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Opposite Effects of PSD-95 and MPP3 PDZ Proteins on Serotonin 5-Hydroxytryptamine$_{2C}$ Receptor Desensitization and Membrane Stability

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PSD-95/Disc large/Zonula occludens 1 (PDZ) domain-containing proteins (PDZ proteins) play an important role in the targeting and the trafficking of transmembrane proteins. Our previous studies identified a set of PDZ proteins that interact with the C terminus of the serotonin 5-hydroxytryptamine (5-HT)$_{2C}$ receptor. Here, we show that the prototypic scaffolding protein postsynaptic density-95 (PSD-95) and another membrane-associated guanylate kinase, MAGUK p55 subfamily member 3 (MPP3), oppositely regulate desensitization of the receptor response in both heterologous cells and mice cortical neurons in primary culture. PSD-95 increased desensitization of the 5-HT$_{2C}$ receptor-mediated Ca^{2+} response, whereas MPP3 prevented desensitization of the Ca^{2+} response. The effects of the PDZ proteins on the desensitization of the Ca^{2+} response were correlated with a differential regulation of cell surface expression of the receptor. Additional experiments were performed to assess how PDZ proteins globally modulate desensitization of the 5-HT$_{2C}$ receptor response in neurons, by using a peptidyl mimetic of the 5-HT$_{2C}$ receptor C terminus fused to the human immunodeficiency virus type-1 Tat protein transduction domain, which disrupts interaction between the 5-HT$_{2C}$ receptor and PDZ proteins. Transduction of this peptide inhibitor into cultured cortical neurons increased the desensitization of the 5-HT$_{2C}$ receptor-mediated Ca^{2+} response. This indicates that, overall, interaction of 5-HT$_{2C}$ receptors with PDZ proteins inhibits receptor desensitization in cortical neurons.

INTRODUCTION

The serotonin 5-hydroxytryptamine (5-HT)$_{2C}$ receptor is a G protein-coupled receptor (GPCR) that is exclusively expressed in the CNS. 5-HT$_{2C}$ receptors are distributed throughout various brain regions, including choroid plexus, cerebral cortex, nucleus accumbens, substantia nigra, and striatum (Abramowski *et al*., 1995). 5-HT$_{2C}$ receptors expressed along ascending dopaminergic pathways play a prominent role in the control of mesocorticolimbic and nigrostriatal dopaminergic systems and constitute an important target for the treatment of neuropsychiatric disorders related to dysfunctions of dopaminergic neurons (Di Giovanni *et al*., 1999; Di Matteo *et al*., 1999, 2001; Gobert *et al*., 2000; Alex *et al*., 2005).

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Abbreviations used: GPCR, G protein-coupled receptor; MAGUK, membrane-associated guanylate kinase; MPP3, MAGUK p55 subfamily member 3; PDZ, PSD-95/Disc large/Zonula occludens 1; PSD-95, postsynaptic density-95; Veli3, vertebrate homologue of Lin7 3.

The functional activity of 5-HT$_{2C}$ receptors is regulated by desensitization and resensitization processes. 5-HT$_{2C}$ receptors undergo agonist-dependent desensitization that is associated with G protein receptor kinase (GRK)2-receptor phosphorylation (Berg *et al*., 2001). This step is followed by the binding of β-arrestins to the receptor, which uncouples the receptor from the G protein and initiates its internalization into endosomes (Marion *et al*., 2004; Schlag *et al*., 2004). This phenomenon not only contributes to receptor desensitization but also to receptor dephosphorylation and recycling to the plasma membrane in a fully resensitized state. 5-HT$_{2C}$ receptors also exhibit agonist-independent activity, which is accompanied by constitutive receptor desensitization and internalization (Barker *et al*., 1994; Marion *et al*., 2004). This process is essential for 5-HT$_{2C}$ receptor-mediated control of dopaminergic systems (De Deurwaerdere *et al*., 2004).

Extensive studies on certain GPCRs, such as β-adrenergic receptors have revealed that interaction of their carboxyl (C)-terminal domain with PDZ domain-containing proteins (PDZ proteins) plays a critical role in the regulation of their desensitization/resensitization and trafficking (Bockaert *et al*., 2004; Hall, 2004; Tilakaratne and Sexton, 2005). The PDZ acronym derives from the three first proteins (PSD-95/Disc large/Zonula occludens-1) in which these domains have been described. To date, PDZ proteins are certainly the most

numerous proteins involved in the scaffolding of multiprotein complexes that contribute to the targeting, trafficking, and the fine-tuning of signaling properties of membrane-bound receptors (Nourry *et al*., 2003). 5-HT_{2C} receptors contain a PDZ recognition motif at their extreme C terminus. Deletion of this PDZ-binding motif delays resensitization of the receptor responses in NIH 3T3 fibroblasts (Backstrom *et al*., 2000). To date, several 5-HT_{2C} receptor-accessory proteins have been identified, including a majority of PDZ proteins (Becamel *et al*., 2002, 2004; Ji *et al*., 2006). The functional significance of association of individual PDZ proteins with the receptor is still unknown.

Here, we have analyzed the function of the three main PDZ-binding partners of the 5-HT_{2C} receptor identified in the mouse brain: postsynaptic density-95 (PSD-95), one of the major postsynaptic density PDZ proteins that belongs to the membrane-associated guanylate kinase (MAGUK) scaffolding protein family; MAGUK p55 subfamily member 3 (MPP3) (also designated as Dlg3), a MAGUK of the P55 subfamily that contains a single PDZ domain; and Veli3, one of the vertebrate homologues of the *Caenorhabditis elegans* PDZ protein Lin7. We show that these PDZ proteins differentially modulate desensitization of the receptor Ca^{2+} response in both heterologous cells and cultured cortical neurons, indicating that the functional activity of 5-HT_{2C} receptors is modulated according to the repertoire of PDZ proteins coexpressed with the receptor. Additional experiments were carried out to examine how the interactions between the 5-HT_{2C} receptor and PDZ proteins globally modulate desensitization of the receptor Ca^{2+} effector pathway in cortical neurons, by using an interfering peptide to block receptor–PDZ protein interactions.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Plasmid Vectors, Antibodies, and Peptides

The cMyc-tagged h5-HT_{2C} receptor (pRK5/cMyc-h5-HT_{2C}) and the pRK5/cMyc-h5-HT_{2CSSA} constructs have been described previously (Becamel *et al*., 2001). The 5-HT_{2C} receptor was subcloned from the pRK5/cMyc-h5-HT_{2C} plasmid into the pCMV-Tag 2B Flag epitope tagging vector (Stratagene, La Jolla, CA) with the BamH1/Sal1 restriction sites, yielding the pCMV/Flag-h5-HT_{2C} construct. The 5-HT_{2CDDV} receptor mutant (substitution of Ser458/459 in the PDZ binding motif with aspartate residues to mimic phosphorylation) was generated by QuikChange mutagenesis (Stratagene), with the forward primer 5′-GCGAAAGGATTGACGATGTGTGATCTCGAGTCGACCTGC-3′ and the reverse primer 5′-AATCCTTTCGCTAACCACACTGGAGGGAT-3′.

The Veli3 construct (pRK7/Veli3) has been described previously (Becamel *et al*., 2002). The cDNA encoding PSD-95 (pGW1/cMyc-PSD-95) was provided by Dr. D. S. Bredt (Department of Physiology, University of California, San Francisco, CA) and the cDNA encoding MPP3 (pRK5/cMyc-MPP3) was a generous gift from Dr. B. Margolis (Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI). PDZ1+2 and PDZ1+2+3 truncation mutants of PSD-95 were engineered using pGW1/cMyc-PSD-95 as matrix. Forward and reverse primers were complementary to the region corresponding to the designated truncation and were used to introduce mutations to insert a stop codon by QuikChange polymerase chain reaction (PCR). The amplified PCR products were cut HindIII/Nhe1 and recloned into the pGW1 vector, resulting in vectors encoding cMyc-tagged fusion proteins (corresponding to amino acid positions 1–262 for pGW1/cMyc-PDZ1+2 and 1–420 for pGW1/cMyc-PDZ1+2+3 in the rat PSD-95 protein). The PDZ3 mutant was made from the pGW1/cMyc-PDZ1+2+3 construct. A new ATG initiation codon + an in-frame cMyc epitope tag upstream the PDZ3 was engineered with the forward primer 5′-GGAAAAGCTTATGGAGCAAAAATTAATATCTGAAGAGGACTTACCAAGGCGGATCGTGATCCATCG-3′ (the cMyc sequence is underlined) and the reverse primer 5′-CATAGCTAGCGCCTGCTCCTCTGTGATCCGCTTATTG-3′. The amplified products were cut HindIII/Nhe1 and ligated into the pGW1 plasmid, yielding pGW1/cMyc-PDZ3 (corresponding to amino acid residues 311–420). All constructs were verified by sequencing.

