1521-0111/12/8101-73–85
MOLECULAR PHARMACOLOGY
U.S. Government work not protected by U.S. copyright
Mol Pharmacol 81:73–85, 2012

Vol. 81, No. 1
73171/3738564

Tyrosine Phosphorylation of the Human Serotonin Transporter: A Role in the Transporter Stability and Function

Balasubramaniam Annamalai, Padmanabhan Mannangatti, Obulakshmi Arapulisamy, Toni S. Shippenberg, Lankupalle D. Jayanthi, and Sammanda Ramamoorthy

Department of Neurosciences, Division of Neuroscience Research Medical University of South Carolina, Charleston, South Carolina (B.A., P.M., L.D.J., S.R.); and Integrative Neuroscience Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland (T.S.S.)

Received April 22, 2011; accepted October 12, 2011

ABSTRACT

The serotonin (5-HT) transporter (SERT) regulates serotoninergic neurotransmission by clearing 5-HT released into the synaptic space. Phosphorylation of SERT on serine and threonine mediates SERT regulation. Whether tyrosine phosphorylation regulates SERT is unknown. Here, we tested the hypothesis that tyrosine-phosphorylation of SERT regulates 5-HT transport. In support of this, alkali-resistant ^{32}P-labeled SERT was found in rat platelets, and Src-tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4,*d*]pyrimidine (PP2) decreased platelet SERT function and expression. In human placental trophoblast cells expressing SERT, PP2 reduced transporter function, expression, and stability. Although siRNA silencing of Src expression decreased SERT function and expression, coexpression of Src resulted in PP2-sensitive increases in SERT function and expression. PP2 treatment markedly decreased SERT protein stability. Compared with WT-SERT, SERT tyrosine mutants Y47F and Y142F exhibited reduced 5-HT transport despite their higher total and cell surface expression levels. Moreover, Src-coexpression increased total and cell surface expression of Y47F and Y142F SERT mutants without affecting their 5-HT transport capacity. It is noteworthy that Y47F and Y142F mutants exhibited higher protein stability compared with WT-SERT. However, similar to WT-SERT, PP2 treatment decreased the stability of Y47F and Y142F mutants. Furthermore, compared with WT-SERT, Y47F and Y142F mutants exhibited lower basal tyrosine phosphorylation and no further enhancement of tyrosine phosphorylation in response to Src coexpression. These results provide the first evidence that SERT tyrosine phosphorylation supports transporter protein stability and 5HT transport.

Introduction

In serotonergic neurons, the serotonin (5-HT) transporter (SERT) mediates reuptake of 5-HT from the synapse, thus regulating 5-HT transmission (Barker and Blakely, 1995). Appropriate expression of SERT is critical in the maintenance of normal 5-HT homoeostasis (Bengel et al., 1998). Altered SERT expression has been implicated in several psychiatric disorders (Murphy et al., 2004). SERT is one of the high-affinity molecular targets for selective serotonin-reuptake inhibitors and drugs of abuse (Ramamoorthy et al., 1993a). SERT function and expression are regulated by gene transcription, as well as translational and post-translational modifications (Ramamoorthy et al., 1993b, 1995, 2011; Jayanthi et al., 1994; Blakely et al., 1997). Much effort has been

This work was supported by the National Institutes of Health National Institute of Mental Health [Grant MH62612] (to S.R); the National Institutes of Health National Institute of General Medical Sciences [Grant GM081054] (to L.D.J.), and in part by the Intramural Research Program of the National Institutes of Health National Institute on Drug Abuse (to. T.S.S).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

http://dx.doi.org/10.1124/mol.111.073171.

ABBREVIATIONS: 5-HT, serotonin; SERT, serotonin transporter; PKC, protein kinase C; CaMK, calcium/calmodulin-dependent protein kinase; MAPK, mitogen-activated protein kinase; PKG, cGMP-dependent protein kinase; GPCR, G protein-coupled receptor; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4,*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine; ECL, enhanced chemiluminescence; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1*H*-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; SP600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one; siRNA, small interfering RNA; HTR, human placental trophoblast cells; KRH, Krebs-Ringer-HEPES; h, human; DAT, dopamine transporter; NET, norepinephrine transporter; TauT, taurine transporter; eGFP, enhanced green fluorescent protein; NE, norepinephrine; DA, dopamine; RIPA, radioimmunoprecipitation assay; PAGE, polyacrylamide gel electrophoresis; β-PMA, phorbol 12-myristate 13-acetate; OV, orthovanadate; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; WT, wild type; ANOVA, analysis of variance; GBR12909, vanoxerine; sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate.

expended on identifying the cellular kinases, phosphatases, and SERT-interacting proteins that are responsible for the regulation of 5-HT uptake (Ramamoorthy et al., 2011). Under basal conditions, SERT proteins are present on the plasma membrane and in intracellular compartments, and this distribution is constitutively regulated by exo- and endocytosis (Ramamoorthy and Blakely, 1999; Samuvel et al., 2005). Under basal conditions, SERT proteins are phosphorylated (Ramamoorthy et al., 1998, 2007; Ramamoorthy and Blakely, 1999; Samuvel et al., 2005). Depending on the stimulus, 5-HT uptake and SERT trafficking may be differentially affected, but are often linked with altered SERT basal phosphorylation by Ser/Thr protein kinases (Ramamoorthy et al., 2011). For example, protein kinase C (PKC) activation or inhibition of calcium/calmodulin-dependent protein kinase (CaMK) and p38 mitogen-activated protein kinase (p38 MAPK) decreases SERT activity and regulates SERT basal phosphorylation (Jayanthi et al., 1994; Ramamoorthy et al., 1998; Ramamoorthy and Blakely, 1999; Samuvel et al., 2005). Conversely, activation of p38 MAPK and cGMP-dependent protein kinase (PKG) stimulates 5-HT uptake and triggers SERT phosphorylation (Samuvel et al., 2005; Ramamoorthy et al., 2007). Furthermore, SERT activity attenuates PKC-induced alterations in SERT phosphorylation and surface expression and its interaction with PP2A catalytic subunit (Ramamoorthy and Blakely, 1999; Bauman et al., 2000). Activation of 5-HT_{1B}, adenosine, and atypical histamine receptors also enhances 5-HT transport (Launay et al., 1994; Daws et al., 2000; Zhu et al., 2004). We have established that PKG-mediated SERT regulation requires phosphorylation of Thr276 and demonstrated a mechanistic basis for a direct link between 5-HT transport and alterations in SERT phosphorylation in response to changes in PKG activity (Ramamoorthy et al., 2007). It is noteworthy that mutations in SERT that affect the PKG/p38 MAPK pathway are associated with psychiatric disorders such as obsessive-compulsive disorder, autism, and Asperger's syndrome (Prasad et al., 2005; Zhang et al., 2007).

Recent studies have revealed that Src kinases, members of the nonreceptor tyrosine kinase family, play an important role in GPCR function (Luttrell et al., 1999). Several Src family members, such as cSrc, Fyn, and Yes, are activated by GPCRs and growth factor receptors (Narisawa-Saito et al., 1999). 5-HT neurons express Src-family kinases as well as tyrosine kinase receptors (Madhav et al., 2001) raising the possibility that stimulation of presynaptic receptors located on 5-HT neurons may regulate SERT via tyrosine kinase-mediated pathways. Although short- and long-term treatment with tyrosine kinase inhibitors or activators regulate SERT function and gene transcription (Helmeste and Tang, 1995; Kekuda et al., 1997; Prasad et al., 1997; Zarpellon et al., 2008), the precise molecular basis underlying tyrosine kinase-mediated SERT regulation is not clear. The SERT sequence contains four tyrosine residues (Tyr47, Tyr142, Tyr350, and Tyr358) that face the inside of the cell and could be potential phosphorylation targets for tyrosine kinase(s) (Ramamoorthy et al., 1993a). To date, information regarding the site(s) of tyrosine phosphorylation within SERT and the role of phosphorylation in SERT functional expression is lacking.

In this study, we tested the hypothesis that SERT tyrosine phosphorylation regulates 5-HT transport and SERT protein expression. We found that 1) SERT exists in a tyrosine-phosphorylated form, 2) inhibition of tyrosine kinase(s) reduces SERT expression levels by facilitating SERT protein degradation, 3) Src-kinase activity up-regulates SERT protein expression with a concomitant increase in 5-HT uptake and tyrosine phosphorylation, and 4) mutation of Tyr47 or Tyr142 abolishes src-induced increases in transport function and phosphorylation of SERT. These data demonstrate that tyrosine phosphorylation of SERT is critical for both appropriate SERT protein expression and 5-HT transport function.

Materials and Methods

Reagents and Antibodies. 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4,*d*]pyrimidine (PP2) and 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (PP3) were obtained from Calbiochem (San Diego, CA). Protein A-Sepharose beads were obtained from Amersham Biosciences (Chalfont St. Giles, Buckinghamshire, UK); ECL reagents, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (EZ link NHS-Sulfo-SS-biotin) and NeutrAvidin agarose were from Thermo Fisher Scientific (Waltham, MA). 5-Hydroxy-[^{3}H]tryptamine trifluoroacetate ([^{3}H]5-HT; 28.1 Ci/mmol), DL-[7-^{3}H (N)] norepinephrine (4.8 Ci/mmol), dihydroxyphenylethylamine 3,4[ring-2,5,6-^{3}H]dopamine (34.6 Ci/mmol), $^{32}PO_4$ carrier-free orthophosphate, and Kodak BioMax Ms-1 films were from PerkinElmer Life and Analytical Sciences (Waltham, MA). Taurine [2-^{3}H] (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Reagents for SDS-polyacrylamide gel electrophoresis and Bradford Protein Assay were obtained from Bio-Rad Laboratories (Hercules, CA). The following antibodies were used: anti-Src (clone GD11) and anti-phospho-Src (Tyr418) were obtained from Millipore (Billerica, MA); anti-calnexin antibody was from AKELA Pharma Inc. (Montreal, QC, Canada). 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059), 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1*H*-imidazole (PD169316), 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene (U0126), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580), and anthra[1,9-*cd*]pyrazol-6(2*H*)-one (SP600125) were obtained from Calbiochem (La Jolla, CA). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). RPMI 1640 medium was from Mediatech (Herndon, VA) Transient FuGENE 6 transfection reagent was obtained from Roche Diagnostics (Indianapolis, IN). All other cell culture media and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). ON-TARGETplus SMARTpool specific to c-Src and ON-TARGETplus nontargeting siRNA were purchased from Thermo Scientific Dharmacon (Lafayette, CO). All other chemicals were obtained from Sigma (St. Louis, MO) or Thermo Fisher Scientific unless otherwise indicated.

Preparation of Platelet-Rich Plasma and Isolation of Platelets. All animal procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996), promulgated by the U.S. National Institutes of Health. After rapid decapitation, rat blood from the trunk was collected in tubes containing acid-citrate-dextrose (Jayanthi et al., 2005). Blood was centrifuged immediately at 190*g* for 15 min at room temperature to obtain platelet-rich plasma. As given in the legend to Fig. 1, the platelet-rich plasma was treated with PP2 or activated orthovanadate at the indicated concentrations and times at 37°C. At the end of the treatment and incubation time, the platelet-rich plasma was centrifuged at 2500*g* for 15 min at 22°C. The resulting platelet pellet was gently resuspended in assay buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, and 20 mM HEPES, pH 7.2) and

used immediately to perform immnuoblotting experiments as described below and by Jayanthi et al. (2005).

