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Colocalization and Regulated Physical Association of Presynaptic Serotonin Transporters with A_3 Adenosine Receptors[S]

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ABSTRACT

Activation of A_3 adenosine receptors (A_3ARs) rapidly enhances the activity of antidepressant-sensitive serotonin (5-HT) transporters (SERTs) in vitro, ex vivo, and in vivo. A_3AR agonist stimulation of SERT activity is lost in A_3AR knockout mice. A_3AR-stimulated SERT activity is mediated by protein kinase G1 (PKGI)- and p38 mitogen-activated protein kinase (MAPK)-linked pathways that support, respectively, enhanced SERT surface expression and catalytic activation. The mechanisms by which A_3ARs target SERTs among other potential effectors is unknown. Here we present evidence that A_3ARs are coexpressed with SERT in midbrain serotonergic neurons and form a physical complex in A_3AR/hSERT cotransfected cells. Treatment of A_3AR/SERT-cotransfected Chinese hamster ovary cells with the A_3AR agonist N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (1 μM, 10 min), conditions previously reported to increase SERT surface expression and 5-HT uptake activity, enhanced the abundance of A_3AR/SERT complexes in a PKGI-dependent manner. Cotransfection of SERT with L90V-A_3AR, a hyperfunctional coding variant identified in subjects with autism spectrum disorder, resulted in a prolonged recovery of receptor/transporter complexes after A_3AR activation. Because PKGI and nitric-oxide synthetase are required for A_3AR stimulation of SERT activity, and proteins PKGI and NOS both form complexes with SERT, our findings suggest a mechanism by which signaling pathways coordinating A_3AR signaling to SERT can be spatially restricted and regulated, as well as compromised by neuropsychiatric disorders.

Introduction

The presynaptic, antidepressant-sensitive 5-HT transporter (SERT, *SLC6A4*) is predominantly responsible for high-affinity 5-HT clearance in the nervous system (Fuller, 1994) and also contributes to 5-HT homeostasis and signaling in non-neuronal tissues, including platelets, gut, adrenal gland, and placenta (Blakely, 2001; Gershon, 2004; Mercado and Kilic, 2010). Numerous studies have found that a common polymorphism in the SERT promoter (i.e., the serotonin-transporter-linked polymorphic region) is associated with altered behavioral traits, brain function, and risk for neuropsychiatric disorders (Homberg and Lesch, 2011). Six rare SERT coding variants have been identified in subjects with obsessive-compulsive disorder (OCD) and autism; re-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; OCD, obsessive-compulsive disorder; GPCR, G-protein coupled receptor; PKGI, protein kinase G, type I; PKGII: protein kinase G, type II; MAPK, mitogen-activated protein kinase; A_3AR, A3 subtype adenosine receptor; CNS, central nervous system; IB-MECA, N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine; HA, hemagglutinin; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; coIP, coimmunoprecipitation; PBS/CM, PBS containing Ca^{2+} and Mg^{2+}; GAD, glutamic acid decarboxylase; ASD, autism spectrum disorder; NOS, nitric-oxide synthetase; PCR, polymerase chain reaction; ANOVA, analysis of variance; DT-2, YGRKKRRQRRRPP-LRK5H; MRS1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate.

markably, each confers elevated constitutive activity of SERT in transfected cells as well as in lymphoblastoid lines derived from affected subjects (Prasad et al., 2005, 2009). Because only a small number of patients with OCD or autism carry the aforementioned SERT coding variants, we have sought to define mechanisms of broader relevance by which SERT expression or activity are augmented, with the intention of identifying additional contributors to 5-HT linked risk determinants of mental illness.

Multiple signaling pathways seem to contribute to the regulation of SERT-mediated 5-HT clearance (Blakely et al., 2005). With respect to SERT stimulation, G-protein coupled receptor (GPCR) stimulation can activate protein kinase GI (PKGI)-linked pathways that rapidly up-regulate SERT activity via increased SERT surface expression (Steiner et al., 2008) and via a p38 mitogen-activated protein kinase (MAPK)-linked pathway that induces a catalytic activation of SERT (Zhu et al., 2004, 2005). This latter pathway can be independently activated through stimulation of proinflammatory cytokine receptors (Blakely et al., 2005; Zhu et al., 2006, 2007). Activation of A_3 subtype adenosine receptors (A_3AR) can increase 5-HT uptake via PKG-linked pathways in peripherally derived cells (Miller and Hoffman, 1994; Zhu et al., 2004). In the CNS (Okada et al., 1999), in vivo microdialysis studies demonstrated A_3AR-dependent reductions of extracellular 5-HT in hippocampus, an effect consistent with our studies that demonstrate A_3AR-dependent stimulation of hippocampal 5-HT clearance (Zhu et al., 2007).We have demonstrated that pharmacologically mediated A_3AR modulation of SERT is lost in A_3AR KO mice, confirming the specificity of the pathways targeted by pharmacological agents with reported A_3AR specificity (Gallo-Rodriguez et al., 1994). In addition to PKGI-dependent A_3AR activation of SERT, we have demonstrated that activation of p38 MAPK enhances SERT catalytic activity (Zhu et al., 2004, 2005, 2006). Together, a full appreciation of the mechanisms by which activation of A_3ARs control trafficking and catalytic activation of SERT proteins requires an understanding of whether regulation is indirect or is mediated by more confined, physical interactions. To date, compartmentalizing mechanisms by which GPCRs can target one or more of these modulators to regulate SERT without influencing other cytosolic and membrane effectors are unknown. Here, we provide evidence that A_3ARs also exist within SERT complexes, suggesting a highly compartmentalized SERT "regulome." Moreover, we find that A_3AR agonists can regulate the abundance of SERT/A_3AR complexes in a PKGI-dependent manner.

