Cocaine Induction of Dopamine Transporter Trafficking to the Plasma Membrane

KARLEY Y. LITTLE, LAWRENCE W. ELMER, HUAILING ZHONG, JOSHUA O. SCHEYS, and LIAN ZHANG

Departments of Psychiatry (K.Y.L., H.Z., L.Z.) and Neurology (L.W.E.), University of Michigan, Ann Arbor, Michigan; and Ann Arbor Veterans Affairs Medical Center, Ann Arbor, Michigan (K.Y.L., L.W.E., J.O.S.)

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Several previous human postmortem experiments have detected an increase in striatal [³H]WIN 35428 binding to the dopamine transporter (DAT) in chronic cocaine users. However, animal experiments have found considerable variability in DAT radioligand binding levels in brain after cocaine administration, perhaps caused by length and dose of treatment and type of radioligand used. The present experiments tested the hypothesis that [³H]WIN 35428 binding and [³H]dopamine uptake would be increased by exposure to cocaine through alterations in DAT cellular trafficking, rather than increased protein synthesis. Experiments were conducted in stably hDAT-transfected N2A cells and assessed the dose response and time course of cocaine effects on [³H]WIN 35428 binding to the DAT, [³H]dopamine uptake, measures of DAT protein and mRNA, as well as DAT subcellular location. Cocaine doses of 10^{-6} M caused statistically significant increases in [³H]WIN 35428 binding and [³H]dopamine uptake after 12 and 3 h, respectively. Despite these increases in DAT function, there was no change in DAT total protein or mRNA. Immunofluorescence and biotinylation experiments indicated that cocaine treatment induced increases in plasma membrane DAT immunoreactivity and intracellular decreases. The present model system may further our understanding of regulatory alterations in DAT radioligand binding and function caused by cocaine exposure.

Clinical studies indicate that human cocaine users dying during exposure to cocaine have increased striatal [³H]WIN 35428 binding to the dopamine transporter (DAT; Little et al., 1993, 1998, 1999; Staley et al., 1994), accompanied by up-regulation of dopamine uptake (Mash et al., 1997). Such functional alterations could be important, perhaps contributing to cocaine-induced binging, withdrawal symptoms, or craving. Beyond drug self-administration, dopamine neurons play a role in other rewarding phenomena, including sex (Everitt, 1990) and eating (Phillips et al., 1993), suggesting that regulatory alterations in DAT function could have interesting implications for understanding the dynamics of a number of motivational and appetitive processes. Recent experiments in cell culture have determined that phosphorylative treatments (Pristupa et al., 1998; Daniels et al., 1999; Melikian and Buckley, 1999) or exposure to the stimulant d-amphetamine (Saunders et al., 2000) dynamically regulate DAT function by changing DAT cellular localization, perhaps invoking mechanisms that might be related to those activated by cocaine.

Understanding the mechanisms involved in DAT binding site changes is important because 1) binding site alterations are the primary alterations documented in postmortem brain from cocaine users; 2) DAT inhibitors/ligands are being developed extensively as both therapeutic and imaging agents for both the DAT as well as dopamine neurons; and 3) DAT regulation may provide broader insights into the pharmacological effects of drugs on transporter binding sites. In addition to our need to uncover cocaine's neurochemical effects that provoke symptoms associated with its dependence, it is possible that new therapeutic or imaging agents (many of which are often DAT uptake inhibitors) might themselves induce adaptations in DAT function, as well as alter cocaine's effect on dopamine uptake. Also, potentially, DAT inhibitor binding sites could be altered through some mechanism that is independent of changes in DAT concentration or function.

Human experiments examining the effects of cocaine on DAT regulation (Hurd and Herkenham, 1993; Hitri et al., 1995; Wilson et al., 1996), as well as a number of animal experiments (Kula and Baldessarini, 1991; Benmansour et al., 1992; Alburges et al., 1993; Koff et al., 1994; Wilson et al., 1994; Hitri et al., 1996; Pilotte et al., 1996; Letchworth et al., 2000), have found variability in DAT binding levels after

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ABBREVIATIONS: hDAT, human dopamine transporter; MAT, monoamine transporter; N2A, neuro2a murine neuroblastoma cells; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; TTBS, Tris-buffered saline/Tween 20; PBS, phosphate-buffered saline; CDP-Star, disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1³,⁷]decan}-4-yl)-1-phenyl phosphate; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; ROD, relative optical density.

cocaine administration, for reasons that remain unclear. Animal experiments have varied a great deal in the dose, route, and length of cocaine administered, the withdrawal time after cocaine exposure, as well as the radioligand used to examine binding changes, and any of these and other factors may have contributed to the varied results. Although the literature is complex, relatively higher cocaine doses and longer treatment courses have seemed necessary to induce increases in DAT binding in animals (Alburges et al., 1993; Koff et al., 1994; Wilson et al., 1994; Hitri et al., 1996; Letchworth et al., 2000). Also, because some radioligands (such as [³H]WIN 35428) seem more likely to demonstrate cocaine-induced increases than others (such as [³H]mazindol), this suggest that changes in DAT compartmentalization, rather than total synthesis, may be involved. Although cocaine interferes with *d*-amphetamine-induced DAT internalization (Saunders et al., 2000), no previous experiments have determined if there are effects of cocaine alone on DAT trafficking.

Previously, the pharmacological resemblance of [³H]WIN 35428 binding to DAT expressed in a cell culture system employing neuro2A cells (N2A, derived from mouse neuroblastoma) to that in human brain, has been documented (Zhang et al., 1998). Preliminary data also indicated that alterations in [³H]WIN 35428 binding occurred after a 24-h exposure to cocaine, a period of time longer than that reported for *d*-amphetamine effects in another model system (after 1 h, there were substantial declines in dopamine uptake; Saunders et al., 2000). The present experiments were designed to determine the dose response and time dependence of cocaine effects on DAT binding sites, the degree to which binding site changes paralleled dopamine uptake changes, and the mechanism involved. Our initial hypothesis was that [³H]WIN 35428 binding and [³H]dopamine uptake would be increased by exposure to cocaine through alterations in DAT cellular trafficking, rather than increased protein synthesis. To test this theory, experiments assessed total DAT protein, DAT mRNA, and the subcellular localization of DAT after cocaine treatment. Follow-up experiments searched for a possible role of dopamine in modulating the effects of cocaine on the DAT.

Materials and Methods

hDAT-Transfected Cell Cultures. As described previously (Zhang et al., 1998), N2A cells were obtained from American Type Culture Collection (Manassas, VA). Briefly, the cells were grown in Opti-MEM I (Invitrogen, Carlsbad, CA)/10% FBS/1% penicillin-streptomycin in 75-cm² flasks. After reaching confluence (about 3 days of growth), the cells were collected by trypsinization and transfected with hDAT-cDNA [provided by Zdenek Pristupa (University of Toronto, Toronto, ON, Canada), cloned into the pcDNA3 plasmid]. Transfection was accomplished by electroporation employing a BTX Electroporation System 600 (Biotechnologies and Experimental Research, Inc., San Diego, CA). After selection with Geneticin over several weeks, cells were grown to confluence and then distributed in 24 well plates and allowed to reattach for 24 h before all assays described below. After cocaine or vehicle treatment as described below, cells were thoroughly washed to remove residual cocaine before assay.

