The Journal of Neuroscience, October 1994, 14(10): 6239–6247

Inactivation of G_i and G_o Proteins in Nucleus Accumbens Reduces Both Cocaine and Heroin Reinforcement

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The pertussis toxin (PTX)-sensitive G proteins G_i and G_o may be implicated in drug reinforcement and addiction, since certain reward-related dopamine and opiate receptor subtypes are coupled to these G proteins, and since chronic exposure to cocaine or morphine alters levels of these G proteins in the nucleus accumbens (NAc). As a direct test of this hypothesis, G_i and G_o proteins in the NAc were selectively inactivated by intra-accumbens injections of PTX in rats self-administering either cocaine or heroin. In control animals, bilateral injections of inactive PTX (0.1 μg/1 μl/side) in the NAc failed to alter baseline rates of cocaine and heroin self-administration. In contrast, the same dose of active PTX produced significant, long-lasting increases (up to 1 month) in the self-administration of both drugs, and shifted the dose–response curves to the right. These results suggest that PTX reduces or shortens the reinforcing efficacy of cocaine and heroin, leading to compensatory increases in drug self-administration.

Similar NAc injections of PTX reduced the level of $G_{i\alpha}$ and $G_{o\alpha}$ subunits as measured by both ADP-ribosylation and Western blot, without affecting levels of $G_{s\alpha}$ or G_β subunits. The effect of the toxin was mainly limited to the NAc, and no evidence of abnormal cell death or gliosis was observed. The onset of changes in self-administration rate coincided with the onset of changes in ADP-ribosylation, suggesting that, initially, the increased drug self-administration results directly from a reduction in functional G_i and G_o proteins. After 28 d, self-administration baselines began to recover while levels of G protein ADP-ribosylation and immunoreactivity remained low, suggesting that adaptive mechanisms are involved at later time points. These results provide direct support for a common role of G_i and G_o proteins in the NAc in the reinforcing and addictive properties of psychostimulant and opiate drugs.

[Key words: pertussis toxin, reward, reinforcement, drug addiction, dopamine, opiate, opioid, ADP-ribosylation]

Received Oct. 20, 1993; revised April 1, 1994; accepted April 21, 1994.

This work was supported by USPHS Grants DA-05107, DA-05379, AFOSR 89-0213, and DA-08227. The technical expertise of Dung Minh Lam and Sara Kossuth is greatly appreciated.

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A large body of evidence suggests that the reinforcing effects of drugs as diverse as psychostimulants and opiates are mediated by a common neurobiological substrate. In particular, psychostimulants and opiates are thought to produce reinforcement by direct or indirect actions on the dopaminergic neurons in the ventral tegmental area (VTA) or on their target neurons in the nucleus accumbens (NAc) (Wise and Bozarth, 1987; Koob and Bloom, 1988; Liebman and Cooper, 1989). In the case of psychostimulants, rats will self-administer amphetamine (Hoebel et al., 1983) and dopamine (Dworkin et al., 1985) directly into the NAc. Although cocaine is not self-administered directly into the NAc (Goeders and Smith, 1983), bilateral injections of dopamine receptor antagonists into the NAc (Phillips et al., 1983; Maldonado et al., 1993), or lesions of the mesoaccumbens dopamine system (Roberts et al., 1977; Roberts and Koob, 1982; Koob and Goeders, 1989), attenuate the reinforcing effects of intravenously self-administered cocaine. These studies provide strong evidence that dopamine receptors in the NAc mediate the reinforcing effects of psychostimulants. In contrast, opiates are self-administered directly into the VTA (Bozarth and Wise, 1981), where they activate dopamine neurons via inhibition of inhibitory interneurons (Johnson and North, 1992), and subsequently can increase dopamine release in the NAc (Leone et al., 1991). This has led some investigators to suggest that dopamine may be a final common neurotransmitter in both opiate and psychostimulant reinforcement (Bozarth and Wise, 1986; Di Chiara and Imperato, 1988).

However, opiates may also act independently of dopamine neurons. This idea is supported by the findings that opiates are self-administered directly into the NAc (Olds, 1982; Goeders et al., 1984), and that NAc injections of opiate antagonists (Vaccarino et al., 1985; Corrigall and Vaccarino, 1988), or lesions of neurons within the NAc (Zito et al., 1985; Dworkin et al., 1988), reduce the reinforcing efficacy of intravenously self-administered opiates. In contrast to the effects on psychostimulant self-administration, lesions of the mesolimbic dopamine projection to the NAc (Pettit et al., 1984) and blockade of dopamine receptors with a moderate dose of a dopamine antagonist (Ettenberg et al., 1982) both fail to affect intravenous heroin self-administration. These latter findings have led to the hypothesis that opiates and dopamine can act directly on different postsynaptic receptors in the NAc, and thus independently influence a common reinforcement substrate within this brain region (Ettenberg et al., 1982; Koob, 1988; Cunningham and Kelley, 1992).

These ideas emphasize a common mechanism for opiate and psychostimulant reinforcement at the neurotransmitter or an-

atomical level. A common reinforcement mechanism at the postreceptor, signal transduction level has also been hypothesized, based on the fact that certain receptor subtypes implicated in opioid and psychostimulant reinforcement are coupled to a common G protein–second messenger pathway (Self and Stein, 1992a). For example, drugs with agonist activity at μ- and δ-opiate receptors (Young et al., 1981; Bertalmio and Woods, 1989) or dopamine D_2-like receptors (D_2, D_3, and D_4) (Woolverton et al., 1984; Wise et al., 1990; Caine and Koob, 1993) can support self-administration behavior. Conversely, antagonists that selectively block μ- and δ-opiate receptors (Stevens et al., 1991; Negus et al., 1993) or dopamine D_2 receptors (Roberts and Vickers, 1984; Britton et al., 1991) block the reinforcing effects of opiates or psychostimulants, respectively. These receptor subtypes belong to a receptor family that inhibits adenylate cyclase activity via the guanine nucleotide binding protein G_i (Gilman, 1987). In addition, members of this receptor family inhibit neuronal activity through G_i or G_o activation of K^+ channels (Brown, 1990) or inhibition of voltage-sensitive Ca^{2+} channels (Dolphin, 1990). It is therefore conceivable that G_i and G_o proteins in reward-related brain regions like the NAc could represent a fundamental signal transduction pathway shared by opiate and psychostimulant reinforcement mechanisms.

