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Regulation of Dopamine Transporter Trafficking by Intracellular Amphetamine

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ABSTRACT

The dopamine (DA) transporter (DAT) mediates the removal of released DA. DAT is the major molecular target responsible for the rewarding properties and abuse potential of the psychostimulant amphetamine (AMPH). AMPH has been shown to reduce the number of DATs at the cell surface, and this AMPH-induced cell surface DAT redistribution may result in long-lasting changes in DA homeostasis. The molecular mechanism by which AMPH induces trafficking is not clear. Because AMPH is a substrate, we do not know whether extracellular AMPH stimulates trafficking through its interaction with DAT and subsequent alteration in DAT function, thereby triggering intracellular signaling or whether AMPH must be transported and then act intracellularly. In agreement with our previous studies, extracellular AMPH caused cytosolic redistribution of the wild-type human DAT (WT-hDAT). However, AMPH did not induce cytosolic redistribution in an uptake-impaired hDAT (Y335A-hDAT) that still binds AMPH. The divalent cation zinc (Zn^{2+}) inhibits WT-hDAT activity, but it restores Y335A-hDAT uptake. Coadministration of Zn^{2+} and AMPH consistently reduced WT-hDAT trafficking but stimulated cytosolic redistribution of Y335A-hDAT. Furthermore, direct intracellular application of AMPH, via a whole-cell patch pipette, stimulated the trafficking of Y335A-hDAT. Taken together, these data suggest that the DAT transport cycle is not required for AMPH-induced down-regulation and that an increase of intracellular AMPH is an essential component of DAT redistribution.

Dopamine (DA) is a monoaminergic neurotransmitter involved in the control of locomotion, cognition, reward, and emotion (Iversen, 1971; Giros and Caron, 1993). The DA transporter (DAT) coordinates the spatial and temporal regulation of dopaminergic neurotransmission by mediating the reuptake of DA into presynaptic neurons. Although diffusion and enzymatic degradation contribute to reducing the synaptic concentration of DA, knockout studies have established DAT as the primary mechanism controlling extracellular DA levels (Giros et al., 1996; Jones et al., 1998). The regulatory mechanisms governing DAT are critical to DA signaling/homeostasis. Altered dopaminergic signaling has been implicated in multiple brain disorders, including Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder, and addiction (Jucaite, 2002).

DAT belongs to the Na^+/Cl^--dependent transporter gene family, which also consists of plasmalemmal carriers for the other monoamines, norepinephrine, epinephrine, and serotonin. Modulation of transporter surface expression may constitute a dynamic component of DA clearance (Melikian and Buckley, 1999; Loder and Melikian, 2003; Sorkina et al., 2005). Indeed, the clearance efficiency of DAT is determined not only by the transport rate of an individual transporter but also by the number of functional transporters on the cell surface. DAT trafficking has been shown to be regulated by receptor signaling, as well as by direct activation of protein kinase C (Zhang and Reith, 1996; Huff et al., 1997; Vaughan et al., 1997; Zhang et al., 1997; Zhu et al., 1997; Pristupa et al., 1998; Mayfield and Zahniser, 2001; Carvelli et al., 2002; Granas et al., 2003; Loder and Melikian, 2003; Moron et al., 2003; Sorkina et al., 2005), and by interaction with cytosolic proteins (Torres et al., 2001; Carneiro et al., 2002; Bjerg-

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ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; AMPH, amphetamine; hDAT, human dopamine transporter; WT, wild type; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PBS-Ca-Mg, PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$; ANOVA, analysis of variance; PKC, protein kinase C.

gaard et al., 2004; Jiang et al., 2004; Lee et al., 2004). In addition, substrates (Saunders et al., 2000; Chi and Reith, 2003; Sorkina et al., 2003; Kahlig et al., 2004) and inhibitors (Daws et al., 2002; Little et al., 2002) also regulate DAT function by a trafficking mechanism.

The psychostimulant AMPH is a substrate for DAT. Thus, AMPH elevates extraneuronal DA concentrations through a variety of mechanisms, including uptake competition and stimulation of DAT-mediated DA efflux (Sulzer et al., 2005). In addition, we and others have demonstrated that AMPH causes DAT redistribution away from the plasma membrane (Saunders et al., 2000; Chi and Reith, 2003; Sorkina et al., 2003; Kahlig et al., 2004). We have also shown that DAT leaves the membrane as an active protein in response to AMPH (Kahlig et al., 2004). Therefore, AMPH decreases DA transport capacity by reducing the number of transporter proteins on the plasma membrane and not through inactivation of surface DAT (Kahlig et al., 2004). It is unclear, however, whether the activity of the transporter is necessary for this response to AMPH. Although AMPH can enter the cell to some extent by lipophilic diffusion, the presence of a catecholamine transporter dramatically increases the rate and extent of AMPH accumulation (Bonisch, 1984; Sitte et al., 1998). Moreover, because the inhibitor cocaine blocks AMPH-induced DAT trafficking (Daws et al., 2002), it is likely that AMPH interacts with the transporter to cause its cell surface redistribution. It is important to understand, however, whether this interaction and subsequent changes in DAT activity are sufficient or if AMPH must cross the membrane in a DAT-mediated active transport process to decrease DAT plasma membrane expression.

