Dopamine transporter cell surface localization facilitated by a direct interaction with the dopamine D2 receptor

Frank JS Lee^{1,6}, Lin Pei^{1,6}, Anna Moszczynska^{1,6}, Brian Vukusic¹, Paul J Fletcher^{1,2,3} and Fang Liu^{1,2,4,5,*}

¹Department of Neuroscience, Centre for Addiction and Mental Health, University of Toronto, Toronto, Ontario, Canada, ²Department of Psychiatry, University of Toronto, Toronto, Ontario, Canada, ³Department of Psychology, University of Toronto, Toronto, Ontario, Canada, ⁴Department of Physiology, University of Toronto, Toronto, Ontario, Canada and ⁵Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada

Altered synaptic dopamine levels have been implicated in several neurological/neuropsychiatric disorders, including drug addiction and schizophrenia. However, it is unclear what precipitates these changes in synaptic dopamine levels. One of the key presynaptic components involved in regulating dopaminergic tone is the dopamine transporter (DAT). Here, we report that the DAT is also regulated by the dopamine D2 receptor through a direct protein–protein interaction involving the DAT amino-terminus and the third intracellular loop of the D2 receptor. This physical coupling facilitates the recruitment of intracellular DAT to the plasma membrane and leads to enhanced dopamine reuptake. Moreover, mice injected with peptides that disrupt D2–DAT interaction exhibit decreased synaptosomal dopamine uptake and significantly increased locomotor activity, reminiscent of DAT knockout mice. Our data highlight a novel mechanism through which neurotransmitter receptors can functionally modulate neurotransmitter transporters, an interaction that can affect the synaptic neurotransmitter levels in the brain.

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Introduction

Neurotransmitter transporters are vital in regulating neurotransmission by facilitating the reuptake of released neurotransmitters. The monoamine neurotransmitter dopamine (DA), which plays a major role in regulating motor control, learning, reward and emotion (Girault and Greengard, 2004;

Wise, 2004), is altered in many neurological and neuropsychiatric disorders (Laruelle *et al*, 1996, 2003; Breier *et al*, 1997; Berke and Hyman, 2000; Lotharius and Brundin, 2002). However, the molecular mechanisms that lead to changes in synaptic DA levels remain elusive.

The dopamine transporter (DAT), a membrane-bound protein that facilitates the reuptake of extracellular DA and is a target for drugs of abuse including cocaine and amphetamine, represents a major presynaptic component involved in regulating dopaminergic tone. The potential involvement of the DAT in various disease states and its role in determining dopaminergic neurotransmission implicates the importance of regulating the DAT itself. Previous studies have shown that the DAT can be regulated through phosphorylation (Kitayama *et al*, 1994; Simon *et al*, 1997; Batchelor and Schenk, 1998; Pristupa *et al*, 1998); however, recent studies have shown that DAT function can be regulated through direct protein–protein interactions by intracellular proteins such as α -synuclein, PICK1 and Hic-5, suggesting that protein–protein interactions is an important mechanism in regulating DAT function (Lee *et al*, 2001; Torres *et al*, 2001; Carneiro *et al*, 2002).

The D2 receptor is a member of the G-protein-coupled receptor (GPCR) family. Two molecular isoforms, termed as D2Long (D2L) and D2Short (D2S), arise from alternative splicing that results in a 29-amino-acid insert within the third cytoplasmic loop of the D2L receptor (Dal Toso *et al*, 1989; Giros *et al*, 1989; Grandy *et al*, 1989). Recent studies suggest that the predominant D2 autoreceptor is expressed by the D2S isoform, whereas the D2L functions postsynaptically (Khan *et al*, 1998; Usiello *et al*, 2000; Centonze *et al*, 2002; Lindgren *et al*, 2003). Numerous studies have shown that presynaptic D2 receptors can function as autoreceptors, which allow an inhibitory feedback mechanism by altering DA synthesis, release, reuptake or a combination of the three in response to increasing levels of extracellular synaptic DA (Onali *et al*, 1992; Meiergerd *et al*, 1993; Parsons *et al*, 1993; Cass and Gerhardt, 1994; O'Hara *et al*, 1996; Iannazzo *et al*, 1997; Pothos *et al*, 1998; Jones *et al*, 1999; Kimmel *et al*, 2001). Although previous studies suggest that D2 receptors may be involved in regulating DAT function (Meiergerd *et al*, 1993; Cass and Gerhardt, 1994; Dickinson *et al*, 1999; Jones *et al*, 1999; Kimmel *et al*, 2001; Mayfield and Zahniser, 2001), the molecular pathway underlying the functional modulation of DAT by the D2 receptor has not been identified. As a member of the GPCR family, any D2 receptor-induced modulations are traditionally thought to be mediated through downstream activation of second messenger cascades. However, the recent identification of direct protein–protein interactions in the functional cross-talk between ligand gated ion channels and GPCRs (Liu *et al*, 2000; Lee *et al*, 2002) provides a new molecular avenue to account for the functional interaction between DAT and D2 receptors. Here, we

*Corresponding author. Department of Neuroscience, Centre for Addiction and Mental Health, 250 College Street, Toronto, Ontario, Canada M5T 1R8. Tel.: +1 416 979 4659; Fax: +1 416 979 4663; E-mail: fang_liu@camh.net

⁶These authors contributed equally to this work

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report a direct protein–protein interaction between the DAT and D2 receptor and the functional consequences of disrupting this interaction.

