Regulation of Dopamine Transporter Function and Cell Surface Expression by D3 Dopamine Receptors*

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D3 dopamine receptors are expressed by dopamine neurons and are implicated in the modulation of presynaptic dopamine neurotransmission. The mechanisms underlying this modulation remain ill defined. The dopamine transporter, which terminates neurotransmission via reuptake of released neurotransmitter, is regulated by receptor- and second messenger-linked signaling pathways. Whether D3 receptors regulate dopamine transporter function is unknown. We addressed this issue using a fluorescent imaging technique that permits real time quantification of dopamine transporter function in living single cells. Accumulation of the fluorescent dopamine transporter substrate trans-4-[4-(dimethylamino)styril]-1-methylpyridinium (ASP+4) in human embryonic kidney cells expressing human dopamine transporter was saturable and temperature-dependent. In cells co-expressing dopamine transporter and D3 receptors, the D2/D3 agonist quinpirole produced a rapid, concentration-dependent, and pertussis toxin-sensitive increase of ASP+4 uptake. Similar agonist effects were observed in Neuro2A cells and replicated in human embryonic kidney cells using a radioligand uptake assay in which binding to and activation of D3 receptors by [3H]dopamine was prevented. D3 receptor stimulation activated phosphoinositide 3-kinase and MAPK. Inhibition of either kinase prevented the quinpirole-induced increase in uptake. D3 receptor activation differentially affected dopamine transporter function and subcellular distribution depending on the duration of agonist exposure. Biotinylation experiments revealed that the rapid increase of uptake was associated with increased cell surface and decreased intracellular expression and increased dopamine transporter exocytosis. In contrast, prolonged agonist exposure reduced uptake and transporter cell surface expression. These results demonstrate that D3 receptors regulate dopamine transporter function and identify a novel mechanism by which D3 receptors regulate extracellular dopamine concentrations.

The D3 dopamine (DA) receptor, a member of the D2-like family of DA receptors, is expressed in limbic brain regions, both presynaptically on DA neurons as well as postsynaptically. The D3 receptor has gained increasing attention as a target for the treatment of schizophrenia, psycho-stimulant abuse, and DA cell neurodegeneration (1–4). Its restricted central nervous system distribution, relative to D2 receptors, suggests that D3 receptor ligands may have fewer side effects than currently available therapeutic agents.

Studies using D3 receptor knock-out mice (5–7) or D3 antisense (8, 9) revealed that D3 receptors regulate extracellular DA in ventral striatum. This effect was attributed to D3 regulation of a long negative feedback loop in which postsynaptic D3 receptors on medium spiny neurons modulate the activity of accumbens output neurons projecting to DA cell bodies in mid-brain (5). However, this hypothesis is incompatible with the effects of DA receptor ligands in tissue preparations in which efferent projections to midbrain DA nuclei are disrupted; modulation of extracellular DA by D3 receptors has been demonstrated in striatal slices (7) and tissue suspensions (10). Pharmacological studies examining the mechanism of such regulation have been precluded by the lack of selective ligands that discriminate between D2 and D3 receptors in vivo (5, 11–13).

DA signaling is terminated by the DA transporter (DAT), an integral membrane protein that re-uptakes DA released into the extracellular space (14). Receptor and second messenger-linked kinase cascades regulate DAT function and cell surface

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4 The abbreviations used are: DA, dopamine; DAT, DA transporter; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MesNa, sodium 2-mercaptoethanesulfonate; TCR, transferrin receptor; h, human; ASP4, trans-4-[4-(dimethylamino)styril]-1-methylpyridinium; YFP, yellow fluorescent protein; GFP, green fluorescent protein; df, degrees of freedom; MEK, MAPK/ERK kinase.

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expression (15). An involvement of D2 DA receptors in regulating DAT function was suggested previously (16–18). Consistent with this hypothesis, DA uptake and transporter-associated currents are increased in oocytes (19) co-expressing human DAT (hDAT) and D2 receptors, and D2 receptor stimulation increases DAT activity in transfected HEK293 cells (20). Recently, modulation of DA uptake by low concentrations of D3-preferring ligands was reported in vivo (10). Such findings suggest that D3 receptors may regulate DA neurotransmission via a DAT-mediated mechanism. However, because the brain regions examined contain D2 and D3 receptors and the ligands employed bind to both receptor subtypes, the role of D3 receptors remains unclear.

The present studies aimed to determine whether D3 receptors regulate DAT function in heterologous expression systems and to identify the intracellular mechanisms mediating this effect. We show that acute D3 receptor activation increases DAT activity in human embryonic kidney and mouse neuroblastoma cells co-expressing hDAT and human D3 receptors (hD3). This acute effect is pertussis toxin-sensitive and requires the activation of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK). Interestingly, sustained D3 receptor activation has opposite effects, decreasing DAT activity. Furthermore, biotinylation studies show that D3 receptor stimulation regulates DAT trafficking by affecting both DAT endocytosis and exocytosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), (S)-(+)-(4aR10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H[1]benzopyrano[4,3,b]-1,4-oxazin-9-ol hydrochloride (PD128907), 2-(4-morpholinyl)-8-phenyl-1,2-benzopyran-4-one hydrochloride (LY294002), 2-[4-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride (LY294002), 2-[1-(3-dimethylamino-propyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X), nomifensine, spiperone, and quinpirole were purchased from Tocris Cookson (Ellisville, MO). ASP+ and R(+)-6-bromo-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide (B135) were obtained from Sigma.

**Cell Culture**—Experiments were conducted in EM4 cells (R. Horlick, Pharmacopeia, Cranberry, NJ), HEK293 cells stably expressing macrophage scavenger receptor (21), and Neuro2A cells (N2A, American Type Culture Collection) unless otherwise indicated. ASP+ experiments were performed in EM4 cells stably transfected with FLAG-hDAT (22) and transiently transfected with GFP-tagged human D3 (hD3) receptors (M. G. Caron, Duke University Medical Center, Durham, NC) and in EM4 or N2A cells transiently co-transfected with GFP-hD3 receptors and either FLAG-hDAT or YFP-hDAT (23). Previous studies have shown that the addition of these tags does not alter the function of these proteins (22–26).

EM4 and N2A cells were maintained in DMEM/F-12 and minimum Eagle's medium (Cellgro®, Mediatech, Inc, Herndon, VA), respectively, and supplemented with 10% fetal bovine serum (Invitrogen). Cells were seeded on day 1 at 1.5 × 10⁵ cells/35-mm glass-bottomed Petri dish (BioOptechs, Butler, PA), and transient transfections were performed on day 2 using Lipofectamine 2000. Experiments were performed 32–48 h after transfection when cells were 70–90% confluent. Cells were grown in a humidified atmosphere at 37 °C and 5% CO₂.

