

Activation of μ -Opioid Receptors in the Dorsal Striatum is Necessary for Adult Social Attachment in Monogamous Prairie Voles

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Despite significant evidence that opioids are involved in attachment by mediating social reward and motivation, the role of opioids in the formation of adult social attachments has not been explored. We used the socially monogamous prairie vole (*Microtus ochrogaster*) to explore the role of endogenous opioids in social bonding by examining partner preference formation in female prairie voles. We hypothesized that μ -opioid receptors (MORs) in the striatum have a critical role in partner preference formation. We therefore predicted that peripheral administration of an opioid receptor antagonist would inhibit partner preference formation, and more specifically, that μ -opioid selective receptor blockade within the striatum would inhibit partner preference formation. To test our hypotheses, we first administered the non-selective opioid antagonist naltrexone peripherally to females during an 18-h cohabitation with a male and later tested the female with a partner preference test (PPT). Females showed a dose schedule-dependent decrease in partner preference in the PPT, with females in the continuous dose group displaying stranger preferences. Next, we administered microinjections of the MOR selective antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) into either the nucleus accumbens shell (NAS) or the caudate-putamen (CP) immediately before a 24-h cohabitation with a male, and later tested the female with a PPT. Females receiving CTAP into the CP, but not the NAS, showed no preference in the PPT, indicating an inhibition of partner preference formation. We show here for the first time that MORs modulate partner preference formation in female prairie voles by acting in the CP. *Neuropsychopharmacology* (2011) **36**, 2200–2210; doi:10.1038/npp.2011.117; published online 6 July 2011

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INTRODUCTION

The opiate system has long been known to mediate the rewarding or positively reinforcing properties of unconditioned stimuli such as food, water, sex, and drugs of abuse (Turkish and Cooper, 1983; Agmo and Berenfeld, 1990; Self and Nestler, 1995; Yeomans and Gray, 1996; Fields, 2007; Leknes and Tracey, 2008). Social stimuli can also be rewarding, and the opiate system has been implicated in maternal motivation, infant separation distress, social solicitation, and social learning (Panksepp *et al*, 1980). In fact, the opiate system was the first brain system to be implicated in social attachment (Panksepp *et al*, 1978). However, the role of the opiate system in the formation of adult social attachments has not been explored.

The opioid hypothesis of social attachment posits that opioidergic tone mediates social reward and the desire for social contact (Panksepp *et al*, 1978). Subsequent experiments have largely supported this prediction. Opioid antagonists increase the motivation for social contact in both rats and Rhesus macaques (Panksepp *et al*, 1985, 1994; Martel *et al*, 1995). At the same time, opioid antagonists decrease the social reinforcement, competence, and learning that occur as a result of social contact (Martel *et al*, 1993; Keverne and Kendrick, 1994; Panksepp *et al*, 1994). Opioid antagonists also prevent the formation of mother–offspring attachments in ewes (Kendrick and Keverne, 1989).

The endogenous opiate system consists of three classes of opioid receptors: μ , κ , and δ ; and their endogenous ligands, endorphin, enkephalin, and dynorphin (Le Merrer *et al*, 2009). The μ -opioid receptor (MOR) mediates a wide variety of natural rewards, including hedonic ‘liking’ of highly palatable food (Pecina and Berridge, 2000). In the social domain, MOR selective agonists decrease separation distress vocalizations in both rats and fowl (Panksepp *et al*, 1980; Warnick *et al*, 2005). The MOR gene influences infant–mother attachment in mice and Rhesus macaques

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(Moles *et al*, 2004; Barr *et al*, 2008). This evidence suggests that the role of the opiate system in social reward may be mediated by MOR.

Over the last two decades there has been remarkable progress in understanding the neurobiology of adult heterosexual attachments from studies focusing on the socially monogamous prairie vole (*Microtus ochrogaster*). Prairie vole mating pairs form long-term attachments (or 'pair bonds'), share a nest, and provide coordinated parental care, with the male providing a similar level of care as the female (Ahern *et al*, 2011). These long-term attachments between mating pairs are stable over time, and can be observed after 2 weeks of separation (Insel and Hulihan, 1995). The roles of oxytocin, vasopressin, dopamine, and CRF in social cognition have been explored using the partner preference test (PPT) as a laboratory proxy for pair bond formation (Lim *et al*, 2004, 2007; Curtis *et al*, 2006; Aragona and Wang, 2009; Ross and Young, 2009). This research has led to neuroanatomical models for the formation of mating-induced partner preferences (Young and Wang, 2004; Young *et al*, 2011). These findings in voles have also led to insights into human social cognition that are relevant to social deficit disorders, such as autism and schizophrenia (Hammock and Young, 2006; McGraw and Young, 2010). This makes the prairie vole the ideal model with which to examine the role of opioids in the formation of adult social attachments.

Here, we manipulated the opiate system to determine its role in the formation of adult social attachments. We hypothesized that the opiate system is necessary for partner preference formation. To test this hypothesis, we systemically administered two dose schedules of naltrexone hydrochloride (NTX), a long-lasting, nonselective opioid antagonist that crosses the blood-brain barrier (Fishman *et al*, 1975; Bhargava *et al*, 1993), to receptive females throughout a cohabitation period with a male partner. Since our results supported our hypothesis, we hypothesized that MOR in the striatum is necessary for partner preference formation. To test this hypothesis, we administered site-specific microinjections of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), a highly selective MOR antagonist (Pelton *et al*, 1986), into either the nucleus accumbens shell (NAS) in the ventral striatum or the caudate-putamen (CP) in the dorsal striatum before a cohabitation period with a male partner. The NAS is an integral part of the pair bonding circuitry and is the site where oxytocin, CRF, and dopamine act to modulate partner preference formation (Young *et al*, 2005); the CP is an anatomically adjacent region of striatum that is involved in goal-directed behavior and habit formation (Balleine and O'Doherty, 2010), but has not been previously implicated in social bonding (Pitkow *et al*, 2001; Young *et al*, 2001; Aragona *et al*, 2003; Liu and Wang, 2003; Lim *et al*, 2007). Finally, we used receptor autoradiography and *in situ* hybridization to show the distribution of MOR in the regions of interest.

