

Reversal of Cocaine-Induced Behavioral Sensitization and Associated Phosphorylation of the NR2B and GluRI Subunits of the NMDA and AMPA Receptors

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Cocaine abusers remain vulnerable to drug craving and relapse for many years after abstinence is achieved. We have recently shown that ondansetron (a 5-HT₃ receptor antagonist) given 3.5 h after each daily cocaine injection reverses previously established behavioral sensitization. The purpose of the present investigation was two-fold. First, as cocaine cannot be used as therapy, we examined whether pergolide (a D₁/D₂ receptor agonist with reduced abuse potential) and ondansetron could reverse behavioral sensitization. Second, we investigated whether these behavioral changes were associated with parallel alterations in expression levels and/or phosphorylation changes in the NR2B and GluRI subunits of the respective NMDA and AMPA receptors. Rats were injected for 5 consecutive days with cocaine or saline followed by 9 days of withdrawal. Starting on withdrawal day 10, animals were given vehicle, pergolide/saline, or pergolide/ondansetron for 5 consecutive days. Following a second 9-day period of withdrawal, all animals were challenged with cocaine for assessment of behavioral sensitization and tissues were collected on the following day for Western blot. Sensitization was associated with increased NR2B expression in the accumbens (NAc) shell and decreased Tyr¹⁴⁷² phosphorylation in the NAc core, as well as increased Ser⁸⁴⁵ phosphorylation of the GluRI subunit in prefrontal cortex, NAc core, and shell. Pergolide/ondansetron treatment, but not pergolide alone, consistently reversed both the behavioral sensitization and the associated changes in the NMDA and AMPA receptor subunits. To the extent that sensitization plays a role in chronic cocaine abuse, a combination of these clinically available drugs may be useful in treatment of the disorder.

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INTRODUCTION

Cocaine dependence is a common and serious condition, and is a substantial public health problem. Total abstinence from cocaine is very difficult to accomplish in the clinic mainly because, even after abstinence is achieved, cocaine addicts remain vulnerable for years to episodes of craving and relapse triggered by stimuli previously associated with drug abuse (Gawin and Ellinwood, 1988; Wolf, 2002; Cornish and O'Brien, 1996). To date, no pharmacological treatment is available that can consistently lead to long-

term cocaine abstinence. In rodents, repeated administration of cocaine results in a progressive and enduring augmentation of locomotor and stereotyped behaviors. This response is termed behavioral sensitization and it has long been considered a model of the intensification of cocaine craving in humans that characterizes addiction and promotes relapse (Wolf, 1998; Robinson and Berridge, 1993). We have previously demonstrated in rats that cocaine sensitization established by seven daily injections and a subsequent 7-day withdrawal period can be reversed by a second series of daily cocaine injections if each of these injections is followed 3.5 h later with the 5-HT₃ antagonist ondansetron (Davidson *et al*, 2002). Furthermore, the same reversal regimen can decrease the breaking point under a progressive-ratio self-administration paradigm (Davidson *et al*, 2002) and reduce the cocaine intake under an oral administration paradigm (Davidson *et al*, 2004). Hence, blockade of 5-HT₃ receptors during the acute cocaine withdrawal period (ie, a few hours after cocaine adminis-

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tration that includes the reduction in the acute pharmacological effects of the drug and the onset of withdrawal responses) appears to be sufficient to reverse behavioral sensitization or self-administration in rodents. However, the neurochemical changes accompanying the ondansetron-induced behavioral-reversal have not been previously determined.

Pharmacological and behavioral studies reveal an association between NMDA receptor-mediated events and behavioral sensitization to cocaine (Gainetdinov *et al*, 2001). In this regard, Karler *et al* (1989) have demonstrated that the development of sensitization in mice is prevented when MK-801, a noncompetitive NMDA receptor antagonist, is administered 30 min before each injection of cocaine. These studies suggest an association between behavioral sensitization to cocaine and NMDA receptor activation. More recently, chronic cocaine treatment has been reported to alter the expression of NMDA receptor subunits during withdrawal in a region- and time-dependent manner (Fitzgerald *et al*, 1996; Loftis and Janowsky, 2002, 2003). These changes may be mediated by alterations in the expression of various NMDA receptor subunits and/or post-translational changes owing to phosphorylation of the subunits (Smart 1997; Kalluri and Ticku, 1999; Westphal *et al*, 1999).

The NMDA receptor is a ligand-gated ion channel composed of the core NR1 subunit and the regulatory NR2 (A–D) subunits. Individual NMDA receptor subunits and splice variant combinations possess different receptor functions (Loftis and Janowsky, 2003; Hollmann and Heinemann, 1994). The NR2B subunit is one of the major NMDA regulatory subunits in the forebrain (Nakanishi, 1992; Seeburg, 1993; Omkumar *et al*, 1996) and midbrain (Ungless *et al*, 2001; Loftis and Janowsky, 2003; Hollmann and Heinemann, 1994), regions known to mediate cocaine responses. The NR2B subunit is found in the postsynaptic density fraction and its activity is regulated through phosphorylation of the Tyr¹⁴⁷² residue (Loftis and Janowsky, 2003; Moon *et al*, 1994; Nakazawa *et al*, 2001).

Activation of NMDA receptors also appears to enhance the sensitivity of postsynaptic neurons to glutamate stimulation through the recruitment of AMPA receptors (Clark and Cull-Candy, 2002). Systemic co-administration of AMPA receptor antagonists with cocaine has also been reported to prevent the development of behavioral sensitization as measured by locomotor activity (Li *et al*, 1997) or cocaine-induced stereotypy (Karler *et al*, 1994). Although rats that develop sensitization do not show any changes in GluR1 levels within 24 h of withdrawal, an increase in levels in the nucleus accumbens (NAc) is evident within 3 weeks of treatment (Churchill *et al*, 1999). Whereas this study established changes in GluR1 levels with withdrawal, it is noteworthy that the activity of the AMPA receptor can be also regulated by phosphorylation of the GluR1 subunit at the Ser⁸³¹ and Ser⁸⁴⁵ residues (Lee *et al*, 2000; Ungless *et al*, 2001). The functional role of phosphorylation at these two sites has been extensively characterized, and phosphorylation at either site potentiates AMPA receptor ion channel function, albeit through separate mechanisms (Banke *et al*, 2000; Derkach *et al*, 1999). For instance, phosphorylation at Ser⁸⁴⁵ enhances AMPA currents by increasing the channel open time probability (Roche *et al*, 1996), whereas

phosphorylation of Ser⁸³¹ increases channel conductance (Derkach *et al*, 1999).

