Membrane potential shapes regulation of dopamine transporter trafficking at the plasma membrane

Ben D. Richardson1,*, Kaustuv Saha1,*, Danielle Krout2, Elizabeth Cabrera1, Bruce Felts2, L. Keith Henry2, Jarod Swant1, Mu-Fa Zou3, Amy Hauck Newman3 & Habibeh Khoshbouei1

The dopaminergic system is essential for cognitive processes, including reward, attention and motor control. In addition to DA release and availability of synaptic DA receptors, timing and magnitude of DA neurotransmission depend on extracellular DA-level regulation by the dopamine transporter (DAT), the membrane expression and trafficking of which are highly dynamic. Data presented here from real-time TIRF (TIRFM) and confocal microscopy coupled with surface biotinylation and electrophysiology suggest that changes in the membrane potential alone, a universal yet dynamic cellular property, rapidly alter trafficking of DAT to and from the surface membrane. Broadly, these findings suggest that cell-surface DAT levels are sensitive to membrane potential changes, which can rapidly drive DAT internalization from and insertion into the cell membrane, thus having an impact on the capacity for DAT to regulate extracellular DA levels.

1 Department of Neuroscience, Evelyn F. and William L. McKnight Brain Institute, University of Florida, Gainesville, Florida 32610, USA. 2 Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota 58203, USA. 3 Medicinal Chemistry Section, Intramural Research Program, National Institute on Drug Abuse, Baltimore, Maryland 21224, USA. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to H.K. (email: habibeh@ufl.edu).
Central nervous system dopaminergic (DAergic) neurotransmission is essential in multiple neurological functions, including cognition, extrapyramidal motor control, the reward pathway and attention. In addition to the timing of vesicular release of dopamine (DA) and the expression profiles of G-protein-coupled DA receptors, one major regulator of DA signalling magnitude and timing is the DA transporter (DAT), which rapidly transports extracellular DAT into the intracellular space for vesicular re-packaging or effluxes DA through reversal of DAT-mediated transport. Commonly abused psychotropic drugs, amphetamine (AMPH), methamphetamine and cocaine achieve their effects either by inducing DA efflux through DAT and/or blocking DA uptake.

The physiological function of DAT to remove DA is coupled to the translocation of one Cl⁻ and two Na⁺ ions, and can even function in the absence of substrate, conducting an uncoupled, cocaine-sensitive, depolarizing current under physiological conditions, which is increased in hyperpolarized states. In addition to direct modulation of transport function, DAT density at the cell membrane, and therefore its functional capacity, is also dynamic. Regulated trafficking mechanisms control surface-membrane DAT levels under physiological conditions and in response to DAT substrates, thus having an impact on DA homeostasis. Cell signalling molecules involved in the regulation of DAT trafficking range from protein kinase C (PKC) and mitogen-activated protein kinase to Akt (ref. 20) among others and determine the presence of DAT in regulated or constitutive pools segregated to specific membrane microdomains. Many DAT substrates also influence DAT trafficking, including DA and AMPH, which decrease DAT surface density and cocaine, which increases DAT surface expression.

Interestingly, AMPH's effects are twofold, as it causes DAT internalization and a DAT-dependent membrane depolarization, which suggests an influence on DAT trafficking via a voltage-dependent mechanism in addition to DAT phosphorylation. Indeed, previous studies using striatal synaptosomes have revealed a reduction in DA uptake in depolarized (elevated KCl) conditions while in vitro preparations have suggested elevated DAT function at hyperpolarized states. However, it is not known whether these changes in functional capacity arise from changes in ionic driving forces, essential for DA transport, changes in DAT protein presence at the membrane or both. While changes in the cell membrane voltage state are only typically considered in terms of neurotransmitter release, action potential generation and timing or in the activity of voltage-sensitive transmembrane proteins, it is possible that changes in membrane potential (MP) alone may rapidly and reversibly affect DAT trafficking to and from the cell surface. Here we use confocal and total internal reflection fluorescence microscopy (TIRFM), biochemistry, electrophysiology and optogenetics to demonstrate the degree to which surface-membrane DAT levels are shaped by and sensitive to MP changes.

Results

MP depolarization reduces membrane DAT levels. AMPH-mediated activation of DAT induces a depolarizing DAT-mediated Na⁺ current and simultaneously causes internalization of cell-surface-membrane DAT. To determine whether AMPH-induced DAT internalization was the result of a psychostimulant-specific action or may be, in part, due to activation of voltage-sensitive mechanisms, we performed live cell TIRFM of yellow fluorescent protein-tagged DAT (YFP-DAT) in Human Embryonic Kidney (HEK) cells when perfused with only extracellular solution (vehicle), 10 μM AMPH or 100 mM KCl (Fig. 1), which depolarized cells by 13.5 and 35.7 mV, respectively (Fig. 1e). The distribution of YFP (yellow fluorescent protein)-DAT at the cell membrane (TIRFM footprint) was unchanged throughout perfusion of vehicle, whereas 10 μM AMPH noticeably altered the YFP signal in the TIRFM footprint within the first 60 s, causing a reduction in surface-membrane high-intensity regions and puncta that did not recover in washout (Fig. 1a,b and Supplementary Fig. 1a), in line with previous reports at longer AMPH treatment durations. Similarly, depolarizing 100 mM KCl-based external solution significantly altered the YFP-DAT distribution in TIRFM footprint; however, the effects occurred rapidly, obvious within 30 s, and typically all YFP puncta and high-intensity regions were absent from the surface membrane after 3 min (Fig. 1a,b,d). In contrast to AMPH, treatment with KCl resulted in the return of YFP signal profile and the reappearance of YFP puncta immediately on washout (Fig. 1a and Supplementary Movie 1). To determine the relative specificity of this effect of depolarization for DAT, we identically depolarized HEK cells transfected with an eYFP-tagged version of an unrelated naturally occurring membrane protein, GPR40, which had a membrane distribution similar to DAT, but its trafficking appeared insensitive to depolarization (Supplementary Fig. 1b,c).

Since the depolarization induced by KCl will likely increase free [Ca²⁺] and trigger the activation of Ca²⁺-dependent signalling molecules, we chose to determine the role of CaMKII and PKC in initiating this depolarization-induced redistribution. The depolarization-induced loss of YFP-DAT signal did not appear affected by the PKC inhibitor, bisindolylmaleimide I (10 μM; Supplementary Fig. 2). However, the KCl depolarization-induced loss of YFP-DAT surface puncta was significantly reduced in the presence of the CaMK inhibitor KN93 (10 μM) relative to the same treatment in the presence of the inactive homologue, KN92 (10 μM; Fig. 1c,d), which produced results similar to KCl treatment alone (Fig. 1a–d). However, because of the KN93-induced attenuation of the depolarization-triggered Ca²⁺ influx (Supplementary Fig. 3), we chose to biochemically inhibit CaMKIIZ specifically and assess membrane DAT using TIRFM by co-expressing a kinase-inactive version of CaMKIIZ, a green fluorescent protein (GFP)-tagged K42R mutant and RFP (red fluorescent protein)-DAT. In response to KCl-induced depolarization, RFP-DAT alone behaved similarly to YFP-DAT; however, when GFP-CaMKIIZ(K42R) was co-expressed, KCl treatment was unable to alter the membrane distribution of RFP-DAT (Fig. 1c,d). These changes in membrane DAT in response to depolarization (100 mM KCl application) and repolarization (washout) suggest that the MP state is capable of bidirectionally shaping the cell-surface distribution of DAT through activation of CaMKIIZ.

