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Intranasal oxytocin reduces pre-courtship aggression and increases paternal response in California mice (*Peromyscus californicus*)

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ABSTRACT

Oxytocin (OXT) is a neuropeptide that can facilitate prosocial behavior and decrease social stress and anxiety but can also increase aggression in some contexts. We investigated whether acute pulses of intranasal (IN) OXT influenced social behavior during social challenges that are likely to occur throughout the lifespan of a wild mouse. To test this, we examined the acute effects of IN OXT in the male California mouse (*Peromyscus californicus*), a monogamous, biparental, and territorial rodent, using a within-subjects longitudinal design. Social challenges included a pre-courtship male-female encounter conducted during the (1) initial aggressive and not the following affiliative phase of courtship, (2) same-sex resident intruder test, and (3) parental care test. Consecutive tests and doses were separated by at least two weeks. Males were treated with intranasal infusions of 0.8 IU/kg OXT or saline controls 5-min before each behavioral test, receiving a total of three treatments of either IN OXT or saline control. We predicted that IN OXT would 1) decrease aggression and increase affiliation during the pre-courtship aggression phase, 2) increase aggression during resident intruder paradigms, and 3) increase paternal care and vocalizations during a paternal care test. As predicted, during pre-courtship aggression with a novel female, IN OXT males displayed less contact aggression than control males, although with no change in affiliative behavior. However, post-pairing, during the resident intruder test, IN OXT males did not differ from control males in contact aggression. During the paternal care test, IN OXT males were quicker to approach their pups than control males but did not differ in vocalizations produced, unlike our previous research demonstrating an effect on vocalizations in females. In summary, during pre-courtship aggression and the paternal care test, IN OXT reduced antisocial behavior; however, during the resident intruder test, IN OXT did not alter antisocial behavior. These data suggest that IN OXT promotes prosocial behavior specifically in social contexts that can lead to affiliation.

1. Introduction

In social species, the quality and quantity of social interactions can be altered based on an individual's life history stage and environment. Throughout the lifespan, many social species encounter many different types of social interactions and must respond appropriately to acquire and maintain resources, mating opportunities, and reproductive fitness. What mechanisms underlie how animals alter social responses? Endogenous hormone and neuropeptide levels are important for bio-behavioral 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 [43, 67, 91, 102, 126]. OXT in the brain is released from large dense core vesicles that each release a large bolus, or pulse of OXT, into the synapse [80] and extracellular space [68]. Natural pulses of OXT are released during social experiences such as physical touch (e.g., being stroked on the back) [108], hearing vocalizations [101], and direct eye gaze between dogs (*Canis lupus familiaris*) and humans [87]. Previous studies manipulating OXT show significant effects on prosocial affiliative behaviors such as trust, social bonding, social recognition, parental care, and anxiolytic behavior in both human and animal models [2, 14, 54, 75, 99, 113]. However, OXT effects appear to be dependent on social group and environment. Prior to mating, OXT administered to males increased affiliative contact with familiar females [3, 28] and increased speed of pair bonding when administered to females [123, 128].

Abbreviations: OXT, Oxytocin; USV, ultrasonic vocalization.

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Post-mating, OXT enhanced aggression in male prairie voles during encounters with same-sex conspecifics [124].

In addition to increasing affiliative behaviors, OXT is involved in aggressive behaviors. In humans, OXT can increase envy, schadenfreude, defensive but not offensive aggression toward a competing outgroup, and domestic violence in men prone to aggression [13, 33, 34, 38, 103]. OXT is also associated with increased mate guarding in rats [63], prairie voles [2], and marmoset monkeys [26]. Furthermore, OXT is associated with increased maternal aggression toward potential predators [18]. In dogs, OXT also increases aggression towards owners but not strangers during a threatening approach test[62] . While the studies above show OXT increasing aggression, none of these studies have examined the effect of OXT on female-male interactions during the earliest phase of courtship, as expressed in aggressive species such as California mice and prairie voles [15, 51]. These data on the role of OXT in 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.

In addition to altering aggression levels, OXT is involved in shaping paternal behavior. In mandarin voles, chemogenetic activation of OXT neurons projecting from the PVN to the VTA and from the PVN to the NAc promoted paternal care [61]. In California mice, OXT plasma levels increase in expectant fathers, decrease in fathers, and increase when a father is separated from his mate and pups [55]. These rodent studies in voles and California mice suggest that social experience may drive important changes to the OXT system. These studies further enhance expectations for OXT to modulate paternal behavior. There is also evidence that OXT influences circuits associated with vocal production and may have a role in father-pup communication [114].

