Dysregulation of Dopamine Transporter Trafficking and Function after Abstinence from Cocaine Self-Administration in Rats: Evidence for Differential Regulation in Caudate Putamen and Nucleus Accumbens

Devadoss J. Samuvel, Lankupalle D. Jayanthi, Senthivelan Manohar, Kolanjiappan Kaliyaperumal, Ronald E. See, and Sammanda Ramamoorthy

Division of Neuroscience Research, Department of Neurosciences, Medical University of South Carolina, Charleston, South Carolina

ABSTRACT

The profound alterations produced by cocaine on dopamine (DA) neurotransmission raise the possibility that dopamine transporter (DAT)-expressing neurons may modify DA transport in response to repeated cocaine exposure to maintain the appropriate efficiency of DA clearance. In this study, we determined the changes in molecular mechanisms of DAT regulation in rats with a history of repeated cocaine self-administration followed by 3 weeks of abstinence. Using ex vivo caudate putamen (CPu) and nucleus accumbens (NAcc) synaptosomal preparations, we found that DA uptake was significantly higher in the CPu and NAcc of cocaine-experienced animals compared with yoked saline animals. Surface distribution, p-Ser phosphorylation, and protein phosphatase 2A catalytic subunit (PP2Ac) interaction of DAT were all altered in the CPu. Maximal velocity ($V_{\text{max}}$) values were elevated both in the CPu and NAcc of cocaine-experienced rats compared with saline controls. Although there was no change in the apparent affinity for DA in the CPu, increased DA affinity was evident in the NAcc. Consistent with elevated DAT activity in cocaine-experienced animals, a higher level of surface DAT, DAT-PP2Ac association, and decreased serine phosphorylation of DAT were observed in the CPu, but not in the NAcc. These results, for the first time, suggest that chronic cocaine self-administration followed by abstinence leads to persisting alterations in normal DAT trafficking and catalytic regulatory cascades in the CPu and NAcc in a brain region-specific manner.

Drug abuse and dependence to psychostimulants (e.g., cocaine) and other drugs of abuse constitutes a major health problem worldwide, with profound social and financial costs. Although cocaine inhibits both the serotonin transporter (SERT) and norepinephrine transporter (NET), several lines of evidence have shown that cocaine produces its primary reinforcing effects by binding to the dopamine (DA) transporter (DAT) and blocking the reuptake of DA into presynaptic terminals, thereby potentiating DA neurotransmission in mesocorticolimbic reward pathways (Wise and Rompre, 1989; Robinson and Berridge, 1993; Koob et al., 1998; McFarland and Kalivas, 2001). The relevance of DAT in DA homeostasis and in the reinforcing effects of cocaine are supported by several studies on cocaine-induced behaviors, and on neurotransmitter synthesis, storage, release, and DA receptor expression resulting from removal of DAT genes in mutant mice, and changes described in human cocaine addicts (Fumagalli et al., 1998; Jones et al., 1998; Sora et al., 2001; Jayanthi and Ramamoorthy, 2005; Jayanthi et al., 2007). Thus, long-term changes in DAT levels, kinetics, or regulation would be expected to greatly influence spontaneous and drug-induced behaviors.

The search for neuroadaptations in DAT expression has focused on studies in human cocaine-dependent users and a variety of animal models of cocaine addiction. Several strategies have been adapted to quantify DAT levels, including...
DAT mRNA in situ hybridization, DAT binding using specific DAT ligands, and in vivo imaging techniques. Based on the existing literature, controversy exists as to whether DAT expression/activity is increased, decreased, or shows no changes after repeated cocaine administration (Ng et al., 1991; Cass et al., 1993; Staley et al., 1994; Mash and Staley, 1997; Little et al., 1998; Zahniser and Doolen, 2001; Chefer and Shippenberg, 2002; Mash et al., 2002; Izenwasser, 2004). Nonetheless, the molecular basis for the alterations in DA transport outlined above remains largely unknown. In addition, DAT proteins can be rapidly regulated by G protein-coupled receptors and protein kinase/phosphatase-linked pathways, including those triggered by activation of protein kinase C (PKC), phosphatidylinositol 3-kinase, mitogen-activated protein kinase, calcium-calmodulin dependent protein kinase II, and PPI/PP2Ac (Carvelli et al., 2002; Foster et al., 2003; Morón et al., 2003; Vaughan, 2004; Bolan et al., 2007; Zapata et al., 2007). Phosphorylation of DAT proteins and DAT-associated proteins are involved in dynamic trafficking-dependent and -independent regulation of DAT function (Foster et al., 2002; 2003; Vaughan, 2004). Recent studies examining the concomitant changes in DA transport and membrane DAT expression suggest that these events are correlated (Melikian, 2004). To our knowledge, there has been no evidence presented for relevant alterations in DAT function, presynaptic cell surface expression, and the degree to which kinase-mediated modulation of DAT phosphorylation and trafficking occur using an animal model of cocaine self-administration. The importance of DAT in the homeostasis of DA in the brain, the abundant evidence of its dysfunction in addiction, and the possible role of DAT as a target for therapeutics raise the question of whether alterations in DA homeostasis in cocaine exposure derive from compromised DAT trafficking and post-translational modifications.

