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Mating and social exposure induces an opioid-dependent conditioned place preference in male but not in female prairie voles (*Microtus ochrogaster*)

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Abstract

In rodents, sexual stimulation induces a positive affective state that is evaluated by the conditioned place preference (CPP) test. Opioids are released during sexual behavior and modulate the rewarding properties of this behavior. Prairie voles (*Microtus ochrogaster*) are a socially monogamous species, in which copulation with cohabitation for 6 h induces a pair bond. However, the mating-induced reward state that could contribute to the establishment of the long-term pair bond has not been evaluated in this species. The present study aimed to determine whether one ejaculation or cohabitation with mating for 6 h is rewarding for voles. We also evaluated whether this state is opioid dependent. Our results demonstrate that mating with one ejaculation and social cohabitation with mating for 6 h induce a CPP in males, while exposure to a sexually receptive female without mating did not induce CPP. In the female vole, mating until one ejaculation, social cohabitation with mating, or exposure to a male without physical interaction for 6 h did not induce CPP. To evaluate whether the rewarding state in males is opioid dependent, the antagonist naloxone was injected i.p. The administration of naloxone blocked the rewarding state induced by one ejaculation and by social cohabitation with mating. Our results demonstrate that in the prairie vole, on the basis of the CPP in the testing conditions used here, the stimulation received with one ejaculation and the mating conditions that lead to pair bonding formation may be rewarding for males, and this reward state is opioid dependent.

Keywords

Sexual reward; Conditioned place preference; Social cohabitation with mating; Opioids and voles

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1. Introduction

Sexuality is an essential aspect of human social behavior and has important implications for physical and psychological well-being. Thus, sexual behavior enhances the formation of enduring relationships, which increases longevity and contributes to an adequate function of the immune and cardiovascular systems, resulting in lower incidence of psychiatric disorders (House et al., 1988; Kiecolt-Glaser and Newton, 2001). Deeper pair relationships promote health, well-being, survival, and even social success. Furthermore, social exclusion and the loss of a partner result in depression and feelings similar to physical pain (Zivin and Christakis, 2007).

Despite the importance of pair bonding, its mechanisms and possible effects on the central nervous system are not completely understood. This limitation is produced by the high complexity of the human nervous system and ethical concerns, which require the use of experimental models to explore these paradigms in the laboratory. The socially monogamous species *Microtus ochrogaster* (prairie vole) is used to study the neuronal mechanisms involved in pair bonding because of the reproductive strategy of this species. Prairie vole couples cohabitate the nest, defend their territory, and display parental behaviors, thus forming solid family structures [reviewed in (Gobrogge and Wang, 2015; Gobrogge, 2014; Lieberwirth and Wang, 2016; McGraw and Young, 2010)]. Pair bonding is established when a male and a female cohabitate, with mating, for at least 6 h, or when they cohabitate, without mating, for 24 h. In both instances, voles show a clear preference for the mating partner and selective aggression to other males or females (Carter et al., 1995; Insel et al., 1995; Wang et al., 1997; Williams et al., 1992).

Pair bond formation and maintenance critically depend on the activation of brain structures that also regulate other hedonic behaviors such as social attachment, maternal care, and sexual behavior, which were reviewed previously (Burkett and Young, 2012; Numan and Woodside, 2010; Numan and Young, 2016). These brain areas include the olfactory bulbs, medial amygdala, bed nucleus of the stria terminalis, medial preoptic area, dorsal raphe, anterior hypothalamus, nucleus accumbens, prefrontal cortex, and the mesocorticolimbic system (Amadei et al., 2017; Cushing et al., 2003; Gobrogge and Wang, 2015; Gobrogge, 2014; Johnson and Young, 2015, 2017; Kirkpatrick et al., 1994; Young et al., 2001; Young et al., 2005).

Mating-induced pair bonding likely involves reinforcing properties associated with mating. In several mammals including humans, it has been demonstrated that sexual behavior endures and is repeated because it induces a positive affective state. Several research groups including ours have demonstrated that when male and female rats, a species that does not form a pair bond, are able to control (pace) the rate of sexual stimulation, sexual behavior induces a positive affective state, evaluated by the conditioned place preference (CPP) test (Agmo and Berenfeld, 1990; Coria-Avila et al., 2006; Coria-Avila et al., 2005; Martinez and Paredes, 2001; Parada et al., 2010; Paredes and Alonso, 1997; Pfaus et al., 2012). It is well documented that the release of opioids during sexual behavior contributes to the rewarding consequences of mating. Administration of the opioid antagonist (naloxone) completely blocks the reinforcing properties of mating in male and female rats (Agmo and Gomez,

1993; Coria-Avila et al., 2008; Mehrara and Baum, 1990; Paredes and Martinez, 2001). From the above described data, parsimony will suggest that mating induces a reward state that could contribute to the establishment of the long-term pair bond in the prairie vole.

