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Gβγ subunit activation promotes dopamine efflux through the dopamine transporter

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Abstract

The dopamine transporter (DAT) is an important regulator of brain dopamine (DA) homeostasis, controlling the intensity and duration of DA signaling. DAT is the target for psychostimulants—like cocaine and amphetamine—and plays an important role in neuropsychiatric disorders, including attention-deficit hyperactivity disorder and drug addiction. Thus, a thorough understanding of the mechanisms that regulate DAT function is necessary for the development of clinical interventions to treat DA-related brain disorders. Previous studies have revealed a plethora of protein–protein interactions influencing DAT cellular localization and activity, suggesting that the fine-tuning of DA homeostasis involves multiple mechanisms. We recently reported that G-protein beta-gamma (Gβγ) subunits bind directly to DAT and decrease DA clearance. Here we show that Gβγ induces the release of DA through DAT. Specifically, a Gβγ-binding/activating peptide, mSIRK, increases DA efflux through DAT in heterologous cells and primary dopaminergic neurons in culture. Addition of the Gβγ inhibitor gallein or DAT inhibitors prevents this effect. Residues 582 to 596 in the DAT carboxy terminus were identified as the primary binding site of Gβγ. A TAT peptide containing the Gβγ-interacting domain of DAT blocked the ability of mSIRK to induce DA efflux, consistent with a direct interaction of Gβγ with the transporter. Finally, activation of a G-protein-coupled receptor, the muscarinic M5R, results in DAT-mediated DA efflux through a Gβγ-dependent mechanism. Collectively, our data show that Gβγ interacts with DAT to promote DA efflux. This novel mechanism may have important implications in the regulation of brain DA homeostasis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Dopamine (DA) is an essential neurotransmitter that plays a major role in the control of locomotor activity and motivated behaviors, including attention, reward and pleasure. A primary component of DA neurotransmission that determines the duration of extracellular DA is the re-uptake of the transmitter into nerve terminals by the DA transporter (DAT), thereby limiting the lifetime of DA signaling in the brain.[1–3] In addition, potent psychostimulants, such as cocaine and amphetamine, produce their reinforcing effects primarily by altering DAT function.[4–6]

Over the past 15 years, we and others have identified several DAT protein–protein interactions.[7] Proteins associated with DAT regulate its distribution, functional properties and modulation by psychostimulants.[8,9] We recently identified the βγ subunit of the heterotrimeric G proteins as a DAT-interacting protein.[10] Biochemical experiments demonstrated that DAT and Gβγ co-immunoprecipitated from mouse striatum. We also found that Gβγ subunits interact directly with the carboxy terminus of DAT. Using a combination of functional assays, we showed that activation of Gβγ subunits results in a decrease of DA uptake in heterologous cells, brain synaptosomes, and *in vivo*. Importantly, we demonstrated that the functional modulation of DAT by Gβγ is not the result of changes in DAT cell surface expression. The novelty of this interaction led us to further investigate the regulation of DAT by Gβγ subunits and the functional implications of this interaction.

Although the primary action of DAT is considered to be the re-uptake of released DA back into the nerve terminal, ample evidence supports the contention that plasma membrane transporters, including DAT, can efflux (outward transport) transmitters from the cytoplasmic side of the membrane to the extracellular space.[11] Although the classical calcium-dependent vesicular DA release mechanism has been well documented, experimental evidence suggests that neurons might also release the transmitter through a carrier-mediated mechanism.[11–13] This phenomenon, referred to as ‘DA efflux’, has been extensively studied as the primary mechanism used by amphetamines to increase extracellular DA.[4] However, endogenous signaling mechanisms leading to DA efflux through DAT have remained elusive. In this study, we provide evidence for the involvement of Gβγ subunits in DAT-mediated DA efflux. The physiological implications of this novel regulatory mechanism are discussed.

MATERIALS AND METHODS

(See Supplementary Information for additional details)

Cell culture

Human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection. HEK293 cells, stably transfected with the human DAT (HEK-DAT), were generated and maintained as reported in Garcia-Olivares *et al.*[10] CHO cells stably transfected with human DAT (CHO-DAT) were generated as previously described.[14] Transfections with pcDNA3.1(+)hM5R (www.cdna.org) were

performed using Lipofectamine-LTX (Lifescience) following the manufacturer's instructions.

Primary culture

Embryonic Sprague–Dawley rats (E15-E16) (Charles River Laboratories, Wilmington, MA, USA) were used for primary cultures by dissecting a region containing the substantia nigra from a coronal section of the mesencephalon. A detailed description of the culture preparation protocol can be found in Supplementary Methods.

