Behavioral and Molecular Effects of Dopamine D₁ Receptor Stimulation during Naloxone-Precipitated Morphine Withdrawal

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Morphine dependence is characterized by somatic and motivational signs of withdrawal that likely contribute to the maintenance of addictive behavior. The nucleus accumbens (NAc) receives extensive dopaminergic input and is an important substrate for mediating these aversive states. In the NAc, the function of the transcription factor cAMP response element binding protein (CREB) and AMPA glutamate receptor subunit, type 1 (GluR1) can be regulated by dopamine (DA) D₁ receptor-mediated phosphorylation (P-CREB, P-GluR1). However, the roles of D₁ receptors, CREB, and GluR1 in morphine dependence are not well understood. Here, we show that somatic signs of naloxone-precipitated withdrawal were associated with increased P-CREB, but not P-GluR1, in the NAc of morphine-dependent rats. The D₁ receptor agonist chloro-APB hydrobromide (SKF 82958) was rewarding in morphine-dependent rats and blocked naloxone-induced place aversions and somatic signs of withdrawal. Surprisingly, SKF 82958 increased P-GluR1, but not P-CREB, in the NAc, and naloxone reduced SKF 82958-mediated P-GluR1 induction specifically in morphine-dependent rats. Together, these results confirm that aversive treatments can increase CREB function in the NAc. Furthermore, they suggest a dependence-associated shift in the molecular mechanisms that regulate the consequences of D₁ receptor stimulation, favoring activation of GluR1 rather than CREB. These data raise the possibility that the rewarding effects of SKF 82958 in morphine-dependent rats involve increased P-GluR1 in the NAc, although the involvement of other brain regions cannot be ruled out. Regardless, these findings suggest for the first time that D₁ agonists might be useful for the treatment of withdrawal symptoms that contribute to the maintenance of opiate addiction in humans.

Key words: nucleus accumbens; CREB; GluR1; AMPA; place conditioning; SKF 82958

Introduction

Opiate withdrawal causes somatic abstinence signs as well as a motivational syndrome characterized by dysphoria and depression (Koob and Le Moal, 1997; De Vries and Shippenberg, 2002) in humans. These negative affective states are thought to contribute to addictive behaviors (Koob and Le Moal, 1997; Markou et al., 1998). The nucleus accumbens (NAc), which receives extensive dopaminergic input, plays a key role in opiate reward (Wise, 1989) as well as the motivational and somatic signs of opiate withdrawal (Koob et al., 1992; Harris and Aston-Jones, 1994). The opioid receptor antagonist naloxone precipitates physical withdrawal signs in morphine-dependent rats and produces behaviors, such as conditioned place aversions, that reflect aversive states mediated within the NAc (Stinus et al., 1990).

Acute morphine decreases formation of cAMP (Childers, 1991), whereas chronic morphine causes super-sensitivity of cAMP pathways in areas including the NAc (Nestler and Aghajanian, 1997).

Precipitated opiate withdrawal unmasks upregulated cAMP pathways, which likely contribute to aversive states. Hypothetically, activation of dopamine (DA) D₁ receptors, which are positively coupled to adenylate cyclase (Deary et al., 1990), during morphine withdrawal could potentiate cAMP-mediated signaling (Chartoff et al., 2003a,b) and exacerbate aversive states. However, the role of DA and related signaling pathways in the NAc in morphine dependence is not well understood. Morphine withdrawal decreases DA in the NAc (Pothos et al., 1991; Diana et al., 1999), and administration of the nonspecific DA agonist apomorphine blocks signs of naloxone-precipitated withdrawal (Harris and Aston-Jones, 1994; Laviolette et al., 2002). Paradoxically, nonspecific dopamine antagonists also block naloxone-induced place aversions (Bechara et al., 1998). In contrast, specific D₂ receptor antagonists elicit withdrawal signs in morphine-dependent rats (Harris and Aston-Jones, 1994; Funada and Shippenberg, 1996), whereas a D₁ antagonist establishes place aversions in morphine-dependent and nondependent rats (Funada and Shippenberg, 1996).

Morphine-induced upregulation of cAMP-mediated signaling pathways likely causes intracellular adaptations including activation of cAMP-dependent protein kinase A (PKA) and phosphorylation of downstream targets including cAMP response element binding protein (CREB) and AMPA glutamate receptor subunit, type 1 (GluR1). Elevated CREB or GluR1 function in the

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NAc appears associated with negative motivational states. For example, viral vector-mediated elevations of CREB or GluR1 within the NAc produce depressive- and aversive-like states in rodents, and reduced drug reward (Carlezon et al., 1998; Kelz et al., 1999; Pliakas et al., 2001). Elevation of GluR1 expression in the NAc reduces drug-seeking behavior (Sutton et al., 2003), an effect also caused by acute drug withdrawal (Stewart and Wise, 1992; Carlezon and Wise, 2003). In contrast, targeted disruption of CREB α and Δ isoforms (Maldonado et al., 1996; Walters and Blendy, 2001) or knock-out of GluR1 (Vekovischeva et al., 2001) in mice reduces the signs of opiate withdrawal. However, there is also evidence that increased glutamate signaling at AMPA receptors in the NAc contributes to the expression of psychomotor sensitization and drug seeking (Pierce et al., 1996; Cornish and Kalivas, 2000), behaviors classically associated with increased motivational impact of drugs of abuse (Robinson and Berridge, 2001). Thus, the relationship between drug-related motivational states and CREB or GluR1 expression in the NAc are not well understood, particularly in animals with drug-induced alterations in intracellular signaling.