**[^3]H**DA Uptake—EM4 cells were transiently transfected with the appropriate vector (FLAG-DAT, myc-hD3, and FLAG-DAT plus myc-hD3 receptor and pcDNA3 plasmids) as indicated in figures and legends. DA uptake was performed as described. Briefly, cells were washed with 1.0 ml of Krebs-Ringer-HEPES (KRH) buffer, pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 10 mM HEPES, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM Tris, and 10 mM d-glucose), containing 0.1 mM ascorbic acid and 0.1 mM pargyline. Cells were then preincubated with the modulators for the indicated times followed by the addition of 20 nM[^3]H**DA to initiate DA uptake for 1 min. Uptake was terminated after 1.0 min of incubation at 22 °C by rapid washings with cold KRH assay buffer. Cells were lysed in 0.1% SDS, and accumulated radioactivity was measured by liquid scintillation. Initial studies revealed that part of the[^3]H**DA signal in cells transfected with hDAT plus hD3 was insensitive to the DAT blocker nomifensine but was blocked by the D3 antagonist spiperone or agonist quinpirole (Fig. 1, A and B, see “Results”). Therefore, for each experiment specific[^3]H**DA binding to the hD3 receptor was obtained from cells transfected with hD3 receptor plasmid alone or DAT plus hD3 receptor plasmids in the presence of 50 μM nomifensine. DAT-specific[^3]H**DA uptake was defined as the accumulation of[^3]H**DA in the presence of 0.1 μM spiperone (no receptor stimulation) or 10 μM quinpirole (maximal receptor stimulation). Note that[^3]H**DA signal obtained in the presence of spiperone or quinpirole was sensitive to DAT blocker nomifensine indicative of DAT-specific[^3]H**DA uptake. Nonspecific[^3]H**DA uptake and binding was defined as the accumulation/binding in the presence of both spiperone (or quinpirole) and nomifensine and was subtracted from total counts. Nonspecific background was also compared with cells transfected with pcDNA3 vector alone.

**Cell-based ELISA for pERK Activation**—A phosphospecific cell-based ELISA (modified from Versteeg et al. (71)) for phosphorylated ERK1/2 (p-ERK) was used to determine whether DA (1 pm to 10 μM) or the DAT substrates, tyramine (1 pm to 100 μM) and ASP+ (1 pm to 100 μM), activate D3 receptors. Flip-in T-rex 293 cells (Invitrogen) stably expressing FLAG-tagged hD3 receptors were used. Cells were seeded in 96-well plates (50,000 cells/cm²) in DMEM supplemented with 10% fetal bovine serum and grown at 37 °C and 5% CO₂ for 24 h. Tetracycline (0.1 μM) was added for an additional 16–20 h to induce hD3 receptor expression. Cells were serum-starved in serum-free media for 2 h prior to substrate addition (100 μM). After 3 min, media were aspirated, and cells were immediately fixed by adding 150 μl of 4% formaldehyde/PBS solution for 30 min. Cells were permeabilized by addition of 0.1% Triton X-100/PBS solution (three times for 10 min). After blocking with 10% BSA in Triton/PBS solution for 1 h, cells were washed with Triton/PBS solution. Primary p-ERK monoclonal antibody (Cell Signaling Technology) was diluted 1:400 in Triton/PBS solution containing 5% BSA, and cells were incubated with primary antibody for 1 h at room temperature. After washing cells three times with PBS/Triton solution, and 1 h of incuba-
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...tion with horseradish peroxidase-conjugated goat anti-mouse 2° antibody (1:1000 dilution; Santa Cruz Biotechnology) in PBS/Triton solution containing 5% BSA, cells were again washed with Triton/PBS solution. Pierce supersignal ELISA Pico substrate (100 μl) was added to each well for 2 min, and the luminescence signal was measured with a BMG PolarStar reader. The values from each experiment were normalized to the basal level for that experiment. The means ± S.E. are shown for these determinations from three independent experiments, each performed in triplicate. The data were analyzed with GraphPad Prism using a one-site sigmoidal dose-response curve.

Live Cell Imaging of ASP+ Uptake—Time-resolved quantification of DAT function in single cells was achieved using the fluorescent, high affinity DAT substrate ASP (27). Immediately prior to experiments, growth media were removed, and cells were washed twice in Krebs-Ringer/HEPES medium (KRH (in mM): 130 NaCl, 1.3 KCl, 2.2 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 10 HEPES, and 1.8 g/liter glucose, pH 7.4). After washing, fresh KRH was added, and the culture dish was mounted on an UltraVIEW™ LCI spinning-disk confocal microscope fitted with a ×60 water objective lens (PerkinElmer Life Sciences).

A within cell design was used to assess the effects of the D2/D3 agonists, quinpirole (0.1–10 μM) and PD12897 (10 μM) on ASP+ uptake. Immediately after mounting of the culture dish, the microscope was focused on the center of a monolayer of cells, and background auto-fluorescence was determined by collecting an image immediately prior to replacement of the KRH buffer with buffer containing ASP+ (10 μM). Vehicle or agonist was added 5 min later, and the slope of ASP+ accumulation was determined over a 1-min period both before and after their addition. Control studies showed that ASP+ uptake by DAT was linear over the first 10 min after ASP+ addition (28). Images were collected every 20 s for 10 min to enable capture of either GFP or YFP (excitation, 488 nm; emission, 525–575 nm) and ASP+ fluorescence (excitation, 488 nm; emission, 607–652 nm). A between cell design was used for control experiments assessing saturability, temperature dependence, and the influence of DAT substrates and blockers on ASP+ uptake. Cells were preincubated with test drug or vehicle for the designated time periods, and total intracellular ASP+ accumulation over a 5-min time period was quantified. Saturation of ASP+ uptake was tested using a range of ASP+ concentrations (5–70 μM), and temperature dependence was determined by measuring total ASP+ accumulation at 22 and 6 °C. The influence of the DAT substrates d-amphetamine (0.1–10 μM), dopamine (0.1–10 μM), or cocaine (3–15 μM) on ASP+ uptake was examined by preincubating cells for 15 min and then adding ASP+ and measuring total intracellular ASP+ accumulation for 5 min in the continued presence of substrate or inhibitor. The sodium dependence was investigated by measuring ASP+ uptake in the presence or absence of the monovalent cation channel-forming peptide gramicidin (10 μg/ml, 10 min of preincubation) (29). The influence of the MAPK inhibitor PD98059 (30), the PI3K inhibitor LY294002 (31), and the protein kinase C inhibitor GF109203X (32) on quinpirole (10 μM)-evoked ASP+ uptake was assessed by pre-incubating cells with vehicle or inhibitor for 15 min. ASP+ was then added, and the effects of the D3 agonist on the rate of intracellular ASP+ accumulation were determined using a within cell design as described above, in the presence or absence of the corresponding kinase inhibitor. The concentrations of inhibitors used were chosen based on reported effective concentrations (30–32).

