Conditioned Mate Guarding Behavior in the Female Rat

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ABSTRACT

Conditioned mate guarding behavior in the female rat

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Mating strategies describe a set of social and sexual behaviors that an individual, or a species uses in order to reproduce. The two ends of the spectrum of mating strategies are promiscuity, where an individual mates with a variety of partners, and monogamy, where individuals form enduring bonds and preferentially copulate with one another. However, much evidence exists, demonstrating that there is a great deal of flexibility within each of these mating strategies. The three chapters of this thesis were designed to understand how far the mating strategy of the female rat could be shifted, from promiscuity to monogamy, by assessing whether or not female rats would display mate guarding behavior. Chapter 1 showed that sexually naïve female rats, if given all of their rewarding sexual experience with the same male, would mate guard that male in the presence of a female competitor through female-female mounting. When female rats mate guarded, it they displayed more Fos induction within areas known to be involved in pair bonding and stress. Chapter 2 showed that the Fos induction seen within bonding regions was within oxytocin and vasopressin neurons. We also show that oxytocin and vasopressin are important for conditioned mate guarding behavior, by peripherally administering either oxytocin or vasopressin to females prior to their first sexual experience then subsequently testing them 4 days later for mate guarding. Both vasopressin and oxytocin were able to facilitate mate guarding. Chapter 3 explored the contribution of histone methylation to creating the enhanced expression of oxytocin and vasopressin we observed in chapter two. By pharmacologically blocking the action of LSD1 demethylases we were able to show that the enhanced expression of oxytocin and vasopressin are essential for the onset of mate guarding. Females treated with the LSD1 inhibitor failed to display mate guarding and did not show an enhanced expression of oxytocin or vasopressin. Together, these data demonstrate the flexibility within the mating strategy of female rats, and that their first experiences with mating and sexual reward influence the subsequent expression of their mating strategy which is sub served by neuromolecular and epigenetic mechanisms.

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LIST OF ABBREVIATIONS

Agg – Aggression

AOB – Accessory olfactory bulb

ARC – Arcuate Nucleus

AVP – Argenine Vasopressin

BLA – Basolateral nucleus of the amygdala

BNST – Bed nucleus of the stria terminalis

CeA – Central nucleus of the amygdala

CS – Conditioned stimululs

D1R – Dopamine receptor 1

DA – Dopamine

DAB - 3,3′-diaminobenzidine

EB – Estradiol Benzoate

Ejac – Ejaculation

FFM – Female-Female mounting

Hc – Hippocampus

HDACi – Histone deacetylase inhibitor

HDMs – Histone demethylases

HMTs – Histone methyl transferases

Hop – Hops and darts

Icv – Intracerebroventricularly

IHC – immunohistochemistry

Init – Initiation

Int – Interception

Ip – intraperitoneal

IR – immunoreactive

LSD1 – Lysine specific demethylase 1

Moun – Mount

mPFC – Medial prefrontal cortex

mPOA - Medial preoptic area

NAcc – Nucleus accumbens

NAcSh – Nucleus accumbens shell

OSR – Operational sex ratio

OT – Oxytocin

OTR – Oxytocin receptor

OVX – Ovariectomy

P – Progesterone

PBS – Phosphate buffered saline

Pres – Presenting

PVN – Paraventricular nucleus

RI – Interferences

Sol – Solicitation

SON – Supraoptic nucleus

SubCu – Subcutaneously

TBS – Tris buffered saline

TS – Time spent

UCS – Unconditioned stimulus

V1aR – Vasopressin 1a receptor

VMH – Ventral medial hypothalamus

VP – Ventral pallidum

GENERAL INTRODUCTION

Introduction to mating strategy

According to the Random Woman in *Sex and the City*, "Monogamy is fabulous. It gives you a deep and profound connection with another human being, plus you don't have to shave your legs as much" (Season 1, Episode 7, 1998). But, what about sex in nature? Although monogamy is the focus of the experiments in this thesis, in nature it is only one of four mating strategies displayed by mammals. Within mammals, diverse strategies appear that are based on social, sexual, and parental bonding between individuals in a group, social structure and hierarchy of individuals, along with context and population dynamics, especially sex ratios and availability of suitable mates. The strategies include monogamy, where the male has several mating attempts with the same female; polygyny, where the male mates with the same group of females in successive mating attempts; polyandry, where the female mates with the same group of males across several mating attempts; and finally, promiscuity, in which males and females mate with a variety of members of the opposite sex across all mating attempts (Clutton-Brock, 1989). Each of these unique strategies is associated with its own set of sexual, social, and defensive characteristics that have evolved in order to increase the reproductive success of the species.

Mating strategies have been characterized by the number of mates obtained by the non-limiting sex of a particular species and were, therefore, thought to be evolved characteristics of a species (Reynolds 1996; Dunbar 1988). One important hypothesis of this line of thought is that different species are largely hardwired from birth to mate in a certain way. This suggests then that a species' sexual behavior and the evolutionary strategies that underlie it are relatively fixed in a stable environment, with stable group characteristics, and should not be that amenable to short-term changes within the lifetime of one individual. However, it is now understood that mating strategies are actually a product of the reproductive strategy of the individual, not a characteristic of the species as a whole (Dunbar 1981). Moreover, instead of a mating strategy being hardwired in the brain as a function of a stable evolutionary process, it has been observed in humans and other species that variation exists both within specific populations and across groups of a certain species (Dunbar 1981). There are many different environmental, reproductive, and social factors that govern the plasticity of a mating strategy's expression within

individuals in a population (reflecting plasticity of an individual's brain development and experience). For example, there are many different ecological conditions that can influence the accessibility to mates, thus creating mating strategy flexibility. As mentioned above, one of the ecological factors that can affect mating strategy is the ratio of sexually receptive males to sexually receptive females at any given time, known as the operational sex ratio (OSR) (Emlen and Oring, 1977). By manipulating this ratio to become biased towards one sex, inter and intrasexual selection as well as mate competition increases (Emlen and Oring 1977; Weir et al., 2011). Other ecological factors that contribute to mating strategy flexibility include risk of predation, resource availability, and temporal distribution of receptive females (Emlen and Oring 1977). By manipulating these factors, different forms of mating strategy and mate guarding emerge within an individual's lifetime, again reflecting plasticity in the ability of the neural systems that subserve sexual behavior to process and interpret the different external factors and alter behavior to meet the new reproductive demands in the wake of new environmental demands.

Mate guarding is used in individuals to prevent another individual or group of individuals of the opposite sex from mating with rival members of the same species. Although the behavior within a species may appear fixed and involve some type of agonistic or aggressive strategy, there are actually many different ways that mate guarding is observed in nature. For example, a male may mate guard a specific female or a group of females throughout the reproductive cycle (Clutton-Brock 1988). This is done in an attempt to ensure the paternity of her offspring belongs to him. In most species, mate guarding of this nature entails a single male guarding the female(s) however, there are instances where a group of males will cooperatively guard a subset of females. Another way mate guarding is observed is through defense of territory or resources. In these cases, males typically guard mating or feeding territories that are within a portion of the female range or that overlap with the female range (Clutton-Brock 1988). By guarding these territories it keeps competitor males away, thus decreasing competition for mating with the females during their reproductive cycle. Although much research has been invested in studying mate guarding, a vast majority of these studies have been done in the context of male behavior. Despite the large gap in the literature describing female mate guarding, there is evidence that females are capable of, and do display mate guarding behavior.

Although a majority of mating strategy research has been done on males, one theory is that

the variation in mating strategy of males is actually dictated by several characteristics of female behavior such as; a need for biparental care of young, the size of female ranges, the size of and stability of female groups, and lastly, the spatial distribution of female populations (Clutton-Brock, 1988). These four characteristics of female behavior can be manipulated to create a need for either; monogamous, polygynous, polyandrous, or promiscuous mating strategies. Although these four mating strategies are all observed amongst mammals, for the purposes of this line of work, monogamy and promiscuity will be the focus of this discussion.

Monogamy is found in about 5% of mammalian species (Clutton-Brock 1988). Monogamous mating strategy does not infer sexual fidelity, although, in most cases mating is usually done predominantly with the bonded partner (Clutton-Brock, 1988). Since male fitness increases with the number of impregnated females, monogamous mating strategy is costly from a male perspective. There are several hypotheses as to why a male would confine himself to mating with one female as opposed to many, one of which being improving the reproductive success of females (Keliman 1977). If the female is unable to rear young on her own and male assistance is required, it would benefit the male to help in her raising the young to ensure their survival. In cases such as this, males can help in many aspects of young rearing including; nest building and maintenance, territory defense, providing food to the lactating female, as well as care and defense of young (Getz and Carter 1981). Territory is also a major contributing factor to development of a monogamous mating strategy. For example, if resources are scarce or if female ranges are very large and the male is unable to defend the territory of multiple females, selection favors the development of a monogamous mating strategy (Wittenberger and Tilson 1980; Emlen and Oring 1977). There are a variety of reasons that monogamous mating strategy may develop, paternal investment and territory constraints being two of them. Although it is counterintuitive at a superficial level that males should develop this strategy, as it goes against increasing their fitness, a monogamous mating strategy favors increased female fitness (Kirkpatrick and Ryan 1991) which thereby aids in the increased fitness of the male's offspring. By being selective in choosing a mate, females can gain the direct benefits of resource access, food, and paternal care, but they also gain indirect benefits such as access to strong genes that will ensure the survival of their offspring (Darwin, 1871).

In contrast, a promiscuous mating strategy would be beneficial as male fitness increases with the number of inseminated females (Trivers, 1972). In a promiscuous mating strategy both

males, and females mate with a variety of partners throughout the mating season, after which, males are not expected to contribute parentally to rearing the young. Although this strategy has some risks associated with it, such as not knowing the paternity of the offspring, it is also associated with many benefits for both males and females (Daly, 1972). For example, by mating with many males, the likelihood of encountering strong traits increases (Clutton-Brock and Harvey 1976). Also, promiscuity increases the chances of fertilization and decreases the likelihood of infanticide since the paternity of the offspring is not known (Hrdy, 1979). So, although promiscuity increases male fitness through multiple impregnations, it can also be beneficial to females.

Monogamous and promiscuous mating strategies appear at two ends of a spectrum of sexual partner choice. However, when selection favors one or the other, it is presumed that both males and females will benefit more from one strategy over the other. The need for monogamous or promiscuous mating strategy arises from different reproductive, social, and ecological constraints that have been placed on the species.

Mating strategy of the rat

Rats (*Rattus norvegicus*) are typically described in the literature as a promiscuous species (McClintock 1984). During a copulatory sequence of group mating in the wild, a male rat will mate with a variety of females and a female rat will mate with a variety of males (McClintock 1984). Throughout copulation, males and females typically take turns and work together to achieve a common goal of increasing the number of intromissions and ejaculations females receive from males at a preferred rate (McClintock 1984). During group mating, males take turns mating with the estrus female. However, turn taking is not done in a random manner. Only one male typically mates with estrus females at a time and the others wait in cue until he has ejaculated (McClintock 1984). However, there are several estrus females that the in-turn male will copulate with before an ejaculation is achieved (McClintock 1984). Although group mating is a cooperative effort, competition between animals still exists in form of female interceptions, when one female positions herself between a male and the female he is pursuing in order to take his intromission or ejaculation. This is usually done during early ejaculations, as that is when the sperm count is the highest (McClintock 1984). Not only does cooperative group sex lead to more intromissions and ejaculations, it allows for both males and females to copulate at their optimal pace (McClintock 1984). For males, the optimal pace of copulation is 1 intromission every 3

minutes (Larsson 1956). This rate is optimal for a male to achieve an ejaculation with fewer intromissions (McClintock 1984). For female rats, the optimal rate of sexual stimulation occurs over a different time span compared to the male, with an ideal pace of 1 intromission every 5-10 minutes. The longer latency between intromissions is ideal for inducing a progestational state (Edmonds et al.1972). When mating in groups, female and male rats are not limited to one partner. The in-turn male can intromit with one female and then move on to a different female for the next intromission, if he so chooses, and will continue to do so until he ejaculates. Once ejaculation is reached, a different male will begin mating with the estrus females. By having group sex, both males and females can have sex at their optimal pace (McClintock 1984). The ability to pace sexual stimulation is important, especially to female rats, because the ability to pace the rate of sexual interaction is rewarding (Martinez et al. 2001). As previously stated, in nature, male and female rats have different optimal pacing rates of sexual stimulation, which result in each sex being able to optimize certain physiological and reproductive-related outcomes. In nature, pacing is achieved by soliciting males when copulation is wanted and by running away when it is not. To take advantage of these optimal rates of copulation in a laboratory setting, specialized unilevel and bilevel "pacing" chambers have been designed for laboratory settings (e.g., Erskine, 1989; Pfaus et al., 1999; Pfaus et al., 2008), in addition to large open fields (McClintock, 1984). Use of these specialized chambers has allowed the rewarding effects of paced copulation to be determined (Martinez et al. 2001).

Natural and Conditioned Partner Preference in the Female Rat

Even though sex is a cooperative effort for rats in the wild, female rats naturally show a sexual preference for certain males. This preference is expressed in a few different ways, one of which is the post ejaculatory quiescence period, the period between receiving an ejaculation and initiating the next copulatory series. If a female rat receives an ejaculation from a subordinate male, she will initiate the next copulatory series faster than if she received an ejaculation from a dominant male. The difference in the length of post ejaculatory quiescence can be attributed to female rats trying to ensure that the paternity of her litter belongs to the dominant male (McClintock 1984). Another way that female rats demonstrate partner preference in the wild is by taking ejaculations from a specific male. Before a male rat ejaculates he vocalizes a 50kHz ultrasonic call, which lets the female rat know that he is about to ejaculate (McGinnis & Vakulenko, 2003; McClintock, 1984). Upon hearing this call the female rat can solicit the male

or intercept him in order to ensure that she receives his ejaculation (McClintock, 1984). Some females in the wild demonstrate a preference to receive ejaculations selectively from a particular male, whereas others seem to choose males randomly (McClintock, 1984). How do such preferences develop in a species considered promiscuous? Can they be learned by experience? What are the brain mechanisms associated with such plasticity in behavioral response?

Much work has been done in a laboratory setting to evaluate the role of learning in female sexual behavior and the development of partner preferences. Previously, it has been shown that female rats will develop conditioned appetitive behaviors, including a conditioned place preference (CPP) for environments associated with a sexual reward (Oldenburger, Everitt, & de Jonge, 1992; Paredes & Alonso 1997). The role of paced mating in forming conditioned place preference was examined by Paredes and Alonso (1997). In this study females were given sexual training and either allowed to control the pace of sex or not allowed to pace at all. After sex, the female rats were exposed to one distinctive side of a CPP box so that they would associate a distinct environment with the sexual reward state induced by paced mating. CPP only developed for the distinct environment that was associated with self-paced mating. Since only animals that were allowed to pace the rate of sex developed a place preference, these results demonstrate that self-paced sex induces sexual reward, and that rats will develop a preference for environments in which they received a sexual reward (Paredes & Alonso, 1997).

After establishing that self-paced sex is rewarding, and that rats will develop a preference for environments that they associate with sexual reward, it was next important to see if rats could also develop a preference for partners that they associate with sexual reward. Olfactory stimuli are known to be important in expression and modulation of sexual behavior in the rat, and their role in the development of conditioned partner preference in the female rat has also been thoroughly investigated (Cain & Paxinos, 1974; Larsson, 1971; McClintock 1978, 1984a; McClintock and Adler, 1978; Coria-Avila et al., 2005). Early studies, done in males, demonstrated that neutral odors (e.g., almond, lemon, wintergreen) that are paired with copulation to ejaculation produce an ejaculation preference where males prefer to ejaculate with a certain partner (Kippin & Pfaus 2001a, 2001b). These findings were extended into females by Coria-Avila et al. (2005), which used paced mating to condition females to prefer a male bearing a neutral odor. In this line of research, female rats were given paced mating trials with a male bearing a neutral (almond) odor on the back of his neck. When put into an open field and given

the choice between mating with a scented male or a non-scented male, female rats solicited the scented male more frequently and chose him more often to provide her first ejaculation. This shows that female rats prefer to copulate and mate with partners that possess familiar cues. Also, by pairing a neutral odor with a sexual reward state, female rats can develop a partner preference for specific males (Coria-Avila et al., 2005).

Brain function and behavior are modified by experience. In Pavlovian conditioning paradigms, a neutral external stimulus is paired with an internal state of emotion, reward, or aversion that is induced by an unconditioned stimulus (UCS; Pavlov, 1927). Once the association is made and the neutral stimulus predicts the unconditioned stimulus, it becomes a conditioned stimulus (CS) that is able to prime or activate the same neural circuits and networks as the UCS (Hebb, 1949; Johnson, LeDoux, & Doyere, 2009). In the studies reviewed above by Coria-Avila et al. and Kippen et al., Pavlovian associations were made between an odor CS, and the unconditioned sexual reward state (UCS) in order to modify the partner preference of the rats. These modifications were accompanied by changes in brain activation to the odor alone in paired versus unpaired males (Kippin, Cain, and Pfaus, 2003) and females (Coria-Avila and Pfaus, 2007). It has been shown that the neural circuits responsible for sexual reward and incentive responding require the interaction of three neurochemical systems, dopamine (DA), hypothalamic oxytocin (OT), and opioids to work in conjunction with one another to inhibit brain structures, like the mPOA, and to sensitize the mesolimbic DA system via disinhibition (Kalivas & Stewart, 1991; Pfaus, Ismail, and Coria-Avila, 2010). In the case of partner preference conditioning, if the neutral odor is used to predict the sexual reward, the odor activates the sexual reward circuit and the neurochemical systems for DA, OT, and opioids, which leads to the development of a partner preference for a scented mate.

Social Monogamy

Sociosexual attachments that form between mates occur in a wide variety of species and are a topic of interest. There are no reported examples of absolute sexual exclusivity in sexually monogamous species in the nature, attachments can form between mates that take the form of parental or social monogamy. Social monogamy is defined, across species, as a preferential and enduring association between sexually mature adults of opposite sexes (Wickler and Seibt 1983). Social monogamy exists in many different taxa including many species of birds, Australian sleepy lizards (*Tiliqua rugosa*), prairie voles (*Microtus ochrogaster*), and also, humans (*Homo*

sapiens) (Birkhead and Moller 1992, 1996; Bull et. al 1998; Hall 1981). Relationships that are structured around social monogamy are characterized by bi-parental care, selective contact, affiliation, mate guarding, and preferential copulation with the partner over a stranger (Gubernick 1994). Although socially monogamous pairs demonstrate preferential copulation with their partners, extra-pair copulations frequently occur (Birkhead and Moller 1992, 1996; Bull et al. 1998).

Mating Strategy of the Prairie Vole

The prairie vole is considered to be the hallmark example of a socially-monogamous mammal. Prairie voles are a species of rodent that inhabit the grasslands of the central United States (Hall, 1981). They form enduring social attachments with their mate, called pair-bonds, which are characterized by nest sharing, mate guarding, bi-parental care, selective contact and affiliation, and having a sexual preference for their partner (Young and Wang 2004). Using radio-telemetry, field studies of prairie voles have shown that pair bonded couples generally remain together throughout their lifespans, and if one dies, approximately 70% of the surviving partners will not bond to a new mate (Young et al., 2010; Young, Young & Hammock, 2005). Pair bonding in prairie voles has also been studied in a lab setting using partner preference testing (Young and Wang 2004). These studies have shown that 24h of cohabitation and mating is sufficient to induce a partner preference (Insel et al. 1995). Partner preference is typically tested in a multi-chambered Plexiglas apparatus that resembles a CPP box. The test subject is placed into a central chamber and is allowed to freely access the two connected chambers, which separately house a tethered partner on either side. If a partner preference has been formed, the test subject will maintain more side-by-side contact with and mate more with the partner than with the stranger (Williams et al. 1992).

