Dopamine D₂ Receptor Regulation of the Dopamine Transporter Expressed in Xenopus laevis Oocytes Is Voltage-Independent

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ABSTRACT

Presynaptic dopamine D₂ receptors (D₂Rs) regulate dopamine transporter (DAT) activity in the brain. A potential mechanism was suggested by the observations that somatodendritic D₂R activation produces hyperpolarization and the velocity of DAT expressed in Xenopus laevis oocytes varies with changes in membrane potential. To investigate whether D₂R regulation of DAT function is voltage-dependent, we coexpressed the long isoform of the human (h) D₂R and the hDAT in oocytes. Most DAT substrates fully activate D₂Rs at concentrations used to measure uptake. Thus, DAT function was compared under conditions of maximal D₂R activation (0.1–10 μM DA) or maximal D₂R blockade (DA + 1 μM (−)-sulpiride). D₂R activation significantly increased [³H]DA uptake into unclamped oocytes expressing relatively lower velocities. Uptake measured with a saturating concentration of DA suggested a D₂R-induced increase in \( V_{max} \). The D₂R-mediated enhancement of DA uptake was not associated with changes in resting membrane potential and was abolished by pertussis toxin pretreatment. Furthermore, in voltage-clamped oocytes, D₂R activation enhanced both DA uptake and DAT-mediated steady-state currents by as much as 70%. Activation of D₂Rs resulted in a 59% increase in cell surface binding of the cocaine analog [³H]WIN 35,428; this effect was also abolished by pertussis toxin pretreatment. Saturation experiments confirmed that D₂R activation was associated with an increased \( B_{max} \) and unchanged \( K_f \) for [³H]WIN 35,428. These results suggest that D₂R-induced up-regulation of DAT activity occurs via a voltage-independent mechanism that depends on Gₛₜ activation and a rapid increase in expression of functional DAT molecules at the cell surface.

The dopamine transporter (DAT) belongs to a large family of neurotransmitter and amino acid transporters that are related functionally by their requirement for extracellular Na⁺ and Cl⁻ (Uhl and Hartig, 1992; Amara and Kuhar, 1993). DAT couples the translocation of dopamine (DA) or other substrates to the driving force of these ions down their electrochemical gradients. This transporter, which is exclusively expressed in DA neurons (Ciliax et al., 1995; Kuhar et al., 1998), plays a critical role in regulating synaptic concentrations of DA (Giros et al., 1996). DAT-mediated reaccumulation of DA into the presynaptic terminals terminates the synaptic actions of DA at its receptors, thus regulating the intensity, duration, and extent of dopaminergic neurotransmission. Thus, presynaptic elements such as receptors or other regulatory proteins that alter or regulate DAT function could have a profound impact on DA-mediated pre- and postsynaptic signaling.

Two DA receptor subtypes were originally proposed based on their ability to either stimulate (D₁R) or inhibit (D₂R) adenyl cyclase activity (Kebabian and Calne, 1979). To date, five distinct DA receptors have been cloned that fall into two classes: “D₁-like” (D₁Rs and D₅Rs) and “D₂-like” (D₂Rs, D₃Rs, and D₄Rs) receptors (see Sokoloff and Schwartz, 1995). Two isoforms of the D₂R have been identified, which are encoded by a single gene and differentially spliced to include (long form) or exclude (short form) 29 amino acids within the third intracellular loop of the receptor (Giros et al., 1989; Monsma et al., 1989). Inhibitory D₂₅-like autoreceptors localized on DA neuronal terminals in brain modulate DA synthesis (Tissari et al., 1983; Wachtel et al., 1989) and release (see Starke et al., 1989). Also, D₂₅-like autoreceptors localized on the soma and dendrites of DA neurons inhibit impulse flow by activating G protein-coupled inwardly rectifying potassium channels (GIRKs), thereby hyperpolarizing the neurons (Lacey, 1993; White, 1996). Compelling evidence that D₂₅-like autoreceptors are D₂₅Rs comes from reports that autoreceptor functions are lost in D₂₅R-
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deficient mice (Mercuri et al., 1997; L’Hirondel et al., 1998), but not in D₃R-deficient mice (Koeltzow et al., 1998).

