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Increased Dopamine Receptor Activity in the Nucleus Accumbens Shell Ameliorates Anxiety during Drug Withdrawal

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A number of lines of evidence suggest that negative emotional symptoms of withdrawal involve reduced activity in the mesolimbic dopamine system. This study examined the contribution of dopaminergic signaling in structures downstream of the ventral tegmental area to withdrawal from acute morphine exposure, measured as potentiation of the acoustic startle reflex. Systemic administration of the general dopamine receptor agonist apomorphine or a cocktail of the D1-like receptor agonist SKF82958 and the D2-like receptor agonist quinpirole attenuated potentiated startle during morphine withdrawal. This effect was replicated by apomorphine infusion into the nucleus accumbens shell. Finally, apomorphine injection was shown to relieve startle potentiation during nicotine withdrawal and conditioned place aversion to morphine withdrawal. These results suggest that transient activation of the ventral tegmental area mesolimbic dopamine system triggers the expression of anxiety and aversion during withdrawal from multiple classes of abused drugs. *Neuropsychopharmacology* (2012) **37,** 2405–2415; doi:10.1038/npp.2012.97; published online 13 June 2012

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INTRODUCTION

Negative affective signs and symptoms, including anxiety, irritability, anhedonia, and dysphoria, are common to withdrawal from all classes of abused drugs, are manifested after the very first exposure to a drug, and increase in intensity with repeated exposures (Koob and Le Moal, 1997; Haertzen and Hooks, 1969). Negative reinforcement theories of drug dependence, such as the 'opponent process' and 'hedonic allostasis' theories (Koob and Bloom, 1988; Solomon and Corbit, 1974), hypothesize that the emotional signs and symptoms of withdrawal contribute to the acquisition and maintenance of drug-taking behavior as well as to relapse following periods of prolonged abstinence.

One interesting prediction of the opponent process view of addiction is that activity in the neural circuits responsible for withdrawal is dependent on prior activation of reward-related circuitry (Koob and Bloom, 1988). Recent work in our laboratory (Radke *et al*, 2011) supported this prediction by demonstrating that targeted infusion of morphine in the ventral tegmental area (VTA) was sufficient to induce anxiety-like withdrawal behaviors. In the same study, we also found that systemic injection of the general dopamine receptor agonist apomorphine relieved withdrawal-induced anxiety (Radke *et al*, 2011). Taken together, this evidence led us to hypothesize that negative affective withdrawal behaviors are triggered by declining levels of dopamine in structures downstream of the VTA. The current studies address this hypothesis by examining the contribution of dopaminergic signaling in these structures to withdrawal from acute morphine exposure.

The hypothesis that expression of negative emotional withdrawal signs involves dopaminergic signaling is supported by further evidence. First, VTA dopamine neurons project to structures known to be involved in negative emotional signs of withdrawal, including the nucleus accumbens (NAc), basolateral amygdala (BLA), central amygdala (CeA), and lateral portion of the bed nucleus of the stria terminalis (lBNST) (Fallon *et al*, 1978; Hasue and Shammah-Lagnado, 2002; Meloni *et al*, 2006). Cellular activity in the CeA and lBNST following drug exposure has also been shown to depend on the activation of dopamine receptors (Valjent *et al*, 2004; Kash *et al*, 2008). Furthermore, dopamine release in these target structures falls below baseline levels during spontaneous opiate withdrawal (Acquas *et al*, 1991; Crippens and Robinson, 1994) and

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following administration of an opioid receptor antagonist such as naloxone (Rossetti *et al*, 1992; Pothos *et al*, 1991; Spanagel *et al*, 1994). Treatments that attenuate withdrawal behaviors have been shown to reverse this decrease in dopaminergic signaling, while treatments that exacerbate withdrawal potentiate it (Spanagel *et al*, 1994; Georges and Aston-Jones, 2003). Finally, studies examining the role dopamine plays in opiate withdrawal behaviors (Bechara *et al*, 1995; Laviolette *et al*, 2002; Chartoff *et al*, 2006, 2009; Radke *et al*, 2011) have found that dopaminergic signaling contributes to increased aversion, aggression, and anxiety during withdrawal (but see, Caillé *et al*, 2003).

To better understand how drug-induced changes in dopaminergic signaling contribute to withdrawal behaviors, we evaluated the effects of intracerebrally infused dopamine receptor agonists on potentiation of the acoustic startle reflex ('withdrawal-potentiated startle') during opiate withdrawal (Harris and Gewirtz, 2004; Rothwell *et al*, 2009; Cabral *et al*, 2009). Increases in startle following drug exposure likely represent the anxiety-like component of the withdrawal state. For example, potentiated startle during withdrawal is relieved by a second drug exposure or administration of anxiolytic compounds (Harris and Gewirtz, 2004; Rothwell *et al*., 2009; Engelmann *et al*, 2009, Radke *et al*, 2011). The timing of the withdrawal-potentiated startle effect following morphine also coincides with the decrease in drug levels in the brain (Barjavel *et al*, 1995; Hipps *et al*, 1976).

Previous work from our laboratory and others (Harris and Aston-Jones, 1994; Chartoff *et al*, 2006; Chartoff *et al*, 2009; Radke *et al*, 2011) has demonstrated that dopamine receptor agonists attenuate withdrawal behaviors. The current experiments used two subtype-specific dopamine receptor agonists, SKF82958 and quinpirole, to examine the individual contributions of D1- and D2-like dopamine receptors, respectively, to this effect. These two classes of dopamine receptors have opposite effects on cell excitability and glutamatergic signaling (Neve *et al*, 2004; Surmeier *et al*, 2007), but have both been shown to contribute to aversive and withdrawal behaviors (Carlezon and Thomas, 2009). We next investigated the location of the dopamine receptors involved in withdrawal-induced anxiety by locally infusing the D1/D2 agonist apomorphine into the shell of the NAc, lBNST, CeA, and VTA. The dorsolateral component of the BNST (dlBNST) was specifically targeted because this region contains the most dense distribution of dopaminergic fibers (Freedman and Cassell, 1994). Two final experiments examined whether reduced dopaminergic signaling is similarly involved in nicotine withdrawal-potentiated startle and conditioned place aversion to morphine withdrawal.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (Harlan, Indianapolis, IN), weighing between 225 and 400 g at the start of the experiment, were housed in groups of four in metal cages with a 12 h light–dark cycle and free access to food and water, except during testing. Animals were acclimated to housing conditions for 2 weeks and then gently handled for two consecutive days. Rats that underwent intracranial cannulation surgery were subsequently housed individually in metal cages. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Drugs

