The Role of Oxytocin and Vasopressin on Vocal Communication and Forming, Maintaining, and Breaking Social Bonds in the California Mouse (*Peromyscus californicus*)

By

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ABSTRACT

In social species, coordination of behavior is crucial for accomplishing complex tasks such as resource acquisition, territorial defense, and parental care. Decades of research has shown that the ability to acquire better food and territory and to provide higher quality parental care improves measures of ultimate fitness. More recently, research has examined the proximate mechanisms that facilitate the ability to perform coordinated social behavior. Having strong social bonds, such as parent-offspring bonds and monogamous pair bonds can facilitate increased coordinated social behavior. This dissertation focuses on two key aspects that help facilitate social bonds: auditory communication and neuropeptides. Using the territorial, monogamous, and biparental California mouse (*Peromyscus californicus*) as a model species this dissertation examines the role of auditory communication, oxytocin, and vasopressin on the formation, maintenance, and breakdown of social bonds. Compared to other model species, California mice have a better understood vocal repertoire which allows for pairing vocal communication with monogamous and biparental social bond behavior. The neuropeptides oxytocin and vasopressin have been shown to influence pair bonding, maternal and paternal care, and social recognition. However, vasopressin may also be involved in promoting anxiety and aggression. The oxytocin and vasopressin systems may play a significant role in rewiring the social brain by reinforcing social interactions as positive or negative. While most studies on oxytocin and vasopressin examine their role in affiliative social preferences, this dissertation examines how acute pulses of oxytocin and vasopressin influence different aspects of social bonds and family-unit coordination. During pre-bonding aggression tests, a medium dose of oxytocin reduces aggression in males. Low and medium doses of vasopressin do not change aggression

in either females or males, but high doses of vasopressin reduce aggression levels, similar to the effect of a medium dose of oxytocin (**Chapter 2**). During the formation of mother-offspring bonds, oxytocin increases maternal vocalizations and increases the efficiency of maternal care. However, for fathers, oxytocin only increases the efficiency of paternal care and does not influence vocalizations (**Chapter 3**). In order to maintain coordination among family units during a challenge, California mice use ultrasonic vocalizations to coordinate behavior and parental care strategy. Oxytocin tends to increase division of labor among parents, increasing the effort of mothers whereas vasopressin increases parent retrievals (**Chapter 4**). Parent-offspring bonds in male but not female juveniles may be maintained by acute pulses of OXT. Finally, there are pronounced differences in social receptivity levels of adolescent and adult California mice that are independent of acute oxytocin pulses. (**Chapter 5**). Collectively, these studies show that OXT may facilitate faster formation of pair bonds and parent-offspring bonds, alter division of labor during parental care tasks, and prevent the breakdown of male juvenile-parent bonds. Unlike OXT, IN AVP administration may inhibit female pair bond formation and does not seem to play an important role in either social bond maintenance or breakdown.

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THESIS OUTLINE

- **Chapter 1: Introduction**

- **Chapter 2:** The role of oxytocin and vasopressin in the formation of social bonds in California mice (*Peromyscus californicus*)
  Experiment 1: determine the role of vasopressin in pre-courtship aggression in both females and males
  Experiment 2: determine the role of oxytocin in pre-courtship aggression, resident-intruder aggression, and the formation of the father-offspring bond and communication
  Experiment 3: Characterize the formation of the mother-offspring bond and communication

- **Chapter 3:** The role of oxytocin in the maintenance of family-unit bonds and communication in California mice (*Peromyscus californicus*)
  Experiment 1: Characterize the vocalizations and parental care strategies of mother and father California mice during a pup separation challenge
  Experiment 2: Determine the effects of oxytocin and vasopressin on the vocalizations and parental care strategies of mother and father California mice during a pup separation challenge

- **Chapter 4:** The role of oxytocin and vasopressin in the breakdown of parent-offspring bonds and dispersal social behavior in California mice (*Peromyscus californicus*)
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INTRODUCTION

Chapter 1: The formation, maintenance, and breakdown of social bonds in California mice (*Peromyscus californicus*)

The formation of social bonds

In social species, the formation of social bonds is a critical step in establishing membership in a social group. Membership in a social group has many benefits including increased protection from predation (Villafuerte & Moreno, 1997; Wrona & Dixon, 1991), food sharing (Carter & Wilkinson, 2016; Jaeggi & Gurven, 2013), warmth (Russo et al., 2017), social learning (Ashton et al., 2019), and help rearing offspring—both in the form of alloparental care (Riedman, 1982) and care from a monogamous and/or biparental partner (Gubernick & Alberts, 1987; Brown et al., 2010). While many studies in the field have shown the ultimate fitness benefits to social bonding, the proximate mechanisms that facilitate the formation of these social bonds is less well understood.

The neuropeptide hormones oxytocin (OXT) and vasopressin (AVP) are two widely conserved molecules that are involved in many social behaviors including pair bonding (Cushing & Carter, 2000; Liu & Wang, 2003), territorial aggression (Gutzler et al., 2010; Wersinger et al., 2002; Beiderbeck et al., 2007), social memory (Lukas et al., 2013; Todeschin et al., 2009), and parental care (Guoynes & Marler, 2020; Pedersen & Prange, 1979; Keverne & Kendrick, 1992; Feldman et al., 2007; Marlin et al., 2016). These two neuropeptides may play an important role in facilitating the formation, maintenance, and/or breakdown of social bonds. Structurally, OXT and AVP have many similarities. Both OXT and AVP have three exons that are spliced into a preprohormone. This preprohormone is then cleaved into either OXT or AVP and neurophysin-I or neurophysin-II, respectively (Ivell & Richter, 1984). OXT and AVP ligands have many molecular similarities, starting with their 9-amino-acid sequences. The sequence for OXT is H_2N-Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6-Pro^7-Leu^8-Gly^9-NH_2, and the sequence for AVP is H_2N-Cys^1-Tyr^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-Gly^9-NH_2,

differing from OXT by only two amino acids at position 3 and 8 (Turner et al., 1951; Tuppy, 1953). While OXT and AVP have the same ring and tail shape, their amino acid residues differ at important binding sites that have high affinity for their respective receptors but can also bind to each other's receptors with relatively high affinity (Vigneaud et al., 1953; Akerlund et al., 1999). These differences form the basis for the different function and regulation of the two peptides and are the reason that OXT and AVP can sometimes have opposite behavioral effects (Ivell & Richter, 1984).

In this series of experiments, we focus on the formation of monogamous bonds and parent-offspring bonds in California mice. Both of these social bond types lead to long-term, stable bonds (Gubernick & Nordby, 1993; Ribble, 2003; Gleason et al., 2012, Kendrick, 2004) and have clear ultimate fitness benefits (Gubernick et al., 1993). Previous research in prairie vole females has shown that OXT administration can potently facilitate pair bonding, but that AVP does not impact female pair bond formation (Insel & Hulihan, 1995; Liu & Wang, 2003). In contrast to females, OXT in males does not, but AVP does facilitate partner preferences (Winslow et al., 1993; Liu et al., 2001; Lim & Young, 2004). Furthermore, viral vector transfer of the V1aR from the monogamous prairie vole into the ventral forebrain or ventral pallidum (VP) of a promiscuous species, the meadow vole, causes male partner preferences in meadow voles (Pitkow et al., 2001; Lim et al., 2004). This suggests a strong sex by ligand interaction in pair bonding behavior. These studies highlight how OXT and AVP can have potent but sex-specific effects on pair bonding.

One gap remaining in the study of OXT and AVP on pair bonding is how an acute pulse of these neuropeptides affects immediate behavior interactions. In the field, a pulse of OXT or AVP may be important to facilitate appropriate social behavior and an interaction that lasts long enough for cohabitation. For example, if aggression is too

high at the first encounter, the animals may part ways and not have the opportunity to pair bond. To address this gap, our studies focus on the very early time points of courtship—when the mice have to overcome neophobia and aggression to socially interact. We also compare the effects of an acute pulse of AVP versus OXT influence these interactions in experiments 1 and 2. We compare and contrast the effects of OXT and AVP on the formation of partner bonds because many brain regions important for bonding behavior, social communication, and parental behavior show differences in OXTR and V1aR expression (Mitre et al., 2016). In the context of new social partners, we hypothesize that, like in prairie voles, OXT and AVP may have different effects on courtship behavior. We predict that an acute pulse of AVP will increase aggression due to the association between AVP and aggression in California mice (Bester-Meredith & Marler, 2001; Bester-Meredith et al., 1999; Bester-Meredith et al., 2005). Based on studies showing a negative relationship between OXT and aggression (Kozhemyakina et al., 2020; Harmon et al., 2002; Winslow & Insel, 2002; Witt et al., 1990), we predict that OXT will decrease aggression during the pre-courtship aggression phase of bonding.

In addition to studying the effects of AVP and OXT on the formation of socio-sexual bonds, we also wanted to explore the effect of OXT on the early formation of parent-offspring bonds. These parent-offspring bonds are critical for offspring survival in these altricial species. Mammalian mothers often provide a critical baseline amount of parental care that includes lactation/feeding, warmth, and protection from threats (Strathearn, 2011; Curley & Champagne, 2016; Kohl et al., 2017). However, in monogamous and biparental mammalian species, fathers often contribute to most of the variable care (Perkeybile et al., 2013). This means that when parental dyads are characterized as "high" or low" on parental care scores, the care from the father is the most significant factor. In California mice specifically, having the father around

dramatically increases the number of offspring that emerge from the nest in the field, from 0.5 without the father to 1.5 with the presence of the father (Gubernick & Teferi, 2000). There is mounting evidence (described below) that OXT is involved in both maternal and paternal care, suggesting that this neuropeptide may be an important neurobiological mechanism involved in promoting the formation of parent-offspring bonds.

In mothers, the OXT system has been shown to have significant effects on both physiology and behavior. Throughout the reproductive cycle, uterine tissue progressively becomes more sensitive to OXT throughout pregnancy, was highest during parturition, and decreased after birth and throughout lactation in both human and non-human animals (Robson, 1934; Bell, 1941; Fuchs & Saito, 1971; Takahashi et al., 1980; Higuchi et al., 1985). This suggested that there were changes in the amount of oxytocin ligand being produced, the number of oxytocin receptors expressed, or both throughout pregnancy. Additionally, early studies showed that synthetic oxytocin injected peripherally was sufficient to induce parturition in pregnant females (Colucci, 1954; Douglas et al., 1955; Mayes & Shearman, 1956). Oxytocin was so potently effective at inducing labor that it continues to be used today to augment the labor process, albeit not without potential side effects for offspring (Mercer et al., 1991; Weisman et al., 2015).

In addition to the physiological effects of OXT on mothers, there are many behavioral effects of OXT in mothers. One study compared the effect of oxytocin to vasopressin, prostaglandins F2 and E2, progesterone on the initiation of maternal care. These studies showed that only oxytocin could induce rapid, persistent maternal care. Prostaglandin F2 could induce rapid but not persistent maternal care and vasopressin could induce persistent maternal care but did so with a longer latency to initiate the

care (Pedersen et al., 1982; Rosenblatt & Siegel, 1981). To test whether oxytocin was necessary for maternal behavior, Fahrbach et al. gave intracerebroventricular (icv) antisera or oxytocin antagonist injections to estrogen-primed virgin rats (1985). Their study found that compared to virgin rats given oxytocin, both the antisera and antagonist blocked the onset of spontaneous maternal behavior. Oxytocin antagonists administered via icv were also sufficient to block maternal behavior after natural delivery in pregnant rats (Van Leengoed et al., 1987). Additionally, lesions of the PVN disrupted the onset of maternal behavior but not the maintenance (Insel & Harbough, 1989; Insel, 1992). Later studies examined the behavioral phenotype of oxytocin null mice compared to wildtype. Oxytocin null mice did not show deficits in parturition but had longer latencies to retrieve pups and spent less time crouching over their pups (Takayanagi et al., 2005). Combined, all of these studies showed strong evidence that oxytocin was both sufficient and necessary for initiation of maternal behavior. However, no studies to date have explored how an acute pulse of OXT immediately influences maternal behavior and communication.

In fathers, there is also evidence that OXT plays a role in the development of father-offspring bonds. In mandarin voles, fathers have higher OXT serum concentrations than virgin males (Yuan et al., 2019). In prairie voles, fathers have more OXT innervation in the hypothalamus and regions of the brain associated with regulation of the heart than nonfathers but had less OXT in the BNST (Kenkel et al., 2014). Similarly, California mice fathers showed less OXTR mRNA in the BNST than nonfathers (Perea-Rodriguez et al., 2015). Furthermore, mandarin vole fathers showed greater OXTR expression than virgins in the mPOA, a brain area associated with paternal behavior (Yuan et al., 2019). In nonhuman primates, OXT may facilitate paternal care in males with sexual experience. In marmosets, fathers have greater OXT

in the hypothalamus than nonfathers (Woller et al., 2012). There is mounting evidence that OXT also plays a positive role in paternal care for human fathers. Fathers given OXT had increased BOLD activation in the caudate nucleus, anterior cingulate cortex, and visual cortex when seeing images of their child (Li et al., 2017). This suggests OXT may promote paternal attention toward their own child. Taken together, these studies suggest that OXT may be influencing the paternal state transition but that the brain region most sensitive to OXT changes may vary by species in some cases. Studies in mandarin voles have also examined how OXT changes between new and experienced fathers. New fathers had higher OXTR in NAcc compared to experienced fathers and OXTR receptor levels in the NAcc of both groups decreased as pups got older (Wang et al., 2018). Additional changes were also observed in the OXTR expression in the MeA; new fathers showed decreases in OXTR expression while experienced fathers showed increased expression (Wang et al., 2018). These studies suggest that there may also be activational roles for OXT in fatherhood that differ with offspring needs and parental experience, however, again, neuropeptide hormone manipulations are needed to establish cause and effect. One goal in this dissertation is to fill this gap by administering OXT to first-time fathers and measuring how it affects paternal care and communication toward offspring.

OXT also plays a role in the production and perception of vocal communication. In mice and other rodents, the majority of their vocalizations occur above 50 kHZ and are called ultrasonic vocalizations (USVs). In OXT knockout mice, OXT null pups emit fewer USVs in response to separation from their mother compared to wildtype mice (Winslow & Insel, 2002). This suggests that OXT may be promoting pups to communicate with their parent. There is also evidence that OXT improves the signal-to-noise ratio in mothers responding to pup calls. OXT mediated temporal inhibition and

excitation in the left auditory cortex of female mice and led to increased pup retrievals (Marlin et al., 2015). This suggests that OXT is changing the perception and social salience of pup calls and leads to increases in maternal care. Furthermore, in humans, more efficient OXT receptor genotypes are better able to discriminate content of language under noisy conditions than less efficient OXT receptor genotypes (Tops et al., 2011). This suggests that across taxa, OXT may play an important neuromodulatory role in promoting sensation, understanding, and behavioral response to social calls.

To test the effect of OXT on the early parent-offspring bond, we focused on the mother-pup dyad and father-pup dyad in first-time mothers and fathers. We chose to look at the mother-pup and father-pup dyad separately in order to separate any effects of having the second parent around to help with labor and so that we could determine whether vocalizations were coming from the father or the mother.

In summary, **our goal is to determine the role of neuropeptides in the formation of two types of family-unit bonds: monogamous pair bonds and parent-offspring bonds.** Our studies in the formation of monogamous pair bonds aimed to assess whether OXT and AVP are involved in overcoming initial aggression toward a potential mate. To complement this, our studies on the formation of parent-offspring bonds aimed to assess how parental USVs, pup USVs, and parental behavior relate to one another, and to determine if OXT moderates these relationships.

The maintenance of social bonds

Once social bonds have been established, they must then be maintained by sustained social interactions. This maintenance can occur simply by cohabitating the same environment (Cushing et al., 2003) or via more complex behaviors such as reciprocity (Carter & Wilkinson, 2013; Freidin et al., 2017), food sharing (Shibata, 2008;

Silk et al., 2013; Yamamoto et al., 2017), cooperative hunting (Samuni et al., 2018; Boesch, 2005), joint territorial defense (Rieger & Marler, 2018), and cooperative alloparental and parental care (Bales et al., 2007; da Silva Mota et al., 2006; McGraw et al., 2010). In many of these cases, release of substrates during social interactions reinforces the relationship. For example, in prairie voles, cohabitation with a member of the opposite sex often results in mating and release of dopamine (Gingrich et al., 2000). This release dopamine is reinforcing the social bonds and is necessary for partner preferences in prairie voles (Aragona et al., 2003). There is also evidence that activation of the OXT and AVP systems is involved in maintaining social bonds (Bosch & Young, 2017; Nair & Young, 2006). In vampire bats, intranasal OXT increased food sharing and social grooming (Carter & Wilkinson, 2015). In human-dog interactions, OXT increases eye-gaze behavior toward a caretaker (Nagasawa et al., 2017; Nagasawa et al., 2015) and increases bias toward positive human facial expressions (Kis et al., 2017; Somppi et al., 2017). In monogamous marmosets, OXT regulates reunion behavior with a mate after a separation challenge (Cavanaugh et al., 2018). Also in marmosets, AVP increases response to infant stimuli (Taylor & French, 2015; Taylor et al., 2020). These studies suggest that release of OXT and AVP during social interactions may be an important neurobiological mechanism that reinforces social bonds and cooperative behavior.

In monogamous species, mothers and fathers must learn to coordinate their behavior to maximize efficiency and having a strong social bond and communication is likely to be key. During stressful events, such as a disruption in the nest or a displacement from the nest, it is crucial for pairs to be able to be able to gather, protect, and thermoregulate their pups. As oxytocin and vasopressin are both highly involved in parental behavior, it is likely that they are involved in promoting communication and social behavior in family groups (Guoynes & Marler, 2021; Guoynes & Marler, 2020;

Bales et al., 2004; Francis et al., 2002). In the wild, California mice must adapt to nest disruptions that may occur from predators such as hawks, owls, and rattlesnakes and wildfires that are relatively common occurrences in their habitat in California. Previous studies in the lab have examined the maintenance of social bonds as they occur during division of labor tasks with predation and intruder risks (Rieger et al., 2018; Rieger et al., 2019; Monari et al., 2021). In this context, pairs can adopt one of three strategies: both approach the intruder, one approaches the intruder, or neither approach the intruder. In this context, OXT promotes pairs to choose the same strategy rather than divide labor (Monari et al., 2021). However, it is not known how OXT and AVP will influence parental care and parental care strategy. In this dissertation, maintenance of the family unit bonds (parent-parent and parent-offspring) is tested in the context of a pup separation challenge. **The goal in these experiments is 1) to determine if there is an association between parental care strategy and vocalizations and 2) to determine whether an acute pulse of OXT or AVP influences how parents coordinate behavior to respond to a parental care challenge**.

The breakdown of social bonds

There are many instances where social bonds naturally break down. Bonds may break down in order to facilitate new mating opportunities, to establish sole residency of a territory or resource, or to prioritize one social relationship over another that has greater fitness gains. In hyenas, dominant mothers determine the social rank of offspring. Mothers always prefer the youngest litter, breaking social bonds with older offspring and attacking them if they start fights with the youngest litter (Holekamp & Smale, 1998; Silk, 2019). In some animals, the breakdown of these social bonds happens seasonally. For example, during short winter days meadow voles are socially tolerant

and live in groups; however, as days get longer, the voles establish their own territory and become aggressive toward former group mates (Beery et al., 2014). Another type of bond breaking down is offspring dispersal. Most studies examine dispersal from a physiological standpoint—having enough nutrition (Massot & Clobert, 1995; Ferrer, 1992), becoming reproductively viable (Wolff, 1992; Gaines & McClenaghan Jr., 1980), or having physical protection due to the presence of a parent (Ekman & Griesser, 2002; Stanton et al., 2020). However, there are likely significant changes happening in the social-behavioral neural network of parents and offspring during dispersal.

As in the formation of maintenance of social bonds, there is evidence for the role of the OXT and AVP systems in the breakdown of social bonds as well. Juvenile and adult expression of OXTR and V1aR shows important differences in key areas of the social behavior neural network. For example, juveniles have higher OXTR and V1aR expression in the olfactory bulb and hippocampus. Adults have higher OXTR expression in the prefrontal cortex, medial amygdala, bed nucleus of the stria terminalis, preoptic area, and ventromedial hypothalamus and higher V1aR expression in the prefrontal cortex, nucleus accumbens, and ventral pallidum (Smith et al., 2017). Lastly, and of particular interest, is the switch from dominance of OXTR in the lateral septum of juveniles to the dominance of V1aR in adults. It is possible that sex steroid changes at puberty influence the plexus of OXT and AVP fibers in the lateral septum and signal the switch from dominance of OXTRs to dominance of V1aRs (Smith et al., 2017). Overall, many of the brain areas mentioned above are important for affiliative behavior, aggressive behavior, motivation, social memory, and reproductive behavior and may provide the molecular basis for differences in juvenile and adult behavior (Caldwell, 2017; Shughrue et al., 1997; Newman, 1999; Shamay-Tsoory & Abu-Akel, 2016; Caldwell & Albers, 2016). However, it is not known which changes from the

juvenile period to adulthood change family-unit social behavior and the breakdown of certain social bonds.

In the context of dispersal and the breaking of parent-offspring bonds, we hypothesize that acute pulses of OXT or AVP will influence social preference for spending time with family unit versus novel social stimuli. In this dissertation, the breakdown of family-unit bonds is tested in the context of parent vs peer preference test and resident-intruder paradigm in sexually inexperienced adolescents and adults. **The goal in these experiments is to determine 1) if OXT or AVP influence the breakdown of parent-juvenile social bonds and 2) if OXT influences peer social behavior after dispersal**.

Summary

The formation, maintenance and breakdown of social bonds are critical for ultimate fitness benefits and require neural substrates to reinforce social behaviors. In this dissertation, my goal is to determine the role of the OXT and AVP systems in supporting the formation, maintenance, and breakdown of social bonds in the monogamous and biparental California mouse. Previous research suggests that the OXT and AVP systems are important for social bonding in mammals. **The goals of this dissertation are to 1) further characterize in the influence of the OXT and AVP on social bonds by looking at previously unexplored time points and 2) to add depth to our understanding social bonds by measuring the effect of OXT and AVP on communication via ultrasonic vocalizations.**

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Chapter 2: The role of oxytocin and vasopressin in the formation of social bonds in California mice (*Peromyscus californicus*)

Experiment 1: Determine the role of vasopressin in pre-courtship aggression in both females and males

Experiment 2: Determine the role of oxytocin in pre-courtship aggression, resident-intruder aggression, and the formation of the father-offspring bond and communication

Experiment 3: Characterize the formation of the mother-offspring bond and communication

ABSTRACT

Oxytocin (OXT) and vasopressin (AVP) are two neuropeptides known for their potent role in social behavior. However, many of the studies that examine the role of OXT and AVP in social bonds do not examine early time points in the formation of social bonds, such as pre-courtship behavior and early parental care behavior. Early courtship behavior may facilitate monogamous pair bonding and early parent-offspring communication and care may increase offspring survival. As such, both of these behaviors are critical in the formation of familial bonds. In these series of experiments, we test the effects of an acute pulse of OXT or AVP on pre-courtship aggression and early parental care behavior. During pre-courtship aggression tests, we hypothesize that an acute pulse of AVP will increase aggression whereas an acute pulse of OXT will decrease aggression in California mice. During father-pup and mother-pup care tests, we hypothesize that an acute pulse of OXT will increase parental care and communication. In **experiment 1**, we tested our hypothesis on the effects of AVP on pre-courtship aggression in females and males using a dose-response study. We found that AVP increased approach and aggression in females at all doses but did not significantly influence approach or aggression in males. In **experiment 2**, we tested our hypothesis on the effects of OXT on pre-courtship aggression in males. We found that OXT decreased the proportion of wrestling aggression in the first ten minutes of the pre-courtship aggression test but did not influence aggression in a resident-intruder test. In **experiments 2 and 3,** we tested for rapid effects of OXT in California mouse mothers and fathers. In **experiment 2,** we tested fathers by administering an acute intranasal (IN) dose of OXT (0.8 IU/kg) or saline to first-time fathers at postnatal day 2-4 and challenged them with a separation test. Fathers had previously been tested in a pre-courtship aggression test four to six weeks prior and a resident-intruder test two to four weeks prior. In this paternal care test, we only recorded the separation and reunion phases. As in the first experiment with mothers only, we primarily observed simple sweep USVs in this second experiment with fathers only, and we only observed pup whines form the pups. Upon reunion, IN OXT males were quicker to approach their pups than control males but did not differ in vocalizations produced. In **experiment 3**, we tested mothers by administering an acute intranasal (IN) dose of OXT (0.8 IU/kg) or saline to first-time mothers at postnatal day 2-4 and challenged them with a separation test with three phases: habituation with pups in a new testing chamber, separation via a wire mesh, and finally reunion with pups. We measured maternal care, maternal USVs, and pup USVs. In mothers, we primarily observed simple sweep USVs, a short downward sweeping call around 50 kHz, and in pups we only observed pup whines, a long call with multiple harmonics ranging from 20 kHz to 50 kHz. We found that IN OXT rapidly and selectively enhanced the normal increase in maternal simple sweep USVs when mothers had physical access to pups (habituation and reunion), but not when mothers were physically separated from pups. Frequency of mothers' and pups' USVs were correlated upon reunion, but IN OXT did not influence this correlation. Finally, mothers given IN OXT showed more efficient pup retrieval/carrying and greater total maternal care upon reunion. Behavioral changes were specific to maternal behaviors (e.g. retrievals) as mothers given IN OXT did not differ from controls in stress-related behaviors (e.g. freezing). unlike in the first experiment with mothers. Overall, these findings highlight the rapid effects and context-dependent effect a single treatment with IN OXT has on both maternal USV production and offspring care.

What Is New:

- During a pre-courtship aggression test, intranasal AVP increases social approach and aggression in females at low, medium, and high doses but showed a nonsignificant trend for decreased aggression in males at high doses
- During a pre-courtship aggression test, intranasal OXT decreases escalation to wrestling aggression but does not influence aggression toward an intruder in a resident-intruder aggression test
- Measured correlations between maternal care and both maternal and pup vocalizations, and shows that vocalizations are functionally correlated with maternal care
- IN OXT rapidly and selectively enhanced the normal increase in maternal simple sweep USVs when mothers had physical access to pups
- IN OXT increased maternal pup retrieval/carrying efficiency and greater total maternal care during a maternal care challenge in first-time mothers
- IN OXT increased paternal responsiveness during a paternal care challenge in first-time fathers but does not influence total paternal care or vocalizations as it does in mothers

Experiment 1: Determine the role of vasopressin in pre-courtship aggression in both females and males

Experiment 1: INTRODUCTION

The formation of monogamous bonds often starts with a period of courtship whereby territorial and aggressive species must reduce their aggression in order to investigate and interact with a potential mate. While the ability to reduce aggression is crucial for pair bond formation, it is not known what neurobiological mechanisms may facilitate this change in social behavior toward a prospective mate.

Vasopressin (AVP) is a neuropeptide known for its role in modulating social behaviors such as aggression (Delville et al., 1996; Stribley & Carter, 1999; Terranova et al., 2017) and affiliation (Winslow et al., 1993; Pitkow et al., 2001; Carter, 2017). Thus, AVP is one candidate neuromodulator that could influence the ability for animals to overcome aggression and form pair bonds. Structurally, AVP ligand shares many similar properties to another nonapeptide known for its role in social behavior, oxytocin (OXT). OXT and AVP have the same ring and tail shape, but where their amino acid residues differ are important binding sites that have high affinity for their respective receptors (du Vigneaud et al., 1953; Akerlund et al., 1999). These differences form the basis for the different function and regulation of the two peptides (Ivell & Richter, 1984). However, cross-reactivity OXT and AVP receptor activation and can make it difficult to determine if observed behavioral effects result from activation of the AVP system, OXT system, or both.

To answer this question, dose-response curves paired with behavior can be used. AVP binds to its receptors, V1aR, V1bR, and V2 with affinities of K(i)=1.4nmol/L, 0.8nmol/L, and 4.3nmol/L, respectively. OXT binds to its receptor, OXTR, with

K(i)=6.8nmol/L affinity. AVP can bind to OXTR, but OXT can only bind to V1aR with reasonable affinity (Akerlund et al., 1999). Experimental studies showed that in order for AVP to show the same binding affinity for OTR as OT, the AVP concentration had to be increased 100-fold (Kimura et al., 1994). This is supported by studies that suggest very high doses of OXT are more behaviorally similar to saline—possibly because of binding to V1aR (vasopressin) receptors (Benelli et al., 1995; Bales et al., 2013; Cardoso et al., 2013). These data provide a compelling reason to conduct a dose-response curve when administering IN AVP.

In male prairie voles, activation of AVP receptors is necessary to form a partner preference, the gold standard test of pair bonding in rodents (Lim & Young, 2004). While activation of the AVP is necessary in male prairie voles, it is not in female prairie voles (Nair & Young 2006). However, in other social contexts, activation of the AVP can also increase aggression. For example, increased AVP in the anterior hypothalamus increases aggression in Syrian hamsters (Caldwell & Albers, 2004), and increased AVP in the central amygdala increases aggression in lactating rats (Bosch & Neumann, 2010). The role of AVP in the context of early pre-courtship aggression has not been elucidated and could either promote affiliation as it does in prairie voles or could promote aggression as it does in hamsters and rats. Based on previous studies in California mice linking vasopressin with aggression (Bester-Meredith & Marler, 2001; Bester-Meredith et al., 1999; Bester-Meredith et al., 2005; Frazier et al., 2006), we predict that an acute pulse of AVP will increase aggression during the pre-courtship aggression phase of bond formation in females and males. However, due to possible cross-reactivity with OXTR binding at high doses of AVP, we predict that the high doses of AVP will be more similar to saline control due to activation of OXT receptors.

There are two main goals of this experiment: 1) to determine if an acute pulse of AVP influences pre-courtship aggression in females and males and 2) to establish a dose-response curve using IN AVP.