In this study, to investigate the significance of DAT-mediated AMPH transport, we examined the ability of AMPH to induce transporter trafficking in an uptake-deficient DAT (Y335A-hDAT) that is targeted normally to the plasma membrane and binds AMPH (Loland et al., 2002). Extracellular AMPH did not cause Y335A-hDAT trafficking, but Zn^{2+}, which stimulates Y335A-hDAT-mediated substrate transport (Loland et al., 2002), restored AMPH-induced DAT trafficking. Moreover, bypassing the transport cycle and directly delivering AMPH inside the cell with a whole-cell patch pipette produced Y335A-hDAT trafficking. Taken together, these data suggest that intracellular AMPH is required for AMPH-induced DAT trafficking.

Materials and Methods

Cell Culture. Mutations in the hDAT synthetic cDNA were generated by polymerase chain reaction-derived mutagenesis using *Pfu* polymerase (Stratagene, La Jolla, CA). WT-hDAT and Y335A-hDAT constructs were fluorescently tagged at the N terminus with GFP in the polycistronic expression vector pCIN4, as described previously (Loland et al., 2002). The constructs were verified by restriction enzyme mapping and DNA sequencing, followed by stable transfection into human embryonic kidney 293 cells with Lipofectamine (Invitrogen, Carlsbad, CA). Stably transfected pools were selected with geneticin as described previously (Javitch et al., 1997). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Confocal Microscopy and Electrophysiology. Stably transfected cells were plated at a density of 10^5 per 35-mm culture dish. Before imaging, attached cells were washed twice with bath solution containing 130 mM NaCl, 10 mM HEPES, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.3 mM KH_2PO_4, and 34 mM dextrose adjusted to pH 7.35. In experiments using Zn^{2+}, 10 μM Zn^{2+} was added to the external solution with the other ions. Cells were visualized with an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) running the confocal imaging software, EZ 2000 (Coord Automatisering, Utrecht, The Netherlands). Images were acquired using 488-nm excitation with a 510-nm long pass filter. To minimize fluorophore bleaching and the cytotoxic effects of confocal microscopy, images were acquired at intervals of at least 1 min. AMPH and DA were bath-perfused at the indicated concentrations.

To deliver AMPH intracellularly to the uptake-deficient Y335A-hDAT, we used a whole-cell patch pipette in the current-clamp mode. To reduce the effect of any extracellular AMPH that leaked from within the cell, cells were bath-perfused during the experiment. A programmable puller (model P-2000; Sutter Instruments, Novato, CA) was used to fabricate quartz recording pipettes with a resistance of 7 MΩ. The recording pipette was filled with an internal solution containing the 120 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, and 30 mM dextrose adjusted to pH 7.35. Ten micromolar AMPH or 10 μM DA was added to the internal patch solution, as indicated. Membrane potentials were recorded in current-clamp mode using an Axopatch 200B (Molecular Devices, Sunnyvale, CA) with a low-pass Bessel filter set at 5000 Hz. The basal confocal image was acquired immediately after establishing the whole-cell configuration. Images were acquired as described above.

Because membrane proteins such as GLUT4 can be localized just under the plasma membrane (Inoue et al., 2003) in a manner that cannot be resolved from plasma membrane expression using optical methods, we measured intracellular fluorescence to quantify DAT cell surface redistribution as described previously (Kahlig et al., 2004). The quantification of intracellular fluorescence was performed by normalizing each respective time point by the total fluorescence in the whole field image to account for photobleaching (Lippincott-Schwartz et al., 1999; Piston et al., 1999). In addition, we also accounted for cytosolic volume changes (Lippincott-Schwartz et al., 1999; Piston et al., 1999). ImageJ software (http://rsb.info.nih.gov/ij/) was used for this analysis. Normalized data were analyzed with Prism software (ver. 3.02; GraphPad Software, Inc.) and reported as mean ± S.E. unless otherwise indicated.