Morphine sulfate was purchased from Mallinckrodt (Hazelwood, MO). SKF82958, quinpirole, and (−)-nicotine hydrogen tartrate salt were purchased from Sigma-Aldrich (St Louis, MO). Apomorphine was purchased from Tocris (Ellisville, MO). Systemically administered drugs were dissolved in 0.9% saline and injected subcutaneously. Intracerebrally infused apomorphine was dissolved in 25%. DMSO in sterile saline. The nicotine solution was titrated to a pH of approximately 7.1 using sodium hydroxide. Throughout the text, 0 mg/kg and 0 μg denote groups given vehicle injection or infusion, respectively. All drug doses are expressed as the weight of the salt, except nicotine which is expressed as the weight of the base.

Intracranial Cannulation and Infusion

Animals were anesthetized with Nembutal (sodium pentobarbital, 75 mg/kg, intraperitoneally), injected with atropine (1 mg/kg, subcutaneously), and secured in a Kopf stereotaxic instrument. Following exposure of the skull, 22-gauge guide cannulae (Plastics One Products, Roanoke, VA) were lowered to the appropriate stereotaxic coordinates measured in mm from Bregma. Jeweler screws were anchored to the skull, and the entire assembly was cemented into place using Loctite 444 Tak Pak Instant Adhesive (Henkel Corporation, Düsseldorf, Germany) and Perm Reline & Repair Resin (Hygenic Corporation, Akron, OH). 'Dummy' cannulae (model C232DC or C313DC; Plastics One Products) were inserted to maintain patency, with the tips flush with the end of the guide cannulae. When necessary, dust caps (model 303DC/1; Plastics One Products) were secured over the dummy cannulae to prevent their removal.

22-gauge guide cannulae (model C313G; Plastics One Products) were implanted bilaterally into the NAc shell (AP: 1.7 mm; ML: ± 1.5 mm; DV: −7.2 mm from Bregma), dlBNST (AP: −0.4 mm; ML: ± 3.7 mm; DV: −4.8 mm from Bregma, inserted at a 15° angle), CeA (AP: −2.2 mm; ML: ± 4.0 mm; DV: −6.4 mm from Bregma), or VTA (AP: −5.3 mm; ML: ± 1.0 mm; DV: −7.2 mm from Bregma). Infusions of 0.3 μl were made over the course of 2 min through 28-gauge infusion cannulae (model C313I; Plastics One Products), with tips that extended 1 mm past the end of the guide. Infusion cannulae were attached with polyethylene tubing to a 5 μl Hamilton microsyringe and were left in place for 1 min following infusions. Infusions were given in a room distinct from the colony and behavioral testing rooms.

Acoustic Startle and Activity

Acoustic startle and activity levels were tested in four identical plastic cages ($17 \times 8.5 \times 11\ cm^3$) resting on compression springs and located within individual ventilated

sound-attenuating chambers. Cage movement resulted in the displacement of a piezoelectronic accelerometer (Model ACH-01, Measurement Specialties, Valley Forge, PA) attached to each cage. Voltage output from the accelerometer was filtered and amplified by a custom-built signal processor, digitized on a scale of arbitrary units ranging from 0 to 1000 (National Instruments SCB100 and PCI-6071E boards), and recorded using Matlab (The MathWorks, Natick, MA). Startle amplitude was defined as the peak accelerometer voltage during the first 200 ms after onset of the startle stimulus. High-frequency speakers (Radio Shack Supertweeters, range = 5–40 kHz) located 10 cm beside each cage delivered the startle stimuli, which were 50 ms bursts of filtered white noise (low pass: 22 kHz, rise decay <5 ms) at intensities of 95 or 105 dB. Ventilating fans elevated background noise to approximately 60 dB.

Each startle test session consisted of a 5 min acclimation period, followed by presentation of 40 startle stimuli (20 each at 95 or 105 dB in semi-random order) with a 30 s fixed inter-stimulus interval. Activity levels were monitored during the acclimation period and throughout the session. For each experiment, acoustic startle was first tested on 2 consecutive drug-free days. After the second day, average startle amplitudes were used to match animals into groups with similar overall mean startle amplitude. Each test day began with a pre-drug exposure, baseline startle session (pretest), and concluded with a final post-drug exposure startle session (post-test).

Conditioned Place Aversion

The place conditioning apparatus consisted of a rectangular plastic cage ($40 \times 20 \times 20\,cm^3$) divided into two sides by a central partition. Each side had a distinct floor texture and wall color: metal bars paired with white walls and wire mesh paired with black striped walls. Each rat's position within the conditioning chamber was monitored by an overhead video camera connected to a computer running ANY-Maze software (Stoelting, Wood Dale, IL).

Rats were acclimated to the conditioning room for 10 min before each experimental session. The experiment began with a 10 min baseline session in which rats were free to move between both sides of the conditioning chamber. Rats with $>75\%$ baseline preference for one side were excluded from further study. The side of the chamber paired with drug treatment was counterbalanced within each experiment, yielding an unbiased procedure in which rats spent on an average 50% of the baseline session on the drug-paired side. Two, daily, 30-min conditioning sessions followed the baseline session. During these sessions, rats were injected with vehicle or drug and confined to one side of the conditioning chamber. A final 10-min test session in which rats were free to move between both sides of the chamber was conducted 24 h after the second conditioning session.

