Nucleus accumbens dopamine mediates amphetamine-induced impairment of social bonding in a monogamous rodent species

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The prairie vole (*Microtus ochrogaster*) is a socially monogamous rodent species that forms pair bonds after mating, a behavior in which central dopamine (DA) has been implicated. Here, we used male prairie voles to examine the effects of drug exposure on pair bonding and related neural circuitry. In our first experiment, amphetamine (AMPH) motivated behavior was examined using a conditioned place preference (CPP) paradigm and was shown to be mediated by activation of D1-like DA receptors. Next, we examined the effects of repeated AMPH exposure on pair bonding. Intact and saline pretreated control males displayed mating-induced partner preferences, whereas males pretreated with AMPH at the doses effective to induce CPP failed to show mating-induced partner preferences. Such AMPH treatment also enhanced D1, but not D2, DA receptor expression in the nucleus accumbens (NAcc). Furthermore, pharmacological blockade of D1-like DA receptors in the NAcc rescued mating-induced partner preferences in AMPH-treated males. Together, our data indicate that repeated AMPH exposure may narrow the behavioral repertoire of male prairie voles via a DA receptor-specific mechanism in the NAcc, resulting in the impairment of pair bond formation.

vole | CPP | D1 Receptor

It is widely accepted that motivated and emotional behaviors that promote fitness are regulated by brain reward circuitry including the mesolimbic dopamine (DA) system (1, 2). Although this system is often implicated in food intake and sexual behavior (3, 4), it has also been implicated in other naturally occurring motivated behaviors, such as social play between juveniles and social bonding between parent and offspring (5–9). Often underrepresented in research are the social bonds formed between adult mates, i.e., pair bonds. Recent investigation using a socially monogamous rodent species, the prairie vole (*Microtus ochrogaster*) (10–12), indicate that a great deal of the neural regulation underlying pair bond formation and maintenance occurs within the nucleus accumbens (NAcc) (13–15)—a mesolimbic brain region critical for mediating motivated behaviors (1, 2, 16).

Although motivational circuitry evolved to promote fitness enhancing behavior such as feeding, mating, and social bonding (1, 17), it is vulnerable to artificial usurpation by drugs of abuse (8). For example, administration of psychostimulant drugs of abuse, such as cocaine and amphetamine (AMPH), results in persistent alterations of mesolimbic DA activity (18, 19). The intense impact on this circuit by these and other addictive drugs has been suggested to decrease the perceived value of natural incentives (20), including those of a social nature (8). Although it is known that drug addicts show impaired social behavior (21), the neural regulation of interactions between drug experience and social attachment is poorly understood. This is because, in part, such interactions are difficult to model in traditional laboratory rodents that do not exhibit social bonding between adult conspecifics.

The neurobiology of such social attachment, specifically pair bonding between adults, has been extensively studied in the prairie vole (10–12), and recently, this species has been established as a viable model to examine the motivational value of AMPH (22). Moreover, both pair bond formation and AMPH reinforcement are mediated, at least in part, by DA transmission within the NAcc (14, 15, 23). Therefore, the present study used the prairie vole model to establish a behavioral assay to study the effects of drug exposure on social bonding and focused on the NAcc DA signaling system to reveal a neural mechanism underlying these behavioral effects.

Results

AMPH-Induced Conditioned Place Preference (CPP) Is Mediated by DA in a Receptor-Specific Manner. The formation of a CPP was defined by a significant increase in time spent in the AMPH-paired cage during the posttest, after 3 days of AMPH conditioning, relative to the pretest. Neither saline injections nor saline containing the two lowest doses of AMPH tested (0.1 and 0.5 mg/kg) altered cage preferences (Fig. 1*A*). However, males conditioned with higher doses of AMPH, including 1.0 ($t = 2.87, P < 0.01$), 3.0 ($t = 3.63, P < 0.01$), or 5.0 mg/kg ($t = 3.03, P < 0.01$), displayed CPP (Fig. 1*A*).