Synthetic small-interfering RNAs (siRNAs) (>95% purity) were purchased from Eurogentec (Seraing, Belgium). The sequences of the three PSD-95 siRNAs are as follows: siRNA1, 5′-CAGCACAUCCCUGGAGAUAUU-3′; siRNA2, 5′-GACAGCAUCCUGUUUGUCAUU-3′; and siRNA3, 5′-GAGAUAAUAGCAUCUACGUUU-3′. The sequences of the three MPP3 siRNAs are as follows: siRNA1, 5′-CUCAGUUAUUUAAUGAAGAUU-3′; siRNA2, 5′-AGCCCUAUGUUAUAUUUGUUU-3′; and siRNA3, 5′-GCCCGAUAACAUCGAUGAGUU-3′. Ca^{2+} imaging experiments were performed with the most efficient siRNAs (siRNA1 and siRNA2 for PSD-95 and MPP3, respectively).

The mouse monoclonal anti-PSD-95 (clone K28/43) antibody was obtained from Upstate Biotechnology (Charlottesville, NC), the rabbit polyclonal anti-Veli3 and anti-Flag antibodies were from Zymed Laboratories (South San Francisco, CA), and the mouse monoclonal anti-Pan-actin was from NeoMarkers (Fremont, CA). The mouse monoclonal anti-cMyc antibody was a gift from Dr. B. Mouillac (Institut de Génomique Fonctionnelle, Montpellier, France). The mouse monoclonal anti-tubulin antibody was a gift from Dr. N. Morin (Centre de Recherches en Biochimie Macromoléculaire, Montpellier, France). The rabbit polyclonal antibodies against 5-HT_{2C} receptors (522 antibody) and MPP3 (CPH8 antibody) have been described previously (Becamel *et al*., 2001; Kantardzhieva *et al*., 2006).

Synthetic peptides (>95% purity) were purchased from Eurogentec. Peptide sequences were as follows: 5-HT_{2C}-Ct, VNPSSVVSERISSV; 5-HT_{2CSSA}-Ct, VNPSSVVSERISSA; 5-HT_{2CDSV}-Ct, VNPSSVVSERIDSV; 5-HT_{2CSDV}-Ct, VNPSSVVSERISDV; 5-HT_{2CDDV}-Ct, VNPSSVVSERIDDV; TAT, YGRKKRRQRRR; TAT-5-HT_{2C}-Ct, YGRKKRRQRRRVVSERISSV; TAT-5-HT_{2CSSA}-Ct, YGRKKRRQRRRVVSERISSA; and TAT-GluR2-Ct, YGRKKRRQRRRVYGIESVKI. The TAT-5-HT_{2C}-Ct peptide and the corresponding peptide lacking the TAT sequence (TAT-empty-5-HT_{2C}-Ct) were synthesized with an additional N-terminal cysteinyl residue and conjugated to 2-[(5-fluoresceinyl)aminocarbonyl]ethyl methanethiosulfonate (MTS-4-fluorescein; Toronto Research Chemicals, North York, Ontario, Canada), to monitor peptide transduction into neurons by fluorescence microscopy. Coupling reaction was performed at 20°C for 24 h (1 mg of peptide + 0.6 mg of MTS-4-fluorescein in 60 μl of acetonitrile and 40 μl of water). Derivatized peptides were purified by reversed phase high-performance liquid chromatography by using a Whatman Partisil ODS 3 Magnum 20 column (10-μm particle size, 22 × 500 mm) with the following conditions: linear gradient of 1%/min of mobile phase B [CF_3CO_2H:MeCN, 0.05:100 (vol/vol)] in mobile phase A [CF_3CO_2H:H_2O, 0.1:100 (vol/vol)] at a flow rate of 10 ml/min. Fractions containing peptides were collected and lyophilized.

Cell Culture and Transfection

COS-7 cells were grown in DMEM supplemented with 10% dialyzed heat-inactivated fetal calf serum and antibiotics. They were transfected at 60–70% confluence either by electroporation for enzyme-linked immunosorbent assay (ELISA), as described previously (Claeysen *et al*., 1999) or by using Lipofectamine 2000 (Invitrogen, Cergy-Pontoise, France) for pull-down, immunoprecipitation, Ca^{2+} imaging, and confocal microscopy experiments, according to the manufacturer's instructions. The DNA ratio used for cotransfection was 1:8 (wild type or mutants 5-HT_{2C} receptor cDNA versus either pGW1/PSD-95, pRK5/cMyc-MPP3, or pRK7-Veli3). Immunofluorescence experiments indicated that under these conditions >95% cells expressing recombinant forms of the 5-HT_{2C} receptor also expressed the cotransfected PDZ protein (our unpublished data).

Primary cultures of cortical neurons were prepared as described previously (Weiss *et al*., 1986). Briefly, cells from the cerebral cortex of 17-d-old Swiss mouse embryos were plated in serum-free medium on either 100-mm culture dishes (20 × 10^6 cells/dish), 12-mm coverslips (2.5 × 10^5 cells/slide) or Lab-Tek II chamber slides (1 × 10^6 cells/well; Nalge Nunc International, Naperville, IL), coated successively with poly-L-ornithine (mol. wt. = 40,000; 15 μg/ml) and 10% fetal calf serum + 1 μg/ml laminin. The culture medium included a 1:1 mixture of DMEM and F-12 nutrient supplemented with 33 mM glucose, 2 mM glutamine, 13 mM $NaHCO_3$, 5 mM HEPES buffer, pH 7.4, 5 IU/ml (5 mg/ml) penicillin-streptomycin, and a mixture of salt and hormones containing 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 60 nM putrescine, and 30 nM Na_2SeO_3. Cultures were maintained for 7 d at 37°C in a humidified atmosphere in 5% CO_2, 94% air and transfected with the cMyc-tagged 5-HT_{2C} receptor alone or cotransfected with cMyc-tagged MPP3 or cMyc-tagged PSD-95 proteins (cDNA ratio, 1:8), by using Lipofectamine 2000. Ca^{2+} imaging experiments were performed 3 d after transfection. At this stage, cultures were shown to contain at least 95% of neurons (Weiss *et al*., 1986).

For the knockdown experiments, COS-7 cells or neurons were cotransfected with the cMyc-tagged 5-HT_{2C} receptor and 200 pmol of either rhodamine-conjugated control, PSD-95, or MPP3 siRNAs by using the JetSI-Endo transfection reagent (Eurogentec). The experiments were performed 24 h after transfection for COS-7 cells and 3 d after transfection for neurons.

RNA Isolation and Reverse Transcription (RT)-PCR

Total RNA extraction (20 × 10^6 neurons) was performed using TRIzol reagent (Invitrogen) as described by the manufacturer. The SUPERSCRIPT first-