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 [3] and can have behavioral effects that persist for 30–50-min after administration [25]. Daily chronic doses of IN OXT (for three weeks during the juvenile-adolescent stage) induce long-term modifications to the OXT ligand and receptor expression in the brain [3, 36, 58]; however, single doses spread out across weeks are presumably less likely to have carry-over effects across tests [64].

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., [97]), including opposite-sex conspecifics (e.g., Pultorak et al., 2017). During pre-courtship aggression with an unfamiliar conspecific, there is a period of assessment and often aggression [51] that we will refer to as the pre-courtship aggression phase. This aggression can be 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) [51]. Once paired, female and male California mice form strong, reliable pair bonds but will still show reliable aggression toward unfamiliar conspecifics [7, 10, [116]117]; such aggression is decreased by an antagonist (V1a) to vasopressin [11], a neuropeptide similar to OXT that is often positively associated with aggression[45] [119]. 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[131], California mice mate 7–14 days after being paired [8, 51, 117]. 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, including huddling with and retrieving pups [5, 7–9, 48, 51, 83, 115], grooming and sniffing pups [76, 77] spending more time at the nest site with pups [66] defending the nest and offspring from potentially predatory conspecifics [98] and producing vocalizations that are correlated with parental care [57].

California mice also have a diverse, well-characterized repertoire of ultrasonic vocalizations (USVs), including simple sweeps, complex sweeps, syllable vocalizations, barks, and pup whines [20, 57, 69, 93, 97]. We previously demonstrated that in mother-offspring interactions, the primary call types were maternal simple sweeps and pup whines; maternal simple sweeps also correlated with both maternal care and pup whines [57]. Moreover, OXT administered to mothers stimulated the production of maternal sweeps but did not affect the whine vocalizations of their pups [57]. 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. Based on this, we predicted a similar response to OXT in fathers involving simple sweeps and pup whines. It is important to note that simple sweeps and pup whines have also been recorded in other social contexts, such as maternal care [57], biparental care [93, 94], and resident intruder tests [98]. Because we were not manipulating the OXT system in the pups and pup whines were not influenced when OXT was increased in mothers, 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 in males. 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.

2. Methods and materials

2.1. Animals

University of Wisconsin-Madison Institutional Animal Care and Use Committee approved this research. We started with 24 male *P. californicus* aged 5–10 months. They were group-housed with same-sex, age-matched cagemates (2–3 per cage; 48 × 27 × 16 cm) under a 14L: 10D light cycle with lights off at 4:00 pm. 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.

2.2. Intranasal oxytocin preparation

Male mice were infused intranasally with either sterile saline or IN OXT (0.8 IU/kg) (Bachem, Torrance, California) [57]. The IN OXT dose is equivalent to doses used in other animal models [4, 58, 85] and is similar to weight-adjusted doses used in clinical studies examining the effects of IN OXT on social deficits in autism [3]. IN OXT was dissolved in saline and prepared in one batch that was aliquoted into small plastic tubes and frozen at 20°C. IN OXT was defrosted just prior to administration. A blunt cannula needle (33-gage, 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 the compound was expelled dropwise through the cannula needle and allowed to absorb into the nasal mucosa (approximately10–20 s). 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 an intracerebroventricular infusion [111]. (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 [78, 79, 89, 90, 110]. Several studies have also shown changes in plasma OXT concentrations that peak between 15 and 30-min post-administration [49, 53]. 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 [41, 42]. Other studies indicate that some of the effects of IN OXT are acting through peripheral mechanisms [29, 81, 95]. 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 [57, 106].

2.3. Behavioral tests

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

2.3.1. Pre-courtship aggression test

Male California mice aged 5–10 months (at the start of behavioral trials) 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. This movement allowed the test to be conducted on neutral territory. 5-min after the dose of OXT or saline, a novel, unrelated female aged 5–10 months was placed into the new home cage. Their interaction was videotaped for 10-min (**Fig. 1A**). This time period was chosen because it allowed enough time to quantify behavioral differences in aggression. After the recording, the male and female continued to be housed together for the remainder of the experiments.