The purpose of the present study was several fold. First, as cocaine cannot be used in treating cocaine abusers, we tested whether or not the combination of pergolide, a D₁/D₂ receptor agonist, and ondansetron could reverse previously established behavioral sensitization if ondansetron is given during the acute pergolide withdrawal period (3.5 h after pergolide). Pergolide has been commonly used to treat Parkinson's disease, restless leg syndrome and other neurological disorders (Olanow *et al*, 2001; Staedt *et al*, 1998) and, to our knowledge, there have been no reports in the literature describing its abuse in these patients. This apparent lack of abuse potential may be anticipated to render this drug as a more acceptable dopamine (DA) agonist for treating cocaine abusers than cocaine itself. In addition to the behavioral outcome, we directly determined whether or not a reversal of behavioral sensitization was associated with changes in expression of the NR2B subunit and the GluR1 subunit of the respective NMDA and AMPA receptors. Finally, as phosphorylation of these subunits plays important role in the receptor functions, we examined whether altered phosphorylation state of the receptor subunits was related to behavioral sensitization and its reversal.

MATERIALS AND METHODS

Animals and Drugs

Male Sprague–Dawley rats, initially weighing 150–200 g (Charles River Laboratories, Raleigh, NC), were acclimated for 1 week in the vivarium before the experiment. Rats were housed in pairs in plastic cages in a humidity- and temperature-controlled room on a 12-h light/dark cycle (lights on 0700–1900 hours); lab chow and water were provided *ad libitum*. All experiments were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee and were performed in accordance with NIH guidelines (NIH publication 865–23). Cocaine HCl (NIDA, Bethesda, MD) was dissolved (20 mg/ml) in 0.9% sterile saline. Ondansetron hydrochloride dihydrate (from the Pharmacy at Duke) stock solution was further diluted with 0.9% saline before injections (0.2 mg/ml). Pergolide (Sigma, St Louis, MO) was dissolved into 10% DMSO at 1 mg/ml and diluted 1:10 before use.

Experimental Groups

One group of rats received cocaine (7.5 mg/kg, s.c.) on day 1 and was ranked according to their behavioral responses to cocaine. Animals were evenly divided among three treatment groups ($n = 12$ –15 rats/group). All three groups received cocaine (40 mg/kg/day, s.c., at 0930 hours) for 4 consecutive days and then underwent withdrawal for 9 days (Table 1). Another group of rats was randomly assigned to three groups ($n = 18$ –22 rats/group) and received 5 consecutive days of saline injections followed by 9 days of withdrawal. On day 10 of withdrawal, one cocaine and saline group each was given 0.1 mg/kg pergolide, followed by a saline injection (s.c.); these groups were termed C-P/S and S-P/S, respectively. A second cocaine (C-P/O) and saline

Table 1 Treatments of the Six Different Experimental Groups

Group ^a	Day 1	Days 2–5	Days 6–14	Days 15–19 ^b	Days 20–28	Day 29
S-D/S	Saline	Saline		D/S		
S-P/S	Saline	Saline	Withdrawal	P/S	Withdrawal	7.5 mg/kg, i.p.
S-P/O	Saline	Saline	For all groups	P/O	For all groups	Cocaine challenge for all groups
C-D/S	7.5 mg/kg cocaine	40 mg/kg cocaine		D/S		
C-P/S	7.5 mg/kg cocaine	40 mg/kg cocaine		P/S		
C-P/O	7.5 mg/kg cocaine	40 mg/kg cocaine		P/O		

^aS, saline; C, cocaine; D, dimethylsulfoxide; P, pergolide (0.1 mg/kg); and O, ondansetron (0.2 mg/kg). All injections on days 1–5 and 15–19 were given s.c., whereas the cocaine challenge on day 29 was given i.p.

^bThe second drug injection followed the first one by 3.5 h.

group (S-P/O) received 0.1 mg/kg pergolide (s.c.) plus 0.2 mg/kg ondansetron (s.c.). A third cocaine (C-D/S) and saline group (S-D/S) received parallel vehicle (DMSO plus saline) injections. The interval between the two daily injections was 3.5 h for all groups and these treatments were given over 5 consecutive days followed by a second 9-day withdrawal period. On the second withdrawal day 10, all rats were acutely challenged with 7.5 mg/kg cocaine (i.p.).

Behavioral Measurements

Behavioral ratings (Ellinwood and Balster, 1974; see Table 2) and photobeam-breaks (ambulation) were monitored in the home cage (28 × 18 × 12 cm). Before behavioral assessment, rats were acclimated to the test room in their home cages for 30 min under normal lighting conditions. The rat cages were placed in Opto-Varimex photobeam monitors (8 × 8 beams; Columbus Instruments, Columbus, OH), and baseline activity was monitored over 15 min. Animals were injected with 7.5 mg/kg cocaine (i.p.) and immediately returned to their home cages where behavior was monitored over the next 60 min. For behavioral ratings, behaviors were evaluated at 5-min intervals for 20 s with 10 s elapsing between observations for separate cages.

Brain Dissection and Protein Extraction

Rats were deeply anesthetized with halothane and decapitated. Brains were rapidly removed and blocked in 1 mm coronal sections for prefrontal cortex (PFC), caudate, NAc core, NAc shell, and amygdala. Specifically, PFC samples were taken at +3.2 to +2.2 mm; caudate, NAc core and shell at +2.0 to +1.0 mm; and amygdala at −2.3 to −3.3 mm (all samples taken anterior–posterior to bregma; see Paxinos and Watson, 1986). Within the striatal section, the cortex, corpus callosum, and olfactory tubercle were removed. A diagonal cut was made above the anterior commissure, the area above this was considered caudate; in the remaining ventral section, the tissue below the commissure was sectioned with the most ventral part designated the NAc shell and the dorsal portion the NAc core. Samples were immediately frozen on dry ice, and stored at −80°C until analyses.

Tissues were homogenized in nine volumes of ice-cold homogenate buffer containing 1% sodium deoxycholate,

Table 2 Behavioral Rating Scale for Stereotyped Behaviors^a

Score	Classification	Definition
1	Asleep	Lying down, eyes closed
2	Almost asleep	Lying down, eyes partially shut
3	Dystonia	Lying down, abnormal posture, tense muscles
4	Inactive	Lying down, eyes open, infrequent sniffing
5	In-place oral behavior	Lying down, oral movements (eg, yawning)
6	Grooming	Grooming of face, body, or groin
7	Normal-active movement	Investigation or sniffing of cage, rearing
8	Hyperactive	Running with rapid jerky positional changes
9	Slow-patterned movement	Repetitive exploration of the cage under normal activity
10	Fast-patterned movement	Intense, rapid repetitive exploration of cage
11	Stereotypy	Types of stereotypy are noted

^aModified from Ellinwood and Balster (1974). Each rat is examined for 20 s at 5 min intervals and its behavior is scored.