As mentioned above, one important characteristic of pair-bonded animals is that they will display mate-guarding behavior. Once it has been determined that a partner preference has formed, voles are used to study selective aggression with a variant of the resident-intruder paradigm (Miczek et al, 1987; Winslow et al, 1993). These studies have shown that pair-bonded voles will aggress selectively against a conspecific, male or female, when the conspecific is placed into the same cage. Although most of these studies have been conducted in males, evidence exists that female prairie voles also show this behavior (Getz et al. 1981).

Neural correlates of bonding in the prairie vole

Extensive neuroanatomical and pharmacological studies have illuminated the anatomical and neurochemical mechanisms bonding behavior in prairie voles. Early studies targeted the vasopressin (AVP) and oxytocin (OT) systems, as these neuropeptides are known to regulate many species-specific social behaviors including sexual behavior, aggression, and maternal care (Argiolas and Melis 2005; Ferris et al., 1984; Kendrick et al., 1987). Profiles of AVP and OT cells and fibers have been mapped in the vole brain, in both monogamous prairie voles and promiscuous montane voles (*Microtus montanus*) (Bamshed et al., 1993). Although AVP and OT neurons and fibers are highly conserved across different species of vole, receptor distribution and regional densities are markedly different (Ross et al., 2009; Young et al., 2010). Prairie voles have higher densities of the AVP V1aR receptor in the bed nucleus of the stria terminalis (BNST), ventral pallidum (VP), central (CeA) and basolateral (BLA) nuclei of the amygdala, and accessory olfactory bulb (AOB) than do their non-monogamous relatives (Insel et al., 1994; Young et al., 1997). Monogamous vole species displayed higher densities of OTR in the BNST, medial prefrontal cortex (mPFC), and nucleus accumbens (NAc) than non-monogamous vole species (Insel et al., 1992; Smeltzer et al., 2006; Young et al., 1997). These differences are stable across lifespan and are receptor specific (Insel at al., 1992).

Pharmacological studies have provided further evidence of the functional significance of AVP and OT in pair bonding voles. Intracerebroventricular (icv) administration of vasopressin 1a receptor (V1aR) antagonist blocked partner preference formation in male prairie voles, where direct icv administration of AVP was able to induce partner preference in males without mating, and in females following 1 hour of cohabitation with a male (Winslow et al., 1993). This effect was blocked if V1a antagonist was administered concurrently. Likewise, icv infusion of OT induced partner preference formation in both sexes, and this effect was blocked by concurrent administration of an OT receptor antagonist (Cho et al., 1999). These studies clearly demonstrate that both AVP and OT are essential in pair bond formation in both sexes of prairie vole.

Epigenetic modifications - histone methylation

Chromatin remodeling is known to be a key component of cell function and differentiation, and is important in brain development and the expression of learned behaviors (Kouzarides, 2007; Wood et al., 2006; Levenson et al., 2005; Dias et al., 2013, Wang et al.,

2013). Histone modifications such as, phosphorylation, acetylation, and methylation, modify chromatin structure and result in unique transcriptional outcomes (Strahl et al., 2000; Kobicek et al., 2004). The modification examined in this thesis focuses on histone lysine methylation. Lysine residues on histone protein tails can be methylated in three different ways: mono, di, or tri, and depending on the site being methylated, lysine methylation can have a bidirectional effect on transcription, where certain patterns lead to transcriptional activation and others to repression (Kobicek et al., 2004). Two enzymes orchestrate the addition or removal of methyl groups from protein tails: histone methyl transferases (HMTs) add methyl groups and histone demethylases (HDMs) remove methyl groups from histone tails.

Epigenetic control of behavior

The complex relationship between genes and behavior has long been an interest of neurobiological research. Through the last decade, many advances have been made in understanding this connection, many of which come from the now-booming field of behavioral epigenetics. By pharmacologically manipulating the enzymes responsible for the addition and removal of epigenetic tags (e.g. methyl) to and from histone proteins, the effects that epigenetics have on behavioral outcomes can be observed directly. For example, researchers have blocked the function of lysine specific demethylase enzymes by pharmacologically administering a demethylase inhibitor to mice (Neelamegam et al., 2012). One hour after drug administration, mice were placed in an arena with two novel objects and allowed to explore both for an equal amount of time. One day later, they were presented with one familiar object, and one novel object. Normally, mice would recognize the familiar object and show a preference for investigating the novel object. Mice treated with the demethylase inhibitor failed to show this preference, thus demonstrating that their ability to form long-term memories was disrupted (Neelamegam et al., 2012).

Epigenetic mechanisms are also beginning to be explored in the context of bonding behavior. Sexually naïve female prairie voles were administered a histone deacetylase inhibitor (HDACi), a drug that has been shown to result in transcriptional activation, before being exposed to a partner vole (Wang et al., 2013). Female prairie voles treated with the HDACi were able to establish a partner preference even in the absence of mating, and displayed an up-regulation of OT receptor and V1a receptor. The observed partner preference formation and OTR and V1aR up-regulation in HDACi treated animals mirrored the behavior and receptor expression in female

voles allowed to mate to form partner preference, demonstrating that there are underlying epigenetic mechanisms to partner preference formation in the female prairie vole.

Goals of the present thesis

There is now a wealth of data (reviewed above) showing that female rats form partner preferences for male rats with distinguishing characteristics (e.g., odor, strain) that are associated with the sexual reward state induced by paced copulation (Pfaus et al., 2012). This Pavlovian conditioning during their early sexual experience appears to shift the mating strategy of female rats from promiscuity to a rudimentary form of monogamy. The aim of the experiments reported in this thesis was to examine this effect in further detail. Can female rats form preferences for the same male, based on individual cues (e.g., major histocompatibility complexes (MHCs)), such that they display mate-guarding behavior? Do these behaviors involve the activation of AVP and/or OT systems in the brain? If so, can the activation of those systems be cued by noncopulatory access to the male? And if they can, are they dependent on epigenetic modification that stems from the initial experience with the sexual reward state? Accordingly, the main hypothesis was that sexually naïve female rats could behave more monogamously and display mate guarding behavior, if all of their sexually rewarding experience was paired with the same male. First it was predicted that mate guarding could develop in female rats, but that it was dependent on the sexual reward state induced by paced copulation. Second, it was predicted that female rats trained monogamously would display increased activation of AVP and OT neurons in response to male-related cues compared to female rats that were trained promiscuously, thus showing that AVP and/or OT are important in the acquisition and facilitation of mate guarding (as they are in voles). Third, it was predicted that epigenetic mechanisms, such as histone methylation, play a role in mediating changes to bonding circuits that occur in response to the pairing of sexual reward with male-related cues, thus resulting in monogamous mate-guarding behavior.

The first set of experiments investigated if female rats could display mate guarding behavior, and the role that sexually rewarding paced copulation played in the acquisition of this behavior. Sexually naïve female rats were given their first 10 paced sexual experiences with the same male (paired females) or with a variety of males (unpaired females). Paired females were then placed into an open field with their paired male and an unpaired competitor female and mate guarding was assessed. Neural correlates of mate guarding behavior were then evaluated using

Fos expression. Follow-up experiments addressed the role of paced copulation by not allowing females (paired or unpaired) to pace the rate of copulation and subsequently assessed mate guarding as described above.

The second set of experiments investigated whether female rats that had been conditioned to display mate-guarding had higher numbers of AVP and/or OT neurons activated by male cues relative to female rats trained promiscuously. Brains from paired and unpaired females were assessed after a behavioral test to examine the co-localization of Fos within AVP or OT neurons in the hypothalamic PVN and SON. To assess whether augmentation of AVP or OT transmission could facilitate the acquisition of mate-guarding behavior, peripheral injections were made during sexually naïve females' first sexual experiences of paced copulation with males. This was followed by a mate-guarding test 4 days later in which a paired female from one treatment group was placed into an open field with her paired male and an unpaired untreated competitor female.

The third study assessed the potential role of histone methylation in creating changes to bonding circuits in response to conditioning by disrupting the actions of demethylase enzymes. This was accomplished by peripheral administration of either a demethylase inhibitor or vehicle to paired female rats prior to each of ten conditioning sessions, with mate-guarding behavior assessed in the open field paradigm between paired demethylase treated females and paired saline treated females, relative to unpaired females. The effects of the demethylase inhibitor on the enhanced expression of bonding circuits within the brain was then investigated.

Conditioned mate-guarding behavior in the female rat

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Introduction

Mating strategies are specific sets of interactions that determine with whom and how often an individual mates. Several mating strategies can be observed in mammals, and each is accompanied by its own set of defining behaviors. For example, in nature, the rat is a described as having a promiscuous mating strategy that is characterized by cooperative and promiscuous group mating in both males (polygamy) and females (polygyny) (McClintock et al., 1979). On the opposite end of the spectrum are prairie voles, one of the few mammals that display social monogamy (Wickler et al., 1983). Prairie voles are considered unique in that they form preferential associations with a specific sexually mature individual of the opposite sex, known as a pair bond (Wickler et al., 1983). Pair bonds are characterized by selective contact and affiliation with the partner over a stranger vole, biparental care of the young, nest sharing, and mate-guarding behavior (Young et al., 2004). Thus, although voles and rats are closely related, they have adapted very different mating strategies due presumably to differences in environmental factors such as population density, sex ratio, and predator range, factors that are critical for the development of social monogamy in the prairie vole (Carter et al., 1995; Klug et al., 2010).

The flexibility of sexual and reproductive strategies in the rat have been revealed in laboratory studies that employ Pavlovian conditioning paradigms (Klug et al., 2010; Coria-Avila et al., 2005; Martinez et al., 2001; Kippen et al., 2001a; Kippen et al., 2001b). When neutral odors (e.g., wintergreen, lemon, almond) are paired with copulation to ejaculation, male rats form a preference to ejaculate with a familiar partner over an unfamiliar one, a phenomena referred to as a conditioned ejaculatory preference (Kippen et al., 2001a; Kippen et al., 2001b). Female rats allowed to control or pace the initiation and rate of copulation form conditioned place preferences for distinctive environments paired with the post-copulatory reward state (Martinez et al., 2001). Paced copulation also supports the conditioning of sexual partner and mate preferences in female rats (Coria-Avila et al., 2005) as measured by selective solicitations and copulations with familiar males relative to novel males in an open field, and a selective preference to receive the familiar male's ejaculations. Thus, rats are capable of displaying rudiments of monogamous sexual partner and mate preference despite being an allegedly promiscuous species. Conversely, both male and female rats learn to avoid partners bearing an odor paired with sexual nonreward. In those studies, rats are paired with a sexually nonreceptive

partner bearing an odor, or are administered the opioid receptor antagonist drug naloxone, prior to their first several sexual experiences. Rats are given alternating access to unscented sexually receptive partners (or administration of saline prior to unscented sexually receptive partners). On a final test, rats are given access to two receptive partners, one scented and the other unscented. In both cases, males and females copulate selectively with the unscented partner (Pfaus et al., 2012). Thus, rats can learn to modify their sexual strategy depending on early sexual learning. However, how far the mating strategy of the rat can be shifted by early sexual experience remains elusive.

The present study investigated whether female rats that have exclusive access to a single male during their first paced sexual experiences might display mate-guarding behavior when paired with that male and a competitor female in an open field. We also examined whether the development of such behavior alters the pattern of expression of the immediate-early gene product Fos in the brain of those females following this interaction compared to the competitor females or partnered females that received nonpaced early sexual experience with a male. Comparative studies between monogamous and non-monogamous vole species has lead to the identification of brain regions that are involved in monogamous responding in voles, including the activation of oxytocin and vasopressin systems in the paraventricular and supraoptic hypothalamic nuclei, the mesolimbic dopamine system, and regions such as the ventral pallidum that integrate those systems into behavioral output (Bamshed et al., 1993; Insel et al., 1992; Insel et al., 1994; Northcutt et al., 2009; Aragona et al., 2003; Liu et al., 2003). The activation of these and other regions related to partner preference, sexual reward, and appetitive sexual responding were examined using Fos immunocytochemistry following the open-field test.

Materials and methods

Animals and surgery

Sexually naïve Long-Evans female rats (200-250g) were obtained from Charles River Canada (St-Constant, QC, Canada). Animals were housed in shoebox cages in groups of two in a colony room on a reversed 12:12h light/dark cycle at approximately 21 °C and given free access to food and water. Female rats were ovariectomized (OVX) bilaterally via lumbar incision. Prior to surgery, female rats were anesthetized using a 1ml/kg intraperitoneal injection of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4ml/kg), mixed in a ratio of

4:3 respectively. Female rats were given 1 week to recover from the procedure prior to the conditioning trials. Throughout the duration of the experiment, female rats were maintained on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 μg in 0.1 ml of sesame oil) 48h prior to testing, and progesterone (P; 500 μg in 0.1 ml of sesame oil) 4h prior to testing.

Sexually naïve male rats (300-350g) were also obtained from Charles River Canada (St-Constant, QC, Canada). They were housed in group cages (4 animals per cage) and housed under conditions identical to those of the female rats.

All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Conditioning apparatus

Conditioning occurred in Plexiglas unilevel pacing chambers (38cm H x 60cm W x 38cm deep) with wire-mesh floors covering a layer of bedding (Coria-Avila et al., 2005). Chambers were bisected by a Plexiglas divider with four holes cut into the bottom which were large enough for the female to crawl through but too small for the male to crawl through (Coria-Avila et al., 2005; Ismail et al. 2011; Erskine et al., 1989).

Conditioning procedure

Conditioning sessions occurred at 4-day intervals, 4h after P injections, during the middle third of the rats' circadian cycle (lights off at 08:00). Females were assigned randomly to one of 2 cohorts. Cohort 1 consisted of self-paced paired and self-paced unpaired females. Cohort 2 contained non self-paced paired and non self-paced unpaired (N=12/group) females. All paired females copulated with the same male across all trials, whereas unpaired females copulated with a variety of males across all trials. Males were placed onto one side of the conditioning chambers and allowed a 5-minute habituation period before each trial. Females were then placed into the opposite side of the conditioning chamber and allowed to have paced sex with the male for 20 minutes. Each group received 10 trials, which were all recorded on video and scored for mate-guarding behavior.

A third cohort of animals was run following the same protocol, however, self-paced (N=15 paired + 15 unpaired) and non self-paced (N=16 paired + 16 unpaired) groups were run within the same test session so a direct comparison could be made between the two conditions.

Mate-guarding test

Four days after the final conditioning trial mate-guarding was assessed using an open-field (123cm x 123cm x 46cm) with a thin layer of bedding (Coria-Avila et al., 2005). Each open-field contained a paired female with her corresponding male and an unpaired competitor female. Before the test, males were placed into the open field for a 5-minute habituation period, after which both the paired and unpaired females were placed into the open-field at 2 diagonal corners. Rats were allowed to copulate freely for a 1-hr period. After the open-field test half of female rats (N=6/group) were perfused and their brains were collected to examine Fos induction as a function of the behavior both of these groups displayed. The remaining animals underwent two reconditioning trials, which followed the same protocol as the conditioning trials. After the second reconditioning, the female rats were exposed to the pacing chamber alone for 1h and then perfused so their brains could be examined for Fos expression in response to the contextual cues alone.

All open-field tests were recorded on video and scored afterward using a computerized event recorder customized for rat sexual behavior in an open-field (Cabilio, 1998). The frequency of solicitations, hops and darts, and defensive responses were recorded, as were the incidents and reflex magnitudes of lordosis when males mounted. Mate-guarding behavior appeared as female-female mounting, typically initiated by the paired female toward the unpaired female competitor. Interceptions and time spent near the male were also recorded for both females.

Perfusions

Animals were euthanized with an overdose of sodium pentobarbital (120mg/kg) administered via intraperitoneal injection. They were then perfused intracardially with 250ml of phosphate-buffered saline (PBS) followed by 250ml of 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 hours. After which brains were placed into a 30% sucrose solution for 48 hours then flash frozen and stored at -80°C until slicing.

Tissue preparation and immunocytochemistry

Brains were divided into 4 groups: both paired and unpaired females perfused after the open-field, and paired and unpaired females perfused after reconditioning (N=5/group). Using a freezing microtome, sections were cut at 30µm and divided into three series, one of which was used for immunocytochemistry to label for Fos expression. The sections were washed in cold

Tris-buffered saline (TBS) and incubated first with 30% hydrogen peroxide (H_2O_2) in TBS for 30 min at room temperature followed by 3% Normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at 4°C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at 4 °C, with biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200). Sections were next incubated sequentially in 0.05% Trition-TBS with 3% NGS for 1 h at 4 °C, and avidin–biotinylated–peroxidase complex (Vectastain *ELITE*ABC KIT, Vector Laboratories Canada; diluted 1:55) for 2 h at 4 °C. Sections were washed in TBS (3 × 5 min) between each incubation. Immunoreactions were stained by sequential treatments with 50 mM Tris for 10 min, 3,3′-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer + H_2O_2) all at room temperature. Reaction was stopped by rinsing (3 x 10 min) in PBS.

Following immunocytochemistry, sections were mounted onto gel-coated slides and cover slipped. Cover slipping procedure started with sequential washes of distilled water followed by washes in 70%, 90%, and 100% alcohol each at room temperature for 1minute duration. Slides were then submerged in Xylenes for 2 hours at room temperature after which they were cover slipped using Permount solution.

Pictures of brain regions were taken using an Olympus light microscope at 10x magnification using Q-Capture pro software. Brain regions were defined using the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Fos positive cells were then counted using Image-J technology within the Ca fields and dentate gyrus of the hippocampus (Hc, Plates 28-36), central nucleus of the amygdala (CeC, Plates 25-31), nucleus accumbens shell (NAc, Plates 10-15), supraoptic nucleus (SON, Plates 21-25), paraventricular nucleus (PVN, Plates 25-26), medial preoptic area (mPOA, Plates 18-24), arcuate nucleus (Arc, Plates 26-35), and ventral medial hypothalamus (VMH, Plates 26-32). Five slices were counted per animal. These slices represented each area rostrally to caudally and were matched among animals to ensure site specificity. Counts were done bilaterally in a total of 5 animals per group that were chosen at random. Total number of Fos positive cells was counted on each section and added together to represent a mean total count per region per animal.

Statistical Analyses

Mate-guarding behavior in the open-field was assessed using independent samples t-tests since group-specific comparisons were planned ahead of time. Cohens d was used to assess effect size. The data collected from Fos-IR cells were analyzed using a one-way analysis of variance (ANOVA). Eta squared was used as a measure of effect size.

Results

Mate-guarding behavior

An independent samples t-test revealed that paired female rats in the self-paced condition (cohort 1) showed a significant increase in female-female mounting, $t(44) = -2.174$, $p = .035$, d=.641, compared to unpaired female rats from the same condition. This increase in female-female mounting was not seen in the non self-paced condition, $t(42) = .305$, $p = .762$ (Figure 1). Other behaviors such as interceptions, solicitations, aggression, time spent in proximity to the male, and the number of times initiating contact with the male, did not reach statistical significance.