2.3.2. Resident intruder test

We continued to use the same male and female pairs as in the pre-courtship aggression test above, but this test occurred 14 days after being paired. Residency in the home cage was established by housing the mice in the same home cage for six consecutive days. This is more than sufficient time to establish residency in males [12, 50, 83, 129]. Immediately before testing, female pair mates were removed from the home cage. 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 the home cage with soiled bedding. This allowed the test to be conducted on the resident test mouse's territory. 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**). This time period was chosen because it minimized the time that the mice were exposed to an aggressive encounter but allowed enough time to quantify behavioral differences in aggression. Aggression in this test happens with relatively short latencies and greater intensities compared to aggression tests in neutral territory (e.g., pre-courtship aggression test) [98]. 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.

2.3.3. 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

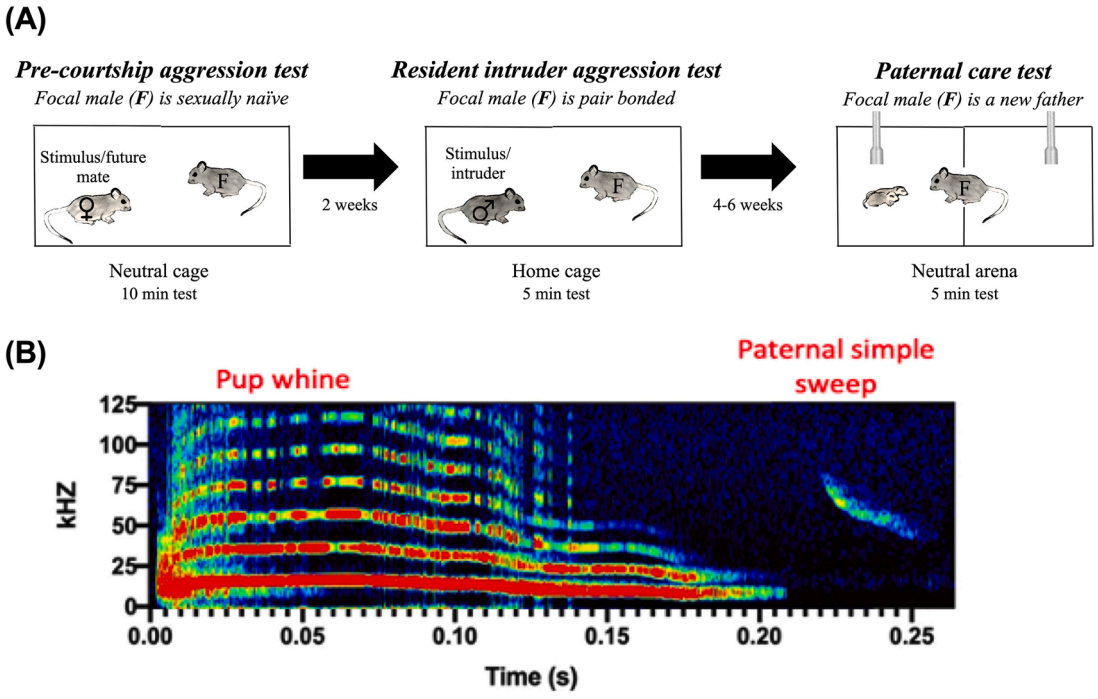

Fig. 1. Experimental design. (A) Timeline of the three behavioral tests throughout the longitudinal study. **(B)** Representative pup whine and paternal simple sweep USVs. Pup whines have multiple harmonics, a peak frequency around 20 kHz, and downward modulation at the end of the call that distinguishes 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.

four to six weeks after the resident-intruder test—on the first or second day after the 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 estrus. The pups were removed from the mother, and the mother was taken out of the home cage. Next, the father and pups in the 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 [57, 93, 97]. 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 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 the 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 were able to interact with each other through the mesh divider 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 paternal care.

2.4. 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. Table 1**. For each of the measures present, we used a continuous sampling method. We measured both frequency and duration for all behaviors but only used duration in the analyses because it was more accurate for discriminating levels of aggression across mice (wrestling and chasing bouts vary widely in duration). In the pre-courtship aggression and resident-intruder tests, we were not only interested in averages of individual aggressive behaviors but were also interested in escalation of aggression as shown by attack latency and the proportion of wrestling (contact) aggression. To calculate proportion of wrestling aggression, we used the following equation: $\left(\frac{\text{wrestling (s)}}{\text{lunging (s)} + \text{chasing (s)} + \text{wrestling (s)}}\right)$.

