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Ack1 is a dopamine transporter endocytic brake that rescues a trafficking-dysregulated ADHD coding variant

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The dopamine (DA) transporter (DAT) facilitates high-affinity presynaptic DA reuptake that temporally and spatially constrains DA neurotransmission. Aberrant DAT function is implicated in attention-deficit/hyperactivity disorder and autism spectrum disorder. DAT is a major psychostimulant target, and psychostimulant reward strictly requires binding to DAT. DAT function is acutely modulated by dynamic membrane trafficking at the presynaptic terminal and a PKC-sensitive negative endocytic mechanism, or “endocytic brake,” controls DAT plasma membrane stability. However, the molecular basis for the DAT endocytic brake is unknown, and it is unknown whether this braking mechanism is unique to DAT or common to monoamine transporters. Here, we report that the cdc42-activated, nonreceptor tyrosine kinase, Ack1, is a DAT endocytic brake that stabilizes DAT at the plasma membrane and is released in response to PKC activation. Pharmacologic and shRNA-mediated Ack1 silencing enhanced basal DAT internalization and blocked PKC-stimulated DAT internalization, but had no effects on SERT endocytosis. Both cdc42 activation and PKC stimulation converge on Ack1 to control Ack1 activity and DAT endocytic capacity, and Ack1 inactivation is required for stimulated DAT internalization downstream of PKC activation. Moreover, constitutive Ack1 activation is sufficient to rescue the gain-of-function endocytic phenotype exhibited by the ADHD DAT coding variant, R615C. These findings reveal a unique endocytic control switch that is highly specific for DAT. Moreover, the ability to rescue the DAT(R615C) coding variant suggests that manipulating DAT trafficking mechanisms may be a potential therapeutic approach to correct DAT coding variants that exhibit trafficking dysregulation.

Dopamine (DA) is a modulatory neurotransmitter critical for locomotion and reward (1), and dopaminergic (DAergic) dysregulation is linked to multiple neuropsychiatric disorders, including Parkinson’s disease, schizophrenia, attention-deficit/hyperactivity disorder (ADHD), and autism spectrum disorder (ASD) (2, 3). Presynaptic recapture, facilitated by the high-affinity DA transporter (DAT), spatially and temporally restricts extracellular DA availability (4–6). Addictive psychostimulants that target DAT and its monoamine transporter homologs for 5HT (SERT) and NE (NET) are either competitive ligands, such as cocaine, or competitive substrates, such as amphetamine (7). Although these drugs interact with DAT, SERT, and NET with equimolar affinity, their binding to DAT is requisite for reward (8, 9). Transporter inhibitors with differential DAT, SERT, and NET specificity are widely used to treat neuropsychiatric disorders (10, 11). However, their therapeutic efficacy differs significantly among patients, consistent with the model that monoamines may differentially contribute to the pathogenesis of these disorders (10, 12). Thus, regulatory mechanisms specific to DAT, SERT, or NET may provide a novel route to develop transporter-specific therapeutics. DAT plasma membrane expression is requisite for efficacious extracellular DA removal and to replenish presynaptic DA stores (13). Indeed, DAT allelic and coding variants have been identified in a variety of neuropsychiatric disorders, including ADHD, ASD, infantile Parkinsonism, and bipolar disorder (14–20), underscoring that even subtle DAT functional changes exert impactful consequences on DAergic neurotransmission. DAT is acutely regulated by membrane trafficking, and either protein kinase C (PKC) activation or AMPH exposure rapidly depletes DAT surface expression (5, 7, 21, 22). Intriguingly, a DAT coding variant, R615C, identified in an ADHD proband, exhibits profound membrane instability due to highly accelerated basal endocytosis (16), suggesting that dysregulated DAT membrane trafficking may contribute to the etiology of DA-related disorders.

Studies from our laboratory (23) and others (24) indicate that a unique negative regulatory mechanism, or “endocytic brake,” stabilizes DAT surface expression. PKC activation releases the endocytic brake, accelerates DAT internalization, and thereby reduces DAT surface levels and function. The cellular basis of this negative regulatory mechanism is completely undefined. Moreover, it is unknown whether the endocytic brake exists in DAergic terminals and whether it is specific to DAT. Activated by cdc42 kinase 1 (Ack1) is a nonreceptor tyrosine kinase that is a major cdc42 effector activated via EGF, PDGF, and m3 muscarinic receptor stimulation (25, 26). Ack1 binds directly to clathrin heavy chain (27, 28) and is enriched in presynaptic terminals (29). Importantly, Ack1 is inactivated by PKC (26), and a recent study demonstrated that Ack1 overexpression suppresses endocytosis (30). Given these attributes, we asked whether...
Ack1 activity is the penultimate step that engages the DAT endocytic brake.