Radioligand Binding Assays. The methods used to assay [³H]WIN 35428 (also known as [³H]CFT) binding have been described previously (Little et al., 1993, 1999). Briefly, [³H]WIN 35428 assays used a 0.32 M sucrose and 10 mM Na_2HPO_4 buffer at pH 7.4

(except one series of [³H]WIN 35428 experiments that compared 50 mM Tris/120 mM NaCl buffer with sucrose/phosphate buffer). Saturation experiments were performed by homologous displacement. A 3 nM radiolabeled concentration of [³H]WIN 35428 (specific activity, 80 Ci/mmol; PerkinElmer, Boston, MA) was used at the equilibrium conditions determined previously (1 h at 2°C), in conjunction with eight WIN 35428 concentrations, from 10^{-10} to 10^{-5} M. Incubations were performed with intact cells, which were terminally washed with buffer for 2 to 3 min. Similar outcomes were obtained when cells were either mechanically scraped and filtered over Whatman GF/B glass fiber filters presoaked in 0.01% polyethylenimine or when solubilized in SDS and the product counted. Nonspecific binding was defined with 30 μM (−)-cocaine. Specific binding with [³H]WIN 35428 averaged 80 to 85% as a fraction of total binding. The cells in one or two wells of each plate were dissolved with 1 ml of 1 N NaOH, and the concentration of protein was determined (Bio-Rad, Hercules, CA). Approximately 0.2 mg of protein was obtained from one well. It should be noted that because only eight concentrations of radioligand were used in the present series of saturation experiments, these conditions were not optimal for the detection of two binding sites. The decision to use only eight concentrations was based on the consistent preference for a one-site model in previous [³H]WIN 35428 binding assays that used 16 radioligand concentrations and the same buffer conditions (Little et al., 1999). Both one-site (Wilson et al., 1996a; Xu and Reith, 1997) and two-site models (Madras et al., 1989; Staley et al., 1994) have been reported for [³H]WIN 35428. Detection of one versus two sites depends on fairly subtle differences in assay characteristics (Coffey and Reith, 1993; Xu and Reith, 1997). WIN 35428 was supplied by Dr. Ivy Carroll (Research Triangle Institute, NC). (−)-Cocaine and dopamine were purchased from Sigma Chemicals (St. Louis, MO).

[³H]Dopamine Uptake Assays. [³H]Dopamine (specific activity, 35 Ci/mmol; PerkinElmer) was added to wells to reach a final concentration of 10 nM and total volume of 0.5 ml (Krebs phosphate buffer), followed by incubation for 5 min (uptake linear) at 37°C on a plate shaker. Uptake was terminated by removing assay medium immediately after incubation with a Brandel 48-well cell harvester, followed by two washes with 1 ml of ice-cold Krebs phosphate buffer. hDAT-N2A cells were then dissolved in 0.8 ml of 1% SDS. The entire liquid content of each well was transferred to a scintillation vial and assayed for radioactivity with 5 ml of CytoScint cocktail (EcoLite; ICN Biomedicals, Costa Mesa, CA) by liquid scintillation counting in an IC spectrometer (model LS 6500; Beckman Coulter, Fullerton,CA). Nonspecific uptake was defined with 30 μM (−)-cocaine. Protein concentrations were again determined using a commercial assay kit (Bio-Rad).

Western Blots. Proteins were extracted, diluted in sample buffer, incubated at 100°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using a 7.5% polyacrylamide solution. Proteins were then electrophoretically transferred to nitrocellulose membranes at 60 mA overnight. Blots were incubated with 5% (w/v) nonfat dry milk in TTBS buffer (0.1% Tween 20, 0.15 M NaCl, and 10 mM Tris-HCl, pH 7.4) for 1 h. Rabbit anti-hDAT carboxy-terminal polyclonal antibody (Chemicon, Inc., Temecula, CA) was diluted with 1% bovine serum albumin in TTBS buffer (1:1000), incubated with the blots for 1 h, and gently washed with TTBS buffer. Alkaline phosphatase-conjugated goat anti-rat IgG (1:5000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) was then incubated with blots for 1 h and washed extensively with double distilled H_2O. The antigen-antibody complex was detected with 5-bromo-4-*O*-chloro-3-indolylphosphate and nitro blue tetrazolium. All steps were performed at room temperature. The relative optical density of protein bands was quantitated using a MCID image analysis system (Microcomputer Imaging Devices, Ottawa, ON, Canada). In addition, a control experiment was performed to determine whether cocaine exposure had any effect on actin, a ubiquitous cell structural component. After quantification of DAT immunoreactivity (IR), control and cocaine treated membranes from three experiments were

stripped and reprobed for actin immunoreactivity (antibody from Chemicon, Inc.), using assay conditions similar to those described above.

DAT Cell Surface Biotinylation. Biotinylation of cell surface-exposed hDAT was performed according to the procedures described by Melikian et al. (1996). Stock solutions of sulfo-NHS-biotine (200 mg/ml in dimethyl sulfoxide; Pierce, Rockford, IL) were stored at $-20°C$ before use. hDAT-N2A cells were grown in six-well plates for 24 to 48 h until the wells were confluent. Cocaine (1 μM) treatment was applied for 24 h before biotinylation. After treatment, cells were washed four times with 1 ml of ice-cold calcium- and magnesium-supplemented PBS (Ca/Mg-PBS, 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.3). Sulfo-NHS-biotin solution (1 mg/ml) in Ca/Mg-PBS was incubated with cells for 1 h at 4°C with agitation. Free sulfo-NHS-biotin was removed by washing with ice-cold 0.1 M glycine in 1 ml Ca/Mg-PBS twice. The reaction was further quenched by incubation with 0.1 M glycine for 30 min and then cells were washed with Ca/Mg-PBS three times. Biotinylated cells were solubilized in 0.5 ml of radioimmuno-precipitation assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate), supplemented with protease inhibitor (1 μM/ml pepstatin, 1 μM/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride) for 1 h at room temperature with gentle shaking. The lysis samples were centrifuged at 20,000g for 30 min at 4°C to pellet nonsolubilized material. Supernatant was incubated with monomeric avidin beads (300 μg of protein/250 μg of beads, Pierce) for 1 h at room temperature to separate biotinylated from nonbiotinylated protein. Beads were washed four times with 1 ml of radioimmunoprecipitation assay buffer and the biotinylated proteins were eluted with 100 μl of Laemmli SDS-PAGE sample buffer for 30 min at room temperature. Samples of total cell lysates (45 μl), lysates after incubation with avidin beads, and the bead eluate were separated by SDS-PAGE and then quantitated, as described above under Western blots. A preliminary control experiment assessed the extracellular specificity of the biotinylation process, by treating whole cells with biotin, followed by centrifugation to separate plasma membrane and cytoplasmic components (initially 2000g for 2 min to pallet debris and undestroyed cells, followed by centrifugation of the collected supernatant at 4000g for 2 min to pellet a crude fraction showing a high content of plasma membrane). The two fractions (2-μg pellet and supernatant) were electrophoresed by SDS-PAGE, then stained with streptavidin-conjugated horseradish peroxidase, which was detected by electrochemical luminescence. Numerous biotinylated bands were present in the plasma membrane fraction and none in the cytoplasmic fraction.

Immunofluorescence/Confocal Microscopy. hDAT-N2A cells were grown on Corning cover slips for 24 to 48 h and then appropriately treated in three ways: with vehicle, with 1 μM cocaine for 24 h, or with 5 μM PMA for 30 min. The cover slips were then fixed with 3.5% formaldehyde for 30 min and permeabilized with 0.2% saponine in phosphate-buffered saline (PBS) for 10 min, repeated once. Incubation was performed using a primary rat anti-hDAT N-terminal monoclonal antibody at 1/80 dilution for 2 h (Chemicon International Inc.). This antibody was used instead of the rabbit anti-hDAT C-terminal polyclonal because it demonstrated lower levels of diffuse background labeling. Slides were then incubated with a secondary lissamine rhodamine-conjugated goat anti-rat antibody at 1/100 dilution for 2 h (Jackson, West Grove, PA). Subcellular localization of immunofluorescently labeled hDAT was determined with an MRC-600 Confocal Imaging System (Bio-Rad). The laser beam was consistently calibrated and centered to obtain maximal brightness with minimal background. Slides were exposed to UV light for limited time periods of similar duration for each treatment group. Each cell with distinct membranal labeling in the same nondividing growth stage was uniformly included. Eight to ten digitized images were made through each cell at 0.1-μm intervals. The image of greatest horizontal extent was quantified using the MCID image analysis system, which determined the relative optical density within a cur-

sor-determined area. Three areas were identified and quantitated for each cell: outer rim (which was always very distinctly labeled), large inclusions (which seemed to include the Golgi), and background cytoplasm. Control nontransfected N2A cells did not display labeling. Slides were quantified from three independent experiments. The results from each experiment were compared; densities obtained from individual cells across experiments were summed. A total of approximately 20 to 30 cells were quantified from each treatment group. The density of DAT immunofluorescence was quantified blind to treatment condition.