5 mM Tris-HCl (pH 7.4), 2 mM EDTA, 10 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin A, and 0.1 mg/ml benzamidine. For phosphorylation analyses, the lysis buffer also contained phosphatase inhibitors (50 mM sodium fluoride, 50 mM sodium pyrophosphate, 20 mM 2-glycerol phosphate, 1 mM *p*-nitrophenyl phosphate, 2 µM microcystin LR). Samples were sonicated on ice for 10–15 s and centrifuged at 12 000g for 2–3 min at 4°C to remove large cell debris. Protein concentrations were determined by Bradford assay (1976). In preparation for Western blot, the lysate was diluted with 6 × sample buffer (48% glycerol, 6% SDS, 17.28 mM β-mercaptoethanol, 12 mM EDTA, 300 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue), boiled for 5 min, and stored at −80°C until assay.

Western Blot

Samples were separated on a 7% SDS-PAGE gel and electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membrane was rinsed with PBS and nonspecific sites were blocked by incubating the

membrane with blocking buffer (PBS-0.05% Tween-20 (PBST) containing 5% nonfat dry milk) overnight at 4°C or for 3 h at room temperature. For detecting total NR2B or GluR1 levels, the membranes were incubated with primary antibody (1:1000 dilution) in blocking buffer for 2 h at room temperature. For detecting site-specific phosphorylation of the GluR1 subunit at the Ser⁸⁴⁵/Ser⁸³¹ residues or phosphorylation of the NR2B subunit at the Tyr¹⁴⁷² residue, the membranes were incubated with primary antibody (Chemicon, Temecula, CA) at 1:1000 dilutions in the PBST buffer containing 5% BSA overnight at 4°C. The membranes were washed sequentially with PBST 4 × for 15 min and reblocked for 1 h with PBST-5% nonfat dry milk, then incubated with peroxidase-labeled secondary antibody at a 1:5000 dilution (Sigma, St Louis, MO) for 2 h at room temperature. The blot was washed with PBS-0.1% Tween-20 4 × for 15 min and developed with Chemiluminescent substrate (Pierce Chemical Company, Rockford, IL).

To control for loading efficiency, the blots for the GluR1, phospho-GluR1 Ser⁸⁴⁵/Ser⁸³¹, NR2B and phospho-NR2B Tyr¹⁴⁷² subunits were stripped and reprobed with α -tubulin (Sigma). The images were scanned with Adobe photoshop (Adobe, San Jose, CA) and quantified with NIH Image J (see <http://rsb.info.nih.gov>). Expression of total NR2B and GluR1 and their phosphorylation levels were evaluated relative to that for α -tubulin (ie, relative density = subunit/ α -tubulin levels). Background correction values were subtracted from each lane to minimize the variability across membranes.

Statistics

The behavioral and Western blot data are presented as means and standard errors of the mean. The results were analyzed with the Statistical Package for the Social Sciences, Version 14.0 (Chicago, IL). The behavioral results were analyzed by repeated-measures ANOVA (RMANOVA) and the interactive term was evaluated by Bonferroni corrected pair-wise comparisons. When the behavioral results were collapsed over the 1 h period following cocaine challenge, a univariate ANOVA was used to examine the results. For Western blots, each set of relative density results for receptor subunits was first normalized to the mean of the S-D/S group before statistical analysis. A univariate ANOVA was applied to determine statistically significant differences between treatment groups for the Western blot experiments. *Post hoc* multiple comparisons for these results were evaluated by Tukey test. A $p < 0.05$ was considered statistically significant.

RESULTS

Reversal of Behavioral Sensitization by Pergolide/Ondansetron

In the present study, animals were given saline or cocaine for 5 days, followed by a 9-day withdrawal period (Table 1) to establish behavioral cocaine sensitization. Animals were then administered DMSO/saline (D/S), pergolide/saline (P/S), or pergolide/ondansetron (P/O) for 5 days and another 9-day withdrawal period was imposed. Subsequently, behavioral sensitization was assessed by locomotor

activity and behavioral rating scores following acute challenge with 7.5 mg/kg cocaine (i.p.) for all animals. When 15-min baseline ambulatory activity was analyzed by RMANOVA, no statistical differences among the groups were discerned (Figure 1a and b). The response to cocaine challenge was next evaluated. Comparison of the response of the three saline controls groups (eg, S-D/S, S-P/S, and S-P/O) to 7.5 mg/kg cocaine at the end of the experiment with RMANOVA found no significant main effects of the time or time by treatment interaction (Figure 1a). This result indicates that prior treatment with saline, DMSO, pergolide, or ondansetron does not differentially influence the cocaine response for the three saline-control groups. Moreover, the response to 7.5 mg/kg cocaine on day 1 of the study for the cocaine groups (eg C-D/S, C-P/S, and C-P/O groups: 367 ± 64 ambulations) was not statistically different from that of the S-D/S group given 7.5 mg/kg cocaine (393 ± 83 ambulations) at the end of the experiment. Hence, the S-D/S, S-P/S, and S-P/O groups were combined into a single control group and compared to the three cocaine groups: C-D/S, C-P/S, and C-P/O (Figure 1b). A RMANOVA revealed significant main effects of time ($F(11,1144) = 11.3$, $p < 0.001$) and a time by treatment interaction ($F(33,1144) = 2.07$, $p < 0.001$). Bonferroni corrected pair-wise comparisons of the interaction revealed that ambulations were higher in C-D/S animals compared to the collapsed saline control group at 10–35 and 55 min following cocaine injection (p 's < 0.001 – 0.018). Although rats treated with C-P/S exhibited fewer ambulations than C-D/S animals at 10 ($p < 0.014$) and 20 min ($p < 0.036$), their responses were still higher than the saline-control groups at 25–45 min (p 's < 0.003 – 0.015). Ambulations in C-P/O-treated animals did not differ from saline control rats at any time point and were lower than those of C-P/S rats at 35 and 45 min (both $p < 0.036$). The ambulation data were collapsed over the 1 h period following cocaine challenge (Figure 1c) and a univariate ANOVA revealed significant main effects of treatment ($F(3,104) = 6.63$, $p < 0.001$). Bonferroni comparisons between all treatment conditions demonstrated that C-D/S rats had significantly higher ambulation levels than saline controls ($p < 0.001$) or C-P/O rats ($p < 0.035$) but not C-P/S rats. Ambulation levels of C-P/S rats tended to be enhanced compared to those of saline controls ($p < 0.071$) but did not differ from those of C-P/O animals. No differences between C-P/O and saline groups were discerned. Collectively, these findings show that, while pergolide alone exerted some influence, the combined pergolide and ondansetron treatment produced the more consistent and robust reversal of behavioral sensitization to cocaine.

