Substrate-Induced Trafficking of the Dopamine Transporter in Heterologously Expressing Cells and in Rat Striatal Synaptosomal Preparations

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ABBREVIATIONS: DAT, dopamine transporter; GAT1, GABA transporter 1; HEK-hDAT, human embryonic kidney 293 cells expressing DAT; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, $\beta$-phorbol 12-myristate 13-acetate; sulfo-NHS-biotin, sulfo-NHS-S-succinimidobiotin; sulfo-NHS-S-S-biotin, sulfo-NHS-S-S-succinimidobiotin.
ABSTRACT

Dopamine transporter (DAT) trafficking was assessed by functional measurements of dopamine uptake and by biotinylation of surface proteins followed by gel electrophoresis and Western blotting. In HEK-293 cells expressing DAT (HEK-hDAT), pretreatment with dopamine (0.1 – 100 µM) followed by wash-out caused reductions in subsequent dopamine uptake (reflected in $V_{\text{max}}$) with effective dopamine concentrations in the 10 – 100 µM range and pretreatment times of 10 – 60 min. Reductions assessed after 60 min pretreatment with 100 µM dopamine corresponded with decreases measured in surface DAT by the noncleavable biotin method, which were caused, at least in part, by enhanced endocytosis as monitored with cleavable biotin. Pretreatment of rat striatal synaptosomes with dopamine (10 and 100 µM) also caused reductions in DAT uptake activity ($V_{\text{max}}$), and again the underlying mechanism appeared to be a diminished presence of DAT at the surface of synaptosomes as measured by the noncleavable biotin method. The co-presence of cocaine during pretreatment with dopamine prevented the downregulation of surface DAT. The present results show that DAT surface residency can be regulated by substrate acting on it, not only in cells heterologously expressing DAT but also in situ in rat brain tissue.
In the brain, the dopamine transporter (DAT) clears extraneuronal dopamine, thereby terminating dopamine action (Iversen, 1971). It is generally thought that the local density of DAT is one factor that determines how much dopamine can be cleared, just as the density of dopamine receptors is crucial for the intensity of dopamine receptor-mediated signaling. In this context, many studies over the past decade have addressed long-term regulation of DAT density in chronic drug studies (for reviews see Kuhar and Pilotte, 1996; Zahniser and Doolen, 2001). The idea that DAT is also susceptible to short-term regulation on a scale of minutes through changes in its presence at the cell surface, is more recent, and originates in studies searching for a link between transporter activity and phosphorylation states. Thus, depending on the mode of treatment, the protein kinase C (PKC) activator β–phorbol 12-myristate 13-acetate (PMA) rapidly increases (Quick et al., 1997) or decreases (Beckman et al., 1999) surface-resident γ–aminobutyric acid (GABA) transporter 1 (GAT1) in oocytes or primary hippocampal cultures. Downregulation of GAT1 is probably the physiologically relevant effect of PKC activation (see Robinson, 2002). In addition, phorbol esters downregulate, or reduce surface activity, of monoamine transporters for serotonin (Qian et al., 1997), norepinephrine (Apparsundaram et al., 1998), and dopamine (Corey et al., 1994; Kitayama et al., 1994; Zhang et al., 1997; Vaughan et al., 1997; Huff et al., 1997) in a variety of systems including heterologous expression systems and brain tissue preparations. It is important to realize that although GAT1 and the monoamine transporters are members of the larger family of Na+,Cl--dependent neurotransporters (Deken et al., 2002), many of their characteristics can differ.

The idea that transporter presence at the cell surface can be acutely regulated by a substrate that acts on it, has been developed much more recently than the trafficking models for neurotransmitters acting on receptors such as β–adrenoceptors (Sibley et al., 1986). Other examples of receptors regulated by agonist on a time scale of minutes are μ–opioid (Keith et al., 1998), δ–opioid (Tsao and von Zastrow, 2000), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (Shi et al., 1999), and somatostatin (Koenig et al., 1997) receptors. In the case of neurotransmitter transporters, emerging evidence supports the possibility of acute
regulation of their density at the cell surface by substrates appropriate for them. Thus, glutamate (10-100 µM for 15-30 min) increases transport activity of excitatory amino acid transporter EAAT1 (also called GLAST) (Munir et al., 1999; Duan et al., 1999) accompanied by increased presence in plasma membranes (Duan et al., 1999). GABA (3-100 µM) increases GAT1 activity through an increase in cell surface expression on the order of minutes (Bernstein and Quick, 1999). Pointing to a substrate effect in the same direction, serotonin (0.1 – 1 µM) counteracts PMA-induced downregulation of surface-resident serotonin transporters on a minute-time scale (Ramamoorthy and Blakely, 1999). The one available study for norepinephrine is an example of a long-term regulation (days), and it suggests the opposite effect on transporter redistribution as evidenced by appreciable decreases in the V$_{\text{max}}$ of norepinephrine uptake and B$_{\text{max}}$ of radioligand binding by norepinephrine (100 - 1000 µM) in preparations of cells heterologously expressing the human norepinephrine transporter (Zhu et al., 2000). Equivocal results have been reported for acute regulation of DAT on the order of minutes by dopamine. In cell systems with DAT, surface DAT is decreased by dopamine (100 µM) (Saunders et al., 2000), not affected by dopamine (10 µM) (Daniels and Amara, 1999), or increased by dopamine (1 µM) (Seamans et al., 2001). It is possible that dopamine exerts a concentration-dependent effect, with high concentrations downregulating and low concentrations upregulating DAT; in such a scheme, intermediate concentrations could lie on the cross-over portion of the dopamine curve where dopamine has no effect. Another possibility relates to the initial density of DAT impacted by dopamine (see Discussion). Results from in vivo voltammetry experiments are consonant with a decrease in cell-surface DAT upon repeated local application of dopamine (every 2 min over a time period of 30 min) into rat striatum and parallel the in vitro effects of repeated bath application of dopamine (10 µM every 5 min for 40 min) on cocaine analog binding to DAT expressing oocytes (Gulley et al., 2002).