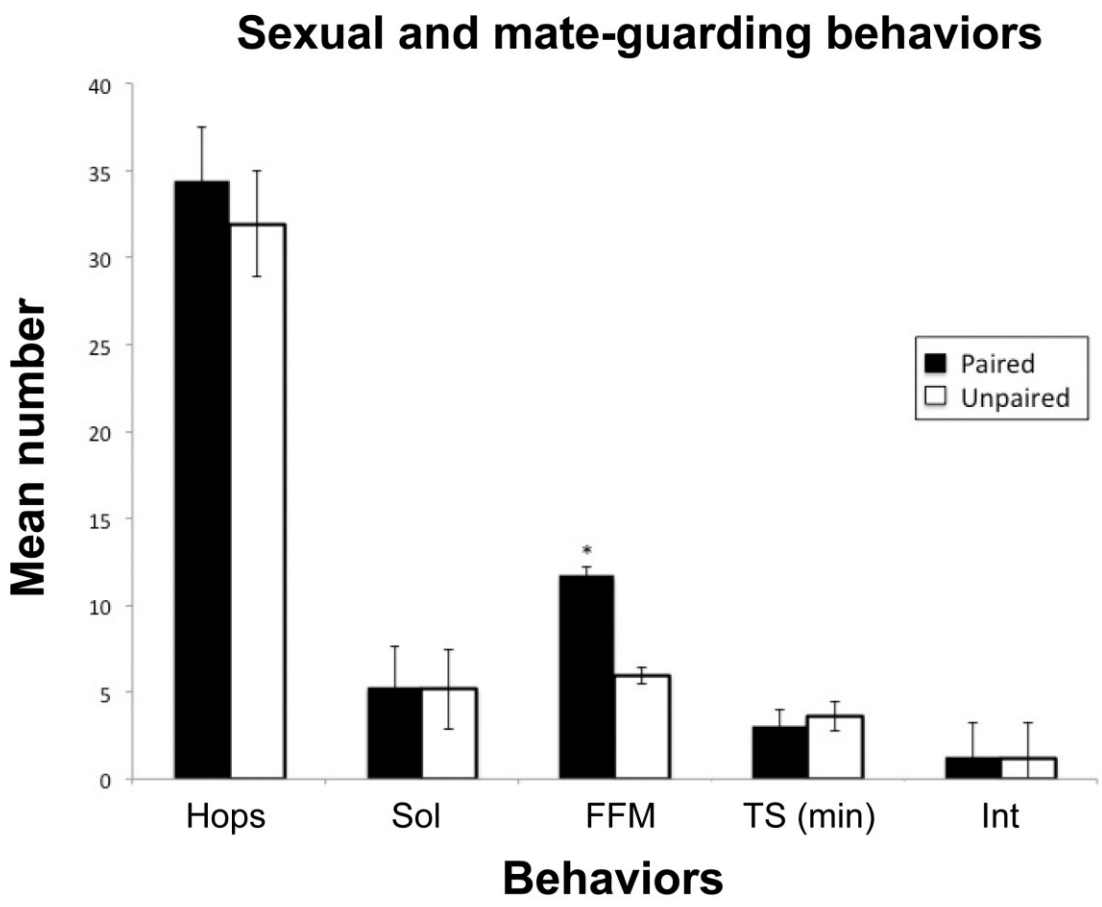

Figure 1:

Paired female rats that were allowed to pace the rate of sexual interaction displayed mate-guarding behavior as expressed by agonistic female-female mounting (FFM) during the open-field test. Behaviors include hops, solicitations (Sol), female-female mounting (FFM), time spent in proximity to the male (TS), and interceptions (Int). Data are means ± SEM. * $p < 0.05$.

Independent samples t-tests were also used to look at behavioral differences between the paired females in both the self-paced, and non self-paced condition (cohort 3). This statistical analysis revealed that paired females in the self-paced condition solicited the male more frequently than the paired females from the non self-paced condition, $t(29) = 4.650$, $p=.000$, d=1.652. Although statistical significance was not reached, there was a trend in favor of paired females in the self-paced condition receiving more ejaculations than paired females in the non self-paced condition, $p=.056$, d=.715, and also, more intromissions, $p=.054$, d=.278 (Figure 2). Interceptions, hops and darts, aggression, female-female mounting, time spent in proximity to the male, and number of times initiating contact with the male were also quantified, but did not approach statistical significance.

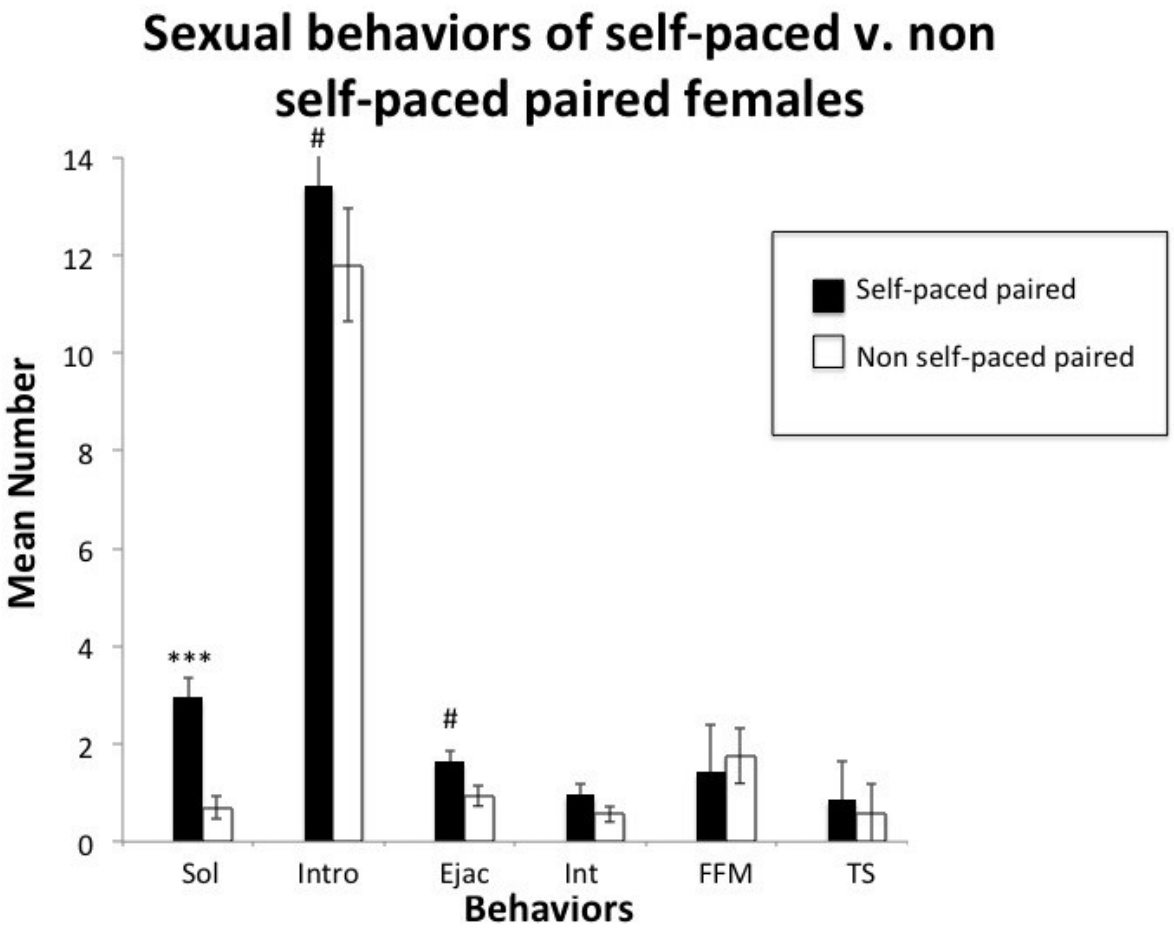

Figure 2:

Paired females in the self-paced condition selectively solicited the male more frequently than paired females in the non self-paced condition in the open-field test. Behaviors include: solicitations (Sol), intromissions (Intr), ejaculations (Ejac), interceptions (Int), female-female mounting (FFM), and time spent in proximity to the male (TS). Data are means ± SEM. *** p <0.001, # p<0.1 (trend).

Brain activation

A one-way ANOVA was used to look at differences in Fos expression between paired and unpaired females after, both, the open-field and exposure to the pacing chamber alone. Paired females from the self-paced condition, perfused after the open-field, showed significantly more Fos expression within the supraoptic nucleus than did unpaired females from the same condition, $F(2,10) = 17.843$, $p=.001$, $\eta^2=.78$ (Figure 3). Although statistical significance was not reached, there was a trend observed in the paraventricular nucleus, with paired females showing more Fos expression than unpaired females, $F(2,9) = 3.560$, $p=.073$, $\eta^2=.44$. In accordance with our hypothesis, the ventromedial hypothalamus, an area known to be activated by copulation alone, showed no differences between paired, and unpaired females in the self-paced group, that were perfused after the open-fields, $p=.988$ (Figure 3).

Additionally, paired females from the self-paced condition, whose brains were collected after exposure to the pacing chamber alone, had significantly more Fos induction within the nucleus accumbens shell than did unpaired females from the same condition, $F(2,12) = 4.608$, $p=.033$, $\eta^2=.434$ (Figure 3). Although statistical significance was not reached, there was a trend in favor of paired females also having more fos expression than unpaired females within the hippocampus, $p=.083$.

Fos expression was also quantified in other regions including the medial preoptic area, central nucleus of the amygdala, and the arcuate nucleus, however, these results did not reach statistical significance.

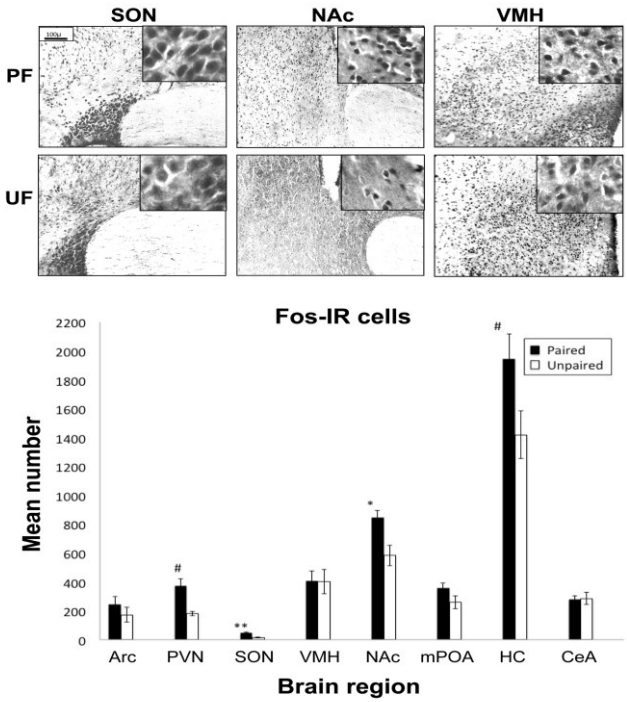

Figure 3: Top: Photomicrographs (40x) showing Fos-IR in the VMH, SON, and NAc following the open-field test (VMH, SON) or exposure to the context alone (NAc) in paired versus unpaired females. Paired females showed significantly more Fos induction, following the open-field, in the SON than did unpaired females. Following exposure, paired females showed significantly more Fos expression within the NAc than did unpaired females. VMH was measured as a control region, no significant differences in Fos were observed. Magnified inserts taken of selected regions at 100x to more clearly display differences in Fos. Bottom: Fos positive cells in different brain regions from paired or unpaired groups Data are means ± SEM. ** $p<.001$, * $p<.05$, # trend. Brain regions include: arcuate (Arc), paraventricular nucleus (PVN), supraoptic nucleus (SON), ventral medial hypothalamus (VMH), nucleus accumbens shell (NAc), medial preoptic area (mPOA), hippocampus (Hc), and central nucleus of the amygdala (CeA).

Discussion

In this experiment, female rats were given their first 10 sexual experiences either with the same male (paired) or with a different male each time (unpaired). When tested in an open-field with the familiar male, paired female rats displayed agonistic mounting of a competitor female, whereas unpaired females did not display this behavior. Female-Female mounting is form of female social behavior, and plays a role in maintaining a dominant social status (Fang and Clemens, 1998). Female rats are more likely to mount an unfamiliar female than a familiar one, especially if the other female is in heat; however, this mounting behavior typically decreases in the presence of a male (Fang and Clemens, 1998). In this study, in presence of the male and a competitor female, the paired female feels dominant to the competitor, and mounts her in an attempt to establish dominance and to chase her away from the male before she can potentially mate with him. This mounting behavior would often occur in succession until the unpaired female had left the vicinity of the male. Although unpaired female rats did mount the paired female, the context in which they did so was markedly different. The few times that unpaired females would mount the paired female, were done out of frustration from not receiving sexual contact from the male.

We also found that mate-guarding behavior only develops if female rats are allowed to pace the rate of copulation, as animals in cohort 1, which were allowed to pace the rate of copulation displayed mate-guarding in the presence of a competitor, but females in cohort 2, which were not allowed to self-pace the rate of copulation, did not. This is supported by previous work by Martinez and colleagues showing that rats will only develop CPP for environments they pair with the sexual reward achieved through self-paced mating (Martinez et al., 1997). Coria-Avilla et al., also demonstrated that female rats will develop a preference to copulate with a scented partner over an unscented one if they are allowed to self-pace the rate of sex with a scented partner (Coria-Avila et al., 2005). The data from this study extend these findings by showing that the sexual reward induced by self-paced mating is so strong that odor is not necessary for a partner preference to form in female rats. If a female rat receives her first 10 paced sexual experiences with the same male, she will recognize that male without the addition of an odor, and will defend her exclusive access to him as a sex partner if a competitor is present. Furthermore, the behavioral differences between the paired females in the self-paced and non self-paced condition indicate that only the self-paced paired females selectively solicit sex from

the male more frequently than those in the non self-paced condition (cohort 3). During conditioning, females in the self-paced condition had many more opportunities to move, and could easily solicit the male within the chamber as it was easy for them to have free access to both sides. This was not the case in the non self-paced condition; the female was often blocked by the male from either entering or leaving his side of the chamber, which likely led to a different and less optimal rate of sexual stimulation. Thus, we argue that females in the self-paced condition experienced a more optimal level of sexual stimulation and reward, leading to a stronger preference for the stimulus male paired with this condition relative to females in the non self-paced condition or unpaired females.

Brain activation was also examined using Fos as a marker of neuronal activation in two conditions, one in response to the expression of mate-guarding behavior, and the other in response to the context (chamber alone)**.** In line with our hypothesis, after the mate-guarding test, paired females showed significantly more Fos-IR within the SON, and there was a trend in the favor of paired females also showing more Fos-IR within the PVN. Both of these brain regions contain OT and AVP cell bodies, and OT and AVP are known to regulate many social behaviors that are associated with pair bonding, such as sexual behavior, aggression, and maternal care (Insel et al., 1992; Insel et al., 1994). Furthermore, it has been shown that OT and AVP play a critical role in pair bonding in monogamous vole species, and that differences in the receptor distribution for both OT and AVP influence the social structure of monogamous and polygamous vole species (Insel et al., 1992; Insel et al., 1994). Alternatively, the increase in Fos-IR within the SON and PVN could signal a reaction to stress (Jezova et al., 1995). Considering that we also observed a trend for a larger number of Fos-IR cells in the hippocampus of paired females, another region involved in stress responding, it is possible that some of the neuronal activation was due to the stress of mate guarding in addition to appetitive responses to the paired male or rewarding copulatory stimulation (Buwalda et al., 2012). However, we note that differences in the number of Fos-IR cells between the groups were not observed in other stress-reactive regions, such as the arcuate nucleus or central amygdala, suggesting that the overall pattern of brain activation is not simply due to the induction of a stress response. Finally, although Fos is used as a marker of neuronal activation, it cannot be used to denote the role of a region or neurochemical system in behavior. As with other forms of brain activation, such as fMRI, the induction of Fos could indicate the activation of either

excitatory or inhibitory pathways, and result from the processing of sensory information that excites or disinhibits different aspects of behavior, or that will inhibit behavior following suitable sensory stimulation (Pfaus and Heeb, 1997). More work is thus required to delineate the role of these regions in mate-guarding behavior.

Female rats in the paired group learned to associate the sexual reward induced by paced mating with a specific male. Because all copulation trials took place within the same chamber, paired female rats also learned that placement in the chamber predicted the presence of her paired male, so, we wanted to look at the Fos induction in response to context exposure alone. Paired females from the self-paced condition, whose brains were collected after exposure to the context alone, showed significantly more Fos induction within the nucleus accumbens shell than did unpaired females from the same condition. The nucleus accumbens has been shown to play a vital role in establishing and maintaining pair bonds in voles (Aragona et al., 2005). Dopamine transmission within the rostral shell of the accumbens promotes pair bond formation, through activation of D2 receptors, and also, plays a role in maintaining pair bonds, by activating D1 receptors, which in turn facilitates selective aggression (Aragona et al., 2005). However, further investigation is required to see if the brain activation seen here is within the dopamine system of this region.

The question of why monogamous behavior has evolved in rodents, namely prairie voles, remains unanswered although several theories exist. It is thought that monogamy arises from the environmental circumstances in which these animals live. Prairie voles, a socially monogamous rodent, occupy the grasslands of the central United States, which is a very harsh environment limited in resources and vole populations are widely dispersed (Hall et al., 1959). The influence of the environment on this behavior is best seen during the shift to winter months, when prairie voles live in closer proximity to one another, called clustering (Getz et al., 1986). During this time of year, more extra pair copulations occur, than in the spring/summer months when they disperse (Getz et al., 1986). In this study, we recreated this change in the female rat's environment artificially by allowing her to copulate with only one partner. This change in environment and specificity of the male may well have created a change in behavior by shifting the female rat's mating strategy more towards a monogamous pattern that resulted in mate-guarding, a behavior that has not previously been reported in rats, as they are typically described as being a promiscuous species (McClintock et al., 1978).

In summary, these data demonstrate that the female rat is not hard-wired to be promiscuous. By pairing sexual reward with a specific male, she can recognize that male based on primary partner cues alone (e.g. major histocompatibility complexes, pheromones, natural odors). Furthermore, when a competitor female is present, paired female rats display mate-guarding in the presence of their paired male. That this change in behavior is accompanied by brain activation in regions associated with the neurohypophyseal hormones oxytocin and vasopressin, dopamine, or regions known to be involved in monogamous responding in other rodent species, suggests that changes in environment, such as partner availability, during a rat's primary experiences with sexual reward induce long-term changes in neuroanatomical systems that underlie partner preference.

Summary of Chapter 1

Chapter 1 examined if the mating strategy of the female rat could be shifted from promiscuity towards monogamy using a Pavlovian conditioning paradigm, where a sexually naïve female had all of her sexually rewarding experience paired with the same male. This shift produced a pattern of mate guarding behavior when paired females were with their paired male and a competitor female. This pattern was expressed mostly in the form of agonistic female-female mounting of the competitor. Unpaired females with the same amount of sexual experience but with different males each test did not display conditioned mate guarding. In a second study, females were not allowed to self-pace the rate of copulation, removing the sexually rewarding reinforcement. In this study, neither paired nor unpaired females displayed mate guarding behavior. Next, a replication was conducted with both self-paced and non-self paced conditions. The data we obtained supported the initial findings: Only paired females from the self-paced group displayed mate guarding behavior with their paired male.

Brain regions activated in response to paired vs. unpaired male cues were examined using immunohistochemical detection of Fos protein. Paired females had more Fos induction within the SON nucleus and NAcSh compared to unpaired females. There were also two trends in favor of paired females displaying more Fos within the Hc and PVN nucleus than unpaired females. It was particularly interesting that these brain regions were active in response to mate guarding, as these regions are known to be involved in bonding and stress in other monogamous rodents. Of particular interest was the Fos induction with the SON and PVN nuclei, as these two regions contain a large number of AVP and OT neurons, the two neuropeptides that are heavily involved in bonding behavior. The studies in Chapter 2 assessed whether these two neuropeptide systems are involved in conditioned mate-guarding in female rats, as they are in female prairie voles.

The role of oxytocin and vasopressin in conditioned mate guarding behavior in the female rat.