SUBJECTS AND METHODS

Animals

Animal subjects were adult prairie voles from 10 weeks to 9 months of age from Emory University's breeding colony

at the Yerkes National Primate Research Center. Care, housing, and colony management were provided as previously described (Olazabal and Young, 2006).

Experiment 1 included 34 sexually naïve female prairie voles between 10 weeks and 9 months of age as experimental subjects. Experiment 2 used experimental subjects within a more restricted age range, and included 58 sexually naïve female prairie voles between 70 and 120 days of age. In all, 44 sexually experienced male prairie voles between 10 weeks and 12 months of age were used as stimulus animals in this study and were re-used wherever possible. Six male and six female prairie voles from experiment 1 were killed 1 week after the experiment and brain tissue was collected for autoradiography and *in situ* hybridization. All procedures used in this study were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Institutional Animal Care and Use Committee of Emory University.

Drugs

NTX (Sigma, MO) was prepared at 1 mg/ml concentration in sterile saline (Hospira, Lake Forest, IL) and injected (i.p.) in a sufficient volume to deliver 7.5 mg/kg. In peripheral studies in voles and rats, 5–10 mg/kg i.p. reflected an intermediate to high dose (Shapiro *et al*, 1989; Giraudo *et al*, 1998; MacDonald *et al*, 2003). CTAP (Sigma) was prepared at 5 µg/µl concentration in sterile saline. This concentration was based on the minimal effective dose from a previously published study in rats (Tang *et al*, 2005) and adjusted based on pilot studies. CTAP has a half-life of >8 h in rat brain and blood (Abbruscato *et al*, 1997).

Experiment 1: Peripheral Opioid Antagonist

Females were estrogen-primed before cohabitation as described previously (Donaldson *et al*, 2010). Females were divided into three groups: control, NTX × 1, and NTX × 3. Each group received an i.p. injection at 0, 6, and 12 h during an 18-h cohabitation. Control females (*N* = 13) received three injections of saline. NTX × 1 females (*N* = 11) received a single injection of NTX followed by two injections of saline in order to allow the drug to dissipate. NTX × 3 females (*N* = 10) received three injections of NTX. Since NTX has a half-life in the blood of 4 h (Bhargava *et al*, 1993), this injection schedule allowed us to probe the effects of both acute and continuous administration of NTX.

Immediately following the first injection, females were cohabitated with an unrelated, sexually experienced stimulus male for 18 h in a novel cage. Since most mating occurs during the first 4 h of cohabitation (unpublished observations), the first 4 h of cohabitation were digitally recorded and were later replayed at 16 × speed using PowerDVD 5 (Cyberlink USA, Fremont, CA) and manually scored for huddling, presence or absence of mating, latency to mate, and number of mating bouts using Stopwatch + (Center for Behavioral Neuroscience, GA; <http://www.cbn-atl.org/research/stopwatch.shtml>). Females that failed to mate within the first 4 h of cohabitation were eliminated from subsequent tests. 'Huddling' was defined as motionless

side-by-side contact. The 'latency to mate' was defined as the time from the beginning of cohabitation to the first mount. A 'mating bout' was defined as a series of mounts where any two mounts were separated by no longer than 60 s. As the NTX \times 1 and NTX \times 3 groups received identical treatment during the first 4 h of cohabitation, cohabitation data from these two groups were combined for analysis.

After 18 h, all subjects were separated from their partners and isolated for 14 h, for a total of 20 h (five half-lives) since the last NTX injection, to allow the drug to dissipate. Females were subsequently tested for partner preference using a traditional partner preference apparatus as described previously (Williams *et al*, 1994; Donaldson *et al*, 2010).

Recordings of the PPT were manually scored using Stopwatch + for time spent huddling with the partner, time spent huddling with the stranger, and center cage crossings as described for cohabitation. A 'center cage crossing' was scored when the entire body passed into a tunnel connected to the center cage.

Experiment 2: MOR Antagonist Infusions into the NAS and CP

Females were divided into two experimental groups and two control groups based on two injection targets: the NAS (1.7 mm rostral, \pm 1.0 mm bilateral, 4.5 mm ventral from bregma; N = 13 experimental, N = 12 control; coordinates from Lim *et al*, 2004) and the CP (1.4 mm rostral, \pm 1.5 mm bilateral, 3.0 mm ventral from bregma; N = 17 experimental, N = 16 control; coordinates from Olazabal and Young, 2006). Bilateral guide cannulae (Plastics One, VA) were surgically implanted as described previously (Olazabal and Young, 2006), except that meloxicam (Vetmedica, St Joseph, MO) was prepared in sterile saline (0.5 mg/ml) and administered (1 mg/kg SQ) during surgery and as needed for analgesia.

Females were estrogen-primed before cohabitation as in Experiment 1. Four days after surgery, bilateral micro-injections of CTAP (1 μ g/0.2 μ l per side) to either the NAS or CP were performed as described previously (Olazabal and Young, 2006). Immediately following the microinjection, females were cohabitated with an unrelated, sexually experienced stimulus male for 23–24 h. Cohabitation was recorded and scored as in Experiment 1. Females that failed to mate within the first 4 h of cohabitation were eliminated from subsequent tests.

After 24 h of cohabitation, all subjects were tested for partner preference using a modified partner preference apparatus as described previously (Ahern *et al*, 2009). During the PPT, 'social immobility' or huddling was defined by the CleverSys SocialScan software (CleverSys, VA) using an algorithm in which time spent in social contact while relatively immobile was counted. Entrance into the center zone was scored as a center cage crossing by SocialScan.

