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# Behavioral Sensitization, Behavioral Tolerance, and Increased [$^3$H]WIN 35,428 Binding in Rabbit Caudate Nucleus After Repeated Injections of Cocaine

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ALOYO, V. J., P. S. PAZDALSKI, A. L. KIRIFIDES AND J. A. HARVEY. *Behavioral sensitization, behavioral tolerance, and increased [$^3$H]WIN 35,428 binding in rabbit caudate nucleus after repeated injections of cocaine.* PHARMACOL BIOCHEM BEHAV **52**(2) 335–340, 1995.—This study examined whether changes in the behavioral response to repeated intravenous injections of cocaine hydrochloride (4 mg/kg, twice daily for 22 days) might be related to alterations in the dopamine (DA) transporter as measured by the binding of the potent cocaine analog [$^3$H]WIN 35,428 to membranes derived from fresh caudate tissue. Rabbits demonstrated both tolerance and sensitization. Tolerance occurred for cocaine elicited convulsions, whereas sensitization occurred to the ability of cocaine to elicit motor activity, facial twitches, and head bobbing. Cocaine-exposed animals demonstrated a significant 17% increase in the $B_{max}$ of specific [$^3$H]WIN 35,428 binding to caudate membranes with no change in $K_d$. The increase in $B_{max}$ was observed at 42 but not 96 h after the last chronic cocaine administration. There was no change in [$^3$H]WIN 35,428 binding at 42 h after a single injection of cocaine. We suggest that the upregulation of the dopamine transporter in the caudate nucleus reflected the mechanisms involved in tolerance rather than sensitization.

Cocaine WIN 35,428 Tolerance Sensitization Caudate Rabbit

COCAINE administration may result in the development of either tolerance (16,17,48,49) or sensitization (14,19,29,30,35) to its behavioral effects. It has been suggested that the dose, route, and frequency of cocaine administration, as well as the environmental context and behavior being measured, may play an important role in determining whether one obtains tolerance or sensitization (17,33,34,37,48); however, the precise conditions that would reliably produce these opposing effects remain unknown. The neurochemical correlates of tolerance and sensitization to cocaine remain equally obscure. Although cocaine blocks the neuronal uptake of 5-HT, norepinephrine (NE), and DA (39), it is thought that cocaine's inhibition of the striatal DA transporter (DAT) is a major factor modulating the behavioral response to cocaine injection (13,40,44).

Tolerance to cocaine results in cross tolerance to WIN 35,428, a potent congener of cocaine (16). Because both cocaine and WIN 35,428 bind to the same site on the DAT (3,23), tolerance to cocaine might be related to changes in the number or affinity of DAT sites. This possibility was examined in the rat (2,46) and in postmortem brain tissue of human cocaine abusers (22,42). These studies reported an upregulation of the DAT in striatum as measured by increases in [$^3$H]cocaine binding (2) and [$^3$H]WIN 35,428 binding (22, 42,46). Unfortunately, behavioral data were not obtained to indicate whether upregulation of the DAT in these studies was

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related to sensitization or tolerance. These changes may not have been related to the development of sensitization, because chronic cocaine injections were reported to produce increased motor activity in BALB/cByJ (19,35), C57BL6/J, and CD-1 mice (35) but to have no effect on [$^3$H]WIN 35,428 binding. However, other strains of mice (DBA/2J and B6AF1/J) demonstrated both sensitization and an increase in [$^3$H]WIN 35,428 binding (19).

In an attempt to resolve some of these issues, we used rabbits to determine whether the chronic administration of cocaine would produce an increase in the density of cocaine binding sites on the DAT and whether such a change would be related to the development of tolerance and/or sensitization. To obtain a more extended behavioral measure of sensitization and tolerance, we rated 11 separate behavioral reactions to cocaine across 22 days of injection. The rabbit was used because of the ease with which one can carry out repeated intravenous injections, thus mimicking the effects of crack cocaine. As an index of cocaine binding sites, we measured the binding of the cocaine analog [$^3$H]WIN 35,428 because it demonstrates cross tolerance to cocaine (16), has the same behavioral profile as cocaine (5,9,41), labels the same site on the DAT as cocaine, and is ideal for binding studies because of its stability, high affinity, and slow rate of dissociation (3,6,23,38,40).