D₁ receptor-mediated phosphorylation of CREB and GluR1 enhances their function (Chao et al., 2002b; Song and Haganir, 2002; Carlezon et al., 2005). Considering that morphine withdrawal increases the function of downstream targets of cAMP signaling in the striatum and NAc (Nye and Nestler, 1996; Gracy et al., 2001; Shaw-Lutchman et al., 2002) and triggers aversive withdrawal signs, we examined whether precipitated morphine withdrawal would activate CREB and GluR1 within the NAc. We also examined whether D₁ agonist-induced stimulation of cAMP signaling during precipitated morphine withdrawal would further increase CREB and GluR1, thereby exacerbating withdrawal signs. Unexpectedly, we discovered dependence-mediated shifts in the consequences of cAMP-mediated signaling.

Materials and Methods

Rats. A total of 261 male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used in this study. Rats weighed 325–375 g at the time of the experiments and were maintained on a 12 h light/dark (7:00 A.M. to 7:00 P.M.) cycle with *ad libitum* access to food and water except during testing. Experiments were conducted in accordance with the 1996 National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and McLean Hospital policies.

Drugs. Naloxone HCl and chloro-APB hydrobromide (SKF 82958) were obtained from Sigma-Aldrich (St. Louis, MO). Lithium chloride (LiCl) was obtained from Fisher Scientific (Pittsburgh, PA). Naloxone was dissolved in 0.9% saline (NaCl) and LiCl, and SKF 82958 was dissolved in ultra-pure distilled water. Drugs were administered subcutaneously to rats in a volume of 1 ml/kg. Placebo and morphine pellets (75 mg morphine base/pellet) were obtained from the National Institute on Drug Abuse (Bethesda, MD). Rats were made dependent on morphine by subcutaneous implantation of two morphine pellets (or two placebo pellets for control) while under isoflurane anesthesia, as described by Gold et al. (1994). The wounds were closed with two stainless-steel skin clips, and a triple-antibiotic ointment was applied.

Place conditioning. For place conditioning studies, an unbiased, three-compartment place conditioning apparatus (Med Associates, St. Albans, VT) was used in which each compartment differed in floor texture, wall coloring, and lighting. On day 0, rats were allowed to freely explore all three compartments for 30 min to test for any inherent bias for one of the three compartments ("screening"). Rats that did not show strong a priori preferences (≥ 18 min) for a compartment during the screening period were then assigned to treatment groups. Rats were implanted subcutaneously with either morphine or placebo pellets on day 1. On day 4, when the rats were morphine dependent (Gold et al., 1994), they were conditioned in two separate sessions. In the morning (10:00 A.M.), rats were

injected subcutaneously with 0.9% saline (1 ml/kg) and confined to one side of the conditioning apparatus for 1 h. In the afternoon (2:00 P.M.), rats were injected subcutaneously with the test drug and confined to the opposite side of the conditioning apparatus for 1 h. The test drug-associated compartment was counterbalanced among rats to eliminate groupwise bias in initial preferences, as described previously (Carlezon, 2003). The following day (day 5), rats were allowed to freely explore all three compartments in a 30 min "test" session. Data are expressed as the following: [time spent in the drug-paired side minus time spent in the saline side after conditioning (testing)] minus [time spent in the drug-paired side minus time spent in the saline side before conditioning (screening)]. Data were analyzed using a three-way ANOVA [dependence $\times$ treatment $\times$ conditioning] with repeated measures on conditioning (before vs after conditioning). Significant effects were followed by simple main effects tests (one-way ANOVA) and *post hoc* Fisher's protected *t* tests.

Somatic withdrawal. Behavioral signs of morphine withdrawal were scored for a 15 min period in clear, 65 cm tall by 25 cm diameter Plexiglas cylinders that contained a small amount of bedding in a quiet, temperature-maintained (68°F) room by an observer blind to the treatments. Rats were implanted on day 1 with morphine or placebo pellets as described above. Two days after implantation (day 3), rats were habituated to the cylinders: they were placed in the cylinders for 15 min, injected subcutaneously with 0.9% saline and returned to the cylinders for another 15 min. On day 4, an experimental treatment was administered instead of saline: rats were placed in the cylinders for 15 min, injected subcutaneously with the test drug, and returned to the cylinders for another 15 min during which time somatic withdrawal behaviors were scored in three 5 min bins. This protocol is a modification of that described previously (Punch et al., 1997). The following behaviors were marked as either present or absent: diarrhea, ptosis, rhinorrhea (reddish-brown discharge from the nose), lacrimation (reddish-brown discharge from the eyes), penile erection, and irritability to touch. The frequency of the following behaviors was quantified: jumping, cage crossing, rearing, digging, flat posture, wet dog shakes, grooming, and teeth chattering. Somatic withdrawal behaviors are reported as a total behavioral score, which is the sum of the presence of, or the total occurrences of, each individual behavior over the 15 min scoring period. Data were analyzed using a two-way ANOVA (dependence $\times$ treatment) with repeated measures on treatment. Significant effects were followed by simple main effects tests (one-way ANOVA) and *post hoc* Fisher's protected *t* tests.