Histology

Animals were deeply anesthetized with Beuthanasia (sodium pentobarbital, 390 mg/kg, intraperitoneally) and perfused intracardially with 0.9% saline, followed by 10% formalin. Brains were subsequently removed and immersed in a 30% sucrose–formalin solution for at least 3 days. Coronal sections (30 μm) from the relevant brain regions were cut, mounted onto gelatin-coated slides, stained with cresyl violet, and scored for correct cannulae placement by an observer who was blind to group assignments.

Experimental Design

Experiment 1: SKF82958 or quinpirole injection during withdrawal from systemic morphine. Rats were injected with either 0 or 10 mg/kg of morphine at 0 h, followed by vehicle ($N=12$) or 10 ($N=8$) or 50 μg/kg ($N=8$) of the D1-like receptor agonist SKF82958 or 10 ($N=11$) or 50 μg/kg ($N=8$) of the D2-like receptor agonist quinpirole 3 h and 30 min later. Startle was tested at 4 h. A crossover design was used so that each rat was injected with the two doses of morphine in a random order over two consecutive test days.

Experiment 2: SKF82958 and quinpirole cocktail injection during withdrawal from systemic morphine. Rats were injected with either 0 ($N=12$) or 10 mg/kg ($N=11$) of morphine at 0 h and received an injection of a cocktail of SKF82958 and quinpirole (0, 10, or 50 μg/kg of each agonist) 3 h and 30 min later. Startle was tested at 4 h. A Latin square design was used so that each rat was injected with the three doses of the dopamine receptor agonist cocktail in a random order over three consecutive test days.

Experiment 3: Apomorphine infusion in local brain structures during withdrawal from systemic morphine. Following bilateral implantation of cannulae into the shell of the NAc, dlBNST, CeA, or VTA, rats were injected with either 0 or 10 mg/kg of morphine at 0 h and received an infusion of apomorphine (0, 1, or 5 μg per side; Willner *et al*, 1985; Hull *et al*, 1986) 3 h and 40 min later. Startle was tested at 4 h. A Latin square design was used so that each rat was infused with the three doses of apomorphine in a random order over three test days. Rats received a total of three test days, each separated by two intervening days to prevent tissue damage. In the NAc, 3 animals were removed for misplaced cannulae or large lesions at the infusion site and 1 animal was removed as an outlier, leaving final sample sizes of 7 (0 mg/kg) and 11 (10 mg/kg). In the dlBNST, 10 animals were removed for misplaced cannulae or large lesions at the infusion site and 1 animal was removed because of a blocked cannula, leaving final sample sizes of 8 (0 mg/kg) and 8 (10 mg/kg). In the CeA, 8 animals were removed for misplaced cannulae or large lesions at the infusion site and 1 animal was removed as an outlier, leaving final sample sizes of 7 (0 mg/kg) and 11 (10 mg/kg). In the VTA, 4 animals were removed for misplaced cannulae, leaving final sample sizes of 8 (0 mg/kg) and 9 (10 mg/kg).

Experiment 4: Apomorphine injection before induction of morphine conditioned place aversion. To determine whether activation of dopamine receptors can relieve another negative affective sign of opiate withdrawal, rats were administered apomorphine (0 or 100 μg/kg) during a 2-day, naloxone-precipitated place conditioning paradigm (Rothwell *et al*, 2009). One vehicle and one drug conditioning session were conducted over 2 days. There were a total of three groups in this

study. To test whether apomorphine alone could produce a place preference, the first group received 0 mg/kg morphine, 100 μg/kg apomorphine, and 0 mg/kg naloxone on the drug-paired side of the conditioning chamber ($N=8$). A second group went through precipitated morphine withdrawal on the drug-paired side (10 mg/kg morphine, 0 μg/kg apomorphine, and 1 mg/kg naloxone) ($N=5$) and a third received apomorphine 10 min before this conditioning procedure (10 mg/kg morphine, 100 μg/kg apomorphine, and 1 mg/kg naloxone) ($N=8$). The second and third groups also received naloxone on the vehicle-paired side of the conditioning chamber to control for any nonspecific aversive effects of naloxone. The timing of the injections is indicated in Figure 4a.

Experiment 5: Apomorphine injection during withdrawal from systemic nicotine. Because repeated nicotine exposure is necessary to observe withdrawal-potentiated startle (Engelmann *et al*, 2009), rats were injected with 0 ($N=10$) or 0.25 mg/kg ($N=10$) nicotine for 7 days. On days 8, 9, and 10, animals were injected with nicotine or saline at 0 h followed by 0, 50, or 100 μg/kg apomorphine hydrochloride 1 h and 50 min later. Startle was tested at 2 h. A Latin square design was used so that rats received each dose of apomorphine once over a series of three consecutive test days.

Data Analysis

Throughout the text and figures, all data are expressed as mean ± SEM. Startle data were collapsed across both intensities (95/105 dB) before further statistical analysis, as the magnitude of withdrawal-potentiated startle does not depend on startle stimulus intensity (Harris and Gewirtz, 2004). As there were no effects of order of treatment in any experiment, data were also collapsed across test days. In each experiment, one-way analysis of variance (ANOVA) was conducted to verify that animal weights and baseline startle amplitudes did not differ between experimental groups. Changes in startle or activity after experimental treatment were calculated as the percent change from baseline on the same day, that is, percent change = ((test−baseline)/baseline) × 100 (Harris and Gewirtz, 2004). Data were evaluated for outliers with the Grubb's extreme studentized deviate test with a significance level of $\alpha=0.01$.

Data from all experiments were analyzed with repeated measures ANOVA followed by *t*-tests corrected for multiple comparisons. Bonferroni adjusted α-levels for each experiment are reported with the results. All statistical analyses were conducted using SPSS (version 17.0) with a type I error rate of $\alpha=0.05$ (two-tailed).