Fluorescent images were processed using MetaMorph or ImageJ software (W. Rasband, National Institutes of Health). For ASP+ uptake, fluorescent accumulation within the cell was measured as the average pixel intensity of time-resolved images. The intracellular fluorescence is the signal contained inside the cell as defined by the plasma membrane. The plasma membrane was identified by either GFP-D3 or YFP-DAT visualization (which are localized mainly in the plasma membrane) or bright field optics (in the case of FLAG-hDAT or N2A cells). Data are expressed as arbitrary fluorescence units or as a percent change in the rate of ASP+ uptake after drug addition. The total intracellular ASP+ fluorescence 5 min after addition of ASP+ was used as a measure of basal uptake (ASP+ accumulation over 5 min, see Figs. 2 and 3). To measure drug-induced effects on uptake, the rate of ASP+ fluorescence accumulation during the minutes before and after drug addition was calculated as the increase in intracellular ASP+ signal over that min. The effect of the drug was calculated as the percent change in the rate of ASP+ accumulation before and after the drug addition. Typically 10–100 cells were used for each experiment. These cells were from at least two culture dishes from three separate transfection experiments. Data are expressed as mean ± S.E. and analyzed by ANOVA followed by the Student-Newman-Keuls test for multiple comparisons between groups. Statistical significance was achieved at p < 0.05.

Immunoblotting of Kinases—Cells were transfected as described above and serum-starved in serum-free media 16–18 h before the assay. On the day of the assay, cells were treated for 15 min with kinase inhibitor or vehicle prior to stimulation with quinpirole (10 μM) for 1 min at 37 °C. After incubation, media were aspirated, and boiling Laemmli buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue) was added directly to the wells. Lysates were then collected and boiled for 10 min. For DAT protein immunoblot analysis, samples were incubated with Laemmli buffer for 30 min at room temperature (see below). Proteins were separated by SDS-PAGE (4–20% Duramide gradient gel; Cambrex, Walkersville, MD) and blotted onto polyvinylidene difluoride (Millipore, Bellerica, MA). The membranes were then incubated for 1 h at room temperature in TBS-T containing 5% nonfat milk. Phosphorylated MAPK was detected using a rabbit polyclonal antibody specific for p44/42 MAPK phosphorylated at residues threonine 202 and tyrosine 204 (p-ERK). Phosphorylated Akt was detected utilizing a rabbit polyclonal antibody that detects levels of Akt only when phosphorylated at serine 473 (p-Akt). To control for differences in protein loading, blots were stripped with 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris, pH 6.8, for 1 h at 50 °C and reprobed with a polyclonal antibody that recognizes total p44/42 MAPK or total Akt. All kinase antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Blots were visualized using a...
torseradish peroxidase-conjugated secondary antibody (Upstate Biotechnology, Inc., Lake Placid, NY) with enhanced chemiluminescence reagents.

For quantification, films were scanned at high resolution using a Scanjet 7400c (Hewlett-Packard, Palo Alto, CA), and band densities were quantified using MetaMorph software (Molecular Devices, Downingtown, PA). The relative amounts of phosphorylated kinases were normalized against those of the corresponding total kinase. Data were analyzed by one-way ANOVA followed by Student-Newman-Keuls Multiple Comparison post hoc test using the GraphPad Prism version 4.0. The significance level for all analyses was $p \leq 0.05$.

**Surface Biotinylation of DAT**—Cell surface biotinylation and immunoblot analyses were performed as described (33) to quantify the amount of plasma membrane DAT protein. EM4 cells (1 $\times$ 10$^5$ cells/well) were seeded into 12-well plates containing DMEM/F-12 medium supplemented with 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 $\mu$g/ml) in an atmosphere of 5% CO$_2$ and 95% O$_2$ at 37 °C. After 24 h, cells were transfected with FLAG-hDAT and myc-hD3 receptor cDNA plasmids (0.9 $\mu$g of D3 receptor and 0.3 $\mu$g of DAT) using Lipofectamine™ 2000 according to the manufacturer’s protocol. In all wells, the total amount of plasmid DNA was adjusted with the corresponding empty vector. Where indicated, cells were treated with different modulators 24 h after transfection, as described in the figure legends. At the end of the treatment, cells were washed two times with ice-cold PBS/Ca-Mg (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 9.6 mM Na$_2$HPO$_4$, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, pH 7.3) and incubated with EZ link NHS-Sulfo-SS-biotin (1 mg/ml) in PBS/Ca-Mg for 30 min at 4 °C. The reaction was quenched by two washes with cold 100 mM glycine in PBS/Ca-Mg and further incubation with 100 mM glycine in PBS/Ca-Mg at 4 °C for 20 min. The cells were then lysed in 500 $\mu$l of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 0.1% sodium deoxycholic acid) containing protease inhibitors (1 $\mu$M pepstatin A, 250 $\mu$M phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, and 1 $\mu$g/ml aprotinin) for 1 h at 4 °C with constant shaking. Lysates were centrifuged at 25,000 $\times$ g for 30 min at 4 °C, and supernatants were incubated with streptavidin beads (100 $\mu$l of beads/400 $\mu$l of cell lysates from one well) for 1 h at room temperature. Beads were washed three times with RIPA buffer, and bound proteins were eluted with 50 $\mu$l of Laemmli buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue) for 30 min at 22 °C. Aliquots from total cell lysates (50 $\mu$l) and unbound fractions (100 $\mu$l) and all (50 $\mu$l) of the streptavidin-bound samples were analyzed by immunoblotting with a monoclonal DAT-specific antibody (Chemicon, Temecula, CA). To validate the surface localization of biotinylated DAT protein, blots were stripped and reprobed with anticalnexin antibody (StressGen Biotechnologies, Victoria, British Columbia, Canada). Band intensities were quantified using NIH Image J software (version 1.32j).

Exposures were precalibrated to ensure quantitation within the linear range of the film, and multiple exposures were taken to validate linearity of quantitation. DAT densities from total, nonbiotinylated (representing the intracellular pool), and biotinylated fractions (representing the surface pool) were normalized using levels of calnexin in the total extract, and values were averaged across five experiments. Data were analyzed by ANOVA followed by the Student-Newman-Keuls test for comparisons between groups.

**Reversible Biotinylation to Determine DAT Internalization**—A reversible biotinylation strategy was used to quantify DAT internalization (34, 35). The rationale for this method is based on initial labeling of the extracellular DAT pool (under trafficking inhibiting conditions at 4 °C). Then after washing out the free biotin label, incubations are conducted under trafficking permissive conditions (22 °C) to allow for internalization of the biotin-labeled DAT. Finally, at the end of the incubation, trafficking is stopped (at 4 °C), and the extracellular DAN-bound striotin is stripped with a reducing agent (MesNa), leaving only the intracellular biotinylated DAT pool, which represents DAT that was initially on the plasma membrane and was internalized during the incubation. Briefly, EM4 cells transiently transfected with FLAG-DAT and myc-hD3 plasmids were prepared as described under surface biotinylation. Cells were cooled rapidly to 4 °C to inhibit protein trafficking by washing with cold PBS. Cell surface proteins were biotinylated with a disulfide-cleavable biotin (sulfo-NHS-SS-biotin; Pierce), and free biotinylating reagent was removed by quenching with glycine. DAT internalization was initiated by incubating the cells with prewarmed KRH buffer containing the vehicle or quinpirole (10 $\mu$m) for 1 or 30 min at room temperature (in the absence of NHS-SS-biotin). At the end of the incubation, the reagents were removed, and fresh pre-chilled KRH buffer was added to stop trafficking. The cells were then washed and incubated twice with 250 $\mu$m MesNa, a reducing agent, in PBS/Ca-Mg for 20 min to dissociate the biotin from cell surface resident proteins via disulfide exchange. To determine the total biotinylated DATs, one dish of biotinylated cells per condition was not subjected to reduction with MesNa and directly processed for extraction following by isolation of avidin beads. To determine MesNa-accessible DAT proteins, another dish of cells was treated with MesNa immediately (at 0 time) following biotinylation at 4 °C to reveal the quantity of surface DAT biotinylation that MesNa can reverse efficiently. Cells were then solubilized in RIPA, and biotinylated DAT were separated by using monomeric avidin beads. Biotinylated proteins were eluted from beads and resolved by SDS-PAGE. DAT proteins in the fractions were visualized as described under surface DAT biotinylation.

