) had a suture tied tightly around the base of the SV, and the SV was then snipped off (Procedure modified from Birkhauser et al., 2012). This procedure was repeated on the other SV. The remaining tissues were placed back into the incision. For males in the sham procedure (SHM), the SVs were brought out of the incision, and then replaced without further manipulation. All males then had the incision sutured closed. Each surgery took less than 20 minutes from initially putting the vole under to when it woke up in the recovery cage. Males were allowed to recover for ten days, at which time there were no external or behavioral signs of surgery.

Mating. To induce estrus and receptivity to males, females were subcutaneously injected with 0.1 mL of 20 μg/mL estradiol benzoate (Fisher BioReagents) once a day for two consecutive days prior to mating. Pairs consisting of unrelated, sexually naïve male and female voles were placed in together in a clean cage and the number of mountings that occurred in the first hour was hand-scored by researchers who were blind to male condition.

Behavioral Tests. Pairs were placed together for 18 hours to induce pair bonding in prairie voles (Williams et al., 1992; Insel et al., 1995). Following cohabitation females were tested in a partner preference test (described below). The following day, we subjected mated female prairie voles to three subsequent behavioral tests (open field test, alloparental care test, and resident intruder test), described in detail below. After behavioral testing, pairs were allowed to breed. Up to two adult offspring of each sex were also subjected to the behavioral tests at PND 70-77.

Partner preference test. Pair bonding was tested through the use of the partner preference test (Slob et al., 1987; Williams et al., 1992; Ahern et al., 2009). In this test, the female's male partner and a stranger male (a male who underwent 18 hours of cohabitation with a different female, unrelated to the partner male or the experimental female) were tethered at opposite ends of 0.6 x 0.2 x 0.2 m box. The female was placed in the middle of the arena. Interactions were video recorded for three hours and scoring was performed using TopScan (version 3.00), as previously described (Ahern et al., 2009). We analyzed how much time the female spent alone, in social contact with her partner, and in social contact with the stranger male.

Open field test. The open field test assesses anxiety-like behavior (Archer, 1973). This assay uses an empty 0.6 x 0.6 x 0.6 m box, into which a prairie vole is placed and allowed to move naturally. Each test was video recorded for 15 minutes from the time the vole was placed into the arena, under overhead illumination. Videos were analyzed using TopScan (version 3.00,

2013). Total time spent in the center or the edge were used for statistical analyses. The edge was defined as the area 0.1 m from the edge of the box on all sides, and the center was everything else.

Alloparental care assay. The alloparental care test evaluates parental behavior towards an unfamiliar pup (Roberts et al., 1996). In this behavioral assay, an adult vole inexperienced with pups was exposed to a single 1-4 day old pup in a 0.3 x 0.3 x 0.2 m box with corncob bedding. The experimental animal had five minutes to interact with the pup, unless it displayed aggressive behavior, at which point the test ended. Behaviors were videotaped for five minutes, and analyzed at a later date using Stopwatch+ (version 1.5.1, 2003) to quantify the latency of time the adult took to approach the pup, the amount of time spent away from the pup, huddling/hovering over the pup, licking/grooming the pup, and carrying the pup. Aggressive behavior was analyzed in a binary format: 0 for no aggression, 1 for aggression.

Resident intruder test. The resident intruder assay measures aggressive behaviors toward an unfamiliar, unrelated conspecific (Koolhaas et al., 2013). The experimental animal was placed into a 0.5 x 0.15 x 0.2 m box with corncob bedding. After one minute the "intruder", a conspecific of the same sex not included elsewhere in the study, was placed into the box and video recording commenced for five minutes under overhead illumination. After testing, both animals were placed into their respective home cages. The video was analyzed at a later date using Stopwatch+ (version 1.5.1, 2003) and quantified the latency of time the experimental animal took to approach the intruder, the amount of time spent alone, and the amount of time

the experimental animal displayed affiliative, defensive, or aggressive behavior towards the intruder.

Weights. Offspring were weighed at three times: birth, weaning (PND 21), and adult (PND 70-77). The weight of the litter was averaged because sex of the pup can not be determined at birth. At weaning age, all offspring were weighed and their weights recorded. At weaning, sex can be determined, so weight was averaged by sex and litter. Individual weight for each animal could not be tracked. At adulthood, only the offspring who went through behavioral testing (maximum two males and two females from each litter) were weighed. Offspring were chosen at random for testing, so the change in weight between weaning and adulthood should be indicative of all animals in the litter.

Statistics. A one-way ANOVA was performed to see if there were significant differences between the SV removed, sham, and control groups for mountings in the first hour, all behavioral tests, time to first litter, and the weights of the offspring at birth, weaning, and adulthood. All statistics were performed in R (version 3.0.1, 2012).

Results

Mountings were not affected by treatment. Using a one-way ANOVA of mountings in the first hour against treatment, there was a significant difference in the number of mountings in the first hour of pairings ($F_{2, 0.05} = 4.470$, $p = 0.0177$), however, post-hoc Tukey test showed there were no significant differences between any of the treatments.

Behavioral Tests.

Adult Females:

Time spent with partner was not affected by treatment: Time spent alone in the partner preference test was tested using a one-factor ANOVA against treatment. There was no significant difference in time spent alone due to treatment ($F_{2, 0.05} = 0.536$, $p = 0.588$). Time spent with partner or stranger were tested similarly, and no significant differences between treatment groups were found ($F_{2,0.05} = 0.863$, $p = 0.428$, $F_{2, 0.05} = 0.582$, $p = 0.562$, respectively).

Anxiety-like behaviors were not affected by treatment: Using a one-factor ANOVA against treatment, we tested the amount of time the experimental female spent in either the center or the edge of the open field arena. Open field behaviors were not significantly different between treatment groups ($p > 0.05$ for both).

Alloparental care was not affected by treatment: Using a one-factor ANOVA against treatment, we tested the behaviors as listed above. Time spent licking/grooming and carrying the pup were significantly different due to treatment ($F_{2, 0.05} = 5.7593$, $p = 0.005453$, $F_{2, 0.05} = 3.235$, $p = 0.0473$, respectively). No other behaviors were significantly different between treatment groups ($p > 0.05$).

Resident intruder behavior was not affected by treatment: Using a one-factor ANOVA against treatment, we tested the amount of time the experimental female spent to approach the intruder female, away from the intruder, non-aggressive, defensive, and

aggressive behavior. Behaviors were not significantly different between treatment groups ($p > 0.05$).

Offspring:

Open field: Using a one-factor ANOVA against the male parent's treatment, we tested anxiety-like behaviors. Neither time spent in the center or edge of the arena was different due to treatment ($F_{2,0.05} = 0.672$, 0.678, respectively, $p > 0.05$).

Alloparental care behavior: Using a one-factor ANOVA against treatment, we tested behaviors associated with caring for a pup. Time spent licking/grooming the pup were significantly different due to treatment, and a post-hoc Tukey HSD test showed a significant difference between offspring of control and surgery groups ($F_{2,0.05} = 4.747$, $p = 0.0103$; $p = 0.0114$). No other measured behaviors were significantly different between treatment groups ($p > 0.05$).

Resident intruder behavior was not affected by treatment: Using a one-factor ANOVA against treatment, we tested the amount of time each offspring spent to approach the conspecific intruder, away from the intruder, non-aggressive, defensive, and aggressive behavior. No behaviors were significantly different between treatment groups ($p > 0.05$).