The opiate system is involved in social attachment, maternal bonding, social learning, and sexual reward (Nelson and Panksepp, 1998; Panksepp et al., 1980). In female voles, the administration of the opioid antagonist naltrexone blocks the formation of a partner preference. Furthermore, administration of the μ -opioid receptor (MOR) selective antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CT-AP) into the caudate-putamen (CP) 24 h before cohabitation with a male inhibits the preference for the sexual partner (Burkett et al., 2011). In addition, inhibition of MORs within the dorsomedial nucleus accumbens shell inhibited partner preference formation without affecting mating behavior (Resendez et al., 2013).

We hypothesized that cohabitation with mating for 6 h, which produces pair bonding (Williams et al., 1992), and sexual stimulation until one ejaculation, which is rewarding in male and female rats (Agmo and Berenfeld, 1990; Martinez and Paredes, 2001), will induce a reward state in male and female voles. Because opioids are involved in sexual reward and social attachment, we propose that the positive affective state induced by one ejaculation and 6 h of mating would be blocked by the administration of an opioid antagonist.

2. Methods

Six *M. ochrogaster* mating pairs were generously donated by Dr. Larry J. Young from his colony at Emory to establish a colony at the Instituto de Neurobiología, Universidad Nacional Autónoma de México. Animals were maintained in a room with controlled light (14:10 light-dark cycle) and temperature (23 °C) conditions. The animals were provided Rabbit diet high fiber 5326 (LABDIET), oats, sunflower seeds, and water *ad libitum*. Adult males and females (3–4 months old) were used in this experiment. Females were bilaterally ovariectomized under deep anesthesia with a mixture of ketamine (Cheminova, 60 mg/kg) and xylazine (Cheminova, 4 mg/kg), and were allowed to recover from the surgery for 1 week. To induce sexual receptivity, females were injected daily with estradiol benzoate (EB, 0.5 μ g/vole, Sigma-Aldrich) for 4 days before the behavioral test. The EB injections continued daily until the end of the CPP tests (12 days total). This treatment consistently induces sexual receptivity in this species (Roberts et al., 1998; Smale et al., 1985). In voles, progesterone is not needed to induce sexual receptivity (Dluzen and Carter, 1979).

All experiments were performed in accordance with the “Reglamento de la Ley General de Salud en Materia de Investigación para la Salud” of the Mexican Health Ministry, which follows NIH guidelines for the use and care of animals in research. The experiments were approved by the Instituto de Neurobiología Animal Care Committee and by the Ethics Committee of the Instituto Nacional de Perinatología. The timeline of the different experiments is depicted in Fig. 1.

2.1. Conditioned place preference (CPP)

The test was performed in a three-compartment acrylic box; the central compartment (22 × 24 × 32 cm) was gray and was connected with the lateral compartments through guillotine doors (23 × 37 × 32). One of the lateral compartments was white and the other was black; thus, the lateral compartments offered distinct color stimuli.

We followed the procedure described by Dr. Wang's research group with slight modifications (Liu et al., 2011; Young et al., 2011). Briefly, the CPP test includes a pretest, six training sessions, and a test. During the pretest (day 1), each vole was placed at the center of the cage (gray compartment) with the guillotine doors closed. After 1 min of habituation, doors were opened to allow the animals to move freely between compartments for 30 min. The time that voles spent in the white and black compartments was recorded. The compartment where the vole spent more time was called the preferred compartment, while the compartment where the vole spent less time was called the reinforced compartment. On day 2, voles were taken directly from their home cages and placed without any stimulation in the preferred compartment for 40 min. On day 3, after the voles performed the corresponding behavioral test (sexual exposure, mating until one ejaculation or social cohabitation with mating for 6 h) in the behavioral cage, subjects were gently withdrawn and placed in the reinforced compartment of the adjacent CPP box for 40 min. This procedure was repeated three times. The test (day 8) was performed following the same procedure as in the pretest. A CPP was defined by a statistically significant increase in the time spent in the reinforced compartment in the test compared to the pretest. This parameter is the most consistently used to evaluate the reinforcing effects of a stimulus after conditioning (Carboni and Vacca, 2003; Schechter and Calcagnetti, 1993; Tzschentke, 2007).

