Physical interaction between the serotonin transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity

B. Chanrion*†‡§¶‖, C. Mannoury la Cour‖, F. Bertaso*†‡§¶, M. Lerner-Natoli*†‡§¶, M. Freissmuth**, M. J. Millan‖, J. Bockaert*†‡§¶††, and P. Marin*†‡§¶

*Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5203, F-34094 Montpellier, France; †Institut National de la Santé de la Recherche Médicale, U661, F-34094 Montpellier, France; ‡Université de Montpellier I, F-34094 Montpellier, France; §Université Montpellier II, F-34094 Montpellier, France; ¶Institut de Génomique Fonctionnelle, Département de Neurobiologie, 141 Rue de la Cardonille, F-34094 Montpellier Cedex 5, France; ‖I.d.R Servier, 78290 Croissy, Paris, France; and **Institute of Pharmacology, University of Vienna, Wahringer Strasse 13a, A-1090 Vienna, Austria

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The spatiotemporal regulation of neurotransmitter transporters involves proteins that interact with their intracellular domains. Using a proteomic approach, we identified several proteins that interact with the C terminus of the serotonin transporter (SERT). These included neuronal nitric oxide synthase (nNOS), a PSD-95/Disc large/ZO-1 (PDZ) domain-containing protein recruited by the atypical PDZ binding motif of SERT. Coexpression of nNOS with SERT in HEK293 cells decreased SERT cell surface localization and 5-hydroxytryptamine (5-HT) uptake. These effects were absent in cells transfected with SERT mutated in its PDZ motif to prevent physical association with nNOS, and 5-HT uptake was unaffected by activation or inhibition of nNOS enzymatic activity. 5-HT uptake into brain synaptosomes was increased in both nNOS-deficient and wild-type mice i.v. injected with a membrane-permeant peptidyl mimetic of SERT C terminus, which disrupted interaction between SERT and nNOS, suggesting that nNOS reduces SERT activity *in vivo*. Furthermore, treating cultured mesencephalic neurons with the mimetic peptide similarly increased 5-HT uptake. Reciprocally, indicating that 5-HT uptake stimulates nNOS activity, NO production was enhanced on exposure of cells cotransfected with nNOS and SERT to 5-HT. This effect was abolished by 5-HT uptake inhibitors and absent in cells expressing SERT mutated in its PDZ motif. In conclusion, physical association between nNOS and SERT provides a molecular substrate for their reciprocal functional modulation. In addition to showing that nNOS controls cell surface localization of SERT, these findings provide evidence for regulation of cellular signaling (NO production) by a substrate-carrying transporter.

PDZ | proteomic | serotonin uptake

Serotonin plays a major role in the regulation of mood, cognition, and motor behavior, and a disruption of serotonergic transmission is implicated in several pathophysiological states, including affective disorders. The activity of serotonergic pathways is critically regulated by the reuptake of 5-HT via the plasma membrane serotonin transporter (SERT), a member of the Na^+/Cl^--dependent transporter family (SLC6) (1, 2). SERT is of major pharmacological and clinical interest inasmuch as it represents the primary target of several widely prescribed antidepressants, including the selective [5-hydroxytryptamine (5-HT)] reuptake inhibitors, citalopram and paroxetine (2–4). Moreover, altered SERT expression or function has been suggested not only in depression, but also in anxious and obsessive-compulsive states, disorders that can likewise be improved by treatment with selective 5-HT reuptake inhibitors (4–6).

Over the last 10 years, it has become evident that monoaminergic and other classes of plasma membrane transporters are not isolated proteins "floating" within the plasma membrane, but rather are components of protein complexes. These generally incorporate an oligomer (possibly formed of dimers) of the transporter that is physically associated with several intracellular proteins modulating its localization, trafficking, and function (1, 7–9). Several SERT-interacting proteins have already been identified principally by use of yeast two-hybrid screens. These include the SNARE protein, syntaxin 1A (10, 11), and secretory carrier membrane protein 2 (SCAMP2) (12), all of which associate to the N-terminal domain of SERT. In addition, SERT also complexes with the LIM domain adaptor protein, Hic-5 (13), the phosphatase 2A catalytic subunit, and α-synuclein, which is highly expressed in the nerve terminals of many brain regions (14). The binding site of these three proteins in the SERT sequence has not been identified. The C terminus of SERT also associates with PICK1 (15) and MacMARCKS, two protein kinase C substrates and/or anchoring proteins (16), but the functional significance of these interactions remains unknown. PICK1 belongs to the PSD-95/Disc large/ZO-1 (PDZ) domain-containing family of proteins. These are indispensable scaffolding components of multiprotein complexes that permit the compartmentalization and trafficking of diverse transporters, ion channels, and membrane-bound receptors (17). PICK1 contains a single PDZ domain at its N terminus that binds with a low selectivity to PDZ binding motifs located at the extreme C termini of numerous synaptic proteins (18). We hypothesized, thus, that the C-terminal sequence of SERT (NAV) may be a nonclassical PDZ binding motif capable of interacting with additional PDZ proteins.

This possibility was explored by use of a proteomic approach based on peptide-affinity chromatography, which allowed for the identification of four novel protein interactions with the C-terminal domain of SERT: two PDZ proteins, neuronal nitric oxide synthase (nNOS) and channel-interacting PDZ protein (CIPP), and two proteins of the coat complex II (COPII), Sec23A and Sec24C (19). Because NO has been implicated in the control of mood and in the etiology of depressive states (4), and

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Abbreviations: 5-HT, 5-hydroxytryptamine; CaM, calmodulin; CIPP, channel-interacting PDZ protein; DAT, dopamine transporter; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MESNA, 2-mercaptoethanesulfonic acid; nNOS, neuronal nitric oxide synthase; PDZ, PSD-95/Disc large/ZO-1; PKG, protein kinase G; SERT, serotonin transporter; WT, wild type.

See Commentary on page 7739.

††To whom correspondence should be addressed. E-mail: joel.bockaert@igf.cnrs.fr.

