

Protein Tyrosine Kinase Inhibitors Alter Human Dopamine Transporter Activity in *Xenopus* Oocytes

SUZANNE DOOLEN and NANCY R. ZAHNISER

Department of Pharmacology and Neuroscience Program, University of Colorado Health Sciences Center, Denver, Colorado

Received August 18, 2000; accepted November 28, 2000 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

The dopamine (DA) transporter (DAT) regulates dopaminergic synaptic transmission by controlling extracellular levels of DA. Thus, understanding signaling mechanisms that alter DAT function is critical for understanding dopaminergic neurotransmission. We have expressed the human DAT (hDAT) in *Xenopus laevis* oocytes to test the hypothesis that protein tyrosine kinases (PTKs) acutely regulate DAT function by altering cell surface expression of the transporter. Using a relatively high concentration of DA (10 μ M), we found that several PTK inhibitors, namely, genistein, lavendustin A, and tyrphostin 25 (10 μ M), decreased DA uptake velocity by 58, 41, and 30% of control, respectively. Furthermore, genistein potentially inhibited DA uptake with a K_i = 68 nM. Kinetic analysis confirmed that genistein decreased the V_{max} of the DAT, with no change in K_m . The effects of PTK inhibition on hDAT-associated currents were

also measured. All three PTK inhibitors attenuated substrate transport-associated currents to similar extents as DA uptake. In contrast, the potent Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) did not significantly inhibit either DA uptake or transport-associated currents. PTK inhibitors decreased hDAT-associated leak currents, however in a more variable manner than for uptake and transport-associated currents. Genistein also decreased cell surface binding of [³H]WIN 35,428 to hDAT by 48% of control. Together, these data provide several lines of evidence suggesting that PTK inhibition rapidly reduces hDAT activity via redistribution of the transporter away from the cell surface. Thus, PTKs likely represent another component of cellular signaling cascades that acutely regulate neurotransmitter transporters.

The neurotransmitter DA plays a critical role in a number of physiological processes mediated by the central nervous system (CNS), e.g., locomotion, cognition, and reward. Clearance of DA from the synaptic cleft is primarily accomplished via reuptake of DA by presynaptic DATs localized on the plasma membrane of dopaminergic neurons. The DAT is a member of a superfamily of neurotransmitter transporters, all of which have 12 putative transmembrane domains and translocate neurotransmitters, or other substrates, into cells against their concentration gradients by coupling transport of the neurotransmitter to the cellular electrochemical Na⁺/Cl⁻ gradient (Amara and Kuhar, 1993). Other members of this superfamily include transporters for γ -aminobutyric acid (GAT1), serotonin (SERT), norepinephrine (NET), glycine, choline, taurine, proline, and betaine (Amara and Kuhar, 1993; Giros and Caron, 1993). Since the DAT plays a crucial role in controlling the concentration of extracellular DA, elu-

cidating mechanisms by which DAT activity is regulated is vital to understanding how dopaminergic neurotransmission in the CNS is controlled.

Many of the transporters in this Na⁺/Cl⁻-dependent neurotransmitter transporter superfamily, including DAT, are subject to acute regulation by signaling cascades that often involve activation of cellular protein kinases (Reith et al., 1997; Beckman and Quick, 1998; Blakely and Bauman, 2000). For example, activation of protein kinase C (PKC) by bath-applied phorbol esters down-regulates the number of cell surface, and thus activity of NETs, GAT1s, and SERTs (Qian et al., 1997; Apparsundaram et al., 1998; Beckman et al., 1998). Likewise, PKC-mediated down-regulation of DAT function has been consistently observed in rat striatal synaptosomes (Copeland et al., 1996) and heterologous expression systems (Kitayama et al., 1994; Huff et al., 1997; Zhang et al., 1997; Pristupa et al., 1998), including *Xenopus* oocytes expressing the hDAT (Zhu et al., 1997). Recent studies have revealed that PKC-mediated inhibition of DAT activity also

This work was supported by National Institute of Health Grant DA04216, a National Research Service Award DA05956 to S.D., and an Instrument Society of America DA00174 to N.R.Z.

ABBREVIATIONS: DA, dopamine; CNS, central nervous system; DAT, dopamine transporter; GAT, γ -aminobutyric acid transporter; SERT, serotonin transporter; NET, norepinephrine transporter; PKC, protein kinase C; hDAT, human dopamine transporter; PTK, protein tyrosine kinase; RPTK, receptor protein tyrosine kinase; BDNF, brain-derived growth factor; FRB, frog Ringer's buffer; 3-PPP, *R*-(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine; [³H]WIN 35,428, β -carbomethoxy-3 β -(4-fluorophenyl)[³H]tropane; EGFR, epidermal growth factor receptor; GBR 12909, 1-[2-(bis[4-fluorophenyl]methoxy)ethyl]-4-[3-phenyl-propyl]piperazine; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine.

occurs via increased transporter endocytosis (Daniels and Amara, 1999; Melikian and Buckley, 1999).

Although PKC-induced transporter regulation is well established, it is not yet clear whether the DAT is acutely regulated by other protein kinases, such as PTKs. PTKs are a vital component in intracellular signaling, and well over a thousand human genes encode for PTKs (for review, see Fruman et al., 1998). Receptor PTKs (RPTKs) are transmembrane receptors with intrinsic tyrosine kinase activity (for review, see van der Geer and Hunter, 1994). RPTKs are well known for controlling cellular growth and differentiation, but they also regulate many other cellular programs. In addition, there are at least nine families of nonreceptor PTKs. Such families include Jak and Src family PTKs (Neet and Hunter, 1996).

Tyrosine kinases appear to regulate Na^+/Cl^- -dependent neurotransmitter transporters via several different molecular mechanisms. Prasad et al. (1997) have shown that tyrosine phosphorylation indirectly up-regulates gene expression of hSERT in JAR placental choriocarcinoma cells. Furthermore, PTKs can alter transporter trafficking. Acute inhibition of PTKs attenuates GAT1 activity concomitant with a redistribution of the transporter away from the cell surface of hippocampal neurons (Law et al., 2000). Conversely, insulin, which activates RPTKs, increases the maximal velocity of NET in SK-N-SH cells (Apparsundaram and Blakely, 1997). In contrast, brain-derived growth factor (BDNF) dose dependently reduces hSERT activity in certain immortalized B-lymphocyte cell lines, whereas nerve growth factor is without effect (Mössner et al., 2000). Striatal dopaminergic neurons possess a number of RPTKs that represent potential targets for growth factor-mediated regulation of the DAT. These include RET receptors for glial cell line-derived factor, trkA receptors for nerve growth factor, trkB receptors for BDNF and neurotrophin-4/5, and trkC receptors for neurotrophin-3 (Hyman et al., 1994; Eggert et al., 1999; Numan and Seroogy, 1999; Taraviras et al., 1999). Pharmacological manipulation with PTK inhibitors suggests the involvement of PTKs in the acute regulation of DA uptake in mouse striatum (Simon et al., 1997). However, the mechanism by which this regulation occurs and the specific PTK(s) involved is not known.