Because AMPH significantly increases DA neurotransmission in prairie voles (24), and DA mediates AMPH reinforcement in other species (23), we next examined DA receptor (DAR) regulation of AMPH-induced CPP in male prairie voles. Subjects were pretested in the CPP paradigm, treated with either saline or saline containing different doses of the nonselective DAR antagonist (haloperidol) before AMPH (1.0 mg/kg) injections during 3 days of conditioning, and then tested for CPP in a posttest. Subjects treated with saline ($t = 2.69, P < 0.01$) or saline containing the two lowest doses of haloperidol (0.1 mg/kg; $t = 3.62, P < 0.01$; 1.0 mg/kg; $t = 3.89, P < 0.01$) before AMPH conditioning displayed AMPH-induced CPP, whereas haloperidol at 5.0 mg/kg blocked AMPH-induced CPP, showing the involvement of DARs in the behavioral effects of AMPH (Fig. 1*B*). To determine which DAR subtype mediates AMPH-induced CPP, we next administered either a D1-like specific antagonist (SCH23390) or a D2-like specific antagonist (eticlopride) before AMPH injections during conditioning. D2-like antagonism did not block AMPH-induced CPP ($t = 3.15, P < 0.01$ for 0.5 mg/kg and $t = 2.60, P < 0.05$ for 5.0 mg/kg eticlopride) but blockade of D1-like receptors eliminated AMPH-induced CPP (Fig. 1*B*), demonstrating that AMPH-induced CPP is mediated by activation of D1-like, but not D2-like, receptors in male prairie voles.

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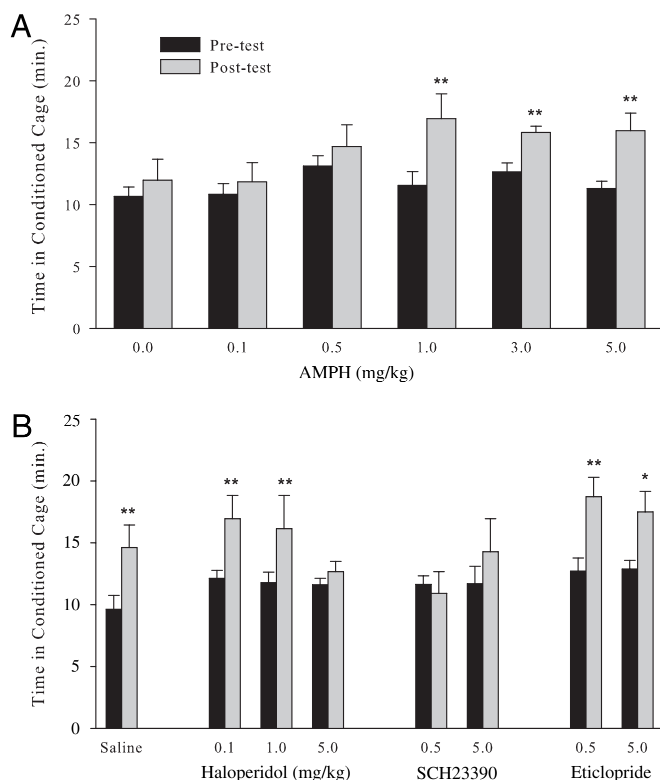

Fig. 1. (*A*) Males that received i.p. injections of saline or the low doses of AMPH (0.1 or 0.5 mg/kg) during 3 days of conditioning did not show CPP. However, males conditioned with AMPH at higher doses (1.0, 3.0, and 5.0 mg/kg) spent significantly more time in the conditioned cage during the posttest than the pretest. (*B*) To test the role of DA receptors in AMPH-induced CPP, males were pretreated with either saline or different doses of a nonselective DA receptor antagonist (haloperidol), D1-like receptor antagonist (SCH23390), or D2-like receptor antagonist (eticlopride) before AMPH injections during 3 days of conditioning. Injections of saline or low doses of haloperidol did not alter AMPH-induced CPP. However, haloperidol injected at 5.0 mg/kg blocked AMPH-induced CPP, demonstrating an involvement of DA receptors in AMPH-induced CPP. Furthermore, injections of the D1-like antagonist at both the low and high doses, but not the D2-like antagonist, blocked AMPH-induced CPP, demonstrating a receptor-specific DA regulation of AMPH-induced CPP. *$P < 0.05$, **$P < 0.01$.