Since drug reinforcement and drug withdrawal in addicted subjects are both characterized by an increase in drug-seeking behavior, it is possible that a common neurobiological substrate mediates the motivational changes associated with the two processes. The idea of a common neurobiological substrate in drug reinforcement and addiction is supported by the finding that a reinforcement-related brain region such as the NAc can also mediate the aversive motivational effects of antagonist-precipitated withdrawal in opiate-addicted rats (Koob et al., 1989; Stinus et al., 1990). The motivational changes associated with drug addiction have been hypothesized to result from cellular adaptations to chronic drug exposure in reinforcement-related brain regions (Koob and Bloom, 1988). In this regard, recent biochemical studies have shown that chronic regimens of morphine or cocaine selectively reduce the amount of $G_{i\alpha}$ and $G_{o\alpha}$ subunits in the NAc, which may suggest, indirectly, that adaptations at the G protein level contribute to certain motivational aspects of addiction (Terwilliger et al., 1991; Nestler, 1992; Striplin and Kalivas, 1993). G_i and G_o proteins in the NAc are thus implicated in both the initial reinforcing and long-term addictive properties of opiates and psychostimulants.

In the present study, the G_i/G_o hypothesis of drug reinforcement was tested directly by inactivating G_i and G_o proteins in the NAc with pertussis toxin (PTX) in rats trained to self-administer either cocaine or heroin. Such inactivation of G_i and G_o proteins was predicted to antagonize the reinforcing effects of cocaine and heroin, as reflected by compensatory increases in drug self-administration rates. PTX selectively inactivates G_i and G_o proteins. PTX therefore blocks signal transduction mediated by receptors that utilize these G proteins (e.g., μ- and δ-opioid and dopamine D_2 receptors) but it does not affect signal transduction mediated by other G proteins. Thus, PTX is a useful tool for specifically implicating G_i and G_o proteins in receptor-mediated responses (see Ui and Katada, 1990).

Materials and Methods

Self-administration

Subjects, surgery, and apparatus. Naive male Sprague–Dawley rats initially weighing 270–300 gm were used for behavioral (Charles River, Raleigh, NC) and biochemical (CAMM, Wayne, NJ) experiments. The animals were housed individually and kept on a 12 hr light/dark cycle (lights on at 7:00 A.M.). Initially, animals were food deprived for 24 hr and trained to press a lever for food pellets (Bioserv, Frenchtown, NJ). Once trained, animals continued to respond for food in 20 min sessions for 3 consecutive days. This procedure was found to hasten the subsequent acquisition of drug self-administration. Under Equithesin anesthesia [Nembutal (8.1 mg/ml), chloral hydrate (43 mg/ml), $MgSO_4$ (21 mg/ml), propylene glycol (40% by vol), EtOH (10% by vol)], each animal was implanted surgically with both a chronic indwelling jugular catheter prepared from Silastic tubing (treated with TDMAC-heparin; Polysciences Inc., Warrington, PA), and 26 gauge bilateral guide cannula (Plastics One, Roanoke, VA), in the NAc. Stereotaxic coordinates with the top of the skull level were +1.7 mm anterior to bregma, ±1.5 mm lateral, and −5.7 mm ventral to dura (Paxinos and Watson, 1982). A dummy cannula cut flush with the guide cannula was left in place throughout the experiment. To prevent clogging, the catheter was flushed daily with 0.2 ml of heparinized, bacteriostatic saline (20 U/ml), and antibiotic ointment was applied to the exit wound. Following a minimum 6 d recovery period, animals were placed in an operant test chamber and connected to a syringe pump system as described by Roberts et al. (1977). A 10 gm lever-press response at the active lever delivered a 0.1 ml intravenous injection of sterile-filtered drug solution in 0.9% saline. A clearly audible tone was sounded during the 6 sec injection interval. Each self-injection response was followed by a 10 sec "time out" period in which the cue light above the active lever was extinguished, and lever-press responses had no programmed consequences. Responding at an inactive lever on the opposite side of the test chamber was monitored for nonspecific increases in lever-press behavior. The test procedures were automatically controlled by an IBM PC 30 computer and Lab Linc I/O panel (Coulbourn Instruments, Lehigh Valley, PA).

Procedure. Two groups of animals were allowed to acquire cocaine (0.75 mg/kg/injection) or heroin (0.03 mg/kg/injection; National Institute on Drug Abuse, Research Triangle Park, NC) in daily, 3 hr self-administration test sessions 6 d a week. These intermediate doses of cocaine and heroin were chosen since they produced roughly equivalent self-administration rates in the maintenance phase. After a minimum of 10 test sessions to acquire self-administration, animals demonstrating stable self-administration baselines (totals varied <10% from the mean of three consecutive sessions) were assigned to either control (heat-inactivated PTX) or experimental (active PTX) groups in a balanced manner, such that the average self-administration baseline was similar for both groups. Awake animals were given bilateral injections of toxin in the nucleus accumbens immediately prior to a test session. The toxin was injected at a dose of 0.1 μg in 1 μl of phosphate-buffered saline (pH 7.4) per side in 100 nl increments over 3 min; the injection was delivered through a 33 gauge bilateral injection cannula (Plastics One, Roanoke, VA), cut to extend 1 mm beyond the guide cannula (6.7 mm ventral to dura); the injection cannula was left in place for 2 min following the injection to allow the toxin to diffuse. The particular lot of PTX used in all of these studies (PT-92AWA, List Biological Labs, Inc., Campbell, CA) exhibited far greater potency, on the order of 10–20×, than several lots tested in pilot experiments.

Self-administration tests continued for a least 14 test sessions following the initial PTX-pretreatment session. At the end of this phase, the injection dose was varied for the next three test sessions to obtain a dose–response curve. The order of dose presentation was 0.375, 1.5, and 0.75 mg/kg/injection in cocaine experiments, and 0.015, 0.06, and 0.03 mg/kg/injection in heroin experiments. Although most animals were not tested for longer periods due to difficulties in maintaining catheter patency, one group of PTX-treated animals was tested for cocaine self-administration for 28 d.