RESULTS

Experiment 1: SKF82958 or quinpirole injection during withdrawal from systemic morphine

To determine whether the effect of apomorphine seen in our previous experiments (Radke *et al*., 2011) was mediated by D1- or D2-like dopamine receptors, rats were injected systemically with the D1-like receptor agonist SKF82958 or the D2-like receptor agonist quinpirole (0, 10, or 50 μg/kg) during spontaneous withdrawal from morphine, and startle

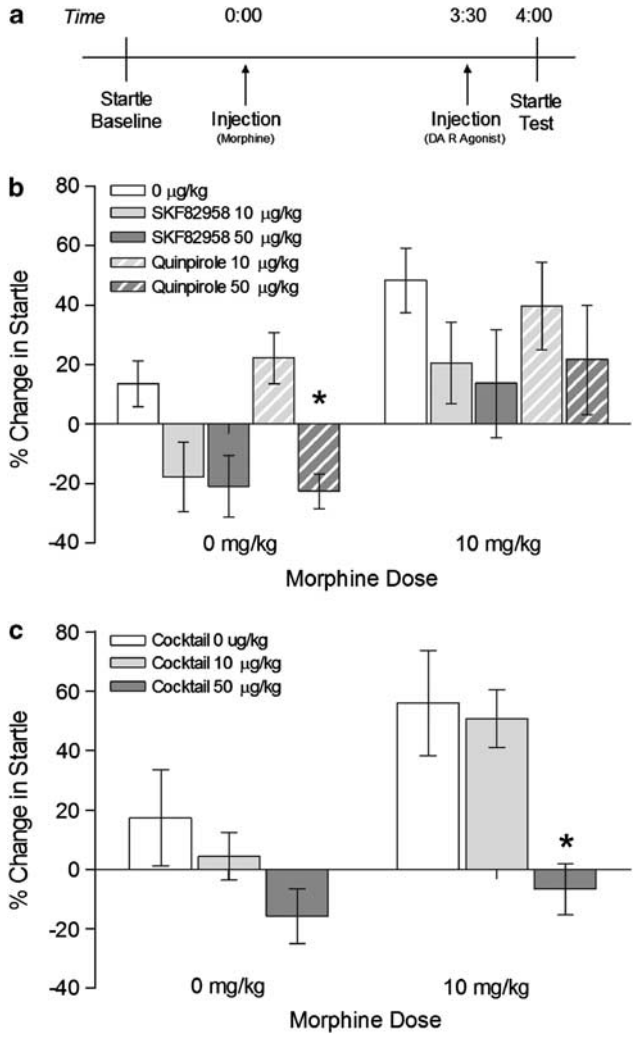

Figure 1 Activation of both D1- and D2-like receptors relieves withdrawal from systemic morphine. (a) Timeline of test day for Experiments 1 and 2. (b) Withdrawal-potentiated startle was not significantly reduced by SKF82958 or quinpirole treatment, although these treatments did have effects on baseline startle. (c) A cocktail of SKF82958 and quinpirole significantly reduced startle potentiation. *$p<0.05$ compared to 0 and 10 μg/kg groups.

was tested 10–30 min later (Figure 1a). Repeated measures ANOVA revealed a significant main effect of morphine ($F_{1,42}=22.904$, $p<0.001$) and a significant main effect of the dopamine receptor agonists ($F_{4,42}=3.562$, $p=0.014$). Neither SKF82958 nor quinpirole significantly decreased morphine withdrawal-potentiated startle at any dose tested (Figure 1b). Quinpirole did, however, significantly reduce startle amplitude on its own (0 μg/kg *vs* quinpirole 50 μg/kg: $t_{18}=3.360$, $p=0.003$; quinpirole 10 μg/kg *vs* quinpirole 50 μg/kg: $t_{17}=3.948$, $p=0.001$; Bonferroni-adjusted α-level = 0.0083).

Experiment 2: SKF82958 and quinpirole cocktail injection during withdrawal from systemic morphine

Because neither SKF82958 nor quinpirole significantly attenuated withdrawal-potentiated startle on its own, the hypothesis that activation of both D1- and D2-like receptors is necessary for dopamine's anxiolytic effects during

withdrawal was tested. The animals received a systemic injection of a cocktail of both SKF82958 and quinpirole (0, 10, or 50 μg/kg of each) 3 h and 30 min after morphine or vehicle injection. Startle was tested at 4 h (Figure 1a). Repeated measures ANOVA revealed a significant main effect of morphine ($F_{1,21} = 8.633$, $p = 0.008$) and a significant main effect of the agonist cocktail ($F_{1.726,36.246} = 9.426$, $p = 0.001$). Potentiated startle was significantly decreased in animals given 10 mg/kg of morphine, followed by 50 μg/kg of the agonist cocktail when compared with the 0 μg/kg ($t_{10} = 3.067$, $p = 0.012$) and 10 μg/kg groups ($t_{10} = 5.271$, $p < 0.001$; Bonferroni-adjusted α-level = 0.0167) (Figure 1c). Although the 50 μg/kg dose of the cocktail also slightly decreased startle amplitude in animals given 0 mg/kg morphine, this effect was not significant.

Experiment 3: Apomorphine infusion in local brain structures during withdrawal from systemic morphine

Experiments 1 and 2 demonstrated that activation of D1- and D2-like receptors attenuates opiate withdrawal-induced anxiety. To identify the location of the receptors involved in mediating this effect, rats were bilaterally implanted with chronically indwelling cannulae targeted at the NAc shell, dlBNST, CeA, or VTA (Figure 2). On the test day, animals were injected with 0 or 10 mg/kg morphine and 3 h and 40 min later infused with apomorphine (0, 1, or 5 μg per side) (Figure 3a). In the NAc shell, repeated measures ANOVA revealed a significant main effect of morphine ($F_{1,16} = 10.954$, $p = 0.004$) and a significant main effect of apomorphine ($F_{1.519,24.309} = 5.204$, $p = 0.020$). Potentiated startle was significantly decreased in animals that received 10 mg/kg of morphine, followed by 5 μg of apomorphine in the NAc shell when compared to the 0 μg ($t_{10} = 3.746$, $p = 0.004$) and 1 μg groups ($t_{10} = 5.752$, $p < 0.001$; Bonferroni-adjusted α-level = 0.0167) (Figure 3b). Repeated measures ANOVA also revealed a significant main effect of morphine in animals treated with apomorphine in the dlBNST ($F_{1,14} = 8.012$, $p = 0.013$), CeA ($F_{1,16} = 24.707$, $p < 0.001$), or VTA ($F_{1,15} = 8.822$, $p = 0.010$). No other significant effects were observed in these groups (Figure 3c and d).