Materials and Methods

Reagents. N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (IB-MECA) was purchased from Sigma-Aldrich (St. Louis, MO); DT-2 was a kind gift from Dr. Wolfgang Dostmann (University of Vermont, Burlington, VT) (Dostmann et al., 2000). Anti-HA-affinity matrix was purchased from Roche (South San Francisco, CA), and anti-myc resin, Streptavidin-coated agarose beads, and EZ-Link sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate were obtained from Thermo Fisher Scientific (Waltham, MA). Trypsin-EDTA, glutamine, and ampicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA); components of modified Eagle's medium and Dulbecco's modified Eagle's medium were obtained from Invitrogen and prepared in the Vanderbilt Media Core. Human SERT-specific mouse monoclonal antibody (ST51-2) was obtained from MAb Technologies (Atlanta, GA). Rodent-specific, goat anti-SERT polyclonal antibody was obtained from Frontier Science (Hokkaido, Japan). Anti-5-HT and anti-A_3AR antibodies were products of Immunostar (Hudson, WI) and Alomone Labs Ltd. (Jerusalem, Israel), respectively. Secondary antibodies for immunostaining and immunoblotting were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other biochemical reagents were of the highest grade possible and obtained from Sigma (St Louis, MO).

Immunohistochemistry Studies. All studies involving mice were conducted under the auspices of an approved protocol of the Vanderbilt University Institutional Animal Care Use Committee. C57BL/6 mice used for immunocytochemistry studies were purchased from Harlan, Inc., (Indianapolis, IN) and housed in Vanderbilt University animal housing facilities, with water and food provided ad libitum. For perfusion-fixation, mice were anesthetized using injection of 100 mg/kg i.p. pentobarbital (Nembutal) and then transcardially perfused with ice-cold 0.1 M PBS, pH 7.4. Fifty milliliters of ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4, was then perfused at a rate of 4 ml/min. Subsequently, brains were removed and fixed in paraformaldehyde buffered overnight at 4°C, followed by another overnight incubation in 30% sucrose in PBS before sectioning. Free-floating, frozen microtome sections (40 μm) were preblocked in 3% normal donkey serum (Jackson ImmunoResearch Laboratories), 0.2% Triton X-100 in PBS for 1 h at room temperature. Primary antibodies targeting A_3ARs (1:100), 5-HT (1:800), or SERT (1:1000) were then applied to sections overnight at 4°C. After washing in PBS, sections were incubated with secondary antibodies (Dylight 488 donkey anti-rabbit IgG for A_3ARs, 1:200; Dylight 549 donkey anti-goat IgG for 5-HT and SERT, 1:200; both from Thermo Fisher Scientific) for 2 h at room temperature. After multiple PBS washes, sections were mounted with Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA). Immunofluorescence was captured using a Zeiss LSM 510 confocal microscope (Vanderbilt University Medical Center Cell Imaging Shared Resource).

Recovery of SERT/A_3AR Complexes from Receptor/Transporter Transfected Cells. Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% L-Glu, and penicillin/streptomycin (100 units $\cdot$ μg^{-1} $\cdot$ ml^{-1}). Transfections were performed using Trans-It reagent (Mirus, Madison, WI). SERT (HA-tagged or nontagged) cDNA and/or A_3AR cDNA (myc- or HA-tagged) were preincubated with transfection reagent per manufacturer's recommendations at ambient temperature for 30 min before adding to plated cells. Typically, 1 μg of SERT construct and/or 0.5 to 1 μg of A_3AR constructs were added to each well of a six-well plate seeded with 5×10^5 cells 24 h earlier. Cells were cultured for 24 to 48 h before biotinylation or generation of detergent extracts for coimmunoprecipitation (coIP) experiments. In some experiments, transfected cells were treated with IB-MECA $\pm$ DT-2 for 10 to 40 min at 37°C before harvest. For coIP experiments, cells were lysed with 1% ice-cold Triton X-100 in PBS buffer containing protease inhibitors and 10 mM *n*-ethylmaleimide. Cell lysates were centrifuged at 20,000*g* for 20 min. In samples cotransfected with HA-SERT/myc-A_3AR, 30 μl of anti-HA antibody-coated resin or 10 μl of anti-myc antibody-coated resin was used to extract protein complexes. Affinity resins (30 μl) were added to cell extracts (0.4 ml) and incubated overnight at 4°C. Subsequently, beads were washed three times with ice-cold lysis buffer and bound proteins were eluted with 50 μl of Laemmli buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue), separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) preblocked with 5% nonfat dry milk in PBS/0.1% Triton X-100. Blots were incubated with either anti-myc antibodies (1:500; for HA-resin-incubated samples), anti-HA, or anti-SERT antibodies (1:500; for myc-resin-incubated samples). Bound antibody was detected with horseradish peroxidase-conjugated, goat anti-mouse secondary antibody, or mouse anti-rabbit secondary antibody (1:10,000; both were from

Jackson ImmunoResearch Laboratories). Horseradish peroxidase signals were developed with ECL-Plus reagents according to manufacturer's recommendations (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Analysis of SERT Surface Expression. For biotinylation studies, cells were washed twice with ice-cold PBS/CM, and incubated with 1 ml/well EZ-Link sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (1 mg/ml in PBS/CM; Thermo Fisher) for 30 min at 4°C. The biotinylation reagent was quenched by two PBS/CM washes, followed by 10 min incubation with 100 mM glycine in PBS/CM, and then an additional two washes with PBS/CM. Cells were then lysed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton-X-100, and 1% sodium deoxycholic acid) containing protease inhibitors (1 μM pepstatin A, 250 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 30 min at 4°C with constant shaking. Lysates were centrifuged at 20,000*g* for 30 min at 4°C and then incubated with Streptavidin beads (30 μl of beads/cell lysate per well) for 45 min at room temperature. Beads were washed three times with radioimmunoprecipitation assay buffer, and bound proteins were eluted with 30 μl of Laemmli buffer for 1 h at room temperature. Samples were centrifuged for 10 min, and supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (10%) as described above. To estimate the relative abundance of proteins in total and surface immunoblots, samples were exposed to Kodak X-ray film and scanned using an AGFA Duoscan T1200. Blots for intracellular proteins (e.g., actin) in this protocol do not reveal significant recovery in biotinylated fractions. Captured images were quantified using NIH Image software (http://rsbweb.nih.gov/nih-image/), using multiple exposures to insure data capture in the linear range of the film.

