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Commentary

Consideration of allosterism and interacting proteins in the physiological functions of the serotonin transporter

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ABSTRACT

The serotonin transporter (SERT) functions to transport serotonin (5-HT) from the extracellular space into neurons to maintain homeostatic control of 5-HT. It is the molecular target for selective serotonin reuptake inhibitor (SSRI) antidepressants. Preclinical research has shown that some SERT inhibitors can bind to two distinct binding sites on the SERT, a primary high affinity binding site and a low affinity allosteric binding site. Mutational studies of the SERT and computational modeling methods with escitalopram resulted in the identification of key amino acid residues important for the function of the allosteric binding site. While this allosteric binding site appears to influence the clinical efficacy of escitalopram under physiological conditions, the molecular mechanism of this effect is still poorly understood and may involve a large network of protein–protein interactions with the SERT. Dynamic interfaces between the SERT and the SERT interacting proteins (SIPs) potentially influence not only the SERT on its uptake function, its regulation, and trafficking, but also on known as well as yet to be identified non-canonical signaling pathways through SIPs. In this commentary, we outline approaches in the areas of selective small-molecule allosteric compound discovery, biochemistry, *in vivo* genetic knock-in mouse models, as well as computational and structural biology. These studies of the intra-molecular allosteric modulation of the SERT in the context of the myriad of potential inter-molecular signaling interactions with SIPs may help uncover unknown physiological functions of the SERT.
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1. Introduction

The serotonin transporter (SERT) is a member of the SLC6 family of neurotransmitter transporters that is structurally comprised of 12 transmembrane (TM) spanning domains and is responsible for the uptake of serotonin (5-HT) from the extrasynaptic space into presynaptic terminals. The SERT serve as a symporter, requiring ions including Na^+, K^+ and Cl^-, that are used as the driving force for transport. The SERT is a target for many of the currently used antidepressant drugs including the selective serotonin reuptake inhibitors (SSRIs).

SSRIs bind to the primary high affinity site, or orthosteric site, of the SERT to inhibit its uptake function, leading to increased extracellular levels of 5-HT. Although this has been regarded as the primary basis for the therapeutic actions of SSRIs, many questions related to the physiological and pathophysiological roles of the SERT as well as its exact role in antidepressant and analgesic treatment remain unanswered. The acute increase in 5-HT caused by SSRIs is not translated into immediate antidepressant effects. Traditional antidepressants, including SSRIs, require at least 2 or more weeks to demonstrate their therapeutic effects. The delayed onset of antidepressant therapeutic effect suggests that adaptive neuronal changes in the brain in addition to elevation of extracellular 5-HT levels are required for treating depression [1–3], but the precise mechanisms underlying this neuroadaptive process are far from well understood and are beyond the aim of this commentary. In this paper, we review the current findings suggesting that the overall machinery responsible for the 5-HT reuptake process by the SERT is more complex than previously anticipated. Firstly, we describe the intra-molecular evidence in the SERT indicating that allosteric ligand interactions may influence the reuptake mechanism and potentially account, at least in part, for some functional differences among SSRIs *in vivo*. Secondly, we discuss data from protein interaction studies at the inter-molecular level and demonstrate that the SERT carries out its main re-uptake function by concomitantly interacting with an extensive cadre of intracellular accessory signaling and scaffolding proteins. This property may not only affect the cellular processing of the SERT but also regulate its function through intracellular signaling cascades.

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2. Allosteric interactions in the SERT

Allosteric mechanisms are a common molecular theme for signal transduction by pharmacological ligands [4–6]. Allosteric binding properties of the SERT were initially reported in imipramine binding experiments [7] and allosteric activities were subsequently demonstrated for all three monoamine transporters [8,9]. Although several allosteric SERT modulators have been reported [10,11], most studies on the characterization of allosteric interactions within the SERT have been described using escitalopram.

Escitalopram is the S-enantiomer of racemic citalopram, which also contains an equal amount of the presumed non-therapeutic enantiomer, R-citalopram [12]. Escitalopram is more active than R-citalopram for its interactions with the SERT, the respective K_i values being 0.8 (S) and 62 nM (R) [13]. Based on preclinical *in vivo* and *in vitro* studies, R-citalopram counteracts the actions of escitalopram without pharmacokinetic interactions [12,14–16]. For example, in a microdialysis study using freely moving rats, extracellular 5-HT levels in the frontal cortex after escitalopram administration showed a greater maximal increase than with citalopram treatment [17]. R-citalopram alone did not affect the 5-HT levels, but counteracted the escitalopram-induced 5-HT increase when co-administered [17]. Similarly, R-citalopram blocked the effect of escitalopram in its potentiation of 5-hydroxytryptophan (5-HTP)-induced behavior in a dose-related manner, while it did not influence the effects of fluoxetine [18]. In a rat chronic mild stress model of depression, escitalopram showed a faster response than citalopram, and this effect was counteracted by R-citalopram [19].