In an effort to delineate the functional status and relevant molecular adaptations of DAT proteins in rats that have been trained to self-administer cocaine followed by abstinence, the present study examined DA transport, functional expression of DAT protein at the plasma membrane, phosphostatus of DAT, and DAT-PP2Ac association. In addition, based on growing evidence for differences in ventral and dorsal striatal function after chronic cocaine (Porrino et al., 2004; Everitt, 1997; Little et al., 1998; Zahniser and Doolen, 2001; Chefer and Shippenberg, 2002) via different regulatory cascades.

**Subjects.** Male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), weighing 275 to 300 g at the start of the experiment, were individually housed in a temperature- and humidity-controlled vivarium on a 12-h light/dark cycle. Rats were maintained on ad libitum water and 20 to 25 g of rat chow (Harlan, Indianapolis, IN) per day. The housing and care of the rats followed the guidelines of the Guide for the Care and Use of Laboratory Rats (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, 1996). Rats were given a minimum of 4 days for adaptation and handling before the start of the experiment.

**Lever Response Training.** Rats were trained to lever press on a fixed ratio 1 schedule of food reinforcement (45-mg pellets; Noyes, Lancaster, NH) in sound-attenuating operant conditioning chambers (30 × 20 × 24 cm in height; MED Associates, St. Albans, VT) during a 16-h overnight lever response training session. The chambers were equipped with two retractable levers, a stimulus light above each lever, a food pellet dispenser between the levers, a house light on the wall opposite to the levers, and a speaker connected to a tone generator (ANL-926; MED Associates). During the session, each lever press on the active lever resulted in the delivery of a food pellet only. Lever presses on the inactive lever had no programmed consequences. After lever response training, food pellets dispensers were removed from the chambers.

**Surgery.** Forty-eight hours after lever response training, rats were anesthetized using a mixture of ketamine hydrochloride and xylazine (66 and 1.33 mg/kg i.p., respectively) followed by equithesin (0.5 ml/kg of a solution of 9.72 mg/ml pentobarbital sodium, 42.5 mg/ml chloral hydrate, and 21.3 mg/ml magnesium sulfate heptahydrate dissolved in a 44% propylene glycol, 10% ethanol solution; i.p.).

Chronic indwelling catheters were constructed using a bent steel cannula with a screw-type connector (Plastics One, Roanoke, VA), SILASTIC tubing (10 cm; i.d. 0.64 mm; o.d. 1.19 mm; Dow Corning, Midland, MI), Prolite polypropylene monofilament mesh (Atrium Medical Corporation, Hudson, NH), and cranialplastic cement, as described previously (Fuchs et al., 2004). The end of the catheter was inserted into the right jugular vein, and it was secured to surrounding tissue with suture. The catheter ran s.c., and it exited on the rat’s back, posterior to the shoulder blades.

To maintain catheter patency, the catheters were flushed once daily for 3 days after surgery with 0.1 ml of an antibiotic solution of cefazolin (10.0 mg/ml; Schein Pharmaceutical, Florham Park, NJ) dissolved in heparinized saline (70 U/ml; Elkins-Sinn, Cherry Hill, NJ) and then with 0.1 ml of heparinized saline. Thereafter, catheters were flushed with 0.1 ml of heparinized saline (10 U/ml) before each self-administration session and with 0.1 ml of the cefazolin solution and 0.1 ml of heparinized saline after each session. Catheter patency was verified periodically by infusing 0.10 to 0.12 ml of methohexital sodium (10 mg/ml, i.v.; Eli Lilly & Co., Indianapolis, IN), which produces a rapid loss of muscle tone only when administered intravenously.