Here, we used PTK inhibitors to test the hypothesis that PTKs acutely regulate DAT activity by altering the number of DATs present on the cell surface. Our results show that inhibition of PTKs, other than Src-family kinases, rapidly decreased hDAT function in *Xenopus* oocytes. Our data are also consistent with the idea that inhibition of PTKs produces a redistribution of hDATs away from the cell surface. Thus, PTKs can rapidly alter the number of functional DATs and thereby may play a role in acutely regulating dopaminergic neurotransmission.

Experimental Procedures

Frogs and Oocyte Preparation. Female *X. laevis* frogs were purchased from either Nasco (Ft. Atkinson, WI) or Xenopus I (Ann Arbor, MI). All animal use procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee, University of Colorado Health Sciences Center. Stage V and VI *X. laevis* oocytes were defolliculated by gentle shaking in OR2 buffer (82 mM NaCl, 2.5 mM KCl, 1.0 mM

MgCl_2 , 5 mM HEPES, pH 7.5) containing 1.4 to 2.0 mg/ml collagenase B (Boehringer-Mannheim, Indianapolis, IN) for 1 to 2 h at room temperature.

hDAT cRNA Preparation and Oocyte Expression. Capped cRNA was transcribed from a linear oocyte expression vector pOTV containing the 1.9-kb hDAT cDNA insert (Sonders et al., 1997) using mMessage mMachine with T7 polymerase (Ambion, Austin, TX). Oocytes were injected with water-diluted cRNA (~10 ng) and maintained at room temperature in frog Ringer's buffer (FRB; 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , and 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 50 $\mu\text{g}/\text{ml}$ gentamycin for 3 to 5 days.

DA Uptake. Individual hDAT-expressing oocytes were placed in a 0.5-ml bath and superfused at a rate of approximately 2 ml/min with FRB at room temperature. For uptake measurements, oocytes were either maintained at resting potential or voltage clamped at -80 mV; similar results were obtained using either protocol. Each oocyte was superfused with a relatively high concentration of DA (10 μM) for 3 min. In experiments testing PTK inhibitors, the inhibitors were superfused for approximately 5 min before DA treatment and were present throughout the DA exposure. After the 3-min DA exposure, the oocytes were quickly washed three times in FRB, transferred to 0.8 ml of 2 mM perchloric acid, and stored for not more than 2 weeks at -4°C . Oocytes were sonicated in the perchloric acid, and the suspension was filtered through 0.22- μm nylon. Intracellular DA accumulation was determined in the filtrate by high performance liquid chromatography using electrochemical detection. Retention times of standards were used to identify peaks, and absolute DA content in samples was determined by comparison to the area of standard peaks. Minimum detection for DA was 0.5 pg/injection. Nonspecific DA uptake was determined in water-injected oocytes and was less than 1% of hDAT-injected oocytes.

Two-Electrode Voltage-Clamp Recording. Currents were measured in oocytes using two-electrode voltage clamp; microelectrodes were filled with 3 M KCl (Sonders et al., 1997). A Warner OC-725B amplifier (Warner Instruments, Hamden, CT) was used with a DigiData 1200 interface. pClamp6 software (Axon Instruments, Foster City, CA) was used to control stimulation parameters, for data acquisition, and for analysis. MacLab data acquisition software (AD Instruments, Castle Hill, Australia) and a MacLab/2e interface were simultaneously used to monitor experiments. Currents were low-pass filtered at 100 Hz and digitized at 2048 Hz.

Oocytes were superfused in a 0.5 ml bath at 2 to 3 ml/min with FRB at room temperature. In experiments designed to measure only hDAT-associated leak currents, oocytes were superfused with FRB containing 96 mM LiCl, instead of 96 mM NaCl. Oocytes were voltage clamped at -60 mV and then subjected to a series of 400-ms steps in membrane potential ranging from -120 mV to $+40$ mV in 10-mV increments. Currents were recorded before and again 1 min after superfusion with 10 μM tyramine or 100 μM *R*-(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine (3-PPP). Steady-state currents were measured and averaged during the last 100 ms of each voltage step. Inward currents induced by the DAT substrate tyramine were measured by performing off-line subtraction of currents measured in the absence of drug from those measured in the presence of drug ($I_{\text{drug}} - I_{\text{buffer}}$). In experiments using the DAT inhibitor 3-PPP to measure inhibition of inward hDAT-associated leak currents, outward 100 μM 3-PPP-induced currents were measured by performing off-line subtraction of the currents measured in the presence of drug from those measured in the absence of drug ($I_{\text{buffer}} - I_{\text{drug}}$). The effects of PTK inhibitors were determined on hDAT-mediated currents by superfusing the inhibitor for 3 to 5 min before addition of either tyramine or 3-PPP and continuing the drug superfusion during the voltage steps. Currents from each oocyte were normalized to the hDAT current measured at -120 mV.

Radioligand Binding to hDAT. hDAT binding sites were quantitated in intact oocytes by superfusion in a 0.5-ml bath with FRB

containing an approximate K_d concentration of 2 β -carbomethoxy-3 β -(4-fluorophenyl)[3 H]tropane ([3 H]WIN 35,428; specific activity 84.5 Ci/mmol; 4 nM) at room temperature for 20 min. Oocytes were then quickly washed three times in FRB, dissolved in 0.25 ml of 2% SDS. Radioactivity was measured by liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 1 μ M GBR 12909 and was $50 \pm 13\%$ of total binding.

Data Analysis. Apparent values for V_{max} , K_m , and K_i were determined using nonlinear regression analysis (GraphPad Prism, San Diego, CA). For statistical analysis, transport-associated currents were compared at -80 mV, whereas leak currents were compared at $+20$ mV. Statistical significance was determined by unpaired Student's t test with a significance criterion of $P < 0.05$.

Materials. Lavendustin A and GBR 12909 were purchased from Sigma/Research Biochemicals International (St. Louis, MO). PP2 was a gift from Dr. Alexander Sorkin (University of Colorado Health Sciences Center, Denver, CO) or purchased from Calbiochem (La Jolla, CA). [3 H]DA and [3 H]WIN 35,428 were purchased from NEN Life Science Products (Boston, MA). All other drugs were purchased from Sigma (St. Louis, MO).