A postulated hallmark for the need of a 2–3 week treatment before the onset of antidepressant action is 5-HT_{1A} receptor desensitization, which can be shown as a recovery of neuronal firing in the dorsal raphe [1,15]. This firing rate recovery is evident after 2 weeks of treatment with escitalopram, but requires a 3-week period when racemic citalopram is used [15]. Subsequently, Mnie-Filali et al. showed that the faster recovery of 5-HT neuronal firing and increased hippocampal neurogenesis elicited by escitalopram were prevented by a co-treatment with R-citalopram [16]. The faster neuroadaptive changes caused by escitalopram compared to other SSRIs may be due to the higher 5-HT levels elicited by escitalopram [14,20]. The enhanced efficacy of escitalopram and the antagonistic effect of R-citalopram on escitalopram are thought to be mediated via interactions at an allosteric binding site on the SERT.

The allosteric binding activity of escitalopram was initially demonstrated by its ability to delay the dissociation of labeled escitalopram from the primary binding site, thus increasing the affinity of the latter [21,22]. Escitalopram and R-citalopram do not appear to show as greater degree of stereoselectivity in this allosteric effect as in the primary site binding, since the EC_{50} values of the two compounds in slowing the dissociation rate of escitalopram are approximately 5 and 25 μM, respectively [13,21]. Given the micromolar concentrations required to demonstrate the allosteric effect as compared to the high affinity primary site binding, its physiological role remains to be understood. It is possible that under *in vivo* conditions, escitalopram may be able to induce SERT conformational changes through its allosteric site with greater sensitivity than under *in vitro* situations. Nonetheless, based on this characterization, escitalopram, paroxetine and R-citalopram have been shown to have allosteric activities, while many other SERT inhibitors including fluoxetine are devoid of allosteric effects [22]. Yet additional evidence corroborates the presence of an allosteric interaction at the SERT. R-citalopram at concentrations (below 100 nM) that are similar to those obtained after therapeutic doses of citalopram [23], can attenuate the association rate of [^{3}H] escitalopram binding to the SERT through an allosteric mechanism [20]. Therefore, escitalopram may elicit a more complete inhibition of 5-HT reuptake due to its dual activity at the primary and allosteric binding sites, leading to higher extracellular 5-HT levels *in vivo* and more rapid 5-HT_{1A} autoreceptor desensitization, and hence greater efficacy and faster onset of action than citalopram [1,14,15].

The existence of an allosteric site has also been corroborated by structural studies showing that key amino acid residues influence primary site versus allosteric binding. These structural studies including mutational and computational methods have been further aided by the crystal structures of the bacterial homolog of the SERT, the leucine transporter [24,25]. The schematic Fig. 1 summarizes these residues with their differential roles. For SSRIs, the primary binding site is likely to be within the deep substrate binding pocket in the SERT, which involves S545, Y95, and I172 residues [26,27]. Escitalopram fits well in this pocket in a reversed

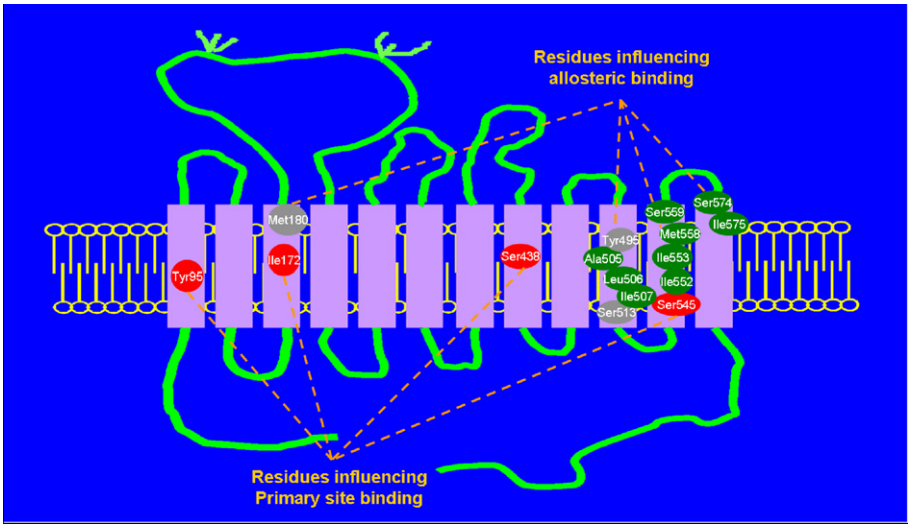

Fig. 1. Schematic representation of the amino acid residues influencing allosteric and primary site binding of the SERT. Allosteric binding can be modulated by the following amino acid residues within TMs 10, 11 and 12 of the SERT: Ala505, Leu 506, Ile507, Ile552, Ile553, Met558, Ser559, Ser574 and Ile575 (labeled in green) [31]. Other SERT residues within TMs 3 and 10, Met180, Tyr495 and Ser513 (labeled in grey), can also partially influence allosteric binding [21]. In comparison, the primary binding site is influenced by a different set of residues, represented by Tyr95, Ile172, Ser438, Ser545 on TMs 1, 3, 8, and 11 (labeled in red) [26,27,30]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

orientation to that of R-citalopram [28,29], with a residue located within the pocket, S438, being particularly important for escitalopram interactions [30].