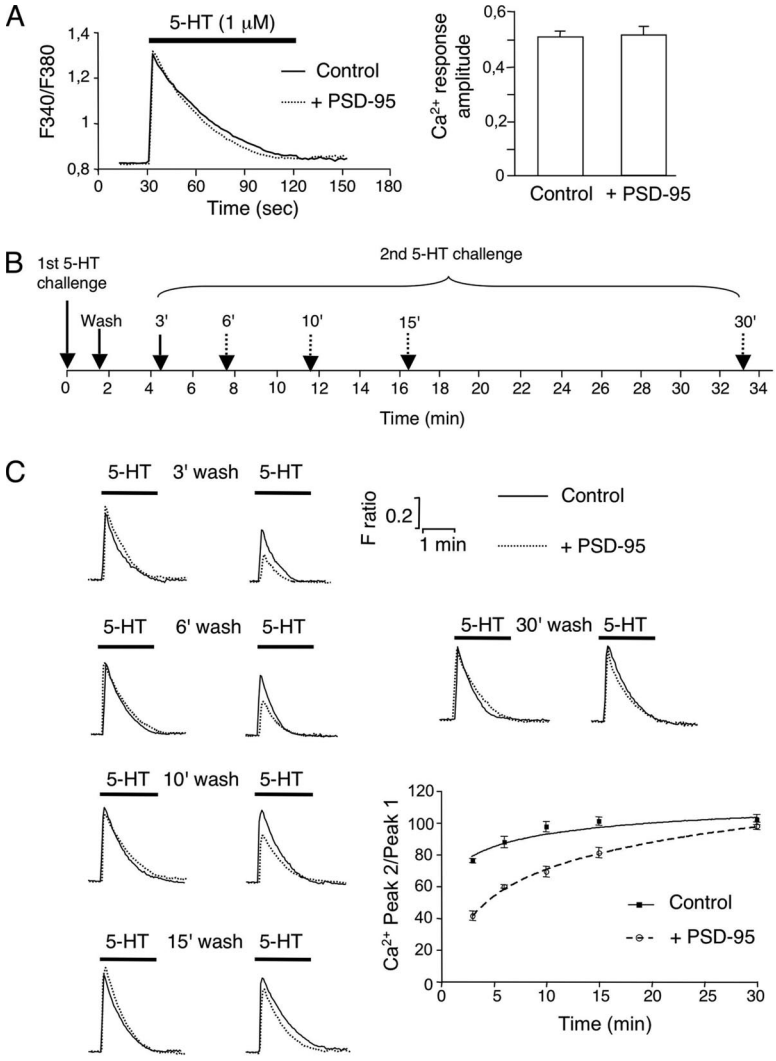

Figure 1. PSD-95 increases the desensitization of the 5-HT$_{2C}$ receptor Ca^{2+} response in COS-7 cells. (A) Representative recordings of variations in intracellular Ca^{2+} levels in response to a 1 μM serotonin 90-s treatment in COS-7 cells transiently transfected with either the cMyc-tagged 5-HT$_{2C}$ receptor alone (solid line) or cotransfected with PSD-95 (dotted line). Before agonist exposure, images were obtained for 30 s to establish a stable baseline Ca^{2+} measurement. Right, mean ± SEM of maximal fluorescence intensity ratios (F340/F380) in 60 cells originating from three different cultures. (B) Schematic representation of protocols used to examine desensitization of the 5-HT$_{2C}$ receptor Ca^{2+} response in COS-7 cells. Cells were challenged twice with 1 μM serotonin for 90 s with 3- to 30-min challenge intervals. (C) Representative recordings of variations in intracellular Ca^{2+} levels in response to two successive applications of serotonin separated by 3-, 6-, 10-, 15-, or 30-min washout periods in COS-7 cells transiently transfected with either the cMyc-tagged 5-HT$_{2C}$ receptor alone or cotransfected with PSD-95. Recordings were interrupted after the first serotonin application, when Ca^{2+} level had returned to baseline and reinitiated 30 s before the second serotonin challenge. Mean values of ratios of the amplitude of the second serotonin Ca^{2+} response to the amplitude of the first response are illustrated for the same challenge intervals. Data are average values from at least 60 cells originating from three independent cultures.

strand synthesis system for RT-PCR (Invitrogen) was used to synthesize cDNA as described by the manufacturer. Thirty cycles of amplifications were performed in a 50-μl reaction volume containing 1.5 μl of each primer (at 10 mM each; sequences available upon request) and 2 μl of RNA as template, and 1 μl of *Taq* polymerase (New England Biolabs, Beverly, MA) and 2.5 μl of dimethyl sulfoxide. The samples were electrophoresed in 3% agarose gels and stained with ethidium bromide.

Peptide Pull-Down Assay

Cultured cells or mice brains were homogenized on ice with lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 1.3% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) (wt/vol), and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Solubilized proteins (5 mg/condition) were incubated with 2 μg of peptides immobilized onto activated CH-Sepharose 4B (GE Healthcare, Orsay, France), as described previously (Becamel *et al.*, 2004). Samples were washed five times with lysis buffer supplemented with 5 M NaCl and once with lysis buffer without NaCl. Proteins retained by affinity were eluted with either SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 30% glycerol, 100 nM dithiothreitol [DTT], and bromophenol blue) for SDS-PAGE and immunoblotting or isoelectrofocusing medium containing 7 M urea, 2 M thiourea, 4% CHAPS, 8 mg/ml preblended ampholines (p*I* = 3.5–9.5; GE Healthcare), 100 mM DTT, 0.2% tergitol NP7, and traces of bromophenol blue for two-dimensional (2-D) electrophoresis. Protein detection, digestion, and identification were carried out using previously described procedures (Becamel *et al.*, 2004).

Immunoprecipitation

CHAPS-soluble proteins from mouse brains or transfected COS-7 cells (1 mg/experiment) were incubated overnight at 4°C with either the anti-5-HT$_{2C}$ receptor 522 antibody or the anti-MPP3 CPH8 antibody (10 mg each). Samples were incubated for 1 h at 4°C with 50 μl of protein A-Sepharose beads (GE Healthcare). After five washes with homogenization buffer, immunoprecipitated proteins were eluted in SDS sample buffer, resolved by SDS-PAGE, and detected by immunoblotting.

Immunoblotting

Proteins resolved by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk diluted in Tris-buffered saline-0.2% Tween and incubated successively with the primary antibodies (anti-Myc, 1:1000; anti-PSD-95, 1:5000; anti-pan actin, 1:2000; anti-tubulin, 1:20; anti-5-HT$_{2C}$ receptor, 1:500; anti-Veli3, 1:11,000; and anti-MPP3, 1:500 in blocking buffer) overnight at 4°C and with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:3000; GE Healthcare) for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence method (ECL detection reagent; GE Healthcare).

Calcium Imaging

Cells were loaded with Fura-2/acetoxymethyl ester (Invitrogen) at a final concentration of 12.5 μM for 30 min at 37°C in Locke's solution containing 140 mM NaCl, 1.2 mM KH_2PO_4, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 1.8 mM $CaCl_2$, and 10 mM glucose. After loading, cells were rinsed twice and incubated for 30 min in dye-free Locke's buffer. Lab-Teks were then placed on the stage of an IX70 Olympus microscope (Olympus, Tokyo, Japan) and continuously superfused with Locke's solution. Imaging of intracellular calcium changes in individual cells treated with agonist was accomplished by ratiometric imaging of Fura-2 fluorescence at 340- and 380-nm excitation using the MetaFluor Imaging system (Molecular Devices, Sunnyvale, CA). Fluorescence was excited by illumination via a 20× water immersion objective with rapid light wavelength switching provided by a DG4 filter wheel

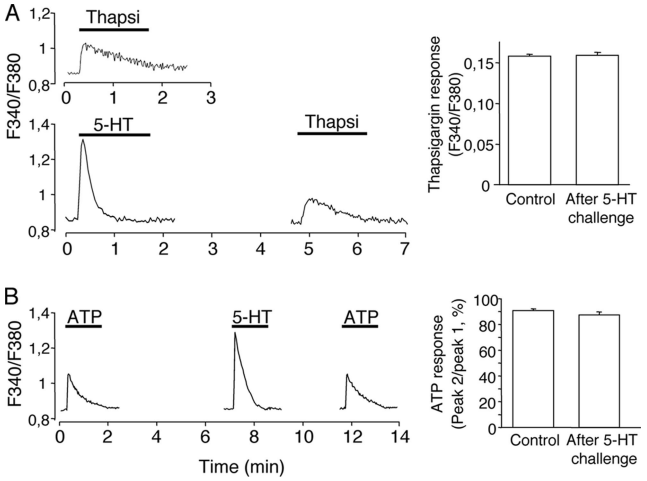

Figure 2. PSD-95-induced inhibition of second serotonin Ca^{2+} responses is not related to intracellular Ca^{2+} store depletion. (A) Representative recordings of variations in intracellular Ca^{2+} levels in response to 10 μM thapsigargin in COS-7 cells cotransfected with the cMyc-tagged 5-HT_{2C} receptor and PSD-95 that have been challenged or not (control) with 1 μM serotonin 3 min before the onset of the thapsigargin treatment. Mean values of thapsigargin response peaks in untreated and serotonin-challenged cells (right) were calculated in 55 cells originating from three independent cultures. (B) Representative recordings of variations of intracellular Ca^{2+} levels in COS-7 cells cotransfected with the cMyc-tagged 5-HT_{2C} receptor and PSD-95 induced by application of first, 10 μM ATP (followed by a 5-min washout), then 1 μM serotonin (followed by a 3-min washout), and finally, ATP. In control experiments, cells were only exposed to two successive ATP applications, separated by a 10-min washout period. This challenge interval was sufficient to obtain fully resensitized ATP responses (our unpublished data). Ratios of Ca^{2+} peak amplitudes between the second and the first ATP response, respectively, were calculated in 60 cells originating from three independent cultures.

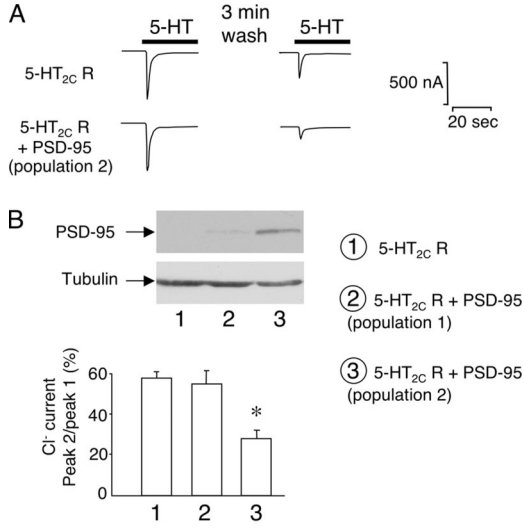

Figure 3. PSD-95 increases the desensitization of 5-HT_{2C} receptors in *X. laevis* oocytes. (A) Representative tracings illustrate Cl^- currents induced by two successive 1 μM serotonin applications (separated by a 3-min washout period) in oocytes injected with cMyc-tagged 5-HT_{2C} receptor cDNA alone or coinjected with PSD-95 cDNA. (B) After recordings, coinjected oocytes were separated into two populations according to the desensitization level of the 5-HT_{2C} receptor-mediated Cl^- current: oocytes exhibiting a desensitization level comparable with that measured in oocytes only injected with the receptor cDNA (population 1) and oocytes exhibiting strongly desensitized secondary Cl^- currents (population 2). Oocytes were then lyzed as described in *Materials and Methods* and expression of PSD-95 was analyzed by immunoblotting. Data represent mean values of ratios of the second to the first Cl^- current amplitude. Each population of oocytes contained ~20 oocytes. *$p < 0.05$ versus oocytes only injected with 5-HT_{2C} receptor cDNA (Student's *t* test).

