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Role of Acetylcholine Transmission in Nucleus Accumbens and Ventral Tegmental Area in Heroin-Seeking Induced by Conditioned Cues

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Abstract

The involvement of cholinergic transmission in heroin self-administration and the reinstatement of heroin-seeking was examined in rats trained to nose-poke for intravenous heroin. Systemic treatment with physostigmine, an inhibitor of acetylcholinesterase, modestly reduced the acquisition and rate of heroin self-administration, and this suppression of heroin intake was reversed by pretreatment with scopolamine but not by mecamylamine. Following 10–14 days of self-administration, rats were left in the home environment for 14 days. Subsequently, rats were evaluated for extinction of nose-pokes during the first hour after being returned to the self-administration apparatus. One hr later a conditioned stimulus (house light, light in the nose-poke hole, sound of the infusion pump) was presented to initiate cue-induced reinstatement. Physostigmine produced a dose-dependent inhibition of cue-induced reinstatement, but only the dose of 0.5 mg/kg significantly decreased nose-poke responding in the extinction test. Chronic treatment with physostigmine (0.1 mg/kg) did not impair performance during acquisition of heroin self-administration. However, during a subsequent reinstatement test conducted in the absence of physostigmine pretreatment, heroin seeking was significantly below that of rats chronically pretreated with saline. To evaluate brain regions mediating the effects of systemic drug treatment on reinstatement, physostigmine was microinjected into the nucleus accumbens (NAc) or ventral tegmental area (VTA). Microinjection of physostigmine into the NAc prior to presenting conditioned cues inhibited the reinstatement of heroin-seeking, without affecting extinction responding. In contrast, microinjection of physostigmine into the VTA augmented the reinstatement induced by conditioned cues and extinction responding. Inactivation of either NAc or VTA by microinjecting tetrodotoxin blocked both extinction responding and cue-induced reinstatement. These data demonstrate that cholinergic transmission influences heroin self-administration and reinstatement. Moreover, cue-induced reinstatement was inhibited by physostigmine in the NAc and potentiated by cholinergic stimulation in the VTA.

Keywords

rat; craving; abstinence; acetylcholinesterase inhibitor; reinstatement; conditioning

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Relapse to heroin use is a major clinical problem in the treatment of heroin addiction (Kreek et al., 2002). Environmental and conditioned stimuli that are associated with the reinforcing effects of heroin are generally thought to play an important role in inducing compulsive drug seeking and relapse (O'Brien, 2004; Lee et al., 2006). Animal studies have utilized conditioned-cue reinstatement of extinguished drug-seeking as a model of craving and relapse to drug use (de Wit and Stewart 1981; Shaham et al., 2003). In these studies, rats trained to self-administer drug show renewed drug-seeking following extinction training when presented with drug-paired stimuli in the absence of the drug. Accordingly, robust heroin-seeking is elicited by conditioned cues (CS) that were previously paired with the heroin self-administration (Gracy et al., 2000; Shalev et al., 2002; Fuchs and See, 2002; Zhou et al., 2004; Zhang et al., 2004; Di Ciano and Everitt, 2004a; Bossert et al., 2006).

The mesolimbic dopamine system projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and is a key neural substrate regulating drug reinforcement (Wise, 2004). The activity of neurons in the NAc is modulated not only by dopaminergic afferents, but also by cholinergic interneurons (Calabresi et al., 2000; de Rover et al., 2002). Although several studies find evidence for altered cholinergic transmission in accumbens following morphine (Rada et al., 1991; Hurd et al., 1990; Rada et al., 1996), remifentanyl (Crespo et al., 2006) or cocaine administration (Smith et al., 2004), the studies differ in the direction or interpretation of the alterations. While most of the acetylcholine in the NAc arises from interneurons, the VTA receives cholinergic input from the laterodorsal and pedunculopontine tegmental nuclei (Ikemoto and Wise 2004). Dopamine neurons and axon terminals express muscarinic and nicotinic cholinergic receptors, and stimulation of these receptors elicits dopamine cell firing, mesolimbic dopamine release and can act to reinforce behavior (Gronier et al., 2000; Nisell et al., 1994; Ikemoto and Wise 2004). Taken together, these findings suggest that cholinergic transmission in the VTA and NAc modulates the mesolimbic dopamine projection and may regulate behaviors elicited by addictive drugs. In the present studies, the effect of acute or chronic systemic administration of the AChE inhibitor physostigmine was examined on heroin self-administration. The effect of physostigmine on extinction responding and conditioned cue-induced reinstatement of nose-poking for heroin was also examined. Finally, the role of acetylcholine in VTA or NAc on the reinstatement of heroin seeking was evaluated by the microinjecting of physostigmine directly into the VTA or NAc.