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Introduction

Socially monogamous animals form enduring associations with one another that are characterized by selective affiliation, contact, and preferential copulation with their partners relative to strangers. These partnerships are called pair bonds (Gubernick et al., 1994). In rodents, the hallmark example of social monogamy is displayed by the prairie vole (*Microtus ochrogaster*), and this rich literature has provided the scientific community with ground breaking and in depth analyses that have laid the groundwork for other studies looking into monogamy, bonding, and mate guarding. Prairie voles inhabit a very harsh environment, with limited food, water, and mate access. It is thought that they adapted a socially monogamous mating strategy in order to increase the likelihood of their survival and reproductive success (Carter et al., 1995; Hall et al., 1959). For example, if a prairie vole happens to come across a potential mate, it would make sense to bond with this mate, since there is not a large likelihood of encountering another one. Bonding would allow for access to a mate, reproduction, would allow for biparental care of the young, and ultimately enable the couple to defend territory and gather resources more efficiently. However, the influence of the environment on bonding behavior in prairie voles can is most clearly observed during the winter months, when voles cluster together to form communal groups (Getz et al., 1993). When living in communal groups, more extra pair copulations occur among pair bonded voles than in spring and summer months when the vole population is more widely dispersed, demonstrating that population density and mate access can create shifts towards promiscuity within the mating strategy of the prairie vole (Getz et al., 1993). In the laboratory, pair bonding is determined when male prairie voles display a preference to spend more time with the first female they copulated with relative to a novel female (Williams et al., 1993), or when male/female prairie voles mate guard the first mate they copulated with (Williams et al., 1993).

The neuroanatomical and neurochemical bases of pair bonding have been elaborately examined in monogamous vs. promiscuous voles. Oxytocin (OT) and vasopressin (AVP) were early targets of this research, as these neuropeptides were known to modulate species-specific social behaviors such as sexual behavior, aggression, maternal care, and olfaction related to conspecific identification (Abel et al., 2001; Arigolas et al., 2005; Becker et al., 2001; Kendrick et al., 1987; Lemon et al., 2006; Pendersen et al., 1979; 1982). Neuroanatomical studies have revealed that, although there are minor species differences in cell bodies and fibers, there are

dramatic differences in regional receptor distribution between monogamous and promiscuous vole species (Ross et al., 2009; Wang et al., 1994; Wang et al., 1995). Specifically, monogamous prairie voles have higher densities of the vasopressin A1 receptor (V1aR), in the BNST, ventral pallidum (VP), central amygdala (CeA), basolateral amygdala (BLA), and accessory olfactory bulbs (AOB) than promiscuous montane voles (Insel et al., 1994; Lim et al., 2004; Smeltzer et al., 2006). Monogamous voles also display higher densities of oxytocin receptor (OTR) in the BNST, medial prefrontal cortex (mPFC), and nucleus accumbens (NaC) than promiscuous vole species (Smeltzer et al., 2006; Young et al., 1999; Insel et al., 1992). These regional differences in V1a and OTR expression have been shown to be stable across lifespan and do not exist in other systems such as the opiate receptor system (Insel et al., 1992).

Pharmacological studies provided direct evidence for the importance of OT and AVP in the formation and maintenance of pair bonding in monogamous voles (Winslow et al., 1995; Insel et al., 1992). By administering a vasopression 1a receptor (V1aR) antagonist intracerebroventricularly (icv), researchers were able to block the onset of pair bonding in male prairie voles. Similarly, direct icv administration of AVP facilitated pair bonding in males even in the absence of mating, and in females after 1h cohabitation (Winslow et al., 1995; Cho et al., 1999). Administration of OTR antagonist, similarly, blocked pair bonding in both males and females, and concurrent administration of OT was able to rescue pair bond formation (Cho et al., 1999). These results clearly demonstrate that both OT and AVP are essential for pair bonding in both sexes of prairie vole.

Rats (*Rattus norvegicus*), on the other hand, are considered promiscuous. They tend to live in large underground burrow systems and mate freely with many different partners throughout several copulatory series (McClintock et al., 1982). Despite this, a growing body of evidence shows that both male and female rats have the ability to display rudiments of monogamy such as sexual partner preferences. For example, through Pavlovian conditioning paradigms, where neutral odors are paired with sexual reward states, female rats have been shown to display preferences for scented partners over unscented ones, and vise versa if rewarding sex is paired with unscented partners (Coria-Avila et al., 2005). We have also reported that changing the conditions under which the promiscuous female rat has her first experiences of sexual reward, notably by shifting mate access, can alter her mating strategies, much like the population density and partner access creates shifts in the prairie vole mating

strategy (Holley et al., 2014; Getz et al., 1993). Female rats given their first 10 paced copulatory experiences with the same male displayed mate guarding behavior in the presence of their paired male and a competitor female. In these studies, mate guarding was defined as any behavior displayed by the female that prevented the male from attending to or mating with the competitor female (Holley et al., 2014). Under this definition several sexual (solicitations, presenting), aggressive (biting, tackling accompanied by vocalizations from the victim), dominant (female-female mounting), social (time spent in proximity, body positioning), and competitive (interceptions) behaviors were included to describe mate guarding (Holley et al., 2014). When female rats mate guarded, they displayed all of these behaviors to varying degrees, however, in our previous study, over time, paired females displayed significantly more female-female mounting relative to unpaired female rats. Paired female rats that were placed into an open field with their paired male and a female conspecific, would mostly remain in the vicinity of the male. However, if the competitor female approached the male, the paired female would run over to her, and mount her in succession until she left the vicinity of the male. We also demonstrated that mate guarding was dependent on paced mating, because females not allowed to pace the rate of copulations during their first sexual experiences, which decreases the reward value of copulation (Paredes et al., 1997), did not display mate-guarding behavior. Likewise, females that received paced copulation with different males each time did not show mate guarding behavior.

This shift in mating strategy was also accompanied by different patterns of neuronal activation within regions of the brain known to play a role in bonding behavior (Holley et al., 2014). For example, female rats conditioned to show mate guarding had significantly more Fos-positive neurons within the PVN and SON (along with other brain regions such as the nucleus accumbens and hippocampus) following exposure to their familiar male relative to females conditioned to show no partner preference. These data suggest that a similar set of neuroanatomic regions are activated by partner cues in monogamous female prairie voles and promiscuous female rats that have been conditioned to show mate guarding behavior. In turn, this opens up the possibility that OT and AVP may play a role in conditioned monogamous behavior.

This purpose of this study was to examine the potential role of OT and AVP in conditioned mate guarding behavior. First we examined whether the Fos induction in the PVN and SON observed in females conditioned to show mate guarding was within OT and/or AVP

neurons. We hypothesized that females that had all of their sexual experience paired with the same male would have more Fos induction within oxytocin and vasopressin neurons than females who had received their sexual experience with a variety of males. Second, we tested whether OT or AVP, injected peripherally prior to female rats first rewarding sexual experience, would facilitate the display of mate guarding behavior on their second sexual experience with the same male, relative to females injected with saline in order to elucidate potential roles that OT and AVP might be playing in the early onset of conditioned mate guarding. We hypothesized that peripheral administration of OT or AVP, but not saline, would facilitate different aspects of mate guarding behavior.

Materials and Methods

Animals and surgery

Sexually naïve Long-Evans female rats (200-250g) were obtained from Charles River Canada (St-Constant, QC, Canada). Animals were housed in pairs in shoebox cages in a colony room on a reversed 12:12h light/dark cycle at approximately 21°C. Animals had free access to food and water. Females were anesthetized using a 1ml/kg intraperitoneal injection of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4ml/kg), mixed in a ratio of 4:3 respectively, prior to bilateral ovariectomy (OVX) using lumbar incisions. Females had 1 week to recover before behavioral tests began. Throughout the duration of the experiment, female rats were maintained on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48h prior to testing, and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4h prior to testing.

Sexually naïve male rats (300-350g) were also obtained from Charles River Canada (St-Constant, QC, Canada). Males were housed in group cages (4 animals per cage) under conditions identical to those of the female rats.

All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Conditioning apparatus

All conditioned took place in Plexiglas unilevel pacing chambers (38cm x 60cm x 38cm) with wire-mesh floors covering a layer of bedding (Coria-Avila et al., 2005; Ismail et al., 2011). Chambers were bisected by a Plexiglas divider with four holes cut into the bottom. Holes were

large enough for the female to crawl through but too small for the male to crawl through (Coria-Avila et al., 2005; Ismail et al., 2011).

Conditioning procedure for animals used for double immunohistochemistry

Females whose brain tissue was used for double IHC in this study were conditioned as follows: Sexually naïve female rats were divided into 2 groups; females who always copulated with the same male (paired females, N=12), and females that copulated with a variety of males across all trials (unpaired females, N=12). All conditioning occurred in a pacing chamber (described above). Five minutes prior to the onset of conditioning, males were placed into one side of the chamber for a 5-minute habituation period. After these five minutes, the female was placed onto the opposite side of the chamber and the 20-minute mating test began. Both groups received a total of 10 trials at 4 day intervals. Four days following the last conditioning session, mate guarding was assessed using an open field (123cm x 123cm x 46cm) with a thin layer of bedding, containing the paired female, her paired male, and an unpaired female. Males were placed into the apparatus first for a 5-minute habituation. After the conclusion of 5 minutes, his paired female and an unpaired female were placed into the open field at opposite corners and a 1h mate guarding test commenced. All trials and open fields were video recorded. Open field videos were coded and then scored blindly for mate guarding behavior.

Perfusions

Animals in the first experiment were euthanized with an intraperitoneal injection of an overdose of sodium pentobarbital (120mg/kg) immediately following their 1h mate guarding test. They were then intracardially perfused with 250ml of phosphate-buffered saline (PBS) followed by 250ml of 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 hours. After which brains were placed into a 30% sucrose solution until they began to sink. Brains were removed from sucrose solution, flash frozen in dry ice, and stored at -80C until used for histochemical procedures.

Tissue preparation and immunocytochemistry

Brains from both paired and unpaired females were cut coronally at 30μm on a freezing microtome and divided into three series, one of which was used for double immunohistochemistry to label for Fos and OT, the second was used to label for Fos and AVP, and the third was stored as a back up. In the primary phase, sections were washed in cold Tris-buffered saline (TBS) and incubated first with 30% hydrogen peroxide (H_2O_2) in TBS for 30 min

at room temperature followed by 3% Normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at 4 °C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at 4 °C. In the secondary phase, sections are incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200), in 3% NGS, and .2% Triton TBS for 1 hour. Following this, sections enter a tertiary phase, where sections are incubated in avidin–biotinylated–peroxidase complex (Vectastain *ELITE*® ABC KIT, Vector Laboratories Canada; diluted 1:55) in 3% NGS, and .05% Triton TBS for 2 h at 4 °C. Sections were washed in TBS (3 × 5 min) between each incubation. Immunoreactions were stained by sequential treatments with 50 mM Tris for 10 min, 3,3′-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer + H_2O_2) all at room temperature. Reaction was stopped by rinsing (3 x 10 min) in PBS. Sections were then transferred into the second primary antibody, either rabbit polyclonal anti-oxytocin (Chemicon, AB911, diluted 1:10,000) or rabbit polyclonal anti-vasopressin (Millipore, AB1565, diluted 1:10,000) in 0.05% Triton-TBS with 3% NGS, and incubated for 72h at 4 °C. After completion of this incubation, secondary and tertiary phases are repeated. However, during the final DAB wash, no nickel chloride was used in order to create a light brown cytoplasmic stain.

Sections were mounted onto gel-coated slides and cover slipped following immunohistochemistry. Cover slipping procedure started with sequential washes of distilled water followed by washes in 70%, 90%, and 100% alcohol each at room temperature for 1minute duration. Slides were then submerged in Xylenes for 2 hours at room temperature after which they were cover slipped using Permount solution. Slides were untouched for a week to allow for the Permount to dry and then cleaned and coded so that all analysis could done blind.

Sections were examined using an Olympus light microscope at 40x magnification. Pictures were taken at 20x and 100x using Q-Capture pro software. Double-labeled cells were identified by eye as any cell having a dark black nucleus (Fos positive) and a light brown cytoplasm (OT or AVP positive). Brain regions were defined using the atlas of Paxinos and Watson (Paxinos & Watson., 2006). Double-labeled cells were counted within the supraoptic nucleus (SON, Plates 21-25), and paraventricular nucleus (PVN, Plates 25-26). Five slices were counted per animal. These slices represented each area rostrally to caudally and were matched among animals to ensure accuracy. Counts were done bilaterally in a total of 5 animals per

group. Total number of double-labeled cells was counted in each section, which were then used to derive the mean number of double-labeled cells within the region for each animal.

Drug preparation

Reagent grade OT (Bachem, H2510) or AVP (Sigma, V-9879) was added to .9% saline solution and diluted to a 5µg/ml concentration.

Conditioning procedure for peripheral administration of OT or AVP

Sexually naïve female rats were divided into 4 groups: paired oxytocin treated females (N=6), paired vasopressin treated females (N=6), paired saline treated females (N=6), and unpaired untreated females (N=18). Although in total there were 18 unpaired untreated females, for the purposes of comparison, unpaired females were only compared to paired females from the treatment group they were run with in the open-field. Females were injected with 5µg/kg of OT, AVP, or saline subcutaneously 15 min prior to the first conditioning trial. Drugs were administered once, prior to the first trial, in order to examine whether augmenting OT or AVP during the first copulation session would facilitate different aspects of bonding during the second copulation session. Male rats were placed into the conditioning apparatus (described above) for a 5-min habituation period prior to the introduction of each female. Females were placed into the compartment adjacent to the one containing the male and given a 30-minute copulation session with the male. Open-field tests were run 4 days after the conditioning session. All open fields contained a paired female from one of the treatment groups, the male she copulated with during the conditioning session, and an unpaired female. The open field was 123cm x123cm x46cm with a thin layer of bedding on the floor [25]. Five minutes prior to the start of the open-field, males were placed into the apparatus for a habituation period, after which females were placed in at diagonal corners. Rats were allowed to copulate freely for a 1h period. After the open-field, female rats were perfused and their brains collected for future use.

All open-field tests were recorded on video, coded, and blindly scored subsequently using a computerized event recorder customized for rat sexual behavior in an open field (Cabilio, 1998). The frequency of solicitations (SOL), hops and darts (HOP), defensive or aggressive responses (AGG) were recorded, as were the incidents and reflex magnitudes of lordosis when males mounted. The frequency of female-female mounting (FFM) and interceptions (INT) were also scored, as was the amount of time spent maintaining body contact with the male. Presenting (PRES) behavior was also scored when the female remained in a receptive position in front of

the male before or after he mounted or intromitted. We also took into account the number of times that a female would position her body in between the male and the opposing female, thus interfering the male's interaction with the female conspecific (RI). Lastly, the number of mounts (MOUN), intromissions (INTRO), and ejaculations (EJAC) received by each female were recorded in order to assess the effectiveness of mate guarding. After conclusion of test, females were euthanized and their brains were collected for double IHC to look for Fos colocalization within either oxytocin or vasopressin.

Statistical Analyses

Mate guarding behavior in the open-field was assessed using independent samples t-tests. For animals whose brains were used for IHC, there were only two groups, paired and unpaired, and one level of comparison. For the pharmacological administration, each group (OT, AVP, and Saline) was represented in each cohort, each treatment group was only compared to the untreated control they were tested with in the open-field, therefore there were only 2 groups with one level of comparison. The data collected from immunohistochemistry were also analyzed using independent samples t-tests, as there were only 2 groups with one level of comparison. Cohen's D was used to evaluate effect size.

Results

Mate guarding behavior after open field of animals used for IHC

Independent samples t-tests, along with Bonferroni corrections, were used to assess mate guarding behavior between paired and unpaired females to establish if a shift in mating strategy had occurred post conditioning. We found that paired females did, in fact, display subsets of mate guarding behavior, and showed significantly more female-female mounting, $t(7.031)=2.415$, $p=.024$, $d=3.4099$, than unpaired females (Figure 1). This finding is consistent with our previously reported findings (Holley et al., 2014). Although other behaviors were examined, they did not approach statistical significance.

Figure 1.

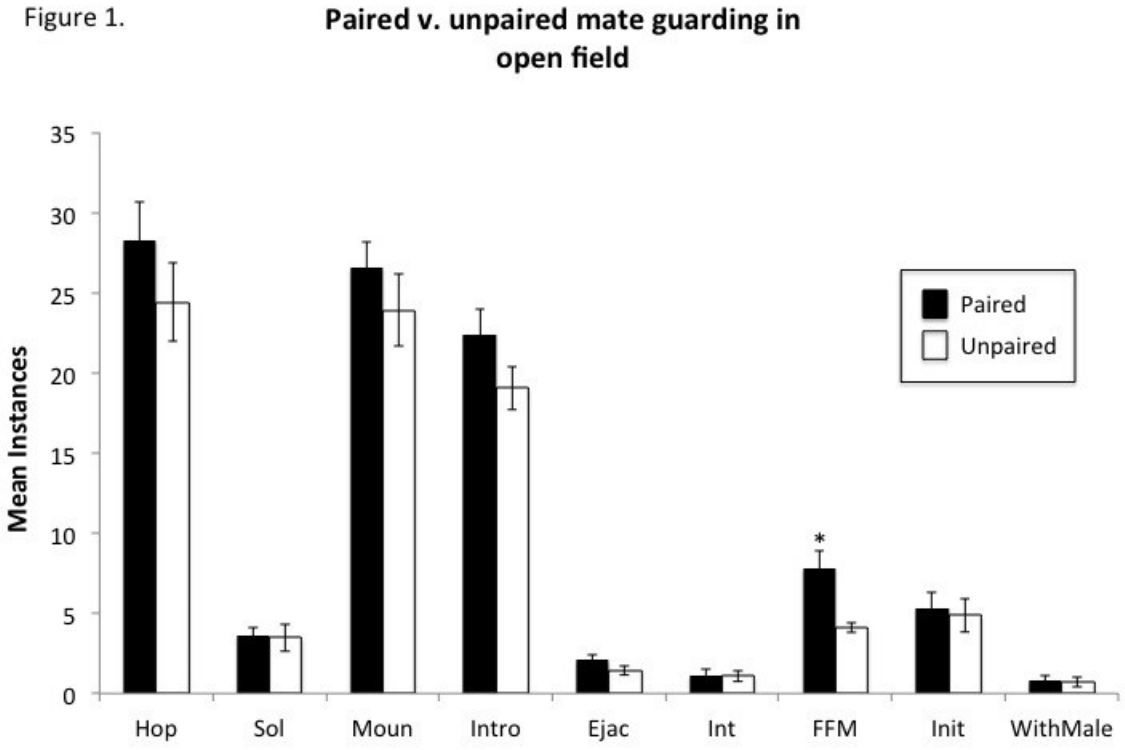

Figure 1:

Mate guarding behavior displayed in open field by paired female compared to unpaired female rats. Behaviors include hops, solicitations (Sol), mounts received (moun), intromissions received (intro), ejaculations received (ejac), interceptions (int), female-female mounting (FFM), number of times initiating closeness to the male (Init), time spent in proximity to the male (WithMale), interference (RI), and presenting (pres). Data are means ± SEM. * $p < 0.05$, ** $p < .01$

Double-labeled Fos and Oxytocin or Vasopressin cell counts

Independent samples t-tests, were used to look at differences in the average number of double-labeled cells within the SON and PVN between paired and unpaired females. Analysis revealed that paired females showed significantly more Fos+OT positive cells within the SON, $t(7.031)=73.14$, $p=.000$, $d=4.44$, and PVN, $t(7.031)=76.9$, $p=.004$, $d=3.17$, than did unpaired females (Figure 2). Paired females also showed more Fos+AVP positive cells within the SON than did unpaired females, $t(2.972)=41.840$, $p=.018$, $d=1.87$ (Figure 3).