Results

DA Uptake. We used several PTK inhibitors, namely, genistein, lavendustin A, tyrphostin 25, and PP2, to test our hypothesis that PTKs acutely regulate DAT function by altering cell surface expression of the transporter. Numerous PTK inhibitors are available that can be used as tools to characterize the effects of PTKs (for review, see Lawrence and Niu, 1998). The flavonoid genistein, which exhibits little specificity, is often used as a general inhibitor of protein kinases. The potency for inhibition of the epidermal growth factor receptor (EGFR) is one basis for comparison among PTK inhibitors. Both genistein and the erbstatin analog PTK inhibitor tyrphostin 25 inhibit the EGFR kinase activity with an $IC_{50} = \sim 3$ μ M. The addition of a glucoside to genistein results in its inactive analog, genistin. Lavendustin A is a more selective PTK inhibitor, with little effect on either protein kinase A or PKC ($IC_{50} > 200$ μ M), and has a substantially higher affinity for the EGFR ($IC_{50} = 11$ nM). PP2 is a potent inhibitor of the Src family of PTKs (Hanke et al., 1996).

To investigate possible effects of PTKs on DAT function, we first measured DA uptake into individual oocytes expressing the hDAT in the absence or presence of these PTK inhibitors. Oocytes were pre-exposed to the PTK inhibitors for approximately 5 min, and exposure to the inhibitors was continued during the 3-min incubation with 10 μ M DA. This is a relatively high concentration of DA and, therefore, changes in uptake are more likely to reflect changes in uptake capacity, rather than affinity. Effects of PTK inhibitors were much more robust and consistent when oocytes were superfused, rather than incubated in a static bath. Although we do not understand the mechanism(s) underlying this observation, all experiments reported here were conducted using superfusion. The velocity of DA uptake in control oocytes was 3.06 ± 0.45 fmol/s/oocyte ($N = 26$ oocytes from four batches of oocytes; Fig. 1). Exposure to genistein inhibited DA uptake into oocytes in a concentration-dependent manner with an apparent K_i value of 68 nM ($pK_i = 7.17 \pm 0.22$ M; $N = 15$ –26 oocytes from four batches of oocytes). At the highest concentration of genistein tested (10 μ M), the velocity of DA uptake was $58 \pm 13\%$ of control. Similarly, two of the other PTK inhibitors tested significantly inhibited the velocity of 10 μ M

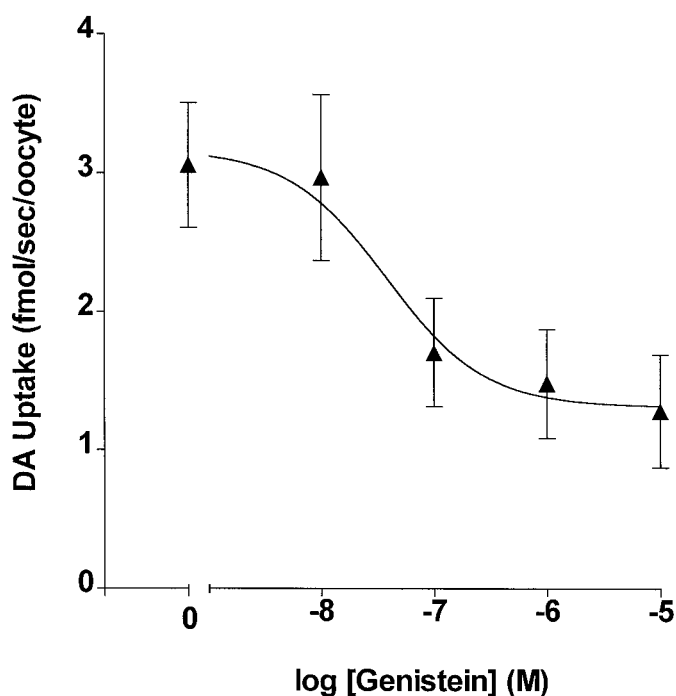


Fig. 1. Concentration-response curve showing the inhibition of specific DA uptake into hDAT-expressing oocytes by the PTK inhibitor genistein. After a 5-min preexposure to FRB or genistein, oocytes were incubated with DA (10 μ M) for 3 min at room temperature in a 0.5-ml bath with a flow rate of 2 ml/min. DA taken up by individual oocytes was quantitated by high performance liquid chromatography/electrochemical detection. Nonspecific DA uptake was defined as the difference in DA accumulated in hDAT- and water-injected oocytes and represented less than 1% of total uptake. Specific DA uptake was inhibited by genistein with an $IC_{50} = 68$ nM. Data represent the mean \pm S.E.M. from 15 to 26 oocytes from four different batches of oocytes.

DA uptake into hDAT-expressing oocytes (Fig. 2). Lavendustin A (10 μ M) reduced uptake to $41 \pm 6\%$ of control ($N = 17$ –18 oocytes from three batches of oocytes), and tyrphostin 25 (10 μ M) reduced uptake to $30 \pm 7\%$ of control ($N = 17$ –19 oocytes from three batches of oocytes). In contrast, the potent Src PTK inhibitor PP2 (1 μ M; Hanke et al., 1996) did not significantly inhibit the velocity of 10 μ M DA uptake (Fig. 2).

To confirm that the decreases in uptake velocities induced by the PTK inhibitors reflected reductions in DAT V_{max} , direct kinetic analysis of DA uptake was conducted in the absence or presence of 10 μ M genistein (Fig. 3). This analysis showed that the K_m was not different between control (7.8 μ M; $pK_m = 5.1 \pm 0.18$ M) and genistein-treated oocytes (4.7 μ M; $pK_m = 5.3 \pm 0.30$ M; $N = 11$ –17 oocytes from three batches of oocytes). However, the V_{max} was decreased by 50% in the presence of 10 μ M genistein (2.8 ± 0.5 fmol/s/oocyte) compared with control (5.6 ± 0.7 fmol/s/oocyte). Thus, the magnitude of the inhibition of the DAT V_{max} by 10 μ M genistein measured with the full kinetic analysis was similar to that seen in Fig. 1 ($58 \pm 13\%$ of control) measured with the single 10 μ M concentration of DA.

hDAT-Associated Currents. DATs generate currents, and these currents provide additional measures by which the functional state of the transporter can be assessed. To investigate possible effects of PTK inhibitors on hDAT-associated currents, we used two-electrode voltage-clamp recording. Although identical results are seen using either DA or tyramine as a substrate in hDAT-expressing oocytes, tyramine was