There is growing evidence that D₂Rs also participate in the presynaptic regulation of DAT activity (Meiergerd et al., 1993; Parsons et al., 1993; Cass and Gerhardt, 1994; Rothblat and Schneider, 1997; Dickinson et al., 1999; Hoffman et al., 1999). In vivo studies showed that chronic cocaine-induced increases in striatal DA uptake were attenuated by pretreatment with a selective D₂R antagonist (Parsons et al., 1993). Similarly, acute or chronic administration of the D₂R antagonist haloperidol reduces DA transport into striatal tissue in vitro, and local administration of haloperidol reduces DA uptake in vivo (Meiergerd et al., 1993; Rothblat and Schneider, 1997). In vivo DA clearance is also decreased in striatum, nucleus accumbens, and prefrontal cortex by the selective D₂R antagonist raclopride but not by the selective D₂R antagonist SCH-23390 (Cass and Gerhardt, 1994). In striatal suspensions, agonist activation of D₂Rs increases DA uptake velocity, an effect blocked by a selective D₂R antagonist (Meiergerd et al., 1993); these results provide the best evidence that the D₂R-mediated effects on DA uptake occur via presynaptic autoreceptors. Consistent with the idea that activation of D₂Rs increases DAT activity, in vivo DA clearance is reduced in D₂R-deficient mice (Dickinson et al., 1999). Thus, D₂Rs regulate DAT function, and this regulation could represent an important mechanism by which dopaminergic neurotransmission is modulated.

The molecular mechanism by which presynaptic D₂Rs regulate DAT function is unknown. However, the activity of human (h) DAT expressed in Xenopus laevis oocytes is sensitive to changes in membrane potential (Sonders et al., 1997). Using electrophysiological and electrochemical techniques, Hoffman et al. (1999) recently concluded that DAT activity in rat substantia nigra is also voltage-dependent. These observations, along with the observation that presynaptic D₂Rs can alter membrane potential through activation of GIRKs (Lacey, 1993), suggest one mechanism by which D₂Rs could regulate DAT function (i.e., changes in membrane potential). Investigating this type of mechanistic question is difficult in native systems, because of the numerous regulatory proteins and neural networks that must be controlled. To address this question in a simpler model system in which membrane potential could be monitored and controlled, we coexpressed the long isoform of the hD₂R and the hDAT in oocytes. We examined D₂R regulation of DAT activity by measuring both DAT-mediated substrate uptake and currents and DAT expression by measuring cell surface binding of [³H]WIN 35,428. We found that D₂R activation enhances DAT function by increasing the number of cell surface transporters via a Gₐ-dependent mechanism and that this effect was independent of changes in membrane potential.

Experimental Procedures

Materials. cDNAs encoding the hDAT and the long isoform of the hD₂R were provided by M. S. Sonders and S. G. Amara (Vollum Institute, Oregon Health Sciences University, Portland, OR), and D. K. Grandy (Dept. of Physiology & Pharmacology, Oregon Health Sciences University, respectively). cDNA encoding the rat GIRK1 (KᵢR₃.1) was provided by H. A. Lenter (California Institute of Technology, Pasadena, CA). Mazindol, GBR 12909, (−)-sulpiride, apomorphine, DA, and pertussis toxin (PTX) were purchased from RBI/Sigma (St. Louis, MO). [³H]DA (3,4-[³H]dihydroxyphenylalanine; specific activity, 21.7 Ci/mmol), [³H](−)-sulpiride (specific activity, 73.7 Ci/mmol), and [³H]WIN 35,428 (2-[β-carbomethoxy-3-(4-fluorophenyl)]-³H]tropane; specific activity, 86.0 Ci/mmol) were purchased from PerkinElmer Life Sciences, Boston, MA. WIN 35,428 was obtained from the National Institute on Drug Abuse (Bethesda, MD). All other drugs were purchased from Sigma Chemical Co.

cRNA Preparation and Oocyte Expression. Capped cRNAs were transcribed from linearized plasmids using standard in vitro transcription reactions (mMessage mMachine; Ambion, Austin, TX). Stage V or VI X. laevis oocytes were manually defolliculated, injected with water-diluted cRNA (−10 ng/oocyte), and maintained at room temperature in frog Ringers’ buffer (FRB), containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5, supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. Oocytes were either coinjected with a mixture of DAT and D₂R cRNAs on the day of oocyte isolation or injected with DAT cRNA on the day of isolation, followed 2 days later by D₂R cRNA injection. Initially to confirm D₂R function, voltage-clamp experiments were performed on oocytes coexpressing DAT and GIRK1 (mixed and injected concurrently). GIRK1-mediated inward potassium currents were measured in FRB buffer containing 50 mM extracellular KCl. D₂R agonists activated GIRK1-mediated currents in a manner consistent with previous reports using the oocyte expression system (Werner et al., 1996; Nelson et al., 1997), and these currents were reversibly blocked by the D₂R antagonist (−)-sulpiride (1 μM).

