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Aashna Sahni April 11, 2020

Investigating the Behavioral, Physiological, and Neural Consequences of Threat to a Social Bond

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An abstract of
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Abstract

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Romantic jealousy is a complex social emotion induced by a threat to a valued relationship due to a rival. Jealousy serves an important adaptive function in keeping a partner in the relationship. However, jealousy can also elicit violent and aggressive behaviors. Therefore, it is important to understand the neurobiological basis of jealousy to provide support to those who experience it. Jealousy occurs in the context of long-term, socially complex relationships with a strong attachment bond. Socially monogamous prairie voles form pair bonds with their partners; this exclusive relationship between partners is characteristic of complex social species like humans. Therefore, voles serve as a model organism to investigate the neural circuitry that evolved into romantic jealousy in humans. In this study, we developed a new model of social threat and investigated the behavioral, physiological, and neural consequences of exposure to a threat to a social bond in male prairie voles. In a pilot experiment, a pair-bonded male and a control male both observed the pair-bonded female interact with the novel male. We observed an increase in the investigative behaviors of the pair-bonded male when the female interacted with a novel male. We further compared the corticosterone levels and c-fos activation in the PVN, lateral septum, amygdala, and BNST between the control and pair-bonded male. We did not observe any significant difference in FOS activation and corticosterone levels between the control and pair-bonded male. We further refined our behavioral test and conducted a choice test in which a pair-bonded male had the choice to investigate either his partner or a stranger female while both the females interacted with a novel male. We observed a significant increase in the male's investigative behavior towards the stranger female and the novel male. This study opens new avenues for studying the psychosocial impact of a social threat in a socially monogamous rodent. Our data from the pilot experiment shows differences in behavior in response to a social threat. Despite the small effect size, it is indicative of a subtle effect that needs to be further refined to observe robust corresponding changes in brain activation as well.

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Introduction

Emotions in Humans and Animals

Emotions are a multifaceted phenomenon and are accompanied by neuronal, physiological, and behavioral changes (Steimer, 2002). Over the last few decades, many theories have been proposed to explain emotions in humans and animals. One of the leading biologically-oriented theories, basic emotion theory, identifies five to seven basic or primary emotions that have evolved to regulate complex social behavior in humans and animals (Panksepp, 1998; Izard, 2007; Adolphs, 2017). These emotions and their underlying neural circuitry are innately wired into the brain through evolution, which directs our actions in ways to promote survival (Davis and Montag, 2018). These basic emotions are thought to be mediated via affective intrinsic processes in the brain. Some basic emotion theorists look at the neural circuitry underlying these emotions (Panksepp, 2005) while others focus more on analyzing the evolving neural circuitry underlying emotional behaviors and their interaction with their environment (LeDoux, 1996, 2012). Basic emotion theorists argue that the emotional brain system processes contribute towards survival in response to environmental challenges and opportunities. Panksepp defines seven basic emotions: seeking, rage, fear, lust, care, panic, and play (Panksepp, 1998). In humans, basic emotions have evolved to help organize and motivate actions that are critical for adaptive responses to immediate challenges on survival and wellbeing (Izard, 2009). For example, an infant expresses joy upon seeing the familiar face of his mother; this expression is essential to survival and development (Izard et al., 1995).

Similarly, in animals, emotions evoke a behavioral response that allow the animal to survive. For example, when a group of chimpanzees was presented with a toy snake, only one of the chimpanzees was aware of the toy snake's position and adapted an ambivalent cautious posture out of fear; this response was followed by other chimpanzees that had never seen the snake and thus demonstrating the effectiveness of emotional communication increasing chances of survival (Menzel, 1988; Numan, 2015). Other emotions such as seeking and rage allow the animal to defend vital resources including food, shelter, territory, mates, or young; individuals compete with one another to obtain these resources (Numan, 2015; Davis and Montag, 2018). Apart from the basic emotions, humans also display other complex emotions that unlike basic emotions don't have a direct role in promoting survival. Romantic jealousy is one such complex social emotion that is commonly observed in humans.

Romantic Jealousy in Humans

Romantic jealousy is a complex social emotion that can be induced by a real or imagined threat to a valued relationship due to a rival. This distressing emotion elicits a behavioral output which is regulated by stress hormones; this response is accompanied by brain processes functioning to protect a valuable resource. Evolutionary psychologists hypothesize that jealousy is an important evolutionary adaptation aimed at keeping a partner in the relationship (Lyons, 2019). Jealousy is a mate-retention tactic that works to maintain a relationship and prevent infidelity (Lyons, 2019; Zheng et al., 2021). However, jealousy also has a maladaptive aspect to it. Jealousy can elicit violent and aggressive behaviors that can be classified under the category of mate-retention or mate-guarding. It is one of the leading causes of domestic violence or an ongoing stressor in many

people's lives (Puente and Cohen, 2003). Jealousy overrides logical inference and leads to irrational behavior such as unrestrained violence. Therefore, it is important to understand the neurobiological basis of jealousy to effectively provide support to those who experience it. Romantic jealousy occurs in the context of long-term, socially complex relationships that consist of a strong attachment bond (Zheng et al., 2021). Such socially complex bonds are characteristic of socially monogamous species.

Social Monogamy and Pair Bonding

Social monogamy is an adaptive strategy adopted by some animals to maximize their fitness. Socially monogamous animals have evolved social behaviors like attraction to potential mates and cooperation in complex animal societies; this increases the individual's ability to gather resources and form social bonds that increase its chances of survival and reproduction (Clutton-Brock, 2009). Intense and exclusive social attachment with a mate is pair bonding. A socially monogamous pair shares a common range or territory and associates with each other for more than one breeding season (Lukas and Clutton-Brock, 2013). Socially monogamous animals also show the evolution of nurturance by caring for the young which is advantageous to the survival of the species. Parents increase the survival of their offspring by guarding, provisioning for, and protecting their offspring from predators; this increases the offspring quality which ultimately increases the reproductive success of the offspring in adulthood (Fietz and Dausmann, 2003; Klug and Bonsall, 2014). Expression of parental care comes hand-in-hand with social attraction and bonding between mates in socially monogamous animals. Many theories have been proposed speculating the evolution of social monogamy. One of the theories suggests social monogamy

evolved as a mate-guarding strategy to prevent males from accessing more than one female (Lukas and Clutton-Brock, 2013). Another theory suggests it evolved as a cooperative strategy between parents to increase the fitness of the offspring and contribute resources to defend the offspring from predation (Plant and Zeleznik, 2015). Both pair bonding and biparental care are unique patterns of social behavior displayed by socially monogamous animals.

Pair Bond Circuitry and Molecules

Oxytocin (OT) and arginine vasopressin (AVP) are important neuropeptides that play a role in regulating a variety of social behaviors including pair bond formation in socially monogamous animals (Plant and Zeleznik, 2015). Both these neuropeptides show evolutionary conservation in their general role in modulating social and reproductive behaviors for at least 700 million years (Donaldson and Young, 2008). OT and AVP are synthesized mainly in the paraventricular (PVN) and supraoptic nucleus (SON) of the hypothalamus (Tabbaa et al., 2016), but AVP can also be synthesized in the bed nucleus of the stria terminalis (BNST). OT neurons project from the hypothalamus to forebrain areas including nucleus accumbens (NAcc) and the amygdala whereas AVP fibers project from the PVN, SON, BNST, and the medial amygdala (MeA) (De Vries, 2004; Ross et al., 2009). AVP is implicated in territorial aggression and selective aggression in rodents (Wang et al., 1997b; Stribley and Carter, 1999; Lim et al., 2004). AVP enhances social recognition and this effect is mediated by vasopressin V1a receptor (V1aR) in the lateral septum (Veenema et al., 2012). Dopamine (DA) is also critical for pair bond formation in socially monogamous rodents, prairie voles (Young and Wang, 2004; Young et al., 2011). D1 and D2 receptors serve opposite functions in pair bond formation; D1 receptors inhibit partner preference formation

whereas D2 receptors induce partner preference (Aragona et al., 2006). This relation is important for pair-bonded males to show partner preference towards their mate and prevent partner preference towards novel females. Partner preference also requires simultaneous activation of both OT and D2 receptors (Liu and Wang, 2003). The stress system also plays a critical role in pair bond formation. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis which results in a cascade of physiological changes that result in the production of glucocorticoids which are corticosterone in rodents (Smith et al., 2013). In response to stress, hypothalamus releases corticotropin-releasing factor (CRF) that induces the release of adrenocorticotropic hormone (ACTH) from the pituitary; then ACTH targets the adrenal cortex to initiate glucocorticoid synthesis (Smith and Vale, 2006). Studies have shown that CRF receptors in the NAcc modulate partner preference in prairie voles (Lim et al., 2007). Furthermore, CRF and D1 receptors are involved in the maintenance of that pair bond by inducing an aversive state when the pair bond is separated or threatened (Resendez and Aragona, 2013).