**Assay of DAT Insertion into the Plasma Membrane by Biotinylation**—The levels of DAT newly inserted into the plasma membrane were measured using protocols similar to those described previously (36). EM4 cells co-expressing FLAG-hDAT and GFP-hD3 receptors were washed with PBS/Ca-Mg and incubated twice with 1 mg/ml sulfo-NHS-acetate (Pierce) in PBS/Ca-Mg for 1 h at 4 °C to block all the free amino groups under nonpermissive trafficking conditions (at 4 °C) (37). After washing away the sulfo-NHS-acetate with cold PBS/Ca-Mg, cell membrane-impermeable sulfo-NHS-biotin in PBS/Ca-Mg containing vehicle or quinpirole (10 $\mu$m) was added to the cells and incubated further for 1 or 30 min at room temperature. Because previously existing surface DAT has been

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... blocked in the previous step, sulfo-NHS-biotin will label only DAT newly delivered to the plasma membrane. To investigate the influence of quinpirole on DAT plasma membrane insertion, sulfo-NHS-acetate-treated cells were incubated with either vehicle or quinpirole (10 μM, 1 or 30 min) with NHS-SS-sulfo-biotin present throughout the incubation period (34, 38). At the end of the incubation period, cells were washed with cold PBS/Ca-Mg containing glycine to quench the excess unbound NHS-SS-biotin, and biotinylated DAT proteins were isolated in streptavidin beads and analyzed as described above. Following determination of DAT in the biotinylated fraction, the blots were stripped and reprobed with transferrin receptor (TfR) (Zymed Laboratories Inc.) and calnexin antibodies (StressGen, Victoria, Canada). Levels of biotinylated TfR, a protein known to recycle rapidly to and from the cell surface, were quantified to confirm that the 22 °C condition permits protein trafficking. Biotinylated calnexin levels were quantified to assess biotinyla-

RESULTS

[^3H]DA Uptake Studies—We first determined the ability of D3 receptor agonist activation to modulate DAT activity as determined by accumulation of[^3H]DA in EM4 cells co-transfected with hD3 receptors and DAT. DA binds with high affinity to monoamine transporters. Recent studies indicated utility. ASP[^3H]DA accumulation was measured during 1 min in the presence of the specified drugs. Initial experiments identified two components of the ^[^3]H signal. One component is nomifensine-sensitive (uptake), and the other is spiperone-sensitive (binding to D3 receptors) (A). This was confirmed in EM4 cells transfected with either the hD3 receptor or hDAT alone, indicating that a significant fraction of the[^3H]DA added bound to hD3 receptors (B). To avoid confounding effects of this D3 receptor binding, subsequent[^3H]DA uptake experiments were carried out in the presence of 0.1 μM spiperone (no receptor activation) or 10 μM quinpirole (maximal D3 receptor activation). These experiments indicated that short (1 min) D3 receptor stimulation increased, whereas sustained (30 min) stimulation decreased[^3H]DA uptake (C). Characterization experiments with different DAT substrates using MAPK phosphorylation as an index of D3 receptor activation indicated that ASP[^3H] in contrast to either DA or tyramine, did not activate D3 receptors at concentrations up to 100 μM (D). ^*, p < 0.05, versus control group, Newman-Keuls post hoc test; **, p < 0.01.

Although we were able to demonstrate modulation of DAT by D3 receptor activation using[^3H]DA uptake, a DAT substrate that binds to and activates the receptor being investigated, the complicated experimental design needed to rule out confounding effects of substrate-receptor interactions is of limited utility. ASP[^+] is a fluorescent analog of MPP[^+] that binds with high affinity to monoamine transporters. Recent studies have shown that it can be used to monitor transporter function in real time in single cells. Therefore, we tested whether ASP[^+] activates D3 receptors by measuring MAPK activation. Addition of ASP[^+] to Flp-in T-rex 293 cells stably expressing FLAG-tagged hD3 receptors did not activate hD3 receptors at concentrations up to 100 μM (Fig. 1D). Consistent with our[^3H]DA...
uptake studies, addition of DA resulted in a concentration-dependent increase in pERK1/2 (EC$_{50}$ = 4.7 nM; Fig. 1D). The DAT substrate tyramine showed a similar maximal effect but lower potency (EC$_{50}$ = 1.4 μM; Fig. 1D). Given these findings, the ASP$^+$ imaging technique was used for subsequent studies.

**Characterization of ASP$^+$ Uptake**—ASP$^+$ rapidly accumulated in the cytoplasm of EM4 cells stably transfected with GFP-hDAT (Fig. 2A) or FLAG-hDAT (not shown). Two distinct phases of incorporation of the ASP$^+$ signal into DAT-expressing cells are observed. Binding of ASP$^+$ to transporters located on the cell surface is rapid (milliseconds to seconds) and is followed by a second slower (seconds to minutes) phase of accumulation of ASP$^+$ signal in the cytoplasm (Fig. 2A and D, and Fig. 3D, vehicle group). Intracellular accumulation is linear for at least 10 min (Figs. 2D and 3D, vehicle group) and is negligible in control EM4 cells not expressing DAT (Fig. 2C). Analogous to $[^3]$HDA assays, ASP$^+$ accumulation was saturable (Fig. 2B) and temperature-dependent (Fig. 2C). Disruption of the sodium gradient with gramicidin (10 μg/ml) blocked ASP$^+$ uptake (Fig. 2, D and E). These data indicate the involvement of an active sodium-dependent uptake system. Incubation of cells with either DA or $d$-amphetamine resulted in a concentration-dependent decrease in ASP$^+$ uptake (Fig. 3D, vehicle group). The transporter blocker cocaine decreased ASP$^+$ accumulation in a concentration-dependent manner (Fig. 3B). These data are in accord with recent findings (40) and confirm the utility of ASP$^+$ as a measure of DAT activity.