DA Uptake Assays. For assays using [³H]DA, oocytes were incubated in 0.5 ml of FRB containing 100 nM [³H]DA for 10 min at room temperature. The oocytes were then washed three times in 5 ml of ice-cold FRB and solubilized in 0.25 ml of 2% SDS. [³H]DA accumulation into individual oocytes was quantified by liquid scintillation spectroscopy. Nonspecific uptake was <2% of total uptake and was determined by incubating water-injected oocytes or cRNA-injected oocytes in the presence of a saturating concentration of mazindol (10 μM).

The dependence of DAT-mediated DA uptake on membrane potential was examined in individual oocytes coexpressing DAT and D₂R cRNAs. In these experiments, oocytes were placed in a recording chamber as described below for two-electrode, voltage-clamp studies, and then superfused with FRB at approximately 4 ml/min. FRB containing 10 μM DA in the absence (D₂R-activated) or presence of 1 μM (−)-sulpiride (D₂R-blocked) was applied for 3 min at room temperature. Membrane potential was monitored continuously. In addition, voltage-clamped (−60 mV) DA uptake was measured in oocytes from the same batch to give a direct within-batch comparison between unclamped and voltage-clamped DA uptake. After 3 min of DA exposure, the electrodes were withdrawn, and oocytes were quickly removed from the bath and washed three times in 5 ml of ice-cold FRB. The oocytes were homogenized by sonication in 2 mM perchloric acid, and the DA content of individual oocytes was quantified by HPLC with electrochemical detection. The nominal detection limit for DA was 0.5 pg/injection. DA was not detected in water-injected oocytes or DAT/D₂R-expressing oocytes that were not exposed to DA (data not shown).

[³H](−)-Sulpiride Binding Assays. Whole-cell D₂R binding was performed in 0.5 ml of FRB buffer containing 5 nM [³H](−)-sulpiride for 15 min at room temperature. Binding was terminated by washing the oocytes three times in 5 ml of ice-cold FRB. Radioactivity was quantified by liquid scintillation spectroscopy. Nonspecific binding was defined as the presence of 1 μM (−)-sulpiride or in water-injected oocytes.

[³H]WIN 35,428 Binding Assays. Indirect saturation radioligand binding to intact oocytes was performed in 0.5 ml of FRB containing 4 nM [³H]WIN 35,428 and unlabeled WIN 35,428 (final concentrations of 5 nM to 1 μM) at room temperature for 20 min. Binding was terminated by washing three times in 5 ml of ice-cold FRB. Radioactivity was quantified by liquid scintillation spectroscopy. Nonspecific binding was defined in the presence of 1 μM GBR.
12909 or in water-injected oocytes. $B_{max}$ and $K_i$ values were estimated by nonlinear regression analysis. To determine the time course for the D$_2$R-mediated up-regulation of DAT, specific binding of 4 nM [H]WIN 35,428 was carried out for 15 min at 0°C after first preincubating oocytes in DA (100 nM) or apomorphine (1 μM) for 0, 5, 30, or 60 min at room temperature.

**PTX Treatment.** Oocytes were incubated in 0.5 μg/ml PTX in FRB for 24 h before assay (Nelson et al., 1997).

**Two-Electrode Voltage-Clamp Recording.** Electrophysiological recordings from oocytes were performed at room temperature using glass microelectrodes filled with 3 M KCl solution and an active bath probe. Oocytes were superfused with FRB buffer at approximately 4 ml/min (bath volume, 0.5 ml). A Warner OC-725B amplifier (Warner Instruments, Hamden, CT) was used with a Digital 1200 interface. pClamp software (Axon Instruments, Foster City, CA) was used to control command voltage parameters, data acquisition, and data analysis. MacLab software and a MacLab/2e interface (AD Instruments, Milford, MA) were also used to simultaneously monitor and record electrophysiological experiments. Analog signals were low-pass-filtered at 100 Hz and digitized at 2048 Hz.