**Cocaine Self-Administration.** After surgery, rats were randomly divided into active cocaine self-administration or yoked saline groups (final n = 43/group). Rats self-administered cocaine for 2-h daily sessions for 10 days along a fixed ratio 1 schedule of cocaine reinforcement (cocaine hydrochloride provided by the National Institute on Drug Abuse, Research Triangle Park, NC). These parameters of cocaine self-administration have been widely used in previous cocaine self-administration experiments, and we chose them for comparability with previous studies. At the start of each session, the rat’s catheter was connected to a liquid swivel (Instech, Plymouth Meeting, PA) via polyethylene 20 tubing that was encased in steel spring leashes (Plastics One). The swivel was suspended above the operant conditioning chamber, and it was connected to an infusion pump (model PHM-100; MED Associates). The house light was illuminated throughout each session. Lever presses on the active lever resulted in a 2-s activation of the infusion pump and a 5-s presentation of the white stimulus light above the active lever. Cocaine hydrochloride was dissolved in sterile saline, it was filtered using a Sartorius GS 0.2 filter, and a 100 ml/ml saline solution was used.

**Materials and Methods.**

**Materials and Reagents.** Phosphoserine antibody, DA, cocaine, and detergents were obtained from Sigma-Aldrich (St. Louis, MO). [3H]Dopamine, protein A-Sepharose beads, ECL reagents, and ECL-enhanced films were obtained from GE Healthcare (Chalfont St. Giles, UK). Sulfo-NHS-SS-biotin and monomeric avidin beads were from Pierce Chemical (Rockford, IL). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Anti-calnexin was obtained from Nventa Biopharmaceuticals (San Diego, CA). Antibody to PP2Ac was from BD Biosciences (San Diego, CA). The DAT antisemur directed against 42 to 59 amino acids corresponding to the N terminus of DAT was used. Other reagents were of the highest grade possible from standard sources.
0.45-μm Ultracleaning Filter Unit (Fisher Scientific, Pittsburgh, PA), and it was delivered at a dose of 0.6 mg/kg (50-μl infusion). After each infusion, responses on the active lever had no consequences during a 20-s time-out period. During the sessions, responses on the inactive (nococaine-paired) lever were recorded, but they had no programmed consequences. Yoked saline rats were placed in test chambers in the same manner as the cocaine self-administration rats, but they received an infusion of saline (50 μl) over 2 s, contingent upon the cocaine infusion received by the self-administering rat in the adjacent self-administration chamber. In addition, yoked saline rats experienced the 5-s presentation of the white stimulus light in a paired manner with the cocaine self-administration rats.

**Abstinence.** After chronic cocaine self-administration or yoked saline infusions, rats from each group underwent abstinence for 21 days. We chose a period of 21 days, because this represents a time well beyond active cocaine reinforcement, when long-lasting changes would be apparent. Furthermore, animals at this time period show robust cocaine-seeking behavior in relapse models (Fuchs et al., 2006). Although the rats were never returned to the self-administration chambers, they were removed from their home cages at the same time each day as during the active self-administration phase, and they were transported to an alternate testing room for a 2-h period to control for handling and daily removal from the vivarium room setting.

**Synaptosome Preparations.** Rats were rapidly decapitated, and the brains were collected in ice-cooled dishes. The CPu and NAcc were rapidly dissected and collected in 10 volumes (w/v) of ice-cold 0.32 M sucrose. The tissue was immediately homogenized using a Teflon-glass homogenizer under chilled water, then it was centrifuged at 1000 g for 15 min at 4°C. The resulting supernatant was centrifuged at 15,000g for 20 min, and the pellet was washed by resuspending in 0.32 M sucrose (Samuvel et al., 2005). The synaptosomes were suspended in regular Krebs-Ringer-HEPES buffer, pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 10 mM HEPES, 1.2 mM MgSO4, 1.2 mM KH2PO4, 5 mM Tris, and 10 mM d-glucose) saturated with 95% O2, 5% CO2. Protein concentration was determined by detergent-compatible protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. The synaptosomal preparation was used immediately for experiments. The CPu or NAcc were pooled from two or four rats based on the experiments conducted, and in such a way, all the experiments were repeated at least three times.

**DA Uptake.** DA uptake was performed as described previously (Samuvel et al., 2005). In brief, 25 to 50 μg of synaptosomes were incubated in 250 μl of Krebs-Ringer-HEPES buffer, pH 7.4, containing 0.1 mM ascorbic acid and 0.1 mM pargyline and 40 nM [3H]DA for 3 min. Synaptosomes were preincubated with the DAT inhibitor nomifensine (100 μM) at 37°C for 10 min followed by the addition of [3H]DA to determine the nonspecific DA uptake. For saturation analysis, [3H]DA was mixed with unlabeled DA from 10 nM to 2 μM. Nonspecific [3H]DA uptake was defined as the accumulation in the presence of 100 μM nomifensine, and it was subtracted from total uptake. Uptake was terminated with the addition of 3 ml of ice-cold phosphate-buffered saline followed by rapid filtration over 0.3% polyethylenimine-coated GF/B filters (Whatman, Clifton, NJ) on a cell harvester (Brandel Inc., Gaithersburg, MD). Filters were washed rapidly with 5 ml of ice-cold phosphate-buffered saline, and radioactivity bound to filter was counted by liquid scintillation counter. Mean values of specific uptake ±S.E.M. of at least three separate experiments were determined.