Histology. At the end of self-administration experiments, animals were injected with chloral hydrate (5 ml/kg, i.p.; 80 mg/ml) and perfused transcardially with 0.9% saline followed by 10% formalin/saline. Brains were dissected, frozen, and sliced in 40 μm sections. The sections were placed on gelatin coated slides, stained with cresyl violet, and examined for cannula placement and abnormal scarring.

Self-administration data analysis. The effects of PTX on self-administration was analyzed by comparing self-administration totals from inactive and active toxin groups using a two-way ANOVA with repeated measures on either test session or dose. Post hoc comparisons were made with Student's t test. An α level of 0.05 or less was considered statistically significant.

Biochemistry

PTX injections. Under Equithesin anesthesia, age-matched rats were given a single bilateral injection of PTX (0.1 μg/μl/side) through 33 gauge injection needles as described above, except that the contralateral side (left or right) received inactive PTX to serve as a control. The dorsal–ventral coordinate of 6.7 mm ventral to dura corresponded to the same injection site as in behavioral experiments. The same lot number of PTX was used for the biochemical and behavioral experiments. The animals were allowed to recover for 3, 7, 14, 21, or 28 d before sacrifice. A no-injection group was included as a 0 d control.

Isolation and homogenization of NAc. Brains were removed rapidly from decapitated rats and cooled immediately in ice-cold physiological buffer (126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM D-glucose, pH 7.4). A coronal section, 0.75–1 mm thick, was obtained by use of a tissue slicer, and 15 gauge punches of the NAc were obtained medial to, and including, the anterior commissure (Terwilliger et al., 1991). Outer punches consisted of the rest of the NAc, septum, olfactory tubercle, and cortical regions lateral and ventral to the NAc. The punches were homogenized (10 mg/ml) in ice-cold "homogenization buffer" containing 50 mM Tris (pH 7.4), 1 mM dithiothreitol, 1 mM EGTA, 10 μg/ml leupeptin, 50 KU/ml aprotinin. Samples were analyzed for protein content by the method of Lowry et al. (1951).

ADP-ribosylation and immunolabeling of G proteins. Brain homogenates were centrifuged at 10,000 × g for 15 min in a microfuge and the pellets were resuspended in the original volume of 100 mM Tris (pH 8.0), 10 mM thymidine, 10 mM isoniazide, 5 mM $MgCl_2$, 2.8 mM dithiothreitol, 2.4 mM benzamidine, 0.8 mM EDTA, 2.5 mM ATP, 2 mM GTP, 4% sucrose, 0.8 μg/ml soybean trypsin inhibitor, and 0.5% Triton X-100. Duplicate aliquots of resuspended pellets (containing 15–30 μg of protein) were subjected to PTX-mediated ADP-ribosylation exactly as described (Nestler et al., 1989) except that ADP-ribosylation reactions were terminated by the addition of trichloroacetic acid to 10%, which dramatically improved the recovery of the ADP-ribosylated G proteins in the final reaction pellets. The pellets were then analyzed by SDS-polyacrylamide gel electrophoresis (with 9% acrylamide, 0.12% bisacrylamide in the resolving gels) as described by Nestler et al. (1989). ADP-ribosylated G protein bands were identified by autoradiography, and levels of ADP-ribosylation were quantitated with a Betagen Betascope blot analyzer. ADP-ribosylation levels were linear over a threefold range of tissue concentration, and were >85% of ADP-ribosylation levels observed with higher NAD or toxin concentrations and longer incubation times. Levels of ^{32}P-ADP-ribose incorporated into $G_{i\alpha}$ and $G_{o\alpha}$ per mg of membrane protein of NAc from control animals were similar to values reported previously (Terwilliger et al., 1991).

Aliquots of brain homogenates (containing 20–40 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis as described above, and proteins in resulting gels were transferred electrophoretically to nitrocellulose papers. The papers were then immunolabeled for G proteins using specific rabbit polyclonal antisera (1:500 dilution) and ^{125}I-labeled goat anti-rabbit IgG (500 cpm/μl; New England Nuclear) exactly as described (Nestler et al., 1990). Alternatively, horseradish peroxidase-conjugated goat anti-rabbit IgG, with chemiluminescence detection, was used with equivalent results. The following antisera (from New England Nuclear) were used: AS/7, which recognizes the two major species of $G_{i\alpha}$ in brain ($G_{i\alpha1}$ and $G_{i\alpha2}$); EC/2, which recognizes a minor species of $G_{i\alpha}$ in brain ($G_{i\alpha3}$) as well as $G_{o\alpha}$; RM/1, which recognizes the two molecular weight species of $G_{s\alpha}$ in brain; and SW/1, which recognizes the two molecular weight species of G_β in brain. Immunolabeled G protein bands were identified by autoradiography or chemiluminescence, and levels of immunolabeling were quantitated by densitometry. Under the immunoblotting conditions used, levels of immunoreactivity for each G protein subunit were linear over at least a threefold range of tissue concentration.

Results

Self-administration

Injections of active or inactive PTX into the NAc did not affect cocaine self-administration on the day of the injection (Fig. 1*A*). However, active PTX produced progressive increases in cocaine self-administration over the next 14 test sessions that reached about 150% of control values from rats treated with inactive PTX [main effect $F(1,11) = 11.264$, $p = 0.006$]. These increases reached statistical significance 6–7 d post-PTX treatment, and appeared to reach a plateau after about 10 d. Most PTX-treated animals self-administered cocaine in highly regular patterns, although some animals initially displayed "burst-like" self-administration patterns. These burst-like response patterns eventually became regular after a few days, while the self-administration rates remained elevated. A significant treatment × test session interaction reflects the differential course of responding of the active and inactive PTX-treated groups [$F(17,187) = 8.017$, $p < 0.001$].