As psychostimulant exposure can serve to restrict the behavioral repertoire of animals in the open field, such that locomotor activity is supplanted by stereotypy (Lyon and Randrup, 1972), we also rated the animal's responses to the cocaine challenge. As with baseline ambulations above, no statistical differences in basal behavioral rating scores among the groups were noted (Figure 1d and e). When the response to cocaine challenge was analyzed by RMANOVA, no significant differences among the S-D/S, S-P/S, and S-P/O treatments were observed (Figure 1d). These three groups were collapsed into a single control group and the data were reanalyzed with respect to the three cocaine groups (C-D/S, C-P/S, and C-P/O; Figure 1e).

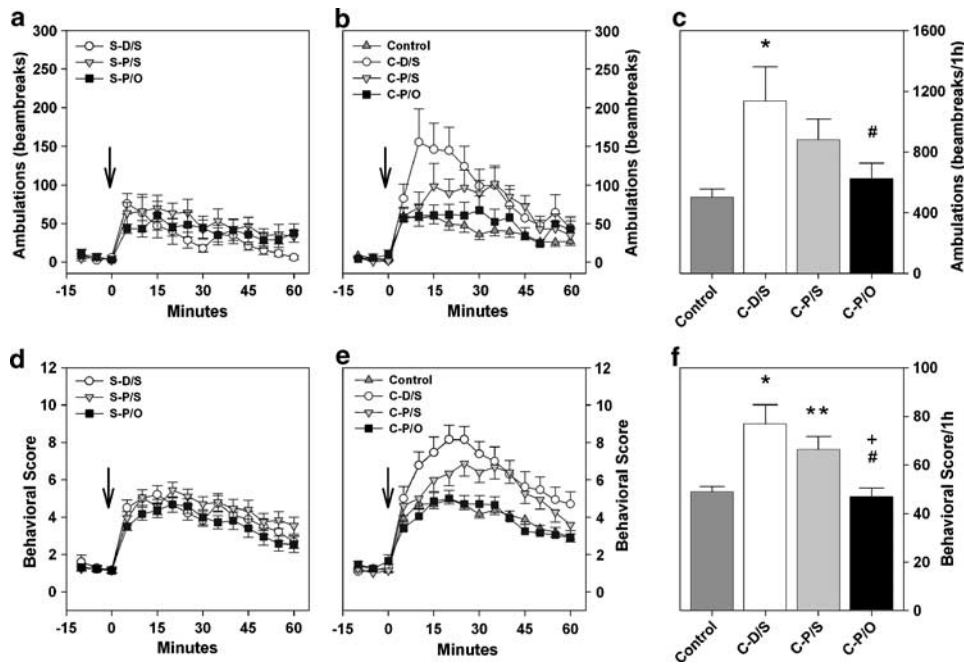


Figure 1 Effects of various treatments on previously established cocaine sensitization. (a, b) Ambulatory activities assessed during baseline and the first hour immediately following cocaine injection. Rats were administered 7.5 mg/kg cocaine (i.p.) and immediately returned to the open field for 1 h. (c) Cumulative ambulations over the 1 h of cocaine challenge. (d, e) Corresponding behavioral rating scores for stereotypy assessed during baseline and immediately after cocaine injection for 1 h. (f) Cumulative behavioral rating score over the 1 h of cocaine challenge. The arrows in panels a, b, d, and e denote when cocaine was given. See Table 1 for description of the treatment conditions ($n = 12$ – 22 rats per treatment group). Control = S-D/S, S-P/S, and S-P/O groups that were collapsed into a single group because they were not statistically different from each other. *, $p < 0.001$ & **, $p < 0.009$ vs combined saline controls; #, $p < 0.035$ (panel c) or $p < 0.001$ (panel f) vs C-D/S group; +, $p < 0.015$ vs C-P/S group, Bonferroni pair-wise comparisons.

RMANOVA revealed significant main effects of time ($F(11,1144) = 31.9$, $p < 0.001$) and a significant time by treatment interaction ($F(33,1144) = 2.04$, $p < 0.001$). Bonferroni corrected pair-wise comparisons demonstrated that C-D/S rats had significantly higher behavioral scores than saline control and C-P/O rats at each time point during the entire observation period after cocaine injection (p 's < 0.001 – 0.002). C-P/S-treated rats also had higher rating scores than saline controls at 15–50 min (p 's < 0.001 – 0.034); however, these scores were less than those for C-D/S animals at 10 ($p < 0.018$) and 20 min ($p < 0.023$). Most importantly, scores of C-P/O rats never differed from saline controls over the entire 1 h period following cocaine challenge, and were significantly decreased relative both to C-D/S rats at all time points (p 's < 0.001 – 0.012) and to C-P/S rats at 5 ($p < 0.034$) and 20–50 min (p 's < 0.001 – 0.012). When the behavioral ratings were collapsed over the 1 h after cocaine injection (Figure 1f) and analyzed with univariate ANOVA, a significant treatment effect was obtained ($F(3,104) = 11.4$, $p < 0.001$). Bonferroni comparisons demonstrated that both C-D/S and C-P/S rats had significantly higher behavioral scores than saline controls (p 's < 0.001 and 0.009 , respectively). C-P/O rats did not differ from the controls and exhibited significantly lower ratings than those for both C-D/S ($p < 0.001$) and C-P/S groups ($p < 0.015$). The C-D/S and C-P/S groups did not differ from each other. Collectively, these results show that established behavioral sensitization, as assessed by both locomotor activity and behavioral ratings, is reversed when the animals are treated with pergolide followed 3.5 h later with ondansetron during the acute withdrawal period.

Pergolide/Ondansetron Effects on NR2B Subunit Expression

To determine whether the pergolide/ondansetron effects on behavioral sensitization were associated with changes in NMDA receptor function, effects of the treatments were compared on NR2B protein expression in various brain regions. Univariate ANOVA failed to reveal significant differences in levels of normalized NR2B protein in the PFC, NAc core, caudate, and amygdala among the six treatment groups (Table 3). By comparison, NR2B protein levels in the NAc shell showed significant differences among the treatment groups ($F(5,44) = 24.4$, $p < 0.001$). Tukey *post hoc* tests demonstrated that the three saline groups (S-D/S, S-P/S, and S-P/O) were not different from each other (Figure 2, left). As a result, these groups were collapsed into a single saline control group and the data were reanalyzed (Figure 2, right). Univariate ANOVA found that total NR2B levels in the NAc shell were significantly different among treatment groups ($F(3,46) = 38.8$, $p < 0.001$). Tukey comparisons showed that levels of the NR2B subunit were higher in the C-D/S, C-P/S, and C-P/O groups than in the saline control (all $p < 0.001$). Importantly, concentrations of the NR2B subunit in the NAc shell were significantly lower in the animals in the C-P/O group than those in the C-D/S ($p < 0.019$) and C-P/S ($p < 0.001$) groups, which did not differ from each other. Taken together, these findings demonstrate that combined pergolide/ondansetron treatment can counteract the effects of chronic cocaine exposure on NR2B expression in the NAc shell.