First litter metrics were not affected by treatment: Using a one-way ANOVA of days before litter against treatment, there was no significant difference between the number of days to the first litter by treatment ($F_{2,0.05} = 0.911$, $p = 0.4103$). There was no significant difference in the number of days to the first litter by the number of copulations in the first

hour ($F_{2,0.05}$ = 1.3729, p = 0.2481). There was also no significant difference between the number of pups in the litter by treatment ($F_{2, 0.05}$ = 0.011, p = 0.989).

Offspring weight was not affected by treatment: Using a two-factor ANOVA of weight at birth by treatment and the number of pups in the litter, there was no interaction between the two ($F_{5,0.05}$ = 1.3006, p = 0.2845). There was no significant difference on the weight of the pups at birth by treatment ($F_{2, 0.05}$ = 1.0335, p = 0.3651); instead, the weight of the pups at birth was dependent on the number of pups in the litter ($F_{5, 0.05}$ = 22.306, p = 2.722 x 10^{-5}). There was no difference in the weight at weaning due to treatment ($F_{2, 0.05}$ = 1.338, p = 0.2739). Using a two-factor ANOVA of weight at weaning by treatment and number born in the litter, there was no significant interaction between the two ($F_{5, 0.05}$ = 0.0695, p = 0.933). The weight at weaning was significantly different due to the number of pups in the litter ($F_{5, 0.05}$ = 24.635, p = 1.262 x 10^{-5}). There was no significant difference of the weight at adulthood in either males or females, respectively ($F_{2, 0.05}$ = 1.725, p = 0.19; $F_{2,0.05}$ = 1.725, p = 0.19).

Discussion

In this study we set out to examine if SVPs would affect copulation, time to pregnancy, fertility, and social behaviors of either the female parent or her offspring, or weight of the offspring. We found no effect of SVPs on any metric measured. However, though we did find significant differences in licking and grooming or carrying of pups during the alloparental care test, these differences in behavior were between females mated to

control males and females mated to SHM or SVX males, or in offspring of control males and offspring of SVX males. This is not conclusive of effects of SVPs, but likely an effect of surgery or anesthesia. The question of how the surgery impacted the males, and in turn impacted the behavior of the mated females and offspring, is one that we can not answer at this time.

We saw no differences in the number of copulations in the first hour, time to pregnancy, or fertility due to SVPs. Other studies have also reported no differences in copulation or time to get pregnant in rodents when SVs were removed (Carballada and Esponda, 1993; Birkhauser et al., 2012). This result suggests that SVPs in rodents may not be essential for fertilization. For example, SFPs produced by the coagulatory glands, but not the SVs, are necessary for fertility in rats (Carballada and Esponda, 1993).

Prairie voles, however, differ from rats and mice, in that they are induced ovulators. In other induced ovulators, such as camels, alpacas, and llamas, a SFP, ovulation-inducing factor/nerve growth factor (OIF/NGFβ), stimulates LH production in the pituitary gland, which triggers ovulation (Paolicchi et al., 1999; Adams et al., 2005; Bogle et al., 2012). As female prairie voles are also induced ovulators, we hypothesized that as a result of removing the SVs, the female prairie voles would not ovulate. However, we saw no difference in time to pregnancy between groups. Future work should examine LH in prairie voles, to see if a surge is detected after mating.

We were interested in examining if SVPs contribute to offspring weight because a recent study in mice found metabolic differences in male offspring of SV removed fathers (Bromfield et al., 2014). We did not see any differences in the weights of the offspring at

birth, weaning, or ten week old adults. However, Bromfield et al. (2014) did not find significant differences in the weight of male offspring until 14 weeks. As our voles were sacrificed at ten weeks, we do not know if some animals would show a rapid weight gain. Future work should allow the voles to live for longer, so their weight can be tracked over additional weeks. Also, it would be useful to subject mice to the same experiment at the same time, to ensure that Bromfield's results are seen across laboratories.

Our results also show no effect of the removal of SVs on pair bond formation or other behaviors measured in the female and her offspring. This does not rule out the possibility that SVPs, or other SFPs from the coagulating glands, prostrate, and epididymis could affect the female's behavior. The role of SFPs in mammals is still largely understudied, however this study suggests that prairie vole SVPs may not play a detectable role in social behaviors, fertility, or early growth of offspring. In the future, determining SFP composition from the removed, sham, and control groups of prairie voles could inform us of which SFPs are produced in the SV, and if the composition of the SFP is different among the groups. If the composition is different, we could look at homologous proteins in other species to make hypotheses about what we expect to be different after removing the SV of prairie voles.

This was the first experiment, to our knowledge, to examine the effects of SVPs on behaviors in mated prairie vole females and offspring. Ultimately we found no difference between offspring from sires that had intact SVs, and transferred SVPs, and offspring from sires that were unable to transfer SVPs due to surgical removal of the SVs. Our results do not necessarily preclude a potential role of SVPs in modifying other behaviors in the female and offspring, nor do our results resolve the potential roles of SFPs from accessory glands.

Acknowledgements

The authors would like to thank Shri Beyagudem, Bryson Deanhardt, and Nicole Wehbie for helping to conduct behavioral assays and Hadley Bryan for scoring videos.

Table 3.1 Reproduction is not influenced by seminal vesicles proteins.

Test (by treatment)	F test	p value
Mountings in 1st hr	4.470	0.0177
Days to 1st litter	0.911	0.4103
# of pups in litter	0.0111	0.989
Weight at birth (litter ave)	1.0335	0.3651
Weight at weaning (litter ave)	1.338	0.2739
Weight at adult (male)	1.725	0.19
Weight at adult (female	1.725	0.19

Table 3.2 Female behaviors are not influenced by seminal vesicle proteins.

Test	Behavior	F test	p value
Alloparental Care	Latency to Approach	2.178	0.123
	Move Away	0.233	0.793
	Licking/Grooming	5.760	0.005
	Huddling/Hovering	2.179	0.123
	Carrying	3.235	0.047
Resident Intruder	Latency to Approach	1.091	0.343
	Alone	0.035	0.966
	Non-Aggressive	0.725	0.489
	Defensive	0.595	0.555
	Aggressive	1.791	0.177
Partner Preference	Alone	0.536	0.588
	Partner	0.863	0.428
	Stranger	0.582	0.562
Open Field	Center	0.546	0.583
	Edge	0.528	0.593

Table 3.3 Offspring behaviors are not affected by seminal vesicle proteins.

Test	Behavior	F test	p value
Alloparental Care	Latency to Approach	1.201	0.304
	Move Away	0.318	0.728
	Licking/Grooming	4.747	0.010
	Huddling/Hovering	1.363	0.26
	Carrying	0.665	0.516
	Aggression		>0.05
Resident Intruder	Latency to Approach	0.327	0.721
	Alone	2.024	0.136
	Non-Aggressive	2.527	0.084
	Defensive	0.629	0.535
	Aggressive	0.521	0.595
Open Field	Center	0.672	0.513
	Edge	0.678	0.510

CHAPTER 4.

My dissertation set out to determine the natural variation in pair bonding and related behaviors in a laboratory population of prairie voles (*Microtus ochrogaster*). The primary goal was to determine underlying neurogenetic mechanisms of social behaviors, and of pair bonding, in particular. I examined vasopressin 1a receptor (V1aR) density in areas of the brain known to contribute to variation in male social behaviors, including pair bonding. Also, I examined if seminal vesicle proteins would influence pair bonding in females, by measuring the amount of time a female spent with her male partner in the partner preference test. Here in this concluding chapter, I will address two primary aspects of this work. First, I will document the key findings of my dissertation. Second, I will discuss the future directions that my studies generate for understanding social behavior from the proximate to ultimate levels of biological analysis.