2.2. Sexual behavior tests

Mating pairs were formed from different breeding pairs. Males were introduced into the acrylic behavioral cage (44 × 23 × 21 cm) 5 min before the females. The following parameters were recorded: number and latency of mounts, intromissions and ejaculations, post-ejaculatory interval (ejaculation latency - intromission latency), and inter-intromission interval (III, ejaculation latency/number of intromissions). Additionally, during each mount or intromission bout, the number of pelvic thrusts (rhythmic movement of the pelvis and hind limbs) was recorded. For females, the lordosis reflex was recorded. A score of "1" was given if the female adopted an immobile posture with concave back flexion, neck extension, elevation of the hindquarters, and tail deviation to facilitate male mounting and intromission. A score of "0" was registered if in response to the mount the female avoided the male, usually by placing her back on the ground or attacking the male. Lordosis Quotient (LQ; number of lordosis/number of mounts) was recorded, see Fig. 2.

2.3. Spontaneous locomotor activity, motor execution, and balance tests

Males were individually placed in the center of an automatized locomotor activity chamber equipped with horizontal and vertical infrared beams (Acuscan Instruments Inc.). Locomotor activity was recorded for 6 h with males having free access to food and water. To determine whether naloxone induced motor alterations, 16 males were randomly assigned to one of the following groups: (a) control ($N = 8$) males that received three injections of 0.9% NaCl (1

ml/kg) and (b) naloxone ($N = 8$) males that were injected three times, in 2-h intervals, with naloxone (5 mg/kg dissolved in 1 ml of 0.9% NaCl). The total distance traveled and time in movement were registered. One week after the locomotor activity test, voles were tested for motor execution and balance using an accelerating rotarod (IITC life Science, Woodland Hills) with five drums (9.5 cm diameter). Briefly, males were trained twice a day for 3 days without drugs. Voles were placed on a drum that was programmed to increase from 4 to 20 rpm in 60 s. In the test, males received NaCl 0.9% or naloxone, and the test was repeated three times at 2-h intervals. The time walking on the top of the rotarod, the speed at which the animal fell, and the distance traveled were recorded.

2.4. Experiment 1. Determine whether one ejaculation induces a reward state

Male and female voles were randomly divided into the following groups: (a) Social exposure (Soc.Exp; $N = 10$ per sex): voles were tested in acrylic behavioral cages ($44 \times 23 \times 21$ cm) equally divided into two compartments by a plastic screen. The male was placed in one side of the cage and the female in the opposite compartment. In this arrangement, voles were allowed to hear, smell, and see the opposite-sex conspecific without physical contact for 30 min. (b) One ejaculation (1E; $N = 11$ males and 10 females); one of the females was eliminated because she spent all the time in one compartment of the CPP test): voles were placed in the acrylic behavioral cage without the division and were allowed to mate until ejaculation. Immediately after the behavioral test, voles were gently placed in the reinforced compartment of the CPP acrylic box (days 3, 5, and 7; Fig. 1A).

2.5. Experiment 2. Determine whether social cohabitation with mating for 6 h induces a reward state

Another group of gonadally intact males and ovariectomized females were randomly assigned to one of the following groups: (a) Control (C; $N = 11$ per sex): males and females were placed in individual acrylic behavioral cages; in this condition, they were not exposed to sexually relevant cues or sexual activity. (b) Social exposure (Soc.Exp; $N = 10$ per sex): same conditions as in experiment 1, Soc.Exp group. (c) Social cohabitation with mating (SCM; $N = 10$ per sex): males and females from different breeding pairs were placed in acrylic behavioral cages and allowed to mate freely. All the behavioral tests lasted 6 h, with voles having free access to food and water. As in experiment 1, voles were gently placed in the reinforced compartment of the CPP acrylic box immediately after the behavioral test (days 3, 5, and 7; Fig. 1B).

2.6. Experiment 3. Determine whether the rewarding properties of mating and social exposure are opioid dependent

Once we determined in experiments 1 and 2 that one ejaculation and 6 h of cohabitation in males induced CPP, in the third experiment, we evaluated whether the preferential μ -opioid receptor antagonist naloxone could block the CPP and whether this drug induced motor alterations. A third group of voles was randomly assigned to one of the following groups: (a) Morphine (M, $N = 12$; 6 males and 6 females): voles that were injected i.p with the opioid agonist morphine (1 mg/kg) as a reinforcer; this dose consistently induces CPP in rats (Agmo and Berenfeld, 1990; Camacho et al., 2009; Mucha and Iversen, 1984; Portillo and Paredes, 2009). (b) Morphine plus naloxone (M + N, $N = 14$; 7 males and 7 females):

subjects received a morphine injection, followed by an i.p. naloxone injection (5 mg/kg) 5 min later, Fig. 1C. A similar dose of naloxone blocked the CPP induced by paced mating in male (Agmo and Berenfeld, 1990; Mehrara and Baum, 1990) and female rats (Paredes and Martinez, 2001) without affecting sexual behavior.

