Published in final edited form as:

Neuropharmacology. 2008 October ; 55(5): 763–770. doi:10.1016/j.neuropharm.2008.06.018.

Calcium/Calmodulin-Dependent Kinase II Regulates the Interaction between the Serotonin Transporter and Syntaxin 1A

Marcia A. Ciccone, Miranda Timmons, Anthony Phillips, and Michael W. Quick*

Department of Biological Sciences, University of Southern California, Los Angeles CA 90089-2520

Summary

Plasma membrane serotonin transporters (SERTs) regulate serotonin (5HT) levels in brain and are a site of action of antidepressants and psychostimulant drugs of abuse. Syntaxin 1A is a component of the synaptic vesicle docking and fusion apparatus and has been shown to interact with multiple plasma membrane neurotransmitter transporters including SERT. Previously we showed that syntaxin 1A regulates the transport stoichiometry of SERT. When not bound to syntaxin 1A, SERT shows both substrate-independent Na⁺ fluxes and substrate-dependent Na⁺ fluxes of variable stoichiometry; these fluxes are eliminated in the presence of syntaxin 1A as Na⁺ flux becomes strictly coupled to 5HT uptake. However, not known are the endogenous signaling molecules that determine the conducting states that SERT exhibits. In the present experiments, we show that inhibitors of calcium/calmodulin-dependent kinase II (CaM kinase II) modulate the stoichiometry of 5HT flux and that this effect requires syntaxin 1A. The modulation correlates with a shift in the affinity of SERT for syntaxin 1A binding. The regulation by CaM kinase II is eliminated by a mutation in the N-terminal domain of SERT. In neonatal thalamocortical neurons that endogenously express SERT and syntaxin 1A, inhibition of CaM kinase II reveals SERT-mediated currents. These data suggest that calcium-mediated signals can serve as a trigger for regulating protein-protein interactions that control SERT conducting states.

Keywords

5-hydroxytryptamine; Protein Interaction; Stoichiometry; Synapse; Transport; Uptake

Introduction

Extracellular levels of serotonin (5HT) are controlled in part by the plasma membrane serotonin transporter (SERT), one member of a family of neurotransmitter transporters that function by coupling the transport of neurotransmitter to the electrochemical gradients for sodium, chloride, and potassium. SERT is a therapeutic target of selective serotonin reuptake inhibitors, tricyclic antidepressants, and appetite suppressants (Olivier et al., 2000). SERT is also a site of action of drugs of abuse such as cocaine and 3,4-methylenedioxymethamphetamine (MDMA; Rudnick and Wall, 1992). Changes in SERT function and expression have been implicated in anxiety, autism, depression, gastrointestinal disorders, obsessive-compulsive disorder, schizophrenia, substance abuse, and suicide (Lesch and Mossner, 1998). SERTs

*To whom correspondence should be addressed at: University of Southern California, 3551, Trousdale Parkway, ADM 304, Los Angeles CA 90089-4012; (phone) 213.740.2531; (fax) 213.740.8887; (email) mquick@usc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

exhibit several conducting states including a 5HT-independent leak conductance, a 5HT-independent transient conductance, and a 5HT-dependent ionic conductance (Mager et al., 1994). Such conductances have led to the idea that SERTs function in part as ion channels in addition to their role as transporters (Cao et al., 1998; Petersen and DeFelice, 1999).

Syntaxin 1A was originally characterized as a component of the machinery involved in transmitter release (Bennett et al., 1993; Söllner et al., 1993). Syntaxin 1A has also been shown to directly regulate Ca^{2+} channels (Sheng et al., 1994; Bezprozvanny et al., 1995), cystic fibrosis-related Cl^- channels (Naren et al., 1997), K^+ channels (Fili et al., 2001) and epithelial Na^+ channels (Qi et al., 1999; Saxena et al., 1999). Syntaxin 1A physically interacts with members of the family of plasma membrane neurotransmitter transporters (Quick et al., 1997; Beckman et al., 1998; Geerlings et al., 2000; Sung et al., 2003), including SERT (Haase et al., 2001) and acts to alter transporter capacity. For the GABA transporter GAT1, syntaxin 1A also affects the rate of substrate permeation (Deken et al., 2000).

Previously, we showed that syntaxin 1A directly interacts with the N-terminal tail of SERT (Quick, 2003). This interaction determines the conducting state that SERT exhibits. When SERT and syntaxin 1A interact, 5HT uptake proceeds in an electroneutral, stoichiometric manner (1 Na^+ , 1 Cl^- , 1 5HT^+ inward; 1 K^+ outward); 5HT-induced ionic currents and 5HT-independent Na^+ leak currents are absent. When SERT is not interacting with syntaxin 1A, 5HT uptake is electrogenic because of the appearance of a voltage-dependent flux of excess sodium (12 additional Na^+ ions per 5HT molecule at -80 mV); 5HT-induced ionic currents and 5HT-independent Na^+ leak currents are present. However, fundamental gaps in our understanding of SERT activity and its regulation remain. These include determining (i) the physiologically relevant molecules that can shift SERT between its conducting states, (ii) the signal transduction pathways through which these molecules act, and (iii) whether these molecules and pathways exert their effects by regulating the association between SERT and syntaxin 1A.

Methods

Constructs and Reagents

For generation of fusion proteins and peptides, cDNAs encoding particular domains were amplified by PCR, subcloned into pGEX (Pharmacia), and expressed in *E. Coli*. GST was removed by thrombin cleavage. Site-directed mutants were made using Altered Sites (Promega). High expression levels of SERT in oocytes was achieved by inserting the alfalfa mosaic virus 5' untranslated region upstream of the start codon, and a poly(50)-A tail downstream of the stop codon. cRNA synthesis was performed using mMessage mMachine (Ambion). An antibody recognizing aa 619 – 653 of SERT, and isoform-specific polyclonal syntaxin antibodies (Naren et al., 1997) were generated by immunizing rabbits with affinity-purified GST constructs. Unless otherwise mentioned, all drugs and chemicals were obtained from Sigma-Aldrich.