Amperometry

To measure amperometric currents, we followed the protocol reported in Hamilton *et al.*[14] CHO-DAT cells were preloaded with 1 μM DA (20 min, 37 °C), and prepared for recording in KRH assay buffer (in mM: 130 NaCl, 1.3 KCl, 1.2 KH_2PO_4, 10 HEPES, 2.2 $CaCl_2$, 10 glucose, 0.1 pargyline, 1 tropolone, 0.1 ascorbic acid, pH 7.4). Amperometric currents were recorded using a carbon fiber electrode (ProCFE, Dagan, Minneapolis, MN, USA) connected to an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). DA efflux was quantified as the maximum value of the amperometric current after the drug application. The data are presented as the mean ± s.e.m.

Efflux assay

CHO-DAT, HEK-DAT cells and neurons were plated in 24-well plates coated with poly-D-Lysine. Cells were loaded (20 min, 30 °C) with 0.02 μM of [^{3}H]-(3,4-[7-3H] dihydroxyphenylethylamine) (DA, 34.8 Ci/mmol; Perkin Elmer, Waltham, CA, USA) or [^{3}H]-Methyl-4-phenylpyridinium (MPP^+, 79.8 Ci/mmol, Perkin Elmer) in KRH buffer. The released [^{3}H]-substrate was collected from the extracellular medium and counts per min (cpm) were obtained using a LS-Counter scintillation counter (Beckman Coulter, Brea, CA, USA). Efflux is expressed as a percentage, relative to baseline levels of extracellular [^{3}H]-substrate in the absence of treatment with Gβγ modulators. Peptides were custom-made by Lifetein-LCC (Somerset, NJ, USA; amino acid sequences shown in Supplementary Table S1). All the data and statistical analysis (two-tailed *t*-test) were performed using SigmaPlot12.5 (Sigma, St. Louis, MO, USA). The data are presented as the mean ± s.e.m. The significance levels were defined as *P*-values<0.05(*) and *P*<0.01(**).

In vitro binding assays

The composition of buffers, binding conditions and peptides sequences are described in Supplementary Methods. Peptides were tagged with Biotin at the N-terminus (Supplementary Table S1). For the resin-based binding assay, peptides (50 ng) were immobilized onto Neutravidin resin (Thermo-Scientific, Waltham, MA, USA). The immobilized peptides were incubated with purified Gβγ (Millipore, Billerica, CA, USA) (50 ng, 30 min, at 4 °C). Unbound Gβγ (flow-through from binding step) was collected and used as a loading control. Samples were analyzed by SDS-PAGE followed by western blotting (Gβ antibody (T-20, Santa Cruz, Dallas, TX, USA)). Densitometry analysis was performed with Imagelab5.0 (Bio-Rad, Hercules, CA, USA). For the plate-based *in vitro* binding assay, peptides were immobilized in neutravidin pre-coated 96-well plates (Thermo-

Scientific). The immobilized peptides were incubated with purified Gβγ (Millipore) (30 min, RT). The quantification of bound Gβγ was determined by immuno-detection using the QuantaBlu kit (Thermo-Scientific), and measured with a plate reader (Tecan, Mannedorf, Switzerland, Exc.420/Emiss. 325 nm). Results are shown as relative binding compared to control group. The data are presented as mean ± s.e.m. Statistics were performed using a two-tail Student's *t*-test with an accepted significance level at $P<0.05$.

RESULTS

Activation of Gβγ promotes DA efflux through DAT

To assess the role of Gβγ subunits in DAT-mediated DA efflux, we investigated the effect of mSIRK, a cell-permeant myristoylated Gβγ-binding/activating peptide,[15–17] on DA efflux using amperometry in CHO cells. As shown in Figure 1a, application of mSIRK (10 μM) elicited a robust outward current attributed to the release of preloaded DA from CHO cells expressing hDAT (CHO-DAT). The maximum increase in the DA current occurred within minutes of mSIRK treatment. Application of mSIRK to untransfected cells (CHO cells without DAT) failed to exhibit oxidative currents (Figure 1a). CHO-DAT cells incubated with a scrambled myristoylated control peptide (scb-mSIRK) or pre-incubated with the DAT inhibitor cocaine (20 μM) prior to mSIRK treatment, did not display DA currents (Figures 1a and b). These results suggest that mSIRK activation of Gβγ results in DAT-mediated DA efflux. As a complementary approach, we tested the effect of mSIRK on CHO-DAT cells preloaded with [^{3}H]-DA. As shown in Figure 1c, CHO-DAT cells displayed minimal, basal release of [^{3}H]-DA without stimulation (see Supplementary Figure S1 for absolute values). When CHO-DAT cells were incubated with mSIRK (10 μM), [^{3}H]-DA efflux increased in a time-dependent manner, reaching maximum efflux in 10 min (Figure 1c). In contrast, scb-mSIRK had no effect on DA efflux (Figure 1c). Stimulation with mSIRK (10 μM, 15 min) resulted in an increase of extracellular [^{3}H]-DA relative to control group (466.9 ± 19.7% to 1524.2 ± 62.0%, in six independent experiments (n = 22), mean ± s.e.m.), with a mean value of 1058.0 ± 80.0% (Supplementary Figure S1). The EC_{50} value for mSIRK was 8.0 ± 2.8 μM (Figure 1d). The effect of mSIRK on [^{3}H]-DA efflux was blocked by the DAT inhibitors cocaine, mazindol, or GBR12935 (Figure 1d and Supplementary Figure S1). Finally, we tested the effect of mSIRK on DA efflux in the presence of two different blockers of Gβγ signaling; the small molecule gallein that binds to Gβγ and disrupts Gβγ signaling to effectors[16,18] and Kpept, a Gβγ scavenger peptide derived from a Gβγ-regulated potassium channel, GRK4,[19,20] fused to a TAT sequence for cell membrane penetration (TAT-Kpept) (Figures 1e and f). Gallein, in a dose-dependent manner, reduced DA efflux induced by 10 μM mSIRK (Figure 1e). Similarly, DA efflux induced by mSIRK (10 μM) was blocked by pre-incubation with 20 μM of the Gβγ scavenger peptide TAT-Kpept (Figure 1f). Moreover, the effects of mSIRK on DA efflux observed in CHO-DAT cells were recapitulated in HEK293 cells expressing DAT (HEK-DAT, Supplementary Figure S1).