Results

Ack1 Negatively Regulates DAT, but Not SERT Endocytosis. Ack1 and its active, autophosphorylated form, pY284-Ack1 (pAck1) (25, 31), were readily detected in both the DAergic cell line SK-N-MC and mouse striatum (Fig. S1A and B). PKC activation significantly decreased pAck1 in both SK-N-MC cells (46.5 ± 3.0% control levels; Fig. S1A) and mouse striatum (78.3 ± 5.2% control levels; Fig. S1B). Likewise, the highly specific Ack1 inhibitor AIM-100 (32) dose-dependently decreased pAck1 in SK-N-MC cells (Fig. S1C) and dramatically decreased mouse striatal pAck1 to 13.2 ± 2.2% control levels (Fig. S1D). Thus, Ack1 is expressed in DAergic cell lines and striatum, and either PKC activation or AIM-100 inactivates Ack1 in both these model systems.

We predicted that if Ack1 imposes the DAT endocytic brake, then Ack1 inactivation would release the brake and decrease both DAT function and surface expression. Indeed, AIM-100 significantly decreased [3H]DA uptake in SK-N-MC cells (IC50 = 50.2 ± 9.9 μM) and striatal slices (Fig. 1 A and B) and significantly reduced DAT surface levels to 72.5 ± 6.4% control levels in mouse striatum (Fig. 1C). DAT surface loss in response to AIM-100 was due to a significant increase in the DAT internalization rate, to 192.9 ± 28.6% control levels (Fig. 1D), demonstrating that Ack1 negatively regulates DAT endocytosis. AIM-100 effects were specific to DAT and had no effect on the SERT endocytic rate measured in SERT-SK-N-MC cells (Fig. 1D; P = 0.89). Interestingly, high AIM-100 concentrations (>20 μM) inhibited DAT function to a much larger degree than what could be attributed to membrane trafficking. DAT loss of function was not due to transmembrane Na+ gradient disruption, as AIM-100 had no effect on Na+-dependent alanine uptake (Fig. S2A). To our surprise, AIM-100 also dose-dependently inhibited SERT function (Fig. S2B), despite exerting no effect on SERT trafficking (Fig. 1D). We noted that AIM-100 bears DAT and SERT pharmacophore properties similar to piperazine derivatives, such as GBR12909 (Fig. S2C). We therefore hypothesized that, in addition to its known function as a high-affinity Ack1 inhibitor, AIM-100 may also be a low-affinity, competitive DAT and SERT inhibitor. Whole cell binding studies revealed that AIM-100 competitively inhibited DAT and SERT binding to [3H]WIN 35428 and [3H]nimipramine, respectively (Fig. S2D), supporting the premise that AIM-100 is a DAT and SERT inhibitor. However, GBR12909 had no effect on pAck1 levels (Fig. S2E), indicating that DAT ligand binding does not globally inactivate Ack1. Moreover, a 10-fold lower AIM-100 concentration that efficaciously decreased p284-Ack1 levels (2 μM; Fig. S1C), also significantly increased DAT internalization rates (Fig. S2F). Thus, distinct endocytic mechanisms regulate DAT and SERT, and Ack1 activity is required to impose the DAT endocytic brake. Moreover, AIM-100 is, coincidentally, a low-affinity, competitive DAT and SERT inhibitor.

Constitutive and Regulated DAT Endocytosis Are Differentially Dependent on Clathrin. Ack1 is recruited to clathrin-coated pits via clathrin heavy chain interactions (27, 28). Thus, we hypothesized that clathrin is required to release the Ack1-imposed brake. To test clathrin-dependence, we acutely inhibited clathrin with pitstop2 and measured DAT internalization ±AIM-100 and ±PMA. Pitstop2 pretreatment significantly attenuated both AIM-100– and PKC-stimulated DAT internalization, but had no effect on basal DAT endocytosis (Fig. 2 A and B), suggesting that stimulated DAT endocytosis is clathrin-dependent, whereas constitutive DAT endocytosis is clathrin-independent. We further used total internal resonance fluorescence microscopy (TIRFM) to examine clathrin and surface DAT under basal conditions, compared with transferrin receptor (TIR), a protein known to undergo robust clathrin-mediated endocytosis. Alexa 594-Tf colocalized markedly with eGFP-clathrin across the plasma membrane, and distinct Tf/clathrin puncta moved away from the TIR field during imaging, consistent with clathrin-mediated endocytosis (Fig. 2C). In contrast, TagRFP-T-DAT was diffusely distributed across the plasma membrane and was enriched in cellular microspikes, with little apparent clathrin colocalization (Fig. 2C). Taken together with the pitstop2 data, these data support that constitutive DAT endocytosis is clathrin-independent, whereas stimulated DAT endocytosis requires clathrin.