Constructs used in the experiments were as follows: Rat SERT (Hoffman et al., 1991) constructs: SERT, wild-type SERT, aa 1 – 653; SERT- $\Delta 10$, SERT lacking aa 2 – 10; SERT- $\Delta 30$, SERT lacking aa 2 – 30; SERT-5A, SERT mutant at positions E14A, D17A, E19A, D20A, E23A; SERT-S13A, SERT mutant at position 13; N-Tail, cytoplasmic N-terminal tail of SERT, aa 1 – 85; N-Tail (1-30), cytoplasmic N-terminal tail of SERT, aa 1 – 30. Syntaxin 1A (Bennett et al., 1993) constructs: Syn1A, full-length syntaxin 1A, aa 1 – 288; Syn1A Δ C, syntaxin 1A lacking the transmembrane domain, aa 1 – 266.

Cell culture

Xenopus oocyte culture is described elsewhere (Quick and Lester, 1994). Oocytes were assayed 7 – 10 days post-injection. The molar ratio of injected SERT:syntaxin 1A cRNA was 1:5. Primary cultures of thalamocortical neurons were prepared from E18 rats as described (Whitworth et al., 2002). Experiments were performed at 6 days *in vitro* (DIV). Membrane permeant drugs were added to the bath 15 minutes prior to assay.

Uptake assays

To assess charge:5HT flux ratios, [^3H]5HT uptake experiments (Quick and Lester, 1994) were performed simultaneously using two-electrode voltage-clamp electrophysiology. [^3H]5HT was superfused into the 60 μl recording chamber for 10 s, and then the flow was stopped; chamber bath exchange was complete in approximately 2 s. Steady-state peak currents elicited by this method were not significantly different from currents recorded under continuous perfusion (data not shown). Peak current was defined as current recorded just prior to 5HT removal, subtracted from the holding current measured in the absence of 5HT. Uptake was terminated by rapid removal of the oocyte from the chamber followed by washes in ice-cold ND96. Total 5HT-induced inward charge was calculated by integrating the 5HT-induced current; this value was matched to [^3H]5HT uptake in the same cell. Specific uptake at a given concentration was determined by subtracting total uptake from uptake measured in uninjected oocytes.

Transport stoichiometry was based upon experiments of Loo et al. (2000), and assessed by voltage clamping oocytes at -40 mV and performing uptake experiments using 22 mM $^{22}\text{Na}^+$, or 10 μM [^3H]5HT. Uptake of each isotope was performed on oocytes from the same batch. SERT-specific uptake was defined as the difference between total uptake and uptake in sister oocytes treated with 50 μM fluoxetine.

SERT antagonist binding assays

Binding to intact oocytes was performed as described (Chang and Weiss, 1999). Individual oocytes were placed in a single well of a 96-well plate containing 40 μl ND96. The assay (60 min at RT) was initiated with the addition of 10 μl $5\times$ [^{125}I]RTI-55 to the well and terminated by four washes in ice-cold ND96. The total wash time for all four wells was 16 s (4 s/well). The final [^{125}I]RTI-55 concentration was 10 nM. Specific binding was determined by the difference between total [^{125}I]RTI-55 binding and non-specific binding ([^{125}I]RTI-55 binding in the presence of 50 μM fluoxetine).

Biochemical assays

For immunoprecipitation experiments, cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail on ice for 60 min. SERT in the cell lysate was immunoprecipitated using anti-SERT antibodies and protein A-agarose at 4°C overnight. Nonimmune serum was used as a negative control. The protein-antibody-agarose complex was then washed three times with ice-cold RIPA buffer. Protein eluted from the agarose by SDS-loading buffer was subject to Western blotting and probed using anti-syntaxin 1A antibodies.

Biotinylation experiments were performed as described (Whitworth et al., 2002). Monoclonal anti-actin antibodies (Sigma) were used to normalize protein levels in each fraction and to verify that the biotinylating reagent was not labeling intracellular proteins (data not shown). Pair-wise, quantitative solution binding assays were performed as described (Naren et al., 1997). For assays involving activated CaM kinase II, binding was performed in the presence of 10 μM purified αCaM kinase II (New England Biolabs, amino acids 1 – 325), 20 mM

HEPES, 10 mM MgCl₂, 2.4 μM calmodulin, 2 mM Ca²⁺, and 100 mM ATP. For the unactivated condition, calmodulin, Ca²⁺, and ATP were absent from the binding assay.

Electrophysiology

Two-electrode voltage clamp was performed using a GeneClamp amplifier and Axoscope 6.0 software (Axon Instruments). Data were digitized at 10 Hz. Control solution was ND96. Electrodes were filled with 3 M KCl and had resistances of 1 – 3 MΩ. Whole-cell neuronal recordings were made using an Axopatch 200B amplifier at 10 kHz, low pass filtered at 2 kHz, and analyzed using pClamp (Axon Instruments). Pipettes were made from borosilicate glass and had resistances of 2 – 4 MΩ when filled. Internal solution contained (in mM): 138 potassium gluconate, 10 glucose, 10 HEPES, 10 KCl, 1 MgCl₂, 1 EGTA, 0.25 CaCl₂, 4 MgATP, 3 NaGTP (pH 7.4); external solution contained (in mM): 146 NaCl, 5 HEPES, 5 KCl, 10 glucose, 1.2 mM MgCl₂, 2.5 CaCl₂ (pH 7.4). Access resistance was monitored throughout the experimental time course. Measurements were made 10 ms from the end of the voltage jump. Potential 5HT receptor activation was minimized by including the 5HT₂ receptor antagonist metergoline (10 μM) and the 5HT_{1A/1B} antagonist pindolol (10 μM) in the bath solution.