Key Findings

I set out to investigate six main hypotheses in my work: 1) that there is quantifiable natural variation in male pair bonding behavior that can be measured using the partner preference test, 2) that mating will alter the expression of social behaviors, 3) that partner preference is correlated with other behaviors (anxiety-like behaviors, alloparental care, and aggression towards same sex intruders), 4) that social behaviors, particularly partner preference, are heritable, 5) that V1aR density is correlated with partner preference, and 6) the possibility that seminal vesicle proteins can affect partner preference. I will briefly review my findings here, and more information can be found in the previous chapters.

Overall, I found that there was much more variation in partner preference within our laboratory population of prairie voles than has been previously reported. As discussed in the introductory chapter, most of our understanding of the different mating strategies employed by prairie voles has come from semi-naturalistic and field studies {Getz et al., 1981; Getz and Hofmann, 1986; Getz et al., 1993; Solomon and Jacquot, 2002; Ophir et al, 2007}. Laboratory research has frequently explored the neurobiology of monogamy, by only using experimental animals that showed a strong partner preference bond, as shown through the use of the partner preference test {Pitkow et al, 2001; Hammock et al, 2005}. Furthermore, it has been assumed, and generally demonstrated, that alloparental care, aggression towards a same-sex intruder, and anxiety-like behaviors are all related to partner preference and that prairie voles change their behaviors in these areas after pair bonding {Winslow et al, 1993; Pitkow et al, 2001}. But my research shows the amount of time males spent with their partner during the partner preference test was not correlated with any other measured behavior. However, males became more aggressive towards a same sex intruder following mating, independent of whether or not they formed a partner preference. One caveat to this observation of the rise in aggressive behavior is that the experimental male animals were tested in the partner preference arena 18 hours after being paired with a female, but the test for aggression against a same sex intruder happened 36 hours after pairing. Therefore, the pair bond may have strengthened over that time period, and the amount of time spent with the partner that we measured may not be the best measurement.

One of the most important findings to come out of this work was my finding that social behaviors, namely partner preference, licking and grooming of pups, aggression

towards a same sex intruder, and anxiety-like behaviors, are not highly heritable, a measure that had previously not been formally tested. My results show that there is very low heritability, not significantly different from zero, for all the behaviors measured. This result is similar to measurements of complex behaviors in other species (Mackay et al., 2009).

In contrast to previously published work that showed V1aR density in the ventral pallidum to be correlated with partner preference and V1aR density in the retrosplenial cortex to be correlated with male sexual fidelity, I found that V1aR density in the ventral pallidum and the rostral area of the retrosplenial cortex were correlated with affiliative behaviors {Ophir et al, 2008; Okhovat et al, 2015}. In my experiments, I used a few tests to quantify affiliative behaviors, and I will discuss two of them here. The first test was the alloparental care test, which quantified the duration of time an adult vole spent caring for an unfamiliar pup. Licking and grooming of the pup was consistently correlated with V1aR density in the retrosplenial cortex of male prairie voles, and affiliative behaviors towards the pup were correlated with V1aR density of the ventral pallidum. The second test was the resident intruder test, which quantified interactions with a same-sex intruder. The more time a male prairie vole spent with the intruder, performing non-aggressive actions such as sniffing, was correlated with V1aR density in the retrosplenial cortex. Studies from other laboratories have found that parental care and other social behaviors are influenced by early life experience (Stone et al., 2010). Of particular interest to my study was the finding that alloparenting in prairie voles is transmitted non-genetically, whereby cross-fostering experiments have shown that alloparental behavior could be predicted by the amount and quality of parental care experienced by the offspring (Perkeybile et al., 2015). Additionally,

alloparenting experience while a juvenile can affect parental care such as licking pups and faster weight gain of offspring in males (Stone et al., 2010). In chapter 2, I described my work where I examined two generations of males, both of which gained alloparental care experience before becoming fathers, as part of the experiment, although they were adults at the time of testing. Based on results from previous studies (Stone et al., 2010; Perkeybile et al., 2015), I predicted that the first generation of males that I tested would have influenced their male offspring's alloparental care behavior. However, I found no significant correlation or heritability between the parents and offspring in the amount of time they spent caring for an unfamiliar pup. Furthermore, when Perkeybile et al. (2015) cross-fostered the offspring, they found that, although alloparental care was influenced by the animals that raised the offspring, oxytocin receptor (OTR) and V1aR binding in the brain was influenced by the genetic parents, albeit in a sex-specific manner (Perkeybile et al., 2015). The regions of the brain that Perkeybile and her team studied were the lateral septum, nucleus accumbens, bed nucleus of the stria terminalis, and central amygdala (Perkeybile et al., 2015). These are different from the regions that Hammock et al. found to be implicated in pro-social behaviors, such as paternal care, juvenile affiliation, partner preference, and resident intruder behaviors. In that study, several regions of the olfactory bulb were found to have a strong relationship with pro-social behaviors (Hammock et al., 2005). I examined none of these regions, because I was looking for a relationship between partner preference and V1aR binding, but I found a significant correlation between alloparental care and both the retrosplenial cortex and the ventral pallidum, neither of which has been implicated in alloparental care before. Alloparenting can also facilitate partner preference formation

(Kenkel et al., 2012). However, I did not see any correlations between the amount of time a male spent caring for an unfamiliar pup and the amount of time a male spent with his partner, which suggests that alloparenting may facilitate partner preference formation, but has no discernable effect on the maintenance of the pair bond, as measured by the amount of time a male spent with his partner during the partner preference test.

Finally, to examine an additional possible mechanism of formation of the pair bond, I tested if females mated to males with surgically removed seminal vesicles would have reduced partner preference. I also examined fertility-related factors and social behaviors of the mated females and their offspring. I found no differences in the measured traits between groups. My findings do not preclude a role of seminal vesicle proteins in contributing to post-mating changes in female behavior; seminal fluid protein(s) that might potentially contribute to the formation of the pair bond could be found in male reproductive organs other than the seminal vesicle, such as accessory glands. Seminal vesicle proteins could affect other behaviors that were not measured or able to be detected in the experiment, as well.

Future Directions

What is the genetic basis of individual variation in social behaviors? Despite ongoing research to understand the neurobiological basis of pair bonding and social behaviors in general, the genetic mechanisms underlying social behaviors in prairie voles has been largely under-studied. Genome-wide association studies in humans with mental disorders have identified hundreds of genes, each having a small effect size, on social behaviors {Levy et al, 2011; Sanders et al, 2011}. In prairie voles, however, the focus has been on a few genes, notably the *avpr1a* gene and the *OXTR* gene {Hammock et al, 2005;

Ophir et al, 2008; Mabry et al, 2011; Keane et al, 2014; Harris et al, 2014; Okhovat et al, 2015; Graham et al, 2016}. The large amount of individual variation in social behaviors that I observed in my study, along with the low estimates of heritability, suggests a major influence by the environment and many genes of small effect underlying variation in social behaviors. The variation in social behaviors is probably due to many genes, each having a small effect on the behavior in question. In order to identify which genes are affecting social behaviors, future work could utilize full genome sequencing on many individual prairie voles to identify SNPs and other polymorphisms that affect the expression of genes that are important for social behaviors. In addition, examining gene expression using RNA-sequencing or quantitative real-time PCR on genes that have been found to be important in human studies, may provide further insight into the genetics of social behavior in prairie voles. For example, *CD38*, which is necessary for normal social behavior in mice would be a potentially interesting gene to examine in prairie voles (Jin et al., 2007). Additional genes to be investigated for their link to social behavior were compiled by McGraw et al. (2010) and include genes in the neurohypophysial peptide system, the dopamine system, the stress axis, sex steroid receptors, and genes for synaptic plasticity (McGraw et al., 2010; McGraw et al., 2012).