To determine whether the rewarding properties of mating and social exposure are opioid-dependent, another set of males was randomly assigned to one of the following groups: (a) Control plus naloxone (C + N; $N = 10$): males were placed alone in the acrylic behavioral cage for 6 h ($N = 7$) or 30 min ($N = 3$) and received one or three injections of naloxone (5 mg/kg) dissolved in NaCl 0.9%. The first drug injection was 10 min before the behavioral test and then every 2 h. It has been reported that naloxone's half-life is 30–90 min (Lewis et al., 2012; Ngai et al., 1976); therefore, this procedure allowed us to block the opioid receptors for the entire test. (b) Social cohabitation with mating for 6 h plus saline (SCM + S, $N = 10$): in this group, males mated with a receptive female for 6 h and received three injections of saline. (c) One ejaculation plus naloxone (1E + N; $N = 8$): males that received one injection of naloxone 10 min before the behavioral test and mated with a nonfamiliar receptive female until one ejaculation. (d) Social cohabitation with mating for 6 h plus naloxone (SCM + N; $N = 10$): males and females from different breeding pairs that mated for 6 h received three naloxone injections, Fig. 1D. Open field test and rotarod were used to determine whether naloxone administration induced motor alterations that could influence the CPP test. For these experiments, we used 8 males that received 0.9% NaCl and 8 voles injected with naloxone, Fig. 1E.

3. Statistical analysis

Data from the time in the reinforced compartment were analyzed by a paired *t*-test. Data from the sexual behavioral tests were not normally distributed and were analyzed by the Kruskal–Wallis test; in case of significant effects, the Mann–Whitney *U* test was used. Open field data were analyzed by a *t*-test, and rotarod data were not normally distributed and analyzed by a Mann–Whitney *U* test. All the statistical tests were performed in the SigmaPlot 11.0 software. Statistically significant differences were considered as $p < 0.05$. Effect size was calculated by Cohen's *d* using the following formula: Cohen's

$d = M1 - M2 / \sqrt{[(\sigma_1^2 - \sigma_2^2)]/2}$. *M* is the mean and σ standard deviation of groups 1 and 2, respectively, and the effect-size $r = d / \sqrt{(d^2 + 4)}$ (Salkind, 2014).

4. Results

4.1. Experiment 1. One ejaculation is reinforcing for the male but not for the female vole

4.1.1. Males—Males from the 1E group spent significantly more time in the reinforced compartment in the test than in the pretest ($T_{(10)} = -2.41$, $P = 0.037$, $d = -1.05$, $r = -0.46$). No significant differences between pretest and tests were found in the Soc.Exp group ($T_{(9)} = -0.725$, $P = 0.487$, $d = 0.22$, $r = -0.11$). Fig. 3A.

4.1.2. Females—Females receiving 1E ($T_{(9)} = -0.83$, $P = 0.43$, $d = -0.26$, $r = -0.13$) or exposed to a male ($T_{(9)} = -2.1$, $P = 0.068$, $d = -0.73$, $r = -0.34$) did not show a significant increase between the pretest and test in the time in the reinforced compartment. Fig. 3B.

4.2. Experiment 2.- social cohabitation with mating is rewarding to the male but not the female vole

4.2.1. Males—SCM males showed a significant increase in the time spent in the reinforced compartment ($T_{(9)} = -5.2$, $P < 0.001$, $d = -1.91$, $r = -0.69$) between test and pretest; however, no significant differences were found in the Soc.Exp males ($T_{(9)} = -2.2$, $P = 0.055$, $d = -0.85$, $r = -0.39$). As expected, males from the C group did not show significant differences between the test and pretest in the time spent in the reinforced compartment ($T_{(10)} = 0.073$, $P = 0.44$, $d = 0.03$, $r = 0.015$), see Fig. 4A.

4.2.2. Females—No significant differences between test and pretest were found in C, Soc.Exp, and SCM females in the spent time in the reinforced compartment ($T_{(10)} = -0.61$, $P = 0.53$, $d = -0.28$, $r = -0.14$; $T_{(9)} = 1.5$, $P = 0.17$, $d = 0.67$, $r = 0.31$; $T_{(9)} = -1.16$, $P = 0.28$, $d = -0.49$, $r = -0.24$ respectively), see Fig. 4B.