Cell Surface Biotinylation. Cell surface biotinylation experiments were performed as described previously (Apparsundaram et al., 1998) with slight modification. Stably transfected cells were seeded in six-well plates (10^6 cells/well) approximately 48 h before the experiment. The cells were washed twice with 37°C bath solution (with or without 10 μM Zn^{2+}), and cells were treated as indicated at 37°C for 1 h. The cells were washed twice with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca-Mg) and treated with sulfo-NHS-biotin (1.5 mg/ml; Pierce Chemical Co., Rockford, IL) in PBS-Ca-Mg on ice for 1 h. The reaction was quenched by washing twice with 4°C PBS-Ca-Mg containing 100 mM glycine (PBS-Ca-Mg-glycine) followed by an incubation with PBS-Ca-Mg-glycine for 30 min on ice. Cells were then washed twice with 4°C PBS-Ca-Mg before lysis with 1 ml of radioimmunoprecipitation assay buffer [20 mM Tris, 20 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 1% Triton X-100; supplemented with protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml pepstatin)] for 30 min on ice with constant shaking. Lysates were centrifuged at 14,000*g* for 30 min at 4°C. The supernatants were isolated and biotinylated proteins were separated by incubation with ImmunoPure Immobilized Streptavidin beads (Pierce Chemical Co, Rockford, IL) for 1 h at room temperature with constant mixing. Beads were washed three times with radioimmunoprecipitation assay buffer, and biotinylated proteins were eluted with Laemmli loading buffer for 30 min at room temperature. Total cell lysates and biotinylated proteins (cell surface) were separated by SDS-polyacrylamide electrophoresis (7.5%) and transferred into polyvinylidene difluoride membranes (Millipore). Polyvinylidene difluoride membranes were

incubated for 1.5 h in blocking buffer (5% dry milk, 0.1% Tween 20 in Tris-buffered saline) and immunoblotted with a rat monoclonal antibody directed against the N terminus of hDAT (1:2000 in blocking buffer; Chemicon Inc., Temecula, CA). Immunoreactive bands were visualized using horseradish peroxidase-conjugated goat anti-rat antibody (1:5000 in blocking buffer; Santa Cruz Biotechnology, Santa Cruz, CA) with ECL Plus on Hypersensitive ECL film (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Band densities were calculated using Scion Image software (Scion Corporation, Frederick, MD) and normalized to the appropriate total extract to control for protein loading. Data were analyzed with Prism software and reported as mean ± S.E.

Results

To investigate the relationship between AMPH-induced DAT trafficking and AMPH transport, we used human embryonic kidney 293 cells stably expressing either GFP-tagged WT-hDAT or uptake-impaired Y335A-hDAT. Mutation of this tyrosine residue in the third intracellular loop of DAT (Tyr-335) does not alter membrane targeting of the transporter or the ability of the transporter to bind AMPH (Loland et al., 2002; Torres et al., 2003). Furthermore, addition of an N-terminal GFP tag to DAT does not alter transporter function or regulation, as assessed by uptake, membrane targeting, PKC-stimulated trafficking, AMPH-induced trafficking, inhibitor binding, and substrate-induced currents (Daniels and Amara, 1999; Sorkina et al., 2003; Kahlig et al., 2004; Garcia et al., 2005). Using confocal imaging, we selected transfected cells with easily visualized plasma membrane fluorescence for analysis. Consistent with previous reports (Loland et al., 2003; Sorkina et al., 2003; Kahlig et al., 2004; Garcia et al., 2005), under basal conditions, both WT-hDAT and Y335A-hDAT were primarily expressed at the plasma membrane (Fig. 1, A and C, left). As expected, 10 μM AMPH caused WT-hDAT to traffic away from the membrane and accumulate intracellularly (Fig. 1A, right) (Saunders et al., 2000; Sorkina et al., 2003; Kahlig et al., 2004). In contrast, AMPH failed to cause the cellular redistribution of Y335A-hDAT (Fig. 1C). To quantify the increase of intracellular DAT, we measured changes in intracellular fluorescence and plotted them as a function of time after addition of AMPH (Fig. 1, B and D). Treatment with vehicle alone did not alter the intracellular distribution of WT-hDAT or Y335A-hDAT (□, Fig. 1, B and D; intracellular fluorescence was 96 ± 2% and 97 ± 3% of the basal values after 60 min, respectively). In WT-hDAT, AMPH produced a significant increase in intracellular fluorescence at all times between 20 and 60 min (*, $p < 0.05$, one-way ANOVA). After 60-min exposure to AMPH, the intracellular fluorescence of WT-hDAT cells increased to 214 ± 33% of basal levels (Fig. 1B, ■). In contrast, in Y335A-hDAT, intracellular fluorescence was unchanged by AMPH exposure (98 ± 5% of basal after 60 min AMPH exposure) (Fig. 1D, ■).