Membrane DAT reduction is CaMKIIZ and dynamin dependent.

To determine the degree to which real-time changes in the YFP-DAT TIRFM footprint were indicative of changes in DAT protein density at the cell membrane, a cell-surface biotinylation assay was used to quantify differences in membrane DAT protein levels. In YFP-DAT HEK cells, compared with vehicle treatment (100%, n = 17), surface DAT (Fig. 2; see Supplementary Fig. 4 for antibody validation and total protein blots) was significantly reduced following a similar 5 min treatment as above with both 50 mM (62 ± 6%, n = 14) and 100 mM (70 ± 5%, n = 17) KCl-based external solution as well as the positive control treatments with AMPH (10 μM; 59 ± 7%, n = 13) and the PKC agonist phorbol myristate acetate (PMA, 2.5 μM; 53 ± 5%, n = 13). The CaMKIIZ dependency of this effect observed in TIRFM studies
Importantly, treatment with 100 mM KCl did not alter surface AMPH-dependent (66 ± 9% vs. respective controls. 

The depolarization- and PMA-dependent (117 ± 6% vs. N) internalization of DAT in HEK cells, whereas expression of CaMKII(K42R) only blocked depolarization- and PMA-dependent (117 ± 3% vs. N) but not AMPH-dependent (66 ± 9% vs. N) internalization (Fig. 2). Importantly, treatment with 100 mM KCl did not alter surface levels of the native or overexpressed, membrane-resident transferrin receptor in comparison with vehicle control, providing further support for the specificity of depolarization-induced downregulation of membrane DAT (Supplementary Fig. 5). Taken together, these data suggest that CaMKIIζ- and dynamin-dependent pathways are involved in depolarization-dependent DAT trafficking at the cell membrane.

**Figure 1 | Depolarization causes CaMK-dependent reduction of membrane YFP-DAT levels in HEK cells.** (a) Representative live cell sequential TIRF microscopy images of YFP-DAT HEK cells 60 s before and throughout 5-min perfusion with vehicle (standard external solution, top row), 10 μM amphetamine (middle row; N = 5, n = 14) or depolarizing 100 mM KCl-based external solution (bottom row) followed by a 5-min washout period. Scale bar, 20 μm. (b) Enlarged insets corresponding to boxes in left-most column before and 150 s after vehicle (top pair), 100 mM KCl (middle pair) and 10 μM amphetamine (bottom pair). Scale bar, 5 μm. (c) TIRF microscopy images of YFP-DAT HEK cells 60 s before and throughout 5-min perfusion, with 100 mM KCl pre-incubated with 10 μM KN92 (top), 10 μM KN93 (middle) or expressing a kinase-inactive CaMKII(K42R). Scale bar, 20 μm. (d) Mean ± s.e.m. Normalized fold change in number of YFP-DAT puncta per cell when perfused with vehicle (open circles; N = 5 experiments, n = 17 cells), 100 mM KCl only (closed circles; N = 6, n = 14), 100 mM KCl when pre-incubated and perfused with 10 μM KN92 (closed circles; N = 5, n = 12) or 10 μM KN93 (open circles; N = 6, n = 23) and RFP-DAT puncta expressed alone (closed squares; N = 9, n = 9) or co-expressing a kinase-inactive GFP-CaMKII(K42R) (open squares; N = 10, n = 12) during 100 mM KCl perfusion. (e) Mean ± s.e.m. Steady-state membrane potential of YFP-DAT HEK cells perfused with vehicle, 10 μM amphetamine or 100 mM KCl (n = 5–7 cells per group). Using independent samples t-test *P < 0.05 for comparison of 100 mM KCl effects with respective controls.
MP depolarization redistributes DAT into early endosomes. To determine the identity of the intracellular destination of depolarization-dependent internalized DAT, fluorescent versions of the endosome markers (which were not apparent at the cell membrane in TIRFM; see Supplementary Fig. 6), EEA1 (TagRFP-T-EEA1) that marks early endosomes or the recycling endosome marker Rab11 (DsRed-Rab11), were expressed in HEK YFP-DAT cells and then treated with standard external solution (vehicle), iso-osmotic 100-mM KCl-based external solution for 5 min or 10 μM AMPH for 5 min as a temporal comparison. Another time point of 60 min AMPH (10 μM) treatment was used as a positive control as it has been shown previously to cause DAT internalization to specific endosomes18,37–39, and would thus allow for comparability to previous work. Average Pearson correlation coefficients per cell for intracellular YFP-DAT and TagRFP-T-EEA1 or DsRed-Rab11 in vehicle (EEA1: 0.17 ± 0.01; Rab11: 0.19 ± 0.01) were significantly less than in cells treated with AMPH at 5 min for EEA1 (0.32 ± 0.03; Fig. 4a,b) and Rab11 (0.47 ± 0.06; Fig. 4c,d), while 60-min AMPH treatment increased YFP-DAT association with EEA1 (0.24 ± 0.01; Fig. 4a,b), but not Rab11 (0.22 ± 0.15; Fig. 4c,d). Similarly, depolarizing conditions significantly enhanced the co-localization of intracellular YFP-DAT with EEA1 (0.30 ± 0.02; Fig. 4a,b) over vehicle and 60-min AMPH treatments, although comparable to the 5-min AMPH treatment. The treatment had no effect on the degree of association of intracellular YFP-DAT with Rab11 (0.16 ± 0.07; Fig. 4c,d) compared with vehicle. While biotinylation and confocal imaging inherently lack the temporal resolution of TIRFM, the collective results indicate that MP depolarization rapidly reduces cell-surface-membrane DAT and internalizes the transporter to intracellular early endosome compartments, suggesting that membrane DAT levels and DAT trafficking may be partially dependent on the MP state and therefore could change rapidly with MP fluctuation on local changes in the activity of receptors, ion transporters and channels.

**Figure 2 | Depolarization-induced reduction in surface-membrane-biotinylated DAT is due to CaMKII-dependent endocytosis.** (a) Representative western blots show the surface DAT present under each indicated condition. (b) Mean ± s.e.m. DAT band density from YFP-DAT HEK cells (control, black bars) or YFP-DAT HEK cells transfected with GFP-C1-CAMKIIζ-K42R (grey bars) or YFP-DAT HEK pre-treated (30 min) with external solution (vehicle) or 80 μM Dynasore (white bars), followed by 30-min treatment with vehicle, vehicle +10 μM KN92 or vehicle +10 μM KN93. Cells normalized to vehicle treatment from at least five independent experiments indicate that AMPH, PMA and depolarization-induced reduction in surface DAT are endocytosis-dependent (Dynasore-sensitive), and both PMA- and depolarization-induced internalization are CaMKIIζ-dependent. Depolarization-induced internalization is sensitive to both pharmacological and molecular inhibition of CaMKII activity. A two-way analysis of variance (ANOVA) with Bonferroni post hoc test was performed to identify significant differences from control (**P<0.05; ***P<0.01; ****P<0.001), a one-way ANOVA with a Dunnett’s post hoc test to determine changes from vehicle treatment (****P<0.0001) and an unpaired t-test to compare KN92 and KN93 treatments (P=0.01). See text for number of experiments.

