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A Direct Interaction of PSD-95 with 5-HT_{2A} Serotonin Receptors Regulates Receptor Trafficking and Signal Transduction*

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The serotonin (5-hydroxytryptamine) 2A receptor (5-HT_{2A}) is an important G protein-coupled receptor (GPCR) that mediates the effects of hallucinogens and is the target of a number of commonly prescribed medications including atypical antipsychotics, antidepressants, and anxiolytics. The 5-HT_{2A} receptor possesses a canonical Type I PDZ-binding domain (*X*-Ser/Thr-*X*-Φ) at the carboxyl terminus and has been predicted, but never demonstrated, to interact with PDZ domain-containing proteins. We discovered that PSD-95, a prototypic PDZ domain-containing protein, directly associates with the 5-HT_{2A} receptor and regulates 5-HT_{2A} receptor-mediated signaling and trafficking in HEK-293 cells. Co-immunoprecipitation studies revealed that the native 5-HT_{2A} receptor, but not a mutant lacking the PDZ-binding domain, interacted directly with PSD-95. The association with PSD-95 enhanced 5-HT_{2A} receptor-mediated signal transduction, a novel action of PSD-95 on GPCRs. The augmentation of 5-HT_{2A} receptor signaling by PSD-95 was not accompanied by alteration in the kinetics of 5-HT_{2A} receptor desensitization but was associated with the inhibition of agonist-induced 5-HT_{2A} receptor internalization. Additional studies demonstrated that 5-HT_{2A} receptor and PSD-95 were co-localized in clusters on the cell surface of HEK-293 cells. Taken together, the present work elucidates novel roles for PSD-95 in regulating the functional activity and intracellular trafficking of 5-HT_{2A} receptors and possibly other GPCRs.

The serotonin (5-hydroxytryptamine) 2A receptor (5-HT_{2A})[1] mediates the effects of many (1) but not all hallucinogens (2, 3) and is the target of a number of commonly prescribed therapeutic agents including atypical antipsychotics, antidepressants, and anxiolytics (4, 5). As with other G protein-coupled receptors (GPCRs) (6–8), elucidating the regulatory mechanisms for 5-HT_{2A} receptor signaling and trafficking provides important insights into the therapeutic mechanisms of drugs that target the 5-HT_{2A} receptor and has implications for the rational design of novel medications (5, 6).

The 5-HT_{2A} receptor, the most abundant serotonin receptor in the cerebral cortex, is enriched in dendritic shafts and, to a lesser extent, asymmetric synapses and dendritic spines of pyramidal neurons primarily from Layers IV and V (9–13). The last four amino acids (VSCV) of the carboxyl terminus of the 5-HT_{2A} receptor constitute a canonical Type I PDZ-binding domain (*X*-Ser/Thr-*X*-Φ) (14). The PDZ-binding domain is located at the carboxyl terminus of a variety of proteins including many GPCRs and is known to associate with PSD-95/Discs-large/ZO-1 (PDZ) domain-containing proteins of which postsynaptic density 95 (PSD-95, also known as synapse-associated protein 90 or SAP-90, is a prototypic member (15, 16). These multi-domain molecules not only target and provide scaffolds for protein-protein interactions but can also modulate the functions of ion channels and receptors with which they associate (16–20). The disruption of the association between PDZ proteins and their targets contributes to the pathogenesis of a number of human diseases (16, 21, 22), most probably because of the failure of PDZ proteins to appropriately target and modulate the actions of the associated proteins.

Na^+/H^+ exchanger regulatory factor (NHE-RF) was the first PDZ domain-containing protein that was reported to modulate the function of a GPCR (*e.g.* the β_2-adrenergic receptor (β_2-AR)) (23). Subsequently, PSD-95 was found to associate with and inhibit the internalization of β_1-ARs and to facilitate the interaction between the β_1-AR and the *N*-methyl-D-aspartate ionotropic glutamate receptor (24, 25). Among the 5-HT_2 class of serotonin receptors, a multi-PDZ domain-containing protein, MUPP1, associates with 5-HT_{2C} receptors *in vitro* and *in vivo* (26). MUPP1 has also been predicted to interact with both the 5-HT_{2A} and the 5-HT_{2B} receptor based on its ability to bind to purified COOH-terminal fusion proteins of both receptors *in vitro* (26). Furthermore, a proteomic approach has recently identified PSD-95 as a component of a multi-protein complex that associates with the 5-HT_{2C} receptor *in vivo* (27). PSD-95, similar to the 5-HT_{2A} receptor, is enriched in asymmetric synapses and dendritic spines of cortical pyramidal neurons (28, 29) and is thus predicted to interact with 5-HT_{2A} receptors. The functional significance of any association between PDZ proteins and 5-HT receptors is unknown because none has been previously reported.

Therefore, we set out to investigate whether PSD-95 interacts with and regulates 5-HT_{2A} receptors. A combination of biochemical and immunocytochemical approaches demonstrated that PSD-95 directly interacts with the 5-HT_{2A} receptor *in vitro*. We also discovered that PSD-95 enhanced 5-HT_{2A} receptor-mediated signaling, inhibited agonist-induced 5-HT_{2A}

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[1] The abbreviations used are: 5-HT_{2A}, serotonin (5-hydroxytryptamine) 2A receptor; GPCR, G protein-coupled receptor; PSD, postsynaptic density 95; PDZ, PSD-95/Discs-large/ZO-1; NHE-RF, Na^+/H^+ exchanger regulatory factor; AR, adrenergic receptor; WT, wild type; GFP, green fluorescent protein; HEK, human embryonic kidney; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; E_{max}, efficacy value; GRK, G protein-coupled receptor kinase; IP, inositol phosphate; PI, phosphoinositide.