Statistics

All summary data are presented as mean ± SEM. Statistical analyses were performed using SPSS (SPSS, Inc). Two-sample comparisons were made using t-tests; multiple comparisons were made using one-way ANOVAs followed by Tukey's honestly significant difference post-hoc test.

Results

To initially investigate the signal transduction cascades that regulate the stoichiometry of SERT conducting states, we focused on the role of calcium. Preliminary experiments in both neurons and expression systems using the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were promising but the results were complicated by significant changes in the trafficking and surface expression of SERT (data not shown). Thus we searched for a calcium-dependent process that had less effect on SERT expression.

Simultaneous measurements under voltage clamp (−80 mV) were made of [³H]5HT uptake and [³H]5HT-induced ionic currents in the same oocyte in order to evaluate the SERT conducting state as was previously performed (Quick, 2003). For direct comparison of charge to 5HT flux, 5HT-induced currents were converted to moles of ion flux based upon Faraday's constant (96,500 coulombs/mol). Then, the amount of charge flux versus the amount of 5HT flux was plotted for each oocyte for a given experimental condition (Figure 1A), or was expressed as a charge:5HT flux ratio for each condition (Figure 1B). In oocytes expressing SERT alone, the ratio of charge flux to 5HT flux was 12.2 ± 1.3. In oocytes expressing SERT and syntaxin 1A, the charge:5HT flux ratio was 0.5 ± 0.3. Acute application of the selective CaM kinase II inhibitor KN-93 (N-[2-[[[3-(4'-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxybenzenesulfonamide) to oocytes expressing SERT alone had no effect on the charge:5HT flux ratio (12.8 ± 1.6) compared to the untreated condition. However, in oocytes expressing both SERT and syntaxin 1A, acute application of KN-93 significantly increased the charge:5HT flux ratio (8.2 ± 3.1). In the KN-93-treated condition, the charge:5HT flux ratio was highly variable across individual oocytes; values ranged from 3.7 to 10.8. The inactive KN-93 analogue KN-92 (2-[N-(4-Methoxybenzenesulfonyl)]amino-N-(4-chlorophenyl)-2-propenyl-N-methylbenzylamine) had no effect on charge:5HT flux ratios of oocytes expressing SERT alone (11.8 ± 2.0) or with syntaxin 1A (0.3 ± 0.5).

These data were consistent with the hypothesis that CaM kinase II could shift SERT between electrogenic and electroneutral stoichiometries. To test this hypothesis directly, radiolabeled substrate fluxes were measured for 5HT and Na⁺ (Figure 1B). Assays were performed in oocytes voltage clamped at -40 mV as described previously (Quick, 2003). Flux was assessed in separate groups of oocytes from the same oocyte batch. In oocytes expressing SERT alone, Na⁺ flux exceeded 5HT flux by a factor of 6.3 ± 0.6 . In oocytes expressing SERT and syntaxin 1A, Na⁺ flux and 5HT flux were statistically equivalent. However, in the presence of KN-93, oocytes expressing SERT and syntaxin 1A showed Na⁺ fluxes that approached the ratio seen in oocytes expressing SERT alone (5.0 ± 1.8). These data suggest that syntaxin 1A is required for regulation of SERT conducting states. We interpret the increased variability in charge:5HT flux ratios in oocytes treated with KN-93 as reflecting increased variability in the proportion of SERT interacting with syntaxin 1A in each cell.

Since syntaxin 1A is a known positive modulator of SERT surface expression (Quick, 2002), we examined the role of KN-93 in regulating this process. Radioligand binding of the high-affinity SERT antagonist RTI-55 was performed on oocytes expressing SERT alone or in combination with syntaxin 1A (Figure 1D). In oocytes expressing SERT alone, SERT-specific binding (binding in the presence of 50 μ M fluoxetine subtracted from total binding) using a saturating concentration of [¹²⁵I]RTI-55 (10 nM) was 12.8 ± 1.1 fmol/oocyte. Specific binding in oocytes expressing SERT and syntaxin 1A was 16.6 ± 0.8 fmol/oocyte, indicating that syntaxin 1A is a positive regulator of SERT surface expression. The addition of KN-93 (or KN-92) to oocytes expressing SERT alone had no effect. In oocytes expressing SERT and syntaxin 1A, KN-93 decreased SERT-specific binding 19% (to 13.4 ± 0.8 fmol/oocyte), comparable to that seen in oocytes expressing SERT alone.

The evidence that CaM kinase II inhibition (i) did not affect the charge:5HT flux ratios in SERT alone-expressing oocytes and that (ii) it affected SERT surface expression in the presence of syntaxin 1A suggested that CaM kinase II might exert its effects by influencing the interaction between SERT and syntaxin 1A. To examine this hypothesis directly, biochemical assays were performed (Figure 2). We performed immunoprecipitation assays from thalamocortical neuron cultures that were either untreated or acutely treated with KN-93. Lysates were precipitated using anti-SERT antibodies and then the precipitated complexes were probed with anti-syntaxin 1A antibodies. A representative immunoblot (Figure 2A) shows the amount of syntaxin 1A in complex with SERT compared to the amount of SERT in the precipitated lysate. Treatment of the cultures with KN-93 had no effect on the amount of SERT precipitated compared to untreated cultures ($107 \pm 16\%$). However, as shown in the graph in Figure 2A, the amount of syntaxin 1A in the immunoprecipitated complex was significantly decreased in cultures treated with KN-93 ($27 \pm 7\%$).