**Figure 3 | Membrane depolarization with KCl increases internalization of JHC 1-064/DAT complexes in HEK cells.** (a) JHC 1-064 binds to surface DAT in HEK cells. Confocal image of YFP signal in YFP-DAT HEK cells (top), with no initial detectable fluorescence emitted between 553 and 617 nm when excited with 561 nm (bottom left, T = 0 min) until after 20-min exposure to 10 mM HJC 1-064 (bottom right) corresponding with YFP fluorescence. (b) Representative confocal images of JHC 1-064/DAT labelling (white) at ~4°C and 5 min following solution change at 37°C. Note the increase at 5 min in the number of white intracellular puncta in the KCl condition. (c,d) Mean ± s.e.m. number of individual JHC 1-064/DAT puncta per cell (c) and normalized (to 4°C) fluorescence intensity (d) in the intracellular space corresponding to vehicle (N = 3 experiments, n = 85 cells, black bars) or 100 mM KCl-containing solution treatment (N = 3 experiments, n = 85 cells, red bars). Independent samples t-tests were used to compare vehicle with KCl effects. Scale bars, 10 μm. ****P<0.0001 using independent samples t-test.
Change in MP state alters cell surface membrane DAT levels.

To further examine whether the membrane distribution of DAT is altered in response to MP changes (depolarization and hyperpolarization), we employed simultaneous single-cell TIRFM and whole-cell patch clamp electrophysiology (Fig. 5a). This technique allowed for time-resolved, bidirectional and precise control of the MP and provided internal controls in adjacent non-clamped cells. Acquisition of TIRFM image sequences (5-s intervals) throughout the course of 5-min duration voltage steps indicated that the surface YFP-DAT signal is stable during whole-cell voltage clamp at −40 mV (typical MP for YFP-DAT HEK cells; Fig. 5b–e), but MP changes from baseline to hyperpolarized (−60 mV; Fig. 5b,f–h) or depolarized (+20 mV; Fig. 5b,i–k) potentials could rapidly (between frames, 5 s duration) increase or reduce, respectively, YFP-DAT puncta in the TIRFM footprint (Fig. 5b). In some cases, the 5-s interframe interval during depolarization was sufficient to eliminate all DAT puncta from the cell surface, and hyperpolarization to −60 mV caused complete recovery of the fluorescent signal profile within 5–10 s (Fig. 5b). The effect of the hyperpolarizing voltage step on the TIRFM footprint intensity of patch-clamped cells rapidly increased, typically plateauing within 5 min, and began to reverse (decrease in intensity) following return of the membrane voltage to −40 mV (Fig. 5f). Continuous clamping of the MP at −40 mV did not significantly alter the YFP-DAT TIRFM footprint intensity at 3 min relative to adjacent cells (n = 4 clamped, four adjacent cells; P > 0.05; Fig. 5c–e). However, when comparing intensity changes between clamped and adjacent cells 3 min into the voltage step, stepping the MP to −60 mV significantly increased the YFP-DAT TIRFM footprint intensity (n = 5 clamped, five adjacent cells; P < 0.01; Fig. 5f–h), while stepping to +20 mV produced the opposite effect (n = 5 clamped, five adjacent cells; P < 0.01; Fig. 5i–k). Further comparison of voltage effects between only cells clamped at −40, −60 or +20 mV also indicates a significant difference in normalized YFP-DAT TIRFM footprint intensity for the −60 mV condition when compared with the −40 mV condition 5 min following the voltage change (Fig. 5l).

Change in MP state alters DAT-mediated current. Since the mere presence of DAT at the cell surface (YFP fluorescence signal) is not necessarily indicative of relative DAT function, we sought to determine whether MP change-induced variations in the surface DAT density (Fig. 5) correlated with the uncoupled DAT-mediated current. To investigate this, as in previous TIRFM experiments, the MP was clamped for 5 min at +20, −40 or −60 mV but was followed by acquisition of a baseline IV curve (Fig. 6a). The subsequent GBR12935-sensitive current was then taken as the DAT-mediated current for each cell for a given condition. Cells clamped at −40 mV (near their endogenous resting MP) had a DAT-mediated current amplitude of −15.3 ± 2.16 pA (n = 8; Fig. 6c–f, black). However, when cells were depolarized to +20 mV for 5 min (Fig. 6c–f, red), the DAT-mediated current (−8.8 ± 1.6 pA, n = 7) was significantly reduced by 42.3% (P < 0.05), and cells hyperpolarized to −60 mV for 5 min (Fig. 6c–f, blue) displayed a 47.7% larger (P < 0.05) DAT-mediated current (−22.6 ± 2.0 pA, n = 5) compared with cells held at −40 mV. For comparison of MP state-dependent changes in YFP-DAT membrane density (Fig. 5) and DAT functional capacity, the average fold change in YFP-DAT TIRFM...
footprint intensity and DAT-mediated current amplitude for each MP-holding potential state are plotted against each other (Fig. 6f), indicating a positive correlation between the two measures. While the cell-surface-membrane DAT levels (TIRFM) are influenced by the MP state, these data imply that functional DAT may be particularly influenced by MP state changes as they are more profoundly affected.

**Neuronal MP changes alter surface-membrane DAT levels.** To determine whether MP influences surface-membrane DAT
density in functional neurons, real-time imaging of membrane DAT (TIRFM fluorescence footprint) was coupled with two characterized non-invasive methods (Supplementary Fig. 7) of transient reliable membrane depolarization and hyperpolarization: 100-mM KCl focal application (Fig. 7a) and archaerhodopsin (Arch) activation (Fig. 7b), respectively. Cultured primary neurons were transfected with TagRFP-T-DAT (RFP-DAT) or CFP-DAT with or without co-transfection with Arch-YFP (Arch-YFP, Supplementary Fig. 7) and were subjected to whole-cell recordings (K-gluconate-based internal solution) or imaging in the presence of tetrodotoxin (TTX) and receptor blocker cocktail (see Methods), unless otherwise indicated. Pressure application of KCl-based external solution induced a reversible 36.9 ± 8.3-mV membrane depolarization in RFP-DAT-expressing neurons (Fig. 7a,c–e), and photo-activation (590 nm) of Arch caused a reversible −23.3 ± 3.2-mV membrane hyperpolarization (Fig. 7a,c–e). In the absence of TTX, Arch activation suppressed action potential firing and induced a rebound burst when the light was turned off (Fig. 7b) and was relatively stable over long pulse durations (Supplementary Fig. 7) used for subsequent imaging experiments. The MP of neurons lacking Arch-YFP expression was unaffected by 590-nm light stimulation.

