Clustered Kv2.1 decreases dopamine transporter activity and internalization

Joseph J. Lebowitz1,2, Jose A. Pino3, Phillip M. Mackie1, Min Lin1, Cheyenne Hurst1, Keeley Divita1, Anthony T. Collins1, Dimitri N. Koutzoumis3, Gonzalo E. Torres3, Habibeh Khoshbouei1,2*

From the 1 Department of Neuroscience, College of Medicine, University of Florida, Gainesville FL, 32610 USA; 2 T32 in Movement Disorders and Neurorestoration, Fixel Center for Neurological Diseases, UF Health, Gainesville FL, 32610 USA; 3 Department of Pharmacology and Experimental Therapeutics, College of Medicine, University of Florida, Gainesville FL, 32610 USA

Running title: Kv2.1 clusters downregulate DAT function

*To whom correspondence should be addressed: Habibeh Khoshbouei: Department of Neuroscience, University of Florida College of Medicine, Habibeh@ufl.edu

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Abstract

The dopamine transporter (DAT) regulates dopamine neurotransmission via reuptake of dopamine released into the extracellular space. Interactions with partner proteins alter DAT function and thereby dynamically shape dopaminergic tone important for normal brain function. However, the extent and nature of these interactions are incompletely understood. Here, we describe a novel physical and functional interaction between DAT and the voltage-gated K+ channel Kv2.1 (potassium voltage-gated channel subfamily B member 1 or KCNB1). To examine the functional consequences of this interaction, we employed a combination of immunohistochemistry, immunofluorescence live-cell microscopy, co-immunoprecipitation, and electrophysiological approaches. Consistent with previous reports, we found Kv2.1 is trafficked to membrane-bound clusters observed both in vivo and in vitro in rodent dopamine neurons. Our data provide evidence that clustered Kv2.1 channels decrease DAT’s lateral mobility and inhibit its internalization, while also decreasing canonical transporter activity by altering DAT’s conformational equilibrium. These results suggest that Kv2.1 clusters exert a spatially discrete homeostatic braking mechanism on DAT by inducing a relative increase in inward-facing transporters. Given recent reports of Kv2.1 dysregulation in neurological disorders, it is possible that alterations in the functional interaction between DAT and Kv2.1 affect dopamine neuron activity.

The dopamine transporter (DAT) reuptakes released dopamine from the extracellular space following an action
potential which terminates the signal and recycles the monoamine (1). Mutations in DAT have been linked to early onset Parkinsonisms in humans, and DAT knockout mice exhibit severely reduced striatal dopamine levels (2–4). Importantly, activation of DAT increases the excitability of dopamine neurons via an inward depolarizing Na⁺ current while also increasing intracellular Ca²⁺ mobilization (5,6). Dopamine neurons have conserved mechanisms to curtail these DAT-mediated responses including transporter internalization and its membrane microdomain redistribution (7–9). Additionally, functional interactions between DAT and its partner proteins have been shown to influence many facets of dopamine neuron physiology, including: neurotransmitter release and reuptake, modulation of excitability, and responses to cellular stress (6,10,11). In the current study, we examine the physical and functional interaction between DAT and Kv2.1, a voltage gated K+ channel critical to the regulation of neuronal excitability and membrane microdomain organization (12–14).

Kv2.1 is a slowly deactivating K⁺ channel that has been extensively studied as a regulator of neuronal excitability and neuroprotective target in hippocampal and cortical neurons (12,15). The activity of Kv2.1 repolarizes neurons following an action potential, and its activation is enhanced in response to cellular stress as a putative homeostatic braking mechanism to decrease neuronal excitability (16). In dopamine neurons, inhibition of Kv2.1 has been shown to increase spontaneous firing activity while also increasing the half-width of action potentials (17). Separate from Kv2.1’s channel activity, Kv2.1 clusters facilitate vesicular release in neuroendocrine cells via an interaction with syntaxin (18). Interestingly, Kv2.1 has been shown to undergo oxidation in the human brain during aging and neurodegenerative disease, leading to non-functional channels distinct from native clusters of non-oxidized channels (19,20). This oxidation of Kv2.1 has been linked to a dysregulation of Ca²⁺ signaling in hippocampal neurons in a rodent model of neurodegenerative pathology (20).

We and others have shown Kv2.1 is a partner protein of DAT as measured by coimmunoprecipitation of the proteins in brain tissue and in heterologous expression systems (21,22). Kv2.1 is one of only two K⁺ channels – in addition to Kv4.3M – shown to interact with DAT via proteomics analysis, despite an extensive variety of expressed K⁺ channels in dopamine neurons (21,23,24). While it is possible that both channels may regulate DAT via a functional interaction, the current study is restricted to the interaction of DAT and Kv2.1. Herein, we report that there is a dynamic functional interaction between Kv2.1 and DAT that can regulate DAT activity and thus dopamine transmission.

**Results**

**Kv2.1 clusters exist at the soma and proximal processes of midbrain dopamine neurons**

Previous reports have shown that Kv2.1 is trafficked into micron-sized clusters along the soma and axon initial segment of cortical and hippocampal neurons (25–27). The clustered channels are hypothesized to serve a nonconducting role, with channels not localized to clusters conducting the signature delayed-rectifier current (28,29). To examine the
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expression and localization of Kv2.1 in dopamine neurons, we utilized double immunofluorescence labeling and 3D confocal microscopy in cultured dopamine neurons (fig 1A) as well as mouse (fig 1B) and rat (fig 1C) brain slices containing the substantia nigra pars compacta. Consistent with previous reports, DAT is expressed throughout the soma and proximal processes of dopamine neurons (fig 1A-C leftmost panels, green in merge). Similar to DAT, Kv2.1 is expressed at the soma and along the proximal processes of dopamine neurons (fig 1A-C middle panels, red in merge). In contrast to DAT's uniform expression pattern, Kv2.1 labeling was localized to discrete clusters, observable as bright red puncta (fig 1A-C). These data confirm the co-expression of Kv2.1 and DAT in dopamine neurons, and indirectly support the previously published association of Kv2.1 and DAT in mouse striatal tissue (21).

Kv2.1 and DAT associate in vivo
Because the previously published co-immunoprecipitation of Kv2.1 and DAT was conducted in mouse tissue, we examined whether an association between endogenous Kv2.1 and DAT can be detected in rat striatal tissue. To this end, we immunoprecipitated DAT and probed for DAT and Kv2.1 in Western blot experiments (supplemental fig 1, antibody information in table 3). Both DAT and Kv2.1 were present in the total input before DAT immunoprecipitation (supplemental fig 1, second and fourth blots). DAT was detected when pulldown using a specific antibody, but no band was detected with a nonspecific IgG control, confirming the specificity of the immunoprecipitation assay (supplemental fig. 1, first blot). Kv2.1 was also detected following DAT immunoprecipitation confirming that the endogenous proteins associate in vivo in rat striatal tissue (supplemental fig 1, third blot). To determine if the interaction between Kv2.1 is dynamically regulated, we next repeated coimmunoprecipitation experiments in a heterologous system with or without activation of DAT.