Results

D2 receptors and DAT form a protein complex

To determine the existence of the D2–DAT complex, we first examined if the DAT could co-immunoprecipitate with the D2 receptor. As depicted in Figure 1A, immunoprecipitation of the D2 receptor from rat striatal tissue resulted in the co-precipitation of the DAT, providing evidence for an interaction between the D2 receptor and DAT. Previous studies have

Figure 1 Identification of an interaction between the D2 receptor and DAT. (A) Immunoprecipitation of the D2 receptor leads to the co-precipitation of DAT from solubilized rat striatal tissue with 50 μ g of striatal extracts loaded as control. (B) Western blot for the DAT reveals affinity purification of the DAT by the GST fusion protein encoding D2_{IL3-2} but not with GST alone, D2_{IL3-1} or D2_{CT} GST fusion peptides. Western blots for the DAT reveal affinity purification of DAT by GST fusion protein encoding D2_{IL3-2-3} (C) and D2_{IL3-2-5} (D). Blots (A), (B), (C) and (D) depict a DAT-immunoreactive band that migrates at ~80 kDa. Western blots for the D2 receptor reveals affinity purification of D2 receptors by GST fusion proteins encoding DAT_{NT} (E) and DAT_{NT1} (F) but not by GST alone, DAT_{CT}, DAT_{NT2} or DAT_{NT3} GST fusion peptides. Blots (E) and (F) depict a D2-immunoreactive band that migrates at ~50 kDa. (G) *In vitro* binding reveals the direct binding of the *in vitro* translated peptide probe, [³⁵S]-D2_{IL3-2}, with GST-DAT_{NT1} but not with GST alone, GST-DAT_{NT2} or GST-DAT_{NT3}. (H) Inhibition of the direct binding of GST-DAT_{NT1} to [³⁵S]-D2_{IL3-2} upon the addition of DAT_{NT1-1} peptide (10 μ M, 30 min) but not with the DAT_{NT1-2} peptide. Apparent molecular weight markers are indicated in the figure.

shown that the intracellular domains of the D2 receptor and DAT contain putative consensus sequences for receptor phosphorylation and potential binding sites for various proteins important for signaling (Ng *et al*, 1994; Li *et al*, 2000; Kim *et al*, 2001; Lee *et al*, 2001; Torres *et al*, 2001; Carneiro *et al*, 2002; Zou *et al*, 2005). Therefore, to determine which regions of the D2 and DAT are involved in the formation of D2–DAT complex, various glutathione-S-transferase (GST) fusion proteins, encoding the third intracellular loop (IL3) and the carboxyl tail (CT) of the D2S receptor (GST-D2_{IL3-1}[K₂₁₁-K₂₄₁], GST-D2_{IL3-2}[E₂₄₂-Q₃₄₄] and GST-D2_{CT}[T₃₉₉-C₄₁₄]; Supplementary Figure 1) were prepared and utilized in affinity purification assays. Only GST-D2_{IL3-2} and not GST-D2_{IL3-1}, GST-D2_{CT} or GST alone, precipitated DAT from solubilized rat striatum (Figure 1B), indicating that the DAT can interact with a discrete region of the D2 receptor third intracellular loop. In order to confirm these results and to further delineate the region of the D2_{IL3-2} involved in the D2–DAT interaction, three GST-fusion proteins (D2_{IL3-2-1}[E₂₄₂-P₂₇₁], D2_{IL3-2-2}[S₂₅₉-I₃₁₁], D2_{IL3-2-3} [E₂₉₇-Q₃₄₄]) encoding truncated regions of D2_{IL3-2} were constructed. Affinity purification assays showed that GST-D2_{IL3-2-3}, but not GST-D2_{IL3-2-1} or GST-D2_{IL3-2-2}, was able to precipitate solubilized DAT (Figure 1C), suggesting that E₂₉₇-Q₃₄₄ region of the D2 receptor is essential for the interaction with the DAT. Given that D2_{IL3-2-2} (S₂₅₉-I₃₁₁) and D2_{IL3-2-3} (E₂₉₇-Q₃₄₄) regions share overlapping sequence (E₂₉₇-I₃₁₁) and GST-D2_{IL3-2-2} failed to interact with DAT, we subsequently examined whether the I₃₁₁-Q₃₄₄ motif is sufficient for the interaction between D2 and DAT. As confirmed in Figure 1D, only GST-D2_{IL3-2-5}(I₃₁₁-Q₃₄₄) but not GST-D2_{IL3-2-4}(E₂₉₇-I₃₁₁) precipitated DAT from solubilized striatal extract, indicating the I₃₁₁-Q₃₄₄ is required to form D2–DAT complex. To locate the interacting site on DAT, GST fusion proteins encoding the amino-terminus (NT) and the CT of the DAT (GST-DAT_{NT}[M₁-D₆₈] and GST-DAT_{CT}[L₅₈₃-V₆₂₀]) were used in affinity purification assays. These results revealed that the sequence encoded by the DAT_{NT} facilitates the interaction with D2 receptors as only the GST-DAT_{NT} but not GST-DAT_{CT} or GST alone was able to pull down D2 receptors (Figure 1E). Further experiments show that GST-DAT_{NT1}[M₁-P₂₆], but not the GST-DAT_{NT2}[A₁₆-T₄₃] or GST-DAT_{NT3}[K₃₅-D₆₈], can successfully pull down D2 receptors from solubilized rat striatum (Figure 1F). Taken together, it appears that the DAT_{NT1}[M₁-P₂₆] region of the DAT and D2_{IL3-2-5}[I₃₁₁-Q₃₄₄] region of the D2 receptor are responsible for mediating the interaction between these two proteins.