Western blotting. To obtain tissue for use in Western blotting experiments, rats were made dependent on morphine as described above. Three days after pellet implantation, at a time when behavioral analyses would have occurred, rats were injected subcutaneously with test drugs (see Results) and killed by live decapitation 30 min later. Rats treated with LiCl were not implanted with pellets. Brains were rapidly removed and frozen in isopentane kept on dry ice. Brains were then sliced on a cryostat (HM 505 E; Microm, Walldorf, Germany) and kept at -20°C until the rostral NAc and caudate putamen (CPu) were exposed (2.20 mm anterior to bregma). Bilateral tissue punches (1 mm²) were taken of the NAc and CPu and placed in Eppendorf (Hamburg, Germany) tubes kept on dry ice. Tissue was sonicated (Sonic Dismembrator 60; Fisher Scientific) in 100 μl of 1% SDS to break apart cell membranes. Protein content was determined using the Bio-Rad (Hercules, CA) DC Protein Assay kit, and the concentration of each sample was adjusted to 2.0 mg/ml protein. NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) and 50 mM dithiothreitol were added to each sample before heating at 70°C for 10 min. Twenty micrograms of each sample were then loaded onto NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) for separation by gel electrophoresis. Proteins were subsequently transferred to polyvinylidene fluoride membrane (PerkinElmer Life Sciences, Boston, MA). Non-specific binding sites on the membranes were blocked for 2 h at room temperature in blocking buffer [5% nonfat dry milk in PBS and 0.1% Tween 20 (PBS-T)]. Blots were then incubated in primary antibody [1:4000 monoclonal anti-Ser¹³³ phospho-CREB (P-CREB), 1:4000 anti-CREB (Cell Signaling Technology, Beverly, MA), 1:3000 anti-Ser⁸⁴⁵ phospho-GluR1 (P-GluR1), or 1:2000 anti-GluR1 (Chemicon, Temecula, CA)] in PBS-T overnight at 4°C . Blots were washed four times

Figure 1. Effect of naloxone dose on morphine withdrawal behaviors. **A**, Treatment of morphine-dependent and placebo-implanted control rats with naloxone dose-dependently elicited conditioned place aversions (means $\pm$ SEM). Place conditioning was performed 3 d after pellet implantation. Significant changes in time spent in drug-paired chamber were as follows: * $p < 0.05$; ** $p < 0.01$, Fisher's protected t tests, 7–13 rats per group. **B**, Treatment of morphine-dependent rats with naloxone dose-dependently elicited somatic withdrawal behaviors (means $\pm$ SEM). Behavioral scoring began 3 d after pellet implantation. * $p < 0.05$ compared with placebo-implanted control treated with same dose of naloxone; [†] $p < 0.05$, ^{††} $p < 0.01$ compared with morphine-dependent rats treated with vehicle (0.00), Fisher's protected t tests.

for 15 min each in PBS-T and incubated in secondary antibody [1:5000 goat anti-rabbit (or anti-mouse) horseradish peroxidase-linked IgG (Vector Laboratories, Burlingame, CA)] for 2 h at room temperature. Blots were washed 4 $\times$ 5 min in PBS-T, followed by immunological detection using Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Antibodies were stripped from the blots by incubation with stripping buffer (62.5 mM Tris, 2% SDS, and 100 mM β -mercaptoethanol), pH 6.8, for 15 min at 50°C. Blots were subsequently reblocked and probed with 1:20,000 anti-actin (Sigma-Aldrich). SeeBlue Plus 2 (Invitrogen) prestained standards were run for molecular weight estimation. P-CREB and CREB bands were detected at 43 kDa, P-GluR1 and GluR1 bands were detected at 100 kDa, and actin was detected at 42 kDa.