The allosteric site of the SERT is influenced by residues within TM domains 10, 11, and 12 (Fig. 1), since mutations of these residues disrupt the allosteric activity of escitalopram: ALI → VFL (ALI/VFL), II → VT (II/VT), MS → SN (MS/SN), and SI → TT (SI/TT) [31]. Additional residues within these TM domains that can influence allosteric binding, albeit to a lesser degree include M180, Y495 and S513 [21]. Moreover, the same mutations of TM10 ALI and TM12 SI residues also abolished the allosteric interaction of R-citalopram on the association binding of escitalopram [13]. Thus, the mutations in residues that disrupt the allosteric activities of escitalopram and R-citalopram provide a structural basis for the existence of an allosteric binding site [13,31]. Based on the co-crystal structure of the leucine transporter, tricyclic antidepressants such as clomipramine and desipramine bind to an extracellular region of the transporter above the substrate binding site, at the tip of extracellular loop (EL) 4 and the extracellular gate, the so-called "vestibule" region due to its shape [24]. This vestibule is reminiscent of an allosteric site since binding by tricyclic clomipramine non-competitively blocks the release of leucine from the substrate-binding site [24]. The allosteric binding for escitalopram might be associated with the extracellular vestibule of the SERT, based on studies with a SERT mutant bearing an engineered Zn^{2+} binding site, namely Leu99His-Ile179His [32]. Thus, these converging data demonstrated the existence of the allosteric binding site for escitalopram, though the exact definition of the allosteric binding pocket awaits the determination of the co-crystal structures of the SERT with escitalopram and other SSRIs.

3. Beyond 5-HT uptake – SERT interacting proteins

Under physiological conditions, many proteins interact with other proteins, which may mediate trafficking, cytoskeletal dynamics and other non-canonical functions of these proteins. The classical function of the SERT is 5-HT uptake activity. Even though its uptake function can easily be demonstrated in recombinant expression systems, under physiological conditions the SERT is unlikely to perform 5-HT transport alone. In fact, the SERT is dynamically regulated in its activities and localization, and can interact with other proteins [33–37], termed SERT interacting proteins (SIPs). In recent years, many new SIPs have been identified [38–41], and it has become evident that the SERT most likely exists *in vivo* as part of a complex directly involving SIPs with other proteins indirectly involved. We review below some of the known SIPs and their interactions with the SERT in the context of understanding novel functions of the SERT.

3.1. Syntaxin 1A

Syntaxin 1A is a plasma membrane protein that is a critical part of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex involved in the regulation of neurotransmitter release [42]. Syntaxin 1A interacts physically with the N-terminal region of the SERT, as shown by both co-immunoprecipitation (co-IP) studies using developing thalamocortical neurons that endogenously express the SERT and syntaxin 1A, and glutathione-S-transferase pull-down assays using recombinant systems [43]. The functional outcome of the SERT and syntaxin 1A interaction is interesting, in that the conducting state of the substrate uptake process is changed from a 5-HT-induced ionic current generation to an electroneutral, stoichiometric process [43]. Recently, Ciccone et al. [44] showed that CaM kinase II can promote the physical interaction between the SERT and syntaxin 1A, which in turn resets the SERT's conducting state to electroneutral 5-HT transport. Thus, calcium signaling can modulate the conducting states of the SERT through syntaxin 1A interactions.

3.2. Myristoylated alanine-rich C kinase substrate related protein (MRP)

MRP is the myristoylated alanine-rich C kinase substrate (MARCKS) related protein (also known as F52 or MacMARCKS). MRP and MARCKS comprise the membrane-associated protein family of protein kinase C (PKC) substrates that have a myristoylated N-terminus and a basic domain for PKC phosphorylation [45]. MRP is expressed in brain and reproductive tissues as well as in macrophages, while MARCKS is widely expressed in the brain and other tissues. The physiological roles of MRP and MARCKS, yet to be clearly defined, include the hypersecretion of mucus, cell spreading, as well as phagocytosis. Using yeast 2-hybrid screening, it was determined that MRP can act as a SIP by interacting with the C-terminal region of the SERT [46]. When expressed in HEK293 cells, MRP not only redistributes to SERT localizations as revealed by confocal microscopy, but also reduces the uptake activity of the SERT by intracellular sequestration. Mochizuki et al. further showed that expression of the C-terminal peptide of the SERT reduced the uptake activity and surface density of the SERT, presumably as the result of blocking the interaction of MRP with the SERT [47]. The exact mechanism has yet to be elucidated, since the recombinant expression of either MRP or the C-terminal peptide of the SERT unexpectedly reduces the surface density of the SERT.