On characterizing the reliability of these tools, we performed simultaneous TIRFM of primary culture neurons (Fig. 7f) during each manipulation (Supplementary Fig. 7b and Fig. 7g,h). TIRFM of neurons expressing RFP-DAT while focally applying 100-mM KCl-based external solution (Supplementary Fig. 7b) for a short duration (45 s) caused a rapid and dramatic reduction (−21.1 ± 8.5%) in the RFP-DAT TIRFM footprint intensity (Supplementary Movie 2), while vehicle application had no effect (Fig. 7g). Similarly, bath application of 100-mM KCl-based external solution also enhanced the internalization of endogenous DAT in primary neurons labelled with JHC 1-064, causing a dramatic increase in JHC 1-064 complexes in the intracellular space (Supplementary Fig. 8). In contrast, simultaneous MP hyperpolarization via activation of Arch by 590-nm light staggered with TIRFM imaging of CFP-DAT (Fig. 7h) indicated that hyperpolarization caused a reversible increase (+9.0 ± 3.5%) in CFP-DAT intensity in the TIRFM footprint timespan, which stabilized after 120 s (Fig. 7h and Supplementary Movie 3). No significant change (−3.8 ± 3.3%) in CFP-DAT TIRFM footprint intensity was observed in neurons lacking Arch-YFP expression (Fig. 7h). These KCl-induced decreases and Arch activation-induced increases in neuronal cell-surface DAT TIRFM signal, which parallel MP state-dependent changes in the surface density of DAT protein (Figs 1–5) and DAT function (Fig. 6) in YFP-DAT HEK cells, were significantly different from their respective controls (depolarization: $P < 0.05$, hyperpolarization: $P < 0.05$; Fig. 7i).
Figure 7 | Neuronal membrane potential rapidly alters membrane-surface DAT level. (a,b) Cultured neuron membrane potential response to (a) vehicle or 100 mM KCl and (b) 590 nm stimulation in neurons with/without YFP-Arch. 0.5 mM TTX omitted in bottom trace. Scale bar, 10 s (a) and 500 ms (b) and 10 mV for (a,b). (c) Membrane potential before, during and after vehicle (black; n = 3) or 100 mM KCl (red; n = 4) and (d) 590 nm-light stimulation of neurons with (blue; n = 13)/without (grey; n = 3) Arch-YFP expression. (e) Resting potential change. (f) TIRFM image of primary culture neuron expressing RFP-DAT. (g) The mean normalized intensity of the surface RFP-DAT during vehicle (black; n = 4) or 100 mM KCl (red; n = 3) application. (h) The mean normalized intensity of the surface CFP-DAT when a 590-nm light pulse was delivered to cells with (blue; n = 5)/without (grey; n = 4) Arch-YFP expression. (i) Normalized somatic membrane RFP- or CFP-DAT fluorescence change for each condition. *P<0.05, **P<0.01 with independent samples t-test.

Discussion
The presence of DAT at the cell membrane is crucial in the regulation of DAergic signalling, timing and magnitude throughout the brain, and thus any alteration in the functional capacity of the transporter may significantly have an impact on neurological functions in which DA is involved. Previous studies have demonstrated that KCl-induced depolarization reduces DA uptake, and that membrane hyperpolarization increases DAT-mediated inward current and DA uptake, albeit with an unknown mechanism. Here we asked whether changes in MP alone may rapidly and reversibly regulate DAT trafficking. One aspect regulating transporter function is that the trafficking of mature DAT to and from the cell membrane is a highly regulated process, which is affected in various disease states and by the activity of DAT-targeting psychostimulants. Using live cell TIRFM and biotinylation on identically treated HEK cells expressing YFP-DAT, we determined that membrane depolarization alone could induce a CaMKIIz- and dynamin-dependent (Figs 1 and 2) rapid reversible (increase in hyperpolarization recovery) reduction in membrane DAT (Fig. 1 and Supplementary Movie 1). This depolarization-induced effect on DAT distribution in the TIRFM footprint was distinctly different when compared with the effects of AMPH, which did not recover as quickly. Another difference between AMPH- and depolarization-induced DAT internalization is the insensitivity of AMPH-induced internalization to the loss of CaMKIIz activity through the coexpression of a dominant-negative, kinase-inactive CaMKIIz (Fig. 2), which, along with the sensitivity of both versions to KN93, may suggest that different isoforms of the kinase may have distinctly different roles in regard to regulating DAT function. Notably, similar fast changes in membrane DAT levels have been reported using this approach with acute AMPH exposure. However, the direction of the AMPH effect on human DAT using the multifaceted approach reported here contrasts with this previous finding and could be due to intrinsic differences between rat and human DAT, AMPH concentrations and/or cell types.

To determine the degree of DAT internalization, with the DAT-specific fluorescent cocaine analogue, JHC 1-064 (ref. 36), we followed the distribution of JHC 1-064 fluorescence (JHC 1-064/DAT complexes; Fig. 3) when cells were left at rest or depolarized. These data suggested that indeed membrane-resident DAT was being more rapidly brought into the intracellular space when depolarized as compared with constitutive internalization (Fig. 3c,d). While biotinylation and confocal imaging inherently lack the temporal resolution of TIRFM, together, results indicate that in contrast to the effects of AMPH on DAT, depolarization resulted in DAT being segregated specifically into early endosome compartments (EEA1), but not recycling endosomes (Rab11; Fig. 4). This divergence in the destination of internalized DAT in cells treated with AMPH versus those simply depolarized again suggests the involvement of differing mechanisms, which may leave initially internalized DAT residing in early endosomes free to transition into rapid recycling endosomes, distinct from recycling endosomes, putatively underlying the faster recovery to the membrane surface during hyperpolarization.

Although few studies have examined DAT activity immediately after depolarization or following the return to the resting hyperpolarized state, our data provide a potential mechanism for the decreased DA uptake in striatal synaptosomes during the fast phase of depolarization-induced DA release. Therefore, to determine any bidirectionality of the KCl effect on DAT trafficking, we used whole-cell voltage-clamp techniques to clamp the MP of YFP-DAT HEK cells while performing TIRFM simultaneously (Fig. 5). Once cells were clamped near their endogenous resting potential (~ 40 mV), the YFP-DAT-TIRFM footprint was relatively similar over time (Fig. 5c–e). However, when stepping the membrane-holding potential from ~ 40 mV to a hyperpolarized potential, an increase in YFP-DAT intensity and puncta number in the TIRFM footprint began immediately
(Fig. 5b) and plateaued after 3 min (Fig. 5f–h). In contrast, when cells were depolarized the opposite effect occurred with a loss of YFP-DAT signal, which paralleled the effects seen in the presence of depolarizing KCl (Fig. 5i–k). In fact, the ~10% change in YFP-DAT intensity directly corresponded to reductions in DAT-mediated (GBR12935-sensitive) current when cells were clamped at depolarized or hyperpolarized potentials (Fig. 6). On the basis of these data and the known electrogenic nature of DAT-mediated DA uptake and efflux, we hypothesize that at the basis of these data and the known electrogenic nature of clamped at depolarized or hyperpolarized potentials (Fig. 6). On the basis of these data and the known electrogenic nature of clamped at depolarized or hyperpolarized potentials (Fig. 6). On the basis of these data and the known electrogenic nature of clamped at depolarized or hyperpolarized potentials (Fig. 6). On the basis of these data and the known electrogenic nature of clamped at depolarized or hyperpolarized potentials (Fig. 6).

With two tools that induced reversible and reliable depolarization (focal KCl application) or hyperpolarization (Arch activation) of Dat levels, similar to those used in previous experiments (Fig. 7a–c), we used TIRFM to monitor fluorescently-tagged DAT expressed in midbrain primary cultures during MP manipulation. The effect of these manipulations on membrane DAT levels were larger than in HEK cells using methods that induced similar voltage differences, implying that these effects are indeed applicable to neuronal populations and results obtained using HEK cells are relevant to shaping conclusions about MP-dependent trafficking of DAT in the nervous system. Together, these data indicate that, while the magnitude of change in membrane DAT levels due to MP changes varies depending on the assay, all changes observed are in a physiologically relevant range and the direction of the effect (increase or decrease) is in agreement across all examinations.