Zn^{2+} has been shown to modulate DAT activity through binding to an endogenous metal binding site (Norregaard et al., 1998; Loland et al., 1999). In WT-hDAT, 10 μM Zn^{2+} decreased the V_{max} for DA uptake by approximately 70% (Loland et al., 2002). Therefore, if active transport of AMPH, and not simply lipophilic diffusion, is required for DAT trafficking, one might expect inhibition of transport by Zn^{2+} to reduce the ability of AMPH to induce trafficking. Indeed, in WT-hDAT cells, 10 μM Zn^{2+} prevented 10 μM AMPH from significantly increasing intracellular fluorescence (Fig. 2, A and B). The intracellular pool of WT-hDAT increased to 129 ± 11% of basal at 60 min (Fig. 2B, ■), an increase that was not significant and was much smaller than the 214% increase obtained in the absence of Zn^{2+} (Fig. 1B).

In contrast to its inhibitory effect on WT-hDAT, 10 μM Zn^{2+} restores DA uptake to the uptake-deficient Y335A-hDAT, with a 24-fold increase in V_{max} (Loland et al., 2002). Here, coapplication of 10 μM AMPH and 10 μM Zn^{2+} led to an increase in the Y335A-hDAT intracellular pool, which became significant by 20 min (Fig. 2, C and D) and reached

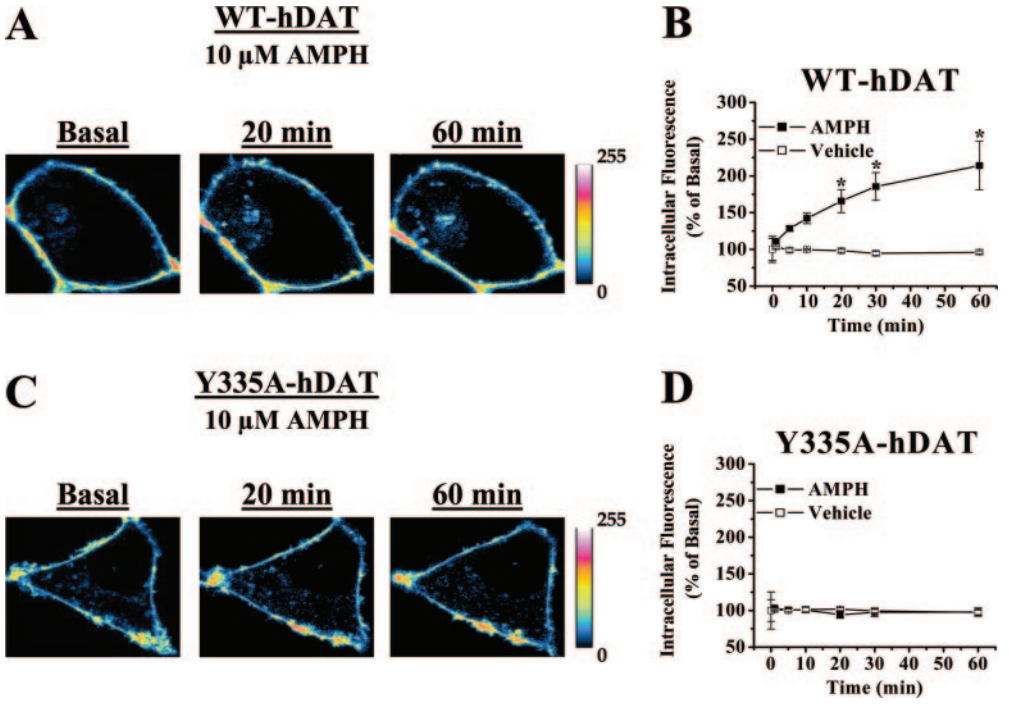

Fig. 1. AMPH induces cell surface redistribution of WT-hDAT but not uptake-deficient Y335A-hDAT. Representative confocal microscopy images illustrating the cellular distribution of either WT-hDAT (A) or Y335A-hDAT (C) during basal conditions (basal), 20 min and 60 min after treatment with 10 μM AMPH. Quantification of the WT-hDAT (B) or Y335A-hDAT (D) intracellular fluorescence measured in nontreated (vehicle, □) or AMPH-treated (AMPH, ■) cells. Data points represent the mean ± S.E. obtained from 15 to 20 cells in five independent experiments (one-way ANOVA followed by Bonferroni's post hoc test; * indicates $p < 0.05$ relative to the basal value for each condition).

165 ± 11% of basal levels after 60 min (Fig. 2, C and D, ■). Treatment with 10 μM Zn^{2+} alone did not alter intracellular levels of WT-hDAT or Y335A-hDAT (Fig. 2, B and D; after 60 min, intracellular fluorescence was 86 ± 4 and 93 ± 3% of the basal value, respectively).