3.3. Myosin IIA

Myosins are cellular motor proteins that, with the consumption of ATP, generate mechanical forces with actin filaments. Among many subtypes, myosin II is the major motor protein involved in actomyosin contractility in muscle and non-muscle cells [48]. Three non-muscle myosin II isoforms have been identified: myosin IIA, myosin IIB, and myosin IIC. These myosin II proteins exist in heteromultimeric complexes that contain duplexes of myosin II heavy chain (MHC), essential light chain (ELC), and regulatory light chain (RLC). These three non-muscle myosin isoforms have both distinct and overlapping roles [49]. Specifically, myosin IIA plays roles in neurite retraction, CXCR4 receptor internalization in association with β-arrestin, as well as tumor invasion and metastasis. In CHO cells transiently co-expressing the SERT and myosin IIA, the two proteins have a direct physical interaction with one another, as demonstrated in co-IP assays, and this physical interaction was also demonstrated using a placental choriocarcinoma JAR cell line, which has endogenous expression of the SERT and myosin IIA [50]. Data from this study [50] also suggest that the interaction between the SERT and myosin IIA is dependent upon N-linked glycosylation of the SERT, a form of post-translational modification important for the folding, oligomerization and stability of the SERT.

3.4. Protein interacting with C kinase 1 (PICK1)

PICK1 is extensively expressed in the CNS, where it is an important scaffolding protein regulating the targeting and functions of many receptors, ion channels and enzymes [51]. PICK1 contains a PDZ (PSD-95/Dlg/ZO1) domain and a BAR (Bin/amphiphysin/Rvs) domain. PICK1 directly interacts with more than 40 proteins via its PDZ domain, while the BAR domain of PICK1 binds to phosphoinositide lipids. A key example of PICK1 function is the regulation of the AMPA receptor where it directly interacts with AMPA receptor subunits via the PDZ domain to control their activity-dependent internalization, leading to altered

synaptic strength [52]. The first small-molecule inhibitor specifically targeting the PDZ domain of PICK1, FSC231, blocked expression of LTP and long-term depression (LTD) in hippocampal CA1 region, confirming the function of PICK1 in modulating synaptic plasticity [53].

PICK1 can also directly interact with the dopamine transporter (DAT) and the norepinephrine transporter (NET) via its PDZ domain, based on yeast two-hybrid and/or biochemical studies [34]. This interaction augments cell surface targeting of endocytotic DAT and enhances its uptake activity. An interaction between PICK1 and the SERT was also reported, although this was weaker than that seen with the DAT or the NET [34]. Chanrion and colleagues (see below) also confirmed that the 15 residue C-terminal peptide of the SERT immobilized on Sepharose recruited PICK1 protein, as detected by western blotting [41]. Thus, although PICK1 is strictly a SIP since it also interacts with the DAT and NET members of the family, it physically interacts with the SERT and the functional consequence of this interaction remains to be elucidated.

3.5. Hydrogen peroxide-inducible clone 5 (HIC5)

The hydrogen peroxide-inducible clone 5 (HIC5) belongs to a focal adhesion family of adaptor proteins that includes paxillin [54]. HIC5 and paxillin have 57% homology, and both have Lin-11, Isl-1, and Mec-3 (LIM) domains. HIC5 and paxillin function as docking proteins in the recruitment of signaling molecules to focal adhesions and within the nucleus, in order to regulate cell spreading and motility, as well as nuclear receptor co-activation. HIC5 is expressed in many brain regions, and together with paxillin may have roles in neurofibrillary tangle function in the hippocampal regions of Alzheimer disease patients [55].

HIC5 physically interacts with all three monoamine transporters (DAT, NET and SERT) through its LIM domain and the C-terminal regions of each of the transporters [56,57]. Thus, like PICK1, HIC5 is not a SIP *per se*, but a protein interacting with all members of the monoamine transporters. The interaction between HIC5 and the SERT was demonstrated in co-IP studies using brain synaptosomes, with this interaction being important in the regulation of uptake function as well as endocytotic translocation of HIC5 [56,57]. The interaction between the SERT and HIC5 can be suppressed by 5-HT binding to the SERT and SERT uptake activity, and is enhanced by the activation of protein kinase C signaling. Moreover, the suppressive effect of 5-HT uptake or binding on the HIC5/SERT interaction is blocked by the SERT inhibitor citalopram [57]. This suggests that the function and regulation of SERT activity are potentially dynamically linked through interacting proteins.

3.6. α-Synuclein and γ-synuclein

Synucleins are small, unfolded soluble cytosolic proteins that include α-synuclein, β-synuclein, and γ-synuclein. They are thought to be involved in the regulation of membrane stability and trafficking. Abnormal α-synuclein has been linked to neurodegenerative disorders including Parkinson's disease and Alzheimer's disease [58]. α-Synuclein is expressed in the serotonergic raphe neurons [59], where the SERT plays a critical role. Co-transfection studies in Ltk^- fibroblast and HEK293 cells showed that α-synuclein negatively modulates 5-HT uptake by decreasing the plasma membrane levels of the SERT [38]. α-Synuclein also physically interacts with the SERT to form heteromeric complexes as demonstrated in co-IP experiments using co-transfected Ltk^- fibroblasts [38]. Recently, γ-synuclein was demonstrated to negatively affect SERT uptake function, although to a lesser degree [39]. Similar to α-synuclein, γ-synuclein but not β-synuclein physically interacts with the SERT as shown in co-IP studies. Both α-synuclein and γ-synuclein co-localize with the SERT in rat raphe neurons in immunostaining studies [39].