Northern Blots. Total RNA was extracted from N2A cell culture with TRIzol reagent (Invitrogen). RNA were separated by electrophoresis through agarose gels containing formaldehyde and transferred to nylon membranes (positively charged) overnight by the capillary method. After transferral, the blots were UV cross-linked. A 45-base oligonucleotide probe (AGA GCA GCA CGA TGA CCA GCA CCA GGC AGG CTG TGA GCT GCC ACC) complementary to bases 710 to 754 of the human dopamine transporter cDNA was synthesized and labeled at the 5′-end with biotin. Hybridization with oligonucleotide probe was carried out at 37°C overnight. The blots then were washed twice in 2× SSC/0.1% SDS, pH 7.0, at room temperature for 5 min each and another two times in 0.5× SSC/0.1% SDS, pH 7.0, at 37°C for 15 min each. The streptavidin/alkaline phosphatase conjugate was used for the detection of biotin-labeled nucleic acid, followed by incubation with CDP-star, an ultra-sensitive chemiluminescent substrate for alkaline phosphatase. The nylon membranes were then exposed for 2 to 7 min to chemiluminescent detection film at room temperature. The relative optical density of RNA bands was again quantitated using the MCID image analysis system.

[³H]Leucine Uptake. hDAT-N2A cells were plated in 24-well plates and treated with 1 μM cocaine or vehicle for 24 h. After treatment, cells were thoroughly washed with dopamine uptake buffer (120 mM NaCl, 4.8 mM KCl, 1.4 mM MgSO4, 16 mM Na2PO4, 11.1 mM dextrose, 1 mm ascorbic acid, 0.03 mm pargyline, and 1.2 mM $CaCl_2$, pH 7.4). [³H]Leucine (150 Ci/mmol; PerkinElmer) was added to wells to reach a final concentration of 1 nM and a total well volume of 0.5 ml, followed by incubation for 7 min at 37°C on a plate shaker. Varying concentrations of leucine were added (10 nM–100 μM) to each group of three wells. Nonspecific uptake was defined with either 10 mM glutamine or lysine (both found equivalent). Uptake was terminated by removing assay medium immediately after incubation, followed by two washes with buffer. Cells were dissolved in 0.55 ml of 2% SDS. The entire content of each well was transferred to a scintillation vial and assayed for radioactivity by liquid scintillation.

Inhibition of Protein Synthesis. Rates of incorporation of [³H]leucine were determined after treatment with varying doses of cycloheximide, in a range of doses (0.1 to 10 μM) previously documented to curtail protein synthesis (Alirezaei et al., 1999). hDAT-N2A cells were grown in six-well culture plates and treated with cycloheximide for 24 h at 37°C. Cells were then washed twice with 1 ml of HEPES buffer (20 mM HEPES, 5.5 mM glucose, 120 mM NaCl, 5.5 mM KCl, 0.9 mM $MgCl_2$, and 1.1 mM $CaCl_2$, pH 7.4) and incubated for 30 min in this medium in the presence of 5 μM leucine. [³H]Leucine (150 Ci/mmol; PerkinElmer) was added to a concentration of 1 μCi/ml and incubated for an additional 30 min. Labeling was terminated by two washes in 1 ml of ice-cold phosphate-buffered saline. Ice-cold trichloroacetic acid (1 ml; 10% w/v) was added to each well. Cells were scraped and suspensions were centrifuged for 10 min at 10,000g. The radioactivity of the supernatant and pellet were counted separately by liquid scintillation. The ratio between [³H]leucine incorporated into proteins (pellet) and [³H]leucine taken up into cells (supernatant) was then determined.

Serum-Free Medium versus FBS. Because others have reported that FBS contains serotonin and norepinephrine (Dibner and Insel, 1981; Cutz et al., 1985), we assayed FBS and found it to contain dopamine and serotonin (levels reported below). Cell expres-

sion systems commonly use FBS because serum-containing medium strongly contributes to the rapid and healthy growth of cells in culture. However, based on the levels discovered in FBS, and the possibility that dopamine or serotonin may have played a role in cocaine regulation (perhaps by decreasing uptake after intracellular transport, an effect which might then be blocked by cocaine), two additional experiments were performed: 1) dose response studies, using 10^{-8} to 10^{-5} M concentrations of dopamine and serotonin; and, 2) cocaine treatment in serum-free medium (Neurobasal medium, N-2 supplement, L-glutamine; Invitrogen).

HPLC Assay (Little et al., 1996). FBS and Opti-MEM samples were assayed using a mobile phase buffer of: 0.1 M citrate, 0.075 M sodium phosphate, 0.75 mM sodium heptanesulfonate, and 17% methanol (v/v), adjusted to pH 4.12. A C18 chromatographic column with 5-μm microparticulate silica gel (Spherisorb ODS-2; Sigma-Aldrich Chromatography) was used. Separations were performed isocratically at a flow rate of 1.0 ml/min employing a pump and controller from Waters (Milford, MA) coupled to an electrochemical detector (potential maintained at 0.35 V; Coulochem model 5100A; ESA, Bedford, MA). Standard calibration curves were prepared as peak area (μV · s/10^{-6}) versus concentration of neurotransmitter (ng/μl) and the concentrations of neurotransmitters and metabolites in samples were then back-calculated. Retention times were distinct for each compound analyzed. Chemicals, along with HPLC grade methyl alcohol and water, were obtained from Sigma Chemical Co.

Data Analysis. Computer analysis of radioligand binding and uptake data were performed with Prism v2.0 (GraphPAD Software, San Diego, CA), using iterative curve-fitting techniques. A coefficient of determination was calculated for each set of data as an indicator of how well the data fit the function, similar to a correlational coefficient. Saturation and competition data were fit to one- or two-component models and evaluated for goodness-of-fit by Sheffé's F test. B_{max}, K_d, and K_i were thus determined. Experiments involving multiple conditions, both cocaine dose and hours of treatment, were analyzed using a repeated-measures, two-way ANOVA model, followed by Tukey's multiple comparison tests. In those experiments involving comparisons between two paired wells, paired Student's t test was used. Experiments involving one treatment time with multiple treatments were analyzed using a one-way ANOVA model, followed by Dunnett's multiple comparison tests. For every experimental issue addressed, at least three independent experiments were performed (the degrees of freedom are noted below with the data for each experiment).

Results

Effects of Cocaine Treatment on [³H]WIN 35428 Binding and [³H]Dopamine Uptake. As seen in Fig. 1, relatively high doses of cocaine were required to induce a change in hDAT binding. At cocaine doses of 10^{-5} or 10^{-6} M, there were clear and statistically significant increases in [³H]WIN 35428 binding. The changes induced in the dopamine transporter were not rapid and required at least 12 h for increases in [³H]WIN 35428 binding to become significant (see Fig. 2). Once established, the [³H]WIN 35428 binding changes persisted at similar levels for 48 h. Figure 3 demonstrates that the changes in binding were paralleled by increases in dopamine uptake V_{max}. The increase seemed to occur at an increased rate compared with the induced binding changes, reaching statistical significance at 3 h. The increases in V_{max} were of a similar magnitude as the [³H]WIN 35428 binding B_{max} increases. There were no changes in affinity for inhibitor or substrate in either binding or uptake experiments.