After PPT, stimulus males were returned to their home cages. Females were euthanized, their brains were collected, and cannula placement was visually confirmed using Nissl staining as previously described (Olazabal and Young, 2006). On the basis of histological analysis, all injections hit the target region and no eliminations were made.

Statistics

For both experiments, categorical data on the presence or absence of mating during the first 4 h of cohabitation were compared between groups using Fisher's exact test. Test subjects that failed to mate were excluded from subsequent analyses. Cohabitation data on huddling, latency to mate, and mating bouts in Experiment 1 were compared between groups using a one-way MANOVA with treatment as the between-subjects factor, while the same measures in experiment 2 were compared using a two (region) by two (treatment) MANOVA. Center cage crossings during the PPT in Experiment 1 were compared between groups to assess relative mobility, using a one-way ANOVA with treatment as the between-subjects factor; the same measure for Experiment 2 used a two (region) by two (treatment) ANOVA. Time spent huddling with the partner *vs* the stranger during the PPT was compared between groups for Experiment 1 using a repeated-measures ANOVA, with treatment as the between-subjects factor and partner or stranger as the repeated measure; the same measure in Experiment 2 was compared using a two (region) by two (treatment) repeated-measures ANOVA. For both experiments, planned *post hoc* within-groups rank-sum tests with Bonferroni correction were run after the ANOVA to isolate the main effect. For both experiments, categorical data on the number of females displaying a partner preference *vs* females not displaying a partner preference in each group were compared using Fisher's Exact Test; individual test subjects met criteria for a 'partner preference' if they spent twice as much time huddling with the partner as with the stranger during the PPT.

MOR Autoradiography

Brains from six male and six female prairie voles were collected and prepared for MOR autoradiography using [Tyr-3,5- 3 H(N)]-DAMGO ([3 H]DAMGO) (PerkinElmer, MA) and analyzed as described previously (Loyd *et al*, 2008). Noncompetitive binding to brain slices was measured using [3 H]DAMGO alone. As a control, competitive binding was measured on adjacent sections using [3 H]DAMGO in the presence of either CTAP or NTX (10 μ M). Competitive binding was used to demonstrate that the ligands bound to the prairie vole MOR as previously described in the rat.

MOR In Situ Hybridization

Sense and antisense 35 S-UTP-labeled RNA probes for MOR mRNA were generated as described previously (Inoue *et al*, 2004), except as follows. Template DNA for the probe was amplified by polymerase chain reaction using a forward primer (5'-GGCTCAACTTGTCCCACGTWGATGGCA ACC-3'), a reverse primer (5'-TGGTTAGTTCATCCACTG TATTRGCCGTGGAG-3') and adult prairie vole brain cDNA. The 1069-bp amplicon (corresponding to 341–1409 of mouse MOR cDNA, Genbank accession number U19380) was sequenced to verify homology with mouse and cloned into pCRII-TOPO vector (Invitrogen, CA) to allow transcription of sense and antisense probes using T7 and SP6 RNA polymerases. Twenty μ m cryosections adjacent to those used for receptor autoradiography were exposed to

the probes as previously described. The slides were exposed to Kodak BioMax MR film for 5 weeks.

RESULTS

Experiment 1: Peripheral Opioid Receptor Antagonist

We hypothesized that the opiate system is necessary for partner preference formation. On the basis of this hypothesis, we predicted that systemic blockade of opioid receptors would inhibit partner preference formation in female prairie voles. To test this prediction, we administered two dose schedules of NTX or saline peripherally to female subjects throughout a cohabitation period with a male partner.

In Experiment 1, female subjects with a wide range of ages were used (10 weeks to 9 months). As such, age was used as a cofactor in all subsequent analyses and did not co-vary with huddling time, latency to mate, mating bouts, center cage crossings, or partner preference.

During the first 4 h of cohabitation, all NTX-injected subjects received identical treatment and were combined for analysis. The proportion of NTX-injected female subjects that mated during cohabitation (16/21) did not significantly differ from that of saline-injected female subjects (10/13) (2×2 Fisher's Exact Test, $p = 1.00$), indicating that NTX did not affect the subjects' decision to mate with the stimulus male. Subjects that did not mate during cohabitation were

eliminated from further analyses. The huddling time and the latency to mate did not differ between subjects receiving saline ($N = 10$) and those receiving NTX ($N = 16$) (one-way MANOVA; huddling, $F = 1.26$, $p = 0.27$; latency, $F = 0.006$, $p = 0.94$) (Figures 1a and b), indicating that NTX treatment did not affect these behaviors. However, NTX did significantly reduce the frequency of mating, as demonstrated by the fact that NTX-injected subjects had significantly fewer mating bouts than saline-injected controls (one-way MANOVA, $F = 8.87$, $p = 0.007$) (Figure 1c).

Center cage crossings during PPT were not statistically different between the saline ($N = 10$), NTX $\times 1$ ($N = 8$), and NTX $\times 3$ ($N = 8$) groups (one-way ANOVA, $F = 2.26$, $p > 0.05$), indicating that treatment did not affect locomotor activity. However, NTX treatment during cohabitation did alter the subjects' subsequent preference for the male partner. There was a significant effect of treatment on time spent with the partner vs the stranger (one-way repeated-measures ANOVA, $F = 4.57$, $p = 0.015$) (Figure 1d). Saline-injected females huddled longer with the partner than the stranger, NTX $\times 1$ females had no significant preference, and NTX $\times 3$ females huddled longer with the stranger (rank-sum tests with Bonferroni correction; $p = 0.017$, $p > 0.05$, and $p = 0.015$, respectively), indicating that only the saline group demonstrated normal partner preference formation. Furthermore, NTX $\times 3$ females were less likely than saline-injected females to meet the criteria for a partner preference. There was a significant difference