Figure 2

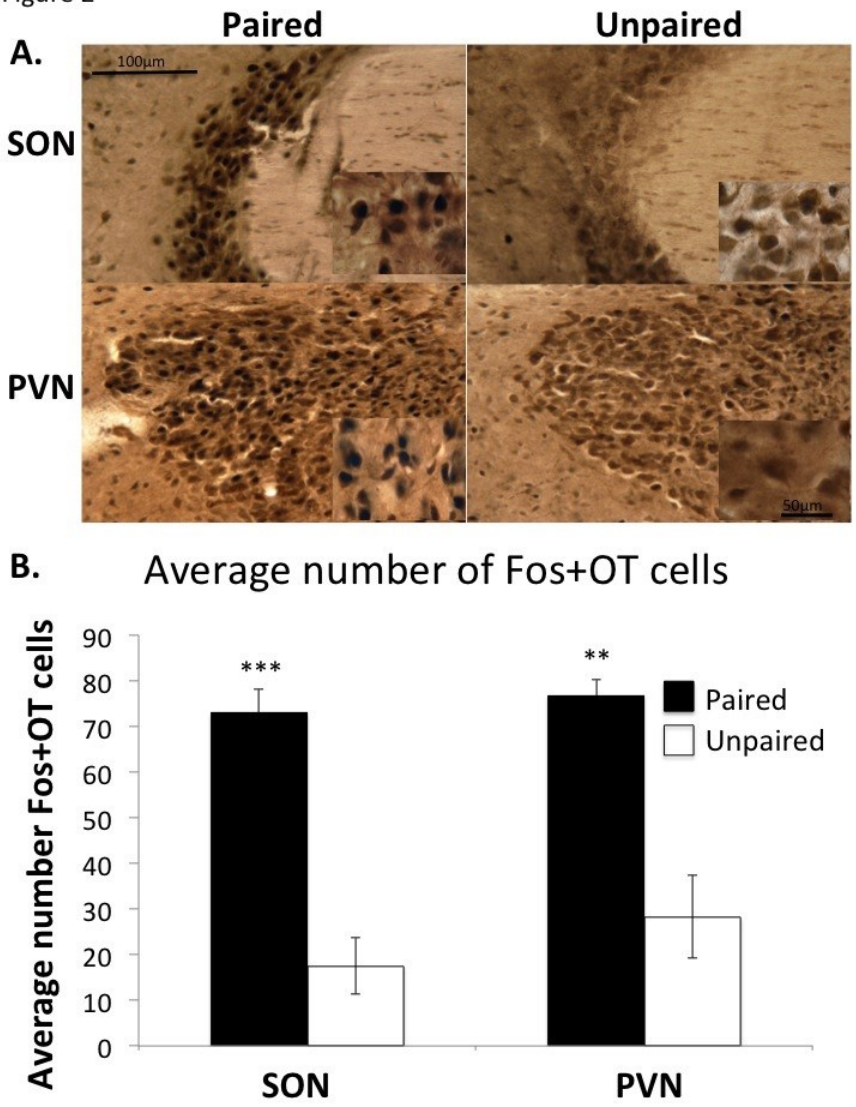

Figure 2:

Top: Photomicrographs (40x) showing OT+Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (OT) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100x to more clearly demonstrate double labeled v. single labeled cells. Bottom: OT+Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from paired (black) or unpaired (white) groups. Data are means ± SEM. * $p < .05$, ** $p < .01$; *** $p < .001$, # trend.

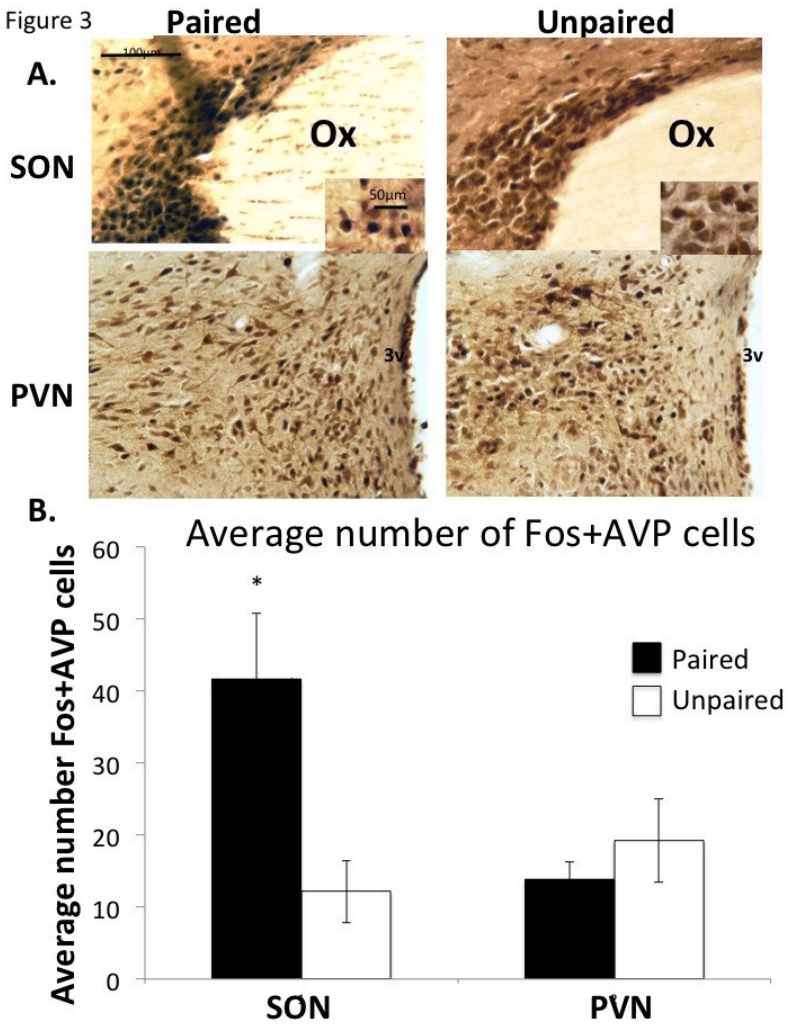

Figure 3: Top: Photomicrographs (40x) showing AVP+Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (AVP) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100x to more clearly demonstrate double labeled v. single labeled cells. Bottom: AVP+Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from paired (black) or unpaired (white) groups. Data are means ± SEM. * $p < .05$, ** $p < .01$; *** $p < .001$, # trend.

Mate guarding behavior after peripheral injection of OT, AVP, or Saline

Independent samples t-test, with Bonferroni correction, were used to examine differences in mate guarding behavior between oxytocin, vasopressin, and saline treated paired females and their respective unpaired females during the open field. Analysis revealed that paired females treated with oxytocin showed significantly more presenting behavior than unpaired females during the open field, $t(3.676)=4.67$, $p=.004$, $d=2.12$ (Figure 4a). Consequently, paired females treated with oxytocin also received more intromissions than did their corresponding unpaired females in the open field, $t(3.101)=16.5$, $p=.011$, $d=1.79$ (Figure 4a). Paired females treated with vasopressin imposed their body between the male and the other female significantly more than did unpaired females in the open-field, $t(3.873)=1.00$, $p=.003$, $d=2.27$ (Figure 4b). No differences in behavior occurred between paired saline treated females and their corresponding unpaired females in the open-field test (Figure 4c).

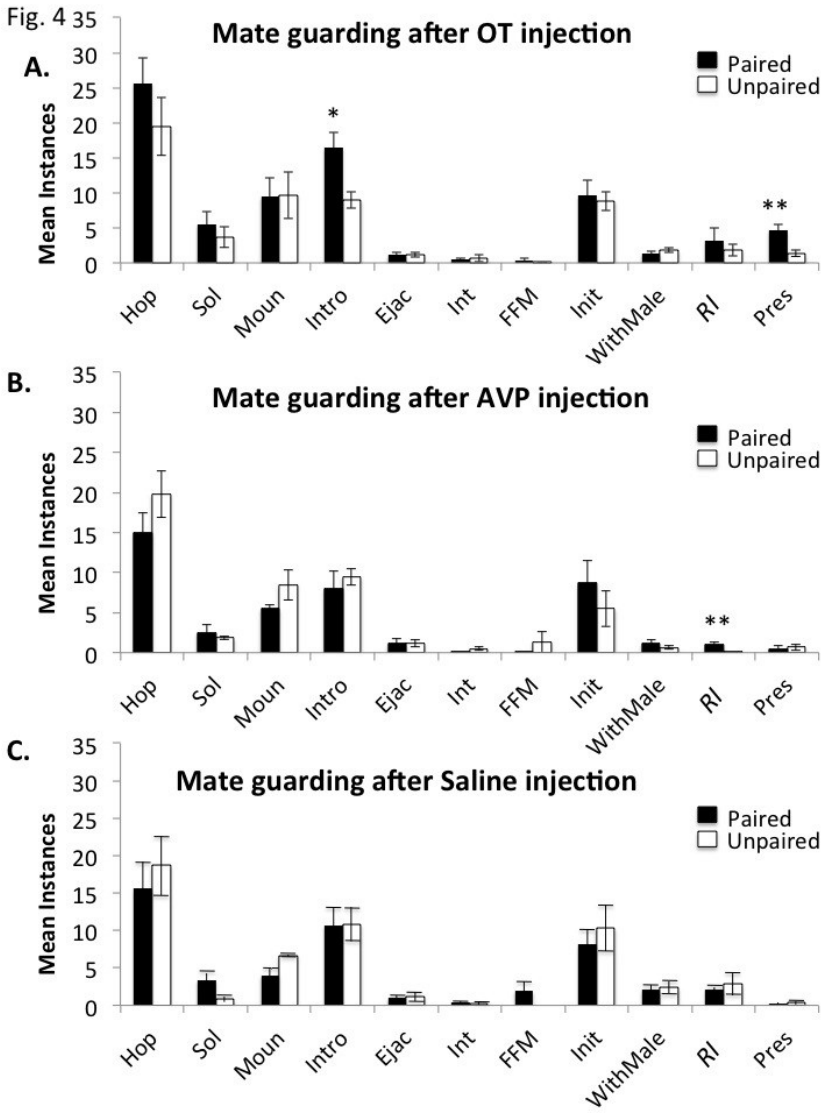

Figure 4: Mate guarding behavior displayed in open field by paired female rats that were treated with; OT, AVP, or Saline. Behaviors include hops, solicitations (Sol), mounts received (moun), intromissions received (intro), ejaculations received (ejac), interceptions (int), female-female mounting (FFM), number of times initiating closeness to the male (Init), time spent in proximity to the male (WithMale), interference (RI), and presenting (pres). Data are means ± SEM. * $p <$ 0.05, ** $p < .01$

Discussion

The results of these experiments replicate and extend those of our previous study showing that paired female rats display conditioned mate guarding behavior of their paired male against a competitor female. The paired females had significantly more double-labeled Fos-OT, within the PVN and SON, and Fos-AVP neurons within the SON, in response to the presentation of the preferred male compared to unpaired females that showed no significant partner preference to any male. In the second experiment, we sought to unravel the role that OT and AVP could be playing in the early onset of conditioned mate guarding, by administration of OT or AVP to sexually naïve females during their first sexual experience. Peripheral administration enhanced different aspects of mate guarding on the second trial relative to females injected with saline: Females given OT on the first trial displayed more presenting near their preferred male on the second trial relative to saline-treated females, whereas females given AVP displayed more interference behavior relative to saline-treated females on the second test. Saline treated females did not differ behaviorally from untreated females on the second trial. These data indicate that OT and AVP neurons are activated in the SON and PVN of female rats that are conditioned to show mate guarding behavior over the course of a rigorous conditioning paradigm. Furthermore, by administering OT and AVP we observed that these two peptides play distinct roles in the early development and manifestation of this behavior.

It has been established that there are remarkably stable regional differences in the distribution of OT and AVP receptors between monogamous and promiscuous vole species that underlie differences in mating strategies between them (Insel et al., 1994; Lim et al., 2004; Smeltzer et al., 2006; Young et al., 1999). By limiting a female rat's rewarding sexual experience to a single male partner, we have been able to shift her behavioral mating strategy towards monogamous mate guarding. This is accompanied by changes to neurochemical systems involved in bonding within the brain (Holley et al., 2014). Although the findings of the first experiment show that hypothalamic OT and AVP neurons are activated more in paired vs. unpaired female rats, this does not provide any information about receptor distribution, which is known to underlie vole behavior. It could be the case that when female rats are conditioned monogamously, OT and AVP cell bodies become more active, releasing more peptide, which sensitizes the system, thus leading to changes in behavioral output, such as mate guarding.

However, further investigation is warranted to examine if this conditioning procedure also alters OT and/or AVP receptor expression.

The double immunohistochemistry data demonstrate that OT and AVP neurons are activated over the course of conditioning; however, they do not reveal the role played by OT or AVP in the development of mate guarding behavior. Therefore, in the second study, we administered OT or AVP to female rats prior to their first sexual experience. We have shown that OT and AVP are activated over the 40 day course of testing, however, we were interested to see if OT and AVP come on board sooner, how they would effect the development of conditioned mate guarding. We hypothesized that peripheral administration would facilitate different aspects of conditioned mate guarding. In line with our hypothesis, treatment with both, OT and AVP, facilitated the onset of mate guarding after one trial alone. However, each of these enhanced different subsets of mate guarding behaviors. Paired females treated with OT showed more presenting around the paired male and consequently they received significantly more intromissions than untreated females. Furthermore, neither AVP nor saline treated females showed an increase in this behavior when compared to controls. In contrast, paired females treated with AVP displayed significantly more interference behaviors than untreated females, however, this effect was very small. These behaviors were not enhanced by OT or saline treatment. The control injection of saline did not enhance any aspect of mate guarding behavior. In previous studies, presenting and interference were observed, but only minimally, and not in consistent enough of a manner to score. However, our previous assessment of mate guarding was made after an extensive conditioning paradigm, where females and males were paired for 10 sessions. In this study, females and males were only paired one time and were sexually naïve prior to this. What these data suggest is that mate guarding could develop as presenting and interference, and then mature into the display of more aggressive and dominant behaviors. This phenomenon of behavioral maturation is true of other complex interactions. For example, when male rats first learn to copulate, protracted anogenital investigation is a key component but this behavior dissipates as the males gain sexual experience. In order to fully understand the roles of OT and AVP in the progression of conditioned mate guarding, further work is warranted to investigate the expression of both neuropeptides at different time points over the conditioning process.

OT and AVP were identified early as neural correlates of bonding in the vole literature due to the fact that they mediate a variety of social behaviors. When we administered OT to female rats in the present study, it mediated presenting behavior, that occurs in proximity to the male, and is described as maintaining a receptive posture in front of the male, allowing her to receive more intromissions. OT and AVP have been conceptualized as having opposing influences on one another (Huber et al., 2005). OT is generally linked to passive social behaviors that require proximity and closeness such as maternal care and maternal bonding (Debiec, 2005; Kendrick et al., 1987; Lemon et al., 2006; Pendersen et al., 1979). In contrast, AVP is known for its role in fear, aggression, social recognition, and sociability (Debiec, 2005; Bychowski et al., 2013, Young et al., 1999). In the present experiment, AVP treatment appeared to facilitate the female's ability to control the male's access to the competitor female by imposing her own body between the two. This type of behavior requires the female to be able to recognize the male and the threat of the competitor female, and to also maintain bodily awareness to interfere with the conspecific's access to the male. Although this study clearly demonstrated that OT and AVP are able to facilitate different aspects of conditioned mate-guarding behavior, we are not able to determine if they are exerting their effects centrally or peripherally due to the nature of the injection. Until very recently, the effects of acute IP administration of OT or AVP on social behavior have been largely uninvestigated despite striking evidence that peripherally-administered OT is able to cross the blood brain barrier (Neumann et al., 2013; Ramos et al., 2013). Taking these findings, and our double label immunohistochemistry data, into account, it is possible that the OT and AVP are exerting their effects on mate-guarding centrally, although we can not definitively draw that conclusion from this line of work.

Mate guarding is a term broadly used in the literature and can be applied to different behaviors across species. For example, in prairie voles, mate guarding is typically synonymous with selective aggression, whereas in long-tailed macaques, mate guarding refers to a male monopolizing a female (Giard-Buttoz et al., 2014; Hall et al., 1959). To be consistent, we operationally defined mate-guarding as the female taking away the choice of the male to mate with the opposing female, which was achieved through a set of aggressive, dominant, sexual, competitive, and social behaviors (Holley et al., 2014). Administration OT or AVP facilitated certain aspects of mate-guarding behavior, however, we did not see a facilitation of female-

female mounting, which we observed in the conditioned group of animals in this study whose brains underwent double IHC, and that we previously reported following 10 sexual experiences with the paired male (Holley et al., 2014). There are several explanations as to why this may be the case. Female-female mounting is not synonymous with mate guarding in female rats, it is just one behavior encompassed under our definition of mate guarding. This is reminiscent of other complex behaviors, such as sexual behavior, which is comprised of a range of appetitive and consummatory behaviors that together describe an animal's sequence of copulatory events. Although each paired animal shows higher instances of each behavior in the mate guarding repertoire than unpaired females, it may be the case that each animal also chooses to employ a certain set of behaviors which they find most effective, which then accounts for individual differences seen across groups. It is important to note, that individual differences in behavioral expression occur in nature and in the laboratory quite frequently, yet are less frequently discussed. Prairie voles are used as an ultimate example of social monogamy within the rodent literature and indeed across taxa. However, even within prairie vole populations, some individuals do not show pair bonding, biparental care, or selective aggression at all, or will display only a subset of these behaviors (Getz et al., 1993; Getz et al., 1992; Cochran and Solomon, 2000; Roberts et al., 1998; Solomon et al., 2008). Individual differences play a role in how rodents mate in both nature and laboratory, and demonstrate how flexible patterns of reproduction are expressed at an individual level, which provides a plausible explanation for why female rats employ different methods of mate guarding. We have observed differences in FFM in paired females that underwent rigorous conditioning over a 40-day period. It could be that during this time, while the female is learning to associate reward with her mate, and bonding with him during each trial, OT and AVP systems both slowly come online, and both are needed ultimately for the display of FFM. In the present study we artificially administered either OT or AVP but not both. Alternatively, in prairie voles, the onset of mate guarding coincides with an increase in the dopamine D1 receptor (D1R) in the nucleus accumbens (Aragona et al., 2005). If D1Rs are selectively blocked in this region, voles fail to display mate guarding behavior (Aragona et al., 2005). Additionally, AVP has also been shown to regulate selective aggression in voles, where central infusion into the lateral ventricles induced aggression towards a conspecific (Winslow et al., 1995). Taken together, these data suggest that OT and AVP are individual pieces in the mate-guarding puzzle, and that dopamine could potentially be a key

component in facilitating other behaviors, such as FFM, which require approach and contact with the competitor. In order to gain a full profile of conditioned mate guarding, much more work is required to examine the behavioral and accompanying neurochemical development of this behavior.

We suggest that OT and AVP are key individual players in the early phase of conditioned mate guarding behavior in female rats. Peripheral administration of OT or AVP is able to facilitate two different aspects of mate guarding behavior typically observed in the early phase, presenting (OT) and interference behavior (AVP). The activation of endogenous OT and AVP systems appears to sensitize following repeated paced copulation with the familiar male. Thus, as with males, patterns of bonding and partner preference are heavily influenced by a female's first experiences with sexual reward. Given that rats are generally reported to have a promiscuous group-mating strategy, we suggest that mating strategies can be shifted from promiscuous toward monogamous by experience with sexual reward that is predicted by a single long-term mate (or cues such as neutral odors worn by potential mates that predict the reward state). The reward state induces induce long-lasting changes in neuroanatomical structure and neurochemical systems that underlie bonding and attention toward partner cues.

Summary of Chapter 2

The experiments in Chapter 2 assessed the role of AVP and OT in the development of conditioned mate-guarding behavior. The first experiment examined whether the Fos induction in the PVN and SON of paired females after they mate guard occurred within OT and/or AVP neurons. Paired females were assessed for mate guarding using an open field paradigm containing her paired male and a female competitor and were found to display FFM relative to unpaired females, in line with our previous findings. Brains were collected from paired and unpaired females in the self-paced condition. Double immunohistochemistry was used to label Fos, a marker of neuronal activation, in cell nuclei, and either OT or AVP within the cytoplasm of the same cell. Paired females displayed significantly more double-labelled cells for Fos/OT in both the SON and PVN than did unpaired females. Paired females also displayed more Fos induction within AVP neurons in the PVN than did unpaired females. These results indicate that over the course of conditioning, when a paired female is subsequently paired with her assigned mate, there is an accompanying enhancement in the activation of OT and AVP neurons.