DAT activation increases its association with Kv2.1
Previous studies from our group and others have shown the cell surface distribution of DAT is regulated by multiple mechanisms including substrate activation of DAT, changes in membrane potential, DAT interaction with its partner proteins, and intracellular kinases (9,10,30,31). To determine whether DAT's association with Kv2.1 was dynamically regulated, we used coimmunoprecipitation with or without pharmacological activation of DAT in overexpressing HEK-293 cells. In the absence of pharmacological stimulation of DAT, Kv2.1 and DAT coimmunoprecipitated (fig 2A). Methamphetamine (METH) activation of DAT (10 µM, 5 min incubation) significantly increased the amount of Kv2.1 coimmunoprecipitated with DAT, suggesting that the association between the two proteins is dynamically regulated by DAT activity (fig 2B, control: 0.2124 ± 0.0416; METH: 0.4821 ± 0.0896; p = 0.0342; unpaired t test; n = 4 independent experiments for each condition). These coimmunoprecipitation data support the hypothesis that there is a functional interaction between Kv2.1 and DAT, however this can be either through a direct interaction and/or via a larger protein complex. To address these possibilities, we next measured the proximity between Kv2.1 and DAT using live cell Förster resonance energy transfer (FRET)
microscopy with and without DAT activation.

**DAT activation directly increases its proximity to Kv2.1 within clusters**

The increased amount of Kv2.1 coimmunoprecipitated with DAT and Kv2.1 supports two potential possibilities: either DAT activation increases its interaction with a protein complex containing Kv2.1 and/or DAT activation increases its interaction with Kv2.1 channels directly. To investigate which of these possibilities underlies the observed increase in DAT and Kv2.1 coimmunoprecipitation, we employed live-cell acceptor photobleaching FRET in cells coexpressing RFP-DAT and GFP-Kv2.1 (fig 2) (32,33). FRET microscopy is based on the transfer of energy between a pair of chromophores (FRET pairs) to estimate the proximity of two fluorescently tagged proteins up to 100 Å (34). All imaging was carried out at a single confocal optical plane near the basal membrane where GFP-Kv2.1 clusters were readily identifiable (fig 3 B). FRET was observed in basal conditions between RFP-DAT and GFP-Kv2.1 (fig 3D, VEH = 8.407 ± 0.552 % n = 116 cells from 10 independent experiments), suggesting these two proteins natively exist within 100 Å of each other. To confirm the specificity of the observed FRET, we repeated the experiment using GFP-Kv2.1 and an intracellular protein (RFP-tagged early endosome antigen 1, RFP-EEA1) (9,35). No FRET was observed between GFP-Kv2.1 and RFP-EEA1 and this was significantly different from the FRET signal measured in each experimental group (fig 3D, p< 0.0001 between RFP-EEA1/GFP-Kv2.1 and all experimental groups n=32 cells from 4 independent experiments; Tukey’s test following one-way ANOVA).

Next, we examined the FRET efficiency between DAT and Kv2.1 following DAT activation. FRET efficiency between RFP-DAT and wt-GFP-Kv2.1 increased ~41% following METH-stimulation of DAT activity when compared to control conditions (fig 3D, METH = 11.88 ± 0.710%; *** p = 0.0006, n = 101 cells from 9 independent experiments, Tukey’s test following one-way ANOVA). Similarly, DAT activation with the endogenous substrate dopamine (1 µM) yielded ~36% increase in the FRET efficiency between RFP-DAT and wt-GFP-Kv2.1 (fig 3D, DA = 11.46 ± 0.953 %, p < 0.0001, n = 61 cells from 7 independent experiments, Tukey’s test following one-way ANOVA). To confirm that this effect was dependent on DAT activation, we repeated this experiment after pretreatment with the DAT blocker nomifensine (10 µM, supplemental fig 2B). When the transporter was blocked, METH did not increase the FRET between the two proteins (NOM/METH = 4.208 ± 0.767%; ns change from control; n= 51 cells (control) and 62 cells (NOM/METH) from 4 independent experiments each, Tukey’s test following one-way ANOVA).

As shown in supplemental figure 2, the increase in proximity between Kv2.1 and DAT was observed irrespective of FRET pair used. These data suggest DAT activation in and of itself directly increases the proximity between Kv2.1 and DAT.

Neuronal activity or cellular stress can induce a loss of Kv2.1 clusters, freeing channels to diffuse throughout the membrane (36,37). Therefore, to examine whether or not Kv2.1 clustering regulates its association with the transporter, we repeated the FRET experiments with the non-clustering s586a mutant Kv2.1 (GFP-s586a-Kv2.1) (38). Unlike what we observed with the wild-type GFP-Kv2.1, activation of the transporter did not increase the FRET efficiency between RFP-DAT and GFP-s586a-Kv2.1,
suggesting that the native localization pattern of Kv2.1 is necessary for its dynamic interaction with DAT (fig 3D, S586A METH = 13.340 ± 0.672%; ns vs GFP-s586a-Kv2.1 vehicle n = 86 and 83 cells for METH and vehicle respectively, each from 6 independent experiments, Tukey’s test following one-way ANOVA). Taken together, these data suggest that the proximity of DAT and Kv2.1 is dynamically regulated by DAT activation, and this regulation is sensitive to the state of Kv2.1 clustering.

**METH-stimulated DAT internalization is inhibited at sites of Kv2.1 clusters**

Next, we examined the functional consequences of increased proximity between Kv2.1 and DAT in cells expressing YFP-DAT and a new mCerulean3-Kv2.1 (mCer3-Kv2.1) generated for this study. Unlike cells expressing YFP-DAT alone, where the transporter molecules are homogenously distributed (6,9,39,7), live cell total internal reflection microscopy (TIRFM) in Kv2.1/DAT cells revealed DAT molecules accumulated in areas of the membrane containing Kv2.1 clusters (fig 4A, bottom left panel, white arrows in inset). This observation effectively segmented DAT at the plasma-membrane into two populations: the Kv2.1 cluster-associated DAT (named as such) and the DAT molecules in the same cell that were not in a region containing a Kv2.1 cluster (non-Kv2.1 cluster associated DAT). To examine whether or not these two DAT populations exhibited unique internalization behavior following activation with METH, dual color live cell TIRFM was utilized to determine Kv2.1 regulation of DAT internalization. Consistent with previous results, YFP-DAT was rapidly internalized following METH-stimulation of DAT activity in cells not expressing mCer3-Kv2.1 (fig 4B, Non-Kv2.1 expressing cell DAT, mean difference: 0.0632 ± 0.025, p= 0.0414, Sidak’s multiple comparisons test following two-way ANOVA). This effect was nearly identical when examining the population of DAT in mCer3-kv2.1 expressing cells not associated with Kv2.1 clusters (fig 4B, Non-Kv2.1 cluster associated DAT, mean difference: 0.0683 ± 0.020, p= 0.0037, Sidak’s multiple comparisons test following two-way ANOVA). Conversely, the population of DAT associated with Kv2.1 clusters proved resistant to internalization and no loss of YFP signal was observed in these regions (fig 4B, Kv2.1 cluster associated DAT, mean difference: -0.0104 ± 0.021, ns, Sidak’s multiple comparisons test following one-way ANOVA). In control experiments, no appreciable DAT internalization was observed following vehicle perfusion (fig 4C, inset). These data suggest Kv2.1 clusters either retain DAT at the membrane by limiting the mobility of the protein, or the DAT molecules are actively trafficked to sites of Kv2.1 clusters following activation.