To examine the effect of mSIRK in a native system, we tested whether mSIRK activation of Gβγ promoted DA efflux through DAT in primary cultures of rat mesencephalic DA neurons. mSIRK induced a dose-dependent increase in DA efflux with an EC_{50} = 7.7 ± 1.0 μM (Figure 2a), a value similar to the EC_{50} obtained in CHO-DAT cells. No effect was

observed with 10 μM of the scrambled control peptide (Figure 2b). Pre-treatment with the Gβγ inhibitor gallein (10 μM) or the DAT inhibitor GBR12935 (0.5 μM) prevented the effect of mSIRK (10 μM) on DA efflux (Figure 2b). The combined results indicate that Gβγ activation in dopaminergic neurons also induces DA efflux through DAT.

Gβγ signaling pathways do not play a role in Gβγ-induced DA efflux

Activation of Gβγ is a potent signal for various cellular signaling pathways.[21] We decided to investigate whether mSIRK effect was through activation of Gβγ signaling pathways or by the direct binding of Gβγ subunits to the transporter. HEK-DAT cells were used to evaluate whether inhibitors of Phospholipase C (U72122), Phospoinositide 3 kinase (Wortmannin), mitogen-activated protein kinases (PD98059 and FR180204), protein kinase C (GÖ6983 and BIM-II) or protein kinase A (KT5720), alter the effect of mSIRK on DA efflux. As shown in Supplementary Table S2, none of the inhibitors tested significantly altered the ability of mSIRK to promote DA efflux.

Gβγ interacts directly with DAT to mediate DA efflux

We previously showed that Gβγ subunits bind directly to the carboxy terminus of DAT to regulate DAT activity.[10] To identify the residues that are necessary for this interaction, peptide fragments of the DAT carboxy terminus were synthesized (Supplementary Table S1 and Figure 3a) and tested for their ability to bind Gβγ. The *in vitro* binding assays demonstrated that Gβγ binds directly to the DATct1 fragment corresponding to S582 through A596 in hDAT (Figures 3b and c). To identify the precise location of the Gβγ-binding site, we made a series of alanine substitutions in DATct1 and performed a binding assay using a plate reader (Figure 3d). As seen in Figure 3e, replacing residues 587–590 with alanine residues in DATct1 was sufficient to abolish binding to Gβγ. Alanine substitutions to residues 583–586 or residues 591–594 in DATct1 did not modify binding to Gβγ (Figure 3e).

To assess the functional significance of the Gβγ-DAT-binding site, we designed a peptide containing the DATct1 fragment fused to the cell-permeant peptide TAT (TAT-DATct1). This peptide did not have an effect on [^{3}H]-DA uptake (Supplementary Figure S2). We investigated whether the TAT-DATct1 peptide would inhibit the effects of mSIRK on DA efflux. We used the TAT-DATct1 peptide, and a control TAT peptide containing a scrambled sequence of the DATct1 fragment (TAT-scbDATct1) in efflux assays in CHO-DAT cells and cultured DA neurons (Figure 4). CHO-DAT cells were first incubated with increasing concentrations of TAT-DATct1 for 10 min before adding mSIRK (10 μM). As shown in Figure 4a, after 15 min, mSIRK incubation resulted in a 10-fold increase in DA efflux. 1 μM of TAT-DATct1 significantly decreased DA efflux and higher concentrations (10 and 50 μM) totally inhibited DA efflux (Figure 4a). In cultured neurons, the TAT-DATct1 peptide (20 μM) completely abolished DA efflux induced by mSIRK (10 μM), whereas the control scrambled peptide had no effect (Figure 4b). Together, these results provide evidence that Gβγ subunits bind specifically to a motif within the carboxy-terminal region to promote DAT-mediated DA efflux.