Experiment 1: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We used 61 female and 58 male *P. californicus* aged 5–10 months. They were group-housed (2–3 per cage; 48 × 27 × 16 cm) under a 14L: 10D light cycle with lights off at 4:00pm. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Mice were randomly assigned to the saline control group (female: N=15; male: N=15), 0.05 IU/kg AVP group (female: N=16; male: N=14), 0.5 IU/kg AVP group (female: N=15; male: N=16), or 5.0 IU/kg AVP group (female: N=15; male: N=13). For pre-courtship aggression testing, opposite-sex mates unrelated by at least two generations were randomly assigned to the focal test mice, and stimulus mice were not re-used.

Intranasal Vasopressin Preparation

Mice were infused intranasally with either sterile saline, 0.05 IU/kg AVP (low dose), 0.5 IU/kg AVP (medium dose), or 5.0 IU/kg AVP (high dose). The IN AVP dose is equivalent to doses used in other animal models (Simmons et al., 2017; Jarcho et al., 2011) and similar to weight-adjusted doses used in clinical studies examining the effects of IN AVP on social behavior (Price et al., 2017; Feng et al., 2020). IN AVP was dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20^{o}C. IN AVP was defrosted just prior to administration. A blunt cannula

needle (33-gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was attached to cannula tubing, flushed, and filled with the compound, then attached to an airtight Hamilton syringe (Bachem, Torrance, California). The animal was scruffed and 25 uL of compound was expelled dropwise through the cannula needle and allowed to absorb into the nasal mucosa (~10-20 seconds).

Behavioral Testing

Throughout the experiment, researchers administering treatments and handling animals were blind to treatment condition. For each test, the same researcher administered all intranasal treatments to reduce variance across administration.

Female and male California mice aged 5-10 months were removed from their home cage (48 × 27 × 16 cm) and were randomly assigned to 25 uL of 0.05 IU/kg AVP, 0.5 IU/kg AVP, 5.0 IU/kg AVP or saline. Immediately after treatment, each mouse was placed in a new home cage (48 × 27 × 16 cm) with fresh bedding. 5-min after the dose of AVP or saline, a novel, unrelated opposite-sex conspecific was placed into the new home cage at the opposite end from the focal test mouse. Their interaction was videotaped for 20-min and behavior was quantified from the videotaped behavior.

Behavior Quantification

All behavior videos were scored twice: once each by two independent observers blind to treatment and in a random order. Scores between observers had to be at least 85% similar and scores between the two observers were averaged for the final output used in statistical analysis. Three main behaviors were measured: number of approaches toward the potential mate, time spent aggressing toward potential mate (a

score that combined lunging, chasing, and wrestling aggression), and time spent allogrooming potential mate, an affiliative behavior.

Data Analysis

For the pre-courtship aggression test, one-way ANOVA tests were conducted to compare the outcomes between saline control and the three AVP treatments. Using GraphPad Prism, pairwise comparisons were also used to determine if there were any differences between the control group and each AVP treatment. Statistical tests for females and males were conducted separately. Data were checked for Grubb's outliers, and this led to removing one medium dose male's aggression score. Final group sizes analyzed for the pre-courtship aggression test were as follows: saline control female, N=15; 0.05 IU/kg AVP female, N=16; 0.5 IU/kg AVP female, N=15; 5.0 IU/kg AVP female, N=15; saline control male, N=15; 0.05 IU/kg AVP male, N=14; 0.5 IU/kg AVP male, N=16; 5.0 IU/kg AVP male, N=13.

Significance level was set at $p < 0.05$ for all analyses and all tests were two-tailed. All reported p-values were corrected using Benjamini-Hochberg false discovery rate corrections to control for multiple comparisons when effect of an X variable was tested for a relationship with multiple Y variables. False discovery rate was set at five percent.

Experiment 1: RESULTS

To determine whether IN AVP influenced social behavior during pre-courtship aggression, we first assessed number of approaches that the focal test mouse made toward the opposite-sex potential mate. In females, ANOVA revealed no significant differences in number of approaches [$F(3, 57)=1.33$, $p=0.27$]. However, pairwise comparisons revealed differences between the control and low dose ($p<0.05$) and the

control and medium dose (p<0.05) but not the control and high dose (p=0.25) (**Fig. 1A)** In males, ANOVA revealed no significant differences in number of approaches, [F(3, 54)=0.87, p=0.46] (**Fig. 1B**). In females, ANOVA revealed no significant differences amount of aggression displayed [F(3, 56)=1.33, p=0.27]. However, pairwise comparisons revealed an increase in aggression with low dose (p<0.01), medium dose (p<0.01) and high dose (p<0.01) (**Fig. 1C).** In males, ANOVA revealed no significant differences in amount of aggression, [F(3, 54)=0.87, p=0.46] (**Fig. 1D**). However, pairwise comparisons revealed that the high dose led to a nonsignificant trend for less aggression (p=0.09). In females, ANOVA revealed no significant differences amount of affiliative allogrooming displayed [F(3, 56)=1.18, p=0.33] (**Fig. 1E**). Likewise, in males, ANOVA revealed no significant differences amount of affiliative allogrooming displayed [F(3, 54)=1.16, p=0.33] (**Fig. 1F**).

Figure 1. Pre-courtship aggression test. (A) Low and medium AVP doses increased female approach **(B)** AVP did not influence male approach. **(C)** Low, medium, and high AVP doses increased female aggression **(D)** AVP did not significantly influence male aggression but showed a nonsignificant trend for the high dose decreasing aggression **(E)** AVP did not influence female allogrooming. **(B)** AVP did not influence male allogrooming. *p<0.05, **p<0.01 for differences between control and AVP.

Experiment 1: DISCUSSION

Forming strong, selective social bonds is critical process for monogamous species like the California mouse. Previous research in prairie voles has implicated activation of the AVP in pair bonding in males, but it is not known whether this mechanism is conserved across other monogamous species (Nair & Young, 2006). In this study, we found that an acute pulse of AVP in male California mice did not increase approaches toward females, increase affiliation, or inhibit aggression during the first 20 min of cohabitation with a female.

In contrast to males, in females, an acute pulse of AVP did influence pre-courtship behavior. IN AVP increased female approach at low and medium doses and increased female aggression at all doses. This suggests that IN AVP may actually inhibit the earliest phases of bond formation via increased aggression. This is consistent with studies in marmosets that show AVP reduces food sharing in family groups (Taylor et al., 2017) and studies in humans that show AVP enhanced dishonesty toward the out-group in females but not males (Feng et al., 2020).

In addition to characterizing the role of an acute pulse of AVP on pre-courtship aggression, this experiment also aimed to determine if different AVP doses led to different behavioral outcomes. We found some support this as only low and medium doses of AVP increased female aggression and in males the high dose of AVP showed a nonsignificant trend for decreased aggression. Together, this suggest that at high doses, IN AVP may be binding to OXTRs to exert their behavioral effects.

Overall, during this first impression of courtship, IN AVP increased aggression in females, a behavior that could potentially increase latency to form pair bonds or make forming pair bonds more difficult. However, in males, there is not strong support for the role of IN AVP on the formation of pair bonds.

Experiment 1: REFERENCES

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Experiment 2: Determine the role of oxytocin in pre-courtship aggression, resident-intruder aggression, and the formation of the father-offspring bond and communication

Experiment 2: INTRODUCTION

In social species, interactions can be altered based on life history stage and environment. Throughout the lifespan, social species encounter many different types of social interactions and must respond appropriately to these social interactions to acquire and maintain resources, mating opportunities, and reproductive fitness. One significant question is determining the mechanisms underlying how animals alter their social responses based on social and environmental context and life stage. Endogenous hormone and neuropeptide levels are important for biobehavioral feedback and to help animals respond appropriately to various social interactions. Oxytocin (OXT), a neuropeptide hormone, is a neuromodulator that may be important for weighing social salience and determining appropriate behavioral response to social stimuli (Shamay-Tsoory & Abu-Akel, 2016; Parr et al., 2018; Yao et al., 2018; Johnson et al., 2017; Egito et al., 2020). Previous studies on OXT show its significant effects on prosocial affiliative behaviors such as trust, social bonding, social recognition, and anxiolytic behavior in both human and animal models (Theodoridou et al., 2009; Kosfeld et al., 2005; Ring et al., 2006; Bales et al., 2003; Blocker et al., 2015; Guestella et al., 2008). In addition to increasing affiliative behaviors, OXT is involved in aggressive behaviors. In humans, OXT can increase envy, schadefreude, defensive but not offensive aggression toward a competing out-group, and domestic violence in men prone to aggression (Shamay-Tsoory et al. 2009; Bethlehem et al., 2015; De Dreu et al., 2016; De Dreu et al., 2010; DeWall et al. 2014). OXT is also associated with increased mate guarding in rats (Holley et al., 2015), prairie voles (Bales & Carter 2003), and marmoset monkeys (Cavanaugh et

al., 2018). Furthermore, OXT is associated with increased maternal aggression toward potential predators (Bosch & Neumann 2012). In canines, OXT also increases aggression towards owners but not strangers during a threatening approach test (Hernadi et al., 2015). These data on the role of OXT on affiliative and aggressive behavior support the hypothesis that social salience and social context are important cues influencing the behavioral effects of OXT. Based on these studies, OXT would be expected to decrease aggression and increase affiliative behavior when a male-female pair is introduced and increase aggression by a resident towards an intruder.

Throughout an animal's lifetime, OXT levels change in response to certain life events such as early life experience, pair bonding, intrasexual aggression, and parenting. This is especially true for monogamous and parental species that require flexibility in response to group membership. In prairie voles, the function of OXT can be altered in response to previous social neglect by their mother during the neonatal period (Bosch and Young, 2017). Prior to mating, OXT increases affiliative contact with familiar females (Cho et al. 1999; Bales et al., 2013) and increases speed of pair bonding in females (Williams et al., 1994; Young & Wang, 2004). Post-mating, OXT enhances aggression in prairie voles during encounters with same-sex conspecifics (Winslow et al. 1993). In California mice, OXT plasma levels increase in expectant fathers, decrease in fathers, and are disrupted when the male is separated from his mate and pups (Gubernick et al., 1995). These rodent studies in prairie voles and California mice suggest that social experience may drive important changes to the OXT system. These studies further enhance expectations for OXT to increase paternal behavior.

To mimic the natural pulses of OXT that may occur during these different social contexts and challenges, acute intranasal OXT (IN OXT) can be used. Previous studies in rodents have shown that IN OXT alters behavior within 5-min of administration

(Bales et al., 2013) and can have behavioral effects that persist for 30-50 min after administration (Carter & Wilkinson, 2015). Daily chronic doses of IN OXT induce long-term modifications to the OXT system (Bales et al., 2013; Guoynes et al., 2018; Del Razo et al., 2020); however, single doses spread out across weeks are presumably less likely to have carry-over effects across tests (Huang et al., 2014).

The California mouse (*Peromyscus californicus*) is a strictly monogamous, biparental rodent species well-suited to examine how OXT modulates vocal production and social behavior across different life stages. California mice show aggression toward unfamiliar conspecifics (e.g. Rieger et al. 2018) including opposite-sex conspecifics (e.g. e.g.Pultorak et al., 2018). During pre-courtship aggression with an unfamiliar conspecific, there is a period of assessment and often aggression (Gleason & Marler, 2010) that we will refer to as the pre-courtship aggression phase. Most of this aggression is in the form of non-contact aggression such as chasing and lunging, but the aggression can escalate to contact forms of aggression such as wrestling. Based on previous experience pairing female and male California mice in the lab, most prospective pairs show some form of aggression (i.e. lunging, chasing) but fewer pairs show contact aggression (i.e. wrestling) (Gleason & Marler, 2010). Once paired, female and male California mice form strong, reliable pair bonds but will still show reliable aggression toward unfamiliar conspecifics (Bester-Meredith & Marler, 2001; Trainor & Marler, 2001; Bester-Meredith & Marler, 2007); such aggression is decreased by an antagonist (V1a) to vasopressin (Bester-Meredith et al. 2005), a similar neuropeptide that is often positively associated with aggression. The period of pre-courtship aggression in the California mice is significantly longer than in other monogamous animal models such as the prairie vole. While prairie voles mate within the first 41 hrs of being paired (Witt et al., 1988), California mice mate 7-14 days after being paired (Bester-Meredith et al.,

2003; Trainor et al., 2001; Gleason & Marler 2010). This longer period of courtship may reflect a longer assessment period for potential mates, as expected in a monogamous species. The first litter of pups is typically born between six and eight weeks after the initial pre-courtship aggression. Once pups are born, both fathers and mothers engage in parental care (Bester-Meredith & Marler, 2001; Bester-Meredith & Marler, 2003; Lee & Brown 2002; Trainor et al., 2003; Trainor & Marler, 2003; Marler et al., 2003; Lee et al., 2007; Frazier et al., 2006; Becker et al., 2010; Gleason & Marler, 2010; Bester-Meredith & Marler, 2012; Johnson et al., 2015; Rieger et al., 2019; Guoynes & Marler, 2021).

California mice also have a diverse, well-characterized repertoire of ultrasonic vocalizations (USVs) including simple sweeps, complex sweeps, syllable vocalizations, barks, and pup whines (Briggs et al. 2011; Kalcounis-Rueppell et al., 2006; Pultorak et al., 2015; Rieger & Marler, 2018; Guoynes & Marler, 2021). A previous study in mother-offspring interactions demonstrated that the primary call types observed were maternal simple sweeps and pup whines; maternal simple sweeps correlated with both maternal care and pup whines (Guoynes & Marler, 2021). Similar to the prevalence of call types in mother-offspring interactions, preliminary recordings between fathers and pups indicated that the primary call types from fathers and pups were also paternal simple sweeps and pup whines, respectively. Moreover, OXT stimulated production of maternal sweeps (Guoynes & Marler 2021). Based on this, we predicted a similar response to OXT in fathers involving simple sweeps and pup whines. It is important to note that paternal simple sweeps and pup whines have also been recorded in other social contexts (Guoynes & Marler, 2021; Rieger et al., 2019; Pultorak et al., 2015; Pultorak et al., 2017). Because we were not manipulating the OXT system in the pups, we did not expect to see an effect of OXT on pup whine USVs.

In the current study, we aimed to address whether acute pulses of IN OXT alter an animal's response to social challenges. We hypothesized that 1) during the pre-courtship aggression phase, IN OXT would reduce aggression, specifically the escalation to contact aggression (i.e., wrestling) in male-female aggression and increase affiliative behavior, 2) during resident intruder paradigms IN OXT would increase aggression towards an intruding male and 3) during a parental care test, similar to the effects in mothers, IN OXT would have a positive effect on paternal care and paternal vocalizations.

Experiment 2: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We used 24 male *P. californicus* aged 5–10 months. They were group-housed (2–3 per cage; 48 × 27 × 16 cm) under a 14L: 10D light cycle with lights off at 4:00pm. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Males were randomly assigned to either the saline control group (N=12) or the OXT group (N=12). The OXT group received three total doses of OXT and the saline group received three total doses of saline (one dose given 5-min before each behavioral test) over eight weeks. For pair bond initiation, 24 female mates unrelated by at least two generations were randomly assigned to the focal test males. For the resident intruder test, 24 unrelated male intruders were randomly assigned to the focal test males. During the paternal care test, pup number across treatments was very similar such that the average number of pups for fathers in the saline control condition was 2.13 ± 0.23 (mean ± SE), and average number of pups for fathers in the OXT condition was 2.25 ± 0.16 (**S. Table 3**).

Intranasal Oxytocin Preparation

Male mice were infused intranasally with either sterile saline or IN OXT (0.8 IU/kg) (Bachem, Torrance, California) (Guoynes & Marler, 2021). The IN OXT dose is equivalent to doses used in other animal models (Bales et al. 2014; Guoynes et al. 2018; Murgatroyd et al. 2016) and similar to weight-adjusted doses used in clinical studies examining the effects of IN OXT on social deficits in autism (Bales et al., 2013). IN OXT was dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20^{o}C. IN OXT was defrosted just prior to administration. A blunt cannula needle (33-gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was attached to cannula tubing, flushed, and filled with the compound, then attached to an airtight Hamilton syringe (Bachem, Torrance, California). The animal was scruffed and 25 uL of compound was expelled dropwise through the cannula needle and allowed to absorb into the nasal mucosa (~10-20 seconds). One person conducted all IN OXT administrations throughout the entire procedure to maintain consistency in handling and IN OXT infusion. We chose to use the method of intranasal administration of IN OXT for two primary reasons. (1) IN OXT is used in clinical studies and is less invasive, does not require special transporters for the molecule, and is presumed to be less stressful compared to intracerebroventricular (Talegaonkar & Mishra 2004). (2) IN OXT shows similar behavioral effects as centrally administered OXT, increases CSF and plasma concentrations of OXT, and reaches the relevant brain areas in both humans and animal models (Neumann et al., 2013; Striepens et al., 2013; Lee et al. 2018; Oppong-Damoah et al., 2019; Lee et al., 2020). Several studies have also shown changes in plasma OXT concentrations that peak between 15 to 30-min post-administration (Freeman et al., 2016; Gossen et al., 2012). These results suggest IN OXT passes through

the blood-brain barrier to exert central effects. In California mice, behavioral effects of IN OXT are consistent with the outcomes of central OXT manipulations suggesting that IN OXT is reaching the brain (Duque-Wilckens et al. 2018, 2020). Other studies indicate that some of the effects of IN OXT are acting through peripheral mechanisms (Churchland & Winkielman, 2012; Quintana et al., 2015; Leng & Ludwig, 2016). Regardless of whether IN OXT is directly targeting the brain, is acting through peripheral mechanisms, or a combination of both, IN OXT has been shown to rapidly alter social behavior in adult California mice (Steinman et al., 2016).

Behavioral Tests

Throughout the experiment, all researchers administering treatments and handling animals were blind to treatment condition. For each test, the same researcher administered all intranasal treatments to reduce variance across handling and administration.

Pre-courtship aggression test

Male California mice aged 5-10 months were removed from their home cage (48 × 27 × 16 cm) and given 25 uL of 0.8 IU/kg OXT or saline. Immediately after treatment, each male was placed in a new home cage (48 × 27 × 16 cm) with fresh bedding. 5-min after the dose of OXT or saline, a novel, unrelated female was placed into the new home cage. Their interaction was videotaped for 10-min (**Fig. 1A**). After the recording, the male and female continued to be housed together for the remainder of the experiments.

Resident intruder test

We continued to use the same male and female pairs as in the pre-courtship aggression test above, but 14 days after being paired. Residency in the home cage was

established by housing the mice in the same home cage for 6 consecutive days. This is more than sufficient time to establish residency in males (Bester-Meredith et al., 1999; Marler et al., 2003; Fuxjager et al., 2010; Zhao et. al 2014). Immediately before testing, female pair mates were removed from the home cage and placed in a new home cage with fresh bedding adjacent to the old home cage with soiled bedding (each 48 × 27 × 16 cm). Male pair mates were given 25 uL of 0.8 IU/kg OXT or saline (same treatment as they received in the pre-courtship aggression test) and placed back in their home cage with soiled bedding. 5-min after administration of OXT, an unrelated, novel male was placed on the far side of the resident's cage. Their interaction was recorded for 5-min (**Fig. 1A**). After the test, the novel male was removed and placed back in his home cage and then the resident male given OXT or saline was removed and placed into the clean home cage with his female pair mate.

Paternal care test with ultrasonic vocalizations (USVs)

This test used the same male and female pairs as in the pre-courtship aggression test and resident intruder test (above) and was conducted three to six weeks after the resident-intruder test—on the first or second day after their first litter was born. Pairs were monitored and checked for pups daily. Testing occurred within 48 hrs of the pups being born during a stage of postpartum estrous. The pups were removed from the mother, and the mother was placed in a new home cage with some soiled bedding from the home cage. Next, the father and pups in their home cage were transferred from the mouse housing room to a behavior testing room capable of recording USVs. This procedure is similar to paradigms previously used in the lab (Guoynes & Marler, 2021; Pultorak et al., 2015; Rieger & Marler, 2018). Testing was done in a custom arena split into two equally sized chambers (45.0 cm × 30.0 cm × 30.0 cm) and contained two symmetrically located circular openings (3.8 cm in diameter, center of opening 7 cm

from the side wall) covered by a wire mesh. Ultrasonic microphones (described below) were placed on each side of the divider. One side of the divider was designated to the focal male, the other to the pup(s). This setup allowed visual, auditory, and olfactory communication between pups and their father, but restricted physical contact between individuals until the mesh wire was removed. In the testing room, fathers were given a third dose of either 25 uL of 0.8 IU/kg OXT or saline (same treatment as they received in the pre-courtship aggression test and aggression test) and placed back into their home cage for 5-min (**Fig. 1A**). At the end of the 5-min waiting period, the pups were moved into the side of the testing chamber near the door, and the fathers were moved into the chamber closest to the wall. They interacted through the mesh divider intact for the first 3-min, then the divider was removed, and the fathers and pups could physically interact for an additional 5-min. Vocalizations and video were recorded for the entire 8-min period. These time periods were chosen because they minimized the time that the pups were away from their mother but allowed enough time to quantify behavioral differences in retrievals.

Behavior Quantification

All behavior videos were scored twice: once each by two independent observers blind to treatment and in a random order. Scores between observers had to be at least 85% similar and scores between the two observers were averaged for the final output used in statistical analysis. For an ethogram describing these different behaviors, see **S. Fig. 1**.

Ultrasonic Vocalization Analysis

Techniques used for recording were similar to those previously used in our laboratory (Pultorak et al. 2017; Rieger & Marler 2018; Guoynes & Marler 2021). USVs were collected using two Emkay/Knowles FG series microphones capable of detecting broadband sound (10–120 kHz). Microphones were placed at the far ends of each of the two chambers. Microphone channels were calibrated to equal gain (− 60 dB noise floor). We used RECORDER software (Avisoft Bioacoustics) to produce triggered WAV file recordings (each with a duration of 0.5 s) upon the onset of a sound event that surpassed a set threshold of 5% energy change (Kalcounis-Rueppell et al., 2010). Recordings were collected at a 250 kHz sampling rate with a 16-bit resolution. Spectrograms were produced with a 512 FFT (Fast Fourier Transform) using Avisoft-SASLab Pro sound analysis software (Avisoft Bioacoustics). The only USVs found in these recordings were pup whines and paternal simple sweeps. Pup whines have a peak frequency around 20 kHz (Johnson et al., 2017; Kalcounis-Rueppell et al., 2018a) and the typical downward modulation at the end of the call often distinguishes these calls from adult syllable vocalizations (Guoynes & Marler, 2021; Nathaniel Rieger, Jose Hernandez, & Catherine Marler, unpublished) (**Figure 1B**). The lower frequencies in the pup whine can also be heard by human ears (below the ultrasonic range). Paternal simple sweeps were categorized by short downward-sweeping vocalizations that sweep through multiple frequencies, typically between 80 kHz and 40 kHz (Kalcounis-Rueppell et al., 2018b) (**Figure 1B**). It is extremely rare for pups to produce simple sweep USVs during PND 0-4 (Rieger, N. S., Hernandez, J. B., and Marler, C. M., unpublished). When young pups produce simple sweeps, they are produced much faster and present completely vertical on the spectrogram (Johnson et al., 2017). This makes these rare pup simple sweeps easy to distinguish from the slower adult simple sweep USVs (**Fig. 1B**). Because of their different spectrogram and acoustic properties,

all USVs could be categorized and counted by combined visual and auditory inspections of the WAV files (sampling rate reduced to 11,025 kHz, corresponding to 4% of real-time playback speed).

Data Analysis

For each behavioral test, nonparametric Mann-Whitney tests were conducted to compare the outcomes between saline control and OXT males. In the pre-courtship aggression test, one OXT mouse was dropped from the analysis because he escaped from the apparatus just prior to testing. Final group size analyzed for the pre-courtship aggression test was N=12 for control males and N=11 for OXT males. In the resident intruder test, final group size analyzed for the pre-courtship aggression test was N=12 for controls and N=12 for OXT males. In the paternal care test, three pairs were removed from behavioral analyses due to accidental deleting of the behavior videos (1 control male, 2 OXT males), and 5 were not tested because of either infanticide or not producing pups within eight weeks of pairing. Final group size analyzed for the behavioral and USV components of the paternal care test was N= 8 for controls and N=8 for OXT.

Correlations between paternal care and USVs were conducted using the program R. To assess for mediation by IN OXT in the relationships between (a) paternal USVs and paternal behavior and (b) paternal behavior and pup USVs, a multivariate comparison was used. Factors included in the model were treatment condition and the interaction between treatment and paternal behavior (e.g. [Paternal behavior] ~ [Paternal USV] + [treatment]).

Significance level was set at $p < 0.05$ for all analyses and all tests were two-tailed. All reported p-values were corrected using Benjamini-Hochberg false discovery rate

corrections to control for multiple comparisons when effect of an X variable was tested for a relationship with multiple Y variables. False discovery rate was set at five percent.

Figure 1. Experimental design. (A) Timeline of the three behavioral tests throughout the longitudinal study. **(B)** Representative pup whine and paternal simple sweep USVs. Pup whines have multiple harmonics, a peak frequency around 20 kHz, and downward modulation at the end of the call that distinguish these calls from adult syllable vocalizations. Paternal simple sweeps have short downward-sweeping vocalizations that sweep through multiple frequencies, typically between 80 kHz and 40 kHz.

Experiment 2: RESULTS

Pre-courtship aggression test

To determine whether IN OXT influenced escalation to contact aggression during pre-courtship aggression, we assessed number of wrestling bouts in male mice given IN OXT versus saline. We found that OXT decreased the proportion of wrestling bouts out of all aggressive behaviors between the male and female during the first 10-min of pre-courtship aggression ($U=33$, $z\text{-}score=2.00$, $p<0.05$) (**Fig. 2A**). Lunging aggression levels made up a relatively small proportion of the aggressive behaviors in both control and OXT males; however, differences arose in proportion of wrestling aggression (highest in control males) and chasing aggression (highest in OXT males) (**Fig. 2B**). Levels of non-contact aggression were relatively similar across groups (lunging aggression: CTRL=1.29± 1.36 and OXT=0.45± 0.37; chasing aggression: CTRL=10.76± 3.73 and OXT=12.80± 3.82) (**S. Table 1**). The biggest difference between treatment groups was amount of time spent engaged in contact aggression (wrestling aggression: CTRL=11.58± 6.22 and OXT=0.77± 0.50) (**S. Table 1**). Thus, the difference in proportion of wrestling of aggression between CTRL and OXT is being driven by time spent wrestling vs. time spent chasing. Other behaviors we did not predict would be affected by IN OXT such as social investigation (body and anogenital sniffing) and

activity (autogrooming, rearing) were measured but not statistically analyzed (**S. Table 1**).

Figure 2. Pre-courtship aggression test. Males given OXT had a significantly smaller proportion of wrestling than control males during the first 10 min of courtship. **(B)** Pie chart showing escalating aggressive behavior (from light: low escalation, to dark: high escalation). *p<0.05 for differences between control and OXT.

Resident intruder aggression test

To determine whether IN OXT influenced escalation to contact aggression during a resident intruder test, we assessed the number of wrestling bouts in males given IN OXT versus saline. Unlike the pre-courtship aggression test, we found that IN OXT did not significantly influence number of wrestling bouts between the males during a 5-min resident intruder test (U=63.50, z-*score*=0.46, *p*=0.637) (**Fig. 3A**). Similar to the pre-courtship aggression test, lunging aggression levels made up a relatively small proportion of the aggressive behaviors in both control and OXT males (**Fig. 3B**). Both chasing and wrestling aggression made up approximately equal proportions of aggressive behavior in the resident intruder aggression test (**Fig. 3B**). Levels of all types of aggression were relatively similar across groups (lunging aggression: CTRL=2.25± 1.00 and OXT=1.63± 0.71; chasing aggression: CTRL=13.63± 5.52 and OXT=11.28± 5.86; wrestling aggression: CTRL=11.47± 4.52 and OXT=10.69± 4.63) (**S. Table 2**). Other behaviors we did not predict would be affected by IN OXT such as social investigation (body and anogenital sniffing) and activity (autogrooming, rearing) were measured but not statistically analyzed (**S. Table 2**).

Figure 3. Resident intruder aggression test. (A) OXT and control males showed no difference in proportion of wrestling during a 5-min resident intruder encounter. **(B)** Pie chart showing escalating aggressive behavior (from light to dark). *p<0.05 for differences between control and OXT.

Paternal care test with ultrasonic vocalizations (USVs)

To determine whether IN OXT would influence behavior during a paternal care challenge we assessed latency to approach pups, pup huddling, and paternal simple sweep USVs in fathers given IN OXT versus saline. Fathers given IN OXT were significantly faster at approaching their pups after a brief separation (U=10.50, *z-score*=2.21, p<0.05) (**Fig. 4A**). Despite initial differences in paternal care response, there were no differences between IN OXT and control males in total time huddling (U=22.50, *z-score*=-0.95, p=0.34) (**Fig. 4B**) or licking pups (U=20, *z-score*=-1.21, p=0.22) (**Fig. 4C**). There was one father in the control group that showed much more paternal care than other control fathers, however, this father was not a Grubb's outlier for paternal care measures. Even if this father is removed from the analysis, the difference between control and OXT is not significant for huddling (U=14.50, *z-score*=1.50, p=0.13) (**Fig. 4B**) or licking (U=12, *z-score*=1.79, p=0.07) (**Fig. 4C**). Neither IN OXT or control fathers engaged in any retrieval behavior throughout the test, so this type of paternal care was not analyzed (**S. Table 3**). There were no differences in number of pups across treatments groups (CTRL=2.13± 1.84 1.84; OXT=2.25± 1.54). Other behaviors related to activity (autogrooming, freezing, rearing) were measured but were not included in the statistical analyses because we did not have *a priori* predictions for these behaviors during the paternal care test (**S. Table 3**).