**Kv2.1 cluster association limits lateral mobility of DAT**

To determine if Kv2.1 clusters retained DAT molecules once associated, live cell fluorescence recovery after photobleaching (FRAP) imaging of RFP-DAT was conducted in the presence of wild-type GFP-Kv2.1 or the GFP-s586a-Kv2.1 mutant and compared to cells expressing RFP-DAT alone (fig 5). FRAP measures lateral membrane mobility of DAT (11,30). Briefly, a region of interest was drawn to selectively bleach DAT molecules in a discrete area of the basal membrane. Re-emergence of RFP signal in the photo-bleached area is indicative of the diffusion of RFP-DAT molecules from unbleached regions of the cell (fig 5A). The amount of signal that is not recovered
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Following photobleaching represents the immobile fraction of DAT (fig 5B). Comparing the immobile fractions across experimental groups revealed a significant increase in the amount of immobile DAT when wild-type GFP-Kv2.1 was present compared with DAT in cells not expressing Kv2.1 (fig 5C, GFP-Kv2.1: 53.69% ± 3.36% n = 9 cells from 7 independent experiments; Non-Kv2.1 expressing cells: 30.72% ± 2.91% n = 17 cells from 8 independent experiments; p = 0.0003, Tukey’s test following one-way ANOVA). In the presence of GFP-s586a-Kv2.1, the measured immobile fraction was nearly identical to that seen in cells not expressing Kv2.1 (GFP-s586a-Kv2.1: 29.44% ± 3.11%, ns vs. non-Kv2.1 expressing cells, p < 0.0001 vs. GFP-Kv2.1; n = 22 cells from 7 independent experiments, Tukey’s test following one-way ANOVA). Taken together, these data suggest decreased DAT internalization at sites of Kv2.1 clusters is likely due to an overall decrease in the membrane mobility of DAT. Additionally, and in agreement with our observed FRET data, the reduction in the lateral mobility of DAT was dependent upon the presence of clustered Kv2.1 channels at the membrane as indicated by the loss of effect with the non-clustering s586a-Kv2.1 mutant channel.

**Kv2.1 expression attenuates DAT-mediated inward current and uptake**

The data presented thus far support the interpretation that the DAT molecules are associated with Kv2.1 clusters at the surface membrane, and these Kv2.1 clusters decrease METH-induced internalization and membrane mobility of DAT. Therefore, we hypothesized the membrane retention of DAT by Kv2.1 clusters is coupled to concomitant changes in DAT-mediated forward transport to prevent runaway transporter activity. Forward transport of substrate through DAT is coupled to an inward current mediated by Na⁺ ions. Substrate activation of DAT allows the inward current associated with forward transport to be measured via whole-cell patch clamp electrophysiology. As reported previously, steady-state DAT currents are measured as the final 100ms of each given voltage step (40,9,41). Therefore, we first tested whether Kv2.1 coexpression influenced DAT-mediated inward current.

In cells expressing Kv2.1 there was a reduction in the nomifensine-sensitive, DAT-mediated inward current at the tested membrane potential (fig 6B, DAT only: -25.77 pA +/- 5.03 pA n=10 cells; DAT/Kv2.1: -12.38 +/- 2.62 pA n=8 cells; p =.0405, two-tailed unpaired t test). In addition, we noticed an apparent voltage-dependent decrease in the transient currents. Because Kahligh et. al. have shown changes in transient currents correlate with surface DAT expression, we tested the hypothesis that Kv2.1 coexpression reduces surface DAT levels (41). To test this hypothesis, we compared DAT levels at the plasma membrane in cells with or without Kv2.1. Biotinylation experiments revealed no difference in surface DAT levels when comparing cells with or without Kv2.1 (DAT only: 3.078 ± 0.299; Kv2.1/DAT: 2.715 ± 0.470, ns difference, two-tailed unpaired t test). These data suggest that retained DAT at sites of Kv2.1 clusters is hypofunctional, a putative homeostatic mechanism to counteract the loss of transporter internalization.

This downregulation of DAT-mediated forward-transport by Kv2.1 was further confirmed using IDT307 (APP+), a fluorescent substrate of monoamine transporters (fig 7B) (42). Similar to the measured decreased in the DAT-mediated inward current, Kv2.1 expression
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significantly decreased IDT307 uptake through DAT (fig 7C, DAT only: 3.551 ±
0.224 fold baseline, n = 33 cells from 7 independent experiments; Kv2.1/DAT:
1.880 ± 0.174 fold baseline, n= 20 cells from 7 independent experiments; p <
0.0001, Tukey’s test following one-way ANOVA). Pre-incubation with nomifensine,
(DAT blocker, 10 µM) prevented IDT307 uptake, confirming the uptake is via DAT
(NOMI: 1.202 ± 0.045 fold baseline, n= 37 cells from 6 independent experiments; p <
0.0001 vs. DAT only; p = 0.0182 vs. Kv2.1/DAT, Tukey’s test following one-way
ANOVA). One potential explanation for a downregulation of forward-transport with
no concomitant loss of available transporters at the membrane is an alteration of the conformational equilibrium of DAT by Kv2.1.

Kv2.1 attenuates DAT function via an alteration of transporter conformational equilibrium

The current working model of dopamine transport states that DAT begins
the transport cycle in on “outward facing” conformation, exposing substrate binding
domains to the extracellular space; upon substrate binding, DAT shunts to an
“inward-facing” conformation to expose the substrate binding domains to the
intracellular space and release the bound molecules into the cell (1). However,
substrate-independent factors such as membrane microdomain composition and protein-protein interactions also impact the proportion of transporters in the outward-
vs. inward-facing conformation at any given time (8,43). To determine the influence of Kv2.1 on DAT’s conformational equilibrium, we employed a novel assay using the fluorescent cocaine analog JHC1-064 (44,45).

DAT blockers, including cocaine and its fluorescent analog JHC1-064 bind DAT molecules that exist in an outward-facing conformation (44,46). To determine the relative levels of inward vs. outward facing DAT, cells expressing YFP-DAT, or cells coexpressing YFP-DAT/mCer3-
Kv2.1 were incubated with JHC1-064, and the ratio of YFP-DAT signal to JHC1-064 was calculated at the stacked membrane region of the cell (fig 8A, C-D). Comparing
the ratio between cells coexpressing DAT & Kv2.1 with those expressing DAT alone revealed a significant increase in the ratio of YFP-DAT signal to JHC1-064 signal (fig
8B, DAT only: 1.527 ± 0.059 n = 44 cells from 5 independent experiments;
Kv2.1/DAT: 1.977 ± 0.113 n = 35 cells from 5 independent experiments; p = 0.0004,
two-tailed unpaired t test) suggesting a relative increase in the level of inwardly-facing transporters in DAT/Kv2.1 cells (fig
8A). Because modifications that shift DAT’s conformational equilibrium towards
more inwardly-facing transporters have been shown to decrease substrate uptake
through the transporter, it is plausible that Kv2.1’s alteration of conformational equilibrium toward more inwardly-facing transporters is responsible for the reduced DAT-mediated inward current and uptake (47).

Discussion

We have determined the extent to which Kv2.1 functionally interacts with DAT
to regulate transporter internalization and its activity, and the degree by which this process is dependent upon clustering of Kv2.1. Using immunofluorescent confocal
microscopy, we confirmed the expression and canonical clustered localization of
Kv2.1 in midbrain dopamine neurons. Co-
immunoprecipitation and live-cell FRET microscopy revealed the interaction
between Kv2.1 and DAT is dynamically regulated, where DAT activation increased the proximity of the two proteins. However,
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no such regulation occurred between DAT and a non-clustering Kv2.1 mutant, supporting the interpretation that Kv2.1 clusters act as discrete sites of DAT regulation. We also found that Kv2.1 cluster association decreased the lateral mobility and the canonical cell surface redistribution of DAT. Functionally, coexpression of Kv2.1 reduced DAT-mediated inward current and substrate uptake, and this was due to a shift in DAT’s conformational equilibrium towards more inward-facing transporters. Our data suggest Kv2.1 clusters may act as an endogenous downregulator of DAT activity specifically at sites of clustered Kv2.1 channels.