2.5. Ultrasonic vocalization analysis

Techniques used for recording were similar to those previously used in our laboratory [57, 94, 97]. 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 [70]. 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 [67, 71], and the typical downward modulation at the end of the call often distinguishes these calls from adult syllable vocalizations ([57]; Nathaniel Rieger, Jose Hernandez, & Catherine Marler, unpublished) (**Fig. 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 [72] (**Fig. 1B**). It is extremely rare for pups to produce simple sweep USVs during PND 0–4 (Nathaniel Rieger, Jose Hernandez, & Catherine Marler, unpublished). When young pups produce simple sweeps, they are produced much faster and present completely vertical on the spectrogram [67]. This makes these rare pup simple sweeps easy to distinguish from the slower adult simple sweep USVs (**Fig. 1B**). Because of 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).

2.6. 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 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 deletion of the behavior videos (1 control male, 2 OXT males), and five 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 conditions 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 the effect of an X variable was tested for a relationship with multiple Y variables. False discovery rate was set at ten percent, making the positive predictive value of significant findings 90 percent [6, 31, 109].

3. Results

3.1. Pre-courtship aggression test

To determine whether IN OXT influenced aggression and escalation to contact aggression during the pre-courtship aggression test, we assessed latency to attack, time spent lunging, chasing, and wrestling, and proportion of time spent wrestling compared to other forms of aggression in male mice given IN OXT versus saline. We found that OXT increased amount of time spent wrestling ($U = 33$, z-*score*=−1.88, $p < 0.05$) (**Fig. 2D**), with a nonsignificant trend for increasing the time to initiate an attack (attack latency) ($U = 36.5$, z-*score*=−1.59, $p = 0.056$) (**Fig. 2A**) and no effect on time spent lunging ($U = 62.50$, z-*score*=1.08, $p = 0.86$) or chasing ($U = 58.50$, z-*score*=0.41, $p = 0.66$) (**Fig. 2B-C**). Additionally, we found that OXT decreased the proportion of wrestling, a form of contact aggression, compared to other aggressive behaviors between the male and female during the first 10-min of pre-courtship aggression ($U = 33$, z-*score*=−1.86, $p < 0.05$) (**Fig. 2E**). Overall, lunging aggression levels made up a relatively small proportion of the