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**D3 Receptor Activation Modulates ASP$^+$ Uptake**—To determine whether D3 receptor activation alters DAT activity, EM4 cells stably expressing FLAG-hDAT were transiently transfected with GFP-tagged hD3 receptors to enable receptor visualization. The rate of accumulation of ASP$^+$ was measured immediately before and after receptor activation. GFP-hD3 receptors were readily expressed and targeted to the plasma membrane (Fig. 3C). Addition of quinpirole (10 μM, Fig. 3D) induced a rapid increase in ASP$^+$ uptake that was apparent within 1 min of agonist exposure (Fig. 3D). Quinpirole also induced a concentration-dependent increase in the rate of ASP$^+$ accumulation in EM4 cells transiently co-transfected with YFP-hDAT and myc-hD3 receptors (Fig. 4A, $F_{3,155} = 4.99, p = 0.0025$; ANOVA). A structurally different D2/D3 agonist, PD128907 (10 μM), also induced a significant increase in ASP$^+$ uptake (33 ± 8%; 95% CI: 20.3, 45.9; $p < 0.001$ versus vehicle, t test; data not shown). The quinpirole (10 μM)-evoked increase in ASP$^+$ uptake was completely blocked by 0.1 μM spiperone (significant quinpirole $\times$ spiperone interaction, $F_{1,227} = 19.64, p < 0.001$, 2-way ANOVA; Fig. 4C). Moreover, quinpirole failed to alter ASP$^+$ uptake in EM4 cells transfected with YFP-hDAT but lacking the D3 receptor indicating that D3 receptor expression is required for this effect. The percent change ± S.E. in the rate of intracellular ASP$^+$ accumulation after addition of 10 μM quinpirole was 33.7 ± 7.2% (n = 56) and −7.5 ± 3.4% (n = 22) in hD3 receptor-transfected and nontransfected cells, respectively (data not shown). EM4 cells transiently transfected with FLAG-hDAT and human D1 DA receptors (D. R. Sibley, NINDS, Bethesda) and the addition of the selective D1 agonist B135 (R(+)-6-bromo-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide, 10 μm) (41) did not alter ASP$^+$ accumulation. The percent change ± S.E. in the rate of intracellular ASP$^+$ accumulation after addition of vehicle or agonist was 5.8 ± 3.2% (n = 54) and 0.8 ± 2.9% (n = 29) for vehicle and B135, respectively, indicating that, in contrast to D3 receptors, D1 receptor activation is ineffective in modulating DAT activity (data not shown).
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To determine whether hD3 receptor modulation of DAT occurs in a neuronal cell line, ASP⁺ uptake was examined in N2A cells transiently co-transfected with YFP-hDAT and myc-D3 receptors. Analogous to EM4 cells, addition of quinpirole (10 μM) to N2A cells co-expressing these proteins significantly increased ASP⁺ uptake (Fig. 4B; F$_{3.93}$ = 5.11, p = 0.003 ANOVA).

Our [³H]DA uptake experiments suggested that prolonged D3 receptor activation down-regulates DAT activity. To determine whether prolonged D3 receptor stimulation decreases DAT activity, as opposed to the effects observed after acute receptor stimulation, EM4 cells transiently transfected with YFP-hDAT and myc-D3 were preincubated with either vehicle or quinpirole (10 μM) for 30 min. After preincubation, cells were washed, and ASP⁺ was added. The rate of accumulation was then measured over 1 min. Consistent with our radioligand uptake and biotinylation studies (see below), preincubation with quinpirole for 30 min induced a significant (t = 2.018, df = 79, p = 0.047, t test) 28.5% decrease in the rate of ASP⁺ uptake.

The mean arbitrary fluorescence units in vehicle and quinpirole-treated cells was 0.508 ± 0.054 (n = 37) and 0.363 ± 0.047 (n = 54 cells, respectively (data not shown).

The D3-mediated Increase in DAT Function Is Pertussis Toxin-sensitive—D3 receptors are G protein-coupled receptors that signal primarily through the G$_i$/G$_o$ class of heterotrimeric G proteins. Pertussis toxin catalyzes ADP-ribosylation of α subunits in the G$_i$ and G$_o$ subfamilies of heterotrimeric G-proteins, thereby preventing G$_i$/G$_o$ proteins from interacting with receptors (42). To test whether the hD3 receptor-mediated modulation of DAT activity requires G$_i$/G$_o$, EM4 cells expressing FLAG-hDAT and GFP-hD3 receptors were incubated with pertussis toxin (100 ng/ml) for 16–24 h, and the ability of quinpirole to increase ASP⁺ uptake was assessed. Preincubation with pertussis toxin prevented the quinpirole-evoked increase in ASP⁺ uptake (Fig. 4D; F$_{2.97}$ = 6.11, p = 0.0032, ANOVA).

D3 Activation Stimulates PI3K and MAPK—D3 receptors signal via MAPK and PI3K in brain and various cell lines (43, 44). The ability of the D3 receptor to stimulate MAPK was evaluated in EM4 cells transiently expressing GFP-hD3 receptors and YFP-hDAT by measuring agonist-induced (quinpirole 10 μM, 1 min incubation) ERK phosphorylation (Fig. 5A). Consistent with our findings in the whole cell ELISA (Fig. 1D), quinpirole evoked a 2.5-fold increase in p-ERK/total MAPK, indicating activation of the MAPK pathway. This increase in p-ERK/total MAPK was prevented by the MEK inhibitor PD98059 (10 μM, 15 min preincubation) (30) but not by the PI3K inhibitor LY294002 (10 μM, 15 min preincubation; data not shown) (31). Stimulation of the PI3K pathway via the D3 receptor was measured by assessing phosphorylation of the ser ine/threonine protein kinase Akt (Fig. 5B). Quinpirole increased p-Akt/total Akt 2.8-fold above vehicle in D3 receptor-transfected cells. This effect was blocked by preincubation with LY294002 (10 μM, 15 min preincubation) but not by the
MAPK inhibitor PD98059 (data not shown). Thus, hD3 receptor activation stimulates both MAPK and PI3K pathways in our cell system.

**D3 Receptor Regulates DAT Function via PI3K- and MAPK-dependent Mechanisms**—To determine the involvement of these kinases in mediating the effects of hD3 receptor activation on DAT activity, EM4 cells co-transfected with YFP-hDAT and GFP-hD3 receptor were incubated with either LY294002 or PD98059 for 15 min prior to quinpirole (10 μM) exposure. The influence of the PKC inhibitor GF109203X on D3 receptor agonist-evoked ASP\(^+\) uptake was also assessed because D3 receptor-mediated MAPK phosphorylation involves an atypical isoform of PKC in some cell lines (45). The concentration of GF109203X used (10 μM) was that previously shown to result in the inhibition of PKC isoforms α, β1, δ, ε, and ζ (32). ANOVA revealed a significant effect of the various kinase inhibitors on quinpirole-evoked ASP\(^+\) accumulation \((F_{4,361} = 14.21, p < 0.0001)\) ANOVA). Post-hoc analysis showed that inhibition of either PI3K or MAPK prevented the quinpirole-induced increase in DAT activity (Fig. 6A). In contrast, the PKC inhibitor was without effect. The effects of both LY294002 \((F_{4,297} = 8.09, p < 0.0001)\) ANOVA) and PD98059 were concentration-dependent \((F_{4,258} = 6.88, p < 0.0001)\) ANOVA; see Fig. 6, C and D).