To examine this idea further, pair-wise *in vitro* binding assays were performed to determine the affinity of the direct interaction between the N-terminal, cytoplasmic tail of SERT and a syntaxin 1A construct lacking its C-terminal membrane anchor. Soluble recombinant N-terminal SERT (GST-N-Tail) was added to soluble recombinant cytoplasmic syntaxin 1A (GST-Syn1A Δ C, in which the GST was cleaved free by thrombin) in the presence or absence of activated CaM kinase II (Figure 2B). In the absence of activated CaM kinase II, the association between the N-terminal tail of SERT and syntaxin 1A was saturable with half-maximal binding of 310 ± 37 nM, comparable to previous estimates (Quick, 2003). In the presence of activated CaM kinase II, half-maximal binding was significantly increased (62 ± 23 nM). Taken together, the biochemical data indicate that CaM kinase II is a positive regulator of SERT and syntaxin 1A binding.

In prior experiments using SERT mutants we identified a region of approximately 20 amino acids in the N-terminal tail of SERT (between amino acids 10 and 30) that are necessary for

the interaction between SERT and syntaxin 1A. We used these mutants in charge:5HT flux experiments to further verify that CaM kinase II exerts its functional effects by regulating SERT and syntaxin 1A interactions (Figure 3A). A SERT mutant lacking amino acids 2 – 10 in the N-terminal tail (SERT-Δ10) was shown previously to bind syntaxin 1A normally. Oocytes expressing SERT-Δ10 and syntaxin 1A showed charge:5HT flux ratios similar to wild-type (0.3 ± 0.3). Treatment with KN-93 caused a significant increase in charge:5HT flux ratios (8.2 ± 1.2), comparable to experiments with wild-type SERT (see Figure 1B). SERT-Δ30 (a mutant lacking the amino acids 2 – 30 of SERT) and SERT-5A, a mutant in which the five negatively charged amino acids between residues 11 and 30 were replaced with alanines (E14A, D17A, E19A, D20A, E23A), were both shown previously to be refractory for syntaxin 1A binding (Quick, 2003). Charge:5HT flux ratios in the absence of KN-93 for these two mutants (co-expressed with syntaxin 1A) were significantly higher than that seen for wild-type SERT and the SERT-Δ10 mutant. Treatment with KN-93 did not affect these ratios. Thus, in mutants that have normal SERT and syntaxin 1A interactions, KN-93 increased the charge:5HT flux ratio; in mutants with substantially diminished ability to bind syntaxin 1A, KN-93 had no effect.

The biochemical data and the charge:5HT flux data obtained with SERT mutants suggested that CaM kinase II is having its functional effects by influencing the interaction between SERT and syntaxin 1A. Thus, as an initial step in determining the site of action of CaM kinase II on SERT, we looked for amino acids within the region of SERT where syntaxin 1A binding occurs (between amino acids 10 and 30). We reasoned that CaM kinase II could be exerting its effects by phosphorylating a serine or threonine residue in that region. In fact, in that stretch of 20 amino acids there is one potential phosphorylation site: a serine at position 13. We mutated that serine to alanine and examined the resulting mutant for charge:5HT flux ratios, syntaxin 1A binding, and regulation by CaM kinase II. In oocytes co-expressing syntaxin 1A with the SERT-S13A mutant, charge:5HT flux ratios (8.8 ± 1.3) were significantly higher than that seen with wild-type SERT or the SERT-Δ10 mutant (Figure 3A). In fact, the results were indistinguishable from those seen in oocytes co-expressing syntaxin 1A with wild-type SERT or the SERT-Δ10 mutant and acutely treated with KN-93. However, acute KN-93 treatment had no additional effect on charge:5HT flux ratios in oocytes co-expressing syntaxin 1A with the SERT-S13A mutant. Direct assessment of transport stoichiometry by radiolabeled flux revealed that the charge:5HT flux in oocytes co-expressing syntaxin 1A with the SERT-S13A mutant was due to a significant increase in Na⁺ flux (5.3 ± 1.3 -fold increase; Figure 3B).

Pair-wise *in vitro* binding assays between syntaxin 1A and SERT (wild-type and the S13A mutant) were performed (Figure 3C). In the presence of activated CaM kinase II, half-maximal binding between syntaxin 1A and the SERT-S13A mutant was 332 ± 38 nM. This was approximately 6-fold less than the binding of SERT and syntaxin 1A in the presence of activated CaM kinase II and comparable to the binding of syntaxin 1A to wild-type SERT in the absence of activated CaM kinase II. In the absence of activated CaM kinase II, half-maximal binding between syntaxin 1A and the SERT-S13A mutant was 315 ± 27 nM, not significantly different from wild-type SERT binding in the absence of CaM kinase II or SERT-S13A binding in the presence of CaM kinase II. Taken together these data demonstrate that in the presence of syntaxin 1A, the SERT-S13A mutant (i) has a phenotype comparable to wild-type SERT treated with CaM kinase II inhibitors and (ii) shows charge:5HT flux ratios and syntaxin 1A binding that is refractory for modulation by CaM kinase II.

The data shown in Figure 1D revealed that in oocytes expressing SERT and syntaxin 1A, KN-93 caused a small but significant decrease in the surface expression of SERT, based on radiolabeled antagonist binding assays. We performed similar experiments with oocytes expressing syntaxin 1A and the various SERT mutants (Figure 3D). Treating oocytes expressing the SERT-Δ10 mutant and syntaxin 1A with KN-93 caused a statistically significant 18% decrease in [¹²⁵I]RTI-55 binding, comparable to that seen with wild-type SERT. This

KN-93-mediated decrease was not seen in oocytes expressing SERT-Δ30, SERT-5A, or the SERT-S13A mutant. These data suggest that the effect of CaM kinase II on SERT stoichiometry and on SERT trafficking is mediated in part through comparable mechanisms.