## METHODS

### *Animals*

Female Dutch Belted rabbits, which were proven breeders, were purchased from Myrtle's Rabbitry (Thompson, TN) and housed in an American Association for the Accreditation of Laboratory Animal Care accredited facility maintained at 23°C, on a 12 L : 12 D cycle with lights on at 0600 h. Rabbits had unrestricted access to rabbit chow and water. These studies were conducted in accordance with the principles and procedures outlined by the NIH Guidelines for Care and Use of Experimental Animals and were approved by our Institutional Animal Care and Use Committee.

### *Drug Injections and Behavioral Ratings*

Rabbits were administered (−)cocaine hydrochloride (a gift of National Institute on Drug Abuse) at a dose of 4 mg/kg (11.8 μmol/kg), via the marginal ear vein in a volume of 2 ml/kg. Acute treatment consisted of a single injection of either cocaine (4 mg/kg) or vehicle (physiologic saline). Chronically treated animals received cocaine or vehicle for 22 days. Injections were given twice daily at 0900 and 1600 h for a total daily cocaine dose of 8 mg/kg. Animals were observed after each injection by two observers and rated for the presence of 11 behavioral responses: pupillary dilation, increased respiration, increased motor activity, thumping with rear legs, Straub tail, hind limb extension, hind limb abduction, head bobbing, body tremor, facial twitch, and convulsions. The data were calculated for each day as the percentage of animals displaying each of these 11 behavioral responses. This procedure was adopted on the basis of previous studies involving chronic cocaine administration to the rabbit (28).

### *Membrane Preparation*

At 42 or 96 h after the last injection of cocaine or vehicle, rabbits were decapitated, their brains were rapidly removed, and the head of the caudate nucleus was dissected free of surrounding brain tissue. Care was taken to exclude the putamen and internal capsule. Only fresh tissue was used in these experiments. The caudate was immediately placed in ice-cold buffer and all subsequent steps were performed at 4°C. All experiments employed a 20-mM sodium phosphate buffer, pH 7.4, at 4°C, containing 0.32 M sucrose. Washed membranes were prepared as previously described (18). Briefly, tissue was homogenized in 10 vol (by weight) of the phosphate-sucrose buffer using a Teflon-glass homogenizer. Homogenates were centrifuged at 40,000 × *g* for 20 min. The resulting pellet was resuspended in 50 vol of the phosphate-sucrose buffer using a Brinkman Polytron (Westbury, NY; 10 s at half power) followed by centrifugation as described earlier. The final pellet was dispersed in phosphate-sucrose buffer using a Polytron.

### *Binding Assay*

Equilibrium binding assays were performed at 4°C in polystyrene tubes containing 0.5 nM [$^3$H]WIN 35,428 (specific activity 81–87 Ci/mmol; New England Nuclear, Boston, MA), increasing concentrations of unlabelled WIN 35,428 (0.1–1000 nM), and washed membranes in a total volume of 1 ml. Nonspecific binding was defined by the addition of 30 μM (−)cocaine. The reaction was initiated by the addition of the membrane fraction obtained from 1 mg original tissue weight, and the incubation was continued for 40 min. To terminate the reaction and to separate bound from free ligand, the mixture was filtered through Whatman (Clifton, NJ) GF/B glass fiber filters (presoaked in 0.15% w/v polyethylenimine) using a Brandel Cell Harvester (Gaithersburg, MD) and washed with three 5-ml aliquots of the phosphate-sucrose buffer. The amount of radioactivity retained on the filter was determined by liquid scintillation counting.

### *Experimental Design and Data Analysis*

The chronic administration of cocaine was carried out with 15 pairs of animals, each consisting of a cocaine-injected animal and its vehicle-injected control. Changes in the percentage of animals displaying each of the 11 behavioral reactions to cocaine across the 22 days of injection were tested for significant trends by means of the $\chi^2$ test (45). Following this 22-day period, 10 pairs of animals were examined at 42 h and five pairs at 90 h after the last injection of cocaine or vehicle. A similar procedure was carried out with an additional five pairs of animals receiving a single acute injection of cocaine or vehicle, and their brains were obtained at 42 h after the last injection. Because only fresh tissue was employed for the binding assays, the schedule of injections was staggered so that membranes could be prepared from one pair of animals at a time. The binding experiments were analyzed using the nonlinear curve-fitting program LIGAND (27) as modified for microcomputers [(24) EBDA/LIGAND; Biosoft, Milltown, NJ). This program provides estimates of the number of binding sites, $B_{max}$, and the dissociation constant, $K_d$. One- and two-site binding models were calculated and compared using the *F*-test. Because the binding data were obtained in separate pairs of animals, a matched pair *t*-test was employed to test for differences in $B_{max}$ and $K_d$ between cocaine- and vehicle-injected animals (45). Significance for all statistical comparisons was set at $p < 0.05$, using a two-tailed test.