Social Monogamy and Mate-Guarding

Another characteristic behavior exhibited by socially monogamous animals is mate guarding. Mate guarding is a defensive strategy maintained by the physical proximity of the males with their females; males display aggression in an attempt to fend off intruders and prevent rival males from mating with his female (Kokko and Morrell, 2005). Mate guarding is a reproductive strategy to maximize an individual's fitness by ensuring paternity. However, some socially monogamous species occasionally display extra-pair copulations (EPC). In habitats with lower population density, individuals develop mating strategies to maximize their fitness. A study in songbirds

showed that older, more attractive (red-black) males seek EPCs and invest less time in their mates (Dowling and Webster, 2017). In contrast, unattractive (brown) males don't seek EPCs; instead, they use an alternate strategy to maximize their fitness – mate guarding (Dowling and Webster, 2017). Mate guarding in rodents is displayed through aggressive behaviors, modulated by the social context in which they occur, and in males is often provoked by the actions of another male (Numan, 2015). The most common trigger for offensive behavior in rats and mice is the intrusion of an unfamiliar male into their territory; offensive aggression is used by these males to attack intruders to defend social resources (Koolhaas et al., 2013; Numan, 2015). The brain regions associated with aggression towards intruders in rodents have been termed as the hypothalamic aggression area (HAA); The HAA is in the hypothalamic region extending from the anterior hypothalamic nucleus (AHN) through the ventromedial nucleus of the hypothalamus (VMN) (Kruk, 1991).

Socially Monogamous Animals as a Model to Understand Jealousy

Socially monogamous animals have naturally occurring social behaviors such as male-female pair-bonding and mate-guarding. Pair bonds are **privileged bonds or those underlying specific, high-value relationships that include a degree of exclusivity**, which are characteristic of species with more complex social structures like humans. All bonds are not equal; studies in humans have shown that disruptions to privileged high-value bonds, such as those between partners are more likely to cause psychosocial stress than disruptions to low-value bonds (Bruce and Kim, 1992). Therefore, socially monogamous species serve as a good model to study romantic jealousy as they can be used to study social behaviors such as pair-bonding and mate-guarding which are

similar to those seen in humans. In the past, very few studies have investigated the neural and behavioral correlates underlying jealousy in socially monogamous animals. One study performed in socially monogamous titi monkeys examined the neuronal and hormonal changes in males when their pair bond was challenged by viewing their female mate with a stranger male. Results from the study showed an increase in cortisol and plasma testosterone levels in males. The study further measured brain activity using a PET scan and showed increased activation in the right LS, left posterior cingulate cortex, left anterior cingulate, and decreased activity in the right MeA in jealous males (Maninger et al., 2017).

Prairie Voles

Prairie voles are rodents that form socially monogamous pair-bonds with their mates and express biparental behavior. Prairie voles form life-long bonds with a single breeding partner; male voles stay with their partners after copulation and nest together communally to raise their offspring (Getz et al., 2003). Male-female prairie voles that are cohabitated for 24 hours without mating or 6 hours with mating display a preference towards their partners (Williams et al., 1992). This is tested through a partner preference test in which the experimental subject chooses between their mate or a novel animal and the time spent with each is used to calculate a preference score; this test is used as a proxy for pair bonding (Ahern et al., 2009). Prairie voles also display intense aggression towards novel males and female conspecifics which is termed selective aggression (Gobrogge and Wang, 2009). Selective aggression is hypothesized to play a crucial role in mate guarding and pair bond maintenance (Gobrogge et al., 2007). Sexually naïve voles don't display

a high degree of aggressive behaviors compared to sexually experienced male voles that display a high degree of aggression towards strangers.

OT and AVP play a critical role in partner preference formation in prairie voles; mating and endogenous release of oxytocin facilitate a partner preference formation in voles. (Williams et al., 1994; Ross and Young, 2009; Johnson et al., 2016). Intracerebroventricular infusion of OT facilitates the formation of partner preference in female prairie voles after 6 hours of cohabitation in the absence of mating; pair bond formation is absent despite 24 hours of cohabitation when an OT receptor antagonist is administered (Williams et al., 1994). Furthermore, AVP is also involved in partner preference formation as well as expression of preference in voles (Plant and Zeleznik, 2015). Prairie voles have higher densities of V1aR binding in the ventral pallidum and amygdala compared to montane voles that are non-monogamous (Wang et al., 1997b; Lim et al., 2004). AVP is also implicated in selective aggression. Studies have shown that mating-induced selective aggression can be stimulated by an external infusion of AVP in the absence of mating (Ferris et al., 1984; Winslow et al., 1993; Plant and Zeleznik, 2015). Moreover, studies have shown that V1aR in the anterior hypothalamus mediates the onset of selective aggression (Gobrogge et al., 2007). Selective aggression is also enduring and males that were pair-bonded for two weeks showed an increase in AVP in the anterior hypothalamus in a resident intruder test (Aragona et al., 2006). Resident-intruder tests are conducted by introducing a conspecific intruder into the male resident's cage. We can expect these neuropeptides to be involved in the expression of jealousy; the PVN is also a region of interest as it is the source of these neuropeptides.

There is an important interaction between the two neuropeptides OT and AVP as well as the HPA axis in the formation of a pair bond in prairie voles. OT, an anxiolytic, has been shown to suppress the stress response whereas AVP, an anxiogenic, has been shown to increase the stress response in prairie voles (Neumann et al., 2000). Studies have shown that stress has sexually dimorphic effects on pair bond formation in prairie voles (DeVries et al., 1996). In males, an increase in corticosterone levels before pairing facilitates the formation of a partner preference whereas, in females, an increase in corticosterone levels before pairing interferes with the formation of a partner preference (DeVries et al., 1995; DeVries et al., 1996). However, increase in corticosterone levels of a female that has already cohabitated with a male does not prevent expression of a preference towards the male. Jealousy or threat to a pair bond is a stressful event; we can expect it to activate the HPA axis which makes the measurement of glucocorticoids of interest to us.

Using C-FOS, which is an immediate-early gene used as a proxy for neuronal activation, studies have looked at brain areas involved in aggression in prairie voles. Male voles cohabitated with a female for 24 hours with mating displayed aggression towards intruders as well as increased Fos-immunoreactivity (Fos-ir) expression in some brain like the BNST; males that mated for 24 hours and displayed a high degree of selective aggression had increased Fos-ir expression in MeA (Wang et al., 1997a; Gobrogge and Wang, 2011). More recent studies have investigated neuronal activation in pair-bonded males that underwent resident-intruder tests compared to males that did not; there was an increased Fos-ir expression in many brain areas such as BNST, PVN, lateral septum (LS), and the media preoptic area (MPOA) (Gobrogge et al., 2007). Overall, many studies have confirmed that MeA and anterior hypothalamus are associated with the display of selective

aggression in prairie voles (Wang et al., 1997a; Gobrogge et al., 2009). We can expect these brain areas to be involved in the expression of behaviors following threat to a pair bond.