Is SERT activity in neurons that endogenously co-express SERT and syntaxin 1A regulated by CaM kinase II? To address this question we performed standard whole-cell patch-clamp experiments in dissociated thalamocortical neurons (Quick, 2003) that have been shown to express SERT during development (Lebrand et al., 1996). SERT-mediated currents were defined as the difference in 5HT-induced steady-state currents recorded in the absence and presence of the high-affinity SERT inhibitor paroxetine. In the basal state, SERT-mediated currents were small or absent in the majority of neurons. This might be expected if SERT currents were suppressed because of SERT interactions with syntaxin 1A. To test whether CaM kinase II plays a role in regulating SERT conducting states in neurons, we performed voltage-jump experiments to trigger calcium influx (brief depolarization to 0 mV followed by current measurements at -120 mV) and measured SERT-mediated currents in the presence of a CaM kinase II inhibitory peptide (CaMK-(281-309); Colbran et al., 1988) in the patch pipette. As shown in Figure 4A for a representative cell, one minute after gaining cell access, application of 5HT failed to elicit an inward current different from control. However, 15 minutes after gaining access, paroxetine-sensitive 5HT-induced steady-state currents were seen. Summary data (Figure 4B) were plotted for the experiments described in Figure 4A. Neurons in which 5HT-induced currents were less than 5 pA at 1 minute were excluded from the analysis. For the group of 7 remaining neurons, 5HT-induced currents were 8 ± 5 pA at 1 minute; at 15 minutes, the currents were 42 ± 7 pA.

We repeated the patch-clamp studies under a variety of experimental conditions. The results are shown in Figure 4C and presented as the ratio of the 5HT-induced peak steady-state current at 15 minutes compared to the 5HT-induced peak steady-state current at 1 minute. Neurons in which 5HT-induced currents were less than 5 pA at 1 minute were excluded from the analysis. With the CaM kinase II inhibitory peptide in the pipette, the currents became 5.2-fold larger in 15 minutes ($n = 7$). With a scrambled peptide control, the ratio was 1.1 ± 0.2 ($n = 6$). In the presence of a standard internal pipette solution, bath application of 10 μ M KN-93 resulted in a 3.6 ± 1.1 -fold increase in currents at 15 minutes ($n = 5$); this was significantly larger than experiments done in the presence of the inactive analogue KN-92 (1.0 ± 0.3 ; $n = 5$). Lastly, we examined the effect of a peptide consisting of the first 30 amino acids of the SERT N-terminal (N-Tail (1-30)). Similar to previous results (Quick, 2003), including this peptide in the patch pipette caused a 4.2-fold increase in the ratio of currents ($n = 6$), consistent with the hypothesis that the peptide disrupts the interaction between SERT and syntaxin 1A. Bath application of KN-93 in the presence of the SERT N-Tail (1-30) peptide had no effect on the ratio (4.1 ± 0.6 ; $n = 5$). These data are consistent with the idea that CaM kinase II, in a syntaxin 1A-dependent manner, regulates SERT-mediated currents in neurons.

Peak steady-state currents are a function of the unitary conductance properties of SERT and its surface expression. Our data from oocytes suggests that the increase currents seen at 15 minutes are the result of a change in the conducting state of SERT. An alternative explanation for the large increase in SERT-mediated currents would be a large increase in SERT surface expression. To attempt to examine that possibility, we performed surface biotinylation experiments on thalamocortical cultures after 15 minute treatment with KN-93 and KN-92 (Figure 4D). Regardless of treatment condition, the amount of SERT found in the biotinylated (surface) fraction was approximately 50% of total cellular SERT. Although this type of experiment cannot rule out a multi-fold increase in surface expression in the neurons specifically measured electrophysiologically, it does suggest a limited role for trafficking in CaM kinase II-mediated regulation of SERT-mediated currents.

Discussion

Serotonin transporters display multiple conducting states. One of these is a substrate-dependent conductance of variable stoichiometry which exceeds that predicted from electroneutral substrate transport. Previously we showed that syntaxin 1A binds the N-terminal tail of SERT and this interaction alters the transport stoichiometry of SERT. In the presence of syntaxin 1A, 5HT transport is electroneutral; in the absence of syntaxin 1A, excess Na⁺ flux makes 5HT transport electrogenic (Quick, 2003). Experimental manipulations that disrupt the interaction between SERT and syntaxin 1A alter transport stoichiometry although the endogenous molecules that might control this process are not known. The present data show that CaM kinase II is one such molecule and suggest a model in which CaM kinase II enhances the binding of SERT to syntaxin 1A resulting in electroneutral 5HT transport. At low levels of CaM kinase II activity, binding of SERT to syntaxin 1A is reduced and 5HT transport is accompanied by increased Na⁺ flux. These data support the hypothesis that calcium-mediated signals serve as a trigger for regulating protein-protein interactions that control SERT conducting states.

Kinases and phosphatases have long been known to regulate the functional activities of SERT. Much of the regulation occurs through alterations in the transport capacity of SERT by mechanisms related to subcellular redistribution of the transporter. For example, protein kinase C (PKC) is associated with decreased surface expression of the transporter (Ramamoorthy and Blakely, 1998). In the basal state, SERT interacts with protein phosphatase 2A. Upon PKC activation, protein phosphatase 2A dissociates from the complex and SERT internalizes (Bauman et al., 2000). PKC appears to exert its effects in part by enhancing macromolecular complexes involving SERT and the membrane skeleton adaptor protein Hic-5 (Carneiro and Blakely, 2006). Protein kinase G affects SERT function by altering intrinsic transporter properties (Kilic et al., 2003) via a p38 mitogen-activated protein kinase (MAPK) pathway and a separate pathway that alters SERT surface expression (Zhu et al, 2004). Calcium and calmodulin have likewise been identified as regulators of SERT function by altering the affinity of 5HT binding to SERT (Jayanthi et al., 1994). The present work identifies a separate mechanism for CaM kinase II-mediated SERT regulation – by regulating the interactions of SERT and syntaxin 1A which act to control SERT stoichiometry. These data build on prior work showing that kinases can regulate the activities of norepinephrine transporters (Sung et al., 2003) and GABA transporters (Beckman et al., 1998) through interactions with syntaxin 1A.