Super-resolution microscopy indicates DAT distribution in the soma and axon–initial-segment of dopamine neurons is irregular and this functional organization of DAT is affected by neuronal activity (48). Similarly, Kv2.1 is asymmetrically targeted to the axon-initial-segment, a neuronal compartment adjacent to the soma that is involved in signal summation and action potential generation (26,49,50), suggesting Kv2.1 activation or inhibition can regulate neuronal firing activity. Consistent with this interpretation, blockade of Kv2.1 in SNc dopamine neurons increased the spontaneous firing frequency while decreasing the magnitude of the afterhyperpolarization of spontaneously-generated action potentials (17,23).

Using both confocal and TIRF microscopy, we found that Kv2.1 and DAT form “co-clusters” at or near the plasma membrane (fig 4A). As predicted by the reported activity-dependent nanodomain distribution of these proteins (48,51), methamphetamine activation of DAT increased the proximity of the two proteins and inhibited canonical DAT internalization. Following METH stimulation of DAT activity, there was a modest increase in surface DAT levels at the membrane nanodomain containing Kv2.1 clusters, as opposed to canonical DAT internalization. The significant reduction in the DAT’s lateral membrane mobility as measured by live cell FRAP microscopy identified a potential mechanism for the decreased DAT internalization at Kv2.1 clusters. Given the impact on DAT conformational equilibrium, ionic current, and internalization behavior, this raises the possibility that DAT is differentially regulated in subcellular compartments based on the relative amount of Kv2.1 clustering present at each site. However, the subcellular distribution of Kv2.1 in distinct regions in dopamine neurons must first be examined to determine if this type of differential regulation can alter DAT behavior in a subcellular-specific manner. This hypothesis is consistent with our work and others reporting that DAT localized to neuronal extensions preferentially adopts a clustered pattern (48,52). Our data suggest Kv2.1 clusters on the membrane of dopamine neurons can influence membrane organization of DAT and its internalization in response to activation. This supports the possibility that the observed clustering of DAT is a result of reorganization induced by Kv2.1 clusters. However, simultaneous super-resolution imaging of both proteins will be necessary to examine this possibility. Interestingly, recent reports have shown Kv2.1 localization is dysregulated in models of neurodegenerative disease, suggesting a vital role for the channel’s clustered distribution in normal neuronal physiology as well as stress-induced pathology (20,51). The degree to which clustered Kv2.1 localization impacts dopaminergic physiology may therefore shed light on the heightened vulnerability to degeneration of.
these neurons. In summary, our data support the conclusion that Kv2.1 and DAT functionally interact, and this interaction can modulate dopamine neurotransmission.

**Experimental Procedures**

**Drugs and chemicals**

Unless indicated, all drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). A summary of all chemicals, drugs and antibodies used in this study can be found in tables 1-4.

**Animals**

All procedures utilizing animals were conducted in accordance with the University of Florida Institutional Animal Care and Use Committee (IACUC) policies and adhered to the guidelines established by the National Institute of Health. Wild-type C57BL/6J mice were obtained from the University of Florida Animal Care Services and were maintained on a 12h light/dark cycle with food and water available *ad libitum* in their home cage.

**Ventral Midbrain Primary Culture and Immunocytochemistry**

Primary cultures of ventral midbrain dopamine neurons were prepared as described (6). Briefly, P0 or P1 wild-type pups of both sexes were anesthetized on ice before rapid decapitation and brain extraction. Whole brains were submerged in ice-cold Hank’s Balanced Salt Solution (HBSS) & allowed to cool for ~10 min before the ventral midbrain was isolated by removing the cerebellum, cortical lobes, and dorsal-most one-third of the mesencephalon. Isolated ventral mesencephalon tissue was transferred into dissociation media (table 2) oxygenated with 95% CO2 & 5% O2 and allowed to incubate for 2 hr at 37°C. Following dissociation, tissue was transferred to a 50 ml conical tube and gently washed two times with glial media (table 2), before being triturated with 5ml of glial media. Tissue was gently triturated using progressively smaller micropipette tips until the solution was homogenous, at which point it was centrifuged for 3 min at 500xg at RT to pellet dissociated cells. Supernatant was removed and the pellet was suspended in glial media (1ml per animal) before being passed through a 70µM cell strainer. Cells from this solution were plated on 12mm glass coverslips coated with 100 µg/ml poly-d-lysine and 5 µg/ml laminin. Following two hours of incubation, coverslips were gently flooded with 2 ml of neuronal media supplemented with GDNF and kynureate as described in table 2. Cells were fed every 4 days by exchanging 1 ml for fresh neuronal media and fixed for immunolabeling on D10IV.

**Immunofluorescence labeling and imaging**

Mice used for immunohistochemistry were four to five week old males; rats used were 4 month old males. Both species were anesthetized with isoflurane until consciousness was lost, and then fitted with custom-made anesthetic masks for continuous isoflurane delivery until time of death. An incision was made just below the sternum to allow access to the diaphragm, which was then severed to terminate the animal and expose the heart. An initial puncture was made in the right atrium before perfusing 10 ml (mice) or 300 ml (rats) of ice-cold phosphate-buffered saline (PBS) followed by 10 ml (mice) or 300 ml
(rats) of freshly prepared 4% paraformaldehyde (PFA) in PBS through the left ventricle. Brains were then extracted and post-fixed in 4% PFA in PBS overnight. Rat tissue was cryoprotected by sequential storage in 10% sucrose until sunk, and then 30% sucrose until sunk. For mouse tissue, 40 µM thick sections containing the ventral midbrain were cut on a Vibratome 1000 plus sectioning system (Ted Pella Inc., Redding CA). Rat brains were mounted and frozen in O.C.T Compound (Fisher Scientific, Hampton, NH) and 35 µM sections were cut using a Leica CM1850 cryostat (Leica Microsystems, Buffalo Grove, IL).

Slices were blocked and permeabilized for 1 hr at 37°C in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and 10% Normal Goat Serum (Lampire Biological Products, Pipersville PA). Sections were immediately transferred to primary antibody solution containing 0.1% Triton X-100 and 5% Normal Goat Serum. The primary and secondary antibodies used are listed in table 3. Following incubation, slices were rinsed 3x 30 min with PBS, and labeled with secondary antibody for 1hr at RT in the same blocking solution used for primary incubation. Slices were then washed for 24 hrs in PBS at room temperature before being mounted onto slides for visualization using Fluoromount-G (Southern Biotechnology, Birmingham AL). The primary and secondary antibodies used are listed in table 3.

Confocal Imaging was carried out on a Nikon A1 laser scanning confocal microscope (Nikon Instruments, Melville NY). Samples were visualized through a 60X 1.4 NA oil-immersion objective (Nikon Instruments, Melville NY). Excitation was achieved with 488nm and 647nm for DAT and Kv2.1 respectively; emission was captured at 525 nm and 685 nm for DAT and Kv2.1 respectively. To prevent non-specific bleed-through, excitation and emission detection was activated sequentially in a non-overlapping series. Image processing, including deconvolution and denoising of the displayed representative images, was carried out using Nikon Elements imaging software (Nikon Instruments, Melville NY). All images presented together in a given panel were manipulated identically.

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A list of all constructs used in this study can be found in table 4. All constructs were generous gifts from the stated source. GFP-tagged Kv2.1 was a generous gift from Dr. Jim Trimmer (UC-Davis) and was used to generate the mCerulean3-Kv2.1 (mCer3-Kv2.1) utilized in this study (25). Briefly, Rat Kv2.1 was digested from GFP-Kv2.1 and subcloned into mCerulean3-C1 (gift from Michael Davidson, Addgene Plasmid #54605) with EcoR1 and SacII restriction enzymes (Markwardt ML et al., 2011). Correct insertion of the construct was verified through Sanger sequencing.