After removal of cocaine (see Fig. 4), cocaine-induced increases in DAT binding sites returned to baseline over the
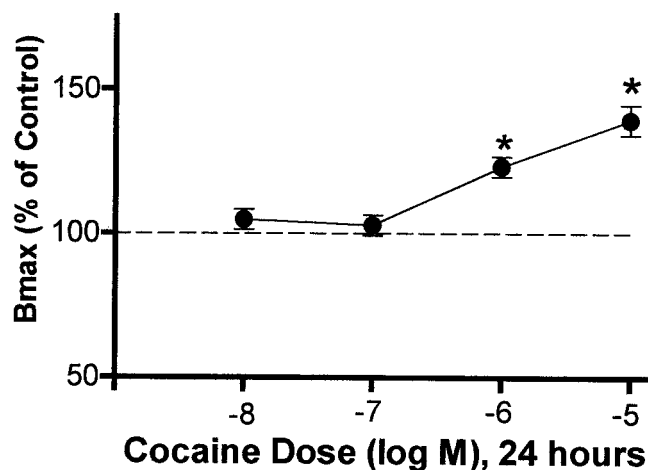

[3H]WIN35428 Binding, hDAT-expressing N2A

Fig. 1. Effects of ($-$)-cocaine on [³H]WIN 35428 binding to hDAT-N2A cells. Cells were grown to confluence, then treated for 24 h with fresh medium containing either ($-$)-cocaine (10^{-8} M to 10^{-5} M) or the same volume of medium only. $n = 5$ pairs of independent saturation experiments for each ($-$)-cocaine concentration. Eight concentrations of [³H]WIN 35428 were used in each experiment, and the data fit to a one-site model. At higher doses, 10^{-6} and 10^{-5} M, cocaine significantly increased [³H]WIN 35428 binding sites (one-way ANOVA, df = 4,23, F = 15.27, $p < 0.0001$, 10^{-5} M different from control, Tukey's post hoc q = 9.79, $p < 0.001$, 10^{-6} M different from control, Tukey's post hoc q = 5.05, $p < 0.05$). There were no effects on binding affinity.
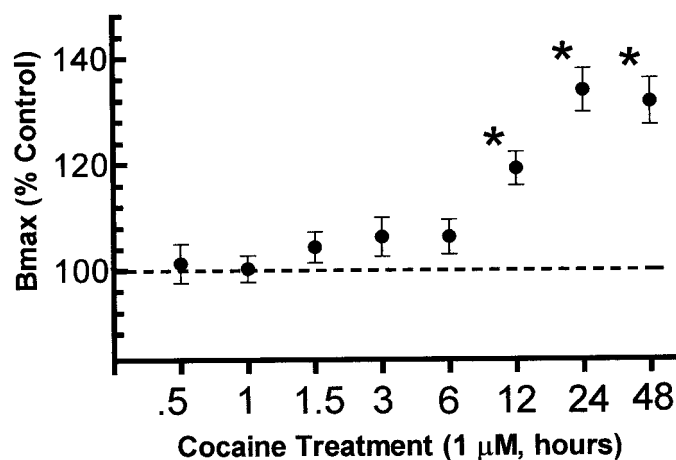

[3H]WIN 35428 Binding, hDAT-expressing N2A

Fig. 2. Time course of cocaine effects on [³H]WIN 35428 B_{max} in hDAT-N2A cells. Cells were grown to confluence, then treated with fresh medium containing either cocaine or the same volume of medium only. Cells were initially treated with cocaine, 10^{-6} M for the indicated time periods, from 0.5 to 48 h. Cells used in the 48-h experiment were washed after 24 h and supplied with fresh cocaine or vehicle. Eight concentrations of [³H]WIN 35428 were used in each experiment, and the data fit to a one-site model. Three to five pairs of independent saturation experiments were performed at each time point, and the raw data was analyzed as matched pairs. A repeated-measures, two-way ANOVA found there were significant cocaine (df = 15,1, F = 3.92, $p = 0.04$) and time effects (df = 15,7, F = 33.3, $p < 0.001$) on [³H]WIN 35428 B_{max}. Follow-up paired t tests found there were significant cocaine effects at 12 (t = 4.87, $p = 0.02$), 24 (t = 6.83, $p = 0.02$), and 48 h (t = 5.15, $p = 0.05$). There were no effects on binding affinity.

next 24 h. Re-exposure to cocaine on the second day after withdrawal, at the same dose, invoked up-regulation again, of a similar magnitude (about 30%), compared with control cells (data not shown).

Because a Tris buffer has been used to assess cocaine effects on DAT regulation in the past (Little et al., 1993, 1998, 1999), and the inclusion of Tris in assay buffer has been found to decrease absolute B_{max} for DAT binding sites (Reith et al., 1984), experiments were performed examining the effect of cocaine exposure on DAT binding in an assay buffer containing 50 mM Tris/120 mM NaCl, instead of the phosphate/sucrose buffer. In these experiments ($n = 3$), [³H]WIN 35428 B_{max} was increased to a degree similar to that found in phosphate/sucrose buffer, 28.5 ± 8% higher versus control cells, which was statistically significant (t = 2.93, df = 4, $p < 0.05$).

Effects of Other DAT Inhibitors Treatment on [³H]WIN 35428 Binding. To assess the specificity of the cocaine effect, hDAT-N2A cells were treated similarly for 24 h with two DAT inhibitors believed to have similar functional effects: methylphenidate and WIN35428. The doses used were near their K_d values for the DAT: 100 nM for methylphenidate and 10 nM for WIN 35428. Both drugs caused similar increases in [³H]WIN 35428 B_{max} compared with vehicle-treated control cells, of 21 ± 4% (methylphenidate, $n = 3$ well plates each) or 26 ± 3% ($n = 3$ well plates each, WIN 35428).

Effects of Cocaine Treatment on Total DAT Concentrations, DAT mRNA, and Subcellular Location. A preliminary biotinylation experiment found that 64% of hDAT was intracellular and 36% on the surface basally in the hDAT-N2A cells [based on quantified relative optical densities (RODs); see Fig. 5A]. Further experiments conducted after 24 h of treatment with 1 μM cocaine, determined that cocaine-induced increases in hDAT function did not involve an increase in synthesis of DAT molecules. Western blots of the DAT showed no increase in total DAT immunoreactivity after cocaine exposure (1 μM for 24 h; see Fig. 5B), but surface hDAT, as measured by biotinylation, was significantly increased (biotinylated DAT IR 0.39 ± 0.03 ROD in vehicle-treated cells versus 0.50 ± 0.04 in cocaine-treated (t = 2.40, df = 7, $p = 0.04$). Consistent with these results suggesting that trafficking mechanisms were involved was a clear visual increase in DAT-immunoreactive labeling of the outer rim of cocaine-treated cells (as shown in Fig. 6). When digitized and quantitated, this effect was found to be statistically significant (see Fig. 7). Conversely, DAT immunofluorescence was mildly decreased intracellularly. Control experiments that involved treating hDAT-N2A cells with PMA (5 μm for 60 min) caused statistically significant increases in cytoplasmic and large inclusion DAT immunofluorescence, with a trend noted toward decreased outer membrane DAT-immunofluoresence. Figure 8 demonstrates the appearance of hDAT immunofluorescence through the extent of a cocaine-treated cell with a series of z-sections.

DAT mRNA levels were found to be similar to control cells 24 h after cocaine exposure (again, 1 μM cocaine dose; see Fig. 9). Other experiments using cycloheximide treatments demonstrated that cocaine-induced increases in [³H]WIN 35428 binding were not blocked by the inhibition of new protein synthesis (Fig. 10B). Also, a control experiment demonstrated that cocaine did not effect [³H]leucine uptake (Fig.
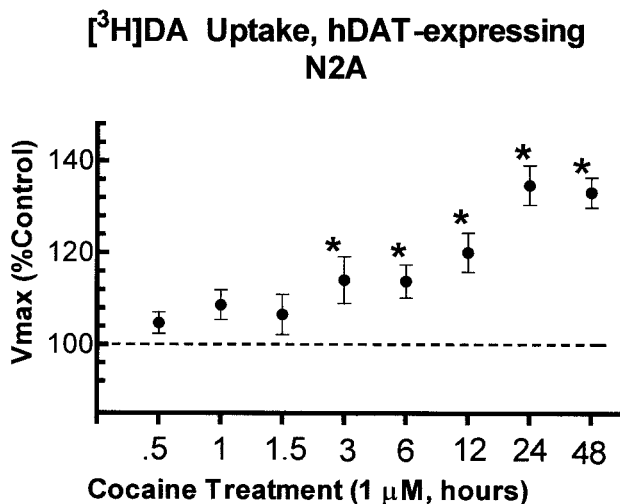
[³H]DA Uptake, hDAT-expressing N2A

Fig. 3. Time course of cocaine effects on [³H]dopamine uptake V_{max} in hDAT-N2A cells. Cells were grown to confluence, then treated with fresh medium containing either cocaine or the same volume of medium only. Cells were initially treated with cocaine, 10^{-6} M for the indicated time periods, from 0.5 to 48 h. Cells used in the 48-h experiment were washed after 24 h and supplied with fresh cocaine or vehicle. Eight concentrations of [³H]dopamine were used in each experiment, and the data were fit to a one-site model. Three to five pairs of independent saturation experiments were performed at each time point, and the raw data was analyzed as matched pairs. A repeated-measures, two-way ANOVA found there were significant cocaine (df = 15,1, $F = 6.88$, $p = 0.01$) and time effects (df = 15,7, $F = 4.38$, $p < 0.001$) on [³H]dopamine V_{max}. Follow-up paired t-tests found there were significant cocaine effects at 3 (t = 3.10, $p = 0.05$), 6 (t = 3.28, $p = 0.04$), 12 (t = 4.00, $p = 0.03$), 24 (t = 6.67, $p = 0.007$), and 48 h (t = 5.24, $p = 0.01$). There were no effects on binding affinity (see Table 1).