This effect of the MP on DAT trafficking sheds light on an additional mechanism by which the activation of hyperpolarizing D2Rs may be altering DA transport. The activation of D2Rs has long been understood to enhance DAT function44, and previous studies have suggested that a D2R activation initiates a signalling cascade to upregulate cell-surface DAT19. Although others have examined how changes in the neuronal MP similarly to those initiated by D2R activation may alter DAT function and found no impact on [3H]-DA uptake44, these experiments were performed at room temperature, which likely attenuates trafficking rates as opposed to the studies here conducted at near-physiological temperatures (37°C). Thus, this methodological difference may explain the discrepancies between that [3H]-DA uptake study44 relative to the data presented here. Nevertheless, collectively this study and previous studies support the involvement of multifaceted regulatory mechanisms for DAT trafficking that are substrate-, kinase- and activity-dependent. The existence of multiple regulatory mechanisms supports the notion that the DAT proteins at the membrane are responsive to diverse regulatory mechanisms. The overriding mechanism for activation of a given trafficking pathway will be determined by the nature of the stimulation and the availability of specific regulatory constituents.

DA signalling is crucial in many neurological functions, as aberrations in DA neurotransmission contribute to multiple neuropsychiatric disorders, including addition3,45. Parkinson’s disease and movement disorders46–48. schizophrenia49,50 and attention-deficit hyperactivity disorder (ADHD)51, all of which have been linked to how extracellular DA may be mishandled by altered DAT expression and function25,30,31. As a result, disease-related deviations from physiological states and variations in neuronal MPs may be altering the functional capacity of DAT by affecting its trafficking to and from the membrane. This dynamic balance of electrophysiological and biochemical processes to regulate subtle but essential aspects of neurotransmission opens a range of possibilities for exploring related aberrations in disease states and in pharmacotherapy targeting this interaction. Broadly, the regulation of protein (DAT) trafficking by the MP may provide additional means by which plasticity (for example, activity-dependent changes) in DAergic and possibly non-DAergic systems is maintained and controlled.

Methods

**Cell culture.** Cell lines. HEK cells overexpressing FLAG-tagged or YFP-tagged human DAT (hDAT), HEK FLAG-DAT (refs 52,53) or YFP-DAT HEK (ref. 54), respectively, were a generous gift from Dr Jonathan Lavish (Colorado University) prepared from HEK293 EM4 as previously described55,56. The addition of the YFP tag and FLAG epitope to hDAT is a widely used construct and has not been shown to alter basic functional properties of the transporter or other transporter-mediated activity56,57. HEK cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin, streptomycin (50 μg/ml) and 3 μg/ml 10% D-glucose. Cells were typically passaged and/or used for electrophysiology or imaging experiments after reaching 60–80% of full confluency (every 2–3 days). To induce expression of constructs not stably expressed in HEK cell lines, HEK293 EM4 cells were transfected using a standard calcium phosphate protocol. Transfected cells were used in experiments 12–36 h after transfection.

**Midbrain primary neuron culture.** All animals were housed in the University of Florida’s McKnight Brain Institute animal care facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The University of Florida’s McKnight Brain Institute animal care facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The University of Florida’s McKnight Brain Institute animal care facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The University of Florida’s McKnight Brain Institute animal care facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The University of Florida’s McKnight Brain Institute animal care facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The University of Florida’s McKnight Brain Institute animal care facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility.

This effect of the MP on DAT trafficking sheds light on an additional mechanism by which the activation of hyperpolarizing D2Rs may be altering DA transport. The activation of D2Rs has long been understood to enhance DAT function44, and previous studies have suggested that a D2R activation initiates a signalling cascade to upregulate cell-surface DAT19. Although others have examined how changes in the neuronal MP similarly to those initiated by D2R activation may alter DAT function and found no impact on [3H]-DA uptake44, these experiments were performed at room temperature, which likely attenuates trafficking rates as opposed to the studies here conducted at near-physiological temperatures (37°C). Thus, this methodological difference may explain the discrepancies between that [3H]-DA uptake study44 relative to the data presented here. Nevertheless, collectively this study and previous studies support the involvement of multifaceted regulatory mechanisms for DAT trafficking that are substrate-, kinase- and activity-dependent. The existence of multiple regulatory mechanisms supports the notion that the DAT proteins at the membrane are responsive to diverse regulatory mechanisms. The overriding mechanism for activation of a given trafficking pathway will be determined by the nature of the stimulation and the availability of specific regulatory constituents.

**Plasmid constructs.** The plasmid coding for the cyan fluorescent protein-tagged DAT was described previously56,57 and was provided as a generous gift from Dr Haley Melikian (University of Massachusetts), and the construct for fatty-acid receptor GPR40-EYFP driven by the cytomegalovirus promoter was a gift from Dr Sergei Zolotukhin and Seth Currin (University of Florida). DrRed-Rab11 WT5, a recycling endosome marker, was a gift from Richard Pagano (Mayo Clinic and Foundation, Addgene plasmid #12679). TagRFP-T-EEA1 (ref. 62), an early endosome marker, was provided by Silvia Corvera (University of Massachusetts Medical School, Addgene plasmid #42635). The GFP-C1–CAMKIIz-Kz42R (ref. 63) was a gift from Tobias Meyer (Stanford University, Addgene plasmid #21221), and the CFP-eArch3.0–eYFP plasmid was a gift from Dr Sergei Zolotukhin and Seth Currin (University of Florida). DrRed-Rab11 WT5, a recycling endosome marker, was a gift from Richard Pagano (Mayo Clinic and Foundation, Addgene plasmid #12679). TagRFP-T-EEA1 (ref. 62), an early endosome marker, was provided by Silvia Corvera (University of Massachusetts Medical School, Addgene plasmid #42635). The GFP-C1–CAMKIIz-Kz42R (ref. 63) was a gift from Tobias Meyer (Stanford University, Addgene plasmid #21221), and the CFP-eArch3.0–eYFP plasmid was a gift from Dr Sergei Zolotukhin and Seth Currin (University of Florida). DrRed-Rab11 WT5, a recycling endosome marker, was a gift from Richard Pagano (Mayo Clinic and Foundation, Addgene plasmid #12679). TagRFP-T-EEA1 (ref. 62), an early endosome marker, was provided by Silvia Corvera (University of Massachusetts Medical School, Addgene plasmid #42635). The GFP-C1–CAMKIIz-Kz42R (ref. 63) was a gift from Tobias Meyer (Stanford University, Addgene plasmid #21221), and the CFP-eArch3.0–eYFP plasmid was a gift from Dr Sergei Zolotukhin and Seth Currin (University of Florida).

To confer optical control of MP hyperpolarization, neuronal cultures were transfected with AAV-CaMKIz-eArch 3.0–EYFP plasmid, a generous gift from Dr Karl Deisseroth (Stanford University). Arch was chosen for its ability to induce large magnitude H-refractory hyperpolarization shifts (10–50 mV). The neuronal MP, which were relatively stable over seconds to minutes with minimal decay when continuously activated40,65.