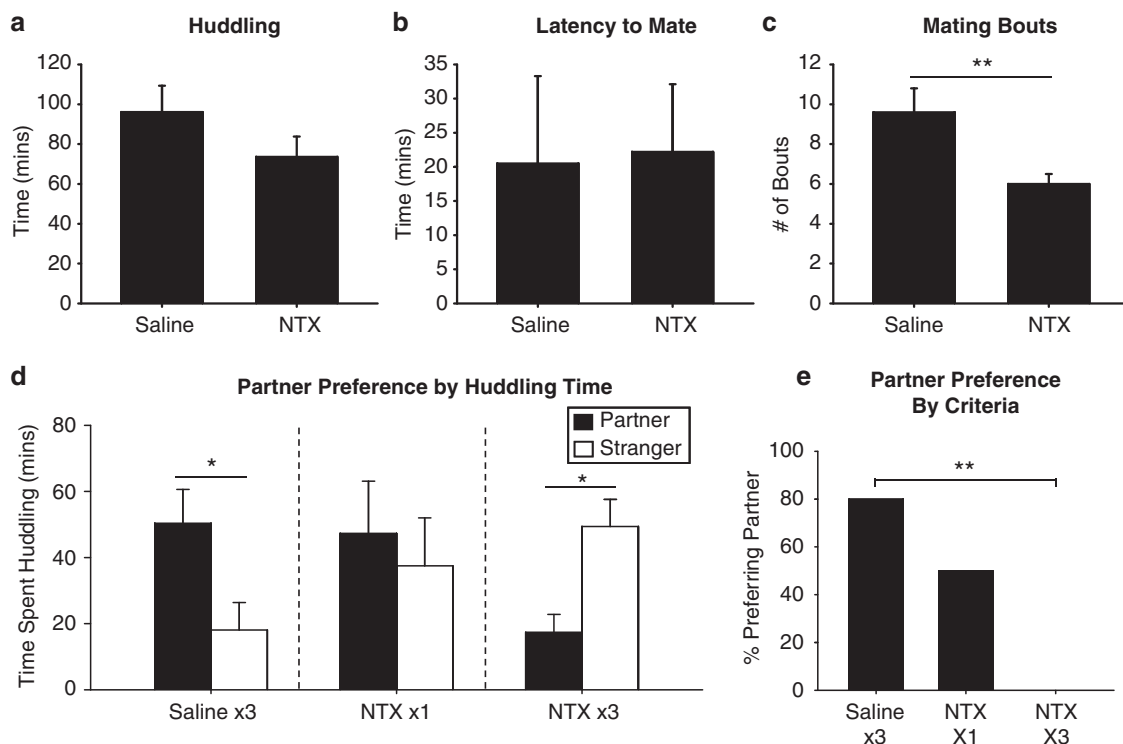


Figure 1 The effects of peripheral NTX administration on cohabitation and partner preference behaviors. In the first 4 h of cohabitation, there were no group differences between saline-treated ($N = 10$) or NTX-treated ($N = 16$) animals in (a) time spent huddling or (b) the latency to mate, but (c) NTX-treated females showed significantly fewer mating bouts. At this time point, both the NTX $\times 1$ group and the NTX $\times 3$ group had received identical treatment and were combined for analyses. (d) After 18 h of cohabitation, partner preference was measured using the PPT. Saline-injected females ($N = 10$) huddled significantly longer with the partner, NTX $\times 1$ females ($N = 8$) had no significant preference, and NTX $\times 3$ females ($N = 8$) huddled significantly longer with the stranger. (e) The percentage of individual females in each group that met the criteria for a partner preference was significantly different between groups. Data are plotted as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

between groups in the proportion of females meeting criteria for a partner preference to females not meeting criteria for a partner preference (2×3 Fisher's Exact Test, $p = 0.002$) (Figure 1e), and *post hoc* tests showed that the saline group was significantly different from the NTX $\times 3$ group (2×2 Fisher's Exact Tests with Bonferroni correction; saline vs NTX $\times 1$, $p = 0.35$; NTX $\times 1$ vs NTX $\times 3$, $p = 0.036$; saline vs NTX $\times 3$, $p = 0.001$). These findings are consistent with our prediction that blockade of opioid receptors would inhibit partner preference formation.

Experiment 2: MOR Antagonist Infusions into the NAS and CP

We hypothesized that MOR in the striatum is necessary for partner preference formation. Previous literature on partner preference formation demonstrated the involvement of the NAS, and as such, we predicted that site-specific blockade of MOR in the NAS, and not the CP, would inhibit partner preference formation. To test this prediction, we administered CTAP or saline to females via microinjection to the NAS or CP before a cohabitation period with a male partner. Since Experiment 1 demonstrated that a single infusion of antagonist was sufficient to disrupt partner preference formation, we administered only a single microinjection of CTAP or saline.

The proportion of female subjects that mated during cohabitation did not significantly differ between groups receiving saline to the NAS (12/12), CTAP to the NAS

(12/13), saline to the CP (16/16), and CTAP to the CP (16/17) (2×4 Fisher's Exact Test, $p = 0.84$), indicating that CTAP did not affect the subjects' decision to mate with the stimulus male. Subjects that did not mate during cohabitation were eliminated from further analyses. There were no significant differences between any of the treatment groups on measures of huddling time (Figure 2a), latency to mate (Figure 2b), or mating bouts (Figure 2c) (2×2 MANOVA, $p > 0.05$) during the first 4 h of cohabitation, suggesting that CTAP did not affect any of these affiliative or sexual behaviors during the critical period of cohabitation when the most mating occurs.