Transfection and Treatments of Human Placental Trophoblast Cells. The HTR cell line (Graham et al., 1993) was kindly provided by Dr. Charles H. Graham (Queen's University, Kingston, ON, Canada). HTR parental cells were cultured in a mixture of RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/100 μg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37°C. Transient transfections were carried out by FuGENE 6 transfection reagent according to the manufacturer's instructions. Cells were seeded on 12-well (15×10^4 cells per well) or 24-well (7.5×10^4 cells per well) plates and maintained for 24 h before transfections. Cells were transfected with different expression plasmids together with transporter cDNAs. HTR cells were transfected with hSERT or human norepinephrine transporter (hNET) or dopamine transporter (hDAT) or taurine transporter (hTauT) plasmids together with eGFP or Src plasmids. In all wells, the total amount of plasmid DNA was adjusted with corresponding empty vector (eGFP). Approximately 40 to 60% of cells were transfected as observed with the expression of eGFP after 48-h transfection. Where indicated, cells were treated with different modulators and used 48 h after transfection.

RNA Interference. The c-Src interference was performed in HTR cells with ON-TARGETplus SMARTpool small interfering RNA (siRNA) specific to c-Src. The unspecific scrambled sequence was used for control transfections. siRNA duplexes (25 nM) were transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions. At 16 h after transfection with siRNAs, cells were transfected with hSERT (100 ng/well). Twenty-four hours later, 5-HT uptake was performed as described previously (Samuvel et al., 2005). In parallel, suppression of c-Src was verified by Western blotting with Src-specific antibodies. The same blot was reprobed with anti-calnexin antibody to determine equal loading and nonspecific effect of siRNAs.

Measurement of Transport Activity. 5-HT uptake was performed as described previously (Samuvel et al., 2005). Transfected HTR cells were washed with KRH assay buffer, pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, and 10 mM D-glucose) containing 0.1 mM ascorbic acid and 0.1 mM pargyline and preincubated with the modulators at 37°C for the indicated times followed by addition of 50 nM [^{3}H]5-HT to initiate SERT activity. 5-HT uptake was terminated after 10-min incubation at 37°C by rapid washings with KRH buffer containing 10 μM imipramine. Cells were lysed with 1% SDS, and the accumulated radioactivity was measured by liquid scintillation counting using a MicroBeta2 LumiJET (PerkinElmer Life and Analytical Sciences). For saturation analysis, [^{3}H]5-HT was mixed with unlabeled 5-HT from 10 nM to 5 μM. Nonspecific 5-HT uptake was defined as the accumulation in the presence of 0.1 μM fluoxetine and was subtracted from total 5-HT uptake. In parallel experiments, hDAT, hNET, and hTauT activities were measured. Cells transfected with hNET, hDAT, or hTauT expression plasmids together with eGFP or Src plasmids were used for transport assays 48 h after transfection by incubating with 50 nM [^{3}H]NE (hNET), [^{3}H]DA (hDAT), or [^{3}H]Tau (hTauT) for 10 min at 37°C. Nonspecific uptake in the presence of 0.1 μM nisoxetine (hNET) or 0.1 μM vanoxerine (GBR12909; hDAT) or in the absence of Na^+/Cl^-(hTauT) was subtracted from the total accumulation to yield specific uptake. All uptake assays were performed in duplicate or triplicate, and mean values of specific uptake S.E.M of at least three separate experiments were determined.

Site-Directed Mutagenesis of Tyrosine Residues in hSERT. Tyrosine residues in hSERT intracellular domains (Tyr47, Tyr142, Tyr350, and Tyr358) were mutated to phenylalanine or alanine residues using the QuikChange site-directed mutagenesis kit as outlined by Stratagene. XhoI/AgeI or XbaI/AgeI fragments containing the mutated site(s) were then ligated into correspondingly cut wild-type hSERT. Mutations were confirmed by sequencing the DNAs on both strands.

Metabolic Labeling and Detection of SERT Tyrosine Phosphorylation. Cells or rat platelets were metabolically labeled with [^{32}P]orthophosphate as described previously (Ramamoorthy et al., 1998; Jayanthi et al., 2005). In brief, cells or platelets were incubated with 1 mCi/ml [^{32}P]orthophosphate for 1 h in phosphate-free media and subjected to treatments as indicated in the figure legends. After washing and solubilization with RIPA lysis buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, and 0.1% sodium deoxycholate) supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, and 250 μM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM sodium fluoride, 50 mM sodium pyrophosphate, and 5 mM activated orthovanadate), SERT proteins were immunoprecipitated with SR-12 SERT-specific polyclonal antibody along with parallel control experiments as described previously (Ramamoorthy et al., 1998; Jayanthi et al., 2005). In our previous studies, the use and specificity of SR-12 SERT antibody were thoroughly characterized (Jayanthi et al., 2005; Samuvel et al., 2005). The SERT immunoprecipitates were captured by protein A-Sepharose resins, washed, and subjected to SDS-PAGE. To detect SERT tyrosine phosphorylation and to remove nontyrosine phospho-residues, the fixed and stained gels were subjected to alkali treatment as described previously (Cheng and Chen, 1981; Golden et al., 1986; Panneerselvam et al., 1987). The gels were incubated with 1 M NaOH for 15 min at room temperature then at 44°C for 60 min. The gels were then washed with 7% acetic acid/7% methanol for 2 h at 22°C. Dried gels were exposed to Kodak BioMax Ms-1 films, and alkali-resistant ^{32}P-tyrosine labeled SERT was detected by autoradiography. Multiple exposures (2–14 days) were evaluated by digital quantitation using ImageJ (http://rsbweb.nih.gov/ij/) software to ensure that results were within the linear range of the film exposure.

Cell Surface Biotinylation and Immunoblotting. Cell surface biotinylation and immunoblotting was performed as described previously (Samuvel et al., 2005). In brief, transfected cells grown in 12-well plates were first washed twice with ice-cold phosphate-buffered saline/Ca^{2+}-Mg^{2+} (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.3). Then cells were incubated with EZ link NHS-Sulfo-SS-biotin (1 mg/ml) for 30 min on ice; then, the excess biotinylating reagents were removed by two washes with 100 mM glycine, and the cells were further incubated with glycine for 20 min. The cells were solubilized using RIPA buffer containing a cocktail of protease and phosphatase inhibitors. Total cellular protein content was determined by the Bradford Protein Assay procedure. Using equal amounts of solubilized proteins, the biotinylated proteins were isolated using NeutrAvidin Agarose resins overnight at 4°C followed by washing. Bound proteins were eluted with 50 μl of Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, and 5% β-mercaptoethanol) for 30 min at room temperature. Aliquots from total extracts, unbound fractions, and all the eluate were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with SERT-specific SR-12 antibody or other antibodies as indicated in figures and legends. The immunoreactive proteins were visualized using ECL or ECL plus reagent. Subsequently, the blots were stripped and reprobed with anti-calnexin antibody to validate the surface biotinylation of plasma membrane proteins as well as loading protein levels. SERT densities from total, nonbiotinylated (representing the intracellular pool), and biotinylated (representing the surface pool) fractions were normalized using levels of calnexin in the total extract. Multiple exposures were evaluated by digital quantitation by using ImageJ software to ensure that results were within the linear range of the film exposure. Because of variations in SERT expression levels that are associated with transient transfections, it was necessary to adjust the time of film exposure. Therefore, direct quantitative comparisons of SERT protein between experiments/figures are not possible.

Biotinylation and SERT Stability Assay. The stability of biotinylated total SERT was determined in HTR cells that were transfected with hSERT plus eGFP vector. All biotinylation procedures were conducted under sterile conditions. Cells were biotinylated for 2 h at 37°C followed by quenching with glycine and washing as described under *Cell Surface Biotinylation and Immunoblotting*. Prewarmed complete growth media with or without PP2 were added to the cells, and the cells were maintained in an incubator (5% CO_2 humidified atmosphere) at 37°C. Further incubations were continued for 6, 12, 24, and 36 h. For the 0 h time-point, biotinylated cells were immediately processed for extraction. At the end of the each incubation period, cells were solubilized in RIPA, biotinylated SERT proteins were separated from nonbiotinylated proteins using NeutrAvidin Agarose resins, and eluted biotinylated proteins were resolved by SDS-PAGE. SERT proteins in the fractions were visualized by immunoblotting with SR-12 SERT antibody and quantified as described under *Cell Surface Biotinylation and Immunoblotting*.

Statistical Analyses. Values are expressed as mean ± S.E.M. As noted in the figure legends, one-way analysis of variance was used followed by post hoc testing (Bonferroni, Dunnett, and Newman-Keuls) for multiple comparisons, and Student's *t* test was performed for paired observations with Prism (GraphPad, San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

Results

The Role of Tyrosine Kinase(s) in Regulating the Function and Expression of SERT in Rat Platelets. Previous studies using human platelets indicated that although acute tyrosine kinase inhibition decreased 5-HT uptake, inhibition of tyrosine phosphatase increased uptake (Helmeste and Tang, 1995; Zarpellon et al., 2008). Because persistent activation or inhibition of cellular signaling cascades can differentially affect SERT regulation (Jayanthi et al., 2005) we initially examined the effects of short and prolonged (30 min versus 24 h) inhibition of protein tyrosine kinase(s) and protein tyrosine phosphatase(s) on SERT activity and expression using rat platelet-rich plasma. Figures 1A shows the effect of tyrosine kinase and tyrosine phosphatase inhibition on 5-HT uptake and SERT protein expression. Incubation of platelet-rich plasma for 24 h at 37°C resulted in a significant loss of both 5-HT uptake and SERT protein compared with that found at 30 min (Fig. 1). Furthermore, incubation of platelet-rich plasma with PP2, a Src-family kinase inhibitor, during the 24-h period, further decreased 5-HT uptake (Fig. 1A) and SERT protein (Fig. 1, B and C). It is noteworthy that addition of activated orthovanadate, a protein tyrosine phosphatase inhibitor, during the 24-h incubation period prevented the decrease in 5-HT uptake (Fig. 1A) and the loss of SERT protein (Fig. 1, B and C). Treatment of activated orthovanadate for 30 min produced a significant increase in 5-HT uptake without affecting SERT protein (Fig. 1).