Results

Motivational and somatic morphine withdrawal signs can be dissociated by naloxone dose

Naloxone-induced place aversions in the place conditioning paradigm depended on an interaction between conditioning (preconditioning vs postconditioning) and dependence (placebo vs morphine) ($F_{(1,54)} = 17.136$; $p < 0.001$), with repeated measures on conditioning (Fig. 1A). Simple main effects tests reveal that morphine-dependent rats displayed significant place aversions in response to all doses of naloxone tested (0.01 mg/kg: $F_{(1,9)} = 9.272$, $p < 0.05$; 0.1 mg/kg: $F_{(1,10)} = 74.841$, $p < 0.001$; 1.0 mg/kg: $F_{(1,7)} = 24.198$, $p < 0.01$), whereas placebo-implanted control rats only showed place aversions in response to the highest dose of naloxone ($F_{(1,6)} = 20.239$; $p < 0.01$). The effect of naloxone on somatic withdrawal behaviors depended on an interaction between dose and dependence ($F_{(3,42)} = 3.447$; $p < 0.05$), with repeated measures on dose (Fig. 1B). All doses of naloxone caused significant increases in somatic withdrawal behaviors in morphine-dependent rats compared with morphine-dependent rats treated with vehicle ($F_{(3,21)} = 8.837$; $p < 0.001$), whereas naloxone did not significantly increase somatic withdrawal behaviors in placebo-implanted control rats ($F_{(3,21)} = 2.395$, NS). However, the lowest dose of naloxone tested (0.01 mg/kg) failed to induce somatic withdrawal signs in morphine-dependent rats that were significantly greater than those observed in placebo-implanted rats also treated with naloxone (0.01 mg/kg).

Figure 2. Effects of naloxone dose on CREB and GluR1 phosphorylation. Morphine-dependent and control rats were treated with naloxone (0.0–1.0 mg/kg, s.c.) 3 d after pellet implants and killed 30 min later. Tissue from the NAc or CPu was subjected to Western blot analysis for P-CREB (**A**, **B**) or P-GluR1 (**C**, **D**) followed by actin as a control. Data are presented as the fold induction of P-CREB/actin or P-GluR1/actin in morphine-dependent rats compared with placebo-implanted control rats for each dose of naloxone (means $\pm$ SEM). * $p < 0.05$; ** $p < 0.01$ compared with naloxone (0.0), Fisher's protected t tests, five to nine rats per group. Insets, Nonimplanted rats were treated with vehicle (H₂O) or LiCl (120 mg/kg, s.c.) and killed 30 min later. Tissue from the NAc or CPu was assessed by Western blot for P-CREB or P-GluR1 followed by actin as a control. Data are expressed as the fold induction of P-CREB/actin or P-GluR1/actin from LiCl-treated rats compared with vehicle-treated rats (means $\pm$ SEM). ** $p < 0.01$ compared with vehicle, Student's t tests, eight rats per group. P, Placebo; M, morphine; V, Veh, vehicle; L, LiCl.

Aversive states are associated with P-CREB induction in the NAc

To identify molecular mechanisms that might underlie morphine withdrawal-induced aversive states, we performed a dose-effect function of naloxone on P-CREB and P-GluR1 induction in the NAc and CPu. The effects of naloxone on P-CREB depended on dose (NAc: $F_{(3,16)} = 8.621$, $p < 0.01$; CPu: $F_{(3,16)} = 5.219$, $p < 0.05$). In both brain regions, levels of P-CREB in morphine-dependent rats treated with naloxone (1.0 mg/kg) compared with placebo control rats treated with naloxone (1.0 mg/kg) were significantly greater than levels of P-CREB in morphine-dependent rats treated with vehicle (naloxone, 0.0 mg/kg) compared with placebo control rats treated with vehicle (Fig. 2A,B). Because naloxone (1.0 mg/kg) induces place aversions in both morphine-

dependent and nondependent rats (Fig. 1A), it is possible that this dose of naloxone could affect P-CREB expression in placebo control rats as well as morphine-dependent rats. Absolute fold inductions ($\pm$ SEM) of P-CREB in the NAc and CPu of rats treated with naloxone (1.0 mg/kg) compared with placebo controls treated with vehicle are as follows: NAc (placebo, 0.891 ± 0.118 ; morphine, 1.420 ± 0.173); CPu (placebo, 0.686 ± 0.109 ; morphine, 1.317 ± 0.324). Levels of CREB protein were unchanged in the NAc and CPu of morphine-dependent rats compared with placebo controls (fold induction in NAc: placebo, 1.001 ± 0.012 SEM; morphine, 1.07 ± 0.061 SEM, $p = 0.29$; fold induction in CPu: placebo, 0.999 ± 0.022 SEM; morphine, 0.965 ± 0.101 SEM, $p = 0.74$, Student's t tests, seven rats per group). Naloxone failed to change levels of P-GluR1 in either the NAc ($F_{(3,26)} = 1.241$, NS) or the CPu ($F_{(3,26)} = 0.631$, NS) (Fig. 2C,D). Absolute fold inductions ($\pm$ SEM) of P-GluR1 in the NAc and CPu of rats treated with naloxone (1.0 mg/kg) compared with placebo controls treated with vehicle are as follows: NAc (placebo, 1.115 ± 0.113 ; morphine, 1.405 ± 0.202) and CPu (placebo, 1.003 ± 0.125 ; morphine, 1.317 ± 0.324). Levels of GluR1 protein were also unchanged in morphine-dependent compared with placebo controls (fold induction in NAc: placebo, 1.00 ± 0.006 SEM; morphine, 0.983 ± 0.074 SEM, $p = 0.82$; fold induction in CPu: placebo, 1.00 ± 0.01 SEM; morphine, 1.013 ± 0.103 SEM, $p = 0.91$, Student's t tests, eight rats per group).