Prairie Voles as a Model to Understand the Evolution of Jealousy

Extensive research in prairie voles in the last few decades has shown that they serve as a good model organism to understand the underlying neural circuitry that has evolved into complex social behaviors in humans such as love and empathy. Walum and Young (2018) have proposed that pair bonding is the evolutionary antecedent of romantic love and pair bonding is an important part of romantic bonds (Walum and Young, 2018). Similarly, Burkett and colleagues have shown evidence of consoling behaviors in prairie voles providing new insights into the evolution of complex empathy-motivated behaviors (Burkett et al., 2016). Prairie voles are complex social species that have a privileged bond with their partners; this exclusive relationship between partners is characteristic of complex social structures like humans. Partners cooperate to invest their resources in nesting together and raise young ones throughout their life. These pair bonds are extremely high-value bonds that are guarded and maintained by the male voles by displaying selective aggression. A threat to these bonds due to separation or death results in a display of emotional distress and aggression; this is induced by a fear-related response resulting in anxiety-related behaviors to protect their mate and maintain their bond (Numan, 2015; Lieberwirth and Wang, 2016). Therefore, **we hypothesized that voles can also serve as a model organism to investigate the neural circuitry that evolved into romantic jealousy in humans.**

Our aim is to develop a new model of social threat that can provide us some insight into understanding how jealousy evolved in humans. In the current study, we investigated the

behavioral, physiological, and neural consequences of exposure to a threat to a privileged social bond in male prairie voles. We conducted a pilot experiment where the experimental male is pair-bonded to a female that interacts with a novel male. The experimental male and a control male both observed the female interact with the novel male. We expected to see an increase in investigative behaviors of the experimental male when the female interacted with a novel male. We further compared the corticosterone levels and c-fos activation between the control and experimental males. Similarly, we expected an increase in plasma corticosterone levels of the experimental male and an increase in fos-ir in areas that have been implicated in stress and aggression in rodents, BNST, LS, PVN, and the amygdala. Finally, we further refined our behavior test and conducted a choice test in which a pair-bonded male had the choice to investigate either his partner or a stranger female while both the females interacted with a novel male. We predicted a greater preference in investigative behaviors directed towards that female mate.

Materials and Methods

Animals

Prairie vole (*Microtus ochrogaster*) were bred and maintained in a colony at the Yerkes National Primate Research Center that were originally derived from field caught specimens from Illinois, USA. After weaning at 20 to 23 days of age, the offspring were separated from their parents and housed in same-sex groups with two or three voles per cage. All animals were housed in ventilated 36cm x 18cm x 19cm plexiglass cages filled with Bed-o'Cobs laboratory animal bedding (The Andersons Inc., Maumee, Ohio) under a 14/10-hour light/ dark cycle (lights on 7:00 AM–

9:00 PM) at 68-72˚C with access to food (rabbit diet; LabDiet, St. Louis, Missouri) and water ad libitum.

All the voles used in our experiments were adults ranging from 2 months to 6 months of age. All voles were used for experimentation after reaching adulthood (between 2 months and 6 months of age). All female voles were ovariectomized and sexual receptivity was induced by daily subcutaneous administration of estradiol benzoate (EB, 2 μg/vole, Fischer) dissolved in sesame oil for 3 days before the behavioral test ensuring a sexually receptive state during the experiment. Before testing began, adult subjects to be tested as male-female mated pairs were co-housed for at least 7 days to allow for the formation of a bond. All behavioral experiments were performed between 10:00 AM and 3:00 PM. In an attempt to minimize stress during the experimental paradigm, a careful cage change regimen was followed: animals were only exposed to new cages at the beginning of the cohabitation and separation periods. Furthermore, some of the bedding from an animal's previous cage was added to its new cage. All breeding, housing, and experimental procedures were approved by the Institutional Animal Care and Use Committee at Emory University. The methods listed below have been established by various members of the Young lab and have been successfully used before.

Pairing Procedure

Adult female voles were paired with an unfamiliar adult male. Twenty-four hours of cohabitation with a female even in the absence of mating is sufficient for the induction of a partner preference, which is a laboratory proxy for pair bond formation (Williams et al., 1992)

Behavioral Tests

To study the behavioral consequences of exposure to a threat to a privileged social bond in prairie voles, threat to a social bond was modeled by physically isolating the male vole from its female partner and introducing a third party (male) that freely interacted with the female partner. The male vole was able to observe the female partner and third party interact.

Pilot Experiment

Twenty-four adult males were randomly distributed into the following two groups: (1) Control (C; N=17) and (2) Experimental (E; N=19). Each experimental male were used as control as well. The experimental setup consisted of a plexiglass chamber divided into three compartments by an opaque plastic divider with small holes (Fig. 1). The holes allowed the males to smell and hear the sexually receptive female in the center chamber but prevented any physical contact. The experimental males were pair-bonded to the sexually receptive female in the center compartment and the control males were pair-bonded to another female that was not a part of the experiment. First, a 30-minute habituation period allowed the experimental and control male time for acclimation to the testing environment. Then during the object condition (90 minutes), the control and experimental males are put in the first and third compartment and the female partner of the experimental male was put in the center compartment of the plexiglass chamber with an object resembling a rodent. Following this condition, the object was replaced with a novel male during the social threat condition (90 minutes). The female was allowed to interact freely

with the object and the novel male during the two conditions; both the experimental and control male were able to observe the female through the plastic screen.

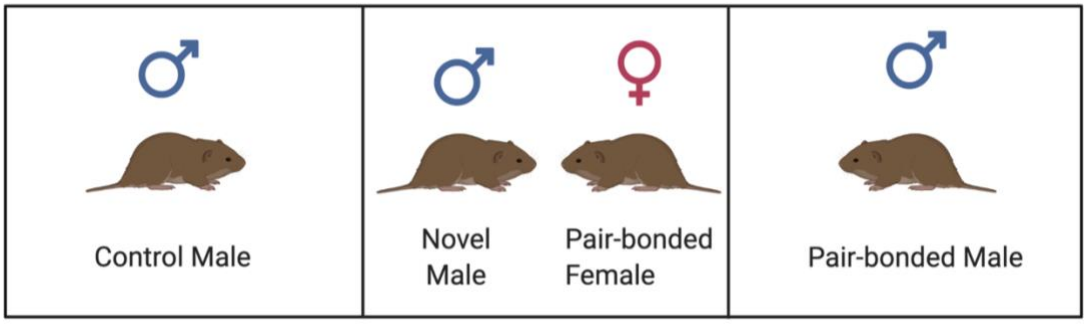


Figure 1: Setup of the behavioral test in the pilot experiment. The experimental male is pair-bonded to the female in the experiment whereas the control male is pair-bonded to another female that is not in this experimental setup.

Choice Experiment

Five adult males were tested in a behavioral assay (N=5) that presented them with a choice to investigate their female partner or a stranger female (Fig. 2). The experimental setup consisted of a plexiglass chamber divided into three compartments by an opaque plastic screen with small holes. The holes allowed the males to smell and hear the sexually receptive female in the center chamber but prevented any physical contact. The experimental females were pair-bonded to the male in the center compartment and the stranger females were pair-bonded to another male that was not a part of the experiment. During the habituation condition (15 minutes), the male is placed in the center compartment of the plexiglass chamber allowing time for acclimation to the testing environment. Next, a female that was pair-bonded to the male was placed in one of the compartments adjacent to the male's; a sexually receptive stranger female that was pair

bonded to another male was placed in the other compartment adjacent to the male's compartment during the females-only condition (30 minutes). Afterwards, sexually naïve, novel males were added to the compartments of both the female mate and stranger females; the novel males could freely interact with the females. During this female-with-male condition (30 minutes), the male in the center compartment had a choice between investigating his partner with a novel male or the stranger female with a novel male.

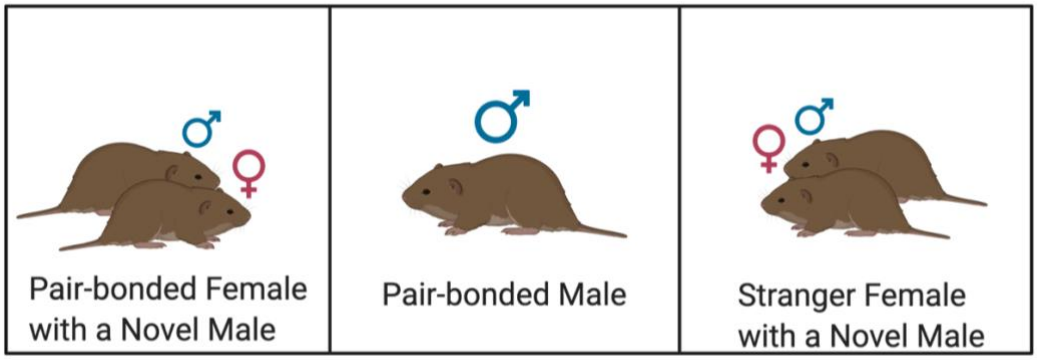


Figure 2: Setup of the behavioral test in the choice experiment. The experimental female is pair-bonded to the male in the experiment whereas the stranger female is pair-bonded to another female that is not in this experimental setup.