In parallel, we used cell surface biotinylation studies to confirm that the changes in intracellular transporter pools measured by confocal imaging were indicative of alterations in DAT cell surface expression. Treatment with 10 μM AMPH for 60 min induced a 46 ± 5% reduction in WT-hDAT cell surface expression compared with control (Fig. 3, A and B). In contrast, coincubation of 10 μM AMPH with 10 μM Zn^{2+} prevented this effect (WT-hDAT density was 119 ± 11% relative to control) (Fig. 3, A and B). Consistent with the confocal imaging data (Fig. 2), AMPH treatment did not reduce cell surface expression of Y335A-hDAT (Fig. 3, C and D). However, in contrast to its effects on WT-hDAT, 10 μM Zn^{2+} restored the ability of 10 μM AMPH to cause Y335A-hDAT trafficking (Fig. 3, C and D); coincubation with Zn^{2+} and AMPH for 60 min resulted in a 27 ± 6% loss of cell surface Y335A-hDAT (Fig. 3, C and D). Treatment with 10 μM Zn^{2+} alone did not alter cell surface expression of WT-hDAT or Y335A-hDAT (Fig. 3, B and D; band densities were 103 ± 16 and 112 ± 8%, respectively).

To bypass the transporter and address whether intracellular accumulation of AMPH is sufficient to drive transporter trafficking, we used the whole-cell current-clamp technique combined with confocal imaging. We used the patch pipette to deliver AMPH into the cell, while monitoring changes in the cellular distribution of DAT by confocal imaging (Fig. 4A). Intracellular delivery of 10 μM AMPH produced an increase in Y335A-hDAT intracellular fluorescence [Fig. 4, C and E; at 20 min, the intracellular fluorescence was 160 ± 9% of that measured immediately after establishing the whole cell configuration (basal)]. In the absence of AMPH in the patch pipette, intracellular fluorescence was stable for at least 20 min after establishing the whole-cell configuration (Fig. 4, B and E; 100 ± 5% of basal after 20 min). Intracellular delivery of 10 μM DA, in contrast to AMPH, did not alter intracellular Y335A-hDAT (Fig, 4, D and E; 103 ± 5% of basal after 20 min).

Discussion

AMPH-induced down-regulation of DAT activity results from reducing transporter cell surface expression (Saunders et al., 2000; Kahlig et al., 2004). A number of signal transduction pathways have been implicated in this modulation. For example, PKC, which has been shown to be activated by AMPH (Giambalvo, 1992a,b), reduces the cell surface expression of DAT, resulting in reduced uptake capacity (Pristupa et al., 1998; Granas et al., 2003; Loder and Melikian, 2003; Sorkina et al., 2005). Moreover, we have shown that insulin signaling maintains DAT cell surface expression and that the serine/threonine kinase Akt/protein kinase B plays a key role in the hormonal modulation of AMPH-induced DAT trafficking (Garcia et al., 2005). The molecular mechanism by which AMPH induces DAT trafficking and whether the transport of AMPH across the plasma membrane is essential for this effect are not known. Although cocaine, a DAT reuptake blocker, inhibits AMPH-induced trafficking (Daws et al., 2002; Little et al., 2002), this might occur by preventing the interaction of AMPH with the transporter. Thus, AMPH-induced trafficking may not require intracellular accumulation of AMPH. Rather, the interaction of AMPH with DAT may alter DAT function, such as an ion conductance, which would in turn alter intracellular signaling. Because cocaine blocks interaction of extracellular AMPH with the transporter as well as its transport, we cannot differentiate between these two mechanisms.

Instead, we have taken advantage of a transporter mutant, Y335A-hDAT, in which transport activity is reduced to less than 1% of WT-hDAT levels but that binds AMPH normally (Loland et al., 2002). In contrast to WT-hDAT, this uptake-