Is there a compensatory role between the neuropeptides and their respective receptors? While the neurobiology of the vasopressin and oxytocin receptors has been examined in many studies (see Table 1.1), the actual ligands themselves have not. A frequent question that I hear, and wonder about, is whether the density of the receptors is playing a compensatory role with regards to the amount of vasopressin hormone available in the brain.

The amount of vasopressin peptide in socially-relevant brain areas could be analyzed and compared with the density of the V1aR in those same brain areas in the same animals. This would provide the first evidence for a compensatory mechanism by the V1aR. Furthermore, my analysis found some previously unobserved correlations between the density of V1aR in the ventral pallidum and retrosplenial cortex, and affiliative behaviors towards other voles.

How does variation in social behavior in the species allow for animals to reproduce and survive? Individual variation in alloparental care has been investigated by the Bales laboratory, who have developed a model for why some parents spend a greater proportion of time licking and grooming than other parents. The model explains how in populations of low density, there is more availability of resources, so parents spend more time with their pups, and young adults of the family stay closer to the natal nest for longer, whereas populations with high density encourage parents to spend less time with their pups, leading to young adults of the family ranging further (Perkeybile and Bales, 2017). These behaviors, over time, help to keep the population at a relatively stable size. For this question, I will focus primarily on partner preference. A simple answer of adaptive function for partner preference would say that the habitat of the prairie vole is so demanding that it requires both parents to care for the young, and that partner preference evolved to keep the parents together. If that were so, all the prairie voles would form strong partnerships. So the question remains, why is there variation in partner preference? Here is where questions of sexual choice, from both the male and female perspective, could be examined. What does a male look for in a good partner? What does a female look for? Some preliminary thoughts on what to look for include parental care, aggressive behavior towards intruders, and sperm quality.

Does pair bonding change over an animal's lifetime? To again focus on partner preference, we know that it happens in a shorter amount of time when accompanied by mating. Additionally, the formation of partner preference is generated by OTR and V1aR, which are in the same brain regions as dopamine 1 (D1) and dopamine 2 (D2) receptors (Liu and Wang, 2003). There is also considerable natural individual variation in the density of V1aR, which was recapitulated in my work in chapter 2 (Phelps and Young, 2003). But how does partner preference change over time? We know that some animals will mate outside of the pair bond, some will not, and some will not form a pair bond at all. Solomon's laboratory showed that males whose partner died will wander, but some of them would later find another partner (Solomon and Jacquot, 2002). This shows that the pair bond is plastic. Are there differences between the strength of the pair bond in the first partnership versus later partnerships? This could be tested in the laboratory using the partner preference test. Future work could also use the partner preference test with the same animals, to see if partner preference with the same animals in the pair bond changes over time.

Why monogamy? Monogamy has arisen several times in the vole phylogeny, (Fitzgerald and Madison, 1983). There is some debate within the prairie vole researcher community about whether the proportion of prairie voles that engage in social monogamy differs by population, and therefore, if the mating system is affected by the available resources. Field research suggested that populations in Kansas and Tennessee differed in the proportion of animals that showed partner preference from the populations in Illinois (Danielson and Gaines, 1987; Swihart and Slade, 1989). The differences did not hold up during laboratory testing, however (Ophir et al., 2007). A future project could examine the

proportion of prairie voles engaging in social monogamy in semi-naturalistic arenas with differing amounts of resources, such as population density, food availability, and presence of predators.

Conclusion

Future experimental plans that come from thinking about both proximate and ultimate questions will allow for a more thorough understanding of pair bonding behavior. Exploring the behavior from multiple angles, such as the evolutionary history, function, genetic mechanisms, and molecular architecture, will also help to discover overlaps between prairie vole and human behaviors. This will help to fully understand social behaviors in humans, and potentially help humans with social detriments due to mental health disorders. As discussed extensively in Chapter 1, impaired social behaviors are a fundamental hallmark of mental health disorder (Volkmar, 2001). The individual variation of symptoms apparent in mental health disorder is mirrored in all social behaviors, even among people without mental health disorders. Investigating individual variation in social behaviors in model organisms, such as prairie voles, can inform researchers about neurobiological and genetic mechanisms, which may eventually translate into treatments for social deficits associated with many mental health disorders.

REFERENCES

Adams, G. P., M. H. Ratto, W. Huanca and J. Singh (2005). "Ovulation-inducing factor in the seminal plasma of alpacas and llamas." Biol Reprod **73**(3): 452-457.

Ahern, T. H., M. E. Modi, J. P. Burkett and L. J. Young (2009). "Evaluation of two automated metrics for analyzing partner preference tests." J Neurosci Methods **182**(2): 180-188.

Anholt, R. and T. Mackay (2010). Principles of behavioral genetics. Amsterdam, Boston, Academic.

Aragona, B. J., Y. Liu, J. T. Curtis, F. K. Stephan and Z. Wang (2003). "A critical role for nucleus accumbens dopamine in partner-preference formation in male prairie voles." J Neurosci **23**(8): 3483-3490.

Aragona, B. J., Y. Liu, Y. J. Yu, J. T. Curtis, J. M. Detwiler, T. R. Insel and Z. Wang (2006). "Nucleus accumbens dopamine differentially mediates the formation and maintenance of monogamous pair bonds." Nat Neurosci **9**(1): 133-139.

Archer, J. (1973). "Tests for emotionality in rats and mice: a review." Anim Behav **21**(2): 205-235.

Association, A. P. (2013). Diagnostic and statistical manual of mental disorders. Arlington, VA, American Psychiatric Association.

Avila, F. W., L. K. Sirot, B. A. LaFlamme, C. D. Rubinstein and M. F. Wolfner (2011). "Insect seminal fluid proteins: identification and function." Annu Rev Entomol **56**: 21-40.

Barber, F. D. (2012). "Social support and physical activity engagement by cancer survivors." Clin J Oncol Nurs **16**(3): E84-98.

Barrett, C. E., A. C. Keebaugh, T. H. Ahern, C. E. Bass, E. F. Terwilliger and L. J. Young (2013). "Variation in vasopressin receptor (*Avpr1a*) expression creates diversity in behaviors related to monogamy in prairie voles." Horm Behav **63**(3): 518-526.

Birkhauser, F. D., C. Schumacher, R. Seiler, L. de Meuron, P. Zehnder, B. Roth, A. Wetterwald, G. N. Thalmann, M. G. Cecchini and U. E. Studer (2012). "Occlusion of seminal vesicles increases sexual activity in a mouse model." Eur Urol **62**(5): 855-862.

Bogle, O. A., M. H. Ratto and G. P. Adams (2012). "Ovulation-inducing factor (OIF) induces LH secretion from pituitary cells." Anim Reprod Sci **133**(1-2): 117-122.

Bromfield, J. J., J. E. Schjenken, P. Y. Chin, A. S. Care, M. J. Jasper and S. A. Robertson (2014). "Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring." Proc Natl Acad Sci U S A **111**(6): 2200-2205.