Next, we assessed whether IN OXT would influence paternal and/or pup USVs behavior during a paternal care challenge. We assessed number of paternal simple sweeps and number of pup whines produced and their correlations with the two types of paternal care observed, huddling and licking. Fathers given IN OXT did not produce more simple sweeps than controls (U=23.50, *z-score*=-0.84, p=0.40) (**Fig. 4D**). There were also no differences in number of pup whines produced in offspring of IN OXT versus control fathers (U=24.50, *z-score*=0.35, p=0.72) (**Fig. 4E**).

Lastly, we examined the relationship between paternal care and paternal and pup USVs and any interactions with OXT treatment. Using a multivariate model controlling for the effects of treatment, we found no main effects of paternal simple sweeps on huddling ($F_{2,16}$=0.21, p=0.65, η^2=0.016) (**Fig. 4H**) or licking ($F_{2,16}$=0.01, p=0.91, η^2=0.00) (**Fig. 4J**). Similarly, we found no main effects of pup whines on huddling ($F_{2,16}$=0.05, p=0.81, η^2=0.00) (**Fig. 4I**) or licking ($F_{2,16}$=0.07, p=0.80, η^2=0.00) (**Fig. 4K**).

Figure 4. Paternal care test. OXT males had shorter latencies to approach their pups than control males **(A)**. OXT males did not show significant differences in huddling **(B)** or licking **(C)** behavior. **(D)** Males given OXT did not make more simple sweeps than control males. Paternal simple sweeps did not correlate with **(E)** huddling or **(F)** licking. **(G)** Pups with OXT versus control fathers showed no differences in number of pup whines produced. There were no correlations between pup whines and **(H)** huddling or **(I)** or licking. *p<0.05 for differences between control and OXT.

Experiment 2: DISCUSSION

Our study assessed the response of male California mice to different challenges that would naturally occur during their lifespan. During contexts in which the social stimuli had the potential to become part of the in-group, a male-female bonded pair, OXT administered to the male promoted prosocial approach through reduced aggression. In contrast, during the resident-intruder aggression test, the social stimuli did not have the potential to become part of the in-group in a strongly territorial species, and OXT did not promote prosocial approach. Finally, in the paternal behavior test, OXT increased paternal motivation to approach pups in this biparental species. We speculate that OXT may function to promote social approach only in contexts that are or are likely to be affiliative-prone.

In the monogamous and territorial California mice, when virgins encounter an unfamiliar individual of the opposite sex, there is both an aggressive response to an unfamiliar conspecific, and possibly novelty, and a potential for pair bond formation.

During the initial 10-min of this interaction, only aggressive behavior was exhibited, with no signs of affiliative behavior characteristic of later stages of courtship (Gleason & Marler, 2010) or as they are bonding (Pultorak et al., 2017); also similar to the behavioral sequence seen in research with other species between male and females prairie voles (Williams et al., 1992; Carter et al., 1995; Cho et al., 1999; Willett et al., 2018; Harbert et al., 2020) and marmosets (Smith et al., 2009). Because we were testing the effect of IN OXT on this early phase of a female-male introduction, we predicted that IN OXT would reduce the escalation to contact aggression but also increase affiliative behavior as described in the introduction. We found similar levels of lunging and chasing behavior in both OXT and control males, but control males engaged in more wrestling aggression, leading to a significantly higher proportion of control males that escalated their aggression to contact aggression. In this context, OXT may increase the rapid social assessment of and approach towards a potential mate, attenuating high levels of aggression. This change in behavior may decrease time to pair bonding and reduce the chance of injury because males are approaching females with less intense aggression. In the time frame of this test, we did not see a transition to affiliative behavior in either OXT or control males. Similar OXT-driven reductions of aggression in mating contexts have been observed in female Syrian hamsters (Harmon et al., 2002). However, this is the first study reporting anti-aggressive effects of OXT during intersexual interactions in males towards females. This anti-aggressive effect of OXT may have been revealed in California mice specifically because they are a highly aggressive species that also has a prolonged courtship phase prior to mating.

In contrast to opposite-sex social interactions, encounters with unfamiliar individuals of the same sex interactions do not have the same potential for affiliative behavior in a highly monogamous and territorial species. While we predicted that IN

OXT would increase escalation to contact aggression in the resident-intruder paradigm, we found that there was no difference in aggression between control and IN OXT treated males. This is consistent with another study that found the same dose of IN OXT used in this study (0.8 IU/kg) did not influence numbers of bites or attack latency in a resident intruder aggression test in California mice (Steinman et al., 2016). It is possible that in a highly territorial and monogamous species there may be selection for a maximum aggressive response to an intruding male. Interestingly, intracerebroventricular injections of vasopressin increased did not increase aggression in a resident-intruder paradigm for male California mice, but a V1a antagonist decreased aggression, further supporting the idea of a maximum level of aggression (Bester-Meredith et al., 2005). Previous studies in less territorial species have found that OXT increases aggression. In house mice, OXTR null mice expressed increased intrasexual aggression (Devries et al., 1997). A study in female rats that manipulated OXT in lateral septum demonstrates that OXT increases and vasopressin decreases aggression towards same-sex intruders (Oliveira et al., 2021). Studies in humans have also shown an association between increased aggression, competition, and OXT (DeWall et al., 2014; Ne'eman et al., 2016; De Dreu, 2012; Fischer-Shofty et al., 2013). However, studies in monogamous marmosets (Cavanaugh et al., 2018), monogamous titi monkeys (Witczak et al., 2018), female and male rats (De Jong et al., 2014; Calcagnoli et al., 2013; Calcagnoli et al., 2015a; Calcagnoli et al., 2015b), house mice primed for aggressive behavior due to social isolation (Tan et al., 2019), and house mice bred for callous traits (Zoratto et al., 2018) found that OXT was associated with reduced intrasexual competition and aggression. Together with our data, these findings suggest that OXT's effect on intrasexual aggression may depend heavily on the species, brain areas activated by OXT, and social context.

In our last test, we aimed to assess whether IN OXT had similar prosocial effects in fathers as it did in California mice mothers (Guoynes & Marler, 2021). We predicted a positive prosocial effect on both paternal behavior and vocalizations. We found that IN OXT decreased paternal latency to approach their pups but did not influence overall level of paternal care. Studies in Mandarin voles have also shown similar effects of OXT on latency to engage in paternal care (Yuan et al., 2019). Reduced latency to approach pups in IN OXT fathers suggests that IN OXT may increase paternal motivation for pup contact without altering the quality of paternal care. This is supported by studies that show activation of the OXT system can increase dopamine and reinforce rewarding behavior (Borland et al., 2018; Borland et al., 2019; Dolen et al., 2013; Martins et al., 2021). However, it is also possible that the decreased latency to approach pups was driven by dampening anxiety during the challenge test. Several studies have also shown that OXT can reduce anxiety and facilitate prosocial approach (Steinman et al., 2019; Williams et al., 2020; Cohen & Shamay-Tsoory, 2018; Domes et al., 2019). Because we did not observe any overall differences in level of paternal care during the test, the effects of OXT on paternal care may be rapid and more likely to influence paternal responsiveness in California mice versus quality of paternal care seen in marmosets (Saito & Nakamura, 2011; Finkenwirth et al., 2016) and human fathers (Naber et al., 2010; Feldman et al., 2010; Gordon et al., 2017; Li et al., 2017; review by Guoynes & Marler, 2020). We again see species variation in the effect of OXT on paternal care, suggesting that differences across species and brain connectivity may have significant impacts on the how OXT will affect paternal care.

In contrast to the positive association between simple sweeps and maternal care, simple sweeps produced by fathers did not have any relationship with paternal care. This could be due to fathers producing a lower number of calls than mothers during the

same testing time frame (mothers produced approximately 1.0 simple sweep/s compared to fathers that produced approximately 0.33 simple sweeps/s) (Guoynes & Marler, 2021). However, it is also possible that fathers are more stressed in the absence of their partner than mothers are and therefore vocalize less. This is supported by findings in several other species that show blunted vocalization in response to heighted stress (Lumley et al., 1999; Chabout et al., 2012; Simola & Granon, 2019; Riaz et al., 2015). Lastly, it is also possible that there are sex differences in the function of simple sweeps in California mice, and that mothers rely more heavily on this call than fathers. Previous research in the lab has shown that while both fathers and mothers show biparental care, there are differences in parental care expression between fathers and mothers. For example, during a very similar paradigm, mothers showed retrieval behavior, unlike fathers in this test (Guoynes & Marler, 2021), and when both parents are together and given a resident intruder challenge in the presence of their pups, fathers were first to approach pups while mothers did significantly more retrieving behavior (Rieger et al., 2019). This suggests that fathers and mothers may divide parental care duties differently and may, therefore, vocalize and communicate differently.

Overall, the social challenges tested during these experiments show that IN OXT increases prosocial approach behavior in affiliative-prone contexts, but not during the context of direct threat or competition. These results align with the social salience hypothesis of OXT (Kemp & Guastella, 2010; Shamay-Tsoory & Abu-Akel, 2016; Peled-Avron & Shamay-Tsoory, 2018). This hypothesis suggests OXT enhances the processing of social stimuli and that this can either lead to affiliative or aggressive behavior depending on the environment, social stimuli, and internal state of the animal. Across the lifespan in a monogamous, territorial species, it is critical to assess social contexts

and balance the costs of aggression and challenges with the benefits of mating opportunities and offspring-rearing. To our knowledge, our study is the first to assess the effect of IN OXT during different life-stage challenges in the same animal. Furthermore, our study was the first to show an effect of OXT dampening aggression during pre-courtship female-male interactions.

Experiment 2: REFERENCES

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Experiment 3: Characterize the formation of the mother-offspring bond and communication

Experiment 3: INTRODUCTION

Quality of maternal care has significant impacts on offspring survival outcomes across many mammalian species (Fairbanks & McGuire, 1995; Nowak et al., 2000; Blomquist, 2013; Lerch-Haner et al., 2008; Francis et al., 1999). These studies underscore the importance of maternal behavior from an evolutionary perspective. However, the proximate mechanisms that reinforce maternal care remain more elusive. Several studies in rodents illustrate that pup whines, high energy calls produced by pups, quickly and reliably elicit maternal care (Allin & Banks, 1972; Hennessy et al., 1980; Shiavo et al., 2020; Portfors, 2007; White et al., 1992; Bell, 2018). Other studies, however, show that mothers are more apt to exhibit care in response to stressful events or disturbances and that pup calls do not influence their care above and beyond the disturbance (Brewster & Leon, 1980). It has also been shown that female house mice prefer calls from their own pups and can locate their own faster than alien pups (Mogi et al., 2017). These studies show that pup whines can elicit changes in maternal behavior. However, the role of maternal vocalizations in this relationship has not been studied. Adult rats, including mothers, make spontaneous vocalizations that typically occur above 50 kHz when presented with drug and social (Knutson et al., 2002; Simola et al., 2012). The association of reward with these calls in rats is interpreted as an indicator of a positive internal state. In mothers, USVs may indicate maternal motivation but could also reinforce maternal care and the mother-offspring bond (Stern, 1989; Cheng, 1992) or reduce maternal anxiety (Arnon et al., 2014).

Complementing the stimulus of maternal and pup vocalizations, the neuropeptide hormone oxytocin (OXT) plays an important role in processing and producing behaviors that support maternal care. OXT modulates many social behaviors including bonding and parental care (Bartz & Hollander, 2006; Lim & Young, 2006;

Maynard et al., 2018; Taylor et al., 2015; Guoynes & Marler 2020). During mammalian birth, OXT increases to stimulate parturition and milk let-down in mothers; this increase was likely co-opted during evolution to also facilitate maternal care (Rosenblatt, 1969; Moltz et al., 1970; Pederson & Prange, 1979; Feldman et al., 2007). Immediately after parturition, rising levels of peripheral estrogen (Siegel & Rosenblatt, 1975) prime the neural substrates that respond to OXT to initiate maternal behaviors in rats (Pedersen & Prange, 1979). Additionally, OXT knockout mice have greater latency to onset of maternal behaviors (Takayanagi et al., 2005). In the brain, OXT antagonists blocked maternal behavior after natural delivery in pregnant rats (Van Leengoed et al., 1987). This reveals that central OXT is important for initiating maternal care in rodents. Acute activation of maternal care by OXT is indirectly supported by an optogenetics study in which dopamine neurons were activated in the anteroventral periventricular nucleus (AVPV) that monosynaptically connects to and activates OXT neurons in the paraventricular nucleus (PVN), resulting in enhanced maternal care (Scott et al., 2015). OXT receptor densities are also important. In rats genetically selected for differences in maternal care, high grooming compared to low grooming females had more OXT receptors in the bed nucleus of the stria terminalis, medial preoptic area, central nucleus of the amygdala, and these differences were observed in maternally-experienced females that were either non-lactating and lactating (Francis et al., 2000; Champagne et al., 2001). Collectively, these studies provide strong evidence that OXT plays an important role in activating and coordinating maternal care.

OXT also plays a role in the production and perception of vocalizations. In mice and other rodents, the majority of vocalizations occur above 20 kHz and are called ultrasonic vocalizations (USVs) (Mulsolf et al., 2010; Scattoni et al., 2009; Kalcounis-Ruepell et al., 2010; Arriaga & Jarvis, 2013). In OXT knockout mice, OXT null pups emit

fewer USVs in response to separation from their mother compared to wildtype mice (Marlin et al., 2015). This suggests that OXT may enhance pup communication with their mother. OXT can also improve the signal-to-noise ratio in mothers responding to pup calls via mediation of temporal inhibition and excitation in the left auditory cortex of female mice, leading to increased pup retrievals (Winslow & Insel, 2002). These data provide evidence that OXT is changing the perception and social salience of pup calls, leading to increased maternal care. Furthermore, in humans, the OXT receptor has a polymorphism (rs53576) with functional significance. The genotype GG (presumably produces more OXTRs compared to AG or AA genotypes) is associated with better ability to discriminate content of language under noisy conditions (Tops et al., 2011). This suggests that across taxa, OXT may play an important neuromodulatory role in promoting sensory processing and behavioral response to social auditory information.

A key social behavior that has previously not been measured is maternal vocalizations. We speculated that mothers modulate vocalization quantity or type when interacting with their offspring and that maternal vocalizations would be associated with maternal care. Moreover, we predicted that OXT would modulating these vocalizations.

The California mouse (*Peromyscus californicus*) is a strictly monogamous, biparental rodent species well-suited to examine how OXT modulates auditory sensory processing, vocal production, and social behavior. California mice have a diverse, well-characterized repertoire of ultrasonic vocalizations (USVs) including simple sweeps, complex sweeps, syllable vocalizations, barks, and pup whines (Briggs et al. 2011; Kalcounis-Rueppell et al., 2006; Pultorak et al., 2015; Rieger & Marler, 2018). Previous recordings between mothers and pups indicated that the primary call types from mothers were maternal simple sweeps and the primary call type from pups were pup

whines. While this suggests that these two call types are important in mother-pup contexts, both maternal simple sweeps and pup whines have also been recorded in other social contexts (Pultorak et al., 2015; Rieger & Marler, 2018; Rieger et al., 2019).

In the current study, we aimed to address the gaps in our understanding of proximate mechanisms that contribute to maternal care by determining the association between maternal vocalizations and maternal care and whether an acute dose of IN OXT in primiparous female California mice could rapidly increase both maternal vocalizations and care. Previous studies have shown that IN OXT alters behavior within five minutes of administration (Bales et al., 2013) and can have behavioral effects that can persist for 30-50 min after administration (Carter & Wilkinson, 2015). As we were not manipulating the OXT system in the pups, we did not expect to see an effect of OXT on pup whine USVs. We hypothesized that 1) maternal care would be associated with maternal USVs and that 2) IN OXT would have a positive effect on maternal care. Specifically, we predicted that during our behavioral paradigm, IN OXT would increase maternal care, increase maternal USV production, and enhance the correlation between pup USVs and maternal USVs and that physical separation would disrupt the pro-social effects of IN OXT.

Experiment 3: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We used 24 primiparous postpartum female *P. californicus* aged 5–10 months because of ages of previously unpaired females available within our colony. Females across this age range also show equivalent corticosterone responses to corticotrophin releasing hormone and dexamethasone challenge (Harris & Saltzman,

2013), suggesting that females within this age range have comparable glucocorticoid responsiveness. Females were pair-housed (1 female, 1 male, and 1-3 pups per cage; 48 × 27 × 16 cm) under a 14L: 10D light cycle. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Female and male mice were randomly paired to an unrelated mouse and were housed in their home cage. After females were visibly pregnant, cages were checked once daily for pups and gave birth in the home cages. Mothers were randomly assigned to either the saline control group (N=11) or the IN OXT group (N=13). The mode of pups per litter was two, and there was a range of pups per litter (1-3). Number of pups was considered for use as a covariate, but in the statistical models, including this variable a) did not explain additional variance and b) reduced the power of the statistical comparison. Pup number across treatments was very similar—average number of pups for mothers in the saline condition was 2.11, and average number of pups for mothers in the OXT condition was 1.91.

Intranasal Oxytocin Preparation

Mothers were infused intranasally with either sterile saline control or IN OXT (0.8 IU/kg) (Bachem, Torrance, California). The IN OXT dose was equivalent to doses used in other rodent species (Bales et al. 2014; Guoynes et al. 2018; Murgatroyd et al. 2016) and similar to weight-adjusted doses used in clinical studies examining the effects of IN OXT on social deficits in autism (Bales et al. 2013). IN OXT was dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20^{o}C. IN OXT was defrosted just prior to administration. A blunt cannula needle (33-gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was attached to cannula tubing, flushed, and filled with the compound, then attached to an airtight Hamilton syringe

(Bachem, Torrance, California). The animal was scruffed and 25 uL of compound was expelled dropwise through the cannula needle, alternating between left and right nostrils for each mouse. Rodents are obligate nose-breathers and thus the solution was quickly absorbed into the nasal mucosa (~10seconds). One person conducted all IN OXT administrations throughout the entire procedure to maintain consistency in handling and IN OXT infusion.

We chose to use the method of intranasal administration of IN OXT for two primary reasons. (1) IN OXT is used in clinical studies and is less invasive, does not require special transporters for the molecule, and is presumed to be less stressful compared to intracerebroventricular (Talegaonkar & Mishra 2004). (2) IN OXT shows similar behavioral effects as centrally administered OXT, increases CSF and plasma concentrations of OXT, and reaches the relevant brain areas in both humans and animal models (Neumann et al., 2013; Striepens et al., 2013; Lee et al. 2018; Oppong-Damoah et al., 2019; Lee et al., 2020). Several studies have also shown changes in plasma OXT concentrations that peak between 15 to 30 min post-administration (Freeman et al., 2016; Gossen et al., 2012). These results suggest IN OXT passes through the blood-brain barrier to exert central effects with minimal stress on the animal. In California mice, behavioral effects of IN OXT are consistent with the outcomes of central OXT manipulations suggesting that IN OXT is reaching the brain (Duque-Wilckens et al., 2020; Duque-Wilckens et al., 2018). Other studies indicate that some of the effects of IN OXT are acting through peripheral mechanisms Churchland & Winkielman, 2012; Quintana et al., 2015; Leng & Ludwig, 2016). Regardless of whether IN OXT is directly targeting the brain, is acting through peripheral mechanisms, or a combination of both, IN OXT has been shown to rapidly alter social behavior in adult California mice (Steinman et al., 2016).

Behavioral Testing and USV Recording

In order to test the effects of IN OXT on acute maternal care, we conducted this experiment in a novel recording chamber where mothers and pups could be briefly isolated from the father. Separation from the mate may be a mild stressor but would occur in natural populations in response to competing demands such as pup care, foraging and defending territories. Moreover, California mice do not exhibit a change in short- or long-term paternal care in response to corticosterone (Harris et al., 2017), show limited correlations between individual baseline corticosterone levels and behavior (Dlugoszet al., 2012), parents exhibit blunted behavioral response to predator odor stress (Chauke et al., 2011), and diel corticosterone cycle between single mothers and paired mothers does not differ (Zhao et al., 2019). While we cannot exclude an effect of a baseline level of stress, it is both a normal experience for these mice in the wild, and we do not expect corticosterone to influence the results of our current experiment above and beyond our IN OXT manipulation.

On postnatal day (PND) two to three, fathers were temporarily removed from the home cage, and the home cage with the mother and her pups was transferred to a behavioral testing room. In the behavioral testing room, mothers were randomly selected to receive 25 microliters of either 0.8 IU/kg IN OXT or saline control intranasally. Immediately after dosing, mothers and pups were placed into one side of a partitioned two-chambered apparatus (45.0 cm × 30.0 cm × 30.0 cm) that contained a circular opening (3.8 cm in diameter, center of opening 7 cm from the side wall) covered by a wire mesh (**Fig. 1A**). This apparatus, like their home cages, had approximately 1/2 inch of aspen shavings covering the entire floor. Microphones sensitive to ultrasonic frequencies (described below) were placed on each side of the divider, such that the

microphones were far enough apart to identify the source (chamber) of the calls (Pultorak et al., 2018) (**Fig. 1A**).

Figure 1. (A) Schematic for experimental design. Mother and pup (PND 2-3) groups were temporarily removed from their home cage and placed in the right side of two-chambered apparatus (five min) to habituate to the testing arena. Next, pups were moved from the right chamber and placed into the left chamber (three min). Lastly, the researchers lifted the mesh gate separating the right and left chambers, allowing mother-pup interactions (five min). Animals not to scale in diagram. **(B) Ultrasonic vocalizations (USVs) on a spectrogram.** Pup whines have multiple harmonics, a peak frequency around 20 kHz, and downward modulation at the end of the call that distinguish these calls from adult syllable vocalizations. Maternal simple sweeps have short downward-sweeping vocalizations that sweep through multiple frequencies, typically between 80 kHz and 40 kHz.

For each test, there were always two researchers present who coordinated activation of the audio software and the video camera at the same time using visual cues. This coordination allowed us to subsequently compare USVs and behavior with temporal precision. The test consisted of three phases that occurred in immediate succession: habituation, separation, and reunion. During the five-min habituation phase mothers and pups were placed together on the right side of the chamber with the partition down and allowed to freely interact with each other. During the three-minute separation phase, the pups were removed from the mother and placed on the other side of the partitioned divider. Mothers remained in the right-most chamber and pups were isolated in the left-most chamber. This setup allowed visual, auditory, and olfactory communication between pups and their mother, but restricted physical contact between individuals until the mesh wire was removed. Lastly, during the five-minute reunion phase, the mesh divider was lifted, and mothers could retrieve and interact with their pups. USVs and video were recorded for the entire 13-minute period. We chose a five-minute initial mother-pup interaction time to allow mothers time to adjust to the chamber and to mirror the time in the reunion phase where we measured latency to

enter the chamber. This time period is important because it is the first time that the mothers and her pups are removed from the home cage and the father, so this time period served as an initial measure of behavior. Results from a pilot study measuring maternal retrievals indicates that five-minutes allowed most mothers to enter the chamber, approach the pups, and engage in maternal behaviors. We shortened the separation phase to three minutes because it was still sufficient to see signs of maternal distress but minimized the time that the pups were away from their mother.

Behavior Quantification

All behavioral videos were scored in random order and by an independent observer blind to treatment. During each video, maternal behaviors (licking and grooming, huddling, and retrieving/carrying) were quantified. Of note, unlike house mice and rats that show several different types of nursing and huddling behavior, California mouse mothers do not show arched back nursing (Bester-Meredith et al., 2017; Johnson et al., 2015). The definitions of behaviors measured are detailed in an ethogram (**S. Fig. 1**). To gain insights into the correlations between maternal behavior and maternal and pup USVs, videos were coded by the exact time that mothers were huddling, licking and grooming, and retrieving/carrying. Huddling was counted when mothers were physically over their pups' bodies (Bester-Meredith & Marler, 2003). Retrieving/carrying was counted when mothers picked up their pups and transferred them to a different location. Using the precise times that mothers engaged in different types of maternal care (or none at all) throughout the 13-minute testing window, we counted the maternal USVs within those windows. This allowed us to determine how maternal behavior was related to maternal USV production.

S. Figure 1. Ethogram with description of behaviors measured.

Ultrasonic Vocalization Analysis

Techniques used for recording were similar to those previously used in our laboratory (Pultorak et al. 2017; Rieger & Marler 2018). USVs were collected using two Emkay/Knowles FG series microphones capable of detecting broadband sound (10–120 kHz). Microphones were placed at the far ends of each of the two chambers. Microphone channels were calibrated to equal gain (− 60 dB noise floor). We used RECORDER software (Avisoft Bioacoustics) to produce triggered WAV file recordings (each with a duration of 0.5 s) upon the onset of a sound event that surpassed a set threshold of 5% energy change (Kalcounis-Rueppell et al., 2010). Recordings were collected at a 250 kHz sampling rate with a 16-bit resolution. Spectrograms were produced with a 512 FFT (Fast Fourier Transform) using Avisoft-SASLab Pro sound analysis software (Avisoft Bioacoustics). The only USVs found in these recordings were pup whines and maternal simple sweeps. Pup whines have a peak frequency around 20 kHz (Johnson et al., 2017; Kalcounis-Rueppell et al., 2018a) and the typical downward modulation at the end of the call often distinguishes these calls from adult syllable vocalizations (Nathaniel Rieger, Jose Hernandez, & Catherine Marler, unpublished) (**Figure 1B**). The lower frequencies in the pup whine can also be heard by human ears (below the ultrasonic range). Maternal simple sweeps were categorized by short downward-sweeping vocalizations that sweep through multiple frequencies, typically between 80 kHz and 40 kHz (Kalcounis-Rueppell et al., 2018b) (**Figure 1B**). It is extremely rare for pups to produce simple sweep USVs during PND 0-4 (Rieger, N. S., Hernandez, J. B., and Marler, C. M., unpublished). When young pups do produce simple sweeps, they are produced much quicker, and present completely vertical on the spectrogram (Johnson et al., 2017). This makes these rare pup simple sweeps easy to

distinguish from the slower adult simple sweep USVs (**Fig. 1B**). Because of their different spectrogram and acoustic properties, all USVs could be categorized and counted by combined visual and auditory inspections of the WAV files (sampling rate reduced to 11,025 kHz, corresponding to 4% of real-time playback speed).

Data Analysis

Statistical analyses were conducted using the program R. Significance level was set at $p<0.05$ for all analyses and all tests were two-tailed. All reported p-values were corrected using Benjamini-Hochberg false discovery rate corrections to control for multiple comparisons when effect of an X variable was tested for a relationship with multiple Y variables. Grubb's outlier test was performed, and outliers for maternal vocalizations and freezing were removed from all analyses. Two mice (one control and one IN OXT-treated mouse) were Grubb's outliers ($p<0.05$) for freezing (likely due to train noise during the test). One control mouse was a Grubb's outlier ($p<0.05$) for both maternal and pup USVs. All analyses used a generalized linear mixed model (GLMM). Treatment condition was used in all models as each female was given one treatment (between-subjects design). Thus, when a relationship between two variables was significant, treatment was left as a moderator in the model even if not significant.

Effects of IN OXT on vocalizations

To assess differences in total USV production across testing conditions, a within-subjects two-way ANOVA was used. To correct for differences in total time of the three testing phases, average number of USVs/second during each phase for each animal was calculated and used to compare the five-minute versus three-minute trials. To assess main effects of treatment, Student's t-test was used in each of the three time points.

To examine the effects of IN OXT in the relationships between maternal simple sweep USVs and pup whine USVs, an interactive multivariate model was used (e.g. [Maternal behavior] ~ [Maternal USV] + [treatment]). Factor included in all models was treatment condition.

Effects of IN OXT on maternal and non-maternal behaviors

For maternal and non-maternal behavioral analysis, behavioral changes after the separation event, were calculated to examine reunion behaviors with and without OXT administration. To examine changes in behavior over time and after the separation challenge with and without OXT, the scores from total duration of each behavior in the reunion phase were subtracted by total duration of each behavior in the habituation phase (Reunion-Habituation). Thus, positive scores indicate more of the behavior was observed during the reunion phase and negative scores indicate more of the behavior was observed during the habituation phase. To compare main effects of IN OXT and saline control on maternal behavior, Students t-tests were used to assess behavioral outcomes (**Fig. 3**). To calculate total maternal behavior, amount of time spent huddling and amount of time spent retrieving were summed. To calculate total non-maternal behavior, amount of time spent autogrooming, rearing, and freezing were summed.

Correlations between maternal care and maternal USVs and maternal care and pup USVs

To assess for mediation by IN OXT in the relationships between (a) maternal USVs and maternal behavior and (b) maternal behavior and pup USVs, a multivariate comparison was used. Factors included in the model were treatment condition and the interaction between treatment and maternal behavior.

Experiment 3: RESULTS

Effects of IN OXT on vocalizations

To determine how testing conditions affected vocal production in mothers and pups, we first assessed number of maternal and pup USVs per second across the habituation, separation, and reunion phases, and in response to IN OXT versus saline. Controlling for within subject analyses and treatment effects, mothers made fewer simple sweeps/second during the separation phase compared to the habituation or reunion phases ($F_{2,20}$=13.00, p<0.00001). (**Fig. 2A**). In the habituation phase, IN OXT mothers produced more simple sweeps than control mothers ($F_{2,20}$=5.83, p<0.03, ΔR^2=0.23*)* (**Fig. 2A**). In the separation phase, IN OXT and control mothers did not differ in number of simple sweeps produced ($F_{2,20}$=1.86, p=0.19, ΔR^2=0.09) (**Fig. 2A**). Similar to the habituation phase, in the reunion phase, IN OXT mothers showed a nonsignificant trend for producing more simple sweeps than control mothers ($F_{2,20}$=3.13, p=0.08, ΔR^2=0.15) (**Fig. 2A**).