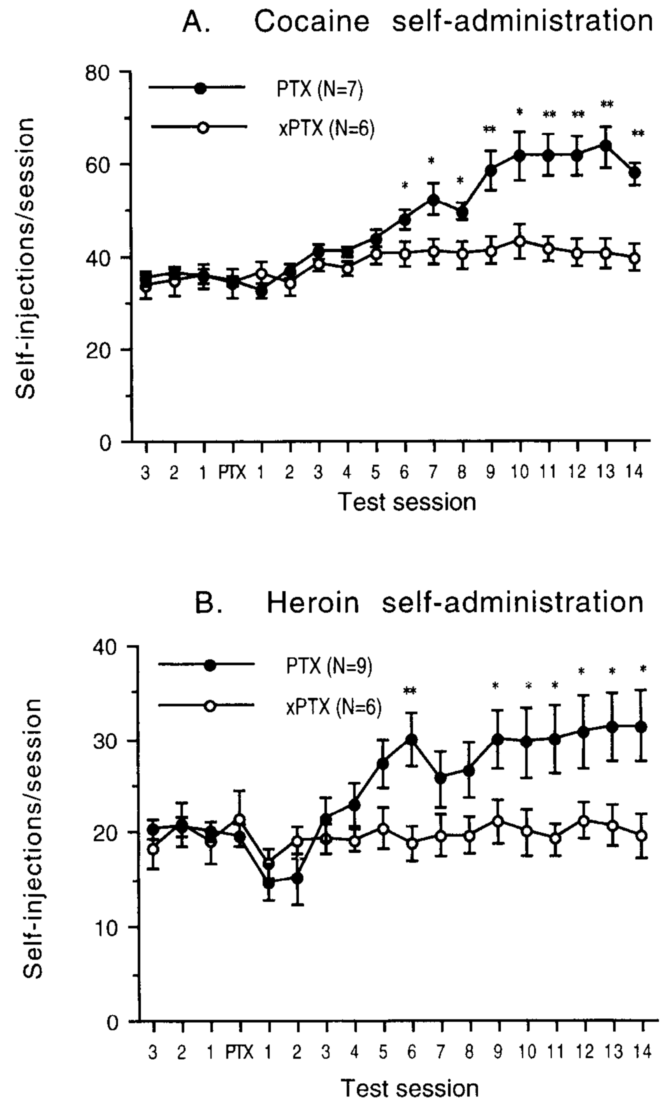

Figure 1. Effects of bilateral PTX injections into the NAc on the maintenance of preestablished intravenous self-administration baselines for cocaine (0.75 mg/kg/injection; *A*) and heroin (0.03 mg/kg/injection; *B*). The data are expressed as the mean ± SEM of self-administration totals from daily 3 hr test sessions. Control rats received similar injections of inactive PTX (*xPTX*). *Asterisks* indicate that values differ from xPTX-treated controls (Student's *t* test): *, $p \leq 0.05$, **, $p \leq 0.01$.

Similar results were obtained for heroin, except that a slight, nonsignificant decrease in heroin self-administration was seen for two test sessions following the PTX injection. These minor decreases were followed by increases in heroin self-administration that reached about 150% of values seen with control rats treated with inactive PTX [Fig. 1*B*; main effect $F(1,13) = 3.698$, $p = 0.077$]. The increased heroin self-administration also reached

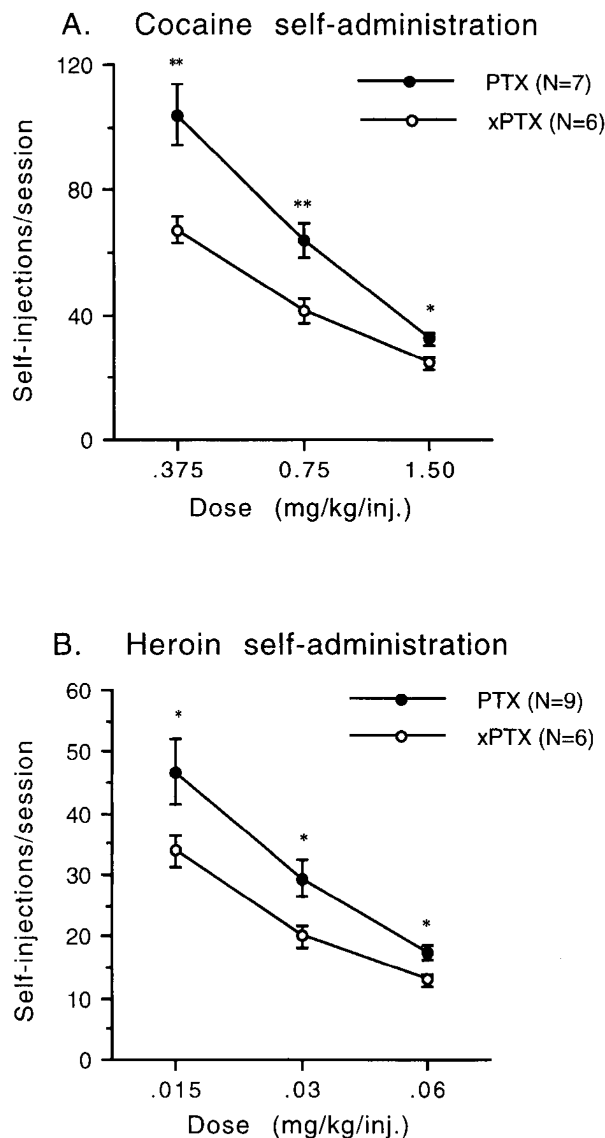

Figure 2. Effects of bilateral PTX injections into the NAc on the dose–response relationship of intravenous cocaine (*A*) and heroin (*B*) self-administration. The data are expressed as the mean ± SEM of self-administration totals as a function of injection dose for daily 3 hr test sessions conducted at the end of maintenance testing. Control rats received similar injections of inactive PTX (*xPTX*). *Asterisks* indicate that values differ from xPTX-treated controls (Student's t test): *, $p \leq 0.05$, **, $p \leq 0.01$.

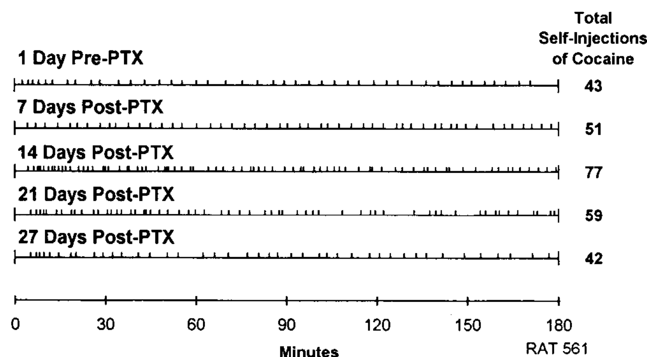

Figure 3. Event records from a representative rat self-administering cocaine (0.75 mg/kg/injection) at various times following a single bilateral injection of PTX into the NAc. The *deflections* mark the time of each self-injection response in 3 hr self-administration tests. This animal exhibited complete recovery from the PTX effect 1 month later, although not all animals showed a similar recovery at this time.

statistical significance about 6 d post-PTX, and was generally maintained throughout the rest of the maintenance test phase. PTX produced a significant treatment × test session interaction in animals self-administering heroin [$F(17,221) = 3.345$, $p < 0.001$], as seen for cocaine.