Direct protein–protein interaction between D2 receptor and DAT

Although these results demonstrated the presence of the D2–DAT complex in rat striatal tissue, it did not clarify whether the D2–DAT complex is mediated by a direct physical coupling or indirect interaction involving accessory binding proteins. *In vitro* binding assay results demonstrated that [³⁵S]D2_{IL3-2} hybridized with GST-DAT_{NT1} but not with GST-DAT_{NT2} or GST-DAT_{NT3}, suggesting a direct D2–DAT interaction (Figure 1G). However, these three GST fusion proteins share overlapping regions of the DAT_{NT} to minimize the chance of disrupting the binding motif. To delineate sequences within the DAT_{NT1} that are involved in the D2–DAT interaction, we synthesized two peptides that are truncated versions of DAT_{NT1}: DAT_{NT1-1}[M₁-V₁₅] and DAT_{NT1-2}[A₁₆-P₂₆]. The *in vitro* binding between the [³⁵S]D2_{IL3-2} and GST-DAT_{NT1}

Figure 2 D2 receptor coexpression upregulates DAT-mediated DA uptake. (A) Representative graph that demonstrates that coexpressing D2S receptor and DAT in HEK-293 cells induces an increase in the $V_{\max}$ for DA uptake accumulation by $34 \pm 10\%$ (t -test, $*P < 0.05$; $n = 6$), with no significant alteration in the estimated K_m values (see text). (B) Pretreatment with $10 \mu\text{M}$ haloperidol for 30 min at 37°C did not affect the upregulation of DAT activity upon coexpression of the D2S receptor. The control group exhibited a $V_{\max}$ of $3.8 \pm 0.1 \text{ pmol}/10^5 \text{ cells}/\text{min}$. Data analyzed by one-way ANOVA, *post hoc* SNK test, $*P < 0.05$, compared with control group, $P < 0.05$, $n = 3$. (C) Immunoblot analysis of lysates from HEK-293 cells ($50 \mu\text{g}$ protein) that were cotransfected with either DAT/pcD or with DAT/D2S revealed no changes in whole-cell DAT protein levels (immunoreactive band that migrates at $\sim 80 \text{ kDa}$), and confirmed D2S receptor expression (a $\sim 50 \text{ kDa}$ immunoreactive band is shown). (D) Overexpression of DAT_{NT1} mini-gene together with DAT and D2S in HEK-293 cells blocked the increase of DAT uptake by D2S receptors, whereas DAT_{NT2} and DAT_{NT3} mini-genes had no effect. The control group exhibited a $V_{\max}$ of $2.2 \pm 0.12 \text{ pmol}/10^5 \text{ cells}/\text{min}$. (E) The DAT_{NT1-1} but not DAT_{NT1-2} mini-gene abolished D2S receptor-induced increase in DAT uptake. The control group exhibited a $V_{\max}$ of $2.6 \pm 0.18 \text{ pmol}/10^5 \text{ cells}/\text{min}$. (F) Coexpression of the D2 mini-gene (MG) encoding amino acids I₃₁₁-Q₃₄₄ is able to inhibit the upregulation of DAT activity by coexpression of the D2S receptor. The control group exhibited a $V_{\max}$ of $2.4 \pm 0.21 \text{ pmol}/10^5 \text{ cells}/\text{min}$. Data in (D), (E) and (F) were analyzed by one-way ANOVA, *post hoc* SNK test, $*P < 0.05$, $n = 3-4$.

is disrupted by co-incubation with the purified DAT_{NT1-1} (M₁-V₁₅) peptide but not with the DAT_{NT1-2}[A₁₆-P₂₆] peptide (Figure 1H). These results provide evidence that the D2–DAT direct interaction is dependent on sequences located at the very beginning of the DAT NT. Taken together, these data support the existence of a direct protein–protein interaction occurring between the D2 receptor and DAT and confirm the role of DAT M₁-V₁₅ in the DAT–D2 direct protein–protein interaction.

D2S receptors enhance DAT-mediated DA uptake through the D2–DAT direct protein–protein interaction

To determine the functional relevance for the D2–DAT interaction, we examined how D2 receptors modulate DAT-mediated DA uptake in HEK-293 cells coexpressing D2S receptors and DAT. As the D2S receptor is reported to be the predominant presynaptic D2 receptor (Khan *et al*, 1998; Uziel *et al*, 2000; Centonze *et al*, 2002; Lindgren *et al*, 2003), we used this isoform in our cotransfection experiments. The DAT-mediated DA uptake was significantly increased in

HEK-293 cells cotransfected with D2S receptors relative to cells cotransfected with the empty expression vector pcD, with the estimated $V_{\max}$ for DAT-mediated [^3H]DA uptake being enhanced by approximately 34% (DAT/pcD: 2.16 ± 0.2 ; DAT/D2S: $2.85 \pm 0.4 \text{ pmol}/10^5 \text{ cells}/\text{min}$; $n = 6$), with no significant change in the estimated K_m of the DAT in D2S coexpressing cells (DAT/pcD: 6.56 ± 2.0 and DAT/D2S: $5.26 \pm 1.5 \mu\text{M}$; $n = 6$) (Figure 2A). Pretreatment of cells coexpressing DAT/D2S with D2 receptor antagonists did not affect the D2 receptor's ability to upregulate DAT activity (Figure 2B). Furthermore, we confirmed that the D2S receptor coexpressed in HEK-293 cells were functional as indexed by cAMP assays measuring the inhibition of forskolin-mediated cAMP production upon D2 receptor activation (Supplementary Figure 2A). The apparent enhancement of cellular DA uptake was not due to a result of increased DAT expression levels, as Western blot analysis of DAT protein levels were not significantly different between the DAT/pcD and DAT/D2S cotransfected cells (Figure 2C). In addition, the estimated $B_{\max}$ of DAT, as indexed by the saturable binding of

[³H]CFT in permeabilized cells, was not significantly different between cells cotransfected with DAT/pcD and DAT/D2S (Supplementary Figure 2B).