Figure 2. Rapid effects of IN OXT on USV production in mothers and pups. (A) All mothers made more simple sweeps when given free access to their pups (during the habituation and reunion phases). Importantly, IN OXT mothers made more simple sweeps when given free access to their pups during habituation and reunion, but not when they were physically apart from pups during separation. **(B)** All pups made more whines when first placed into the chamber during the habituation phase. There were no effects of maternal IN OXT on pup USVs. Pups made more whines during the habituation phase than the separation and reunion phases. There was no effect of IN OXT treatment on pup whines during habituation, separation or reunion. Mediation analysis of the relationship between maternal USVs, pup USVs and treatment in (**C**) the habituation phase showed no simple effects of maternal USVs, no simple effects of treatment, but IN OXT showed a nonsignificant trend for the two-way interaction between maternal simple sweep USVs and treatment. **(D)** The separation phase showed no simple effects of maternal USVs, no simple effects of treatment, and no effect of interaction. (**E**) The reunion phase showed a significant positive correlation between maternal simple sweeps and pup whines, no simple effect of treatment, and no effect of interaction. Correlation line in black is the average slope across treatment groups; magenta and teal lines are the slopes for the saline and OXT treatments, respectively . *p<0.05 for differences across time conditions; *p<0.05 for differences between control and OXT; #p<0.10 for differences between control and OXT.

Across the three phases, pup USVs showed no effect of IN OXT but did show changes in vocal production frequency across the testing phases. Controlling for within-subject analyses, pups made more whines during the habituation phase than the separation and reunion phases ($F_{2,20}$=25.26, p<0.0000001) **(Fig. 2B)**. Pups with IN OXT and control mothers did not differ in number of USVs produced in the habituation phase ($F_{1,20}$=0.35, p=0.56, ΔR^2=0.02), separation phase ($F_{1,20}$=1.64, p=0.22, ΔR^2=0.08) or the reunion phase ($F_{1,20}$=0.68, p=0.42, ΔR^2=0.04) (**Fig. 2B**).

Next, we examined the relationship between number of maternal simple sweeps and pup whines using a model with treatment as a covariate. There were no significant correlations between maternal and pup USVs in either the habituation ($F_{1,20}$=1.63, p=0.22) (**Fig. 2C**) or the separation phase ($F_{1,20}$= 0.03, p=0.86) (**Fig. 2D**). However, during the reunion phase, maternal simple sweeps and pup whines positively correlated ($F_{1,20}$=7.51, p<0.02, ΔR^2=0.21). For each pup whine, there were approximately 1.17 maternal simple sweeps (**Fig. 2E**). There were no simple effects of OXT on the correlation between maternal-pup USVs: habituation ($F_{1,20}$=0.25, p=0.62) (**Fig. 2C**), separation ($F_{1,20}$= 1.27, p=0.27) (**Fig. 2D**), reunion ($F_{1,20}$=0.15, p=0.69) (**Fig. 2E**). Finally, our model also assessed the two-way interaction between maternal simple sweep USVs and treatment. IN OXT showed a nonsignificant trend for the two-way interaction between maternal simple sweep USVs and treatment, with IN OXT animals having a more positive slope (non-significant trend) than controls ($F_{1,20}$=3.71, p=0.069) (**Fig. 2C**). Slope for IN OXT-treated mothers did not differ from controls in either the separation ($F_{1,20}$=0.07, p=0.79) (**Fig. 2D**) or reunion ($F_{1,20}$=2.90, p=0.10) (**Fig. 2E**) phases.

Effects of IN OXT on maternal and non-maternal behaviors

To assess the impact of IN OXT on maternal care following separation from pups, we assessed latency to enter the chamber with pups and then calculated change in maternal care from habituation to reunion to measure changes in other types of maternal behavior. This allowed us to determine how IN OXT affected response to pup separation above and beyond any initial effects of IN OXT observed in the habituation phase. In the beginning of the reunion phase, IN OXT mothers showed a non-significant trend for a shorter latency to approach pups ($F_{1,20}$=3.63, p=0.10, ΔR^2=0.16) (**Fig. 3A**). We also tested for main effects of IN OXT in several maternal and non-maternal behaviors. Negative scores mean that the behavior occurred more frequently during habituation and positive scores mean that the behavior occurred more frequently during reunion. Mothers tended to retrieve/carry more during the habituation phase and huddle more during the reunion phase. Mothers given IN OXT did not show any differences in huddling from controls ($F_{1,20}$=0.62, p=0.44) (**Fig. 3B**). However, mothers given IN OXT showed a significantly more positive change in retrieval/carrying behavior from the habituation to the reunion phase ($F_{1,20}$=6.71, p<0.05, ΔR^2=0.26) (**Fig. 3C**). This is driven by high rates of retrieval in control mothers during habituation. High rates of retrieval are associated with less efficient maternal care in mother rats in home and novel environments (Beach & Jaynes, 1956; Smart & Preece, 1973; Aguggia et al., 2013) and virgin and mother mice in novel environments (Dunlap et al., 2020; Liu et al., 2006). Thus, the IN OXT mothers are likely more efficient at maintaining offspring care during this disruption. While mothers from both groups increased huddling behavior post-separation from pups, control mothers decreased retrieval/carrying behavior while IN OXT mothers maintained a consistent level of retrieval/carrying behavior. The net increase in maternal care for IN OXT mothers from habituation to reunion ($F_{1,20}$=*6.6*, *p<0.02*, ΔR^2=0.26) **(Fig. 3D**) is therefore largely due to changes in retrieval behavior.

Figure 3. Rapid effects of OXT on change in maternal and non-maternal behavior from habituation to reunion. *Maternal behaviors:* **(A)** There was a non-significant trend for mothers given IN OXT to have a shorter pup approach latency. **(B)** There were no treatment differences for maternal huddling. **(C)** Mothers given IN OXT showed a significantly greater decrease in retrieval/carrying behavior from the habituation to the reunion phase. **(D)** IN OXT had a net positive effect on total maternal care relative to controls from the habituation to reunion phases. *Non-maternal behaviors:* **(E)** There were no treatment effects for change in autogrooming, **(F)** rearing, or **(G)** freezing. **(H)** There was no net change in non-maternal behaviors from the habituation to reunion. Correlation line collapsing across treatment groups. *p<0.05; #p<0.10.

In order to determine if IN OXT was acting on neural systems that were specific to maternal care, we also tested for main effects of IN OXT on measures of activity (autogrooming, rearing) and stress/anxiety (freezing). From habituation to reunion, there were treatment differences in autogrooming ($F_{1,20}$=0.32, p=0.58) **(Fig. 3E)**, rearing ($F_{1,20}$=1.23, p=0.28) **(Fig. 3F)**, or freezing ($F_{1,20}$=0.03, p=0.85**) (Fig. 3G)**. When summing all activity and stress-related behaviors, there was no net effect on non-maternal behaviors from habituation to reunion ($F_{1,20}$=1.94, p=0.18) **(Fig. 3H)**. In this context, IN OXT is not influencing general, non-maternal behaviors in response to an offspring separation event. The individual means and SEMs for each behavior in each phase are also reported in **S. Table 1.**

S. Table 1. **Means and SEMs of behaviors measured.**

Correlations of maternal care with maternal USVs and pup USVs

To determine whether maternal simple sweeps were associated with specific maternal and investigative behaviors during each of the three testing phases, we correlated number of maternal USVs, which were always simple sweeps, during each phase with the corresponding behavior while controlling for moderation by IN OXT administration. In the habituation phase, maternal simple sweeps positively correlated with licking behavior ($F_{2,20}$=12.04, p<0.007, ΔR^2=0.34) **(Fig. 4B)**. There was also a

nonsignificant trend for a correlation between maternal simple sweeps and huddling ($F_{2,20}$=4.48, p=0.072, ΔR^2=0.18) **(Fig. 4A)**, and no effect associated with retrieving/carrying ($F_{2,20}$=0.44, p=0.51, ΔR^2=0.02) **(Fig. 4C)**. There was also, however, a significant moderation by IN OXT in the relationship between maternal retrievals and maternal simple sweeps. During habituation, mothers given IN OXT carried/retrieved pups less than mothers given saline ($F_{2,20}$=*9.95*, p<0.005, ΔR^2=0.33) **(Fig. 4C)** but note that overall, IN OXT mothers had consistent retrieval levels across the test **(Fig. 2B)**. In the separation phase, there was no correlation between time the mother spent at the mesh divider and maternal simple sweep USVs ($F_{2,20}$=1.2, p=0.58, ΔR^2=0.058) **(Fig. 4D).** Other maternal behaviors could not be assessed because of the mesh divider between mothers and pups. In the reunion phase, maternal simple sweeps positively correlated with maternal retrievals/carrying ($F_{1,20}$=7.65, p<0.037, ΔR^2=0.26) **(Fig. 4G)**. Other maternal behaviors did not correlate with maternal simple sweeps in this phase: huddling ($F_{1,20}$=0.48, p=0.99, ΔR^2=0.02) **(Fig. 4E)** and licking ($F_{1,20}$=0.001, p=0.98, ΔR^2=0.00) **(Fig. 4F)**. Thus, overall, there were associations between maternal simple sweeps and maternal care, but the maternal behavior that correlated with maternal simple sweeps varied depending on context.

Fig. 4. Correlations between maternal simple sweep USVs and maternal care. (A-C) Habituation. (A) There was a nonsignificant trend correlation between maternal simple sweeps and huddling. **(B)** Maternal simple sweeps positively correlated with licking behavior. **(C)** Maternal simple sweeps did not correlate with retrieving/carrying. Mothers given IN OXT carried/retrieved pups less than mothers given saline**. (D) Separation.** There was no correlation between time mothers spent at the mesh divider and maternal simple sweep USVs. **(E-G) Reunion. (E)** Maternal simple sweeps did not correlate with huddling or **(F)** licking. **(G)** Maternal simple sweeps positively correlated with maternal retrievals. Correlation line in black is the average slope across treatment groups; magenta and teal lines are the slopes for the saline and OXT treatments, respectively. *p<0.05; #p<0.10.

Next, we correlated pup whine USVs with specific types of maternal behavior to see if these pup calls were associated with a specific maternal response. During the habituation phase, there was a significant positive correlation between pup whines and maternal huddling ($F_{1,20}$=8.93, p<0.024, ΔR^2=0.30) **(Fig. 5A)**, but not with maternal licking ($F_{1,20}$=3.26, p=0.174, ΔR^2=0.13) **(Fig. 5B)** or retrieval/carrying behavior ($F_{1,20}$=1.14, p=0.26, ΔR^2=0.04) **(Fig. 5C)**. During the separation phase, pup whines did not correlate with time that the mother spent at the mesh gate ($F_{1,20}$=0.89, p=0.36, ΔR^2=0.04) **(Fig. 5D)**. Lastly, during the reunion phase, pup USVs positively correlated with retrieving/carrying ($F_{1,20}$=9.94, p=0.016, ΔR^2=0.34) **(Fig. 5G)** but was not correlated with either huddling ($F_{1,20}$=0.05, p=0.823, ΔR^2=0.003) **(Fig. 5E)** or licking ($F_{1,20}$=0.893, p=0.36, ΔR^2=0.043) **(Fig. 5F)**. Across all correlations of USVs and maternal care, significant correlations occurred, but neither maternal simple sweep USVs nor pup whine USVs consistently correlated with a specific type of maternal care.

Fig. 5. Correlations between pup whine USVs and maternal care. (A-C) Habituation. (A) There was a significant positive correlation between pup whines and maternal huddling. **(B)** There was no correlation between pup whines and maternal licking or (**C)** pup whines and maternal retrieval/carrying behavior. **(D) Separation.** Pup whines did not correlate with time that the mother spent at the mesh gate. **(E-G) Reunion. (E)** There was no correlation between pup whines and maternal huddling or **(F)** pup whines and maternal licking. **(G)** Pup USVs positively correlated with maternal retrieving/carrying. Correlation line in black is the average slope across treatment groups; magenta and teal lines are the slopes for the saline and OXT treatments, respectively. *p<0.05.

Experiment 3: DISCUSSION

Maternal care and communication have lifelong consequences for (Champagne & Meaney, 2006; Champagne & Curley, 2009; Cutuli et al., 2015; Champagne & Curley, 2016). Therefore, it is important to elucidate the proximate hormonal mechanisms that increase maternal care and communication. OXT is known for its potent role in maternal physiology, neurophysiology, and social behavior, but whether OXT could

rapidly change vocal production and behavior in mothers remained unknown. We aimed to fill these knowledge gaps by testing maternal response and effects of IN OXT during a potentially challenging and stressful pup separation paradigm.

We predicted that maternal care activity would be associated with vocal production and that IN OXT would amplify this effect. We found support for this hypothesis as all mothers produced more simple sweep USVs per second during both the habituation phase and reunion phase when mothers had access to tactile pup experience. We speculate that maternal sweeps decreased during the separation phase because mothers no longer had physical access to pups. The relative reduction of maternal simple sweeps during the separation phase may suggest that simple sweeps occur more frequently during social contact or may reflect a difference in internal state. In support of the social salience theory (Shamay-Tsoory & Abu-Akel, 2016; Kemp & Guastella, 2010; Egito et al., 2020; Bartz et al., 2011; Ramsey et al., 2019), IN OXT amplified the effect of the tactile pup experience, leading to an increase above and beyond the observed increase in control mothers during the habituation phase (and a trend in reunion phase). To our knowledge, this is the first study reporting context-dependent IN OXT-moderated changes in USV production that were associated with physical access to a social stimulus (review by Marler & Monari, 2020).

There are several possible functions for simple sweep USV production in the context of maternal care. Increased simple sweep USVs during maternal care may result from a higher state of arousal that is regulated by the autonomic nervous system (Porges, 2009). This is supported by evidence in prairie voles, where vocal features covary with heart rate—longer vocalizations were associated with increased vagal tone and more calm behavior whereas shorter vocalizations were associated with decreased vagal tone and more anxious behavior (Stewart et al., 2015). Alternatively, increases in

simple sweep USVs may be associated with a positive affective state (review by Simola & Granon, 2019). In rats, 50 kHz USVs (similar kHz as California mouse simple sweeps) have been associated with positive affective state (Portfors, 2007), and in California mice, simple sweeps typically occur during affiliative contexts (Pultorak et al., 2018). This adds to a growing body of literature that aims to elucidate California mouse call type with function. Complex sweeps predict pair mate social behavior (Pultorak et al. 2017); syllable USVs are associated with aggression (shorter calls) and female preference (longer calls) (Rieger et al., 2019); both syllable USVs (shorter calls) and barks are associated with increased aggression (Rieger et al., 2019); pup whines can also elicit paternal retrievals (Chary et al., 2015). As expected, we did not observe any syllable vocalizations or barks and only a handful of complex sweeps across all tests as these calls tend to be associated with aggression or adult interactions. Because IN OXT increased maternal simple sweep USVs, and in other studies, IN OXT has increased both vagal tone (Higa et al., 2002) and positive affective state (Dolen et al., 2013; Hung et al., 2017; Baracz & Cornish, 2013), we suggest that maternal simple sweeps are most likely to be associated with changes in affective state.

In contrast to mothers, we did not expect to see a treatment effect with regards to pup vocalizations because only the mothers were treated. As expected, we did not observe differences in pup vocalizations between pup with control mothers and IN OXT mothers. This suggests that effects of IN OXT on the mother do not directly and rapidly influence pup behavior. Overall, pups called the most when first placed into the new chamber with their mothers at the rate of 0.94 whines per second, and then called at a steady rate of 0.33 whines per second during separation and reunion. This suggests that pup vocal production does not vary by social contact in the same way as maternal vocalizations. Instead, pup vocal production may be a function of their thermal

challenge, as indicted by previous studies on rat pup USVs demonstrating pups increase USVs when first separated from their nest and given thermal challenges (Blumberg & Alberts 1990, Blumberg & Sokoloff 2001). After losing a certain amount of heat energy, number of pup whines produced may decrease to balance energy conservation and venous blood return to the heart.

We predicted a correlation between maternal simple sweeps and pup whines. We expected that the correlation between mother-pup USVs would be driven by the mother's vocalizations and/or behavior, as the pups' ear canals have not opened at PND 2-3, likely rendering them deaf (Fox, 1965). Mother and pup USVs did not correlate during habituation, possibly because pup whine USVs were highest during this phase (**Fig. 2B**) or possibly because the removal from the home cage at the start of the test disrupted the coordination between mothers and pups. There was also a nonsignificant trend for IN OXT to improve the correlation between mother and pup USVs. If the removal from the home cage at the start of the test disrupted the coordination between mothers and pups, IN OXT may be mediating this negative effect by increasing the salience of pup whine stimuli (Valtcheva & Froemke, 2019), allowing mothers to more effectively and efficiently cope with the challenge. During reunion, we found support for our initial prediction: maternal simple sweeps and pup whines positively correlated. Mothers may be more responsive to pup whines during the reunion phase because the pups have been without care for a longer period of time. An alternative explanation is that this synchrony occurred out of necessity because, with the second chamber open, the mice had double the space in the reunion phase compared to the habituation phase. In lambs and ewes, mother-offspring vocalizations have been shown to be important for recognition and location purposes (Sèbe et al., 2007; Searby & Jouventin, 2003) with young lambs only being able to distinguish their

mother via low frequency calls but using high frequency calls during vocal exchanges (Sèbe et al., 2010). Mother-offspring vocalizations that are contingent on each other's vocalizations have also been observed across cultures in humans (Bornstein et al., 2015). To our knowledge, this is the first reporting of correlations between maternal USVs and offspring USVs in an animal model.

We also wanted to explore whether maternal simple sweeps or pup whines are correlated with specific types of maternal behavior. During habituation, USVs from both mothers and pups were associated with greater maternal care but were not associated with the same maternal behavior; maternal simple sweeps positively correlated with maternal licking behavior and pup whines positively correlated with maternal huddling behavior. During reunion, we saw a different relationship between USVs and maternal behavior, suggesting that if pup calls and not maternal responsiveness are driving these correlations, the pup whines are not eliciting a specific type of maternal care. During reunion, both maternal simple sweeps and pup whines positively correlated with maternal retrieval/carrying behavior. This suggests that after a separation event, pup whines may drive maternal retrieval/carrying behavior, similar to the finding previously reported in fathers (Chary et al., 2015], and that maternal simple sweeps may be a reliable signal supporting maternal responsiveness. Studies in the literature show support for this effect being driven by maternal responsiveness in mice (D'Amato et al., 2005; D'Amato & Populin, 1987) and other studies show that the effect can also be driven by the pups (Curry et al., 2013; Wöhr, et al., 2010).

Finally, based on the prosocial effects of OXT, we predicted that an acute dose of IN OXT would increase maternal care. Our results show that in the context of our paradigm, IN OXT maintains maternal care rather than overtly increasing it. Mothers given IN OXT showed consistent number of retrievals pre- and post-separation while

control mothers significantly decreased number of retrievals performed post-separation, leading to greater maintenance of total maternal care for IN OXT mothers. These findings are consistent with other studies in the literature in sheep (Keverne & Kendrick, 1992), mice (Rich et al., 2014), rats (Francis et al., 2002; Bosch & Neumann, 2012), and humans (Feldman et al., 2010) but highlight that OXT can also increase maternal behavior within minutes of administration. We did not find that IN OXT led to a significant decrease in latency to approach pups after separation, but we found a non-significant trend. This supports previous findings in the literature that OXT has been associated with a reduction in the latency to retrieve or start maternal behavior (Pedersen et al., 2006; Liu et al., 2019) though several other studies have not reported an effect of OXT on latency to retrieve pups (Watarai et al., 2020; Rich et al., 2014; Macbeth et al., 2010). Notably, we did not find any simple effects of IN OXT on nonmaternal behaviors during the habituation, separation, or reunion phases. This suggests that IN OXT specifically influences maternal behavior and not general activity (autogrooming, rearing) or anxiety (freezing) in female California mice. If IN OXT is dampening the stress/anxiety response, it is specific to maternal anxiety. This is important to note because one hypothesis regarding the effects of IN OXT is that it primarily functions as an anxiolytic agent versus a pro-social capacity across a variety of contexts and species (Yoshida et al., 2009; Waldherr & Neumann, 2007; de Oliviera et al., 2012). In certain contexts, OXT can also have anxiogenic effects (Guzman et al., 2013; Duque-Wilckens et al., 2018). However, we do not find that OXT is promoting anxiety in this context. Our results suggest that in this context, IN OXT has a specific effect on maternal care behavior that is not explained by differences in the non-maternal activities related to activity or stress.

In summary, these data are consistent with the concept that IN OXT rapidly and selectively increases maternal vocalizations and maintains maternal care. This data also highlights the importance of social contact for normal communication and care and enhancement by IN OXT. Overall, we propose that higher levels of OXT in mothers function to increase efficiency and maintain maternal care, particularly during challenges.

Experiment 3: REFERENCES

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Chapter 2: SUMMARY

The goal of this chapter was to elucidate the role OXT and AVP in pair bond formation and determine the role of OXT in parent-offspring bond formation. Overall, the results from these experiments suggest that IN AVP may inhibit bond formation in females due to increased pre-courtship aggression. In contrast, IN OXT decreased pre-courtship aggression in males and IN AVP did not influence male pre-courtship aggression. This suggests that activation of AVP system in females may inhibit pair bond formation whereas activation of the OXT system in males may promote pair bond formation. With regards to parent-offspring bonds, activation of the OXT increased both maternal care and communication toward offspring. However, in fathers, activation of the OXT led to a much subtler effect—increasing paternal responsiveness but influencing overall paternal care or communication.

Chapter 2: FIGURES & TABLES

Experiment 1:

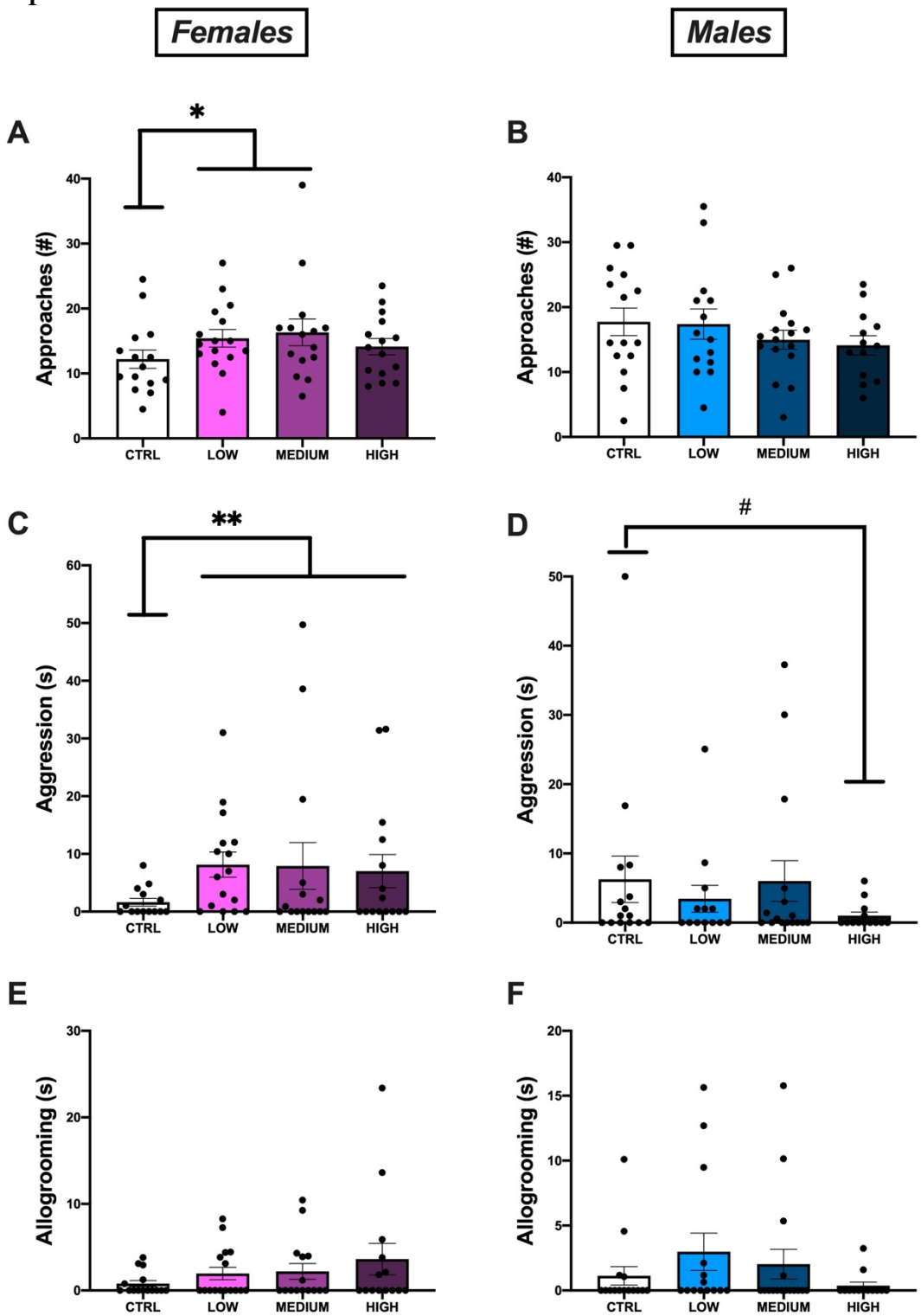

Figure 1. Rapid effects of IN AVP treatment on social behavior during a pre-courtship aggression test.

Experiment 2:

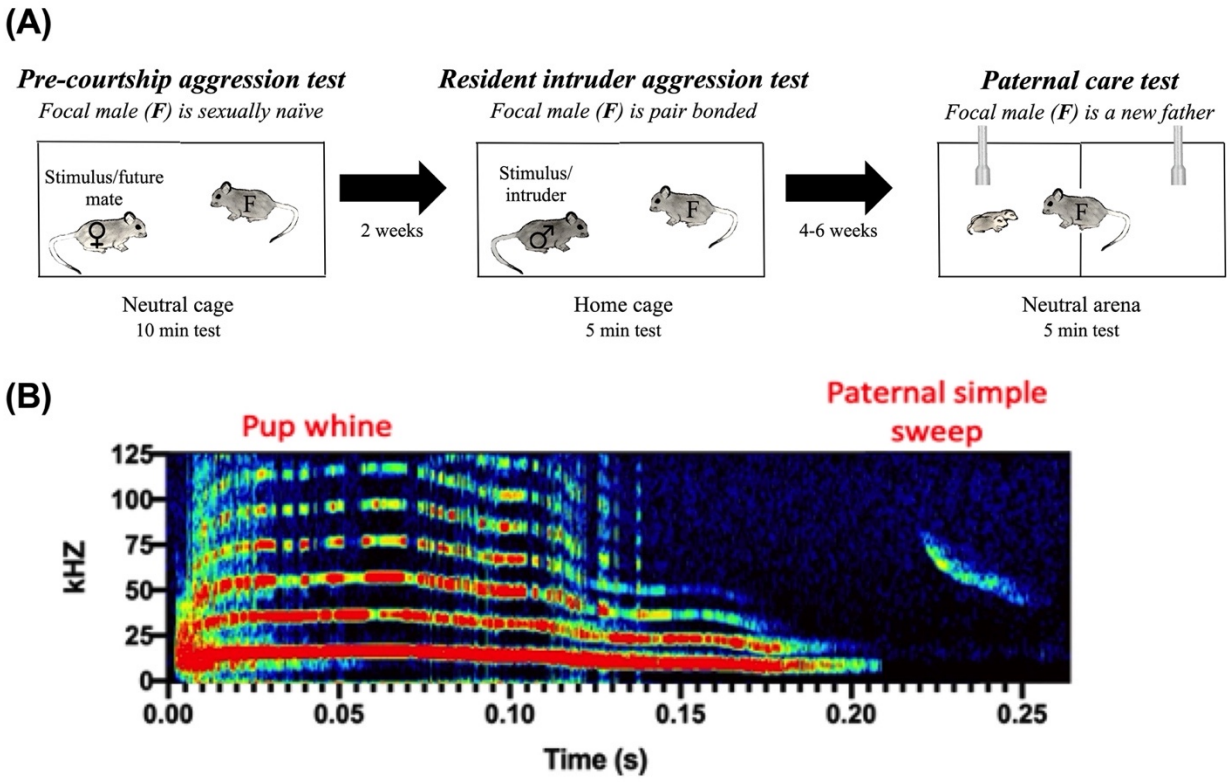

Figure 1. (A) Schematic for experimental design. (B) Ultrasonic vocalizations (USVs) on a spectrogram.

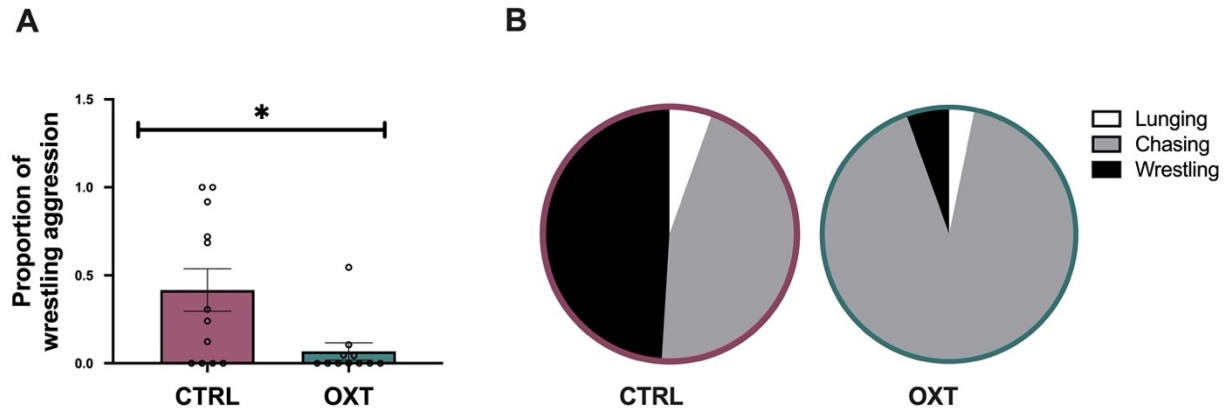

Figure 2. Rapid effects of IN OXT on aggression during a pre-courtship aggression test.