Statistical Analyses. All data derive from experiments replicated a minimum of three times. Statistical analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA) with a significance level set at $P < 0.05$ for all experiments. Specific analyses are given in the Figure legends.

Results

Coexpression of SERT and A_3AR in Mouse Brain. Little information exists regarding the regional distribution of A_3ARs (Dixon et al., 1996; Yamano et al., 2007), and there are no data relevant to A_3ARs within serotonergic neurons. Initially, we performed reverse transcription-PCR analysis of midbrain RNA and could readily detect an amplification product matching that expected for A_3ARs (B. Thompson, personal communication). To gain cellular resolution of A_3AR protein in the mouse midbrain, we probed sections from C57BL/6 mice using a recently developed A_3AR antibody (Alomone Labs Ltd), alone or in combination with antibodies targeting 5-HT or SERT. As shown in Fig. 1, A to C, A_3AR labeling was evident in serotonergic dorsal raphe neurons as revealed by colabeling of receptor expressing cells with 5-HT. SERT immunoreactivity is enriched in serotonergic axons and could be colocalized with A_3AR immunoreactivity (Fig. 1, D–F), although other sites of A_3AR expression were also evident, consistent with evidence that A_3ARs are also expressed by glutamatergic neurons (Macek et al., 1998). It is noteworthy that incubations of sections without primary antibodies or with the peptide constituting the A_3AR epitope failed to demonstrate either cell body or fiber labeling (Supplemental Fig. 1). Finally, sections costained A_3AR and glutamic acid decarboxylase (GAD) as a marker of raphe GABAergic terminals on 5-HT neurons (Fig. 1, G–I) failed to demonstrate colabeling.

Physical Association of SERT with A_3AR in Transfected CHO Cells. Efforts to perform coimmunoprecipitation of SERT and A_3AR from midbrain extracts were unsuccessful (as is Western blotting of A_3ARs), probably as a result of the insensitivity of our A_3AR antibody. Use of other commercial A_3AR antibodies was similarly unsuccessful. Thus, to assess SERT/A_3AR associations, we turned to epitope-tagged transporter/receptor-transfected CHO cells, a model system that supports regulation of SERT activity by transfected A_3ARs (Zhu et al., 2004). HA-tagged SERT (HA-SERT) and myc-tagged A_3AR (myc-A_3) cDNAs were individually or cotransfected and then immunoprecipitated and blotted from detergent extracts as described under *Materials and Methods*. As shown in Fig. 2, A and B, SERT immunoprecipitates were found to include A_3ARs. A_3ARs were not recovered from extracts of cells transfected with A_3AR cDNA but lacking HA-SERT. Detergent extracts of separately transfected cells that were mixed after membrane solubilization also did not support recovery of A_3AR proteins with SERT-directed antibodies. These findings are consistent with an endogenous formation of receptor/transporter complexes, as opposed to an artifactual association arising during extraction. Similar results (Fig. 2C-D) were obtained when we reversed the targets for immunoprecipitation and immunoblotting (IP anti-myc (A_3AR), blot anti-HA (SERT)) or used nontagged SERT in transfections (data not shown).

A_3AR Agonist IB-MECA Enhances Recovery of SERT Complexes in a PKGI-Dependent Manner. To examine whether A_3AR/SERT complexes are constitutive or subject to regulation, we treated receptor/transporter-transfected CHO

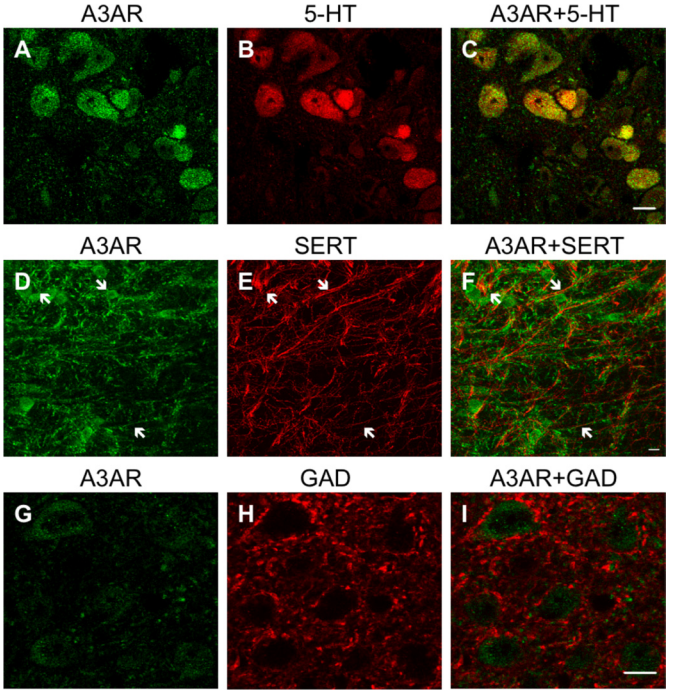

Fig. 1. Colocalization of A_3AR and SERT in mouse midbrain serotonergic neuron. C56BL/7 mice were perfused and fixed for immunostaining of A_3AR and 5-HT (A–C), A_3AR and SERT protein (D–F), or A_3AR and GAD protein (G–I) in the medial aspects of the dorsal raphe nucleus. A, D, and G, A_3AR staining; B, 5-HT staining; E, SERT staining; C, F, and I, overlap of A_3AR and 5-HT (C) or SERT (F) and lack of costaining with GAD (I). Arrows in A to C identify examples of colocalization of A_3AR and 5-HT in cell bodies and axons. Arrows in D to E identify examples of colocalization of A_3AR and SERT in axons. Scale bar, 10 μM.