3.7. Protein phosphatase 2A

The phosphorylation states of signaling proteins provide an essential regulatory mechanism in the cell and are determined by the counteracting functions of kinases and phosphatases. There are four subtypes of serine/threonine protein phosphatases: PP1, PP2A, PP2B, and PP2C. Protein phosphatase 2A (PP2A) is one of the major subtypes, and plays an important role in the regulation of cell cycle, signal transduction, DNA replication and gene expression [60]. The interaction between PP2A and the SERT has been reviewed previously [35,37]. In SERT immunoprecipitates from stably transfected HEK293 cells, okadaic acid-sensitive phosphatase activity is enriched, and PP2A can be detected by western blotting. This interaction of PP2A with the SERT is through its catalytic subunit (PP2Ac), as shown in both transfected cells and brain tissue. In addition, PP2A inhibitors such as okadaic acid and calyculin A can increase SERT phosphorylation and decrease its 5-HT uptake function in transfected HEK293 cells.

3.8. Protein kinases

The regulation of the SERT by protein kinases has also been reviewed previously [33,35,37]. In transfected HEK293 cells, activation of protein kinase C (PKC) by the phorbol ester activators β-PMA and β-PdBu rapidly (within 30 min) inhibits 5-HT uptake by the SERT that is likely due to a decrease of in the level of SERT protein at the cell surface, since both processes can be blocked by PKC inhibitors such as staurosporine. The SERT can be phosphorylated at its serine/threonine sites by PKC or protein kinase A (PKA). Under basal conditions in transfected cells, the SERT is phosphorylated, and the phosphorylation level may be either further enhanced by activators of PKC and PKA, or reversed by inhibitors of PP2A. Interestingly, PKA activation does not affect SERT uptake function, suggesting that PKA- and PKC-induced SERT phosphorylation may be independent pathways [37].

The regulation of the SERT by protein kinase G (PKG) and p38 mitogen-activated protein kinase (MAPK) has also been established [37]. Adenosine A_3 receptor stimulation increased SERT uptake function in rat basophilic leukemia (RBL-2H3) cells and mouse midbrain and hippocampal synaptosomes. This effect is thought to be mediated by both a PKG-dependent surface density increase of the SERT, and p38-MAPK-dependent activation of SERT intrinsic activity [37]. As confirmation, these effects were blocked by inhibitors of the A_3 receptor, PKG and p38 MAPK kinase, and were not present in synaptosomes from A_3 receptor knockout mice [37]. The SERT is also co-localized with PKGIα, a cytosolic subtype of PKG, in immortalized rat serotonergic raphe neurons (RN46A) and PKGIα can physically interact with the hSERT in a specific manner as shown in co-IP studies using HEK293 T cells [61]. Interestingly, A_3 receptors are coexpressed with SERT in midbrain serotonergic neurons and can form a physical complex with the human SERT in cotransfected cells, which is enhanced by A_3 receptor activation in a PKG1-dependent manner [62]. Moreover, this study shows that a hyperfunctional coding variant of the A_3 receptor, L90V, identified in subjects with autism spectrum disorder, can cause dysregulation of the A_3-SERT complexes [62], suggesting that SIPs may be part of the underlying mechanism in SERT-related neuropsychiatric diseases. Other kinases potentially involved in SERT regulation include phosphatidylinositol 3-kinases, protein tyrosine kinases, and Ca^{2+}/calmodulin-dependent kinase II [35].

Extracellular 5-HT levels in a native tissue environment can be dynamically and differentially modulated as the result of regulation

of the SERT by protein kinases. For example, the extracellular 5-HT levels in the striatum, midbrain, and cerebellum are regulated by PKC-α, whereas in the pons medulla the 5-HT level is regulated by p38-MAPK. Recently, it was reported that the 5-HT level in cerebral cortex is co-regulated by S-100 β protein via protein–protein interaction with the SERT via cAMP/PKA pathways [63].

3.9. Secretory carrier membrane protein 2 (SCAMP2)

The secretory carrier membrane protein (SCAMP) family consists of five integral proteins that each have four transmembrane domains. SCAMP proteins are involved in the cellular processes of exocytosis and endocytosis, and thus their localization includes the Golgi apparatus, the trans-Golgi network, recycling endosomes, and synaptic vesicles (for a review, see [64]). SCAMP2, which has a highly conserved E-peptide region between transmembrane domains 2 and 3, plays a critical role in the regulated exocytosis seen in endocrine cells and synaptic terminals [65]. SCAMP2 has also been proposed to serve as a plasma membrane platform to facilitate key events during SNAP receptor (SNARE)-mediated exocytosis.