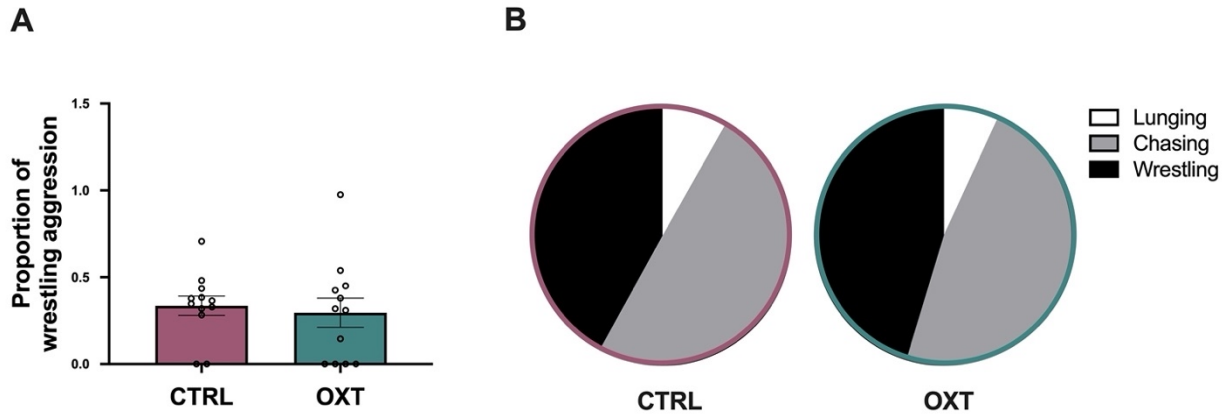

Figure 3. Rapid effects of IN OXT on aggression during a resident-intruder aggression test.

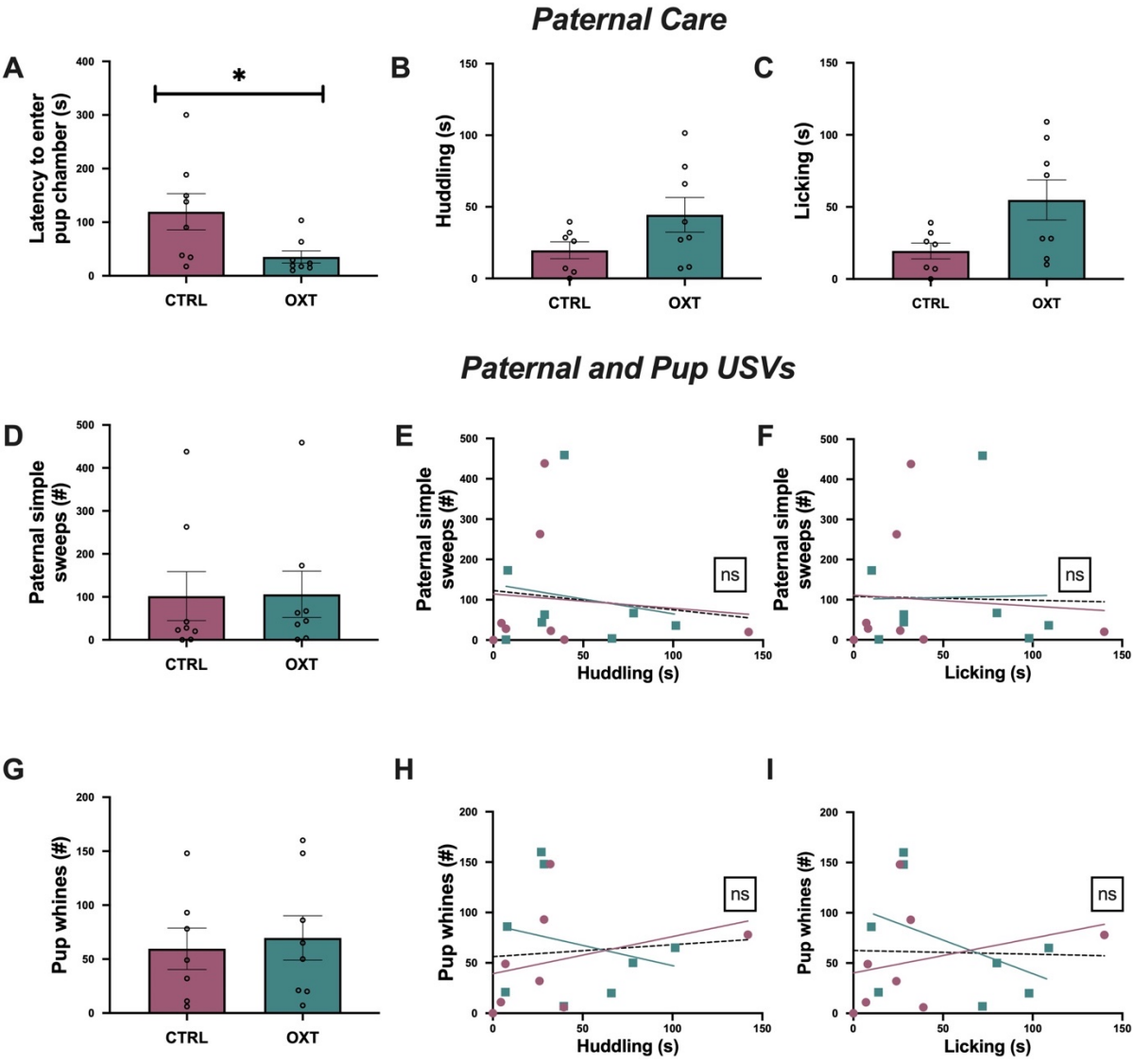

Figure 4. Rapid effects of IN OXT on paternal care and paternal and pup USVs.

Behavior	**Type of test behavior was measured in**	**Description**
Lunging	Pre-courtship, resident intruder	When the mouse lifts both front paws up and quickly extends them out toward another mouse.
Chasing	Pre-courtship, resident intruder	When the mouse runs behind the stimulus mouse at a high rate of speed and follows with less than a tail's length distance.
Wrestling	Pre-courtship, resident intruder	When the mouse and the stimulus mouse have physical contact accompanied by at least two of the following: putting body weight on top of the other mouse, tail thrashing, biting, audible vocalizations.
Body sniffing	Pre-courtship, resident intruder	Sniffing that occurs within a whisker's length of segment of the body other than the anogenital region.
Anogenital sniffing	Pre-courtship, resident intruder	Sniffing that occurs within a whisker's length of the anogenital region of another mouse.
Autogrooming	Pre-courtship, resident intruder, paternal care	Licking, biting, or scratching their own body, at any location.
Rearing	Pre-courtship, resident intruder, paternal care	Both front paws lift up towards the wall of the cage. Counted as an additional event if front paws touch the ground again and lift back up.
Freezing	Paternal care	When the mouse does not move at all but has eyes alert/open. Usually ears are up, and they do not make any other movements. Considered a second event once mouse moves for at least 2 seconds and before freezing again.
Latency to approach pups	Paternal care	Amount of time that it takes the father to first reach his pups after the partitioned mesh door is opened.
Huddling	Paternal care	Amount of time that the father spends over the pups. At least 50% of the

		body of pup must be covered by the father to count.
Licking and grooming	Paternal care	Amount of time that the father spends licking and grooming pups.
Retrieving/carrying	Paternal care	Amount of time that the father spends with a pup in his mouth while locomoting in the chamber. It is counted regardless of whether locomotion is toward or away from home bedding and cotton ball.

S. Figure 1. Ethogram with description of behaviors measured in each test.

Behavior	**Treatment**	**Mean**	**SEM**
Lunging	CTRL	1.29	± 1.36
	OXT	0.45	± 0.37
Chasing	CTRL	10.76	± 3.73
	OXT	12.80	± 3.82
Wrestling	CTRL	11.58	± 6.22
	OXT	0.77	± 0.50
Body sniff	CTRL	36.38	± 10.00
	OXT	58.59	± 10.90
Anogenital sniff	CTRL	38.33	± 11.22
	OXT	47.82	± 12.74
Autogrooming	CTRL	22.08	± 8.55
	OXT	26.09	± 8.50
Rearing	CTRL	60.42	± 11.93
	OXT	73.91	± 18.24

S. Table 1. All behaviors measured during the pre-courtship aggression test.

Experiment 3:

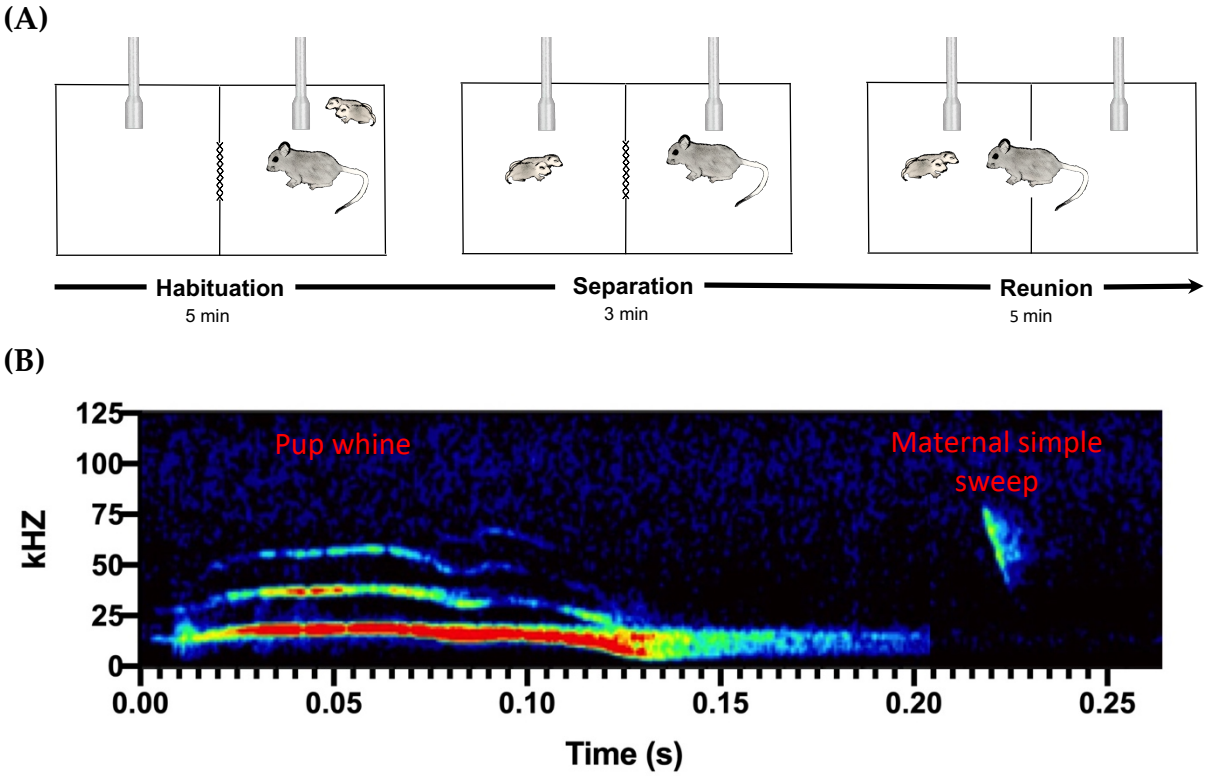

Figure 1. (A) Schematic for experimental design. (B) Ultrasonic vocalizations (USVs) on a spectrogram.

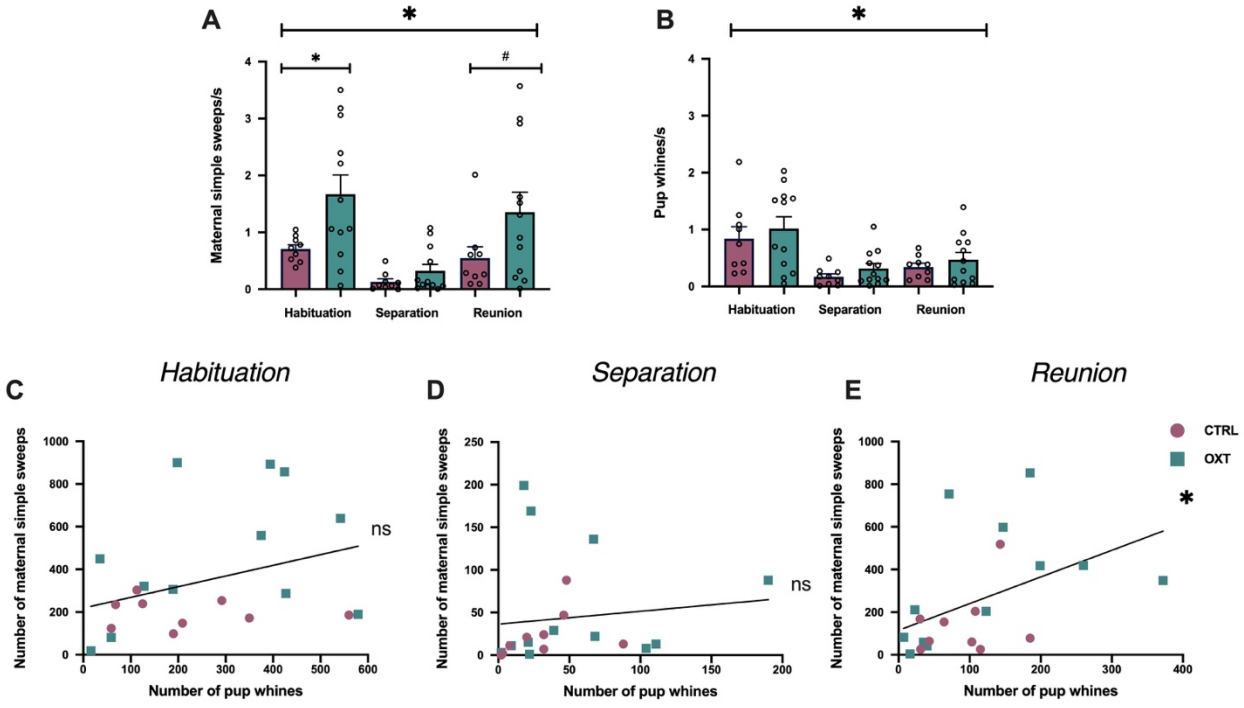

Figure 2. Rapid effects of IN OXT on USV production in mothers and pups.

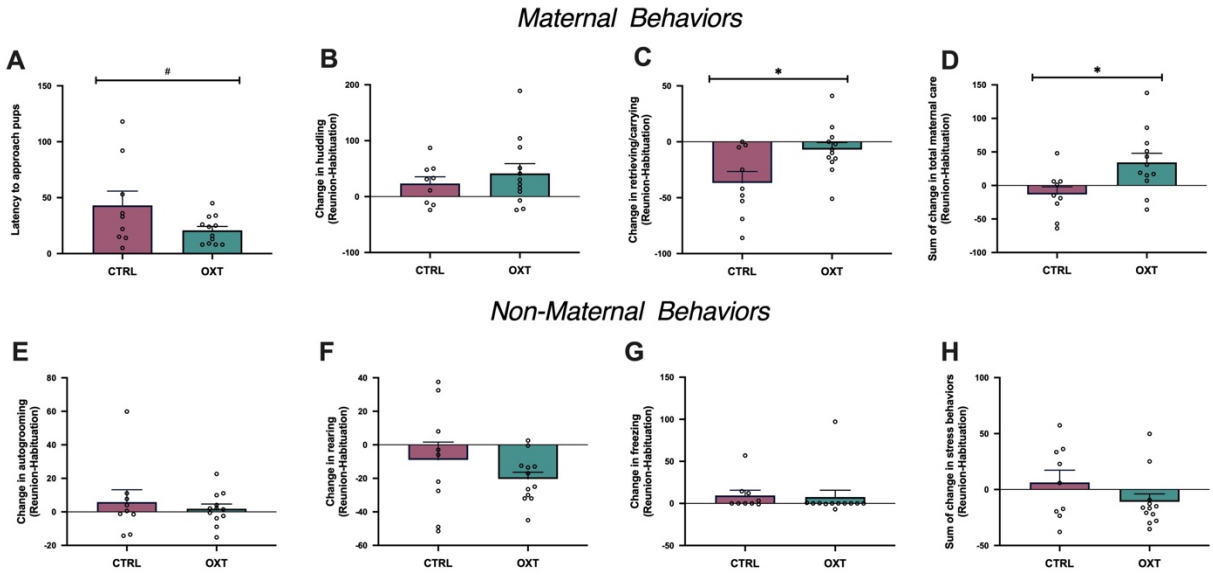

Figure 3. Rapid effects of OXT on change in maternal and non-maternal behavior from habituation to reunion.

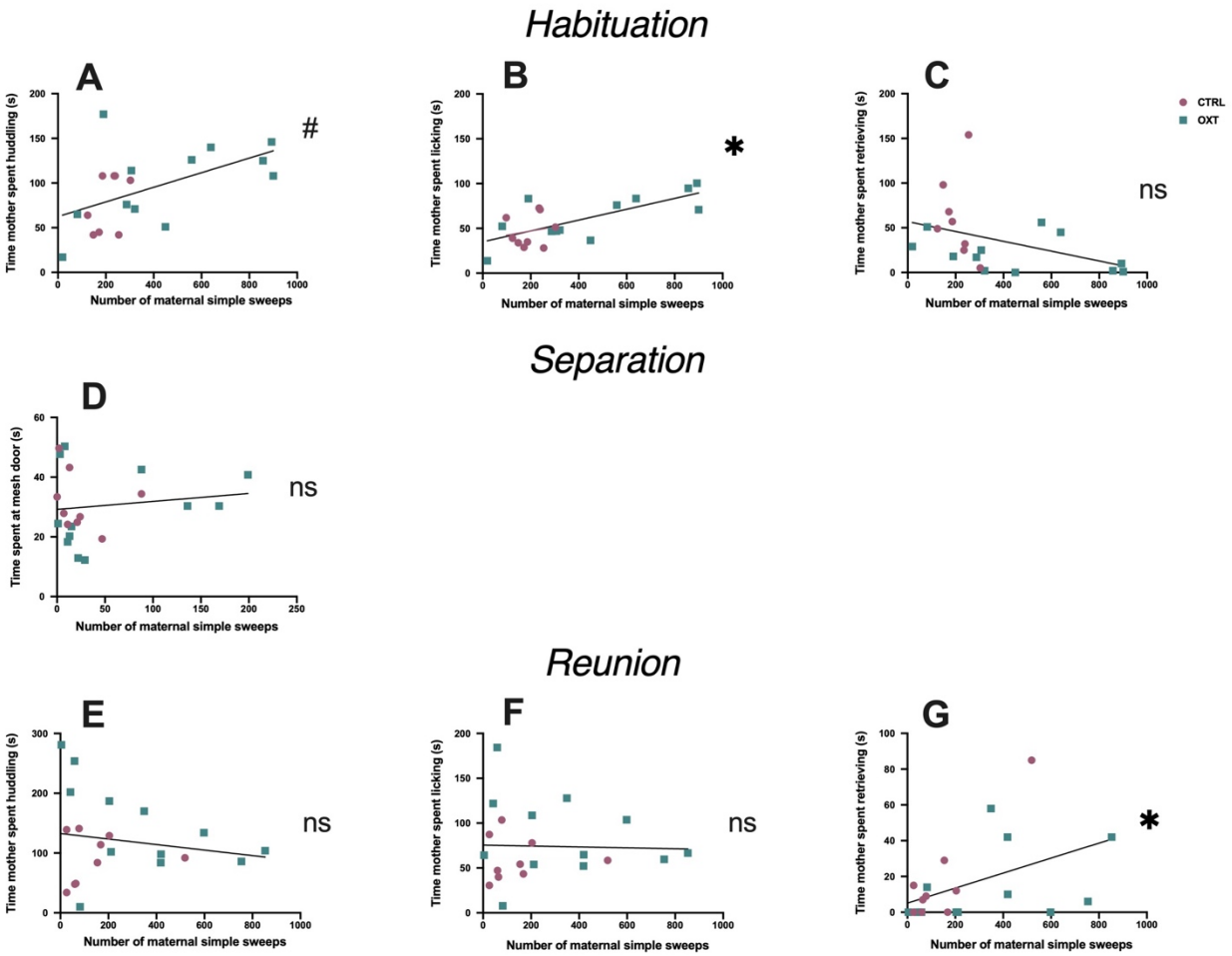

Fig. 4. Correlations between maternal simple sweep USVs and maternal care. (A-C) Habituation.

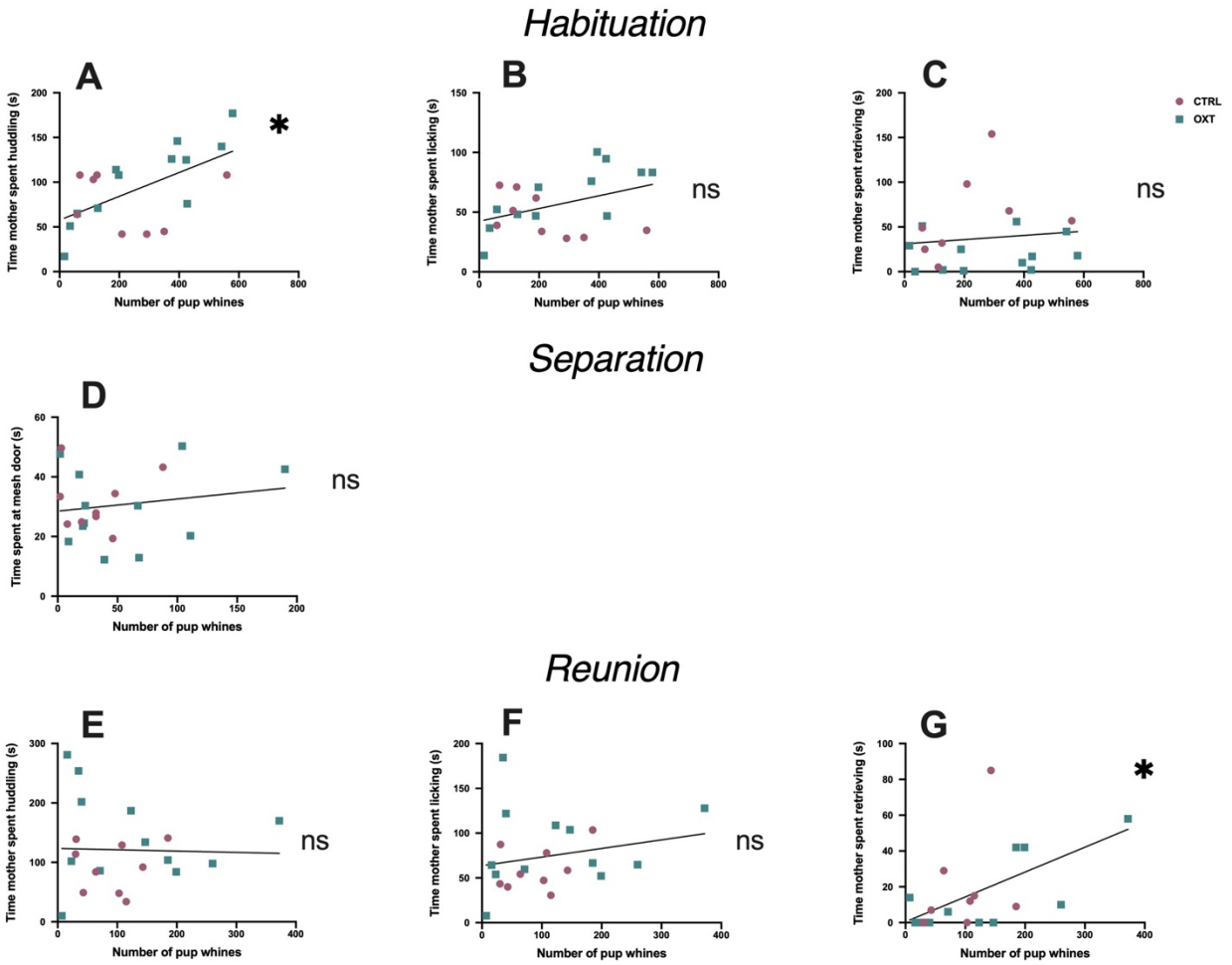

Fig. 5. Correlations between pup whine USVs and maternal care. (A-C) Habituation.

Behavior	Maternal/Non-maternal	Description
Huddling	Maternal	Amount of time that the mother that the mother spends over the pups. At least 50% of the body of pup must be covered by the mother to count.
Licking and grooming	Maternal	Amount of time that the mother that the mother spends licking and grooming pups.
Retrieving/carrying	Maternal	Amount of time that the mother with a pup in her mouth while locomoting in the chamber. Is counted regardless of whether locomotion is toward or away from home bedding and cotton ball.
Time spent at mesh	Maternal	Amount of time that the mother spends in front of (within a whisker's length distance) or climbing on the mesh door that partitions her chamber and the pup chamber.
Latency to approach pups	Maternal	Amount of time that it takes the mother to first reach her pups after the partitioned mesh door is opened.
Autogrooming	Non-maternal	Licking, biting, or scratching their own body, at any location.
Freezing	Non-maternal	When the mouse does not move at all but has eyes alert/open. Usually their ears are up, and they do not make any other movements. Considered a second event once the mouse moves for at least 2 seconds and before freezing again.
Rearing	Non-maternal	Both front paws lift up towards the wall of the cage. Counted as an additional event if front paws touch the ground again and lift back up.

S. Figure 1. Ethogram with description of behaviors measured.

Behavior	Phase	Treatment	Mean	SEM
Huddling	Habituation	CTRL	77.50	± 10.69
		OXT	101.33	± 13.23
	Reunion	CTRL	92.22	± 13.78
		OXT	142.67	± 22.57
Licking and grooming	Habituation	CTRL	46.83	± 5.95
		OXT	62.76	± 7.50
	Reunion	CTRL	60.30	± 8.08
		OXT	84.67	± 13.43
Retrieving/carrying	Habituation	CTRL	61.00	± 15.69
		OXT	21.33	± 6.73
	Reunion	CTRL	17.44	± 9.00
		OXT	14.33	± 6.00
Time spent at mesh	Separation	CTRL	31.52	± 3.25
		OXT	29.48	± 3.80
Latency to approach pups	Reunion	CTRL	38.00	± 12.96
		OXT	14.42	± 2.58
Autogrooming	Habituation	CTRL	7.81	± 1.84
		OXT	7.72	± 1.54
	Separation	CTRL	0.41	± 0.30
		OXT	2.34	± 1.83
	Reunion	CTRL	12.81	± 6.51
		OXT	9.60	± 2.49
Freezing	Habituation	CTRL	0.11	± 0.11
		OXT	0.71	± 0.58
	Separation	CTRL	1.26	± 0.87
		OXT	0.62	± 0.39
	Reunion	CTRL	9.64	± 6.20
		OXT	8.27	± 8.09
Rearing	Habituation	CTRL	52.44	± 4.48
		OXT	48.33	± 5.74
	Separation	CTRL	45.89	± 4.30
		OXT	42.96	± 5.04
	Reunion	CTRL	37.61	± 7.27
		OXT	27.96	± 7.67

S. Table 1. Means and SEMs of behaviors measured.

Chapter 3: The role of oxytocin in the maintenance of family-unit bonds and communication in California mice (*Peromyscus californicus*)

Experiment 1: Characterize the vocalizations and parental care strategies of mother and father California mice during a pup separation challenge

Experiment 2: Determine the effects of oxytocin and vasopressin on the vocalizations and parental care strategies of mother and father California mice during a pup separation challenge

ABSTRACT

An advantage to forming social bonds is an enhanced ability to coordinate behavior with another individual in order to maximize efficiency or accomplish more complex tasks. In this chapter, we assess the association between ultrasonic vocalizations produced by parents and their parental care strategy after a nest disruption. We hypothesized that vocalizations may be used to help pairs coordinate behavior and care for offspring. In experiment 1, we found support for this hypothesis. Number of simple sweeps produced was greatest when both parents were present and there was a nest disruption when they had very young offspring compared to when parents were alone or when there was a nest disruption, but pups were near weaning age. Frequency of complex sweeps and syllable vocalizations calls did not differ by group composition. We also found an association between time spent engaged in biparental, uniparental, and non-parental care strategies and vocal production. Simple sweeps positively correlated with time spent engaged in a uniparental care strategy; SV length negatively correlated with time using a biparental care strategy; complex sweeps positively correlated with number of times parents switch parental care strategy. In experiment 2, we hypothesized that giving parents an acute pulse of intranasal oxytocin or vasopressin would alter their vocal communication and parental care. We predicted that OXT would enhance communication and parental care whereas vasopressin would disrupt parental communication and care. We found that IN OXT decreased parental SVs, increased time pairs spent not doing any parental care and altered division of labor—increasing maternal huddling and decreasing paternal huddling. IN AVP did not influence production of USVs or parental care strategy but did increase total parent retrievals. These results suggest that an acute pulse of OXT may shift the burden of parental care on mothers and that an acute pulse of AVP decreases parental efficiency.

What Is New:

- Number of simple sweeps produced depends on family unit composition. Female and male pairs make more simple sweep calls when in the chamber together, regardless of pup presence. Complex sweeps and SV number and length were not influenced by family unit composition.
- When caring for pups after a nest disruption, simple sweeps positively correlated with time spent engaged in a uniparental care strategy; SV length negatively correlated with time using a biparental care strategy; complex sweeps positively correlated with number of times parents switch parental care strategy.
- IN OXT decreases number of SVs produced, time spent engaged in parental care, and biases the burden of parental care toward mothers
- IN AVP does not influence vocal production but may decrease parental efficiency by increasing total parent retrievals

Experiments 1 & 2: INTRODUCTION

The maintenance of social bonds in family units is critical for the ability to efficiently coordinate behavior and accomplish tasks. In monogamous and biparental species, successfully raising offspring requires contributions from both parents to maximize offspring viability into adulthood (Gubernick & Teferi, 2000). However, less is known about how parents communicate and coordinate behavior to efficiently accomplish this task. Two potential mechanisms that may enhance social perception and lead to more efficient coordination are through enhanced communication and/or changes to circulating hormone levels.