4.3. Experiment 3. Mating and social exposure rewarding properties are opioid dependent

To determine whether the rewarding state induced by one ejaculation and social exposure with mating for 6 h in males is opioid dependent, we used the opioid antagonist naloxone. First, we determined if our naloxone dose could block the CPP induced by the opioid agonist morphine. Our data showed that voles treated with morphine show a clear CPP (increased time in the reinforced compartment $T_{(11)} = -5.8$, $P < 0.001$, $d = -2.32$, $r = -0.76$). Naloxone blocked the CPP induced by morphine administration (time in the reinforced compartment $T_{(11)} = 0.5$, $P = 0.62$, $d = -0.19$, $r = -0.09$), Fig. 5.

Data from the CPP test showed that naloxone by itself did not induce CPP because control males treated with naloxone (C + N) showed no significant differences in the amount of time in the reinforced compartment ($T_{(9)} = 0.94$, $P = 0.37$, $d = 0.37$, $r = 0.18$). Males that ejaculated once and received naloxone (1E + N) did not show an increase between test and pretest in the time spent in the reinforced compartment ($T_{(7)} = 0.4$, $P = 0.69$, $d = 0.18$, $r = 0.09$). Similarly, naloxone injections blocked the CPP induced by the social cohabitation with mating (SCM + N) because no significant differences were found between the pretest and test in the time spent in the reinforced compartment ($T_{(9)} = -1.47$, $P = 0.18$, $d = -0.42$, $r = -0.2$). Naloxone blockade of CPP induced by sexual stimulation was not due to the aversive effects of repeated injections because male voles from the SCM + S group that received three saline injections showed a clear CPP (time in the reinforced compartment $T_{(9)} = -2.6$, $P = 0.03$, $d = -1.65$, $r = -0.63$), Fig. 6.

4.4. Sexual behavior

Male sexual parameters are shown in Tables 1 and 2. We first compared the execution of sexual behavior in voles that ejaculated once with those treated with naloxone before allowed to ejaculate once. As observed in Table 1, naloxone administration did not induce alterations in sexual behavior. Male voles consistently mated during the total duration of the

test and in all three behavioral tests. Data from males that ejaculated one time showed that naloxone administration decreases only the number of pelvic movements during the intromission. However, no significant differences were found in the rest of the parameters analyzed (Table 1). As well, when we compared the voles that mated for 6 h without injections and males that received three naloxone administrations no significant differences were found (Table 2).

During the sexual behavior tests, females from experiment 1 showed high levels of receptivity evaluated by the Lordosis Quotient (LQ) in test 1 (71.9 ± 8.4), test 2 (92.8 ± 2.8), and test 3 (94.9 ± 2.5). Similarly, all females that mated for 6 h (experiment 2) were sexually receptive, as indicated by the LQ (test 1, 83 ± 5.5 ; test 2, 90.8 ± 3.4 ; and test 3, 83.7 ± 3).

4.5. Spontaneous locomotor activity, motor execution, and balance tests

Data from the open field test showed that naloxone did not induce motor alterations. No significant differences between voles treated with NaCl 0.9% and naloxone were found in the total distance traveled and in the time in movement during the three behavioral tests. Similarly, naloxone administration did not induce alterations in fine motor coordination, as evaluated in the rotarod test. During training, all males showed similar performance (data not shown). No significant differences between groups were found in the time walking on top of the rotarod, the speed at which subjects fell, and the distance traveled in each of the three tests (Table 3).

5. Discussion

Data from the present study clearly show that in male voles, one ejaculation or mating for 6 h that leads to pair bonding formation is rewarding. This reward state depends on the sexual interaction because males placed alone or exposed to a receptive female, without the possibility of mating, for 30 min or 6 h did not develop CPP.

Different lines of evidence indicate that opioids are involved in sexual behavior (Coria-Avila et al., 2016; Paredes, 2014; Szechtman et al., 1981; van Furth et al., 1995). Administration of the mixed mu/delta opioid receptor agonist d-Ala2-Met5-enkephalinamide during copulation facilitates ejaculation (Agmo and Paredes, 1988). Thus, the release of endogenous opioids during sexual activity can facilitate ejaculation and induce the reinforcing properties of this behavior (Pfaus et al., 2001). In male and female rats, the reward state induced by mating is mediated by opioids. Systemic and intra-cerebral administration of naloxone consistently blocks the CPP induced by mating (Agmo and Berenfeld, 1990; Ismail et al., 2009; Paredes and Martinez, 2001). Similarly, in humans, opioids play a fundamental role in sexual behavior. Administration of opioids such as heroin and morphine induces sensations similar to those produced by orgasm, followed by a period of relaxation and sedation (Mirin et al., 1980; Pfaus and Gorzalka, 1987); and administration of naloxone decreases the subjective pleasure induced by the orgasm (Murphy et al., 1990).