MATERIALS AND METHODS

Subjects

The subjects were 168 male Sprague Dawley rats (280–300 g, Experimental Animal Center of Zhejiang Province, Hangzhou, China), housed individually in the home cages in a temperature-controlled ventilated colony room with a reversed 12 hr light/dark cycle (lights onset 1900 h, offset 0700 h). *Ad libitum* food and water was provided in the home cage. All experiments were conducted in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory rats (NIH Publications No. 80-23 revised 1996).

Drugs

Heroin (diacetylmorphine HCl) was obtained from National Institute of Forensic Science (Beijing, China). The heroin dose (0.05 mg/kg/injection) used for the self-administration experiment was chosen on the basis of previous studies (Zhou et al., 2004; Zhang et al., 2004). The heroin was dissolved daily in sterile physiological saline at a concentration of 0.2 mg/ml. Physostigmine sulfate (RBI, Natick, MA) used for intraperitoneal administration was dissolved in 0.9% saline, and for intracranial injection was dissolved in artificial CSF consisting

of (in mM): 148 NaCl, 2.7 KCl, 1.2 CaCl₂, and 0.85 MgCl₂ (pH adjusted to 7.5). Tetrodotoxin (Qingdao Institute of Marine Biology, China) was dissolved in artificial CSF.

Apparatus

A total of 32 Plexiglas custom-made operant boxes (Ningbo Addiction Research and Treatment Center, China) were used for this experiment and each was enclosed in a sound-attenuating plywood chamber. Each operant box was equipped with two nose-poke operandum (ENV-114M, Med Associates, Lafayette, IN) located 5 cm above the floor of the box. There was a green LED light inside each nose-poke hole. A house light (28 V, 0.1 mA, ENV-215M, Med Associates) was situated on the wall above the nose-pokes. Drug solution was delivered through Tygon tubing, protected by a leash assembly (PHM-120, Med Associates) and suspended through the ceiling of the chamber from a plastic fluid swivel (PHM-115, Med Associates). The leash assembly was modified to fit a custom-made fluid connector fixed on the animal's jacket. The Tygon tubing was attached to a syringe pump (PHM-100, Med Associates) that delivered fluid at a speed of 1.2 ml/min using a 10 ml syringe. Experimental events were controlled by an IBM-compatible PC, using a MED Associates interface and running self-programmed software written in Borland Delphi 6.0 (OBSM v4.0, operant behavioral schedule manager).

Surgery

All rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.; Serva). Atropine sulfate and penicillin B were given at the time of surgery. All the rats were implanted with chronically indwelling intravenous catheters. A silicon catheter (Silastic; length 3.5 cm, 0.5 mm inner diameter, 0.94 mm outer diameter) was inserted into the right external jugular vein so that the tip reached the right atrium and was secured with thread. The other end of the catheter (10 cm, PE20) was passed subcutaneously to an incision on the back of the body where it exited into the custom-made fluid-connector fixed to a jacket. The catheters were flushed daily with 0.2 ml saline–heparin solution (25 U/ml heparin) to maintain catheter patency. To prevent infection, the rats were treated post-surgically with penicillin B for 5 days. All the rats were allowed to recover for at least 7 days.

In some experiments bilateral guide cannulae (20-gauge, Small Parts Inc., Roanoke, VA) were implanted in the NAc (1.4 mm anterior to bregma, 1.0 mm lateral to midline, and 7.1 mm ventral to the surface of the cortex) or VTA (6.0 mm posterior to bregma, 1.0 mm lateral to midline, and 7.8 mm ventral to the surface of the cortex using a 10° angle) according to the atlas of Paxinos and Watson (1986). Guide cannulae were lowered into place and attached to the skull via dental acrylic. Obdurators extended 0.5 mm beyond the tip of each cannula to prevent obstruction by debris.

Microinjection procedure

Subjects were microinjected before the reinstatement testing. Obdurators were removed and bilateral infusion cannulas (33 gauge) were inserted, extending 1 mm beyond the tip of the guide cannulas. All injections into the VTA and accumbens were delivered by using a microinjection pump (MD-1001, Bioanalytical System Inc., IN) in a volume of 0.5 µl over 5 minutes.