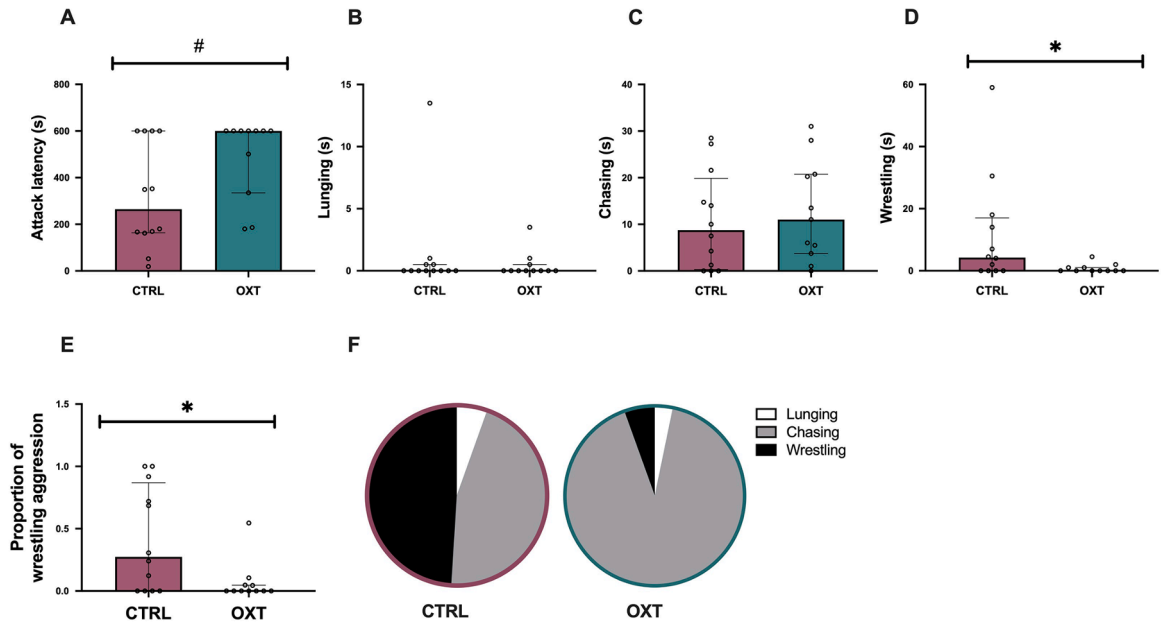

Fig. 2. Pre-courtship aggression test. (A) There was a nonsignificant trend for males given OXT to increase latency to attack a potential mate. **(B)** There was no difference in lunging duration between OXT and CTRL groups. **(C)** There was no difference in chasing duration between OXT and CTRL groups. **(D)** Males given OXT wrestled for less time than controls. **(E)** Males given OXT had a significantly smaller proportion of wrestling than control males during the first 10 min of courtship. **(F)** Pie chart showing escalating aggressive behavior (from light: low escalation, to dark: high escalation). Values shown in bar graphs represent the median and interquartile range. Values in pie charts are group means for each behavior. *$p<0.05$, #$p<0.1$ for differences between control and OXT.

aggressive behaviors in both control and OXT males. Differences across groups arose in the proportion of wrestling aggression (higher in control males) and chasing aggression (higher in OXT males) (Fig. 2F). Levels of non-contact aggression had relatively similar medians across groups (lunging aggression: CTRL=0 and OXT=0; chasing aggression: CTRL=8.75 and OXT=11) (**S. Table 2**). The biggest difference between treatment groups was the median amount of time spent engaged in contact aggression (wrestling aggression: CTRL=4.25 and OXT=0) (**S. Table 2**). Thus, males given OXT are less likely to escalate aggression to contact aggression over the course of the interaction.

3.2. *Resident intruder aggression test*

To determine whether IN OXT influenced aggression and escalation to contact aggression during the resident intruder test, we assessed latency to attack, time spent lunging, chasing, and wrestling, and