Tyrosine Phosphorylation of SERT in Rat Platelets. If tyrosine kinase is necessary for the regulation of SERT expression and function, it is possible that tyrosine kinase phosphorylates SERT on tyrosine residue(s). We next sought to determine whether SERT is phosphorylated on tyrosine residues in rat platelets. Our effort using anti-phosphotyrosine antibodies (freeform or coupled to agarose) yielded unsuccessful results because of the presence of IgG at the location of SERT band interfering with immunoblot analysis using SERT or p-Tyr antibodies. We also reasoned that certain proteins known to contain phosphorylated tyrosine(s) may not be recognized by anti-phosphotyrosine antibody be-

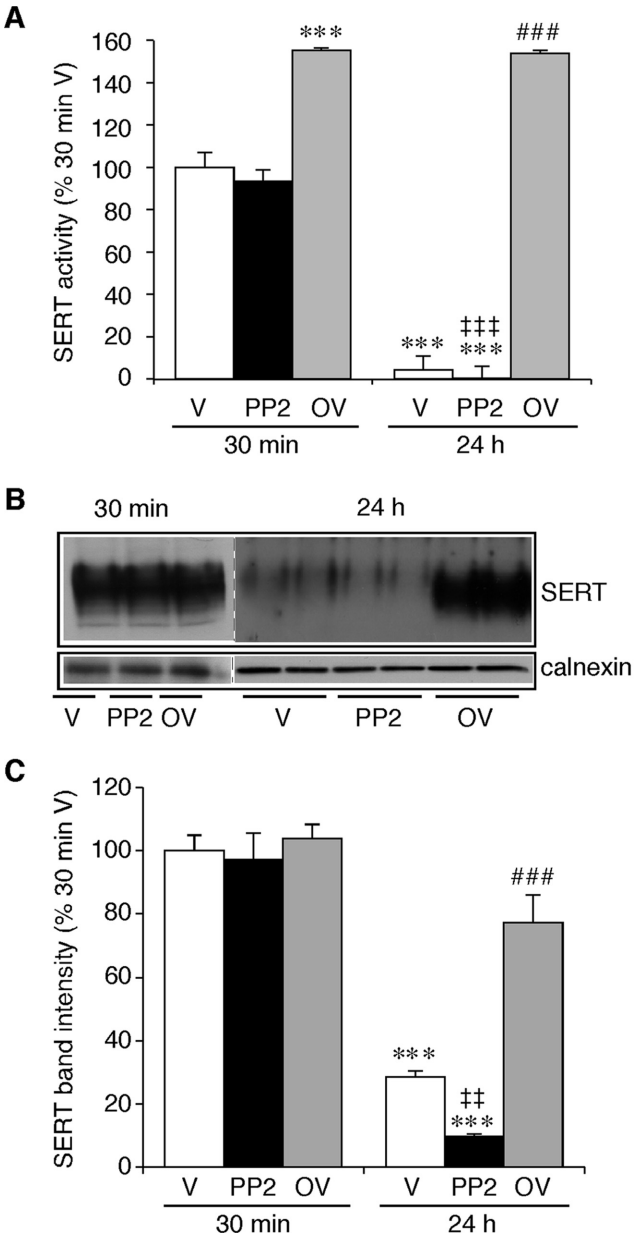

Fig. 1. Effect of pharmacological manipulation of tyrosine kinase/phosphatases on 5-HT uptake and SERT protein in platelets: platelet-rich plasma (from rats) was incubated with vehicle (V) or 10 μM PP2 or 50 μM activated orthovanadate (OV) for either 30 min or 24 h at 37°C and used for (A) 5-HT uptake assays or (B) immunoblot analysis. A, platelets were isolated and 5-HT uptake was measured as described under *Materials and Methods*. Nonspecific uptake in the presence of 0.1 μM fluoxetine was subtracted from the total accumulation to yield specific uptake. The results are expressed as percentage of uptake measured in vehicle treated platelets and represent the mean ± S.E.M. of three experiments. Specific 5-HT uptake in vehicle treated platelets was 232 ± 17 fmol/10^6 platelets/min (100%). ***, $p < 0.001$ compared with 30-min vehicle control; ‡‡‡, $p < 0.001$ compared with 24-h vehicle control; ###, $p < 0.001$ compared with 24 h vehicle control (one-way ANOVA with post hoc Newman-Keuls test). B, in parallel, isolated platelets were solubilized and subjected to SDS-PAGE followed by immunoblotting with SR-12 SERT antibody as described under *Materials and Methods*. Representative SERT immunoblots (~100 kDa) of three separate experiments are shown. Immunoblotting of calnexin is also shown. C, quantitative analysis of SERT band densities. SERT proteins were quantified using ImageJ, and the densities of the SERT band from three separate experiments are presented as mean ± S.E.M. ***, $p < 0.001$ compared with 30 min vehicle control; ‡‡‡, $p < 0.001$ compared with 24-h vehicle control; ###, $p < 0.001$ compared with 24-h vehicle control (one-way ANOVA with post hoc Newman-Keuls test).

cause of steric hindrance of the recognition site. In addition, SERT is also constitutively phosphorylated on serine and threonine residues, and thus differentiation of tyrosine phosphorylation with metabolic labeling studies is difficult. It is known that phosphodiester linkage to tyrosine is more resistant to alkali treatment than phosphothreonine and phosphoserine (Cheng and Chen, 1981; Golden et al., 1986). Therefore, alkali treatment was performed to hydrolyze the phosphoserine and phosphothreonine residues and detect only phosphotyrosine in SERT. Similar strategies have been adapted to detect tyrosine phosphorylated proteins by others and us from platelets and brain tissue (Cheng and Chen, 1981; Golden et al., 1986; Panneerselvam et al., 1987). As a proof of concept, Fig. 2A shows the effect of alkali treatment on SERT phosphorylation. We have reported previously that short-term PKC activation triggers SERT phosphorylation on Ser residues and that prolonged PKC activation triggers

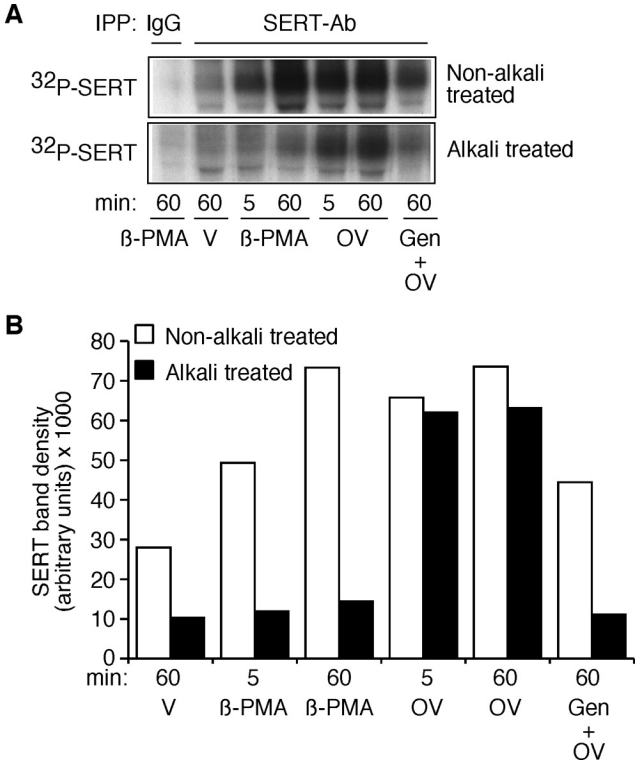

Fig. 2. Tyrosine phosphorylation of SERT in rat platelets. A, rat platelets were metabolically labeled with [^{32}P]orthophosphate for 45 min at 37°C and treated with vehicle and the indicated regulatory agents [1 μM β-PMA, 10 μM activated orthovanadate (OV), and 10 μM genistein (Gen)]. The tyrosine kinase inhibitor genistein was added 15 min before addition of activated orthovanadate and further incubation at 37°C was carried out for the indicated times. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described under *Materials and Methods*. Two identical sets of SDS-PAGE were conducted. Gels were stained and fixed with 40% (v/v) methanol and 10% (v/v) acetic acid. After fixing, one gel was dried (non–alkali-treated) and another gel was incubated with 1 M NaOH at 44°C for 60 min (alkali-treated) followed by washing and drying as described under *Materials and Methods*. Both dried gels were exposed to Kodak BioMax Ms-1 films, and total and alkali-resistant ^{32}P-tyrosine labeled SERT (~100 kDa) was detected by autoradiography as described under *Materials and Methods.* Parallel experiments were performed using irrelevant IgG to validate specificity of SERT-specific antibody SR-12. Note that there was no ^{32}P-labeled SERT band (~100 kDa) when irrelevant IgG is used. Also note that alkali treatment removed β-PMA-triggered Ser and Thr phosphorylation of SERT but not genistein-sensitive and -activated orthovanadate-induced SERT phosphorylation (A and B). Experiments were repeated with essentially equivalent results.

both Ser and Thr phosphorylation in rat platelets. Consistent with our published results (Jayanthi et al., 2005), when metabolically ^{32}P-labeled platelets were exposed to β-PMA for 5 and 60 min, ^{32}P-SERT (~100 kDa) increased in a time-dependent manner (Fig. 2, A and B). Treatment with the protein tyrosine phosphatase inhibitor, activated orthovanadate (OV), increased the level of ^{32}P-SERT that is sensitive to the protein tyrosine kinase inhibitor genistein (Fig. 2, A and B). Similar to genistein, another Src-kinase inhibitor, PP2 also inhibited activated orthovanadate-induced ^{32}P labeling of SERT (data not shown). However, alkali treatment of the gel resulted in 1) reduced ^{32}P labeling of SERT in vehicle-treated cells and 2) complete loss of β-PMA-mediated, but not activated orthovanadate-induced, ^{32}P labeling of SERT (Fig. 2, A and B). These results indicate that alkali treatment removed ^{32}P labeling from Ser and Thr residues of ^{32}P-SERT and established the validity of alkali treatment procedure to visualize SERT-Tyr phosphorylation. Furthermore, it also revealed that SERT proteins in rat platelets are phosphorylated on tyrosine residue(s).

Pharmacological Inhibition of Src-Kinase Reduces SERT Activity in HTR Cells. The data presented in Figs. 1 and 2 suggest that the protein tyrosine kinase-linked pathway regulates activity and stability of SERT protein, perhaps via tyrosine phosphorylation of SERT. To further characterize the involvement of tyrosine kinase, to identify the site of tyrosine phosphorylation in SERT, and finally to verify its functional importance in 5-HT uptake, we used HTR cells as a heterologous expression system. Human placenta expresses SERT (Balkovetz et al., 1989). Although HTR cells are derived from human placenta (Graham et al., 1993), they do not express endogenous SERT or NET (S. Ramamoorthy and L. D. Jayanthi unpublished observations) and serve as a suitable endogenous cell model to study amine transport regulation (Mannangatti et al., 2011). We expressed hSERT in HTR cells and studied the effect of PP2 on 5-HT uptake. Figures 3, A and B show that PP2 produces a time- and concentration-dependent decrease in 5-HT uptake. Time course studies revealed a significant inhibition of 5-HT uptake after 6-h treatment with 10 μM PP2 and further inhibition after 24-h treatment (Fig. 3A). When PP2 pretreatment time was fixed at 24 h, significant reductions in 5-HT uptake were observed at 0.5 μM with further decreases at 10 and 20 μM (Fig. 3B). Treatment with PP3 (20 μM for 24 h), the inactive analog of PP2, did not alter SERT activity (Fig. 3B). In parallel experiments, we determined constitutive levels of endogenous Src and its active form, phospho-(Tyr^{416})Src. Figure 3C shows that HTR cells express both Src and pSrc. PP2 treatment decreased the level of pSrc.