**D3 Receptor Regulation of DAT Function Does Not Require an Intact DAT N Terminus**—The DAT N-terminal domain contains several target phosphorylation sites for kinases, including MAPK and PKC (46–48). Therefore, we investigated whether the N-terminal domain of DAT was required for the effects of D3 receptors on DAT activity. Quinpirole increased the rate of ASP\(^+\) uptake in EM4 cells transfected with hD3 receptor and a truncated FLAG-hDAT lacking the first 55 N-terminal amino acids \(\Delta N_{1-55}\). The effect of quinpirole on uptake was similar to that observed in cells expressing hD3 receptors and wild type FLAG-hDAT (Fig. 6B, 22.2 ± 2.8% versus 25.6 ± 2.1% in N-terminal truncated versus wild type FLAG-hDAT, respectively). The \(\Delta N_{1-55}\) mutant DAT shows normal uptake activity and insertion of the transporter in the cell membrane.\(^5\)

\(^5\) N. Sen and J. A. Javitch, unpublished results.
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**A Cell Surface Labeling**

**B Insertion in the Plasma Membrane**

**C Internalization**

![Image](58x104 to 563x733)

**FIGURE 7.** D3 receptor activation modulates DAT trafficking. A, time-dependent effects of D3 receptor activation on DAT surface expression. EM4 cells were co-transfected with FLAG-hDAT and GFP-hD3 receptors. After 24 h, cells were washed once with KRH buffer and incubated with vehicle or quinpirole (10 μM) for 1 or 30 min at room temperature. Surface DAT was marked by biotinylation. Immunoblot analysis of DAT expression using biotinylation procedures (top panel, representative blot from six independent experiments shown) and quantitative DAT surface and intracellular band density (bottom panel) were performed as described under “Experimental Procedures.” The data represent the mean ± S.E. of six separate experiments. Incubation with quinpirole induced a rapid (1 min) increase in cell surface DAT. However, prolonged incubation (30 min) produced opposite effects, decreasing the amount of cell surface DAT and increasing its redistribution to the intracellular compartment. *, significantly different from vehicle-treated controls cells (p < 0.05, Newman-Keuls), †, significantly different from 1-min quinpirole-treated cells (p < 0.05, Newman-Keuls).

**Asterisks** indicate significant changes in biotinylated DAT compared with vehicle treatment at 22 °C (p < 0.05, one-way ANOVA with Bonferroni post-hoc analysis). Note that the blots corresponding to 1-min incubations were exposed for longer times than the ones incubated 30 min to be able to visualize DAT proteins. Therefore, direct quantitative comparison of DAT protein insertion between 1 and 30 min is not possible. B, time-dependent effects of D3 receptor activation on DAT plasma membrane insertion. EM4 cells were co-transfected with FLAG-hDAT and GFP-hD3 receptors. After 24 h, cells were washed once with PBS/Ca-Mg, and free amino groups were blocked with sulfo-NHS-acetate at 4 °C. After this blocking step, cells were incubated with biotinylating reagent at 22 °C in the presence or absence of quinpirole (10 μM) for 1 or 30 min. The isolation and quantification of biotinylated DAT newly inserted to the plasma membrane were performed as described under “Experimental Procedures.” Western blots of DAT, TfR, and calnexin from avidin beads eluates are also shown. Each blot is representative of three separate experiments. Quantitative analysis of DAT band densities for three experiments is given as mean ± S.E. (bottom panel). Asterisks indicate significant changes in biotinylated DAT compared with vehicle treatment at 22 °C (p < 0.05, one-way ANOVA with Bonferroni post-hoc analysis). Note that the blots corresponding to 1-min incubations were exposed for longer times than the ones incubated 30 min to be able to visualize DAT proteins. Therefore, direct quantitative comparison of DAT protein insertion between 1 and 30 min is not possible. C, time-dependent effects of D3 receptor activation on DAT internalization. EM4 cells were co-transfected with FLAG-hDAT and GFP-hD3 receptors. After 24 h, cells were biotinylated with sulfo-NHS-acetate at 4 °C and incubated with quinpirole (10 μM) for the vehicle or for 1 or 30 min. Following MesNa treatment, biotinylated (internalized) DATs were isolated and analyzed as described under “Experimental Procedures.” A representative immunoblot from five separate experiments is shown (top panel). The internalized DAT band densities from five separate experiments are presented as mean ± S.E. (bottom panel). Asterisks indicate a significant change in DAT internalization by quinpirole treatment compared with vehicle treatment (p < 0.05, one-way ANOVA with Bonferroni post-hoc analysis). In parallel experiments, cells were kept at 4 °C throughout the procedure, and biotinylated DAT was analyzed before (total DAT biotinylated on the surface) and after MesNa treatment (background, the efficiency of MesNa cleaving surface biotin-SS-linked proteins). Note that MesNa treatment in control experiments performed at 4 °C where endocytosis was arrested revealed a background signal of ~5–10% from total amount of DAT (top panel).

**D3 Receptor Activation Regulates DAT Subcellular Distribution**—Transporter trafficking allows rapid up- and down-regulation of transporter expression at the cell surface and is implicated in the regulation of transport activity (49). Because D3 receptor activation is able to either increase (1 min of activation) or decrease (30 min of activation) uptake of DA and the fluorescent DAT substrate ASP (49), we examined whether D3 receptor activation regulates DAT trafficking.

To determine the effects of short (1 min) versus prolonged (30 min) hD3 receptor stimulation on DAT trafficking, biotinylation studies, which permit quantification of cell surface and intracellular DAT, were conducted. Consistent with the uptake data, incubation of cells for 1 min with the D3 receptor agonist significantly increased biotinylated DAT (52.3 ± 4.4%, p < 0.05) and reduced nonbiotinylated DAT, indicating an increase in cell surface DAT (Fig. 7A). Whereas DAT surface expression and activity were higher after 1 min of exposure to quinpirole (10 μM), DAT surface expression and activity significantly decreased following 30 min of quinpirole exposure. In agreement with [3H]DA and ASP uptake data, prolonged (30 min) incubation with quinpirole decreased the amount of biotinylated DAT (26.4 ± 4.2%, p < 0.05), and this was accompanied by an increase in nonbiotinylated DAT protein. Less than 0.5% of total calnexin was present in streptavidin-bound fractions suggesting that cells were intact and intracellular proteins were not significantly biotinylated. Treatment with quinpirole did...
not alter the total amount of DAT protein as measured by immunoblotting.