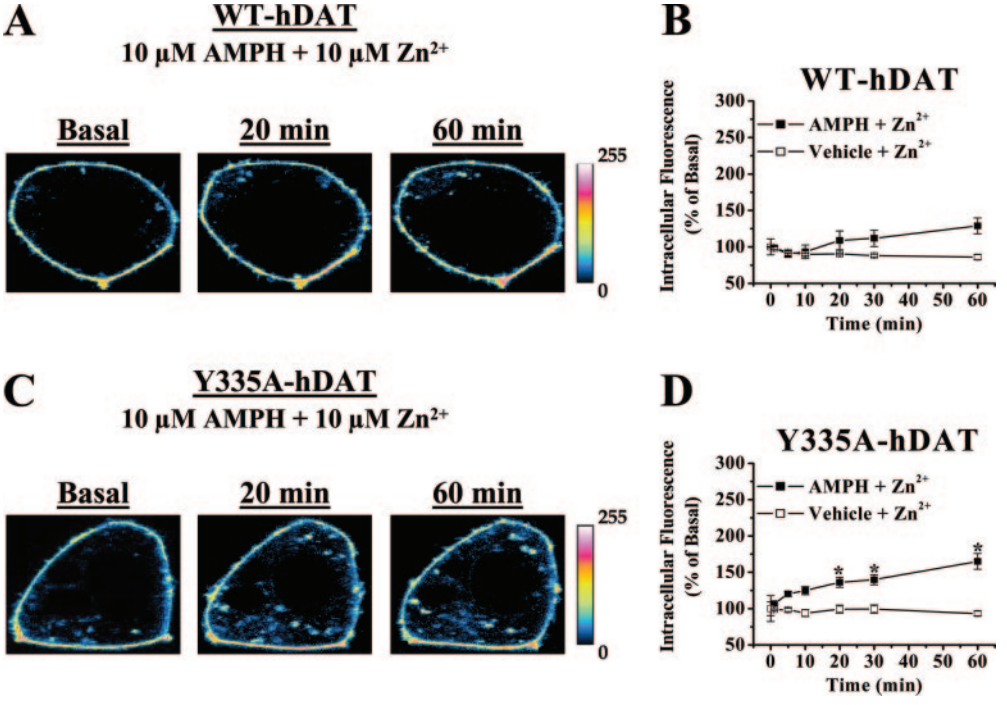

Fig. 2. Zn^{2+} regulation of transport activity alters DAT trafficking in response to AMPH. Representative confocal microscopy images illustrating the cellular distribution of either WT-hDAT (A) or Y335A-hDAT (C). The "basal" image was obtained in the presence of 10 μM Zn^{2+} and the "20 min" and "60 min" images were obtained after application of 10 μM AMPH in the continued presence of 10 μM Zn^{2+}. Quantification of the intracellular fluorescence of WT-hDAT (B) or Y335A-hDAT (D) measured under basal conditions (vehicle, □) or AMPH-treated (AMPH, ■) cells, as in A and C. Data points represent the mean ± S.E. obtained from 15 to 20 cells in five independent experiments (one-way ANOVA followed by Bonferroni's post hoc test; * indicates $p < 0.05$ relative to the basal value for each condition).

deficient mutant does not undergo trafficking in response to AMPH (Figs. 1 and 3), demonstrating that the lipophilic diffusion of AMPH into the cells is not sufficient to cause transporter trafficking.

DAT contains three endogenous residues (His-193, His-375, and Glu-396) that coordinate the binding of Zn^{2+} (Norregaard et al., 1998; Loland et al., 1999). Zn^{2+} binding to this site produces a potent, noncompetitive inhibition of WT-hDAT uptake (Norregaard et al., 1998; Loland et al., 2002; Meinild et al., 2004). In contrast to this inhibition of WT-hDAT, Zn^{2+} dramatically stimulates uptake activity in Y335A-hDAT (Loland et al., 2002). Therefore, we used the differential effects of Zn^{2+} on the activity of WT-hDAT and Y335A-hDAT to further explore whether transport of AMPH is implicated in its stimulation of DAT trafficking.

Zn^{2+} inhibited AMPH-induced trafficking of WT-hDAT (Figs. 2 and 3), consistent with its inhibition of uptake. We demonstrated, by means of confocal imaging and cell surface biotinylation, that Zn^{2+} restored the ability of AMPH to induce trafficking of Y335A-hDAT (Figs. 2 and 3). This is consistent with Zn^{2+} stimulation of uptake in this mutant and the possibility that AMPH must be accumulated intracellularly. It is noteworthy that Zn^{2+} alone did not affect WT-hDAT or Y335A-hDAT trafficking (Figs. 2 and 3), indicating that uncoupled ion conductances elicited by Zn^{2+} (Meinild et al., 2004) do not cause transporter trafficking.