To investigate whether the observed D2 receptor-mediated enhancement of DA uptake is the consequence of D2–DAT protein complex formation, in HEK-293 cells coexpressing D2S and DAT, we overexpressed mini-genes encoding DAT sequences that are responsible for the interaction with D2 receptors. Overexpression of the mini-gene encoding DAT_{NT1} but not DAT_{NT2} or DAT_{NT3} blocked the ability of the D2S receptor to enhance DAT-mediated DA uptake in cells coexpressing the D2S and DAT (Figure 2D). Coexpression of these mini-genes with DAT alone did not affect DAT activity. Furthermore, overexpression of the DAT_{NT1-1} mini-gene, which encodes a peptide that has been shown to disrupt the D2–DAT interaction (Figure 1H), significantly blocked the D2S receptor-mediated enhancement in DAT uptake (Figure 2E), providing evidence that sequences M₁–V₁₅ of the DAT mediates the direct protein–protein interaction of D2–DAT and facilitates the D2S-dependent enhancement of DAT-mediated DA uptake. In addition, coexpression of a mini-gene that corresponds to sequences with the D2 third intracellular loop (I₃₁₁–Q₃₄₄) exhibited an ability to block the upregulation in DAT activity by the coexpression of the D2S receptor (Figure 2F).

As the region within the D2 receptor that was identified to facilitate the direct interaction with DAT is common to both the D2S and D2L variants of the D2 receptor, we predicted that coexpression of the D2L receptor would also induce an upregulation of DAT activity. Surprisingly, cells transfected with D2L did not exhibit a significant increase in DAT activity yet was able to demonstrate significant co-immunoprecipitation (Supplementary Figure 2D and E).

Although D2 receptor antagonist pretreatment indirectly demonstrates that the increase in DAT activity upon coexpression of the D2S receptor is not attributable to D2 receptor activation (Figure 2B), it still does not address whether D2 receptor activation or inhibition has any effect on the physical coupling between the DAT and the D2 receptor. To clarify this issue, we examined the co-immunoprecipitation of DAT with the D2 receptor from striatal tissue of rats injected with either quinpirole (D2 receptor agonist; 1.0 mg/kg) or haloperidol (D2 receptor antagonist; 0.1 mg/kg). Western blots revealed that rats treated with either quinpirole or haloperidol exhibited no significant change in either total DAT levels (Figure 3A and D) or D2 levels (Figure 3A and C). Furthermore, the co-immunoprecipitation of D2 receptor with the DAT was not affected by quinpirole or haloperidol administration (Figure 3B and E). In addition, treatment of HEK-293 cells coexpressing the DAT and D2S receptor with 1 μ M phorbol

Figure 3 The D2–DAT physical interaction is agonist independent. Male Wistar rats (250–300 g) were injected s.c. with saline, 1.0 mg/kg quinpirole or 0.1 mg/kg haloperidol (Hooper *et al*, 1997; Horvitz *et al*, 2001; Kapur *et al*, 2003). One hour after injection rats were killed, brains extracted and striatum dissected. **(A)** Western blot analysis reveals that there were no changes in either DAT levels or D2 levels between control, quinpirole or haloperidol treated rats (one-way ANOVA; DAT, $P=0.538$ and D2, $P=0.130$; $n=4$). **(B)** Furthermore, co-immunoprecipitation results reveal no significant change in the D2–DAT interaction between rats treated with quinpirole or haloperidol compared with control (one-way ANOVA, $P=0.104$, $n=4$). Quantification of the Western blots for the D2, DAT and D2–DAT are shown in **(C)**, **(D)** and **(E)**, respectively.

12-myristate 13-acetate (PMA) to induce the activation of PKC did not affect the co-immunoprecipitation of DAT with the D2 receptor (Supplementary Figure 2F).

D2 receptors recruit DAT to plasma membrane through the D2–DAT direct protein–protein interaction

The observed enhancement of DA uptake by coexpression of D2S receptors, an effect not associated with an increase in DAT protein levels, suggests the possibility that the observed increase in DAT function may result from the recruitment of an intracellular pool of DAT to the plasma membrane. Confocal immunofluorescence microscopy of HEK-293 cells expressing the DAT alone (Figure 4A, top left panel) indicated that the DAT is expressed quite diffusely throughout the cell. Upon coexpression with D2S receptors, however, the widespread diffuse intracellular distribution of DAT was substantially diminished and instead, DAT immunoreactivity was located primarily at the cell surface (Figure 4A, bottom left panel). However, treatment of cells coexpressing DAT and the D2S receptor with the interfering TAT-DAT_{NT1-1} peptide (10 μ M, 30 min), DAT localization became more diffuse with less distinct cell surface localization and significantly more intracellular distribution (Figure 4B, middle panel). Both DAT_{NT1-1} and DAT_{NT1-2} peptides were rendered cell-permeant by incorporating the cell membrane transduction TAT domain of the human immunodeficiency virus type 1 (HIV-1) (Schwarze *et al*, 1999; Aarts *et al*, 2002). Dansyl chloride was conjugated to the TAT-DAT_{NT1-1} and TAT-DAT_{NT1-2} peptides to verify the intracellular accumulation of TAT peptides by fluorescence microscopy (data not shown). Both TAT and TAT-DAT_{NT1-2} peptides had no significant effect on DAT cell surface localization (Figure 4B, top and bottom panels).