Center cage crossings during PPT were not statistically different between groups (two-way ANOVA, $p > 0.05$), indicating that treatment did not affect locomotor activity. However, CTAP treatment in the CP during cohabitation did alter the subjects' subsequent preference for the male partner. There was a significant region-by-treatment interaction effect on time spent with partner vs stranger (two-way repeated-measures ANOVA, $F = 5.23$, $p = 0.026$) (Figure 2d). Groups receiving CTAP to the NAS or saline to either brain region huddled longer with the partner than the stranger (rank-sum tests with Bonferroni correction; $p < 0.001$, $p = 0.005$, and $p < 0.001$, respectively), demonstrating normal partner preference formation. However, females receiving CTAP to the CP had no significant preference ($p > 0.05$). Furthermore, there was a significant difference between groups in the proportion of females meeting criteria for a partner preference to females

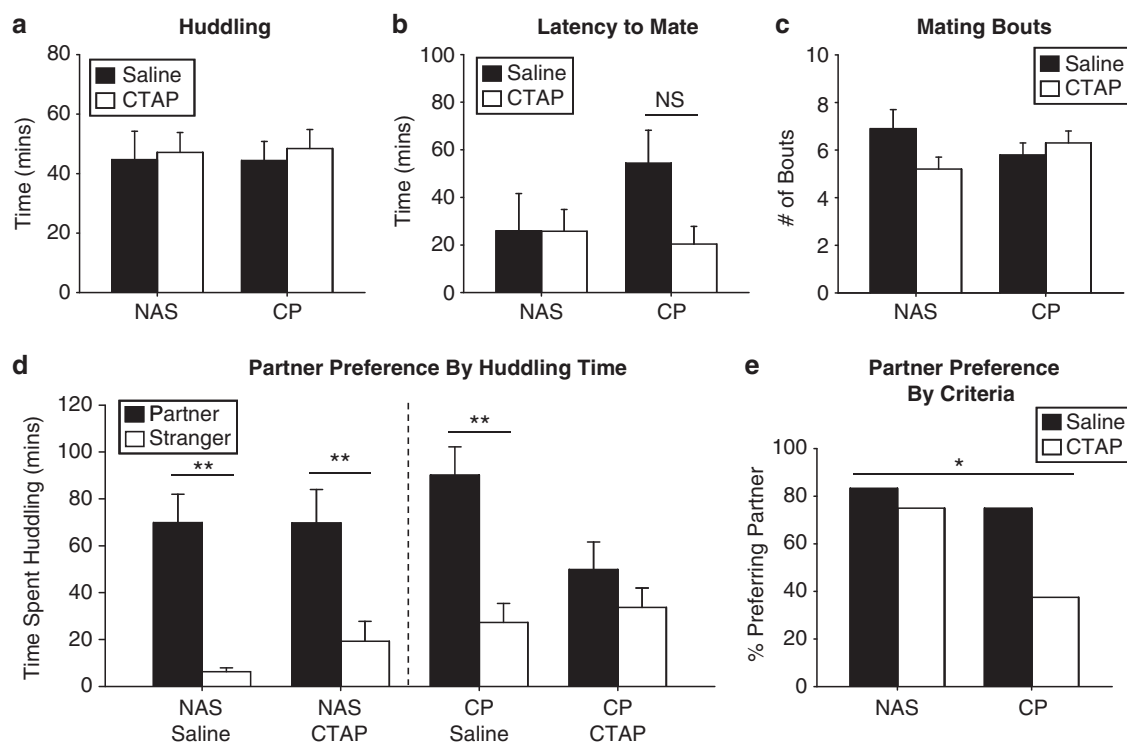


Figure 2 The effect of site-specific injections of CTAP on cohabitation and partner preference behaviors. In the first 4 h of cohabitation, there were no group differences in (a) time spent huddling, (b) the latency to mate, or (c) the number of mating bouts between females receiving saline to the NAS ($N = 12$), CTAP to the NAS ($N = 12$), saline to the CP ($N = 16$), or CTAP to the CP ($N = 16$). (d) After 24 h of cohabitation, partner preference was measured using the PPT. Females receiving CTAP or saline to the NAS, or receiving saline to the CP, huddled significantly longer with the partner. Females receiving CTAP to the CP huddled equally with the partner and stranger. (e) The percentage of individual females in each group that met criteria for a partner preference was significantly different between groups. Data are plotted as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$. NS, not significant.

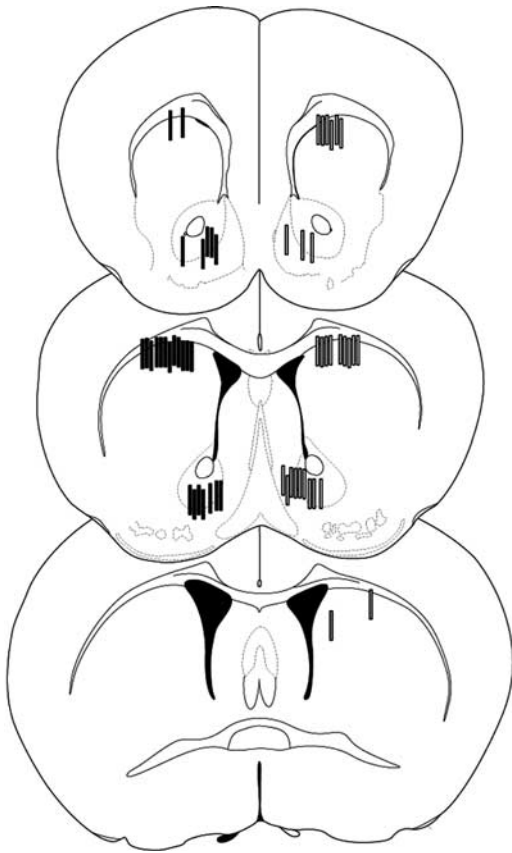


Figure 3 Cannula placement for CTAP microinjection studies. Marks represent one side of the bilateral injection path of the internal cannula in each animal as verified using histology. The line represents the entire 0.5 mm projection path of the internal cannula from the implanted guide cannula, with microinjections entering the brain at the bottom tip. Marks on the left (black boxes) show saline injection sites targeting the CP (dorsal) and NAS (ventral). Marks on the right (open boxes) show CTAP injection sites targeting the CP (dorsal) and NAS (ventral).

not meeting criteria for a partner preference (2×4 Fisher's Exact Test, $p = 0.05$) (Figure 2e). These findings show that, contrary to our prediction, MOR blockade in the CP (and not the NAS) inhibits partner preference formation.