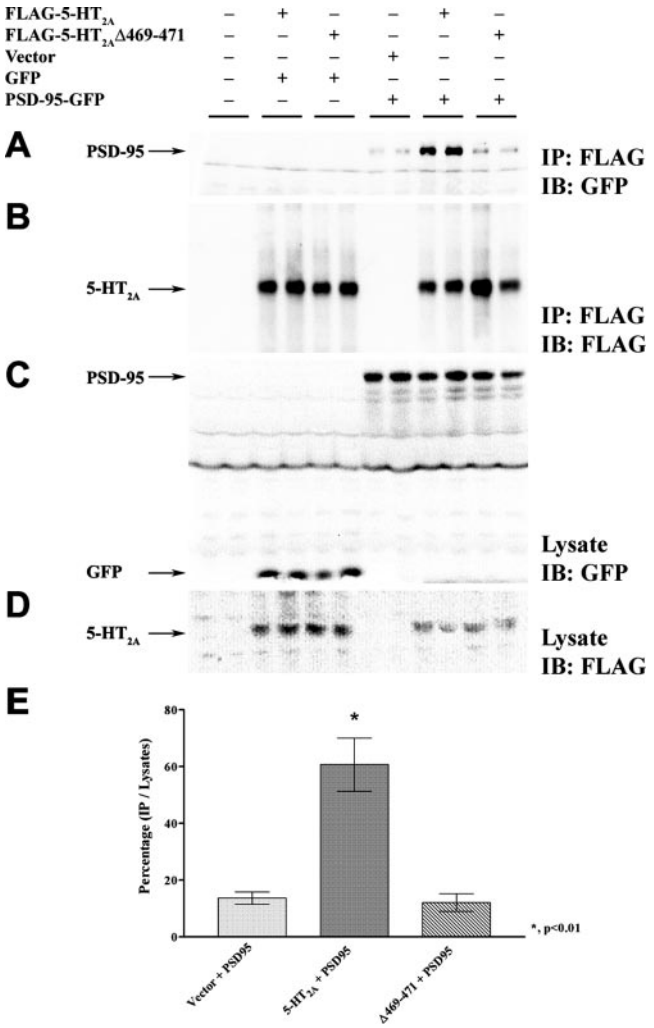

FIG. 1. **PSD-95 interacts with native 5-HT$_{2A}$ receptors but not with mutant 5-HT$_{2A}$ receptors lacking the PDZ binding motif.** For these experiments, HEK-293 cells were either nontransfected (*lanes 1* and *2*) or transiently co-transfected with FLAG-5-HT$_{2A}$ + GFP (*lanes 3* and *4*), FLAG-5-HT$_{2A}$Δ469–471 + GFP (*lanes 5* and *6*), pcDNA3 + PSD95-GFP (*lanes 7* and *8*), FLAG-5-HT$_{2A}$ + PSD95-GFP (*lanes 9* and *10*), or FLAG-5-HT$_{2A}$Δ469–471 + PSD95-GFP (*lanes 11* and *12*). FLAG-tagged native or mutant 5-HT$_{2A}$ receptors were immunoprecipitated by a monoclonal FLAG antibody conjugated to Sepharose beads. PSD95-GFP or GFP was detected by a polyclonal GFP antibody, and FLAG-5-HT$_{2A}$ or FLAG-5-HT$_{2A}$Δ469–471 was detected by a polyclonal FLAG antibody on Western blots. The 5-HT$_{2A}$ receptor and PSD-95 appear as 62- and 95-kDa proteins, respectively. Representative immunoblots from a single experiment that has been replicated three times with equivalent results are shown. *A–B*, immunoblots from immunoprecipitates. *C–D*, immunoblots from cell lysates. *E*, quantification of PSD-95 levels on immunoblots from three independent experiments. The average levels of PSD-95 in immunoprecipitates from cells co-expressing vector, FLAG-5-HT$_{2A}$, or FLAG-5-HT$_{2A}$Δ469–471 + PSD-95-GFP were normalized to the corresponding levels in cell lysates. *IB*, immunoblotting.

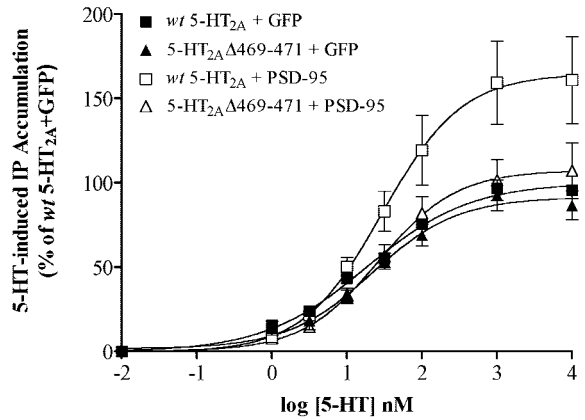

FIG. 2. **PSD-95 enhances 5-HT$_{2A}$ receptor-mediated signal transduction.** For these experiments, HEK-293 cells were transiently co-transfected with FLAG-5-HT$_{2A}$ + GFP (■), FLAG-5-HT$_{2A}$Δ469–471 + GFP (▲), FLAG-5-HT$_{2A}$ + PSD-95-GFP (□), or FLAG-5-HT$_{2A}$Δ469–471 + PSD-95-GFP (△). Dose-response PI hydrolysis and saturation binding assays were performed on cells from the same batch of transfection with efficacies (E_{max} values) determined from PI hydrolysis normalized to receptor expression (B_{max} values in fmol/mg; see "Experimental Procedures"). The average results (±S.E.) from three independent experiments are shown. EC_{50} and relative E_{max} values are shown in Table I.

receptor internalization, and promoted 5-HT$_{2A}$ receptor clustering on the plasma membrane. Thus, this work demonstrates a novel role for PSD-95 in modulating the activity of a GPCR. These findings have important implications for elucidating the roles of PDZ domain-containing proteins in regulating the functions of the 5-HT family of receptors and possibly other GPCRs.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Reagents—The construct coding for FLAG-tagged wild type (WT) rat 5-HT$_{2A}$ receptor (FLAG-5-HT$_{2A}$) was modified from a similar construct that has been described previously (30) by adding an amino-terminal cleavable signal peptide sequence [MKTIIALSYIFCLVFA (31)] derived from influenza hemagglutinin. The addition of such a signal sequence has been shown to increase the expression of GPCRs (31). FLAG-5-HT$_{2A}$Δ469–471 was constructed by using FLAG-5-HT$_{2A}$ as the template and by introducing a premature stop codon at residue 469, the third to the last amino acid of the carboxyl terminus, using site-directed mutagenesis (5′-primer: 5′-CGTGAATGAAAAGGTT**TGA**TGTGTGTGATCTAGAGGGCCC-3′, and 3′-primer: 5′-GGGCCCTCTAGATCACACACACA**TCA**AACCTTTTCATTCACG)-3′ (QuikChange). Deletion of amino acid residues 469–471 effectively ablates the PDZ-binding domain of the 5-HT$_{2A}$ receptor. PSD-95-GFP (a generous gift from D. Bredt) has been described elsewhere (32). The EGFP-C2 vector (Clontech, Palo Alto, CA) contained the GFP coding sequence. The constitutively active Gα_q mutant (Q229L) (a generous gift from D. Siderovski) has been described elsewhere (33). All of the constructs containing inserts in the appropriate orientation were verified by automated sequencing (Cleveland Genomics, Cleveland, OH).

5-Hydroxytryptamine creatinine sulfate (5-HT), quipazine, clozapine, chlorpromazine, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride, CHAPS, and sodium orthovanadate were acquired from Sigma. [^{3}H]Ketanserin and *myo*-[^{3}H]inositol were purchased from PerkinElmer Life Sciences.

Transfection of HEK-293 Cells—Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, penicillin (100 units/ml), and streptomycin (100 mg/ml) (Invitrogen) at 37 °C and 5% CO_2. Transient transfection with FuGENE 6 (Roche Applied Sciences) using a FuGENE 6 to DNA ratio of 5:1 was performed according to the manufacturer's recommendations. A total of 6 μg of DNA was used in each co-transfection, 4 μg of which were vector, 5-HT$_{2A}$ receptor, or mutant 5-HT$_{2A}$ receptor DNA and 2 μg of which were DNA encoding either PSD-95-GFP or GFP.