cells with the A_3AR-selective agonist IB-MECA (Gallo-Rodriguez et al., 1994). We used a concentration of IB-MECA (1 μM) shown previously to rapidly enhance SERT activity (Zhu et al., 2007). Anti-HA (SERT) immunoprecipitates of non-stimulated cells (Fig. 3A) contained readily detectible myc-labeled A_3ARs as noted above. IB-MECA treatment of cells for 10 min induced an enhanced recovery of A_3AR/SERT complexes that was time-dependent and blocked by pretreatment with 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191; 1 μM), a specific A_3AR antagonist (Figs. 3, A and B, and 4, A and B). Total A_3AR levels were unchanged by agonist or antagonist treatments. A similar elevation of A_3AR/SERT complexes after IB-MECA treatments was detected when the coimmunoprecipitation paradigm was reversed to isolate A_3AR complexes, blotting for HA-SERT (Fig. 3, C and D).

To determine whether the effects of IB-MECA derive from the PKGI-linked signaling pathway, we conducted IB-MECA treatment of receptor/transporter cotransfected cells in the presence of PKGI-specific antagonist DT-2 (Dostmann et al., 2000). In initial experiments, we found that DT-2 at concentrations at or below 0.3 μM failed to alter recovery of basal SERT/A_3AR complexes (data not shown). However, DT-2 (0.1 μM) significantly attenuated the stimulatory effect of IB-MECA on the level of receptor/transporter complexes in co-immunoprecipitations (Fig. 3C–D).

Transient and PKGI-Dependent Elevations in SERT Surface Expression by IB-MECA Stimulation of A_3AR/SERT Cotransfected Cells. Our previous work demonstrated that activation of A_3AR can induce a PKG-dependent increase in surface expression of SERT (Zhu et al., 2004). To determine whether IB-MECA-induced increase in recovery of A_3AR/SERT complexes correlates temporally with elevated SERT surface levels, we conducted biotinylation experiments, blotting total and cell surface fractions for SERT immunoreactivity. As seen in previous studies of transiently

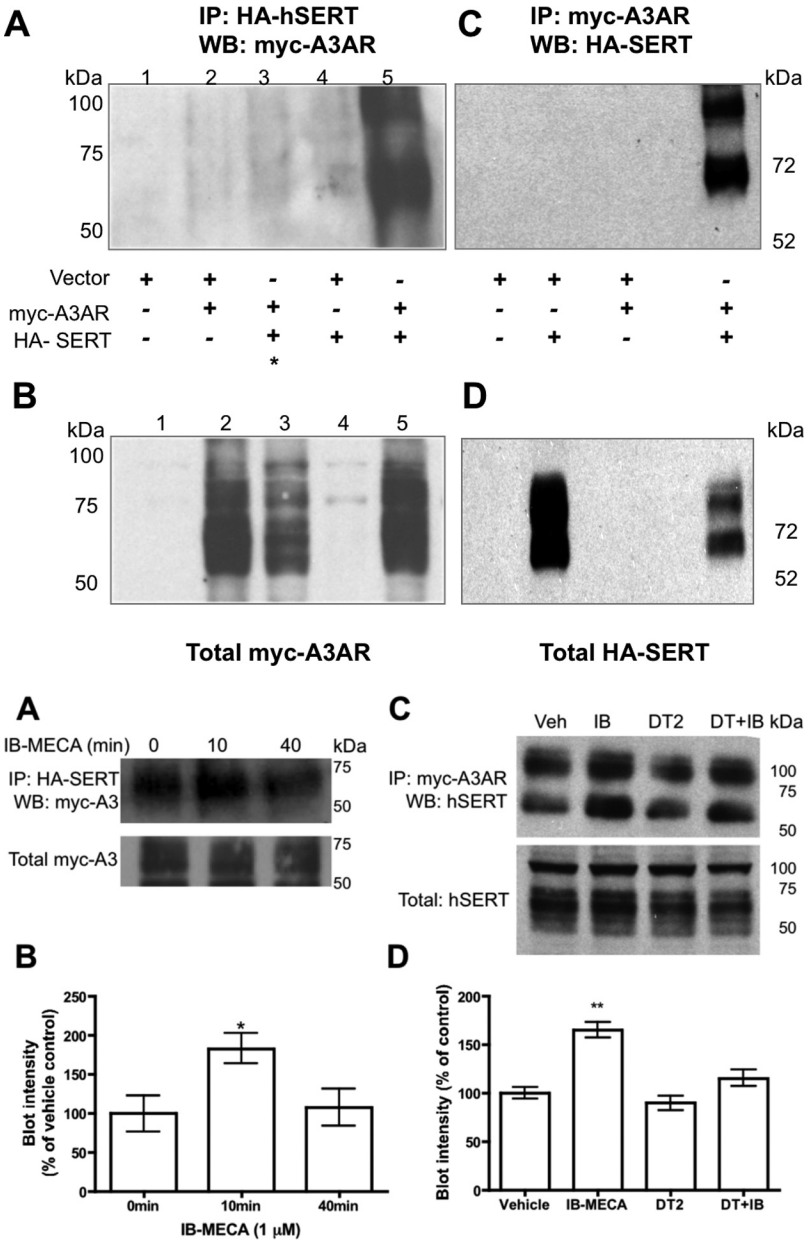

Fig. 2. Physical association of SERT with A_3AR. A and B, CHO cells were transfected with vector (pcDNA) and either myc-A_3AR or HA-hSERT individually or contransfected with either myc-A_3AR or HA-hSERT. Total myc-A_3AR or coIP of myc-A_3AR/HA-SERT complexes was eluted from anti-HA beads and detected by Western blot (WB) analysis using anti-myc antibody. Lane 3, HA-SERT and myc-A3AR were individually transfected and detergent extracts were mixed before coIP; lane 5, sample was from direct cotransfection of HA-SERT and myc-A3AR. C and D, CHO cells were transfected with vector (pcDNA), myc-A_3AR, or HA-hSERT individually or in combination, and complexes were collected on anti-Myc beads. Blots were probed with anti-HA antibody. A and C, signals from coimmunoprecipitation; B and D, signals from total extracts of experiments shown in A and C.