Heroin self-administration training and withdrawal procedure

During each daily 4 h self-administration training session, the rats were moved from their home cages to the self-administration chambers and their connectors were attached to the infusion lines. Each session started with the illumination of the green light inside the active nose-poke hole. The rats received a single heroin infusion (50 µg/kg per infusion) following completion

of the ratio requirement in the active nose-poke. An escalating fixed ratio schedule of reinforcement was used in present experiment. Thus, during each daily session the FR1 for the first 5 heroin infusions, FR2 for the second 5 infusions, FR3 for the third 5 infusions, FR4 for the fourth 5 infusions and FR5 for the last 5 infusions. Based on our previous experiments, this schedule supports rapid and reliable heroin self-administration at a heroin dose of 50 µg/kg per injection (Zhou et al., 2004; Zhang et al., 2004).

Each infusion was paired with a 5 s illumination of the house light and in combination with the noise of the infusion pump, therefore serving as discrete CS paired with the drug infusion. A timeout period was imposed for 30 s, during which the responding produced no programmed consequences but was still recorded. Illumination of the green light in the active nose-poke signaled the end of the 30 s timeout period. Responding in the inactive nose-poke produced no programmed consequences. The sessions ended after 4 h or 25 heroin injections, whichever occurred first. The rats were put back into their individual home cages shortly after the session. Each rat received one heroin self-administration session per day for a total of 10 or 14 days. Subsequent to daily self-administration, the rats remained undisturbed in their individual home cages for 14 days, and did not have access to heroin, the heroin-associated cues or the heroin self-administration context.

Extinction and cue-induced reinstatement of drug-seeking

All rats underwent an identical sequence of behavioral training and withdrawal procedures. On the test day, all rats were tested for extinction and reinstatement of the drug seeking in the original self-administration context and each animal was tested only once (Zhang et al 2004, Zhou et al., 2005). During the extinction session, the connectors were attached to the swivels but not to the infusion lines. The rats were brought to the self-administration chambers and were given 1 h extinction session in the absence of the discrete cues previously associated with heroin infusions. Following one hr of extinction, the discrete CS (house light, light in the nose-poke hole, pump noise) were presented for 5 s, after which each active nose-poke response resulted in another presentation of the CS. Nose-pokes during this phase of CS reinstatement were accumulated over 60 min.

Locomotor test after withdrawal

After two weeks withdrawal from heroin self-administration, the rats (n=36) were habituated for one hr in photocell chambers (AccuScan Instruments, Inc. Columbus, OH). Each box contained a series of 16 photocell beams measuring horizontal distance traveled. Beam breaks were continuously counted and recorded once every 5 min by a PC running VersaMax/Digiscan System Software (AccuScan Instruments, Inc.). After one hr habituation in the chamber, the rats were injected i.p. with physostigmine or saline. Horizontal locomotor activities traveled were recorded for 2 hrs.

Histology and Statistics

Once all testing was completed, rats were anesthetized and transcardially perfused with phosphate-buffered saline, followed by 4% polyformalin solution. The brains were sectioned on a Cryostat Microtome (Leica CM1850, Germany) in the coronal plane at a thickness of 50 µm and were stained with Cresyl Violet. Infusion-cannula tip placement was located using light microscopy and was mapped onto a schematic diagram of the rat brain (Paxinos and Watson, 1986). The microinjection guide cannulae were located within the shell and medial core subcompartment of the NAc or within the central portion of the VTA, generally at the rostral edge of the interpeduncular nucleus.

The data from the self-administration and reinstatement tests were analyzed separately. The number of infusions or responses for active and inactive holes during self-administration were

analyzed using a one-way ANOVA or two-way (hole, treatment) ANOVA with repeated measure. Total nose-pokes during reinstatement were analyzed using a two-way ANOVA with repeated measure over active and inactive nose-pokes. Locomotor data were analyzed using one-way ANOVA. A Newman-Keuls multiple-comparison with an alpha level of 0.05 was used for *post hoc* comparisons between group means.

Specific experiments

Experiment 1—Rats (n=40) were tested for the effect of physostigmine on heroin self-administration. The rats were trained heroin self-administration for 10 days (e.g. see figure 2A). The rats were randomly assigned to one of 5 groups (n=8 in each group) and were injected the vehicle, 0.02, 0.1, 0.5 or 2.5 mg/kg physostigmine (i.p.) at 10 min prior to the training session on day 11. Another twenty-four rats (n=8 each group) were pretreated with scopolamine (0.5 mg/kg, s.c.), mecamylamine (0.5 mg/kg, s.c.), or saline at 10 min before the injection of 0.5 mg/kg of physostigmine (i.p.) on the day 11 of heroin training session.