**Electrophysiology.** HEK cells and cultured neurons were visualized with a ×60 objective on an inverted Nikon Ti Eclipse microscope (Nikon, Melville, NY). All currents and MPs were recorded via an Axoclamp 200A amplifier using the whole-cell configuration after forming a high-resistance seal in the cell-attached configuration (~1 MΩ). All recordings were digitized with a Digidata 1440A at 10 kHz, and a 5-kHz low-pass Bessel filter was applied during acquisition. An additional 2-kHz Gaussian filter was applied to all traces for presentation only.
The standard external solution for electrophysiology experiments using HEK cells was the same used in all microscopy and biochemical experiments and contained (in mM): 135 NaCl, 10 HEPES, 1.5 CaCl2, 1.2 MgSO4, and 1.3 KH2PO4, with a pH of 7.35 and osmolarity of 275–290 mOsm. Pipettes for whole-cell recordings were pulled from borosilicate glass on a P-2000 laser-based puller (Sutter Instruments, Novato, CA). Pipettes used for recording the MP (3–6 MΩ) were filled with the external solution (in mM) to the following: 130 K-gluconate, 10 KCl, 10 HEPES, 1 EGTA, 2 MgCl2, and 0.1 CaCl2 adjusted to pH 7.35 and osmolarity of 262 mOsm. For recording of DAT-mediated whole-cell currents, pipettes were filled with (in mM) the following: 120 CsCl, 30 dextrose, 10 HEPES, 1.1 EGTA, 2 MgCl2, and 0.1 CaCl2 adjusted to pH 7.35 and osmolarity of 264 mOsm. All experiments were performed at 37 °C. To determine DAT-mediated current and IV changes at different holding potentials, a stable IV (−100 to +100 mV steps) was generated after 5 min of continuously holding the cell at the given potential (−60, −40 or +20 mV), and then the DAT blocker GBR12935 (20 μM) was added to the bath and subsequent IVs were measured every 30–60 s. To determine the DAT-mediated current amplitude, the IV in the presence of GBR12935 (Fig. 6c; protocol #2; grey traces) was subtracted from the preGBR12935 (Fig. 6c; protocol #1; red trace) to account for bleaching and was plotted as a fraction of the initial internal intensity of all pixels. For determination of the mean intracellular intensity, the entire image and the Pearson correlation coefficient for the two channels was again normalized to the intensity of the entire image to account for bleaching and was plotted as a fraction of the initial internal fluorescence in the original control 4 °C image57. For manual counts of intracellular JHC 1-064 puncta and clarity for display (Fig. 3 and Supplementary Fig. 8), all images were processed identically.

Microscopy. All microscopy analyses were performed at 37 °C, and cells were washed twice with external solution as described above before all experiments. For all imaging experiments, cells/neurons were set on 35-mm glass-bottom dishes (MatTek, Ashland, MA) with glass thickness of 0.13–0.16 mm (TIRFM) or 0.085–0.13 mm (confocal). Wide-field fluorescence images were acquired identically to TIRFM images; however, a Lambda LS Xenon Arc Lamp provided the light source that bypassed the additional TIRFM mirror set and was passed through appropriate excitation (Ex)/emission (Em) filters and dichroic mirror. Microscopy data were analysed in the Nikon’s NIS Elements software.

TIRFM. TIRFM imaging of HEK cells and neurons plated on poly-d-lysine-coated dishes was performed at 37 °C using Nikon Eclipse TE-2000-U inverted microscope, with a × 60 1.49 numerical aperture (NA) objective and equipped with a multilens solid-state laser system (470, 514, and 561 nm) and appropriate filter combinations (Ex: 451 nm/Em: 457 nm, TagRFP; Ex: 561 nm/Em: 584 nm and CFP: Ex: 445 nm/Em: 475 nm), similar to as previously described57. TIRFM was achieved via the ‘through-the-objective’ laser guidance method with the laser incident angle set to 76°, which is greater than the critical angle of 62° and generated an evanescent field depth between 66 and 72 nm depending on the wavelength and temperature. Images were captured with an objective heater (20/20 Technology Inc.). Image exposure time was coupled with laser excitation duration at 200–300 ms, and laser intensity was maintained at 40–60% of maximum intensity, but neither changed throughout the course of a given experiment. Images were detected digitally using an attached CoolSNAP HQ2 CCD camera and acquired on a computer hard drive at 5–10 s intervals. For imaging of HEK cells, baseline images were acquired during perfusion of standard external solution before changing the solution to 100-mM KCl-based external solution (osmotically balanced) or 10 μM AMPH, prepared as described above, or throughout the entirety of being held in the whole-cell configuration. For simultaneous imaging and TIRFM, membrane-internalizing potentials (+20, −40 and −60 mV) were determined in preliminary experiments to approximate endogenous resting potential of these cells (−40 mV), to mimic the effects of 100-mM KC depolarization (+20 mV) and to oppose depolarizing effects of −20 mV with a similar magnitude of change (−60 mV). For quantification of surface fluorescence intensity of isolated HEK cells and primary culture neurons, regions of interest were created, including the TIRFM footprint of each HEK cell in its entirety or the neuron’s soma. For all image sequences, a background ROI similar to the size of a cell was placed in a region devoid of cells/fluorescence and was subtracted from the entire image and recalculated for each frame. The mean intensity (in arbitrary fluorescence units) over time was monitored and plotted/analysed as a fraction of the baseline intensity (the mean raw intensity of all frames within 30–60 s before initiation of indicated manipulation) and used for analysis. Bleaching was controlled for in two ways. The first was the inclusion of a vehicle group and/or non-patched adjacent cells for each assay and recording, because of the high variability in the change in baseline fluorescence over time ranging from −3.0 to +2.1% per min (average 0.4 ± 0.3% per min), a correction factor or rate was determined for each cell and was used to account for this change in each cell. Since the bleaching rate with the current TIRFM imaging parameters was linear, a linear fit was generated for 120 s before a solution change and was used to determine that the rate of bleaching was extrapolated over the entire 12–15 min experiment. This projected rate of change in fluorescence intensity due to bleaching was then accounted for during each experiment.

Confocal microscopy and JHC 1-064 labelling of DAT. Imaging of YFP-DAT (ex: 514 nm, em: 540/30 nm), mCherry, diRed and JHC 1-064 (all ex: 561 nm, em: 585/65 nm) was performed using the Nikon A1R confocal system mounted on a Nikon Eclipse Ti-E inverted microscope (Nikon) using a × 60.1.9 NA Plan-Apo objective (Nikon). For YFP-DAT and endosome co-localization experiments, YFP- DAT HEK cells grown on glass-bottom dishes and transfected with TagRFP-T-EEA1 or DiRed-Rab11 were treated with either 100 μM KC for 5 min, 10 μM AMPH for 1 h or with standard external solution throughout all the experiments at 37 °C. After the treatment, the dishes were placed on ice and washed with the ice-cold standard external solution, then washed two more times with PBS solution and then treated with 3% paraformaldehyde. Cells were then washed and imaged using identical imaging parameters (for example, laser power, gain, and so on) immediately in PBS. For co-localization analysis, a region of interest (ROI) was drawn over the intracellular space of each cell in the raw image and the Pearson correlation coefficient for the two channels was calculated on a cell-by-cell basis in NIS Elements (Nikon). For clarity and image display only, a single-count 3 × 3 pixel matrix smooth was applied, and intensity of all pixels was enhanced by 40%.

The fluorescent cocaine analogue, JHC 1-064, which has a high affinity for DAT, was used as previously described to selectively label membrane-resident DAT33,36,38,66. When YFP-DAT HEK cells had reached 60–80% confluency after 2–3 days or midbrain primary culture neurons had reached DIV 5, they were treated for 5 min with either 20–30 μM of the inhibitor. Following acquisition, the cold solution was removed and replaced with fresh 4 °C external solution. Immediately, the dish was placed on the stage, and cells were selected and a baseline image was acquired. Following acquisition, the cold solution was removed and replaced with either vehicle or KC-based external solution at 37 °C, and images were acquired every 5 min. Imaging parameters (for example, laser power, gain, pinhole, and so on) were identical for images of HEK cells and were used for the imaging series on the neuron. For analysis of changes in cell size) was again normalized to the intensity of the entire image to account for bleaching and was plotted as a fraction of the initial internal fluorescence in the original control 4 °C image57. For manual counts of intracellular JHC 1-064 puncta and clarity for display (Fig. 3 and Supplementary Fig. 8), all images were processed identically.