Fig. 3. Recovery of A_3AR-hSERT complexes is A_3AR-regulated and PKGI-dependent. A and B, CHO cells were cotransfected with myc-A_3AR and HA-SERT and treated with IB-MECA (1 μM) for 0, 10, or 40 min, followed by collection of complexes on anti-HA resin. Samples were blotted with anti myc antibody to detect A_3AR receptor. A, representative immunoblot. B, quantitation from multiple experiments in A ($n = 4$). C and D, CHO cells were cotransfected with myc-A_3AR and hSERT, and treated with the PKGI membrane-permeant peptide inhibitor DT-2 (0.1 μM) for 10 min followed by incubation with IB-MECA (1.0 μM) for an additional 10 min. Cells then were lysed with 1% Triton X-100 as detailed under *Materials and Methods* and collected on anti-myc beads. Western blotting was performed with anti-SERT antibody. C, representative immunoblot. D, quantification from multiple experiments in C ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ versus 0 min or vehicle control (one-way ANOVA with Dunnett's multiple comparison test).

transfected cells (Zhu et al., 2004), we observed an elevation of surface SERT (but not total SERT) with 10 min of stimulation that returned to nonstimulated level by 40 min (Fig. 5, A and B). Pretreatment of cells with A_3AR MRS1191 completely abolished IB-MECA- induced surface SERT elevation (Fig. 4, C and D). Surface fractions blotted for SERT demonstrated the expected enrichment of heavily glycosylated 90- to 100-kDa protein, whereas total extracts were more enriched for less heavily glycosylated 50- to 60-kDa forms (Fig. 5A). As with the PKGI dependence of IB-MECA elevations in A_3AR/SERT complexes, IB-MECA increased surface SERT protein could be blocked by DT-2 (Fig. 5, C and D).

A_3AR Variant L90V Found in Subjects with Autism Spectrum Disorder Enhances Recovery of A_3AR Complexes and SERT Surface Expression. Multiple hyperactive SERT coding variants have been identified in subjects with ASD (Prasad et al., 2009). Because these variants are rare, we have sought evidence for genetic variation in SERT modulatory genes that might also produce anomalous elevations of wild-type SERT (Campbell et al., 2009). We identified an A_3AR coding variant L90V in subjects with ASD that shows a more prolonged, agonist-dependent increase in both cGMP levels and SERT activity (N. Campbell, C.-B. Zhu, K. Lindler, R. Blakely, and J. Sutcliffe, manuscript in preparation). To examine the impact of the L90V-A_3AR variant on basal and regulated SERT protein associations and surface expression, we cotransfected myc- A_3AR or myc-L90V-A_3AR with SERT and stimulated receptors with IB-MECA. In total extracts of coimmunoprecipitation experiments, we observed no impact of the L90V variant on A_3AR receptor expression with or without IB-MECA exposure (Fig. 6A). However, and consistent with effects on uptake, whereas IB-MECA enhanced recovery of wild-type and L90V A_3AR/SERT complexes equally after 10 min of exposure, L90V-A_3AR/SERT complexes remained significantly elevated above vehicle controls at 40 min of IB-MECA exposure, and by 40 min, wild-type A_3AR/SERT complex levels returned to those seen vehicle treatments (Fig. 6A-B). As with wild-type A_3AR at 10 min, pretreatment of IB-MECA-treated cells with either MRS1191 or DT-2 abolished the effects of IB-MECA on L90V-A_3AR-SERT associations at 40 min (Fig. 6, C and D). Finally, the prolonged impact of the A_3AR L90V variant on recovery of receptor/transporter complexes was mirrored by a sustained effect of IB-MECA on SERT surface expression (Fig. 6, E and F).