Caldwell, H. K., H. J. Lee, A. H. Macbeth and W. S. Young, 3rd (2008). "Vasopressin: behavioral roles of an "original" neuropeptide." Prog Neurobiol **84**(1): 1-24.

Carballada, R. and P. Esponda (1993). "Structure of the vaginal plugs generated by normal rats and by rats with partially removed seminal vesicles." J Exp Zool **265**(1): 61-68.

Carter, C. S., E. M. Boone, H. Pournajafi-Nazarloo and K. L. Bales (2009). "Consequences of early experiences and exposure to oxytocin and vasopressin are sexually dimorphic." Dev Neurosci **31**(4): 332-341.

Carter, C. S., A. C. DeVries and L. L. Getz (1995). "Physiological substrates of mammalian monogamy: the prairie vole model." Neurosci Biobehav Rev **19**(2): 303-314.

Christensen, D. L., J. Baio, K. Van Naarden Braun, D. Bilder, J. Charles, J. N. Constantino, J. Daniels, M. S. Durkin, R. T. Fitzgerald, M. Kurzius-Spencer, L. C. Lee, S. Pettygrove, C. Robinson, E. Schulz, C. Wells, M. S. Wingate, W. Zahorodny, M. Yeargin-Allsopp, C. Centers for Disease and Prevention (2016). "Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years--Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012." MMWR Surveill Summ **65**(3): 1-23.

Cukierski, M., J. Sina, S. Prahalada and R. Robertson (1991). "Effects of seminal vesicle and coagulating gland ablation on fertility in rats." Reprod Toxicol **5**(4): 347-352.

Curley, J. P., S. Davidson, P. Bateson and F. A. Champagne (2009). "Social enrichment during postnatal development induces transgenerational effects on emotional and reproductive behavior in mice." Front Behav Neurosci **3**: 25.

Cushing, B. S., J. O. Martin, L. J. Young and C. S. Carter (2001). "The effects of peptides on partner preference formation are predicted by habitat in prairie voles." Hormones and Behavior **39**(1): 48-58.

Danielson, B. J. and M. S. Gaines (1987). "Spatial Patterns in 2 Syntopic Species of Microtines – *Microtus ochrogaster* and *Synaptomys-Cooperi*." Journal of Mammalogy **68**(2): 313-322.

Dawson, G., S. Rogers, J. Munson, M. Smith, J. Winter, J. Greenson, A. Donaldson and J. Varley (2010). "Randomized, controlled trial of an intervention for toddlers with autism: the Early Start Denver Model." Pediatrics **125**(1): e17-23.

Druart, X., J. P. Rickard, S. Mactier, P. L. Kohnke, C. M. Kershaw-Young, R. Bathgate, Z. Gibb, B. Crossett, G. Tsikis, V. Labas, G. Harichaux, C. G. Grupen and S. P. de Graaf (2013). "Proteomic characterization and cross species comparison of mammalian seminal plasma." J Proteomics **91**: 13-22.

Dubois-Dauphin, M., C. Barberis and F. de Bilbao (1996). "Vasopressin receptors in the mouse (Mus musculus) brain: sex-related expression in the medial preoptic area and hypothalamus." Brain Res **743**(1-2): 32-39.

Eapen, V., R. Crncec and A. Walter (2013). "Clinical outcomes of an early intervention program for preschool children with Autism Spectrum Disorder in a community group setting." BMC Pediatr **13**(1): 3.

Fanous, A. H., B. Zhou, S. H. Aggen, S. E. Bergen, R. L. Amdur, J. Duan, A. R. Sanders, J. Shi, B. J. Mowry, A. Olincy, F. Amin, C. R. Cloninger, J. M. Silverman, N. G. Buccola, W. F. Byerley, D. W. Black, R. Freedman, F. Dudbridge, P. A. Holmans, S. Ripke, P. V. Gejman, K. S. Kendler, D. F. Levinson and C. Schizophrenia Psychiatric Genome-Wide Association Study (2012). "Genome-wide association study of clinical dimensions of schizophrenia: polygenic effect on disorganized symptoms." Am J Psychiatry **169**(12): 1309-1317.

Fitzgerald, R. W. and D. M. Madison (1983). "Social-Organization of a Free-Ranging Population of Pine Voles, *Microtus pinetorum*." Behavioral Ecology and Sociobiology **13**(3): 183-187.

Getz, L. L., C. S. Carter and L. Gavish (1981). "The Mating System of the Prairie Vole, *Microtus ochrogaster*: Field and Laboratory Evidence for Pair-Bonding." Behavioral Ecology and Sociobiology **8**(3): 189 - 194.

Getz, L. L. and J. E. Hofmann (1986). "Social organization in free-living prairie voles, *Microtus ochrogaster*." Behav Eco Socio **18**(4): 275-282.

Getz, L. L., B. McGuire, T. Pizzuto, J. E. Hofmann and B. Frase (1993). "Social Organization of the Prairie vole (*Microtus ochrogaster)*." Journal of Mammalogy **74**(1): 44-58.

Gingrich, B., Y. Liu, C. Cascio, Z. Wang and T. R. Insel (2000). "Dopamine D2 receptors in the nucleus accumbens are important for social attachment in female prairie voles (*Microtus ochrogaster*)." Behav Neurosci **114**(1): 173-183.

Gobrogge, K. and Z. Wang (2015). "Neuropeptidergic regulation of pair-bonding and stress buffering: Lessons from voles." Horm Behav **76**: 91-105.

Gobrogge, K. L. and Z. W. Wang (2011). "Genetics of aggression in voles." Adv Genet **75**: 121-150.

Graham, B. M., N. G. Solomon, D. A. Noe and B. Keane (2016). "Male prairie voles with different *avpr1a* microsatellite lengths do not differ in courtship behaviour." Behav Processes **128**: 53-57.

Ha, S., I. J. Sohn, N. Kim, H. J. Sim and K. A. Cheon (2015). "Characteristics of Brains in Autism Spectrum Disorder: Structure, Function and Connectivity across the Lifespan." Exp Neurobiol **24**(4): 273-284.

Hadfield, J. D. (2010). "MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package." Journal of Statistical Software **33**(2): 1-22.

Hammock, E. A., M. M. Lim, H. P. Nair and L. J. Young (2005). "Association of vasopressin 1a receptor levels with a regulatory microsatellite and behavior." Genes Brain Behav **4**(5): 289-301.

Hammock, E. A. and L. J. Young (2005). "Microsatellite instability generates diversity in brain and sociobehavioral traits." Science **308**(5728): 1630-1634.

Harony, H. and S. Wagner (2010). "The contribution of oxytocin and vasopressin to mammalian social behavior: potential role in autism spectrum disorder." Neurosignals **18**(2): 82-97.

Harris, M. N., R. M. Alvarez, B. Keane, A. D. Talib, M. J. Eiswerth and N. G. Solomon (2014). "The role of *avpr1a* microsatellite length on reproductive success of female *Microtus ochrogaster*." Behaviour **151**(8): 1185-1207.

Henderson, C. R. (1984). Applications of linear models in animal breeding, University of Guelph.

Hopkins, B. R., I. Sepil and S. Wigby (2017). "Seminal fluid." Curr Biol **27**(11): R404-R405.

Huguet, G., E. Ey and T. Bourgeron (2013). "The genetic landscapes of autism spectrum disorders." Annu Rev Genomics Hum Genet **14**: 191-213.

Insel, T. R. (2010). "The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior." Neuron **65**(6): 768-779.