Vocalizations are costly to produce—costing not only energy but also increasing chance of predation—and therefore must serve an important function in the behavior of the mouse. At the most basic level, this information may be relaying information regarding the sender's internal state (stress vs. pleasure) as seen in rat vocalizations (Portfors, 2007). However, some of the vocalizations may also function to communicate directives to their partner, indicating where their partner should go or what their partner should do as in species like meerkats and prairie dogs (Townsend et al., 2011; Rauber & Manser, 2018; Kiriazis & Slobodchikoff, 2006). Pairs with stronger bonds may have more efficient communication, and this may lead to greater fitness outcomes for their pups. Most studies on vocalizations focus on their role in courtship and mating (D'amato, 1991; Egnor & Seagraves, 2016; Portfors & Perkel, 2014; Kanno & Kikusui, 2018). Other studies have also examined the role infant USVs in eliciting care from their parents (Litvin et al., 2007; Zeskind et al., 2011; Mogi et al., 2017), but no studies to date have examined the role of parent and offspring call during a parental care in biparental species.

In biparental species, having two parents involved in infant care can have several advantages. For example, one parent can be feeding or providing thermoregulation for offspring while the other parent can forage for food or defend against a predator or conspecific. Previous studies in the lab have examined the division of labor when bonded pairs are challenged with a resident intruder. These studies have shown that IN OXT drives pairs to use a more similar strategy (Monari et al., 2021) and that shorter SVs predict more aggressive behavior toward a resident-intruder (Rieger et al.,2019). There are three possible parental care strategies: biparental care where both parents are actively caring for the pups; uniparental care where only one parent is caring for the pups and the other parent is engaged in another behavior such as territory vigilance or eating; and no parental care where both parents are engaged in another behavior such as territory vigilance or eating. Most pairs will use a combination of all three strategies throughout the day but may be inclined to use one more than the other under certain contexts. We hypothesize that hormone levels may influence how much time parents spend in each parental care strategy.

The neuropeptide hormones oxytocin (OXT) and vasopressin (AVP) are two neuromodulators that may be involved in the maintenance of social bonds. In particular, OXT is known for its role in decreasing anxiety (Windle et al., 1997; Missig et al., 2010; Peters et al., 2014) and enhancing the social salience of cues (Shamay-Tsoory & Abu-Akel, 2016). AVP, on the other hand, is known for its role in increasing anxiety (Bielsky et al., 2004; Neumann & Landgraf, 2012). Based on this, we predict that OXT will enhance parental communication and lead to more efficient parental care while AVP will disrupt parental communication and lead to less efficient parental care.

Experiment 1: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We used 18 pairs of *P. californicus* aged 6-12 months and their offspring (litter numbers 3-10). Family units were -housed in breeding cages with parents and offspring (48 × 27 × 16 cm) under a 14L: 10D light cycle with lights off at 1:00pm. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Behavioral Testing

Using a within-subjects design to assess the relationship between family group composition and vocal production, family units underwent five different behavioral conditions. On postnatal day 2-4 parents were assigned to either a "pups absent" or "pups present" trial first which would last 5 min. After the first trial, parents were tested in second trial type. Two days later, on postnatal day 4-6, mothers and fathers were taken from their home cage and tested separately in "mom only" and "dad only" trials that last 5 min each. It was randomly assigned which parent would be tested first. Lastly, on postnatal day 24-26, parents were tested in a final "pups absent" trial. For a schematic detailing the experimental design, see **Fig. 1**.

During the "pups present" trial, parental care strategy was measured. Time spent engaged in a biparental care strategy was recorded when both parents were engaged in care of offspring, regardless of type of care (huddling, grooming, retrieving). Time spent engaged in a uniparental care strategy was recorded when only one parent was engaged in care of offspring, regardless of type of care (huddling, grooming, retrieving) and which parent was caring for offspring. Time spent engaged in a non-

parental care strategy was recorded when neither parent was engaged in care of offspring.

Behavioral Analysis

Our goal in this experiment was to determine if vocalizations correlated with parental care strategy. Mothers and fathers can either choose to both care for the pups, have one parents care for the pups, or neither parent care for the pups. Here, we break this behavioral decision-making into three different strategies: biparental care (both parents engaged in parental care but can be doing the same of different tasks), uniparental care (only one parent is caring for pups), or no parental care (neither parent caring for pups). Each parental dyad spends at least some engaged in each of the three strategies, so we measured time spent in biparental, uniparental, and no parental care as a continuous measure for each pair. Parental behaviors included huddling, licking, and retrieving. Uniparental care was grouped into one measure, regardless of whether the mother or father was the caregiver.

Ultrasonic Vocalization Analysis

Techniques used for recording were similar to those previously used in our laboratory (Pultorak et al., 2017; Rieger et al., 2018). USVs were collected using two Emkay/Knowles FG series microphones capable of detecting broadband sound (10–120 kHz). One microphone was placed at the center of the chamber. Microphone channels were calibrated to equal gain (− 60 dB noise floor). We used RECORDER software (Avisoft Bioacoustics) to produce triggered WAV file recordings (each with a duration of 0.5 s) upon the onset of a sound event that surpassed a set threshold of 5% energy change (Kalcounis-Rueppell et al., 2010). Recordings were collected at a 250 kHz

sampling rate with a 16-bit resolution. Spectrograms were produced with a 512 FFT (Fast Fourier Transform) using Avisoft-SASLab Pro sound analysis software (Avisoft Bioacoustics). In these recordings, we identified pup whines, simple sweeps, complex sweeps, and syllable vocalizations. Pup whines have a peak frequency around 20 kHz (Kalcounis-Rueppell et al., 2018; Johnson et al., 2017) and the typical downward modulation at the end of the call often distinguishes these calls from adult syllable vocalizations (Nathaniel Rieger, Jose Hernandez, & Catherine Marler, unpublished). The lower frequencies in the pup whine can also be heard by human ears (below the ultrasonic range). Simple sweeps were categorized by short downward-sweeping vocalizations that sweep through multiple frequencies, typically between 80 kHz and 40 kHz (Kalcounis-Rueppell et al., 2018). Complex sweeps were categorized by being above 90 kHz and having multiple inflection points (Kalcounis-Rueppell et al., 2018). Syllable vocalizations were categorized by long-duration vocalizations with multiple harmonics that occur in bouts, or syllables, typically between 20 kHz and 40 kHz (Kalcounis-Rueppell et al., 2018). It is extremely rare for pups to produce simple sweeps, complex sweeps, or syllable vocalizations during PND 0-4 (Rieger, N. S., Hernandez, J. B., and Marler, C. M., unpublished), therefore, we categorized these calls as adult-only calls. Because of their different spectrogram and acoustic properties, all USVs could be categorized and counted by combined visual and auditory inspections of the WAV files (sampling rate reduced to 11,025 kHz, corresponding to 4% of real-time playback speed).

Data Analysis

Statistical analyses were conducted using the program GraphPad Prism. Significance level was set at $p<0.05$ for all analyses and all tests were two-tailed.

Repeated measures ANOVAs were used to assess differences in call type frequency in different social paradigms (i.e. "pups present", "pups absent", "mom only", and "dad only" trials). To account for differences in number of adults between trials, number of calls per adult in the chamber was used. To assess correlations between parental care strategy and vocalization type, simple linear regression models were conducted.

Experiment 1: RESULTS

Effects of social environment on call types frequency

In order to assess whether vocal production changed as a result of social group composition, a one-way ANOVA with multiple comparisons was conducted. Simple sweep production was greatest when both parents were present. There was no difference in the young pups vs. no young pups trial (p=0.99). However, more simple sweeps per adult were recorded in the no young pups vs mom only trial (p<0.001), dad only trial (p<0.001), and no old pups trial (p<0.001) (**Fig. 2A**). There were no differences in the number of complex sweeps per adult produced across the five trials (p=0.31) (**Fig. 2B**). There were no differences in the number of complex sweeps per adult produced across the five trials (p=0.31) (**Fig. 2B**). There were no differences in the number of SVs per adult produced across the five trials (p=0.64) (**Fig. 2C**). There were no differences in the length of SVs per adult produced across the five trials (p=0.70) (**Fig. 2D**).

Figure 2. (**A**) Simple sweep production was greatest when both parents were present. There were no differences in simple sweep output in the young pups vs. no pups trial, but there were significantly fewer sweeps in the mom only, dad only, and no old pups trials than the no young pups trial (**B**) Complex sweep production did not differ with social composition. (**C**) SV production did not differ with social composition. (**D**) SV length did not differ with social composition. ***p<0.0001, **p<0.001.

Correlations between parental care strategy and USVs

In order to assess the relationship between parental care strategy and USV type, linear regressions were conducted. Syllable vocalization length was negatively correlated with time spent in a biparental care strategy, ($F_{1,12}$=5.68, p<0.05, ΔR^2=0.32) (**Fig. 3A**), but did not correlate with time spent engaged in a uniparental care strategy (p=0.33) (**Fig. 3B**), no parental care strategy (p=0.13) (**Fig. 3C**), or number of times pairs switched their parental care strategy (p=0.25) (**Fig. 3D**). Simple sweeps showed a nonsignificant trend for being correlated with time spent engaged in a uniparental care strategy ($F_{1,15}$=4.03, p=0.06, ΔR^2=0.21) (**Fig. 4B**), but did not correlate with time spent in a biparental care strategy, (p=0.32) (**Fig. 4A**), no parental care strategy (p=0.51) (**Fig. 4C**), or number of times pairs switched their parental care strategy (p=0.39) (**Fig. 4D**). Complex sweeps were positively correlated with number of times pairs switched their parental care strategy ($F_{1,15}$=6.98, p<0.05, ΔR^2=0.32) (**Fig. 5D**), but were not correlated with time spent in a biparental care strategy, (p=0.19) (**Fig. 5A**), uniparental care strategy (p=0.23) (**Fig. 5B**), or no parental care strategy (p=0.82) (**Fig. 5C**).

Figure 3. (**A**) Biparental care and SV length were negatively correlated. (**B**) Uniparental care and SV length were not correlated (**C**) No parental care and SV length were not correlated (**D**) Number of times switching parental care strategy and SV length were not correlated.

Figure 4. (**A**) Biparental care and simple sweep production were not correlated. (**B**) Uniparental care and simple sweep production were positively correlated (**C**) No parental care and simple sweep production were not correlated (**D**) Number of times switching parental care strategy and simple sweep production were not correlated.

Figure 5. (**A**) Biparental care and complex sweep production were not correlated. (**B**) Uniparental care and complex sweep production were not correlated (**C**) No parental care and complex sweep production were not correlated (**D**) Number of times switching parental care strategy and complex sweep production were positively correlated.

Experiment 2: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We used 34 pairs of *P. californicus* aged 6-12 months and their offspring (litter numbers 2-10). Family units were -housed in breeding cages with parents and offspring (48 × 27 × 16 cm) under a 14L: 10D light cycle with lights off at 1:00pm. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Pairs were randomly assigned to be administered saline control (N=12), 0.5 IU/kg IN OXT (N=11), or 0.5 IU/kg IN AVP (N=11). Both parents in each treatment group were administered the IN dose.

Intranasal Oxytocin and Vasopressin Preparation

Both father and mother were infused intranasally with 25 uL of either sterile saline, IN OXT (0.5 IU/kg), or IN AVP (0.5 IU/kg) (Bachem, Torrance, California) (Guoynes & Marler, 2021). The IN OXT dose is equivalent to doses used in other experiments in this dissertation (see Chapters 2 and 4). IN OXT and IN AVP were dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20^{o}C. Treatments, including saline control, were defrosted just prior to administration. A blunt cannula needle (33-gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was attached to cannula tubing, flushed, and filled with the compound, then attached to an airtight Hamilton syringe (Bachem, Torrance, California). The animal was scruffed and 17.5 uL of compound was expelled dropwise through the cannula needle and allowed to absorb into the nasal mucosa (~10-20 seconds). One person conducted all IN administrations throughout the entire procedure to maintain consistency in handling and IN infusion.

Behavioral Testing

Using a within-subjects design to assess the relationship between family group composition and vocal production, family units underwent two different behavioral conditions. On postnatal day 2-4 one parent was randomly assigned a black tail mark and the other parent was randomly assigned a red tail mark to distinguish the parents apart for behavioral analysis. Next, both parents were infused intranasally with 25 uL of either sterile saline, IN OXT (0.5 IU/kg), or IN AVP (0.5 IU/kg). Pairs were given the same treatment (ex. both the mother and father were given IN OXT) in their home cage. Five minutes after IN dose, both parents were removed from the home cage and placed in the testing chamber without their pups for a trial that lasted 5 min. This first trial is referred to as the "pups absent" trial. After the "pups absent" trial, the pair's pups were immediately placed in the testing chamber with the parents and tested in a second 5 min trial. This second trial is referred to as the "pups present" trial.

During the "pups present" trial, parental care strategy was measured. Time spent engaged in a biparental care strategy was recorded when both parents were engaged in care of offspring, regardless of type of care (huddling, grooming, retrieving). Time spent engaged in a uniparental care strategy was recorded when only one parent was engaged in care of offspring, regardless of type of care (huddling, grooming, retrieving) and which parent was caring for offspring. Time spent engaged in a non-parental care strategy was recorded when neither parent was engaged in care of offspring. Parental behaviors such as huddling, grooming, and retrieving were also scored for both mothers and fathers (scored by red tail and black tail to conceal sex of the parent).

Behavioral Analysis

As in experiment 1, we broke down behavioral decision-making into three different strategies: biparental care (both parents engaged in parental care but can be doing the same of different tasks), uniparental care (only one parent is caring for pups), or no parental care (neither parent caring for pups). Each parental dyad spends at least some engaged in each of the three strategies, so we measured time spent in biparental, uniparental, and no parental care as a continuous measure for each pair. Parental behaviors included huddling, licking, and retrieving. Uniparental care was grouped into one measure, regardless of whether the mother or father was the caregiver. Huddling, grooming, and retrieving were scored separately for each parent.

Ultrasonic Vocalization Analysis

Techniques used for recording were similar to those previously used in our laboratory (Pultorak et al., 2017; Rieger et al., 2018). USVs were collected using two Emkay/Knowles FG series microphones capable of detecting broadband sound (10–120 kHz). One microphone was placed at the center of the chamber. Microphone channels were calibrated to equal gain (− 60 dB noise floor). We used RECORDER software (Avisoft Bioacoustics) to produce triggered WAV file recordings (each with a duration of 0.5 s) upon the onset of a sound event that surpassed a set threshold of 5% energy change (Kalcounis-Rueppell et al., 2010). Recordings were collected at a 250 kHz sampling rate with a 16-bit resolution. Spectrograms were produced with a 512 FFT (Fast Fourier Transform) using Avisoft-SASLab Pro sound analysis software (Avisoft Bioacoustics). In these recordings, we identified pup whines, simple sweeps, complex sweeps, and syllable vocalizations. Pup whines have a peak frequency around 20 kHz (Kalcounis-Rueppell et al., 2018; Johnson et al., 2017) and the typical downward

modulation at the end of the call often distinguishes these calls from adult syllable vocalizations (Nathaniel Rieger, Jose Hernandez, & Catherine Marler, unpublished). The lower frequencies in the pup whine can also be heard by human ears (below the ultrasonic range). Simple sweeps were categorized by short downward-sweeping vocalizations that sweep through multiple frequencies, typically between 80 kHz and 40 kHz (Kalcounis-Rueppell et al., 2018). Complex sweeps were categorized by being above 90 kHz and having multiple inflection points (Kalcounis-Rueppell et al., 2018). Syllable vocalizations were categorized by long-duration vocalizations with multiple harmonics that occur in bouts, or syllables, typically between 20 kHz and 40 kHz (Kalcounis-Rueppell et al., 2018). It is extremely rare for pups to produce simple sweeps, complex sweeps, or syllable vocalizations during PND 0-4 (Rieger, N. S., Hernandez, J. B., and Marler, C. M., unpublished), therefore, we categorized these calls as adult-only calls. Because of their different spectrogram and acoustic properties, all USVs could be categorized and counted by combined visual and auditory inspections of the WAV files (sampling rate reduced to 11,025 kHz, corresponding to 4% of real-time playback speed).

Data Analysis

Statistical analyses were conducted using the program R. Significance level was set at $p<0.05$ for all analyses and all tests were two-tailed. All analyses used a generalized linear mixed model (GLMM) to assess differences across treatment groups.

Experiment 2: RESULTS

Effects of neuropeptide treatment on USV production

To determine the effect of IN OXT and IN AVP administration on USV production, we assessed the total number of each call type produced during the five-minute test when parents were reunited with their pups. Number of simple sweeps produced was not influenced by neuropeptide treatment (p=0.82) (**Fig. 1A**). Likewise, number of complex sweeps produced was not influenced by neuropeptide treatment (p=0.47) (**Fig. 1B**). However, IN OXT treatment decreased number of SVs produced, ($F_{1,32}$=4.24, p<0.05, ΔR^2=0.12) (**Fig. 1C**).

Fig. 1. (**A**) There was no effect of either OXT or AVP on simple sweep production. (**B**) There was no effect of either OXT or AVP on complex sweep production. (**C**) IN OXT treatment decreased number of SVs produced. *p<0.05.

Effects of neuropeptide treatment on parental care strategy and behavior

We were also interested in the relationship between IN OXT and IN AVP on parental care and parental care strategy. There was a nonsignificant trend that IN OXT increased amount of time parents engaged in no parental care ($F_{1,29}$=4.08, p=0.053, ΔR^2=0.12) (**Fig. 2A**). Neither IN OXT or IN AVP influenced time spent engaged in a uniparental care strategy (p=0.86) (**Fig. 2B**) or biparental care strategy (p=0.18) (**Fig. 2C**). We next assessed type of parental care that pups received. We found no treatment differences in total huddling behavior across treatments (p=0.78) (**Fig. 3A).** However, there were significant treatment differences in retrievals ($F_{1,29}$=8.61, p<0.01, ΔR^2=0.23), with IN OXT decreasing retrievals and IN AVP increasing retrievals (**Fig. 3B**). Lastly, we wanted to examine to the division of labor in huddling and retrieving between the mother and father in each treatment group. We found that IN OXT increased maternal huddling, ($F_{1,29}$=4.77, p<0.05, ΔR^2=0.14) (**Fig. 4A**)**.** IN OXT also showed a nonsignificant trend for but decreasing paternal huddling ($F_{1,29}$=4.01, p=0.055, ΔR^2=0.12) (**Fig. 4B**)**.** IN OXT decreased maternal retrieving ($F_{1,29}$=6.61, p<0.05, ΔR^2=0.19) (**Fig. 4C**), but did not influence paternal retrieving (p=0.25)**.**

Fig. 2. (**A**) There was nonsignificant trend showing pairs given IN OXT spent more time not engaged in parental care behaviors. (**B**) There was no effect of either OXT or AVP time spent engaged in uniparental care or (**C**) biparental care. #$p<0.10$.

Fig. 3. (**A**) There was no effect of treatment on total huddling behavior (**B**) Treatment with IN OXT reduced retrieval behavior and treatment with AVP increased retrieval behavior. **$p<0.01$.

Fig. 4. (**A**) IN OXT increased maternal huddling. (**B**) There was a nonsignificant trend for IN OXT decreasing paternal huddling. (**C**) IN OXT treatment decreased maternal retrieving. (**C**) There was no effect of neuropeptide treatment on paternal retrievals. *$p<0.05$, #$p<0.10$.

Experiments 1 & 2: DISCSUSSION

Understanding how two different animals can coordinate behavior to accomplish a common goal is an intriguing question. We hypothesized that both vocalizations and neuropeptides would influence the ability of pair bonded mice to coordinate behavior and parental care. In our first experiment, we tested the how production of USVs changed across different social paradigms that could occur in family units. We found that simple sweep production was the call type that altered during different social paradigms. Production of complex sweeps, and syllable vocalizations did not change significantly across trials with just one parent or trials where pups were present or absent. This suggests that simple sweep production may be one way that parents communicate with one another during a stressful challenge.

We hypothesized that levels of OXT and AVP may underlie pair communication and parental care. Here, we found that IN OXT but not AVP influenced parental communication and care. Acute pulses of OXT decreased parent communication in the form of SVs. Interestingly, we also did not find an increase in simple sweeps USVs as we did in the maternal study in Chapter 2. This could be because in this test, we cannot separate the mother and father ultrasonic vocalizations, so it is possible that the

combination of maternal and paternal call dilutes the effect of the maternal calls. Alternatively, the increase in maternal simple sweeps could be specific to when the mother is alone with her pups and does not occur in the presence of the father.

IN OXT also disrupted division of labor among parents, shifting a greater burden of care to the mothers. This could either be due to enhanced maternal motivation to care for pups as is seen in other species such as marmosets (Gordon et al., 2011) or to fathers decreasing their contributions to parental care. Because there were no differences in total amount of huddling that pups with OXT-treated parents received, we hypothesize that IN OXT may be increasing maternal motivation to huddle rather than decreasing paternal motivation to huddle.

Experiments 1 & 2: REFERENCES

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Chapter 3: SUMMARY

The goal of this chapter was to characterize how OXT and AVP influence the maintenance of family social bonds through parental communication and coordination of parental care. We found that vocalizations correlate with parental care strategy and may have important implications in the ability for pairs to efficiently coordinate parental care. We found that OXT but not AVP influenced parental communication and care. Acute pulses of OXT decreased parent communication and disrupted division of labor among parents suggesting it may have a negative role in the maintenance of family unit bonds.

Chapter 3: FIGURES & TABLES

Experiment 1:

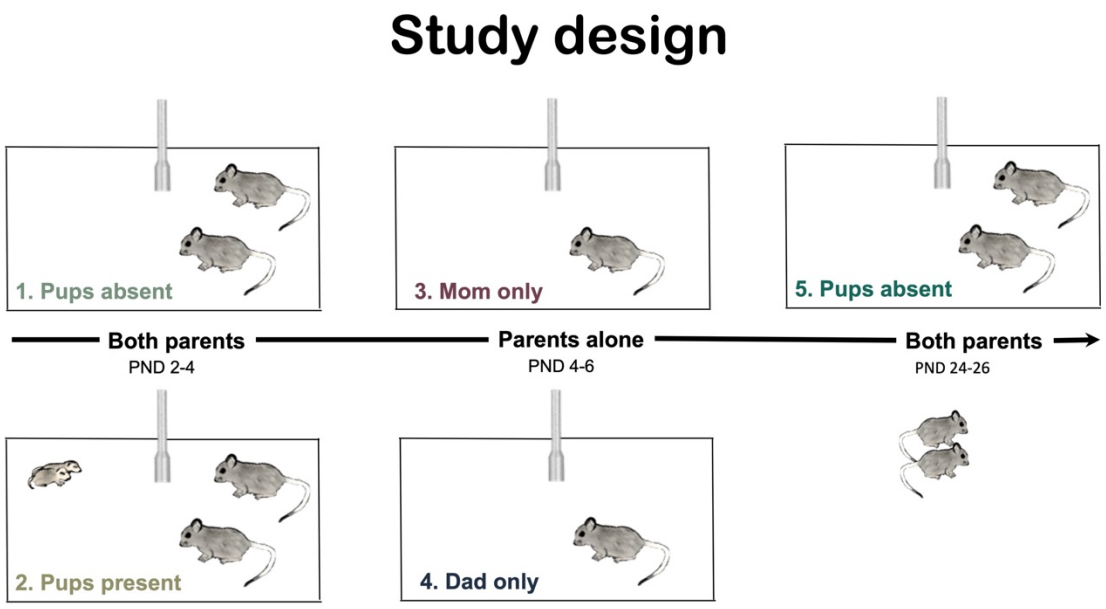


Figure 1. Schematic for behavioral testing.

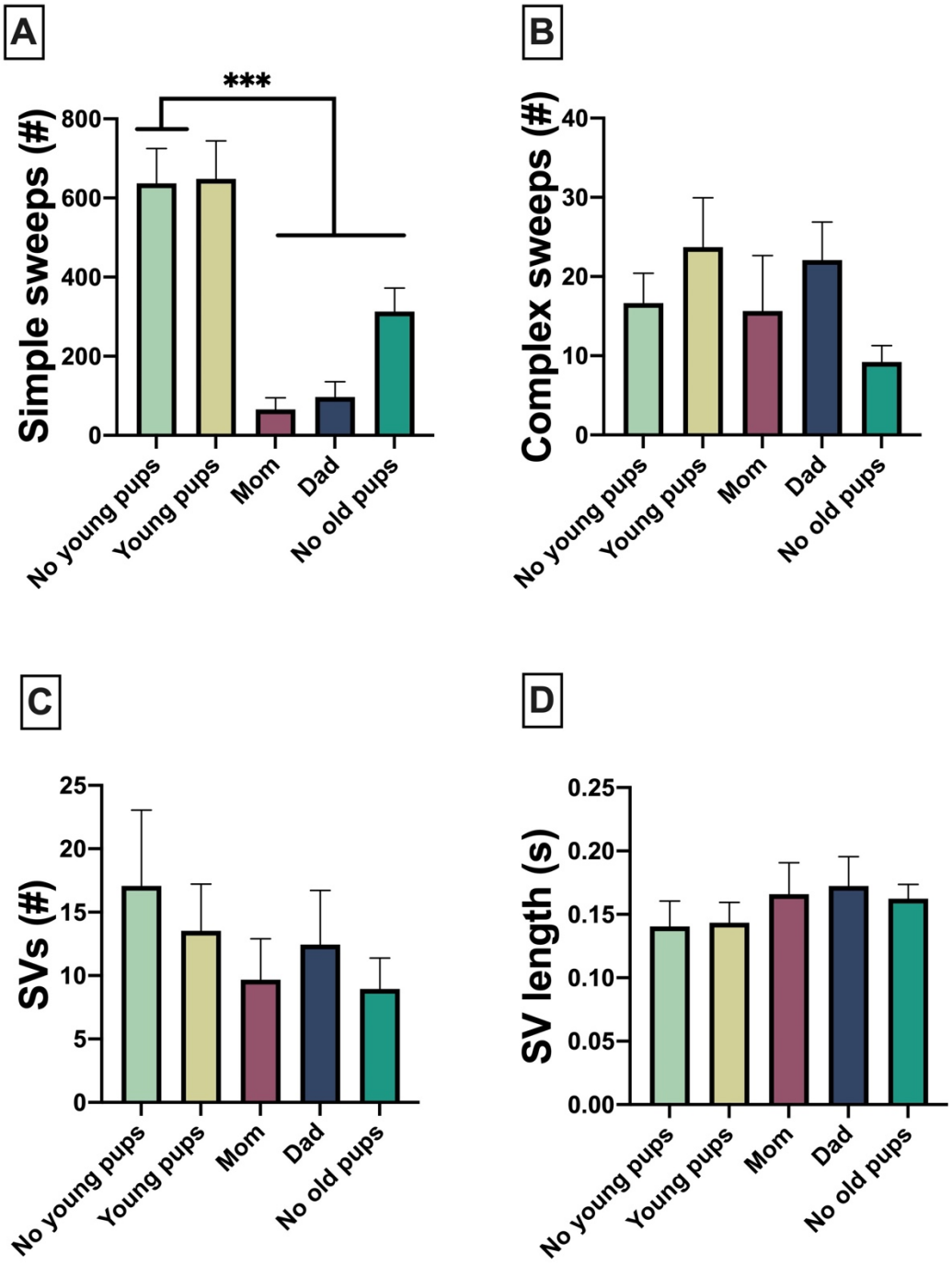


Figure 2. (**A**) Simple sweep production was greatest when both parents were present. There were no differences in simple sweep output in the young pups vs. no pups trial, but there were significantly fewer sweeps in the mom only, dad only, and no old pups trials than the no young pups trial (**B**) Complex sweep production did not differ with social composition. (**C**) SV production did not differ with social composition. (**D**) SV length did not differ with social composition. ***$p<0.001$.

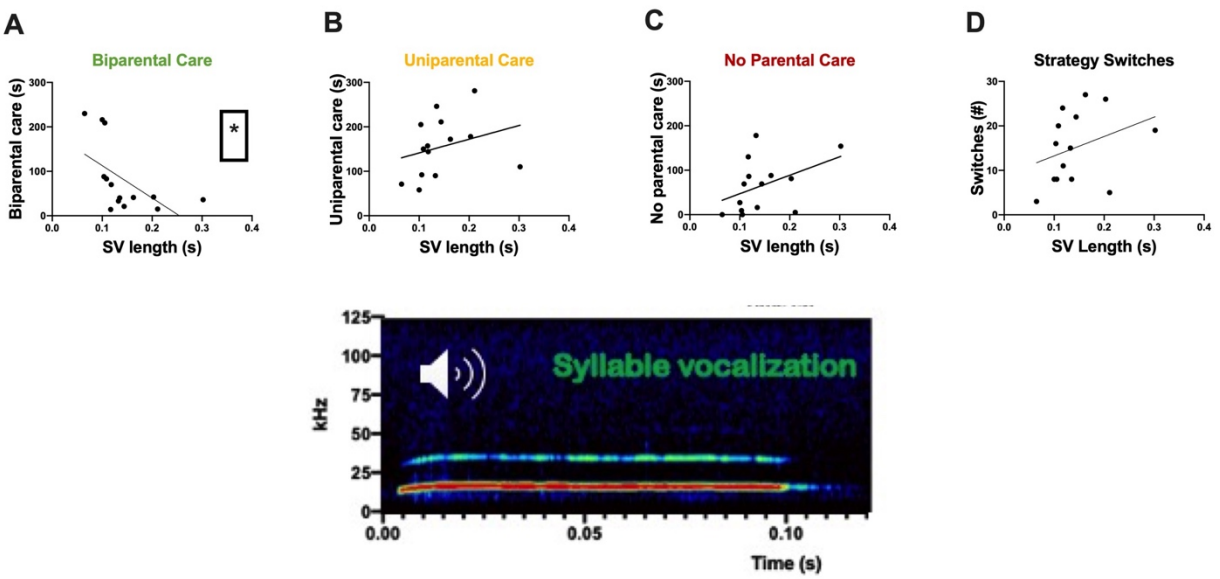


Figure 3. (**A**) Biparental care and SV length were negatively correlated. (**B**) Uniparental care and SV length were not correlated (**C**) No parental care and SV length were not correlated (**D**) Number of times switching parental care strategy and SV length were not correlated. *p<0.05.