(Sutter Instrument, Novato, CA) and detected by a charge-coupled device camera under the control of MetaFluor software. Before agonist stimulation, images were obtained for 30 s to establish a stable baseline Ca^{2+} measurement. Our standard protocol consisted of two sequential applications of agonist, separated by 3- to 30-min washouts, as depicted in Figure 1B. Each individual Ca^{2+} trace in the figures is a representative response for a given field of cells. Ca^{2+} responses were averaged from 50 to 80 cells from at least three experiments performed on different cultures, and mean values are expressed as the ratio of the amplitude of the second response to the amplitude of the first response. Statistical significance between mean values was assessed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test or Dunnett's test.

Xenopus Oocyte Electrophysiology

Ovary lobes were surgically excised from *Xenopus laevis* under 0.1% tricaine anesthesia. Follicle layers were removed from oocytes by a 1 mg/ml collagenase A treatment in OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) for 90 min. Stage V and VI oocytes were microinjected with 20 ng of cDNA/oocyte encoding the cMyc-tagged 5-HT_{2C} or 5-HT_{2CSSA} receptor with/without the cDNA of PSD-95 (ratio between receptor and PSD-95 cDNAs, 1:8). Oocytes were stored in an incubator at 18°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.45, supplemented with 2.5 mM sodium pyruvate and 10 μg/ml gentamicin). Two days after injection, two-electrode voltage-clamp recordings were performed to analyze the 5-HT_{2C} receptor-induced Cl^- currents, by using a GeneClamp500 amplifier and pCLAMP software version 7 (Molecular Devices). Whole-cell Cl^- currents, induced by responses to two successive 1 μM serotonin applications (30 s each), separated by 3- to 10-min washout periods in ND96, were monitored at a holding potential of −60 mV.

Oocytes coinjected with 5-HT_{2C} receptor and PSD-95 cDNAs were divided into two populations according to the level of response recovery measured 3 min after a first serotonin challenge (population 1: Cl^- currents [I_{Cl}] amplitude ratio ~50%; and population 2: I_{Cl} amplitude ratio <30%). Each pool of oocytes was lysed in a solubilizing buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, and 5 mM $Na_4P_2O_7$ and then centrifuged at 10,000 × *g* at 4°C for 5 min. The upper aqueous phase was collected and subjected to SDS-PAGE (gels were loaded with ~3 oocytes/lane) and assayed for PSD-95 expression.

Cell Surface ELISA

Colorimetric cell surface ELISA was performed at room temperature under nonpermeant conditions as described previously (Kniazeff *et al*., 2004). COS-7 cells grown in 96-well culture dishes were washed once with phosphate-buffered saline (PBS), and fixed for 10 min with 4% paraformaldehyde in PBS, washed twice and then blocked for 30 min in PBS containing 1% fetal calf serum (blocking solution). Cells were incubated for 30 min with primary antibodies (polyclonal anti-Flag [1:3000] in blocking solution), washed five times for 20 min with blocking solution, and then incubated for 30 min with secondary antibodies (horseradish peroxidase-conjugated secondary antibodies, 1:5000). After three 5-min washes with blocking solution and three 5-min washes in PBS, chemiluminescence substrate was added to the wells (Supersignal ELISA Femto; Pierce Chemical, Rockford, IL) and immunoreactivity was detected at 492 nm with a Wallac Victor2 luminescence counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Control experiments were performed by omitting the primary antibody or using nontransfected cells, and this background was subtracted from the mean values. Values were also normalized to total protein amount. For each data point, three experiments were averaged, and results were analyzed using ANOVA, followed by Student-Newman-Keuls test.

Immunocytochemistry and Confocal Microscopy

Cells were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. They were washed three times with 0.1 M glycine and permeabilized with 0.1% (wt/vol) Triton X-100 for 5 min. Cells were then incubated with blocking buffer (10% bovine serum albumin [BSA] in PBS) for 30 min at 37°C and incubated overnight at 4°C with the primary antibody (anti-Flag, 1:1000; anti-Myc, 1:1000; anti-PSD-95, 1:1000; and anti MPP3, 1:500) in PBS supplemented with 3% BSA. Cells were washed three times with blocking buffer and incubated for 1 h at room temperature with Alexa green-labeled or Cy3-labeled anti-mouse or anti-rabbit antibodies (1:2000 dilution in PBS + 3% BSA). After three washes, the cells were mounted on

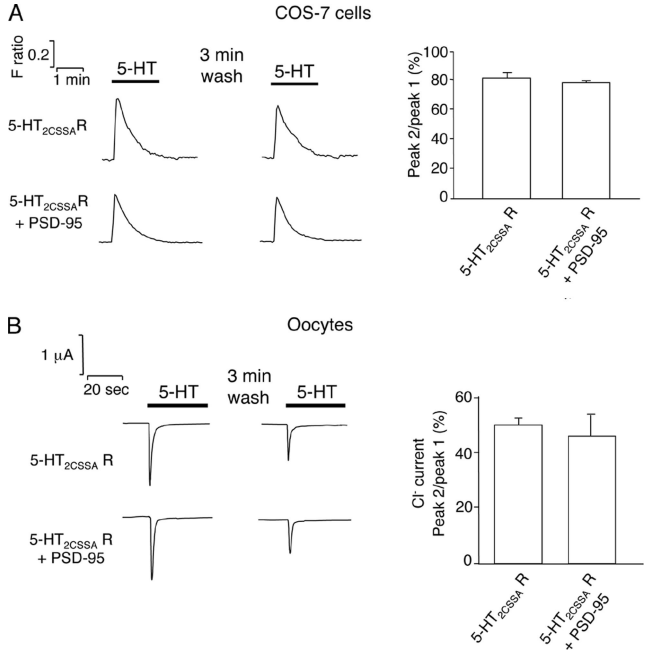

Figure 4. PSD-95-induced modulation of 5-HT$_{2C}$ receptor desensitization is mediated by PDZ-based interactions. (A) Representative recordings of variations in intracellular Ca^{2+} levels in response to two successive applications of 1 μM serotonin separated by a 3-min washout in COS-7 cells transiently transfected with either the cMyc-tagged 5-HT$_{2C}$ receptor mutated on its PDZ binding motif (5-HT$_{2CSSA}$) alone or cotransfected with PSD-95. Data represent mean values of ratios of second to first Ca^{2+} peak responses, calculated from at least 60 cells originating from three independent cultures. (B) Representative Cl^- currents induced by two successive 1 μM serotonin applications (separated by a 3-min washout period) in oocytes injected with the cMyc-tagged 5-HT$_{2CSSA}$ receptor cDNA alone or coinjected with PSD-95 cDNA. Data represent mean values of ratios of the second to the first Cl^- current amplitude measured in a population of 15 oocytes. Note that the ratios of second to first Cl^- current amplitudes were in the same range in all oocytes coinjected with the 5-HT$_{2CSSA}$ receptor and PSD-95 cDNAs as that measured in oocytes expressing only the 5-HT$_{2CSSA}$ receptor.

glass slides in Mowiol 4.88. Observation of fluorescent staining was performed with either a Leitz DMRB microscope (Leica, Wetzlar, Germany) equipped for epifluorescence to monitor knockdown of neuronal PDZ proteins or a Zeiss LSM 510 META confocal system (Carl Zeiss, Jena, Germany) to analyze 5-HT$_{2C}$ receptor expression at the plasma membrane. Series of optical sections were collected with a step of 0.40 μm. Images were collected sequentially to avoid cross-contamination between the fluorochromes and scanned at 1024 $\times$ 1024 pixel resolution.

RESULTS

Effect of PSD-95 on Desensitization of the 5-HT$_{2C}$ Receptor-mediated Ca^{2+} Response in Heterologous Cells

We first examined whether interaction of the 5-HT$_{2C}$ receptor with PSD-95, one of the major postsynaptic scaffolding proteins, alters the efficacy of receptor signal transduction in transfected COS-7 cells. The 5-HT$_{2C}$ receptor is a G_q-coupled receptor that activates phospholipase C and induces intracellular Ca^{2+} mobilization. The effect of PSD-95 coexpression on 5-HT$_{2C}$ receptor-mediated Ca^{2+} responses was thus assessed by performing Fura-2 Ca^{2+} imaging. Receptor/PSD-95-cotransfected cells exhibited robust and transient Ca^{2+} peaks of similar amplitudes and decay rates (identical half-lives of ~30 s and a return to basal Ca^{2+} level in <90 s) as cells expressing only receptor in response to a first serotonin application (Figure 1A). No difference in the latency of the Ca^{2+} response was observed between both cell populations. These results indicate that PSD-95 does not modify the efficacy of 5-HT$_{2C}$ receptor signaling triggered by a single serotonin challenge.