**Immunoprecipitations and Immunoblotting.** DAT immunoprecipitations and immunoblotting were performed from detergent extracts of synaptosomes as described previously (Bauman et al., 2000). Additional experiments to test specificity were carried out with irrelevant IgG, preimmune serum, or protein A-Sepharose alone. Protein samples (20 μg) were subjected to 4 to 15% linear gradient SDS-PAGE, they were blotted to polyvinylidene difluoride membrane, and then they were probed with specific antibodies as indicated in the figures and legends. Immunoreactive bands were visualized by chemiluminescence (ECL reagent; GE Healthcare). In our initial immunoblot analysis, we optimized the linearity of DAT and PP2A band detection 1) by using various protein concentrations from CPu and NAcc extracts with a fixed concentration of antibodies, 2) with a fixed concentration of extract with various dilutions of antibodies, 3) with multiple exposures of immunoblots, and finally 4) by comparing internal control such as calnexin. These multiple internal controls were adapted to ensure that the antibody/protein loaded and band development on the film were within the linear range. Band densities were quantified by scanning and analyzed using National Institutes of Health ImageJ version 1.32j (http://rsb.info.nih.gov/ij/ software).

**Surface Biotinylation.** Synaptosomes (500 μg) were treated with sulfo-NHS-biotin (1 mg/ml protein) for 30 min at 4°C in ice-cold Krebs-bicarbonate buffer. Subsequently, the samples were washed with the same buffer containing 100 mM glycine, and the pellet was resuspended in radioimmunoprecipitation assay lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg pepstatin, and 250 μM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM sodium fluoride, 50 μM sodium pyrophosphate, 5 mM sodium orthovanadate, and 1 μM okadaic acid). The resuspended synaptosomes were triturated 10 times through a 25-gauge needle, and then they were centrifuged at 40,000g for 20 min. The biotinylated proteins were separated from clear solubilize by incubating with monoemeric avidin beads for 4 h at 4°C. Beads were washed three times with radioimmunoprecipitation assay buffer, and bound biotinylated proteins were eluted with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 5% beta-mercaptoethanol) for 20 min at 22°C. Aliquots from total extracts (20 μg), unbound fractions (20 μg), and entire eluted fractions were separated by SDS-PAGE (10%), transferred to membrane, and probed with DAT-specific antibody. DAT-reactive proteins were visualized using ECL plus reagent followed by exposure to Hyperfilm-ECL (GE Healthcare). Multiple exposures of immunoblots were taken to ensure that the band development on the film was within the linear range. Band densities were quantified by scanning, and they were analyzed using National Institutes of Health ImageJ version 1.32j (http://rsb.info.nih.gov/ij/) software. Subsequently, the blots were stripped and reprobed with anti-calnexin antibody to validate the surface biotinylation of plasma membrane proteins. DAT densities from total, nonbiotinylated (representing the intracellular pool), and biotinylated (representing the surface pool) fractions were normalized using levels of calnexin in the total extract (Samuvel et al., 2005).

**Statistical Analyses.** All values are expressed as mean ± S.E.M. For both cocaine self-administration and biochemical data, statistical differences between the means of the two groups were determined by Student’s t test. A value of p < 0.05 was considered statistically significant.

**Results**

**Self-Administration.** The two experimental groups did not significantly differ in body weight (mean ± S.E.M. calculated for the last 3 days of self-administration: cocaine, 327.2 ± 3.5 g and saline, 329.6 ± 3.4 g; t94 = 0.52, p = 0.61). The number of i.v. infusions (cocaine or yoked saline) calculated for the last 3 days of self-administration was 33.5 ± 1.8/session, and the cocaine group self-administered 20.5 ± 1.1 mg/kg/session. Figure 1 shows active and inactive lever responding averaged over the last 3 days of self-administration for both experimental groups. As expected, animals that actively self-administered cocaine showed significantly higher active lever responding than yoked saline-
treated animals ($t_{94} = -3.52, p < 0.001$), and they showed selectively higher responding on the active lever, with saline-yoked animals exhibiting higher pressing on the inactive lever relative to the cocaine group ($t_{94} = 4.08, p < 0.001$).