To test whether the induction of P-CREB in the NAc and CPu of morphine-dependent rats by naloxone (1.0 mg/kg) is attributable to an induction of aversive-like states, as opposed to alterations in locomotor activity or other nonspecific behaviors, separate rats were treated with a dose of LiCl (120 mg/kg) known to cause dysphoria (Shippenberg et al., 1988; Tomasiewicz et al., 2006). LiCl significantly increased P-CREB in the NAc ($p < 0.001$, Student's t test) (Fig. 2A, inset) but not the CPu (Fig. 2B, inset). Likewise, LiCl did not affect P-GluR1 levels in either the NAc or CPu (Fig. 2C,D, insets).

Systemic administration of a dopamine D₁ receptor agonist is rewarding in morphine-dependent rats

To determine whether morphine dependence alters the motivational impact of dopamine D₁ receptor stimulation, rats with subcutaneous implants of either morphine or placebo pellets were treated with the D₁ receptor agonist SKF 82958 and tested in the place conditioning paradigm. The doses of SKF 82958 that we chose were based on previous behavioral studies demonstrating that D₁ agonists prevent cocaine-induced reinstatement of cocaine-seeking behavior (Self et al., 1996b). The effects of SKF 82958 depended on conditioning ($F_{(1,52)} = 11.778$; $p < 0.01$) (Fig. 3A). Simple main effects tests reveal that SKF 82958 (1 mg/kg) was only rewarding in morphine-dependent rats ($F_{(1,12)} = 12.113$; $p < 0.01$).

Dopamine D₁ receptor agonists block affective and somatic signs of morphine withdrawal

To test the hypothesis that activation of D₁ receptors during morphine withdrawal would exacerbate signs of morphine withdrawal, morphine-dependent rats were treated with naloxone plus SKF 82958. A low dose of naloxone (0.01 mg/kg) was used to selectively precipitate affective signs of morphine withdrawal, which were measured using place conditioning. Naloxone failed to induce place aversions in morphine-dependent rats in the presence of any dose of SKF 82958 (0.1, 0.3, 1.0 mg/kg) (Fig. 3B). Furthermore, SKF 82958 dose-dependently elicited place preferences in the presence of naloxone (0.01 mg/kg) in morphine-

Figure 3. Effects of the dopamine D₁ receptor agonist SKF 82958 on place conditioning in morphine-dependent rats. Rats were implanted with placebo or morphine pellets and 3 d later were conditioned with SKF 82958 in the place conditioning paradigm. **A**, SKF 82958 (0.1, 0.3, 1 mg/kg, s.c.) caused place preferences in morphine-dependent rats at the highest dose tested. **B**, SKF 82958 administered in a mixture with naloxone (nal; 0.01 mg/kg, s.c.) was motivationally neutral in nondependent rats and blocked naloxone-induced place aversions in morphine-dependent rats at all doses tested. SKF 82958 (1.0 mg/kg) plus naloxone (0.01 mg/kg) caused place preferences in morphine-dependent rats. Data are expressed as the change in time spent in the drug-paired side after conditioning (means $\pm$ SEM). Significant changes in time spent in the drug-paired chamber are as follows: $^{\dagger}p < 0.05$, naloxone alone (data from Fig. 1A); $*p < 0.05$; $**p < 0.01$, naloxone plus D₁ agonist, Fisher's protected t tests, 7–13 rats per group.

dependent rats ($F_{(2,43)} = 3.662$; $p < 0.05$). Simple main effects tests reveal that SKF 82958 (1.0 mg/kg) was rewarding in morphine-dependent rats treated with naloxone ($F_{(1,7)} = 13.329$; $p < 0.05$). For reference, the effects of naloxone (0.01 mg/kg) alone on place conditioning are shown (see “0” dose of D₁ agonist in Fig. 3B).