Activation of a G-protein-coupled receptor induces DA efflux through a Gβγ-dependent mechanism

Finally, we examined whether the activation of a G-protein-coupled receptor was sufficient to induce DAT-mediated DA efflux through a mechanism involving Gβγ. CHO-DAT cells were transfected with the human muscarinic acethylcholine receptor 5 (hM5R), as a previous study showed DAT-mediated DA release in DAT-expressing SH-SY5Y cells following activation of Gαq-muscarinic receptors with carbachol.[22] Preliminary efflux experiments in CHO-DAT cells transfected with hM5R showed less baseline efflux of the DAT substrate [^{3}H]-MPP$^+$ when compared to [^{3}H]-DA; consequently, in these experiments, we preloaded the cells with [^{3}H]- MPP$^+$ and measured carbachol-induced efflux of [^{3}H]-MPP$^+$. Activation of the hM5R receptor with increasing concentrations of carbachol (20 min, RT) resulted in significant efflux of [^{3}H]-MPP$^+$ (Figure 5a). In a previous study, the effect of carbachol on DAT-mediated efflux was associated with PKC activation.[22] We evaluated if bisindolymaleimide-II (BIM-II), a potent PKC-inhibitor, altered the carbachol-induced efflux (Cchol 100 μM). BIM-II (1 μM) reduced efflux by ~ 30%. However, the addition of the Gβγ inhibitor gallein (25 μM) together with BIM-II completely reduced carbachol-stimulated MPP$^+$ efflux (Supplementary Figure S3). Since the effect of carbachol on [^{3}H]-MPP$^+$ efflux was abolished in the presence of gallein or in the presence of the Gβγ-binding peptide TAT-DATct1 (Figure 5b), our results suggest a major contribution from Gβγ. Taken together, these results suggest that the activation of M5R leads to DA release through DAT and that this process is dependent on Gβγ binding.

DISCUSSION

In this report, we demonstrate that the activation of an endogenous signaling pathway results in DA release through DAT. First, in heterologous expression systems and neurons, Gβγ activation by mSIRK increased DA efflux in a time and dose-dependent manner. This Gβγ-dependent DA efflux was blocked by DAT inhibitors, indicating that efflux occurs through DAT. Moreover, mSIRK-induced efflux could be blocked by the Gβγ inhibitor, gallein or Gβγ scavenger peptides, revealing Gβγ is responsible for the DA efflux. Next, we established two lines of evidence supporting the notion that Gβγ-induced DA efflux through DAT results from a direct interaction between the transporter and Gβγ subunits. First, inhibitors of Gβγ signaling pathways did not alter the ability of mSIRK to evoke DA efflux. Second, we showed that Gβγ binds specifically to residues 582–596 within the carboxy terminus of DAT and, more importantly, a TAT peptide containing this DAT-binding site (TAT-DATct1) attenuated mSIRK-induced DA efflux. Finally, we determined that the activation of muscarinic receptors results in DA efflux through a Gβγ-dependent and DAT-mediated process. Thus, taken together, the current study establishes that binding of Gβγ subunits to DAT induce DA efflux through the transporter.

The primary function of DAT is to clear DA from the extracellular space and limit the magnitude and duration of DA signaling in the brain. Deletion of the DAT gene in mice results in a approximately six-fold increase in extracellular DA leading to profound behavioral changes characterized by locomotor hyperactivity.[23] However, it has become evident over the past decade that DAT function is not limited to DA uptake. Ample evidence

indicates that plasma membrane neurotransmitter transporters—including DAT—can efflux transmitters from the cytoplasm to the extracellular space.[24] Indeed, non-vesicular release of glutamate, GABA or glycine through transporter-mediated efflux are mechanisms that contribute to neurotransmitter release under physiological and pathological conditions.[25] In the case of DAT, DA efflux through the transporter has been studied in the context of amphetamine action.[26] Different models have been proposed to describe the amphetamine-induced DA efflux through DAT. The first proposed model was the facilitated exchange diffusion mechanism,[27] which predicts that the transport of AMPH into the cell would facilitate the outward flux of DA. In this model, amphetamine translocation through the transporter (outward to inward) would increase the availability of the inward-facing carrier for DA binding and facilitate reverse transport of DA. This model has been challenged by experimental evidence revealing that the efflux of DA mediated by AMPH is more complex that the simple thermodynamic reversal of the carrier.[13,24,28,29] A channel-like transporter mode has been proposed to explain the outward flux of DA.[30] As currently understood, AMPH-induced DA efflux depends on intracellular concentrations of Na^+,[31] phosphorylation events at the N-terminus[32,33] and, more recently, interactions with anionic lipids like PIP_2.[14] However, despite significant efforts aimed at elucidating the amphetamine-mediated DA efflux through DAT, the identification of physiological mechanisms leading to DAT-mediated DA efflux is yet to be established. Previously, we demonstrated the functional effect of the DAT/Gβγ interaction as a reduction in DA uptake.[10] Here we propose that GPCR activation leads to free Gβγ-subunits near DAT, which bind to the carboxy terminus of the transporter driving it into an efflux mode. Because uptake and efflux have been shown to be independent processes that are unlikely to take place simultaneously, increasing the equilibrium towards carriers in efflux mode would result in an apparent reduction in uptake, which is what we have observed experimentally.