Cannula placement was verified and recorded as described (Figure 3). No subjects had injection sites outside the target regions.

MOR *In Situ* Hybridization and Autoradiography

Both antisense cRNA hybridization and noncompetitive binding with [3 H]DAMGO revealed a pattern in the dorsal and ventral striatum consistent with what is seen in rats and mice (Figures 4a–d) (Mansour *et al*, 1987; Sharif and Hughes, 1989; Thompson *et al*, 1993; Kaufman *et al*, 1995). Interestingly, the MOR autoradiography and *in situ* hybridization results predicted the region of striatum involved in partner preference formation. MOR radioligand binding and MOR mRNA were densest in the dorsal striatum and dorsal aspects of the ventral striatum. Although MOR binding in the NAS was above background, binding in the NAS was lower than in any other part of the striatum. In fact, the borders of the ventral and ventrome-

dial aspects of the NAS are easy to discern on the autoradiogram on the basis of receptor binding alone. Thus, it is perhaps unsurprising that MOR antagonists exert their effects on social bonding in the dorsal striatum.

Sections processed for competitive binding as a control using NTX (Figure 4e) or CTAP (Figure 4f) all resulted in no signal above background, suggesting that [3 H]DAMGO lacks nonspecific binding in vole tissue. Thus, all binding was specific and the noncompetitive binding is equivalent to the specific binding to MOR. Furthermore, the strong overlap between the MOR autoradiography and MOR mRNA hybridization suggests that DAMGO is binding to MOR in vole, as reported in rat. Taken together, these results provide complementary evidence that both CTAP and NTX compete for binding sites with DAMGO on the MOR in vole.

DISCUSSION

In our initial study exploring the role of MOR in partner preference formation, we used a systemic administration paradigm, resulting in the blockade of both peripheral and central opioid receptors. This blockade dose-dependently abolished the formation of a partner preference, which is consistent with the hypothesis that opioid receptor activation is necessary for partner preference formation. However, the decreased mating and apparent partner aversion at the highest dose are not fully explained by this hypothesis. If this treatment had affected only partner preference formation, we would expect that mating would not differ between groups, and that treated females would have no preference for the partner or stranger. The presence of these side-effects indicates that global opioid receptor blockade affects additional systems that are responsible for other behaviors and may be aversive (Parker and Rennie, 1992; Skoubis *et al*, 2001). Nonetheless, this experiment provided important information regarding the direction of the effect of global receptor blockade on partner preference, and justified investigation into the role of opioid receptors in specific regions of the brain.

The central microinjections of CTAP were designed to produce long-lasting selective blockades of MOR, to lend spatial resolution to the observed peripheral effect, and to dissociate the blockade of partner preference formation from the effects of decreased mating and partner aversion. Microinjections of CTAP directly into the CP prevented partner preference formation without evoking a partner aversion. Importantly, this manipulation also did not result in any differences in sexual behavior. This suggests that pharmacological blockade of MOR in the CP prevents a necessary component of the pair bonding circuitry from functioning properly, and that this necessary element does not exert its effects through changes in sexual motivation or through conditioned aversion.

Multiple lines of evidence support the regional specificity of this effect to the CP. First, CTAP injections into the NAS, a nearby region of striatum, had no effect on partner preference formation, suggesting that the effects were not due to local diffusion. Second, the density of MOR in the striatum (as revealed by autoradiography) and of mRNA for these receptors (as revealed by *in situ* hybridization) is