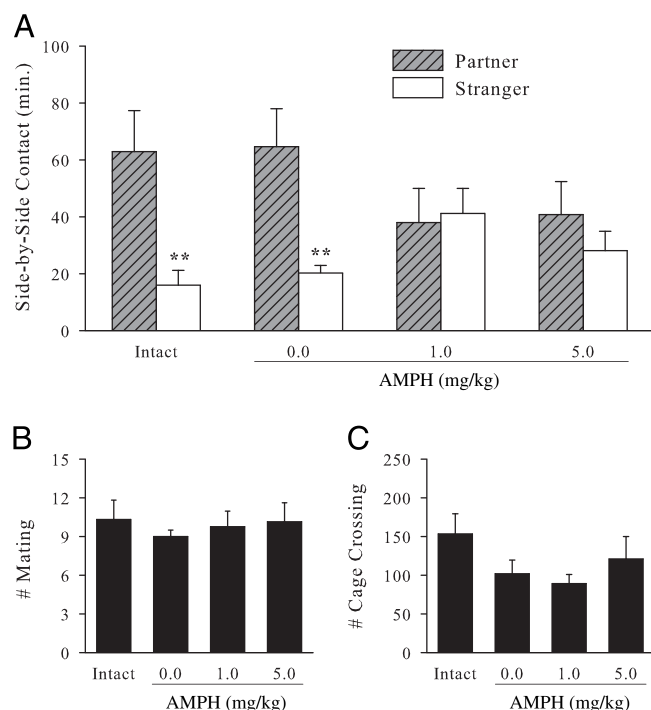

Fig. 2. (*A*) After 24 h of mating, intact males displayed partner preferences by spending significantly more time in side-by-side contact with a familiar mate versus a strange female. This mating-induced partner preference was also displayed by males that received 3 days of pretreatment with saline. However, 3 days of pretreatment with AMPH at 1.0 or 5.0 mg/kg (doses of AMPH that induced CPP) blocked mating-induced partner preferences. No group differences were found in the frequency of mating bouts during the first 6 h of mating (*B*) or in the number of cage crossings during the partner preference test (*C*). **$P < 0.01$.

AMPH Experience Alters Mating-Induced Partner Preference Formation. Although AMPH-induced CPP required activation of D1-like receptors (Fig. 1*B*), we have previously shown that activation of D1-like receptors prevents mating-induced pair bond formation (14). Therefore, we hypothesized that AMPH pretreatment would interfere with mating-induced pair bonding in male prairie voles. Males were divided into four groups that received no injections (intact), saline injections, or injections of 1.0 or 5.0 mg/kg AMPH for 3 days (an injection paradigm sufficient to induce CPP). On the fourth day, all males were paired with a sexually receptive female for 24 h and then tested for partner preferences. Consistent with previous studies (14, 25–27), intact males and males that received saline injections for 3 days before mating showed mating-induced partner preferences (intact males; $t = 3.05, P < 0.01$, saline injected males; $t = 3.21, P < 0.01$; Fig. 2*A*). However, males that were pretreated with either dose of AMPH for 3 days before mating failed to show partner preferences (Fig. 2*A*). Importantly, AMPH pretreatment did not affect mating frequency during the cohabitation period ($F_{(3, 26)} = 0.26, P = 0.85$; Fig. 2*B*) or locomotor activity during the partner preference test ($F_{(3, 26)} = 2.34$, $P = 0.10$; Fig. 2*C*), indicating that AMPH directly interfered with mating-induced partner preferences.