Altered plasma membrane expression levels of DAT following D3 activation could arise from altering DAT endocytosis or exocytosis or both. To examine the influence of hD3 receptor activation on DAT plasma membrane insertion (exocytosis), cells were biotinylated in the presence and absence of quinpirole for either 1 or 30 min after previously blocking existing surface DAT. Cells were first treated with sulfo-NHS-acetate to block all sulfo-NHS-biotin-reactive amino groups on the cell surface, thereby preventing labeling of DAT already present on the cell surface. Cells were then biotinylated in the presence and absence of quinpirole for either 1 or 30 min under trafficking permissive temperature (22 °C). Thus the amount of biotinylated DAT represents DAT that was newly inserted (exocytosis) on the plasma membrane. The sulfo-NHS-acetate preincubation step was effective in preventing biotinylation of preexisting surface DAT. Cells were first treated with sulfo-NHS-acetate to block all sulfo-NHS-biotin-reactive amino groups on the cell surface DAT. Cells were then biotinylated in the presence and absence of quinpirole for either 1 or 30 min under trafficking permissive temperature (22 °C). Thus the amount of biotinylated DAT represents DAT that was newly inserted (exocytosis) on the plasma membrane. The sulfo-NHS-acetate preincubation step was effective in preventing biotinylation of preexisting surface DAT at the beginning of the incubation (Fig. 7B, lanes 2 versus 1 and 6 versus 5) indicating that biotinylated DAT represents transporter newly delivered to the plasma membrane during the incubation. In vehicle-treated cells, the amounts of biotinylated DAT and TIR were greater at 22 °C (Fig. 7B, lanes 3 and 7) as compared with biotinylation performed at 4 °C (Fig. 7B, lanes 2 and 6). The changes in DAT and TIR biotinylation at 22 °C were not because of biotinylation of intracellular proteins (because of loss of plasma membrane integrity) because levels of calnexin, an intracellular protein, were unaltered in the biotinylated fraction (Fig. 7B, top panel). Thus, the changes in biotinylated DAT observed at different time points after warming the cells to 22 °C represents DAT that was newly delivered to the plasma membrane. Quinpirole did not affect TIR insertion. When biotinylation was performed at 22 °C in the presence of quinpirole for 1 min, a significant increase in the amount of biotinylated DAT was seen (Fig. 7B, top panel, lane 3 versus 4, and bottom panel). However, after 30 min of incubation with quinpirole, the amount of biotinylated DAT was reduced relative to vehicle (Fig. 7B, top panel, lanes 7 versus 8, and bottom panel). These results suggest that whereas shorter (1 min) quinpirole treatment increases DAT plasma membrane insertion, following prolonged quinpirole exposure, less DAT is ultimately located at the plasma membrane. Although this procedure is routinely used to study transporter insertion into the plasma membrane (50), it should be noted that this procedure assumes that DAT inserted in the plasma membrane is biotinylated before it is again internalized. If the efficiency of biotinylation is low then changes in the rate of internalization could potentially increase the time a DAT molecule is on the surface and thereby increase the efficiency of biotinylation. Finally, although it is tempting to infer greater amounts of biotinylated DAT and TIR after 30 min as compared with 1 min of incubation (Fig. 7B, lanes 3 and 4 versus 7 and 8), different exposure times of both blots preclude direct comparisons between time points. For the same reasons, we cannot directly compare DAT protein levels across different experiments (i.e. across Fig. 7, A–C).

Next we sought to examine the internalization of DAT after 1 and 30 min of quinpirole exposure. Using a reversible biotinylation strategy, DAT internalization was determined by estimating surface biotinylated DAT that moves to an intracellular compartment. The amount of biotinylated DAT protein was determined for the surface (cells left at 4 °C without cleavage of surface biotin, total biotinylated DAT (Fig. 7C, top panel, lane 1)) and intracellular pools (after MesNa treatment, internalized biotinylated DAT (Fig. 7C, top panel, lanes 3–6)). Control experiments performed at 4 °C, a condition in which endocytosis was arrested, revealed a background signal of ~2–4% after MesNa treatment (Fig. 7C, top panel, lane 2). This likely represents surface DAT from which biotin was not completely cleaved by MesNa. Following 1 min of quinpirole exposure, the level of biotinylated DAT in MesNa-treated fractions was decreased compared with vehicle-treated control (Fig. 7C, top panel, lanes 5 and 6, and bottom panel). However, this effect did not reach statistical significance. This may be due to the low level of DAT internalization relative to background at the 1-min time period. However, a significant increase in the amount of biotinylated DAT in MesNa-resistant fractions was observed in cells treated with quinpirole for 30 min at 22 °C compared with the respective vehicle-treated control (Fig. 7C, top panel lanes 3 and 4, and bottom panel), indicating an increase in internalized DAT.

The amount of biotinylated protein that is internalized is affected not only by the rate of endocytosis but also by the rate at which internalized protein is recycled back to the plasma membrane. For this reason it is important to interpret the results of the internalization assay together with the results obtained in the assay for DAT plasma membrane insertion. Taken together these data demonstrate that acute D3 receptor activation increases cell surface DAT, DAT plasma membrane insertion, and increases uptake activity, whereas prolonged receptor stimulation results in decrease in uptake activity by decreasing DAT plasma membrane insertion and enhancing DAT internalization.

**DISCUSSION**

D3 receptors regulate extracellular DA concentrations in limbic brain regions, an effect that has been attributed to alterations in DA release (7). Evidence that D3 receptor ligands modulate DA uptake was provided recently (10). In vivo and in vitro studies showed that the preferential D3 agonist, PD128907, enhanced the rate of DA clearance in nucleus accumbens by increasing the maximal transport velocity. Furthermore, in vitro studies revealed decreased DA uptake in response to a preferential D3 antagonist. However, the presence of D2 and D3 receptor types in brain and the limited specificity of the ligands used precluded definitive identification of the receptor types mediating this effect.

By using cell expression systems in which D2 receptors are absent, we show that D3 receptors regulate DAT function. The D2/D3 receptor agonist, quinpirole, induced a concentration-dependent increase in uptake of the high affinity DAT substrate, ASP⁺, in EM4 cells co-expressing DAT and hD3 receptors. Similar effects were observed in a neuronal cell line co-expressing these proteins and in response to PD128907; a structurally different D3 agonist. In cells lacking D3 receptors, no agonist-induced increase in ASP⁺ accumulation was seen. The results obtained with the ASP⁺ technique were con-
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Supported using a [3H]DA uptake assay in which the DA receptor antagonist, spiperone, was used to prevent binding to and activation of D3 receptors by [3H]DA. These data provide the first clear evidence that D3 receptor stimulation is sufficient to regulate DAT function. Moreover, the results indicate that the effects of D3 receptor activation on DAT activity are time-dependent.

Pertussis toxin prevented the increase in ASP+ uptake produced by quinpirole indicating that D3 receptors regulate DAT via coupling to Gs proteins. D2/D3 receptor agonists activate MAPK via a pertussis toxin-sensitive mechanism (44, 51). D3 receptors also activate PI3K and PKC. Cross-talk between these kinase cascades is implicated in D3 receptor-mediated MAPK activation (43, 45). Evidence that these kinases regulate DAT function and cell surface expression (52–56) prompted us to hypothesize that their activation may be one mechanism mediating the increase in DAT function produced by D3 receptor stimulation.