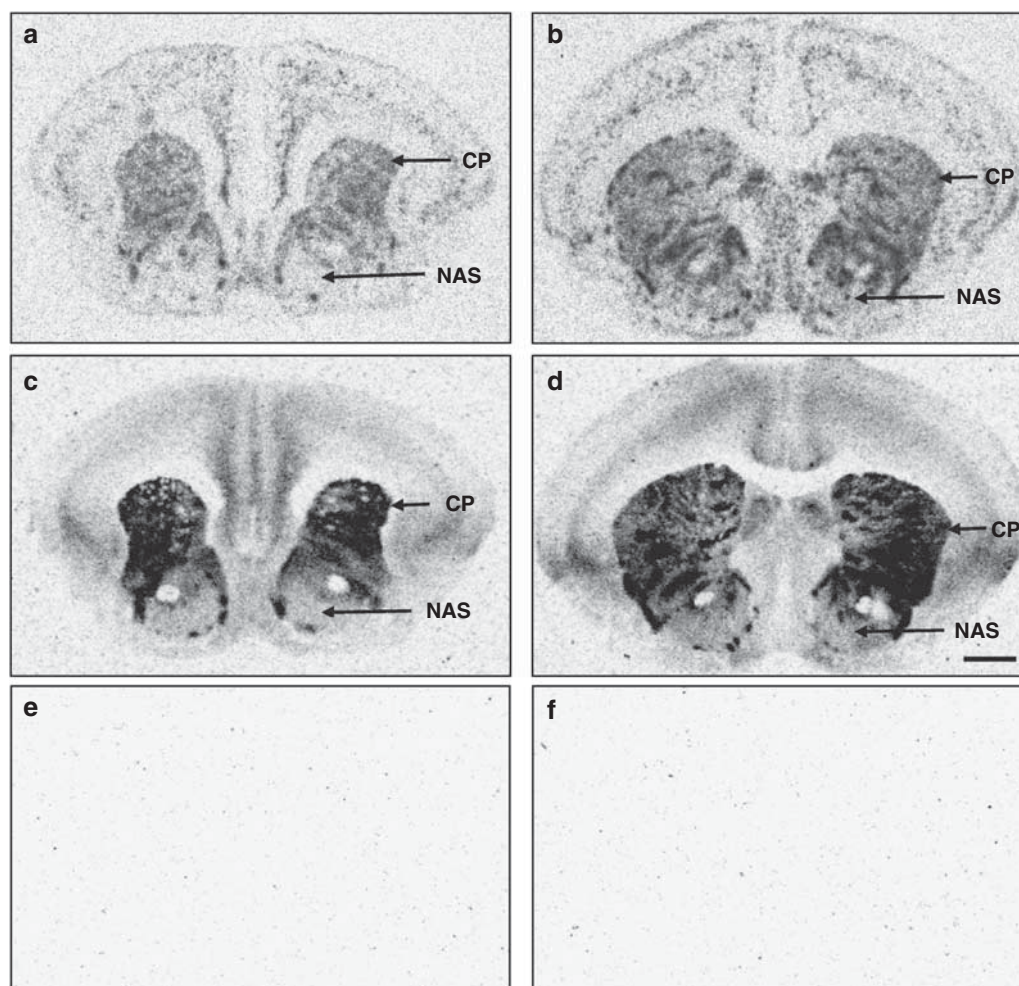


Figure 4 MOR *in situ* hybridization and receptor autoradiography in the prairie vole striatum. (a, c, e) show one set of adjacent sections proximal to the stereotactic coordinates targeting the NAS, and (b, d, f) show a second set of adjacent sections proximal to the stereotactic coordinates targeting the CP. *In situ* hybridization in (a) the NAS and (b) the CP shows the mRNA signal for MOR using the antisense probe. Receptor autoradiography in (c) the NAS and (d) the CP shows the total binding of tritiated DAMGO to MOR in the absence of competitor. Co-incubation of tritiated DAMGO with (e) NTX or (f) CTAP completely abolishes binding, demonstrating that these drugs bind to the prairie vole MOR as has been reported in the rat. Scale bar: 1 mm.

drastically higher in the dorsal striatum than in the NAS, consistent with the experimental results.

The bioactivity of the drugs used in this study was confirmed using autoradiography. [^3H]DAMGO showed strong binding in brain regions, which also expressed high levels of mRNA for the MOR. CTAP and NTX each completely disrupted binding of [^3H]DAMGO to the tissue, suggesting a lack of nonspecific binding. Taken together, these data show that CTAP and NTX are competing for binding sites with DAMGO on the MOR in vole.

Neuroanatomical Connections Between Dorsal and Ventral Striatum

The involvement of the mesolimbic dopamine pathway in partner preference formation, including elements of the prefrontal cortex and ventral striatum, has been well established. Dopamine and CRF in the NAS, and oxytocin in the prelimbic cortex and NAS, are all involved in partner preference formation (Young *et al*, 2001; Aragona *et al*, 2003; Lim *et al*, 2007). Although it remains an empirical

question whether the mesolimbic dopamine pathway and the CP act in a serial or parallel manner to modulate partner preference formation, there are a few candidate sites where integration could occur between dorsal and ventral striatum (Deutch *et al*, 1993; Ikemoto, 2007). The endogenous ligand for MOR is enkephalin, and in the striatum, enkephalin is primarily expressed in medium spiny neurons of the indirect pathway (Le Moine and Bloch, 1995; Steiner and Gerfen, 1998). Activation of these indirect pathway neurons modulates the output of corticostriatopallidothalamic 'reentrant' loops through both the dorsal and ventral striatum (Steiner and Gerfen, 1998). Reentrant loops through the ventral striatum terminate in regions of prefrontal cortex that are more dorsal than where they begin, which can lead to information from ventral striatum being passed forward into dorsal striatum. In a broad sense, this suggests that the prefrontal cortex may link the dorsal and ventral striatal pair bonding circuits. Perhaps more directly, both the dorsal and ventral striatum are reciprocally connected to the ventral tegmental area (VTA). In rats, the dorsal striatum projects primarily to a sub-population

of dopaminergic neurons in the VTA and substantia nigra pars compacta (SNPC) that projects back into dorsal striatum. Conversely, the NAS projects to a sub-population in the VTA and SNPC, which in turn projects to both dorsal and ventral striatum (Conrad and Pfaff, 1976; van Domburg and ten Donkelaar, 1991). This pattern of projections leads to the prediction that dopaminergic transmission from the VTA and SNPC may link the dorsal and ventral striatal pair bonding circuits. Interestingly, both of these proposed circuits suggest that the dorsal striatum is downstream of the ventral striatum with regard to pair bonding.

The projection neurons from the dorsal striatum to the VTA and SNPC are of particular interest. These projection neurons are contained within the patch compartments of the patch-and-matrix structure of the dorsal striatum, which are well known for their high levels of MOR expression (Jimenez-Castellanos and Graybiel, 1989; Desban *et al*, 1993). MOR in these compartments is predominantly extrasynaptic and responds to enkephalin released locally to modulate neuronal activity (Wang *et al*, 1996; Steiner and Gerfen, 1998). Medium spiny neurons of the indirect pathway, which contain the endogenous ligand for MOR, also express dopamine D2 receptors, the same receptors that act to enhance partner preference formation in the ventral striatum (Aragona *et al*, 2003). These observations suggest the possibility that the dopamine and opiate systems interact to modulate pair bonding, either within the dorsal striatum or via its projections to the VTA–SNPC or both.

The possibility of an interaction in partner preference formation between the dopamine and opiate systems of the dorsal striatum, as suggested by neurophysiology, has not been excluded by previous literature. Only two previous studies have examined the role of the dorsal striatum in partner preference formation. One study showed that an oxytocin antagonist in the CP does not prevent partner preference formation, indicating that the oxytocin receptors in the CP are not necessary for this process (Young *et al*, 2001). Another showed that the nonselective dopamine receptor agonist apomorphine does not enhance partner preference formation when injected into the CP; however, this could be due to co-activation of dopamine D1 receptors, which act to inhibit partner preference formation in the NAS (Aragona *et al*, 2003). The key experiments more fully establishing the role of dopamine in partner preference formation have not involved the dorsal striatum (Gingrich *et al*, 2000; Aragona *et al*, 2006).