## RESULTS

### *Behavioral Reactions to Cocaine*

Rabbits demonstrated a significant development of tolerance (Fig. 1A) and sensitization (Fig. 1B) to several effects of

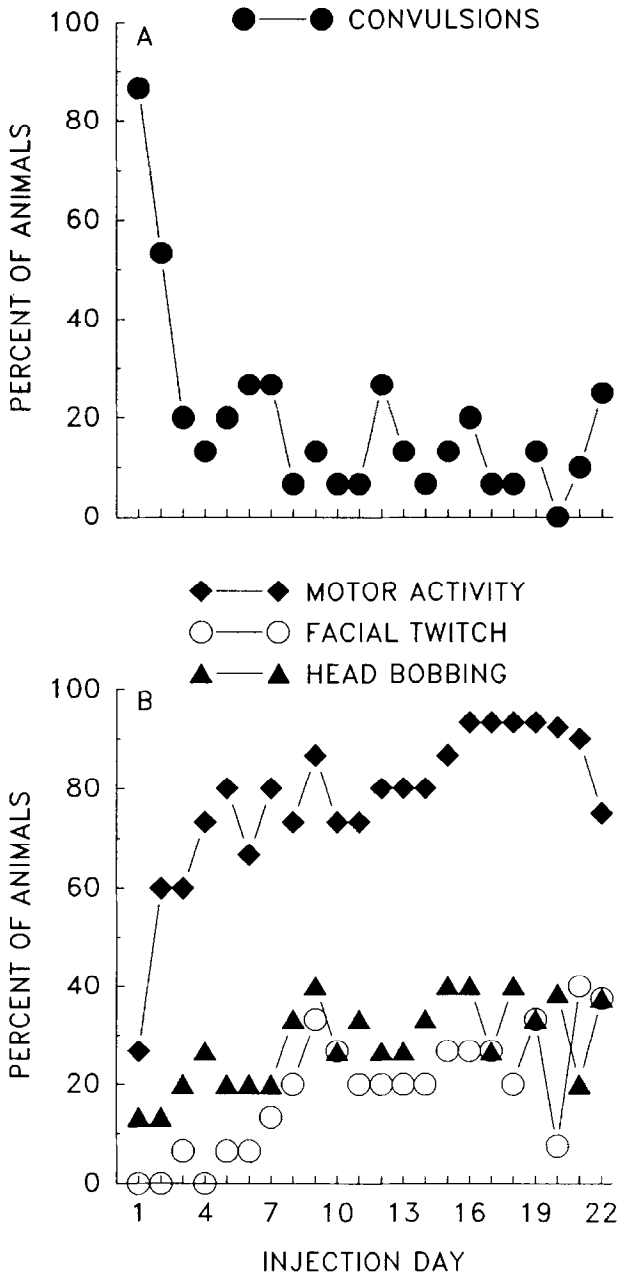


FIG. 1. Development of tolerance (A) and sensitization (B) to the behavioral effects of cocaine. Adult female Dutch Belted rabbits were administered cocaine HCl at a dose of 4 mg/kg, IV, twice daily for 22 days. Data are plotted as the percentage of animals displaying a particular behavioral response on each of the 22 injection days. The decreases in behavioral reactions shown in A and the increases in behavioral reactions shown in B were significant at $p < 0.05$.

cocaine ($p < 0.05$ for each behavior). There was a large and rapid development of tolerance as measured by the percentage of animals demonstrating convulsions (Fig. 1A). The percentage of animals demonstrating convulsive behavior on the first day of cocaine injections was 87%; this rapidly declined to 20% by day 3 and varied between 0 and 27% thereafter.