In the brain, DA release has been proposed to occur through two primary mechanisms: a fast transient, calcium-dependent, ‘quantal’ mechanism that occurs rapidly over milliseconds and results from neuronal firing and a slower, sustained, ‘efflux’ mechanism that occurs over minutes and has been suggested to be carrier-mediated.[12,34] Consistent with a physiological role for DAT-mediated efflux, several studies have documented DA efflux through DAT in the striatum and somatodendritic regions upon activation of G-protein-coupled receptors.[22,35] More recently, a rare DAT mutation, Ala559Val, has been found in individuals with ADHD, suggesting that efflux may play a role in DA homeostasis and alterations in efflux might be involved in brain conditions.[36] Characterization of this DAT mutation revealed normal transporter levels and uptake activity, but anomalous DA efflux. Cells expressing the DAT A559Val mutant exhibited spontaneous DA efflux, supporting the contention that efflux is not exclusive to the actions of releasers, such as amphetamine,[37] and that uptake and efflux mechanisms through DAT are functionally independent. In this context, our study is an important step towards the identification and characterization of intracellular signaling mechanisms associated with DA efflux through DAT.

Gβγ subunits participate in a number of signaling pathways involving ion channels and enzymes such as PLC, PI3K and MAPK.[21] Although we have evidence of the direct binding of Gβγ to the DAT carboxy terminus, we sought to determine if Gβγ-dependent DA efflux occurs secondarily to the activation of Gβγ-mediated signaling pathways. In our studies,