Insel, T. R., S. Preston and J. T. Winslow (1995). "Mating in the monogamous male: behavioral consequences." Physiol Behav **57**(4): 615-627.

Insel, T. R., Z. X. Wang and C. F. Ferris (1994). "Patterns of brain vasopressin receptor distribution associated with social organization in microtine rodents." J Neurosci **14**(9): 5381-5392.

Jin, D., H. X. Liu, H. Hirai, T. Torashima, T. Nagai, O. Lopatina, N. A. Shnayder, K. Yamada, M. Noda, T. Seike, K. Fujita, S. Takasawa, S. Yokoyama, K. Koizumi, Y. Shiraishi, S. Tanaka, M. Hashii, T. Yoshihara, K. Higashida, M. S. Islam, N. Yamada, K. Hayashi, N. Noguchi, I. Kato, H. Okamoto, A. Matsushima, A. Salmina, T. Munesue, N. Shimizu, S. Mochida, M. Asano and H. Higashida (2007). "CD38 is critical for social behaviour by regulating oxytocin secretion." Nature **446**(7131): 41-45.

Kanazawa, T., M. Ikeda, S. J. Glatt, A. Tsutsumi, H. Kikuyama, Y. Kawamura, N. Nishida, T. Miyagawa, R. Hashimoto, M. Takeda, T. Sasaki, K. Tokunaga, J. Koh, N. Iwata and H. Yoneda (2013). "Genome-wide association study of atypical psychosis." Am J Med Genet B Neuropsychiatr Genet **162B**(7): 679-686.

Keane, B., S. Parsons, B. J. Smucker and N. G. Solomon (2014). "Length polymorphism at the *avpr1a* locus is correlated with male reproductive behavior in a natural population of prairie voles (*Microtus ochrogaster*)." Behavioral Ecology and Sociobiology **68**(12): 1951-1964.

Kenkel, W. M., J. Paredes, J. R. Yee, H. Pournajafi-Nazarloo, K. L. Bales and C. S. Carter (2012). "Neuroendocrine and behavioural responses to exposure to an infant in male prairie voles." J Neuroendocrinol **24**(6): 874-886.

Kleiman, D. G. (1977). "Monogamy in mammals." Q Rev Biol **52**(1): 39-69.

Koolhaas, J. M., C. M. Coppens, S. F. de Boer, B. Buwalda, P. Meerlo and P. J. Timmermans (2013). "The resident-intruder paradigm: a standardized test for aggression, violence and social stress." J Vis Exp(77): e4367.

Lee, H. J., A. H. Macbeth, J. H. Pagani and W. S. Young, 3rd (2009). "Oxytocin: the great facilitator of life." Prog Neurobiol **88**(2): 127-151.

Levinson, D. F., J. Shi, K. Wang, S. Oh, B. Riley, A. E. Pulver, D. B. Wildenauer, C. Laurent, B. J. Mowry, P. V. Gejman, M. J. Owen, K. S. Kendler, G. Nestadt, S. G. Schwab, J. Mallet, D. Nertney, A. R. Sanders, N. M. Williams, B. Wormley, V. K. Lasseter, M. Albus, S. Godard-Bauche, M. Alexander, J. Duan, M. C. O'Donovan, D. Walsh, A. O'Neill, G. N. Papadimitriou, D. Dikeos, W. Maier, B. Lerer, D. Campion, D. Cohen, M. Jay, A. Fanous, P. Eichhammer, J. M. Silverman, N. Norton, N. Zhang, H. Hakonarson, C. Gao, A. Citri, M. Hansen, S. Ripke, G. C. Schizophrenia

Psychiatric, F. Dudbridge and P. A. Holmans (2012). "Genome-wide association study of multiplex schizophrenia pedigrees." Am J Psychiatry **169**(9): 963-973.

Lim, M. M., A. Z. Murphy and L. J. Young (2004). "Ventral striatopallidal oxytocin and vasopressin V1a receptors in the monogamous prairie vole (*Microtus ochrogaster*)." J Comp Neurol **468**(4): 555-570.

Lim, M. M. and L. J. Young (2004). "Vasopressin-dependent neural circuits underlying pair bond formation in the monogamous prairie vole." Neuroscience **125**(1): 35-45.

Liu, Y. and Z. X. Wang (2003). "Nucleus accumbens oxytocin and dopamine interact to regulate pair bond formation in female prairie voles." Neuroscience **121**(3): 537-544.

Lukas, M., I. Toth, S. O. Reber, D. A. Slattery, A. H. Veenema and I. D. Neumann (2011). "The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice." Neuropsychopharmacology **36**(11): 2159-2168.

Lung, O. and M. F. Wolfner (2001). "Identification and characterization of the major *Drosophila melanogaster* mating plug protein." Insect Biochem Mol Biol **31**(6-7): 543-551.

Mabry, K. E., C. A. Streatfeild, B. Keane and N. G. Solomon (2011). "*avpr1a* length polymorphism is not associated with either social or genetic monogamy in free-living prairie voles." Anim Behav **81**(1): 11-18.

Mackay, T. F., E. A. Stone and J. F. Ayroles (2009). "The genetics of quantitative traits: challenges and prospects." Nat Rev Genet **10**(8): 565-577.

Manolio, T. A., F. S. Collins, N. J. Cox, D. B. Goldstein, L. A. Hindorff, D. J. Hunter, M. I. McCarthy, E. M. Ramos, L. R. Cardon, A. Chakravarti, J. H. Cho, A. E. Guttmacher, A. Kong, L. Kruglyak, E. Mardis, C. N. Rotimi, M. Slatkin, D. Valle, A. S. Whittemore, M. Boehnke, A. G. Clark, E. E. Eichler, G. Gibson, J. L. Haines, T. F. Mackay, S. A. McCarroll and P. M. Visscher (2009). "Finding the missing heritability of complex diseases." Nature **461**(7265): 747-753.

McGraw, L. A., J. K. Davis, J. J. Lowman, B. F. ten Hallers, M. Koriabine, L. J. Young, P. J. de Jong, M. K. Rudd and J. W. Thomas (2010). "Development of genomic resources for the prairie vole (*Microtus ochrogaster*): construction of a BAC library and vole-mouse comparative cytogenetic map." BMC Genomics **11**: 70.

McGraw, L. A., J. K. Davis, P. J. Thomas, N. C. S. Program, L. J. Young and J. W. Thomas (2012). "BAC-based sequencing of behaviorally-relevant genes in the prairie vole." PLoS One **7**(1): e29345.

McGraw, L. A., S. S. Suarez and M. F. Wolfner (2015). "On a matter of seminal importance." Bioessays **37**(2): 142-147.

McGuire, B. and L. L. Getz (2010). "Alternative male reproductive tactics in a natural population of prairie voles *Microtus ochrogaster*." Acta Theriologica **55**(3): 261-270.

Murgatroyd, C., A. V. Patchev, Y. Wu, V. Micale, Y. Bockmuhl, D. Fischer, F. Holsboer, C. T. Wotjak, O. F. Almeida and D. Spengler (2009). "Dynamic DNA methylation programs persistent adverse effects of early-life stress." Nat Neurosci **12**(12): 1559-1566.

Neubaum, D. M. and M. F. Wolfner (1999). "Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently." Genetics **153**(2): 845-857.

Okhovat, M., A. Berrio, G. Wallace, A. G. Ophir and S. M. Phelps (2015). "Sexual fidelity trade-offs promote regulatory variation in the prairie vole brain." Science **350**(6266): 1371-1374.