Experiment 2—Twelve rats (n=6 per group) were tested for the effect of physostigmine on the acquisition of heroin self-administration. Ten min prior to the each daily training session, one group of rats received the physostigmine, while another group was injected with saline (1 ml/kg, i.p.). The dose of physostigmine (0.1 mg/kg, i.p.) was selected based on the results from experiment #1 as a dose subthreshold for affecting self-administration in well-trained rats (e.g. see figure 1). The training was carried out for 10 days. The rats in the two groups were then tested for the reinstatement of drug seeking after the 14 days withdrawal. During the withdrawal period and reinstatement test, rats were not administered physostigmine.

Experiment 3—Following 14 d of heroin self-administration and 14 d of withdrawal as described above, rats (n=6 per group) were tested for the effects of physostigmine on the reinstatement of heroin-seeking. After 2 weeks in the home cage, the rats were injected with physostigmine (0.02 mg/kg, 0.1 mg/kg or 0.5 mg/kg) or saline 10 min prior to re-introducing the animal into training chamber to extinction. One hr later, the rats were presented with the CS and each subsequent nose-poke in the hole previously paired with heroin elicited the CS without a heroin injection. CS-induced reinstatement was observed for 1 hr after initial cue presentation.

Experiment 4—Following 10 d of heroin self-administration and 14 d of withdrawal as described above, bilateral microinjections were made into NAc or VTA as described above into a total of 56 rats. Rats were placed into the self-administration chambers 10 min after intra-NAc or -VTA physostigmine (1.5 µg/side, 0.5 µl), physostigmine (5 µg/side, 0.5 µl), TTX (0.3 ng/side, 0.5 µl) or vehicle (aCSF 0.5 µl). Rats were divided into 6 groups of 7 rats each and tested only once. The extinction and cue-induced reinstatement of drug seeking was examined as described above.

RESULTS

Systemic physostigmine and heroin self-administration

In Experiment 1, the rats were trained to self-administer heroin for 10 days, and on day 11 rats were pretreated 10 min prior to beginning heroin self-administration with physostigmine (0.02, 0.1, 0.5 or 2.5 mg/kg, i.p.) or saline. The dose of 2.5 mg/kg physostigmine produced seizures in 2 of the 8 animals tested. Therefore, while these data are shown in figure 1, they were not included in the statistical analysis. A repeated measures two-way ANOVA showed a significant dose X hole interaction for total nose-pokes (figure 1A; $F_{(3, 28)} = 4.06$, $p = 0.016$) and a one-way ANOVA revealed a significant effect of dose on total injections received (figure 1B; $F_{(3, 28)} = 3.90$, $p = 0.018$). *Post hoc* comparisons revealed a significant reduction in active nose-pokes

and injections of heroin by 0.5 mg/kg physostigmine compared with saline. The inhibitory effect of physostigmine was primarily in the first 30 min of the trial as revealed in the representative time course of active nose-pokes shown in figure 1E.

Figures 1C and D show that the physostigmine-induced inhibition of total responses and number of injections, respectively, was reversed by pretreatment with the muscarinic antagonist scopolamine (0.5 mg/kg), but not by pretreatment with the nicotinic antagonist mecamylamine (0.5 mg/kg). A repeated measures two-way ANOVA showed a significant main effect of group for total nose-pokes (figure 1C; $F_{(2,21)} = 3.8$, $p = 0.039$), and a one-way ANOVA revealed a significant effect of group on total injections received (figure 1D; $F_{(2,21)} = 4.1$, $p = 0.032$). *Post hoc* comparisons revealed a significant increase in active nose-pokes and injections of heroin by pretreatment with scopolamine (0.5 mg/kg) compared with physostigmine alone.

To determine whether physostigmine could attenuate the acquisition of heroin self-administration, rats in Experiment 2 were pretreated with physostigmine (0.1 mg/kg, i.p.) or saline 10 min prior to each daily self-administration training session. Although not statistically significant, figure 2 shows that there was a tendency towards an inhibition of active nose-pokes and heroin infusions on the first day of self-administration training. Figure 2A shows the development of significant discrimination between the nose poke hole associated with heroin administration (active responses) and the inactive (non-drug-paired) responses. A three-way repeated measures ANOVA revealed that while there was a significant main effect difference between active and inactive holes ($F_{(1,10)} = 28.67$, $p < 0.001$) and a session X hole interaction ($F_{(1,10)} = 39.0$, $p < 0.001$), there were no significant group main or interaction effects. Figure 2B shows the data from Experiment 2 as infusions received in each session, and while a two-way repeated measures ANOVA revealed a main effect of session ($F_{(9,90)} = 7.14$, $p < 0.001$) there was no main effect of physostigmine or interaction.