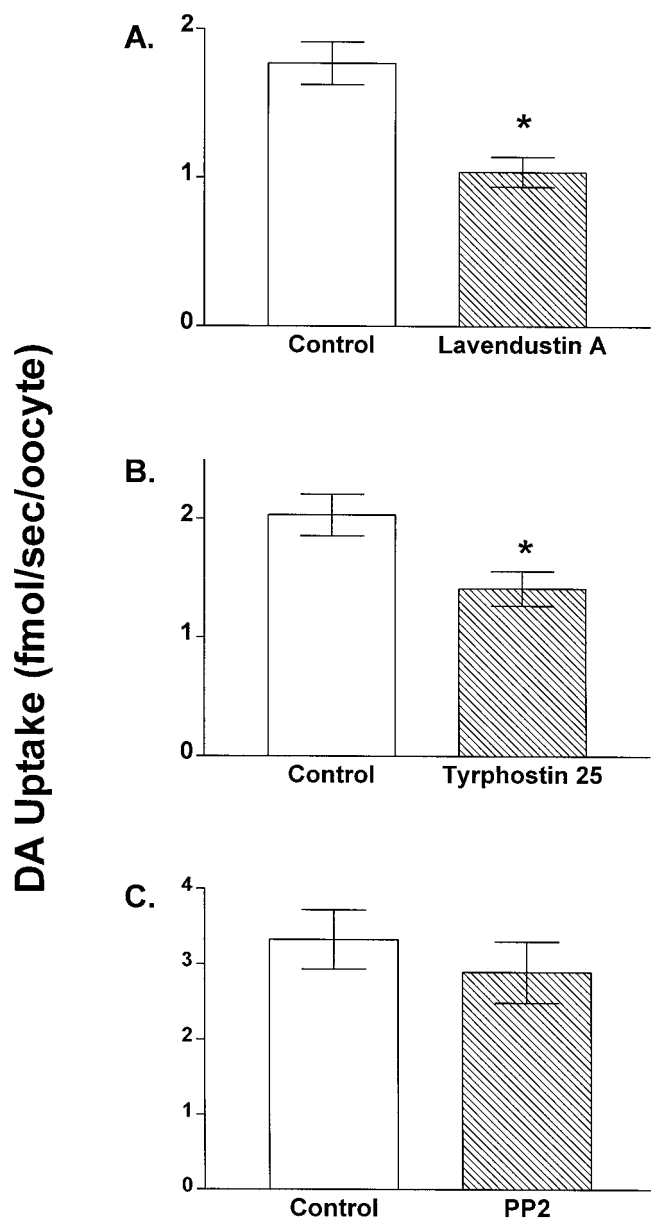


Fig. 2. The PTK inhibitors lavendustin A and tyrphostin 25, but not the potent Src family PTK inhibitor PP2, inhibit specific DA uptake into individual hDAT-expressing oocytes. See Fig. 1 for experimental details. PTK inhibitors tested were 10 μ M lavendustin A (A), 10 μ M tyrphostin 25 (B), and 1 μ M PP2 (C). Data represent the mean \pm S.E.M. from 17 to 19 oocytes from three different batches of oocytes. * $P < 0.05$, control versus treated, unpaired Student's t test.

used here because it is more stable in solution over time. The current-voltage (I-V) relationship produced by DAT substrates in hDAT-expressing oocytes is comprised of at least two separate currents (Sonders et al., 1997). An inward transport current, induced by translocation of the positively charged substrate and Na^+ , predominates at more hyperpolarized membrane potentials (-120 to -20 mV). In addition, a smaller outward current is produced by both DAT substrates and inhibitors. This current results from blockade of a constitutively active inward cation leak over all voltages tested (-120 to $+40$ mV). However, since DAT substrates such as tyramine produce the two opposing currents, blockade of the leak is more readily evident at more depolarized potentials ($+10$ to $+40$ mV).

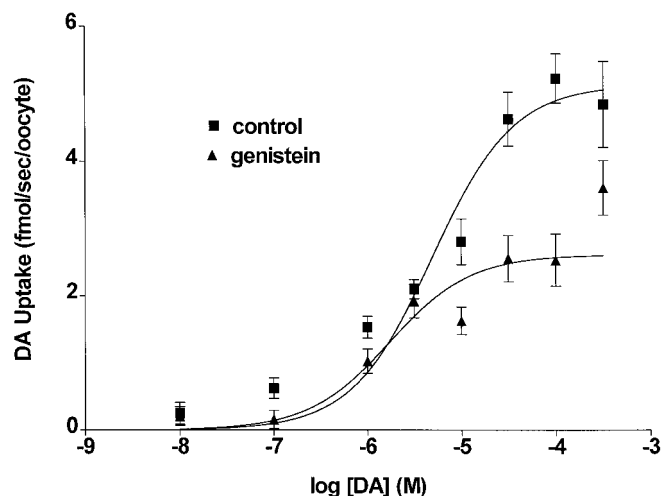


Fig. 3. Acute exposure to genistein reduces the maximal velocity, but not the affinity, of hDAT expressed in oocytes. Kinetic analysis of specific DA uptake into individual hDAT-expressing oocytes was conducted in the absence (control) or presence of 10 μ M genistein. See Fig. 1 for experimental details. Data represent the mean \pm S.E.M. from 11 to 17 oocytes from three different batches of oocytes. Kinetic parameters derived from curve fitting of these data are given under Results.

Individual hDAT-expressing oocytes were clamped at -60 mV, and I-V relationships were generated using the voltage jump protocol described under *Experimental Procedures*. Tyramine-induced currents in oocytes superfused with 10 μ M genistein were significantly inhibited at hyperpolarized potentials (Fig. 4A). For example, currents measured at -80 mV in the presence of 10 μ M genistein were only $23 \pm 19\%$ of control currents ($N = 5$ oocytes from three batches of oocytes). However, at more depolarized potentials where blockade of the leak current is more readily observed, genistein did not significantly alter tyramine-induced currents. The inactive analog of genistein, genistin (10 μ M), had no effect on transport-associated currents at hyperpolarized membrane potentials but appeared to induce a small change at more depolarized potentials (Fig. 4B). Lavendustin A also significantly inhibited tyramine-induced currents (Fig. 5A). At -80 mV, tyramine-induced currents in the presence of 10 μ M lavendustin A were $35 \pm 22\%$ of control currents ($N = 9-10$ oocytes from three batches of oocytes). In contrast to the effects of genistein, lavendustin A also significantly inhibited tyramine-induced currents at more depolarized potentials. For example, currents measured at $+20$ mV were $35 \pm 7\%$ of control currents. These results suggest that lavendustin A inhibited not only the transport-associated current, but also the leak current. Tyramine-induced currents were also significantly inhibited by 10 μ M tyrphostin 25 at hyperpolarized membrane potentials (Fig. 5B). Similar to genistein, the predominant effect of tyrphostin 25 was to inhibit the transport-associated current. At -80 mV currents in the presence of tyrphostin 25 were $50 \pm 9\%$ of control currents ($N = 9-10$ oocytes from three batches of oocytes), whereas tyrphostin 25 only slightly decreased currents at depolarized potentials (currents at $+20$ mV were $77 \pm 16\%$ of control currents). The Src inhibitor PP2 (10 μ M) had no measurable effect on hDAT-associated currents at any voltage tested (Fig. 5C).

To more fully investigate the effect of PTK inhibitors on hDAT-mediated leak currents, currents induced by the DAT inhibitor 3-PPP were measured in LiCl-substituted FRB.