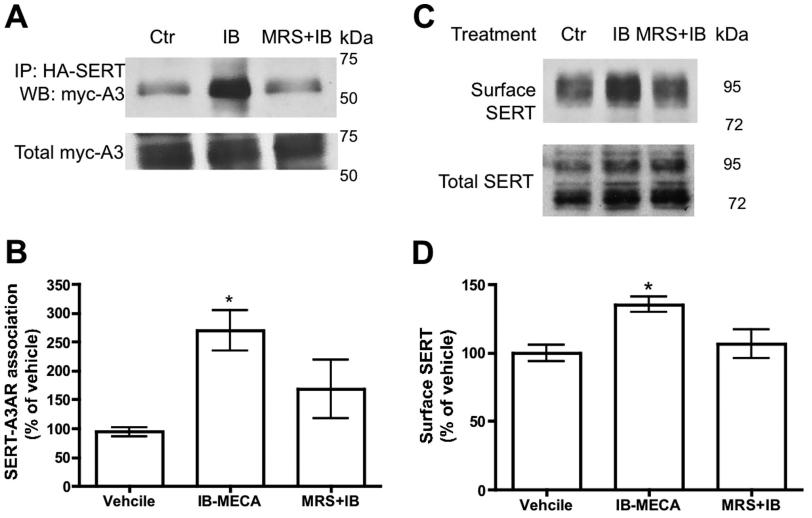

Fig. 4. Elevated A_3AR-SERT recovery and SERT surface expression produced by IB-MECA are blocked by the specific A_3AR antagonist MRS1191 (MRS). A, CHO cells were cotransfected with myc-A_3AR and HA-SERT and treated with IB-MECA (1 μM) ± MRS1191 (1 μM) for 10 min, followed by coIP with anti-HA beads. Samples were blotted with anti-myc antibody. A, representative immunoblot. B, quantitation of multiple experiments from A ($n = 4$). C, CHO cells were cotransfected with myc-A_3AR and hSERT and treated with IB-MECA (1 μM) ± MRS1191 (1 μM) for 10 min, followed by biotinylation as described under *Materials and Methods*. Samples were blotted with antibody targeted to hSERT. C, representative experiment. D, quantitation of multiple experiments from C ($n = 3$). *, $p < 0.05$ versus vehicle control (one-way ANOVA with Dunnett's multiple comparison test).

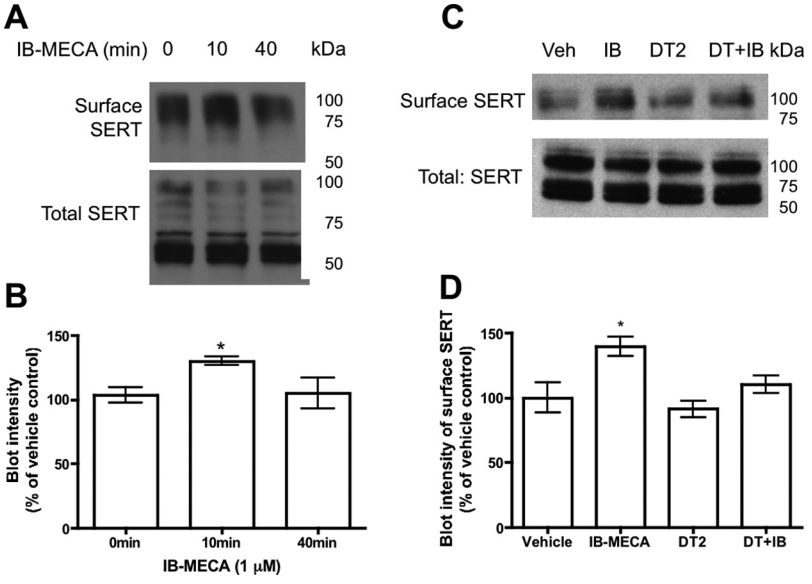

Fig. 5. Elevated SERT surface expression produced by IB-MECA requires PKGI activity. A and B, CHO cells were cotransfected with myc-A_3AR and hSERT and treated with IB-MECA (1 μM) for 10 and 40 min, followed by cell surface biotinylation and blotting with anti-SERT antibody. A, representative immunoblot. B, quantitation from multiple experiments from A ($n = 4$). C and D, CHO cells were cotransfected with myc-A_3AR and hSERT and treated with DT-2 (0.1 μM) for 10 min followed by incubation with IB-MECA (1.0 μM) for an additional 10 min. Cells were then biotinylated as described under *Materials and Methods*, lysed with 1% Triton X-100, and collected on Streptavidin beads as detailed under *Materials and Methods*. Western blots were performed with anti-SERT antibody. C, representative immunoblot. Veh, vehicle. D, quantification from multiple experiments from C ($n = 4$). *, $p < 0.05$ versus vehicle control (one-way ANOVA with Dunnett's multiple comparison test).

Discussion

SERT activity is known to be regulated at both transcriptional and post-translational levels (Blakely et al., 1998; Bauman et al., 2000) with evidence derived from kinase/phosphatase inhibitors and activators on transfected cell lines (Ramamoorthy and Blakely, 1999), cultured pulmonary endothelial and smooth muscle cells (Ren et al., 2011), platelets (Carneiro and Blakely, 2006), nerve terminal preparations (Zhu et al., 2007), brain slices (Ansah et al., 2003), as well as in the CNS in vivo (Daws and Toney, 2007). In recent years, we have focused on receptors that regulate SERT via PKG and p38 MAPK signaling pathways (Zhu et al., 2004, 2007; Blakely et al., 2005). With respect to the current report, we demonstrated that in both RBL-2H3 and transiently transfected CHO cells, A_3ARs have the capacity to rapidly regulate SERT trafficking and catalytic activity, respectively (Zhu et al., 2004). In addition, Zhu et al. (2004) demonstrated that A_3AR stimulation of SERT requires phospholipase C, Ca^{2+}, NOS, guanylyl cyclase, and PKG. More recently, we identified PKG-dependent regulation of SERT by A_3ARs in mouse CNS nerve terminal preparations (synaptosomes), regulation absent in synaptosomes prepared from A_3AR knockout mice (Zhu et al., 2007). Our current report provides evidence that A_3ARs are expressed in midbrain serotonergic neurons, where they can be colocalized with SERT. Moreover, we provide evidence that A_3ARs and SERT can form regulated, detergent-resistant complexes in receptor/transporter cotransfected cells.

Although multiple physiological and behavioral studies point to A_3AR-dependent actions of adenosine, evidence of A_3AR localization in the CNS is limited. Indeed, some investigators have questioned whether the A_3AR is expressed in the brain at all (Rivkees et al., 2000), and the Allen Brain Atlas project (http://www.brain-map.org) detects little if any A_3AR mRNA in brain by in situ hybridization. Zhou et al. (1992) originally cloned A_3AR cDNA from rat striatal mRNA but in reverse transcription-PCR studies found only a low level expression of the receptor in cortex, striatum, and olfactory bulb, being more highly expressed in testis and lung. Salvatore et al. (2000) described somewhat higher levels of A_3AR mRNA in whole-brain extracts by Northern analysis, though still much lower than in peripheral tissues. Yaar et al. (2002) found significant and discretely localized expression of β-galactosidase in the CNS of A_3AR promoter reporter mice, although cautious interpretation of the distributions reported in these studies is warranted because of the small size of the promoter fragment used and the differing patterns evident in different reporter lines.