The mechanism by which CaM kinase II exerts its effects on SERT and syntaxin 1A is currently being investigated. A straightforward hypothesis is that CaM kinase II phosphorylates SERT on serine 13, resulting in a change in the binding of SERT to syntaxin 1A. However, we have yet to measure SERT phosphorylation at the serine 13 site. It is also possible that CaM kinase II is not exerting its modulatory effects through phosphorylation but rather through direct binding to SERT. For example, it has been shown that autophosphorylated CaM kinase II directly binds syntaxin 1A and this interaction influences syntaxin 1A's binding to other partners (Ohyama et al., 2002). The fact that CaM kinase II binds syntaxin 1A also raises the possibility that the CaM kinase II effect is mediated through syntaxin 1A and not SERT and that the results seen with the SERT-S13A mutant are due to disruption to the syntaxin 1A binding site on SERT in a way that prevents further modulation by CaM kinase II.

The ability of CaM kinase II to regulate the interaction between SERT and syntaxin 1A, leading to a change in SERT conductance states in neurons that endogenously express the transporter, raises the question as to the potential physiological significance of this regulation. We know that transporter currents have a physiological role. Glutamate transporter-mediated chloride currents regulate membrane potentials and affect synaptic signaling (Otis et al., 1997). Dopamine transporter-mediated chloride currents modulate membrane potential (Carvelli et

al., 2004) and firing rates (Ingram et al., 2002). We showed previously that 5HT-independent leak currents and 5HT-induced transport-associated currents mediated by SERT act to depolarize the cell's membrane potential; interaction with syntaxin 1A inhibits depolarization (Quick, 2003). The present work suggests that CaM kinase II may act as a switch to counterbalance cell excitability. With high levels of neuronal activity, calcium influx would increase CaM kinase II activity leading to syntaxin 1A binding to SERT and inactivation of depolarizing Na⁺ fluxes. During periods of inactivity, and thus reduced calcium influx, syntaxin 1A binding to SERT would be lessened and SERT-mediated Na⁺ fluxes would increase.

Acknowledgements

This work was supported by NIH Grant MH74034.

References

- Bauman AL, Apparsundaram S, Ramamoorthy S, Wadzinski BE, Vaughan RA, Blakely RD. Cocaine and antidepressant-sensitive biogenic amine transporters exist in regulated complexes with protein phosphatase 2A. *J Neurosci* 2000;20:7571–7578. [PubMed: 11027216]
- Beckman ML, Bernstein EM, Quick MW. Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. *J Neurosci* 1998;18:6103–6112. [PubMed: 9698305]
- Bennett MK, Garcia-Ararras JE, Elferink LA, Peterson K, Fleming AM, Hazuka CD, Scheller RH. The syntaxin family of vesicular transport receptors. *Cell* 1993;74:863–873. [PubMed: 7690687]
- Bezprozvanny I, Scheller RH, Tsien RW. Functional impact of syntaxin on gating of N-type and Q-type calcium channels. *Nature* 1995;378:623–626. [PubMed: 8524397]
- Cao Y, Mager S, Lester HA. H⁺ permeation and pH regulation at a mammalian serotonin transporter. *J Neurosci* 1997;17:2257–2266. [PubMed: 9065487]
- Carneiro AMD, Blakely RD. Serotonin-, protein kinase C-, and Hic-5-associated redistribution of the platelet serotonin transporter. *J Biol Chem* 2006;281:24769–24780. [PubMed: 16803896]
- Carvelli L, McDonald PW, Blakely RD, DeFelice LJ. Dopamine transporters depolarize neurons by a channel mechanism. *Proc Natl Acad Sci USA* 2004;101:16046–16051. [PubMed: 15520385]
- Chang Y, Weiss DS. Channel opening locks agonist onto the GABA_C receptor. *Nat Neurosci* 1999;2:219–225. [PubMed: 10195213]
- Colbran RJ, Fong YL, Schworer CM, Soderling TR. Regulatory interactions of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca²⁺/calmodulin-dependent protein kinase II. *J Biol Chem* 1988;263:18145–18151. [PubMed: 2848027]
- Deken SL, Beckman ML, Boos L, Quick MW. Transport rates of GABA transporters: regulation by the N-terminal domain and syntaxin 1A. *Nat Neurosci* 2000;3:998–1003. [PubMed: 11017172]
- Fili O, Michaelievski I, Bledi Y, Chikvashvili D, Singer-Lahat D, Boshwitz H, Linial M, Lotan I. Direct interaction of a brain voltage-gated K⁺ channel with syntaxin 1A: functional impact on channel gating. *J Neurosci* 2001;21:1964–1974. [PubMed: 11245681]
- Geerlings A, Lopez-Corcuera B, Aragon C. Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A. *FEBS Lett* 2000;470:51–54. [PubMed: 10722844]
- Haase J, Killian AM, Magnani F, Williams C. Regulation of the serotonin transporter by interacting proteins. *Biochem Soc Trans* 2001;29:722–728. [PubMed: 11709063]
- Hoffman BJ, Mezey E, Brownstein MJ. Cloning of a serotonin transporter affected by antidepressants. *Science* 1991;254:579–580. [PubMed: 1948036]
- Ingram SL, Prasad BM, Amara SG. Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons. *Nat Neurosci* 2002;5:971–978. [PubMed: 12352983]
- Jayanthi LD, Ramamoorthy S, Mahesh VB, Leibach FH, Ganapathy V. Calmodulin-dependent regulation of the catalytic function of the human serotonin transporter in placental choriocarcinoma cells. *J Biol Chem* 1994;269:14424–14429. [PubMed: 8182048]