Table 3 Effects of Various Treatments on Total NR2B Protein in Brain

Brain area ^a	Treatment condition ^b					
	S-D/S	S-P/S	S-P/O	C-D/S	C-P/S	C-P/O
PFC	1.000 ± 0.246	0.968 ± 0.257	0.822 ± 0.184	0.924 ± 0.274	1.008 ± 0.281	1.020 ± 0.288
NAc core	1.000 ± 0.084	1.091 ± 0.151	0.839 ± 0.093	0.876 ± 0.145	0.889 ± 0.202	0.824 ± 0.137
Caudate	1.000 ± 0.226	1.195 ± 0.257	0.925 ± 0.273	0.885 ± 0.248	1.208 ± 0.187	1.167 ± 0.185
Amygdala	1.000 ± 0.121	1.071 ± 0.139	0.980 ± 0.134	0.843 ± 0.219	0.970 ± 0.171	1.074 ± 0.131

^aPFC, prefrontal cortex; NAc, nucleus accumbens. The following amounts of proteins were loaded: PFC—14 µg/lane, NAc core—17.5 µg/lane, caudate—17.5 µg/lane, and amygdala—32.5 µg/lane.

^bSee Table 1 for abbreviations for the treatment conditions ($n = 7-11$ samples from individual rats per treatment condition).

^cMeans ± standard errors of the mean. For each brain area, the relative density of NR2B immunoreactivity to that of α -tubulin was determined for each sample and subsequently normalized to the S-D/S group mean.

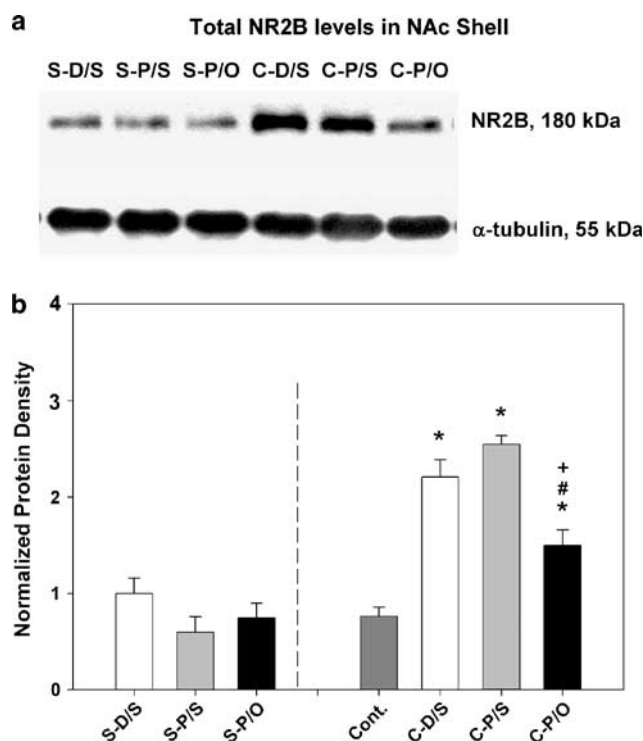


Figure 2 Effects of various treatments on total NR2B levels in the NAc shell. (a) Representative Western blot of NR2B and α -tubulin expression. Each lane represents a different rat from six different groups (17.5 µg protein/lane). (b) Blots were scanned, and the relative density of total NR2B immunoreactivity to that of α -tubulin was determined for each sample. Individual density results were subsequently normalized to the S-D/S group mean. See Table 1 for descriptions of the six experimental groups ($n = 7-11$ samples from individual rats per treatment condition). As no significant differences among the S-D/S, S-P/S, and S-P/O groups were discerned, they were collapsed into a single saline control group (Cont.) and the results analyzed against the three cocaine groups. *, $p < 0.001$ vs saline control group; #, $p < 0.019$ vs C-D/S group; +, $p < 0.001$ vs C-P/S group, Tukey test.

To further relate biochemical changes to reversal of behavioral sensitization, the status of Tyr¹⁴⁷² phosphorylation of the NR2B subunit was examined in the PFC, NAc core, NAc shell, caudate, and amygdala (Table 4). Significant differences were only observed in the NAc core (Figure 3). As expression of phospho-NR2B protein was

similar among the S-D/S, S-P/S, and S-P/O groups, the data from these treatments were collapsed into a single saline control group (Figure 3, left) and analyzed against the three cocaine treatment conditions (Figure 3, right). Univariate ANOVA revealed significant treatment effects on levels of phospho-NR2B ($F(3,33) = 4.33$, $p < 0.011$), and Tukey comparisons revealed that the levels in C-D/S and C-P/S rats were reduced significantly compared to C-P/O rats ($p < 0.040$ and 0.044). Whereas these levels were also reduced compared to that in saline controls, the differences did not reach statistical significance. Finally, the C-P/O and saline control groups were not different from each other. These findings show that pergolide/ondansetron treatment during cocaine withdrawal can normalize altered Tyr¹⁴⁷² phosphorylation of the NR2B subunit.

Pergolide/Ondansetron Effects on GluR1 Subunit Expression

To determine whether the effects of pergolide/ondansetron on reversal of behavioral sensitization are also associated with alterations in AMPA receptors, total GluR1 expression was evaluated. No significant effects among the six treatment conditions were noted in the PFC, NAc shell and core, caudate, or amygdala (Table 5). Similarly, no distinctions among the various treatment groups in the different brain regions were observed for levels of the Ser⁸³¹-phospho-GluR1 subunit (data not shown). By comparison, levels of the Ser⁸⁴⁵-phospho-GluR1 subunit were increased in the PFC, NAc shell, and core (Figure 4), but not in the caudate or amygdala (Table 6). As the levels of Ser⁸⁴⁵-phospho-GluR1 were similar among the S-D/S, S-P/S, and S-P/O conditions (Figure 4b, d, f, left), these data were collapsed into a single saline control group and the data were reanalyzed (Figure 4b, d, f, right). Univariate ANOVA detected significant group differences in levels of the phospho-GluR1 protein in the PFC ($F(3,30) = 5.31$, $p < 0.005$), NAc shell ($F(3,32) = 8.42$, $p < 0.001$), and core ($F(3,34) = 5.49$, $p < 0.003$). Tukey comparisons for PFC samples revealed that the C-P/S group exhibited significantly enhanced Ser⁸⁴⁵-phospho-GluR1 levels relative to the saline control ($p < 0.031$) and C-P/O ($p < 0.030$) groups, whereas the C-D/S group showed marginal enhancement compared to the saline control and C-P/O groups (p 's < 0.060 and 0.054 , respectively). No differences were