Because dopamine is also involved in the production of motor behaviors, activity levels (ie, cage displacement during the 5 min acclimation period before startle stimulus presentation) were measured during each startle session. Changes in activity after agonist infusion were calculated as the percent change from baseline on the same day. No increases in activity were observed following infusion of apomorphine into the dlBNST, CeA, or VTA. In the NAc, there was a main effect of infusion that approached significance ($F_{1.449,23.190} = 2.923$; $p = 0.088$). Activity levels following infusion of 5 μg apomorphine into the NAc were increased equally in animals treated with saline and morphine (saline = 16.41%; morphine = 16.91%).

Experiment 4: Apomorphine injection before induction of morphine conditioned place aversion

To determine whether activation of dopamine receptors can relieve other signs of opiate withdrawal, rats were place-conditioned during naloxone-precipitated morphine withdrawal following 0 or 100 μg/kg apomorphine (Figure 4a). Repeated measures ANOVA revealed a significant session × group interaction ($F_{2,18} = 3.621$, $p = 0.048$). Follow-up analyses revealed that animals that went through precipitated morphine withdrawal with 0 μg/kg apomorphine developed a significant aversion to the drug-paired side of the chamber, whereas those receiving 100 μg/kg apomorphine

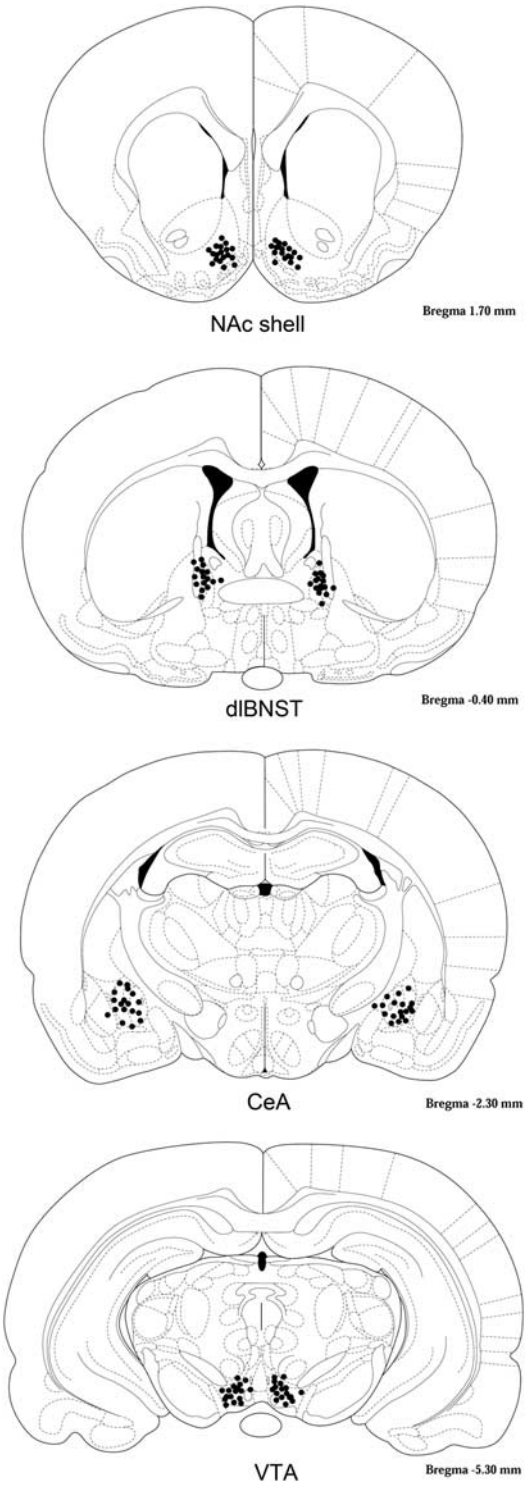

Figure 2 Cannula tip placements for Experiment 3. Tip locations for animals with correct placements are indicated with black circles.