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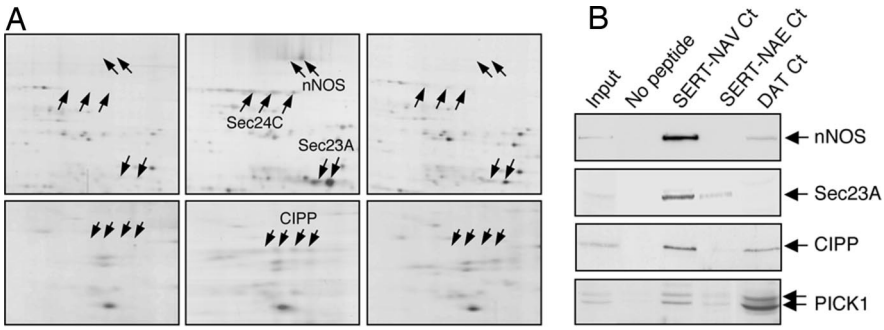

Fig. 1. The SERT C terminus associates with PDZ proteins and subunits of the COPII coat complex. (*A*) Protein extracts from mouse brain (10 mg protein) were incubated with 10 μg of either a synthetic peptide that incorporated the 15 C-terminal residues of mouse SERT (SERT-NAV Ct) or a peptide in which the C-terminal valine was replaced by a glutamate (SERT-NAE Ct) and immobilized on Sepharose beads or with Sepharose beads only. Areas of interest of 2D gels representative of three experiments performed independently are illustrated. The arrows indicate the position of spots that were specifically recruited by SERT C terminus. (*B*) Brain extracts (1 mg protein) were incubated with the indicated Sepharose-immobilized peptides (10 μg each), and proteins recruited by affinity chromatography were analyzed by Western blotting with specific antibodies. The data are representative of three independent experiments.

because the activity of SERT can be modulated by the NO–cGMP pathway (20, 21), we subsequently focused on the potential functional consequences of the physical association between SERT and nNOS.

Results

The SERT C Terminus Associates with PDZ Proteins and Subunits of the COPII Coat Complex. To identify proteins that interact with the putative PDZ binding motif located at the extreme C terminus of SERT, we used a proteomic approach that has already proved efficiency and sensitivity for characterizing specific PDZ binding partners of serotonin receptors (22). This approach was based on peptide-affinity chromatography by using a synthetic peptide encompassing the 15 C-terminal amino acids of SERT as bait, followed by separation of affinity-purified proteins by 2D electrophoresis. To specifically identify PDZ proteins that bind to the SERT C terminus, we carried out differential analyses of gels obtained with the SERT C-terminal peptide (SERT-NAV Ct) and a peptide in which the C-terminal valine, which is critical for interaction with target PDZ proteins (17), was substituted by a hydrophilic residue (SERT-NAE Ct). Two trains of spots detectable in gels obtained with the SERT-NAV Ct peptide were absent in both gels obtained with the SERT-NAE Ct peptide and control gels obtained after incubating brain extracts with beads not coupled to the peptides (Fig. 1*A*). These proteins were identified by MALDI-TOF MS as nNOS (or NOS-1), the only one of the three NOS isoforms that possesses a PDZ domain at its N terminus (23), and InaD-like protein [also designated as a CIPP; see supporting information (SI) Table 1]. The latter protein contains four PDZ domains and was previously identified as a binding partner of Kir4.0 potassium channel family members, *N*-methyl-D-aspartate receptor NR2 subunits, neurexins, neuroligins, acid-sensing ionic channels, and the 5-HT_{2A} receptor (22, 24, 25). Specific binding of these proteins to the C-terminal peptide of SERT but not to the modified peptide was confirmed by immunoblotting (Fig. 1*B*). Comparing the 2D protein pattern of gels obtained with the SERT-NAV Ct peptide and control gels indicated that the SERT C terminus also recruited two components of the COPII coat complex involved in protein export from the endoplasmic reticulum to either the endoplasmic reticulum–Golgi intermediate compartment or the Golgi complex: namely, Sec24C, one of the cargo-binding subunits of COPII; and Sec23A, one of the Sec23 subunits that, together with the Sec24 subunits, forms the membrane-proximal layer of the complex (19). Both Sec24C and Sec23A bound more weakly to the SERT-NAE Ct peptide, compared with the SERT-NAV Ct peptide (Fig. 1), indicating that the extreme C terminus of SERT contributes to the recruitment of the COPII coat complex.

PICK1 was not detected in our proteomic screen. Further, although immunoblotting experiments indicated that the C-terminal peptide of SERT did indeed recruit PICK1, consistent with previous findings (15), this interaction was weaker than the interaction between PICK1 and a dopamine transporter (DAT) C-terminal peptide (Fig. 1*B*). In contrast, we observed a much stronger association between nNOS or CIPP and the SERT C terminus, compared with the DAT C terminus (Fig. 1*B*).

Physical Interaction of nNOS with SERT Inhibits 5-HT Uptake in HEK293 Cells. To investigate the consequence of the SERT/nNOS interaction on SERT function, HEK293 cells were transiently transfected with YFP-tagged SERT and nNOS. nNOS could be coimmunoprecipitated with a YFP antibody (see SI Fig. 5), indicative of an interaction between SERT and nNOS in cotransfected cells. In HEK293 cells coexpressing nNOS and SERT, maximal 5-HT uptake was decreased ($-34 \pm 3\%$, $n = 4$), compared with cells transfected with SERT alone (Fig. 2*A*). nNOS overexpression also induced a slight, but not significant, increase in the apparent affinity of SERT for 5-HT transport ($K_m = 1.9 \pm 0.5$ μM in cells coexpressing SERT and nNOS vs. 3.0 ± 0.8 μM for cells transfected with SERT alone; $n = 4$, $P > 0.05$). In contrast, coexpression of nNOS with DAT in HEK293 cells did not alter dopamine uptake, consistent with the much weaker interaction between nNOS and DAT, compared with nNOS/SERT interaction (see SI Fig. 6). The inhibitory influence of nNOS on SERT did not depend on the catalytic activity of nNOS. Thus, neither A23187-mediated Ca^{2+} entry that activated nNOS in transfected HEK293 cells (as assessed by cGMP production) nor N^{ω}-nitro-L-arginine methyl ester (L-NAME), an nNOS inhibitor that prevented A23187-induced NO production, modified the effect of nNOS coexpression on 5-HT uptake (Fig. 2 *B* and *C*). Rather, the reduction of 5-HT uptake measured in cells coexpressing SERT and nNOS reflected a physical association between both proteins because it was undetectable in cells expressing a mutant SERT (C-terminal valine mutated into glutamate) incapable of interacting with nNOS (Fig. 2*D*).