Using single-cell PCR, Lopes et al. (2003) identified A_3AR mRNA in rat hippocampal neurons and by Western blot analysis also detected A_3AR protein in nerve terminal membranes. These effects are consistent with our findings of A_3AR immunoreactivity in nonserotonergic and non-GABAergic fibers in the dorsal raphe, possibly derived from descending glutamatergic inputs. In monitoring effects of caffeine on extracellular 5-HT levels in hippocampus in the presence of A1 and A2 subtype antagonists and the SSRI fluoxetine, Okada et al. (1999) were the first to suggest a role for A_3ARs in presynaptic modulation of 5-HT reuptake. We have provided evidence that the A_3AR agonist IB-MECA rapidly enhances 5-HT transport in mouse midbrain synaptosomes and enhance 5-HT clearance rates in vivo (Zhu et al., 2007). Consistent with these findings, immunolabeling of mouse midbrain sections revealed A_3AR immunoreactivity that colocalized with both 5-HT and SERT labeling of raphe cell bodies and fibers, respectively.

Specificity of antisera is always important to document and even more so with the apparent low level expression of the A_3AR, as seen with many CNS GPCRs. Although staining for all targets was absent with the omission of primary antibodies (Supplemental Fig. 1) and our A_3AR antibody

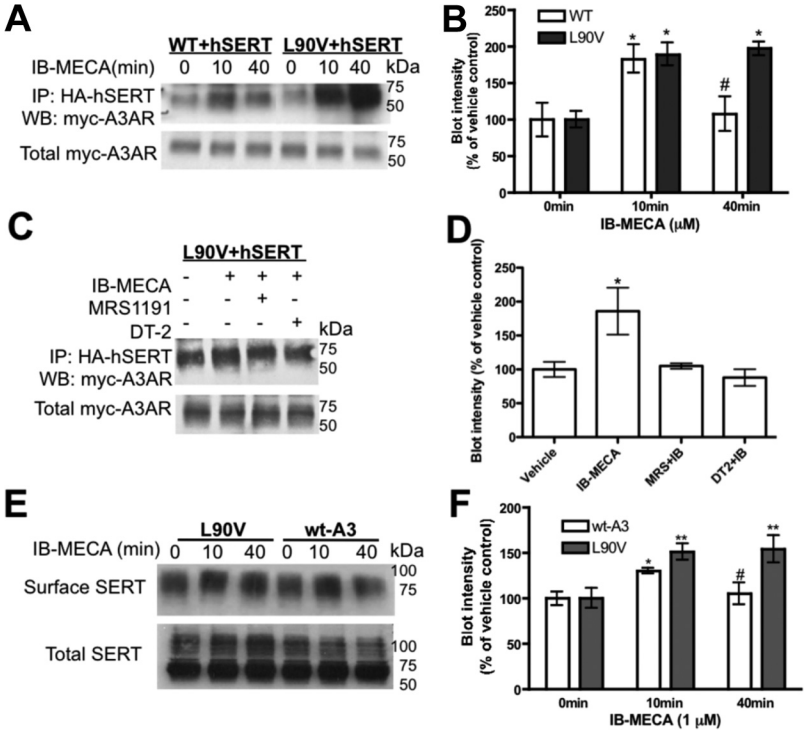

Fig. 6. L90V A_3AR exhibits prolonged enhancement of recovery of SERT-A_3AR complexes and SERT surface expression after A_3AR agonist stimulation. A, wild-type or L90V Myc-A_3AR were cotransfected with HA-hSERT and treated with IB-MECA (1 μM) for 0, 10, or 40 min. Complexes were recovered on HA beads, and eluants were probed with anti-myc antibody. B, quantitation of multiple experiments from A ($n = 3$). *, $p < 0.05$ versus vehicle control; #, $p < 0.05$ versus L90V at 40 min (two-way ANOVA with Bonferroni post tests). C, L90V myc-A_3AR+HA-hSERT-transfected CHO cells were treated with MRS1191 (MRS, 1 μM) or DT2 (0.1 μM) for 10 min followed by a 40-min incubation with either vehicle or IB-MECA (1 μM). Blot was immunoprecipitated with HA beads and probed with anti-myc antibody. D, quantification of multiple Western blots from C ($n = 4$). *, $p < 0.05$ versus vehicle control (one-way ANOVA with with Dunnett's multiple comparison test). E, CHO cells transfected with wild type A_3AR or L90V A_3AR and hSERT were treated with vehicle or IB-MECA (1 μM) for 10 to 40 min then biotinylated on ice for 40 min. Western blots were performed using anti-SERT antibody. F, quantification of multiple experiments from E ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ versus vehicle control; #, $p < 0.05$ versus L90V at 40 min (two-way ANOVA with Bonferroni post tests).

detects human nontagged A_3AR in transfected cells (data not shown), we were unable to document consistent loss of A_3AR immunoreactivity using sections from A_3AR knockout mice. The A_3AR antibody targets the 3rd intracellular loop (from 216 to 230 amino acids; http://www.alomone.com) of A_3AR, whereas the deletion of A_3AR in A_3AR knockouts targets the N-terminal half of the receptor [up to the third transmembrane domain (Salvatore et al., 2000)], thus leaving the antibody recognition intact. In addition, the mouse and human A_3ARs exhibit alternatively spliced mRNAs that encode a truncated protein and that preserve the C-terminal 179 amino acids of the receptor, including the epitope for our A_3AR antibody. This alternatively spliced product of the A_3AR gene seems to be widely expressed, including in the CNS (Burnett et al., 2010). To better address A_3AR specificity issues, we also coincubated our receptor antibodies with an A_3AR peptide and found a complete absence of staining in cell bodies or fibers. In addition, we double-stained sections with antibodies to GAD, a marker of GABAergic nerve terminals and demonstrated a lack of overlap with A_3AR staining. Together, these findings provide the best evidence achievable with current reagents that A_3AR proteins are coexpressed with SERT in vivo.