In the present study, we demonstrated that administration of the opioid antagonist naloxone blocked the CPP induced by one ejaculation and 6 h of mating in male voles. Moreover, naloxone did not induce alterations in locomotor activity and fine motor coordination, as

evaluated in the open field and rotarod tests, which could influence the outcome of the CPP test. Our data is also in agreement with studies performed in rats demonstrating that naloxone did not induce aversion, at least at the dose tested (Agmo and Berenfeld, 1990). Previous studies have demonstrated that naloxone (5 mg/kg) did not interfere with affiliative behaviors in voles. Females and males that were placed together for 2 h showed huddling, and naloxone administration did not decrease huddling duration (Shapiro et al., 1989).

Naloxone did not induce severe alterations in mating behavior. In males that ejaculated once, naloxone treatment only decreased the number of pelvic movements during the intromission, and no significant differences were found in the other parameters analyzed. In male voles that mated for 6 h and received three drug injections, naloxone did not modify any of the parameters. The results of sexual behavior are also in agreement with previous observations indicating that the same dose of naloxone in rats did not modify sexual behavior (Forsberg et al., 1987). Thus, opioid blockade did not alter locomotor activity or mating behavior, indicating that opioids released during mating in voles induce a reward state evaluated by CPP.

Our data showed that exposure to a vole of the opposite sex, without sexual interaction for 6 h, did not induce CPP. Although males increased the time spent in the reinforced compartment after the test ($P = 0.055$), it was not statistically significant. In this species, pair bonding is induced after 24 h of cohabitation, without mating, with a vole of the opposite sex (Williams et al., 1992). This suggests that neural pathways involved in bonding can be activated without sexual interaction in prairie voles. Sexual reward may function as a catalyst that accelerates bonding, and not necessarily as a crucial factor that will induce a pair bond. Further studies are needed to evaluate whether exposure to a conspecific for longer periods (24 h), without mating, induces a reward state in voles. The sensory cues during the 24 h period of exposure could increase the release of opioids, thus inducing a reward state in male and female voles.

Our data show that in female voles, the stimulation received in the different groups; mated to one ejaculation, social exposure, and social exposure with mating for 6 h did not induce a reward state under the conditions used and based on the CPP test. It has been demonstrated that sexual reward in rats depends on mating conditions (Arzate et al., 2011; Coria-Avila and Pfau, 2007; Parada et al., 2012; Paredes and Alonso, 1997; Paredes and Martinez, 2001; Paredes and Vazquez, 1999). Mating can have aversive consequences for females. Thus, female rats that mated for long periods and received several intromissions and ejaculations show a decrease in the lordosis response and an increase in rejection behaviors (Bermant and Westbrook, 1966; Hardy and Debold, 1971, 1972). The aversive properties of mating decrease considerably when females pace the sexual contacts (Bermant and Westbrook, 1966; Paredes and Alonso, 1997; Peirce and Nuttall, 1961). In pacing conditions, female rats receive fewer intromissions and the inter-intromission interval increases; in this way, females avoid overstimulation that can induce aversive properties of mating. Pacing also induces physiological changes that favor reproduction. When females mate in pacing conditions and receive 10 intromissions or one ejaculation or mate for 1 h, a clear CPP, indicative of a reward state, is observed (Arzate et al., 2013; Paredes and Alonso, 1997; Paredes and Vazquez, 1999). In the present study, females were not able to pace the sexual interaction

because they mated in a regular cage without the possibility of escaping from the male. Female voles and mice can reject the male by showing aggressive behavior, but probably this is not enough to avoid the unwanted sexual overstimulation. One study in voles evaluated the effects of pacing the sexual interaction on different reproductive outcomes (McCracken et al., 2015). Female voles that mated in pacing conditions had the same litter size as females that did not pace the sexual interaction. No significant differences were found in the latency to initiate mating, in the duration of mating, or in the amount of time that females spent close to the male compared to females that did or did not pace the sexual interaction. Unfortunately, the research group did not evaluate the number and latencies to mount, intromit, and ejaculate and could not calculate the inter-intromission interval and parameters that are different between pacing and no pacing. Thus, in the present testing conditions female voles were not able to pace the sexual interaction and avoid the aversive properties of mating. Further studies will need to determine if female voles that pace the sexual interaction develop a reward state, as it occurs in rats.