Coexpression of nNOS with SERT in HEK293 cells did not alter whole-cell SERT expression (Fig. 2*E*). However, biotinylation experiments indicated that the amount of SERT at the cell surface (biotinylated) was significantly decreased ($-45 \pm 4\%$, $n = 3$, $P < 0.01$) in cells coexpressing nNOS and SERT, compared with cells transfected with SERT alone (Fig. 2*E*). Correspondingly, there was an increase in intracellular SERT (not biotinylated) in cells coexpressing nNOS and SERT, com-

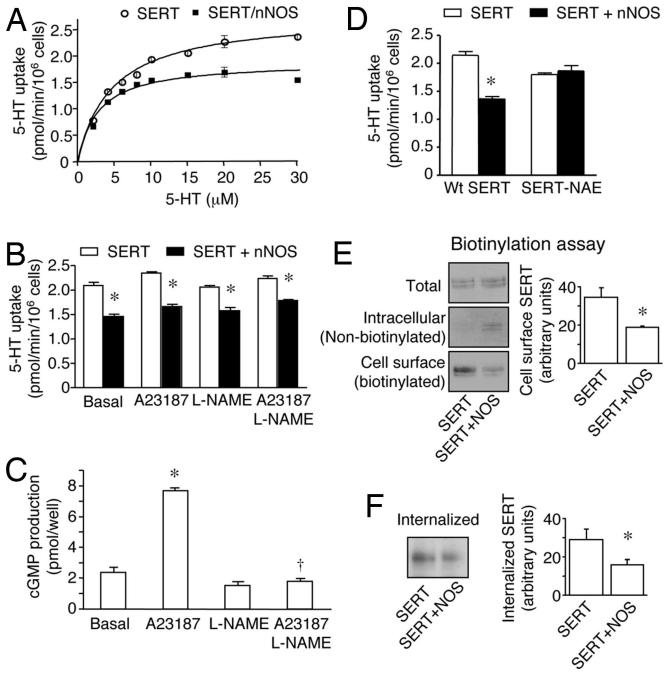

Fig. 2. nNOS coexpression inhibits 5-HT uptake and decreases plasma membrane SERT density in HEK293 cells. (*A*) Effect of nNOS coexpression with SERT on [^{3}H]5-HT uptake. Nonspecific uptake was determined in the presence of fluoxetine (10 μM, data not shown). (*B*) Lack of influence of NO production on the inhibition of 5-HT transport in cells coexpressing nNOS. Cells were treated for 10 min in the absence or presence of A23187 (10 μM) and/or L-NAME (100 μM) before commencing the [^{3}H]5-HT transport assay in the presence of 20 μM of 5-HT. Data represent the means ± SEMs of values obtained in three different experiments performed in quadruplicate (also applies for *C* and *D*). *, $P < 0.05$ vs. cells expressing SERT alone (ANOVA, followed by Student-Newman-Keul's test). (*C*) Production of NO in cells coexpressing SERT and nNOS. Cells were exposed to the treatments indicated in *B* in the presence of 1 mM 3-isobutyl-1-methylxanthine, and then intracellular cGMP was determined as an index of NO synthesis. *, $P < 0.0001$ vs. basal; †, $P < 0.0001$ vs. A23187-treated cells. (*D*) Lack of inhibitory effect on 5-HT transport of nNOS coexpression in cells transfected with a SERT mutant incapable of interacting with nNOS (SERT-NAE). *, $P < 0.05$ vs. cells expressing SERT alone. (*E*) Effect of nNOS coexpression on cell surface expression of SERT. Biotinylated SERT was detected by Western blotting by using a monoclonal anti-GFP antibody. Intensities of bands in immunoblots were measured by densitometry. Data are the means ± SEMs of three determinations performed on different cultures. $P < 0.05$ vs. cells expressing SERT alone. (*F*) Lack of effect of nNOS expression on SERT internalization. Cells biotinylated with sulfo-NHS-SS-biotin were incubated for a 90-min period at 37°C to allow biotinylated SERT internalization, and the remaining cell surface biotin was quenched by using the nonpermeant sulfydryl reagent MESNA. Internalized SERT was detected as described in *E*. *, $P < 0.05$ vs. cells expressing SERT alone.