The present study was undertaken to further characterize the effect of dopamine on DAT trafficking in both a heterologous cell system and rat striatal synaptosomal preparations in terms of time- and concentration-dependency. Transporter trafficking was assessed by functional
measurements of dopamine uptake and by biotinylation of surface proteins followed by gel electrophoresis and Western blotting.

Methods

DAT expressing cells. A clone (pCDNA1:hDAT) containing a human (h) DAT gene isolated from human substantia nigra was generously given to us by Drs. A. Janowski, A. Eshlemen, and K.A. Neve (Oregon Health Sciences University, Portland, OR). Subsequently, the 3.5 kb hDAT insert was subcloned into pCDNA3.1 (Invitrogen, Carlsbad, CA) which contains a neomycin-resistance gene that is expressed in mammalian cells as a selective marker. The orientation of the hDAT-insert was confirmed by restriction digestion analysis with Bam HI. Stable pools of HEK-293 cells expressing DAT (HEK-hDAT) were obtained and grown as described previously (Chen et al., 2001; 2002).

Synaptosomal preparations of rat striatum. Male Sprague-Dawley rats (250-350 g, 2-3 months of age) were derived from Harlan Sprague-Dawley (Indianapolis, Ind., USA). Striatal tissue was dissected and homogenized in ice-cold 0.32 M sucrose and centrifuged at 1,000g for 10 min. The supernatant fraction was centrifuged at 17,000g for 20 min. The pellet was resuspended in “uptake buffer A” (122 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 10 mM dextrose, 1 mM CaCl₂, 0.01 mM nialamide, and 15 mM Na⁺ and 8 mM phosphate from a mixture of primary and secondary phosphate buffer, pH 7.4).

[^3H]DA uptake. HEK-hDAT cells were seeded in 96-well plates (white plate with clear bottom, Corning Costar Co., Cambridge, MA) at 50,000 cells per well approximately 48 h before the uptake experiment and grown to 80% confluency. To improve adhesion of this cell line to plate, the wells were treated for 40 min at room temperature with a novel polyethyleneimine-based polymer (AB-30, 12.5 µg/ml in ethanol). The polymer was synthesized by Dr. W.J. Dunn III, Dept. Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, IL, according to the procedure of Bledi et al. (2000). For the trafficking experiment, cells were incubated with dopamine at 37°C in serum-free medium for 1 hr. The culture medium was removed from each well in the plate. Cells were washed at room temperature with 200 µl of “KRH buffer” (130 mM
NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 1.8 g/L glucose, 0.1 mM tropolone, pH 7.4). Subsequently, 100 µl of KRH buffer was added to each well. Uptake assays were initiated by the addition of 100 µl of KRH buffer containing [³H]dopamine (54.8 Ci/mmol, NEN Life Sciences Products, Boston, MA, for a final concentration of 10 nM) and varying concentrations of unlabeled dopamine (0, 30, 100, 300, 1,000, 3,000 and 10,000 nM). Plates were incubated on a shaker at 21°C for 8 min. Uptake was terminated by addition of 100 µl of ice cold phosphate-buffered saline). The assay medium was removed by inverting the plate and shaking off the fluid. This was followed by two washes with 200 µl of ice cold wash buffer per well, and addition of 100 µl of Hi-Safe cocktail (Wallac Scintillation Products, Wallac UK, Milton Keynes). The plate was covered with a clear adhesive-backed sheet and counted in a Wallac counter (Gaithersburg, MD).

For the experiments with rat striatal synaptosomes, suspensions prepared as described above were incubated with dopamine at 37°C for 1 hr in uptake buffer A, and then washed with the same buffer twice in centrifugation steps (17,000 g for 20 min) at 4°C. The subsequent uptake assays were performed in a total volume of 0.4 ml in borosilicate glass culture tubes (12 × 75 mm). The experiments were divided in sets of 24 assay mixtures for harvesting in the 24-pin Brandel cell harvester (Gaithersburg, MD) with Whatman GF/C filters pretreated with 0.05% (w/v) poly-L-lysine. Incubations were started with intervals of 10 seconds between samples in a water bath with shaking, and termination of reaction occurred with same intervals. The uptake measurement was carried out in triplicate at room temperature and consisted of making additions to each tube in the shaker bath resulting in a content of 0.3 ml of buffer A with 4 nM [³H]dopamine and concentrations of unlabeled dopamine varying between 0.03 – 10 µM, followed, approximately 5 min later, by a 4-min incubation in the additional presence of 100 µl of treated synaptosomal preparation. Uptake was stopped by addition of 4 ml of ice-cold buffer A, and accumulated radioactivity was separated from free by filtration with the Brandel harvester. The filters were suspended in 5ml of CytoScint fluid (ICN, CA) for 6 hours and assayed for radioactivity by liquid scintillation counting (Beckman model LS 6000IC spectrometer).
Nonspecific uptake was defined with 100 µM cocaine. Protein content was determined by the DC Protein Assay (Bio-Rad).