Taken together, these results suggest that Zn^{2+} regulation of AMPH-induced Y335A-hDAT trafficking is due to modulation of transporter activity. However, whether this is due to intracellular accumulation of AMPH or to alterations in in-

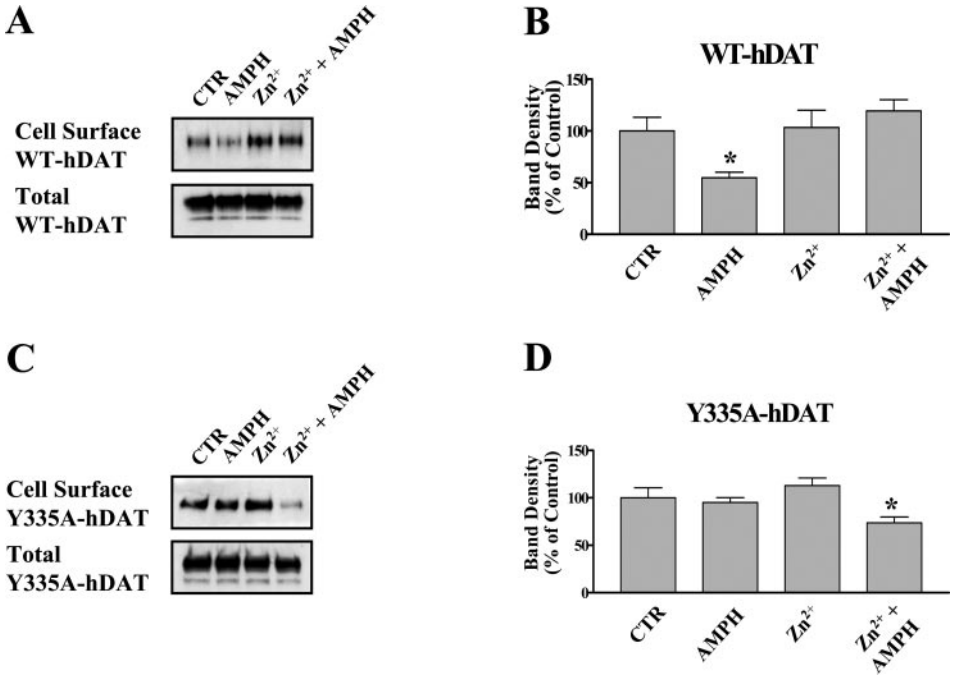

Fig. 3. Zn^{2+} attenuates AMPH-induced trafficking of WT-hDAT but rescues AMPH-induced trafficking of Y335A. Representative immunoblots showing biotinylated cell extracts (cell surface) and total extracts (total) of WT-hDAT (A) or Y335A-hDAT (C) after incubation with the indicated treatment for 60 min. Biotinylated band densities were quantified for WT-hDAT (B) and Y335A-hDAT (D). Normalized values are reported as mean ± S.E. obtained from at least three independent experiments (one-way ANOVA followed by Newman-Keuls post hoc test; * indicates $p < 0.05$ relative to the control).