Coexpression of Src-Kinase Increases SERT Activity in HTR Cells. Additional experiments were conducted to specifically test the physiological role of Src kinase in regulating SERT activity. HTR cells were transfected with either hSERT plus Src or the eGFP vector. Total Src, phospho-(Tyr416)Src, and SERT activity were examined 24 h later. Figure 3C confirms higher levels of total and active forms of phospho-(Tyr416)Src in Src-transfected cells and shows that PP2 treatment decreased the level of active pSrc. 5-HT transport in cells transfected with hSERT + Src was higher than that observed in cells transfected with hSERT + eGFP (Fig. 3D). PP2, but not PP3, blocked the Src-induced increase in 5-HT uptake. PP2 at 0.5 μM completely blocked the Src-

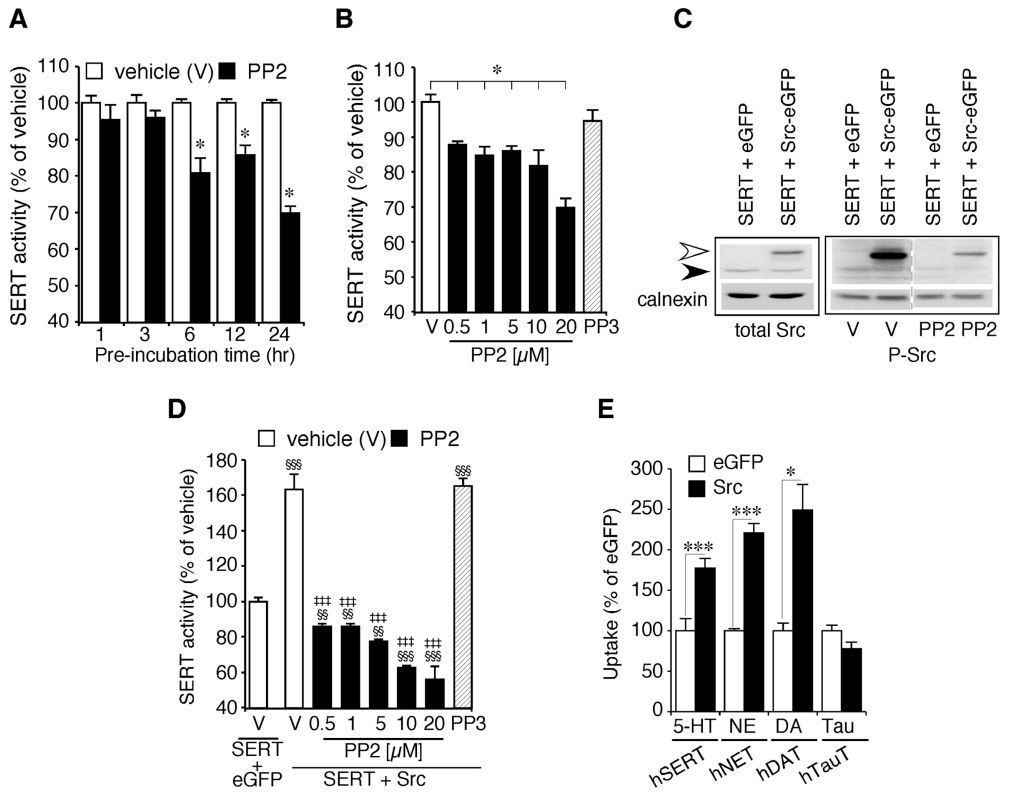

Fig. 3. Effect of PP2 (time and concentration) and Src cotransfection on 5-HT transport in HTR cells. HTR cells transiently expressing SERT were treated with vehicle or 20 μM PP2 for the times indicated (A) or treated with PP2 at the indicated concentrations (B) for 24 h. In parallel, the PP2 inactive analog PP3 (20 μM) was incubated for 24 h (B). After treatments, 5-HT uptake was assayed as described under *Materials and Methods*. HTR-cells transfected with hSERT plus either eGFP (empty vector) or Src-eGFP was analyzed for total and p-Src expression (C) or 5-HT transport (D) as described under *Materials and Methods*. Representative Western blots from three separate experiments show expression levels of total and p-Src and calnexin (C). In C, top arrowhead (unfilled) represents Src-eGFP transfected cells, bottom arrowhead (filled) represents endogenous Src, and vertical dashed line indicates that the lanes were excised [vehicle and PP2 (20 μM)] from the same gel/Western blot. A, B, and D, the results are expressed as percentage of uptake measured in vehicle (V) treated cells and represented as mean ± S.E.M. of three experiments performed in triplicate. Specific 5-HT uptake in vehicle treated hSERT + eGFP cells was 1184 ± 64 fmol/10^6 cells/min (100%). *, $p < 0.05$ compared with vehicle control (V); §§, $p < 0.01$; §§§, $p < 0.001$ compared with eGFP-vehicle control (V); ‡‡‡, $p < 0.001$ compared with Src-vehicle control (V) (one-way ANOVA with Newman-Keuls post hoc analysis). E, HTR cells transfected with indicated transporter cDNA plus either eGFP (empty vector) or Src-eGFP and assayed for uptake of corresponding substrates as given under *Materials and Methods*. Nonspecific uptake in the presence of 0.1 μM fluoxetine (hSERT), 0.1 μM nisoxetine (hNET), or 0.1 μM GBR12909 (hDAT) or in the absence of Na^+/Cl^- (hTauT) was subtracted from the total accumulation to yield specific uptake. The results are expressed as percentage of uptake measured in transporter + eGFP transfected cells and represent the mean ± S.E.M. *, $p < 0.05$; ***, $p < 0.001$ compared with transporter + eGFP control (Student's t test).

induced increase in 5-HT uptake. A further increase in PP2 concentration not only blocked Src-induced increases in 5-HT uptake but also resulted in 5-HT uptake lower than that in cells transfected with hSERT + eGFP control. There was no change in 5-HT uptake in cells transfected with hSERT + inactive Src compared with that in cells transfected with hSERT + eGFP (data not shown). The magnitude of stimulation of 5-HT uptake varied between experiments with a minimum of 50% to a maximum of 100%. To determine the selectivity of the Src-kinase effect, HTR cells were transfected with NET, DAT, or TauT [other members of the Na^+/Cl^--dependent transporter gene (SLC6) family] plus eGFP or Src, and uptake of NE, DA, or Tau was measured. NET-mediated NE uptake and DAT-mediated DA uptake were significantly higher in Src-cotransfected cells compared with eGFP-cotransfected cells. However, hTauT-mediated Tau uptake was not significantly altered by Src coexpression (Fig. 3E).

Specific Knockdown of Src-Kinase by siRNA Reduces SERT Activity in HTR Cells. To confirm the validity of Src kinase inhibitor PP2 and to further confirm the specific involvement of Src kinase in the regulation of SERT activity, we used short interfering RNA targeting Src kinase and determined the effect of loss of function of Src on 5-HT uptake in HTR-cells. Forty-eight hours after transfection, total Src as well as phospho-Src were significantly reduced in cells transfected with 25 nM Src-siRNA and hSERT compared with cells transfected with nonspecific scrambled siRNA and hSERT (Fig. 4A). As controls for specificity, Src-siRNA and/or scrambled siRNA transfection did not affect the total expression of calnexin (Fig. 4A). Thus, the specific and robust reduction of total and active Src protein by siRNAs provides a reliable method for analyzing the specific role of Src in the regulation of 5-HT uptake. We found a significant decrease in 5-HT uptake in cells transfected with hSERT and Src-siRNA relative to cells transfected with hSERT and nonspecific scrambled siRNA (Fig. 4B). These results are consistent with results from our pharmacological approach using PP2 to block Src (Fig. 3) and strongly support the idea that endogenous Src plays a crucial role in the regulation of SERT activity.

Src-Kinase Alters SERT Kinetics. Kinetic parameters of 5-HT uptake in HTR cells transfected with hSERT + eGFP and hSERT + Src are presented in Fig. 5. Treatment with PP2 produced a highly significant reduction (~50%) in the

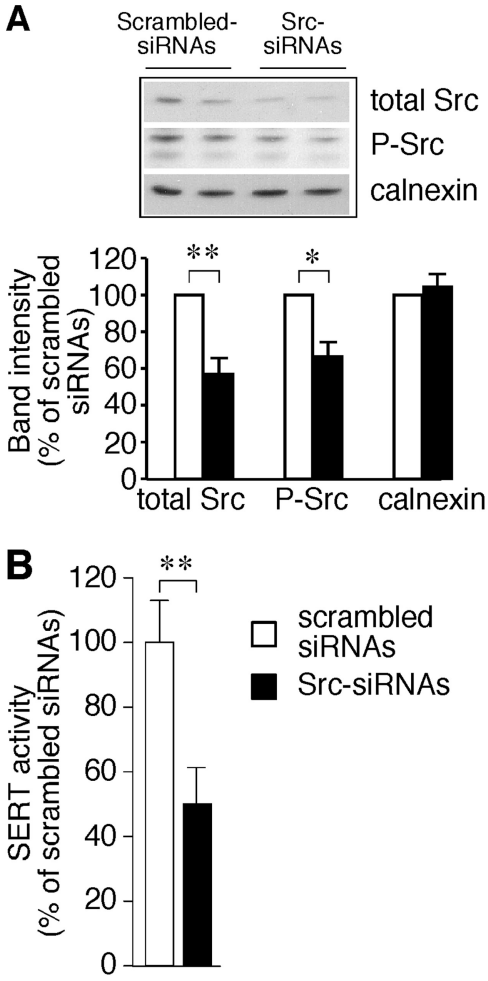

Fig. 4. Effect of siRNA knock-down of Src expression on 5-HT transport. HTR cells were transiently transfected with hSERT plus 25 nM siRNAs targeted to Src or control scrambled siRNAs. After 48 h, cells were used for analysis of total and p-Src expression (A) and 5-HT transport (B) as described under *Materials and Methods*. A, representative Western blot shows the expression levels of total and phospho-Src and calnexin. Band densities are presented in the lower panel as mean ± S.E.M. **, $p < 0.004$ ($n = 6$); *, $p < 0.025$ ($n = 4$) compared with scrambled siRNAs (Student's t test). B, 5-HT uptake values (mean ± S.E.M.) are shown. **, $p < 0.01$ ($n = 9$) compared with scrambled siRNAs (Student's t test). Specific 5-HT uptake in scrambled siRNAs transfected cells was 1289 ± 194 fmol/10^6 cells/min (100%).

maximal velocity (V_{max}) and a moderate increase in the affinity for 5-HT. V_{max} was decreased from 2875 ± 105 fmol/10^6 cells/min (vehicle) to 1360 ± 39 fmol/10^6 cells/min (PP2). Corresponding K_m values are 424 ± 47 nM (vehicle) and 276 ± 28 nM (PP2). The V_{max} was significantly higher with a moderately decreased affinity in hSERT- + Src-transfected cells. The corresponding values for hSERT- + Src-transfected cells were, V_{max}, 6254 ± 502 fmol/10^6 cells/min and K_m 580 ± 128 nM. Thus, the Src-induced stimulation of 5-HT uptake was due primarily to an increase in V_{max}.

Src-Kinase Up-Regulates SERT Protein Expression. Expression of Src kinase not only increased 5-HT uptake (Fig. 3) but also increased total SERT expression (Fig. 6A). Consistent with the inhibitory effect of PP2 on 5-HT uptake (Fig. 3), PP2 (10 μM or 20 μM) treatment for 24 h decreased total, intracellular, and surface SERT expression in cells transfected with SERT + eGFP (Fig. 6B). In addition, Src-induced increases in the expression of total, intracellular, and surface SERT protein were also sensitive to PP2 (Fig. 6C). These results further suggest that Src-kinase regulates both SERT protein expression and function.

Inhibition of Serine/Threonine Protein Kinases Does Not Block Src-Mediated SERT Up-Regulation. Because Src is a possible upstream regulator of several Ser/Thr protein kinases, and several of these kinases have been linked to SERT regulation, we tested whether SERT stimulation by Src is linked to Ser/Thr protein kinases. Cells transfected with hSERT + eGFP or hSERT + Src were treated with or without kinase inhibitors for 24 h, and the Src effect on SERT activity was determined. Table 1 summarizes the influence of various kinase inhibitors on the Src effect on SERT. Consistent with the above results (Fig. 3D), coSrc expression produced a PP2-sensitive stimulation of SERT activity. Treatment with inhibitors of Src, PKC, PI3K, p38 MAPK, and CaMKII significantly reduced 5-HT uptake in hSERT- + eGFP-transfected and hSERT- + Src-transfected cells. ERK inhibitor produced a nonsignificant reduction in 5-HT uptake in both in hSERT- + eGFP-transfected and hSERT- + Src-transfected cells. However, the Src-dependent stimulatory effect was not altered by the presence of these inhibitors, suggesting that SERT up-regulation by Src is not linked to p38 MAPK, ERK, PKC, CaMKII, or PI3K-signaling.