AMPH Experience Elevates D1 Receptors in the NAcc. Given that AMPH preexposure impaired partner preferences and both AMPH-induced CPP (see above) and pair bonding (14) are regulated by NAcc DA, we hypothesized that AMPH would significantly alter mesolimbic DA circuitry in male prairie voles. Brains of subjects from the above-mentioned behavioral experiment were processed for in situ labeling of DA marker mRNAs. Males treated with AMPH (1.0 mg/kg) showed a significant increase in D1 receptor (D1R; $t = 3.06$, $P < 0.01$), but not D2 receptor (D2R), mRNA labeling within the NAcc, compared to males receiving saline pretreatment (Fig. 3 *A*–*C*). However, no group differences were found in the density of mRNA labeling for tyrosine hydroxylase (TH), DA transporter (DAT), or D2Rs within the ventral tegmental area (VTA) - the brain region that provides the primary dopaminergic input to the NAcc (Fig. 3 *D*–*G*). Increased expression of D1Rs in the NAcc was further confirmed by Western blotting ($t = 1.90$, $P < 0.05$; Fig. 3 *H* and *I*). Together, these data indicate that AMPH exposure has receptor- and site-specific effects on the mesolimbic DA system of male prairie voles—increasing the level of D1Rs in the NAcc.

D1 Receptors in the NAcc Mediate AMPH Impairment of Partner Preferences. We have previously shown that, in male prairie voles, activation of D1Rs within the NAcc prevented partner preference formation (14), and the current study demonstrates that AMPH exposure up-regulates D1Rs in the NAcc (Fig. 3). Therefore, we tested the hypothesis that AMPH-induced impairment of partner preferences is mediated by D1Rs within the NAcc. Males received stereotaxic cannulation aimed bilaterally at the NAcc shell (Fig. 4*A*). Artificial cerebrospinal fluid (CSF) alone or CSF containing