Muller et al. [40] reported that SCAMP2 directly binds to and interacts with the SERT. These authors first identified SCAMP2 from a human brain cDNA library as a novel SERT-interacting protein using the yeast two-hybrid system. The physical interaction was confirmed in co-IP assays using both a recombinant system and native rat brain tissue. Functionally, co-expression of SCAMP2 with the SERT caused a decrease in SERT surface density and a concomitant reduction in 5-HT uptake activity in HEK293 cells. A mutation within the conserved E-peptide region of SCAMP2, Cys^{201} to Ala, abolished SCAMP2-mediated down-regulation of the SERT without affecting the physical interaction between the SERT and SCAMP2. In addition, the role of SCAMP2 in regulating the surface distribution of the SERT is thought to be at least partially independent of its role in regulating exocytosis.

3.10. Neuronal nitric oxide synthase (nNOS)

Neuronal nitric oxide (NO) synthase (nNOS) is widely expressed in the brain with localization at synaptic spines, and is the predominant enzyme for the generation of NO in neurons [66]. nNOS is the only NOS member that contains an N-terminal PDZ-binding domain [66]. nNOS plays a role in learning, memory, and neurogenesis, and has been associated with various CNS disorders such as depression, Parkinson's disease, and Alzheimer's disease [66].

Using a proteomic approach involving peptide-affinity chromatography of extracts of mouse brain to identify multiple SIPs interacting with the 15 amino acid residue C-terminal peptide of mouse SERT, nNOS, and the channel-interacting PDZ protein (CIPP) were identified [41]. In HEK293 cells, co-expression of nNOS with the SERT decreased both SERT cell surface density and 5-HT uptake [41]. This suggests a tonic inhibition of SERT activity by nNOS under physiological condition *in vivo*. In addition, in these co-transfected HEK293 cells, 5-HT was able to activate nNOS, resulting in increased cGMP production. The effect of 5-HT was mediated through the uptake activity of the SERT, since the SSRIs, citalopram and paroxetine, prevented the nNOS-activating effect of 5-HT [41].

It should be emphasized that the inhibition of SERT activity by nNOS via direct physical interaction is independent of the upregulation of SERT activity by NO through the cGMP/PKG/p38-MAPK pathway, as mentioned earlier [37,61]. In addition, the reciprocal interaction between the SERT and nNOS reported by Chanrion and colleagues [41] could be of particular physiological importance in that this may be a potential activity-dependent acuity tuning mechanism for serotonergic neurotransmission.

A possible model is as follows: under basal physiological conditions, SERT uptake activity is tonically inhibited by nNOS via physical interaction, to allow sufficient background or tonic extracellular 5-HT levels. When 5-HT release is dramatically increased in response to neuronal activities, SERT uptake activity also increases. This activates nNOS leading to an increased NO production. The increase in NO levels then up-regulates SERT uptake function, resulting in timely termination of elevated 5-HT-induced neurotransmission. Thus, the reciprocal interplay between the SERT and nNOS potentially serves to temporally (and perhaps spatially) facilitate activity-dependent 5-HT neurotransmission.

3.11. Protein interaction network of the SERT

In living organisms, proteins exist and function within complex networks in which the activity of one member can directly or indirectly affect the functions of many other members. Protein interaction networks have been studied on a system- or whole organism-level, which gives rise to the term "interactome" [67]. One of the searchable interactome databases BioGRID registers three protein interactors for the SERT (SLC6A4): syntaxin 1A (brain), Sicca syndrome antigen A (autoantigen Ro; calreticulin), and major histocompatibility complex class I antigen-binding protein p88. As additional SIPs are entered into the databases, the interaction network of the SERT will expand considerably. For example, BioGRID registers at least 16 interactors for PICK1 and at least 37 interactors for syntaxin 1A (brain). Thus, as directly interacting SIPs and indirect protein interactor cascades are eventually incorporated in interactome databases, novel functions of the SERT will be amenable to study at a holistic level.

4. SIPs and allosteric mechanisms may reveal novel aspects of SERT functions

Based on the data reviewed in the previous sections, it can be surmised that there exist multiple regulatory/modulatory mechanisms for the SERT. At the intra-molecular level, the primary substrate-binding site can be modulated by allosteric interactions. At the inter-molecular level, the surface expression, trafficking, phosporylation state, the intrinsic uptake function and conducting state of the SERT are regulated through various dedicated or promiscuous SIPs. These interactions are simplistically depicted in Fig. 2. Understanding the roles of these interactions and regulation mechanisms under physiological conditions as well as disease states warrants additional study.

The interactions reported to date between the SERT and its SIPs have focused on SIPs modulating SERT function. It is likely that the interactions are reciprocal, in that the SERT in its basal or active state may modulate SIP function. Additionally, some SIPs may serve as important links to other proteins. SCAMP2, for example, physically interacts with NHE5, promoting its cell-surface targeting, and is also important for regulated exocytosis [64]. Thus the SERT can be directly associated with proteins involved in neurotransmitter release (such as syntaxin 1A), or endosomal regulation, leading to the intriguing possibility that 5-HT release and its reuptake are not independent processes, and are co-regulated by the same or overlapping protein complexes.