Ophir, A. G., P. Campbell, K. Hanna and S. M. Phelps (2008). "Field tests of cis-regulatory variation at the prairie vole *avpr1a* locus: association with V1aR abundance but not sexual or social fidelity." Horm Behav **54**(5): 694-702.

Ophir, A. G., S. M. Phelps, A. B. Sorin and J. O. Wolff (2007). "Morphological, genetic and behavioral comparisons of two prairie vole populations in the field and laboratory." Journal of Mammalogy **88**(4): 989-999.

Ophir, A. G., S. M. Phelps, A. B. Sorin and J. O. Wolff (2008). "Social but not genetic monogamy is associated with greater breeding success in prairie voles." Animal Behaviour **75**(3): 1143-1154.

Paolicchi, F., B. Urquieta, L. Del Valle and E. Bustos-Obregon (1999). "Biological activity of the seminal plasma of alpacas: stimulus for the production of LH by pituitary cells." Anim Reprod Sci **54**(3): 203-210.

Paxinos, G. and K. Franklin (2013). The Mouse Brain In Stereotaxic Coordinates, 4th Ed. 32 Jamestown Road, London NW1 7BY, UK, 225 Wyman Street, Waltham, MA 02451, USA, 525 B Street, Suite 1800, San Diego, CA 92101-4495, USA, Elsevier, Inc.

Paxinos, G. and C. Watson (1998). The Rat Brain in Stereotaxic Coordinates, 4th Ed. 525 B Street, Suite 1900, San Diego, CA 92101-4495, USA, Academic Press, Inc.

Paxinos, G. and C. Watson (2006). The Rat Brain in Stereotaxic Coordinates. 30 Corporate Drive, Suite 400, Burlington, MA 01803, USA, 525 B Street, Suite 1900, San Diego, CA 92101-4495, USA, 84 Theobald's Road, London WCiX 8RR, UK, Elsevier Academic Press.

Perkeybile, A. M. and K. L. Bales (2017). "Intergenerational transmission of sociality: the role of parents in shaping social behavior in monogamous and non-monogamous species." J Exp Biol **220**(Pt 1): 114-123.

Perkeybile, A. M., N. Delaney-Busch, S. Hartman, K. J. Grimm and K. L. Bales (2015). "Intergenerational transmission of alloparental behavior and oxytocin and vasopressin receptor distribution in the prairie vole." Front Behav Neurosci **9**: 191.

Persico, A. M. and T. Bourgeron (2006). "Searching for ways out of the autism maze: genetic, epigenetic and environmental clues." Trends Neurosci **29**(7): 349-358.

Phelps, S. M. and L. J. Young (2003). "Extraordinary diversity in vasopressin (V1a) receptor distributions among wild prairie voles (*Microtus ochrogaster):* patterns of variation and covariation." J Comp Neurol **466**(4): 564-576.

Pitkow, L. J., C. A. Sharer, X. Ren, T. R. Insel, E. F. Terwilliger and L. J. Young (2001). "Facilitation of affiliation and pair-bond formation by vasopressin receptor gene transfer into the ventral forebrain of a monogamous vole." J Neurosci **21**(18): 7392-7396.

Poiani, A. (2006). "Complexity of seminal fluid: a review." Behav Ecol Sociobiol **60**(3): 289-310.

Ratto, M. H., Y. A. Leduc, X. P. Valderrama, K. E. van Straaten, L. T. Delbaere, R. A. Pierson and G. P. Adams (2012). "The nerve of ovulation-inducing factor in semen." Proc Natl Acad Sci U S A **109**(37): 15042-15047.

Ripke, S., C. O'Dushlaine, K. Chambert, J. L. Moran, A. K. Kahler, S. Akterin, S. E. Bergen, A. L. Collins, J. J. Crowley, M. Fromer, Y. Kim, S. H. Lee, P. K. Magnusson, N. Sanchez, E. A. Stahl, S. Williams, N. R. Wray, K. Xia, F. Bettella, A. D. Borglum, B. K. Bulik-Sullivan, P. Cormican, N. Craddock, C. de Leeuw, N. Durmishi, M. Gill, V. Golimbet, M. L. Hamshere, P. Holmans, D. M. Hougaard, K. S. Kendler, K. Lin, D. W. Morris, O. Mors, P. B. Mortensen, B. M. Neale, F. A. O'Neill, M. J. Owen, M. P. Milovancevic, D. Posthuma, J. Powell, A. L. Richards, B. P. Riley, D. Ruderfer, D. Rujescu, E. Sigurdsson, T. Silagadze, A. B. Smit, H. Stefansson, S. Steinberg, J. Suvisaari, S. Tosato, M. Verhage, J. T. Walters, C. Multicenter Genetic Studies of Schizophrenia, D. F. Levinson, P. V. Gejman, K. S. Kendler, C. Laurent, B. J. Mowry, M. C. O'Donovan, M. J. Owen, A. E. Pulver, B. P. Riley, S. G. Schwab, D.

B. Wildenauer, F. Dudbridge, P. Holmans, J. Shi, M. Albus, M. Alexander, D. Campion, D. Cohen, D. Dikeos, J. Duan, P. Eichhammer, S. Godard, M. Hansen, F. B. Lerer, K. Y. Liang, W. Maier, J. Mallet, D. A. Nertney, G. Nestadt, N. Norton, F. A. O'Neill, G. N. Papadimitriou, R. Ribble, A. R. Sanders, J. M. Silverman, D. Walsh, N. M. Williams, B. Wormley, C. Psychosis Endophenotypes International, M. J. Arranz, S. Bakker, S. Bender, E. Bramon, D. Collier, B. Crespo-Facorro, J. Hall, C. Iyegbe, A. Jablensky, R. S. Kahn, L. Kalaydjieva, S. Lawrie, C. M. Lewis, K. Lin, D. H. Linszen, I. Mata, A. McIntosh, R. M. Murray, R. A. Ophoff, J. Powell, D. Rujescu, J. Van Os, M. Walshe, M. Weisbrod, D. Wiersma, C. Wellcome Trust Case Control, P. Donnelly, I. Barroso, J. M. Blackwell, E. Bramon, M. A. Brown, J. P. Casas, A. P. Corvin, P. Deloukas, A. Duncanson, J. Jankowski, H. S. Markus, C. G. Mathew, C. N. Palmer, R. Plomin, A. Rautanen, S. J. Sawcer, R. C. Trembath, A. C. Viswanathan, N. W. Wood, C. C. Spencer, G. Band, C. Bellenguez, C. Freeman, G. Hellenthal, E. Giannoulatou, M. Pirinen, R. D. Pearson, A. Strange, Z. Su, D. Vukcevic, P. Donnelly, C. Langford, S. E. Hunt, S. Edkins, R. Gwilliam, H. Blackburn, S. J. Bumpstead, S. Dronov, M. Gillman, E. Gray, N. Hammond, A. Jayakumar, O. T. McCann, J. Liddle, S. C. Potter, R. Ravindrarajah, M. Ricketts, A. Tashakkori-Ghanbaria, M. J. Waller, P. Weston, S. Widaa, P. Whittaker, I. Barroso, P. Deloukas, C. G. Mathew, J. M. Blackwell, M. A. Brown, A. P. Corvin, M. I. McCarthy, C. C. Spencer, E. Bramon, A. P. Corvin, M. C. O'Donovan, K. Stefansson, E. Scolnick, S. Purcell, S. A. McCarroll, P. Sklar, C. M. Hultman and P. F. Sullivan (2013). "Genome-wide association analysis identifies 13 new risk loci for schizophrenia." Nat Genet **45**(10): 1150-1159.