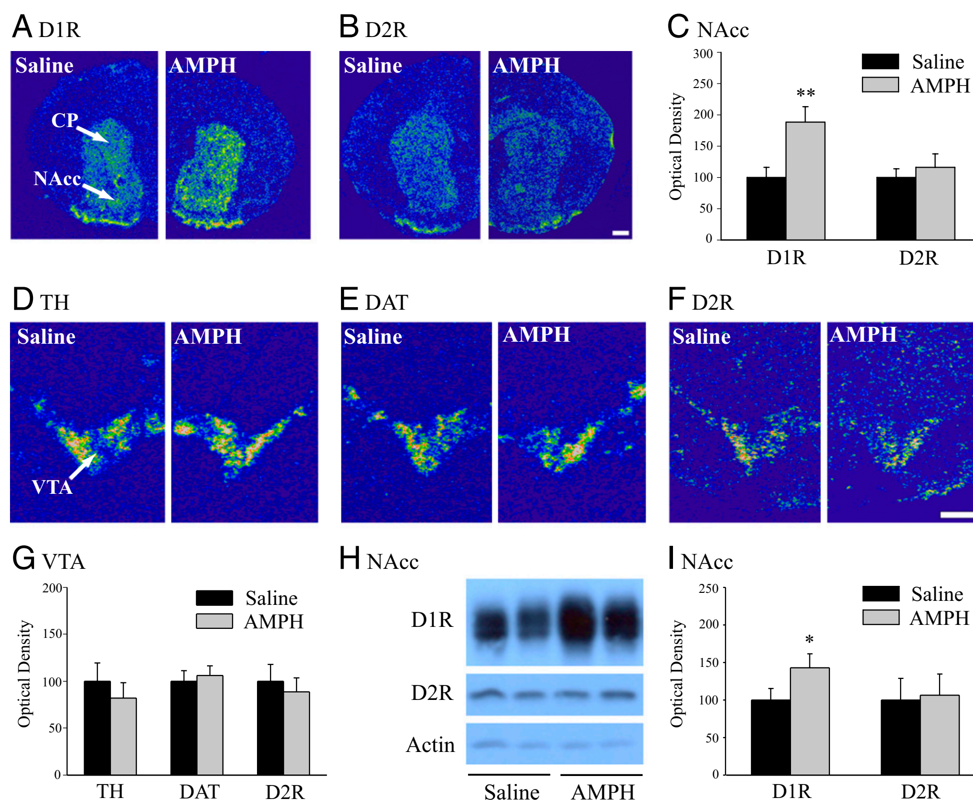

Fig. 3. Photo images showing in situ labeling of D1R (*A*) and D2R (*B*) mRNA in the NAcc and caudate putamen (CP) of male prairie voles that received i.p. injections of saline or AMPH (1.0 mg/kg) for 3 days. AMPH treatment significantly increased the density of D1R, but not D2R, mRNA in the NAcc. Because this increase was equivalent across the NAcc core and shell, data were collapsed across subregions (*C*). Photo images illustrating mRNA labeling of tyrosine TH (D), DAT (*E*), and D2Rs (*F*) in the VTA of the prairie vole brains. AMPH treatment did not affect the density of those DA marker mRNAs in the VTA (*G*). In the NAcc, AMPH treatment significantly increased the protein density of D1Rs, but not D2Rs, as measured by Western blotting (*H* and *I*). $*P < 0.05$, $**P < 0.01$. (Scale bar, 1 mm.)

different doses of the D1-like receptor antagonist SCH23390 was injected into the NAcc before AMPH injections (1.0 mg/kg) during 3 days of conditioning. Thereafter, subjects were paired with a female for 24 h and then tested for partner preferences. As with the experiment above (Fig. 2), AMPH exposure prevented mating-induced partner preferences in males that received intra-NAcc injections of CSF or a low dose of SCH23390 (Fig. 4*B*). However, males that were injected with a high dose of SCH23390 (100 ng/kg) displayed partner preferences ($t = 2.55$, $P < 0.05$), indicating that D1R blockade in the NAcc eliminated the AMPH-induced impairment of partner preference formation (Fig. 4*B*). No group differences were found in mating frequency during the cohabitation or locomotor activity during the partner preference test.

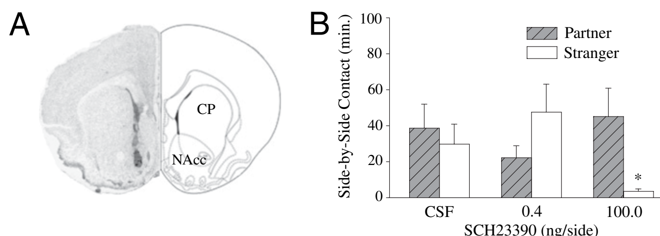

Fig. 4. (*A*) A photo image and schematic drawing illustrating the injection site in the NAcc of the male prairie vole brain. We focused on the NAcc shell because this specific subregion mediates mating-induced partner preferences. (*B*) Males that received intra-NAcc injections of CSF or a low dose of D1 receptor antagonist (SCH23390; 0.4 ng/side) before AMPH injections displayed AMPH-impairment of partner preferences. However, intra-NAcc injections of a high dose of SCH23390 (100 ng/side) rescued mating-induced partner preferences in AMPH-treated males. $*P < 0.05$.

Discussion

In this study, we replicate our previous finding that AMPH exposure induces CPP in male prairie voles (22) and demonstrate that D1R activation in the NAcc is necessary for this behavior, a result consistent with studies in other rodent species (28). This finding, together with earlier studies, suggests that distinct DAergic mechanisms in the NAcc regulate AMPH- and partner-motivated behaviors: AMPH-motivated behavior (CPP) is mediated by D1R, whereas partner-motivated behavior (partner preferences) is facilitated by D2R activation and inhibited by D1R activation within the NAcc (13–15). [It is important to note that, in the NAcc, D2R activation mediates drug-induced CPP under certain conditions (29) and D1R activation is involved in other socially-motivated behaviors, such as those directed toward offspring (7, 30)].