**Assessment of surface DAT by biotinylation.** Non-cleavable biotin coupled to the highly reactive N-hydroxysuccinimide ester group, a membrane-impermeant probe, was used to covalently tag any surface-resident protein with free amino groups. The cells were grown on 100-mm dishes pretreated with AB-30 (see above). 80%-Confluent cells were incubated with dopamine at 37°C in serum-free medium for 1 hr. Cells were washed two times with ice-cold phosphate-buffered saline supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ (“PBS/Ca/Mg”) and treated with sulfo-NHS-biotin (Pierce, Rockford, IL), diluted with ice-cold PBS/Ca/Mg at a final concentration of 1 mg of protein/ml, and cells were gently shaken for 1 hour at 4°C. The free sulfo-NHS-biotin was then removed by rapidly washing two times with ice-cold 100-mM glycine containing PBS/Ca/Mg followed by two washes with PBS/Ca/Mg. The biotinylated cells were solubilized while shaking for 30 min at 4°C in 1 ml of “RIPA buffer” (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Na deoxycholate). The samples were centrifuged at 20,000 x g to pellet nonsolubilized material, and protein determinations were performed on the supernatant with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard. A sample of this supernatant was removed for estimation of “total DAT”. The biotinylated proteins in the supernatant were separated from nonbiotinylated proteins by batch affinity chromatography with monomeric avidin beads (250 µl beads/300 µg protein). Before use, the beads were blocked with 2 mM biotin in PBS for 15 min at room temperature and then beads were washed three times in 1 ml of 0.1 M glycine, pH 2.8. The beads were re-equilibrated in PBS by washing four times in 1 ml of PBS. These beads were then incubated with supernatant for 1 hour at room temperature with consistent rotation, centrifuged, and samples of the supernatant were stored for estimating “intracellular DAT”. The beads were washed four times in 1 ml of RIPA buffer. The biotinylated proteins were eluted from the beads by incubation for 30 min at room temperature with an equal volume of Laemmli SDS-polyacrylamide gel electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% mercaptoethanol and 0.05% bromophenol blue). The
samples were subjected to gel electrophoresis and western blotting as follows. The proteins were separated on a 8% polyacrylamide gel and transferred to nitrocellulose membrane (Hybond-ECL, Amersham, Piscataway, NJ) using a Trans-Blot Cell (Bio-Rad Laboratories) overnight at 0.1 A and a transfer buffer composed of 25 mM Tris and 192 mM glycine, 20% (v/v) methanol, pH 8.3. The nitrocellulose membrane was treated with blocking solution (1% bovine serum albumin in Tris-HCl saline containing 0.5% Tween-20) at room temperature for 1 hour. It was then incubated in a solution of the primary antibody, a rat anti-hDAT monoclonal antibody (Chemicon, Temecula, CA), diluted (0.4 µg/ml) with the blocking buffer overnight at 4°C. The blots were washed 5 times with the blocking solution at room temperature. Subsequently, it was incubated with secondary antibody, anti-rat IgG, peroxidase-linked species-specific whole antibody (Santa Cruz Biotechnology, Inc, CA) diluted 1:40,000 with the blocking buffer for 1 hour at room temperature. The blot was washed again as above and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) as described by the producer’s protocol book. The synaptosomal non-cleavable biotinylation procedure was as described above for cells except that unbound biotin was removed from synaptosome suspensions by centrifugation. A rabbit anti-rat DAT antibody 16B (1:800) was used for Western blotting. The antibody was provided by Dr. R.A. Vaughan, Dept. of Biochemistry and Molecular biology, University of North Dakota School of Medicine and Health Sciences.

Cleavable biotin was used in the internalization assays. Cell surface protein was biotinylated with 0.5 mg/ml cleavable sulfo-NHS-S-S-biotin (Pierce, Rockford, IL) for 1 hr at 4°C. Cells were then incubated in serum-free medium prewarmed to at 37°C for 1 hr and supplemented with appropriate ligands as indicated in Results section. Subsequently, cells were incubated at 4°C with two changes of strip buffer (50 mM 2-mercaptoethanesulfonic acid in 50 mM Tris-HCl, pH 8.7, 100 mM NaCl, and 2.5 mM CaCl₂). Cells were then solubilized as described above.

Densitometric scanning of western blots was done on the FlourChem digital imaging system (Alpha Innotech Corp., San Leandro, CA). On each gel, samples were compared that came from the biotinylation procedure applied to the same amount of cells (regardless of
pretreatment) as determined by the DC protein assay (Bio-Rad). The current experiments were performed within the linear range of the densitometry reading of the DAT band as a function of the amount of protein applied according to control experiments with varying amounts of protein load per lane. Densitometry data were captured as total signal in a rectangular area encompassing the band of study corrected for background; the same rectangular area was used for estimates of the same band in other lanes of the gel. Results from different scans were uniform.