Co-immunoprecipitation and Immunoblot—At 24 h after co-transfection, HEK-293 cells were split into 6-well plates and cultured first in DMEM supplemented with 5% dialyzed FBS for 24 h and then in DMEM without serum for an additional 24 h. Cells were washed at 4 °C twice with PBS and once with Buffer A (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM $Na_4P_2O_7$, and 2 mM sodium orthovanadate, pH 7.5). Cells were lysed by incubation on a rocker platform at 4 °C for 15 min in Buffer A containing 1.5% CHAPS and EDTA-free Complete protease inhibitor tablets (Roche Applied Sciences). A portion of the cell lysates was saved for subsequent determination of total protein expression levels, and the remainder was incubated with Sepharose beads conjugated to a monoclonal FLAG antibody (Sigma) at 4 °C for 2 h. The beads were washed at 4 °C for three times in Buffer B (50 mM HEPES, 150 mM NaCl, 0.3% Triton X-100, and 10% glycerol, pH 7.5). Immunoprecipitated proteins were then eluted from the beads by resuspension in 2× SDS sample buffer. Samples were heated for 5 min at 67 °C, and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed according to standard

TABLE I
PSD-95 potentiates 5-HT$_{2A}$ receptor signaling

Agonist potency (EC_{50}) and relative agonist efficacy (normalized E_{max}) were determined from phosphoinositide hydrolysis assays as described under "Experimental Procedures." The results represent the average of three independent experiments with E_{max} values normalized for receptor expression from saturation binding assays performed in parallel. Comparison to FLAG-5-HT$_{2A}$ + GFP: *, $p < 0.05$; NS, no statistically significant difference.

	Agonist potency EC_{50} (pEC_{50} ± S.E.)	Agonist efficacy relative E_{max} ± S.E.
FLAG-5-HT$_{2A}$ + GFP	18.1 nM (1.26 ± 0.07)	1.00 ± 0.02
FLAG-5-HT$_{2A}$ 469-471 + GFP	21.6 nM (1.33 ± 0.15)^{NS}	0.92 ± 0.05^{NS}
FLAG-5-HT$_{2A}$ + PSD-95-GFP	29.4 nM (1.47 ± 0.19)^{NS}	1.65 ± 0.14*
FLAG-5-HT$_{2A}$ 469-471 + PSD-95-GFP	26.6 nM (1.42 ± 0.16)^{NS}	1.07 ± 0.07^{NS}

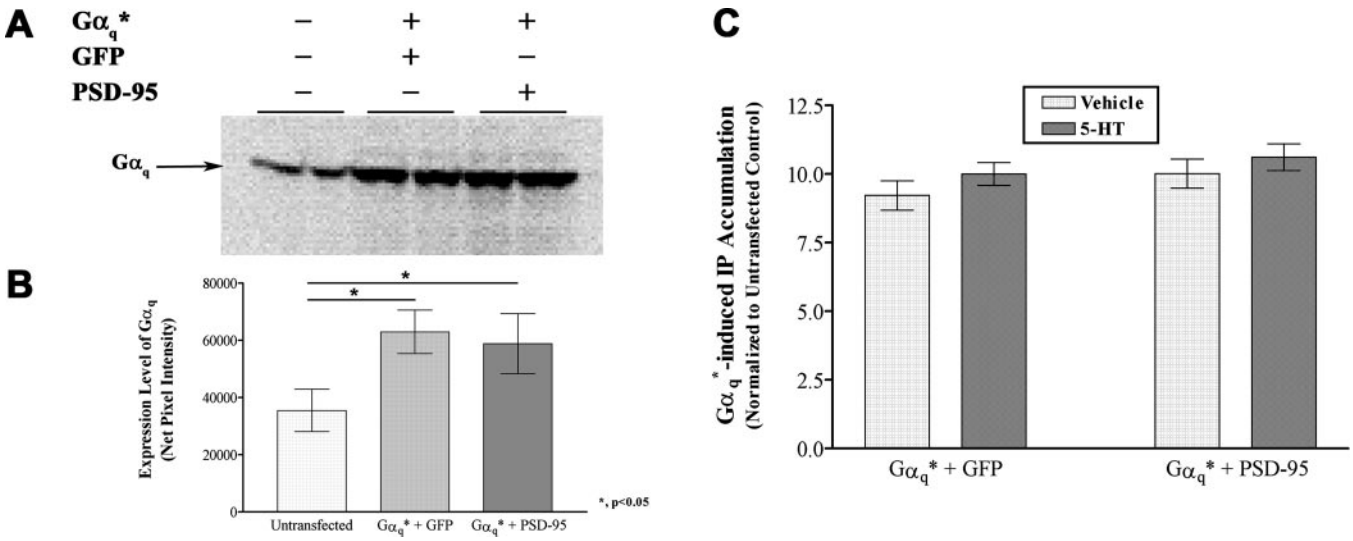

FIG. 3. **PSD-95 does not augment Gα$_q$ signaling independent of the 5-HT$_{2A}$ receptor.** For these experiments, a constitutively active form of Gα$_q$ (Q229L or Gα$_q^*$) was co-transfected with either GFP or PSD-95-GFP. *A*, representative immunoblot of total Gα$_q$ from all of the sample groups (in duplicate). *B*, quantification of the net pixel intensities of bands from three independent experiments showed equal levels of total Gα$_q$ expression in the presence or absence of PSD-95, both of which were significantly higher than that from nontransfected cells because of the overexpression of Gα$_q^*$. *C*, PSD-95 (PSD-95 + Gα$_q^*$) did not elevate Gα$_q^*$-stimulated IP accumulation over the base line (PSD-95 + GFP) in the absence of 5-HT$_{2A}$ receptors. Results shown represent the mean (±S.E.) of three independent experiments.

methods (30, 34). A polyclonal GFP antibody (1:2000) (Abcam, Cambridge, United Kingdom) was used to detect PSD-95-GFP or GFP. A rabbit polyclonal FLAG antibody (1:1500) was used to detect FLAG-tagged WT and mutant 5-HT$_{2A}$ receptors. The constitutively active Gα$_q$ mutant and the endogenous WT Gα$_q$ in cell lysates were detected by a rabbit polyclonal Gα$_q$ antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA). The levels of PSD-95-GFP expression from immunoprecipitates and cell lysates were quantified using a chemiluminescence imaging system (Kodak Scientific Imaging System, Eastman Kodak Co.). The levels of PSD-95-GFP expression from immunoprecipitates were subsequently normalized to corresponding levels in cell lysates.

Agonist-mediated Internalization—At 24 h after co-transfection, HEK-293 cells were split into 24-well plates containing poly-L-lysine-coated coverslips and maintained in DMEM supplemented with 5% dialyzed FBS for 24 h and then in DMEM without serum for an additional 24 h. Cells were treated for 15 min at 37 °C with 5-HT at a final concentration of 10 μM. Treatment was terminated by placing cells at 4 °C, removing media, and fixing with 4% paraformaldehyde. Immunocytochemistry was performed, confocal images were acquired (see "Immunocytochemistry, Confocal Microscopy, and Image Quantification"), and internalization was quantified using the MetaView imaging software (Universal Imaging, Downingtown, PA).