Biotinylation assay. For biotinylation assays, YFP-DAT HEK cells or parental HEK cells were plated on 24-well poly-D-lysine-coated dishes and either GEP-G1-CaMKIIζ-K24R or pTIR-Pam(Cherry)1, as previously described58. Forty-eight to ninety-six hours after plating, cells were pre-treated (30 min) with external solution (vehicle) or 80 μM Dynasore, followed by 30-min treatment with vehicle, vehicle +10 μM KN92 or vehicle +10 μM KN92. Cells were then washed and treated for 5 min with either vehicle, 10 μM AMPH, 2.5 μM PMA, iso-osmotic 100 mM KC1, 100 mM KC1 + KN92 or 100 mM KC1 + KN93. Cell-surface proteins were then biotinylated and analysed via western blot analysis as described previously59. Total protein concentrations for each sample were determined using the Pierce BCA protein assay kit (Thermo Scientific), and the resulting values were used to load equal amounts of protein for each sample when conducting SDS-PAGE. Blots of total and surface protein (Supplementary Figs 4,c and 5c for original blots) were probed with an N-terminal-targeted anti-DAT monoclonal antibody (1:1,000; mAb16 (ref. 70); a gift from Dr Roxanne Vaughan of the University of North Dakota) or an anti-transferrin receptor antibody (1:1,000; C2F2, BD Biosciences), followed by HRP-conjugates of the appropriate antibodies (GE Healthcare) or Image Studio (LI-COR). Prism5 (GraphPad) was used for statistical analysis following normalization of surface values to vehicle and determination of the vehicle variance by normalizing to AMPH.

Drug/solution application and optical stimulation. In electrophysiology, microscopy and biochemical experiments, increased KCl concentrations (100 mM) were achieved by replacing NaCl in the standard external solution or ACSF with KCl. For imaging of HEK cells, vehicle (standard external solution), the KCl-based external solution or 10 μM AMPH was applied via bath perfusion using a laminar flow insert for 35-mm dishes at a rate of 2 ml min−1. For neuronal recordings and TIRF microscopy, vehicle or KCl-based external solution was applied via pressure perfusion with a piezo (any manufacturer) connected to a pressure controller (Kent Scientific). The perfusion pipette was positioned 50 μm from the cell body. HEK293 or YFP-DAT HEK cells were exposed to either 80 μM dynasore54 (Thermo Fisher Scientific), 10 μM KN92, 10 μM KN93 or 10 μM BIM.
(all from EMD Millipore) for 25–30 min before and throughout treatment with vehicle or 100 mM KCl to maintain the respective inhibition of dynamin, CaMK and PKC throughout each imaging or biotinylation experiment as indicated. For steady-state photo-activation of eArch3.0 in cultured neurons, 590-nm light was generated from a light-emitting diode (LED) source (Thorlabs) and coupled to an optical fibre and placed at a 45 degree angle, with the tip 150–200 μm from the cell body. The output at the fibre tip (200 μm diameter) was regulated via a potentiometer on the externally-triggered LED driver and was calibrated so that the light power density at the tip was 15 mW mm−2. Changes in MP in response to eArch 3.0 activation were determined by taking the average MP over 50 ms before light onset and the last 50 ms of a 1-s light pulse.

Data analysis. All data were analysed with Microsoft Excel, IBM SPSS, Prism5 or Igor Pro. Statistical analyses used for comparison are identified in the legend, and all values are the mean ± s.e.m., unless otherwise stated.