**Cocaine-Induced Increases in DAT Activity in the CPu and NAcc.** To investigate the effect of cocaine-self administration followed by abstinence on DA clearance, specific DAT-mediated DA uptake was analyzed and compared with yoked saline rats using CPu and NAcc synaptosomal preparations. As shown in Fig. 2, DAT-mediated DA uptake was significantly elevated in both the CPu and NAcc in cocaine-treated rats compared with saline-yoked rats (131.2 ± 2.9 and 185.6 ± 24.3%, respectively). Because nomifensine has effects on the norepinephrine transporter, we tested an additional group of animals using the selective DAT blocker GBR 12909 (0.2 μM). As with nomifensine, elevated DAT activity was evident in both the CPu and NAcc in cocaine-treated rats when GBR 12909 was used as DAT blocker (data not shown). For the DAT blocker nomifensine, GBR 12909- and NaCl-insensitive DA transport was not altered in either the CPu or the NAcc in both groups (data not shown). Our initial optimizing assay conditions for nomifensine (100 μM)-sensitive DAT activity in CPu and NAcc synaptosomes showed linear DA uptake with respect to uptake time, 1 to 15 min, and protein, 5 to 75 μg, using 50 nM labeled DA (data not shown). Therefore, we used a 5-min uptake time using 30 μg of synaptosomal proteins in all subsequent DAT assays.

**Cocaine-Induced Changes in the Kinetic Properties of DAT Are Differentially Altered in the CPu and NAcc.** The kinetic parameters of the DAT (Michaelis-Menten constant, $K_m$, and maximal velocity, $V_{max}$) were determined in the CPu and NAcc (Fig. 3). In the CPu, after abstinence from cocaine self-administration, the $V_{max}$ value was increased (yoked saline, 146.1 ± 8.8 pmol/mg protein/min; cocaine, 231.5 ± 28.9 pmol/mg protein/min; $p < 0.05$), with no significant changes in the $K_m$ value ($K_m$ values: yoked saline, 248.1 ± 48.6 nM; cocaine, 352.7 ± 60.3 nM). In the NAcc, the $V_{max}$ value was also significantly higher (yoked saline, 301.1 ± 18.6 pmol/mg protein/min; cocaine, 408.3 ± 35.6 pmol/mg protein/min; $p < 0.05$), but with a significant reduction in the $K_m$ value in the cocaine group relative to yoked saline rats ($K_m$ values: yoked saline, 381.7 ± 36.6 nM; cocaine, 96.4 ± 29.2 nM; $p < 0.05$).

**Cocaine-Induced Alterations in DAT Plasma Membrane Expression, but Not Total DAT Protein Expression.** The elevated $V_{max}$ value for DA transport found in the CPu and NAcc in rats with a history of cocaine self-administration may be caused by alterations in trafficking events, whereby more functional DAT is available on the neuronal cell surface for DA uptake. Alternatively, an increase in total DAT biosynthesis could increase both total and surface cell membrane DAT. To investigate these possibilities, we measured cell surface DAT by surface biotinylation and immunoblotting. In a previous study, surface biotinylation strategy has been successfully used to determine the cell surface distribution of DAT and other amine transporters (Ramamoorthy et al., 1998). Figure 4 illustrates the total expression of DAT (82 kDa) and the levels of surface DAT in cocaine and yoked saline rats. There was no significant difference in the level of total DAT protein expression in either the CPu or NAcc between cocaine and yoked saline rats (Fig. 4A). However, after abstinence from cocaine self-administration, there was an increase in the amount of immunoreactive DAT pro-

---

**Fig. 1.** Active and inactive lever responding (mean ± S.E.M.) during self-administration. Responses on the active and inactive levers exhibited by rats are averaged for the last three days of cocaine self-administration ($n = 43$) or yoked saline infusions ($n = 43$). *, $p < 0.001$, compared with saline.

**Fig. 2.** DA transport in synaptosomes from NAcc and CPu after abstinence from cocaine self-administration. Dopamine uptake in synaptosomes isolated from CPu and NAcc. Synaptosomes were prepared from the CPu and NAcc derived from rats that self-administered cocaine or received yoked saline infusions, followed by 3 weeks of abstinence. Synaptosomes (30 μg of protein) were preincubated for 5 min at 37°C, and then they were assayed for 5 min using 40 nM [3H]DA. Non-specific uptake was defined as the uptake in the presence of 100 μM nomifensine and subtracted from the total accumulation to yield specific DAT-mediated DA uptake. The results are expressed as percentage of saline, and data represent mean ± S.E.M. of three experiments. Tissue from the CPu or NAcc were pooled from three rats from the saline and cocaine groups. *, $p < 0.001$, compared with saline (CPu); #, $p < 0.005$, compared with saline (NAcc) by a two-tailed Student’s $t$ test.