Behavioral Coding

Top-down videos of the male voles were recorded from both the behavioral tests. In the pilot experiment, the videos were viewed blinded to the experimental and control group. First 10 minutes of each phases (object present and male present) were coded for investigative behaviors such as sniffing and digging. Investigative behaviors have been defined as behaviors displayed when the animal approaches the plastic screen to observe the animal in the compartment across

the plastic screen. This experiment was coded using The Observer XT (Noldus, Wageningen, The Netherlands). In the choice experiment, the animals were tracked using DeepLabCut (Mathis et al., 2018) , which is an automated tool that can track and label the body parts of a moving animal; we used to point between the ears of the animal to track its movement. The time spent by the male vole investigating the females' compartments was characterized by the proximity of the male to the plastic screen separating the male's chamber from the female's chamber (Fig. 3). For each experiment, 1/6th of the breadth of the cage was chosen as the radius from the center of the plastic screen. The duration recorded with the male's head within that radius was considered as investigative behavior.

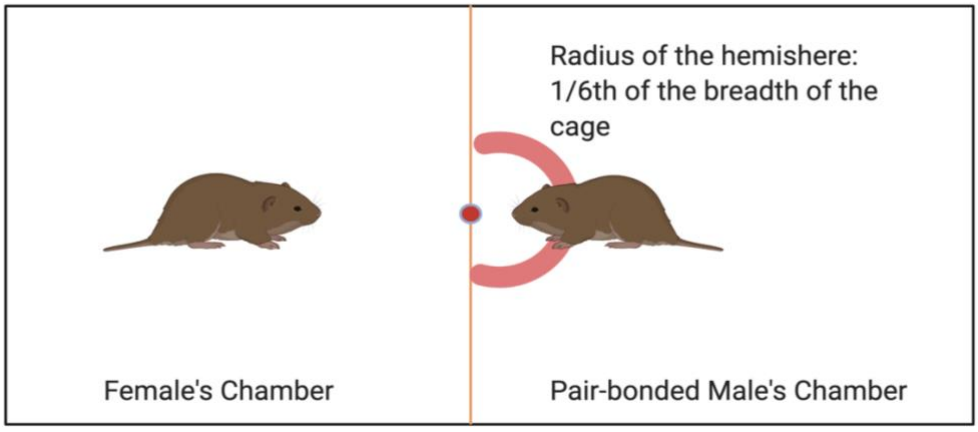


Figure 3: Measurement of investigative behaviors

Physiology

Collection of Plasma and Tissue

In the pilot experiment, male voles were deeply anesthetized with an overdose of isoflurane and blood was sampled from the right atrium which was immediately placed on ice in EDTA-coated

tubes. The blood was centrifuged at 4˚C at 5000 rpm, for 5 minutes to obtain the plasma, which was aliquoted and stored at -80˚C until assayed for corticosterone. Following the collection of blood, voles were immediately perfused transcardially with phosphate-buffered saline (PBS, pH 7.4; Teknova, Hollister, CA), followed by 4% paraformaldehyde (Polysciences, Warrington, PA) at 4 mL/minute using a perfusion pump (Easy-Load II Masterflex; ColePalmer, Vernon Hills, IL). Following the perfusion, the brains were removed and soaked overnight in 4% paraformaldehyde at 4 °C overnight, and finally stored in PBS containing 30% sucrose until sectioning.

Sectioning

Perfused brains were sliced into 40 µm sections using a sliding microtome (Microm HM 450, Microm International, Walldorf, Germany) with a freezing stage at -20˚C (Physitemp BFS30TC, Physitemp Instruments, Clifton, NJ) and were stored in 1x PBS with 0.1% sodium azide until immunohistochemical staining.

Corticosterone Analysis

Plasma corticosterone levels were measured using a commercially available kit (Tecan, Germany) as follows. Plasma samples stored at 80˚C were thawed and 10µL of samples were added into the wells of the microtiter plate and 100µL of enzyme conjugate was added to the well plates, mixed, and incubated for 60 minutes. The wells were rinsed 3 times with diluted Wash solution (30 mL of concentrated Wash Solution with 1170 mL distilled water) after which 50µL of substrate solution was added to each well and was incubated for 15 minutes at room temperature. Afterwards, 25 µL of Stop solution was added to each well to stop the enzymatic reaction. After

10 minutes, the absorbance (Optical Density) of the solution in each well at 450 nm (reading) and at 620 - 630 nm (background subtraction) was determined using a microtiter plate reader.

Histology

Immunohistochemistry

Sections were washed 3 times in PBS for 5 minutes each, incubated for 15 minutes in a freshly-made solution of 0.1% sodium borohydride and washed 3 times in PBS for 5 minutes each. Further, the sections were incubated in PBS containing 0.2% Triton-X (PBST; Sigma-Aldrich, St. Louis, MO) and 5% normal goat serum (Fitzgerald, Acton, MA) for 2 hours at room temperature. The sections were then incubated overnight in PBS containing 0.2% Triton-X, 0.5% normal goat serum and, the primary rabbit polyclonal anti-FOS antibody (Synaptic Systems, Germany) at a dilution of 1:1,000 on an orbital shaker at 4°C. Following primary incubation, sections were washed 3 times in PBS for 5 minutes each and incubated in PBS containing 0.2% Triton-X, 5% normal goat serum, and the secondary goat anti-rabbit IgG antibody (A-11008, Thermo Fisher Scientific) one hour at room temperature. After secondary incubation, sections were washed 3 times with PBS for 5 minutes each and were mounted on slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Once dried, mounted sections were coverslipped using Fluoromount-G™ Mounting Medium.

Image Analysis

Sections were imaged at 20x magnification using a fluorescent microscope (Keyence, BZ-X710 series, Japan); using the Rat Brain atlas (Paxinos and Watson, Seventh Edition), we defined the margins enclosing the region of interest. Threshold for DAPI and FOS positive cells was chosen to best detect all the positive cells and the FOS positive cells were counted using an online machine learning tool, DeepFLaSH (Segebarth et al., 2018). Binary segmentation maps were created manually from FOS images corresponding to the region of interest using ImageJ. These segmentation maps and the corresponding original image were then uploaded to DeepFLaSH to either train a new model from scratch (100 epochs, threshold= 0.5, roi_size= 10) or adapt to an existing CNN-model library (50 epochs, threshold=0.5, roi_size= 10) through transfer learning. The FOS images were uploaded, and segmentation maps of the FOS signals were produced based on the training (Fig. 4). These segmentation maps were further uploaded on ImageJ and the FOS positive cells were counted using the Analyze Particles function. FOS positive cells in all regions were measured in square pixels. All areas measured in square pixels were converted to an arbitrary area for better representation.

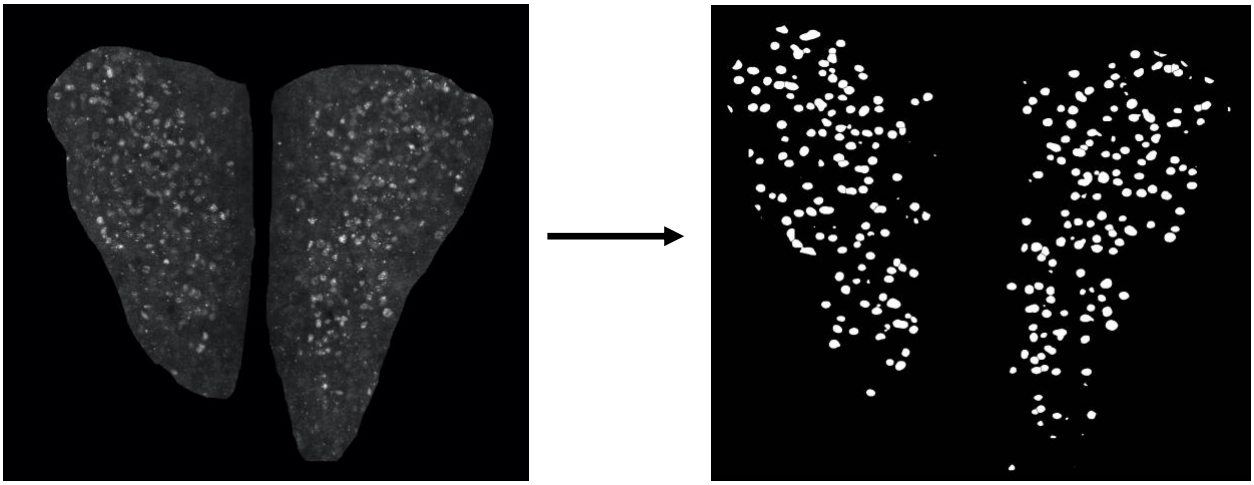


Figure 4: Image of C-FOS activation in the PVN (left) which is converted into a segmentation map of C-FOS activation (right) by DeepFLaSH.