Quantification of cell surface DAT, using cell ELISA assays, revealed an approximately 20% increase in DAT plasma membrane localization by co-expression of D2S receptors (Figure 4C). Furthermore, this process could be blocked by overexpression of the DAT_{NT1-1} but not the DAT_{NT1-2} mini-gene. Similarly, in midbrain neurons infected with both DAT and D2 adenoviruses, application of the TAT-DAT_{NT1-1} peptide significantly reduced DAT membrane expression, whereas the TAT-DAT_{NT1-2} peptide did not affect DAT membrane expression (Figure 4D). These data suggest that the D2 receptor enhances DAT membrane expression through the D2–DAT direct protein–protein interaction and the enhanced DAT-mediated DA uptake may be a result of increased DAT plasma membrane expression.

The enhancement of DAT-mediated DA uptake and plasma membrane expression is dependent on membrane fusion exocytosis

Previous studies have shown that *Clostridium* tetanus toxin (TeTx) selectively cleaves vesicle-associated membrane protein (VAMP) and prevents exocytosis (Maletic-Savatic and Malinow, 1998; Hua and Charlton, 1999). We repeated DA uptake and cell surface ELISA experiments after impairing the fusion of intracellular vesicles with the plasma membrane using TeTx. We confirmed that pretreatment with TeTx (100 ng/ml) for 48 h cleaved the v-SNARE synaptobrevin/VAMP2 in neurons. VAMP2 cleavage occurred upon preincubation with TeTx (Figure 5A) and under the same TeTx pretreatment condition, the cleavage of VAMP2 significantly decreased both the DAT membrane expression (Figure 5B)

Figure 4 Disruption of the D2–DAT interaction blocks DAT upregulation by D2S coexpression. (A) Confocal microscopy of HEK-293 cells expressing D2S, DAT or both. Cells cotransfected with the DAT and empty expression vector pcD exhibited diffuse immunolabelling of the DAT throughout the cell (top left panel). Coexpression of the D2S receptor and DAT revealed significant colocalization of both proteins localized at the cell surface (lower panel). D2S receptor localization does not appear to be significantly different between cells coexpressing the D2S receptor with either the empty expression vector pcD (top middle panel) or with DAT (lower middle panel). (B) Confocal microscopy of HEK-293 cells coexpressing the D2S receptor and DAT treated with TAT, TAT-DAT_{NT1-1} or TAT-DAT_{NT1-2} (10 μ M, 30 min). Whereas cells treated with both TAT and TAT-DAT_{NT1-1} (top and bottom panels) exhibited distinct DAT localization at the cell surface, treatment with the TAT-DAT_{NT1-1} led to significant reduction in distinct DAT cell surface localization, with more diffuse intracellular localization (middle panel). (C) Quantification of DAT cell surface localization in HEK-293 cells coexpressing D2S receptor and DAT reveals an approximately 21% increase in DAT cell surface localization, compared with cells coexpressing DAT with pcD, an effect blocked only by the coexpression of DAT_{NT1-1} (one-way ANOVA, *post hoc* SNK test, * P < 0.05, n = 6). (D) Quantification of DAT cell surface localization in midbrain neurons infected with DAT and D2 recombinant adenovirus. Neurons pretreated with TAT-DAT_{NT1-1} but not with TAT-DAT_{NT1-2} peptides (10 μ M, 30 min) exhibited significantly decreased DAT cell surface localization (one-way ANOVA, *post hoc* SNK test, * P < 0.05, n = 6).

Figure 5 D2 receptors regulate DAT plasma membrane localization. Upregulation of DAT-mediated DA uptake and cell surface localization is mitigated by tetanus toxin (TeTx) treatment. (A) Western blot of VAMP2 from solubilized midbrain neurons pretreated with TeTx (100 ng/ml) for 48 h. Lysates from untreated neurons exhibited VAMP2 immunoreactivity that is absent in TeTx-treated neurons. Tetanus toxin decreased the DAT-mediated DA uptake (control group exhibited a $V_{\max}$ of 42 ± 7 pmol/ 10^5 cells/min) (B) and DAT membrane expression (C) in midbrain neurons (t -test, $*P < 0.05$, $n = 3-4$). (D) Biotinylation of HEK-293 cell surface proteins after treatment with 10 μ M TAT peptides for 30 min at 37°C reveals an increase in DAT at the cell surface upon coexpression of the D2S receptor. This increase was blocked by treatment with the TAT-DAT_{NT1-1} peptide. (E) Endocytosis of cell surface proteins upon treatment with 10 μ M TAT control or TAT-DAT_{NT1-1} peptides for 30 min at 37°C reveals no significant difference in DAT endocytosis levels upon TAT treatment. Stripping efficiency of cell surface biotin was measured in parallel compared with control cells. Both blots in (D) and (E) depict a ~80 kDa DAT-immunoreactive band.

and DAT-mediated uptake (Figure 5C) in midbrain neurons infected with both D2 and DAT adenoviruses. Tetanus toxin pretreatment did not show significant effect on DAT membrane expression or DAT uptake in cultured midbrain neurons infected with DAT adenovirus only (data not shown). These data suggest that D2 receptors may modulate DAT function through a membrane fusion-dependent exocytotic process.

To corroborate these results, we performed a series of biotinylation experiments to address whether the increase in DAT at the cell surface is due to increased plasma membrane insertion or decreased endocytosis. Using HEK-293 cells cotransfected with either DAT/pcD or DAT/D2S, cells were pretreated with either TAT control peptide or TAT-DAT_{NT1-1} peptide upon which cell surface proteins were biotinylated. As shown in Figure 5D, cells cotransfected with D2S exhibited higher levels of DAT at the cell surface compared with cells cotransfected with DAT/pcD. However, upon treatment with TAT-DAT_{NT1-1}, there was a significant decrease in DAT at the cell surface in cells coexpressing DAT/D2S. To measure endocytosis, cells were first labeled with biotin and then treated with TAT peptides, after which all cell surface biotin was cleaved, leaving only proteins that were endocytosed still labeled with biotin. In these experiments, there was no significant difference in the levels of DAT endocytosis in cells that were treated with TAT control peptide and the TAT-DAT_{NT1-1} peptide (Figure 5E). These data suggest that D2–DAT interaction facilitates the insertion

of DAT into the plasma membrane as opposed to decreased endocytosis.