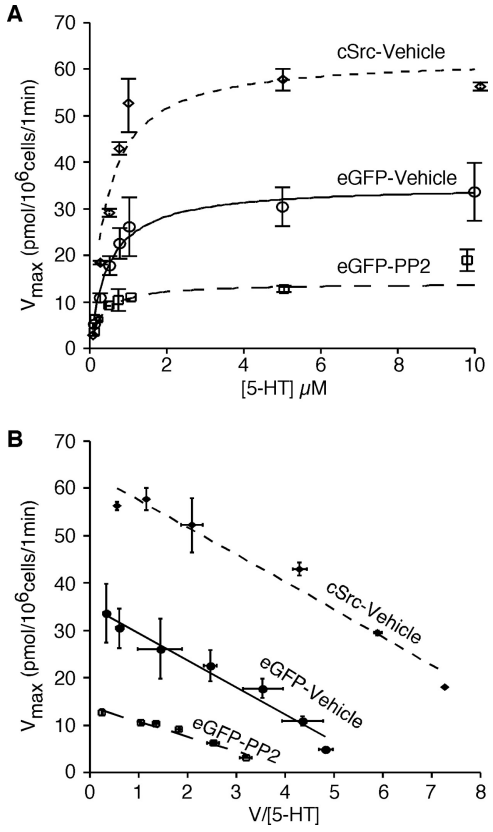

Fig. 5. 5-HT uptake kinetics in vehicle and PP2-treated cells expressing SERT alone or SERT plus Src. HTR cells were cotransfected with hSERT + eGFP or hSERT + Src. After 24 h, cells were washed and exposed to vehicle or 20 μM PP2 for 24 h (hSERT + eGFP cells) and vehicle (hSERT + Src cells). After treatments, uptake of 5-HT was measured over a concentration range of 0.01 to 10 μM using a 10-min uptake period as described under *Materials and Methods*. Values represent the mean ± S.E.M. of three independent experiments. A, nonlinear curve fits of data for uptake used the generalized Michaelis-Menten equation (KaleidaGraph; Synergy Software, Reading, PA). B, an Eadie-Hofstee plot of transformation of the data.

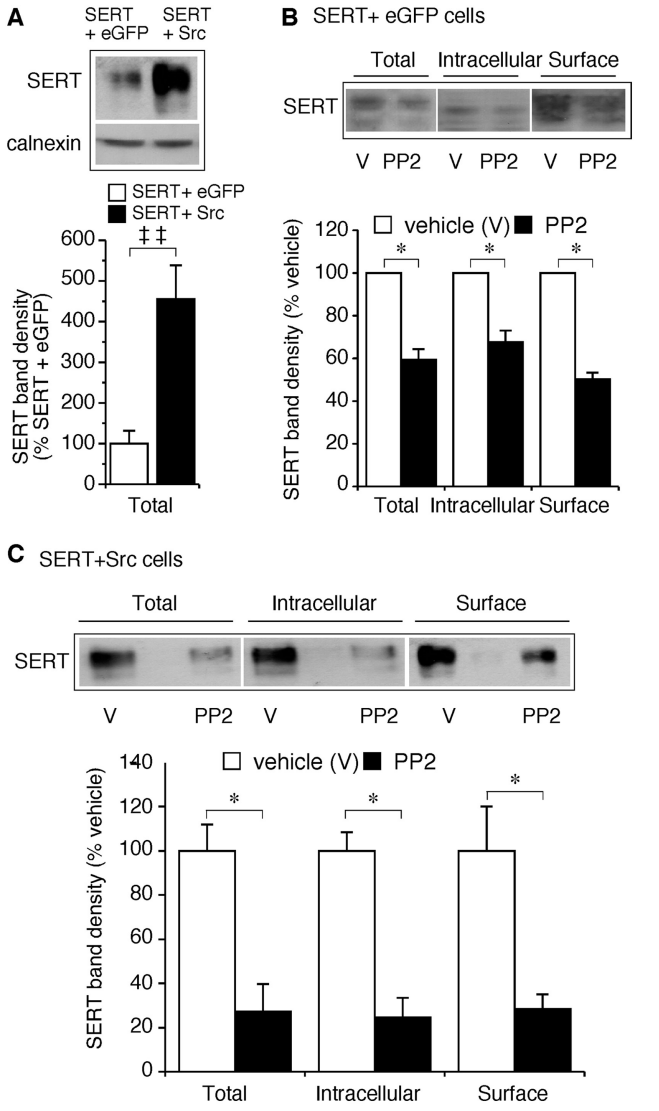

Fig. 6. Effect of PP2 treatment and Src expression on SERT expression. HTR cells were transfected with hSERT + eGFP or hSERT + Src. After 24 h, cells were extracted for the analysis of total SERT protein expression (A) or exposed to vehicle or 10 μM PP2 for 24 h and subjected to biotinylation at 4°C with sulfo-NHS-SS-biotin followed by immunoblotting with SR-12 SERT antibody (B and C) as described under *Materials and Methods*. A representative immunoblot of three separate experiments shows total SERT band densities from hSERT- + eGFP- or hSERT- + Src- transfected cells (A). Total SERT proteins were quantified using ImageJ, and the densities of SERT band from three experiments (mean ± S.E.M) are presented in the lower panel (‡‡, $p < 0.004$ compared with hSERT + eGFP by Student's t test). Representative SERT immunoblots of three separate biotinylation experiments are shown (B and C). Total, intracellular and surface SERT proteins were quantified using ImageJ, and the densities of SERT band from three experiments are presented in the lower panel as means ± S.E.M. *, $p < 0.04$ compared with vehicle (V) by Student's t test.

Inhibition of Protein Tyrosine Kinase(s) Decreases SERT Protein Stability. To determine whether inhibition of kinase(s) by PP2 alters SERT protein stability, the longevity of cell surface delivered and biotinylated SERT was measured in the presence and absence of PP2 as a function of time. For these assays, biotinylation was performed at 37°C for 2 h to tag the entire recycling SERT pool with biotin. The excess biotinylating reagent was removed and quenched with serum-containing media, followed by incubations at 37°C in fresh complete culture media with vehicle or PP2. Our previously reported studies suggested that performing biotinylation processes at 37°C for 1 h results in biotinylation of ~90% of recycling SERT pool (Jayanthi et al., 2005; Samuvel et al., 2005). One dish of biotinylated cells was immediately processed for extraction followed by isolation by avidin beads to reveal the quantity of biotinylated SERT at 0 h. At 6, 12, 24, and 36 h, the cells were solubilized and lysates were collected. Biotinylated proteins from extracts were isolated using avidin beads and the presence of biotinylated SERT was quantified by immunoblotting with SERT antibody. The level of biotinylated hSERT protein that gradually decreased with time, however, seemed more stable, decreasing modestly by ~37% over a 36-h period (Fig. 7A). On the other hand, in the presence of PP2, the level of biotinylated hSERT protein was diminished markedly as a function of time by ~78% at 36 h of treatment (Fig. 7A). Analysis of the time course revealed that biotinylated SERT in vehicle control cells was stable, with a half-life of 55 ± 7 h. However, in the presence of PP2, accelerated disappearance of biotinylated SERT was evident, with a reduced half-life of 16 ± 0.1 h ($p < 0.006$). Although rate of constitutive hSERT degradation fits better with one-phase decay model, the optimal fit to the data was achieved by using second-phase decay model for PP2-induced hSERT degradation (Fig. 7B). These results provide evidence for the participation of protein tyrosine kinase-linked pathways in the regulation of SERT protein stability.

Mutations of Tyrosine Residues in SERT Differentially Affect Basal and Src-Induced 5-HT Uptake and SERT Protein Expression and Stability. Four inward-facing tyrosine residues (Tyr47, Tyr142, Tyr350, and Tyr358) in SERT could be potential phosphorylation targets for tyrosine kinase(s). Therefore, we investigated whether phosphorylation of these residue(s) was necessary for SERT functional expression and Src-mediated SERT up-regulation. We constructed Y47F, Y142F, Y350F, and Y358F single-point SERT mutants, and a combined Y47F/Y142F/Y350F/Y358F mutant. Equal amounts of wild-type hSERT and hSERT mutant cDNAs were transfected in HTR cells along with Src or the empty vector (eGFP). Figure 8 summarizes the effect of Tyr mutations on basal and Src-mediated SERT activity and protein expression relative to wild-type hSERT. In the absence of Src coexpression, total as well as intracellular and surface expression levels of Y47F or Y142F were higher compared with WT-SERT expression (Fig. 8, D–F). In addition, when coexpressed with Src, Y47F and Y142F mutants were expressed at higher levels, similar to those observed for WT-SERT (Fig. 8, D-F). Figure 8A shows 5-HT uptake by cells transfected with WT or Y47F or Y142F in the absence of Src coexpression (e.g., in eGFP-transfected cells). After normalization of 5-HT uptake to SERT surface expression (Fig. 8E), we found that the Y47F and Y142F mutants exhibit reduced 5-HT uptake levels compared with WT activity (Y47F, 33 ± 2%; Y142F, 44 ± 4% of WT activity) (Fig. 8A). It is noteworthy that although Src-induced increases in SERT protein expression were evident (Fig. 8, D–F), the Src-induced increase in 5-HT transport function was completely absent in Y47F- or Y142F-expressing cells (Fig. 8B). The increase of 5-HT transport in Src-transfected cells compared with that of eGFP-transfected cells is shown in Fig. 8B (WT, 150 ± 5%; Y42F, 106 ± 6%; and Y142F, 91 ± 6%). Similar experiments were conducted in cells expressing Y350F, Y358F single mu-

TABLE 1

Effect of protein kinase inhibitors on basal and Src- triggered SERT activity

HTR cells were co-transfected with hSERT with eGFP or hSERT with Src. After 24 h, cells were washed and exposed to corresponding vehicle or the following agents: PP2, 20 μM; PD169316, 10 μM; PD98059, 10 μM; SB2033580, 10 μM; staurosporine, 50 nM; KN93, 2 μM, or LY294002, 10 μM. After 24-h pretreatment, uptake of 5-HT (40 nM) was measured using a 5-min uptake period as described under *Materials and Methods*. Values are the averages from three independent experiments, and the mean values ± S.E.M. are given. Significant differences were determined by Student's *t* test.

Treatments	Kinase Target	Effect of Kinase Inhibitors		Src Effect in the Presence of Inhibitors
		hSERT/eGFP	hSERT/Src	
		% vehicle		*% vehicle (% stimulation)*
Vehicle	None	100.00 ± 0.9	100.00 ± 11.5	144.27 ± 6.4[a] (44%)
PP2	Src-family kinases	44.52 ± 1.5[b]	38.42 ± 0.5[c]	53.42 ± 2.0[d] (−47%)
PD169316	p38 MAPK	89.78 ± 1.5[b]	84.76 ± 2.9[c]	134.12 ± 4.2[e] (34%)
PD98059	ERKs	80.20 ± 10.9[f]	76.02 ± 5.3[f]	154.37 ± 13.7[e] (54%)
SB2033580	P38 MAPK	101.38 ± 4.3[f]	94.26 ± 2.7[f]	137.86 ± 4.4[e] (38%)
Staurosporine	PKCs	49.08 ± 1.1[b]	58.37 ± 1.4[c]	159.92 ± 6.2[e] (60%)
KN93	CaMKII	73.75 ± 1.3[b]	85.23 ± 4.4[c]	148.72 ± 4.5[e] (49%)
LY294002	PI3K	87.69 ± 4.9[b]	86.77 ± 3.0[c]	166.97 ± 10.5[e] (67%)

KN93, 2-(*N*-[2-hydroxyethyl])-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylamine.
[a] Significantly different from the levels observed under vehicle conditions from hSERT/eGFP cells.
[b] Significantly different from levels observed under vehicle conditions from hSERT/eGFP cells.
[c] Significantly different from the levels observed under vehicle conditions from hSERT/Src cells.
[d] Significantly different from the Src-mediated stimulatory levels observed under vehicle conditions from hSERT/Src cells.
[e] Significantly different from the levels observed with the application of kinase modulator from hSERT/eGFP cells.
[f] Not statistically different from the levels observed under vehicle conditions.