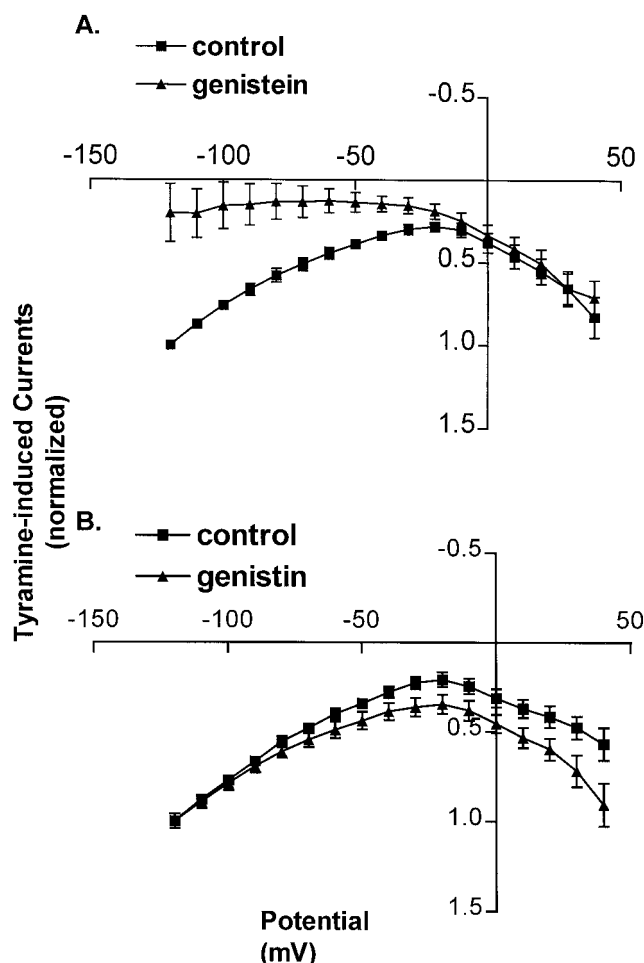


Fig. 4. Genistein, but not the inactive analog genistin, inhibits hDAT transport-associated currents. Individual hDAT-expressing oocytes were superfused at room temperature with FRB at a flow rate of 2 ml/min. Two-electrode voltage-clamp experiments were performed using the voltage step protocol described under *Experimental Procedures*. I-V relationships are shown for hDAT-mediated currents elicited by superfusion with the DAT substrate tyramine (10 μ M) in the absence (control) and presence of 10 μ M genistein (A) or in the absence and presence of 10 μ M genistin (B). Oocytes were superfused with drugs beginning 3 to 5 min before tyramine. Steady-state currents were measured 1 min after tyramine superfusion was begun. Subtractive currents for $I_{\text{tyramine}} - I_{\text{buffer}}$, normalized to the currents at -120 mV, are shown. Data represent mean \pm S.E.M. for five to nine oocytes from three batches of oocytes for each treatment.

DAT inhibitors such as 3-PPP block the leak current but do not induce the transport-associated current. Li^+ ions are conducted more readily than Na^+ ions via the hDAT cation leak (Sonders et al., 1997). Therefore, the outward current resulting from the block of the leak conductance by 3-PPP is amplified in the LiCl-substituted FRB, compared with currents measured in the presence of NaCl. Currents induced by 100 μ M 3-PPP were markedly inhibited in the presence of 10 μ M genistein (Fig. 6). 3-PPP-induced currents were 50 to 80% of control at all membrane potentials tested, with the exception of 0 mV where control and genistein curves intersect. The specificity of this effect may be questioned because, as noted above, the inactive analog genistin (10 μ M) also appeared to inhibit the leak current (Fig. 4B). Nonetheless, the 3-PPP data in the LiCl-substituted FRB strongly suggest that, in addition to inhibiting transport-associated currents,

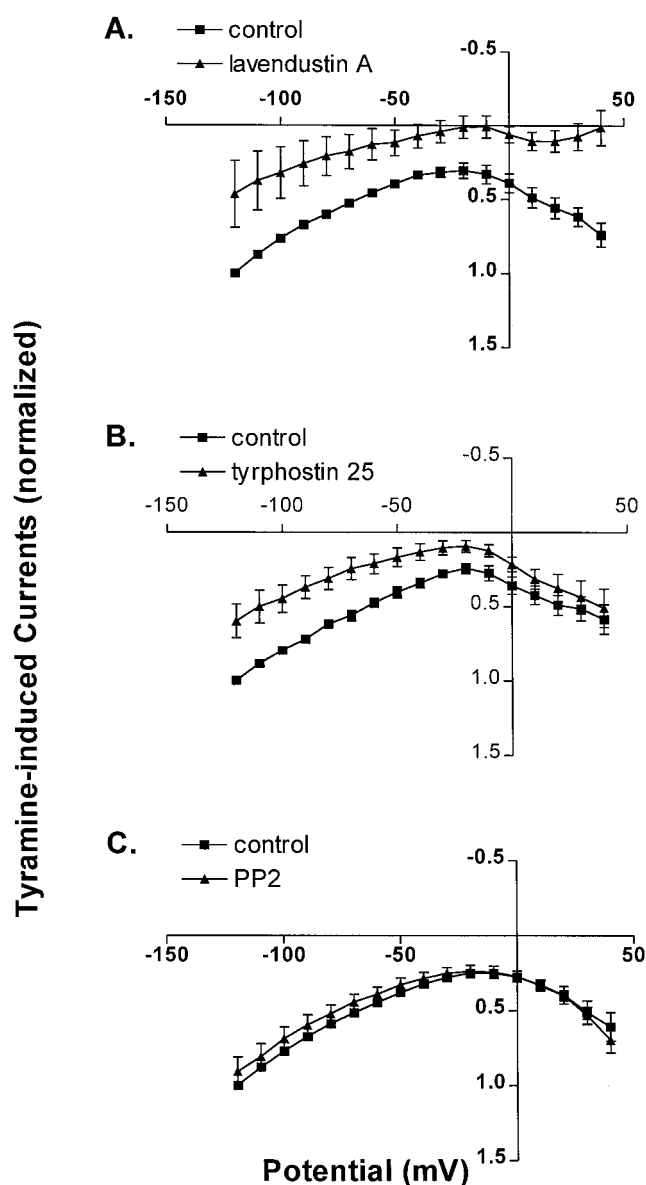


Fig. 5. PTK inhibitors lavendustin A and tyrphostin 25, but not the potent Src family PTK inhibitor PP2, inhibit hDAT-associated currents. See Fig. 4 for experimental details. I-V relationships are shown for hDAT-mediated currents elicited by 10 μ M tyramine in the absence (control) and presence of 10 μ M lavendustin A (A), 10 μ M tyrphostin 25 (B), or 1 μ M PP2 (C). Data represent mean \pm S.E.M. for 9 to 10 oocytes from three batches of oocytes.