**Data analysis.** $K_m$ and $V_{max}$ values for $[^3H]$DA uptake were obtained with the nonlinear computer fitting program KELL as described by us previously (Li et al., 2002). All results are expressed as mean ± SEM. Statistical analysis consisted of one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. The accepted level of significance was 0.05.

**Results**

**$[^3H]$Dopamine uptake by HEK-hDAT cells pretreated with substrate.** Because increases in $[^3H]$dopamine uptake had been reported following treatment for 10 min with 1 µM dopamine in HEK-hDAT cells in a preliminary report of Seamans et al. (2001), varying concentrations of dopamine were used at different preincubation times (Fig. 1). In these experiments, HEK-hDAT cells were pretreated with dopamine at 37°C, followed by wash-out. No effects were seen with 1 µM dopamine for 10 min or increasing times up to 60 min (Fig. 1A). Concentrations of 10 and 100 µM dopamine were effective in reducing $[^3H]$dopamine uptake after a 60-min pretreatment period, which could be shortened to 40 or 20 min while still displaying an inhibitory effect (Figs. 1B and C). Pretreatment with dopamine for 1 h at 37°C, followed by wash-out, showed a concentration-dependent effect on $[^3H]$dopamine uptake: no changes with concentrations of dopamine between 0.1 and 1 µM, and statistically significant reductions with concentrations in the 10-100 µM range (Fig. 2A). The effect of pretreatment with 10, 50, and 100 µM dopamine for 1 h was further characterized (Table 1). $K_m$ values for
[3H]dopamine uptake were not affected, whereas increasing dopamine concentrations during pretreatment caused progressive decreases in V_max values (Table 1).

Because previous reports have described the effect of the substrate d-amphetamine (Saunders et al, 2000; Daws et al., 2002), this drug was included here for comparison. HEK-hDAT cells were pretreated with d-amphetamine (4 µM) for 1 h at 37°C; the resulting reduction in [3H]dopamine uptake was blocked by the co-presence of cocaine (4 µM) whereas cocaine itself (4 µM) was ineffective (Fig. 2B).

**Trafficking of HEK cell surface DAT assessed by biotinylation.** Biotinylation of cell surface protein with noncleavable sulfo-NHS-biotin showed a 30% decrease in surface DAT after an 1-h treatment with dopamine (100 µM) at 37°C (Table 2 top half). In order to check the responsivity of the current system to phorbol esters well known to down-regulate DAT, PMA (10 µM) was applied and found to decrease surface DAT by approximately 53% (Table 2 top half). A faint 30- and 50-kDa band was present in addition to 75-kDa DAT. Densitometric scanning did not reveal statistically significant changes in these bands from the same gels that showed an effect from dopamine or PMA pretreatment on DAT, arguing in favor of the specificity of the DAT response.

In order to assess whether dopamine reduces surface DAT by enhancing endocytosis, first surface proteins were biotinylated with cleavable sulfo-NHS-S-S-biotin, and then cells were incubated with dopamine (100 µM) or PMA (10 µM) for 1 h at 37°C. Subsequently, surface biotin was cleaved off with 2-mercaptoethanesulfonic acid, cells were lysed, and internalized biotinylated proteins were captured with avidin beads and probed on polyacrylamide gels by Western blotting with anti-DAT antibody (Fig. 3A). No bands other than 75-kDa DAT were observed. The total protein fraction was obtained by taking a sample of the cell lysate before treatment with avidin beads, and this sample was examined on gels (“total DAT”, Table 2 bottom half, Fig. 3A) along with a sample of cells taken before pretreatment and carried through the entire procedure of stripping and avidin-capturing (“strip of untreated cells”, Table 2 bottom half). The latter strip, as a fraction of total DAT, showed a very small amount of DAT (2.8% of total, Table 2, see also Fig. 3A), indicating the near-complete cleavage of surface biotin.
Pretreatment for 1 h with dopamine or PMA showed 40% and 59%, respectively, of total DAT being endocytosed. This is an increase over the constitutive 18.5% endocytosis seen in the same 1-h period in the absence of activating compound (Table 2, Fig. 3A). The dopamine effect, 40% - 18% = 22% (Table 2), appears to be smaller than the effect on [3H]dopamine uptake (~ 30-40%, Figs. 1 and 2 and Table 1). In this comparison, it should be kept in mind that the % endocytosis data are expressed as a fraction of total DAT (which is the sum of surface and intracellular DAT), whereas the uptake effect, if fully caused by loss of surface DAT, would be related to endocytosed DAT as % of surface DAT. In separate experiments we have estimated that 54% of total DAT resides at the surface in HEK-293 cells (manuscript in preparation), and therefore 22% endocytosis of total DAT can be calculated as percent of surface DAT as follows: (22/54) x 100 = 41%. This is in the range of the observed dopamine uptake effects.

**[3H]Dopamine uptake by rat striatal synaptosomes pretreated with substrate.**

Pretreatment of rat striatal synaptosomes with dopamine for 1 h at 37°C, followed by wash-out, showed a concentration-dependent effect on [3H]dopamine uptake: no change with 1 µM dopamine, and statistically significant reductions (24% and 34%) at concentrations of 10 and 100 µM (Table 3 second column). Saturation analysis showed that K_m values for [3H]dopamine uptake were not affected, whereas pretreatment with dopamine concentrations of 10 and 100 µM caused a decrease in V_max of approximately 34% (Table 3 third and fourth column). Preliminary experiments indicated the inhibitory effect of pretreatment with 100 µM dopamine could be achieved also with a shorter preincubation time of 10 min.