SERT inhibitors not only inhibit 5-HT uptake activity, but can also affect interactions of the SERT with SIPs. Carneiro et al. reported that 5-HT decreased the SERT/HIC5 association, while citalopram not only enhanced SERT/HIC5 association but blocked the ability of 5-HT to decrease this association [57]. Chanrion et al. [41] also reported that SERT uptake can activate the catalytic activity of nNOS in a calmodulin-dependent manner that can be blocked by the SERT inhibitors, citalopram or paroxetine.

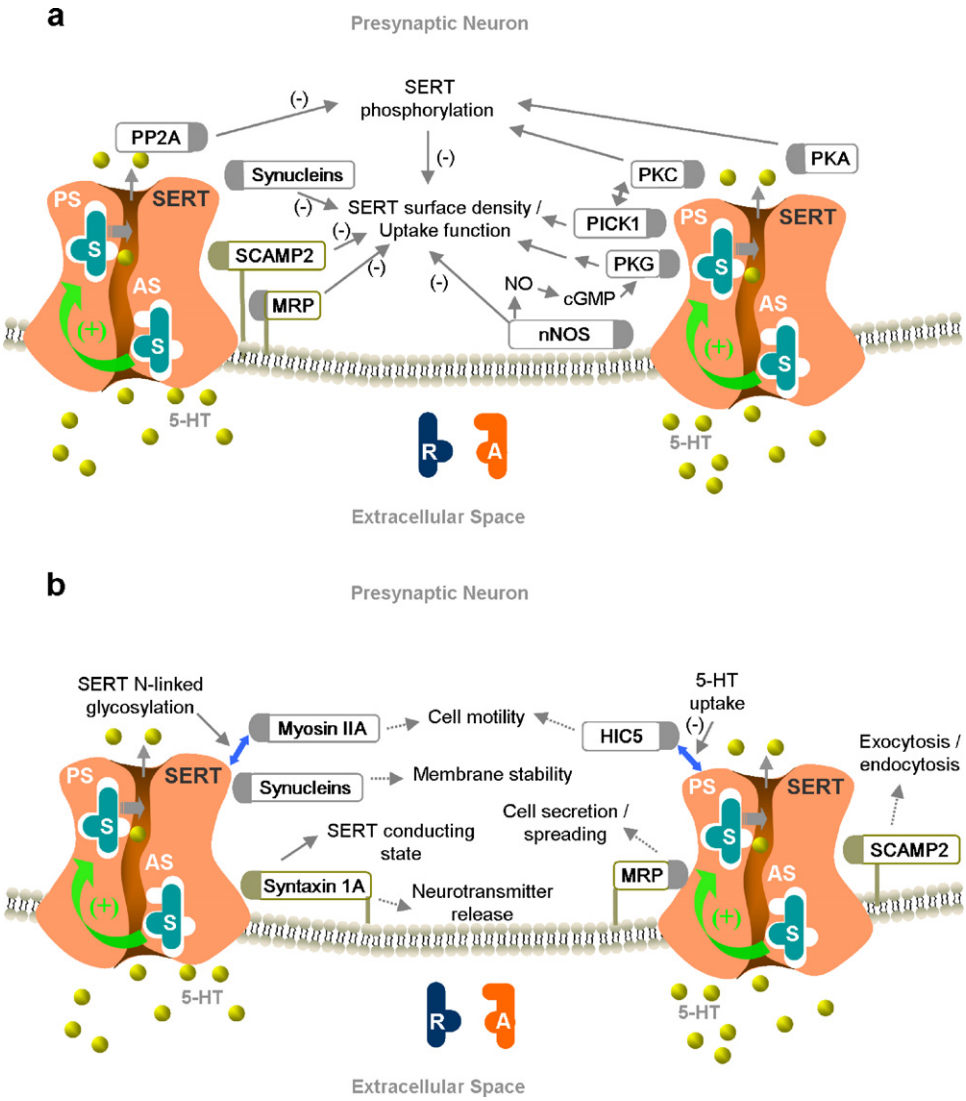

Fig. 2. Allosteric interactions lead to altered inhibitor binding at the primary site, changed extracellular 5-HT levels, in context of SERT interacting proteins (SIPs). In presynaptic terminals, the serotonin (5-HT) transporter (SERT) serves to transport 5-HT from the extracellular space back into presynaptic neurons. The SERT has a primary site (PS), and an allosteric site (AS) for the inhibitors escitalopram (S) and R-citalopram (R). A hypothetically selective allosteric modulator (A) is also shown. The SERT has physical binding contacts with SERT interacting proteins (SIPs) including HIC5, nNOS, synucleins (α- and γ-), protein phosphatase PP2A, protein kinases PKA/PKC/PKG, PICK1, myosin IIA, syntaxin 1A, SCAMP2, and MRP. (Note the contact locations between the SERT and SIPs in the drawings are arbitrary for demonstration purpose.) Between the SERT and SIPs, some interactions modulate the surface density and/or uptake function of the SERT (a), while other interactions may hypothetically lead to signaling through individual functions of SIPs, as shown by dotted arrows (b). Escitalopram binding to an allosteric site enhances its binding affinity at the primary site (as suggested by the decreased dissociation rate of escitalopram from the primary site), resulting in a greater increase in extracellular 5-HT levels. R-citalopram (R), which has much lower affinity than escitalopram for the primary binding site, may bind to an allosteric site and in doing so interfere with the ability of escitalopram to allosterically enhance its own affinity (as suggested by the reduced association rate of escitalopram in the presence of R-citalopram), resulting in a lower extracellular 5-HT increase. A selective allosteric modulator (A) may only bind to an allosteric site, potentially modulating binding at the primary site. Abbreviations: A, selective allosteric modulator; PS, primary site; AS, allosteric site; S, escitalopram; R, R-citalopram; SERT, serotonin transporter; SIPs, SERT interacting proteins. The diagram showing allosteric interactions is based on one by Sanchez [14].