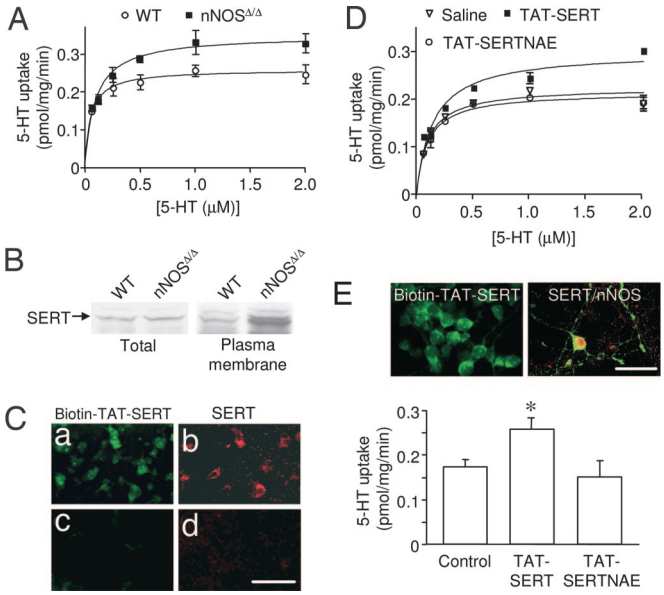

Fig. 3. Cerebral 5-HT uptake is increased in nNOS-deficient and WT mice treated with a peptide preventing the association of SERT with nNOS. (*A*) [^{3}H]5-HT uptake in synaptosomes from nNOS$^{\Delta/\Delta}$ compared with WT mice. Nonspecific uptake was determined in the presence of 1 μM of citalopram. Data represent the means ± SEMs of values obtained in synaptosomes from four WT and four nNOS$^{\Delta/\Delta}$ mice. (*B*) SERT expression in whole-brain and plasma membrane-enriched fractions from WT and nNOS$^{\Delta/\Delta}$ mice. Densitometric analysis indicated a 42 ± 3% ($n = 3$) decrease of SERT in the plasma membrane fraction from WT mice, compared with nNOS$^{\Delta/\Delta}$ mice, and no change in total SERT expression. (*C*) Visualization of intraneuronal accumulation of biotinylated TAT-SERT peptide. (*a* and *b*) Avidin and SERT labeling in the raphe nucleus of animals injected with biotinylated TAT-SERT, respectively. (*c*) Avidin staining in animals treated with saline. (*d*) Control immunostaining in biotinylated TAT-SERT-injected animals omitting primary (anti-SERT) antibody. (Scale bar: 50 μm.) (*D*) 5-HT uptake in synaptosomes from mice, which had received two i.v. injections of the TAT-SERT peptide or the TAT-SERTNAE control peptide (500 μg each, separated by a 90-min interval) or saline. The data illustrated are representative of four experiments each performed on different animals. (*E*) [^{3}H]5-HT uptake in cultured mesencephalic neurons in the absence (control) or presence of either TAT-SERT or TAT-SERTNAE (10 μM, 3 h). Nonspecific uptake was determined in the presence of 50 nM fluoxetine. Data represent the means ± SEMs of values obtained in three experiments. *, $P < 0.05$ vs. control (ANOVA, followed by Dunnett's test). The images illustrate intracellular accumulation of biotinylated TAT-SERT peptide in neurons (*Left Upper*) and a merge image (*Right Upper*) of neurons immunolabeled with SERT (red channel) and nNOS antibodies (green channel). (Scale bar: 50 μm.)

pared with SERT alone (Fig. 2*E*; see also control experiment in SI Fig. 7). Consistent with these findings, we detected intracellular localization of SERT in cells coexpressing nNOS and SERT, whereas SERT was primarily localized at the plasma membrane in cells only transfected with SERT (see SI Fig. 5). Moreover, [^{3}H]citalopram binding was significantly decreased in the plasma membrane fraction of cells coexpressing SERT and nNOS, compared with cells only transfected with SERT (B_{max} = 1.19 ± 0.03 and 1.52 ± 0.07 pmol/mg protein, respectively; $n = 4$, $P < 0.01$; see SI Fig. 8*A*).

We next determined whether the decreased localization of SERT at the surface of cells cotransfected with nNOS resulted from an increase in its internalization rate. Cells were exposed to Sulfo-NHS-SS-biotin for 30 min at 4°C and then incubated for an additional 90-min period at 37°C to allow biotinylated SERT internalization. The remaining cell surface biotin was quenched by using the nonpermeant sulfhydryl reagent 2-mercaptoethanesulfonic acid (MESNA). As shown in Fig. 2*F*, the amount of internalized SERT (biotinylated SERT resistant to MESNA reduction) was lower in cells coexpressing SERT and nNOS, compared with cells only transfected with SERT. Note that the decrease in internalized SERT measured in cells coexpressing nNOS was of similar magnitude as the reduction of cell surface SERT (compare Fig. 2 *E* and *F*). Accordingly, we concluded that the reduction of the density of plasma membrane SERT in cells coexpressing nNOS did not result from an increase in its internalization rate, but rather from an inhibition of its export to the plasma membrane.

The Interaction Between nNOS and SERT Reduces 5-HT Uptake in Mouse Brain. To determine whether the activity of SERT is modulated by nNOS within the mouse brain, we compared the velocity of 5-HT uptake in synaptosomes from wild-type (WT) and nNOS-deficient (nNOS$^{\Delta/\Delta}$) mice. As shown in Fig. 3*A*, maximal 5-HT transport was significantly enhanced in synapto-

somes prepared from nNOS$^{\Delta/\Delta}$ mice compared with WT mice (V_{max} = 350 ± 15 and 260 ± 10 fmol/mg protein in nNOS$^{\Delta/\Delta}$ and WT mice, respectively; $n = 4$, $P < 0.05$). In contrast, there was no significant change in the affinity of SERT for 5-HT (K_m = 53 ± 11 and 76 ± 21 nM for nNOS$^{\Delta/\Delta}$ and WT mice, respectively; $P > 0.05$). Note that the apparent SERT affinity for 5-HT transport was higher in mouse brain synaptosomes than that measured in transfected HEK-293 cells, consistent with previous reports (26, 27). The increased 5-HT uptake measured in synaptosomes from nNOS$^{\Delta/\Delta}$ mice was probably not related to the lack of NO synthesis. Accordingly, treating synaptosomes with *S*-nitroso-*N*-acetyl penicillamine, an NO-releasing compound, did not alter synaptosomal 5-HT uptake (see SI Fig. 9).