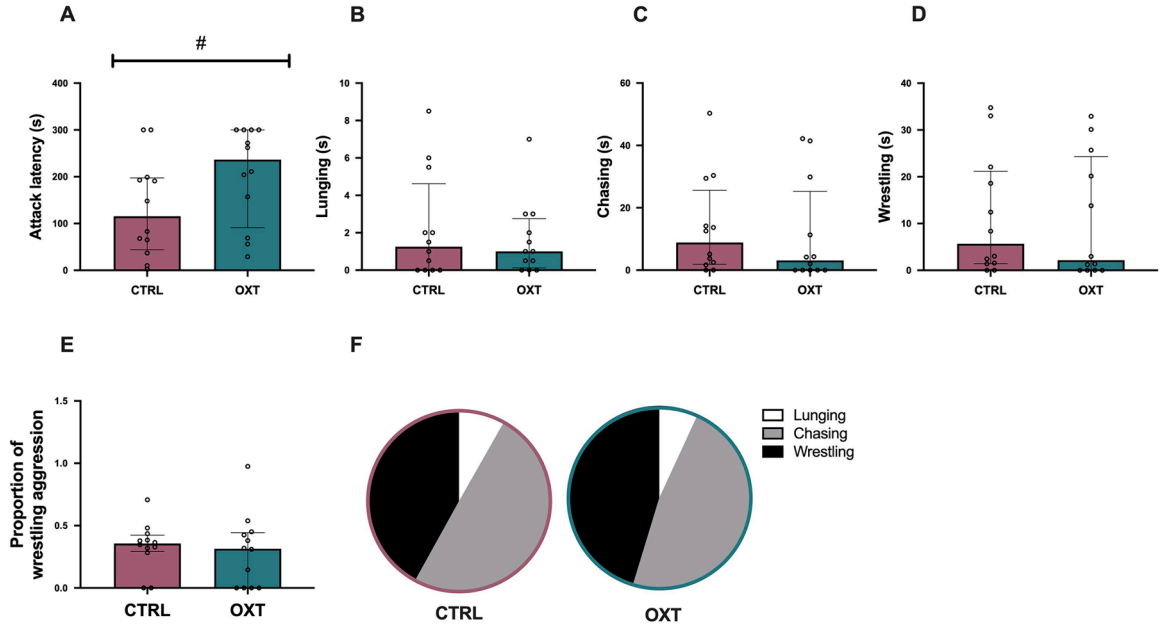

Fig. 3. Resident intruder aggression test. (A) There was a nonsignificant trend for males given OXT to increase latency to attack a resident intruder. **(B)** There was no difference in lunging between OXT and CTRL groups. **(C)** There was no difference in chasing between OXT and CTRL groups. **(D)** There was no difference in wrestling between OXT and CTRL groups. **(E)** OXT and control males showed no difference in proportion of wrestling during a 5-min resident intruder encounter. **(F)** Pie chart showing escalating aggressive behavior (from light to dark). Values shown in bar graphs represent the median and interquartile range. Values in pie charts are group means for each behavior. *$p<0.05$, #$p<0.1$ for differences between control and OXT.

proportion of time spent wrestling compared to other forms of aggression. Similar to the pre-courtship aggression test, we found that IN OXT males had a nonsignificant trend for increasing attack latency ($U = 43$, z-*score*=−1.31, $p = 0.095$), and we also found that OXT did not influence time spent lunging ($U = 69.50$, z-*score*=1.08, $p = 0.86$) or chasing ($U = 56$, z-*score*=−0.33, $p = 0.37$). (Fig. 3**A-C**). However, unlike the pre-courtship aggression test, we found that IN OXT did not significantly influence wrestling duration ($U = 60$, z-*score*=0, $p = 0.50$) or proportion of wrestling bouts ($U = 63.50$, z-*score*=0.36, $p = 0.637$) between the males during a 5-min resident intruder test (Fig. 3**D-E**). 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. 3**F**). Both chasing and wrestling aggression made up approximately equal proportions of aggressive behavior in the resident intruder aggression test (Fig. 3**F**). Levels of all types of aggression had relatively similar medians across groups (lunging aggression: CTRL=1.25 and OXT=1; chasing aggression: CTRL=8.86 and OXT=3.11; wrestling aggression: CTRL=5.69 and OXT=2.16) (**S. Table 3**). As there were no significant effects of IN OXT, aggression of males in the resident-intruder aggression was not influenced by OXT.

3.3. 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, pup licking, and paternal simple sweep USVs in fathers given IN OXT versus saline. Paternal simple sweeps could occur simultaneously or separately with all other behaviors, and pup huddling and licking could also occur simultaneously or separately. Fathers given IN OXT were significantly faster at approaching their pups after a brief separation ($U = 10.50$, z-*score*=−2.01, $p < 0.05$) (Fig. 4**A**). Despite initial differences in paternal care response, there were no significant differences between IN OXT in other aspects of paternal care. The difference between control and OXT was not significant for huddling ($U = 14.50$, *z-score*=−1.12, $p = 0.13$) (Fig. 4**B**), but showed a nonsignificant trend for increased licking ($U = 12$, *z-score*=−1.48, $p = 0.07$) (Fig. 4**C**). Neither IN OXT nor control fathers engaged in any retrieval behavior throughout the test, so this type of paternal care was not analyzed (**S. Table 4**). There were no differences in number of pups across treatments groups ($U = 29$, *z-score*=3.72, $p = 0.99$) (see **S. Table 4**).

Next, we assessed whether IN OXT would influence paternal and/or pup USVs and 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,