Disruption of the D2–DAT interaction in midbrain neurons decreases DA reuptake

To verify the functional relevance for the observed direct protein–protein interaction between the D2 receptor and DAT in a relevant cellular milieu, we examined the effect of perturbing the interaction in primary cultures of rat midbrain neurons on DAT-mediated DA reuptake. Owing to the low expression of both DAT and D2 receptors, we infected midbrain neurons with DAT and D2 receptor recombinant adenoviruses. D2–DAT interaction was disrupted by treating midbrain neurons with the TAT-DAT_{NT1-1} interfering protein peptide. Application of the TAT-DAT_{NT1-1} peptide to midbrain neurons significantly decreased both D2–DAT protein–protein coupling (Figure 6A), and DAT-mediated DA uptake (Figure 6B), whereas the TAT-DAT_{NT1-2} peptide did not have any significant effect. These results suggest that the D2 receptor can functionally interact with DAT and that disruption of this interaction leads to a significant decrease in DAT-mediated DA reuptake in neurons.

Disruption of D2–DAT direct protein–protein interaction induces hyperlocomotor activity

The impaired D2–DAT interaction leads to decreased DAT-mediated DA uptake, which can cause an accumulation of synaptic DA. Therefore, we predicted that disruption of the D2–DAT interaction would induce hyperlocomotor activity. Thus, we examined locomotor activity in mice treated with TAT-DAT_{NT1-1} peptide, which interferes with the D2–DAT interaction in neurons (Figure 6A). Consistent with previous studies that have shown that systemically injected TAT peptide penetrates the blood–brain barrier 1 h after injection (Schwarze *et al*, 1999; Aarts *et al*, 2002), we have also observed TAT localization in mice brains following intraperitoneal injections (Supplementary Figure 3). Correlating with the observation that TAT-DAT_{NT1-1} decreases DAT-mediated DA reuptake (Figure 6B), striatal synaptosomal DA uptake experiments from mice injected with purified TAT-DAT_{NT1-1} displayed significantly decreased DA uptake compared with synaptosomes for mice injected with TAT control peptides (Figure 6C). Furthermore, mice injected with purified TAT-DAT_{NT1-1} displayed significantly increased distance traveled and rearing compared with mice treated with TAT peptide or TAT-DAT_{NT1-2} (Figure 6C–F). These observations are similar to the hyperactivity exhibited by DAT knockout mice (Giros *et al*, 1996) that exhibited a ~2.5-fold increase in spontaneous activity in a 3 h period, compared with wild-type mice. In our study, TAT-DAT_{NT1-1}-injected mice exhibited a ~1.5-fold increase in horizontal locomotor activity compared with mice injected with TAT control peptide.

Discussion

The regulation of DAT has important functional consequences, given the role of synaptic dopamine in neuronal motor control, cognition, event prediction and emotion (Girault and Greengard, 2004; Wise, 2004). Our present study provides evidence for a direct protein–protein interaction between the D2 receptor and DAT. The D2–DAT coupling may constitute an important event that is involved in

Figure 6 Disruption of the D2–DAT interaction leads to decreased DA reuptake in neurons. **(A)** Neurons treated with TAT-DAT_{NT1-1} but not TAT-DAT_{NT1-2} or TAT alone (10 μM, 30 min) exhibited a significant decrease in the co-immunoprecipitation of DAT with the D2 receptor. **(B)** Midbrain neurons infected with DAT and D2 recombinant adenovirus exhibited a significant decrease in [³H]DA uptake after pretreatment with TAT-DAT_{NT1-1} peptides but not with TAT-DAT_{NT1-2} peptides. Data were analyzed by one-way ANOVA, *post hoc* SNK test (**P* < 0.05, *n* = 3–6). **(C)** Striatum of mice injected i.p. with peptides (TAT, TAT-DAT_{NT1-1}, 3 nmol/g) were harvested and used to prepare synaptosomal preparations to measure [³H]DA uptake. Mice injected with TAT-DAT_{NT1-1} exhibited a significant decrease in DA uptake compared with mice injected with TAT control peptides (*t*-test, **P* < 0.05, *n* = 5). TAT peptides effect on locomotor behavior over a 3-h session was also measured. **(D)** and **(E)** show distance traveled (cm); **(F)** and **(G)** show vertical activity as a measure of rearing. Measurements shown in **(D)** and **(F)** are the mean total distance/counts over the 3-h test period. **(E)** and **(G)** illustrate the time course of TAT, TAT-DAT_{NT1-1} and TAT-DAT_{NT1-2} administration (3 nmol/g, i.p.). One-way ANOVA, followed by *post hoc* SNK test, showed significant treatment effects on distance traveled and rearing (*n* = 8, ***P* < 0.01 TAT versus TAT-DAT_{NT1-1}).