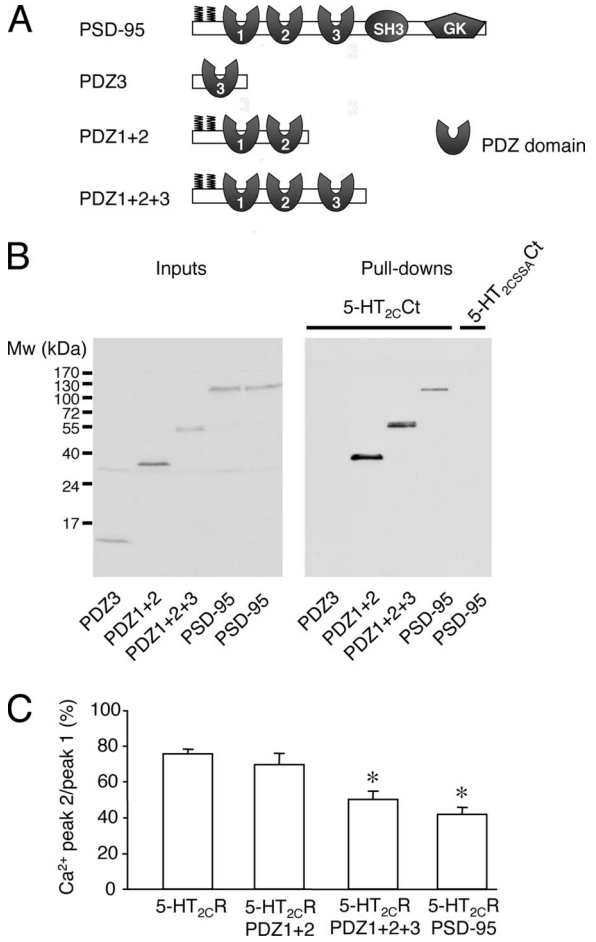

Figure 5. PSD-95 binds to the 5-HT$_{2C}$ receptor via its first two PDZ domains, but the third PDZ domain is required for PSD-95-induced increase in receptor desensitization. (A) Schematic representation of the domain structures of PSD-95 and of the PSD-95 mutants tested. SH3, Src homology 3 domain; GK, guanylate kinase domain. (B) Protein extracts from COS-7 cells transfected with constructs encoding cMyc-tagged fusion proteins corresponding to either the first two PDZ domains of PSD-95 (PDZ1+2), the third PDZ domain (PDZ3), the three PDZ domains (PDZ1+2+3), or the entire protein were incubated with a peptide corresponding to the 14 C-terminal residues of the 5-HT$_{2C}$ receptor immobilized onto Sepharose beads (5-HT$_{2C}$-Ct). Protein extracts from COS-7 cells transfected with the cMyc-tagged PSD-95 construct were also incubated with an immobilized mutant peptide in which the C-terminal valine residue was mutated into an alanine (5-HT$_{2CSSA}$-Ct). Proteins retained by affinity were run on SDS-PAGE, transblotted onto nitrocellulose, and probed with an anti-cMyc antibody. Inputs (left gel) represent 2.5% of total protein amount used for the pull-downs (right gel). (C) COS-7 cells transfected with the indicated constructs were challenged twice with 1 μM serotonin, with a 3-min washout between the serotonin challenges. Data represent mean values of recovery ratios calculated by dividing the height of the second Ca^{2+} peak by the height of the first peak, calculated from at least 60 cells originating from three independent cultures. *$p < 0.01$ versus cells only transfected with the 5-HT$_{2C}$ receptor.

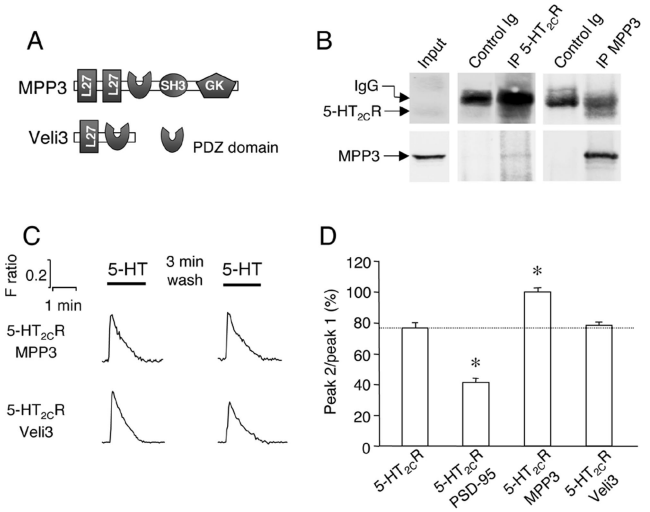

Figure 6. PDZ proteins differentially modulate the desensitization of the 5-HT_{2C} receptor-mediated Ca^{2+} response. (A) Schematic representation of the domain structures of MPP3 and Veli3. SH3, Src homology 3 domain; GK, guanylate kinase domain; L27, L27 domain. (B) Association of MPP3 protein with 5-HT_{2C} receptors in mice brain. Solubilized protein extracts from mice brain were immunoprecipitated with either the anti-5-HT_{2C} receptor or the anti-MPP3 antibody. Coimmunoprecipitated proteins were analyzed by Western blotting using antibodies raised against the MPP3 protein and the 5-HT_{2C} receptor. Input (CHAPS-soluble extract) represents 10% of the total protein used for the immunoprecipitation. (C) Representative recordings of variations in intracellular Ca^{2+} levels in response to two successive applications of 1 μM serotonin separated by a 3-min washout period in COS-7 cells cotransfected with the cMyc-tagged 5-HT_{2C} receptor and either MPP3 or Veli3. (D) COS-7 cells transfected with the indicated constructs were challenged twice with serotonin. Data represent mean values of ratios of the second to the first Ca^{2+} peak response, calculated from at least 60 cells originating from three independent cultures. The dotted line represents the desensitization level measured in cells only expressing the receptor (control). *$p < 0.01$ versus cells transfected only with the 5-HT_{2C} receptor.

We next examined the effect of PSD-95 on the dynamics of desensitization/resensitization of the 5-HT_{2C} receptor response by performing Fura-2 Ca^{2+} imaging again, because previous findings revealed that these processes are rapid, occurring within a time frame of several minutes (Backstrom *et al*., 2000). We used a protocol consisting of two successive serotonin challenges (90 s each) separated by washout periods of increasing duration (3–30 min; Figure 1B), and we then calculated recovery ratios by dividing the amplitude of the second response by the amplitude of the first. In COS-7 cells transfected with only 5-HT_{2C} receptors, a second serotonin challenge after a 3-min washout period, produced an attenuated (desensitized) Ca^{2+} response, with an amplitude of 75.4 ± 2.3% of the initial response (Figure 1C). Thereafter, the 5-HT_{2C} receptor Ca^{2+} response recovered rapidly, a fully resensitized response (97.5 ± 3.2% of the initial response) being achieved after a 10-min washout period (Figure 1C). In cells coexpressing PSD-95, the amplitude of Ca^{2+} responses evoked by a second serotonin application was markedly reduced, relative to cells transfected with the receptor alone, for challenge intervals ranging from 3 to 15 min (Figure 1C). The decreased recovery ratio in PSD-95–cotransfected cells was not because of depletion of intracellular Ca^{2+} stores. Indeed, a first application of serotonin to COS-7 cells cotransfected with the receptor and PSD-95 did not reduce intracellular Ca^{2+} mobilization induced by a subsequent exposure (after a 3-min washout) to 10 μM thapsigargin, a Ca^{2+} ATPase inhibitor (Figure 2A). Thapsigargin-evoked responses with or without serotonin pretreatment were compared in different cells because this compound induces irreversible depletion of inositol trisphosphate-sensitive intracellular Ca^{2+} stores. In the same manner, Ca^{2+} mobilization induced by 10 μM ATP in COS-7 cells via endogenous G_q-coupled P2Y receptors (Ishii *et al*., 1996) was not altered by a serotonin pretreatment (after a 3-min washout; Figure 2B). It is important to note that COS-7 cells exhibited reduced thapsigargin and ATP-evoked Ca^{2+} responses for washout periods shorter than 3 min (our unpublished data), precluding genuine determination of receptor desensitization rate at shorter challenge intervals. Thus, the standard challenge interval protocol of our study was chosen as 3 min for further experiments.