The effect of PTX on the cocaine dose–response relationship is shown in Figure 2*A*. PTX increased cocaine self-administration at three different injection doses when compared to controls [main effect $F(1,11) = 17.020$, $p = 0.002$], while the inverse relationship between the injection dose and self-administration rate was maintained. Both the injection dose [$F(2,22) = 70.979$, $p < 0.001$] and the treatment × dose interaction [$F(2,22) = 4.441$, $p = 0.024$] produced significant effects on the number of self-injections taken, reflecting a greater effect of PTX at the lower injection doses. The effect of PTX on the heroin dose–response relationship is shown in Figure 2*B*. PTX also increased heroin self-administration at all three injection doses [main effect $F(1,13) = 5.237$, $p = 0.04$], and maintained the inverse relationship between the injection dose and self-administration rate. While the effect of injection dose on self-administration rate was significant [$F(2,26) = 65.805$, $p = < 0.001$], the treatment × dose interaction failed to reach significance [$F(2,26) = 1.835$, $p = 0.18$], reflecting a similar effect of PTX at all three heroin injection doses. However, closer inspection of the data suggests that the PTX-induced increases in self-administration are relatively larger at lower injection doses for both cocaine and heroin, when response rates are higher.

The effects of PTX on cocaine and heroin self-administration are illustrated for representative animals in Figures 3 and 4, respectively. These animals were allowed to self-administer drug for up to 27 d post-PTX treatment. The PTX-induced increases in self-administration were characterized by shortened intervals between successive self-injections. In these and other animals there was evidence of partial recovery from the toxin's effects after 1 month, although many animals did not show recovery from PTX at this time.

PTX failed to increase nonreinforced responding at the inactive lever, where response rates generally were low (0–2 responses/session) both before and after PTX treatment. Some of the PTX-treated animals exhibited heightened excitability when handled, most evident after a self-administration test session. PTX also produced minor weight loss (<10% body weight) in some animals within the first few days after treatment, but the weight loss did not coincide with the onset of the self-administration increases. Two animals in the cocaine experiment and one in the heroin experiment died a few days after PTX treatment, possibly due to seizure activity. In this regard, PTX reportedly facilitates morphine-induced seizures and lethality in mice (Lufty et al., 1991). One animal's catheter in the cocaine experiment lost its patency. These four animals were excluded from the analysis.

Figure 5 is a photomicrograph of a coronal brain section showing a typical bilateral cannula placement in the NAc. The brain slice is from a PTX-treated rat from the cocaine self-administration experiment, stained with cresyl violet. There was no indication of abnormal scarring or gliosis at the injection site

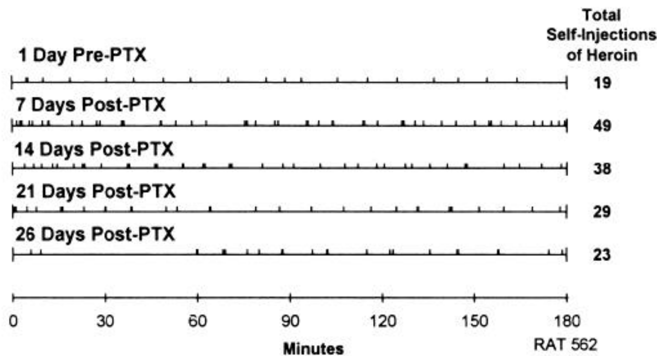

Figure 4. Event records from a representative rat self-administering heroin (0.03 mg/kg/injection) at various times following a single bilateral injection of PTX into the NAc. The *deflections* mark the time of each self-injection response in 3 hr self-administration tests. This animal exhibited complete recovery from the PTX effect 1 month later, although not all animals showed a similar recovery at this time.

in this or other animals. Histological analysis of cannula placements found that all animals had accurately placed cannulae in the anteromedial nucleus accumbens.

Biochemistry

The efficacy and time course of PTX effects on G proteins in the NAc were assessed by subjecting NAc extracts to ^{32}P-ADP-ribosylation in the presence of excess PTX *in vitro*. It was found that the *in vivo* administration of PTX reduced the amount of substrate available for G protein ADP-ribosylation *in vitro* when compared to the contralateral side injected with inactive PTX (Fig. 6*A*). The ^{32}P label was specifically incorporated into two distinct bands corresponding to the molecular weights for the α subunits of G_i (40–41 kDa) and G_o (39 kDa; see Milligan, 1988). The effect of PTX was apparent for both the G_i and G_o bands. The PTX-induced reduction in G protein ADP-ribosylation was slow in onset, reached statistical significance at 7 d, and persisted for up to 28 d (Table 1). At 14 d, the PTX effect was still mainly limited to the injection site, since dissections of tissue immediately surrounding the inner NAc punch ("outer punches") showed a lower and more variable reduction in G_o and G_i ADP-ribosylation of 10% and 15%, respectively, compared to the reduction for G_o and G_i of 56% and 51% observed in the NAc punch.

The effect of PTX on G proteins was assessed further by measuring levels of G protein immunoreactivity by Western blotting. Levels of $G_{i\alpha1,2}$ and $G_{o\alpha}$ immunoreactivity were reduced by PTX injections after 14 d (Fig. 6, Table 1), although the reduction in the level of ADP-ribosylation (G_i, 56%; G_o, 51%) was greater than the reduction in the amount of the G protein subunits ($G_{i\alpha1,2}$, 27%; $G_{o\alpha}$, 36%) at this time point. After 28 d, equivalent reductions were seen in $G_{o\alpha}$ ADP-ribosylation and immunoreactivity. In contrast, PTX injections had no effect on levels of $G_{s\alpha}$ or G_β immunoreactivity.