Data Analysis

The data are presented as means with the standard error of the mean (SEM) for all analyses and figures. A probability value of $P > 0.05$ was considered to be statistically significant. In the pilot experiment, the duration of time spent by the experimental male and the control male investigating the object with female in the control condition and novel male with female in the experimental condition was analyzed using a paired t-test. The control and experimental male were viewing the same chamber throughout the pilot experiment and were considered paired. A two-sample paired t-test was conducted on the average of the corticosterone levels between the control and experimental groups. A two-way ANOVA was conducted on the average of FOS-positive cell counts between the control and experimental group in PVN, BNST, LS, and Amygdala followed by a paired t-test for each of the brain areas. In the choice experiment, the behavioral data analyzed by DeepLabCut was further quantified in the R statistical software package version

3.6.3 (R Project for Statistical Computing, Vienna, Austria) and a paired t-test was conducted. All statistical tests were run on Prism-GraphPad version 9.0.2.

Results

Pilot Experiment

In the pilot experiment, animals that underwent the same experiment during which the pair-bonded and control male view the same compartment with the female, were considered as paired. Results from the behavioral test showed that compared to the control male, the pair-bonded male spends a significantly greater time investigating the pair-bonded female when she was interacting with a conspecific, $P<.0001$. However, the control male that is not pair-bonded to the female in the behavioral test did not spend more time investigating the female when she was interacting with a conspecific, $P=0.219$. Despite the significant increase in pair-bonded males' investigation behavior, there is considerable variability in the data with a small effect size (Fig. 5) when compared to the duration of control male's investigative behaviors. Due to these factors, there wasn't a pronounced difference in behavior between the control and pair-bonded male and the behavioral assay required modification.

A two-way ANOVA test on the neuronal data suggested that the C-FOS activation was not significantly different (Fig. 6) between the pair-bonded male and the control male and there was no significant difference in the C-FOS activation between the pair-bonded male and the control male in the different brain areas we investigated (BNST, LS, PVN, Amygdala), two-way ANOVA;

brain area F (3, 30) = 59.21; P<.0001; treatment F (1, 10) = 0.4889; P=0.500; interaction F (3, 19) = 0.6993; P= 0.564. Since our behavioral test was a pilot experiment, we wanted to look for any subtle changes in brain activation that could be further investigated in improved behavioral paradigms. To that effect, we also conducted paired t-tests on each of the brain areas between the control and experimental condition. Results from the t-test showed that there were no significant differences in C-FOS activation between the control and experimental conditions in the BNST (P=0.151), LS (P=0.561), Amygdala (P=0.752), and PVN (P=0.981).

Finally, on examination of the endocrine response, results from a paired t-test showed that there was no significant difference (Fig. 7) between the corticosterone levels of the pair-bonded male and the control male that were both investigating the compartment with pair-bonded or stranger female respectively interacting with a conspecific, P=0.729.

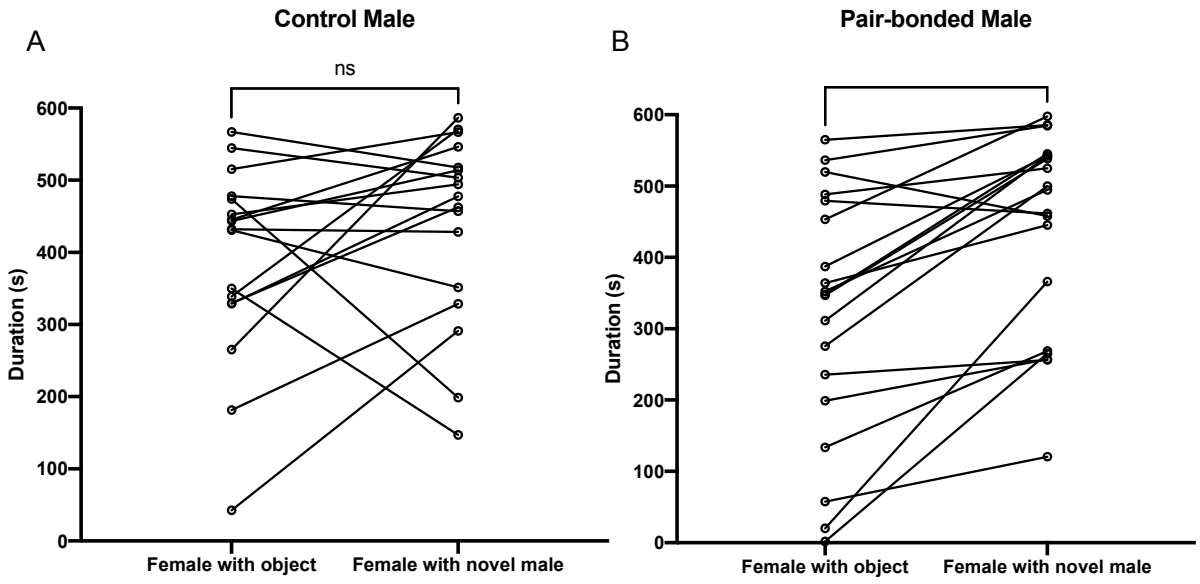
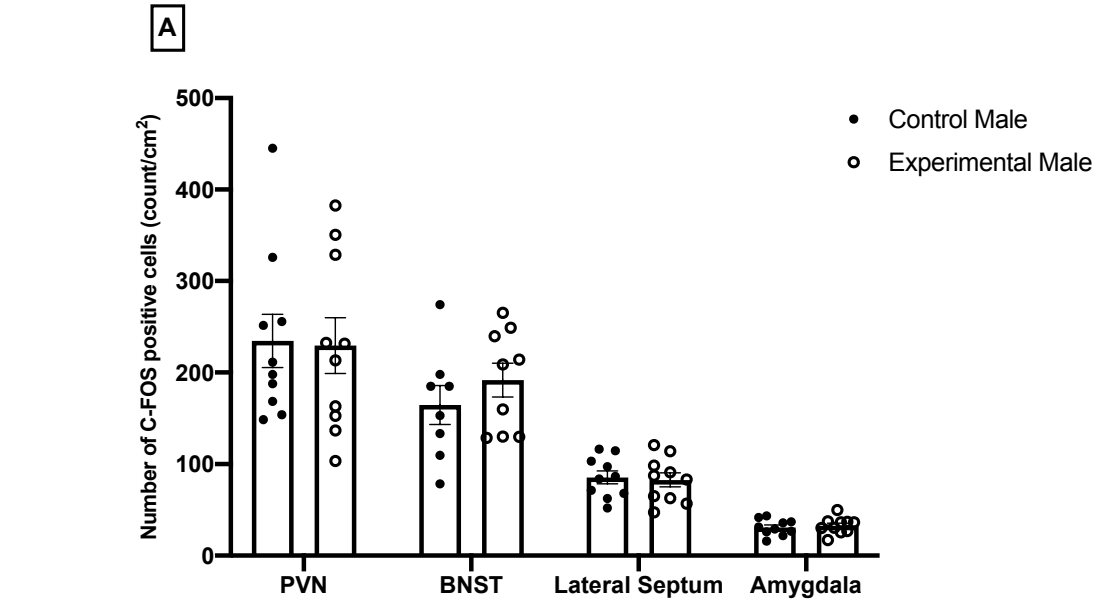
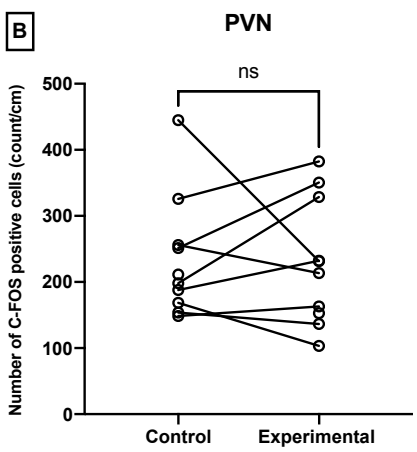
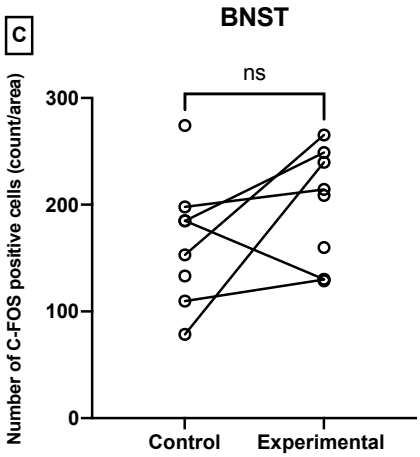
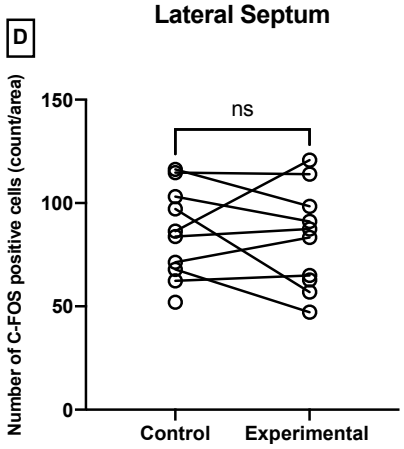