**Trafficking of surface DAT assessed by biotinylation of rat striatal synaptosomes.**

Biotinylation of surface proteins in rat striatal synaptosomes with noncleavable sulfo-NHS-biotin showed a 21-27% decrease in surface DAT after an 1-h treatment with dopamine (100 µM) at 37°C (Table 4 two sets of experiments, see also Fig. 3B). PMA (10 µM) also decreased surface DAT (33%, Table 4). The involvement of DAT in the effect of dopamine was supported by the observation of full blockade by cocaine which had no effect by itself (Table 4, Fig. 3). Cocaine was applied at a dose (100 µM) expected to be in excess of and thus compete for 100 µM dopamine upon consideration of approximate K_i values for DAT of 0.4 µM for cocaine and 8 µM.
for dopamine (Reith et al., 2001). Another indication for DAT being responsible for the signal detected with anti-DAT antibody on gels loaded with striatal fractions comes from the extremely low signal obtained for cerebrocortical synaptosomes worked up and assayed under the same conditions (Table 4, Fig. 3B). Concanavalin A (250 µg/ml) blocked the effect of dopamine in reducing surface DAT in rat striatal synaptosomal fractions (Table 4, Fig. 3B). As in HEK-hDAT cells treated with non-cleavable biotin, a faint 30- and 50 kDa band was present. Again, there were no statistically significant changes in the densitometry reading of these bands from the same gels that showed an effect from dopamine or PMA pretreatment on DAT, arguing in favor of a selective effect on DAT.

Discussion

Most results regarding acute effects of dopamine on surface DAT activity come from experiments with cells heterologously expressing DAT, which indicate an increase in activity after pretreatment with 1 µM dopamine (Seamans et al., 2001), no effect from exposure to 10 µM dopamine (Daniels and Amara, 1999), and a decrease following treatment with 100 µM dopamine (Saunders et al., 2000). The possibility for a biphasic effect depending on dopamine concentration was tested in the present work and not borne out by the results which show only decreases in surface DAT uptake activity starting at 10 µM dopamine in experiments with dopamine concentrations ranging from 0.1 to 100 µM. The reductions in uptake became significant at pretreatment times of 20 min or longer. The results from biotinylation experiments support the idea that the uptake reduction is mediated by a decrease in DAT expression at the cell surface. This decrease at 100 µM dopamine agrees with the effect described only qualitatively in the study by (Saunders et al., 2000) for a cell system (EM4) resembling ours (HEK-293). The lack of effect of 10 µM dopamine in the study by (Daniels and Amara, 1999) could be due to the use of a different expression system, Madin-Darby canine kidney (MDCK) cells, or to the additional presence of a green fluorescent protein tag at the N-terminus of DAT. More information is required in order to explain the difference between the lack of effect of pretreatment with 1 µM dopamine in the present experiments and the increase in surface DAT.
activity described in the preliminary report of (Seamans et al., 2001). Most likely, the cell system used was the same, HEK-293 cells, if the experiments in the work of Seamans et al. (2001) utilized the same cells as described by the same group in the oligomerization work (Torres et al., 2003). It is possible a His\textsubscript{6} (HHHHHH) or HA (YPYDVPDYA) tag was present, potentially impacting DAT trafficking. Another possibility is that the effect of dopamine depends on the density of DAT at the surface. Thus, the nucleus accumbens has a lower density of DAT than the striatum and is also more resistant to dopamine-induced down regulation of DAT function (Gulley et al., 2002).

The observed reduction in surface DAT activity in the present HEK-hDAT cells is likely due to decreased surface expression as monitored by reduced surface biotinylated DAT. The reduction in surface DAT by dopamine could be due to enhanced endocytosis or reduced recycling to the plasma membrane. The present results with cleavable biotin strongly implicate enhanced endocytosis, in agreement with the general idea that recycling is constitutive and generally unaffected by receptor agents (Koenig and Edwardson, 1997). For DAT, an ongoing debate concerns the fate of the protein after endocytosis and entrance into the endosomal compartment: recycling to plasma membranes or progression to degrading lysosomes. Cell environment and tags attached to DAT are possible factors determining the outcome of recycling vs. degradation (Daniels and Amara, 1999; Melikian and Buckley, 1999; Loder and Melikian, 2003). Interestingly, in stably transfected PC12 cells where DAT recycling plays a major role, PKC not only enhances endocytosis but also reduces recycling (Loder and Melikian, 2003), indicating that the recycling step can be manipulated. More experiments are needed in our HEK-hDAT system to determine whether the dopamine effect is fully explained by enhanced endocytosis, and how much recycling occurs in comparison to degradation.