regulating synaptic DA levels and, thus, normal dopaminergic neurotransmission. It is tempting to speculate that this interaction provides a mechanism by which a population of DAT can be targeted to active dopaminergic terminals by tethering to a distinct pool of presynaptic D2S receptors. Moreover, even in the presence of D2 antagonists, D2 receptor coexpression induces upregulation of DAT activity, which suggests that the D2–DAT interaction is not dependent on D2 receptor activation. However, this does not preclude the possibility that the D2–DAT interaction may be regulated by protein phosphorylation or other post-translational events. *In vivo* voltammetry studies have demonstrated that the D2 receptor can increase DAT-mediated DA clearance (Meiergerd *et al*, 1993; Cass and Gerhardt, 1994), and the coexpression of D2

receptors and DAT in oocytes has also demonstrated an increase in [³H]DA uptake upon D2 receptor activation, paralleled by an increase in DAT at the cell surface (Mayfield and Zahniser, 2001). These studies show an upregulation in DAT clearance upon D2 receptor activation that is likely to be mediated by downstream signaling events. However, our study characterizes a direct D2–DAT interaction that facilitates DAT upregulation independent of D2 receptor activation. Interestingly, a recent study by Wu *et al* (2002) suggests that uptake regulating dopamine autoreceptors operate continuously, whereas downregulation of DA release by autoreceptors is linked to low stimulation frequency. Moreover, *in vivo* electrochemistry studies reveal that D2-null mice exhibit decreased DAT function, as indexed by

a 50% decrease in DA clearance compared with wild-type mice (Dickinson *et al*, 1999).

We have characterized that the DAT directly couples to the third intracellular loop of D2 receptors. Although we have almost exclusively used the D2S isoform in experiments involving transfected cells or GST fusion proteins, as the DAT coupling site located on the D2 receptor occurs in an area common for both D2S and D2L, we would not have been surprised if the D2L receptor was also capable of enhancing DAT uptake via a direct protein–protein interaction with DAT. However, we have shown that cells coexpressing DAT/D2L did not exhibit a significant increase in DAT uptake, yet are capable of interacting with the DAT (Supplementary Figure 2D and E). It is currently unclear what role the D2L isoform has with respect to the D2–DAT interaction, but the D2L receptor may potentially regulate the functional effects of the D2–DAT interaction by acting as a negative modulator. Nevertheless, the predominant presynaptic localization of both the D2S receptor and the DAT would strongly suggest that the D2S receptor is the physiologically relevant isoform capable of physically interacting with the DAT, a concept that would be consistent with previous findings that D2L and the D2S serve distinct physiological functions owing in part to differential localization (Khan *et al*, 1998; Usiello *et al*, 2000; Centonze *et al*, 2002; Lindgren *et al*, 2003). Although D2 receptor knockout mice do not exhibit elevated dopamine levels (Dickinson *et al*, 1999), which at the surface would appear to contradict our model, this may be accounted for by the diversity of proteins capable of regulating the DAT (e.g. PICK1, Hic-5 and α -synuclein). Even though D2S receptor coexpression has a significant effect on DAT function, transporters are capable of reaching the cell surface in the absence of the D2 receptor. Thus, we speculate that a proportion of D2 receptors can interact with a pool of DAT, which allows for directed insertion of transporters to the plasma membrane and allow for the proteins to be localized in close spatial proximity to be regulated by downstream signaling cascades.

The direct D2–DAT interaction provides a molecular pathway by which D2 receptors regulate DAT and may also contribute to our understanding of how both these dopaminergic proteins may be involved in disorders involving hyperdopaminergia. Disruption of this interaction may lead to impaired clearance of extracellular DA and may possibly lead to hyperdopaminergia. In fact, we have shown that disruption of the D2–DAT direct protein–protein interaction results in hyperlocomotion in mice, reminiscent of DAT knockout mice (Giros *et al*, 1996). After being injected with the TAT-DAT_{NT1-1} peptide, which is capable of disrupting the D2–DAT interaction, mice appear to remain hyperactive for a period of 90 min, consistent with a previous study that describe a waning of intracellular TAT peptide accumulation after 90 min (Schwarze *et al*, 1999; Aarts *et al*, 2002). Given our *in vitro* data that demonstrate that the TAT-DAT_{NT1-1} peptide can disrupt the D2–DAT interaction in neurons, leading to a reduction in DA reuptake and that striatal synaptosomes prepared from mice injected with TAT-DAT_{NT1-1} exhibit decreased DA uptake, we speculate that the TAT-DAT_{NT1-1} peptide treated mice exhibit hyperlocomotor activity owing to an increase in synaptic DA levels in the striatum from disrupting the D2–DAT interaction. A caveat to this conclusion is that i.p. administration allows for the peptide to be delivered throughout the brain, thus introducing the

possibility that the behavior may have resulted from an effect in a different region of the brain. However, striatal synaptosomes prepared from mice injected with TAT-DAT_{NT1-1} exhibited decreased DA uptake, which we cautiously attribute to the disruption of the D2–DAT interaction in the striatum.

Our present study provides the first evidence showing the direct functional interaction between the D2 receptor and DAT. We have shown that the recruitment of DAT to the plasma membrane, which is essential for DAT functionality, is promoted by the D2–DAT interaction. We speculate that the existence of the direct protein–protein interaction may play a role in positioning proteins in close spatial proximity, which allows for rapid and specific regulation (e.g. co-trafficking, protein phosphorylation) that can modify downstream signaling. The direct D2–DAT interaction not only sheds light on the molecular pathways involved in the regulation of DAT by the D2 receptor, but may also contribute to our understanding of how both these dopaminergic proteins may be involved in the etiology of diseases that are characterized with hyperdopaminergia.

Materials and methods

Plasmids

The DAT, D2S and D2L constructs were generated from human cDNA library (Grandy *et al*, 1989; Pristupa *et al*, 1994) and inserted into the mammalian expression vector pcD-PS.