Electrophysiological recording from DAT-expressing oocytes has been described in detail previously (Sonders et al., 1997). Briefly, oocytes were superfused with FRB buffer, and membrane potential was clamped at −60 mV. DAT-mediated currents were examined using a voltage jump protocol in which steady-state currents were measured at each of 17 command voltages (10-mV increments from −120 to +40 mV). Membrane potential was held at each voltage for 400 ms. Drug-induced DAT-mediated currents were determined offline by subtracting control currents from those induced by various drug conditions ($I_{drug} – I_{control}$, by convention). The resulting subtractive currents were then plotted as a function of membrane potential, thus generating current-voltage (I-V) curves for each drug response.

**Results**

**Effects of D$_2$R Activation on DAT-Mediated DA Uptake.** In initial experiments, the effect of D$_2$R activation on 100 nM [H]DA uptake was examined in oocytes coexpressing DATs and D$_3$Rs. Because most DAT substrates are D$_2$R agonists, [H]DA uptake was measured under conditions of complete D$_2$R blockade (100 nM [H]DA in the presence of 1 μM (−)-sulpiride; D$_2$R-blocked) or maximal D$_2$R activation (100 nM [H]DA in the absence of (−)-sulpiride; D$_2$R-activated). In these experiments, the effects of D$_2$R activation on [H]DA uptake depended on DAT expression level as assessed by measuring the uptake of 100 nM [H]DA. In oocytes expressing relatively low DA uptake velocities (<2 fmol/s/oocyte), [H]DA uptake increased significantly by 22% in response to D$_2$R activation (Fig. 1A). In contrast, in batches of oocytes expressing relatively higher velocities (>3 fmol/s/oocyte), D$_2$R activation produced no consistent effects on [H]DA uptake (Fig. 1B). Whole-cell D$_2$R number, as measured by 5 nM [H]((−)-sulpiride binding, ranged from 1.8 to 4.6 fmol/oocyte. However, no correlation was observed between D$_2$R expression level and D$_2$R regulation of DAT activity.

Experiments were conducted to determine whether the D$_2$R-dependent increase in DAT activity was associated with D$_2$R-mediated changes in membrane potential. DAT and D$_2$R expression levels, again measured with 100 nM [H]DA uptake and 5 nM [H]((−)-sulpiride binding, respectively, were similar to those reported above in Fig. 1A. In these experiments, accumulation of a DAT-saturating concentration of unlabeled DA (10 μM) was also measured in individual oocytes under D$_2$R-blocked versus D$_2$R-activated conditions while simultaneously monitoring membrane potential. In addition, before DA application, apomorphine (1 μM) was applied so that membrane potential could be monitored under D$_2$R-activated conditions in the absence of substrate translocation. After washout of apomorphine, under D$_2$R-blocked conditions, uptake of 10 μM DA was approximately 44 fmol/s/oocyte. D$_2$R activation increased DA uptake by approximately 50% (Fig. 2A). However, the D$_2$R-mediated increase in uptake was not associated with changes in membrane potential (D$_2$R-blocked = −31 ± 2 mV; D$_2$R-activated = −33 ± 2 mV). The D$_2$R-mediated increase in DA uptake was abolished in oocytes pretreated with PTX (bath applied; 0.5 μg/ml; data not shown), suggesting that activation of G$_i/o$ proteins is involved. Voltage-clamped DA uptake experiments were also conducted to address the voltage dependence of the D$_2$R-mediated increase in DA uptake. In oocytes with membrane potential clamped at −60 mV, uptake of 10 μM DA was approximately 45 fmol/s/oocyte (Fig. 2B). D$_2$R activation increased DAT-mediated DA uptake by approximately 70%. Thus, because membrane potential was clamped at −60 mV, the D$_2$R-mediated increase in DA uptake did not require changes in membrane potential.