In order to evaluate if OT and AVP are contributing to mate guarding in female rats, OT, AVP, or saline were administered to paired females prior to their first conditioning trial. If OT and AVP are important for conditioned mate guarding, then the administration of one or the other should enhance the expression of mate guarding after the females' first sexual experience. Females treated with OT and AVP both displayed elements of mate guarding after one trial alone. Saline treatment was not sufficient to induce mate guarding after one trial. Females treated with OT displayed significantly more presenting behavior, where they remain in a receptive posture in front of the male, than did unpaired females. AVP and Saline treatment did not affect this behavior. In contrast, females treated with AVP displayed more conspecific blocking or interference behavior, where they would impose their body between the male and the competitor female, than did unpaired females. Treatment with OT or Saline did not enhance this behavior. These data suggest that OT and AVP are important for the onset of conditioned mate guarding, as they were both able to facilitate this behavior in one trial alone. Also, the data demonstrate that there is a developmental aspect to conditioned mate guarding, where early forms of this behavior are demonstrated by presenting and interference behavior, and more mature forms are expressed as female-female mounting. The experiments of Chapter 3 examine

whether epigenetic changes underlie the sensitization of OT and AVP neurons in the development of this behavior.

Lysine specific demethylase enzyme inhibitor disrupts sexually conditioned mate guarding in female rats.

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Introduction

Despite being allegedly promiscuous, female rats have been shown to display a great deal of flexibility within their mating patterns. We have shown previously that if female rats have all of their self-paced, rewarding, sexual experience paired with the same male, they will mate guard that male in the presence of a competitor female (Holley et al., 2014; 2015). Female rats from the control condition that had their self-paced rewarding sexual experience paired with a variety of males did not display this behavior (Holley et al., 2014; 2015). Interestingly, when we subsequently examined bonding circuits within the brain, including oxytocin (OT) and vasopressin (AVP), we saw an increase in activation of both OT and AVP neurons within the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei in females that displayed mate guarding behavior but not in controls (Holley et al., 2015). Peripheral administration of OT or AVP also facilitated different aspects of mate-guarding behavior, whereas control vehicle treatment did not, demonstrating that OT and AVP are necessary for the full expression of this behavior (Holley et al., 2015). Taken together, these findings demonstrate that Pavlovian conditioning can be used as a mechanism for pushing the mating strategy of the female rat from promiscuity, to monogamy, and that changes in expression to OT and AVP neural circuitry underlie this behavioral output. However, what mechanisms are responsible for creating this enhancement of OT and AVP expression remain elusive. In this study, we sought to investigate the potential role that epigenetic modifications to histones, namely methylation, play in creating this change in expression of neural circuitry.

Sexual conditioning takes place over many weeks, and is reliant on the formation of stable associations between a conditioned and unconditioned stimulus (Pavlov, 1927). It has been long accepted that long-term memory formation requires gene transcription, and more recently, epigenetic modifications to chromatin structure have been shown to be vital in regulating gene expression (Reik et al., 2007). Recently, studies have revealed that there is a dynamic regulation to chromatin structure in response to learning and memory (Chwang et al., 2006; Levenson et al., 2004; Gupta et al., 2010; Gupta-Agarwal et al., 2012; for review see: Kramer et al., 2013). One such learning-induced chromatin remodeling mechanism is histone methylation. Histone methylation is plastically regulated in the rat hippocampus in response to fear conditioning (Gupta-Agarwal et al., 2012; Gupta et al., 2010). Patterns of histone methylation can act bidirectionally, meaning that some patterns result in transcriptional

activation, whereas others result in transcriptional repression, and both repressive and activational can occur simulatenously (Kouzarides et al., 2007; Suganuma et al., 2002). For example, in the context of conditioned learning, histone H3 lysine 4 trimethylation, can act as an activating modification, whereas histone H3 lysine 9 methylation has been shown to act as a repressive modification (Gutpa-Agarwal et al., 2012; Gutpa et la., 2010). Although these two forms of methylation have different outcomes, activation and repression, both have been shown to increase in response to fear conditioning (Gutpa-Agarwal et al., 2012; Gutpa et al., 2010).

Although conditioning underlies the bonding we have previously observed in female rats, histone modifications also play a defining roll in naturalistic bonding behavior observed in other rodent species, namely prairie voles. Wang and colleagues, treated prairie voles, a monogamous rodent, with histone deacetylase inhibitors (HDACi), and found that this treatment facilitated partner preference formation, and also, lead to an up-regulation of oxytocin receptor (OxtR) and vasopressin 1a receptor (V1aR) in the nucleus accumbens (Wang et al., 2013). Similar to treatment with HDACi, treatment with various demethylase inhibitors has been shown to affect gene expression within the brain (Sun et al., 2010; Metzger et al., 2005). Taken together, these data demonstrate that epigenetics play a role in naturally occurring bonding behavior, and also, in conditioned and learned behavior. Therefore, an epigenetic basis of conditioned monogamy in female rats can be suggested. To test this hypothesis, we used an LSD1 (lysine specific demethylase 1) demethylase inhibitor, to disrupt the methylation process during conditioning. We predict that if histone methylation is important in conditioned mate guarding, then by disrupting the methylation process we would consequently disrupt the development of this behavior and result in no enhanced activation of oxytocin or vasopressin neurons, as we have observed previously.

Materials and methods

Animals and surgery

Sexually naïve Long-Evans female rats (200-250g) were obtained from Charles River Canada (St-Constant, QC, Canada). Animals were housed in shoebox cages in groups of two in a colony room on a reversed 12:12h light/dark cycle at approximately 21 °C and given free access to food and water. Female rats were ovariectomized (OVX) via bilateral lumbar incision.

Proceeding surgery, female rats were anesthetized with 1ml/kg of ketamine hydrochloride (50mg/ml) and xylazine hydrochloride (4ml/kg), mixed in a ratio of 4:3 respectively, administered via intraperitoneal injection. Female rats were given 1 week to recover from surgery prior to the conditioning trials. Throughout the duration of the experiment, female rats were maintained on hormone replacement by subcutaneous injections of estradiol benzoate (EB; 10 µg in 0.1 ml of sesame oil) 48h prior to testing, and progesterone (P; 500 µg in 0.1 ml of sesame oil) 4h prior to testing.

Sexually naïve male rats (300-350g) were also obtained from Charles River Canada (St-Constant, QC, Canada). They were housed in group cages (4 animals per cage) and housed under conditions identical to those of the female rats.

All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Conditioning apparatus

Conditioning occurred in unilevel Plexiglas pacing chambers (38cm H x 60cm W x 38cm deep) with wire-mesh floors covering a layer of bedding [Genaro]. Chambers were bisected by a Plexiglas divider with four holes cut into the bottom which were large enough for the female to crawl through but too small for the male to crawl through [6,17,18].

Drug Preparation

LSD1 demethylase inhibitor (LSD1) was purchased from Calbiochem (LSD1 inhibitor IV; RN-1, HCL; cat# 489479). 10mg of drug was dissolved in 2mL of saline using a syringe.

Conditioning procedure

Conditioning sessions occurred at 4-day intervals, 4h after P injections, during the middle third of the rats' circadian cycle (lights off at 08:00). Females were assigned randomly to one of 3 groups. Paired females treated with LSD1, paired females treated with vehicle alone, or unpaired females that received no treatment (N=12/group). All paired females copulated with the same male across all trials, whereas unpaired females copulated with a variety of males across all trials. 1h prior to testing, paired females were given an intraperitoneal injection of either LSD1 (10mg/kg) or vehicle (.9% saline). 1h time frame was chosen based off of microdialysis data demonstrating that 1h is sufficient time for LSD1 to cross the blood brain barrier (Neelagam et al., 2012). Males were placed onto one side of the conditioning chambers and allowed to habituate for 5-minutes before each trial. Females were then placed into the

opposite side of the conditioning chamber and allowed to have paced sex with the male for 20 minutes. Each group received 10 trials, which were all recorded on video.

Throughout the course of this study, the health of the animals was assessed daily. Rats eating and drinking was monitored and body weights were taken bi-daily to ensure drug treatment did not impair the health of the animals. This was done to ensure the overall health and comfort of the rats, and also, to ensure that any effects were due to the action of the drug and not to failing health or abnormal behavior.

Mate-guarding test

Four days after the final conditioning trial mate guarding was assessed using an open-field (123cm x 123cm x 46cm) with a thin layer of bedding [Genaro]. Each open-field contained either a paired female, from either LSD1 or vehicle groups, along with her corresponding male and an unpaired competitor female. Before the test, males were placed into the open field for a 5-minute habituation period, after which both the paired and unpaired females were placed into the open-field at 2 diagonal corners. Rats were allowed to copulate freely for a 1-hr period. After the open-field test female rats were perfused and their brains were collected to examine Fos induction within oxytocin and vasopressin neurons.

All open-field tests were recorded on video and scored afterward using a computerized event recorder customized for rat sexual behavior in an open-field [Cabilio]. Mate guarding is operationally defined as any behavior done with the intent of taking away the choice of the male to mate with the other female, and can be achieved through a variety of aggressive, dominant, sexual, competitive, and social behaviors as described in our previous work (Holley et al., 2014a; 2014b).

Perfusions

Animals were euthanized with an overdose of sodium pentobarbital (120mg/kg) administered via intraperitoneal injection. They were then perfused intracardially with 250ml of phosphate-buffered saline (PBS) followed by 250ml of 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 4 hours, then placed into a 30% sucrose solution for 48 hours, following which, they flash frozen and stored at -80°C until slicing.

Tissue preparation and immunocytochemistry

Brains from both paired and unpaired females (N=5 brains/group) were cut coronally at 30μm on a freezing microtome and divided into three series, one of which was used for double

immunohistochemistry to label for Fos and OT, the second was used to label for Fos and AVP, and the third was stored as a back up. In the primary phase, sections were washed in cold Tris-buffered saline (TBS) and incubated first with 30% hydrogen peroxide (H2O2) in TBS for 30 min at room temperature followed by 3% Normal goat serum (NGS) in 0.05% Triton-TBS for 90 min at 4 °C, with rabbit polyclonal anti-Fos (Fos ab5, Calbiochem, Mississauga, ON; diluted 1:40,000) in 0.05% Triton-TBS with 3% NGS for 72 h at 4 °C. In the secondary phase, sections are incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories Canada, Burlington, ON; 1:200), in 3% NGS, and .2% Triton TBS for 1 hour. Following this, sections enter a tertiary phase, where sections are incubated in avidin–biotinylated–peroxidase complex (Vectastain *ELITE*® ABC KIT, Vector Laboratories Canada; diluted 1:55) in 3% NGS, and .05% Triton TBS for 2 h at 4 °C. Sections were washed in TBS (3 × 5 min) between each incubation.

Immunoreactions were stained by sequential treatments with 50 mM Tris for 10 min, 3,3′-diaminobenzidine (DAB) in 50 mM Tris (0.1 ml of DAB/Tris buffer, pH 7.8) for 10 min, and 8% nickel chloride (400 µl per 100 ml of DAB/Tris buffer + H2O2) all at room temperature. Reaction was stopped by rinsing (3 x 10 min) in PBS. Sections were then transferred into the second primary antibody, either rabbit polyclonal anti-oxytocin (Chemicon, AB911, diluted 1:10,000) or rabbit polyclonal anti-vasopressin (Millipore, AB1565, diluted 1:10,000) in 0.05% Triton-TBS with 3% NGS, and incubated for 72h at 4 °C. After completion of this incubation, secondary and tertiary phases are repeated. However, no nickel chloride was added to the final DAB wash in order to create a light brown cytoplasmic stain.

Following staining, sections were mounted onto gel-coated slides and cover slipped following immunohistochemistry. Cover slipping procedure started with sequential washes of distilled water followed by washes in 70%, 90%, and 100% alcohol each at room temperature for 1minute duration. Slides were then dipped 50 times in Citrisolve at room temperature after which they were cover slipped using Permount solution. Slides were set aside for a week to allow for the Permount to dry and then cleaned and coded so that all analysis would be conducted blind.

Sections were examined using an Olympus light microscope at 400x magnification. Pictures were taken at 200x and 1000x using Q-Capture pro software. Double-labeled cells were identified by eye as any cell having a dark black nucleus (Fos positive) and a light brown

cytoplasm (OT or AVP positive). Brain regions were defined using the atlas of Paxinos and Watson (Paxinos & Watson; 2006). Double-labeled cells were counted within the supraoptic nucleus (SON, Plates 21-25), and paraventricular nucleus (PVN, Plates 25-26) as these two regions are where OT and AVP neuronal bodies reside. Five slices were counted per animal. These slices represented each area rostrally to caudally and were matched among animals to ensure accuracy. Counts were done bilaterally in a total of 5 animals per group. Total number of double-labeled cells was counted in each section, which were then used to derive the mean number of double- labeled cells within the region.

Statistical Analyses

Mate-guarding behavior in the open-field was assessed using independent samples t-tests since paired females were only compared directly to the unpaired female they were tested in the open-field with, rendering us with two groups and one level of comparison. Cohen's d was used to assess effect size. The data collected from Fos-IR cells were analyzed using a one-way analysis of variance (ANOVA) with post-hoc t-tests between individual groups corrected for elevated experiment-wise error using the Bonferroni method. Eta squared was used as a measure of effect size.

Results

Mate guarding behavior in the open-field

Independent samples t-tests were used to compare mate guarding behavior between treatment groups and their respective controls. Analysis revealed that during the open field test, paired vehicle treated females initiated more contact with the male (INIT) than did the unpaired females they were tested with $t(10) = 2.791$, $p = .028$, d=1.609 (Figure 1). Paired females treated with LSD1 failed to show any measure of mate guarding behavior and did not differ behaviorally from vehicle treated females. Control behaviors, such as hops and darts (HOP), were assessed in order to evaluate sexual receptivity to ensure all animals were sexually receptive and behaving normally. No statistical differences were found indicating that all animals were sexually receptive and behaving in a comparable manner (p=.561).

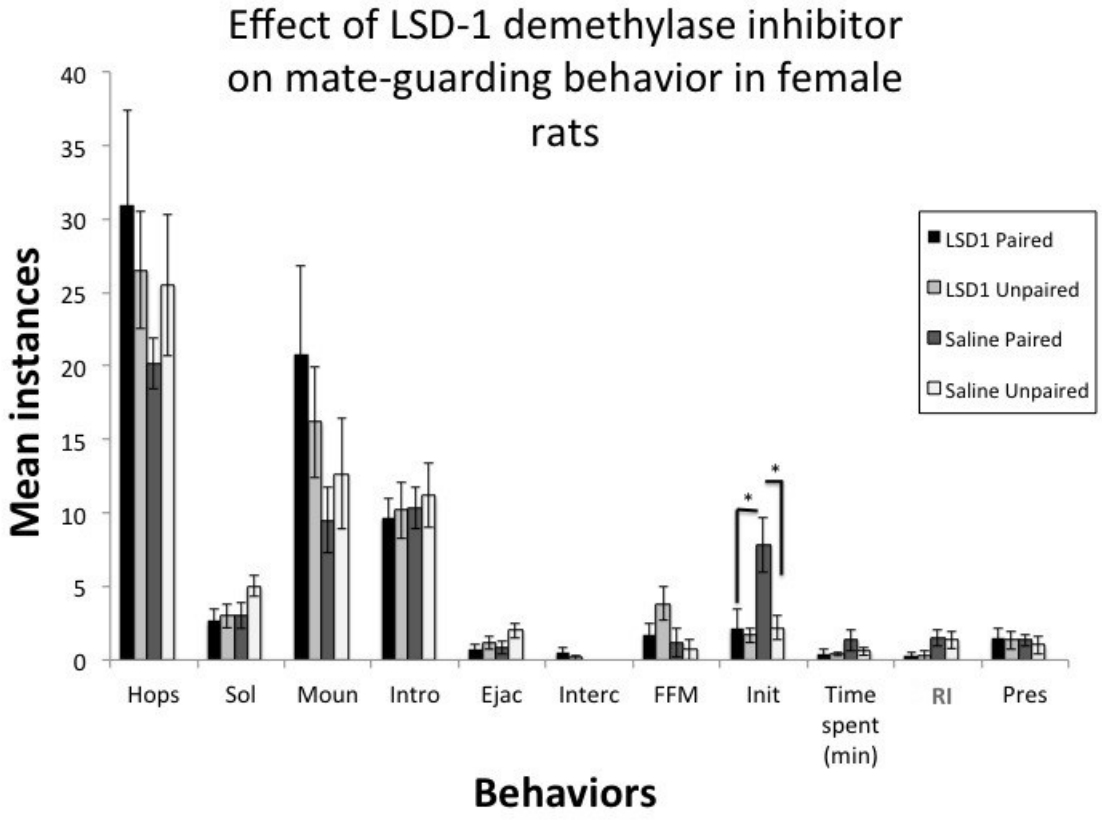

Figure 1:

Mate guarding behavior displayed in open field by paired female rats that were treated with either LSD1 or Saline. Behaviors include hops, solicitations (Sol), mounts received (moun), intromissions received (intro), ejaculations received (ejac), interceptions (interc), female-female mounting (FFM), number of times initiating closeness to the male (Init), time spent in proximity to the male (TimeSpent), interference (RI), and presenting (pres). Data are means ± SEM. * $p <$ 0.05, ** $p < .01$

Double-label cell counts

A one-way ANOVA was used to examine differences in Fos induction within OT and AVP neurons between paired LSD1, paired vehicle, and unpaired untreated females. In line with our hypotheses, we found that vehicle treated females had more Fos and OT neurons within the SON, $F(2,12) = 97.063$, $p=.000$, $\eta^2=.942$, and the PVN, $F(2,12) = 70.903$, $p=.000$, $\eta^2=.922$, than their respective controls (Figure 2). Paired vehicle treated females also had more FOS and AVP colocalized neurons within the SON, $F(2,12) = 25.895$, $p=.000$, $\eta^2=.812$, and within the PVN, $F(2,12) = 10.836$, $p=.002$, $\eta^2=.644$ (Figure 3). We found that LSD1 treated females did not differ from controls in their expression of OT (Figure 2) or AVP (Figure 3) within the SON or PVN, $p>.5$ for all measures.