HEK-293 culture conditions and transfection

HEK-293 cells were cultured in α -MEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and maintained in incubators at 37°C, 5% CO₂. One day before transfection, cells were split onto poly-D-lysine coated plates at 50% confluency. For calcium phosphate transfections, DAT:D2S cDNA ratio of 1:5–1:10 to maximize coexpression. Cells were utilized 2 days post transfection.

[³H]DA uptake analysis

Measurement of DA uptake was performed on intact HEK-293 cells or neurons as previously described (Lee *et al*, 2001). For more details, see the Supplementary data.

GST fusion proteins and mini-genes

Dopamine D2_{CT}, D2_{IL3-1}, D2_{IL3-2}, D2_{IL3-2-1}, D2_{IL3-2-2}, D2_{IL3-2-3}, D2_{IL3-2-4}, D2_{IL3-2-5} and DAT_{CT}, DAT_{NT1}, DAT_{NT2}, DAT_{NT3}, DAT_{NT1-1}, DAT_{NT1-2} cDNA-encoding fragments were amplified by PCR from full-length human cDNA clones. To confirm appropriate splice fusion and absence of spurious PCR-generated nucleotide errors, all constructs were sequenced. GST fusion proteins were prepared from bacterial lysates as described by the manufacturer (Amersham).

Co-immunoprecipitation and protein affinity purification (pull-down) assays

Co-immunoprecipitation, affinity pull-down and Western blot analyses were performed as previously described (Liu *et al*, 2000; Lee *et al*, 2002). For details, see the Supplementary data.

In vitro binding assays

Glutathione beads carrying GST fusion proteins (DAT_{NT1}, DAT_{NT2} and DAT_{NT3}) or GST (10–20 μ g each) alone was incubated with [³⁵S]methionine-labeled D2-IL3-2 probe, respectively. The beads were then washed 4–6 times with PBS containing 0.5% Triton X-100 and eluted with 10 mM glutathione elution buffer. Eluates were separated by SDS–PAGE and visualized by autoradiography.

Laser confocal microscopy

As described previously (Lee *et al*, 2001), HEK-293 cells that were transiently transfected with DAT, D2S cDNA (as indicated). For details, see the Supplementary data.

Cell-ELISA assays

Cell-ELISA assays (colorimetric assays) were performed essentially as previously described (Lee *et al*, 2002). For details, see the Supplementary data.

Primary cultures of midbrain neurons

Midbrains dissected from postnatal day 2 rats were prepared in ice-cold HBSS. Tissue is placed in cold neurobasalA/B27 medium (Invitrogen) supplemented with 0.5 mM L-glutamine and 10 ng/ml bFGF (culture medium) and mechanically dissociated either through trituration through a fire-polished Pasteur pipette. The single-cell suspensions were counted and plated at desired densities onto poly-D-lysine (100 µg/ml)-coated tissue culture plates of varying formats or onto coated glass coverslips for microscopy. Cultures are incubated at 37°C in a 5% CO₂ incubator in neurobasalA/B27 medium for 10–14 days before infecting with recombinant adenovirus of D2 and DAT.

Recombinant adenovirus construction and infection

Recombinant adenoviruses were formed by cotransfecting human cDNAs encoding the DAT in the shuttle vector pDC315 (Microbix) with replication-deficient adenovirus type 5 DNA into HEK-293 cells. The recombinant adenoviruses containing the DAT cDNAs were isolated, confirmed by PCR, plaque-purified, expanded and titered. For infection, primary midbrain cultures were infected with 10–20 plaque-forming units per neuron of recombinant adenovirus in 500 µl culture medium. Cultures were supplemented with 1.5 ml of fresh medium 1 h after infection.

TAT peptides construction

The midbrain cultures were treated with TAT-DAT peptides (1 h, 10 µM) before [³H]DA uptake measurement. TAT peptides were synthesized by Chemicon, with a dansyl tag at the NT to facilitate visualization of the intra-neuronal accumulation of the peptides. Peptides are rendered cell permeant by fusing to the cell membrane transduction domain of the human immunodeficiency virus type 1 TAT protein (YGRKKRRQRRR), as previously described (Aarts *et al*, 2002). The TAT peptide was applied to primary cultures directly (10 µM) for 30 min. Primary cultures were examined by fluorescence microscopy.

Locomotor activity

Tests of locomotor activity were conducted in four clear Plexiglas activity chambers (Med Associates Inc., St Albans, VT) measuring

43 cm long, 43 cm wide and 30 cm high. An array of 16 × 16 photodetectors, spaced 2.5 cm apart, and positioned 2.5 cm above the floor of the chamber was used to detect horizontal locomotor activity as distance traveled. Before testing, all mice were first habituated to the apparatus by placing them in the activity chambers for 1 h on three consecutive days. On test days, mice were placed in the activity chamber for 1 h before receiving an i.p. injection of TAT, TAT-DAT_{NT1-1} or TAT-DAT_{NT1-2} peptide (3 nmol/g in a 0.3 ml bolus). Locomotor activity was immediately measured after injection for the next 180 min.

Biotinylation experiments

Experiments were performed as previously described (Loder and Melikian, 2003; Sorkina *et al*, 2005). For more details see the Supplementary data.

Synaptosome preparations

Synaptosomal uptake was performed as described previously with modifications (Moron *et al*, 2002; Ansah *et al*, 2003). For more details see the Supplementary data.

Statistical analysis

All values are provided as means ± s.e.m. For comparisons between two groups, *t*-test (two-tailed) was performed. For comparisons of more than two groups, one-way ANOVA followed by Student–Newman–Keuls *post hoc* analysis was performed. Unless otherwise noted, significance level was set at 0.05.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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