Phosphoinositide Hydrolysis, Radioligand Binding, and Desensitization Assays—Phosphoinositide (PI) hydrolysis and saturation radioligand binding assays were performed on cells from the same transfection. At 24 h following transfection, HEK-293 cells were either 1) split into poly-L-lysine-coated 24-well plates and maintained in DMEM supplemented with 5% dialyzed FBS for 24 h and then in serum-free and inositol-free DMEM for an additional 24 h before performing PI hydrolysis assay or 2) kept in DMEM supplemented with 5% dialyzed FBS for 24 h and then in serum-free DMEM for additional 24 h before harvesting cell membranes for binding assay. As described previously, agonist efficacy (E_{max}) and potency (EC_{50}) were determined from dose-response PI hydrolysis assays and kinetic binding parameters (B_{max} and K_d) were determined from saturation binding assays with [^{3}H]ketanserin (35). The resulting E_{max} values were then normalized to B_{max} values with results replicated in at least three separate experiments. PI hydrolysis data were analyzed by nonlinear regression using the GraphPad Prism software (San Diego, CA), and saturation binding data were analyzed using GraphPad Prism or the weighted nonlinear least-squares curve-fitting program LIGAND (36). Agonist-dependent desensitization of 5-HT$_{2A}$ receptor-mediated accumulation of inositol phosphate (IP) was performed as described previously (34, 35).

Immunocytochemistry, Confocal Microscopy, and Image Quantification—Dual-labeling immunocytochemistry was performed essentially as described previously (30). HEK-293 cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 at 4 °C, blocked with 5% milk, incubated with primary antibodies overnight at 4 °C and then with secondary antibodies, conjugated to the appropriate Alexa Fluor dyes (Molecular Probes, Eugene, OR), and finally mounted on glass slides. FLAG-tagged constructs were visualized using a monoclonal FLAG antibody (1:1500) (Stratagene). GFP-tagged constructs were visualized with a rabbit polyclonal GFP antibody (1:2000) (Abcam, Cambridge, United Kingdom). Images of cells were acquired digitally using a Zeiss 410 confocal microscope (Oberkochen, Germany) without saturation of pixel intensities and were subsequently analyzed using MetaView (Universal Imaging) as previously detailed (30, 34, 35, 37, 38). For each cell in which 5-HT$_{2A}$ receptor internalization was quantified, two measurements were made: 1) total cellular immunofluorescence and 2) intracellular immunofluorescence. The extent of 5-HT$_{2A}$ receptor internalization was then defined as the percentage of the total 5-HT$_{2A}$ receptor immunofluorescence, which was intracellular. For each sample group, at least 50 cells were quantified in a blinded fashion. In prior studies (30, 37), we have demonstrated that this method provides quantitative estimates of internalization that are essentially equivalent to results obtained by biochemical techniques such as surface biotinylation.

Statistical Analysis—Data from co-immunoprecipitation and internalization studies were analyzed using the unpaired Student's *t* test

with statistical significance defined as $p < 0.05$. Statistical significance of the PI hydrolysis data was analyzed by GraphPad Prism using the two-tailed paired Student's *t* test and was defined as $p < 0.05$.

RESULTS

5-HT$_{2A}$ Receptors Directly Interact with PSD-95 via a Type I PDZ-binding Motif—Because the 5-HT$_{2A}$ receptor contains a canonical Type I PDZ-binding domain and is found in the postsynaptic densities of cortical neurons where PSD-95 is also enriched (12), we predicted that 5-HT$_{2A}$ receptors and PSD-95 might directly associate. To test this hypothesis, we performed co-immunoprecipitation experiments in which 5-HT$_{2A}$ receptors and PSD-95 were co-transfected in HEK-293 cells. We probed for GFP-tagged PSD-95 following lysis of transiently transfected HEK-293 cells and immunoprecipitation of FLAG-tagged WT or mutant 5-HT$_{2A}$ receptors. Wild type FLAG-5-HT$_{2A}$ receptors were co-immunoprecipitated robustly with PSD-95-GFP (Fig. 1*A*, *lanes 9* and *10*), indicating that 5-HT$_{2A}$ receptors and PSD-95 directly interact *in vitro* (see Fig. 1*E* for quantification).

To identify the responsible binding motif(s) on the 5-HT$_{2A}$ receptor, we constructed a mutant 5-HT$_{2A}$ receptor (5-HT$_{2A}$Δ469–471) that lacks the PDZ-binding domain. Ablating the PDZ-binding domain attenuated the interaction between FLAG-5-HT$_{2A}$ and PSD-95-GFP to control levels (Fig. 1, *A*, *lanes 11* and *12 versus lanes 7* and *8*, and *E*), demonstrating that the PDZ-binding domain of the 5-HT$_{2A}$ receptor (residues 469–471, SCV) mediates the association with PSD-95.

To further establish the specificity of the interaction between the 5-HT$_{2A}$ receptor and PSD-95, we performed a number of controls. First, we showed that cells co-transfected with PSD-95-GFP and an empty vector (Fig. 1, *A*, *lanes 7* and *8*, and *E*) yielded minimal pull-down of PSD-95. Second, no PSD-95 was immunoprecipitated from nontransfected cells (Fig. 1*A*, *lanes 1* and *2*) or from cells co-expressing 5-HT$_{2A}$ receptors (WT or mutant) and GFP (Fig. 1*A*, *lane 3–6*). These findings demonstrated the specificity of PSD-95 association with 5-HT$_{2A}$ receptors. Finally, the significantly higher levels of PSD-95 that co-immunoprecipitated with WT 5-HT$_{2A}$ receptors (Fig. 1*A*, *lanes 9* and *10*) resulted neither from variations in the expression of WT or mutant 5-HT$_{2A}$ receptors in immunoprecipitates (Fig. 1*B*, *lanes 3–6* and *lanes 9–12*) or in cell lysates (Fig. 1*D*, *lanes 3–6* and *lanes 9–12*) nor from variations in PSD-95 expression (Fig. 1*C*, *lanes 7–12*; see Fig. 1*E* for quantification). Therefore, we conclude that PSD-95 specifically associates with 5-HT$_{2A}$ receptors by interacting with the PDZ-binding domain of the 5-HT$_{2A}$ receptor.