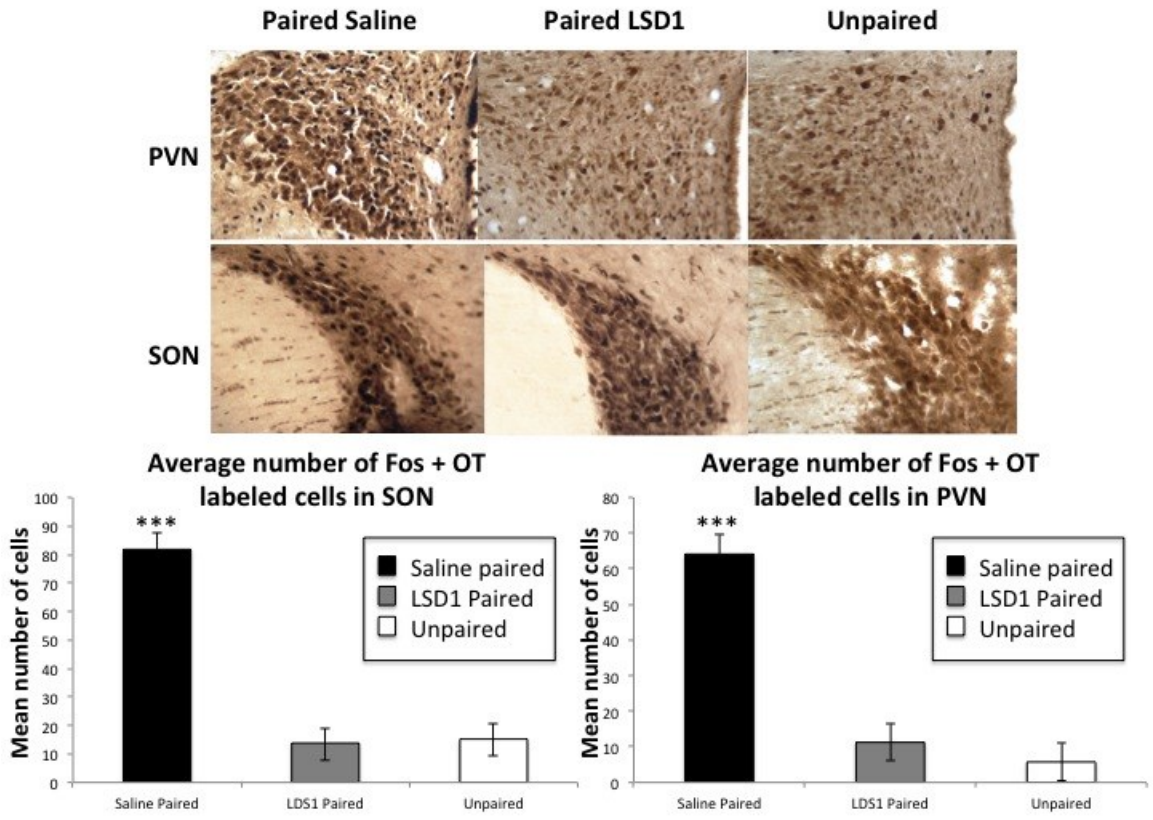

Figure 2:

Top: Photomicrographs (40x) showing OT+Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (OT) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100x to more clearly demonstrate double labeled v. single labeled cells. Bottom: OT+Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from saline paired (black), LSD1 paired (grey), or unpaired (white) groups. Data are means ± SEM. * $p < .05$, ** $p < .01$; *** $p < .001$, # trend.

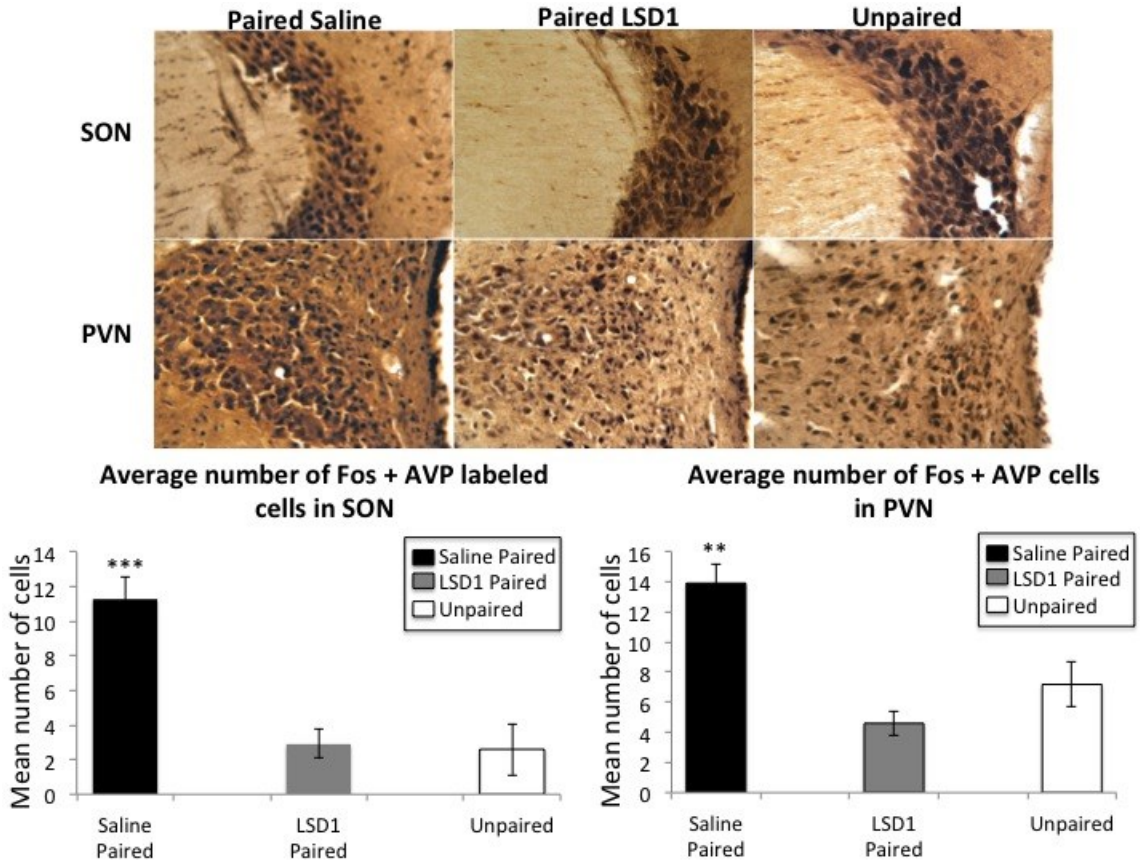

Figure 3:

Top: Photomicrographs (40x) showing AVP+Fos-IR in the SON and PVN following the open-field test. Cells positive for both markers have a light brown cytoplasmic stain (AVP) with a dark black nucleus (Fos). Magnified inserts taken of selected regions at 100x to more clearly demonstrate double labeled v. single labeled cells. Bottom: AVP+Fos positive cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from saline paired (black), LSD1 paired (grey), or unpaired (white) groups. Data are means ± SEM. * $p < .05$, ** $p < .01$; *** $p < .001$, # trend.

Discussion

Here we demonstrate an epigenetic regulation of conditioned monogamous behavior in female rats. First, we demonstrated that globally blocking the action of LSD1 demethylase enzymes, through use of a LSD1 demethylase inhibitor, blocks the acquisition of conditioned mate guarding behavior in female rats that we have previously observed (Holley et al., 2014; 2015). Control females, treated with vehicle alone, still displayed mate guarding behavior (Fig 1). Next, we examined oxytocin and vasopressin neuronal activation, which we have previously shown is increased in response to monogamous conditioning (Holley et al., 2015), and found that LSD1 treatment also blocked the enhanced expression of Fos protein within both OT (Fig 2) and AVP (Fig 3) neurons. Vehicle treated controls, which displayed mate guarding, also displayed enhanced OT (Fig 2) and AVP (Fig 3) neuronal activation. These data provide evidence that the conditioned shift in mating strategy, from promiscuity towards monogamy, that we observed in female rats in response to sexual conditioning is subserved by, and dependent on, an epigenetically-regulated increase in activation of OT and AVP neurons.

We have reported previously that if sexually naïve female rats are given all of their sexual experience with the same male, they will mate guard their assigned partner in the presence of a female competitor (Holley et al., 2014; 2015). We have defined mate guarding as any behavior performed by the female in an attempt to block the male from copulating with a competitor female (Holley et al., 2014; 2015). Under this definition, we include several aggressive, dominant, sexual, social, and competitive behaviors, and during mate guarding, a female rat may employ any subset of these behaviors to mate guard (x-axis, Fig 1). In this study, paired female rats treated with LSD1 failed to display mate guarding of their assigned male, whereas control females, treated with saline, did display mate guarding in the form of number of times they initiated closeness to the male. This measure is commonly used in the vole literature to assess partner preference in the laboratory. Researchers conclude that a pair bond has been established when the test vole spends more time maintaining side-by-side contact with the partner than with a novel vole (Gubernick et al., 1994). Treatment with LSD1 did not affect control behaviors such as hops and darts (Fig 1) or receptivity, demonstrating the effect was specific to behaviors, associated with mate guarding, that are acquired through the conditioning process. In previous studies (Holley et al., 2014; 2015) we reported an increase in female-female mounting (FFM) in paired females when compared to unpaired females. We did not see this

increase in FFM in the saline paired females in this study. This subset of saline paired females mate guarded by initiating time around the male when compared to unpaired controls. Mate guarding, like other complex behaviors, is an umbrella term used to describe a specific subset of behaviors that are used to prevent the male from accessing a competitor female, it is not synonymous with FFM. Paired females display the encompassed mate guarding behaviors more than unpaired females, and we have observed the use of all of these behaviors to varying degrees of significance across studies (Holley et al., 2014; 2015). The display of mate guarding in female rats is shaped largely by the behavior of the competitor female. We have previously observed FFM in groups of paired females when unpaired females would refuse to leave the vicinity of the male regardless of the presence of the unpaired female (Holley et al., 2014). However, during behavioral observation in the present study, the presence of the saline paired female in the vicinity of the male was enough to lead to the unpaired female to leave the male. LSD1 paired females behaved indistinguishably from unpaired females and no mate guarding was observed.

Neuroanatomical and pharmacological studies have provided strong evidence that both OT and AVP are crucial for bonding in prairie voles (Ross et al., 2009; Wang et al., 1995; Insel et al., 1994; Smeltzer et al., 2006; Insel et al., 1992; Winslow et al., 1995). Previously, we have demonstrated that, female rats conditioned monogamously showed an increased activation of OT neurons within the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, and increased activation of AVP within the SON (Holley et al., 2015). Through pharmacological manipulation, where we peripherally administered OT and AVP prior to paired females first conditioning trials, we were able to demonstrate that OT and AVP are able to facilitate the onset of conditioned mate guarding behavior and shape the early display of this behavior (Holley et al., 2015). These data demonstrate that OT and AVP expression are naturally enhanced through our conditioning process, and that these neuropeptides are important for the display and development of mate guarding in female rats. Therefore, since we saw that LSD1 treatment was able to block the acquisition of conditioned mate guarding, we were interested to see if it did so by preventing the enhanced expression of OT and AVP we had previously observed. We examined OT and AVP activation in the PVN and SON by examining nuclear Fos colocalization with either OT or AVP cytoplasmic markers. We found that LSD1 treatment, did in fact, block the enhancement of both OT and AVP within the brain, whereas saline paired females retained this enhanced

expression (Figures 2 and 3). Histone modifications are a key element in gene regulation through chromatin remodeling. LSD1 demethylases have been shown to demethylate repressive histone marks thus leading to transcriptional activation (Metzger et al., 2005). The up-regulation of OT and AVP observed in response to monogamous conditioning would require changes in gene expression and the data from this study strongly supports the role of LSD1 histone demethylases as, at least, a partial mechanism in creating these changes. Due to the nature of the type of injection used, we can not determine whether the LSD1 inhibitor was acting centrally or peripherally. However, the LSD1 demethylase inhibitor used here has been shown to cross the blood brain barrier in under and hour, and has also been shown to disrupt central processes such as long-term memory formation (Neelamegam et al., 2012). Although LSD1 demethylase enzymes have been shown to act centrally they also can act peripherally, so it could be that the effect we are seeing is due to LSD1 demethylase enzyme function being blocked in a perhipheral location (Neelamegam et al., 2012; Metzger 2005, Yang et al., 2007). However, since LSD1 inhibitor treatment did not create any symptoms of sickness in treated animals, did not disrupt control behaviors, and blocked the activation of Fos in OT and AVP neurons in the hypothalamus, the effect is most likely central.

Taken together with our previous findings, this study shows that conditioned mate guarding behavior is under the epigenetic regulation of at least two mechanisms of conspecific social and sexual bonding: the activation of OT and AVP within the hypothalamus. That an LSD1 demethylase inhibitor blocked both the behavior and activation of OT and AVP neurons suggests a critical process of demethylation that regulates the expression of genes related to OT and AVP expression and binding in response to sexual reward-related processes.

Summary of Chapter 3

This study assessed whether epigenetic histone modifications, specifically, histone methylation, played a role in the development of conditioned mate guarding behavior, and in altering the activation of OT and AVP neurons in response to male cues. An LSD1 histone demethylase inhibitor (LSD1) was used to inhibit LSD1 class demethylase enzymes during paired and unpaired female rats' initial sexual experiences. It was hypothgesized that if histone methylation is important in creating these changes to OT and AVP systems, then by disrupting the ability of the brain to establish the proper pattern of methylation, the enhanced activation of OT and AVP would be blocked, as would conditioned mate guarding behavior. Rats were administered either LSD1 or saline prior to 10 conditioning trials with either the same male (paired females) or a different male every test (unpaired females). On the final test, paired or unpaired females were placed into an open field with their paired male and a female competitor to assess mate guarding. Paired females treated with LSD1 failed to show any measure of mate guarding behavior in the open field whereas paired females treated with saline did display mate guarding, in the form of number of times they initiated closeness to their paired male, relative to unpaired females.

Next, the ability of the LSD 1 treatment to block the activation of OT and AVP neurons within the PVN and SON was assessed using double-labeling of Fos within OT or AVP neurons. LSD1 treatment prevented the enhanced activation of Fos in OT and AVP within the SON and PVN of paired females. Saline treatment did not block this expression. Unpaired females did not display the enhanced expression. These data demonstrate that the activation (and perhaps also sensitization) of OT and AVP is essential for the onset of conditioned mate guarding behavior in female rats, and that epigenetic histone methylation is an important part of these neural changes.

GENERAL DISCUSSION

In a classic Darwinian account of patterns of reproduction, mating strategies were viewed as a feature of the species as a whole, and therefore thought to be relatively stable in a fixed environment (Reynolds 1996; Dunbar 1988). However, a more modern interpretation acknowledges individual differences and places mating strategies as a function of individual variation in reproductive capacity, experience, and behavior (Dunbar, 1981). Conceptualized in this manner, an understanding of mating strategies must account for observable individual differences within and across species. Using this definition, mating strategies are understood to be more plastic, and can shift freely in accordance with environmental, social, and evolutionary pressures (Dunbar, 1981).

The overarching goal of the experiments reported in this thesis was to explore the flexibility of the mating strategy of the female rat and to see whether their promiscuous pattern of copulation and partner preference could be shifted toward monogamy. This was accomplished by creating artificial environmental pressure in the form of limited mate access during first sexual experiences using a Pavlovian conditioning paradigm that allowed the females to associate salient features of a male rat with their first experiences of paced copulation and sexual reward. Accordingly, the conditioning of monogamous mate guarding behavior in paired female rats was described along with neural and genetic mechanisms underlying this shift in behavior.

The experiments of Chapter 1 were designed to characterize mate guarding behavior in female rats and to understand the role that sexual reward played in the development of this behavior. The ability to self-pace copulation has been shown to be rewarding to female rats (Coria-Avila et al., 2006; Paredes et al., 1997). If female rats are allowed to pace the rate of sexual stimulation they receive, and this self-paced sex is paired with specific environments, males, or neutral odors, females will form preferences for these stimuli, thus demonstrating that self-paced sex is rewarding (Coria-Avila et al., 2006; Paredes et al., 1997). The fist study showed that if sexually naïve female rats were given all of their sexually rewarding experience with the same male they would mate guard that male in the presence of a female competitor.

Before we could evaluate mate guarding, we needed to define what this behavior would be in rats. Mate guarding is a complex behavior that is displayed in a wide variety of ways across species (Clutton-Brock, 1988). It was defined operationally as any behavior performed by a female that takes away the choice of the male to mate with a competitor female (Holley et al.,

2014). Under this definition, we included several aggressive, dominant, social, sexual, and competitive behaviors that can be used by a female to achieve this goal. Aggressive behavior was considered to be any instance that one female was attacking the other female where the female being attacked was vocalizing loudly, which is an indication that what is being observed is stressful, and not just play. Female-female mounting (FFM), is a known dominance behavior in rats, and is known to play a role in a maintaining dominant status (Fang and Clemens, 1998). Solicitations occur freely during sex and are a means of females obtaining stimulation from a specific partner (McClintock, 1978). When a female rat solicits sex from a partner, she will face him and then abruptly turn and run in the other direction, prompting the male to pursue and copulate with her (McClintock, 1978). Interceptions were included as a competitive behavior and occur when one female runs between the male and a female he is pursing to shift his focus on to her, ultimately resulting in her receiving his stimulation or ejaculation and not the other female (McClintock, 1978). In the prairie vole literature, partner preferences are determined by giving mated voles a choice between the partner they copulated with and a novel partner. It is believed that two voles have bonded when the choosing vole maintains more side-by-side contact with the familiar partner over a novel vole (Gubernick et al., 1994). For this reason, we included time spent in proximity to the male and the number of times the female initiates contact with the male. Lastly, after observing animals in the recorded mate guarding sessions, it was noticed that female rats were engaging in two behaviors we had not initially thought to include, presenting and conspecific blocking which interfered with the competitor female's ability to make contact with the male. During early experiences, paired females were observed to hover in a receptive position in front of the male, even after they had been mated with, when typically they would receive stimulation and then dart away. This was described as presenting behavior (McClintock, 1978; Holley et al., 2015). We also observed that female rats would position their bodies between the male and the competitor female, should the competitor female begin to approach the male. This was described as conspecific blocking or interference behavior (Holley et al., 2015). Both behaviors were observed more frequently during the early phases of conditioning relative to later phases, and there were individual differences in the number and timing of these behaviors.

In order to assess the effects of rewarding sex on mate guarding, females were given 10 self-paced sexual trials with either the same male every trial (paired females) or a variety of

males across trials (unpaired females). After the conclusion of the final trial, each paired females was placed into an open field with her paired male and a competitor female from the unpaired group was then introduced into the open field. During the open field test, paired females engaged in female-female mounting nearly twice as much as unpaired females (Holley et al., 2014, Fig 1). Although unpaired females would mount the paired female, it appeared to be done out of sexual frustration when she was not receiving sexual stimulation, whereas when the paired female would mount the unpaired female, it was to remove her from the vicinity of the male (Holley et al., 2014). Although FFM is a readily observed dominance behavior, it is typically done in the context of the home cage to maintain dominance. Typically FFM actually decreases in the presence of a male, whereas in this case, the presence of the male, with an approaching female competitor, facilitates FFM behavior (Fang and Clemens, 1998; Holley et al., 2014). Although FFM is used by females to maintain dominance over other females, it is possible that it is observed more strongly here because of the presence of the paired male. In fact, this behavior sensitized over trials, to the point that it became a fast method of assuring dominance and reducing the solicitations of the competitor. This rapid onset of mate guarding is consistent with what is observed in prairie voles in similar circumstances. Sexually naïve male prairie voles are actually highly affiliative. It is only after they copulate with a female and bond with her that they being to show monogamous behavior and selective aggression towards conspecifics (Gobrogge et al., 2009).

Previous studies have shown that sexual reward is critical for the induction of partner preference in rats and voles. For example, if the sexual reward of copulation is reduced by the opioid antagonists naloxone or naltrexone, partner preferences are diminished dramatically in both male and female rats (Coria-Avila et al., 2008; Ismail et al., 2009) and male prairie voles (Burkett et al., 2011). Similarly, conditioned mate-guarding was dependent on self-paced sexual stimulation. In the second study, following the same protocol as the first study, females were assigned to paired or unpaired groups, however, this time neither group was allowed to pace the rate of copulation. When tested for mate guarding behavior in an open field, paired females did not display any rudiment of mate guarding, suggesting that self-paced sex is essential in order for female rats to bond and display mate guarding (Holley et al., 2014).

Because mate guarding had not previously been characterized in female rats, it was important to replicate the findings from the first two studies in order to validate what had been

observed. Replicating the methods used in the first two studies, both self-paced and non self-paced conditions were repeated and mate guarding in each condition was assessed. Similarly to what had previously been observed, paired females from the self-paced group showed an increase in female-female mounting (Holley et al., 2015) whereas paired females from the non self-paced condition failed to show any measure of mate guarding behavior. Replicating the first two experiments provided a strong foundation for the thesis and the phenomenon, demonstrating clearly that female rats can employ mate guarding behavior when their first rewarding sexual experiences are with one male. Therefore, subsequent studies were conducted on paired and unpaired females that were conditioned using self-paced copulation.