In line with the increase in the maximal velocity of cerebral 5-HT transport in nNOS$^{\Delta/\Delta}$ mice, immunoblotting experiments revealed an increase of SERT in the plasma membrane-enriched fraction from the nNOS$^{\Delta/\Delta}$ mouse brain, compared with WT mice, whereas whole-brain SERT expression was not modified by nNOS gene inactivation (Fig. 3*B*). Moreover, [^{3}H]citalopram binding was significantly increased in the plasma membrane fraction derived from the nNOS$^{\Delta/\Delta}$ mouse brain (B_{max} = 42.1 ± 1.4 and 31.3 ± 1.5 fmol/mg protein in nNOS$^{\Delta/\Delta}$ and WT mice, respectively; $n = 4$, $P < 0.01$; see SI Fig. 8*B*). By contrast, no significant change in the affinity of SERT for [^{3}H]citalopram was observed between nNOS$^{\Delta/\Delta}$ and WT mice (K_D = 0.84 ± 0.14 and 0.63 ± 0.12 nM, respectively; $n = 4$, $P > 0.05$). These results suggest that native nNOS may diminish the cell surface localization of SERT in the brain and, correspondingly, reduce 5-HT reuptake.

We next investigated whether the inhibition of cerebral SERT activity by nNOS reflected their physical association by use of a peptide comprising the nine C-terminal residues of SERT to competitively occlude the interaction of the SERT C terminus with nNOS. The peptide was N-terminally fused to the transduction domain of the Tat protein from the HIV type 1 (TAT-SERT) to permit its intracellular delivery (28). Pull-down experiments showed that the TAT-SERT peptide disrupted the association of SERT with nNOS *in vitro*, whereas a peptide in which the C-terminal valine was substituted by glutamate (TAT-SERTNAE) did not prevent this interaction (see SI Fig. 5*A*). Several lines of evidence indicated that the TAT-SERT peptide, but not the TAT-SERTNAE peptide, also efficiently prevented interaction between SERT and nNOS in living cells. Treatment of HEK293 cells coexpressing SERT and nNOS with TAT-SERT strongly decreased the amount of nNOS coimmunoprecipitated with SERT. Moreover, this treatment induced a redistribution of SERT to the plasma membrane, similar to that observed in cells only expressing SERT, whereas TAT-SERTNAE did not modify cellular distribution of SERT (see SI Fig. 5). Thus, TAT-SERTNAE was used as control in further experiments.

Adult mice (25 g) were i.v. injected twice with a 500-μg dose of TAT-SERT, TAT-SERTNAE, or saline. The two consecutive injections were separated by a 90-min interval, and experiments were performed 90 min after the second injection. i.v. injection of biotinylated TAT-SERT resulted in strong avidin labeling in all brain regions, including the raphe nuclei, and in SERT-positive neurons (Fig. 3*C* and SI Fig. 10), consistent with the reported efficiency of TAT-fused peptides to enter the brain following systemic administration (28). The 3-h pretreatment with the TAT-SERT peptide significantly increased maximal 5-HT uptake into synaptosome, compared with saline (28 ± 7%; $n = 4$, $P < 0.05$), whereas TAT-SERTNAE was inactive (Fig. 3*D*). These observations are reminiscent of the increased 5-HT uptake seen in synaptosomes from nNOS$^{\Delta/\Delta}$ vs. WT mice and indicate that the inhibitory influence of nNOS on 5-HT reflects its physical interaction with SERT. Consistently, treating mesencephalic neuron cultures, which contain ≈5% of SERT/nNOS-positive neurons, with TAT-SERT (10 μM, 3 h) increased 5-HT uptake by 33 ± 7% ($n = 4$), whereas TAT-SERTNAE had no effect (Fig. 3*E*).

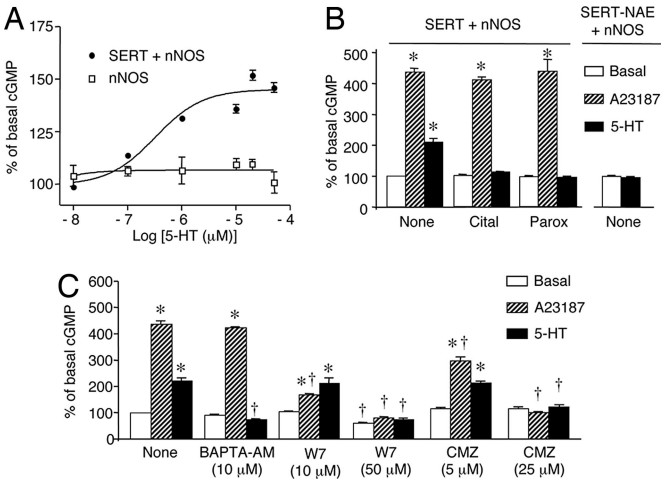

Fig. 4. 5-HT uptake by SERT enhances the activity of coexpressed nNOS in HEK293 cells. (*A*) HEK293 cells transfected with nNOS alone or cotransfected with SERT and nNOS were treated for 10 min with the indicated concentrations of 5-HT plus 1 mM 3-isobutyl-1-methylxanthine. Note that cGMP was not detectable in cells not transfected with nNOS (data not shown). (*B*) Cells were preincubated for 10 min in the absence or presence of the 5-HT reuptake inhibitors citalopram (Cital, 3 μM) or paroxetine (Parox, 10 μM) before 5-HT (20 μM) or A23187 (10 μM) exposure. (*C*) Cells cotransfected with SERT and nNOS were treated for 10 min in the absence or presence of A23187 (10 μM), 5-HT (20 μM), BAPTA-AM (10 μM), W7 (10–50 μM), and calmidazolium (CMZ; 5–25 μM). In experiments using BAPTA-AM, W7, or CMZ, they were added to cells 10 min before A23187 (10 μM) or 5-HT (20 μM). In *A–C*, data expressed in percentage of basal cGMP level measured in nNOS/SERT cotransfected cells in the absence of any treatment (96 ± 15 fmol/10^6 cells) represent the means ± SEMs of values obtained in three experiments performed in triplicate on different sets of cultured cells. *, $P < 0.01$, compared with cGMP level in the corresponding condition in the absence of A23187 or 5-HT (ANOVA, followed by Student-Newman-Keul's test). †, $P < 0.01$, compared with the corresponding value measured in the absence of BAPTA-AM, W7, or CMZ.