Differential DAergic regulation of CPP and partner preference formation is most likely achieved by differences in the degree of stimulus-evoked DA concentration activating different DAR subtypes. Due to differences in binding affinities, robust elevations in DA concentration are required to activate low affinity D1Rs, whereas modest increases in DA concentrations preferentially activate high affinity D2Rs (31). In prairie voles, AMPH evokes much greater increases in DA concentration (24) compared to that evoked by mating (15, 25). These data suggest that relatively modest increases in DA concentration during social interactions (15, 25) allow specific activation of high affinity D2Rs and thus facilitate pair bond formation. Conversely, robust increases in DA concentration following AMPH administration is

likely sufficient to activate low affinity D1Rs, thereby facilitating AMPH-induced CPP.

Receptor-specific behavioral regulation by DA is consistent with extracellular electrophysiological studies in freely moving rats showing that drug and natural stimuli are processed by distinct neuronal populations within the NAcc (32). Although such electrophysiological studies are unable to identify the subtype of DA receptors expressed on individual neurons, anatomical studies demonstrate that the NAcc is comprised of projection neurons that express either D1Rs or D2Rs with very little coexpression (33). Psychostimulants preferentially activate intracellular signaling pathways downstream from D1Rs (as determined by increased phosphorylation of signaling molecules) (34) and we have previously shown that increased activation of these signaling pathways prevents partner preference formation (35). Thus, although DA transmission within the NAcc plays an important role in the response to AMPH and the formation of partner preferences, these behaviors are likely mediated through distinct microcircuitries that comprise striatal projection systems (33, 36). This is of particular interest because pair bond formation is mediated by activation of D2R-expressing neurons that preferentially project to the ventral pallidum (33), another brain region important for pair bonding (26).

In this study, we provide evidence that AMPH experience prevents mating-induced pair bonding. As the partner preference tests in these experiments were performed 48 h after the last AMPH exposure (i.e., after the drug had been completely metabolized), these data suggest a persistent effect of AMPH on social bonding. One potential mechanism, consistent with the effects of AMPH in other species (37, 38), by which AMPH may impair pair bonding is through the increase of D1R expression within the NAcc. This notion is supported by the antagonistic role of NAcc D1Rs in partner preference formation (14, 24) and by the current data demonstrating the rescue of mating-induced partner preference by intra-NAcc D1R blockade in AMPH-treated animals (Fig. 4). Interestingly, we have previously shown that, in male prairie voles, up-regulation of NAcc D1Rs after 2 weeks of pair bonding facilitates selective aggression toward conspecific strangers, including sexually receptive females, suggesting that this neural plasticity is an evolved mechanism by which male voles maintain already established pair bonds (14). The current study suggests that AMPH artificially triggers this neuroplasticity, leading to AMPH-impairment of pair bonding. It is also possible that AMPH-treated males may associate the female with an aversive state of AMPH withdrawal, and thus this negative association might underlie the AMPH impairment of pair bonding. A previous study demonstrated that withdrawal from an escalating dose schedule of d-amphetamine impaired sexual behavior in male rats (39). In our study, however, voles from different treatment groups exhibited similar mating bouts (Fig. 2*B*), indicating that they were probably not in a state of withdrawal from amphetamine. Nevertheless, this possibility warrants further investigation.

It is well established that drug-induced neural plasticity is fundamental to drug addiction (40). Psychostimulants dramatically alter structural plasticity in the mesolimbic DA system (41) and there is evidence to suggest that such changes are more enduring in D1 expressing neurons (37). Moreover, electrophysiological studies have shown that cocaine experience can reduce subsequent neuroplasticity within the NAcc (42). Indeed, drug-induced neural reorganization of the NAcc attenuates the naturally occurring neural reorganization following novel exposure to complex environments (43). Therefore, although drug addiction is widely recognized as a disorder of learning and memory (1, 40, 44), it is often underappreciated that a drug-induced loss of the potential for additional alteration in mesolimbic circuits may also be essential for addiction. Such a reduction in plasticity may limit a behavioral repertoire to drug seeking. It is therefore not surprising that AMPH-treated voles demonstrated reduced pair bonding behavior. The current effects of AMPH exposure on pair bonding are consistent with previous studies illustrating the deleterious effects of psychostimulants on other social behaviors, including maternal behavior (45–47) and social play (48–50). Together, such work provides the promise that examination of how drug and social stimuli interact in the brain may significantly extend our understanding of the strong interactions between social behavior and drug use in humans (21).