Figure 7 shows an extended time course for the effect of PTX on both cocaine self-administration and ADP-ribosylation of G proteins. The onset of PTX-induced increases in cocaine self-administration coincides with the onset of PTX-induced decreases in ADP-ribosylation. At 3 d post-PTX treatment, neither cocaine self-administration nor ADP-ribosylation was significantly altered, although small changes were detected in both. In addition, no changes were detected in levels of $G_{i\alpha1,2}$ (not shown) and $G_{o\alpha}$ (Table 1) immunoreactivity at this time. At 7, 14, and 21 d post-PTX treatment, cocaine self-administration rates showed progressive increases, while the reduction in ADP-ribosylation remained stable. At 28 d post-PTX, when cocaine self-administration rates began to recover, levels of ADP-ribosylation and immunoreactivity of $G_{i\alpha1,2}$ (not shown) and $G_{o\alpha}$ (Table 1) remained decreased, possibly even to a greater extent than that observed after 14 d.

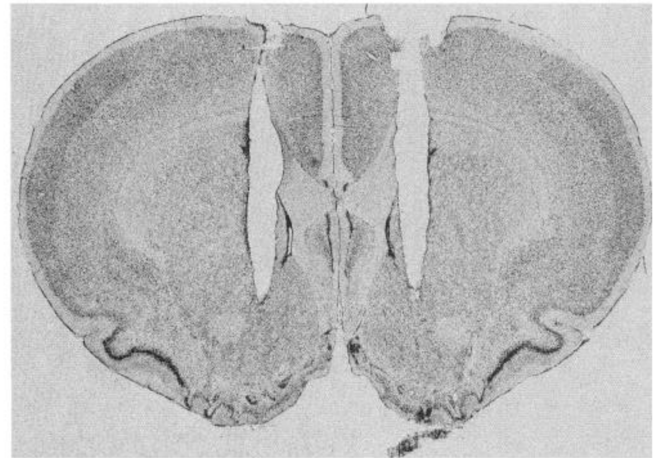

Figure 5. Photomicrograph showing a typical bilateral guide cannula placement in the anteromedial NAc (stained with cresyl violet). Injections were given through 33 gauge injection cannulas that extended 1 mm beyond the 26 gauge guide cannulae. The coronal section is from an animal injected with PTX approximately 1 month prior to sacrifice. In this particular animal, PTX increased cocaine self-administration to 180% of its pre-PTX baseline.

Discussion

Previous studies have used PTX to implicate the inhibitory G proteins G_i and G_o in central dopamine D_2 receptor mechanisms. For example, PTX was found to attenuate D_2 receptor–mediated inhibition of adenylate cyclase (Weiss et al., 1985; Boyajian et al., 1989) and inhibition of spontaneous cell firing (Bickford-Wimer et al., 1990) in striatal neurons, as well as D_2

Table 1. Effect of intra-NAc PTX on G protein ADP-ribosylation and immunoreactivity

	Days after PTX injections			
	0	3	14	28
G protein ADP-ribosylation in NAc				
$G_{o\alpha}$	100 ± 3 (6)	89 ± 14 (6)	44 ± 4*** (6)	38 ± 6* (6)
$G_{i\alpha}$	103 ± 4 (6)	82 ± 13 (6)	49 ± 6*** (6)	39 ± 7* (6)
G protein ADP-ribosylation in outer punch				
$G_{o\alpha}$	117 ± 8 (5)		90 ± 33 (6)	
$G_{i\alpha}$	104 ± 6 (5)		85 ± 30 (6)	
G protein immunoreactivity in NAc				
$G_{o\alpha}$	110 ± 13 (6)	98 ± 17 (6)	64 ± 11* (6)	40 ± 7* (5)
$G_{i\alpha}$	95 ± 6 (6)		73 ± 7** (12)	
G_β	110 ± 13 (12)		91 ± 7 (12)	
$G_{s\alpha}$	102 ± 9 (12)		92 ± 4 (12)	

Data are expressed as mean percentage of control ± SEM (*N*). *, **, and *** indicate that actual values differ ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, by paired *t* test) from the contralateral side treated with inactive PTX.

A: ADP-ribosylation

B: Immunoblotting

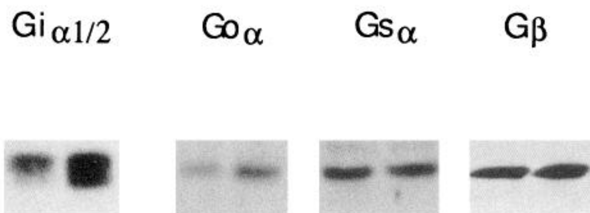

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Figure 6. Autoradiograms showing the effects of unilateral PTX (+) injections into the NAc on levels of ADP-ribosylation (*A*) and immunoreactivity (*B*) of G protein subunits within the NAc. The contralateral side received control injections of inactive PTX (−). *A*, Two major ADP-ribosylated bands are resolved under these conditions: a 40–41 kDa band that corresponds to $G_{i\alpha}$, and a 39 kDa band that corresponds to $G_{o\alpha}$ (Nestler et al., 1989). *B*, The apparent M_r of the immunolabeled bands shown in the figure are, for $G_{i\alpha 1,2}$, 40–41 kDa; $G_{o\alpha}$, 39 kDa; $G_{s\alpha}$, 47 and 42 kDa; and G_β, 35–36 kDa.

autoreceptor–mediated inhibition of dopamine cell firing in the substantia nigra (Innis and Aghajanian, 1987). At the behavioral level, D_2 agonists were found to produce ipsilateral rotation in rats given unilateral striatal injections of PTX, indicating that D_2 receptors utilize inhibitory G proteins to stimulate locomotion (Marin et al., 1991). Furthermore, striatal PTX injections attenuate apomorphine-induced stereotypy (Fujita et al., 1985) and exacerbate apomorphine-induced postural asymmetry (Kelly et al., 1987), and PTX injections in the ventral tegmental area block the soporific effects of D_2 autoreceptor stimulation (Bagetta et al., 1989), implicating inhibitory G proteins in these D_2 receptor–mediated behaviors.