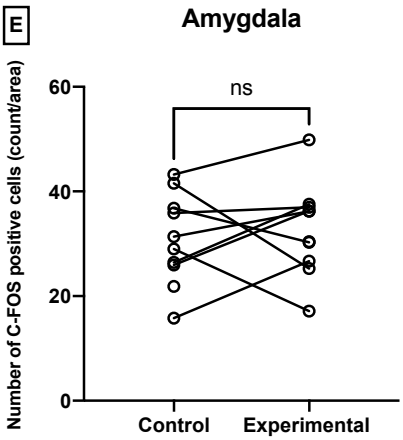
Figure 5: Increase in duration of investigative behaviors in the presence of female mate and a novel male. A: Male prairie voles (n=17) don't show any significant difference in time investigating the compartment with a novel sexually-receptive female and a novel male than one with a novel female and a novel object, P=0.219. **B:** Male prairie voles (n=19) spend more time investigating the compartment with their sexually-receptive, pair-bonded female mate and a novel male than one with their female mate and a novel object, *** P<.0001.


A
Number of C-FOS positive cells (count/cm2)
500
400
300
200
100
0
Control Male
Experimental Male
PVN
BNST
Lateral Septum
Amygdala

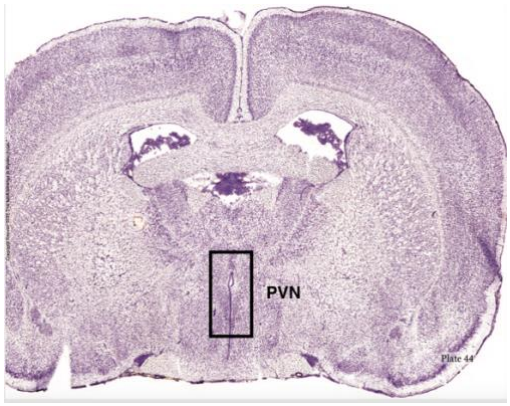

B
PVN
ns
Number of C-FOS positive cells (count/cm)
500
400
300
200
100
0
Control
Experimental


C
BNST
ns
Number of C-FOS positive cells (count/area)
300
200
100
0
Control
Experimental


D
Lateral Septum
ns
Number of C-FOS positive cells (count/area)
150
100
50
0
Control
Experimental


E
Amygdala
ns
Number of C-FOS positive cells (count/area)
60
40
20
0
Control
Experimental

F


PVN
Plate 44

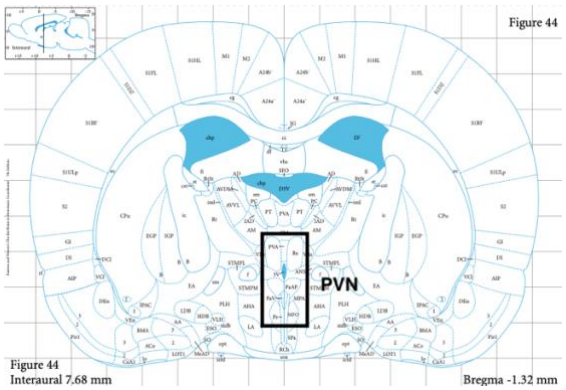
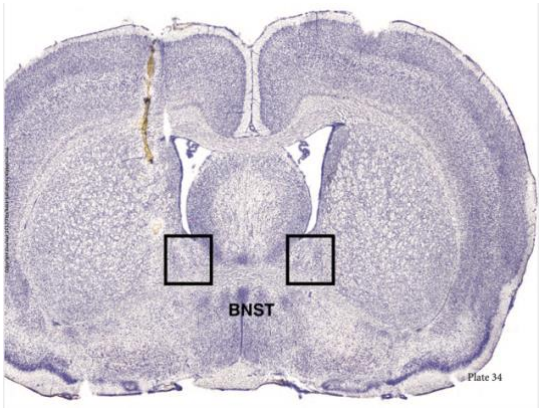


Figure 44
PVN
Figure 44
Interaural 7.68 mm
Bregma -1.32 mm


BNST
Plate 34

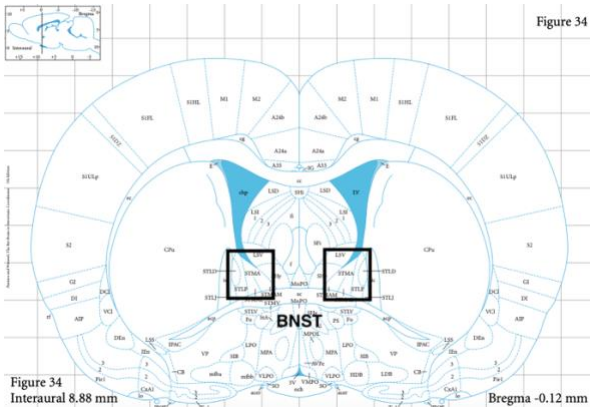
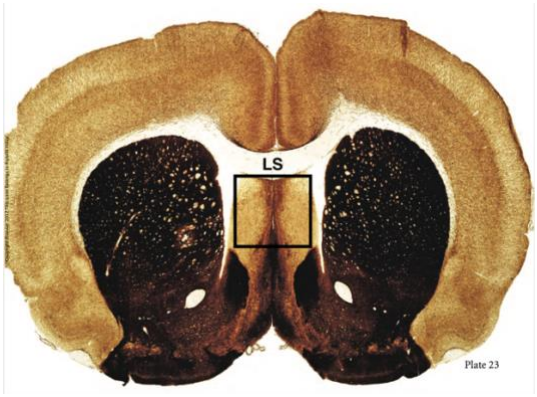


Figure 34
BNST
Figure 34
Interaural 8.88 mm
Bregma -0.12 mm


LS
Plate 23

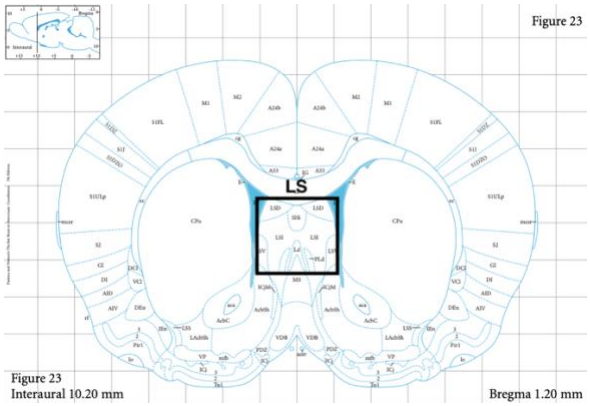
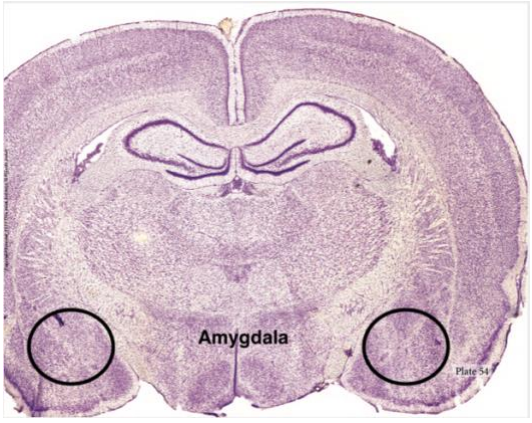