Materials and Methods

Subject. Subjects were sexually naive male prairie voles from a laboratory breeding colony. Subjects were weaned at 21 days of age and housed in same-sex sibling pairs in plastic cages ($12 \times 28 \times 16$ cm) where water and food were provided ad libitum. All cages were maintained under a 14:10 light-dark cycle, and the temperature was approximately 20 °C. All subjects were about 90 days of age when tested. Stereotaxic cannulation and site-specific infusion of DA drugs have been described in detail elsewhere (14).

Behavioral Testing. CPP testing was conducted as previously described (22) with the following exceptions. The initial cage preference for each subject was determined in a 30-min pretest on day 1. Subjects were then conditioned, during 40 min sessions, with AMPH to the nonpreferred cage and saline to the preferred cage (both AMPH and saline injections were given on the same day, 6 h apart) for 3 consecutive days (days 2–4). Thereafter, subjects were tested (posttest) for the presence of a CPP on day 5.

Partner preference testing was conducted as previously described (14). Briefly, the testing apparatus consisted of a central cage ($12 \times 28 \times 16$ cm) joined by hollow tubes (7.5×16 cm) to two parallel identical cages each housing a stimulus animal. The stimulus animals were the familiar "partner" (female mate of the subject) and an unfamiliar "stranger" (a female that had not previously encountered the subject) that were loosely tethered within their separate cages without direct contact to each other. At the initiation of the 3-h test, the subjects were placed into the central cage and allowed to move freely throughout the apparatus. Behavior was recorded using a time-lapse video recording system. Experimenters blind to the manipulation reviewed the tape and recorded subject's behavior. A partner preference was defined as the subject spending significantly more time in

Table 1. cDNA probes for DA marker mRNA labeling in the prairie vole brain

cDNA	Designer	Antisense: Linearization enzyme	Antisense: Transcription enzyme	Sense: Linearization enzyme	Sense: Transcription enzyme
Rat D1R (480 bp)	Dr. O. Civelli, University of California, Irvine, CA	Hind III	T7	EcoR I	SP6
Rat D2R (495 bp)	Dr. O. Civelli, University of California, Irvine, CA	EcoR I	T7	Xba	SP6
Rat DAT (532 bp)	Dr. H. Akil, University of Michigan, Ann Arbor, MI	EcoR I	T7	BamH I	SP6
Vole TH (247 bp)	Dr. M. Kabbaj, Florida State University, Tallahassee, FL	BamH I	T7	Xho I	SP6

side-by-side contact with the partner than the stranger, as indicated by a paired samples *t* test (27).

In Situ Hybridization and Western Immunoblotting. Specific antisense riboprobes (Table 1) were used for D1R, D2R, TH, and DAT in situ mRNA labeling. Labeling was conducted with ^{35}S-labeled probes and sense mRNA controls for each DA marker as previously described (51). For Western blotting analysis, DAR protein was extracted from the supernatant of NAcc tissue punches and assayed as described previously (52).

Data Quantification and Analysis. CPP and partner preferences were determined by paired samples *t* tests. Group differences in mating bouts during the first 6 h of pairing with a female and cage entries during the partner preference test were analyzed by ANOVA. The optical densities of D1R and D2R mRNA labeling in the NAcc as well as TH, DAT, and D2R mRNA labeling in the VTA were quantified from autoradiograms using a computerized image program (NIH IMAGE 1.64). Data were presented as percent change of the mean of the saline control group and group differences were analyzed by *t* tests. Finally, optical densities of D1R and D2R labeling on the x-ray film from the Western blotting experiments were analyzed by *t* tests.