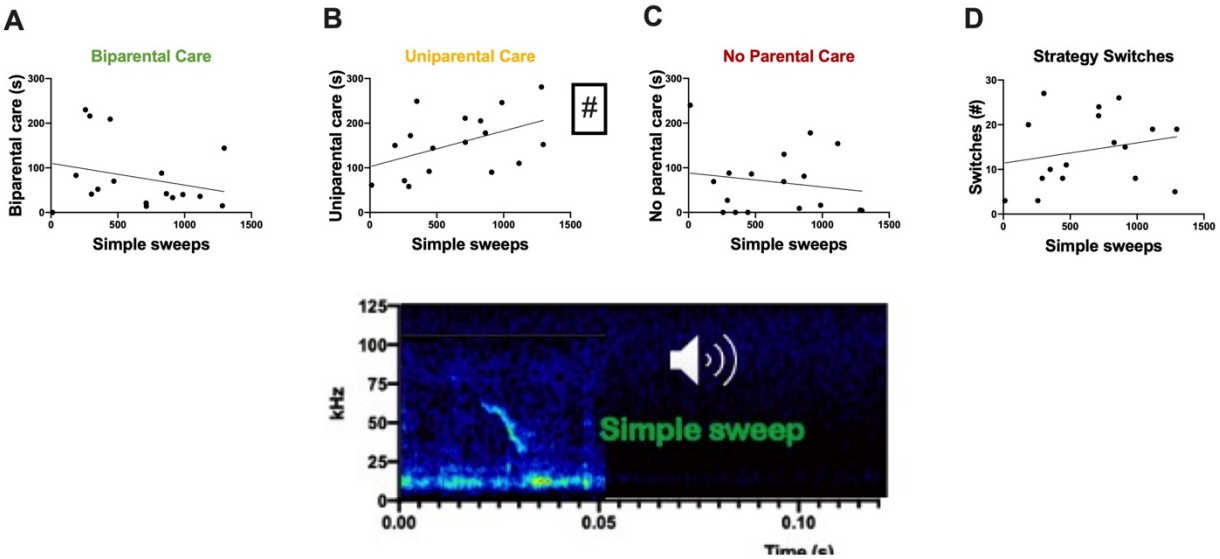
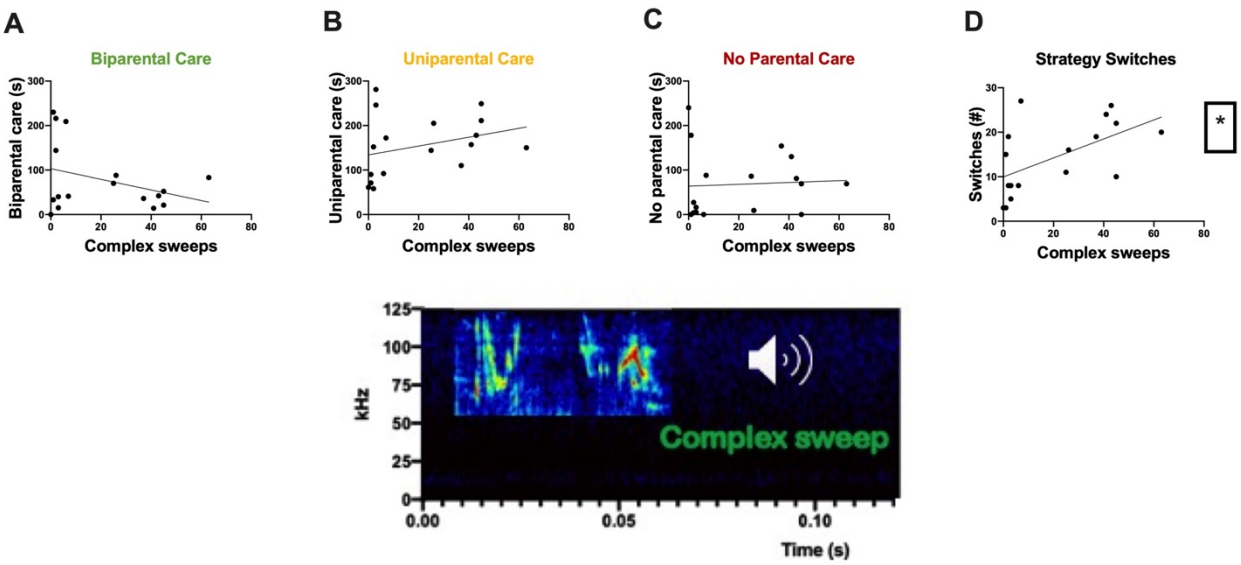


Figure 4. (**A**) Biparental care and simple sweep production were not correlated. (**B**) Uniparental care and simple sweep production were positively correlated (**C**) No parental care and simple sweep production were not correlated (**D**) Number of times switching parental care strategy and simple sweep production were not correlated. #p<0.10.



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Experiment 2:

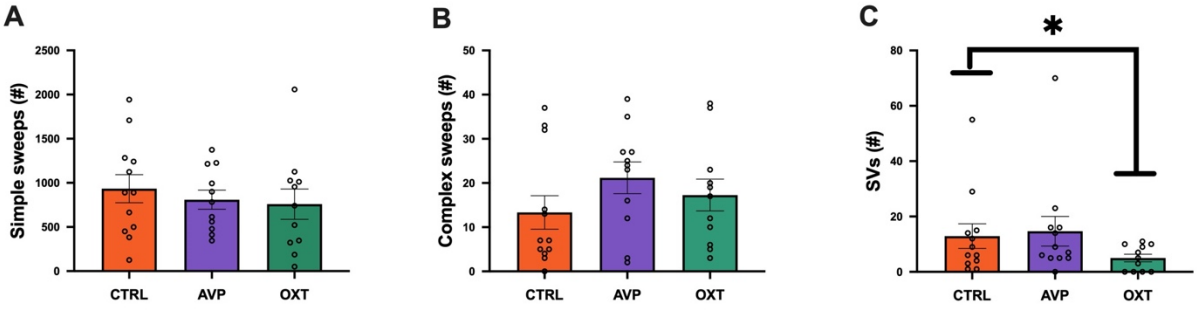

Fig. 1. (**A**) There was no effect of either OXT or AVP on simple sweep production. (**B**) There was no effect of either OXT or AVP on complex sweep production. (**C**) IN OXT treatment decreased number of SVs produced. *p<0.05.

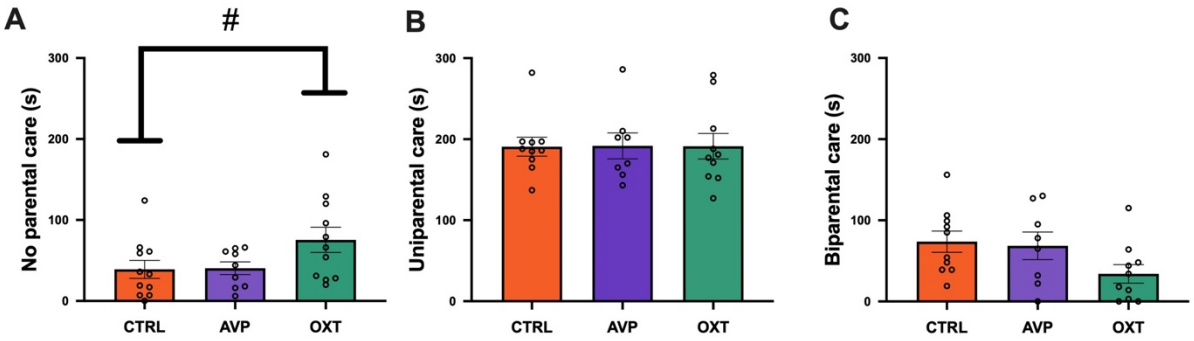

Fig. 2. (**A**) There was nonsignificant trend showing pairs given IN OXT spent more time not engaged in parental care behaviors. (**B**) There was no effect of either OXT or AVP time spent engaged in uniparental care or (**C**) biparental care. #p<0.10.

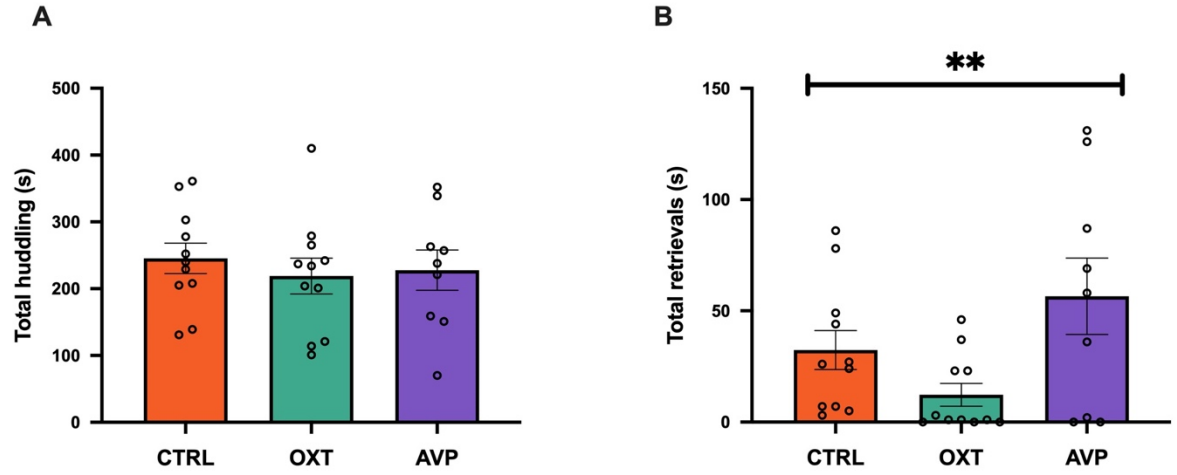

Fig. 3. (**A**) There was no effect of treatment on total huddling behavior (**B**) Treatment with IN OXT reduced retrieval behavior and treatment with AVP increased retrieval behavior. **p<0.01.

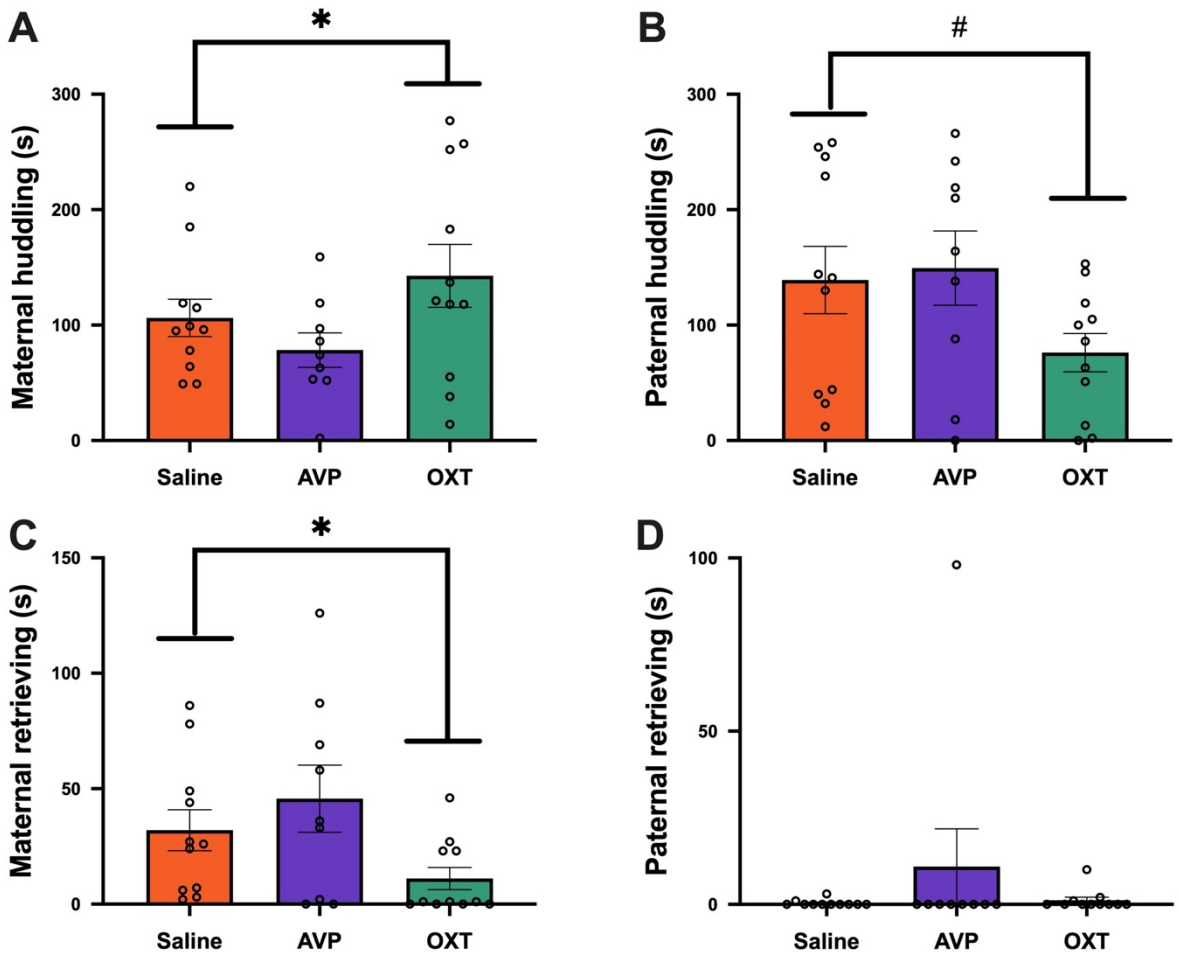

Fig. 4. (**A**) IN OXT increased maternal huddling. (**B**) There was a nonsignificant trend for IN OXT decreasing paternal huddling. (**C**) IN OXT treatment decreased maternal retrieving. (**C**) There was no effect of neuropeptide treatment on paternal retrievals. *$p<0.05$, #$p<0.10$.

Chapter 4: The role of oxytocin and vasopressin in the breakdown of parent-offspring bonds and dispersal social behavior in California mice (*Peromyscus californicus*)

Experiment 1: Characterize social preferences and exploratory behavior of juveniles near weaning age

Experiment 2: Characterize how developmental age influences social receptivity in mice living away from their parents

ABSTRACT

Oxytocin (OXT) and vasopressin (AVP) are two neuropeptides known for their potent role in social behavior. Throughout development, both the OXT and AVP undergo many changes in brain areas that are relevant to social behavior. Parallel to these changes in the brain are profound changes to the social behavior and social preferences that occur between juveniles, adolescents, and adults. In this series of experiments, we hypothesize that by activating the OXT and AVP systems with an acute pulse of OXT or AVP, we will influence social preference. In juveniles, we predicted an acute pulse of OXT would increase preference for parents and an acute pulse of AVP would decrease preference for parents. In adolescents and adults, we predicted that an acute pulse of OXT would decrease aggression toward resident intruders. In **experiment 1**, we tested our hypothesis on the effects of OXT and AVP juvenile social preference. We found that OXT increased juvenile male but not female preference for their parents over peers and that AVP had no effect on social preference. In **experiment 2**, we tested our hypothesis on the effects of OXT on resident-intruder aggression in adolescents and adults. We found no effect of OXT on aggressive behavior in either adolescents or adults but did find that adult females and males were more aggressive toward resident intruders than adolescents.

What Is New:

- Juvenile California mice have a preference for their parents over their peers and an empty chamber and a preference for their peers over and empty chamber.
- An acute pulse of IN OXT increases male preference, but not female preference, for their parents. This suggests OXT may be a mechanism that inhibits male dispersal from the nest.
- IN OXT does not influence aggression toward age- and sex-matched resident intruders
- Despite the same life experience, adolescent California mice show much less aggression toward intruders than adult California mice

Experiment 1: Characterize social preferences and exploratory behavior of juveniles near weaning age

Experiment 1: INTRODUCTION

Across a wide variety of species, the developmental stage of adolescence is a highly conserved (Spear, 2007). Mammals with shorter lifespans and less complex social environments enter puberty more quickly—rodents enter puberty in a matter of weeks whereas humans and other primate species often take years (Brenhouse & Andersen, 2011). In social species with longer lifespans, it may be important to have a longer buffer period between puberty and adulthood. This buffer period allows individuals to explore social reward challenges, especially away from their parents and in peer groups, that may later help them attain higher social ranks and greater mating opportunities (Steinberg, 2008). However, the neurobiological mechanisms that prevent parent-offspring bond breakdown and keep juveniles and adolescents close to the nest are not well understood.

One possibility is that changes in sex steroid levels associated with peri-pubescence influence the OXT and AVP systems to cause a change in social behavior. Estrogen and testosterone have both been shown to have interactions with OXT and AVP. Estrogen and testosterone both increase OXTR binding but have no effect on V1aR binding, and castration affects AVP ligand but has no effect on OXT ligand (Tribollet et al., 1990). Binding of ER-beta by estrogen drives OXT transcription, increasing levels of OXT in the cytoplasm (Shughrue et al., 2002; Choleris et al. 2003; Acevedo-Rodriguez et al., 2015). Studies have also shown that ER-beta increases AVP transcript expression in the PVN (Nomura et al., 2002). Additionally, binding of ER-alpha by estrogen drives transcription of OXTRs and is important for regulating the expression of OXTRs (Young

et al., 1998; Yamamoto et al., 2006). Both the OXT and AVP system are known for their role in social behavior and social preference across a wide variety of species (Kent et al., 2013; Landon et al., 2020; Lukas et al., 2011; Lukas & Neumann, 2014; Wood et al., 2014), therefore, changes to these systems during the peri-adolescent period may facilitate the changes to the processing of social stimuli and social preference. Based on OXT promoting pair bond formation and AVP inhibiting pair bond formation in California mice (**chapter 2**), we hypothesize that an acute pulse of IN OXT will increase juvenile preference for their parents whereas AVP will decrease preference for their parents

To test this hypothesis, we use a three-chambered choice test and have juveniles close to weaning age choose between spending time with their parents, an age-matched novel female and male peer, or an empty chamber. Furthermore, to test to whether IN OXT and IN AVP are specific to juvenile social behavior, we will also test the effect of IN OXT and IN AVP on two nonsocial tasks that assess anxiety and exploration: the elevated plus maze and the novel object task. We predict that since these tasks do not involve social stimuli, we will not see an effect of either IN OXT or AVP.

Experiment 1: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We used 99 *P. californicus* aged 24-26 days. They were -housed in breeding cages with their parents and siblings (48 × 27 × 16 cm) under a 14L: 10D light cycle with lights off at 1:00pm. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Juvenile mice were randomly assigned to treatments in Group 1 and Group 2. In Group 1 sample sizes were: N=8 (CTRL female), N=8 (OXT female), N=7 (AVP female), N=7

(CTRL male), N=8 (OXT male), N=6 (AVP male). In Group 2 sample sizes were: N=10 (CTRL female), N=10 (OXT female), N=9 (AVP female), N=8 (CTRL male), N=9 (OXT male), N=9 (AVP male).

Intranasal Oxytocin and Vasopressin Preparation

Mice were infused intranasally with 17.5 uL of either sterile saline, IN OXT (0.5 IU/kg), or IN AVP (0.5 IU/kg) (Bachem, Torrance, California) (Guoynes & Marler, 2021). The IN OXT dose is equivalent to doses used in other experiments in this dissertation (see Chapters 2 and 3). IN OXT and IN AVP were dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20°C. Treatments, including saline control, were defrosted just prior to administration. A blunt cannula needle (33-gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was attached to cannula tubing, flushed, and filled with the compound, then attached to an airtight Hamilton syringe (Bachem, Torrance, California). The animal was scruffed and 17.5 uL of compound was expelled dropwise through the cannula needle and allowed to absorb into the nasal mucosa (~10-20 seconds). One person conducted all IN administrations throughout the entire procedure to maintain consistency in handling and IN infusion.

Behavioral Tests

In order to assess juvenile social preference and exploration, we assigned juvenile mice to one of two groups. Regardless of group assignment, each mouse was weaned from its home cage 24 hours prior to testing and placed in a new home cage alone. In Group 1, mice were tested in the parent-peer preference test. In Group 2, mice were tested in

the elevated plus maze test and then tested in the novel object test. For an informational graphic on experimental groups and timeline, see **Fig. 1**

Parent- peer preference test

Mice assigned to Group 1 were tested in the parent-peer preference test. This test uses a three-chambered apparatus (91 cm x 46 cm x 43 cm) divided into three equal chambers. Each side chamber had a wire mesh partition at the back (30 cm x 10 cm) where stimulus animals could be presented. Juveniles were given IN treatment in their home cage five minutes prior to behavioral testing. For each test, the parents of the focal mouse were randomly assigned to either the left or right side of the chamber and unrelated, age-matched peers (one male, one female) were placed on the other side of the chamber. Five minutes prior to the start of the test, juvenile mice were given their randomly assigned IN dose of treatment in their home cage. At the start of the test, the mouse was placed in the center chamber of the testing apparatus and behavior was recorded for 30 min. Mice had to visit both sides of the chamber in the first 10 min of the test in order to be scored.

Elevated plus maze test

Mice assigned to Group 2 were first given the elevated plus maze test and then the novel object test (see below). The maze consisted of two open and two enclosed opaque arms, each 67 cm long and 5.5 cm wide. The arms were elevated 1 m above the floor. Juveniles were given IN treatment in their home cage five minutes prior to behavioral testing. At the start of the test, each mouse was placed into the center of the maze and its behavior was scored for 5 min. Any animals that jumped off the open arms of the maze were captured and placed back into the center of the maze. If a subject jumped off the maze 3 times, the test was stopped. Trained observers blind to conditions scored behavior live for duration of time in the open and closed arms and number of crosses

through the center of the maze. Total duration of this test was 5 min. Following testing animals were tested in novel object test (see below).

Novel object test

Immediately following the elevated plus maze test, mice were placed on the far side of a glass arena (50cm x 30cm x 30cm) that contained an 5cm x 5cm x 5cm metal cube that was novel to them, and behavior was recorded for 10 min. Number of approaches to novel object and time spent engaging with novel object was measured.

Data Analysis

For each behavioral test, ANOVA tests were conducted to compare the outcomes between saline control, OXT, and AVP treatment. Significance level was set at $p < 0.05$ for all analyses and all tests were two-tailed.

Experiment 1: RESULTS

Parent- peer preference test

To assess the effects of an acute pulse of OXT or AVP on juvenile social preference for their parents versus novel peers, we conducted a three-chambered social preference test. Regardless of treatment, juvenile females had a preference for their parents over their peers $F(1, 22)=25.09$, $p<0.0001$, and their peers over the empty chamber $F(1, 22)=55.38$, $p<0.0001$ (**Fig. 2A**). Likewise, regardless of treatment, juvenile males had a preference for their parents over their peers $F(1, 20)=37.16$, $p<0.0001$, and their peers over the empty chamber $F(1, 20)=25.15$, $p<0.0001$ (**Fig. 2B**). To test for effects of IN OXT and AVP on social preference, we created a preference score by subtracting each individual's preference for their peers from their preference for their parents. There were no effects of treatment on social preference in females, $F(2, 20)=1.38$, $p=0.27$ (**Fig.**

2C). However, IN OXT increased juvenile male preference for their parents $F(2, 18)=4.26$, $p<0.05$ (**Fig. 2D**).

Figure 2. (**A**) Both female and (**B**) male juveniles had a preference for their parents compared to their peers or the empty chamber and had a preference for their peers over an empty chamber. (**C**) Neither IN OXT nor IN AVP influenced social preference in female juveniles. (**D**) IN OXT increased juvenile male preference for their parents above and beyond their natural preference for their parents.

Elevated plus maze test

To assess the effects of an acute pulse of OXT or AVP on juvenile exploration, we measured behavior in an elevated plus maze task. There were no treatment effects of time spent on the open arms for females ($p=0.61$) or males ($p=0.27$) (**Fig. 3A**). There were also no treatment effects of number of crosses through the center of the apparatus for females ($p=0.77$) or males ($p=0.51$) (**Fig. 3B**).

Novel object test

To assess the effects of an acute pulse of OXT or AVP on juvenile response to novelty, we measured behavior in a novel object task. There were no treatment effects of latency to approach novel object for females ($p=0.38$) or males ($p=0.38$) (**Fig. 3C**). There were also no treatment effects of time spent investigating the novel object for females ($p=0.98$) or males ($p=0.37$) (**Fig. 3D**).

Figure 3. (**A**) Neither IN OXT not IN AVP influenced time spent on open arms (**B**) Neither IN OXT not IN AVP influenced number of crosses through the center of the elevated plus maze (**C**) Neither IN OXT nor IN AVP influenced latency to approach novel object (**D**) Neither IN OXT not IN AVP influenced time spent investigating novel object.

Experiment 1: DISCUSSION

Dispersal from the natal territory is critical step as it allows animals to establish their own territory and mating opportunities. Both the OXT and AVP system experience

changes in the transition from the juvenile period to adulthood, making these neuropeptides good candidate molecules for facilitating changes to the processing of social stimuli (Parr et al., 2018; Johnson et al., 2017; Steinman et al., 2019; Egito et al., 2020, Caldwell, 2017; Zhuang et al., 2021). The goal of this study was to determine if the neuropeptides OXT and AVP influence juvenile influence the breakdown of parent-offspring bonds by assessing their effect on social preference in juveniles.

We predicted that OXT would increase juvenile preference for their parents by promoting familiar social bonds and that AVP would decrease preference for their parents in favor of dispersal. We found partial support for this prediction. During the late juvenile phase (PND 24-26), we found that juvenile California mice have a marked preference for their parents over social novelty (novel peers) and social isolation. This is unlike other rodent species such as mice and rats that prefer social novelty over familiar individuals (Smith et al., 2017; Jin et al., 2020; Smith et al., 2018). We also found that an acute pulse of OXT enhanced this preference for parents in males only. This suggests that in males, OXT may be one mechanism that keeps males close to the natal territory and prevents them from dispersing. In wild, juvenile mice will still get natural pulses of OXT from their mothers when they nurse and drink milk (UvnäsMoberg et al., 2020). If mothers allow their sons to nurse for longer, they may be reinforcing the parent-offspring bond and preventing it from breaking down. Interestingly, this same mechanism does not appear to influence juvenile female dispersal, suggesting that other neurobiological mechanisms may play a greater role in females.

Unlike OXT, we predicted the AVP would be involved in the breakdown of parent-offspring and cause a loss of preference for the parents. However, we did not find support for this hypothesis. IN AVP did not influence either female or male juvenile social preference, suggesting that activation of the AVP system in juveniles is

not sufficient to cause parent-offspring bond breakdown. It is possible that AVP did not have an effect at this age of mouse because of the development of the AVP system. Adults have higher expression of the V1a receptor in the prefrontal cortex, nucleus accumbens, ventral pallidum, and lateral septum than juveniles (Smith et al., 2017). Many of these brain areas are important for sexual behavior and aggression, so it is possible that if we tested the role of an acute pulse of AVP at later time points in adolescence or early adulthood we would see an effect.

Because OXT and AVP are known for their role in social behavior specifically, we predicted that the effects of IN OXT and IN AVP treatment would be exclusive to social behavior (Group 1) and would not influence general exploratory behavior (Group 2). In line with our predictions, we found that treatment with IN OXT or IN AVP did not influence behavior in the elevated plus maze or the novel object task. This is consistent with other studies in prairie voles (Bales et al., 2013) that also show no effect neuropeptide treatment on nonsocial tasks. These results suggest that OXT is exerting its effects specifically through social behavior neural networks.

To our knowledge, this study is the first to examine the effects of neuropeptides on juvenile dispersal and the breakdown of parent-offspring bonds. Our results highlight the importance of acute pulse of OXT in juvenile male preference for their parents but highlight the

Experiment 1: REFERENCES

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Experiment 2: Characterize how developmental age influences social receptivity in mice living away from their parents

Experiment 2: INTRODUCTION

At birth, most mammals lack the coordination, strength, and social experience to be successful in aggressive encounters. Therefore, early stages of development are devoted to increasing physical coordination and strength (Walton et al., 1992; Le Roy et al., 2001). In preparation for independence and adulthood, the peri-adolescent phase of development is marked by changes to hormone and neuropeptide systems that support physical, social, and cognitive development (Cameron, 2004; Spear, 2000; Griffin, 2017; Gee et al., 2018). Hormone changes and social experience during this peri-adolescent phase are critical for expression of appropriate aggression phenotypes in adulthood (Susman et al., 1987; Ramirez, 2003; Bell, 2018; Fragkaki et al., 2018; Sachser et al., 2018).

In many group-living animals, expressing appropriate adult aggression requires a fine balance between optimizing mating opportunities and reducing chance of injury. For these species, practicing aggression in the form of social play may be particularly important because it can help animals make contextual adjustments of their actions based on feedback from play partners (Pellis & Pellis, 2017). Additionally, communal rearing has been shown to improve competition (Fischer et al., 2018). In juveniles, social play activates the opioid reward system (Zhao et al., 2020; Chang et al., 2019; Vanderschuren et al., 2016) and is enhanced when animals have experienced social isolation (Ikemoto & Panksepp, 1992; Guerra et al., 1999; Holloway & Suter, 2004). Deprivation of social play can lead to maladaptive adult resident-intruder interactions (Van den Berg et al., 1999) and defeat-induced social avoidance in both adult females and males (Kyle et al., 2019). This suggests that juveniles have a high drive for social

play and have neurobiological mechanisms that reinforce the behavior (Trezza et al., 2019). Species that show juvenile social play include rats (Veenema et al., 2013; Auger & Olesen, 2009), Syrian and golden hamsters (Kyle et al., 2019; Cheng et al., 2008), dogs (Kottferová et al., 2020), cats (Bateson & Barrett, 1978; Delgado & Hecht, 2019), chimpanzees (Cordoni & Palagi, 2011; Shimada & Sueuer, 2014), and humans (Fry, 2005; Viega et al., 2020). However, there are several species that display high levels of adult aggression, but do not show robust juvenile social play—some of these species include California mice and prairie voles, two monogamous species. This suggests that the development of aggression may rely on species-specific competition demands and be driven by differences in hormone and neuromodulator levels.