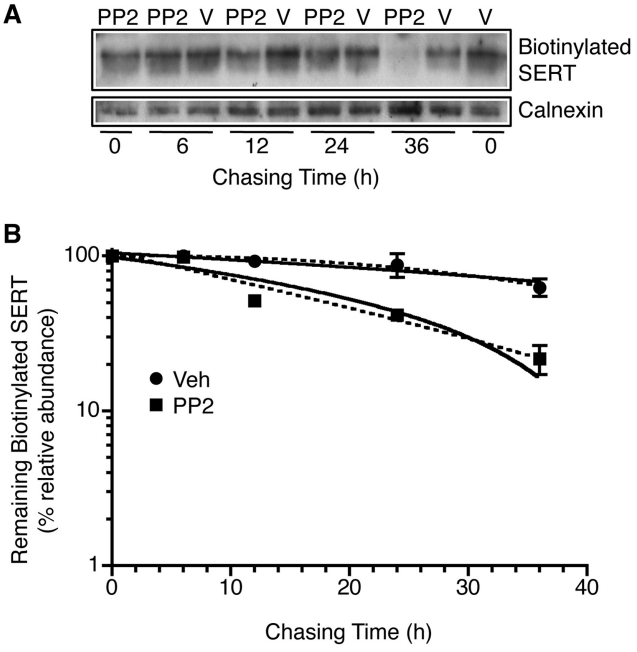

Fig. 7. Effect of PP2 on SERT protein stability. HTR cells transiently expressing SERT were biotinylated for 2 h at 37°C. After washing and quenching excess biotinylating reagent, vehicle or PP2 (20 μM) was added in complete media and incubated for 6, 12, 24, or 36 h. For 0 h, biotinylated cells were immediately processed for extraction to reveal the quantity of biotinylated SERT. At the end of treatment, cells were washed and extracted with RIPA buffer. The biotinylated proteins from RIPA extracts were isolated using avidin beads, and the presence of biotinylated SERT was analyzed by immunoblotting with SERT antibody as described under *Materials and Methods*. A representative immunoblot shows biotinylated SERT bands (A). Immunoblotting of calnexin is also shown (A). Biotinylated SERT band densitometric intensities were expressed as a percentage of band density at 0 h time point and plotted as a function of time. Curve fits by exponential decay using one-phase (solid lines) and two-phase (dotted lines) decay models are shown (B). The results are representative of the mean ± S.E.M. of three experiments.

tants, and the Y47F/Y142F/Y350F/Y358F combination. However, these mutated proteins were not expressed at detectable levels even when Src was coexpressed. Therefore, further analysis of these mutants was not possible. Next, we studied the influence of PP2 on Y47F and Y142F mutants. PP2 treatment for 24 h produced a significant reduction in 5-HT uptake in cells expressing Y47F and Y142F mutants, similar to that observed in cells expressing WT-hSERT (WT, 54 ± 4%; Y47F, 64 ± 2%; and Y142F, 54 ± 3% of corresponding vehicle) (Fig. 8C). The higher level of protein expression found in Y47F and Y142F could arise from higher stability of SERT protein. As shown in Fig. 9, analysis of biotinylated SERT over 36 h revealed that Y47F and Y142F mutants exhibit more stability compared with WT-hSERT (biotinylated SERT remaining after 36 h (percentage of level at 0 h): WT, 42 ± 11%; Y47F, 81 ± 5%; and Y142F, 80 ± 8%). In agreement with the effect of PP2 on 5-HT uptake (Fig. 8C), biotinylated SERT protein levels were decreased significantly (60–70%) after 36-h PP2 treatment similar to WT (biotinylated SERT remaining after 36 h in presence of PP2 (percentage of level at 0 h): WT, 14 ± 6%; Y47F, 34 ± 6%; and Y142F, 24 ± 4%) (Fig. 9B).

Phosphorylation of SERT Tyr47 and Tyr142 Is Required for Src-Induced Increases in 5-HT Uptake. Having shown that tyrosine mutations affect both basal and Src-induced 5-HT uptake (Fig. 8, A and B), we next investigated the impact of Tyr mutations on alkali-resistant basal and Src-induced SERT phosphorylation in HTR cells. SDS-PAGE and alkali treatment followed by visualization of ^{32}P-labeled SERT by autoradiography is shown in Fig. 10A. Similar to platelets (Fig. 2), we detected an alkali-resistant ^{32}P-labeletd SERT band in cells expressing WT-SERT but not in control experiments with nontransfected cells or use of irrelevant IgG (Fig. 10A). Because coexpression of Src and muta-

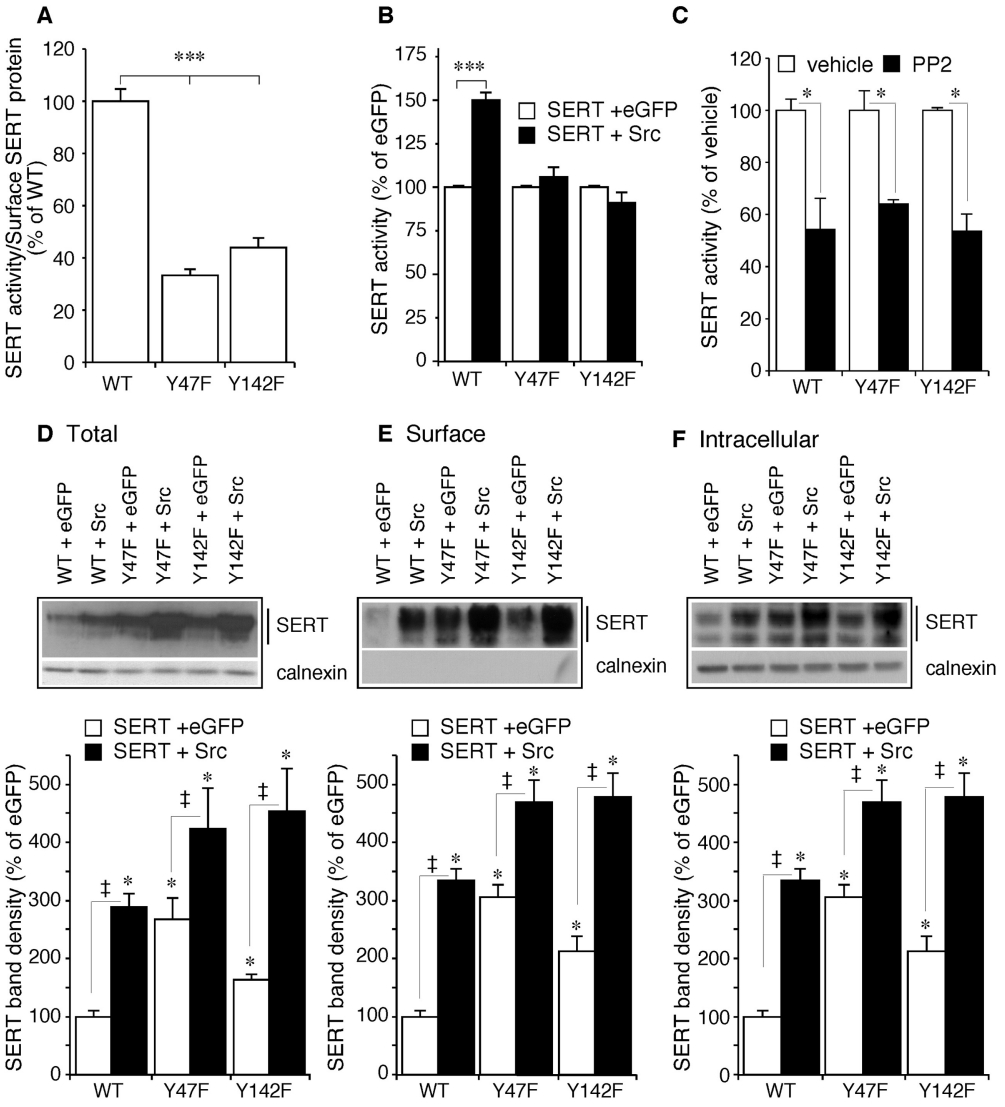

Fig. 8. Effect of intracellular tyrosine point mutations on basal and Src induced up-regulation of 5-HT uptake and SERT protein expression. HTR cells were transiently transfected with WT, Y47F, or Y142F SERT mutants as indicated along with eGFP or Src. Cells were used for 5-HT uptake after 48 h after transfection (A–C) or surface biotinylation experiments (D–F) as described under *Materials and Methods*. In A, 5-HT uptake was normalized to SERT surface expression obtained from E. ***, $p < 0.001$ compared with WT (one-way ANOVA with Dunnett's test). In B, the results are expressed as percentage of 5-HT uptake by cells transfected with SERT mutants plus eGFP or Src compared with that of WT transfected cells. ***, $p < 0.001$ compared with WT + eGFP. In C, transfected cells were pretreated with vehicle or 10 μM PP2 for 24 h, and the results were expressed as percentage of 5-HT uptake compared with vehicle. *, $p < 0.05$ compared with vehicle. Specific 5-HT uptake in vehicle-treated hSERT cells was 1371 ± 65 fmol/10^6 cells/min (100%). Representative SERT immunoblots show total (D), surface (E), or intracellular SERT (F), and the densities of SERT bands from three separate experiments are presented as mean ± S.E.M (bottom). *, $p < 0.05$ compared with WT + eGFP-transfected cells; ‡, $p < 0.05$ Src transfections compared with corresponding eGFP transfections (one-way ANOVA with Bonferroni test).

tions of Tyr residues affect SERT protein expression levels differently (Fig. 8, D–F), we analyzed SERT protein levels in parallel experiments by immunoblot analysis of extracts from cells not labeled with ^{32}P. Alkali-resistant ^{32}P-labeled SERT normalized to total SERT protein expression is presented in Fig. 10B. In cells (eGFP-transfected) expressing Y47F and Y142F, Tyr phosphorylation of SERT was significantly less compared with that of WT-SERT (Fig. 10B) (reduced basal SERT-Tyr phosphorylation, percentage of WT: Y47F, 38 ± 3%; and Y142F, 41 ± 3%). The Tyr phosphorylation of SERT was significantly elevated in cells coexpressing WT- SERT and Src (percentage of WT: eGFP, 203 ± 16%). However, Src-induced SERT-tyrosine phosphorylation was significantly reduced in cells expressing Y47F and Y142F compared with that of WT-SERT (Fig. 10B) (percentage of WT-Src: Y42F, 69 ± 6%; and Y142F, 60 ± 16%).

Discussion

We measured the impact of pharmacological and genetic modulation of Src-tyrosine kinase on SERT functional expression in platelets and HTR-hSERT cells using established biochemical paradigms. Assays were chosen that would provide insights into the contribution of Src activity to 5-HT uptake, SERT protein expression, trafficking, stability, and phosphorylation. Our functional studies establish that inhibition of Src kinase decreases SERT activity and expression by increasing SERT protein turnover. Although reduction of Src expression by siRNAs decreased 5-HT transport, coexpression of Src with SERT increased 5-HT transport and surface and total SERT expression. Our studies provide a direct correlation between SERT functional expression and Src activity, and they constitute the first evidence that Src-mediated up-regulation of 5-HT transport (catalytic function) and SERT protein expression is associated with the tyrosine phosphorylation of SERT protein.