To determine whether activation of D₁ receptors could also affect somatic signs of morphine withdrawal, rats were coadministered a moderate dose of naloxone (0.1 mg/kg) shown to induce both place aversions and somatic withdrawal signs (Fig. 1A,B) and SKF 82958 (1.0 mg/kg). The effects of SKF 82958 on the sum of all naloxone-induced somatic signs depended on an interaction between treatment and dependence ($F_{(2,28)} = 17.315$; $p < 0.0001$) (Fig. 4A). Simple main effects tests reveal that treatment did not effect total somatic withdrawal behaviors in placebo-implanted control rats ($F_{(2,14)} = 1.273$, NS) but did have a significant effect on behavior in morphine-dependent rats ($F_{(2,14)} = 79.754$; $p < 0.0001$). SKF 82958 significantly reduced the expression of somatic withdrawal behaviors compared with naloxone alone, but rats treated with naloxone and SKF 82958 still displayed more withdrawal behaviors than rats treated with SKF 82958 alone. To further examine which specific somatic withdrawal behaviors were affected by activation of D₁ receptors, we analyzed wet dog shakes, teeth chattering, and cage crossings (Fig. 4B–D). Wet dog shakes and teeth chattering are quite specific for opiate withdrawal, whereas cage crossing is a more general locomotor effect. We found that the effects of SKF 82958 on wet dog shakes, teeth chattering, and cage crossings depended on interactions between treatment and dependence ($F_{(2,28)} = 9.865$, $p < 0.001$; $F_{(2,28)} = 13.523$, $p < 0.0001$; $F_{(2,28)} = 3.728$, $p < 0.05$, respectively). Simple main effects tests reveal that treatment did not effect any behavior in placebo-implanted rats, whereas SKF 82958 significantly reduced the expression of naloxone-induced wet dog shakes and teeth chattering. Naloxone-induced cage crossings in morphine-dependent rats were not affected by SKF 82958.

D₁ receptor-mediated induction of P-GluR1 in the NAc of morphine-dependent rats is attenuated by naloxone

To test whether the behavioral effects of SKF 82958 in morphine-dependent rats are correlated with changes in the function of

Figure 4. Effects of SKF 82958 on somatic withdrawal signs. Rats were implanted with placebo or morphine pellets and 3 d later were treated subcutaneously with a mixture of naloxone (nal; 0.1 mg/kg) and SKF 82958 (SKF; 1.0 mg/kg), and somatic withdrawal behaviors were scored for 15 min. SKF 82958 reduced the total incidence of somatic withdrawal behaviors (**A**) as well as wet dog shakes (**B**) and teeth chattering (**C**) but not cage crossings (**D**) (means $\pm$ SEM). $^{**}p < 0.05$ compared with effects of naloxone alone in morphine-dependent rats; $^{††}p < 0.01$ comparing bracketed groups, Fisher's protected *t* tests, eight rats per group.

CREB and GluR1 in the NAc and CPu, levels of P-CREB and P-GluR1 were measured in response to systemic administration of the D₁ agonist. Interestingly, SKF 82958 failed to significantly increase P-CREB in either the NAc ($F_{(5,48)} = 0.328$, NS) or CPu ($F_{(5,47)} = 1.207$, NS) under any condition tested (Fig. 5*A,B*). In contrast, SKF 82958 significantly increased P-GluR1 levels in both the NAc ($F_{(5,51)} = 14.786$; $p < 0.0001$) and CPu ($F_{(5,51)} = 3.883$; $p < 0.01$) (Fig. 5*C,D*). *Post hoc* analysis showed that, in the NAc, SKF 82958 significantly induced P-GluR1 in all treatment groups compared with P-GluR1 levels in placebo-implanted rats treated with vehicle. Interestingly, naloxone decreased SKF 82958-mediated P-GluR1 expression in morphine-dependent rats. Hence, P-GluR1 levels in morphine-dependent rats treated with naloxone and SKF 82958 were not significantly different from P-GluR1 levels in morphine-dependent rats treated with naloxone alone. In the CPu, SKF 82958 significantly increased P-GluR1 levels only in nondependent (placebo) rats.

Discussion

"Unmasking" of opiate-induced increases in cAMP-mediated signaling likely contributes to behavioral signs of withdrawal (Nestler and Aghajanian, 1997). The current studies were designed to examine whether two targets of cAMP signaling, CREB and GluR1, would be activated in response to withdrawal-associated aversive states and whether activation of D₁ receptors would potentiate cAMP signaling in the NAc and exacerbate withdrawal-associated aversive states. Previous work has demonstrated that activation of CREB and GluR1 within the NAc contributes to aversive effects (Carlezon et al., 1998; Kelz et al., 1999; Pliakas et al., 2001). We found that P-CREB, but not P-GluR1, was increased in morphine-dependent rats treated with a high dose of naloxone that elicits both motivational and somatic ab-

Figure 5. Effects of SKF 82958 on CREB and GluR1 phosphorylation in morphine-dependent rats. Morphine-dependent and control rats were treated subcutaneously with a mixture of naloxone (0.01 mg/kg) and SKF 82958 (1.0 mg/kg) 3 d after pellet implants and killed 30 min later. Tissue from the NAc or CPu was subject to Western blot analysis for P-CREB (**A, B**) or P-GluR1 (**C, D**) followed by actin as a control. Data are presented as the fold induction of P-CREB/actin or P-GluR1/actin for each treatment compared with control (vehicle-treated nondependent rats) (means $\pm$ SEM). $^{*}p < 0.05$; $^{**}p < 0.01$ compared with vehicle control; $^{††}p < 0.01$ compared with morphine plus naloxone; $^{\S}p < 0.05$ comparing bracketed groups, Fisher's protected *t* tests, 4–14 rats per group (each experiment always contained multiple nondependent vehicle controls). P, Placebo; M, Morph, morphine; V, vehicle; S, SKF, SKF 82958; N, Nal, naloxone.