We next examined whether PSD-95 also modulates the desensitization of the Ca^{2+} response mediated by 5-HT_{2C} receptors expressed in *Xenopus* oocytes, by recording I_{Cl} currents with the two-electrode voltage-clamp technique. Similarly to what we observed in COS-7 cells, a second serotonin application yielded a decreased I_{Cl}, compared with the initial response, which depended on the challenge interval (I_{Cl} amplitude ratio, calculated by dividing the amplitude of the second current response by the first current response, was 58 ± 3% for a 3-min challenge interval). Coinjecting oocytes with the cDNA encoding PSD-95 did not significantly modify the mean I_{Cl} amplitude induced by a first serotonin challenge (Figure 3A). Surprisingly, in coinjected oocytes, the I_{Cl} ratio was either unaffected or markedly reduced, in comparison with the oocytes injected with receptor alone. We postulated that this variability was related to differential PSD-95 expression. Thus, oocytes were divided into two populations according to their I_{Cl} ratio. Immunoblotting experiments revealed a strong expression of PSD-95 in oocytes exhibiting a reduced I_{Cl} ratio, whereas negligible PSD-95 amounts were found in oocytes with I_{Cl} ratios similar to those measured in oocytes solely injected with the cDNA encoding 5-HT_{2C} receptor (Figure 3B). These results indicate that the coexpression of PSD-95 evokes an inhibition of the 5-HT_{2C} receptor response recovery in oocytes and that modulation of the 5-HT_{2C} receptor activity by PSD-95 does not depend on the expression model used.

Characterization of the PDZ-based Interaction Involved in Modulation of 5-HT_{2C} Receptor Desensitization by PSD-95

Unlike for the wild-type receptor, secondary responses evoked by serotonin were not inhibited by the coexpression of PSD-95 in COS-7 cells transfected with the 5-HT_{2C} receptor mutated on the terminal valine residue (5-HT_{2CSSA}) (Figure 4A), which is critical for interaction with target PDZ proteins, including PSD-95 (Figure 5B). This mutation did not modify the amplitude or the decay rate of the Ca^{2+} response induced by the initial serotonin challenge. In the same manner, PSD-95 did not alter the desensitization level of I_{Cl} in oocytes pretreated with serotonin that had been injected with 5-HT_{2CSSA} receptor cDNA (Figure 4B). These observations indicate that the effect of PSD-95 on the desensitization of the 5-HT_{2C} receptor Ca^{2+} response depends on PDZ-based interactions. PSD-95 contains three PDZ domains. Previous studies have revealed that the first two PDZ domains (PDZ1/2) and the third PDZ domain (PDZ3) of PSD-95 bind to different target PDZ binding motifs (Niethammer *et al*., 1998). To determine which PDZ domain(s) is responsible for the binding to the 5-HT_{2C} receptor C

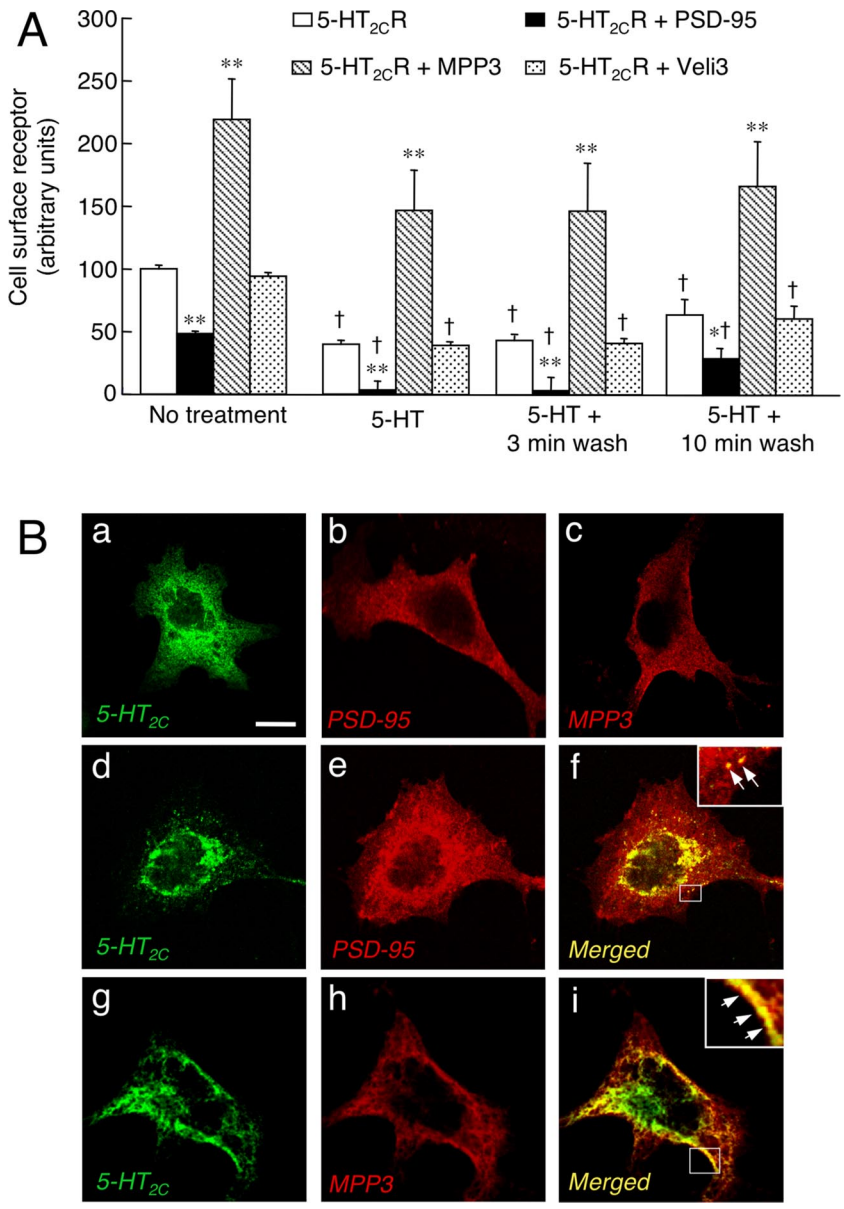

Figure 7. PDZ proteins differentially affect cell surface expression of the 5-HT$_{2C}$ receptor. COS-7 cells were transiently transfected with either the Flag-tagged 5-HT$_{2C}$ receptor alone, or cotransfected with either PSD-95, MPP3, or Veli3 constructs. (A) Quantification of cell surface expression of receptors was performed by ELISA in either untreated cells or cells exposed to serotonin (for 90 s). When indicated, serotonin exposure was followed by a 3- or 10-min washout. Data are average values obtained in three experiments performed in quadruplicate on different sets of cultured cells. * and **p $<$ 0.01 and p $<$ 0001, respectively, versus cells transfected only with 5-HT$_{2C}$ receptors; †p $<$0.01, versus corresponding untreated cells. (B) Immunofluorescence staining of Flag-tagged 5-HT$_{2C}$ receptors (green channel), cMyc-tagged MPP3 and PSD-95 (red channel) in single (a–c) or cotransfected (d–i) COS-7 cells is illustrated. Cells were examined by confocal laser scanning microscopy. Representative images from several independent experiments are shown. Merged images were magnified to show colocalization of 5-HT$_{2C}$ receptors with PSD-95 in cytoplasmic punctate structures (f) and their colocalization with MPP3 at the cell surface (i). Bar, 10 μm (a–i).

terminus and which is involved in the modulation of receptor desensitization, truncated forms of PSD-95 corresponding either to the first two PDZ domains (PDZ1+2) or PDZ3 were expressed in COS-7 cells, and their interaction with the 5-HT$_{2C}$ receptor C terminus was examined in pull-down experiments using a 5-HT$_{2C}$ receptor C-terminal peptide as bait. As shown in Figure 5B, the 5-HT$_{2C}$ receptor C terminus robustly bound to PDZ1+2, but it did not detectably associate to PDZ3. We next examined the ability of PDZ1+2 to modulate desensitization of 5-HT$_{2C}$ receptor Ca^{2+} responses. Interestingly, cotransfecting PSD-95 PDZ1+2 did not decrease the Ca^{2+} response evoked by a second serotonin challenge (Figure 5B), indicating that other PSD-95 regions are responsible for the modulation of the 5-HT$_{2C}$ receptor signaling. Moreover, secondary serotonin responses were decreased in cells cotransfected with a PDZ1+2+3 construct (PSD-95 truncated after the third PDZ domain) to a similar extent as that observed in cells expressing the entire PSD-95 protein.