In parallel with the cell experiments, the present results with rat striatal synaptosome-containing fractions indicate a reduction in surface DAT activity following exposure to dopamine. These data are consonant with preliminary results reported for repeated local application of dopamine into rat striatum in vivo, leading to a reduction in surface DAT activity (Gulley et al., 2002). Results from biotinylation of surface proteins with noncleavable sulfo-NHS-biotin in the
current work with synaptosomes suggest that reduced surface expression underlies the observed decrease in DAT activity. The dopamine-mediated decrease in surface DAT could blocked (Table 4, Fig. 3) by the co-presence of concanavalin A which has been used previously with the aim to inhibit endocytosis of DAT (Saunders et al., 2000) based on the report that concanavalin A prevents the formation of the low-affinity form of β-adrenergic receptors that is associated with internalization (Toews et al., 1984). Be that as it may, it is to be noted that caution is needed in interpreting biotinylation results for synaptosomal preparations. One might expect, in these preparations, the presence of broken membrane fragments from axons, and from nerve endings that did not successfully reseal during preparation; these fragments would be biotinylated, captured in the avidin step, and therefore included in the “surface” fraction. One could also think that DAT is not regulated by dopamine pretreatment in these fragments as translocation appears to be required for the dopamine effect (see next paragraph). However, the present results indicate that the presence of such biotinylated fragments is not major, because the effect of dopamine pretreatment (100 µM) on subsequent [³H]dopamine uptake (34%, Table 3) is only slightly larger than the effect on surface expression of DAT as measured by biotinylation (21-27%, Table 4). In addition, the effects of dopamine and PMA monitored by biotinylation in HEK-hDAT cells are largely retained in synaptosomes (compare Tables 2 and 4), arguing in favor of the validity of the biotin approach for studying synaptosomal fractions. The biotinylation technique applied to brain tissue appears to be a viable measure reflecting, for a large part, surface DAT. In the absence of other tools for assessing surface proteins in synaptosomal fractions, biotinylation allows measurement of DAT trafficking in a physiologically relevant environment.

In the experiments with cocaine for blockade of dopamine-induced trafficking, an excess of cocaine was used compared with the Kᵢ value for cocaine in order to compete for dopamine in relation to the Kᵢ value for dopamine. In this context, it can be noted that the Kᵢ for dopamine (8 µM under the present conditions in synaptosomes) is much greater than the Kᵢ for dopamine uptake (0.16 µM, Xu et al., 1995), because a step after initial dopamine recognition in the translocation process becomes saturated more rapidly than dopamine binding itself upon raising the concentration of dopamine. This step likely is the reorientation step (see also Chen et al.,
2001). We have previously suggested that this reorientation step may be relatively less rate limiting in cell lines heterologously expressing DAT, reflected by higher $K_m$ values for dopamine uptake (Reith et al., 1996).

Under what conditions would one expect extracellular concentrations of 10 to 100 µM of dopamine that are required for downregulation of DAT? With the ongoing debate about precise measurement of extracellular dopamine concentrations this is a difficult question. Thus, Bungay et al. (2003) convincingly show that dopamine measurements by microdialysis likely reflect underestimates, with discrepancies between measured and true values increasing as a function of the thickness of the trauma layer that results from probe implantation. This thickness is unknown, but it could be great enough to underestimate dopamine concentrations by a factor of 10 or more, yielding perhaps basal extracellular dopamine concentrations of 150 – 485 nM (Bungay et al., 2003). With this in mind, one could interpret 50- to 65-fold changes in caudate and accumbal dialysate dopamine observed following 5 mg/kg (s.c.) of d-amphetamine (Kuczenski and Segal, 1992) as representing 8 - 32 µM extracellular concentrations; peak effects following DAT blockers such as cocaine (30 mg/kg i.p.) or nomifensine (10 mg/kg i.p.) in the same study would represent 1 – 10 µM extracellular dopamine. It is therefore possible that DAT trafficking could occur, to some extent, upon in vivo administration of DAT substrates and blockers. It is also possible that downregulation of DAT by high levels of extracellular dopamine represents a protective mechanism against dopamine which is known to be neurotoxic upon intrastriatal injection (Rabinovic et al., 2000). For a description how dopamine release can be stimulated by glutamate at the same time that dopamine uptake is inhibited through the arachidonic acid signaling pathway, please see Reith et al. (1997). Conceivably, this mechanism could massively enhance extracellular dopamine under conditions of glutamate excitotoxicity.

The ability of cocaine to attenuate the reduction in dopamine uptake induced by pretreatment with d-amphetamine in HEK-hDAT cells (Fig. 1B) and to block the dopamine effect on surface DAT in rat striatal synaptosomes (Fig. 3, Table 4) suggests that dopamine translocation by DAT is a prerequisite for the trafficking effect. Alternatively, cocaine has a direct effect on DAT trafficking, perhaps by inhibiting transporter internalization or promoting exocytosis as
suggested by (Saunders et al., 2000) and Daws et al. (2002). Indeed, cocaine by itself has been shown to increase DAT surface expression in HEK-hDAT cells, enhance \[^3H\]dopamine uptake into rat nucleus accumbens synaptosomes ex vivo, and enhance DAT-mediated dopamine clearance in rat striatum in vivo (Daws et al., 2002). It is possible that cocaine-induced enhancement of DAT surface expression in the nucleus accumbens also underlies the early observation of Missale et al. (1985) of an increased $V_{\text{max}}$ of \[^3H\]dopamine uptake into accumbal slices prepared ex vivo, in contrast to the reduction in uptake $V_{\text{max}}$ in striatal slices. We observed increases in surface DAT in HEK-hDAT cells upon treatment with 1 or 10 $\mu$M cocaine for 24 h, as reported by Little et al. (2002) for neuro2A-hDAT cells, but in the present experiments with acute cocaine exposure we did not observe changes in surface DAT expression. Under our conditions, cocaine’s action appears to be limited to blockade of substrate effects on DAT trafficking by prevention of substrate translocation. Differences between the present conditions and those in the study of Daws et al. (2002) include the absence and presence, respectively, of a yellow fluorescent protein tag on DAT in HEK-hDAT cells, and the in vitro vs. ex vivo or in vivo cocaine treatment protocol in the rat studies. The present results with cocaine suggest that regulation of DAT by dopamine requires DAT activity or accumulation of dopamine into the cytosol. More experiments are needed to distinguish these possibilities.