PTX has also been used to implicate inhibitory G proteins in opioid receptor mechanisms. PTX reportedly blocks central μ- and δ- (but not κ-) opioid receptor–mediated inhibition of catecholamine release (Werling et al., 1989; see also Childers, 1991). PTX also blocks μ and δ receptor–mediated electrophysiological activity (Aghajanian and Wang, 1986; Crain et al., 1987; Dunwiddie and Su, 1988) and inhibition of cAMP formation (Duman et al., 1988; Makman et al., 1988; see also Childers, 1991). Behaviorally, intracerebral PTX injections were shown to block morphine analgesia, an indication that central inhibitory G proteins play a role in opioid antinociception (Hoehn et al., 1988; Sanchez-Blazquez and Garzon, 1988; Bodnar et al., 1990; Parolaro et al., 1990; Chang et al., 1991).

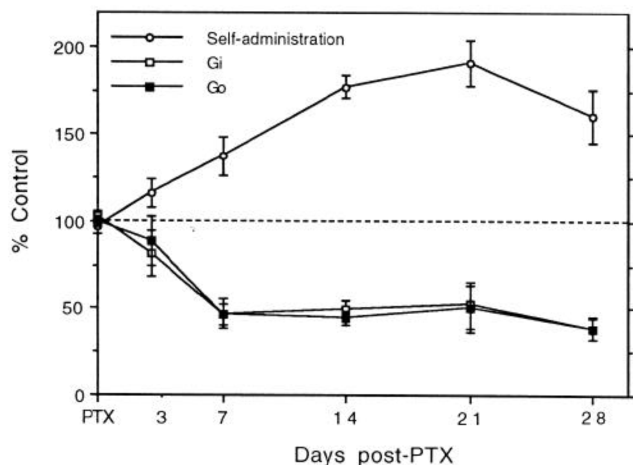

Figure 7. Time course of PTX-induced increases in cocaine self-administration and inactivation of G_i and G_o proteins in the NAc as assessed by ADP-ribosylation. The data for cocaine self-administration are expressed as the mean ± SEM percentage of individual pre-PTX baselines ($N = 6$); the data for ADP-ribosylation experiments are expressed as the mean ± SEM percentage of the contralateral NAc injected with inactive PTX ($N = 6$ for each time point).

In the present study, PTX injections in the NAc increased both cocaine and heroin self-administration. Since the effect of PTX on self-administration is identical to the effect of reducing the self-administered dose of drug, or to pretreating animals with a dopamine or opiate antagonist (Koob and Goeders, 1989), it is suggested that PTX reduces or shortens the reinforcing efficacy of the cocaine and heroin injections, causing animals to compensate by increasing their drug intake. An antagonist-like mechanism of action for PTX is supported further by the finding that PTX shifted the dose–response curves for cocaine and heroin self-administration to the right. Another possible interpretation is that the PTX-induced increases in drug self-administration were caused by some nonspecific rate-increasing influence unrelated to the drug's reinforcing efficacy. The nonspecific interpretation is unlikely for the following reasons. Response rates at the inactive lever were not increased, suggesting that increased self-administration did not reflect an increase in "accidental" lever presses due to psychomotor activation. Also, animals treated with PTX self-administered drug in highly regular response patterns (Figs. 3, 4); such precisely timed response patterns suggest that the rate-increasing effect of PTX is not due to generalized increases in lever pressing (e.g., stereotypy), but rather to reward-related factors that affect the duration of drug reinforcement. Finally, PTX shifted the dose–response curves to the right without disrupting the inverse dose–response relationship; such rightward shifts of self-administration dose–response curves are usually taken to reflect reward-related decrements (Self-and Stein, 1992a). Therefore, the PTX-induced increases in cocaine and heroin self-administration are best interpreted as resulting from decreases in drug reinforcement.

Inactivated PTX failed to alter cocaine and heroin self-administration. This indicates that enzymatic activity is required for PTX to produce its effects. In ADP-ribosylation experiments, active PTX injected into the NAc reduced the amount of 39–41 kDa PTX substrates available for *in vitro* ribosylation,

when compared to the contralateral side injected with inactive PTX. Since the molecular weights of the PTX-labeled substrates are the same as those for $G_{i\alpha}$ and $G_{o\alpha}$ protein subunits, and $G_{i\alpha}$ and $G_{o\alpha}$ are the only substrates for PTX in brain tissue (Neer et al., 1984; Sternweiss and Robishaw, 1984), it is concluded that the *in vivo* PTX injections ADP-ribosylated and, consequently, reduced the amount of functional G_i and G_o proteins in the NAc. Thus, a reduction of functional G_i and G_o proteins in the NAc led to increases in cocaine and heroin self-administration.

The increased drug self-administration would not appear to have been caused by a neurotoxic effect of PTX, since the brains from rats receiving active PTX did not exhibit abnormal gliosis or scarring at the injection site (Fig. 5). In addition, PTX-induced cell death would expectedly lead to a generalized reduction in G protein subunits, and yet only specific PTX substrates ($G_{i\alpha}$ and $G_{o\alpha}$), and not $G_{s\alpha}$ or G_{β}, were reduced (Table 1). While the possibility of cell death was not eliminated completely, our conclusion is supported further by the finding that kainic acid lesions of NAc neurons actually produce decreases (rather than increases) in both cocaine and heroin self-administration (Zito et al., 1985). It is also unlikely that PTX produced its effect outside the NAc, since brain tissue surrounding the NAc punch showed only slight, nonsignificant decreases in ADP-ribosylation, and since intracerebral PTX injections are reported to remain highly localized (Van der Ploeg et al., 1991). In previous reports, NAc injections of dopamine or opioid antagonists were found to produce similar increases in cocaine and heroin self-administration (Phillips et al., 1983; Vaccarino et al., 1985; Corrigall and Vaccarino, 1988; Maldonado et al., 1993), with the effectiveness of these antagonists lost or reduced when injected more dorsally in the caudate-putamen or in the cerebral ventricles. Taken together, these results suggest that the effects of PTX on cocaine and heroin self-administration are due to a PTX-induced blockade of dopamine and opiate receptor–activated G_i and G_o proteins in the NAc. However, these findings do not preclude the possibility that PTX injections into other, more distal reward-related brain regions could similarly attenuate dopamine and opiate receptor–mediated reinforcement. In view of this possibility, we have recently reported that pretreatment with PTX blocks the reinforcing effects of intracranial morphine injections in the hippocampus and VTA (Self and Stein, 1993). Moreover, PTX injections in these brain regions also could reduce the reinforcing effects of intravenous heroin.