Behavioral Function of Dorsal Striatum in Partner Preference Formation

There is an increasingly broad range of evidence in human and nonhuman primate studies showing that the dorsal striatum is involved in socially relevant, functional roles such as positive and negative emotions (Lane *et al*, 1997), response to emotional faces (Morris *et al*, 1996), romantic love (Bartels and Zeki, 2000; Aron *et al*, 2005), reward expectation (Kawagoe *et al*, 1998), and reward learning (O'Doherty, 2004; Delgado *et al*, 2005). The dorsal striatum is well positioned to integrate information pertaining to cognition and reward with motor control circuits and to potentiate specific motor outputs (Balleine *et al*, 2007).

The results of this study provide evidence that the dorsal striatum in prairie voles has a necessary role in partner preference formation. However, precisely what functional component of social bonding is mediated by MOR in the dorsal striatum remains unknown.

Given the highly diverse functions of the CP and the widespread expression of MOR in this region, it is unlikely that MOR in the CP mediates a function that is specific to partner preference formation or to attachment in general. Partner preference formation is a complex social behavior that is mediated by many brain regions that serve both social and non-social functions without anatomical segregation of information flow (Young *et al*, 2005). For instance, the mesolimbic dopamine system is necessary for partner preference formation, but is also involved in a wide variety of non-social rewards. The social functions of these structures are likely to be differentiated through expression of receptors for neuropeptides that are domain-specific to social information, such as oxytocin. Instead, MOR in the CP is likely to serve a more general function that is necessary for partner preference formation but not specific to it.

Partner preference formation is a type of social learning, and therefore these experiments show that CTAP in the CP causes an impairment of social learning. Although MOR in the CP is necessary for partner preference formation, we do not exclude the possibility that the effects on learning are not specific to the social domain. Some other possibilities for ways that MOR in the CP may mediate partner preference formation can be eliminated. During the cohabitation period, CTAP microinjections in the CP did not result in differences in huddling behavior, presence or absence of mating, latency to mate, or mating bouts, suggesting that the drug had no effect on social or sexual motivation or hedonics while it was active.

Recent literature on goal-directed learning and habit formation in rats suggests another possible mechanism by which dorsal striatal MOR may mediate partner preference formation (Balleine and O'Doherty, 2010). Goal-directed learning is characterized by associations between response and outcome that are sensitive to changes in the value of rewards or the probability of receiving them. In contrast, habitual learning is characterized by associations between stimulus and response that persist despite changes in reward value or outcome. During the initial acquisition period of training on a stimulus–response–outcome paradigm, rats are normally sensitive to changes in the outcome, including devaluation of the reward or changes in the causal result of the response; however, after a period of overtraining, the responding becomes insensitive to the outcome, and rats will select a response despite devaluation or a decrease in the probability of reward (Adams, 1982; Dickinson *et al*, 1998). This evidence supports the idea that early training is dominated by response–outcome learning, while overtraining leads to habitual performance driven by stimulus–response associations (Dickinson, 1994).

This shift from goal-directed learning to habitual learning is highly analogous to partner preference formation, and shares some neural correlates (Balleine and O'Doherty, 2010). In rats, goal-directed learning involves the prelimbic cortex and downstream dorsomedial striatum, and is influenced by outcome values, which are calculated in part

in the amygdala and ventral striatum. In contrast, habitual learning involves the dorsolateral striatum. All of these regions have been implicated in partner preference formation and contain receptors whose activation is necessary for partner preference formation (Young *et al*, 2005; Modi and Young, 2011).

The cohabitation period used in the laboratory setting to induce partner preference formation could be considered a type of training, where a subject is repeatedly trained to associate the stimulus of the partner with social and sexual reward. Thus, partner preference formation would be divisible into an early and late component. The early component would be characterized by sociosexual goal-directed behaviors and require recruitment of regions involved in sociosexual goal-directed behavior, such as the prelimbic cortex, dorsomedial striatum, ventral striatum, and amygdala. This period may be comparable to the early period of cohabitation during which the majority of mating occurs (unpublished observations) but partner preferences have not yet formed (Young *et al*, 2005). Over time, habitual learning processes in the dorsolateral striatum would take over, and the subject's behavior would become insensitive to changes in both devaluation of the reward (as represented by habituation to the sexual stimulus value of the partner) and to decreases in the frequency of rewards (as represented by a decrease in mating frequency). This habit formation would result in a preference for the partner despite the presence of new receptive mates in a PPT. This interesting theoretical possibility is not addressed by these experiments, but could be addressed in future experiments by varying the time of antagonist infusion over the cohabitation period.

Conclusion

These experiments are the first manipulations of the opiate system in relation to partner preference formation. The results provide strong evidence for two novel discoveries: first, that MOR is a necessary element of the pair bonding circuitry in female prairie voles; and second, that the CP is the location where MORs exert their effects on partner preference formation. Previous studies in female prairie voles have focused on the NAS as the striatal component of the pair bonding circuitry (Young and Wang, 2004). The CP represents an anatomically and physiologically parallel region of striatum that has not previously been implicated in partner preference formation. The involvement of the opiate system in the formation of adult social attachments is of particular importance as opioid antagonists, including NTX, are being used increasingly in humans as a treatment for alcoholism, opioid dependence, obesity, and fibromyalgia (Ablin and Buskila, 2010; Anacker and Ryabinin, 2010; Greenway *et al*, 2010; Lobmaier *et al*, 2010; Soyka and Rosner, 2010). Furthermore, the role of MOR in this behavior provides a vital link between the pair bonding literature and previous literature on maternal behavior, which further supports the hypothesis that the pair bonding circuitry evolved from the same mechanisms governing maternal behavior that are present in all mammals (Donaldson and Young, 2008). Although μ -opioids have long been known to have an important role in other kinds of attachment (Panksepp *et al*, 1978; Kendrick and Keverne,

1989), only now do we have evidence of the role the opiate system has in the formation of adult social attachments.

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DISCLOSURE

The authors declare no conflict of interest.

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