**Effects of D$_2$R Activation on DAT-Mediated Currents.** Two-electrode, voltage-clamp experiments were per-
formed with oocytes coexpressing DAT and D₂R proteins to determine whether D₂R activation alters DAT-mediated ionic conductances. DAT expression levels in the oocytes used in these experiments were similar to those in Fig. 1A. In DAT-expressing oocytes voltage-clamped at −60 mV, DA (10 μM) induced low nA inward currents that returned to baseline after substrate washout (Fig. 3A). Neither DA (10 μM) nor (−)-sulpiride (1 μM) had effects on water-injected controls (Fig. 3B). In oocytes coexpressing D₂R and DAT proteins under D₂R-blocked conditions, 10 μM DA induced inward currents that were similar to those observed in oocytes expressing only DAT (Fig. 3, A and C). Sulpiride alone had no effect on baseline currents (Fig. 3C). After a 10-min washout period, DA-induced D₂R activation enhanced DAT-mediated inward currents significantly compared with D₂R-blocked conditions (Fig. 3D).

Using a voltage-jump protocol, steady-state currents were recorded over a range of holding potentials. Consistent with previous findings (Sonders et al., 1997), at least two distinct currents were mediated by DAT (see Fig. 4A). The first was a "transport-associated" inward current, which was induced by DAT substrates and was predominant at hyperpolarized holding potentials (−20 to −120 mV). The second was a constitutively active inward leak current, which was blocked by DAT substrates and apparent at more depolarized potentials (−10 to +40 mV). Similar to DA uptake assay conditions, DAT-mediated currents were compared under D₂R-blocked versus D₂R-activated conditions (Fig. 4A). Subtractive currents (I_{drug} − I_{control}) were plotted as a function of holding potential, and the current-voltage (I-V) relationships were compared. D₂R activation by 10 μM DA enhanced inward-directed, transport-associated currents across a wide range of hyperpolarized potentials (−20 to −120 mV). Similar results were obtained when tyramine (10 μM) was used to induce DAT-mediated currents either in the absence (1 μM (−)-sulpiride) or the presence of D₂R activation (100 nM apomorphine; data not shown). Figure 4B shows the mean data for the effect of D₂R activation on DAT-mediated currents at holding potentials of −90 or +20 mV. On average, at a holding potential of −90 mV, transport-associated currents were increased by 70% by D₂R activation (Fig. 4B). The block of the leak current by DAT substrates was apparent at more depolarized potentials (−20 to +40 mV). Although D₂R activation had modest effects on the leak current at depolarized potentials as high as 20 mV, the robust increases in DAT-mediated currents at hyperpolarized potentials suggests that the primary effect of D₂R activation is on transport-associated ionic conductances. When the effect of D₂R activation on DAT-mediated currents was expressed as the percentage change from control (D₂R-blocked) and plotted function of holding potential, the D₂R-induced increase in DAT-mediated currents was relatively constant across a wide range of hyperpolarized potentials (−20 to −120 mV; Fig. 4C). At the most depolarized potentials, where the leak current predominates, currents were reduced slightly by D₂R activation (Fig. 4C).

**D₂R Activation Increased Cell Surface Binding of [³H]WIN 35,428.** A potential mechanism for the D₂R-mediated increases in DAT function might involve an increase in the number of DAT molecules trafficked to the cell surface. To address this question, the effects of D₂R activation on whole-cell binding of the cocaine analog [³H]WIN 35,428 was determined. Indirect saturation curves using an approximate K_D concentration of [³H]WIN 35,428 (4 nM) were generated in the absence (1 μM apomorphine in the presence of 1 μM sulpiride) or presence (1 μM apomorphine) of D₂R activation (Fig. 5A). D₂R activation resulted in a 60% increase in B_max values (D₂R-blocked = 26 ± 3.2 fmol/oocyte; D₂R-activated = 42 ± 2.6; N = 3–5 oocytes/group from three batches). The increase in [³H]WIN 35,428 binding was not associated with a concomitant change in K_i values (D₂R-blocked = 26 ± 10.3 nM; D₂R-activated = 18 ± 0.9) for WIN 35,428. The effect of PTX pretreatment was also tested on [³H]WIN 35,428 binding under D₂R-blocked versus D₂R-activated conditions (Fig. 5B). Activation of D₂Rs by 1 μM apomorphine resulted in a 59% increase in specific binding of [³H]WIN 35,428. Similar to the results for DA uptake, the D₂R-mediated increase in [³H]WIN 35,428 binding was abolished by 24 h pretreatment with PTX (Fig. 5B). The time course of the up-regulation of specific 4 nM [³H]WIN 35,428 binding was determined after activating D₂Rs with DA (100 nM) or apomorphine (1 μM) for various times (5, 30, or 60 min). [³H]WIN 35,428 binding increased maximally by 40% after 5 min of D₂R activation and remained relatively constant for up to 60 min (Fig. 5C). Furthermore, activation of D₂Rs by either DA or apomorphine increased [³H]WIN 35,428 binding to the same extent.
Discussion
There is growing evidence that D_2R-regulate brain DAT activity. However, the complexities of intact neuronal preparations make investigations into the molecular mechanisms involved difficult. In the present study, we used the X. laevis oocyte expression system to coexpress the hD_2R (long isoform) and the hDAT to address the hypothesis that D_2R regulate DAT activity by altering membrane potential. D_2R activation enhanced DAT-mediated substrate translocation, as well as ionic conductances associated with substrate translocation. However, the D_2R-mediated increases in DAT activity were independent of changes in membrane potential. D_2R activation also increased the number of whole-cell binding sites detected with [3H]WIN 35,428, suggesting that D_2R activation enhances DAT activity by increasing the cell surface expression of DAT. These D_2R-mediated effects likely depend on G_i/o signaling, because pretreatment with PTX abolished both the enhanced function and cell surface binding of DAT.