The effects of PTX on drug self-administration and G protein ADP-ribosylation were delayed about 6–7 d, which agrees with other behavioral studies using central PTX injections (Parenti et al., 1986; Hoehn et al., 1988; Chang et al., 1991). The tight correlation between the time of onset of the behavioral and biochemical changes suggests that, at least initially, the increased drug self-administration may result directly from a reduction in functional G_i/G_o proteins. However, the PTX-induced increases in self-administration were greatest at about 3 weeks postinjection, whereas reductions in G proteins persisted and may even have increased further after 4 weeks, a time when self-administration rates began to return to baseline (Fig. 7). The recovery of PTX-induced increases in self-administration could possibly be explained by secondary adaptive changes that act to physiologically compensate for a primary inactivation of G proteins. In contrast to the long-term effects of PTX on ADP-ribosylation in the NAc reported here, Steketee et al. (1992) reported almost complete recovery from the reduction in ADP-ribosylation at 30 d following VTA injections of PTX. Reasons for this difference are unknown; one explanation could be a faster turnover of G_i and G_o proteins in the VTA compared to the NAc.

It is, of course, possible that the PTX-induced increases in cocaine and heroin self-administration are not due directly to primary reductions in G_i and G_o protein function per se, but rather to some secondary effect of the toxin-induced decrease in G proteins that then functionally antagonizes reinforcement mechanisms. One secondary effect of intrastriatal PTX injections is to increase the amount of proneurotensin and preproenkephalin A mRNAs 24 hr later (Augood and Emson, 1992). However, these effects would not explain the present findings, since the changes in neuropeptide message appear several days before the changes in drug self-administration. Moreover, increased levels of these peptides would tend to facilitate rather than attenuate reinforcement, since striatal neurotensin facilitates dopamine release (Tanganelli et al., 1989), and enkephalins are themselves self-administered (Belluzzi and Stein, 1977; Goeders et al., 1984). Whether or not other secondary effects of reduced G_i and G_o protein function contribute to the increased drug self-administration is not known.

The PTX-induced reductions in $G_{i\alpha}$ and $G_{o\alpha}$ ADP-ribosylation were accompanied by more slowly developing reductions in levels of $G_{i\alpha}$ and $G_{o\alpha}$ immunoreactivity. The fact that PTX treatment decreases the total amount of $G_{i\alpha}$ and $G_{o\alpha}$ subunits is surprising, since the toxin is believed to inactivate the proteins functionally by ADP-ribosylating specific cysteine residues, and not to reduce directly the total amount of these proteins. In fact, one might expect the opposite result, that PTX-induced reductions in G_i and G_o function would be followed by adaptive increases in the levels of these proteins to compensate for their reduced functional activity. Analogous to our results that functional inactivation of G_i and G_o by PTX is followed by a reduction in the amount of these proteins, it is interesting to note that *in vivo* administration of PTX decreased mRNA levels of $G_{i\alpha 1}$ 15–20 d later in a rat adipocyte model (Ramkumar and Stiles, 1990). Thus, PTX can reduce the amount of functional G_i/G_o proteins by a primary inactivation (ADP-ribosylation) mechanism, followed by a secondary reduction in G_i expression. Further studies in brain tissue are needed to test whether PTX reduces levels of G_i and G_o in brain by decreasing their expression or increasing their breakdown.

Chronic treatment with cocaine or opiates has been shown to reduce $G_{i\alpha}$ and $G_{o\alpha}$ subunits selectively in the NAc (Terwilliger et al., 1991; Nestler, 1992; Striplin and Kalivas, 1993). No other brain region studied had a similar responsivity both to cocaine and morphine. When taken together with behavioral evidence implicating the NAc in motivational processes (Koob and Bloom, 1988), these findings thus are consistent with the idea that long-term drug-induced reductions in G_i and G_o proteins in the NAc may be implicated in the motivational changes underlying drug addiction (Nestler, 1992). If so, PTX-induced inactivation of these G proteins in the NAc might also facilitate the development of drug addiction. Such facilitation could be reflected in the motivational symptoms reported by human addicts, such as drug craving. However, the PTX-induced increases in drug self-administration reported here may not reflect increases in drug craving, since increases in drug craving do not necessarily lead to increases in drug self-administration under fixed-ratio schedules of reinforcement (Roberts and Richardson, 1992). Future studies using PTX in animal models of drug craving and

relapse to self-administration might clarify whether a reduction in G protein levels in the NAc is an important intracellular event underlying these processes.

Finally, it should be noted that agonists acting selectively at dopamine D_1 receptors also have reinforcing effects (Self and Stein, 1992b), which appropriately are blocked by D_1, but not D_2, antagonists (Self et al., 1993). These reward-related D_1 receptors may be located in the NAc (Maldonado et al., 1993). Because D_1 receptors activate G_s proteins that lead to an increase in cAMP formation, one is presented with the paradox that drug reinforcement may be mediated by opposing signal transduction pathways in the NAc—one pathway involving activation of G_s and the other activation of G_i/G_o. This apparent inconsistency—that opposite signal transduction pathways in the NAc may be activated to induce reinforcement—might be reconciled if the two pathways operated in separate populations of neurons. This possibility is supported by the finding that striatal D_1 and D_2 receptors, which activate G_s and G_i/G_o signal transduction pathways, respectively, appear to be expressed largely by separate neuronal populations that project to distinct brain regions (Gerfen, 1992). Alternatively, it is conceivable that dopamine D_1 and D_2 receptor-mediated reinforcement mechanisms utilize different second messenger systems in the same neurons, since some central dopamine D_1 receptors may be coupled to second messenger systems other than cAMP formation (Andersen et al., 1990). In any case, the fact that both G_s and G_i/G_o signal transduction pathways modulate cAMP levels, together with the finding that chronic cocaine or opiate exposure upregulates the cAMP system in the NAc, almost certainly implicates the NAc cAMP system in a general way in drug reinforcement and addiction.

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