5-HT Uptake by SERT Enhances the Activity of Coexpressed nNOS. We next examined the possibility that the association of SERT with nNOS would reciprocally modulate the catalytic activity of nNOS. Exposure to 5-HT increased cGMP production in a concentration-dependent manner in HEK293 cells coexpressing nNOS and SERT. In contrast, cGMP production was not affected in cells transfected with nNOS only (Fig. 4*A*). Further, 5-HT-mediated cGMP production was inhibited by L-NAME (data not shown), indicating that it resulted from activation of nNOS. Consistent with the notion that the activation of nNOS by 5-HT depends on its uptake by SERT, 5-HT-induced cGMP production was abolished in cells treated with the 5-HT reuptake inhibitors citalopram or paroxetine, which did not, in contrast, alter the stimulation of nNOS activity induced by A23187 (Fig. 4*B*). Moreover, 5-HT did not influence cGMP production in cells coexpressing nNOS and a SERT mutant incapable of interacting with nNOS (Fig. 4*B*). The latter observation indicates that stimulation of nNOS by SERT-mediated 5-HT uptake depends on its physical interaction with nNOS.

nNOS is a Ca^{2+}-calmodulin (Ca^{2+}-CaM)-dependent enzyme generally activated by elevations of cytosolic Ca^{2+} (29). Nonetheless, 5-HT (10 μM, up to 10-min exposure) did not induce a detectable increase in cytosolic Ca^{2+} concentration in HEK293 cells coexpressing SERT and nNOS, as assessed by Fura-2 Ca^{2+} imaging (data not shown). However, 5-HT-induced NO production was markedly reduced in cells pretreated with BAPTA-AM

(50 μM), an intracellular Ca^{2+} chelator, indicative of an intracellular Ca^{2+} requirement for nNOS activation by 5-HT uptake (Fig. 4*C*). In contrast, chelating intracellular Ca^{2+} did not inhibit A23187-induced NO production. Both 5-HT- and A23187-mediated NO production in HEK293 cells coexpressing SERT required CaM, but exhibited differential sensitivity to CaM inhibitors, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and calmidazolium. Thus, treating cells with either W7 (10 μM) or calmidazolium (5 μM) inhibited A23187-induced nNOS activation, but did not alter the response to 5-HT. Only 50 μM of W7 or 25 μM of calmidazolium prevented 5-HT-mediated NO production (Fig. 4*C*). Collectively, these results indicate that activation of nNOS physically associated with SERT by 5-HT uptake requires CaM bound to Ca^{2+}, but does not necessitate a global increase in cytosolic Ca^{2+} concentration.

Discussion

The homeostasis of serotonergic neurotransmission and the long-term treatment of psychiatric disorders such as depression, impulsive states, and anxiety are intimately related to the spatiotemporal regulation of SERT activity (1). SERT is acutely regulated by various protein kinase and phosphatase-dependent signaling cascades that modulate both its functional activity and internalization (21, 30), whereas a physical interaction of SERT with intracellular proteins primarily affects its subcellular compartmentalization (8, 12–14).

Here, using a proteomic approach, we demonstrated that nNOS, a protein coexpressed with SERT in the majority of dorsal raphe nucleus serotonergic neurons [in cell bodies, axons, and terminals (31)], interacts (directly or indirectly by an intermediate protein partner) with SERT, and that this interaction provides a molecular substrate for a negative modulation of SERT activity by nNOS. Accordingly, an inhibitory influence of nNOS was absent in cells transfected with nNOS and a SERT mutant with which it was unable to interact, whereas the maximal velocity of 5-HT uptake into brain synaptosomes and mesencephalic neurons was enhanced by treatment with a peptidyl mimetic of the transporter C terminus that competitively prohibits interaction between SERT and nNOS. Further, the nNOS-mediated inhibition of SERT activity was related to a decrease in transporter cell surface density, reflecting an inhibition of its trafficking to the plasma membrane, rather than an increase in its rate of internalization. This inhibition may result from a competition between nNOS and transport molecules (e.g., proteins of the COPII complex) for interaction with SERT. Accordingly, the C-terminal residue of SERT, which was critical for interaction with nNOS, also contributed to the recruitment of Sec24C and Sec23A, two COPII subunits.

Several lines of evidence support functional interactions among SERT and the nitrergic signaling pathway (4, 20, 21). Nonetheless, the regulation of SERT activity observed in previous studies depended on the catalytic activity of nNOS and NO production, in contrast to the herein-documented role of physical association in the inhibitory influence of nNOS on SERT function. Notably, the influence of NO signaling on SERT function may depend on the cellular environment, as well as the source and concentration of NO. On the one hand, activation of the NO/cGMP/PKG cascade on the stimulation of A3 adenosine and histamine receptors in a rat basophilic leukemia cell line (RBL-2H3) and human platelets, respectively, resulted in increased 5-HT uptake (20, 21, 32). On the other hand, NO donors were found to reduce 5-HT reuptake in transfected COS-7 cells by triggering *S*-nitrosylation of SERT (33). Conversely, in the transfected HEK293 cells and synaptosomes used here, we did not detect an inhibition of SERT function on NO production. Indeed, A23187, a Ca^{2+} ionophore that induced NO production in HEK293 cells, did not affect 5-HT reuptake, which was likewise insensitive to the nNOS inhibitor, L-NAME. Moreover, the NO donor, *S*-nitroso-*N*-acetyl penicillamine, did not modify the kinetic properties of synaptosomal 5-HT uptake. Finally, the augmentation of cerebral 5-HT uptake observed in nNOS-deficient mice was likely due to the loss of the interaction between SERT and nNOS, rather than the suppression of nNOS catalytic activity. Indeed, inactivation of the gene encoding nNOS elicited an enhancement of synaptosomal 5-HT uptake similar to that obtained following peripheral delivery of a membrane-permeant peptide abrogating the interaction between SERT and nNOS.

Collectively, these data emphasize the complexity of the spatiotemporal regulation of 5-HT reuptake by nitergic mechanisms, involving contrasting modes of control, both dependent on, and independent of, nNOS catalytic activity. These include "phasic" modulation mediated by posttranslational modifications (phosphorylation or *S*-nitrosylation) of SERT by NO and "tonic" modulation mediated by an interaction between SERT and nNOS.