PSD-95 Potentiates 5-HT$_{2A}$ Receptor-mediated Signal Transduction—We next examined whether the association between PSD-95 and the 5-HT$_{2A}$ receptor has functional consequences on 5-HT$_{2A}$ receptor signal transduction. Because the 5-HT$_{2A}$ receptor is a G$_q$-coupled receptor that activates phospholipase C (PLC) (39), we measured inositol phosphate (IP) accumulation resulting from 5-HT$_{2A}$ receptor-mediated PI hydrolysis (Fig. 2) and determined in parallel the expression of 5-HT$_{2A}$ receptors by immunoblot analysis (Fig. 1, *B* and *D*) and saturation binding assays. We then normalized the functional activity of the 5-HT$_{2A}$ receptor (*i.e.* IP accumulation) to the expression level of the 5-HT$_{2A}$ receptor (*i.e.* B_{max}), thus accounting for minor variations in 5-HT$_{2A}$ receptor activation that might have resulted from variations in receptor expression. Assays were also performed under the conditions of minimal receptor reserve (35).

PSD-95 significantly enhanced WT 5-HT$_{2A}$ receptor-mediated signaling when compared with the base line (*i.e.* 5-HT$_{2A}$ receptors co-transfected with GFP) (Fig. 2, WT 5-HT$_{2A}$ + PSD-95 *versus* WT 5-HT$_{2A}$ + GFP) (Table I). The augmentation of signaling by PSD-95 was not seen when PSD-95 was co-transfected with the 5-HT$_{2A}$Δ469–471 mutant (Fig. 2, 5-HT$_{2A}$Δ469–471 + PSD-95) (Table I). Blocking the direct physical interaction between PSD-95 and 5-HT$_{2A}$ receptors thus inhibited the ability of PSD-95 to augment 5-HT$_{2A}$ signaling. Interestingly, PSD-95 did not alter agonist potency (Table I), and there was no apparent alteration in the constitutive activity of any of the receptor constructs (measured as detailed by Shapiro *et al.* (40); data not shown). These results indicate that PSD-95 via a direct physical interaction with 5-HT$_{2A}$ receptors augments 5-HT$_{2A}$ receptor-mediated signaling.

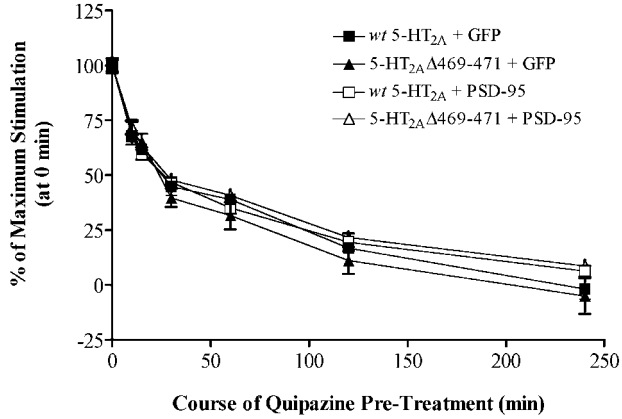

FIG. 4. **PSD-95 does not affect 5-HT$_{2A}$ receptor desensitization in HEK-293 cells.** For these experiments, cells were transiently co-transfected with FLAG-5-HT$_{2A}$ + GFP (■), FLAG-5-HT$_{2A}$Δ469–471 + GFP (▲), FLAG-5-HT$_{2A}$ + PSD-95-GFP (□), or FLAG-5-HT$_{2A}$Δ469–471 + PSD-95-GFP (△). At 48 h following transfection, cells were incubated for 24 h with serum-free media and then pretreated for 15, 30, 60, 120, and 240 min with 300 μM quipazine and subsequently stimulated with 10 μM 5-HT for 1 h before PI hydrolysis assay was performed (see "Experimental Procedures"). 5-HT$_{2A}$ receptor-mediated IP accumulation at each time point was normalized to that in cells that were not desensitized by quipazine (at 0 min). Results from three independent experiments showed no statistically significant difference among the sample groups.

To investigate the possibility that PSD-95 augments the PLC signaling pathway by associating with downstream effectors (*i.e.* Gα$_q$ and PLC), we examined whether PSD-95 increased IP accumulation in cells expressing a constitutively active form of Gα$_q$ (Q229L and Gα$_q^*$), which activates PLC directly (33). In the absence of 5-HT$_{2A}$ receptor expression, PSD-95 neither altered the Gα$_q^*$-stimulated IP accumulation when compared with GFP alone (Fig. 3*C*) nor affected the relative expression of Gα$_q^*$ (Fig. 3, *A* and *B*). These findings strongly suggest that PSD-95 enhances intracellular signaling at the level of receptor-effector coupling and not via a receptor-independent augmentation of Gα$_q$ signaling.

PSD-95 Does Not Affect the Agonist-dependent Desensitization of 5-HT$_{2A}$ Receptors—To determine the potential cellular mechanisms subserving the PSD-95-mediated augmentation of 5-HT$_{2A}$ receptor signaling, we examined whether PSD-95 alters the time course of 5-HT$_{2A}$ receptor desensitization. We have previously reported that exposure to agonists inhibits the activation of the 5-HT$_{2A}$ receptor by the process of desensitization whereby receptors on the cell surface no longer respond to agonists and fail to efficiently signal downstream pathways (41). PSD-95 did not attenuate agonist-dependent 5-HT$_{2A}$ receptor desensitization when compared with the base line (Fig. 4, WT 5-HT$_{2A}$ + PSD-95 *versus* WT 5-HT$_{2A}$ + GFP). Likewise, disrupting the association between PSD-95 and 5-HT$_{2A}$ receptors had no effect on agonist-dependent desensitization of 5-HT$_{2A}$ receptors (Fig. 4, 5-HT$_{2A}$Δ469–471 + PSD-95). Hence, PSD-95 does not enhance 5-HT$_{2A}$ receptor signaling by attenuating the desensitization of 5-HT$_{2A}$ receptors.