Roberts, R. L., B. S. Cushing and C. S. Carter (1998). "Intraspecific variation in the induction of female sexual receptivity in prairie voles." Physiology & Behavior **64**(2): 209-212.

Roberts, R. L., A. Zullo, E. A. Gustafson and C. S. Carter (1996). "Perinatal steroid treatments alter alloparental and affiliative behavior in prairie voles." Horm Behav **30**(4): 576-582.

Robles, T. F. and J. K. Kiecolt-Glaser (2003). "The physiology of marriage: pathways to health." Physiol Behav **79**(3): 409-416.

Rogers, S. J., A. Estes, C. Lord, L. Vismara, J. Winter, A. Fitzpatrick, M. Guo and G. Dawson (2012). "Effects of a brief Early Start Denver model (ESDM)-based parent intervention on toddlers at risk for autism spectrum disorders: a randomized controlled trial." J Am Acad Child Adolesc Psychiatry **51**(10): 1052-1065.

Ross, H. E. and L. J. Young (2009). "Oxytocin and the neural mechanisms regulating social cognition and affiliative behavior." Front Neuroendocrinol **30**(4): 534-547.

Sansone, G. R., C. A. Gerdes, J. L. Steinman, J. T. Winslow, J. E. Ottenweller, B. R. Komisaruk and T. R. Insel (2002). "Vaginocervical stimulation releases oxytocin within the spinal cord in rats." Neuroendocrinology **75**(5): 306-315.

Schizophrenia Psychiatric Genome-Wide Association Study, C. (2011). "Genome-wide association study identifies five new schizophrenia loci." Nat Genet **43**(10): 969-976.

Shapiro, L. E. and D. A. Dewsbury (1990). "Differences in affiliative behavior, pair bonding, and vaginal cytology in two species of vole (*Microtus ochrogaster* and *M. montanus*)." J Comp Psychol **104**(3): 268-274.

Slob, A. K., L. W. de Klerk and T. Brand (1987). "Homosexual and heterosexual partner preference in ovariectomized female rats: effects of testosterone, estradiol and mating experience." Physiol Behav **41**(6): 571-576.

Solomon, N. G. and J. J. Jacquot (2002). "Characteristics of resident and wandering prairie voles, *Microtus ochrogaster*." Canadian Journal of Zoology-Revue Canadienne De Zoologie **80**(5): 951-955.

Solomon, N. G., A. R. Richmond, P. A. Harding, A. Fries, S. Jacquemin, R. L. Schaefer, K. E. Lucia and B. Keane (2009). "Polymorphism at the *avpr1a* locus in male prairie voles correlated with genetic but not social monogamy in field populations." Mol Ecol **18**(22): 4680-4695.

Stein, E. R. and B. W. Smith (2015). "Social support attenuates the harmful effects of stress in healthy adult women." Soc Sci Med **146**: 129-136.

Stone, A. I., D. Mathieu, L. Griffin and K. L. Bales (2010). "Alloparenting experience affects future parental behavior and reproductive success in prairie voles (*Microtus ochrogaster*)." Behav Processes **83**(1): 8-15.

Swihart, R. K. and N. A. Slade (1989). "Differences in Home-Range Size between Sexes of *Microtus ochrogaste*r." Journal of Mammalogy **70**(4): 816-820.

Terleph, T. A., N. Jean-Baptiste and M. Bamshad (2004). "Mechanisms and time course for induction of paternal behavior in prairie voles (*Microtus ochrogaster*)." Journal of Mammalogy **85**(6): 1124-1129.

Tram, U. and M. F. Wolfner (1999). "Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster*." Genetics **153**(2): 837-844.

Tsuda, M. C., N. Yamaguchi and S. Ogawa (2011). "Early life stress disrupts peripubertal development of aggression in male mice." Neuroreport **22**(6): 259-263.

Veenema, A. H., R. Bredewold and I. D. Neumann (2007). "Opposite effects of maternal separation on intermale and maternal aggression in C57BL/6 mice: link to hypothalamic vasopressin and oxytocin immunoreactivity." Psychoneuroendocrinology **32**(5): 437-450.

Volkmar, F. R. (2001). "Pharmacological interventions in autism: theoretical and practical issues." J Clin Child Psychol **30**(1): 80-87.

Wang, Z., G. Yu, C. Cascio, Y. Liu, B. Gingrich and T. R. Insel (1999). "Dopamine D2 receptor-mediated regulation of partner preferences in female prairie voles (*Microtus ochrogaster*): a mechanism for pair bonding?" Behav Neurosci **113**(3): 602-611.

Weaver, I. C., N. Cervoni, F. A. Champagne, A. C. D'Alessio, S. Sharma, J. R. Seckl, S. Dymov, M. Szyf and M. J. Meaney (2004). "Epigenetic programming by maternal behavior." Nat Neurosci **7**(8): 847-854.

Williams, J. R., K. C. Catania and C. S. Carter (1992). "Development of partner preferences in female prairie voles (*Microtus ochrogaster*): the role of social and sexual experience." Horm Behav **26**(3): 339-349.

Williams, J. R., T. R. Insel, C. R. Harbaugh and C. S. Carter (1994). "Oxytocin administered centrally facilitates formation of a partner preference in female prairie voles (*Microtus ochrogaster*)." J Neuroendocrinol **6**(3): 247-250.

Winslow, J. T., N. Hastings, C. S. Carter, C. R. Harbaugh and T. R. Insel (1993). "A role for central vasopressin in pair bonding in monogamous prairie voles." Nature **365**(6446): 545-548.

Wolff, J. O. and A. S. Dunlap (2002). "Multi-male mating, probability of conception, and litter size in the-prairie vole (*Microtus ochrogaster*)." Behavioural Processes **58**(1-2): 105-110.

Wolff, J. O., S. G. Mech, A. S. Dunlap and K. E. Hodges (2002). "Multi-male mating by paired and unpaired female prairie voles (*Microtus ochrogaster*)." Behaviour **139**: 1147-1160.

Wong, C. L., K. H. Lee, K. M. Lo, O. C. Chan, W. Goggins, W. S. O and P. H. Chow (2007). "Ablation of paternal accessory sex glands imparts physical and behavioural abnormalities to the progeny: an in vivo study in the golden hamster." Theriogenology **68**(4): 654-662.

Xu, W., S. Cohen-Woods, Q. Chen, A. Noor, J. Knight, G. Hosang, S. V. Parikh, V. De Luca, F. Tozzi, P. Muglia, J. Forte, A. McQuillin, P. Hu, H. M. Gurling, J. L. Kennedy, P. McGuffin, A. Farmer, J. Strauss and J. B. Vincent (2014). "Genome-wide association study of bipolar disorder in Canadian and UK populations corroborates disease loci including SYNE1 and CSMD1." BMC Med Genet **15**: 2.

Young, K. A., K. L. Gobrogge, Y. Liu and Z. Wang (2011). "The neurobiology of pair bonding: insights from a socially monogamous rodent." Front Neuroendocrinol **32**(1): 53-69.

Young, L. J. and Z. Wang (2004). "The neurobiology of pair bonding." Nat Neurosci **7**(10): 1048-1054.

Young, L. J., J. T. Winslow, R. Nilsen and T. R. Insel (1997). "Species differences in V1a receptor gene expression in monogamous and nonmonogamous voles: behavioral consequences." Behav Neurosci **111**(3): 599-605.