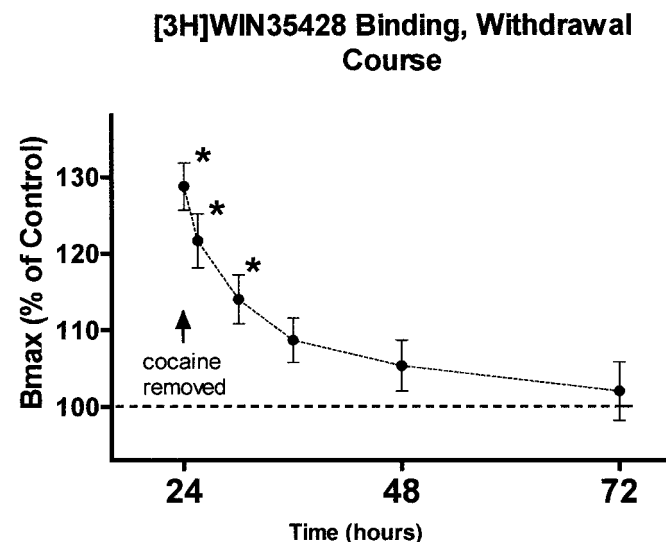## [3H]WIN35428 Binding, Withdrawal Course

Fig. 4. Withdrawal time course for [³H]WIN 35428 binding to hDAT-N2A cells after cocaine treatment. Paired well plates were initially prepared, then plates treated as indicated with cocaine (1 μM for 24 h) or vehicle, and cocaine then removed after 24 h. At the successive time intervals indicated, 25 to 72 h, a pair was assayed. Eight concentrations of [³H]WIN 35428 were used in each experiment, and the data were fit to a one-site model. $n = 4$ to 6 pairs of independent saturation experiments for each time point. Increased [³H]WIN 35428 binding dissipated over a 24-h time period [one-way ANOVA, df = 6,31, $F = 3.27$, $p < 0.05$; 25 h (1 h withdrawal) sig. different from control, Tukey's post hoc q = 5.622, $p < 0.05$]. Follow-up paired t tests found significant cocaine effects compared with vehicle at 24 h (no withdrawal, $t = 8.710$, $p < 0.01$), 25 h (1-h withdrawal, $t = 6.846$, $p < 0.05$), and 30 h (6-h withdrawal, $t = 5.382$, $p < 0.05$).

10A), which depends on an ubiquitous plasma membrane amino acid transporter.

Possible Role of Phosphorylation. As shown in Fig. 11, cells treated with cocaine (1 μM $\times$ 24 h, and demonstrating increased DAT function) showed no change in their responsiveness to treatment with PMA, which has been shown to cause internalization of DAT via protein kinase C phosphorylation (Pristupa et al., 1998).

Possible Involvement of Dopamine and Serotonin as Intracellular Messengers. Because it was possible that the effect of cocaine depended on reversing the basal suppression of hDAT expression by low levels of dopamine present in serum, further experiments incubating hDAT-N2A cells with dopamine for 24 h in concentrations from 10^{-8} to 10^{-5} M were performed. An inhibiting effect on [^{3}H]WIN 35428 binding was found only at the highest concentration (see Table 1). There was no evidence of cell toxicity, as measured by total protein, which was equivalent to controls at all four concentrations of dopamine. The presence of residual dopamine possibly contributed to the decrease in [^{3}H]WIN 35428 binding seen at 10^{-5} M, although dopamine concentrations were probably much diluted. Incubation with the same concentration range of serotonin found no diminishment in [^{3}H]WIN 35428 B_{max} or affinity, nor any change in total protein levels. One other indicator that dopamine played no role in cocaine-induced trafficking, hDAT-N2A neurons grown in serum-free medium (which is dialyzed to remove any trace levels of dopamine), demonstrated a similar pattern of cocaine-induced DAT increases.

HPLC assay found that fetal bovine serum contained considerably lower levels of dopamine (0.125 $\pm$ 0.077 μM, three samples) and serotonin (3.57 $\pm$ 1.33 μM, three samples) than required to suppress hDAT levels in the above experiments.

When used with N2A cells, the levels of dopamine and serotonin in FBS were further diluted to 1/10 with Opti-MEM (which did not contain measurable dopamine or serotonin). Average levels of dopamine and serotonin in FBS present in cell cultures over 24 h were probably even lower than initial levels; recent experiments have shown that catechol-O-methyltransferase actively degrades monoamines in several cell cultures expressing recombinant DAT cDNA (Eshleman et al., 1997). In any case, as shown in Fig. 12, cocaine treatment (again 1 μM for 24 h) increased [^{3}H]WIN 35428 B_{max} in serum-free medium to a level comparable with that found in preparations that included FBS.

Discussion

The present experiments extend our earlier report in N2A cells (Zhang et al., 1998), and model features that have been discovered in postmortem specimens from human cocaine users, including increased [^{3}H]WIN 35428 binding (Little et al., 1993, 1999; Staley et al., 1994), increased [^{3}H]dopamine
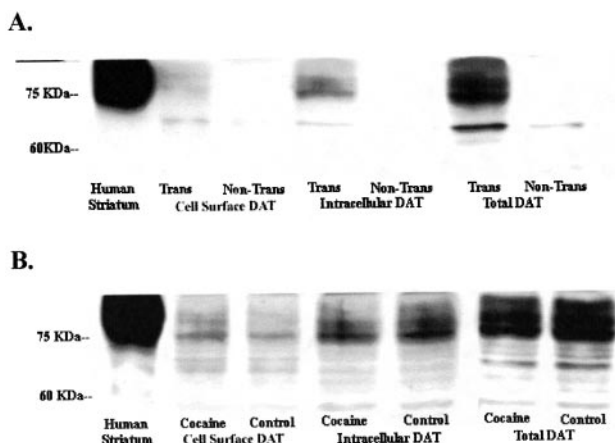

Fig. 5. hDAT immunoreactivity after Western blotting with hDAT antibody: A, computer scanned image illustrating the results of transfection: Non Trans = N2A without pcDNA3-hDAT transfection. A striatal sample from human striatum is also included for comparison. B, scanned image illustrating hDAT immunoreactivity after cocaine exposure, 1 μM $\times$ 24 h (cell surface = biotinylated fraction). There was an increase in the density of the cell surface band after cocaine treatment. This was consistently present in four independent experiments: cell surface DAT immunoreactivity (IR) 0.39 $\pm$ 0.03 (ROD) control versus 0.50 $\pm$ 0.04 cocaine (t = 2.40, df = 7, p = 0.04). Intracellular DAT IR was not significantly decreased: 0.69 $\pm$ 0.05 control versus 0.60 $\pm$ 0.08 cocaine (t = 0.69, df = 8, p = 0.36). There was no effect of cocaine treatment on total hDAT IR: 1.67 $\pm$ 0.03 control versus 1.70 $\pm$ 0.04 cocaine (t = 0.85, df = 8, p = 0.42). The gel image is overexposed to highlight the surface DAT comparison, but total IR was also quantitated on film with shorter exposures to assure that the films were not saturated.
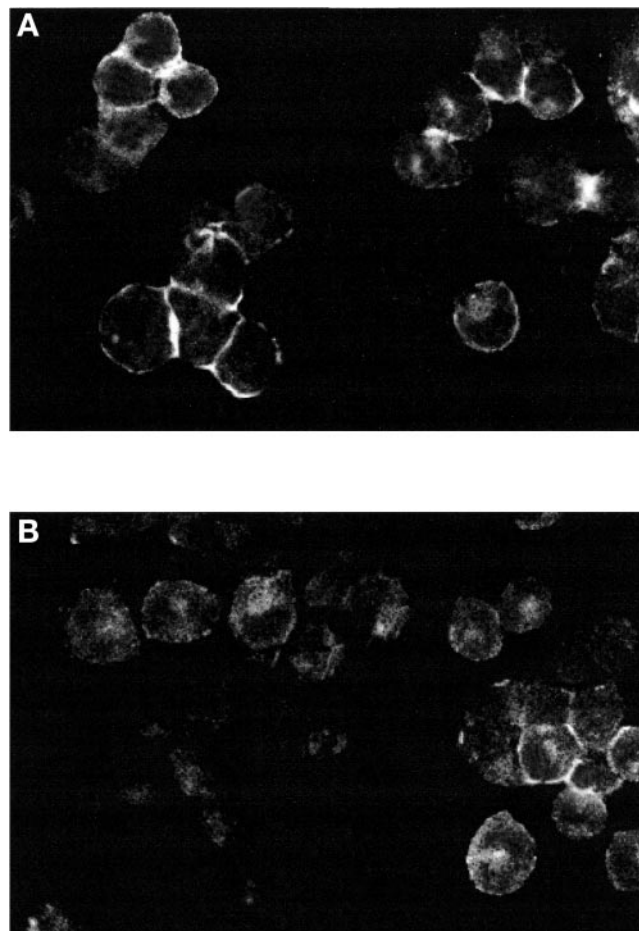