Table 4 Effects of Various Treatments on Tyr¹⁴⁷² Phosphorylation of the NR2B Subunit

Brain area ^a	Treatment condition ^b					
	S-D/S	S-P/S	S-P/O	C-D/S	C-P/S	C-P/O
PFC	1.000 \pm 0.606	2.151 \pm 0.728	1.811 \pm 0.793	2.009 \pm 0.435	3.098 \pm 0.904	3.008 \pm 0.956
NAC shell	1.000 \pm 0.084	1.091 \pm 0.151	0.839 \pm 0.093	0.876 \pm 0.145	0.889 \pm 0.202	0.824 \pm 0.137
Caudate	1.000 \pm 0.493	1.041 \pm 0.394	1.184 \pm 0.246	1.345 \pm 0.321	1.342 \pm 0.260	1.397 \pm 0.373
Amygdala	1.000 \pm 0.318	1.232 \pm 0.313	1.207 \pm 0.202	1.383 \pm 0.334	1.404 \pm 0.471	0.990 \pm 0.186

^aPFC, prefrontal cortex; NAC, nucleus accumbens. The following amounts of proteins were loaded: PFC—28 μ g/lane, NAC shell—35 μ g/lane, caudate—30 μ g/lane, and amygdala—65 μ g/lane.

^bSee Table 1 for abbreviations for the treatment conditions ($n=6-7$ samples from individual rats per treatment condition).

^cMeans \pm standard errors of the mean. For each brain area, the relative density of Tyr¹⁴⁷²-phospho-NR2B immunoreactivity to that of α -tubulin was determined for each sample and subsequently normalized to the S-D/S group mean.

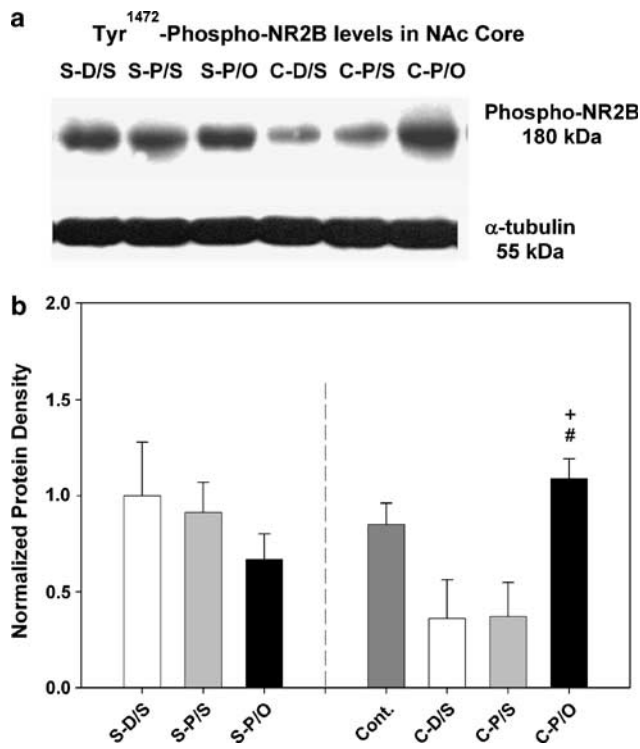


Figure 3 Effects of various treatments on Tyr¹⁴⁷²-phospho-NR2B levels in the NAc core. (a) Representative Western blot of phospho-NR2B and α -tubulin expression. Each lane represents a different rat from six different groups (35 μ g protein/lane). (b) Blots were scanned, and the relative density of phospho-NR2B immunoreactivity to that of α -tubulin was determined for each sample. Individual density results were subsequently normalized to the S-D/S group mean. See Table 1 for descriptions of the six experimental groups ($n=6-7$ samples from individual rats per treatment condition). As no significant differences among the S-D/S, S-P/S, and S-P/O groups were discerned, they were collapsed into a single saline control group (Cont) and the results analyzed against the three cocaine groups. #, $p < 0.040$ vs C-D/S group; +, $p < 0.044$ vs C-P/S group, Tukey test.

discerned between C-D/S and C-P/S rats or between saline control and C-P/O rats. In the NAc core and shell, *post hoc* comparisons demonstrated that Ser⁸⁴⁵-phospho-GluR1 levels were significantly enhanced in the C-D/S treatment conditions compared to the saline control (p s < 0.006 and 0.001 , respectively) and the C-P/O groups (p 's < 0.004 and

0.001). The C-D/S and C-P/S groups were not statistically different in either brain area. Together, these results show that pergolide/ondansetron treatment can consistently reverse changes in the status of Ser⁸⁴⁵ phosphorylation of the GluR1 subunit, whereas levels of the phospho-Ser⁸³¹ GluR1 subunit are not modified by any of these treatment conditions.

DISCUSSION

We have previously demonstrated that behavioral sensitization to cocaine, established by 7 daily injections and a subsequent 7-day withdrawal period is reversed by combined cocaine plus ondansetron treatment if the 5-HT₃ antagonist is given 3.5 h after each cocaine injection (Davidson *et al*, 2002). Treatment with ondansetron during this acute cocaine withdrawal period also inhibits self-administration the next day (Davidson *et al*, 2002, 2004). In the present study, we have substituted cocaine with a nonabused, clinically used DA agonist, pergolide (Olanow *et al*, 2001; Staedt *et al*, 1998). This direct DA agonist, given in combination with ondansetron 3.5 h later, reverses long-term behavioral sensitization to cocaine. These results suggest that daily administration of ondansetron during acute withdrawal from a DA agonist (eg, pergolide or cocaine) may provide a means of disassociating previously established relationships between long-term cocaine sensitization dynamics and the acute 'stimulant' effects of DA agonists. In addition to the putative lack of significant abuse potential, pergolide was selected in the present study for several reasons. First, rat studies have shown a dose similar to the one in our own experiments to reverse cocaine sensitization when given in combination with memantine (Li *et al*, 2000). Second, pergolide can substitute for cocaine in drug discrimination procedures (Witkin *et al*, 1991). Finally, the drug can activate DA-mediated behaviors (increased locomotor activity or contralateral turning) in rats after unilateral 6-hydroxydopamine lesion or reserpine pretreatment (Arnt, 1985; Clemens *et al*, 1993). As the behavioral effects of pergolide typically last for 3–4 h in rats (eg, Clemens *et al*, 1993), the 0.1 mg/kg dose (i.p.) may be expected to lead to initial DA activation with subsequent acute agonist withdrawal effects within this time interval. This prediction is consistent with our present findings.