Effects of MPP3 and Veli3 Proteins on the Desensitization of 5-HT$_{2C}$ Receptor-mediated Ca^{2+} Responses

We investigated whether MPP3, another major PDZ binding partner of the 5-HT$_{2C}$ receptor identified in our previous studies, modulated receptor function in the same way. We first demonstrated that, like PSD-95 (Becamel *et al.*, 2004), MPP3 interacts with the 5-HT$_{2C}$ receptor in vivo. Indeed, MPP3 protein coimmunoprecipitated with the 5-HT$_{2C}$ receptor in brain samples, and, conversely, the receptor was coimmunoprecipitated with a MPP3 antibody (Figure 6B). We next examined the effect of MPP3 overexpression on the desensitization of the receptor Ca^{2+} effector pathway. The amplitude and the rate of decay of the initial Ca^{2+} response evoked by a first serotonin challenge were identical in MPP3-cotransfected COS-7 cells relative to cells expressing only 5-HT$_{2C}$ receptors. After a 3-min washout period, the mean recovery ratio was 100% in receptor/MPP3-cotransfected cells (Figure 6, C and D), whereas the recovery ratio of cells transfected with the receptor alone for the same wash-

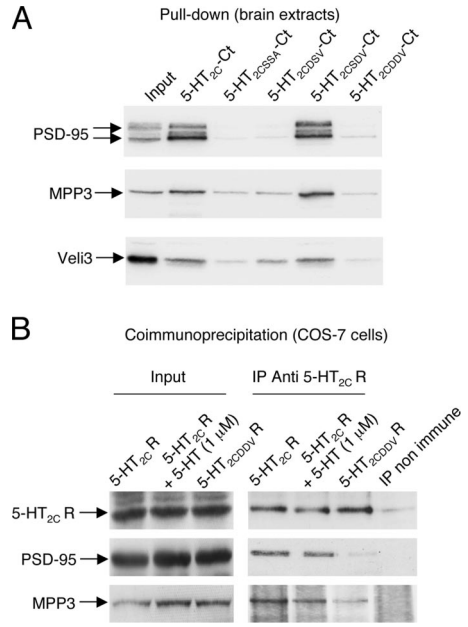

Figure 8. Phosphorylation of serine residues located in the 5-HT_{2C} receptor PDZ binding motif does not differentially affect its interaction with PDZ proteins. (A) Mice brain extracts were incubated with immobilized peptides encompassing the 14 C-terminal residues of the 5-HT_{2C} receptor or mutant peptides in which residues of the PDZ ligand were mutated (V460A, S458D, S459D, or S458D/S459D). Proteins retained by affinity were analyzed by immunoblotting using antibodies raised against PSD-95, MPP3, and Veli3. (B) Proteins of COS-7 cells cotransfected with the 5-HT_{2C} receptor and either PSD-95 or MPP3 and treated or not with 1 mM serotonin for 15 min were immunoprecipitated with the anti-5-HT_{2C} receptor 522 antibody. Coimmunoprecipitated proteins were analyzed by Western blotting by using antibodies raised against cMyc, PSD-95, and MPP3. Inputs represent 2 and 10% of the total protein amount used for pull-downs and immunoprecipitations, respectively. The data illustrated are representative of three independent experiments.

out duration was only 75% (Figures 1C and 6D). This indicates that MPP3 prevents desensitization of the 5-HT_{2C} receptor Ca^{2+} response, contrasting to the effect of PSD-95. We also examined the effect of the coexpression of Veli3, another major PDZ protein that associates with the receptor in vivo (Becamel *et al*., 2002). We found that desensitized 5-HT_{2C} receptor Ca^{2+} responses were undistinguishable between cells coexpressing Veli3 and cells expressing only receptor (Figure 6, C and D). Together, these results indicate that PDZ proteins produce a differential modulation of desensitization of 5-HT_{2C} receptor Ca^{2+} signaling.

Effects of PDZ Proteins on Cell Surface Expression of 5-HT_{2C} Receptors

To determine whether these differential effects are related to opposite regulation of receptor trafficking in and out of the plasma membrane, we examined the effect of coexpressing each PDZ protein on cell surface expression of the 5-HT_{2C} receptor by using an ELISA assay and immunocytochemistry in combination with fluorescence confocal microscopy. In unstimulated cells, coexpression of PSD-95 and MPP3 decreased and increased, respectively, the 5-HT_{2C} receptor cell surface expression, whereas Veli3 did not modify its cellular distribution compared with cells transfected only with 5-HT_{2C} receptors, as assessed by ELISA (Figure 7A). The differential effects of both MAGUKs on receptor expression at the plasma membrane were confirmed by dual-labeling immunofluorescent confocal microscopy. Flag-tagged 5-HT_{2C} receptors showed a wide distribution on membrane-type structures, including mainly intracellular membranes, in cells expressing the receptor alone (Figure 7B, a). In cells coexpressing the Flag-tagged 5-HT_{2C} receptor and PSD-95, most of receptor immunostaining was found to be intracellular, with a prominent labeling in the perinuclear region and punctate structures, where the receptor was colocalized with PSD-95 (Figure 7B, d–f). In contrast, when coexpressed with MPP3, a large fraction of the Flag-tagged 5-HT_{2C} receptor was recruited to the plasma membrane, exhibiting strong colocalization with MPP3 (Figure 7B, g and h).

We next quantified cell surface expression of the Flag-tagged 5-HT_{2C} receptor after an acute agonist exposure and within the time frame of resensitization of the receptor-mediated Ca^{2+} response observed in our Fura-2 imaging experiments, by using the ELISA assay. Serotonin stimulation of cells transfected only with 5-HT_{2C} receptors decreased the pool of cell surface receptors (Figure 7A). A dramatic internalization of 5-HT_{2C} receptors in response to serotonin application was seen in cells coexpressing the receptor and PSD-95 (Figure 7A). This effect of PSD-95 is reminiscent to the increased desensitization observed in the Ca^{2+} imaging experiments in the presence of this PDZ protein. In contrast, in cells coexpressing the receptor and MPP3, which displayed elevated cell surface receptor expression before treatment, we observed only a slight and not significant agonist-induced internalization of the receptor (Figure 7A). Cotransfecting cells with Veli3 did not modify receptor redistribution after agonist exposure (Figure 7A). Partial recovery in the level of cell surface receptors was observed after a 10-min serotonin washout (Figure 7A). This recovery was of similar magnitude in cells expressing either the 5-HT_{2C} receptor alone, or the receptor with PSD-95, suggesting that association of the receptor with PSD-95 does not alter the rate of receptor recycling to the plasma membrane. Together, our results indicate that the interaction of 5-HT_{2C} receptors with PSD-95 facilitates constitutive and agonist-dependent receptor internalization, whereas their association with MPP3 increases the membrane stability of the receptor.

Effect of 5-HT_{2C} Receptor Phosphorylation on Receptor-PDZ Protein Interactions

A previous study indicated that 5-HT_{2C} receptors are phosphorylated on two serine residues located in the PDZ binding motif (SSV) upon agonist treatment and that phosphorylation of the serine at −2 position results in a loss of the interaction between the receptor and the multi-PDZ protein MUPP1 (Parker *et al*., 2003). To determine the effect of the phosphorylation of these serine residues on the interaction with PSD-95, MPP3, and Veli3, we first performed pull-down experiments by using mutated peptides in which serine residues were substituted with aspartates to mimic phosphorylation. Substituting the serine located in the −2 position in the receptor C-terminal tail with an aspartate strongly decreased interaction of the receptor C terminus with PSD-95, MPP3, and Veli3 (Figure 8A). In contrast, mutating the serine at the −1 position did not alter the recruitment of either one of these proteins, consistent with the findings of Parker *et al*. (2003). The double substitution (5-HT_{2CDDV}) yielded an even stronger inhibition of the interactions, compared with that found with the single mutation at the −2 position (Figure 8A). The decreased ability of the 5-HT_{2CDDV} receptor to bind to PDZ proteins in living cells was confirmed by coimmunoprecipitation experiments