- Kilic F, Murphy DL, Rudnick G. A human serotonin transporter mutation causes constitutive activation of transport activity. *Mol Pharmacol* 2003;64:440–446. [PubMed: 12869649]
- Lebrand C, Cases O, Adelbrecht C, Doye A, Alvarez C, El Mestikawy S, Seif I, Gaspar P. Transient uptake and storage of serotonin in developing thalamic neurons. *Neuron* 1996;17:823–835. [PubMed: 8938116]
- Lesch KP, Mossner R. Genetically driven variation in serotonin uptake: is there a link to affective spectrum, neurodevelopmental, and neurodegenerative disorders. *Biol Psychiatry* 1998;44:179–192. [PubMed: 9693390]
- Loo DD, Eskandari S, Boorer KJ, Sarkar HK, Wright EM. Role of Cl^- in electrogenic Na^+ -coupled cotransporters GAT1 and SGLT1. *J Biol Chem* 2000;275:37414–37422. [PubMed: 10973981]
- Mager S, Min C, Henry DJ, Chavkin C, Hoffman BJ, Davidson N, Lester HA. Conducting states of a mammalian serotonin transporter. *Neuron* 1994;12:845–859. [PubMed: 8161456]
- Naren AP, Nelson DJ, Xie W, Jovov B, Pevsner J, Bennett MK, Benos DJ, Quick MW, Kirk KL. Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. *Nature* 1997;390:302–305. [PubMed: 9384384]
- Ohyama A, Hosaka K, Komiya Y, Akagawa K, Yamauchi E, Taniguchi H, Sasagawa N, Kumakura K, Mochida S, Yamauchi T, Igarashi M. Regulation of exocytosis through Ca^{2+} /ATP-dependent binding of autophosphorylated Ca^{2+} /calmodulin-activated protein kinase II to syntaxin 1A. *J Neurosci* 2002;22:3342–3351. [PubMed: 11978810]
- Olivier B, Soudijn W, van Wijngaarden I. Serotonin, dopamine and norepinephrine transporters in the central nervous system and their inhibitors. *Prog Drug Res* 2000;54:59–119. [PubMed: 10857386]
- Otis TS, Kavanaugh MP, Jahr CE. Postsynaptic glutamate transport at the climbing fiber-Purkinje cell synapse. *Science* 1997;277:1515–1518. [PubMed: 9278516]
- Petersen CI, DeFelice LJ. Ionic interactions in the *Drosophila* serotonin transporter identify it as a serotonin channel. *Nature Neurosci* 1999;2:605–610. [PubMed: 10404179]
- Qi J, Peters KW, Liu C, Wang JM, Edinger RS, Johnson JP, Watkins SC, Frizzell RA. Regulation of the amiloride-sensitive epithelial sodium channel by syntaxin 1A. *J Biol Chem* 1999;274:30345–30348. [PubMed: 10521407]
- Quick MW, Lester HA. Expression of excitability proteins in oocytes. In: Conn, PM., editor. *Methods in Neuroscience: Ion Channels of Excitable Cells*. San Diego: Academic Press; 1994. p. 261–279.
- Quick MW, Corey JL, Davidson N, Lester HA. Second messengers, trafficking-related proteins, and amino acid residues that contribute to the functional regulation of the rat brain GABA transporter GAT1. *J Neurosci* 1997;17:2967–2979. [PubMed: 9096133]
- Quick MW. Role of syntaxin 1A on serotonin transporter expression in developing thalamocortical neurons. *Int J Dev Neurosci* 2002;20:219–224. [PubMed: 12175857]
- Quick MW. Regulating the conducting states of a mammalian serotonin transporter. *Neuron* 2003;40:537–549. [PubMed: 14642278]
- Ramamoorthy S, Blakely RD. Phosphorylation and sequestration of serotonin transporters differentially modulated by psychostimulants. *Science* 1998;285:763–766. [PubMed: 10427004]
- Rudnick G, Wall SC. The molecular mechanism of "ecstasy" [3,4-methylenedioxy-methamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. *Proc Natl Acad Sci USA* 1992;89:1817–1821. [PubMed: 1347426]
- Saxena S, Quick MW, Tousson A, Oh Y, Warnock DG. Interaction of syntaxins with the amiloride-sensitive epithelial sodium channel. *J Biol Chem* 1999;274:20812–20817. [PubMed: 10409621]
- Sheng ZH, Rettig J, Takahashi M, Catterall WA. Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* 1994;13:1303–1313. [PubMed: 7993624]
- Sollner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 1993;75:409–418. [PubMed: 8221884]
- Sung U, Apparsundaram S, Galli A, Kahlig KM, Savchenko V, Schroeter S, Quick MW, Blakely RD. A regulated interaction of syntaxin 1A with the antidepressant-sensitive norepinephrine transporter establishes catecholamine clearance capacity. *J Neurosci* 2003;23:1697–1709. [PubMed: 12629174]
- Whitworth TL, Herndon LC, Quick MW. Psychostimulants differentially regulate serotonin transporter expression in thalamocortical neurons. *J Neurosci* 2002;22:RC192. [PubMed: 11756522]

Zhu CB, Hewlett WA, Feoktistov I, Biaggioni I, Blakely RD. 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* 2004;65:1462–1474. [PubMed: 15155839]

Figure 1. A CaM kinase II inhibitor modulates the stoichiometry of SERT transport