The function of SERT is rapidly regulated by several Ser/Thr protein kinases, including PKC, CaMKII, p38 MAPK, and PKG (Ramamoorthy et al., 2011). In addition to the role Ser/Thr protein kinases play in the regulation of SERT, evidence suggests that a family of tyrosine protein kinases also regulate SERT activity. Several structurally distinct tyrosine kinase inhibitors, such as genistein, herbimycin A, and 2,5-dihydroxycinnamate, inhibit 5-HT uptake in platelets (Helmeste and Tang, 1995; Zarpellon et al., 2008). However, the exact sequence of molecular events that occurs during the

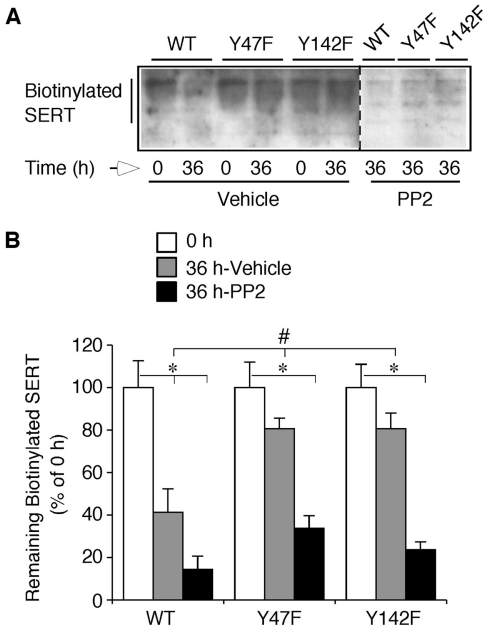

Fig. 9. Effect of Y47F and Y142F mutation on basal and PP2 induced SERT stability. HTR cells transiently expressing WT hSERT, Y47F-hSERT, or Y142F-hSERT were biotinylated and pretreated with vehicle or PP2 (20 μM) for 36 h as described in Fig. 7. The biotinylated proteins from RIPA extracts were isolated using avidin beads, and the presence of biotinylated SERT was analyzed by immunoblotting with SERT antibody as described under *Materials and Methods*. A representative immunoblot shows biotinylated SERT bands (A). Biotinylated SERT band densitometric intensities were expressed as a percentage of 0 h time point (B) and presented as mean ± S.E.M. *, $p < 0.05$ compared with corresponding 0 h; #, $p < 0.05$ compared with WT 36-h vehicle (one-way ANOVA with Bonferroni test).

SERT regulatory process and whether SERT tyrosine phosphorylation regulates SERT activity are unknown.

In this study, we found that SERT activity and SERT protein levels in platelets decline over a 24-h incubation period. Whereas the decline of SERT activity and SERT protein was further exacerbated by Src-kinase inhibition, inhibiting tyrosine phosphatase protected against loss of SERT activity and SERT protein. Platelets have been shown to be enriched with Src kinases (Golden et al., 1986). Our studies suggest that Src-induced tyrosine phosphorylation of SERT regulates 5-HT uptake and SERT protein stability. Indeed, our phosphorylation studies established that SERT proteins are phosphorylated on tyrosine(s) in platelets and in HTR heterologous cell preparations. In agreement with our studies, Zarpellon et al. (2008) provided evidence that Src-family tyrosine kinases regulate SERT function and SERT phosphorylation on tyrosine residues. Therefore, it is possible that regulating Src-mediated tyrosine phosphorylation of SERT or protecting from dephosphorylation by sustaining the tyrosine phosphorylated form of SERT might affect SERT protein stability and hence the function.

Our strategy for gaining a better understanding of the mechanisms of tyrosine kinase-mediated SERT regulation has been to identify potential tyrosine phosphorylation residue(s) within SERT and characterize SERT-Tyr mutants that might provide insight into the SERT regulatory process. Toward that end, we used HTR cells as a heterologous expression model. Pharmacological blockade of Src-kinase with PP2 as well as reduction of Src-kinase using siRNA-mediated suppression inhibited 5-HT uptake. Furthermore, coexpression of Src-kinase increased 5-HT uptake suggesting a role for Src-kinase in SERT regulation. Although the decrease in 5-HT uptake after PP2 treatment is associated with a significant reduction in SERT V_{max} and a moderate increase in 5-HT affinity, a higher SERT V_{max} and a moderate decrease in 5-HT affinity is observed with Src coexpression. In parallel studies, Src inhibition resulted in decreased SERT protein expression that is reflected in the total, surface, and intracellular amount of SERT. On the other hand, Src coexpression resulted in increased SERT expression that is reflected in the total, surface, and intracellular SERT pools. Inhibition of PKC, CaMKII, p38 MAPK, ERK, or PI3K did not influence Src-induced SERT activity. The combined pharmacological and genetic approaches revealed the involvement of Src-kinase in SERT regulation at the level of protein stability and activity. Studies also suggest that different tyrosine kinase inhibitors differentially affect SERT at the level of activity and transcription (Prasad et al., 1997). Our results do not completely rule out the possibility that the use of a different tyrosine kinase modulator or that in different cell types, the sequence of SERT regulation may differ from that observed in rat platelets and HTR cells. Such differences could reflect the specific nature of signaling pathways in different environments. Nevertheless, we found that inhibition of tyrosine kinase and phosphatase affects SERT activity and protein in platelets, a physiologically relevant native

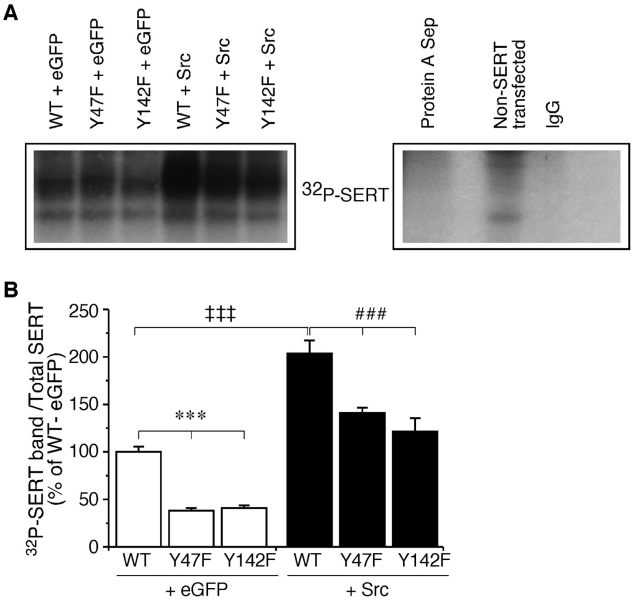

Fig. 10. Effect of intracellular tyrosine point mutations on basal and Src-induced tyrosine phosphorylation of SERT: HTR cells were transiently cotransfected with SERT-expression plasmids as indicated with eGFP or Src. Forty-eight hours after transfection, cells were metabolically labeled with [^{32}P]orthophosphate for 60 min at 37°C. RIPA extraction, immunoprecipitation, SDS-PAGE, alkali treatment, and autoradiography were performed as described under *Materials and Methods*. Parallel experiments were performed using protein A Sepharose, untransfected cells, and irrelevant IgG to validate specificity of SERT specific antibody SR-12. A representative autoradiogram of alkali-treated gel from three experiments is shown (A). Note: SERT tyrosine point mutants Y47F and Y142F express in different levels compared with WT. Autoradiogram in A was not normalized to total SERT protein expressions (see Fig. 9). The relative intensity of alkali-resistant ^{32}P-labeled SERT that was normalized with total SERT protein is shown in B. Data are presented as mean values ± S.E.M. ***, $p < 0.001$ compared with WT + eGFP; ‡‡‡, $p < 0.001$ compared with WT + eGFP; ###, $p < 0.001$ compared with WT + Src by one-way ANOVA with Bonferroni test.

preparation in which the SERT gene is absent. Therefore, it is reasonable to focus our inspection of possible regulation at the level of SERT protein stability. Our biotinylation-chase strategy, which assessed the fate of fully matured SERTs that have been recycled to plasma membrane, revealed that SERT protein is quite stable, with a half-life of 55 h. Previously published results indicate that in vivo SERT has a longer half-life of 3.4 days in rat (Vicentic et al., 1999). It is noteworthy that inhibition of Src-kinase reduced the half-life of SERT protein to 16 h, suggesting a role of Src-kinase in SERT protein stability. It is noteworthy that analysis of PP2-induced SERT degradation revealed two-phase decay suggesting processes that require multiple steps. Several factors may have contributed to the multiphase decay of SERT. For example, sequential and synergic effects of SERT phosphorylation on multiple tyrosine residues (Tyr47, Tyr142, Tyr350, and Tyr358) may influence SERT decay at multiple levels. It is also possible that tyrosine phosphorylation of SERT could regulate SERT interaction with other proteins or other unknown processes that could regulate SERT stability at different levels. Future studies will address these possibilities.

In agreement with platelet studies, SERT is phosphorylated on tyrosine residues in HTR-cells heterologously expressing hSERT. Both transport function and tyrosine phosphorylation of SERT are disrupted by Y47F and Y142F mutations. It is noteworthy that although the Src-mediated increase in SERT expression is intact, src did not increase the activities of these mutant variants. Several explanations may account for the lack of regulation of 5-HT transport. It has been proposed that interactions of the N terminus with the internal loops of amine transporters and the GABA transporter regulate substrate efflux, influx, and substrate permeation (Deken et al., 2000; Quick, 2003; Sucic et al., 2010). Phosphorylation of N-terminal residues in DAT have been linked with amphetamine-mediated efflux and conformation adaptations (Khoshbouei et al., 2004; Guptaroy et al., 2009). In SERT, interaction of syntaxin 1A with the N terminus governs SERT expression, conducting state and substrate permeation (Quick, 2003). It is noteworthy that S13A disrupts CaMKII-dependent regulation of SERT-syntaxin 1A interaction and SERT activity and is linked to phosphorylation of Ser13 (Ciccone et al., 2008). Our previous study (Zhang et al., 2007) indicates that modification of Thr276 in SERT via PKG-dependent phosphorylation alters the transmembrane domain 5 conformation that leads to increased catalytic activity. Our recent study revealed that activated p38 MAPK phosphorylates NET on the Thr30 residue in response to cocaine binding, and Thr30 phosphorylation enhances NE uptake and surface levels of NET (Mannangatti et al., 2011). Therefore, conformational changes resulting from Tyr47 and Tyr142 phosphorylation and/or dephosphorylation of p-Tyr47 and p-Tyr142 may alter SERT function with or without the influence of transporter-interacting proteins. It is also possible that Src regulates SERT phosphorylation and functions through direct binding to SERT. Zarpellon et al. (2008) have identified the presence of Src-kinase in SERT immunoprecipitates from human platelets suggesting that SERT and Src exist as a complex.

Because Y42F and Y142F mutants contain other potential phosphorylation sites (e.g., Tyr350 and Tyr358), the remaining SERT tyrosine phosphorylation revealed in the Y42F and Y142F mutants may be associated with Tyr350 and Tyr358 phosphorylation. However, Y350F or Y358F mutants alone failed to support normal levels of SERT protein expression. Moreover, the higher level of SERT expression found in Y42F and 142F mutants was also reduced by combined mutation of Y350F and Y358F (data not shown). Given, however, that mutation of Tyr350 and Tyr358 diminished SERT protein expression, it is tempting to speculate that phosphorylation of Tyr350 and Tyr358 may regulate SERT protein stability. Because Y42F and Y142F mutants affect SERT protein expression, there may be a synergistic mechanism existing in regulating 5-HT uptake and SERT stability via Src-mediated phosphorylation of Tyr42, Tyr142, Tyr350, and Tyr358 residues. Our data, supportive of this notion, reveal that 1) PP2 inhibits Y47F and Y142F activity, 2) Y47F and Y142F mutant SERT proteins are more stable than WT, and 3) PP2 reduces protein stability of Y47F and Y142F mutants. As a result of inadequate expression levels of Y350F and Y358F mutants, resolution of the role of Tyr350 and Tyr358 was precluded.