It has been established previously that, when expressed individually in oocytes, DAT or D_2R proteins exhibit their expected activities and pharmacological profiles. For example, the translocation kinetics of the hDAT expressed in oocytes has been characterized in detail (Sonders et al., 1997) and found to be consistent with those observed in mammalian cell lines expressing hDAT (Giros et al., 1992; Eshleman et al., 1995). We observed similar results in oocytes coexpressing hDAT and hD_2R proteins. Initially, we confirmed D_2R function by coexpressing hD_2R with rat GIRK1 (Kir3.1). Agonist stimulation of D_2R-activated GIRK1-mediated potassium currents in a manner consistent with previous reports (Werner et al., 1996; Nelson et al., 1997). The D_2R-activated potassium currents were completely and reversibly blocked by the D_2R antagonist (−)-sulpiride. Thus, in most subsequent experiments, D_2R expression was confirmed using [3H](−)-sulpiride binding, rather than GIRK1 coexpression.

Because most DAT substrates are agonists at D_2Rs, D_2Rs were already maximally activated in the presence of DA (here either 100 nM [3H]DA or 10 μM unlabeled DA). Thus, baseline DAT activity was measured in the presence of DAT substrate and 1 μM (−)-sulpiride. In oocytes expressing lower DAT velocities (<2 fmol/s/oocyte, measured with 100 nM [3H]DA), uptake was enhanced significantly by D_2R activation. These results demonstrate that hDAT activity can be regulated by hD_2Rs and are consistent with reports suggesting that D_2R ligands modulate rodent brain DAT function (Meiergerd et al., 1993; Parsons et al., 1993; Cass and Gerhardt, 1994; Rothblat and Schneider, 1997; Dickinson et al., 1999; Hoffman et al., 1999). However, it is unclear why a D_2R-mediated increase in DA uptake did not occur when DAT velocities were relatively higher (>3 fmol/s/oocyte, measured with 100 nM [3H]DA). It is unlikely that substrate depletion reduced the effect of D_2R activation on DA uptake in these experiments, because identical results were obtained using a saturating concentration of DA (10 μM; data not shown). Regulation of the γ-aminobutyric acid (GABA) transporter GAT1 by phorbol esters has been shown to depend on the level of protein expression (Quick et al., 1997). Thus, it is possible that, in oocytes expressing a high number of DAT molecules, the mechanism(s) and/or signaling cascades linking D_2Rs and DATs may be limiting, thereby saturating DAT or D_2R protein-protein associations and leading to diminished regulatory potential. However, the magnitude of D_2R-activated [3H]DA uptake was not correlated with the level of D_2R expression as assessed by [3H](−)-sulpiride binding.