Experimental Design. Experiment 1a established a dose–response curve for AMPH-induced CPP. Subjects were pretested in the CPP apparatus on day 1, randomly assigned into one of six experimental groups that received i.p. (ip) injections of saline containing different concentrations of AMPH [0 (n = 12), 0.1 (n = 8), 0.5 (n = 9), 1.0 (n = 12), 3.0 (n = 12), or 5.0 mg/kg (n = 13)] during 3 days (day 2–4) of conditioning, and then tested for CPP in a posttest on day 5.

Experiment 1b revealed the roles of DA receptors in AMPH-induced CPP. Subjects were pretested in the CPP apparatus and randomly assigned into one of eight experimental groups that received a s.c. (sc) injection of saline (n = 10) or saline containing different concentrations of a nonselective DA receptor antagonist [haloperidol; 0.1 (n = 8), 1.0 (n = 8), or 5.0mg/kg (n = 8)], or a D1-like specific (SCH23390; 0.5 (n = 7) or 5.0 mg/kg (n = 7)] or D2-like specific DA receptor antagonist [eticlopride; 0.5 (n = 8) or 5.0 mg/kg (n = 8)]. Thirty minutes later, a threshold dose of AMPH (1.0mg/kg), which induced CPP in Experiment 1a, was used for AMPH conditioning. After 3 days of AMPH conditioning, all subjects received a CPP posttest.

Experiment 2 examined whether AMPH experience interfered with pair bonding. Subjects were randomly assigned into one of three experimental groups that received i.p. injections of saline (n = 8) or saline containing 1.0 mg/kg (n = 8) or 5.0 mg/kg (n = 7) AMPH once per day for 3 consecutive days—a paradigm which induced CPP in male prairie voles. On the fourth day, subjects were paired with an estrogen-primed female for 24 h (14), and were then tested in a 3-h partner preference test. To control for the potential effects of injections on pair bonding, a fourth experimental group of intact males that did not receive any injections (n = 6) was paired with estrogen-primed females for 24 h and then tested for partner preferences. All behavioral tests were video-taped for the verification of mating. The duration of the subjects side-by-side contact with the partner and the stranger were quantified. In addition, frequencies of mating bouts during the first 6 h of pairing and locomotor activity (indicated by cage-crossing) during the 3-h partner preference test were quantified. After the partner preference test, subjects were immediately killed. All brains were harvested, frozen on dry ice, and stored at −80 °C for in situ hybridization labeling of DA marker mRNAs.

Experiment 3 examined whether AMPH-impaired pair bonding was associated with changes in mesolimbic DA activity. Brains from the subjects that received saline (n = 8) or 1.0 mg/kg AMPH (n = 8) in Experiment 2 were cut on a cryostat into coronal sections (14 μm thickness) that were thaw mounted onto Superfrost/plus slides (Fisher Scientific). Brain sections at 98-μm intervals were processed for in situ hybridization labeling of D1R, D2R, TH, and DAT mRNAs. Because voles experienced with 1.0 mg/kg AMPH showed an increase in D1R, but not D2R, mRNA labeling in the NAcc, as compared to saline-injected controls, two additional groups of subjects were generated that received injections of saline (n = 6) or 1.0 mg/kg AMPH (n = 6), 24 h of mating, and then partner preference testing, as described above. Subjects were decapitated and brains were sliced on a cryostat at 300 μm thickness. Tissue punches bilaterally taken from the NAcc were processed for D1R and D2R Western blotting.

Experiment 4 examined whether activation of D1-type receptors in the NAcc was responsible for AMPH-impairment of pair bonding. Subjects were implanted with guide cannulae bilaterally aimed at the NAcc shell. After 3 days of recovery, they were randomly assigned into one of three experimental groups in which they received intra-NAcc injections of CSF (200 nL/side, n = 11), or CSF containing 0.4 (n = 6) or 100 ng/side (n = 7) SCH23390. Thirty minutes later, they received i.p. injections of 1.0 mg/kg AMPH. This procedure was repeated for 3 consecutive days. On the fourth day, subjects were paired with an estrogen-primed female for 24 h and then tested for partner preferences.

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