The present study also demonstrates that SERT-mediated 5-HT uptake enhances the enzymatic activity of nNOS in cells coexpressing SERT and nNOS, revealing a reciprocal functional interaction between these protein partners. This observation is consistent with previous findings indicating that, on local injection into the hippocampus, selective 5-HT reuptake inhibitors and tricyclic antidepressants diminish the activity of nNOS (4). Further, it reveals a phenomenon whereby 5-HT can modify cellular signaling not by recruitment of serotonergic receptors, but indirectly by SERT on its reuptake by cells. Interestingly, another type of receptor-independent signaling by 5-HT, termed "serotonylation," was previously reported in platelets. Serotonylation consists in the transamidation of 5-HT to the small GTPases, RhoA and Rab4, leading to α-granule exocytosis from platelets (34).

A physical interaction between SERT and nNOS was a prerequisite for the activation of nNOS on 5-HT uptake. Interestingly, NO production induced by 5-HT uptake was not mediated by an influx of Ca^{2+}. This process contrasts to the classical mechanism involved in nNOS activation by stimuli known to increase intracellular Ca^{2+}, including glutamatergic agonists. Nevertheless, nNOS activation on 5-HT uptake was prevented by BAPTA-AM and CaM inhibitors, consistent with the Ca^{2+}-CaM dependency of the enzyme. One possibility is that 5-HT uptake results in a SERT-mediated allosteric conformational change of nNOS, which increases its affinity for CaM, thereby permitting its interaction with CaM even at low (basal) Ca^{2+} concentration. Another NOS isoform, endothelial NOS, can similarly be activated in a CaM-dependent manner without requirement of elevation of cytosolic Ca^{2+} concentration, a process that results from endothelial NOS phosphorylation by the protein kinase Akt (35).

What might be the significance of an induction of NO release on 5-HT uptake? An activation of nNOS mediated by 5-HT uptake may acutely up-regulate SERT by a PKG- and p38-transduced increase in its phosphorylation state (21). Conversely, nNOS activation might participate in a negative feedback loop responsible for long-term regulation of SERT activity. In this hypothesis, NO signaling initiated by 5-HT uptake, which is known to enhance the activity of the transcription factor, CREB, will recruit many target genes, including nNOS (36). According to the present findings, increased nNOS levels would lead to intracellular sequestration of SERT, thereby preventing excessive 5-HT uptake and enhancing 5-HT neurotransmission.

In conclusion, this study demonstrates that the physical association of nNOS with SERT provides a molecular substrate for a reciprocal modulation of their functional activity and reveals an intracellular signaling pathway initiated by 5-HT that does depend on the engagement of serotonergic receptors, but is mediated by its reuptake by SERT. A loss of the inhibitory influence of nNOS on the activity of SERT in serotonergic terminals may conceivably be involved in the pathogenesis of psychiatric disorders, including depressive states (37) and en-

hanced aggressiveness and impulsivity, as reported, for instance, in $nNOS^{\Delta/\Delta}$ mice (38).

Methods

Cell Cultures and Transfection. HEK293 cells grown in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed FCS and antibiotics were transfected by electroporation by using the V nucleofactor kit (Amaxa Biosystems, Gaithersburg, MD). Each transfection sample contained 0.5 μg of pEYFP/SERT or pEYFP/SERT-NAE constructs and/or 2 μg of pRK5/nNOS plasmid and 10×10^6 cells in 100 μl of V nucleofactor solution according to the manufacturer's instructions. Experiments were carried out 48 h after transfection. Immunocytochemistry experiments indicated that >50% of cells were transfected with SERT or with SERT and nNOS, and that 62% of YFP-positive cells coexpressed nNOS (nine fields containing 150 cells originating from three independent cultures counted). No cells were found to be transfected with nNOS only.

Cultured mesencephalic neurons were prepared from 14-day-old Swiss mouse embryos as previously described (39) and were used at 9 days *in vitro*.

Preparation of Synaptosomes. Brains from WT and $nNOS^{\Delta/\Delta}$ mice were gently homogenized in 10 volumes of ice-cold buffer containing Tris·HCl (50 mM, pH 7.4), sucrose (0.32 M), EDTA (0.5 mM), and a mixture of protease inhibitors (Roche Diagnostics, Indianapolis, IN) by using a glass-teflon homogenizer. The homogenates were centrifuged at $1,000 \times g$ for 10 min at 4°C, and the resulting supernatant was centrifuged at $12,500 \times g$ for 20 min. The final pellet (crude synaptosomal fraction) was gently resuspended in Krebs–Ringer bicarbonate medium containing 150 mM NaCl, 4.2 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM Hepes (pH 7.4), 5 mM glucose, and the protease inhibitor mixture and saturated with 95% air/5% CO_2. Protein concentration was determined by using the bicinchoninic acid method.

5-HT Uptake Assays. HEK293 cells grown in 96-well culture dishes were washed in Hepes buffer [150 mM NaCl, 4.2 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, and 10 mM Hepes (pH 7.4)] and incubated for 10 min at 37°C in Hepes buffer containing 100 μM pargyline, 100 μM ascorbic acid, and the indicated treatments. 5-HT uptake was initiated by the addition of [^{3}H]5-HT (20 nM) and increasing concentrations of cold 5-HT ranging from 1–30 μM. The reaction (10 min at 37°C) was terminated by three washes with ice-cold Hepes buffer, and cells were lysed in 1% SDS. The radioactivity incorporated into cells was determined by scintillation counting. Nonspecific 5-HT uptake was determined in the presence of 10 μM of fluoxetine.

For 5-HT uptake into cultured mesencephalic neurons, cultures were pretreated as indicated above, and uptake was initiated by the addition of 100 nM [^{3}H]5-HT. Nonspecific 5-HT uptake was determined in the presence of 50 nM fluoxetine.