Acetylcholine regulates the heroin-seeking elicited by conditioned cues after abstinence

Following day 10 of daily heroin self-administration, the rats in Experiment 2 were placed in their home cages for 14 days without receiving heroin or physostigmine. Rats were then returned to the heroin self-administration chamber. Nose-pokes were recorded, but were without programmed consequence. Nose-pokes over the first 60 min were operationally defined as extinction responding, and figure 2C shows that significant nose-poking occurred (comparing active to inactive responses for both extinction $F_{(1,10)} = 77.41$, $p < 0.001$ and reinstatement of CS $F_{(1,10)} = 135.5$, $p < 0.001$), and that daily pretreatment with physostigmine during the heroin self-administration training did not affect extinction responding. In contrast, drug seeking induced by the CS in the physostigmine pretreatment group was significantly lower than that of the saline group (main effect of physostigmine, $F_{(1,10)} = 14.60$, $p = 0.003$, and interaction $F_{(1,10)} = 17.30$, $p = 0.002$).

Experiment 3 evaluated the effect of physostigmine pretreatment 10 min prior to the reinstatement trial occurring 2 wks following the last daily heroin self-administration session. Both extinction (figure 3A) and cue-induced (figure 3B) responses were significantly reduced by physostigmine. However, while the minimum effective dose for cue-primed reinstatement was 0.1 mg/kg (i.p.), the minimum effective dose for extinction responding was 0.5 mg/kg (i.p.). Both extinction responding and CS-induced effects were evaluated by a separate two-way repeated measures ANOVA (extinction: dose $F_{(3,20)} = 15.95$, $p < 0.001$, hole $F_{(1,20)} = 72.42$, $p < 0.001$, interaction $F_{(3,20)} = 9.38$, $p < 0.001$; CS: dose $F_{(3,20)} = 14.22$, $p < 0.001$, hole $F_{(1,20)} = 133.0$, $p < 0.001$, interaction $F_{(3,20)} = 12.10$, $p < 0.001$). The effects of physostigmine on horizontal locomotor activity were examined by injecting physostigmine (0.02, 0.1 or 0.5 mg/kg, i.p.) or saline in rats after withdrawal from heroin self-administration (Figure 4). A one-way ANOVA showed a significant effect of treatment of physostigmine on the horizontal

locomotion activity (for first hour: $F_{(3, 31)}=7.7$, $p=0.001$; for second hour: $F_{(3, 31)}=8.2$, $p<0.001$). *Post hoc* comparison revealed a significant decrease in horizontal locomotor activity by treatment with 0.5 mg/kg physostigmine during first hr period, but a significant increase in locomotor activity by treatment with 0.02 mg/kg physostigmine.

Different effect of physostigmine in the accumbens versus VTA on drug-seeking

In Experiment 4, physostigmine was infused into the NAc prior to placing rats into the self-administration chamber 14 days following 10 days of daily heroin self-administration. Physostigmine inhibited drug-seeking induced by the CS, but did not alter extinction responding (figure 5A). In contrast, both extinction responding and drug-seeking induced by CS was blocked by inactivation of the NAc with TTX. Both extinction responding and CS-induced effects were evaluated by separate two-way repeated measures ANOVA (extinction: drug $F_{(3,24)} = 3.5$, $p < 0.031$, hole $F_{(1,24)} = 81.65$, $p < 0.001$, interaction $F_{(3,24)} = 2.73$, $p < 0.066$; CS: drug $F_{(3,24)} = 17.03$, $p < 0.001$, hole $F_{(1,24)} = 115.15$, $p < 0.001$, interaction $F_{(3,24)} = 15.47$, $p < 0.001$).

Either drug seeking induced by CS or extinction responding was increased significantly after physostigmine (5 µg/side, 0.5 µl) microinjection into the VTA (figure 5B). In contrast, intra-VTA microinjection of TTX blocked the induction of either extinction responding or drug-seeking by CS. Both extinction responding and CS-induced effects were evaluated by separate two-way repeated measures ANOVA (extinction: drug $F_{(3,24)} = 4.42$, $p = 0.013$, hole $F_{(1,24)} = 27.07$, $p < 0.001$, interaction $F_{(3,24)} = 5.72$, $p = 0.004$; CS: drug $F_{(3,24)} = 10.08$, $p < 0.001$, hole $F_{(1,24)} = 157.46$, $p < 0.001$, interaction $F_{(3,24)} = 8.86$, $p < 0.001$).