Table 5 Effects of Various Treatments on Total GluR1 Protein in Brain

Brain area ^a	Treatment condition ^b					
	S-D/S	S-P/S	S-P/O	C-D/S	C-P/S	C-P/O
PFC	1.000 ^c ±0.289	1.003±0.314	1.086±0.199	0.557±0.132	0.730±0.238	0.668±0.246
NAc core	1.000±0.317	1.234±0.134	1.108±0.268	1.257±0.288	1.018±0.306	1.207±0.267
NAc shell	1.000±0.454	1.647±0.473	2.077±0.491	1.680±0.408	2.226±0.219	1.005±0.282
Caudate	1.000±0.382	1.187±0.354	1.092±0.451	1.280±0.413	1.364±0.582	1.476±0.272
Amygdala	1.000±0.247	1.139±0.165	1.108±0.204	1.136±0.224	0.755±0.174	1.051±0.101

^aPFC, prefrontal cortex; NAc, nucleus accumbens. The following amounts of proteins were loaded: PFC—14 μg/lane, NAc core—17.5 μg/lane, NAc shell—17.5 μg/lane, caudate—15 μg/lane, and amygdala—32.5 μg/lane.
^bSee Table 1 for abbreviations for the treatment conditions (*n* = 6–7 samples from individual rats per treatment condition).
^cMeans ± standard errors of the mean. For each brain area, the relative density of GluR1 immunoreactivity to that of α-tubulin was determined for each sample, and was subsequently normalized to the S-D/S group mean.

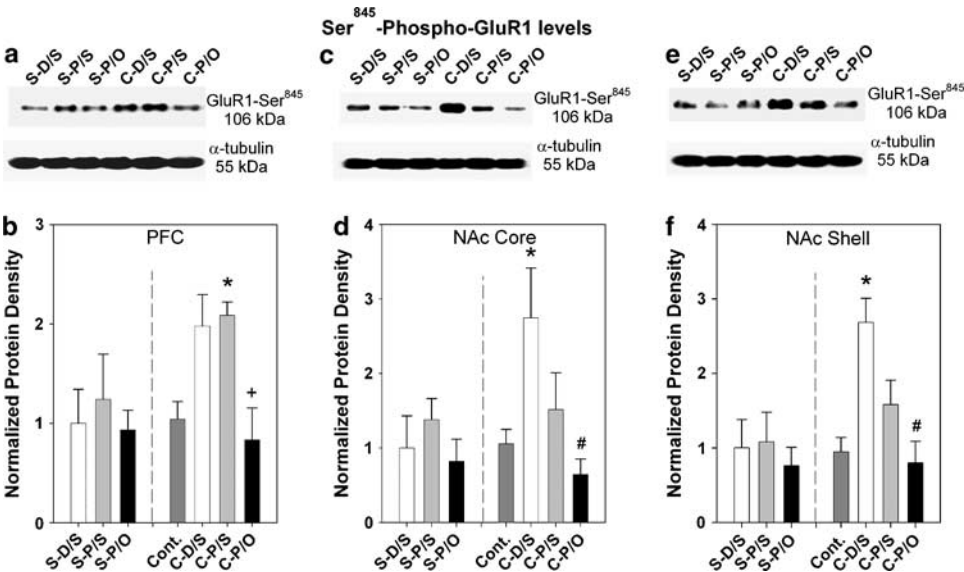


Figure 4 Effects of various treatments on Ser⁸⁴⁵-phospho-GluR1 levels in the PFC and NAc core and shell. (a, c, and e) Representative Western blots of Ser⁸⁴⁵-phospho-GluR1 and α-tubulin in each brain area. Each lane represents a different rat from six different groups (PFC—28 μg protein/lane, NAc shell and core—35 μg protein/lane). (b, d, and f) Blots were scanned, and the relative density of phospho-GluR1 immunoreactivity to that of α-tubulin was determined for each sample. Individual density results for each brain area were subsequently normalized to the S-D/S group mean. See Table 1 for descriptions of the six experimental groups (*n* = 5–7 samples from individual rats per treatment condition). As no significant differences among the S-D/S, S-P/S, and S-P/O groups were discerned for each brain area, the three groups were collapsed into a single saline control group (Cont) for statistical analyses. *, *p* < 0.031 (panel b), *p* < 0.006 (panel d), or *p* < 0.001 (panel f) vs saline control group; #, *p* < 0.004 (panel d) or *p* < 0.001 (panel f) vs C-D/S group; +, *p* < 0.030 vs C-P/S group.

Table 6 Effects of Various Treatments on Ser⁸⁴⁵ Phosphorylation of the GluR1 Subunit

Brain area ^a	Treatment condition ^b					
	S-D/S	S-P/S	S-P/O	C-D/S	C-P/S	C-P/O
Caudate	1.000 ^c ±0.097	0.780±0.164	0.920±0.163	0.814±0.247	0.632±0.223	0.572±0.209
Amygdala	1.000±0.151	0.923±0.181	1.121±0.149	1.268±0.166	1.052±0.160	1.103±0.149

^aThe following amounts of proteins were loaded: caudate—15 μg/lane and amygdala—32.5 μg/lane.
^bSee Table 1 for abbreviations for the treatment conditions (*n* = 6–7 samples from individual rats per treatment condition).
^cMeans ± standard errors of the mean. For each brain area, the relative density of Ser⁸⁴⁵-phospho-GluR1 immunoreactivity to that of α-tubulin was determined for each sample, and subsequently normalized to the S-D/S group mean.

Compared to combined pergolide/ondansetron regimen, the sensitization-reversing effects of pergolide alone were not as robust as those of the pergolide/ondansetron combination. Moreover, pergolide alone was not sufficient to consistently reverse the sensitization-associated changes in NR2B or GluR1 protein expression. Although pergolide has been reported to reduce craving in hospitalized cocaine abusers during the early withdrawal period (Malcolm *et al*, 1991, 1994), the inability of pergolide itself to reverse the long-term NMDA and AMPA receptor subunit changes in the present report is consistent with previous studies showing that this drug is ineffective in maintaining long-term abstinence in outpatients (Malcolm *et al*, 2000; Levin *et al*, 1999). Thus, induction of an 'acute DA withdrawal state' and its antagonism by delayed 5-HT₃ antagonist administration may provide a potential maintenance treatment strategy for achieving long-term cocaine abstinence in chronic abusers. Previously, Li *et al* (2000) reported that pergolide could also reverse cocaine sensitization if the NMDA/5-HT₃ antagonist memantine (Rammes *et al*, 2001) is given before the DA agonist. It is noted, however, that while both ondansetron and memantine are approved for the clinic (eg, anti-emetic and anti-Parkinson treatments, respectively), ondansetron produces much fewer and less serious side effects (eg, constipation) than memantine and other NMDA antagonists (eg, hallucinations and nightmares). The combination of ondansetron and pergolide (or other mixed D₁/D₂ agonists) may be associated with an improved therapeutic outcome in cocaine abusers owing to reduced side effects and increased therapeutic compliance. In future studies, we plan to evaluate whether the efficacy of the pergolide/ondansetron regimen can be further enhanced through changes in pergolide dose and/or by altering the time interval between the two drugs.