Cdc42 Negatively Regulates DAT, but Not SERT, Endocytosis. Ack1 is a major cdc42 effector, suggesting that cdc42 may contribute to the DAT endocytic brake, upstream of Ack1. To test this possibility, we measured DAT surface levels in DAT SK-N-MC cells and striatal DAergic terminals following acute treatment with two structurally distinct cdc42 inhibitors, casin and pirl1. Both casin and pirl1 significantly reduced DAT surface levels in SK-N-MC cells (Fig. 3 A and B), and casin significantly decreased surface DAT in mouse striatum (Fig. 3C). DAT surface loss in response to cdc42 inhibition was due to profound DAT endocytic acceleration (238.0 ± 15.5% control levels, Fig. 3D). In contrast, pirl1 did not significantly affect SERT internalization (Fig. S3A). We further tested whether PKC and cdc42 impact DAT surface stability in independent or convergent manners. Pretreatment ±casin

Fig. 1. Ack1 activity stabilizes DAT at the plasma membrane. (A) [3H]DA uptake. DAT SK-N-MC cells were treated with the indicated AIM-100 concentrations for 30 min at 37 °C, and [3H]DA uptake was measured as described in SI Methods. Data are expressed as percent specific DA uptake ± SEM (n = 12). (B) Ex vivo slice uptake. Striatal slices were treated with 20 μM AIM-100, 60 min, 37 °C and [3H]DA uptake was assessed as described in SI Methods. *P < 0.05, Student’s t test, n = 6 hemislices obtained from two independent mice. (C) Ex vivo slice biotinylation. Striatal slices were treated with 20 μM AIM-100 for 30 min at 37 °C, and surface proteins were isolated by biotinylation as described in SI Methods. (C, Upper) Representative immunoblots. (C, Lower) Average DAT surface levels expressed as percent vehicle-treated levels ± SEM. **P < 0.01, Student’s t test, n = 3. (D) Internalization assays. DAT and SERT internalization rates were measured in SK-N-MC cells ±20 μM AIM-100 as described in SI Methods. (D, Upper) Representative immunoblots showing the total DAT and SERT surface pools at t = 0 (T), strip control (S), and internalized protein during either vehicle (V) or AIM-100 (A) treatments. (D, Lower) Average internalization rates expressed as percent vehicle-treated ± SEM. *P < 0.02, Student’s t test; n = 5 (DAT); n = 3 (SERT).
We next used two efficacious hAck1-targeted shRNAs, 10 and 12 (Fig. S4), to test whether Ack1 is required to (i) engage the DAT endocytic brake and (ii) stimulate DAT endocytosis by PKC activation or cdc42 inhibition. The most efficacious hAck1 shRNA, 10, significantly increased basal DAT endocytosis to 138.7 ± 12.3% control levels (Fig. 4B), consistent with Ack1’s requisite role as the DAT endocytic brake. Moreover, Ack1 depletion with either shRNA 10 or 12 significantly attenuated stimulated DAT endocytosis, either via PKC stimulation (Fig. 4C) or cdc42 inhibition (Fig. 4D). In sum, these results support that Ack1 is required to engage the DAT endocytic brake.

Although perturbing Ack1 enhanced DAT endocytosis, we next asked whether there is a direct causal link between Ack1 inactivation and either cdc42 inhibition or PKC activation to release the DAT endocytic brake. To test this, we coexpressed DAT with either wild-type, constitutively active (S445P), or kinase dead (K158A) Ack1 isoforms (33) (see Fig. S4B for Ack1 mutant overexpression profiles). We predicted that if Ack1 inactivation were required to release the DAT endocytic brake, then S445P-Ack1 would block accelerated DAT internalization in response to either PKC activation or cdc42 inhibition. Wild-type Ack1 overexpression had no effect on basal or accelerated DAT endocytosis in response to PKC activation or cdc42 inhibition (Fig. 5 B–D). In
contrast, S445P-Ack1 significantly attenuated both PKC-stimulated (Fig. 5C) and pirl1-stimulated (Fig. 5D) DAT internalization. K158A-Ack1 had no significant effect either basal ($P = 0.30$) or pirl1-stimulated ($P = 0.30$) DAT internalization (Fig. 5 B and D), but significantly inhibited PKC-stimulated DAT endocytosis (100.1 ± 5.2% control level, Fig. 5C). Although the K158A mutant lacks kinase activity (34), it was unknown, a priori, whether this mutant would exert a dominant negative effect. Ack1 activation is required for targeting to clathrin-coated pits (30). Thus, it is not surprising the kinase dead mutant failed to exert a dominant effect on DAT internalization. Taken together, these results provide a causal link between upstream PKC or cdc42 stimuli and Ack1 inactivation as requisite steps in releasing the DAT endocytic brake.