References

1. Schultz, W. Multiple dopamine functions at different time courses. *Annu. Rev. Neurosci.* 30, 259–288 (2007).
2. Nisouloud, A. Dopamine and the regulation of cognition and attention. *Prog. Neurobiol.* 67, 53–83 (2002).
3. Koob, G. F. & Volkow, N. D. Neurocircuity of addiction. *Neuropsychopharmacology* 35, 217–238 (2010).
4. Jaber, M., Robinson, S. W., Missale, C. & Caron, M. G. Dopamine receptors and brain function. *Neuropsychopharmacology* 35, 1503–1519 (1996).
5. Mundorf, M. L., Troyer, K. P., Hochstetler, S. E., Near, J. A. & Wightman, R. M. Vesicular Ca2+(2−) participates in the catalysis of exocytosis. *J. Biol. Chem.* 275, 9136–9142 (2000).
6. Beaulieu, J.-M. & Gainetdinov, R. T. The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63, 182–217 (2011).
7. Jaber, M., Jones, S., Girod, B. & Caron, M. G. The dopamine transporter: a crucial component regulating dopamine transmition. *Mov. Disord.* 12, 629–633 (1997).
8. Sitte, H. H. et al. Carrier-mediated release, transport rates, and charge transfer induced by amphetamine, tyramine, and dopamine in mammalian cells transfected with the human dopamine transporter. *J. Neurochem.* 71, 1289–1297 (1998).
9. Kahlig, K. M. et al. Amphetamine induces dopamine efflux through a dopamine transporter channel. *Proc. Natl Acad. Sci. USA* 102, 3495–3500 (2005).
10. Khoshbouei, H., Wang, H., Lechleiter, J. D., Javitch, J. A. & Galli, A. Amphetamine-induced dopamine efflux. A voltage-sensitive and intracellular Na+ dependent mechanism. *J. Biol. Chem.* 278, 12070–12077 (2003).
11. Amara, S. G. & Sonders, M. S. Neurontransmitter transporters as molecular targets for addiction. *Drug Alcohol Depend.* 51, 87–96 (1998).
12. DeFelice, L. J. & Galli, A. Electrophysiological analysis of transporter function. *Adv Pharmacol.* 42, 186–190 (1998).
13. Sonders, M. S., Zhu, S. I., Zahnis, N. R., Kavanaugh, M. P. & Amara, S. G. Multiple ion conductances of the human dopamine transporter: the actions of dopamine and psychostimulants. *J. Neurosci.* 17, 960–974 (1997).
14. Ingram, S. L., Prasad, B. M. & Amara, S. G. Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons. *Nat. Neurosci.* 5, 971–978 (2002).
15. Schmitt, K. C. & Reith, M. E. α. Regulation of the dopamine transporter: aspects relevant to psychostimulant drugs of abuse. *Ann. N Y Acad. Sci.* 1187, 316–340 (2006).
16. Mortensen, O. V. & Amara, S. G. Dynamic regulation of the dopamine transporter. *Eur. J. Pharmacol.* 479, 159–170 (2003).
17. Pristupa, Z. B. et al. Protein kinase-mediated bidirectional trafficking and functional regulation of the human dopamine transporter. *Synapse* 30, 79–87 (1998).
18. Sorkin, A. et al. Human dopamine transporter gene expression of dopamine transporter-mediated internalization of the dopamine transporter is mediated by a clathrin-dependent mechanism. *Neurotoxicology* Mar. 1–13 (2013).
19. Grant, B. D. & Donaldson, J. G. Pathways and mechanisms of endocytic recycling, *Nat. Rev. Mol. Cell Biol.* 10, 597–608 (2009).
20. Meiergerd, S. M., Patterson, T. A. & Schenk, J. O. D2 receptors may modulate the function of the striatal transporter for dopamine: kinetic evidence from studies in vitro and in vivo. *J. Neurochem.* 61, 764–767 (1993).
21. Prasad, B. M. & Amara, S. G. The dopamine transporter in mesencephalic cultures is refractory to physiological changes in membrane voltage. *J. Neurosci.* 23, 7561–7567 (2001).
22. Kalivas, P. W. Neurobiology of cocaine addiction: implications for new pharmacotherapy. *Am. J. Addict.* 16, 71–78 (2007).
23. Vernier, P. et al. The degeneration of dopamine neurons in Parkinson’s disease: insights from embryology and evolution of the mesostriatocortical system. *Ann. N Y Acad. Sci.* 1035, 231–249 (2004).
24. Li, X. et al. Clinical and molecular characterisation of hereditary dopamine transporter deficiency syndrome: an observational cohort and experimental study. *Lancet Neurol.* 10, 54–62 (2011).
25. Meiergerd, S. M., Patterson, T. A. & Schenk, J. O. D2 receptors may modulate the function of the striatal transporter for dopamine: kinetic evidence from studies in vitro and in vivo. *J. Neurochem.* 61, 764–767 (1993).
26. Prasad, B. M. & Amara, S. G. The dopamine transporter in mesencephalic cultures is refractory to physiological changes in membrane voltage. *J. Neurosci.* 23, 7561–7567 (2001).
27. Kalivas, P. W. Neurobiology of cocaine addiction: implications for new pharmacotherapy. *Am. J. Addict.* 16, 71–78 (2007).
28. Vernier, P. et al. The degeneration of dopamine neurons in Parkinson’s disease: insights from embryology and evolution of the mesostriatocortical system. *Ann. N Y Acad. Sci.* 1035, 231–249 (2004).
29. Lodge, D. J. & Grace, A. A. Developmental pathology, dopamine, stress and ADHD. *Nat. Rev. Neurosci.* 29, 207–213 (2008).
30. Cordeiro, Q., Siqueira-Roberto, J. & Vallada, H. Association between the SLC6A3 A1343G polymorphism and schizophrenia. *Arq. Neuropsiquiatr.* 68, 716–719 (2010).
51. Madras, B. K., Miller, G. M. & Fischman, A. J. The dopamine transporter: relevance to attention deficit hyperactivity disorder (ADHD). Behav. Brain Res. 130, 57–63 (2002).
52. Hastrup, H., Karlín, A. & Javitch, J. A. Symmetrical dimer of the human dopamine transporter revealed by cross-linking Cys-306 at the extracellular end of the sixth transmembrane segment. Proc. Natl Acad. Sci. USA 98, 10055–10060 (2001).
53. Hastrup, H., Sen, N. & Javitch, J. A. The human dopamine transporter forms a tetramer in the plasma membrane: cross-linking of a cysteine in the fourth transmembrane segment is sensitive to cocaine analogs. J. Biol. Chem. 278, 45445–45458 (2003).
54. Kahlig, K. M., Javitch, J. A. & Galli, A. Amphetamine regulation of dopamine transport. Combined measurements of transporter currents and transporter imaging support the endocytosis of an active carrier. J. Biol. Chem. 279, 8966–8973 (2004).
55. Goodwin, J. S. et al. Amphetamine and methamphetamine differentially affect dopamine transporters in vitro and in vivo. J. Biol. Chem. 284, 2978–2989 (2009).
56. Eshleman, A. J., Henningsen, R. A., Neve, K. A. & Janowsky, A. Release of dopamine via the human transporter. Mol. Pharmacol. 45, 312–316 (1994).
57. Saha, K. et al. Intracellular methamphetamine prevents the dopamine-induced enhancement of neuronal firing. J. Biol. Chem. 289, 22246–22257 (2014).
58. Fog, J. U. et al. Calmodulin kinase II interacts with the dopamine transporter C terminus to regulate amphetamine-induced reverse transport. Neuron 51, 417–429 (2006).
59. Sorkina, T., Doolen, S., Galperin, E., Zahniser, N. R. & Sorkin, A. Oligomerization of dopamine transporters visualized in living cells by fluorescence resonance energy transfer microscopy. J. Biol. Chem. 278, 28274–28283 (2003).
60. Miranda, M., Wu, C. C., Sorkina, T., Korstjens, D. R. & Sorkin, A. Enhanced ubiquitylation and accelerated degradation of the dopamine transporter mediated by protein kinase C. J. Biol. Chem. 280, 35617–35624 (2005).
61. Choudhury, A. et al. Rab proteins mediate Golgi transport of caveolae-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. J. Clin. Invest. 109, 1541–1550 (2002).
62. Navaroli, D. M. et al. Rabenosyn-5 defines the fate of the transferrin receptor following clathrin-mediated endocytosis. Proc. Natl Acad. Sci. USA 109, E471–E480 (2012).
63. Shen, K. & Meyer, T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science 284, 162–166 (1999).
64. Subach, F. V. et al. Photoactivatable mCherry for high-resolution two-color fluorescence microscopy. Nat. Methods 6, 153–159 (2009).
65. Zhang, F. et al. The microbial opsins family of optogenetic tools. Cell 147, 1446–1457 (2011).
66. Rickhag, M. et al. Membrane permeable C-terminal dopamine transporter peptides attenuate amphetamine-evoked dopamine release. J. Biol. Chem. 288, 27534–27544 (2013).
67. Kahlig, K. M. et al. Regulation of dopamine transporter trafficking by intracellular amphetamine. Mol. Pharmacol. 70, 542–548 (2006).
68. Dahal, R. A. et al. Computational and biochemical docking of the irreversible cocaine analog RTI 82 directly demonstrates ligand positioning in the dopamine transporter central substrate-binding site. J. Biol. Chem. 289, 29712–29727 (2014).
69. Felts, B. et al. The two Na+ sites in the human serotonin transporter play distinct roles in the ion coupling and electrogenicity of transport. J. Biol. Chem. 289, 1825–1840 (2014).
70. Foster, J. D., Pananuosorn, B., Cervinski, M. A., Holden, H. E. & Vaughan, R. A. Dopamine transporters are dephosphorylated in striatal homogenates and in vitro by protein phosphatase 1. Brain Res. Mol. Brain Res. 110, 100–108 (2003).

Acknowledgements
We thank Dr Min Lin for assistance and direction in preparation of midbrain neuron primary culture and Sean Olson for his assistance in preparing plasmid DNA. This study was funded by NIH grant # DA026947, NS071122, OD020026 and the NIDA-IRP.

Author contributions
B.D.R. and K.S. performed TIRF and electrophysiology experiments, and B.D.R., K.S. and E.C. performed confocal microscopy experiments, with guidance from H.K., M.-F.Z. and A.H.N. Biochemical experiments were performed by D.K. and B.D.R. under guidance from H.K., K.S., and B.F. The project was initiated by H.K., B.D.R. and J.S. Together, B.D.R., K.S., and L.K.H. performed TIRF and electrophysiology experiments, and B.D.R., K.S. and L.K.H. performed electrophysiology experiments. Combined measurements of transporter currents and transporter imaging support the endocytosis of an active carrier. J. Biol. Chem. 279, 8966–8973 (2004).

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Richardson, B. D. et al. Membrane potential shapes regulation of dopamine transporter trafficking at the plasma membrane. Nat. Commun. 7:10423 doi: 10.1038/ncomms10423 (2016).