When female voles mate for 6 h, they form a clear partner preference and bonding. This suggests that in females, mating, which releases oxytocin and dopamine (Johnson et al., 2017; Ross et al., 2009; Wang et al., 1999; Young et al., 2008), allows the females to associate the cues of the partner with reward pathways, even though the initial experience may not be rewarding in the context of CPP. Therefore, “sexual reward” *per se* is not essential for pair bonding, but mating does trigger a set of neurochemical and electrophysiological processes that make the partner rewarding (Johnson et al., 2016; Johnson et al., 2017), and females show a conditioned partner preference according to the salience of the partner's social cues which do not extend to the context around them, in this case, the conditioning box. Thus, in female voles' sexual behavior until one ejaculation or for 6 h may indeed be rewarding, but CPP is not sensitive enough to reveal a reward state. Male and sexual behavior can be a very powerful incentive for the female even when there is no reward or when the value of the reward associated with sex is reduced.

6. Conclusion

We conclude that in male voles, the formation of pair bonding by sexual stimulation to one ejaculation or by cohabitation with mating for 6 h is rewarding. This positive affective state is opioid dependent. The positive state induced by mating may increase the probability that copulation will occur again with that partner. The partner-specific characteristics (olfactory visual or auditory cues) can be associated with the sexual reward, which in turn will allow the vole to recognize and choose a partner and exclude other potential mates (Coria-Avila et al., 2016; Johnson et al., 2017; Johnson and Young, 2015; Young and Wang, 2004). This process results in long-lasting preference (pair bonding) for the sexual partner. Future studies will need to evaluate whether mating in pacing conditions could induce positive affective (reward) states in female voles.

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Fig. 1.

Schematic representation of the experiments. Timeline of experiment 1 (A), 2 (B), and 3 (C–E). Females were ovariectomized and treated with estradiol benzoate (EB) for four consecutive days before the conditioned place preference test.

Fig. 2. Schematic representation of the sexual parameters in male voles. A) anogenital investigation; B) mount; C) intromission; D) ejaculation; and E) post-ejaculatory interval, time elapsed between the ejaculation and the first intromission after ejaculation.

Fig. 3. Time in the reinforced compartment in social exposure (Soc.Exp) and one ejaculation (1E) groups in male (A) and female voles (B). Data are expressed in seconds as the mean $\pm$ S.E.M.

*Different from pretest in the same group. $P < 0.05$.

Fig. 4.

Time in the reinforced compartment in control (C), social exposure (Soc.Exp), and social cohabitation with mating (SCM) groups in male (A) and female voles (B). Data are expressed in seconds as the mean $\pm$ S.E.M.

*Different from pretest in the same group. $P < 0.05$.

Fig. 5. Time in the reinforced compartment in voles treated with morphine (M) or morphine plus naloxone (M + N). Data are expressed in seconds as the mean $\pm$ S.E.M.
*Different from pretest in the same group. $P < 0.05$.

Fig. 6.

Time in the reinforced compartment in different groups of voles. Control males treated with naloxone (C + N), males with social cohabitation with mating for 6 h plus NaCl injections (SCM + S), males that cohabitated and mated with a receptive female for 6 h and received three naloxone injections (SCM + N), and males that were allowed to mate until one ejaculation and received one naloxone injection (1E + N). Data are expressed in seconds as the mean $\pm$ S.E.M.

*Different from pretest in the same group. $P < 0.05$.

Table 1

Sexual behavior parameters recorded in voles that mated until the male ejaculated once (1E; males from experiment 1) and from males that received a naloxone injection before allowed to ejaculate once (1E + N; males from experiment 3). Males mated on days 3 (test 1), 5 (test 2), and 7 (test 3) of the CPP test. Males mated always with the same female. The following parameters were recorded: number of mounts and intromission, pelvic movements in mounts and intromissions, latency to the first mount, intromission and ejaculation, and inter-intromission interval (III).

	1E			1E + N		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
No. of mounts	3.7 ± 1.3	4.9 ± 1.4	3.5 ± 1.1	3.7 ± 1.3	1.6 ± 0.7	3.3 ± 1.1
No. of intromissions	5.3 ± 1	6.2 ± 1.4	9 ± 1.9	4.3 ± 2	2.9 ± 0.8	3.9 ± 0.9
Pelvic mov. in mounts	8.2 ± 3.3	10.8 ± 4.5	8 ± 2.6	8.3 ± 4.9	3.4 ± 1.3	3.7 ± 0.4
Pelvic mov. in intromissions	30.2 ± 4.1	32.5 ± 5.2	47.2 ± 7.3	18.3 ± 5.6	15.9 ± 2.6 [#]	24.7 ± 5.5 [#]
Mount lat.	332.9 ± 141.5	145 ± 68	198.6 ± 156.9 [*]	104.3 ± 22.1	105.9 ± 16	41.9 ± 8.9 ⁺
Intromission lat.	485.7 ± 263.6	174.1 ± 68.3	212.5 ± 155.5	173.3 ± 51.6	146.1 ± 25	60.5 ± 11.4
Ejaculation lat.	1019 ± 314.2	673.2 ± 147.4	793.8 ± 267.9	329.8 ± 50.4	254.6 ± 39.3	632.4 ± 207.6
III	204.7 ± 38.9	125 ± 27.1	142.2 ± 89.1	176.5 ± 86.2	120.8 ± 27.8	231.5 ± 106.8

Data are expressed as mean ± S.E.M and were analyzed by Kruskal–Wallis test; in case of significant effects, Mann–Whitney U test was used. Comparisons were done between tests in the same group and between groups in the same behavioral test. Mount, intromission, and ejaculation latencies are reported in seconds.