DA uptake into DAT-expressing oocytes has been shown to be voltage-dependent (Sonders et al., 1997). Consistent with this conclusion, Hoffman et al. (1999) found that activation of D_2Rs or GABA_B receptors in rat substantia nigra hyperpolarizes DA neurons and increases DAT-mediated DA clearance. Thus, a potential mechanism underlying the D_2R-mediated increase in DA uptake would be a D_2R-dependent change in the resting membrane potential. In brain, activa-

![Image](https://example.com/image.png)
Fig. 4. Effects of D₂R activation on DAT-mediated currents in oocytes coexpressing D₂R and DAT proteins. A, representative data from a single oocyte demonstrating enhanced substrate-associated currents by D₂R activation. The I-V plot shows steady-state subtractive currents as a function of membrane potential in response to instantaneous jumps in voltage (−120 to +40 mV; 10-mV increments) from a holding potential of −60 mV. DA-induced DAT-mediated currents were obtained under D₂R-blocked (1 μM (−)-sulpiride) versus D₂R-activated (10 μM DA) conditions. Boxes indicate potentials for which mean data are shown in panel B. B, DAT-mediated currents were increased significantly by D₂R activation at hyperpolarized holding potentials (−90 mV shown; represents transport-associated current) but not at depolarized potentials (+20 mV shown; represents the block of tonic leak current by DAT substrates). Mean values ± S.E.M. are shown for N = 14 oocytes from four batches (*p < 0.05 single factor ANOVA). C, effect of D₂R activation on DAT-mediated currents expressed as the percentage change from control and plotted as a function of holding potential. The effect of D₂R activation on the substrate-associated current, observed at negative holding potentials, was voltage-independent.
A. 

**D2R Blocked**

**D2RActivated**

B. 

Specific[^3H]WIN 35,428 Binding (pmol/oocyte)

- **D2R Blocked**
- **D2R Activated**

C. 

% Increase Over Control

- **5**
- **30**
- **60**

Fig. 5. Effects of D2R activation on whole-cell binding of[^3H]WIN 35,428 to DAT in oocytes coexpressing D2R and DAT proteins. A, D2R activation increased the number of cell surface DAT binding sites. Indirect saturation curves were generated with 4 nM[^3H]WIN 35,428 at room temperature under D2R-blocked (1 μM apomorphine in the presence of 1 μM (−)-sulpiride) versus D2R-activated conditions (1 μM apomorphine). Curve fitting revealed that D2R activation increased B\text{max} without altering K\text{d} (see Results). Mean values ± S.E.M. are shown for N = 3–5 oocytes/group from three batches. B, the D2R-mediated increase in specific binding of 4 nM[^3H]WIN 35,428 was abolished by PTX pretreatment. GBR 12909 (1 μM) or uninjected oocytes were used to define nonspecific binding. Mean values ± S.E.M. are shown for N = 10–16 oocytes from two batches (\(p < 0.01\) single factor ANOVA). PTX was bath applied (0.5 μg/ml) for 24 h. Mean values ± S.E.M. are shown for N = 6–8 oocytes from a single batch. C, the D2R-mediated increase in[^3H]WIN 35,428 binding was maximal after 5 min of receptor activation. D2Rs were activated by either DA (100 nM) or apomorphine (1 μM) for 5, 30, and 60 min at room temperature. After each period of activation, specific[^3H]WIN 35,428 binding (4 nM) was determined at 0°C. The effects of DA and apomorphine were identical, and the data were combined for illustration. Mean values ± S.E.M. are shown for N = 5 to 6 oocytes/group from four batches.

The results from experiments measuring DAT-associated currents also argue that D2R activation of DAT is voltage-independent. DAT substrates induce transport-associated currents, which are Na\textsuperscript{+} - and voltage-dependent (Sonders et al., 1997; Sitte et al., 1998). In voltage-clamped oocytes coexpressing D2R and DAT, we found that activation of D2Rs by DA significantly enhanced transport-associated inward currents. Similar to our findings with DA uptake, enhanced substrate-associated currents were only observed in oocytes expressing lower DAT velocities. The D2R-mediated increase in DAT currents were observed at holding potentials between −20 and −120 mV, and the current magnitude increased as a function of increasing hyperpolarization. Although steady-state currents at hyperpolarized potentials were voltage-dependent under both D2R-blocked and -activated conditions, the percentage increase in steady-state currents resulting from D2R activation was relatively constant over this range of holding potentials, equaling approximately 50%. This finding suggests that the increased transport-associated current induced by D2R activation is voltage-independent.