Parental and YFP-DAT stably expressing human embryonic kidney (HEK-293; EM4 clonal line) cells were cultured as previously described (53,54). Cells were grown in Dulbecco’s modified minimum essential medium (Corning, 10-017-CV) to ~85% confluency in 25 cm² flasks before transient transfections and plating. For the generation of Kv2.1/DAT cells, parental HEK cells were transiently transfected with GFP-Kv2.1 and RFP-DAT, or YDP-DAT HEK cells were transiently transfected with mCer3-Kv2.1 in flasks. Transfections were carried out with polyethylenamine (PEI, Polysciences Inc., Warrington PA) at a 2:1 ratio (10µg plasmid:20 µg PEI). The PEI/DNA solution was made in 1 ml of sterile ddH₂O and allowed to rest at room temperature for 20 minutes before being added into 5 ml of standard media in flasks. These cells were then split the following day onto either coverslips or glass bottom dishes, and used 24-48 hrs following plating.

Co-immunoprecipitation of Kv2.1 and DAT

Brain tissue samples from Sprague Dawley rats (60-90 days old) were homogenized with a glass/Teflon homogenizer (20 x strokes) in 10 volumes of ice-cold lysis buffer [125 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA and Hapes (20 mM, pH 7.6)], containing protease inhibitors (1 µM leupeptin, 150 nM aprotinin, 1 µM E-64, 500 µM AEBSF, 1 mM PMSF and 0.5mM EDTA; Calbiochem). The homogenates were incubated on a microtube rotator at 4°C for 1 h in the presence of 1% (v/v) Triton X-100, and insoluble material was removed by centrifugation at 16000 x g, 15 min at 4°C. The supernatants obtained (protein lysates) were collected, measured for protein concentration, and used in the immunoprecipitations and immunoblots. HEK cells overexpressing DAT and KV2.1 were homogenized in ice-cold lysis buffer containing protease inhibitors and 1% (v/v) Triton X-100, and protein lysates were obtained as described above.

Immunoprecipitations were performed using 0.5 mg total protein. To immunoprecipitate DAT from rat brain protein lysates and HEK cells overexpressing DAT and KV2.1, the homogenates were incubated overnight at 4°C with a polyclonal anti-DAT antibody (table 3, SantaCruz C-20, dilution 1:100) and a monoclonal anti-DAT antibody (Millipore MAB369, dilution 1:100) respectively, followed by the addition of 80 µL of a mixture of protein A and protein G Sepharose beads (2 h at 4 °C in a rotatory shaker). The immunoprecipitated proteins were recovered by centrifugation at 10000 x g for 1min (4 °C), washed four times with ice-cold lysis buffer supplemented with 1% (v/v) Triton X-100, and pellets were resuspended in 40µL of 4X protein sample buffer containing β-
mercaptoethanol. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using the Bio-Rad system. For immunodetection, the nitrocellulose membranes were first blocked for 1 h in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20) containing 5% fat-free dry milk (blocking buffer), and then incubated with the indicated primary antibody for 1 h at RT in blocking buffer, washed three times for 5 min each, and incubated with an HRP-conjugated secondary antibody (dilution 1:5000) for 1 h at RT in blocking buffer. After all antibody incubations, membranes were washed three times with TBS-T buffer and immunoreactive bands were visualized using the ClarityTM Western ECL Substrate (BIO-RAD).

**Surface DAT determinations**

HEK cells transfected with DAT and KV2.1 were cultured in 4-well plates washed three times with ice-cold PBS and then each well was incubated with gentle agitation for 30 min at 4°C with 1 ml of 1.5 mg/ml sulfo-NHS-SS-biotin prepared in biotinylation buffer (150mM NaCl, 2mM CaCl2, 10mM triethanolamine, pH 7.8). The reaction was quenched by incubating the cells for an additional 15 min at 4°C with 50 mM glycine in PBS. Cells were then washed three times with ice-cold PBS and solubilized at 4°C for 1 h in ice-cold lysis buffer containing 1% Triton X-100 and protease inhibitors. The protein lysates were then divided into two aliquots: one for pull-down biotinylated proteins (400µL) and the other to determine total DAT and KV2.1 (100µL). The biotinylated proteins were precipitated incubating the biotinylated protein lysates for 1h at 4°C with 80µL of ultralink-immobilized avidin beads (50% slurry in lysis buffer, Pierce). Finally, 40µL of 2X sample buffer was added to each protein sample in order to analyze DAT and KV2.1 expression by SDS-PAGE on 10% Tris-HCl polyacrylamide gels and immunoblotting using antibodies against DAT and KV2.1 as outlined in table 3.

**Live-cell FRET**

Kv2.1/DAT cells were plated in 35 mm glass bottom dishes (MatTek, Ashland MA) 24-48h before imaging. Prior to imaging, cells were briefly washed three times with imaging buffer, the constituents of which can be found in table 1. All cells were imaged at a single confocal plane near the basal membrane. For nomifensine pretreatment, cells were incubated in 10 µM nomifensine in imaging buffer for 10 minutes at 37°C prior to imaging. For drug treatment, cells were selected a priori and 200 µl of external solution with or without methamphetamine (METH) was added to 800 µl of external in the dish to achieve the desired final concentration. METH or vehicle was allowed to diffuse for 40 seconds before cells were imaged throughout the following seven minutes. To measure FRET, three frames were imaged prior to photobleaching to determine the basal GFP-Kv2.1 intensity. Next, RFP-DAT (photoacceptor) was bleached at least 80% in a target region containing GFP-Kv2.1 clusters. Three subsequent images were taken and the following formula was applied to calculate the level of FRET: (GFP_{post} – GFP_{pre}) / GFP_{post} where GFP_{F} represents the average GFP-Kv2.1 fluorescence signal (AFU) captured...
in the ROI where RFP-DAT signal was bleached. Only cells that exhibited ≥ 80% RFP-DAT bleaching threshold were analyzed. All images were background subtracted using a locally drawn background ROI.

**Live-cell FRAP**

Kv2.1/DAT cells were plated and washed prior to imaging as above in FRET experiments. As described above, images were acquired at an optical plane near the basal membrane where both DAT and Kv2.1 localization were visually appreciable over the entire cell surface. Previous reports suggest a 3-5 µM circular region of interest (ROI) at the basal membrane reliably measures the lateral mobility of DAT within the membrane (11,30). Therefore, a 5 µM circular ROI was selected for photobleaching with a reference ROI identical in size placed on either a region of the cell that was not bleached or a nearby cell expressing RFP-DAT that was not bleached. Five consecutive images were taken prior to photobleaching to determine the baseline level of RFP-DAT signal. The region within the ROI was then selectively photobleached, and RFP-DAT signal was then measured in the photobleached region for 3.5 minutes to determine the rate of fluorescence recovery. Background subtraction was carried out prior to all measurements using a local background ROI. To determine the percent of DAT in the immobile fraction, the normalized final signal was subtracted from baseline and converted to a percentage.