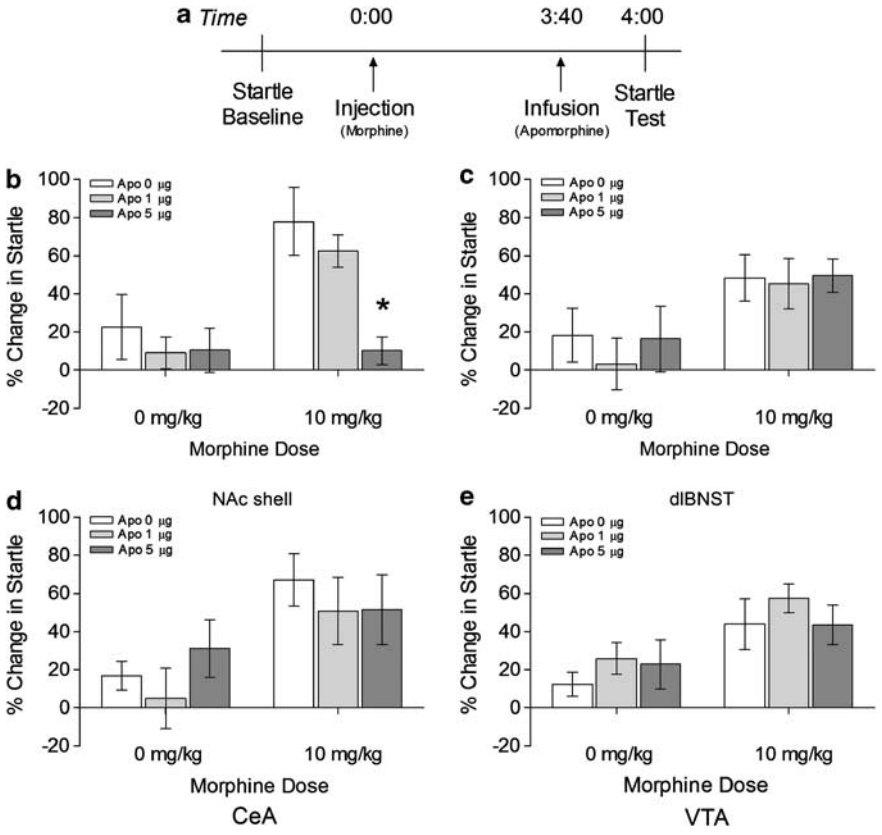

Figure 3 Withdrawal from systemic morphine is dependent on dopaminergic signaling in the shell of the nucleus accumbens. (a) Timeline of test day for Experiment 3. (b) Startle potentiation was attenuated by apomorphine infusion into the NAc shell (*$p<0.05$ compared to 0 and 1 µg groups). (c) Startle potentiation was not reduced by apomorphine infusion into the dorsolateral component of the bed nucleus of the stria terminal (dlBNST). (d) Startle potentiation was not reduced by apomorphine infusion into the central amygdala (CeA). (e) Startle potentiation was not reduced by apomorphine infusion into the ventral tegmental area (VTA).

did not (0 µg/kg: $t_4 = 3.192$, $p = 0.033$; 100 µg/kg: $t_7 = 0.117$, $p = 0.910$) (Figure 4b). The 100 µg/kg dose of apomorphine also did not produce a place preference when administered alone ($t_7 = 0.099$, $p = 0.924$).

Experiment 5: Apomorphine injection during withdrawal from systemic nicotine

To test whether dopaminergic mechanisms also contribute to withdrawal from another drug of abuse, nicotine withdrawal-potentiated startle was induced by systemically injecting rats with 0 or 0.25 mg/kg of nicotine for 7 days (Engelmann *et al*, 2009). On the test day, animals received nicotine, followed by a systemic injection of apomorphine hydrochloride (0, 50, or 100 µg/kg) 1 h and 50 min later and startle was tested at 2 h (Figure 5a). Repeated measures ANOVA revealed a significant main effect of apomorphine ($F_{2,36} = 9.494$, $p<0.001$). Potentiated startle was significantly decreased in animals given 0.25 mg/kg of nicotine, followed by injection of 50 µg/kg ($t_9 = 3.124$, $p = 0.012$) and 100 µg/kg apomorphine ($t_9 = 3.151$, $p = 0.012$; Bonferroni-adjusted α-level = 0.0167) (Figure 5b). There were no significant differences between the 50 and 100 µg/kg nicotine-apomorphine groups. Startle was also decreased in animals receiving 0 mg/kg nicotine and 100 µg/kg apomorphine when compared to the 0 µg/kg ($t_9 = 4.399$, $p = 0.002$) and 50 µg/kg groups ($t_9 = 3.253$, $p = 0.010$; Bonferroni-adjusted α-level = 0.0167). Administration of 50 µg/kg apomorphine following 0 mg/kg nicotine did not

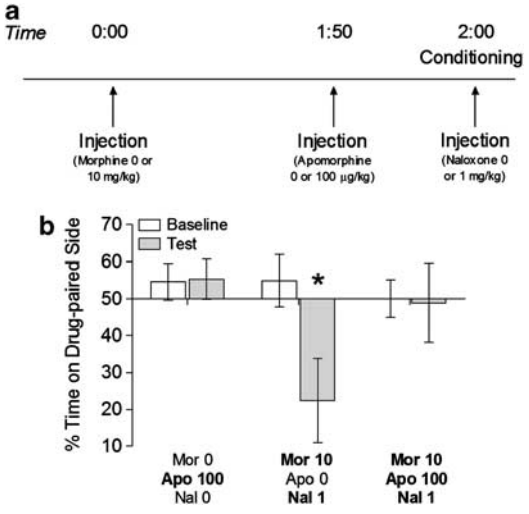

Figure 4 Dopamine receptor activation prevents conditioned place aversion to morphine withdrawal. (a) Timeline of conditioning days for Experiment 4. (b) Aversion to the drug-paired side of the conditioning chamber was relieved by apomorphine injection (*$p<0.05$ compared to baseline).

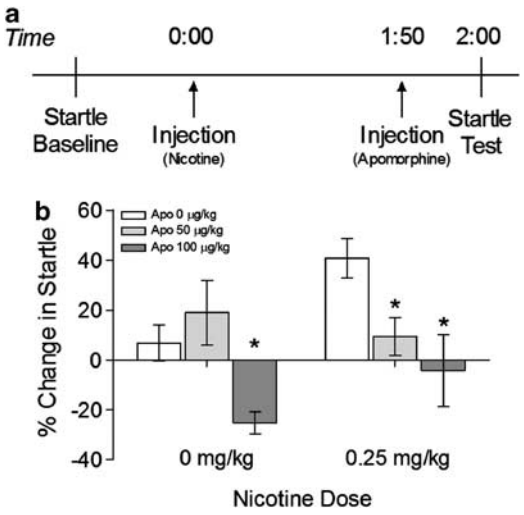

Figure 5 Withdrawal from systemic nicotine is dependent on dopaminergic signaling. (a) Timeline of test day for Experiment 5. (b) Startle potentiation was attenuated by apomorphine injection (*$p<0.05$ compared to 0 µg/kg group). See text for discussion of significant effects in 0 mg/kg group.

significantly change startle, demonstrating that the lower dose of apomorphine attenuated withdrawal-potentiated startle without affecting baseline startle.