DISCUSSION

The present data show that drugs regulating cholinergic transmission can modulate heroin-seeking induced by environmental stimuli that were previously associated with heroin reward. Thus, systemic administration of physostigmine reduced cue-induced reinstatement; an effect mimicked by potentiating cholinergic transmission selectively in the NAc. This effect is likely the result of muscarinic receptor stimulation since it was reversed by pretreatment with scopolamine. In contrast with the NAc, potentiating cholinergic transmission in the VTA augmented both extinction responding and cue-induced reinstatement of nose-poking. Systemic administration of physostigmine produced more modest reductions in the rate of heroin self-administration. However, while the effect of daily treatment with physostigmine only slightly attenuated the acquisition of heroin self-administration, this pretreatment markedly reduced subsequent cue-induced reinstatement 14 days after the last administration of physostigmine and heroin.

Methodological concerns

It is important to note that the rate reducing effect of high systemic doses (0.5 and 2.5 mg/kg, i.p.) physostigmine may result in part from nonspecific effects of systemic cholinergic activation, as described by Wilson and Schuster (1973) in primates, and indicated here by the induction of seizures in 2 of 8 rats administered 2.5 mg/kg physostigmine. However, the fact that intra-NAc administration of physostigmine produced similar reductions supports a direct effect on cholinergic transmission in an area of the brain known to regulate heroin reward (Wise, 2002; Di Chiara, 2002). A potential concern with experimental design is that the extinction trial and cue reinstatement trial were conducted sequentially, yet all pretreatments were administered prior to the extinction trial (e.g. one hr prior to the cue trial). Thus, brain levels of physostigmine would be expected to be elevated during the extinction relative to cue trial. Nonetheless, the inhibitory effect of physostigmine was generally greater on cue-induced responding than during the extinction trial, indicating that the present data may be

underestimating the relative inhibitory efficacy of cholinergic activation on cue-induced reinstatement. Similarly it is important to note that the rate of responding during cue-induced reinstatement was higher than during extinction, thus allowing a significant reduction in cue-induced responding to be more readily identified. In a related issue, the facts that heroin self-administration was limited to a maximum of 25 infusions per session and most rats achieved this maximum, could have limited the ability to identify rate-increasing effects of cholinergic drugs. Another potential concern is that without a full heroin dose-response curve, the rate increasing effect of scopolamine plus physostigmine could have resulted from decreased, not increased rewarding value of heroin, in which case, the elevated rate of self-administration resulted from the animal attempting to overcome the reduced rewarding value of each heroin injection. Finally, although the microinjection procedure was consistent with relatively restricted injection spread, it is possible that the effects induced by intracranial physostigmine could result, in part, from diffusion to adjacent brain areas, notably diffusion along the cannula track dorsal to the NAc or VTA.

Cholinergic transmission in the nucleus accumbens

A primary finding of this study was that microinjection with physostigmine into NAc inhibited the heroin seeking induced by conditioned cues; thereby suggesting that cholinergic interneurons in the accumbens are capable of regulating reinstatement. Similar to the reinstatement circuitry in rats trained to self-administer cocaine (Di Ciano and Everitt, 2001; Kalivas and McFarland, 2003), inactivation of the NAc with TTX in the present study reveals a critical role for the nucleus accumbens in reinstatement in heroin trained subjects. The reduction in reinstatement by intra-accumbens physostigmine is consistent with previous reports that blocking AChE in the accumbens also inhibits morphine-induced locomotor sensitization and conditioned place preference (Hikida et al., 2003). Although cholinergic interneurons are a likely source of acetylcholine transmission in the accumbens, exactly how enhanced cholinergic transmission in the NAc inhibits reinstatement is difficult to discern from the literature describing electrophysiological effects of acetylcholine agonists on accumbens spiny cells. However, based on the blockade of the inhibitory effect of systemically administered physostigmine by scopolamine, it seems likely that the rate decreasing effect of intra-NAc physostigmine may also rely on stimulating muscarinic receptors. Arguing against this possibility, stimulating M₁ muscarinic receptors promotes glutamatergic NMDA currents (Calabresi et al., 2000) and acts to presynaptically inhibit GABA transmission (de Rover et al., 2002); both effects that should promote, not inhibit behavioral responding according to the majority of the studies employing cue-primed reinstatement of drug seeking (McLaughlin and See, 2003; Kalivas and McFarland 2003; Di Ciano and Everitt, 2004b; Bossert et al. 2006).