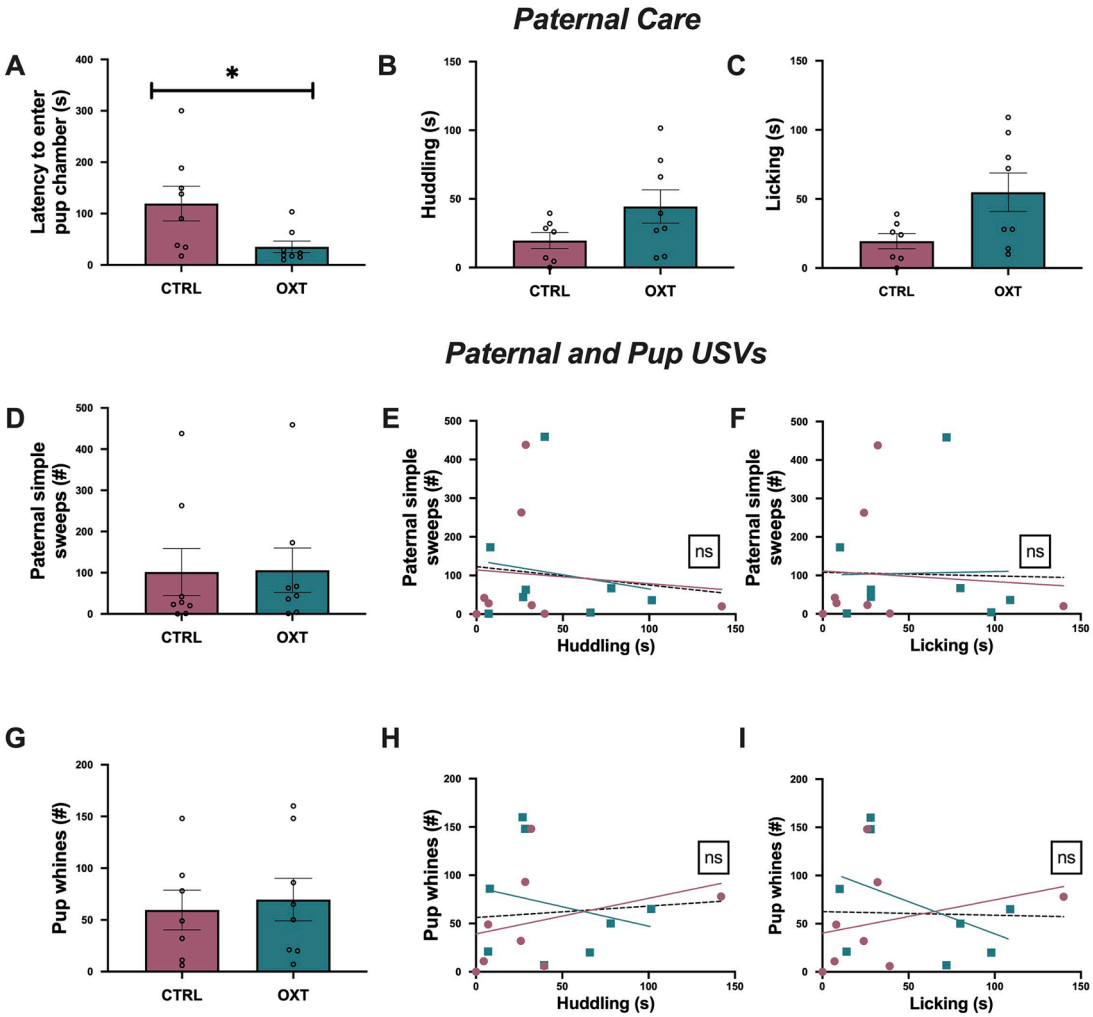

Fig. 4. Paternal care test. OXT males had shorter latencies to approach their pups than control males **(A)**. OXT males did not show significant differences in huddling **(B)** or licking **(C)** behavior. **(D)** Males given OXT did not make more simple sweeps than control males. Paternal simple sweeps 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. Values shown in bar graphs represent the median and interquartile range. *$p<0.05$ for differences between control and OXT.

huddling and licking. Fathers given IN OXT did not produce more simple sweeps than controls ($U = 23.50$, *z-score*=−0.25, $p = 0.40$) (Fig. 4**D**). There were also no differences in number of pup whines produced in offspring of IN OXT versus control fathers ($U = 24.50$, *z-score*=0.58, $p = 0.72$) (Fig. 4**E**).

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 ($F_{2,16} = 0.21$, $p = 0.65$, $\eta^2 = 0.016$) (Fig. 4**H**) or licking ($F_{2,16} = 0.01$, $p = 0.91$, $\eta^2 = 0.00$) (Fig. 4**J**). Similarly, we found no main effects of pup whines on huddling ($F_{2,16} = 0.05$, $p = 0.81$, $\eta^2 = 0.00$) (Fig. 4**I**) or licking ($F_{2,16} = 0.07$, $p = 0.80$, $\eta^2 = 0.00$) (Fig. 4**K**).

4. Discussion

Throughout an animal's lifetime, OXT levels change in response to certain life events such as early pre- and postnatal experience, pair bonding, intrasexual aggression, and parenting [19]. Our study assessed the response of male California mice to a single OXT pulse immediately prior to different challenges that would naturally occur during its lifespan. During potentially affiliative contexts—tested here during early phases of courtship—OXT administered to males reduced antisocial approach toward the female through reduced aggression. In contrast, during contexts with little to no potential for affiliative behavior—tested here in a resident-intruder paradigm—OXT did not reduce antisocial 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 related to affiliative behavior, such as during formation of a pair bond or parent-offspring interactions when cooperative social units form.