Figure 23
LS
Figure 23
Interaural 10.20 mm
Bregma 1.20 mm


Amygdala
Plate 54

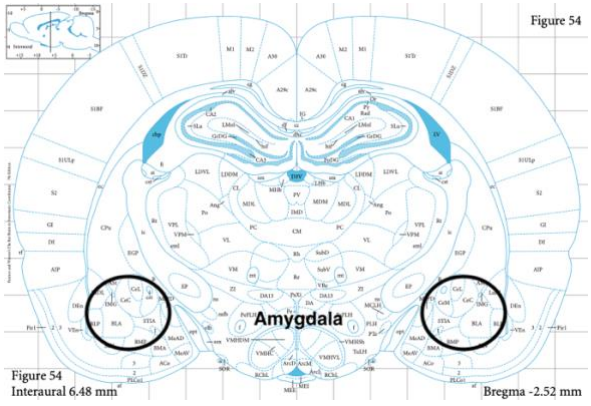


Figure 54
Amygdala
Figure 54
Interaural 6.48 mm
Bregma -2.52 mm

Figure 6: No difference in brain activation between the pair-bonded male and the control male. Male prairie voles that are pair-bonded to the sexually-receptive female interacting with a novel male do not show increased C-FOS activation in the BNST (P=0.151), lateral septum (P=0.561), Amygdala (P=0.752), and the PVN (P=0.981) compared to the control male that is not pair-bonded to the sexually-receptive female interacting with a novel male. In graphs C-F, for each of the brain areas, the lines connect the control and experimental male that were viewing the same compartment. Panel F shows the neuroanatomical positions (rectangular/circular frames) at which images were captured for Fos expression analysis in each of these regions using illustrations from The Rat Brain atlas (Paxinos and Watson, Seventh Edition).

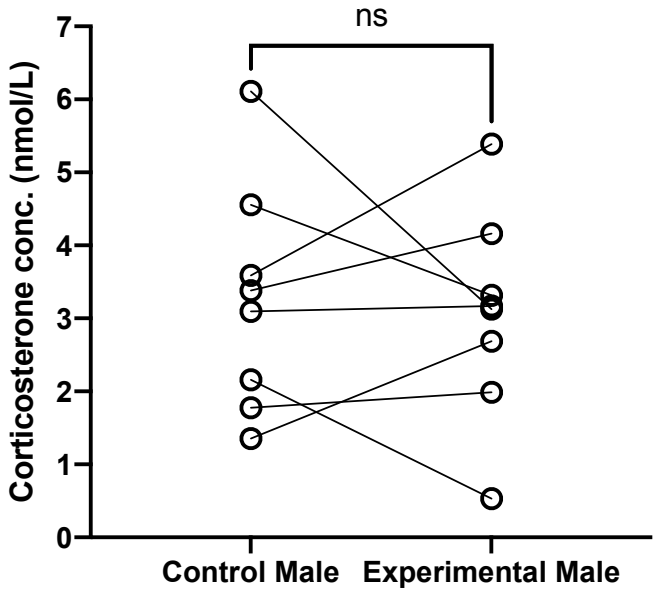


Figure 7: No difference in the endocrine responses of the pair-bonded male and the control male. Male prairie voles investigating the compartment with their sexually-receptive, pair-bonded female mate and a novel male don't show any significant difference in corticosterone

levels than the control male that is investigating the compartment with a novel sexually-receptive female and a novel male, P=0.729. The lines in the graph connect the control and experimental male that were viewing the same compartment.

Choice experiment

Results from the choice experiment showed that compared to the control male, the pair-bonded male does not have a greater preference towards investigating his female mate or the stranger female, P=0.176. In the following phase, when both the females (partner and stranger) are interacting with a conspecific, the pair-bonded male spends a significantly greater time investigating the stranger female with the novel male P=0.0319. During the habituation phase when the male is getting acclimated to the environment (Fig 8.), there no significant difference in the time spent investigating the two empty compartments (P=0.0892). However, we can see that there is a slight preference towards the stranger female's compartment which could have influenced the data in the following phases.

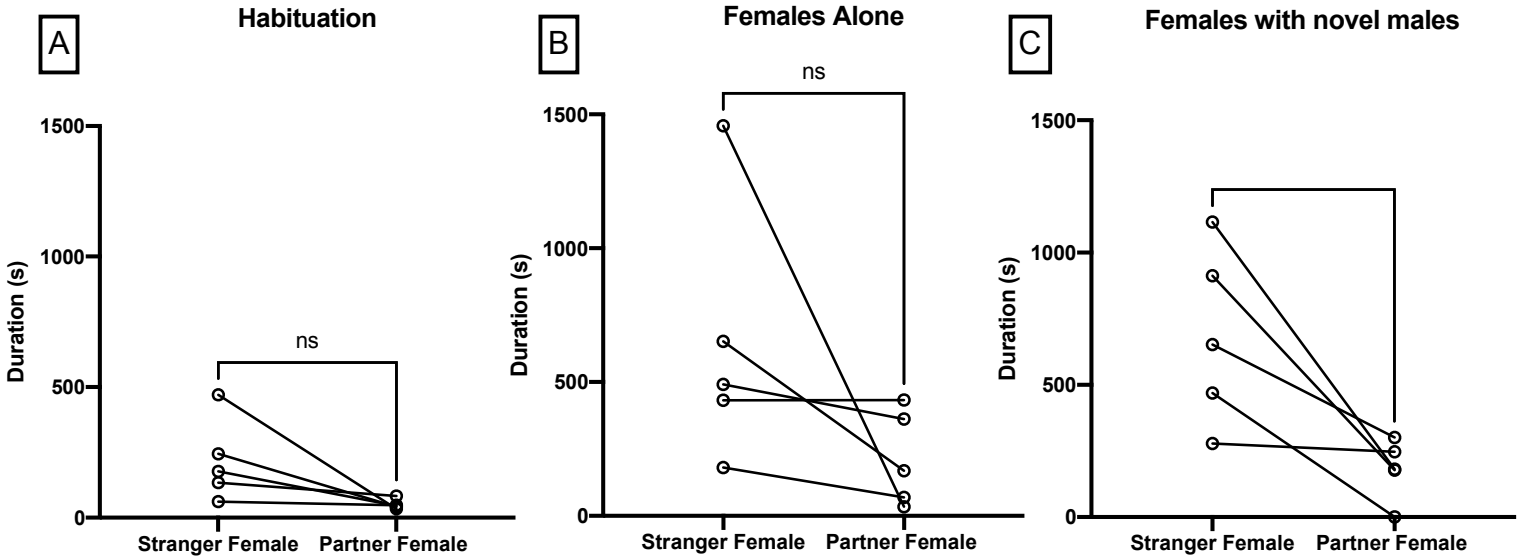


Figure 8: Increased duration of investigative behaviors towards a novel female and a novel male. A: There is no significant difference between the time the male partner spent investigating the two empty compartments during the habituation phase, P=0.0892. **B:** There is no significant difference between the time the male partner spent investigating his female partner and the stranger female after they were added to their respective compartments, P=0.176. **C:** The male partner spent a significantly greater time investigating the stranger female's compartment than his female mate's after a novel male was added to both the females' compartments, *P<0.05.