Motor activity demonstrated sensitization as reflected by a large rise in the percentage of animals displaying this behavior from 27% on day 1 to 73% on day 4, and then rose gradually to >90% by day 16 (Fig. 1C). There was also a small, though significant, increase in the percentage of animals demonstrating facial twitches (from 0% on day 1 to 33% on day 9) and head bobbing (13.3% on day 1 to 40% on day 9).

A number of autonomic, skeletal, and behavioral responses to cocaine failed to demonstrate any significant change across the 22 days of injection: thumping of the hind limbs (range 53–80%), hind limb extension (range 0–27%), body tremor (range 0–33%), hind limb abduction (range 0–13%), and Straub tail (range 0–40%). Pupillary dilation and increased respiration occurred in all animals from the first to the last day of injection.

### *Binding of [$^3$H]WIN 35,428 to Caudate Membranes*

The saline- and cocaine-treated animals of Fig. 1 were sacrificed in pairs at 42 or 96 h after the last injection, and [$^3$H]WIN 35,428 binding parameters were determined by homologous displacement assays using freshly prepared caudate membranes. The binding data were then analyzed by LIGAND to provide estimates of $K_d$ and $B_{max}$. This analysis was carried out on the 10 pairs of animals assayed at 42 h after the last chronic injection, the five pairs of animals assayed at 90 h after the last chronic injection, as well as the five pairs of animals assayed at 42 h after an acute injection. Analysis of the binding data for all of these animals, by LIGAND, indicated a preference for a one-site fit. The average $K_d$ and $B_{max}$ for the 20 control animals were 2.5 ± 0.1 nM and 345 ± 16 fmol/mg of tissue, respectively.

The binding data obtained for the 10 pairs of rabbits at 42 h after the last chronic injection revealed that there was a significantly ($p < 0.01$) higher $B_{max}$ value for the cocaine-treated animals as compared with the corresponding $B_{max}$ values for their paired saline-injected controls (Fig. 2A). The increase in $B_{max}$ at 42 h occurred in eight of the 10 animals, with the mean difference being 52 ± 13 fmol/mg tissue, which was a 17 ± 5% increase from control values. A representative Scatchard plot of binding data obtained from a pair of animals at 42 h after cessation of chronic cocaine and saline injections is presented in Fig. 3. The linearity of the two Scatchard plots was consistent with a single class of binding sites. As can be seen in Fig. 3, the rabbit that had received chronic cocaine injections demonstrated a higher $B_{max}$ than its control (as indicated by differences in the x-axis intercepts) but no change in $K_d$ (as reflected by parallel slopes). In this example, the $B_{max}$ value of the rabbit receiving chronic cocaine (437 fmol/mg) was 19% higher than that of its saline control (366 fmol/mg).

A single acute injection of cocaine had no significant effect on the $B_{max}$ for [$^3$H]WIN 35,428 binding at 42 h after injection (Fig. 2A). The significant increase in $B_{max}$ observed at 42 h after the last chronic injection of cocaine was no longer present by 90 h (Fig. 2A). The affinity ($K_d$) for [$^3$H]WIN 35,428 binding was not significantly altered by either acute or chronic cocaine treatment (Fig. 2B).

## DISCUSSION

The results of this study indicate that the repeated, twice daily, intravenous injection of cocaine for 22 days resulted in an increase in the number of [$^3$H]WIN 35,428 binding sites ($B_{max}$) with no change in affinity ($K_d$). Because both cocaine and WIN 35,428 bind to the same site on the DAT (3,6,