---

The elevated $V_{max}$ value for DA transport found in the CPu and NAcc in rats with a history of cocaine self-administration may be caused by alterations in trafficking events, whereby more functional DAT is available on the neuronal cell surface for DA uptake. Alternatively, an increase in total DAT biosynthesis could increase both total and surface cell membrane DAT. To investigate these possibilities, we measured cell surface DAT by surface biotinylation and immunoblotting. In a previous study, surface biotinylation strategy has been successfully used to determine the cell surface distribution of DAT and other amine transporters (Ramamoorthy et al., 1998). Figure 4 illustrates the total expression of DAT (82 kDa) and the levels of surface DAT in cocaine and yoked saline rats. There was no significant difference in the level of total DAT protein expression in either the CPu or NAcc between cocaine and yoked saline rats (Fig. 4A). However, after abstinence from cocaine self-administration, there was an increase in the amount of immunoreactive DAT pro-
Dopamine Transporter Trafficking after Chronic Cocaine

Fig. 3. Kinetic characteristics of DA transport mediated by DAT in CPu and NAcc synaptosomal preparations after abstinence from cocaine self-administration. Synaptosomes (30 μg) from CPu (A) and NAcc (B) were preincubated at 37°C for 5 min followed by DA uptake measured over a concentration range of 0.01 to 2 μM using a 5-min uptake period. In parallel, nonspecific uptake at each concentration of DA used (in the presence of 100 μM nomifensine) was subtracted from total uptake to calculate DAT-mediated DA uptake. Values (mean ± S.E.M.) are from three independent experiments (each experiment used CPu or NAcc tissue pooled from three or four rats). Nonlinear curve fits of data for uptake used the generalized Michaelis-Menten equation (KaleidaGraph; Synergy Software, Reading, PA) to obtain \( V_{\text{max}} \) and \( K_m \) values for DA uptake. Data were analyzed using two-tailed Student’s \( t \) test.

Discussion

Long-term changes in DAT levels, kinetics, or regulation would be expected to greatly influence spontaneous and drug-induced behaviors. Cocaine binds with DAT and inhibits DA reuptake in both dorsal (caudate putamen) and ventral (nucleus accumbens) striatum, which primarily receive their dopaminergic inputs from the substantia nigra and ventral tegmental area, respectively. The present study fo-
cused on changes after 3 weeks of abstinence from cocaine self-administration to ascertain lasting adaptive changes well after the period of drug taking. We found cocaine-induced increases in DA uptake in both the CPu and NAcc. More importantly, the data presented here provide novel evidence that elevated DAT activity in the CPu and NAcc is accompanied by distinctly different changes in the biochemical, kinetic, and regulatory properties of DAT, and protein-protein interactions. These changes may have profound implications for understanding dysfunctional DA regulation that may underlie susceptibility to relapse to renewed cocaine seeking and drug consumption. DA transport is a property of the DAT protein at the cell surface, and to date, the reported ligand binding studies have not actually discriminated cytoplasmic from surface pools because available radiolabeled antagonists are membrane-permeant. Thus, the major limitation across these studies is the lack of assessment of functional DAT activity and plasma membrane expression.

We used i.v. cocaine self-administration, an animal model with a high degree of face validity, to determine long-lasting changes in DAT function after a history of cocaine exposure. The 3-week abstinence period provided a withdrawal period well beyond the time of active cocaine taking, and it represents a point when animals with a history of drug taking are highly susceptible to reinstatement to cocaine seeking (Grimm et al., 2001; Fuchs et al., 2006). Assessment of striatal tissue obtained after abstinence showed enhanced DAT activity in both the CPu and NAcc. Analysis of DA transport kinetics indicated a significant increase in the transport capacity ($V_{\text{max}}$) in the CPu, and the NAcc. However, the apparent affinity for DA remained unchanged in the CPu, whereas in the NAcc, higher apparent DA affinity to DAT was noted. Microdialysis studies have shown that the higher DAT $V_{\text{max}}$ resulted in lower level of extracellular DA found in rats that had 24-h cocaine access followed by withdrawal (Mateo et al., 2005). Previous reports have shown enhanced potency of cocaine to inhibit DAT after daily noncontingent injections of cocaine for 3 days (Izenwasser and Cox, 1992). However, Mateo et al. (2005) observed reduced efficiency of cocaine to block DAT in the core subregion of the NAcc in rats that had 24-h access to cocaine followed by withdrawal. Our observations of change in the apparent substrate affinity and the demonstrated altered cocaine efficacy to inhibit DAT suggest altered intrinsic transport properties. However, further studies are needed to determine the physiological relevance of altered DAT affinity in the NAcc. Surface biotinylation studies indicated the presence of a higher amount of cell surface DAT in the CPu, but unaltered surface DAT density in the NAcc. Taken together, these results demonstrate that increased DA uptake in the CPu and NAcc is associated with a composite change in DAT catalytic properties and subcellular distribution.