Fig. 6. Digital image displaying hDAT immunofluorescence pattern detected in hDAT-N2A cells using confocal microscopy. A, after (−)-cocaine treatment, 1 μM for 24 h. B, after vehicle treatment. Compared with vehicle, a number of cells demonstrate more intense labeling of the outer membrane (as viewed at 200×), and less internal DAT immunofluorescence. The intensity of fluorescence rather than area was measured. The entire plasma membrane was outlined at each level, including bright and less intense regions for each cell, by a technician who was blind to treatment condition. For cells that were confluent, the region of interest outlined was divided midway through the section that seemed joined.
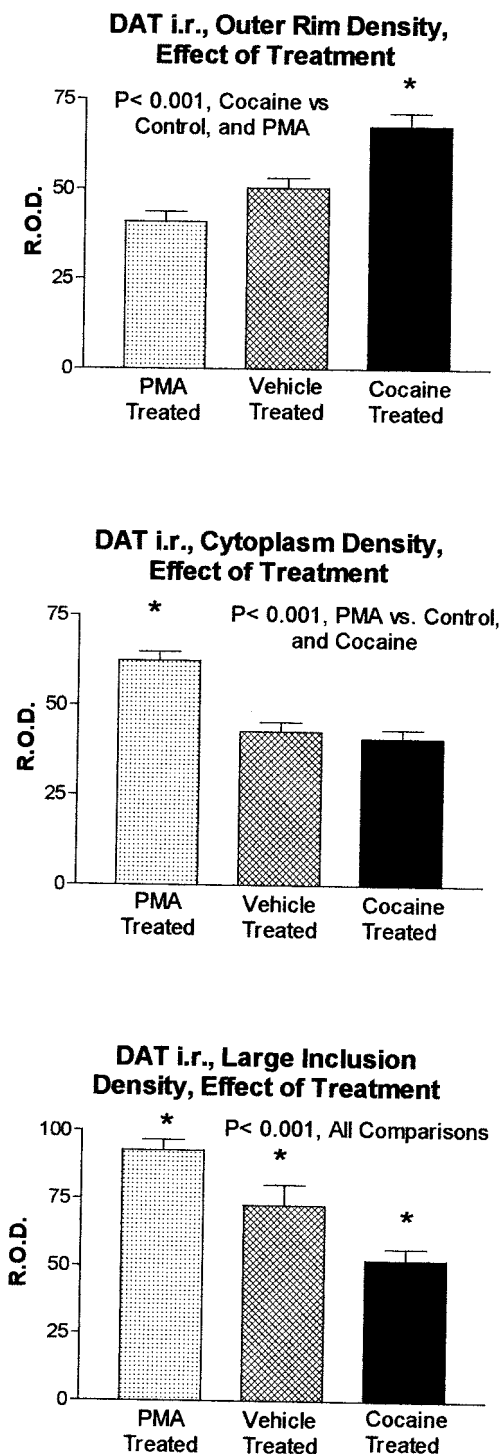
DAT i.r., Outer Rim Density, Effect of Treatment

P< 0.001, Cocaine vs Control, and PMA

DAT i.r., Cytoplasm Density, Effect of Treatment

P< 0.001, PMA vs. Control, and Cocaine

DAT i.r., Large Inclusion Density, Effect of Treatment

P< 0.001, All Comparisons

Fig. 7. Quantification of digitized hDAT immunofluorescence patterns detected in hDAT-N2A cells after PMA treatment, vehicle treatment, and (−)-cocaine treatment. The images quantified were accumulated from three pairs of independent experiments, each involving the sampling of optical densities from 20 to 30 cells by a technician blind to experimental condition. There was an increase in outer membrane and a decrease in large inclusion immunofluorescence after cocaine (1 μM for 24 h), and an increase in cytoplasm and large inclusion immunofluorescence after PMA treatment (5 μM for 30 min). Rim, one-way ANOVA (F = 19.23, df = 2, 60, $p < 0.001$); cocaine versus vehicle, Dunnett's post hoc, q = 3.93, $p <$ 0.01. Cytoplasm, one-way ANOVA (F = 22.34, df = 2, 60, $p < 0.001$); PMA versus vehicle, Dunnett's post hoc, q = 5.51, $p < 0.01$. Large inclusions, one-way ANOVA (F = 13.98, df = 2, 60, $p < 0.001$); cocaine versus vehicle, Dunnett's post hoc, q = 2.64, $p < 0.05$, PMA versus vehicle, Dunnett's post hoc, q = 2.66, $p < 0.05$.

uptake (Mash et al., 1997), without increases in DAT mRNA (Little et al., 1998).

A number of independent measures demonstrated the presence of increased, functional DAT after cocaine treatment. This increase seemed restricted to the cell surface, based on biotinylation and confocal microscopic results. The lack of change in DAT mRNA or total protein levels, and the failure of cyclohexamide treatments that markedly inhibited protein synthesis to block the effects of cocaine, further indicate there was no change in total number of DAT molecules. Other control experiments indicate that the effect of cocaine was specific to the DAT because cocaine treatment caused no changes in [^{3}H]leucine uptake or actin protein levels. The magnitude of the increases detected with the various measures were similar at 24 h, with a 37% increase recorded in dopamine V_{max}, a 34% increase in [^{3}H]WIN 35428 B_{max}, a 35% increase in outer membranal labeling using confocal microscopy, and a 28% increase in biotinylated hDAT. The small differences probably reflect to some degree differences between measures in their selectivity for membrane-inserted hDAT versus internalized hDAT. Although the decrease in nonbiotinlyated DAT after cocaine treatment was not statistically significant on Western blots, this probably reflects the much larger intracellular volume and greater absolute amounts of intracellular DAT, compared with that of the plasma membrane, and would have been expected to demonstrate smaller percentage changes after cocaine treatment.