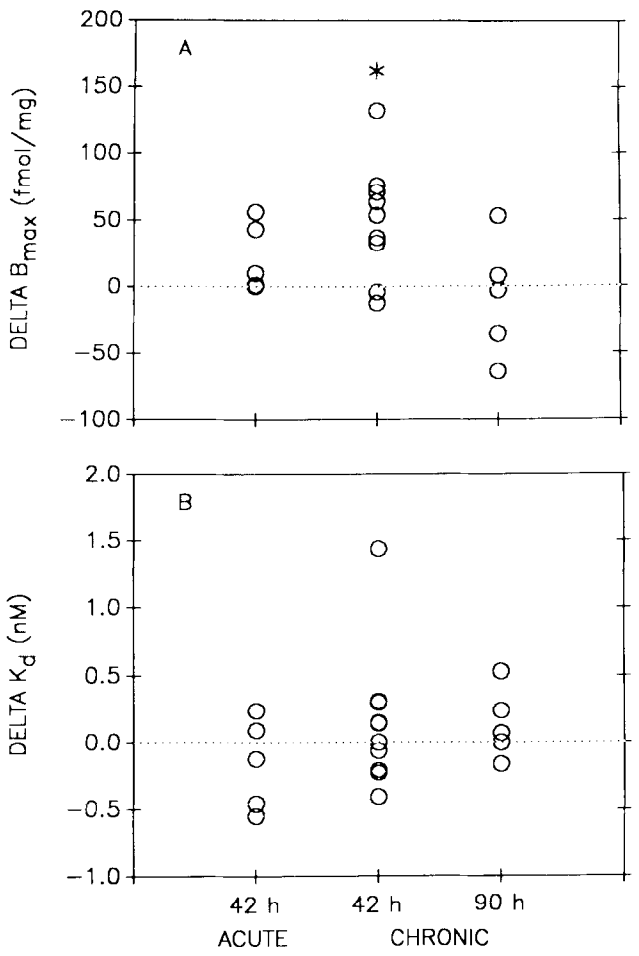


FIG. 2. Chronic cocaine transiently increased the $B_{max}$ for [$^3$H]WIN 35,428 binding without altering its $K_d$. The $B_{max}$ and $K_d$ for [$^3$H]WIN 35,428 binding to rabbit caudate membranes were determined 42 h after a single injection or 42 and 90 h following the last chronic injection of cocaine or saline. Pairs of saline- and cocaine-treated rabbits were sacrificed at the same time, and the binding parameters were determined in parallel assays. The difference (cocaine minus saline) in $B_{max}$ values (A) or $K_d$ values (B) is plotted for each pair of animals. *Significant difference from controls as measured by a matched pair $t$-test ($p < 0.01$).

13,23,36,38), these results suggest that chronic administration of cocaine produces an upregulation of the DAT. A similar conclusion was reached based on the findings of an upregulation of [$^3$H]cocaine or [$^3$H]WIN 35,428 binding sites in caudate tissue of rats (2,46) and some strains of mice (19) after repeated injections of cocaine, and an upregulation of [$^3$H]WIN 35,428 binding sites in postmortem caudate tissue obtained from human cocaine abusers (22,42).

Cocaine produces an increase in the extracellular content of DA (8,12,14,29,31,32), presumably because of its ability to bind to the DAT and thus prevent the neuronal reuptake of DA (13,38). Indeed, the extracellular concentration of cocaine in striatum has been demonstrated to be highly correlated with the resulting extracellular concentration of DA (29). It is well established that the brain can partially compensate for the blockade of postsynaptic DA receptors by an increase in receptor density with no change in receptor affinity (43). Our data and those of others (2,22,42,46) demonstrate that the presynaptic DAT is capable of similar adaptive changes as reflected by an increase in the number of transporter sites with no change in their affinity for cocaine or its analogs. However, the behavioral results of this study indicate that a functional role for this upregulation of DAT in the caudate nucleus remains unclear.

The behavioral measures employed in this study demonstrated that animals can develop both sensitization and tolerance to cocaine depending on the behavioral reaction being examined. Although previous studies have reported the occurrence of sensitization (14,19,29,30,35) and tolerance (16,17, 48,49), our study is unique in demonstrating that both changes can occur in the same animal with an intermittent injection procedure. However, these behavioral results make it difficult to determine whether the behavioral role of DAT upregulation in the caudate nucleus is related to sensitization or tolerance. The difficulty lies in part with our lack of knowledge concerning the anatomical substrates for many of the behaviors that have been measured in this and other studies (16,17,35,48,49), the dependence of behavioral outcomes on the dose and frequency of cocaine administration (33,34,37,48), and the fact that chronic cocaine can produce homeostatic changes in other transmitter systems (10), which may in turn modulate the expression of DA-mediated behaviors. Thus, convulsive activity appears to be mediated, at least in part, by $D_1$ dopamine receptors (4,15), but this involvement may be minor (47) and not fully dependent on dopaminergic activity in the caudate nucleus. Although DA is well known to be importantly involved in motor activity, this behavior appears to be primarily mediated by the nucleus accumbens rather than the caudate nucleus (11). These differences in the anatomical regions involved in the expression of various DA-mediated behaviors may be crucial for resolving the basis for the development of sensitization and tolerance. Though the literature is complex, there are suggestions that sensitization and tolerance to chronic cocaine exposure are due to different homeostatic responses of the DAT mediated by different regions of the dopaminergic system. For example, it has been reported that the regulation