Cholinergic transmission in the VTA

A great deal of research has been conducted on the capacity of cholinergic transmission to modulate the dopamine projection from the VTA to the nucleus accumbens, and microinjecting carbachol or an AChE inhibitor into the VTA produced conditioned place preference and sustained self-administration (Ikemoto and Wise, 2002). As these effects were inhibited by either nicotinic or muscarinic antagonists, both receptor subtypes may be important. It has been proposed that the primary reinforcing properties of nicotine may arise from stimulating of nicotinic receptors in the VTA (Nomikos et al, 2000; Maskos et al., 2005). Thus, stimulating nicotinic receptors in the VTA, specifically receptors containing the $\alpha 7$ subunit, increases dopamine cell firing, primarily via a presynaptic action on glutamatergic afferents to dopamine neurons (Gronier et al., 2000; Klink et al., 2001). The role of muscarinic receptors in the VTA is mixed. There is a clearly defined presynaptic inhibitory effect on cholinergic afferents (Yeomans et al., 2000), but also a possible direct stimulatory effect on dopamine cells (Gronier et al., 2000).

The augmented cue-induced reinstatement and extinction responding produced by promoting cholinergic transmission in the VTA is consistent with activating dopamine neurons. It was recently shown that blocking glutamatergic receptors in the VTA prevents morphine induced conditioned place preference (Harris et al., 2004) or cue-induced reinstatement in rats trained to self-administer heroin (Bossert et al., 2004). Similarly, cue-induced reinstatement was inhibited by inactivating the VTA with TTX (figure 5). The fact that systemic physostigmine inhibited both extinction and cue responding indicates that the facilitating effects of intra-VTA physostigmine are countermanded by inhibitory affects elsewhere in the circuitry regulating extinction responding and cue-induced reinstatement. Consistent with this possibility, cholinergic stimulation in a dopamine axon terminal field, the NAc inhibited cue-induced reinstatement.

Systemic physostigmine inhibits the development of cue-induced reinstatement

The capacity of systemic physostigmine given daily with heroin self-administration to inhibit the development of cue-induced reinstatement was surprising (see figure 2C). The dose of physostigmine employed (0.1 mg/kg) had no effect on stable heroin self-administration and only slightly reduced the acquisition of self-administration on the first day of drug exposure. However, physostigmine given during heroin training inhibited drug seeking induced by cue during subsequent testing 2 weeks later. Impairment in heroin seeking was not likely attributable to nonspecific motor effects, because it was stimulus specific (i.e. extinction responding was not impaired) and this dose of physostigmine alone did not alter locomotor activity. Thus, physostigmine appeared to blunt the learning required to form strong associations between drug and discrete environmental cues (Goldberg, 1976; See, 2002; Di Ciano and Everitt, 2004a). One potential site of action whereby physostigmine may be producing this effect is in the nucleus accumbens where increased cholinergic transmission by physostigmine or inhibition of neuronal activity by TTX reduced cue-primed reinstatement (figure 5A).

Effects of acetylcholine transmission system on drug reinforcement have been shown before (Wilson and Schuster, 1973; Mark et al., 1999; Rasmussen et al., 2000; Smith et al., 2004; Crespo et al., 2006). We showed in rats trained to self-administer heroin that physostigmine at higher dose decreased the rate of heroin administration. These findings corroborate the previous results others showing that increasing acetylcholine transmission in NAc suppressed both cocaine- and morphine-induced conditioned place preference (Hikida et al., 2003), and that systemic administration of muscarinic agonists inhibit rates of cocaine self-administration (Rasmussen et al., 2000). However, these data are at apparent odds with a number of other studies, including findings by Smith et al. (2004) that neurotoxic cholinergic lesions in the NAc-ventral pallidum reduced cocaine self-administration, and two studies showing that M₅ receptor deficient mice self-administer less cocaine (Thonsen et al., 2005) and show reduced morphine reward in a conditioned place preference paradigm (Basile et al., 2002). One potential reason for the differences between the present data and these studies is that the latter studies rely either on constitutive loss of cholinergic transmission (e.g. deletion of the muscarinic5 receptor gene) or neurotoxic lesions, while the current study involved acute, reversible manipulations of cholinergic transmission.