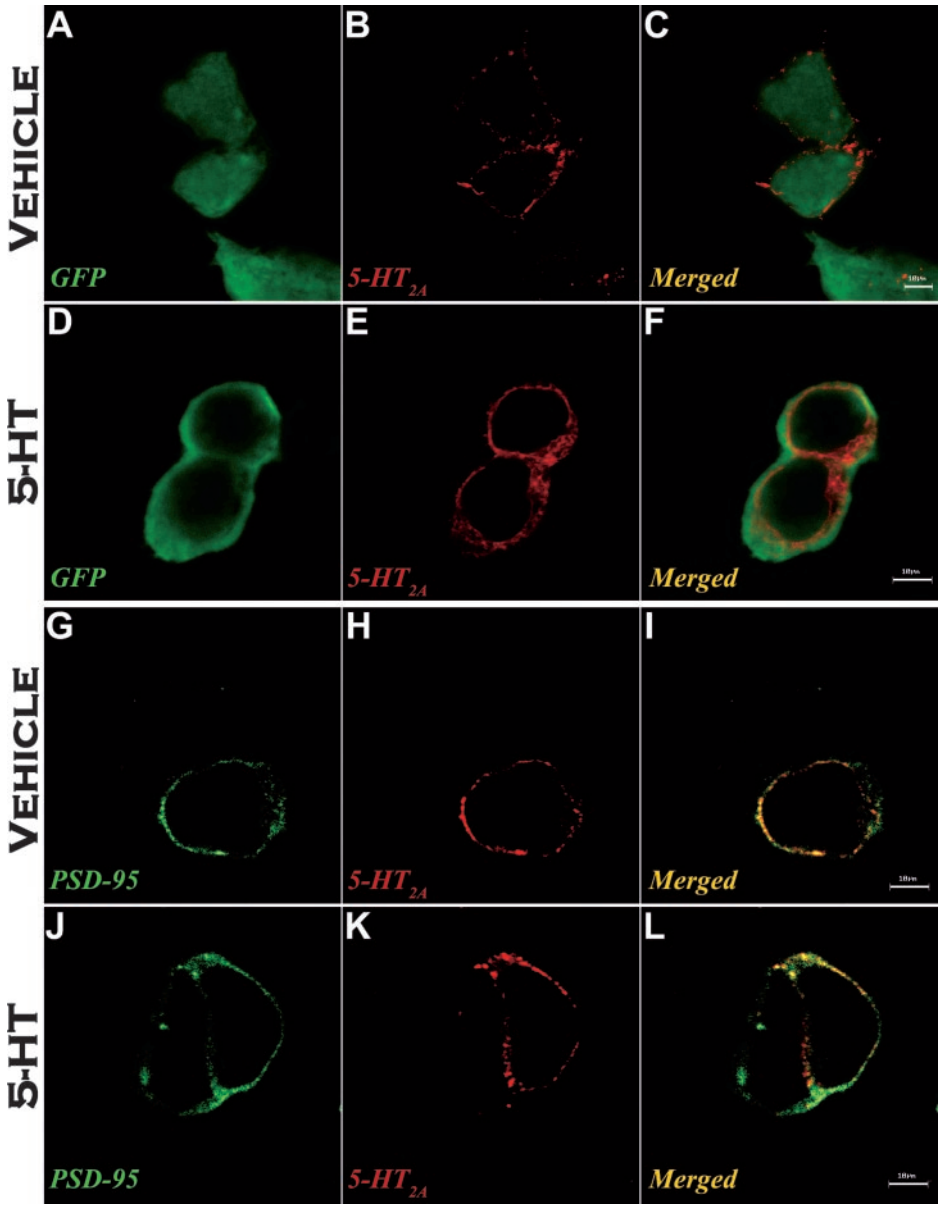

FIG. 5. **PSD-95 inhibits agonist-mediated internalization of 5-HT$_{2A}$ receptors.** For these studies, cells were transiently co-transfected with FLAG-5-HT$_{2A}$ + GFP (*A–F*) or FLAG-5-HT$_{2A}$ + PSD-95-GFP (*G–L*). At 48 h following transfection, cells were incubated with serum-free media for 24 h and then exposed to the vehicle (*A–C* and *G–I*) or 10 μM 5-HT (*D–F* and *J–L*) for 15 min followed by dual-labeling immunofluorescent confocal microscopy (see "Experimental Procedures"). Representative images from several independent experiments are shown. GFP (*A* and *D*) or GFP-tagged PSD-95 (*G* and *J*) are shown in the *green channel*. FLAG-tagged native 5-HT$_{2A}$ receptors (*H* and *K*) are shown in the *red channel*, and merged images are shown in *panels C*, *F*, *I*, and *L*. Scale bars are shown in the merged panels.

PSD-95 Inhibits the Agonist-induced Internalization and Promotes the Cell Surface Clustering of 5-HT$_{2A}$ Receptors—We next investigated whether PSD-95 affects the targeting and trafficking of 5-HT$_{2A}$ receptors. We have previously demonstrated that acute exposure to agonists facilitates the redistribution of 5-HT$_{2A}$ receptors from the plasma membrane to the intracellular pool via receptor internalization (30). As expected, 5-HT induced the rapid internalization of WT 5-HT$_{2A}$ receptors (Fig. 5, *A–C versus D–F*; see Fig. 7 for quantification). Interestingly, PSD-95 significantly inhibited the agonist-induced 5-HT$_{2A}$ receptor internalization (Figs. 5, *G–I versus J–L*, and 7) and promoted the co-clustering (*i.e.* formation of puncta containing both proteins) of 5-HT$_{2A}$ receptors with PSD-95 on the cell surface (Figs. 5, *G–I* (*cross-section view*), and 8, *A–C* (*top view*)).

As an essential control, we found that disrupting the PDZ-binding domain of the 5-HT$_{2A}$ receptor blocked the ability of PSD-95 to inhibit 5-HT$_{2A}$ receptor internalization (Figs. 6, *A–C versus D–F*, and 7). Furthermore, disrupting the PDZ-binding domain attenuated 5-HT$_{2A}$ receptor clustering on the cell surface (Figs. 6, *A–C* (*cross-section view*), and 8, *D–F* (*top view*)). Interestingly, whereas WT 5-HT$_{2A}$ receptors appeared to co-cluster with the membrane-bound PSD-95, mutant 5-HT$_{2A}\Delta 469-471$ receptors did not facilitate the formation of PSD-95-immunoreactive puncta on the cell surface (Figs. 5, *G–I versus J–L*, and 6, *A–C versus D–F*). Taken together, these findings suggest that PSD-95 augments 5-HT$_{2A}$ receptor-mediated signaling at least in part by inhibiting the internalization and promoting the cell surface clustering of 5-HT$_{2A}$ receptors.

DISCUSSION

The major findings of this work are that PSD-95 directly interacts with 5-HT$_{2A}$ receptors *in vitro*, thereby augmenting 5-HT$_{2A}$ receptor signaling, inhibiting the agonist-dependent internalization, and promoting the cell surface clustering of the 5-HT$_{2A}$ receptor. PSD-95 was recently reported to be among the several PDZ domain-containing proteins that physically interact with the closely related 5-HT$_{2C}$ receptor *in vivo* (27), although the physiological significance of the interaction between 5-HT receptors and PDZ domain-containing proteins is largely unknown. Although there is precedence for GPCR signaling being enhanced by another PDZ domain-containing protein (23), this is the first report that PSD-95, a prototypic PDZ