Our results do not rule out the possibility that in the absence of post-translational modifications, tyrosine residues at 42, 142, 350, and 358 are essential for proper SERT function and expression. Despite these complexities, the fact that semiconservative mutation of Tyr to Phe (or Ala; data not shown) was sufficient to 1) influence basal functional expression of SERT and 2) abolish Src-induced increases in 5-HT uptake and SERT protein expression suggests that phosphorylation of these tyrosine residues in vivo is a crucial determinant of Src effect and is essential for optimal SERT expression and function, in terms of both protein stability and transporter catalytic function. Further investigations are required to resolve the signaling pathways involved in SERT protein stability and/or function, and the manner by which the phosphorylation of Tyr42, Tyr142, Tyr350, and Tyr358 residues collaborate to produce appropriate functional expression of SERT.

Acknowledgments

We thank Drs. David Shalloway (Cornell University, Ithaca, NY) and John Woodward (Medical University of South Carolina, Charleston, SC) for providing c-Src-eGFP cDNAs.

Authorship Contributions

Participated in research design: Jayanthi and Ramamoorthy.

Conducted experiments: Annamalai, Mannangatti, Arapulisamy and Ramamoorthy.

Performed data analysis: Ramamoorthy.

Wrote or contributed to the writing of the manuscript: Shippenberg, Jayanthi, and Ramamoorthy.

References

Balkovetz DF, Tiruppathi C, Leibach FH, Mahesh VB, and Ganapathy V (1989) Evidence for an imipramine-sensitive serotonin transporter in human placental brush-border membranes. *J Biol Chem* **264:**2195–2198.

Barker EL and Blakely RD (1995) Norepinephrine and serotonin transporters: Molecular targets of antidepressant drugs, in *Psychopharmacology: The Fourth Generation of Progress* (Bloom FE and Kupfer DJ eds) pp 321–333, Raven Press, New York.

Bauman AL, Apparsundaram S, Ramamoorthy S, Wadzinski BE, Vaughan RA, and Blakely RD (2000) Cocaine and antidepressant-sensitive biogenic amine transporters exist in regulated complexes with protein phosphatase 2A. *J Neurosci* **20:**7571–7578.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mössner R, Westphal H, and Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxymetamphetamine ("ecstasy") in serotonin transporter-deficient mice. *Mol Pharmacol* **53:**649–655.

Blakely RD, Ramamoorthy S, Qian Y, Schroeter S, and Bradley C (1997) Regulation of antidepressant-sensitive serotonin transporters, in *Neurotransmitter Transporters: Structure, Function, and Regulation* (Reith ME ed) pp 29–72.

Cheng YS and Chen LB (1981) Detection of phosphotyrosine-containing 34,000-dalton protein in the framework of cells transformed with Rous sarcoma virus. *Proc Natl Acad Sci USA* **78:**2388–2392.

Ciccone MA, Timmons M, Phillips A, and Quick MW (2008) Calcium/calmodulin-dependent kinase II regulates the interaction between the serotonin transporter and syntaxin 1A. *Neuropharmacology* **55:**763–770.

Daws LC, Gould GG, Teicher SD, Gerhardt GA, and Frazer A (2000) 5-HT(1B) receptor-mediated regulation of serotonin clearance in rat hippocampus in vivo. *J Neurochem* **75:**2113–2122.

Deken SL, Beckman ML, Boos L, and Quick MW (2000) Transport rates of GABA transporters: regulation by the N-terminal domain and syntaxin 1A. *Nat Neurosci* **3:**998–1003.

Golden A, Nemeth SP, and Brugge JS (1986) Blood platelets express high levels of the pp60c-src-specific tyrosine kinase activity. *Proc Natl Acad Sci USA* **83:**852–856.

Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N, and Lala PK (1993) Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* **206:**204–211.

Guptaroy B, Zhang M, Bowton E, Binda F, Shi L, Weinstein H, Galli A, Javitch JA, Neubig RR, and Gnegy ME (2009) A juxtamembrane mutation in the N terminus of the dopamine transporter induces preference for an inward-facing conformation. *Mol Pharmacol* **75:**514–524.

Helmeste DM and Tang SW (1995) Tyrosine kinase inhibitors regulate serotonin uptake in platelets. *Eur J Pharmacol* **280:**R5–R7.

Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals* 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.

Jayanthi LD, Ramamoorthy S, Mahesh VB, Leibach FH, and Ganapathy V (1994) Calmodulin-dependent regulation of the catalytic function of the human serotonin transporter in placental choriocarcinoma cells. *J Biol Chem* **269:**14424–14429.

Jayanthi LD, Samuvel DJ, Blakely RD, and Ramamoorthy S (2005) Evidence for biphasic effects of protein kinase C on serotonin transporter function, endocytosis, and phosphorylation. *Mol Pharmacol* **67:**2077–2087.

Kekuda R, Torres-Zamorano V, Leibach FH, and Ganapathy V (1997) Human serotonin transporter: Regulation by the neuroprotective agent aurintricarboxylic acid and by epidermal growth factor. *J Neurochem* **68:**1443–1450.

Khoshbouei H, Sen N, Guptaroy B, Johnson L, Lund D, Gnegy ME, Galli A, and Javitch JA (2004) N-terminal phosphorylation of the dopamine transporter is required for amphetamine-induced efflux. *PLoS Biol* **2:**E78.

Launay JM, Bondoux D, Oset-Gasque MJ, Emami S, Mutel V, Haimart M, and Gespach C (1994) Increase of human platelet serotonin uptake by atypical histamine receptors. *Am J Physiol* **266:**R526–R536.

Luttrell LM, Daaka Y, and Lefkowitz RJ (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* **11:**177–183.

Madhav TR, Pei Q, and Zetterström TS (2001) Serotonergic cells of the rat raphe nuclei express mRNA of tyrosine kinase B (trkB), the high-affinity receptor for brain derived neurotrophic factor (BDNF). *Brain Res Mol Brain Res* **93:**56–63.

Mannangatti P, Arapulisamy O, Shippenberg TS, Ramamoorthy S, and Jayanthi LD (2011) Cocaine upregulation of the norepinephrine transporter requires threonine 30 phosphorylation by p38 mitogen-activated protein kinase. *J Biol Chem* **286:**20239–20250.

Murphy DL, Lerner A, Rudnick G, and Lesch KP (2004) Serotonin transporter: gene, genetic disorders, and pharmacogenetics. *Mol Interv* **4:**109–123.

Narisawa-Saito M, Silva AJ, Yamaguchi T, Hayashi T, Yamamoto T, and Nawa H (1999) Growth factor-mediated Fyn signaling regulates alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression in rodent neocortical neurons. *Proc Natl Acad Sci USA* **96:**2461–2466.

Panneerselvam K, Ramamoorthy S, and Balasubramanian AS (1987) Phosphorylation of the lysosomal enzyme binding receptor protein from monkey brain and evidence for a tyrosine kinase activity associated with it. *Biochem Biophys Res Commun* **147:**927–935.

Prasad HC, Zhu CB, McCauley JL, Samuvel DJ, Ramamoorthy S, Shelton RC, Hewlett WA, Sutcliffe JS, and Blakely RD (2005) Human serotonin transporter variants display selective insensitivity to protein kinase G and p38 mitogen activated protein kinase. *Proc Natl Acad Sci USA* **102:**11545–11550.

Prasad PD, Torres-Zamorano V, Kekuda R, Leibach FH, and Ganapathy V (1997) Functional link between tyrosine phosphorylation and human serotonin transporter gene expression. *Eur J Pharmacol* **325:**85–92.

Quick MW (2003) Regulating the conducting states of a mammalian serotonin transporter. *Neuron* **40:**537–549.

Ramamoorthy JD, Ramamoorthy S, Papapetropoulos A, Catravas JD, Leibach FH, and Ganapathy V (1995) Cyclic AMP-independent up-regulation of the human serotonin transporter by staurosporine in choriocarcinoma cells. *J Biol Chem* **270:**17189–17195.

Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang-Feng T, Chang AS, Ganapathy V, and Blakely RD (1993a) Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc Natl Acad Sci USA* **90:**2542–2546.

Ramamoorthy S and Blakely RD (1999) Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. *Science* **285:**763–766.

Ramamoorthy S, Cool DR, Mahesh VB, Leibach FH, Melikian HE, Blakely RD, and Ganapathy V (1993b) Regulation of the human serotonin transporter. Cholera toxin-induced stimulation of serotonin uptake in human placental choriocarcinoma cells is accompanied by increased serotonin transporter mRNA levels and serotonin transporter-specific ligand binding. *J Biol Chem* **268:**21626–21631.

Ramamoorthy S, Giovanetti E, Qian Y, and Blakely RD (1998) Phosphorylation and regulation of antidepressant-sensitive serotonin transporters. *J Biol Chem* **273:**2458–2466.

Ramamoorthy S, Samuvel DJ, Buck ER, Rudnick G, and Jayanthi LD (2007) Phosphorylation of threonine residue 276 is required for acute regulation of serotonin transporter by cyclic GMP. *J Biol Chem* **282:**11639–11647.

Ramamoorthy S, Shippenberg TS, and Jayanthi LD (2011) Regulation of monoamine transporters: Role of transporter phosphorylation. *Pharmacol Ther* **129:**220–238.

Samuvel DJ, Jayanthi LD, Bhat NR, and Ramamoorthy S (2005) A role for p38 mitogen-activated protein kinase in the regulation of the serotonin transporter: evidence for distinct cellular mechanisms involved in transporter surface expression. *J Neurosci* **25:**29–41.

Sucic S, Dallinger S, Zdrazil B, Weissensteiner R, Jørgensen TN, Holy M, Kudlacek O, Seidel S, Cha JH, Gether U, et al. (2010) The N terminus of monoamine transporters is a lever required for the action of amphetamines. *J Biol Chem* **285:**10924–10938.

Vicentic A, Battaglia G, Carroll FI, and Kuhar MJ (1999) Serotonin transporter production and degradation rates: studies with RTI-76. *Brain Res* **841:**1–10.

Zarpellon A, Donella-Deana A, Folda A, Turetta L, Pavanetto M, and Deana R (2008) Serotonin (5-HT) transport in human platelets is modulated by Src-catalysed Tyr-phosphorylation of the plasma membrane transporter SERT. *Cell Physiol Biochem* **21:**87–94.

Zhang YW, Gesmonde J, Ramamoorthy S, and Rudnick G (2007) Serotonin transporter phosphorylation by cGMP-dependent protein kinase is altered by a mutation associated with obsessive compulsive disorder. *J Neurosci* **27:**10878–10886.

Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, and Blakely RD (2004) Adenosine receptor, protein kinase G, and p38 mitogen-activated protein kinase-dependent up-regulation of serotonin transporters involves both transporter trafficking and activation. *Mol Pharmacol* **65:**1462–1474.

Address correspondence to: Sammanda Ramamoorthy, Department of Neurosciences, Division of Neuroscience Research, 173 Ashley Ave., BSB 403, Medical University of South Carolina, Charleston, SC 29425. E-mail: rama@musc.edu