Discussion

The goal of this study was to characterize the impact of a threat to a pair bond on behavior, neuronal activation and endocrine response. To do so, we physically separated socially monogamous prairie voles and examined the effects of a social threat (a stranger male in contact with the female) to an exclusive bond in male prairie voles. The results of the behavioral test

from the pilot experiment demonstrate an increase in the time spent by the male partner investigating his female partner's compartment in the presence of a male conspecific compared to the condition where the female is with a novel object. This shows that in the presence of an unfamiliar intruder with the female mate, the pair-bonded male engages in a high degree of investigative behaviors such as sniffing and digging towards the compartment with the partner female and the intruder (Wang et al., 1997a; Gobrogge et al., 2007; Ahern et al., 2011; Young et al., 2011). After the formation of a pair bond, male voles display selective aggression towards unfamiliar conspecifics, which is important to maintain the bond between the mated prairie voles (Resendez et al., 2016). However, it is possible that the presence of an intruder conspecific near the female mate results in a social threat that elicits mate-guarding behavior; this can cause a higher degree of selective aggression towards the sexual competitor that is present with the female mate. In the same experiment, the control male also spends most of its time investigating the center chamber. We suspect that because the female was in estrous, it made it an enticing opportunity for the control male to investigate. Since the control male spent most of its time investigating the female, it wasn't possible for the experimental male to spend significantly more time investigating than the control male. Due to this ceiling effect, there was not a pronounced difference in behavior and the behavioral assay required modification.

The corticosterone levels between the partner male and the control male were not significantly different in the pilot experiment. The HPA axis in prairie voles is exceptionally active even under basal conditions; this makes their corticosterone levels much higher than those measured in other rodent species like rats (DeVries et al., 1996). Limited research has been done to identify

the physiological and behavioral responses to various stressors in prairie voles (Smith et al., 2013). Since both the control and experimental males were observing the same compartment (with the female mate and a novel male), the experience may have been provocative for both the males, which could have caused elevated corticosterone levels in both the control and experimental male. Furthermore, we were unable to measure the baseline corticosterone levels before the behavioral tests. This is because repeated blood collection from the vein in the tail is not possible as voles' tails are much smaller than tails in other rodents. Furthermore, the procedure of blood collection in voles can elevate corticosterone levels as well. It is possible that in our behavioral paradigm, corticosterone levels were not an appropriate measurement to determine the stress induced by the presence of a novel male with the female mate. The methods need to be refined such that baseline corticosterone levels can be accounted for as well.

The C-FOS activation was not significantly different between the control and experimental male in the pilot experiment. The C-FOS activation was not significantly different between the BNST, LS, BLA, and PVN. To accurately assess changes in brain activity induced by the social stressor, it is important to manipulate the control animals and minimize the differences in the experimental design such as familiarity with the experimenter or odors of other males (Kollack-Walker and Newman, 1995; Martinez et al., 2002). Therefore, it is possible that in our behavioral paradigm, the Fos immunohistochemistry is not able to differentiate between the neuronal activation caused by social stress in experimental males and stressors experienced by the control male who is also viewing the same compartment. Furthermore, the areas investigated in this study are sensitive to stress and show activity for aggressive behaviors. This suggests the possibility that

our paradigm induced some stress and aggression in the control male as well which resulted in C-FOS activation in the brain regions we investigated. Moreover, the results from the endocrine response also suggest that there was no significant difference between the control and experimental male's response to the social stressor. Some studies have suggested that there is an overlap between the brain areas that are active during a social stimulus and those that are involved in the response to stress (Martinez et al., 2002). This possibly explains why these brain areas can be active during a social stimulus in the control male or in response to a stressor in the experimental male which may not be differentiated by our immunohistochemical techniques. Not many studies have investigated the effects of social threat in pair-bonded species, so we examined whole areas rather than differentiating between subdivisions of those areas. This possibly could have masked subtle differences in brain activation in specific regions that we missed. More specific brain areas and subpopulations of neurons in those areas need to be compared in future studies.

In the pilot experiment, the behavioral paradigm was set such that both the experimental and control male were looking at the center compartment with the female and novel male. The pair-bonded male was viewing his female mate interact with a novel male, whereas the control male was viewing two novel voles interacting with each other. This could have resulted in different stressors for the control and experimental male. Therefore, when we designed the choice experiment, the behavioral paradigm was set such that the pair-bonded male had a choice between his female mate and a novel female. We expected a stronger effect in this paradigm as compared to the pilot experiment where the male had only one compartment to investigate. In

the choice experiment, the pair-bonded male had a much stronger preference for the stranger female's compartment than its female partner's compartment. The absence of a robust preference for the female mate suggests that the male may have been seeking a novel social stimulus over the familiar pair-bonded partner. This behavior is not commonly observed, and it has been shown that prairie voles prefer their partners over a novel female in partner preference tests after the formation of a pair bond (Williams et al., 1992). However, our paradigm does not allow for extensive physical contact between the experimental male and the females as the male is unable to access the females. Physical contact with the partner in the form of huddling is essential for displaying a preference towards the partner (Ahern et al., 2009); lack of physical contact can significantly affect the duration spent in the proximity of the partner (Beery et al., 2018). Furthermore, a combination of sociosensory cues such as visual, physical, and olfactory are important in the social recognition of the partner (Walum and Young, 2018). Therefore, it is possible that in our behavioral setup the male was unable to access his female mate and display preference. This could have resulted in a preference towards the novel female and a greater preference when two novel voles were present (stranger female with a novel male). Moreover, the duration of cohabitation prior to testing between the male-female mated pair was a week; this duration may be insufficient for the male to display intense aggression towards rejecting a potential new mate in estrous (Gobrogge et al., 2009; Young et al., 2011).

It is also noteworthy that although prairie voles are socially monogamous, male voles sometimes seek additional reproductive opportunities with other females (Ophir et al., 2008). When limited resources like receptive females are present, males can adopt alternative reproductive strategies

allowing them to maximize their own reproductive success. It is unclear which ecological and social pressures result in seeking EPC (Rice et al., 2018). The significant increase in the time spent investigating the stranger female with the novel male suggests another possibility that the pair-bonded male was seeking additional reproductive opportunities with the stranger female. A greater preference towards one compartment over the other during the habituation phase could also suggest a side bias for no apparent goal or reason, which is commonly observed in other rodent species such as mice (Treviño and Medina-Coss y León, 2020). This could also be a contributing factor in a greater preference in investigating the stranger female's compartment. Therefore, our data suggest that we need to run this test on more animals to rule out a side bias. The behavioral paradigm could also be modified to allow the male partner to clearly see his female mate and allow him to go in close proximity to his mate to effectively characterize social threat.

There are a few important limitations to our data. Firstly, the male's investigative behavior was characterized by the proximity of the male to the wall separating the male's compartment from the female's compartment. While proximity can tell us about the position of the male during a certain period, it does not necessarily tell us if the male was investigating the compartment or not. Furthermore, proximity also does not tell us about the type of behavior the male was displaying. Through this method, we were unable to measure the vigor with which the male was trying to approach the wall separating him from the females' compartments. Secondly due to the absence of a baseline measurement for corticosterone levels and FOS activity for the pair-bonded male in our choice experiments, we were unable to compare any physiological or neuronal data

between the female alone and female with a novel male conditions. Third, the absence of physical proximity or a visual component in our choice experiment could be a reason for the male's behavior. It is also possible that our paradigm was not able to elicit a social threat strong enough for the male to respond. Fourth, we need to look into more specific brain regions that have been implicated in social stress, mate-guarding behavior, and selective aggression such as the central nucleus of the amygdala which projects to different sites involved in mediating a stress response (Kalin et al., 2004), medioventral BNST which is implicated in stress-induced social vigilance, and the medial preoptic area which responds to acute stress (Zhang et al., 2021). Finally, partner preference tests need to be conducted after cohabitation and only those males that display a strong preference for their partners should be chosen for the behavioral tests; we would expect the effects of a social threat would depend on the strength of a pair bond. Future experiments might use a transparent screen instead of an opaque divider that would allow the male to see his female mate interacting with a novel male. Furthermore, we will examine more specific brain areas and subpopulation of neurons in those areas.

Despite these limitations, to our knowledge, these are the first data to explore these behavioral and neuronal changes in a socially monogamous rodent species. This study opens new avenues for studying the psychosocial impact of a social threat in a socially monogamous rodent. Our data from the pilot experiment shows differences in behavior in response to a social threat. Despite the small effect size, it is indicative of a subtle effect that needs to be further refined to observe robust corresponding changes in brain activation as well. Linking these robust behavioral changes as well as increased activation in brain areas associated with mate-induced aggression and

emotional processing is critical to our understanding of the ancient neurobiological processes that evolved to result in jealousy in humans.

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