In contrast with the lack of effect of membrane potential on D2R-mediated enhancement of DAT function, pretreatment with PT abolished the effects of D2R activation on both DAT function and binding (see below). D2Rs couple to PTX-sensitive Gi/o proteins to inhibit adenylyl cyclase, voltage-gated calcium channels, and protein kinase C (PKC), as well as to activate GIRKs and mitogen-activated protein kinase (see Giambalvo and Wagner, 1994; Sokoloff and Schwartz, 1995; Watts et al., 1998). The long isoform of the D2R, which we investigated here, can selectively activate G\textsubscript{i/o} proteins to inhibit adenylyl cyclase, voltage-gated calcium channels, and protein kinase C (PKC), as well as to activate GIRKs and mitogen-activated protein kinase (see Giambalvo and Wagner, 1994; Sokoloff and Schwartz, 1995; Watts et al., 1998). The long isoform of the D2R, which we investigated here, can selectively activate G\textsubscript{i/o} proteins to inhibit adenylyl cyclase, voltage-gated calcium channels, and protein kinase C (PKC), as well as to activate GIRKs and mitogen-activated protein kinase (see Giambalvo and Wagner, 1994; Sokoloff and Schwartz, 1995; Watts et al., 1998). However, it remains to be determined whether the D2R regulation of DAT involves G\textsubscript{i} and/or G\textsubscript{o} proteins. Furthermore, D2R actions can be mediated by both G\textsubscript{o} and G\textsubscript{bg} subunits (Watts et al., 1998; Choi et al., 1999). In primate
brain the short isoform of the D_2R predominates in DA neurons (Khan et al., 1998). Signal transduction mechanisms can differ between the long and short isoforms (Sokoloff and Schwartz, 1995; Choi et al., 1999). Nonetheless, our findings of enhanced DAT function, mediated by the long isoform of the D_2R in the oocyte expression system, agrees with the observations of D_2R-mediated up-regulation of DA clearance in rodent brain.

Recent results suggest that DAT trafficking plays a prominent role in the regulation of transporter activity. For example, activation of PKC by phorbol esters decreases DAT function by reducing the number of functional transporters on the cell surface (Zhang et al., 1997; Zhu et al., 1997; see Pristupa et al., 1998). Furthermore, the phorbol ester-induced loss of cell surface DAT is associated with increased endocytotic trafficking of the transporter (Daniels and Amara, 1999; Melikian and Buckley, 1999). Here, we used whole-cell [3H]WIN 35,428 binding to estimate the number of cell surface DATs. There are no hydrophilic radiolabeled DAT ligands available for detection of DAT exclusively at the cell surface. However, [3H]WIN 35,428 binding to DAT is negligible at low Na+ concentrations (Reith and Coffer, 1993), such as are present intracellularly in oocytes [6 mM (Barish, 1983)]. We found that D_2R activation increased the number of cell surface DATs by 60%, as measured by [3H]WIN 35,428 binding. This D_2R-mediated increase in [3H]WIN 35,428 binding was maximal after 5 min of receptor activation, suggesting that redistribution of DAT to the cell surface occurs rapidly. The rapid redistribution of DAT is also evident from electrophysiological studies showing enhanced DAT-mediated currents within 1 min of D_2R activation. These results are consistent with a hypothesis that D_2Rs regulate cell surface expression of DAT, thereby providing a rapid, local feedback mechanism for transporter regulation. Whether the net increase in cell surface transporters reflects reduced DAT endocytosis or increased trafficking to the cell surface remains to be determined.

In conclusion, the increase in the number of [3H]WIN 35,428 binding sites, together with the enhanced DAT uptake and currents, suggest strongly that the D_2R-induced up-regulation of DAT function results from an increase in the number of functional DAT molecules expressed at the cell surface. D_2R-mediated changes in membrane potential do not seem to be involved in this regulation of DAT function, whereas D_2R-mediated changes in G_{i/o} signaling do. It is possible that common mechanisms are involved in receptor-mediated regulation of neurotransmitter transporters. Receptors that couple to PKC via G proteins alter the activity of the norepinephrine and GABA transporters by inducing a rapid redistribution of these transporters (Apparsundaram et al., 1998; Beckman et al., 1999). Whatever the molecular mechanism for D_2R/DAT interactions, these findings support the idea that presynaptic D_2Rs can alter not only DA neuronal firing, synthesis, and release, but also DA uptake.

References


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