Regulation of surface expression of transporters by their substrates or blockers does not appear to follow the general trend documented for receptors, where agonists commonly downregulate, and antagonists upregulate, their presence at the plasma membrane. Even within the same family of \(\text{Na}^+,\text{Cl}^-\)-dependent neurotransmitter transporters, the serotonin transporter and GAT1 respond to external substrate in an opposite manner (increase in surface presence, Bernstein and Quick, 1999; Ramamoorthy and Blakely, 1999) compared with the DAT (decrease in surface residency, this study). For the serotonin transporter, PKC-mediated phosphorylation has been shown to be involved in the trafficking effect exerted by substrate (Bernstein and Quick, 1999; Ramamoorthy and Blakely, 1999); for GAT1, PKC-mediated effects are also well-known (Beckman et al., 1998; 1999) and perhaps implicated in regulation of trafficking by substrate (Bernstein and Quick, 1999). PKC activation downregulates DAT (Zhu et al., 1997; Pristupa et
al., 1998; Little et al. 2002; this study) and preliminary evidence suggests that exposure to dopamine promotes the dephosphorylated form of DAT (Vrindavanam et al., 1996). In addition, there is evidence that PKC-mediated phosphorylation is involved in DAT reversal upon exposure to substrate (Cowell et al., 2000). PKC-mediated downregulation of surface transporters is common to the serotonin transporter, GAT1 and DAT, but the substrate effect on the former two transporters has a direction opposite to that on DAT. This indicates different underlying mechanisms even if PKC-mediated phosphorylation of transporter is involved in all these cases. The report by Chang et al. (2001) and a recent study by Granas et al. (2003) cast doubt as to whether PKC-mediated phosphorylation of DAT affects its ability to traffic. It is possible that phosphorylation is involved of a protein associated with DAT.

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Rabinovic AD, Lewis DA, and Hastings TG (2000) Role of oxidative changes in the degeneration of dopamine terminals after injection of neurotoxic levels of dopamine. *Neurosci.* **101:**67-76.


Legends to figures

**Fig. 1.** $[^3]$Hdopamine uptake into HEK-hDAT cells after pretreatment at varying times (10-60 min) with (A) 1 µM dopamine (DA), (B) 10 µM DA, or (C) 100 µM DA. After pretreatment at 37°C, drug was removed and uptake of 10 nM $[^3]$Hdopamine was monitored for 8 min at room temperature. Results are expressed as % of uptake after pretreatment without dopamine (% control) and are mean ± S.E.M. for 4 independent experiments. *P<0.01 (compared with control, Tukey-Kramer multiple comparisons test).

**Fig. 2.** $[^3]$Hdopamine uptake into HEK-hDAT cells after (A) pretreatment for 1 h with dopamine (0.1-100 µM) or (B) d-amphetamine (4 µM) with and without cocaine (4 µM). After pretreatment at 37°C, drug was removed and uptake of 10 nM $[^3]$Hdopamine was monitored for 8 min at room temperature. Results are expressed as % of uptake after pretreatment without dopamine (% control) and are mean ± S.E.M. for 4 independent experiments. *P<0.05, **P<0.01, ***P<0.001 (compared with control, Tukey-Kramer multiple comparisons test).

**Fig. 3.** Biotinylation experiments with (A) HEK-hDAT cells and (B) rat brain synaptosomes. Shown are the molecular weight areas on the gels where DAT migrates for typical experiments. Averages of all experiments are shown in (i) Table 2 in which the noncleavable biotin results correspond to the three lanes at the left in panel A in this figure and the cleavable biotin results correspond to the remaining lanes in panel A, and (ii) Table 4 in which the striatal results correspond to the first eight lanes from left to right in panel B in this figure and the cortex data correspond to the final lane at the right hand side. See text in Tables 2 and 4 for details.
Table 1. [³H]dopamine uptake into HEK-293-hDAT cells after treatment with dopamine for 1 h.

<table>
<thead>
<tr>
<th>Dopamine concentration µM</th>
<th>K_m µM</th>
<th>V_max pmol/mg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.61± 0.02</td>
<td>3.98 ± 0.14</td>
</tr>
<tr>
<td>10</td>
<td>0.58 ± 0.01</td>
<td>2.96 ± 0.18*</td>
</tr>
<tr>
<td>50</td>
<td>0.58 ± 0.02</td>
<td>2.53 ± 0.22**</td>
</tr>
<tr>
<td>100</td>
<td>0.60 ± 0.03</td>
<td>2.26 ± 0.35**</td>
</tr>
</tbody>
</table>

Cells were pretreated for 1 h at 37°C with the indicated dopamine concentrations, dopamine was removed, and uptake of 10 nM [³H]dopamine was monitored for 8 min at room temperature. Results are mean ± S.E.M. of 4 independent experiments carried out in triplicate.