stinence signs of withdrawal. Unexpectedly, systemic administration of the DA D₁ agonist SKF 82958 reduced motivational and somatic signs of morphine withdrawal and was rewarding on its own in morphine-dependent rats, although it had no behavioral effects in nondependent rats. Furthermore, SKF 82958 increased P-GluR1, but not P-CREB, in the NAc. However, SKF 82958-stimulated P-GluR1 expression was decreased in morphine-dependent rats cotreated with naloxone. These findings suggest that the behavioral and molecular consequences of D₁ receptor activation are altered by morphine dependence.

Dissociation of motivational and somatic withdrawal signs

We dissociated motivational and somatic signs of morphine dependence by precipitating withdrawal with several naloxone doses, similar to previous work (Schulteis et al., 1994). A low dose of naloxone resulted in significant place aversions with few overt signs of somatic withdrawal, whereas higher doses of naloxone elicited both place aversions and robust signs of somatic withdrawal. The ability to measure motivational signs of withdrawal independently of somatic signs allowed us to examine molecular correlates of each in the NAc and CPu, a brain region implicated in motor processing (Mink, 1996). We found that neither CREB nor GluR1 protein levels were altered in morphine-dependent rats. This is in contrast with a previous report that chronic mor-

phine decreases CREB immunoreactivity in the NAc, with no change in the CPu (Widnell et al., 1996). However, the total amount of morphine administered and the times at which CREB levels were examined differ between these studies, making comparisons difficult.

We found that a high dose of naloxone increased P-CREB levels in the NAc and CPu of morphine-dependent rats. A high dose of LiCl caused an induction of P-CREB in the NAc but not the CPu. Similar doses of LiCl produce aversive-like effects in behavioral assays including place conditioning (Shippenberg et al., 1988), conditioned taste aversion (Swank and Bernstein, 1994), and intracranial self-stimulation (Tomasiewicz et al., 2006). These findings suggest that increased CREB function in the NAc contributes to the aversive aspects of morphine withdrawal, whereas increased CREB function in the CPu might contribute to increased locomotor activity associated with withdrawal (Punch et al., 1997). This confirms and extends previous work indicating that CREB in the NAc is associated with depressive- and aversive-like states (Carlezon et al., 2005).

Despite finding that a low dose of naloxone selectively elicited motivational signs of morphine withdrawal, we did not detect a parallel induction of P-CREB or P-GluR1. This suggests that low doses of naloxone might be insufficient to activate signaling pathways required for phosphorylation of CREB and GluR1. Both PKA and glutamate-mediated increases in Ca²⁺ are necessary for CREB activation, but only PKA can induce GluR1 phosphorylation at Ser⁸⁴⁵ (Sheng et al., 1991; Chao et al., 2002a). Thus, naloxone-induced P-CREB in morphine-dependent rats might reflect synergism between signaling from withdrawal-induced glutamate release (Sepulveda et al., 1998) and increased cAMP. Other studies have shown that low doses of naloxone induce *c-fos* and Fos-related antigens (FRAs) in the NAc of morphine-dependent rats (Walters et al., 2000; Gracy et al., 2001). Fos can be activated by Ca²⁺-mediated signaling and is not necessarily reliant on P-CREB (Lerea and McNamara, 1993), suggesting different sensitivities and functions for these various proteins.

Dependence-associated effects of D₁ receptor activation

The D₁ receptor agonist SKF 82958 elicited place preferences in morphine-dependent, but not nondependent, rats. Other studies have shown that the effects of D₁ receptor agonists on place conditioning in drug-naïve rats are variable: a low dose of SKF 82958 can produce place preferences (Abrahams et al., 1998), whereas other D₁ agonists fail to establish place conditioning or cause place aversions (White et al., 1991). Our findings are consistent with increasing evidence that chronic drug treatment regulates the physiological and behavioral impact of D₁ receptor stimulation (Bonci and Williams, 1996; Laviolette et al., 2002). For example, DA does not appear to be necessary for the acute rewarding effects of morphine, but it is required for reward during withdrawal (Bechara et al., 1998; Hnasko et al., 2005) (but see Shippenberg and Herz, 1988).

SKF 82958 blocked place aversions established by a low dose of naloxone in morphine-dependent rats. Because there is some evidence that high doses of SKF 82958 can have effects at D₂ receptors (Ruskin et al., 1998), we tested the more selective D₁ agonist SKF 81297 [(±)-6-chloro-PB hydrobromide] in pilot studies and found a similar blockade of naloxone-induced place aversions in morphine-dependent rats (data not shown). SKF 82958 produces D₁-selective effects at doses similar to those used in this study (Self et al., 1996a), suggesting that our results are attributable primarily to D₁ receptor stimulation.