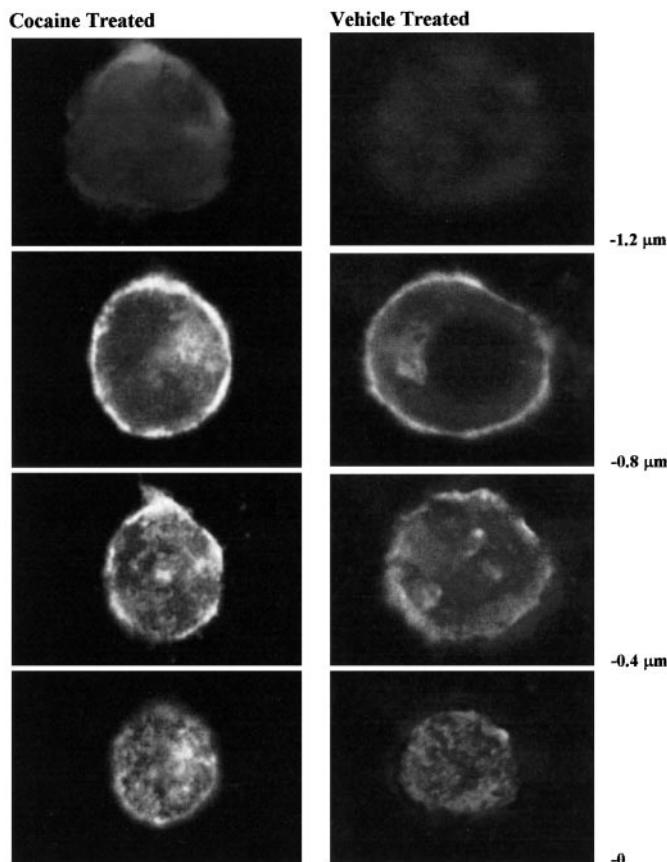The present results can be compared with those reported

Fig. 8. A series of x-y sections at different z levels in cocaine- and vehicle-treated cells. Confocal images (at intervals of 0.4 μm) demonstrate increased peripheral hDAT immunofluorescence after cocaine treatment.
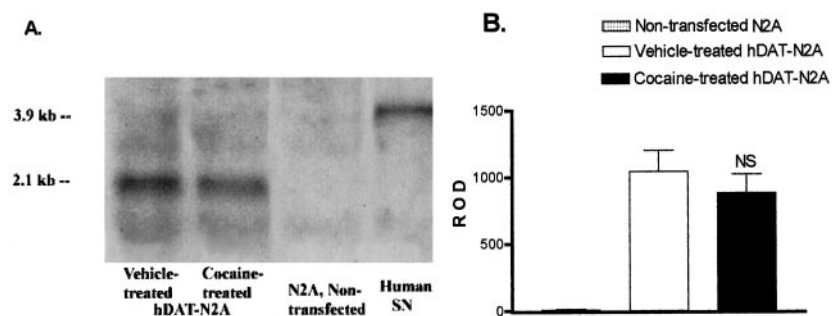
Fig. 9. A, digital image illustrating hDAT mRNA bands detected on electrophoretic gel: (−)-cocaine treatment, 1 μM for 24 h; NT, N2A cells without hDAT transfection; SN, sample from human substantia nigra. B, graph depicting the results of three independent experiments. Gel results were digitized and the relative optical densities for each band quantitated and compared. There was no effect of cocaine treatment on hDAT mRNA band density (cocaine-treated versus control, t = 0.768, df = 4, p = 0.48).

by Saunders et al. (2000) using hDAT-FLAG expressed in human embryonic kidney 293-EM4 cells. These investigators found that 2 μM d-amphetamine increased internalization of surface DAT within 1 h on confocal microscopy and in whole-cell current recordings. We have also noted an opposite effect of d-amphetamine on hDAT binding compared with (−)-cocaine (Little and Zhang, 1998).

Compared with the results of Saunders et al. (2000), cocaine effects on [3H]WIN 35428 binding were slower developing in the present experiments, suggesting that detection of changes in [3H]WIN 35428 binding may require a greater relative shift in the distribution of DAT to the cell surface than other measures, perhaps because of some limits to its selectivity for plasma membrane versus intracellular DAT fractions. Consistent with this explanation, cocaine- induced increases in [3H]dopamine uptake were more rapid. In our experiments, we emphasized [3H]WIN 35428 binding as our primary measure of (−)-cocaine effect because it represents the DAT measure of most practical use in clinical studies.

It is possible that markedly distinct mechanisms are at play in the processes noted here versus those caused by d-amphetamine as described by Saunders et al (2000). However, cocaine-induced redistribution of DAT to the surface might involve similar pathways and seem slower in hDAT-N2A cells than DAT internalization in d-amphetamine-treated EM4 cells simply because of relative differences in basal state DAT distribution patterns for the two types of cells. In contrast to hDAT-N2A cells, EM4 cells displayed very little internal DAT fluorescence basally. Thus, the ki-

netics of surface versus intracellular distribution seem to favor a membrane locale in EM4 cells, an ideal situation for measuring shifts toward intracellular accumulation. In contrast, in the hDAT-N2A cells, which basally display more intracellular hDAT, redistribution effects may take longer to detect because of the more even distribution between the two compartments.

Daniels et al. (1999) have recently described PMA-induced trafficking of a green fluorescent protein-tagged DAT expressed in Madin-Darby canine kidney cells. Similar to the cells used by Saunders et al. (2000), these cells display little basal intracellular DAT fluorescence, which markedly increases after PMA treatment. In contrast, Melikian and Buckley (1999), using the same hDAT-pcDNA3 construct as presently described, transfected into a neuronal cell PC12, reported that 63% of hDAT IR was intracellular in studies employing biotinylation and sucrose gradient ultracentrifugation. Together, these results suggest that tagged DAT may have a shorter half-life after internalization and increased lysosomal degradation rates compared with untagged DAT. Supporting this idea, Daniels and Amara (1999) found that 0.1 μM PMA treatment for 1 h caused extensive DAT-green fluorescent protein fluorescence colocalized with AC-17 (anti-lysosomal glycoprotein antibody)-labeled vesicles and a considerable loss of total DAT IR on Western blots. Conversely, Melikian and Buckley (1999), working with untagged DAT, found that 30 min of 1 μM PMA induced internalization of DAT IR that was nearly all concentrated in the transferrin-labeled recycling compartment, not in the EEA1-labeled slow
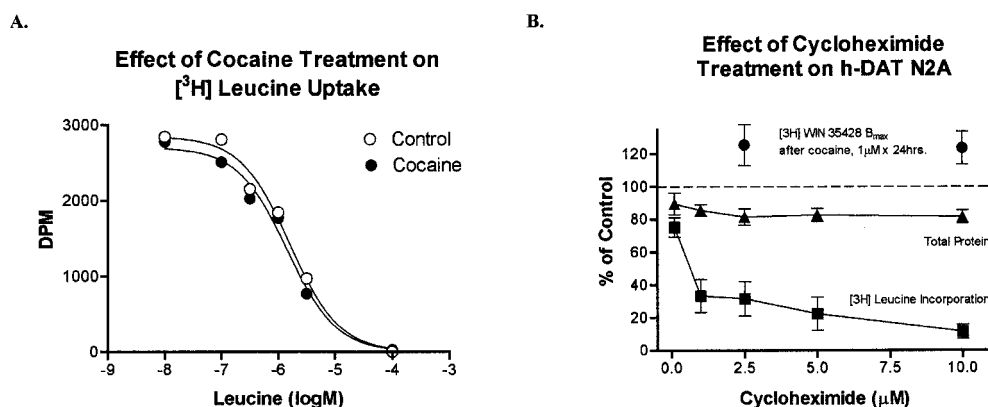

Fig. 10. A. Effects of (−)-cocaine treatment(1 μM for 24 h) on [3H]leucine uptake in hDAT-N2A cells, from one of three independent experiments. Eight concentrations of [3H]leucine were used in each experiment, and the data were fit to a one-site model. There was no significant effect of (−)-cocaine on V_{max} or K_m value. B, effects of cycloheximide treatment on protein synthesis in hDAT-N2A cells. Three or four pairs of independent saturation experiments were performed at each cycloheximide dose. A range of cycloheximide doses markedly inhibited [3H]leucine protein incorporation and mildly decreased total protein, but none blocked cocaine-induced increases in [3H]WIN 35428 binding. Comparing vehicle-treated, cocaine-treated (1 μm × 24 h), and cocaine + cycloheximide-treated cells at each cycloheximide doses: 2.5 μM dose: F = 6.854, df = 2,8, p < 0.05, by one-way ANOVA, Tukey's post hoc; cocaine versus vehicle, q = 4.970; p < 0.05; cocaine + cyclo versus vehicle, q = 5.132; 10.0 μM dose: F = 29.58, df = 2,7, p < 0.01, Tukey's post hoc; cocaine versus vehicle, q = 10.27, p < 0.01; cocaine + cyclo, q = 7.932; p < 0.01.
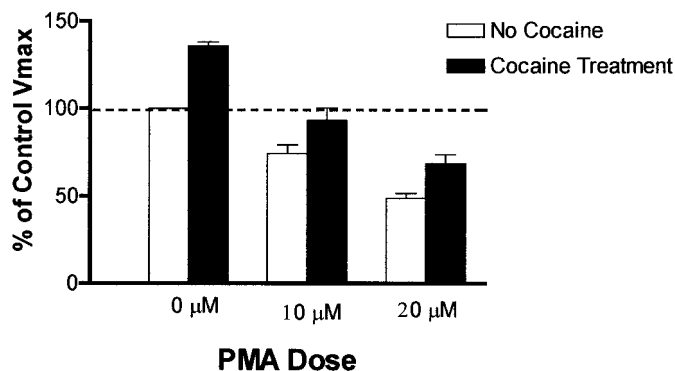
[3H]DA Uptake, Effects of PMA and Cocaine