inhibitors of known Gβγ effectors did not alter mSIRK-induced DA efflux. These findings combined with the fact that the Gβγ-inhibitor gallein and the Gβγ-binding peptide DATct1 inhibited mSIRK-mediated efflux, suggest that Gβγ activation and binding directly promotes efflux. Together, our findings provide evidence that Gβγ activation regulates DAT activity by increasing DA efflux, and suggests that Gβγ promotes an efflux-willing state of the transporter.[32] We also provide initial evidence that the activation of a GPCR can lead to a Gβγ-dependent DA efflux. Furthermore, the cellular responses that occur following activation of a Gαq-coupled receptor are the result of multiple signaling pathways mediated by Gα and 'free' Gβγ. Gαq signaling activates PLC, leading to a reduction of PIP_2, elevation of intracellular Ca^{2+}, and activation of downstream kinases, including PKC. However, further studies are needed to determine the degree that these subsequent events in Gαq signaling can regulate DAT function. Nevertheless, our data suggest that Gβγ is a major contributor to the M5 receptor activation of DAT-mediated efflux. Thus far, DAT-mediated DA efflux has been associated with the pharmacological effects of amphetamine.[4] The present study suggests that the Gβγ-induced DA efflux may be an important mechanism to control dopaminergic tone under physiological conditions. More studies examining the mechanisms responsible for DA efflux in the brain are warranted. Ultimately, a better understanding of this mechanism and its physiological significance will increase our knowledge of the molecular details regulating DA homeostasis and may provide insights into neurological and psychiatric disorders involving DA dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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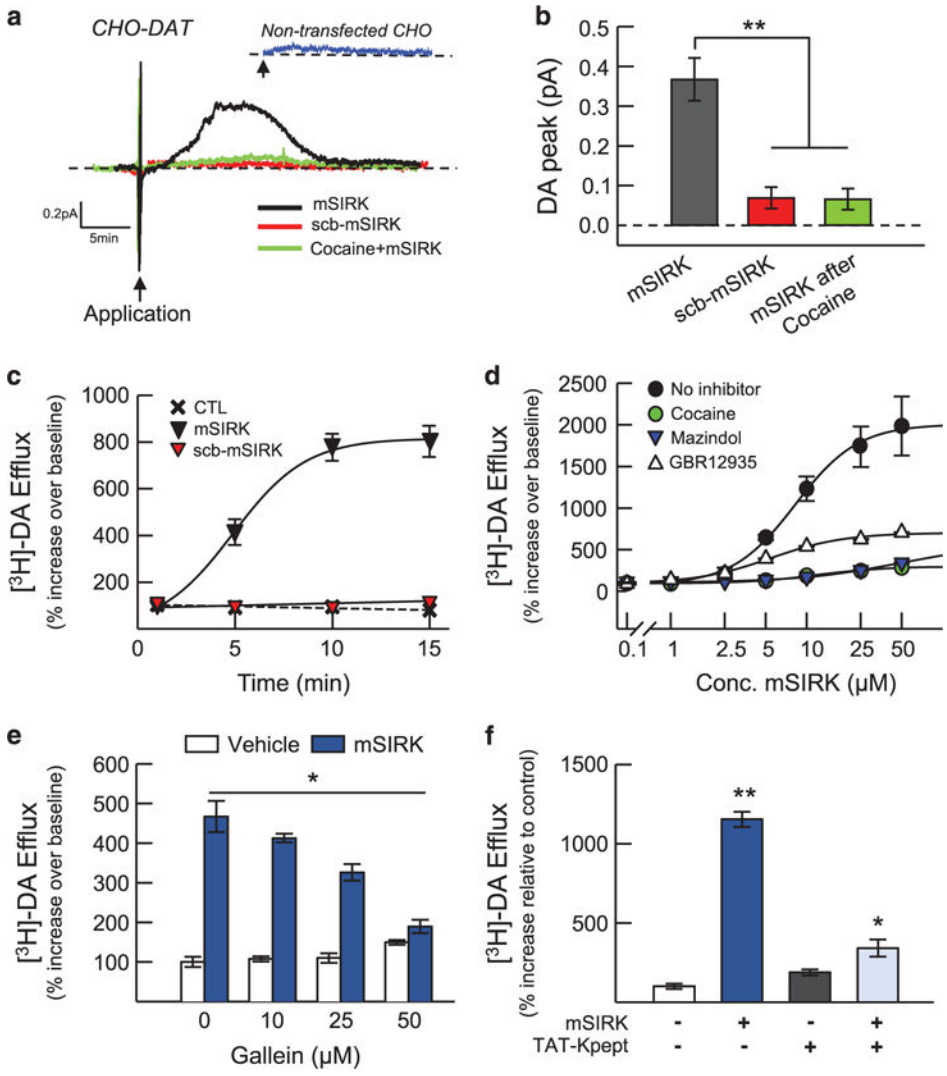

Figure 1.

Activation of Gβγ subunits by mSIRK promotes DA efflux in heterologous cells. (**a**) Amperometric currents obtained from DA-preloaded CHO and CHO-DAT cells after bath application of mSIRK (10 μM) and scb-mSIRK (10 μM). DAT was blocked with cocaine (20 μM) for 5 min followed by the application of mSIRK (10 μM). (**b**) Maximum DA current induced in CHO-DAT cells by mSIRK ($n = 9$), scb-SIRK ($n = 3$) and mSIRK after cocaine ($n = 3$). (**c**–**f**) Efflux assay in CHO-DAT cells preloaded with 0.02 μM [^{3}H]-DA. (**c**) Time-course of [^{3}H]-DA efflux in CHO-DAT cells with and without (CTL) mSIRK (20 μM), and scb-mSIRK (20 μM) ($n = 3$). (**d**) mSIRK dose response in the presence of cocaine (20 μM), mazindol (50 μM) or GBR12935 (10 μM) ($n = 6$). (**e**) Effect of Gβγ inhibition on the mSIRK-induced DA efflux. CHO-DAT cells were incubated with mSIRK (10 μv) in the absence or presence of gallein (10, 25, 50 μv) ($n = 3$). (**f**) Effect of the Gβγ scavenger peptide TAT-Kpept (20 μM) on mSIRK-induced DA efflux ($n = 3$). In **d**, **e** and **f**, released [^{3}H]-DA was measured 10 min after mSIRK. *P<0.05, **P<0.01

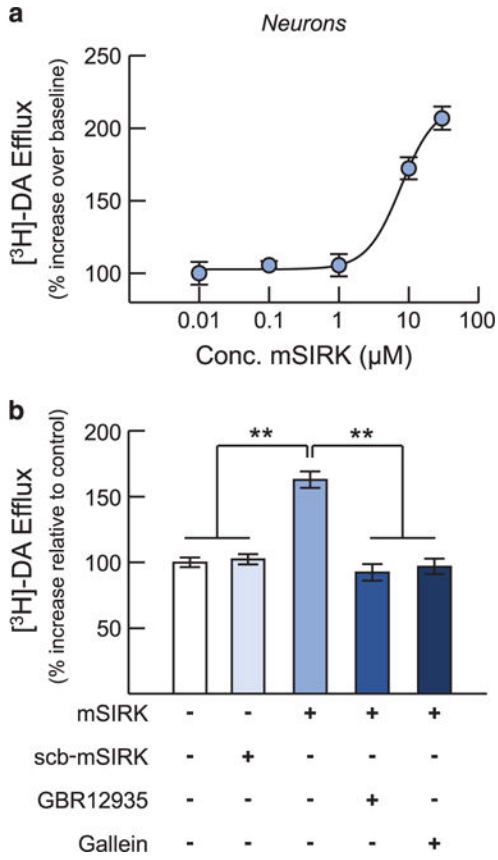

Figure 2.