^{*} Different from test 1 in the same group. $P < 0.05$.

⁺ Different from test 2 in the same group. $P < 0.05$.

[#] Different from 1E in the same test. $P < 0.05$.

Table 2

Sexual behavior parameters in voles that mated for 6 h. Data from the three behavioral tests are reported in males from the social cohabitation with mating (SCM) group (experiment 2) and males in SCM that were injected with naloxone (SCM + N; from experiment 3). The same parameters as those in Table 1 were recorded in addition to the number of ejaculations and the post-ejaculatory interval (PEI).

	SCM			SCM + N		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
No. of mounts	21.6 ± 2.9	21.4 ± 4	26.7 ± 4.9	20 ± 5	11.4 ± 3.2	13.8 ± 7.3
No. of intromissions	18.4 ± 2.6	26 ± 4	28.5 ± 3.3	20.4 ± 3.5	19.9 ± 1.8	26.2 ± 6.8
No. of ejaculations	2.9 ± 0.3	3.4 ± 0.5	3.5 ± 0.3	3.5 ± 0.5	3.4 ± 0.3	3.7 ± 0.4
Pelvic mov. in mounts	47.1 ± 8.2	48.2 ± 8.6	51.2 ± 15	49.6 ± 12.3	19.8 ± 4.6	28.6 ± 15.3
Pelvic mov. in intromissions	98.4 ± 12.8	116.3 ± 16.8	131 ± 15.5	90.7 ± 18.2	78.9 ± 7	93.6 ± 16.2
Mount lat.	830.7 ± 312.8	158.9 ± 41.6	64.8 ± 21	609.1 ± 326	149.2 ± 47	365.2 ± 155.5
Intromission lat.	905.9 ± 303.5	541.5 ± 350.4	248.4 ± 121.4	735.9 ± 327.1	207.4 ± 65.8	481.1 ± 178.8
Ejaculation lat.	1634.8 ± 379.2	766.3 ± 386.3	684.4 ± 255.7	1629.4 ± 340.4	629.6 ± 148.3	781.9 ± 225.3
PEI	1291.9 ± 229	1688 ± 701.2	1143.5 ± 213.4	1548.1 ± 446.3	1593 ± 296.6	1479.8 ± 296.6
III	245.9 ± 77.8	53.2 ± 16.8	35.7 ± 11.3	107.1 ± 25.8	37 ± 12	36 ± 10.5

Data are expressed as mean ± S.E.M and were analyzed by Kruskal–Wallis test. No significant differences were found in any of the parameter analyzed between tests in the same group and between groups in the same behavioral test. Mount, intromission, and ejaculation latencies are reported in seconds.

Spontaneous locomotor activity and motor execution tests. A) Open field test demonstrates that naloxone did not induce locomotor alterations, and no significant differences were found between males treated with NaCl and those treated with naloxone in the total distance and time in movement after the three injections. B) Rotarod test shows that naloxone administration did not induce alterations in the time walking on top of the rotarod (s), speed at which subjects fell off the cylinder (rpm), and distance traveled (m).

Table 3

A						
Total distance (cm)			Time in movement (s)			
Test	1	2	3	1	2	3
NaCl	7142.3 ± 2224	3017.2 ± 1027.7	2033.7 ± 753.8	1699.4 ± 329	1027 ± 166	892.6 ± 110
Naloxone	6483.1 ± 1874	3298.4 ± 1422.8	2455 ± 931	1360.6 ± 294	831.6 ± 236	773.7 ± 188.4

B						
Time walking on the top of the rotarod (s)			Speed at which subjects fell (rpm)		Distance traveled (m)	
Test	1	2	3	1	2	3
NaCl	55.1 ± 4.9	58.3 ± 1.7	57.1 ± 2.9	28.0 ± 2	29.3 ± 0.7	28.8 ± 1.2
Naloxone	60	59.4 ± 0.6	60	30	29.4 ± 0.6	30
					1.75	1.68 ± 0.6
						1.75

Data are expressed as mean ± S.E.M; eight animals were included in each group.