The neuropeptide oxytocin (OXT) is involved in processing the salience of social cues and may play an important role in social behavior after dispersal (Shamay-Tsoory & Abu-Akel, 2016; Caldwell, 2017). In aggressive rats, adolescent treatment with OXT increased attack latency, suggesting that OXT may attenuate aggressive response (Kozhemyakina et al., 2020). However, other studies have found a positive association between OXT and expression of play and aggression. In mandarin voles, paternal deprivation decreased play fighting and number of OXT immunoreactive cells in the PVN, suggesting that OXT in the PVN may play an important role in promoting juvenile social play (Wang et al., 2012). Similarly, in female and male rats, post-weaning isolation increased aggression and upregulated OXT mRNA in the PVN but decreased OXTR expression in the NAcc (de Moura Oliveira et al., 2019). Together, these studies suggest that altering the OXT system may have subtle effects on the expression of aggression and social receptivity.

In this experiment, our goal is to better characterize the developmental effects of an acute pulse of OXT using a resident-intruder paradigm. Based on the decreased

aggression observed in adolescent rats and previous experiments conducted in this dissertation, we predict that OXT will increase social receptivity toward resident intruders compared to saline controls. As California mice are territorial and aggressive as adults, we also predict that adolescents will be less aggressive toward intruders than adults.

Experiment 2: METHODS

Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All mice were housed in the same room under a 14L: 10D light cycle with lights off at 4:00pm. We used 24 female *P. californicus* aged 5–10 months (adult female group), 24 male *P. californicus* aged 5–10 months (adult male group), 24 female *P. californicus* aged 30-45 days (adolescent female group), and 24 female *P. californicus* aged 30-45 days (adolescent male group). Four days prior to testing, two siblings were taken from their home cage that housed 3–4 mice per cage (48 × 27 × 16 cm) and placed in a new glass aquarium cage (50cm x 30cm x 30cm) to establish residency. Siblings were randomly assigned to either the saline control (N=12 per age group) or OXT group (N=12 per age group) on the day of behavioral testing. Resident intruders were unrelated by at least two generations, age-matched, sex-matched, and randomly assigned to the pairs of siblings.

Intranasal Oxytocin Preparation

Sibling mice were infused intranasally with either sterile saline or IN OXT (0.8 IU/kg) (Bachem, Torrance, California) (Guoynes & Marler, 2021). The IN OXT dose is equivalent to doses used in other animal models (Bales et al. 2014; Guoynes et al. 2018; Murgatroyd et al. 2016) and similar to weight-adjusted doses used in clinical studies examining the effects of IN OXT on social deficits in autism (Bales et al., 2013). IN OXT was dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20^{o}C. IN OXT was defrosted just prior to administration. A blunt cannula needle (33-gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was attached to cannula tubing, flushed, and filled with the compound, then attached to an airtight Hamilton syringe (Bachem, Torrance, California). The animal was scruffed and 25 uL of compound was expelled dropwise through the cannula needle and allowed to absorb into the nasal mucosa (~10-20 seconds). One person conducted all IN OXT administrations throughout the entire procedure to maintain consistency in handling and IN OXT infusion.

Resident-Intruder Aggression Test

To assess the effects of an acute pulse of OXT on aggressive behavior, same-sex siblings were housed for three days in a glass arena (50cm x 30cm x 30cm) used in Rieger & Marler, 2018. Three days is sufficient for California mice to establish residency in the arena (Bester-Meredith et al., 1999; Marler et al., 2003; Fuxjager et al., 2010; Zhao & Marler, 2014). Sibling mice were randomly assigned either a red or green tail mark to differentiate each other during behavioral scoring. Resident intruders always had a black tail mark. On day four, one sibling was given 0.8 IU/kg intranasal OXT and the other sibling was given intranasal saline control. Adult females and males were given 25 μl whereas juvenile females and males were given 17.5 μl to control for weight

differences between the age groups. Five minutes after intranasal administration, an age- and sex-match intruder was placed into the arena and the interaction was videotaped for twenty minutes (**Figure 1**). Each video was later scored for lunging, chasing, and wrestling behavior. All behavior videos were scored twice: once each by two independent observers blind to treatment and in a random order. Scores between observers had to be at least 85% similar and scores between the two observers were averaged for the final output used in statistical analysis.

Data Analysis

Using a multivariate model in R, we assessed the relationship between treatment and age on aggressive behavior, (e.g. [Aggressive behavior] ~ [Treatment] + [Age]). Significance level was set at $p < 0.05$ for all analyses and all tests were two-tailed.

Figure 1. Experimental design. Resident mice were randomly assigned to receive either 0.8 IU/kg intranasal oxytocin (green dot) or intranasal saline control (orange dot). Five minutes later, an age- and sex-matched intruder (black dot) was placed in the cage and the interaction was taped for twenty minutes.

Experiment 2: RESULTS

To determine whether IN OXT influenced social behavior during a resident intruder aggression test, we analyzed the data for main effects of IN OXT treatment on three measures of aggression: lunging, chasing, and wrestling. Females did not show any main effects of OXT treatment on lunging (p=0.53), chasing (p=0.29), or wrestling (p=0.99) (**Figure 2A, C, E**). Likewise, males did not show any main effects of OXT treatment on lunging (p=0.89), chasing (p=0.67), or wrestling (p=0.94) (**Figure 2B, D, F**).

In addition to analyzing the data for main effects of OXT, we were also interested in examining how developmental age influenced aggression. There were age differences in aggression in females. Adult females lunged more [$F(2,31)=4.31$, $p<0.05$,

ΔR^2=0.121] (**Figure 2A**), chased more [$F(2,31)$=5.06, p<0.05, ΔR^2=0.136] (**Figure 2C**), but did not wrestle more (p=0.87) (**Figure 2E**) than juvenile females. Adult males showed a nonsignificant trend for chasing more than juvenile males [$F(2,34)$=3.13, p=0.086, ΔR^2=0.084] (**Figure 2D**), but showed no difference in lunging (p=0.44) (**Figure 2B**) or wrestling (p=0.80) (**Figure 2F**).

Figure 2. Aggressive behavior during a 20-min resident-intruder paradigm with an age-matched intruder. (**A-F**) There were no main effects of IN OXT on aggression. (**A)** Adult females lunged at intruders more than juvenile females. (**B**) Adult and juvenile males showed no difference in lunging toward intruders. (**C**) Adult females chased intruders more than juvenile females. (**D**) Adult and juvenile males showed no difference in chasing intruders. (**E**) and (**F**) Both females of males showed no difference in wrestling bouts, regardless of age. *p<0.05, #p<0.10.

Experiment 2: DISCUSSION

Appropriate expression of aggression toward conspecifics is critical step in the social development of many animals. In this study, we examined the role of IN OXT on aggression toward intruders in sexually naïve adult and adolescent female and male California mice. We predicted that an acute pulse of OXT may increase social receptivity toward a stranger and reduce territorial aggression. Overall, our results did not support a role for OXT in the expression of aggression toward intruders, regardless of sex or age. These finding are similar to our results in mated adult male mice (see Chapter 2, experiment 2). This suggests that developmental changes that occur once the mouse has fully developed likely play a stronger role in the development of territorial aggression compared to OXT.

Although we did not find main effects of OXT treatment, we did find main effects of age on aggression. Both adult and adolescent mice in this study had the same life experience history (sexually naïve, housed with siblings), but both adult females and adult showed greater aggression toward a resident-intruder than their adolescent

counterparts. Changes in testosterone and estrogen levels that occur in fully mature adults may be responsible for these age-dependent effects on aggression (Schulz et al., 2009; Herting et al., 2014; Grotzinger et al., 2018). A recent study in male California mice pre-pubertal castration made adult males more likely to show social defeat after an aggressive encounter, but adult replacement of testosterone or dihydrotestosterone reversed these effects (Wright et al., 2020). This suggests that adult expression of aggression may rely heavily on activation by androgens.

Overall, this study contributes to our understanding of when and how an acute pulse of OXT influences prosocial behavior and bonding in California mice. While previous experiments in this dissertation suggest that OXT plays a role in the formation of pair bonds and parent-offspring bonds, the maintenance of family-unit bonds, and the breakdown of male juvenile- parent bonds, OXT does not seem to influence peer sociality in California mice.

Experiment 2: REFERENCES

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Chapter 4: SUMMARY

The goal of this chapter was to characterize how OXT and AVP influence the breakdown of family social bonds and novel interactions with peers. We found strong evidence for the role OXT but not AVP influencing male preference for their parents over novel peers. This suggests that pulses of OXT, possibly coming from their mother's milk, may be one mechanism that inhibits dispersal in males. However, in females, neither OXT nor AVP influenced social preference between parents and peers. This suggests that mechanisms inhibiting dispersal may be different in females and males. Lastly, we tested both adolescent and adult California mice in a resident-intruder paradigm and found no effects of OXT on social receptivity toward strangers in a resident-intruder a paradigm. This suggests that social salience and species-typical behavior are important factors in the ability of OXT to influence prosocial behavior.

Chapter 4: FIGURES & TABLES

Experiment 1:

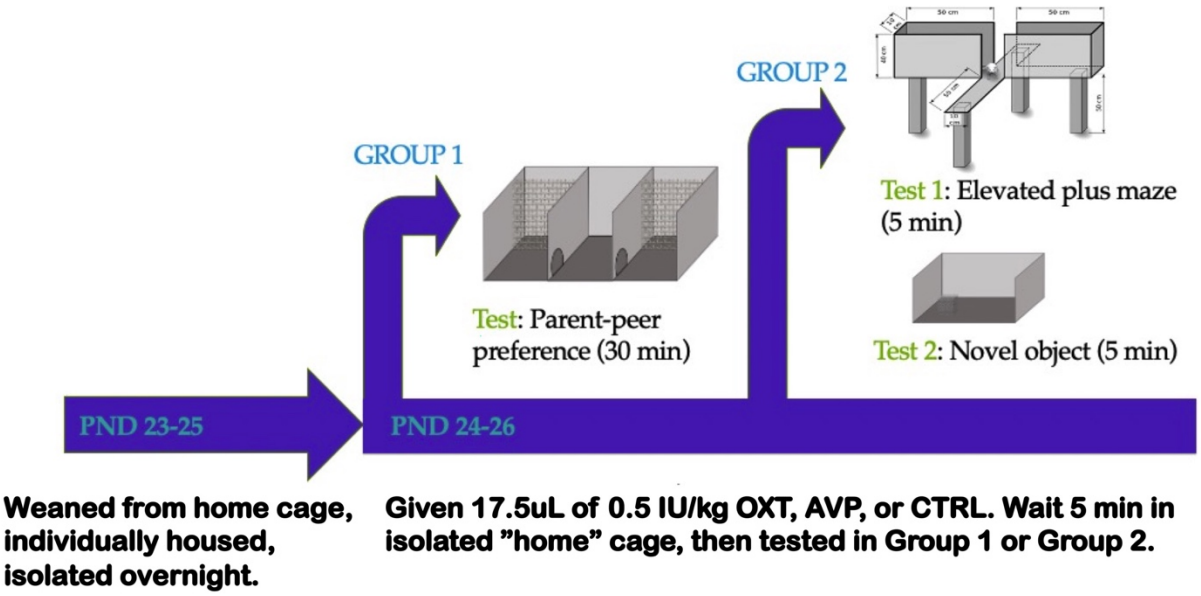

Figure 1. Experimental design. Juvenile mice were randomly assigned to either Group 1 or 2.

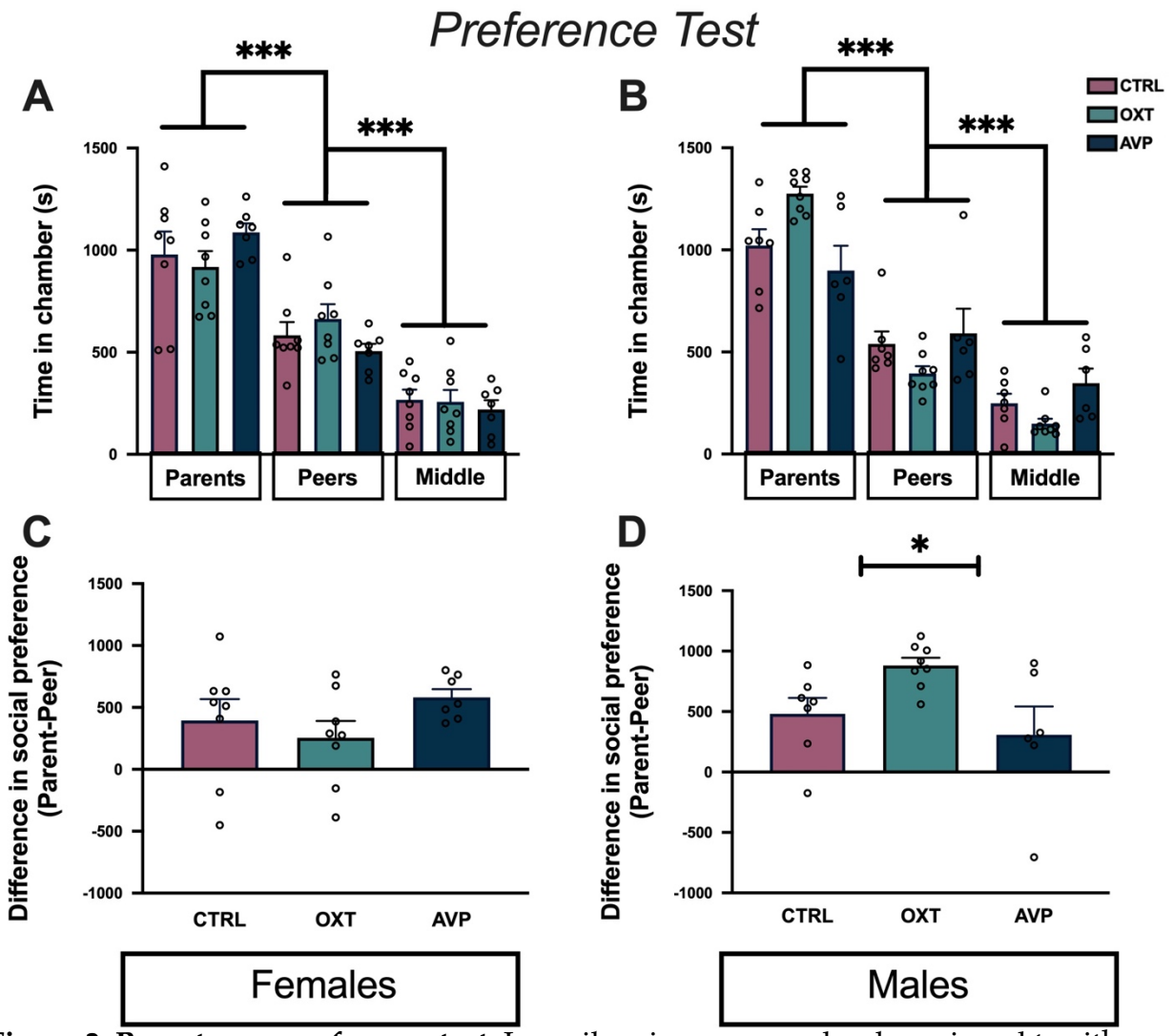

Figure 2. Parent-peer preference test. Juvenile mice were randomly assigned to either Group 1 or 2.

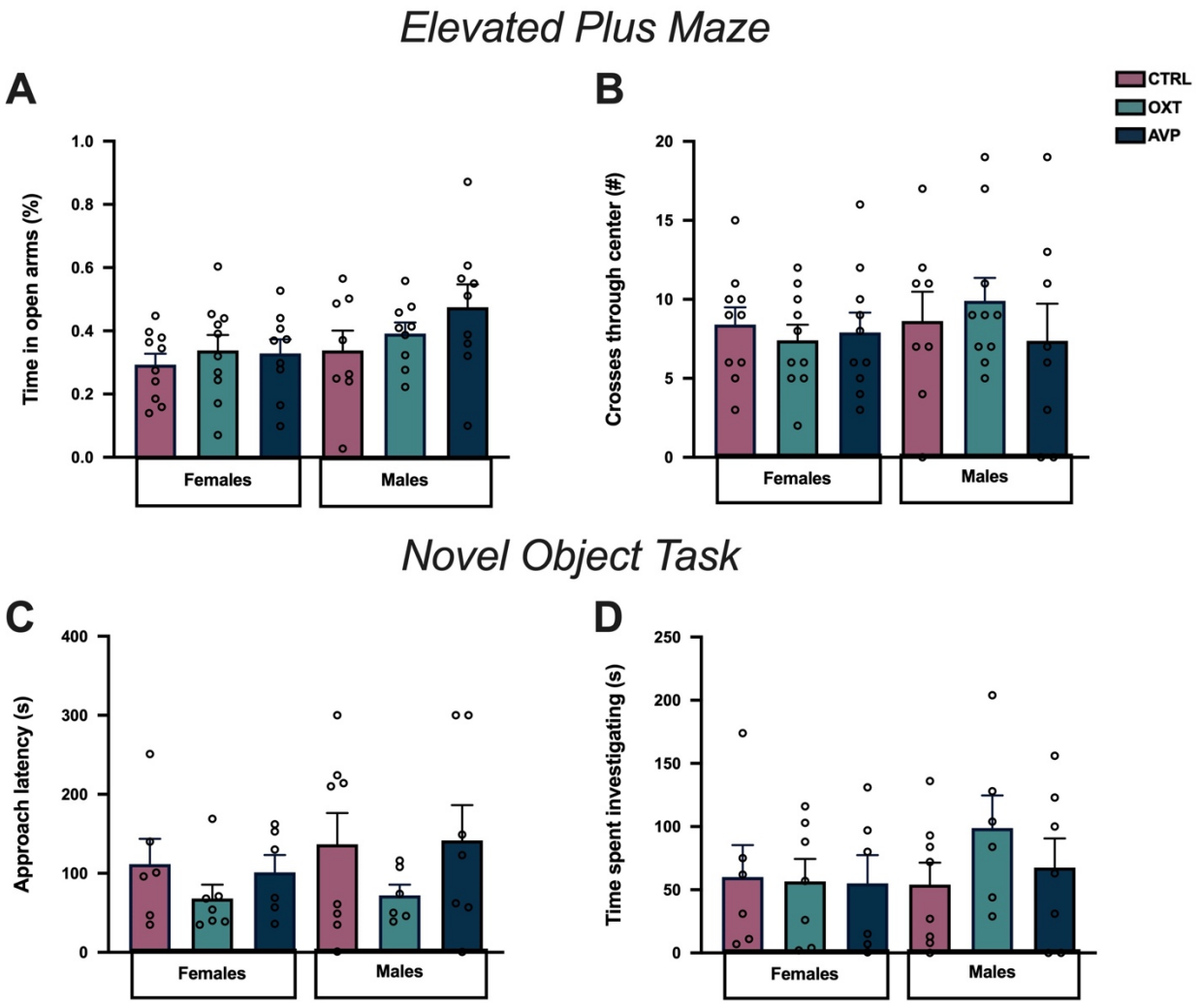

Figure 3. Elevated plus maze and novel object tests. There was no effect of treatment on exploratory behavior in either the elevated plus maze or the novel object test

Experiment 2:

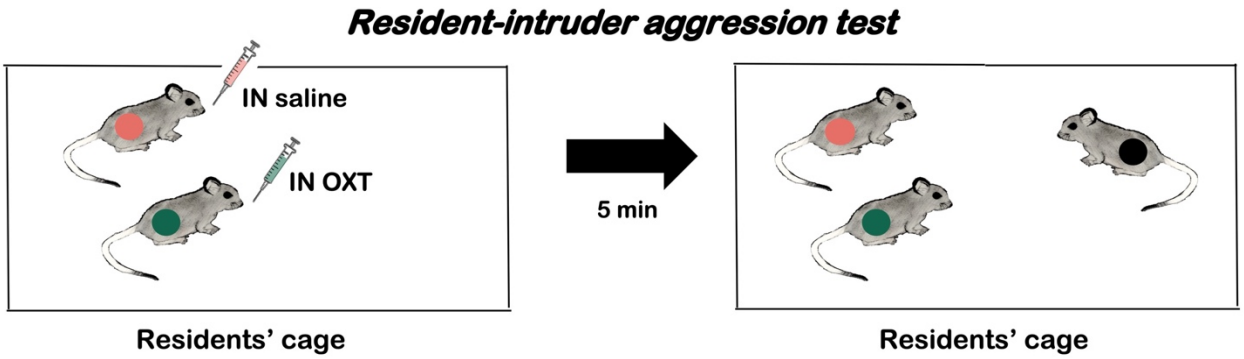

Figure 1. Experimental design. Resident mice were randomly assigned to receive either 0.8 IU/kg intranasal oxytocin (green dot) or intranasal saline control (orange dot). Five minutes later, an age- and sex-matched intruder (black dot) was placed in the cage and the interaction was taped for twenty minutes.

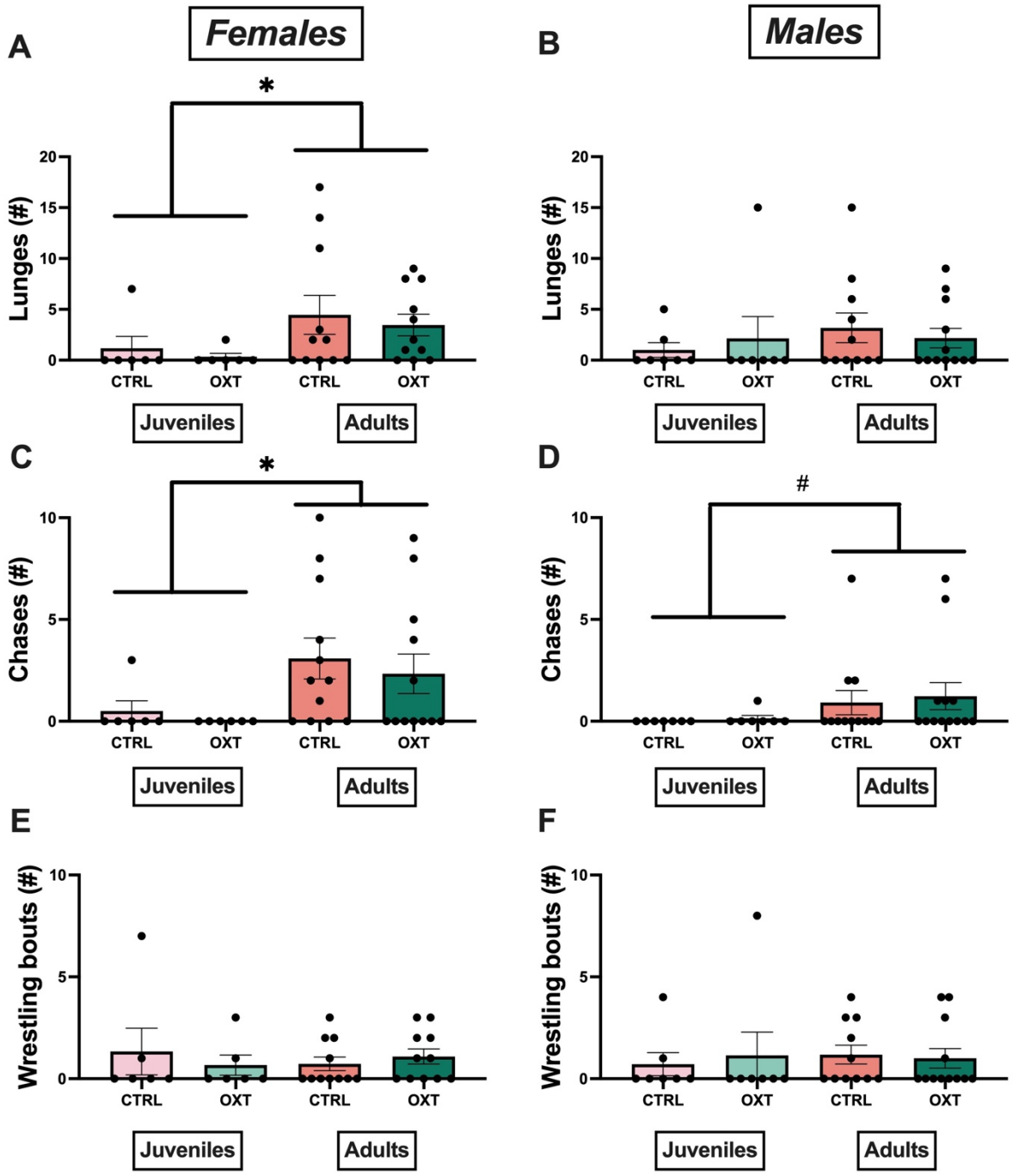

Figure 2. Aggressive behavior during a 20-min resident-intruder paradigm with an age-matched intruder. (A-F) There were no main effects of IN OXT on aggression. **(A)** Adult females lunged at intruders more than juvenile females. **(B)** Adult and juvenile males showed no difference in lunging toward intruders**. (C)** Adult females chased intruders more than juvenile females. **(D)** Adult and juvenile males showed no difference in chasing intruders. **(E)** and **(F)** Both females of males showed no difference in wrestling bouts, regardless of age. *$p<0.05$, #$p<0.10$.

CONCLUSIONS

The neuropeptides OXT and AVP and their analogs have been shown to influence social behavior across fish, amphibians, reptiles, birds, and mammals. In general, OXT promotes pair bonding, maternal and paternal care, and social recognition. AVP also influences bonding and paternal care but may also be involved in promoting aggression. An emerging hypothesis in the literature is that changes in OXT and AVP may play a significant role in rewiring the social brain by reinforcing social interactions as positive or negative. Most of the studies that inform this hypothesis examined social preference but did not examine whether oxytocin and vasopressin kickstarted the initiation of social bonds during first meetings or whether OXT and/or AVP supported the maintenance or breakdown of social bonds. The studies in this dissertation helped fill some of these gaps.

We characterized the behavioral effects of OXT and AVP on interactions during early courtship of a pair and found that an acute pulse OXT inhibits the escalation to contact aggression in male California mice but that an acute pulse of AVP may actually increase aggression in female but not male California mice during pre-courtship. These findings highlight that pulses of OXT and AVP may have opposite effects on early pair bonding behavior and that there are sex differences in response to AVP.

We also characterized the behavioral effects of OXT in first-time mothers and fathers to examine the role of OXT in early parent-offspring bonds. We found that a pulse of OXT enhanced communication and parental care in mothers but had only a moderate effect of increasing paternal responsiveness to pups and no effect on communication in fathers. These findings highlight sex differences in response to OXT treatment in the formation of family-unit bonds.

Few studies have examined the role of OXT and AVP in the maintenance of socials bonds. To address this gap, we examined the role of communication and pulses of OXT and AVP on parents' ability to coordinate behavior under mildly stressful conditions. Vocal communication is often overlooked in studies in rodents, but measuring vocal communication is likely an important part of social perception and maintenance of social bonds in group living animals. Compared to other model species, California mice have a better understood vocal repertoire which allows for pairing vocal communication with behavior. We used this feature of California mice to assess how pairs responded to a mildly stressful pup separation paradigm. We found that vocalizations are associated with parental care strategy and change with changing social conditions. This suggests that vocalizations are directly relevant to social behavior within family units. Furthermore, our studies suggest that simple sweeps are particularly relevant to parental communication.

For maintenance of social bonds within the family unit, acute pulses of OXT may actually inhibit parent coordination. An acute pulse of OXT decreased parental SV production, increased the time offspring had without parental care, and caused mothers to take on a greater load of parental care. This suggests that while OXT may enhance pair bond formation, it may hinder the maintenance of social bonds. We did not find strong support for the role AVP in the maintenance of social bonds but did find that when parents were given an acute pulse of AVP, they increased the amount of time they spent retrieving. This could be interpreted as less efficient parental care and may be indicative of greater anxiety in parents with higher AVP levels. To our knowledge, these are the first studies that examine how OXT and AVP influence coordination of parental care in a monogamous species.

California mice are also a well-suited model to understand the breakdown of social bonds because both female and male juveniles disperse from the nest, suggesting that the parent-offspring bond breaks down. To our knowledge, the studies in this dissertation are the first to examine the role of OXT and AVP in the breakdown of these family-unit bonds. We found sex differences for the role of OXT on maintain juvenile-parent bonds with males being more sensitive to acute pulses of OXT than females. Lastly, when we tested the role of OXT on adolescent and adult social receptivity to peers, we found no effect of OXT. Taken together with experiments throughout this dissertation, this suggests that OXT is only important for the maintenance of juvenile-parent bonds and the initiation potential mating opportunities with conspecifics of the opposite-sex and does not influence social receptivity toward strangers.

Understanding how the neuropeptide systems influence communication, and the initiation, maintenance, and breakdown of social bonds is important for understanding the basic neurobiological mechanisms of social bonding behavior. This dissertation provides critical insights into the role of OXT and AVP in modulating behavior during each of these social stages.

	Initiation of pair bonding	**Initiation of parental care**	**Maintenance of family unit coordination**	**Breakdown of parent-offspring bonds (from perspective of juveniles)**
Females	Inhibited by AVP	OXT enhances maternal care and communication	OXT decreases communication and changes division of labor increasing maternal load of parental care	No effect of OXT or AVP
Males	Enhanced by OXT; not influenced by AVP	OXT enhances paternal responsiveness but does not influence overall care or communication		OXT enhances parent-offspring bond

Table 1. Summary of main findings in dissertation.