To our knowledge, ours are the first studies to identify a complex between a GPCR and SERT. It is noteworthy that D2 subtype DA receptors have been found to associate with and regulate activity of DAT proteins (Lee et al., 2009), suggesting that receptor/neurotransmitter transporter complexes may be a more general phenomenon. Whereas D2/DAT receptor complexes seem to be insensitive to D2 agonist stimulation, the A3AR agonist IB-MECA can regulate A_3AR/SERT complexes in a PKGI-dependent manner. With respect to a signaling network triggered by A_3ARs, PKGIα and SERT colocalize in transformed serotonergic cells line (RN46A) and physically associate in receptor/kinase cotransfected cells (Steiner et al., 2009). DT-2 is a peptide inhibitor that shows a nearly 1000-fold selectivity for PKGI isoforms versus PKGII (12.5 nM versus 9.1 μM) (Dostmann et al., 2000; Steiner et al., 2009). Because activation of PKG produces an increase in SERT activity that is accompanied by elevated surface expression (Zhu et al., 2004; current study), we speculate that the formation or stabilization of A_3AR/SERT complexes is an important facet of PKGI-dependent, 5-HT uptake enhancement. It is noteworthy that A_3AR enhancement of SERT has been found to require NOS activity and neuronal NOS has been found to be associated with SERT in mouse brain (Chanrion et al., 2007), suggesting that a large SERT regulatory complex assembles to achieve efficient A_3AR-dependent modulation of the transporter. Studies that prevent PKGI- and NOS-dependent SERT/A_3AR associations, likely using A_3AR and/or SERT mutants that disrupt their interactions, are needed to determine the spatial and temporal control of SERT by the A_3AR/NOS/PKGI pathway. We have reported previously that peripheral activation of the native immune system that induces an elevation in pro-inflammatory cytokines in both the periphery and brain rapidly elevates CNS SERT activity (Zhu et al., 2010). This effect requires p38 MAPK activity, and A_3AR stimulation of SERT also requires concurrent p38 MAK activation. Additional studies are needed therefore to assess whether components of both PKGI and p38 MAPK signaling pathways assemble with SERT and whether such complexes could be independently regulated.

With respect to molecular mechanisms that can facilitate assembly of an A_3AR-linked signaling pathway with SERT, the LIM domain scaffolding protein Hic-5 is known to associate with platelet SERT. Hic-5 dissociates from internalized SERT after PKC activation (Carneiro and Blakely, 2006). The fibrinogen receptor, integrin αIIbβ3, a structural and signaling component of focal adhesions, also associates with platelet SERT and enhances transporter surface expression (Carneiro et al., 2008). Other reported SERT-interacting proteins include PICK1, syntaxin 1A, SCAMP2, α-synuclein, Rab4, and vimentin (for review, see Mercado and Kilic, 2010). We and others have demonstrated that PKGIα and the catalytic subunit of the Ser/Thr protein phosphatase 2A regulate SERT phosphorylation (Ramamoorthy and Blakely, 1999; Bauman et al., 2000; Zhang and Rudnick, 2011) and are physically associated with the transporter (Bauman et al., 2000; Steiner et al., 2009). Finally, neuronal NOS, an essential signaling molecule in A_3AR-triggered PKGI and p38 MAPK-dependent activation (Zhu et al., 2004) is a SERT-associated protein (Chanrion et al., 2007). In the context of evidence presented here that A_3ARs interact with SERT, we propose that SERT trafficking, localization, and catalytic activation require assembly of a much larger and regulated macromolecular complex in which compromised interactions could affect risk for disorders associated with altered 5-HT signaling.

To explore the hypothesis that A_3AR/SERT complexes could be influenced by disease-associated mechanisms, we seek to determine whether the A_3AR coding variant L90V, recently identified in subjects with ASD (Campbell et al., manuscript in preparation), could alter receptor modulation of SERT trafficking or its assembly into a receptor/transporter complex. The L90V variant produces elevated basal cGMP levels in transfected cells compared with wild type A_3AR, and upon IB-MECA stimulation, leads to a more sustained enhancement of cGMP production and 5-HT uptake (Campbell et al., manuscript in preparation). We found that A_3AR agonist treatment of both wild type and L90V A_3AR transfected cells leads to a time-dependent increase in receptor/transporter complexes and an increase in SERT surface expression. Consistent with the enhanced cGMP signaling and 5-HT uptake stimulation of the L90V variant, cells transfected with the mutant receptor demonstrated a maintained stimulation of receptor/transporter complexes and SERT surface expression at a time when these measures had returned to basal levels in cells transfected with wild-type A_3ARs. Because these effects are dependent on PKGI activation, we believe that the impact of the L90V variant on SERT arises from an elevated efficiency of receptor/G-protein coupling, possibly as a consequence of more limited receptor desensitization. Alhough further research is needed to fully elucidate this mechanism, they provide an example of how enhanced SERT activity need not arise from intrinsic changes in SERT structure such as we have found in subjects with ASD (Prasad et al., 2009) but can also be established through functional changes in the SERT regulatory network.

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Authorship Contributions

Participated in research design: Zhu, Lindler, Hewlett, and Blakely.

Conducted experiments: Zhu, Lindler, and Campbell.

Performed data analysis: Zhu, Lindler, and Blakely.

Wrote or contributed to the writing of the manuscript: Zhu, Lindler, Campbell, Sutcliffe, Hewlett, and Blakely.

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