Conclusion

In conclusion, the present study confirms a role for cholinergic transmission in the brain processes that underlie heroin seeking. Differential effects on heroin seeking were found when cholinergic transmission was selectively augmented in the VTA versus NAc. Thus, along with previous demonstration of the role of the basolateral amygdala (Fuchs and See, 2002), these data demonstrate involvement of the VTA and NAc in the brain circuitry mediating cue-induced heroin seeking. This circuitry is consistent with studies on nuclei mediating cue-

primed reinstatement of cocaine-seeking (Kalivas and McFarland 2003; Shalev et al., 2002), and pose the possibility that a common circuit may underlie reinstatement and relapse in opioid and psychostimulant addiction.

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Abbreviation

AChE	acetylcholinesterase
CS	conditioned stimuli
NAc	Nucleus accumbens
VTA	ventral tegmental area

Figure 1.

Systemic injection of physostigmine inhibits the maintenance of the heroin self-administration. **(A)** Physostigmine was injected (i.p.) 10 min prior to beginning access to heroin on day 11 of training. Data are shown as mean $\pm$ s.e.m. nose-pokes in the hole associated with heroin (active) and the hole not associated with heroin (inactive). * $p < 0.05$, compared with the vehicle control. **(B)** Infusions earned during the heroin self-administration. * $p < 0.05$, compared with the vehicle control. **(C)** Scopolamine (0.5 mg/kg) or mecamylamine (0.5 mg/kg) was injected 10 min before injection of physostigmine (0.5 mg/kg) on day 11 of training. Data are shown as mean $\pm$ s.e.m. nose-pokes. * $p < 0.05$, compared to the physostigmine treated group. **(D)** Infusions earned during the heroin self-administration. * $p < 0.05$, compared to the physostigmine treated

group. **(E)** Representative individual response records of heroin self-administration on day 11 after pretreatment with physostigmine or saline control. Each vertical tick mark indicates an active response. Note the lower response during the first 30 min of heroin training in the rat pretreated with 0.5 mg/kg physostigmine, and the reduction in responding throughout the session after 2.5 mg/kg physostigmine.

Figure 2.

Effects of daily pretreatment with physostigmine on the acquisition of heroin self-administration and reinstatement of drug-seeking after withdrawal in Experiment 2. **(A)** Mean $\pm$ s.e.m. active and inactive responses (nose-pokes) during heroin self-administration for 10 consecutive days in rats pretreated 10 min prior to each training session with physostigmine or saline control. **(B)** Heroin injections earned during the training sessions. **(C)** When tested after 14 days withdrawal, drug-seeking elicited by the conditioned cues decreased in rats previously treated with physostigmine during heroin self-administration, while extinction responding was not different between the two groups.

* $p < 0.05$, compared to the vehicle control.

Figure 3.

Systemic injection of physostigmine inhibits heroin seeking induced by conditioned cues. **(A)** Mean $\pm$ s.e.m. extinction responses over 60 min when rats were placed in the heroin self-administration context after 14 days of withdrawal. **(B)** Mean $\pm$ s.e.m. nose-pokes over 60 min induced by conditioned cues begun immediately after collecting data in panel A. **(C)** Time course of the active nose-poke responses.

* $p < 0.05$, compared to the vehicle control.

Figure 4. Effect of physostigmine treatment on horizontal locomotor activity after 14 days withdrawal. The rats were allowed to habituate to their novel environment. One hour later, rats were treated with physostigmine (0.02, 0.1, 0.5 mg/kg) or saline and immediately placed back in the AccuSan chamber and observed for two hours. **(A)** Each time point represents the mean $\pm$ s.e.m. photocell beam breaks per 5 min period. **(B)** Histograms represent the total horizontal locomotor activity during the one hr period following physostigmine treatment. * $p < 0.05$, compared with the vehicle control.

Figure 5.

Effect of intra-NAc or intra-VTA treatment with physostigmine (1.5 µg/side), physostigmine (5 µg/side) or TTX (0.3 ng/side) on reinstatement of drug seeking induced by conditioned cues. **(A)** Bilateral injection of physostigmine into the NAc inhibited the drug-seeking induced by the conditioned cues, but did not decrease extinction responding. The extinction responding and drug-seeking induced by conditioned cues were decreased by TTX infused into the NAc. **(B)** Bilateral injection of physostigmine (1.5 µg/side) into the VTA increased the drug-seeking induced by conditioned cues, physostigmine (5 µg/side) increased both extinction responding and drug-seeking induced by conditioned cues, while bilateral injection of TTX inhibited the extinction responding and drug seeking induced by conditioned cues.

* $p < 0.05$, compared to the vehicle control.