**YFP-DAT internalization using Total Internal Reflection Fluorescent Microscopy**

Total Internal Reflection Fluorescent microscopy (TIRFM) was conducted on a Nikon Ti Eclipse inverted microscope equipped with 445nm and 514nm solid-state lasers (Coherent Inc., Santa Clara CA) fed through a 60X 1.49 NA Apo TIRF objective (Nikon Instruments, Melville NY). Images were captured using a CoolSNAP HQ2 CCD camera (Photometrics, Tuscon AZ). Kv2.1/DAT cells were plated in 35 mm glass bottom dishes (MatTek, Ashland MA) 24-48h before imaging. Cells were briefly washed 3 times with external solution before being placed in the stage holder, at which point fresh external solution was continuously perfused at 37° C. Perfusion was carried out at ~3 ml/minute using an automatic peristaltic pump (Instech Laboratories, Plymouth Meeting PA). Cells were imaged at a rate of 0.3 Hz for one minute to determine basal fluorescence levels for both proteins before switching to perfusion of imaging buffer containing 10 µM METH. Cells were then continually imaged at 0.3 Hz for 5 minutes to observe changes in fluorescence intensity. Control cells were perfused with imaging buffer and imaged as above. Analysis was carried out using Nikon Elements (Nikon Instruments, Melville NY). Briefly, binary masks were generated using automatic fluorescence thresholding to distinguish areas of the membrane occupied by mCer3-Kv2.1 and YFP-DAT. The binary layer for Kv2.1 associated-DAT was defined as regions of the YFP-DAT binary mask that also contained the mCer3-Kv2.1 binary mask. Conversely, YFP-DAT internalization in cells not expressing Kv2.1 was measured using the entirety of the basal membrane. The change in YFP-DAT fluorescence intensity in each region was normalized to the average level...
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observed in that region during the baseline period. All images were background subtracted using a local background ROI.

**Whole-cell Electrophysiology**

Recordings were performed on a Nikon FN-1 upright microscope (Nikon Instruments, Melville NY) and Narishige mounting adaptor (Narishige International USA, Amityville NY) mounted on an anti-vibration table (TMC, Peabody MA). Gravity perfusion lines were run through an eight-channel valve controller (Warner Instruments, Hamden CT) and set to a flow rate of ~2 ml/min. Kv2.1/DAT cells were plated on uncoated 12mm glass coverslips (Chemglass Life Sciences, Vineland NJ) 48-72h prior to recording. Once placed into the recording chamber (Warner Instruments, Hamden CT), coverslips were continuously perfused with fresh external solution. Borosilicate pipettes (World Precision Instruments, Sarasota FL) were pulled on a Sutter P2000 laser puller (Sutter Instrument, Novato CA). The pipettes resistance were between 1.5 to 3.5 MOhm. The constituents of the internal recording solution are listed in Table 1. All currents were recorded from cells with high-resistance seal (> 600 MOhm in whole-cell mode) and currents were acquired using an Axoptach 200B Amplifier and Digidata 1440A digitizer run through Axon pCLAMP 10 (Molecular Devices, Sunnyvale CA). To determine the DAT-mediated inward current, a voltage-current (I/V) curve was generated from -120 mV to -40 mV in 10mV steps (250 µs) from a holding potential of -100 mV. Current values were calculated during the final 100 ms of each voltage step’s I/V’s were generated for cells under basal conditions and 2.5 min after perfusion with 10 µM METH. All traces shown are following subtraction of current in the presence of a DAT blocker (nomifensine, 10 µM) as previously described.(53).

**IDT-307 uptake imaging**

YFP-DAT cells were transiently transfected with mCer3-Kv2.1 as above and plated onto uncoated 12mm glass coverslips. Coverslips were placed into an imaging chamber (Warner Instruments RC-26G) mounted on a Nikon Eclipse FN-1 and visualized with a Nikon 40X 0.8NA NIR APO objective (Nikon Instruments, Melville NY). Solution exchange was achieved using a Warner VC-8 automated perfusion system at a rate of ~2 ml/min (Warner Instruments, Hamden CT). Solution was maintained at 37°C using a dual channel temperature controller (Warner Instruments, Hamden CT). Excitation was achieved using a SpectraX light engine (Lumencor, Beaverton OR) controlled by Nikon Elements (Nikon Instruments, Melville NY). Images were acquired at 1 Hz using an Andor Xyla 4.2 PLUS with a constant exposure time of 100ms and a conversion gain of 4 (Andor Technology, Belfast UK). All videos were captured with a constant pixel size (0.16 µM per pixel) but the field of view was cropped to minimize the final image size. For IDT-specific signal, the 488nm LED was used and cells were imaged for 30s to determine the basal fluorescence signal detected. Solution was then changed to imaging buffer containing 1 µM IDT307 (Sigma-Aldrich) and imaged for 2.5 min. As a control, cells were imaged identically as described with the addition of 10 µM Nomifensine (Sigma Aldrich) to the imaging buffer. Analysis was conducted in
Live cell JHC1-064 imaging

Cells were handled and plated as described above for FRET and TIRF imaging. To label the outwardly-facing conformation of DAT molecules at the membrane, cells were incubated in 100 nm of JHC1-064, a fluorescent cocaine analog (44). JHC1-064 incubation was carried out at 4°C to prevent transporter internalization as previously described (9). Cells were washed 3 times with 4°C external and imaged at room temperature using a 60X 1.4 NA oil-immersion objective (Nikon Instruments, Melville NY). To acquire images, a random point was chosen and the z-plane was set (using Nikon perfect focus) to capture the cells’ stacked membrane. Image scans containing 16 surrounding fields of view were generated and stitched together to make one large image (field of view overlap – 0.1%). Cells expressing Kv2.1 and DAT or DAT alone were cropped into individual images and deconvolved using the Nikon Elements automatic 2D deconvolution plugin. For JHC1-064 binding quantification, an ROI was drawn around the stacked membrane of the cell and the ratio of YFP-DAT signal (AFU) to JHC1-064 signal was calculated for each cell.

Statistics

Statistical analysis was run on Prism 8.0 (Graphpad Software, La Jolla CA). An α of 0.05 was used to determine statistical significance. Specific tests used for each figure including the n and p-values are described in the figure legends and the Results section. All tests were run under the assumptions of a normal distribution and similar variance among experimental groups. Data are presented as the mean ± SEM

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Conflict of interest: The author’s declare that they have no conflicts of interest with the contents of this article
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Footnotes

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The abbreviations used here are: DAT, dopamine transporter; GFP/eGFP, enhanced green fluorescent protein; RFP, Tag-red fluorescent protein; mCer3, mCerulean3; METH, methamphetamine; NOM, nomifensine; FRET, Forster resonance energy transfer; FRAP, fluorescence recovery after photobleaching; TIRFM, total internal reflection microscopy; HEK, human embryonic kidney cell; PBS, phosphate buffered saline;
### Table 1: Imaging and electrophysiology solutions

| Chemical | MW  | Concentration | Cat #                      |
|----------|-----|---------------|----------------------------|
| NaCl     | 58.44 | 135 mM        | S7653 (Sigma-Aldrich)     |
| KCl      | 74.55 | 5 mM          | P9541 (Sigma-Aldrich)     |
| CaCl2    | 147.2 | 1.8 mM        | 223506 (Sigma-Aldrich)    |
| MgCl2    | 95.2  | 1.8 mM        | 8147330100 (Merck Millipore) |
| Dextrose | 180.16 | 10 mM        | D9434 (Sigma-Aldrich)     |
| Hepes    | 238.8 | 10 mM         | H3375 (Sigma-Aldrich)     |

| Chemical | MW  | Concentration | Cat #                      |
|----------|-----|---------------|----------------------------|
| KCl      | 74.55 | 120 mM        | P9541 (Sigma-Aldrich)     |
| HEPES    | 238.8 | 10 mM         | H3375 (Sigma-Aldrich)     |
| Dextrose | 180.16 | 30 mM        | D9434 (Sigma-Aldrich)     |
| EGTA     | 380.35 | 1.1 mM       | E3889 (Sigma-Aldrich)     |
| CaCl2    | 147.2 | 0.1 mM        | 223506 (Sigma-Aldrich)    |
| MgCl2    | 203.3 | 2 mM          | M9272 (Sigma-Aldrich)     |

Table 1. External and Internal solution constituents
### Table 2. Primary culture solution constituents