Fig. 11. Effects of both (−)-cocaine and PMA treatment: the cocaine treatment was performed as described in other experiments. hDAT-N2A cells were treated with PMA for 15 min at final concentrations of 0, 10, and 20 μM before uptake assay. Both treatments had the expected effects without any apparent interference.

endosome compartment (which is prelysosomal). Against the possibility that the antibodies used in the current experiments labeled another protein in addition to DAT were the control experiments with nontransfected N2A cells, which demonstrated only background immunofluorescence.

Moderate doses of dopamine or serotonin did not effect the DAT. If an inhibiting effect had existed, then the presently described cocaine increases might have reflected a reversal of an intracellular dopamine effect. Further indicating that dopamine was not involved in the presently described trafficking phenomenon, hDAT-N2A neurons grown in serum-free medium also demonstrated cocaine-induced up-regulation. In a previous report, Saunders et al. (2000) found that 100 μM dopamine for 1 h increased DAT internalization in a manner similar to 2 μM d-amphetamine. These results seem consistent with the present findings, which found that high doses of dopamine decreased [3H]WIN 35428 binding, after a longer treatment period.

The quantification of immunofluorescence by confocal microscopy can be confounded by a number of factors including inadequate matching of the 3D plane, differences in cell growth, and the fading of immunofluorescence, among others (Nagelhus et al., 1996). Although the present experiments may not have eliminated all the variability caused by these factors, the quantification of different treatment groups was carefully balanced. The cocaine-induced increase in plasma membrane and decrease in large inclusion-associated im-

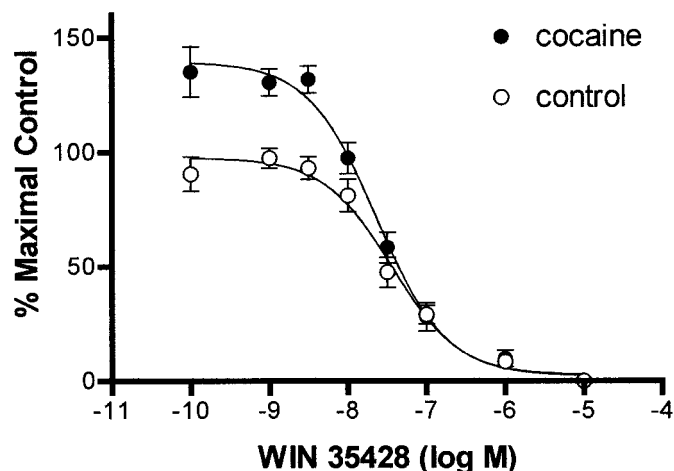## [3H]WIN 35428 Binding, Effect of Cocaine, Serum Free Media

Fig. 12. Effect of (−) cocaine treatment (1 μM, 24 h) on [3H]WIN 35428 binding similarly in serum-free medium. Homologous displacement curves above (above data averaged from three pairs of independent experiments) demonstrated a similar increase after cocaine treatment as found in cells grown in FBS containing medium. The [3H]WIN 35428 B_{max} was increased 29 ± 6% versus paired controls, but K_d was unchanged.

munofluoresence were consistent with the biotinylation results, which found increased cell surface DAT, no increase in total DAT, and a trend toward decreased intracellular DAT.

Previous experiments have alternatively used either sucrose/phosphate buffer or Tris buffer (Madras et al., 1989; Little et al., 1993; 1998), with some evidence suggesting that a two-site model is more likely in low sodium buffers, perhaps partly because of binding to filter paper (Chen et al., 1997). Although Tris decreases total DAT binding (Reith et al., 1984), the current experiments found that cocaine-induced [3H]WIN 35428 binding site increases can be detected in either TRIS or sucrose/phosphate buffer. In different experiments, [3H]WIN 35428 binding displayed K_d values ranging from 5 to 21 nM (see Table 1), but K_d values were never different in cocaine-treated cells compared with similarly plated control cells, and were similar to the K_d values detected in human brain experiments (16.3 ± 4.3 nM, Little et al., 1999). Dopamine uptake affinity in the hDAT-N2A cells has varied in the 200 to 500 nM range in our laboratory over a period of several years, and the range of [3H]dopamine

TABLE 1

Effect of dopamine and serotonin exposure for 24 h

Only dopamine at 10^{-5} M decreased [3H]WIN 35428 B_{max} (one-way ANOVA, df = 3,15, F = 15.06, $p < 0.001$; 10^{-5} M different from 10^{-6} M, Tukey's post hoc q = 7.36, $p < 0.01$). Coincubation of dopamine (10^{-5} M) with cocaine (10^{-6} M) for 24 h did not block the dopamine-mediated decrease. Cocaine treatment had no effect on K_d or total protein. Similar concentrations of serotonin did not diminish [3H]WIN 35428 binding at any serotonin concentration.

Concentration	Dopamine Treatment (24 h)			Serotonin Treatment (24 h)		
	[3H]WIN 35428 B_{max}	K_d	Total Protein	[3H]WIN 35428 B_{max}	K_d	Total Protein
M	% of paired control	nM	μg/well	% of paired control	nM	μg/well
10^{-8}	91 ± 8	21 ± 1.0	209 ± 20	91 ± 9	19 ± 0.3	274 ± 7
10^{-7}	103 ± 4	20 ± 0.8	214 ± 22	94 ± 2	18 ± 1.1	270 ± 5
10^{-6}	95 ± 4	22 ± 1.3	205 ± 11	102 ± 5	19 ± 0.7	280 ± 8
10^{-5}	55 ± 5	20 ± 0.7	215 ± 22	94 ± 7	19 ± 1.3	284 ± 7
Control		19 ± 0.3	213 ± 15		19 ± 1.2	286 ± 9

uptake K_m values reported in the current experiments are somewhat lower than have been reported in other DAT-expressing cells. However, in all the studies reported here, control cells were grown and plated in equal numbers as treated cells, for each saturation experiment, and no sign of cocaine effect on K_m or K_d values was found.

Although a comparison of dose concentrations and time scales between cells in culture and living animals or humans is inexact, the present results parallel animal experiments finding that relatively large cumulative doses of cocaine (generally more than 200 mg/kg/rat) are required to consistently cause DAT up-regulation (Alburges et al., 1993; Koff et al., 1994; Wilson et al., 1994; Hitri et al., 1996; Letchworth et al., 2000). However, the 1 μM cocaine dose repeatedly administered in the present study is within the range reported in blood or brain from human cocaine users (generally 0.1 to 1 μM range, Foltin and Fischman, 1991; Isenschmid et al., 1992; Lukas et al., 1996).

In summary, cocaine treatment of hDAT-N2A cells models features detected in postmortem striatal samples from human cocaine users and suggests a possible mechanism for the observed increases in [3H]WIN 35428 binding. Cocaine regulation of DAT trafficking in hDAT-N2A neurons did not require the mediation of dopamine, but it is possible that intact dopamine neurons incorporate mechanisms for adjusting DAT function based on dopamine levels. The present model system should prove useful in better understanding the complex relationship between alterations in DAT radioligand binding and DAT protein trafficking. Confusion about these phenomena probably contributes to the current conflicting interpretations of existing in vitro and in vivo radioligand experiments.

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Address correspondence to: Karley Y. Little, M.D., Laboratory of Affective Neuropharmacology/116-A, Ann Arbor VAMC, 2215 Fuller Rd., Ann Arbor, MI 48105. E-mail: kylittle@umich.edu