Activation of Gβγ subunits by mSIRK promotes DA efflux in dopaminergic neurons. (**a**) Dose-dependent effect of mSIRK on DA efflux in primary cultures of DA neurons. [^{3}H]-DA efflux was measured 15 min after application of mSIRK (10 μM) (n = 3). (**b**) Effect of mSIRK (10 μM) and control peptide (scb-mSIRK, 10 μM) on [^{3}H]-DA efflux. mSIRK-induced efflux was tested in presence of the DAT inhibitor GBR12935 (0.5 μM) or the Gβγ inhibitor gallein (10 μM) (n = 4). ** P<0.01.

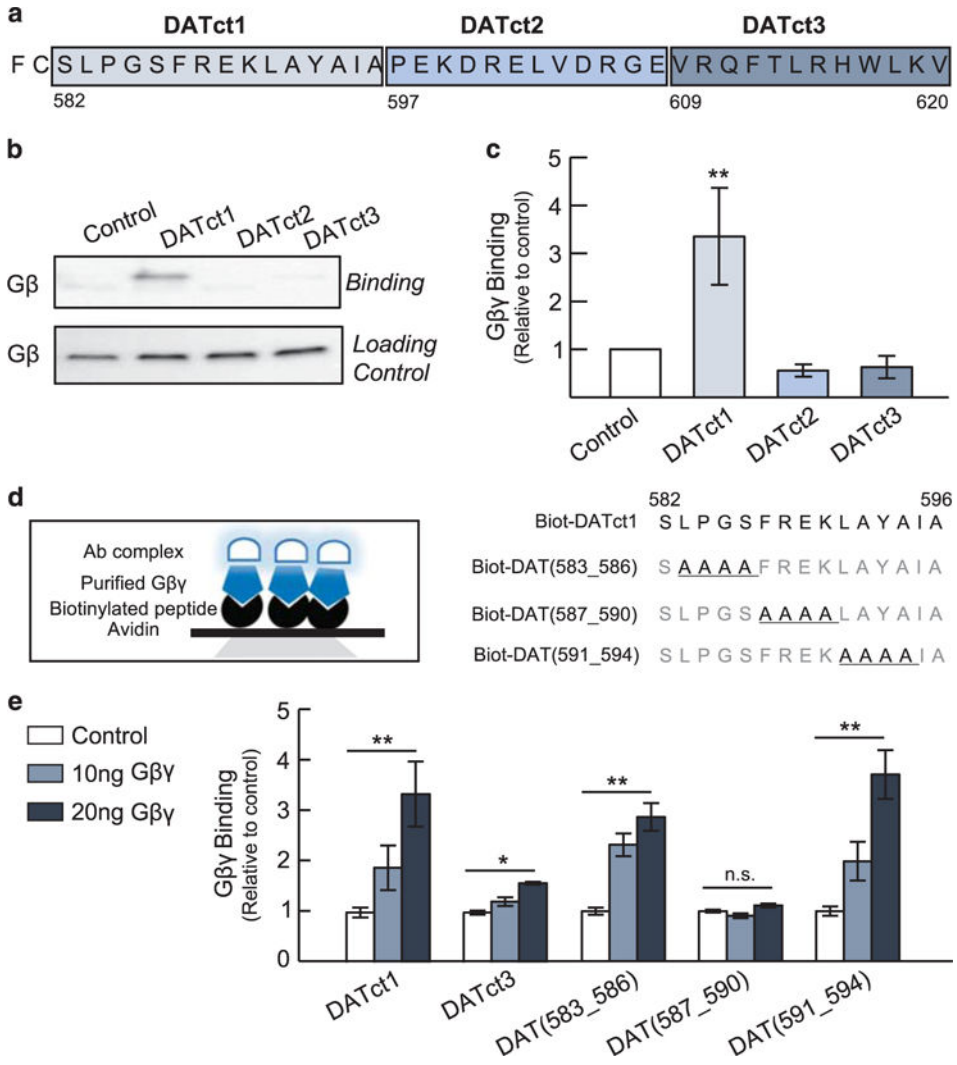

Figure 3.