#### Dissociation Media

| Chemical          | Concentration | Cat #                      |
|-------------------|---------------|---------------------------|
| Cysteine*         | 963 μM        | C7352 (Sigma-Aldrich)     |
| NaCl              | 23.2 mM       | S7653 (Sigma-Aldrich)     |
| KCl               | 1.08 mM       | P9333 (Sigma-Aldrich)     |
| NaHCO3            | 5.2 mM        | S5761 (Sigma-Aldrich)     |
| NaH2PO4.H2O       | 400 μM        | S8282 (Sigma-Aldrich)     |
| MgSO4             | 200 μM        | M7506 (Sigma-Aldrich)     |
| EDTA              | 100 μM        | EDS (Sigma-Aldrich)       |
| Glucose           | 5 mM          | D9434 (Sigma-Aldrich)     |
| HCl               | 0.005 N       | H1758 (Sigma-Aldrich)     |
| Phenol red        | As supplied   | P0290 (Sigma-Aldrich)     |
| Papain*           | 20 units / ml | PAP (Worthington Biochem) |
| Kynureninic acid  | 100 mM        | K3375 (Sigma-Aldrich)     |

#### Glial Medium

| Component                  | Stock concentration | Volume for 1X | Cat #                      |
|----------------------------|---------------------|---------------|---------------------------|
| MEM w/out Glutamine        | As supplied         | 65 ml         | 11090 (Gibco)             |
| Fetal bovine serum         | As supplied         | 50 ml         | 100-106 (Gemini Bio-Products) |
| Insulin                    | 25 mg/ml            | 100 ul        | I6634 (Sigma-Aldrich)     |
| Dextrose                   | 45% in ddH2O        | 3.8 ml        | D9434 (Sigma-Aldrich)     |
| GlutaMAX                   | NA                  | 1.23 ml       | 35050061 (Gibco)          |
| Penn/Strep                 | 5000 units/ml       | 10ml          | P4333 (Gibco)             |

#### Neuronal Media

| Component (*Day 0 only)   | Stock concentration | Volume for 1X | Cat #                      |
|---------------------------|---------------------|---------------|---------------------------|
| Neurobasal A Plus         | As supplied         | 25 ml         | A3582901 (Gibco)          |
| B-27 Plus                 | As supplied         | 500 μl        | A3582801 (Gibco)          |
| Glutamax                  | As supplied         | 250 μl        | 35050061 (Gibco)          |
| GDNF*                     | 0.25 ng/μl          | 40 μl         | 450-10 (PeproTech)        |
| Kynurenic acid*           | 0.5 M               | 20 μl         | K3375 (Sigma-Aldrich)     |
### Table 3: Primary and Secondary antibodies

| Figure | Primary Target | 1st Company & Cat # | Species | IgG subtype | Secondary Antibody | Company & Cat # |
|--------|----------------|---------------------|---------|-------------|--------------------|-----------------|
| 1A-C   | DAT            | Millipore MAB369    | Rat monoclonal | IgG2A    | Goat anti-Rat (H+L), Alexafluor 488 | Thermo Fisher, A-11066 |
|        | Kv2.1          | NeuroMab K89/34     | Mouse monoclonal | IgG1     | Goat anti-Mouse IgG1, Alexafluor 647 | Thermo Fisher, A-21240 |
| 2      | DAT (pulldown) | Santa Cruz (C-20)   | Goat polyclonal | --       | --                 | --              |
|        | DAT (immunoblot) | Santa Cruz (C-20) | Goat polyclonal | --       | Goat anti-Rat (H+L), HRP | Thermo Fisher, 31470 |
|        | Kv2.1 (immunoblot) | K89/34 | Mouse monoclonal | IgG1     | Goat anti-Mouse (H+L), HRP | Thermo Fisher, 31430 |
| 3      | DAT (pulldown) | Millipore MAB369    | Rat monoclonal | --       | --                 | --              |
|        | DAT (immunoblot) | PhosphoSolutions 434-DATEL2 | Rabbit polyclonal | --       | Goat anti-Rabbit (H+L), HRP | Thermo Fisher, 31460 |
|        | Kv2.1 (immunoblot) | K89/34 | Mouse monoclonal | IgG1     | HRP                | Thermo Fisher, 31430 |
| 5      | DAT            | PhosphoSolutions 434-DATEL2 | Rabbit polyclonal | --       | Goat anti-Rabbit (H+L), HRP | Thermo Fisher, 31460 |
Table 4 - Plasmids

| Protein            | Fluorophore      | Vector        | Source                          |
|--------------------|------------------|---------------|---------------------------------|
| Kv2.1              | GFP              | RBG4          | J. Trimmer                      |
| s586a-Kv2.1        | GFP              | RBG4          | J. Trimmer                      |
| Kv2.1              | mCerulean3       | mCerulean3-C1 | M. Davidson (Vector - Addgene)  |
| DAT                | Tag-RFP          | pTagRFP-T-C1  | H. Melikian                     |
| DAT                | EYFP             | stable expressing (pEYFP-N1) | J. Javitch |
| Transferrin Receptor | mCerulean    | mCerulean    | M. Davidson (Addgene)          |
| EYFP               | EYFP             | pEYFP-N1      | Clontech                        |
| mCerulean3         | mCerulean3       | mCerulean3-C1 | M. Davidson (Addgene)          |
| FRET8              | mCerulean / mVenus | pEGFP-C1     | D. Piston                       |
| EEA1               | Tag-RFP          | pcDNA3        | S. Corvera (Addgene)           |

Table 4. Plasmid constructs
Figure 1. Kv2.1 is expressed at the soma and proximal processes of murine dopamine neurons. Double immunofluorescence labeling of DAT (leftmost column, green in merge) and Kv2.1 (middle column, red in merge) in murine dopamine neurons. All images are 2D maximum intensity projections of 3D reconstructed confocal z-stack. In mouse cultured neurons (A) and labeled mouse (B) and rat (C) brain slices, DAT labeling is diffuse along the entirety of the soma and proximal processes, whereas Kv2.1 is localized exclusively to large clusters. (scale: 10 µm)
Figure 2: DAT activation increases the transporter's association with Kv2.1. A. Representative blot showing the coIP between GFP-Kv2.1 and YFP-DAT before (control) or after DAT activation (METH, 10 µM for 5 min). DAT was a pulled down using an n-terminus targeted antibody (MAB369) and detected with an antibody targeting the second extracellular loop (DATEL2). No Kv2.1 was detected in cells expressing DAT alone (right blot, DAT), or when nonspecific IgG beads were used (right blot, IgG lanes). B. Quantification of the ratio of Kv2.1 signal to DAT signal observed in basal (control) and DAT activation (METH 10 µM) conditions. Activation of DAT results in an increase in the observed ratio, indicative of increased association between the two proteins (p = 0.0342, unpaired t test; n = 4 independent experiments for each condition)
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Figure 3: Disruption of Kv2.1 clustering prevents METH-induced increases in its proximity to DAT. A. Representative confocal images at a single optical plane where FRET was conducted showing wild-type GFP-Kv2.1 localization in comparison to the non-clustering mutant GFP-s586a-Kv2.1. (scale = 25 µM) D. Normalized FRET efficiencies. All graphs normalized to the leftmost experimental group. Comparing basal conditions (vehicle) and DAT activation (METH (10 µM) or DA (1 µM)) revealed an increase in FRET efficiency between RFP-DAT and Kv2.1 (top left graph, vehicle vs. METH p = 0.0066, n = 96 and 92 cells from 10 and 9 independent experiments for vehicle and METH respectively; vehicle vs. DA p < 0.0001, n = 61 cells from 6 independent experiments, Tukey’s test following one-way ANOVA). No significant FRET increase was observed under the same conditions when Kv2.1 clustering was disrupted (top right graph, n = 83 and 86 cells for vehicle and METH respectively, each from 6 independent experiments, Tukey’s test following one-way ANOVA). To confirm the specificity of the FRET observed, GFP-KV2.1 was expressed with a non-interacting protein tagged with an identical chromophore (RFP-EEA1), in which conditions no FRET was observed (bottom graph, p < 0.0001 between all experimental groups, n = 32 cells from 4 independent experiments)
**Figure 4:** DAT internalization is reduced at sites of Kv2.1 clusters. 