DISCUSSION

The experiments described here investigated the role of dopamine in opiate, as well as nicotine, withdrawal. Our previous studies demonstrated that anxiety during withdrawal from acute opiate exposure is dependent on reduced opioid receptor activity in the VTA (Radke *et al*, 2011). Collectively, the results of the present experiments support the hypothesis that a drop in dopamine receptor activation is the next step in this process. The ability of the general dopamine receptor agonist apomorphine and a cocktail of SKF82958 and quinpirole to prevent completely the expression of opiate withdrawal-potentiated startle suggests that this behavior is dependent on reduced activity at *both* D1- and D2-like receptors. The effects of apomorphine on nicotine withdrawal-potentiated startle also raise the possibility that changes in dopaminergic activity may represent a shared mechanism involved in the withdrawal syndromes of other classes of abused drugs. Finally, the prevention of conditioned place aversion with the same dose of apomorphine used to reduce morphine withdrawal-potentiated startle (Radke *et al*, 2011) confirms that dopamine's involvement extends to multiple facets of the opiate withdrawal syndrome.

Because systemic administration of dopamine receptor agonists decreased baseline startle in Experiment 1, a unique contribution of either dopamine receptor subtype alone cannot be ruled out. The effects on baseline startle were surprising, as previous reports have shown dopamine receptor agonists to increase startle (Davis and Aghajanian, 1976; Meloni and Davis, 1999; Meloni and Davis, 2000). Decreases in baseline likely do not account for the results of Experiment 4, as the effect of apomorphine on nicotine withdrawal-potentiated startle was apparent following 50 µg/kg apomorphine, a dose that did not affect baseline startle. In addition, the tendency of the dopamine receptor agonists to decrease baseline startle was modest when compared to the decrease in potentiated startle observed in the morphine/nicotine animals, and this difference was not likely due to a floor effect, as other treatments (eg, presentation of a 'prepulse') can cause much greater inhibition of startle than was observed here (Gewirtz and Davis, 1995).

In addition to the systemic effects observed, local infusion of apomorphine into the shell of the NAc attenuated withdrawal-potentiated startle in Experiment 3. Apomorphine infusion into the dlBNST, the CeA, or the VTA, on the other hand, did not affect withdrawal-potentiated startle. This result implicates changes in dopaminergic signaling in the shell of the NAc in the expression of withdrawal following systemic morphine exposure. There was a trend for infusion of apomorphine into the NAc shell to increase locomotor activity as well. This increase in locomotor activity cannot explain the decreased startle potentiation seen in these animals, as treatments that increase locomotor activity in saline-injected animals to a similar or greater degree (eg, intra-NAc apomorphine in the current experiment or intra-VTA morphine infusion in previous studies; Radke *et al*, 2011) do not cause significant decreases in baseline startle. In addition, Cousens *et al*. (2011) have recently shown that increased locomotor activity immediately before presentation of a startle stimulus does not affect startle amplitude.

Our results suggest that expression of anxiety and aversion during opiate withdrawal likely coincides with a relative decrease in activation of the mesolimbic dopamine system. Decreased dopaminergic activity has been shown to contribute to the production of negative emotional states (Stinus *et al*, 1990; Liu *et al*, 2008; Nestler and Carlezon, 2006) and manipulation of dopaminergic signaling attenuates conditioned place aversion (Bechara *et al*, 1995; Laviolette *et al*, 2002; Chartoff *et al*, 2006; but see Caillé *et al*, 2003) and other signs of opiate withdrawal (Harris and Aston-Jones, 1994; Rodríguez-Arias *et al*, 1999). Importantly, the initial increase in NAc dopamine release following acute exposure to 10 mg/kg of morphine would have largely dissipated at the time at which spontaneous withdrawal-potentiated startle is observed (Di Chiara and Imperato, 1988; Rothwell *et al*, 2009). The current finding that apomorphine infusion into the NAc shell prevents the expression of withdrawal-induced anxiety also agrees with the idea that reduced activity within the VTA-to-NAc dopamine projection contributes to negative emotional signs of withdrawal (Diana *et al*, 1995, 1999).

The results of the SKF82958 and quinpirole studies support the conclusion that diminishing activity at both D1- and D2-like receptors, probably in the NAc shell, is involved in the expression of withdrawal. Both of these receptor subtypes are found in the NAc, although D1-like receptors are more abundant than D2-like receptors (Boyson *et al*, 1986). While investigations of the contributions of D1- *vs* D2-like receptors are limited, there is evidence that activation of either receptor subtype can attenuate the somatic signs of opiate withdrawal (Harris and Aston-Jones, 1994; Walters *et al*, 2000; Chartoff *et al*, 2006). D1-like receptor agonists also prevent opiate withdrawal-induced

aggression (Tidey and Miczek, 1992; Rodríguez-Arias *et al*, 1999) and conditioned place aversion (Chartoff *et al*, 2006), although this latter effect appears to be mediated by receptors in the VTA (Chartoff *et al*, 2009). Combined activation of both D1- and D2-like receptors has also recently been shown to prolong the pauses between bouts of cocaine intake in self-administering rats (Suto and Wise, 2011). This finding suggests that anxiety during periods of drug withdrawal and the motivation to consume additional drug may rely on similar mechanisms. One intriguing possibility is that the cooperative effects of D1- and D2-like receptor agonists on opiate withdrawal and cocaine self-administration are mediated by a heterodimeric D1–D2 dopamine receptor signaling complex. A D1–D2 heterodimer found in the NAc requires activation of both receptors to stimulate intracellular signaling (Rashid *et al*, 2007), which could explain why only the dopamine receptor agonist cocktail was effective both in the current study and in the experiments by Suto and Wise (2011).