* P<0.01 compared with 0 µM dopamine (Tukey-Kramer test following 1-way ANOVA).
** P<0.001 compared with 0 µM dopamine (Tukey-Kramer test following 1-way ANOVA).
Table 2. Trafficking of surface DAT in HEK-hDAT cells upon treatment for 1 h with dopamine (100 µM) or PMA (10 µM).

<table>
<thead>
<tr>
<th>Method</th>
<th>DAT at surface (% control)</th>
<th>DAT endocytosed (% of total DAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Noncleavable biotin method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control treatment</td>
<td>100 ± 5</td>
<td></td>
</tr>
<tr>
<td>dopamine treatment</td>
<td>70.0 ± 4.7*</td>
<td></td>
</tr>
<tr>
<td>PMA treatment</td>
<td>46.9 ± 5.4**</td>
<td></td>
</tr>
<tr>
<td><strong>Cleavable biotin method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strip of untreated cells</td>
<td>2.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>endocytosed DAT, control treatment</td>
<td>18.5 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>endocytosed DAT, dopamine treatment</td>
<td>40.0 ± 2.5*</td>
<td></td>
</tr>
<tr>
<td>endocytosed DAT, PMA treatment</td>
<td>59.0 ± 7.3**</td>
<td></td>
</tr>
</tbody>
</table>

For the noncleavable biotin experiments, cells were pretreated for 1 h at 37°C with 100 µM dopamine or 10 µM PMA, drug was removed, and noncleavable biotinylation was applied. For the cleavable biotin method, cell surface protein was biotinylated prior to treatment for 1 h at 37°C with 100 µM dopamine or 10 µM PMA; biotin was stripped with 2-mercaptoethanesulfonic acid from non-endocytosed protein, and subsequently remaining biotinylated protein was isolated from cell extracts. “Control incubation” signifies incubation without dopamine or PMA for the same time as with dopamine or PMA. The “glutathione strip of untreated cells” assesses incomplete stripping which would cause non-endocytosed DAT to show up as endocytosed DAT. Results are mean ± S.E.M. of 3 independent experiments.

* P<0.05 compared with control (Tukey-Kramer test following 1-way ANOVA).
** P<0.01 compared with control (Tukey-Kramer test following 1-way ANOVA).
Table 3. $[^3H]$Dopamine uptake into rat striatal synaptosomes after treatment with dopamine for 1 hr.

<table>
<thead>
<tr>
<th>Dopamine concentration (µM)</th>
<th>$[^3H]$dopamine uptake at 4 nM substrate (% control)</th>
<th>$[^3H]$dopamine uptake</th>
<th>$[^3H]$dopamine uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 4</td>
<td>131 ± 4</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>1</td>
<td>95.5 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>76.2 ± 1.8*</td>
<td>126 ± 5</td>
<td>12.1 ± 0.3*</td>
</tr>
<tr>
<td>100</td>
<td>66.1 ± 2.3*</td>
<td>130 ± 13</td>
<td>12.2 ± 0.8*</td>
</tr>
</tbody>
</table>

Synaptosomes were pretreated for 1 h at 37°C with the indicated dopamine concentrations, dopamine was removed, and uptake of 4 nM $[^3H]$dopamine was monitored for 4 min at room temperature. Results are mean ± S.E.M. of 3 independent experiments carried out in triplicate.

* P<0.01 compared with 0 µM dopamine (Tukey-Kramer test following 1-way ANOVA).
Table 4. Trafficking of surface DAT in rat striatal synaptosomes upon treatment for 1 h with dopamine (100 µM), PMA (10 µM), concanavalin A (Con A, 250 µg/ml), or cocaine (100 µM), contrasted against the assessment of DAT in cerebrocortical synaptosomes.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>DAT at surface (%) control</th>
<th>DAT at surface (% of value in striatum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>102 ± 4</td>
<td></td>
</tr>
<tr>
<td>dopamine</td>
<td>73.1 ± 1.5*</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>66.6 ± 7.1*</td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>103 ± 5</td>
<td></td>
</tr>
<tr>
<td>dopamine</td>
<td>78.6 ± 2.0*</td>
<td></td>
</tr>
<tr>
<td>dopamine plus cocaine</td>
<td>101 ± 4</td>
<td></td>
</tr>
<tr>
<td>dopamine plus Con A</td>
<td>100 ± 4</td>
<td></td>
</tr>
<tr>
<td>cocaine</td>
<td>103 ± 6</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>3.3 ± 0.6**</td>
<td></td>
</tr>
</tbody>
</table>

Rat striatal synaptosomes were pretreated for 1 h at 37°C with 100 µM dopamine, 10 µM PMA, 250 µg/ml concanavalin A, or 100 µM cocaine. For control treatment, treatment was with vehicle. After removal of drug, the noncleavable biotinylation method was applied. Cerebrocortical synaptosomes were prepared and subjected to control treatment the same way as striatal synaptosomes. Results are mean ± S.E.M. of 3 independent experiments carried out in triplicate.

* P<0.01 compared with control (Tukey-Kramer test following 1-way ANOVA).
** P<0.01 compared with Striatum control (Student’s t-test).