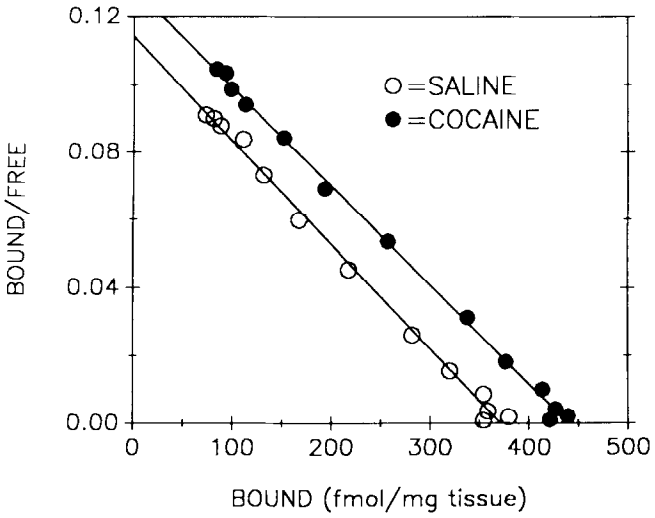


FIG. 3. Representative Scatchard plot of [$^3$H]WIN 35,428 binding to caudate membranes at 42 h after the chronic injection of either cocaine or saline. The two rabbits were assayed in parallel.

of DA uptake in the caudate nucleus occurs by a different mechanism from that in nucleus accumbens (26); this finding is consistent with reports of a heterogeneity of the DAT between caudate nucleus and nucleus accumbens (20,21).

Although the data are complex, recent reports suggest that the cocaine-induced upregulation of the DAT in caudate is mediated by $D_2$ dopamine receptors (25,31). For example, the tolerance induced by continuous cocaine administration was associated with $D_2$ dopamine autoreceptor supersensitivity with no effect on postsynaptic $D_2$ dopamine receptors (17), whereas procedures producing sensitization had no effect. Moreover, the upregulation of the DAT produced by chronic cocaine administration was blocked by a $D_2$ dopamine receptor antagonist, pimozide (31). Finally, the increased uptake of DA produced by the acute administration of the DA agonist quinpirole was blocked by the $D_2$ dopamine receptor antagonist sulpiride and the increased uptake of DA produced by depolarization was blocked by both acute and chronic administration of the $D_2$ receptor antagonist haloperidol (25). It was concluded that the increased extracellular content of DA produced by cocaine leads to an activation of presynaptic $D_2$ dopamine receptors, and that this in turn produces an upregulation of the DAT. The upregulation of the DAT in the caudate would in turn increase the reuptake of DA and reduce the ability of cocaine to increase extracellular DA, thus accounting for the development of tolerance. In partial confirmation of this hypothesis, the increase in extracellular DA in caudate produced by cocaine has been reported to undergo tolerance (12).

In contrast, sensitization as measured by motor activity was concluded to be associated with a downregulation of the DAT in the nucleus accumbens but not in the caudate nucleus (7). Moreover, in contrast with changes in the caudate nucleus, the nucleus accumbens demonstrated an increased release of DA during chronic cocaine treatment (14). Moreover, when a schedule of cocaine injection was employed that produced sensitization of a caudate-mediated behavior (stereotypy), there was also an increase in the ability of cocaine to increase extracellular DA in the caudate nucleus (1). This suggests that sensitization is due, at least in part, to a downregulation of the DAT that in turn decreases the ability of DA neurons to take up DA, and thus allows cocaine to produce an increased extracellular content of DA, resulting in the sensitization of behaviors.

We propose that the increase in [$^3$H]cocaine and [$^3$H]WIN 35,428 binding in caudate that has been reported in this and other studies (2,22) represents a compensatory presynaptic response to the repeated blockade of the DAT produced by the chronic administration of cocaine. Thus, tolerance to the behavioral effects of cocaine is due to an upregulation of the DAT, which in turn increases the ability of DA neurons to take up DA and thus reduces the ability of cocaine to elevate extracellular DA content in the caudate nucleus.

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