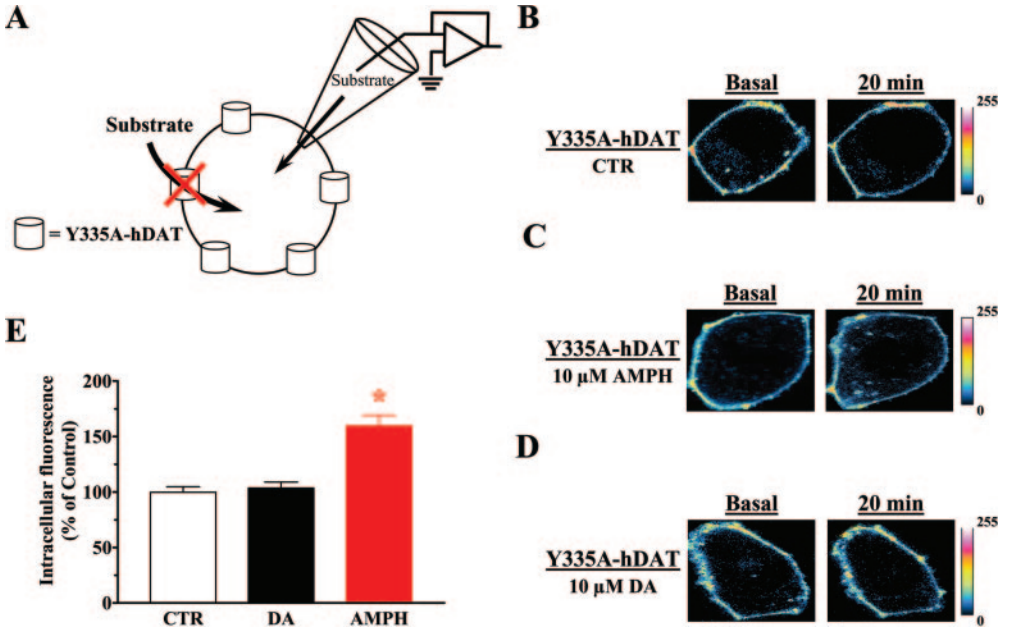

Fig. 4. Intracellular delivery of AMPH stimulates Y335A-hDAT trafficking in the absence of Zn^{2+}. A, the cartoon illustrates the configuration used for the experiments in which a whole-cell patch pipette in current-clamp mode delivered AMPH or DA intracellularly. Representative confocal microscopy images illustrating the cellular distribution of Y335A-hDAT immediately after establishing the whole cell mode (basal) and 20 min after intracellular perfusion with vehicle (CTR) (B), 10 µM AMPH (C), or 10 µM DA (D). Drugs were intracellularly administered via whole-cell patch pipette. E, quantification of the Y335A-hDAT intracellular fluorescence after 20 min of perfusion. Data points represent the mean ± S.E. obtained from three independent experiments for each condition (one-way ANOVA followed by Bonferroni's post hoc test; * indicates $p < 0.05$ relative to the control).

tracellular signaling as a result of DAT function cannot be determined by these data. To discriminate between these possibilities, we used the uptake-impaired Y335A-hDAT mutant and delivered AMPH directly into the cell via the whole-cell patch pipette, thereby bypassing the need for DAT-mediated AMPH transport (Fig. 4A). With pipette delivery, the cytosolic concentration of AMPH would be expected to equilibrate with the patch pipette solution relatively rapidly. This concentration of internal AMPH clearly is more effective than the concentration reached by diffusion alone, as direct delivery of intracellular AMPH via the patch pipette induced trafficking of Y335A-hDAT (Fig. 4C). This suggests that the accumulation of intracellular AMPH is sufficient to promote trafficking and that DAT activity-related signaling alone is not sufficient. Moreover, the membrane voltage, as determined during current clamp, was not significantly altered by intracellular AMPH (data not shown), suggesting the absence of alterations in signaling pathways as a consequence of ionic conductance stimulation in this paradigm.

Intracellular AMPH may induce trafficking via three nonexclusive mechanisms. First, it is possible that the intracellular concentration of AMPH reached with active transport (or by pipette perfusion), but not by lipophilic diffusion, directly modulates signaling pathways. For example, we have shown that AMPH inhibits serine/threonine kinase Akt/protein kinase B (Garcia et al., 2005), and this might simply require intracellular AMPH and not DAT activity per se. Moreover, AMPH has been shown to activate extracellular signal-regulated kinase 1/2 (Choe et al., 2002) and PKC (Giambalvo, 1992a,b) and might directly modulate the activity of intracellular signal transduction cascades to regulate DAT membrane expression.

Second, the interaction of intracellular AMPH with DAT itself may promote a conformational state of the transporter, such as reverse transport, that supports its trafficking. Although the inability of intracellular DA to cause DAT trafficking (Fig. 4D) argues against such a mechanism, we cannot exclude the possibility that intracellular AMPH promotes a different DAT conformation than does intracellular DA. The lack of an effect of intracellular DA is somewhat puzzling, given that extracellular DA can also induce DAT trafficking (Saunders et al., 2000; Chi and Reith, 2003), although it does so at higher concentrations (Saunders et al., 2000) and is cell-type dependent (Daniels and Amara, 1999). Our data suggest that the mechanisms of AMPH- and DA-induced trafficking might differ.

Third, our data may also be explained by the weak base action of AMPH, which may reduce the acidic gradients necessary to maintain rapid endosomal recycling back to the plasma membrane (Sulzer and Rayport, 1990; Johnson et al., 1993), resulting in the accumulation of intracellular transporter pools in the face of constitutive recycling (Loder and Melikian, 2003; Melikian, 2004).

It is unlikely that direct phosphorylation of DAT is involved in AMPH-induced trafficking. Although methamphetamine has been shown to induce phosphorylation of the DAT N terminus, this phosphorylation is not necessary for DAT trafficking (Granas et al., 2003; Cervinski et al., 2005) but is essential for AMPH-induced DA efflux (Khoshbouei et al., 2004).

In summary, AMPH uses DAT to gain entry into the cell, and our data suggest that intracellular AMPH stimulates DAT trafficking. The physiological relevance of our findings is supported by animal studies demonstrating that administration of AMPH at doses reported to cause hyperactivity and stereotyped behaviors leads to extracellular AMPH concentrations equal to or higher than 10 μM (Clausing et al., 1995), the concentration we used in our studies. Furthermore, seminal studies by Fleckenstein and colleagues (Kokoshka et al., 1998; Fleckenstein et al., 1999) demonstrated that a single dose of amphetamines leads to a long-lasting decrement in DA uptake in the face of preserved DAT protein expression, a finding that may be explained by AMPH-induced cell surface redistribution of DAT. Identifying the intracellular target of AMPH may enable new therapies for AMPH abuse by enabling the modulation of DAT cell surface expression and activity.

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