Synaptosomes (0.5 mg per assay) were preincubated with 100 μM of pargyline and 100 μM of ascorbic acid in 0.5 ml of Krebs–Ringer bicarbonate buffer for 10 min at 37°C. 5-HT transport assays (10 min at 37°C) were initiated by the addition of [^{3}H]5-HT (20 nM) and increasing concentrations of cold 5-HT ranging from 0.05–2 μM. The assays were terminated by filtering the samples through 0.3% polyethylenimine-coated glass fiber filters (Whatman GF/C; Whatman, Clifton, NJ) by using a Brandel (Gaithersburg, MD) cell harvester. The filters were washed three times with 1.5 ml of Krebs–Ringer bicarbonate buffer containing 1 μM of citalopram. Nonspecific [^{3}H]5-HT uptake was determined in the presence of 1 μM of citalopram.

Kinetic data were calculated from four independent experiments performed in quadruplicate by using Eadie–Hofstee plots. Data analysis and statistics were carried out by using the Prism 4 software (GraphPad Software, San Diego, CA). The Mann–Whitney test was applied unless otherwise indicated. P values <0.05 were considered as significant.

For the other methods used in this study, see *SI Materials and Methods*.

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1. Torres GE, Gainetdinov RR, Caron MG (2003) *Nat Rev Neurosci* 4:13–25.
2. Jayanthi LD, Ramamoorthy S (2005) *Aaps J* 7:E728–E738.
3. White KJ, Walline CC, Barker EL (2005) *Aaps J* 7:E421–E433.
4. Millan MJ (2006) *Pharmacol Ther* 110:135–370.
5. Ozaki N, Goldman D, Kaye WH, Plotnicov K, Greenberg BD, Lappalainen J, Rudnick G, Murphy DL (2003) *Mol Psychiatry* 8:933–936.
6. Millan MJ (2003) *Prog Neurobiol* 70:83–244.
7. Sitte HH, Freissmuth M (2003) *Eur J Pharmacol* 479:229–236.
8. Deken SL, Beckman ML, Quick MW (2001) *Trends Neurosci* 24:623–625.
9. Torres GE (2006) *J Neurochem* 97(Suppl):3–10.
10. Quick MW (2002) *Int J Dev Neurosci* 20:219–224.
11. Quick MW (2003) *Neuron* 40:537–549.
12. Muller HK, Wiborg O, Haase J (2006) *J Biol Chem* 281:28901–28909.
13. Carneiro AM, Blakely RD (2006) *J Biol Chem* 281:24769–24780.
14. Wersinger C, Rusnak M, Sidhu A (2006) *Eur J Neurosci* 24:55–64.
15. Torres GE, Yao WD, Mohn AR, Quan H, Kim KM, Levey AI, Staudinger J, Caron MG (2001) *Neuron* 30:121–134.
16. Jess U, El Far O, Kirsch J, Betz H (2002) *Biochem Biophys Res Commun* 294:272–279.
17. Nourry C, Grant SG, Borg JP (2003) *Sci STKE* 197:RE7.
18. Dev KK (2004) *Nat Rev Drug Discov* 3:1047–1056.
19. Bonifacino JS, Glick BS (2004) *Cell* 116:153–166.
20. Miller KJ, Hoffman BJ (1994) *J Biol Chem* 269:27351–27356.
21. Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD (2004) *Mol Pharmacol* 65:1462–1474.
22. Becamel C, Gavarini S, Chanrion B, Alonso G, Galeotti N, Dumuis A, Bockaert J, Marin P (2004) *J Biol Chem* 279:20257–20266.
23. Kone BC, Kuncewicz T, Zhang W, Yu ZY (2003) *Am J Physiol Renal Physiol* 285:F178–F190.
24. Kurschner C, Mermelstein PG, Holden WT, Surmeier DJ (1998) *Mol Cell Neurosci* 11:161–172.
25. Baron A, Deval E, Salinas M, Lingueglia E, Voilley N, Lazdunski M (2002) *J Biol Chem* 277:50463–50468.
26. Just H, Sitte HH, Schmid JA, Freissmuth M, Kudlacek O (2004) *J Biol Chem* 279:6650–6657.
27. Samuvel DJ, Jayanthi LD, Bhat NR, Ramamoorthy S (2005) *J Neurosci* 25:29–41.
28. Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang YT, Salter MW, Tymianski M (2002) *Science* 298:846–850.
29. Bredt DS, Snyder SH (1990) *Proc Natl Acad Sci USA* 87:682–685.
30. Ramamoorthy S, Blakely RD (1999) *Science* 285:763–766.
31. Simpson KL, Waterhouse BD, Lin RC (2003) *J Comp Neurol* 466:495–512.
32. Launay JM, Bondoux D, Oset-Gasque MJ, Emami S, Mutel V, Haimart M, Gespach C (1994) *Am J Physiol* 266:R526–R536.
33. Bryan-Lluka LJ, Papacostas MH, Paczkowski FA, Wanstall JC (2004) *Br J Pharmacol* 143:63–70.
34. Walther DJ, Peter JU, Winter S, Holtje M, Paulmann N, Grohmann M, Vowinckel J, Alamo-Bethencourt V, Wilhelm CS, Ahnert-Hilger G, Bader M (2003) *Cell* 115:851–862.
35. Fulton D, Gratton JP, Sessa WC (2001) *J Pharmacol Exp Ther* 299:818–824.
36. Riccio A, Alvania RS, Lonze BE, Ramanan N, Kim T, Huang Y, Dawson TM, Snyder SH, Ginty DD (2006) *Mol Cell* 21:283–294.
37. Karolewicz B, Szebeni K, Stockmeier CA, Konick L, Overholser JC, Jurjus G, Roth BL, Ordway GA (2004) *J Neurochem* 91:1057–1066.
38. Nelson RJ, Trainor BC, Chiavegatto S, Demas GE (2006) *Neurosci Biobehav Rev* 30:346–355.
39. Marey-Semper I, Gelman M, Levi-Strauss M (1995) *J Neurosci* 15:5912–5918.