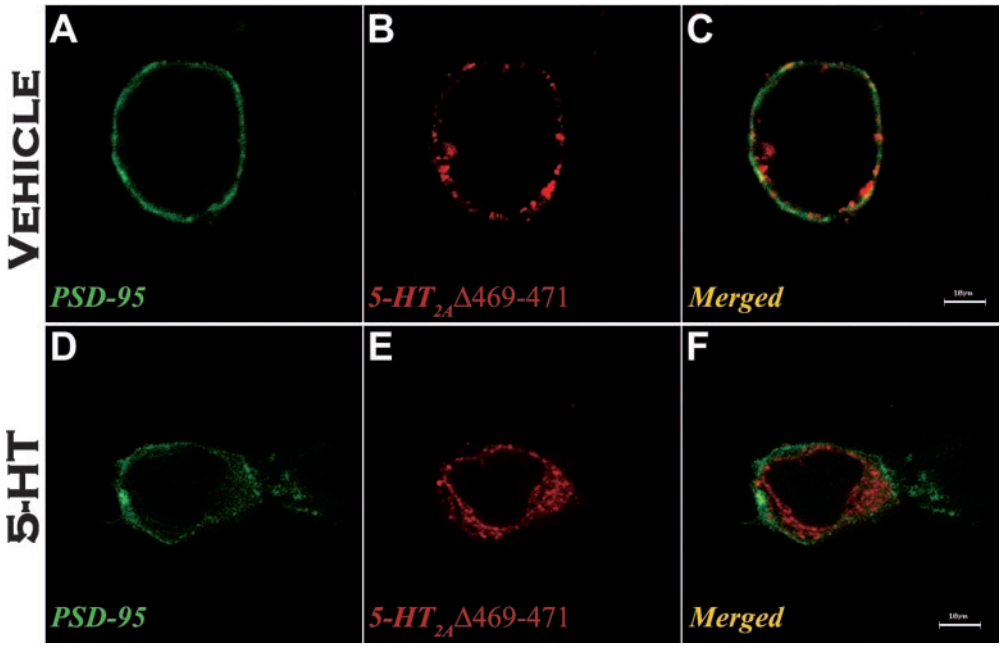

FIG. 6. **Disrupting PSD-95 association with the 5-HT$_{2A}$ receptor restores the agonist-dependent internalization of 5-HT$_{2A}$ receptors in HEK-293 cells.** For these studies, cells were transiently co-transfected with FLAG-5-HT$_{2A}$Δ469–471 and PSD-95-GFP. At 48 h following transfection, cells were incubated in serum-free medium for 24 h and then exposed to vehicle (*A–C*) or 10 μM 5-HT (*D–F*) for 15 min followed by dual-labeling immunofluorescent confocal microscopy (see "Experimental Procedures"). Representative images are shown. GFP-tagged PSD-95 (*A* and *D*) are shown in the *green channel*. FLAG-tagged mutant 5-HT$_{2A}$ receptors that lack the PDZ-binding domain (*B* and *E*) are shown in the *red channel*, and merged images are shown in *panels C* and *F*. Scale bars are shown in the merged panels.

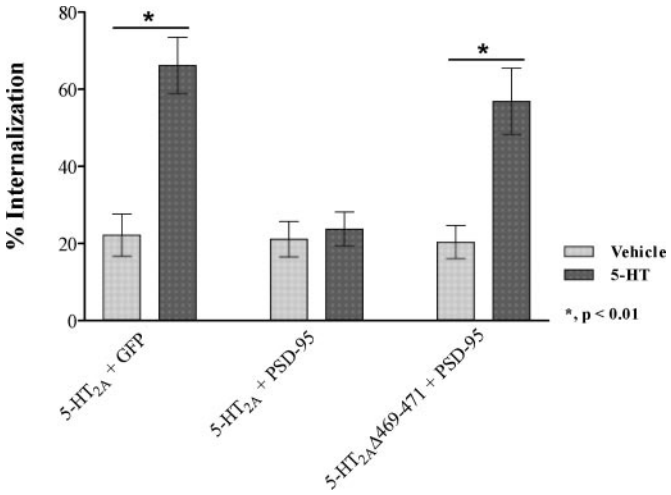

FIG. 7. **PSD-95 inhibits agonist-mediated 5-HT$_{2A}$ receptor internalization-quantification.** Data shown represents the mean ± S.E. of the percent of internalization from at least three independent experiments. The number of cells in which 5-HT$_{2A}$ receptor internalization was quantified (also see "Experimental Procedures") for each condition is the following: FLAG-5-HT$_{2A}$ + GFP (vehicle: n = 52; 5-HT: n = 61), FLAG-5-HT$_{2A}$ + PSD95-GFP (vehicle: n = 89; 5-HT: n = 86), and FLAG-5-HT$_{2A}$Δ469–471 + PSD95-GFP (vehicle: n = 67; 5-HT: n = 69).

domain-containing protein and a major synaptic protein, enhances the signaling of any GPCR. Taken together, these results imply that interactions with PDZ domain-containing proteins may represent a generalized mechanism for modulating GPCR-mediated signal transduction.

PSD-95 is known to associate with a variety of target proteins, including the NMDA receptor (14), β_1-AR (24), neuroligin (42), cysteine-rich interactor of PDZ three (43), and citron (44). Superficially, the association of PSD-95 with the 5-HT$_{2A}$ receptor is reminiscent of PSD-95 interactions with the β_1-AR. Most notably, the extreme carboxyl terminus of the β_1-AR (Ser-Lys-Val) and the 5-HT$_{2A}$ receptor (SCV) shares the same Type I PDZ-binding motif (*X*-Ser/Thr-*X*-Φ) and could conceivably interact with the same PDZ domain on PSD-95. Moreover, PSD-95 inhibits the agonist-induced internalization of both the β_1-AR (24) and the 5-HT$_{2A}$ receptor. On the other hand, we have discovered significant differences. In particular, PSD-95 augments 5-HT$_{2A}$ receptor signaling but has no effect on β_1-AR signaling (24).

The precise mechanism by which PSD-95 augments 5-HT$_{2A}$-mediated signaling is unknown but is probably because of a direct physical interaction. Thus, PSD-95 potentiates the signaling of wild-type 5-HT$_{2A}$ receptors but not mutant 5-HT$_{2A}$ receptors lacking the PDZ-binding domain. Additionally, PSD-95 does not augment basal (constitutive) 5-HT$_{2A}$ receptor signaling, alter agonist potency, perturb the kinetics of agonist-dependent 5-HT$_{2A}$ receptor desensitization, or affect the ability of a constitutively active Gα_q (Gα_q-Q229L) to activate PLC. Taken together, these results are consistent with the notion that PSD-95 augments receptor-effector signaling via a direct physical interaction between 5-HT$_{2A}$ receptors and PSD-95. Based on the present finding that PSD-95 augments 5-HT$_{2A}$ receptor signaling, it is reasonable to speculate that PSD-95 provides scaffolding for G$_q$ and PLC, thereby facilitating the interaction between the 5-HT$_{2A}$ receptor and these downstream signaling molecules. Studies to test this hypothesis are in progress.