(A) Charge and substrate flux in individual SERT-expressing oocytes. Moles of ion flux (charge) and moles of 5HT ($10\ \mu\text{M}$) flux were determined simultaneously in individual oocytes expressing SERT alone (filled circles) or with syntaxin 1A (open circles). Subsets of these groups were treated with $10\ \mu\text{M}$ final concentration KN-93 (square symbols) 30 minutes prior to assay. The holding potential was $-80\ \text{mV}$. Data from individual oocytes are plotted. (B) Summary charge:5HT flux data for oocytes treated as in (A). Oocytes expressing SERT alone (filled bars) or with syntaxin 1A (open bars) were subsequently left untreated or treated with $10\ \mu\text{M}$ KN-93 or KN-92 and assayed. Data are from three separate experiments, 18–24 oocytes per condition. Comparisons denoted by an asterisk represent statistically significant differences ($p < .05$). (C) Stoichiometry of flux. Oocytes treated as in (A) were subjected to uptake using radiolabeled $^{22}\text{Na}^+$ ($22\ \text{mM}$; filled bars) or $[^3\text{H}]\text{5HT}$ ($10\ \mu\text{M}$; hatched bars). The holding potential was $-40\ \text{mV}$. Data represent SERT-specific uptake (difference between total uptake

and uptake in sister oocytes treated with 50 μ M fluoxetine) from 13 – 16 oocytes/condition from one oocyte batch. (D) Binding of a SERT antagonist in intact oocytes. Oocytes expressing SERT alone (filled bars) or with syntaxin 1A (open bars) were subsequently left untreated or treated with 10 μ M KN-93 or KN-92 and then subjected to [125 I]RTI-55 (10 nM) binding. Specific binding was calculated by subtracting total binding from nonspecific binding performed in the presence of 50 μ M fluoxetine. The data shown are from one experiment, 5 oocytes per condition. Comparable results were obtained in two other experiments.

Figure 2. A CaM kinase II inhibitor regulates the physical interaction between SERT and Syntaxin 1A

(A) Immunoprecipitation assays. Thalamocortical cell cultures were untreated or treated with 10 μ M final concentration KN-93 for 30 minutes. Cell lysates were immunoprecipitated using anti-SERT antibodies and subjected to Western blotting to examine SERT and syntaxin 1A immunoreactivity (see immunoblot). Data from three experiments are plotted in the graph. Syntaxin 1A immunoreactivity is shown relative to SERT immunoreactivity from the same condition. Comparisons denoted by an asterisk represent statistically significant differences ($p < .05$). (B) Solution binding assays. Recombinant syntaxin 1A cytosolic domain (0.4 nmole) was bound to increasing concentrations of GST-N-Tail and immunoblotted using a syntaxin

1A-specific antibody. Binding was performed in the presence (open circles) or absence (open squares) of activated CaM kinase II (see Materials and Methods). Percent of syntaxin 1A bound relative to its binding to 1.0 μ M GST-N-Tail was determined from three separate experiments.

Figure 3. An N-terminal SERT mutant shows ion fluxes that are unaffected by CaM kinase II inhibition

(A) Charge-flux ratios. Experiments were performed as in Figure 1A for oocytes expressing syntaxin 1A with four different SERT mutants described on the abscissa (open bars). Subsets of these groups were treated with 10 μ M final concentration KN-93 (filled bars) 30 minutes prior to assay. Data are from three separate experiments, 7 – 10 oocytes per condition per experiment. Data denoted with an asterisk represent statistically significant differences ($p < .05$) compared to data from SERT- Δ 10, untreated. (B) Stoichiometry of flux. Oocytes expressing syntaxin 1A and either wild-type SERT or the SERT-S13A mutant were treated as in (A) and then subjected to experiments as described in Figure 1C. Data represent SERT-specific uptake from 17 – 26 oocytes/condition from two oocyte batches. Comparisons denoted by an asterisk represent statistically significant differences ($p < .05$). (C) Solution binding assays. Experiments were performed as in Figure 2B using increasing concentrations of wild-type (open circles) or mutant GST-N-Tail fusion proteins (squares). Binding with the SERT-S13A mutant was performed in the absence (filled squares) or presence (open squares) of activated CaM kinase II. Data are from three separate experiments. (D) Binding of a SERT

antagonist to intact oocytes. Oocytes as in (A) were subjected to [125 I]RTI-55 (10 nM) binding assays as described in Figure 1D. The data shown are from one experiment, 5 oocytes per condition. Comparable results were obtained in two other experiments. Data denoted by the asterisk are significantly different ($p < 0.05$) than untreated sister oocytes.

Figure 4. CaM kinase II regulates SERT-mediated currents in thalamocortical neurons
 (A) Representative traces of whole-cell patch clamp recordings produced by a voltage step measured 1 min (top traces) or 15 min (bottom traces) after gaining cell access. Cells were perfused with 10 μ M 5HT or 10 μ M 5HT + 100 nM paroxetine with pipette solution containing 10 μ M CaM kinase II inhibitory peptide CaMK-(281-309). (B) Summary data from experiments as in (A), n = 7. Only those neurons that showed 5HT-mediated currents greater than 5 pA at 1 min were included in the analysis. Comparisons denoted by an asterisk represent statistically significant differences (p < .05). (C) Summary data for experiments as in (A), but under various experimental manipulations. The ratio of currents measured at 15 min compared to 1 min is plotted for experiments using a pipette solution containing 10 μ M CaM kinase II inhibitory peptide (peptide), a scrambled control peptide (scrambled), or a SERT peptide containing the first 30 N-terminal amino acids (N-Tail (1-30)). For some experiments 10 μ M KN93 or KN 92 was added to the bath at the 1 min time point. Only those neurons that showed 5HT-mediated currents greater than 5 pA at 1 min were included in the analysis. (D) Surface biotinylation of thalamocortical neuron cultures. Cultures were untreated or treated with 10 μ M KN93 or KN92 15 min prior to assay. The immunoblot shows the biotinylated fraction

from one experiment. The graph represents the biotinylated fraction as a percentage of total SERT immunoreactivity in the lysate. Data are from three separate experiments.