Considering the SIP-interacting domains of the SERT in relation to its uptake function, a rare human SERT mutant (Ile425Leu) found in autism patients with rigid-compulsive traits, retains uptake function (though a gain of function is also present) but has a deficit in trafficking due to altered SERT interaction with PP2A [68]. Ile425Leu may reside in a domain that is independent of, or allosteric to the substrate-binding site.

At protein complex level, the SERT may have different binding sites to which small molecules could bind and modulate its 5-HT uptake activity versus other functions linked to SIPs. The allosteric binding site related to the effects of TM 10, 11, and 12 mutations, as mapped by escitalopram binding, represents one example. Interaction of escitalopram with the SERT at the allosteric site modulates SERT binding activity at the primary site, as shown in dissociation and association studies (see Introduction). These interactions are presented in the context of SIPs in a schematic model in Fig. 2, depicting that allosteric interactions potentially lead to altered inhibitor binding and altered extracellular 5-HT levels. Some interactions between the SERT and SIPs can also modulate the surface density and/or uptake function of the SERT, as shown in Fig. 2a. The interactions between the SERT and some other SIPs are less well defined but may hypothetically lead to signaling through individual functions of those SIPs (Fig. 2b).

5. Discrete allosteric modulators of SERT

The understanding of the mechanisms and *in vivo* consequences of the allosteric interactions between the SERT and SSRIs like

escitalopram or R-citalopram, is complicated by both compounds having potent primary site binding interactions [13]. For GPCRs, many selective allosteric compounds without primary (orthosteric) site activities have served as valuable tool compounds for both pharmacological intervention and in enhancing the understanding of the physiological and pathophysiological roles of GPCRs. Examples include the positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) for metabotropic glutamate receptors and muscarinic acetylcholine receptors [69,70]. Similarly, for nicotinic acetylcholine receptors including the $\alpha4\beta2$ and $\alpha7$ subtypes, allosteric modulators can prove to have therapeutic benefits in areas of high unmet medical need such as pain, Alzheimer's disease and schizophrenia [71–73].

A selective allosteric compound devoid of primary site binding activities is a key to better characterizing the physiology and pathophysiology of allosteric interactions. A series of naphthalene derivatives of citalopram have been synthesized to explore the spatial requirements of the primary and allosteric site interactions. A citalopram derivative with a 1-naphthyl group replacing the 4-fluorophenyl ring is a relatively selective allosteric modulator. Its enantiomers are allosteric modulator (EC_{50} values of 14–19 μM, for S- and R-enantiomers), while their affinities for the primary site are weak (K_i values of 213 and 97 nM, for the S- and R-enantiomers, respectively) [74]. Compared to citalopram, these derivatives do not have the same degree of stereoselectivity for the primary binding site, as the S-enantiomers are weaker than escitalopram. However, their allosteric activities are comparable to those of escitalopram, in the low micromolar range.

In addition to directly inhibiting 5-HT uptake, escitalopram exerts its pharmacological efficacy by also acting as an allosteric SERT inhibitor. Structural evidence for allosteric interactions has depended on heterologous recombinant expression in cells. Confirmation from animals with a SERT allosteric site disrupted will aid immeasurably in providing a physiological context for these *in vitro* studies. Studies in citalopram insensitive (I172M knock-in) mice [75] showed that transgenic mice bearing the I172M mutation, that abolishes citalopram binding without impacting that of 5-HT, possess normal basal 5-HT levels and transport rates but lack the response to citalopram in terms of increased 5-HT and Raphe neuron firing modulation. Knock-in mice bearing the mutations ALI → VFL (ALI/VFL), II → VT (II/VT), MS → SN (MS/SN), and SI → TT (SI/TT) [76] under development (M.G.C. unpublished) will provide an additional level in understanding the allosteric properties of the SERT.

6. Conclusions

The primary function of the SERT is 5-HT uptake. It also interacts with various SIPs that may affect its uptake function. The SERT and its interacting partners possibly exist *in vivo* as part of a protein interaction network, a SERT interactome. The allosteric interactions discovered between the SERT and escitalopram have helped explain some of the preclinical and clinical properties of escitalopram. Further characterization of the nature of SERT allosteric modulation with respect to uptake, regulation, and trafficking, together with the study of various dynamic inter-molecular interactions within the SERT protein network may provide novel physiological insights into SERT function that will extend understanding of the nuances of 5-HT signaling in human disease states.

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