**A.** TIRFM image of a Kv2.1/DAT cell (above), and YFP-DAT signal before (below, left) and two minutes after exposure to 100 µM METH (below, right). (scales = 25 µm; inset scale = 5 µm) **B.** Normalized YFP-DAT signal measured during the final 30s in cells coexpressing mCer3-Kv2.1 (two leftmost groups) and cells expressing YFP-DAT only (rightmost group) following treatment with METH or imaging buffer alone. YFP-DAT signal in cells co-expressing Kv2.1 was segregated on the basis of mCer3-Kv2.1 cluster presence as indicated. Non-Kv2.1 cluster associated DAT and DAT in cells that did not express Kv2.1 exhibited a significant loss of fluorescence at the membrane, indicative of internalization (Non-Kv2.1 cluster associated DAT VEH-METH: p = 0.0037; DAT only cells VEH-METH: p = 0.0414; Sidak’s multiple comparison test following two-way ANOVA). Kv2.1 cluster associated DAT did not exhibit a decrease in fluorescence at the membrane in response to METH treatment. **C.** Line graph showing changes in the YFP-DAT fluorescence levels at the plasma membrane for Kv2.1 cluster associated DAT (blue line), Non-Kv2.1 cluster associated DAT (green line) and DAT only cells (black line). Non-Kv2.1 cluster associated in showed no difference in internalization rate from that in non-Kv2.1 expressing cells, though both groups differed significantly at the indicated time points for Kv2.1 cluster associated DAT. (** - p<0.01, Tukey’s multiple comparison test following two-way ANOVA).
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**Figure 5: Kv2.1 cluster association limits DAT membrane mobility.** Representative fluorescence recovery after photobleaching (FRAP) time series showing the basal membrane of a Kv2.1/DAT cell (top panel) or cell expressing DAT only (lower panel). White circle outlines the photobleached region followed by the time-dependent recovery of RFP signal, representing the lateral mobility of unbleached RFP-DAT molecules. (scales = 5 µm) **B.** The fluorescence recovery profiles for cells only expressing RFP-DAT (black line), or cells coexpressing RFP-DAT with wt-GFP-Kv2.1 (grey line) or RFP-DAT with s586a-GFP-Kv2.1 (green line). **C.** Quantification of immobile DAT fraction defined by the decreased fluorescent recovery. The cells expressing only RFP-DAT and cells coexpressing RFP-DAT plus the non-clustering s586a-GFP-Kv2.1 showed a significant fluorescent recovery following photobleaching leading to a significantly lower immobile fraction for RFP-DAT in cells coexpressing wt-GFP-Kv2.1 (wt vs. non Kv: p = 0.0003; wt vs. s586a: p < 0.0001; n = 9 cells (wt), 17 cells (non-Kv), and 22 cells (s586a) from 7 (wt), 8 (non-Kv), and 7 (s586a) independent experiments; Tukey’s test following one-way ANOVA)
Figure 6: Kv2.1 expression decreases DAT-mediated inward current without reducing surface DAT levels. A. Representative recordings obtained in whole-cell patch clamp configuration with a voltage step from –120 mV to –40 mV showing the DAT-mediated inward current in YFP-DAT only cells (top) or cells coexpressing YFP-DAT and mCer3-Kv2.1 (bottom). The DAT-mediated inward current is defined as basal whole-cell current minus the current after nomifensine (10 µM) application, canonically described as nomifensine-sensitive current B. Bar graph shows the DAT mediated inward current at -120 mV in cells expressing DAT alone (black bar) or in the cells coexpressing DAT plus Kv2.1 (teal bar) (p = 0.0405, n = 8-10; unpaired t-test) C. Representative immunoblot showing surface DAT (biotinylated fraction) and total DAT (total fraction) from cells with or without Kv2.1 coexpression. D. Comparison of surface DAT levels in cells expressing DAT only or cell coexpressing Kv2.1 and DAT. The surface DAT level was determined by calculating the ratio of DAT signal from the biotinylated fraction to total DAT levels. Coexpression of Kv2.1 did not alter the surface DAT level compared to cells without Kv2.1 (p = 0.5496, n = 3, unpaired t-test).
Figure 7: Kv2.1 expression decreases DAT-mediated uptake. A. Representative images of the stacked-membrane of YFP DAT cells (right panel) or cells expressing both YFP_DAT and Kv2.1 (middle panel). (scale = 20 µm) B. Representative baseline fluorescence signal (before, left) and the fluorescence signal after IDT-307 uptake (post, left) (scale = 20 µm) C. IDT-307 uptake (shown as fold increased above baseline signal) in cells expressing DAT alone or coexpressing Kv2.1. Cells coexpressing Kv2.1 and DAT showed a significantly lower IDT-307 uptake (DAT only: 3.385 ± 0.247, n = 33 cells from 7 independent experiments; Kv2.1/DAT: 1.846 ± 0.180, n = 20 cells from 7 independent experiments; p < 0.0001, Tukey’s test following one-way ANOVA). Pretreatment with the DAT blocker nomifensine significantly reduced IDT307 uptake compared to both experimental groups (NOMI: 1.202 ± 0.045, n = 37 cells from 6 independent experiments; p < 0.0001 vs. DAT only, p = 0.0182 vs. KV2.1/DAT, Tukey’s test following one-way ANOVA). D. Time course of the IDT-307 uptake for all experimental groups. The presence of Kv2.1 significantly reduced the uptake of IDT-307 compared with cells expressing DAT alone (p = 0.0002, F(1,45) = 15.95, two-way ANOVA)
Figure 8: DAT adopts an inward-facing conformation in the presence of Kv2.1.  

A. Schematic of the JHC1-064 binding paradigm used to determine the relative conformational preference of DAT. JHC1-064 binds to the outwardly facing conformation of DAT. An increase in the ratio of YFP-DAT signal to JHC1-064 signal suggest a higher proportion of the transporters exist in an outwardly facing conformation.  

B. Quantification and comparison of the ratio of YFP-DAT signal to JHC1-064 signal in cells expressing DAT alone vs. cells coexpressing DAT with Kv2.1. The YFP-DAT:JHC1-064 ratio was increased in cells coexpressing Kv2.1 compared with cells expressing DAT alone (p = 0.0004, n = 44 cells (DAT only) and 35 cells (DAT/Kv2.1) from 5 independent experiments, unpaired t test).  

C, D. Representative images of YFP-DAT signal (lower left panels), mCer3-Kv2.1 signal (upper right panels) and JHC1-064 signal (upper right panels) in either a DAT only cell (C) or a DAT/Kv2.1 cell (D). (scale = 5 µM)
Figure 9: Proposed model for the interaction between Kv2.1 and DAT. We hypothesize that in basal conditions, some transporters are associated with Kv2.1 clusters. This association can be due either to a direct interaction with Kv2.1, or within a protein complex containing the cytoskeletal elements known to stabilize Kv2.1 clusters ("cytoskeletal fence"). DAT in Kv2.1 clusters is stabilized in an inward facing conformation, resulting in decreased uptake. When activated, additional DAT molecules become ensnared at Kv2.1 clusters decreasing the canonical cell surface redistribution of transporters in response to transporter activation.
Clustered Kv2.1 decreases dopamine transporter activity and internalization
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