Having observed a shift in mating strategy from promiscuity towards monogamy in the female rat, the brain regions involved were the next to be studied. Here the induction of Fos protein was used as an immunohistochemical marker of neuronal activation within different cell groups in the brain. The neuroanatomical basis of monogamy has been investigated elaborately within the prairie vole literature. For example, studies comparing neuroanatomy between monogamous and non-monogamous vole species has lead to the identification of brain regions that are involved in monogamous responding, including the activation of AVP and OT systems in the hypothalamic PVN and SON, the mesolimbic dopamine system, and regions such as the ventral pallidum that integrate those systems into behavioral output (Bamshed et al., 1993; Insel et al., 1992; Insel et al., 1994; Northcutt et al., 2009; Aragona et al., 2003; Liu et al., 2003). Brain regions were chosen based on the findings in this literature, in addition to regions known to be involved in stress responding, such as the hippocampus and arcuate nucleus (Buwalda et al., 2012). Finally, the ventromedial hypothalamus was used as a control region because it is involved in the induction of lordosis (female sexual receptivity in rodents) by estradiol. Because both paired and unpaired females were receptive, we expected to see no differences in Fos induction within this region (Pfaff et al., 1979).

In line with our hypotheses, we observed significantly more Fos induction within the SON and NAc shell (NAcSh) in paired females than in unpaired females. There were also trends in the favor of paired females having more Fos induction within the hippocampus (Hc) and PVN compared to unpaired females. These data provided a critical step in understanding the neurochemical mechanisms of conditioned mate guarding behavior. Given the role of the hippocampus in stress responding (Buwalda et al., 2012), the Fos induction in paired vs.

unpaired females was an indication that having to defend their mate against a female competitor caused a stress response. The activation of Fos in the NAcSh in paired females is consistent with its role in prairie voles in pair bond maintenance. An elaborate study by Resendez et al. (2012) demonstrated that kappa opioid receptors within the NAc are essential in the display of selective aggression, which is also synonymous with mate guarding in prairie voles. By administering kappa opioid receptor antagonists to bonded voles, they were able to block the display of selective aggression towards a conspecific, demonstrating that receptors in this region are essential for pair bond maintenance. Finally, the greater activation of Fos within the SON and PVN of paired vs. unpaired females suggests that AVP and/or OT neurons may also play a role in mate guarding, as they do in prairie voles.

As previously mentioned, the activation of AVP and OT neurons are crucial to pair bonding in voles, and these neuropeptide transmitters mediate a variety of species-specific social behaviors involved in bonding such as maternal care, aggression, and sexual behavior (Insel et al., 1992; Insel et al., 1994). Given the greater Fos induction within the SON and PVN of paired vs. unpaired females, it was important to determine whether AVP and OT neurons were activated. This was accomplished using double immunohistochemistry to co-label cells with Fos and cells with AVP or OT. Brains from females in both groups were collected and cut into three series. One series was stained for Fos and OT, the second for Fos and AVP, and the third was saved for back up tissue. By using this method we could visualize OT and AVP neuronal activation within the same brain. In line with our hypotheses, analysis revealed that paired females showed more Fos induction within OT neurons in the SON and PVN and also more Fos induction with AVP neurons in the PVN only. These data suggest that, over the course of conditioning, AVP and OT neurons are sensitized to the cues of the male and are linked, via conditioning, to an expectancy of sexual reward.

Although Fos induction suggests that the neurons are activated, they do not allow speculation about whether neurotransmitter is actually released into the brain or posterior pituitary. Microdialysis studies are required to determine whether circulating levels of AVP and/or OT increase within the brain. Nevertheless, these data strongly suggest a role for AVP and OT in conditioned mate guarding behavior. Although OT and AVP are crucial components in monogamous behavior in prairie voles, there are no differences in OT and AVP neuronal bodies or fibers between monogamous and promiscuous vole species (Ross et al., 2009; Wang et

al., 1995; Wang et al., 1996). There are, however, significant species differences in oxytocin receptor vasopressin 1a receptor between monogamous and promiscuous vole species. For example, monogamous voles have higher densities of V1aR in the bed nucleus of the strail terminalis (BNST), ventral pallidum (VP), central nucleus of the amygdala (CeA), basolateral nucleus of the amygdala (BLA), and accessory olfactory bulb (AOB) then promiscuous vole species (Insel et al., 1994; Lim et al., 2004; Smeltzer et al., 2006; Wang et al., 1997; Young et al., 1997). Regarding OTR, monogamous voles display higher densities within the BNST, medial prefrontal cortex (mPFC), and nucleus accumbens (NAc) than promiscuous vole species (Insel et al., 1992; Smeltzer et al., 2006; Young et al., 1996). These differences in receptor distribution were found to be stable across the lifespan of the animal, and were specific to OT and AVP systems (Insel et al., 1992). Given that OT and AVP are known to underlie social behaviors involved in bonding, OTR and V1aR differences are thought to underlie the different reproductive strategies observed across vole species (Hammock et al., 2002). Taken together with the present data, it can be speculated that differences would also be observed in the activation of AVP and OT cell bodies due to sensitization of OT and AVP systems over the course of conditioning. Prairie voles have evolved to be monogamous, and these differences in receptor distribution are a product of many generations that have been shaped by monogamous behavior over the course of evolution. The female rats in the present studies come from a single inbred population, thus any changes to the OT and AVP systems that arise are due to the conditioning process and not evolutionary pressures. Further work is warranted to investigate any changes that might be occurring to receptor densities and receptor binding in female rats.

Once it was determined that OT and AVP neurons became more active in paired females than in unpaired over the course of conditioning, it was of interest to examine whether pharmacological stimulation of OT or AVP during the early phase of conditioning could advance the conditioning process. It was hypothesized that if OT or AVP was administered peripherally to sexually naïve female rats prior to their first sexual experience, they would display rudiments of mate guarding behavior on the second trial, relative to females treated with the saline control. Sexually naïve female rats were assigned to paired and unpaired groups, with the paired groups further subdivided into 3 treatment groups: OT, AVP, or saline. All paired females received one subcutaneous injection of their assigned drug 15 minutes prior to their first self-paced sexual experience, and were then allowed to copulate for 30 minutes with a male. Four days following

this test, females were assessed for mate guarding in an open field. In line with our hypotheses, OT and AVP treatment did facilitate mate guarding. Saline treatment was not sufficient to induce mate guarding, as we had predicted.

Although OT and AVP treatments both facilitated mate guarding, they each enhanced a different subset of behaviors. Treatment with OT, but not AVP, enhanced presenting behavior. Treatment with AVP, but not OT, enhanced conspecific blocking or interference behavior. These data suggest that early on mate guarding is expressed through behaviors such as blocking or interference and presenting. However, as mate guarding behavior matures over trials, a third system for FFM is sensitized and expressed earlier into the session. This change or maturation of motor patterns is observed in other complex behaviors, e.g., the sexual behavior of male rats. For instance, early on, while males are still trying to figure out the mechanics of sexual responding with a receptive female, they display high amounts of anogential investigation. As they gain more and more experience, anogenital investigation dissipates and the number of mounts and intromissions increases. Future studies are required to disentangle the early contributions of learning and neuropeptides. This could be achieved through a time-point study, where females are tested for mate guarding at different intervals throughout the conditioning process, and then sacrificed so their brain can be used for double immunohistochemistry to stain for Fos and OT, AVP, or a third neurotransmitter (e.g., dopamine; see below). This would reveal the progression of conditioned mate guarding behavior, and would also reveal the time course for the enhancement of OT and AVP systems, relative to a third system that may bring about FFM.

Although early on, OT and AVP may have opposing effects (during which OT mediates behaviors that involve proximity and closeness and AVP mediates more aggressive behaviors and social recognition; Ferris et al., 1984; Kendrick et al., 1987), it must be remembered that OT or AVP was administered alone in the present study, and not together. There are several studies that have emerged demonstrating that OT and AVP actually do not just work separately, but in conjunction with one another in the formation of partner preferences. For example, studies have been conducted centrally co-administering AVP and an OTR antagonist. AVP administration enhanced partner preference formation, however, co-administration of OTR antagonist blocked the effect of AVP treatment (Liu et al., 2001). Similarly, if OT is administered centrally in conjunction with a V1aR antagonist, the action of OT is blocked as well (Cho et al., 1999). Presenting behavior occurs in proximity to the male, and was therefore enhanced by OT

treatment, whereas interference behavior, which requires social recognition and approach, was enhanced by AVP treatment. Female-female mounting requires social recognition, a degree of aggression, and also, proximity to the male. So, it is highly likely that both neuropeptide systems are required in order for this behavior to occur, which plays into the hypothesis that FFM could be a matured form of conditioned mate-guarding.

Additionally, OT and AVP are not the only neuropeptide systems known to be involved in pair bonding. More recently, data have emerged demonstrating the importance of dopamine (DA) in not only pair bond formation, but also in the onset of selective aggression, which is synonymous with mate guarding in prairie voles. In prairie voles, the onset of mate guarding coincides with an increase in the dopamine D1 receptor (D1R) in the NAc (Aragona et al., 2009). If D1Rs are selectively blocked in this region, voles fail to display mate guarding behavior (Aragona et al., 2009). Additionally, AVP has also been shown to regulate selective aggression in voles, where central infusion into the lateral ventricles induced aggression towards a conspecific (Winslow et al., 1995). In support of this idea, preliminary studies not included in this thesis have shown increased double labeling of Fos with tyrosine hydroxylase, the rate-limiting enzyme for DA, within the zona incerta and VTA of paired relative to unpaired females. Although promising, in order to truly determine the contributions of OT, AVP, and DA to conditioned mate guarding, particularly to the development of FFM, several elaborate pharmacological manipulations where certain peptides are administered while the action of opposing receptors is blocked would have to be conducted. If such studies were to show a role of DA, but not OT or AVP, then a triple dissociation would be found in the role of each neurochemical system, with two coming into focus early and the third later on in the development of conditioned mate-guarding.

Overall, the data from Chapter 2 demonstrate that OT and AVP are contributing to conditioned mate guarding behavior in female rats. After lengthy conditioning, there is an increase in the activation of both of these neuropeptide systems in female rats who mate guard. Also, both OT and AVP injection are sufficient to induce two separate aspects of mate guarding after one sexual trial alone. At this point it was of particular interest to understand how these systems might sensitize and whether epigenetic histone modifications might contribute to the observed enhancement of mate-guarding behavior in general, and OT and AVP neuronal activation in particular.

The increasing sophistication of behavioral epigenetic techniques over the last decade has illuminated the reciprocal relationship between the genomic regulation of behavior and stimulus-bound changes in the genome through experience (For review: Kramer et al., 2013). Several lines of evidence suggested that examining the role of histone modifications would be justified, especially in terms of Pavlovian learning. For example, epigenetic histone modifications work to regulate memory through learning paradigms. It is commonly accepted that long-term memory, ranging from 1 day to several months, requires gene transcription (Johansens et al., 2011; Dunning et al., 2003; Lisman et al., 2012; review: Kramer et al., 2013). Learning dependent gene transcription is a complex process, involving many different mechansims, however, histone methylation has been identified as an important component. Patterns of histone methylation can act bidirectionally, meaning that some patterns result in transcriptional activation, whereas others result in transcriptional repression, and both repressive and activational can occur simulatenously (Kouzarides et al., 2007; Suganuma et al., 2002). For example, in the context of conditioned learning, histone H3 lysine 4 trimethylation, can act as an activating modification, whereas histone H3 lysine 9 methylation has been shown to act as a repressive modification (Gutpa-Agarwal et al., 2012; Gutpa et la., 2010). Although these two forms of methylation have different outcomes, activation and repression, both have been shown to increase in response to fear conditioning (Gutpa-Agarwal et al., 2012; Gutpa et al., 2010).

A second line of evidence providing us with a foundation to pursue an epigenetic portion of this mechanism was that Wang et al. (2013) had recently demonstrated a role of epigenetic histone modifications in bonding in prairie voles. Prairie voles treated with histone deacetylase inhibitors (HDACi), a subset of drugs shown to up-regulate gene transcription, displayed partner preference formation, and also, an up-regulation of oxytocin (Oxtr) and vasopressin 1a (V1aR) receptors in the nucleus accumbens following treatment. Although the data from Wang et al. was convincing, histone deacetylase inhibitors are drugs that block the action of histone deacetylases, which results in constitutive transcription and gene expression. The action of this drug leaves histone proteins in an open conformation, meaning that the DNA wrapped around them is always accessible to transcriptional machinery. Therefore, it was predictable that treatment with an HDACi would facilitate pair bonding and up-regulate OTR and V1aR. This only suggests that histone acetylation could be part of the bonding mechanism, however, it does not definitively provide that information. For these reasons, histone methylation, instead of other mechanisms,

was examined using a class of drugs called histone demethylase (HDM) inhibitors (HDMi), which block the action of HDM enzymes. Although histone methylation is bidirectional, and can occur in a wide variety of patterns (mono, di, or tri), thus making it harder to understand mechanistically, it was rationalized that if histone methylation was part of the mechanism creating an enhanced expression of OT and AVP in paired females brains, then its disruption would block the ability of the brain to establish the correct methylation pattern that leads to this outcome and therefore block the onset of conditioned mate guarding along with the increased activation of Fos within OT and AVP neurons.

The first step in being able to test the role of histone demethylases, was to acquire the proper histone demethylase inhibitor. The commercial availability of HDMi is currently limited. These drugs have emerged within the last decade, and identifying one that has been validated to work, and also that crosses the blood brain barrier, was difficult. Only one available drug fit these criteria: an HN-1 LSD1 demethylase inhibitor (LDS1). Microdialysis studies had demonstrated that less than 1h after peripheral administration, LSD1 was able to cross the blood brain barrier and remained at constant levels even past 24 hours (Neelamegan et al., 2012). Furthermore, mice that were administered this drug, showed a disruption in their ability to form long-term memories, as assessed through an object recognition task (Neelamegan et al., 2012).

Utilizing the paradigm established in Chapter 1, sexually naïve female rats were assigned to paired vs. unpaired groups and conditioned accordingly. However, females were further divided into two treatment groups: females treated with LSD1 1h prior to test, and females treated with saline 1h prior to test. After the conclusion of conditioning trials, mate guarding was assessed using the open field test established in chapter one. Brains were collected following the open-field in order for us to be able to use them for double IHC to assess how treatment altered OT and AVP activation. In line with our hypotheses, LSD1 treated females did not display any mate guarding behaviors and behaved indistinguishably from unpaired females. Saline treated females did display mate guarding, in the form of time she initiated contact with her paired male, a commonly used measure of partner preference in the vole literature (Gubernick et al., 1994).

Although paired female rats treated with saline did display conditioned mate guarding, they did not display FFM, which was observed previously after 10 conditioning trials. However, it must be remembered that mate guarding, like other complex behaviors, is an umbrella term

used to describe a specific subset of behaviors that are used to prevent the male from accessing a competitor female. As such it is not synonymous with FFM. Paired females typically display a composite mf mate guarding behaviors across studies more than unpaired females, and they have been observed to use all three behaviors to varying degrees in each subset of animals tested (Holley et al., 2014; 2015). Moreover, the behavior of the competitor female largely impacts the display of mate guarding by the paired female. For example, FFM has been observed in groups of paired females when unpaired females would refuse to leave the vicinity of the male regardless of the presence of the unpaired female (Holley et al., 2014). However, during behavioral observation in the present study, the presence of the saline treated paired female in the vicinity of the male was enough to lead to the unpaired female to leave.

Once it was determined that treatment with LSD1 was able to disrupt the development of conditioned mate guarding behavior, it was of interest to examine whether it also reduced the activation of OT and/or AVP neurons. Using double IHC as described in Chapter 2, cells in the PVN and SON were labeled for Fos and either OT or AVP. Treatment with LSD1 blocked the enhanced expression of Fos within OT and AVP neurons of paired females, whereas paired females treated with saline showed this expression. These data provide convincing evidence that the enhanced activation of OT and AVP neurons is essential for the onset of conditioned mate guarding, and demonstrate that LSD1 demethylase enzymes are at least one critical component to the mechanism in bringing about this change in expression.

Although these data are convincing, they open up a number of possibilities to fully understand the role of epigenetics in creating these changes to neural circuitry. Due to the nature of the injection, it could not be determined if LSD1 was acting centrally or peripherally. LSD1 demethylase enzymes have been shown to act centrally, however, they also can act peripherally, so it could be that the effect we are seeing is due to LSD1 demethylase enzyme function being blocked in a perhipheral location (Neelamegam et al., 2012; Metzger 2005, Yang et al., 2007). However, the fact that Fos was not enhanced in OT or AVP neurons suggests strongly that LSD1 treatment had a central action. LSD1 demethylases demethylate lysine residues 4 (H3K4) and 9 (H3K9) on histone 3 tails. Follow up studies using western blots along with antibodies directed against H3K4 or H3K9, on proteins extracted from SON and PVN brain punches, would be necessary to examine whether drug treatment affected histone methylation in these areas. Another important aspect to consider is that LSD1 demethylases do not typically act alone and

are usually part of molecular complexes in conjunction with HDAC enzymes (Shi et al., 2005; Humphrey et al., 2001; Shi et al., 2003; You et al., 2001). Therefore, to truly understand the total epigenetic mechanisms at play here, much more work is warranted to examine the role of HDACs in addition to other classes of histone demethylases.

One interesting implication of these studies, and one that would be beneficial to empirically evaluate, is the contribution of this shift in mating strategy to subsequent generations. Many emerging lines of evidence show that epigenetic modification to chromatin can be inherited transgerationally, as can methylation patterns to particular DNA regions (Dias et al., 2014a; Crews et al., 2007; review: Dias et al., 2014b). One of the short-comings of this line of work is that it fails to address empirically any ultimate causation or ultimate effects that this type of behavioral shift might have. There are several ways that these issues could partially be addressed. First, it would be interesting to see how this type of conditioning affects methylation patterns and mRNA expression in select brain regions across subsequent generations. Although this would not provide the breadth of an evolutionary study, it could shed light onto whether this type of conditioning can induce transgenerational changes that might contribute to the continued evolution of the species. In order to assess this, it would be necessary to characterize the changes to chromatin conformation and DNA methylation more fully. This is being done in current follow-up studies. It would then be possible to breed animals that have been conditioned to mate guard and evaluate chromatin structure and DNA methylation patterns several generations later in order to see whether those alterations are conserved.

Another valuable direction to take would be to obtain mRNA from brain punches of paired and unpaired females and compare these to other monogamous animal species across taxa to see if conditioning is creating changes to gene expression associated with monogamy in other species, not just rodents. It has been shown that the social behavioral network and mesolimbic reward systems are highly conserved across vertebrates, as are the chemical profiles for the dopamine system, sex steroid hormone signaling, neuropeptide systems, and the spatial receptor distribution patterns of OT, AVP, and DA systems (O'Connell and Hoffman, 2012). Because these neural networks are highly conserved, it is likely that rats possess the ability to express bonding circuits similarly to other monogamous vertebrates, and the data of this thesis suggest that they can adapt to do so should a situation arise where mate guarding is beneficial to survival or where sexual stimulation and reward is provided by only a few competent males.

Over the course of this thesis, conditioned mate guarding behavior in female rats has been characterized on behavioral, neuromolecular, and epigenetic levels. Although there are still many different avenues this fruitful line of research can take, these data provide strong evidence that: (1) female rats are capable of displaying mate guarding; (2) OT and AVP in large part underlie this shift in behavior; and (3) epigenetic histone methylation is an important component in bringing about these neural and ultimately behavioral changes.

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