**Ack1 Activity Restores Normal Trafficking to a DAT Coding Variant in an ADHD Proband.** A recent study reported that a DAT coding variant, R615C, identified in an ADHD proband, lacks endocytic braking, resulting in enhanced basal endocytosis and inability to undergo PKC- and AMPH-stimulated endocytosis (16). We asked whether constitutive Ack1 activation could restore the endocytic brake and thereby rescue the DAT(R615C) gain-of-function endocytic phenotype. DAT(R615C) expressed in SK-N-MC cells internalized significantly faster than wild-type DAT (Fig. 5 E and F) and was defective in PKC-stimulated endocytosis (Fig. S3B), consistent with the previous report (16). Remarkably, S445P-Ack1 significantly decreased DAT(R615C) basal endocytosis to wild-type DAT levels (Fig. 5D), but did not restore PKC-stimulated endocytosis (Fig. S3B).

**Discussion**

Reuptake inhibitors are used to treat a variety of neuropsychiatric disorders, including depression, obsessive-compulsive disorder, and ADHD (10, 35). These agents are differential selective for SERT, NET, and DAT, and their clinical efficacy varies considerably across the population (10, 12). Transporter-specific cellular regulation has the potential to lead to novel and selective therapeutic approaches that manipulate transporters intrinsically, rather than systemically.
Fig. 6. Model for a PKC-sensitive, Ack1-mediated DAT endocytic brake. Under basal conditions, the cdc42-activated Ack1 pool imposes an endocytic brake upon the plasma membrane DAT population, permitting slow, clathrin- and dynamin-independent DAT endocytosis. PKC activation inactivates Ack1 and releases the DAT endocytic brake, facilitating rapid, clathrin- and dynamin-dependent DAT internalization and intracellular sequestration.

7. Torres GE, Ginetdinov RR, Caron MG (2003) Plasma membrane monoamine trans-
8. Thomsen M, Han DD, Gu HH, Caine SB (2009) Lack of cocaine self-administration in
mice expressing a cocaine-insensitive dopamine transporter. J Pharmacol Exp Ther
331(1):204-211.
porters: Molecular function of important drug targets. Trends Pharmacol Sci 27(7):
375-383.
human brain is associated with bipolar disorder. Neuropsychopharmacology 36(8):
1644-1655.
15. Maez-Robinson MS, et al. (2008) Anomalous dopamine release associated with a hu-
tation in the dopamine transporter gene associates dopamine dysfunction with au-
some spectrum disorder. Mol Psychiatry 18(12):1315-1323.
18. Bowton E, et al. (2014) SLCA3 coding variant Ala559Val found in two autism pro-
bands alters dopamine transporter function and trafficking. Transl Psychiatry 4:e464.
daughter albinism and ADHD. J Clin Invest 127(10):3117-3120.
20. Mergy MA, et al. (2014) The rare DAT coding variant Val559 perturbs DA neuron func-
tion, changes behavior, and alters in vivo responses to psychostimulants. Proc Natl Acad Sci USA 111(44):E4779-E4788.
endocytotic determinants: Carboxy terminal residues critical for basal and PKC-stimu-
25. Linseman DA, Heidenreich KA, Fisher SK (2001) Stimulation of M3 muscarinic recep-
tors induces phosphorylation of the Cdc42 effector activated Cdc42Hs-associated ki-

27. Thomsen M, Han DD, Gu HH, Caine SB (2009) Lack of cocaine self-administration in
mice expressing a cocaine-insensitive dopamine transporter. J Pharmacol Exp Ther
331(1):204-211.
porters: Molecular function of important drug targets. Trends Pharmacol Sci 27(7):
375-383.
33. Pinsoneault JK, et al. (2011) Dopamine transporter gene variant affecting expression in
human brain is associated with bipolar disorder. Neuropsychopharmacology 36(8):
1644-1655.
34. Maez-Robinson MS, et al. (2008) Anomalous dopamine release associated with a hu-
porters: Molecular function of important drug targets. Trends Pharmacol Sci 27(7):
375-383.
40. PINSONEAULT JK, et al. (2011) Dopamine transporter gene variant affecting expression in
human brain is associated with bipolar disorder. Neuropsychopharmacology 36(8):
1644-1655.
41. Maez-Robinson MS, et al. (2008) Anomalous dopamine release associated with a hu-
porters: Molecular function of important drug targets. Trends Pharmacol Sci 27(7):
375-383.
45. Tamminga CA, et al. (2002) Developing novel treatments for mood disorders: Accel-
47. PINSONEAULT JK, et al. (2011) Dopamine transporter gene variant affecting expression in
human brain is associated with bipolar disorder. Neuropsychopharmacology 36(8):
1644-1655.