It is possible that altered DAT kinetic properties and sub-

Fig. 4. Expression and subcellular distribution of DAT in the CPu and NAcc after abstinence from cocaine self-administration. A, DAT immunoblot using CPu and NAcc synaptosomes. Surface biotinylation of synaptosomes with sulfo-NHS-SS-biotin was performed as described in the text. Aliquots (20 μg) of total and nonbiotinylated fractions and entire biotinylated fractions were subjected to SDS-PAGE followed by immunoblotting with affinity-purified DAT antibody. A representative DAT (82-kDa) immunoblot of three separate experiments is shown. B, quantitative analysis of DAT band densities. The 82-kDa DAT protein band intensities were quantified using National Institutes of Health ImageJ version 1.32j as described under Materials and Methods. The densities of DAT band from three separate experiments were shown (each experiment used CPu or NAcc tissue pooled from three or four rats). Data are presented as mean ± S.E.M. percentage change from the saline group. *, $p < 0.005$, compared with saline (CPu) by a two-tailed Student's $t$ test.
cellular distribution after abstinence from cocaine self-administration may arise from altered DAT phosphorylation and DAT association with PP2Ac. We have previously shown that amine transporters exist as a complex with PP2Ac in native and transfected cells (Bauman et al., 2000). Increased DAT phosphorylation by activation of PKC and/or inhibition of PP2A and PP1 have been suggested for inhibition of DAT activity concomitant with transporter sequestration and/or DA efflux (Foster et al., 2002; Khoshbouei et al., 2004; Vaughan, 2004). Consistent with this possibility, the current findings that the decreased level of DAT serine phosphorylation with a higher amount of PP2Ac association with DAT in the CPu suggest that the DAT-associated PP2Ac dephosphorylate phospho-DAT to retain the DAT on the cell surface and to increase DA uptake after cocaine self-administration. Alternatively, chronic cocaine occupancy on DAT may induce long-lasting conformational changes within the DAT protein that allow it to be a better substrate for PP2Ac and a poorer

**Fig. 5.** Status of DAT serine phosphorylation in the CPu and NAcc from saline- and cocaine-abstinence rats. A, representative immunoblot showing the DAT phosphorylation status on serine residue(s) from CPu and NAcc synaptosomes. Detergent extraction, immunoprecipitation, SDS-PAGE, and immunoblot were performed as described under Materials and Methods. B, bottom blot is the top blot after treatment with phosphatase that eliminated immunoreactivity DAT protein band of phospho-Ser-phosphorylated DAT protein by anti-phospho-Ser (p-Ser-DAT). C, parallel experiments using irrelevant IgG, preimmune serum, or protein-Sepharose A were performed to validate the specificity of DAT-specific antibody in immunoprecipitations. Note that no p-Ser DAT is seen in CPu extract using irrelevant (preimmune) IgG or protein-Sepharose A. D, quantitative analysis of DAT band densities (82 kDa). Protein band intensities were quantified using National Institutes of Health ImageJ version 1.32j as described under Materials and Methods. The densities of DAT band from three separate experiments were shown (each experiment used CPu or NAcc tissue pooled from three or four rats). Data are presented as mean ± S.E.M. percentage of change from the saline group. #, p < 0.005, compared with saline (CPu) by a two-tailed Student’s t test.

**Fig. 6.** DAT interaction with PP2Ac in CPu and NAcc from saline and cocaine-abstinence rats. The synaptosomes were solubilized and immunoprecipitated with DAT-specific antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis for PP2Ac as described under Materials and Methods. A, representative immunoblots are shown for DAT/PP2Ac associations from the CPu and NAcc from saline and cocaine rats. B, bar plot displays averaged PP2Ac protein band (36-kDa) densities from three separate experiments. Data are presented as mean ± S.E.M. percentage of the saline group. #, p < 0.005, compared with saline (CPu) by a two-tailed Student’s t test.
substrate for protein kinases. Although the mechanisms remain unclear, acute exposure to DAT inhibitors (e.g., cocaine and GBR12935) has been shown to influence DAT phosphorylation in vitro (Cervinski et al., 2005). DAT-associated PP2Ac may also regulate interactions of other proteins or regulatory factors with DAT. It is, therefore, possible that reduced DAT phosphorylation on serine residues may perturb DAT function by inducing more functional DAT on the surface. Thus, dysregulation of DAT phosphorylation on serine residues may provide a signal for elevated DAT activity in the CPUs.