Immunoblotting experiments revealed that quinpirole increased phosphorylated ERK1/2 confirming that hD3 receptor stimulation activates MAPK in our cell system. In agreement with previous findings, D3 receptor stimulation resulted in phosphorylation of Akt, a major target of PI3K (57, 58). Akt phosphorylation was prevented by the PI3K-inhibitor, LY294002 (31), confirming PI3K mediation. LY294002 pretreatment did not prevent quinpirole-evoked MAPK phosphorylation suggesting that D3 receptor activation can stimulate MAPK in a PI3K-independent manner.

Pretreatment with PD98059, a selective MEK inhibitor (30, 59), abolished the quinpirole-evoked increase in ASP+ uptake. The PI3K inhibitor produced a similar effect. It is important to note that within a cell design was used such that uptake after agonist addition was compared with that immediately before agonist addition in the same cell, with the kinase inhibitor present during the whole experiment. Furthermore, quantification of YFP-DAT during ASP+ studies revealed no alteration in cell surface localization following incubation of cells with the kinase inhibitors alone. Therefore, the decreased response to hD3 receptor activation cannot be attributed to effects of inhibitors on basal DAT function. Rather it reflects the ability of inhibitors to prevent the effects of D3 receptor stimulation and indicates that the observed functional interaction of the D3 receptor with DAT is dependent on both MAPK and PI3K activation. Blockade of conventional and atypical PKC isoforms by GF109203X did not alter significantly the effects of D3 receptor activation. Together, these findings indicate a specific involvement of MAPK and PI3K in mediating D3 regulation of DAT and that parallel activation of both MAPK and PI3K pathways is necessary for the hD3 receptor-induced increase in DAT activity. Furthermore, although D3 receptors may couple to PKC (45), this action is not critical for the effect of D3 receptor activation on DAT activity.

DAT may be directly phosphorylated by these kinases, resulting in modulation of transport activity. Alternatively, the kinases may phosphorylate other regulatory proteins that modulate DAT. The DAT N terminus contains several target phosphorylation sites (47, 48) and is phosphorylated in response to PKC activation (46, 47) and amphetamine (60). However, this phosphorylation does not play a role in DAT trafficking in response to PKC activation or amphetamine (47, 60). Similarly, our studies with an N-terminal truncated DAT show that an intact N terminus is not essential for the modulation of DAT function by hD3 receptor activation.

Increased ASP+ uptake occurred seconds after agonist addition, suggesting mediation by a post-translational mechanism. Transporter activity can be post-translationally modulated resulting in changes in apparent substrate affinity and/or the maximal transport rate. Although these mechanisms may contribute to monoamine transporter regulation (61), a more common post-translational mechanism of DAT regulation involves its redistribution to or from the plasma membrane (62–64). Constitutive DAT trafficking has been reported (50). It has been suggested that under basal conditions DAT traffics between the membrane and cytosol. Following internalization, it is either degraded or recycled back to the membrane. Therefore, we examined the influence of D3 receptor activation on DAT trafficking.

Biotinylation experiments, which used an identical protocol to that in ASP+ uptake experiments, confirmed that brief D3 stimulation resulted in a significant increase in cell surface DAT that paralleled the increase in DAT function. These increases were accompanied by a significant decrease in intracellular DAT. These data suggest that acute D3 receptor activation increases DAT redistribution from the intracellular compartment to the cell surface, resulting in increased transport capacity.

We observed that prolonged hD3 receptor activation decreased cell surface DAT, as determined by cell surface biotinylation, and decreased ASP+ and [3H]DA uptake. Thus, although acute D3 activation leads to increased DAT function, prolonged D3 receptor activation leads to DAT down-regulation. A recent study found that repeated systemic administration of a preferential D3 agonist decreased maximal DA transport velocity. DAT binding density was unchanged (65). Interestingly, this effect was absent in D3 knock-out mice suggesting D3 receptor mediation. In addition, there is evidence that D3 receptor stimulation can block the toxic effects of high doses of cocaine in vivo (66). Together with our data, these findings suggest that repeated or sustained activation of D3 receptors may have effects opposite from those of acute activation.

The effect of acute D3 agonist activation on DAT activity was very rapid. Similarly, in electrochemical studies where DAT regulation by D2/D3 receptor agonists has been observed, very short (30 s) incubation times were used (17, 67). With longer exposure periods, as is typically the case with studies employing radiolabeled substrates, inconsistent effects are seen (15, 68). Therefore, sustained D3 receptor stimulation may induce adaptations that oppose those of acute receptor stimulation. The mechanisms underlying these adaptations will require further study.

The amount of cell surface DAT is determined by the balance between transporter internalization and recycling back to the plasma membrane (50). Changes in cell surface DAT can be caused by altering the endocytotic pathway, the exocytotic pathway, or both. For example, a recent study suggested that...
amphetamine induces a rapid increase in surface DAT that is associated with its increased delivery to the plasma membrane (69) and is followed by a redistribution away from the cell surface at later time points (22). In contrast, the PKC-evoked decreases in surface DAT involves both increased internalization and reduced recycling (50). We investigated the contribution of the endocytotic and exocytotic recycling pathways to D3 receptor-induced DAT cellular redistribution. Long (30 min) agonist exposure increased DAT internalization and decreased DAT recycling. These findings are consistent with the cell surface labeling studies and indicate that prolonged D3 receptor stimulation decreases cell surface DAT by increasing the rate of DAT internalization and decreasing the rate of recycling back to the plasma membrane. By contrast, after 1 min of agonist exposure, an increased exocytosis rate was observed, with a nonsignificant decrease in internalization. It is important to note that the biotinylation technique may lack sufficient sensitivity or time resolution to detect significant decreases in endocytosis that occur within a 1-min time scale. This caveat notwithstanding, our results suggest that different mechanisms underlie the effects of short and prolonged D3 receptor stimulation on DAT trafficking. Furthermore, the increase in cell surface expression observed in response to brief receptor stimulation is in accord with an increasing body of evidence that transporter trafficking can be rapidly regulated (69, 70).

In summary, these data provide the first unequivocal evidence that D3 receptors up-regulate DAT activity, an effect that requires both MAPK and PI3K activation. The D3 receptor-mediated enhancement of DAT activity is associated with redistribution of DAT from the intracellular compartment to the cell surface, an effect that results, at least in part, from increased DAT exocytosis. Prolonged receptor stimulation decreases DAT function and cell surface expression. The rate of DAT endocytosis is increased and exocytosis is decreased. These data indicate that different mechanisms may be recruited following acute versus sustained D3 receptor activation resulting in opposite effects on DAT function.

These findings provide potential insights regarding the cellular mechanisms by which D3 receptors regulate DA transmembrane and maintain DA homeostasis. Transporter substrates promote DAT redistribution from the plasma membrane to the cytosol (22). This effect appears paradoxical because decreased cell surface DAT expression decreases DA clearance from the extracellular fluid. Our studies, however, suggest that if DA is available to transiently stimulate D3 receptors, transporter internalization will decrease in the vicinity of these receptors, resulting in rapid clearance of extracellular DA and prevention of prolonged receptor activation. When DA release occurs in areas in which D3 receptors are lacking or at low density, substrate-induced DAT internalization would be unopposed, resulting in a greater half-life of extracellular DA, facilitating diffusion farther from its release site.

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