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 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 [51] or bonding [94]. This is also similar to the behavioral sequence seen in research in male and female prairie voles during bond formation [24, 28, 59, 120, 122] and marmosets [105]. 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 were more likely to escalate to contact aggression via wrestling behavior. The nonsignificant trend for increased attack latency with OXT treatment also supports the idea that OXT is reducing escalation of aggression. In this context, OXT may increase the rapid social assessment of 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 using less aggression when interacting with females. 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 [60]. However, this is the first study reporting the 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 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 number of bites or attack latency in a resident intruder aggression test in California mice [106]. It is possible that in a highly territorial and monogamous species, there may be selection for a set maximum aggressive response to an intruding male. Interestingly, intracerebroventricular injections of vasopressin 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 [11].

While we injected only males with OXT in the current study, other studies have examined effects in females. Aggression levels naturally vary by sex, and OXT complicates the sex-dependent effects on aggression because females and males often have different responses to OXT. For example, developmental exposure to intraperitoneal injection of OXT on postnatal day 1 increased same-sex aggression in female prairie and mandarin voles but not male prairie and mandarin voles [2, 65]. These studies suggest early exposure to sex steroids sets the stage for different reactivity to OXT modulation. In adult prairie voles given lever-press access to a familiar "mate" or a stranger, males showed greater stranger-directed aggression than females [118]. In both females and males, however, OXTR genotype influenced stranger-aggression such that carriers of the C allele (as opposed to carrying the T/T genotype) at intronic OXTR locus NT213739 showed less aggression toward strangers [118]. Carriers of the C allele showed greater OXTR binding and thus likely had greater OXT activity [1, 74]. This suggests that OXT may decrease aggression in both females and males. Because our study used a relatively low dose of OXT, we cannot rule out the possibility that at higher doses, we would see a decrease in aggression in male-male encounters. Lastly, in California mice, there is a relationship between pair bonding, OXT, sex, and coordinated response to aggression. Females but not males alter their approach toward a conspecific intruder in response to a single IN OXT dose (Rieger & Marler unpublished data). This suggests that OXT may be more likely to modulate aggression in females versus males, but more studies are needed.

In addition to sex differences, previous studies reveal species differences in same-sex aggression tests. For example, in less territorial species—such as house mice, rats, and humans—there are different effects of OXT on aggression. In house mice, OXTR null male (females were not tested) mice expressed increased intrasexual aggression [37]. OXT manipulations in the lateral septum of female rats demonstrated that OXT increased and vasopressin decreased aggression towards same-sex intruders [132]. Studies in humans have also shown a positive association between increased aggression, increased competition, and increased OXT [32, 38, 47, 88]. However, studies in monogamous marmosets [26], monogamous titi monkeys [125], female and male rats [21–23, 35], house mice primed for aggressive behavior due to social isolation [112], and house mice bred for callous traits (i.e., not responsive to emotions of others) [130] 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 [57]. 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 (although this was supported by a nonsignificant trend). Studies in Mandarin voles have also shown similar effects of OXT on latency to engage in paternal care [127]. Reduced latency to approach pups in IN OXT fathers suggests that IN OXT may increase paternal motivation for pup contact without strongly influencing the quality of paternal care. Other studies also showed that OXT influenced motivation because activation of the OXT system increased dopamine and reinforced rewarding behavior [16, 17, 39, 84]. 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 [30, 40, 107, 121]. 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 [46, 100] and human fathers ([44, 52, 86]; [133]; review by [56]). 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 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) [57]. However, it is also possible that fathers are more stressed in the absence of their partners than mothers are and therefore vocalize less. This is supported by findings in several other species that show blunted vocalization in response to heightened stress [27, 82, 96, 104]. 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 parental care, there are differences in parental care expression between fathers and mothers. For example, in a very similar paradigm, mothers showed retrieval behavior, but fathers did not [57]. This finding highlights the sex-specific roles for retrieval behavior in the absence of one parent. 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 [98]. 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 or reduces antisocial behavior in affiliative-prone contexts, but not during the context of direct threat or competition. These results generally align with the social salience hypothesis of OXT [73, 92, 102], with the caveat that social context is important. 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.

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Supplementary materials

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