In spite of elevated DAT activity in the NAcc, DAT surface density, DAT serine phosphorylation, and DAT-PP2Ac association remain unaltered, suggesting a trafficking-independent pathway, perhaps via adaptations in DAT intrinsic properties. Supportive of these observations, changes in DAT cell surface expression have been reported following phosphatidylinositol 3-kinase and mitogen-activated protein kinase 1/2 modulation, but not by PKC activation, even though there was an inhibition of DA uptake (Lin et al., 2003). In contrast, Morin et al. (2003) demonstrated that mitogen-activated protein kinase-mediated DAT up-regulation is a combination of both trafficking-dependent and -independent cascades. These findings suggest that changes in transporter activity cannot always be accounted for by changes in surface transporter level and that they may be different based on the type and/or isoform of kinase involved, and differences between cell environments. Analogous to this hypothesis, it is possible that the differences observed between the CPu and NAcc with regard to DAT kinetics, subcellular distribution, phosphorylation, and PP2A association may account for alterations in different signaling pathways specific to NAcc after abstinence from cocaine self-administration, reflecting the distinct functional properties of DAT in the CPu and NAcc. The role of other DAT-interacting proteins and phosphorylation of DAT on threonines and tyrosine residues cannot be ruled out as contributing factors in the observed changes in DAT functional up-regulation. For example, syntaxin 1A, PICK1, Hic-5, PKC, and α-synuclein have all been documented in trafficking-dependent and -independent regulation of DAT (Torres et al., 2003; Zahniser and Sorkin, 2004; Jayanthi et al., 2007). Future studies involving identification of the role of such putative modifiers of DAT regulatory pathways are warranted to fully understand how DAT proteins are perturbed from normal dynamic regulation in a long-lasting manner due to chronic cocaine exposure.

The different regulatory and kinetic transport characteristics we identified in the CPu and NAcc also suggest the potential for functionally distinct populations of DAT that could be spatially and temporally regulated via different presynaptic receptors. Protein phosphorylation and dephosphorylation are common rapid and reversible means of transducing signals from cell surface receptors into varied cellular responses. It should not be surprising that DA neurons are able to use phosphorylation to control DA neurotransmission by altering DAT activity. Activation and deactivation of DAT via phosphorylation and dephosphorylation can affect the availability of synaptic DA, which regulates dopaminergic signaling. In this regard, the current study has identified for the first time the perturbation of these normal DAT regulatory mechanisms after abstinence from chronic cocaine self-administration. Although we assessed changes in DAT at a critical withdrawal time point (3 weeks) when enduring neuro-adaptations are manifested, future studies will be needed to assess dynamic changes in DAT function at multiple time points both during and after cocaine exposure. Our results do not rule out the possibility that, under certain experimental conditions, our current finding of DAT dysregulation in our animal model could proceed through alternative pathways. Depending on the route and time of cocaine intake, different withdrawal training used, and species of subjects used, DAT regulation or trafficking in response to chronic cocaine intake may differ from what we have observed in CPu and NAcc of abstinence rats followed by cocaine self-administration. Such changes could reflect differential involvement of signaling pathways to regulate DAT functions.

The unique neuroadaptations of DAT in dorsal versus ventral striatum observed in the current study may account for differences in ascending dopaminergic modulation of pathways that trigger and maintain drug-seeking behavior. The regionally specific effects of DAT regulation are of particular interest in light of growing evidence for dorsal striatal-based changes as an important mechanism underlying compulsive drug seeking (Gerdeman et al., 2003; Everitt and Robbins, 2005). Although the current results provide evidence for previously unidentified changes in DAT protein after abstinence from cocaine self-administration, whether these neuroadaptations in DAT account for dysregulation of motivational behavior associated with cocaine addiction remains to be explored. Because cocaine also binds with NET and SERT, we cannot exclude the possibility that dysregulation of NET and SERT also exists after abstinence from cocaine self-administration.

References
Dopamine Transporter Trafficking after Chronic Cocaine


Address correspondence to: Dr. Sammanda Ramamoorthy, Division of Neuroscience Research, Department of Neurosciences, Medical University of South Carolina, 173 Ashley Ave., BSB 403, Charleston, SC 29425. E-mail: rama@musc.edu