Increased glutamatergic neurotransmission in the NAc and ventral tegmental area have been proposed as a mechanism underlying behavioral sensitization to repeated cocaine administration (Churchill *et al*, 1999). Considerable evidence supports a role for NMDA receptors in mediating behavioral, electrophysiological, and neurochemical responses to cocaine (Pierce and Kalivas, 1997; Smith *et al*, 1993; Nestler *et al*, 1993). Repeated cocaine administration is reported to cause region-specific and time-dependent alterations in NR2B expression during withdrawal (Loftis and Janowsky, 2002; Yamaguchi *et al*, 2002; Scheggi *et al*, 2002; Fitzgerald *et al*, 1996). Our findings support the idea for changes in the NAc shell, as NR2B expression was upregulated by ~100% in this brain region in cocaine-treated rats up to 3 weeks after cocaine withdrawal (ie C-D/S vs S-D/S groups). These results suggest that regionally specific long-term changes in NMDA receptor protein expression may contribute to difficulties encountered with the reversal of behavioral sensitization. Importantly, we have demonstrated that the combined pergolide plus ondansetron treatment can reverse not only previously established behavioral cocaine sensitization in rats but also the associated increases in the total NR2B expression in the NAc shell.

Activity of NMDA receptors can be regulated not only by changes in subunit expression but also by alterations in the phosphorylation status of the subunits. Tyrosine phosphor-

ylation of the NR2B subunit is important both for the regulation of its channel activity and for intracellular signaling through interaction of the receptor with SH2 domain-containing molecules (Loftis and Janowsky, 2003). These events can contribute to potentiation of receptor responses and the activation of specific signal transduction pathways. Although overall protein levels of the NR2B subunit were not changed by any treatments in the NAc core, phosphorylation of the Tyr¹⁴⁷² residue was selectively decreased in rats sensitized to cocaine (ie C-D/S and C-P/S groups). By comparison, no significant alteration in the phosphorylation status of the subunit was detected in other brain regions under our experimental conditions.

In the present study, we did not observe any alterations in total GluR1 expression in the NAc shell and core, PFC, caudate, or amygdala following a 23-day withdrawal period. Churchill *et al* (1999) also found that rats withdrawn for 21 days from chronic cocaine injections exhibited no changes in the GluR1 subunit levels, although a subgroup selected *a posteriori* for greater than 20% increases in the cocaine sensitivity following the cocaine injections ('sensitized') exhibited increases in NAc GluR1 levels. We also did not observe a similar correlation between the individual cocaine sensitivity and total GluR1 expression levels in any of the cocaine-treated animals. Overall, the present experiments and those from Churchill and co-workers suggest that cocaine sensitization may not be associated with robust and consistent long-term changes in total GluR1 levels in the brain.

In contrast to total protein levels, cocaine sensitization was associated with a selective increase in Ser⁸⁴⁵ phosphorylation of GluR1 subunit in the PFC, NAc core, and NAc shell (compared to the Ser⁸³¹ residue). Furthermore, reversal of behavioral sensitization was accompanied by normalization of these phosphorylation changes. Other investigators have also reported changes in phosphorylation status of the GluR1 subunit during behavioral sensitization. Thus, phosphorylation of the Ser⁸⁴⁵, but not the Ser⁸³¹ residue, is significantly enhanced in the PFC and NAc by cocaine treatment (Snyder *et al*, 2000; Wolf *et al*, 2003). Phosphorylation on the GluR1 Ser⁸⁴⁵ residue is mediated primarily through protein kinase A activation (Roche *et al*, 1996). This alteration may lead to changes at glutamate synapses in the NAc as D₁ stimulation enhances phosphorylation of GluR1 subunit at a PKA site and increases surface expression of the subunit (Wolf *et al*, 2003; also see above). Recently, Kalivas *et al* (2005) have suggested that drug addiction may be associated with a shift in signaling through DA receptors such that D₁ receptor signaling predominates over D₂ receptor-mediated events. Thus, the enhanced phosphorylation of Ser⁸⁴⁵ on the GluR1 subunit may reflect augmented activation of protein kinase A through the enhanced D₁ neurotransmission.

There may be various ways by which 5-HT₃ receptor blockade during acute cocaine or pergolide withdrawal can lead to reversal of previously established cocaine sensitization. Acute cocaine withdrawal is often associated with symptoms of anhedonia, anergia, depression, and anxiety in human cocaine abusers (Gavin and Ellinwood, 1988), and the intensity of these aversive withdrawal symptoms is a strong predictor of poor treatment outcome (Mulvaney *et al*, 1999; Kampman *et al*, 2001). In animals, Koob and Le

Moal (2001) have suggested that aversive 'allosteric dysregulation' during repeated acute cocaine withdrawal may contribute to the maintenance of long-term cocaine sensitization and abuse. To the extent that the 5-HT₃ receptor system can directly or indirectly (eg, via substance P) play a major role in various aversive responses (Gavioli *et al*, 1999), specific 5-HT₃ blockade during DA withdrawal may lead to disruption of the neural mechanisms underlying repeated cocaine abuse. Anatomically, D₁ and 5-HT₃ receptors in the PFC are codistributed in the inhibitory GABA interneurons (Vincent *et al*, 1995; Jakab and Goldman-Rakic, 1998), where 5-HT₃ stimulation can rapidly activate these inhibitory neurons under adverse conditions (Jakab and Goldman-Rakic, 2000). In addition, 5HT₃ terminal receptors have been shown to mediate local stimulatory actions of 5-HT on DA release in the medial PFC (Chen *et al*, 1992), striatum (Blandina *et al*, 1989), and NAc (De Deurwaerdere *et al*, 1998). Thus, modulations of the DA-5-HT₃ interactions in the PFC, NAc core, and shell may form a basis for reversal of the sensitization-associated behavioral and NMDA/AMPA subunit changes by a pergolide/ondansetron treatment regimen.

In conclusion, the present study demonstrates that long-term behavioral sensitization to cocaine, established via chronic injections followed by a chronic withdrawal period, is associated with region-specific alterations in NR2B subunit expression and phosphorylation, as well as, changes in the phosphorylation status of the GluR1 subunit. Importantly, combined pergolide/ondansetron treatment was sufficient to reverse not only the established behavioral sensitization to cocaine but also the associated alterations in NR2B and GluR1 subunits of the respective NMDA and AMPA receptors. To the extent behavioral sensitization contributes to chronic cocaine abuse in humans, a combined pergolide/ondansetron treatment regimen may provide a means of long-term maintenance treatment for cocaine abusers.

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