Characterization of the Gβγ binding motif within the DAT carboxy-terminus. (**a**) Schematic representation of peptides corresponding to the carboxy terminus of hDAT. (**b**) Representative ($n = 4$) *in vitro* binding assay (I.B.). Biotinylated peptides were immobilized with Neutravinagarose resin, followed by incubation with purified Gβγ. Bound Gβγ was detected by SDS-PAGE followed by Gβ-immunoblotting. In lower panel, flow-through from binding assay represents the unbound Gβγ present in all samples as loading control. (**c**) Densitometric analysis of the *in vitro* binding assays ($n = 4$). Relative binding of Gβγ to biotinylated peptides was calculated relative to control group (no peptide bound) (**d**) Depiction of *in vitro* binding assay and sequences of DATct1 peptides. (**e**) Purified Gβγ was added to peptides immobilized on an avidin-coated microplate. For detection of bound Gβγ, a Gβ pan-antibody, Protein A-HRP-conjugated antibody and QuantaBlu kit was used. All results are shown as relative binding to group without Gβγ ($n = 3$). *P<0.05, **P<0.01.

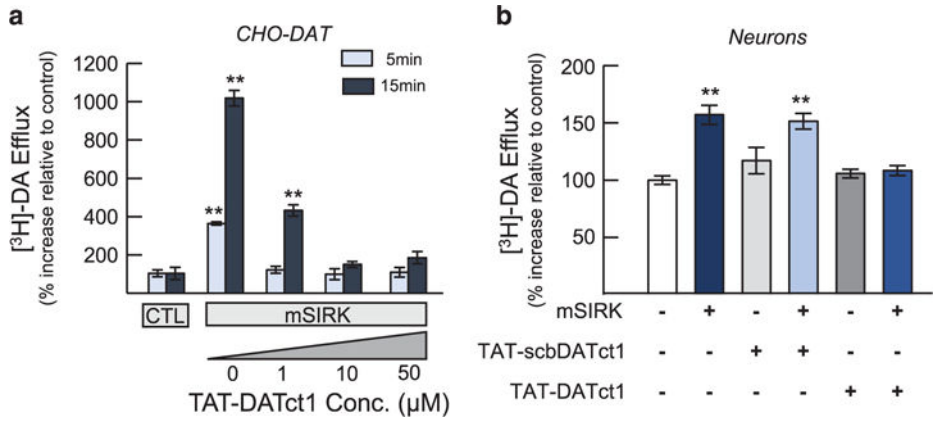

Figure 4.

A TAT-DATct1 peptide containing the DAT-Gβγ binding motif inhibits mSIRK-stimulated DA efflux. (**a**) Efflux of [^{3}H]-DA in CHO-DAT cells after application of mSIRK (10 μM) and increasing concentrations of TAT-DTct1 peptide ($n = 3$). (**b**) Preloaded dopaminergic neurons with [^{3}H]-DA were incubated for 15 min with different combinations of TAT-DATct1 peptide (20 μM) or TAT-scbDATct1 control peptide (20 μM) followed by mSIRK (20 μM) ($n = 5$). ** $P<0.01$.

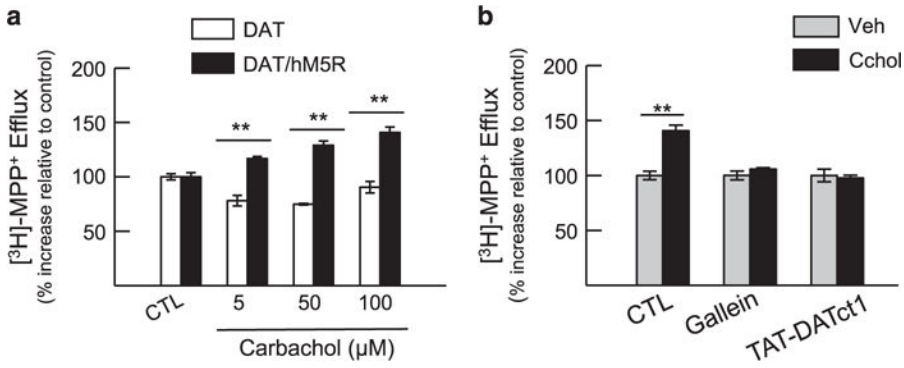

Figure 5.

Muscarinic receptor activation induces Gβγ-dependent DAT-mediated efflux. (**a**, **b**) [^{3}H]-MPP$^+$ efflux assay in CHO-DAT and CHO-DAT cells expressing hM5R. (**a**) [^{3}H]-MPP$^+$ efflux after the addition of increasing concentrations of carbachol. (n = 4). (**b**) Effect of gallein (20 μM) and TAT-DATct1 peptide (20 μM) on [^{3}H]-MPP$^+$ efflux induced by carbachol (Cchol, 100 μM) (n = 3, $^{**}P$<0.01).