PTK inhibitors such as genistein inhibit the hDAT-mediated leak current.

[^3H]WIN 35,428 Binding to Cell Surface DATs. A decrease in the V_{max} of the DAT with no change in K_m suggests that PTK inhibitors regulate DAT function by reducing the number of functional transporters on the cell surface. To address this more directly, we measured [^3H]WIN 35,428 binding to intact hDAT-expressing oocytes in the absence or presence of 10 μ M genistein. Specific [^3H]WIN 35,428 binding is dependent on the presence of a relatively high Na^+ concentration (Reith and Coffey, 1993; S. Doolen and N. R. Zahniser, unpublished observations). Since the Na^+ concentration inside the oocyte is very low (6 mM; Barish, 1983) compared with the extracellular FRB (96 mM), [^3H]WIN

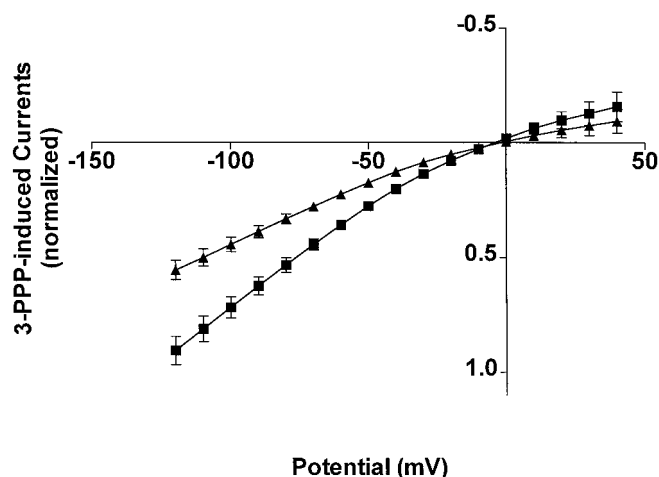


Fig. 6. Acute exposure to genistein also inhibits an hDAT-mediated leak conductance. Individual oocytes were superfused with FRB containing 96 mM LiCl, substituted for 96 mM NaCl. See Fig. 4 for experimental details. I-V relationships are shown for the inhibition of the hDAT-mediated leak conductance by superfusion with the DAT inhibitor 3-PPP (100 μ M) in the absence (control, \blacksquare) and presence of 10 μ M genistein (\blacktriangle). Subtractive currents for $I_{\text{buffer}} - I_{\text{3PPP}}$ are shown. Data represent mean \pm S.E.M. for five oocytes from two batches of oocytes.

35,428 binding is a good indicator of hDATs on the cell surface of intact oocytes. Compared with intact control oocytes, specific [3 H]WIN 35,428 binding was significantly inhibited to intact oocytes superfused with FRB containing 10 μ M genistein (control: 29.4 ± 4.9 fmol/oocyte, $N = 26$; +genistein: 14.3 ± 2.7 fmol/oocyte, $N = 28$; Fig. 7). Thus, consistent with the reduced V_{max} for DA uptake, genistein produced a $52 \pm 9\%$ reduction in specific [3 H]WIN 35,428 binding to cell surface hDATs.

Discussion

Moment-to-moment control of DAT function is crucial for regulation of extracellular DA concentrations and, therefore, is important in all CNS processes that involve dopaminergic neurotransmission. In the present studies we found evidence

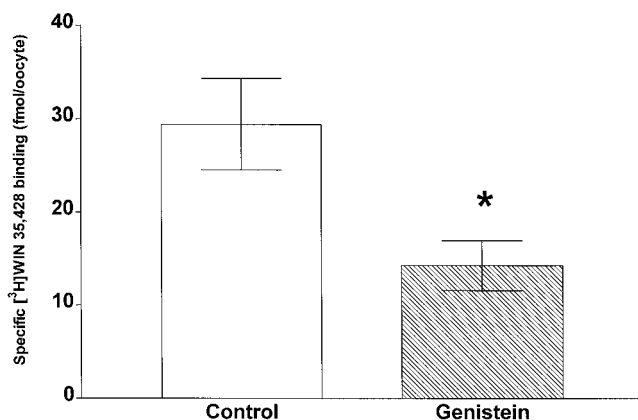


Fig. 7. Acute genistein exposure decreases specific [3 H]WIN 35,428 binding to hDAT in intact oocytes. Individual hDAT-expressing oocytes were superfused with FRB containing 4 nM [3 H]WIN 35,428 for 20 min at room temperature either in the absence (control) or presence of 10 μ M genistein. Nonspecific binding was determined in the presence of 1 μ M GBR 12909 and represented $50 \pm 13\%$ of total binding. Data represent the mean \pm S.E.M. from 26 to 29 oocytes from three different batches of oocytes. * $P < 0.05$, control versus treated, unpaired Student's t test.

that acute PTK inhibition attenuates function of hDAT expressed in oocytes, suggesting that PTKs can rapidly alter DAT activity. Furthermore, several lines of evidence suggest that this regulation occurs via a change in the number of functional DATs on the cell surface. First, three general PTK inhibitors decreased the velocity of DA uptake. Second, DAT transport-associated currents were reduced by a similar magnitude as DA uptake by these PTK inhibitors, but not by an inactive analog. Third, hDAT-associated leak currents were also inhibited by PTK inhibitors, albeit in a more variable manner. Last, genistein inhibited radioligand binding to cell surface hDATs, suggesting that genistein induces a rapid decrease in the number of hDATs present on the cell surface.

Genistein attenuated DA uptake into individual *Xenopus* oocytes with a decrease in V_{max} and no change in K_m , consistent with a reduction in the number of functional hDATs on the cell surface. This inhibition of DA uptake into oocytes occurred in a concentration-dependent manner with high affinity ($\text{IC}_{50} = 68$ nM). Genistein also attenuated hDAT-mediated currents at hyperpolarized membrane potentials where transport-associated currents predominate, whereas the inactive analog genistin had no effect on transport-associated currents. Like genistein, both lavendustin A and tyrphostin 25 significantly inhibited DA uptake and hDAT-mediated currents at hyperpolarized membrane potentials. Among these three general PTK inhibitors (all tested at 10 μ M), genistein had the highest efficacy, producing the greatest inhibition of both uptake and transport-associated currents. Lavendustin A had intermediate efficacy. Tyrphostin 25 produced the smallest effects. Thus, these inhibitors reduced both functional measures of hDAT activity, uptake and transport-associated currents, to similar extents. Interestingly, the potent inhibitor of Src-family kinases PP2 did not inhibit hDAT uptake or currents. Although genistein is a more general protein kinase inhibitor, it appears to elicit changes similar to the more selective PTK inhibitors lavendustin A and tyrphostin 25. Furthermore, if the changes produced by genistein were a result of inhibition of PKC, an increase in DAT activity would be expected. Together, these data suggest that PTKs, other than Src-family PTKs, regulate hDAT function. In support of this hypothesis preliminary data suggest that hDAT function is increased by the tyrosine phosphatase inhibitor orthovanadate (data not shown).