The development of negative emotional signs of withdrawal involves 'between-systems' adaptations in neural structures mediating negative affective states, such as the amygdala and BNST (Koob and Bloom, 1988; Stinus *et al*, 1990; Harris *et al*, 2006; Smith and Aston-Jones, 2008). These between-systems adaptations may be initiated by morphine's effects on the mesolimbic dopamine system. Although infusion of a dopamine receptor agonist into the CeA and dlBNST did not attenuate opiate withdrawal-potentiated startle, these results only demonstrate that a reduction in dopaminergic activity is not necessary for anxiety. It is therefore possible that increased dopamine release in one or both of these structures following morphine exposure is responsible for their recruitment during opiate withdrawal (Stinus *et al*, 1990; Nakagawa *et al*, 2005; Harris *et al*, 2006). Dopaminergic signaling in the BLA, which is necessary for the acquisition of fear-potentiated startle (Nader and LeDoux, 1999; Greba and Kokkinidis, 2000; Greba *et al*, 2001; Fadok *et al*, 2009), may also trigger extended amygdala activity. Following opiate exposure, dopamine levels rise and fall in portions of the extended amygdala (Di Chiara and Imperato, 1988; Acquas and Di Chiara, 1992; Spanagel *et al*, 1992; Wise *et al*, 1995; Carboni *et al*, 2000), possibly triggering the release of corticotropin-releasing factor and norepinephrine (Guiard *et al*, 2008; Kash *et al*, 2008).

Alternatively, changes in dopaminergic signaling in the NAc may be responsible for the recruitment of the amygdala and the BNST during opiate withdrawal. This could occur via direct projections from the NAc shell to the BNST (Nauta *et al*, 1978; Usuda *et al*, 1998) or an indirect pathway from the NAc shell to the ventral pallidum to the amygdala (Nauta *et al*, 1978; Haber *et al*, 1985; Usuda *et al*, 1998). Consistent with this latter possibility, electrical stimulation of the ventral pallidum modulates the amplitude of the acoustic startle reflex (Li *et al*, 1999). In addition, both the NAc shell and ventral pallidum project to the pedunculopontine tegmental nucleus, a structure that projects to the startle circuit and has been shown to play a role in spontaneous morphine withdrawal (Swanson *et al*, 1984; Koch *et al*, 1993; Usuda *et al*, 1998; Vargas-Perez *et al*, 2009). A role for the ventral pallidum seems particularly likely given that it is a target of the NAc shell neurons that co-express both D1- and D2-like receptors (Haber *et al*, 1985; Lu *et al*, 1997). Clearly, further research is necessary to determine the involvement of dopaminergic coupling of the VTA and extended amygdala in the production of anxiety following acute opiate exposure.

Our final finding that apomorphine attenuated nicotine withdrawal-potentiated startle raises the possibility that the mesolimbic dopamine system may play an important role in the emotional component of withdrawal from a wider range of drugs of abuse, a hypothesis that is supported by a number of findings in the literature. Activation of the mesolimbic dopamine system is a feature shared by all classes of abused drugs (Di Chiara and Imperato, 1988) and, much like opiate withdrawal, the electrophysiological activity and neurochemical output of dopamine neurons is reduced during withdrawal from ethanol (Rossetti *et al*, 1991, 1992; Diana *et al*, 1993; Shen 2003; Rada *et al*, 2004), nicotine (Hildebrand *et al*, 1998; Rada *et al*, 2001; Liu and Jin, 2004), and other stimulants (Parsons *et al*, 1991; Robertson *et al*, 1991; Rossetti *et al*, 1992). Furthermore, reduced dopaminergic signaling in the NAc is associated with emotional signs of nicotine withdrawal (Cryan *et al*, 2003; Paterson *et al*, 2007). Further confirmation that the mesolimbic dopaminergic system contributes to withdrawal from other addictive drugs would cohere with a growing body of literature demonstrating that it is also involved in a wide variety of aversive behaviors, including conditioned place aversion (Acquas *et al*, 1989; Calcagnetti and Schechter, 1991; Schechter and Meechan, 1994; Liu *et al*, 2008), fear conditioning (Borowski and Kokkinidis, 1996; Nader and LeDoux, 1999; Pezze and Feldon, 2004; Fadok *et al*, 2009; Muschamp *et al*, 2011), anxiety (Fride and Weinstock, 1988; Barrot *et al*, 2002; Barrot *et al*, 2005; Meloni *et al*, 2006; Rezayof *et al*, 2009; Richard and Berridge, 2011; Zweifel *et al*, 2011), intrinsic aversion to gustatory cues (Roitman *et al*, 2008), conditioned taste aversion (Mark *et al*, 1991; Fenu *et al*, 2001), responses to stress (Geyer and Segal, 1974; Herman *et al*, 1982; Inglis and Moghaddam, 1999; Belda and Armario, 2009), and responses to nociceptive stimulation (Gear *et al*, 1999; Becerra *et al*, 2001; Barrot *et al*, 2002).

Drug exposure involves intrinsic withdrawal episodes that likely contribute to the development of dependence, and these 'daily' withdrawals occur spontaneously after every drug exposure (Dole *et al*, 1966; Koob and Le Moal, 1997; Kreek 2000; Baker *et al*, 2004). The current experiments simulated these conditions by administering discrete injections of morphine and allowing withdrawal to occur spontaneously. These studies therefore offer insight into the neural mechanisms of the type of withdrawal states that participate in the acquisition and maintenance of addictive behavior. The finding that anxiety during withdrawal is dependent on changes in dopamine signaling suggests that the positive reinforcing effects of drugs and the negative affective state that develops as the effects of the drug wear off are initiated by the same circuitry. These results therefore imply that positive and negative sources of reinforcement are mutually interdependent components of drug-taking behavior. Dopamine is hypothesized to serve to make neutral stimuli motivationally relevant (Berridge and Robinson, 1998; Wise, 2004) and these studies emphasize that this is true regardless of whether a stimulus carries a positive or negative valence.

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DISCLOSURE

The authors declare no conflict of interest.

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