We have also found that PSD-95 inhibits the agonist-induced 5-HT$_{2A}$ receptor internalization. A possible mechanism by which PSD-95 inhibits 5-HT$_{2A}$ receptor internalization is that PSD-95 recruits and anchors multiple proteins including the 5-HT$_{2A}$ receptor to the plasma membrane, assembling a complex that in turn prevents the internalization of component molecules. For example, PSD-95 is known to assemble β_1-AR and NMDA receptor in the same complex (24). Consistent with reports of cell surface co-localization between MUPP1 and 5-HT$_{2C}$ receptor (26) and between PSD-95 and β_1-AR (24), 5-HT$_{2A}$ receptors also co-cluster with PSD-95 as the distribu-

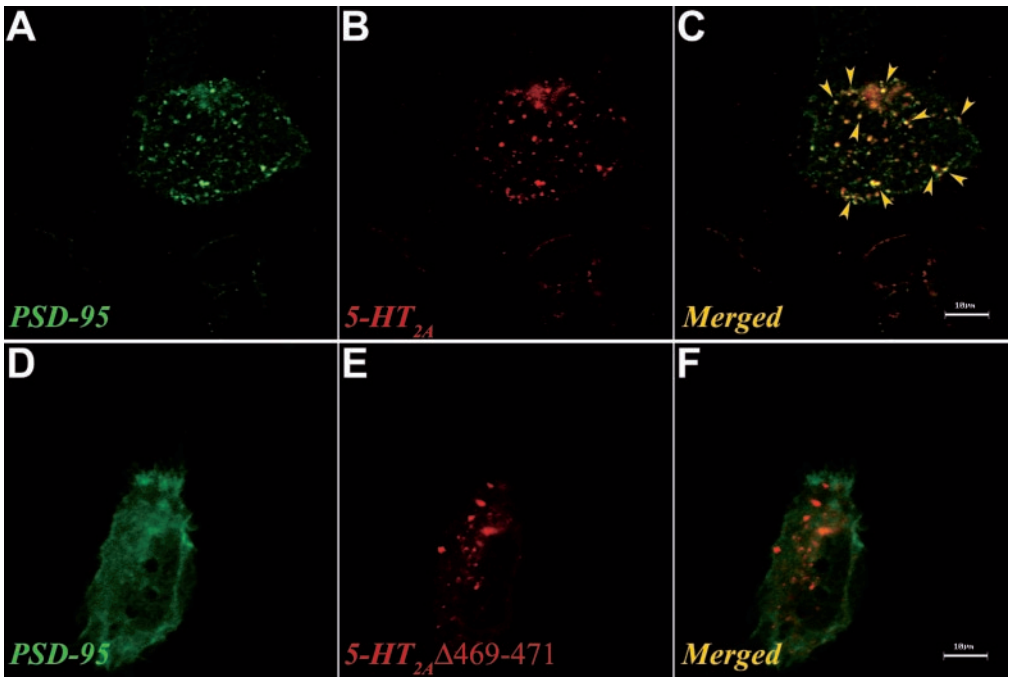

FIG. 8. **PSD-95 co-localizes with 5-HT$_{2A}$ receptors on the cell surface.** For these studies, cells were transiently co-transfected with FLAG-5-HT$_{2A}$ + PSD95-GFP (*A–C*) or FLAG-5-HT$_{2A}$Δ469–471 + PSD95-GFP (*D–F*). At 48 h following transfection, cells were incubated with serum-free media for 24 h and dual-labeling immunofluorescent confocal microscopy was subsequently performed (see "Experimental Procedures"). Images shown here were confocal sections taken at the cell surface as previously detailed (30) and represent images from experiments that were replicated at least three times. GFP-tagged PSD-95 is shown in the *green channel* (*A* and *D*), and FLAG-tagged native (*B*) and the PDZ-binding domain-lacking mutant (*E*) 5-HT$_{2A}$ receptors are shown in the *red channel*. Merged images are shown in *panels C* and *F*. Co-localization is denoted by *orange arrows*. Scale bars are shown in the panels marked *merged*.

tion of PSD-95 on the plasma membrane appears more diffuse in the absence of its association with the 5-HT$_{2A}$ receptor. The co-clustering between 5-HT$_{2A}$ receptors and PSD-95 may reflect the assembly of a multi-protein signaling complex on the cell surface that does not easily internalize following agonist exposure. The net effect in neurons would most probably be an enhancement of 5-HT$_{2A}$-mediated signaling in regions where 5-HT$_{2A}$ receptors and PSD-95 are co-localized.

It is worth noting that PSD-95 inhibits the internalization of the 5-HT$_{2A}$ receptor but does not affect the agonist-dependent desensitization, considering that these regulatory mechanisms for GPCRs (*i.e.* internalization, desensitization, and resensitization) are frequently linked. We have previously demonstrated that both the desensitization and the resensitization of the 5-HT$_{2A}$ receptor are not affected by various well characterized inhibitors of clathrin-mediated endocytosis in HEK-293 cells (35, 41). In addition, it is unlikely that the PDZ-binding domain is involved in agonist-dependent GPCR desensitization because disruption of the PDZ-binding domain does not prevent 5-HT$_{2C}$ receptors from undergoing desensitization (45).

Another potential mechanism for PSD-95-mediated inhibition of 5-HT$_{2A}$ receptor internalization could involve competition for the same binding motif with regulatory molecules that facilitate 5-HT$_{2A}$ receptor internalization. Studies of PSD-95/Kir2.3 and NHE-RF/β_2-AR associations suggest that phosphorylation of the serine or threonine residue at the −2 position of the canonical PDZ-binding domain by G protein-coupled receptor kinases (GRK) may be the regulatory mechanism with which PSD-95 competes (23, 46–48). One such counter-regulatory molecule is GRK5. Upon agonist stimulation, GRK5 phosphorylates the serine residue of the PDZ-binding domain of both β_1-AR and β_2-AR, thereby diminishing receptor association with PDZ proteins (PSD-95 and NHE-RF, respectively) and facilitating receptor internalization (47, 48). Neither GRK5 nor GRK2 is likely to compete with PSD-95 for binding sites on the 5-HT$_{2A}$ receptor, however, since we have demonstrated that 5-HT$_{2A}$ receptors are regulated by GRK2- and GRK5-independent processes (35).

Because PSD-95 becomes membrane-bound following palmitoylation (49), the preferential localization of PSD-95 to the plasma membrane makes it unlikely that PSD-95 could enhance 5-HT$_{2A}$ receptor recycling (50), a possibility that might otherwise confound the interpretation of the present observation. It would be interesting to examine in the future whether an alternatively spliced isoform of PSD-95 (PSD-95β) that lacks palmitoylation sites in the amino terminus and is not membrane-bound (51) could inhibit 5-HT$_{2A}$ receptor internalization.

In summary, we have discovered a novel role for PSD-95 for regulating the activity of GPCRs. PSD-95 augments 5-HT$_{2A}$ receptor-mediated signaling in a way that requires a direct physical interaction between 5-HT$_{2A}$ receptors and PSD-95. This study demonstrates that PSD-95, a component of the neuronal protein sorting apparatus, regulates 5-HT$_{2A}$ receptor signaling at least in part by anchoring 5-HT$_{2A}$ receptors to the cell surface in clusters that are not easily internalized by agonists and possibly by facilitating 5-HT$_{2A}$ receptor interaction with downstream signaling molecules. Because 5-HT$_{2A}$ receptors are responsible for the actions of atypical antipsychotic drugs (4, 5, 37, 52) and most (1, 5) but not all (2, 3) hallucinogens, elucidating the cellular machinery responsible for targeting and regulating 5-HT$_{2A}$ receptors will enhance our understanding of how diverse classes of drugs exert their profound effects on human perception, emotion, and cognition.

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