Rats treated with the highest dose of SKF 82958 plus naloxone

exhibited place preferences equivalent to those observed with SKF 82958 alone in morphine-dependent rats. One possible explanation for this effect is that the rewarding properties of D₁ agonists overshadow the aversive effects of withdrawal induced by a low dose of naloxone. This seems unlikely because doses of SKF 82958 that were not rewarding on their own in morphine-dependent rats also block naloxone-induced place aversions. Also, it has been shown previously that apomorphine blocks withdrawal-induced place aversions but fails to block LiCl-induced place aversions in morphine-dependent rats (Laviolette et al., 2002), suggesting that the rewarding effects of DA receptor activation do not override all aversive stimuli.

The effects of D₁ receptor stimulation during morphine withdrawal were not limited to attenuation of motivational signs: SKF 82958 also reduced somatic withdrawal signs. This is intriguing because previous data indicate that systemic apomorphine reduced somatic withdrawal signs, whereas intra-NAc administration of a D₁ agonist potentiated one somatic withdrawal sign (wet dog shakes) (Harris and Aston-Jones, 1994). We found that systemic administration of SKF 82958 blocked both wet dog shakes and teeth chattering, raising the possibility that effects of SKF 82958 on morphine withdrawal do not occur exclusively within the NAc.

Mechanisms of D₁ agonist effects in morphine-dependent rats

We examined whether D₁ receptor activation would exacerbate aversive states during morphine withdrawal and whether this effect would be associated with increased cAMP signaling in the NAc. Unexpectedly, SKF 82958 failed to reliably induce P-CREB under any conditions. This is in contrast to our previous findings in primary cultures of striatal neurons, in which we observed robust SKF 82958-mediated P-CREB induction during naloxone-precipitated withdrawal from chronic morphine treatment (Chartoff et al., 2003b). Dissociated striatal cultures lack endogenous dopaminergic input; the fact that DA denervation leads to supersensitivity of D₁ receptor signaling (Chartoff et al., 2001) could explain the ability of SKF 82958 to induce P-CREB in striatal cultures. It is possible that SKF 82958 might activate P-CREB *in vivo* at higher doses or by other routes of administration (Haberny et al., 2004). Alternatively, the stress of handling and injections might have contributed to P-CREB induction in control rats (Inglis and Moghaddam, 1999), such that it was not possible to observe additional D₁ receptor-mediated increases in P-CREB.

In contrast, SKF 82958 significantly induced P-GluR1 in the NAc, but not the CPu, of morphine-dependent rats. GluR1 phosphorylation can increase synaptic expression of GluR1 receptors and enhance AMPA transmission, including influx of Ca²⁺ (Song and Haganir, 2002; Mangiavacchi and Wolf, 2004). Interestingly, increased glutamatergic transmission and subsequent Ca²⁺ influx in the NAc is generally associated with aversive states (Kelz et al., 1999; Chartoff et al., 2006). Our findings suggest that, in drug-dependent animals, the downstream consequences of increased P-GluR1 activity or AMPA receptor function are fundamentally altered. For example, the effect of increased AMPA transmission might be different in rats with dependence-associated alterations in intracellular signaling cascades that regulate the function of ion channels, which could cause dramatic alterations in the output of NAc neurons. These types of changes have been observed during cocaine withdrawal (Zhang et al., 1998) and may contribute to altered behavioral sensitivity to AMPA receptor stimulation in cocaine-experienced rats (Pierce

et al., 1996; Cornish and Kalivas, 2000). Indeed, some aspects of stimulant-induced sensitization are correlated with increased synaptic expression of GluR1 receptors (Boudreau and Wolf, 2005).

Although the behavioral effects of SKF 82958 were the same in morphine-dependent rats and dependent rats treated with naloxone, the molecular effects differed. SKF 82958 increased P-GluR1 selectively in the NAc of morphine-dependent rats, but the D₁ receptor-mediated increase in P-GluR1 was significantly reduced in dependent rats treated with naloxone. P-GluR1 is rapidly dephosphorylated during AMPA receptor activation in the NAc (Snyder et al., 2003); because glutamate release is increased in the NAc during morphine withdrawal (Sepulveda et al., 1998), a concomitant increase in AMPA receptor activation and GluR1 dephosphorylation would be expected. It is thus possible that the rewarding effects of SKF 82958 observed in morphine-dependent rats and those treated with naloxone are mediated in different brain regions. For example, during morphine withdrawal, D₁ receptor stimulation in the ventral tegmental area leads to increased DA cell activity, which might alleviate withdrawal-associated aversive states (Bonci and Williams, 1996). Future studies involving brain microinjections may clarify whether D₁ agonist-induced increases in P-GluR1 within the NAc are critical for establishing place preferences in morphine-dependent rats. Microinjection studies are beyond the scope of the present work because they will likely involve numerous D₁ receptor-containing brain regions. Regardless, these findings suggest for the first time that D₁ agonists might be useful for the treatment of withdrawal symptoms that contribute to the maintenance of opiate addiction in humans.

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