In previous studies using mouse striatal synaptosomes, genistein inhibited DA uptake, but lavendustin A (up to 50 μ M) and tyrphostin 25 (up to 100 μ M) did not (Simon et al., 1997). The difference between these and the present findings may be due to the presence of different PTKs and/or different phosphorylation states of the proteins involved in brain synaptosomes versus oocytes. Alternatively, there may be differences between mouse DAT and hDAT in terms of susceptibility to phosphorylation by PTKs.

Although PTK inhibitors altered both DA uptake and transport-associated currents with a similar order of potency, their effects were more variable at depolarized membrane potentials where the constitutive leak current is predominant. In normal FRB hDAT-mediated leak currents were significantly inhibited by lavendustin A but not by genistein. The results with genistein suggest that despite decreases in uptake velocity and transport-associated currents, the transporter may still be present on the cell surface and/or that

heterogeneous pools of hDAT proteins may mediate transport and leak currents. However, these possibilities are unlikely because of our results using LiCl-substituted buffer and the DAT inhibitor 3-PPP, the most definitive way to amplify leak currents and to study them in isolation from transport currents. In these experiments, genistein clearly reduced the magnitude of the leak current, consistent with the idea that there are fewer functional transporters on the cell surface.

It is not completely unexpected that identical PTK inhibitor-induced changes in the transport-associated and leak currents were not observed here. Substrates clearly have differential effects on transport-associated and leak currents; substrates increase transport-associated current, whereas they decrease leak currents (Sonders et al., 1997; Sitte et al., 1998). Studies in our laboratory using hDAT-expressing oocytes have shown an apparent discrepancy between changes in hDAT function and leak currents following pharmacological manipulation (Mayfield and Zahniser, 2000). Furthermore, a difference in ion selectivity between transport-associated and leak currents suggests multiple permeation pathways within a single transporter (Sonders and Amara, 1996). Thus, it is plausible that the different PTK inhibitors have unique effects on leak current permeation in the hDATs remaining on the cell surface and therefore, the magnitude of the leak current may not reflect the actual number of cell surface hDATs after PTK inhibitor treatment.

According to Michaelis-Menten kinetics, a decrease in V_{\max} with no change in K_m may be the result of a reduction in the number of active transporters. The simplest explanation for this would be that PTK inhibition decreases the number of active hDATs on the cell surface. The genistein-mediated decrease in [^3H]WIN 35,428 binding to cell surface hDATs supports this conclusion. Changes in membrane potentials or ion gradients could also cause a decrease in V_{\max} . We ruled out altered membrane potentials by performing all of our electrophysiological and some of our uptake measurements under voltage clamp. Also, we observed no change in resting membrane potential in unclamped oocytes after genistein treatment (data not shown), suggesting that genistein does not alter ion gradients. Although it is possible that PTK inhibitors could induce a conformational change that altered the binding site such that binding was decreased but no change in the number of cell surface DATs occurred, the PTK inhibitor-induced reduction in [^3H]WIN 35,428 binding and functional DAT measures collectively suggest a redistribution of active hDATs away from the cell surface.

PKC activation similarly decreases DAT V_{\max} , with no change in K_m , the hDAT-associated transport current and hDAT cell surface binding (Kitayama et al., 1994; Huff et al., 1997; Zhang et al., 1997; Zhu et al., 1997; Pristupa et al., 1998). PTK inhibition of hDAT activity could occur via increased endocytosis, similar to the mechanism by which PKC activation regulates hDAT activity (Daniels and Amara, 1999; Melikian and Buckley, 1999). Law et al. (2000) recently found that acute exposure to PTK inhibitors (genistein and K252a) attenuates the function of GAT1 in hippocampal neurons via a redistribution of the transporter away from the cell surface. Since both GAT1 and DAT belong to the same superfamily of Na^+/Cl^- -dependent neurotransmitter transporters, it is possible that PTK inhibitors regulate GAT1 and DAT by a common mechanism. Interestingly, however, the

effects of PTK inhibitors and PKC activators on GAT1 function were additive, suggesting multiple independent pathways accomplish down-regulation of the transporter by these different protein kinases.

Although we have shown that PTK inhibitors can rapidly regulate hDAT, the identity of the PTK(s) involved is not yet known. We have ruled out the involvement of Src family kinases as the potent Src inhibitor PP2 did not alter hDAT function. PTK-mediated regulation of GAT1 is produced in hippocampal neurons by BDNF (Law et al., 2000). Thus, although we cannot rule out numerous receptor and cytosolic PTKs, it is conceivable that DAT regulation can occur in dopaminergic neurons via BDNF activation of trkB receptors. However, further studies using neurons must be conducted to confirm this hypothesis.

Whether PTK inhibition regulates hDAT function by altering the tyrosine phosphorylation state of the hDAT itself or another protein involved in hDAT trafficking is also not known. A correlation between PKC-mediated direct phosphorylation of the hDAT and decreased transport V_{\max} has been observed (Huff et al., 1997). Furthermore, the PTK-mediated redistribution of GAT1 away from the cell surface is associated with increased phosphorylation of the transporter (Law et al., 2000). However, a previous study examining overall phosphotyrosine levels in mouse striata did not detect an effect of genistein on the amount of phosphoproteins from 60 to 80 kDa, where DAT would have been expected (Simon et al., 1997). Thus, although more sensitive methods may ultimately reveal direct tyrosine phosphorylation of hDAT, it is not yet clear whether PTK inhibitors regulate hDAT function by decreasing hDAT phosphorylation. Alternatively, PTK inhibitors may regulate hDAT function by inhibiting the phosphorylation state of another protein that interacts with the hDAT. Indeed, the same study revealed differences in the level of unidentified 110- and 180-kD phosphoproteins (Simon et al., 1997). Furthermore, GAT1 trafficking is influenced by the phosphorylation state of Munc18, a syntaxin 1A binding partner, which regulates the association of syntaxin 1A with the GAT1 (Beckman et al., 1998).

Post-translational modulation of DAT is of physiological and therapeutic importance. Growth factors well known for controlling cell cycle differentiation and survival of dopaminergic neurons may also participate in acute control of dopaminergic neuronal function. If receptor PTK inhibition produces down-regulation of DAT function, stimulation of these kinases by growth factors may acutely increase DAT activity and DA clearance, thereby transiently decreasing synaptic activity. Understanding regulation of the DAT will provide new insights regarding normal dopaminergic function. This will ultimately enhance our understanding of pathological conditions in which DA is implicated, such as Parkinson's disease, schizophrenia, and drug addiction.

Acknowledgment

We thank Dr. Alexander Sorkin for helpful discussions and for critically reading this manuscript.

References

- Amara SG and Kuhar MJ (1993) Neurotransmitter transporters: Recent progress. *Annu Rev Neurosci* 16:73–93.
- Apparsundaram S and Blakely RD (1997) Role of phosphoinositol-3-kinase in the

- acute regulation of human norepinephrine transporters. *Soc Neurosci Abstr* **23**:1132.
- Apparsundaram S, Schroeter S, Giovanetti E and Blakely RD (1998) Acute regulation of norepinephrine transport: II. PKC-modulated surface expression of human norepinephrine transporter proteins. *J Pharmacol Exp Ther* **287**:744–751.
- Barish ME (1983) A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J Physiol (Lond)* **342**:309–325.
- Beckman ML, Bernstein EM and Quick MW (1998) Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. *J Neurosci* **18**:6103–6112.
- Beckman ML and Quick MW (1998) Neurotransmitter transporters: Regulators of function and functional regulation. *J Membr Biol* **164**:1–10.
- Blakely RD and Bauman AL (2000) Biogenic amine transporters: Regulation in flux. *Curr Opin Neurobiol* **10**:328–336.
- Copeland BJ, Vogelsberg V, Neff NH and Hadjiconstantiou M (1996) Protein kinase C activators decrease dopamine uptake into striatal synaptosomes. *J Pharmacol Exp Ther* **277**:1527–1532.
- Daniels GM and Amara SG (1999) Regulated trafficking of the human dopamine transporter. *J Biol Chem* **274**:35794–35801.
- Eggert K, Schlegel J, Oertel W, Wörz C, Krieg JC and Vedder H (1999) Glial cell line-derived neurotrophic factor protects dopaminergic neurons from 6-hydroxydopamine toxicity in vitro. *Neurosci Lett* **269**:178–182.
- Fruman DA, Meyers RE and Cantley LC (1998) Phosphoinositide kinases. *Annu Rev Biochem* **67**:481–507.
- Giros B and Caron MG (1993) Molecular characterization of the dopamine transporter. *Trends Pharmacol Sci* **14**:43–49.
- Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA and Connelly PA (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. *J Biol Chem* **271**:695–701.
- Huff RA, Vaughn RA, Kuhar MJ and Uhl GR (1997) Phorbol esters increase dopamine transporter phosphorylation and decrease transport V_{max} . *J Neurochem* **68**:225–232.
- Hyman C, Juhasz M, Jackson C, Wright P, Ip NY and Lindsay RM (1994) Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. *J Neurosci* **14**:335–347.
- Kitayama S, Dohi T and Uhl GR (1994) Phorbol esters alter functions of the expressed dopamine transporter. *Eur J Pharmacol* **268**:115–119.
- Law RM, Stafford A and Quick MW (2000) Functional regulation of γ -aminobutyric acid transporters by direct tyrosine phosphorylation. *J Biol Chem* **275**:23986–23991.
- Lawrence DS and Niu J (1998) Protein kinase inhibitors: The tyrosine-specific protein kinases. *Pharmacol Ther* **77**:81–114.
- Mayfield RD and Zahniser NR (2000) Dopamine D₂ receptor regulation of the dopamine transporter expressed in *Xenopus* oocytes is voltage-independent. *Mol Pharmacol* **59**:113–121.
- Melikian HE and Buckley KM (1999) Membrane trafficking regulates the activity of the human dopamine transporter. *J Neurosci* **19**:7699–7710.
- Mössner R, Daniel S, Albert D, Heils A, Okaladnova O, Schmitt A and Lesch K-P (2000) Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). *Neurochem Int* **36**:197–202.
- Neet K and Hunter T (1996) Vertebrate non-receptor protein-tyrosine kinase families. *Genes Cells* **1**:147–169.
- Numan S and Seroogy KB (1999) Expression of trkB and trkC mRNAs by adult midbrain dopamine neurons: A double-label in situ hybridization study. *J Comp Neurol* **403**:295–308.
- Prasad PD, Torres-Zamorano V, Keduda R, Leibach FH and Ganapathy V (1997) Functional link between tyrosine phosphorylation and human serotonin transporter gene expression. *Eur J Pharmacol* **325**:85–92.
- Pristupa ZB, McConkey F, Liu F, Man HY, Lee FJS, Wang YR and Niznik HB (1998) Protein kinase-mediated bidirectional trafficking and functional regulation of the human dopamine transporter. *Synapse* **30**:79–87.
- Qian Y, Galli A, Ramamoorthy S, Risso S, DeFelice LJ and Blakely RD (1997) Protein kinase C activation regulates human serotonin transporters in HEK-293 cells via altered cell surface expression. *J Neurosci* **17**:45–57.
- Reith ME and Coffey LL (1993) Cationic and anionic requirements for the binding of 2 beta-carbomethoxy-3 beta-(4-fluorophenyl)[³H]trifluoromethane to the uptake carrier. *J Neurochem* **61**:167–177.
- Reith MEA, Xu C and Chen N-H (1997) Pharmacology and regulation of the neuronal dopamine transporter. *Eur J Pharmacol* **324**:1–10.
- Simon JR, Bare DJ, Ghetti B and Richter JA (1997) A possible role for tyrosine kinases in the regulation of the neuronal dopamine transporter in mouse striatum. *Neurosci Lett* **224**:201–205.
- Sitte HH, Huck S, Reither H, Boehm S, Singer EA and Pifl C (1998) Carrier-mediated release, transport rates, and charge transfer induced by amphetamine, tyramine, and dopamine in mammalian cells transfected with the human dopamine transporter. *J Neurochem* **71**:1289–1297.
- Sonders MS and Amara SG (1996) Channels in transporters. *Curr Opin Neurobiol* **6**:294–302.
- Sonders MS, Zhu S-J, Zahniser NR, Kavanaugh MP and Amara SG (1997) Multiple ionic conductances of the human dopamine transporter: The actions of dopamine and psychostimulants. *J Neurosci* **17**:960–974.
- Taraviras S, Marcos-Gutierrez CV, Durbec P, Jani H, Grigoriou M, Sukumaran M, Wang LC, Hynes M, Raisman G and Pachnis V (1999) Signaling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. *Development* **126**:2785–2797.
- van der Geer P and Hunter T (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Biochem* **10**:251–337.
- Zhang L, Coffey LL and Reith MEA (1997) Regulation of the functional activity of the human transporter in a neuronal cell line. *Mol Brain Res* **59**:33–73.
- Zhu S-J, Kavanaugh MP, Sonders MS, Amara SG and Zahniser NR (1997) Activation of protein kinase C inhibits uptake, currents and binding associated with the human dopamine transporter expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther* **282**:1358–1365.

Send reprint requests to: Dr. Suzanne Doolen, 4200 E. 9th Ave., Box C-236, Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80262. E-mail: suzanne.doolen@uchsc.edu