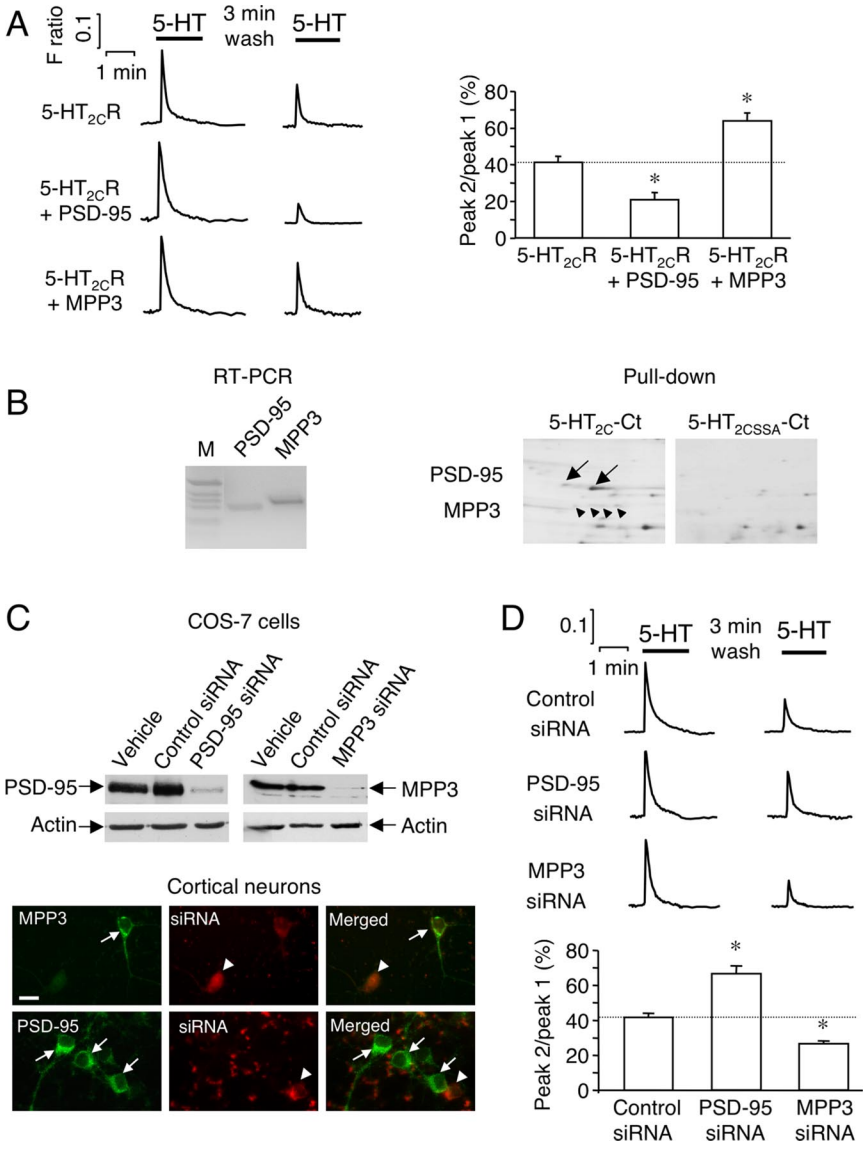

Figure 9. PDZ proteins induce opposite regulation of the desensitization of the 5-HT$_{2C}$ receptor Ca^{2+} response in cortical neurons. (A) Representative recordings of variations in intracellular Ca^{2+} concentration in response to two successive applications of 1 μM serotonin separated by a 3-min washout period in cultured cortical neurons overexpressing the cMyc-tagged 5-HT$_{2C}$ receptor and either MPP3 or PSD-95 proteins. The histogram represents mean values of ratios between the second and the first Ca^{2+} peak response in the corresponding neurons, calculated from at least 60 cells originating from three independent cultures. *$p < 0.01$, versus neurons that do not overexpress PDZ proteins. (B) Identification of PSD-95 and MPP3 as two major PDZ binding partners of 5-HT$_{2C}$ receptors in the cultures. Left, RT-PCR showing expression of PSD-95 and MPP3 mRNAs in neurons. M, DNA markers. Right, areas of 2-D gels showing the recruitment of the two PDZ proteins by the 5-HT$_{2C}$ receptor C-terminal peptide (5-HT$_{2C}$-Ct) in protein extracts from cultured neurons (5 mg of protein per condition) but not by a control peptide mutated on the PDZ binding motif (5-HT$_{2CSSA}$-Ct). Arrows indicate the positions of spots corresponding to PSD-95, arrowheads those of spots corresponding to MPP3. The data representative of three experiments are illustrated. (C) siRNA knockdown of PSD-95 and MPP3. The efficiency and the specificity of each siRNA was tested by Western blotting in COS-7 cells cotransfected with PSD-95 or MPP3 and either the corresponding siRNA or a control siRNA and by immunocytochemistry in neurons transfected with either one of these siRNAs. Bar, 30 μm. (D) Neurons cotransfected with the cMyc-tagged 5-HT$_{2C}$ receptor and either PSD-95, MPP3, or control siRNAs were challenged twice with serotonin. Data represent mean values of ratios of the second to the first Ca^{2+} peak response, calculated from at least 60 cells originating from three independent cultures. *$p < 0.01$ versus neurons transfected with the control siRNA.

(Figure 8B). Nevertheless, we showed that agonist stimulation of 5-HT$_{2C}$ receptors only slightly but not significantly decreased receptor interaction with PDZ proteins (Figure 8B), as assessed by densitometric analysis of four independent experiments (our unpublished data). In any case, our results indicate that receptor phosphorylation does not differentially affect its association with PSD-95, MPP3 or Veli3.

Role of the Interactions between 5-HT$_{2C}$ Receptors and PDZ Proteins in Cortical Neurons

To determine whether PSD-95 and MPP3 modulate 5-HT$_{2C}$ receptor function in a more physiological cellular background, Ca^{2+} imaging experiments were performed in primary cultures of mice cortical neurons overexpressing either one of these MAGUK proteins. Because these neurons exhibited no detectable Ca^{2+} increases in response to serotonin application, they were cotransfected with the receptor. Similar to what was observed in COS-7 cells, overexpression of PSD-95 and MPP3 in cortical neurons increased and decreased the desensitization of the 5-HT$_{2C}$ receptor Ca^{2+} response, respectively (Figure 9A). We next examined whether these MAGUK proteins were endogenously expressed in cultured cortical neurons. RT-PCR experiments revealed that both PSD-95 and MPP3 mRNAs were present in these cultures (Figure 9B). Moreover, peptide affinity chromatography experiments using the 5-HT$_{2C}$ receptor C-terminal peptide as bait followed by the separation of proteins retained by affinity onto 2-D gels allowed us to identify PSD-95 and MPP3 as two major receptor PDZ binding partners in cultured neurons (Figure 9B), consistent with our previous findings obtained in adult mouse brains (Becamel *et al.*, 2002). To complement overexpression experiments, we used the siRNA methodology to knockdown the expression of PSD-95 or MPP3 proteins in cultured cortical neurons. Among the three different siRNAs tested for each PDZ protein, one siRNA strongly reduced expression of cotransfected PSD-95 protein or MPP3 protein, respectively, in COS-7 cells (Figure 9C, top). A scrambled siRNA sequence (control siRNA) did not affect PSD-95 or MPP3 expression. Thus, these siRNAs were effective and specific against heterologously expressed PSD-95 or MPP3 proteins. Their efficiency toward native PSD-95 and MPP3 expression in neu-

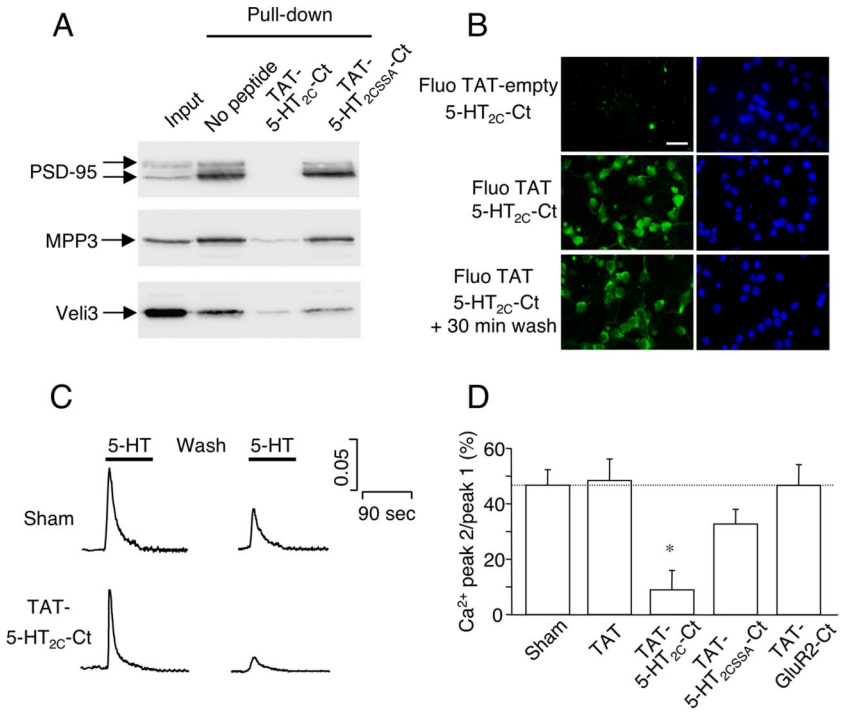

Figure 10. Disrupting 5-HT_{2C} receptor/PDZ protein interactions increases desensitization of receptor Ca^{2+} response in cortical neurons. (A) The TAT-5-HT_{2C}-Ct peptide but not the TAT-5-HT_{2CSSA}-Ct peptide prevents the interaction of Veli3, PSD-95 and MPP3 with the 5-HT_{2C} receptor C-terminus. Brain protein extracts were incubated with the immobilized 5-HT_{2C} receptor C-terminal peptide in the absence or presence of either the TAT-5-HT_{2C}-Ct peptide or the TAT-5-HT_{2CSSA}-Ct peptide (10 mM each). Proteins retained by affinity were analyzed by immunoblotting. The data representative of three experiments are illustrated. (B) Visualization of intracellular accumulation of MTS-fluorescein (Fluo)-derived TAT-5-HT_{2C}-Ct peptide and the corresponding peptide lacking the Tat protein transduction domain (TAT-empty) in the cultured neurons (10 μM each; 30-min incubation). Fluorescence was visualized in cells fixed immediately after the incubation period (following thorough washout of peptide), or 30 min after washing the peptide from the bath (bottom). Transduction of peptides into all the cultured neurons was assessed by counterstaining cell nuclei with Hoechst 33,258 (right). Representative images of five experiments are depicted. Bar, 50 μm. (C) Representative recordings of variations in intracellular Ca^{2+} levels in response to two successive applications of 1 μM serotonin, separated by a 3-min washout period, in cortical neurons transfected with the cMyc-tagged 5-HT_{2C} receptor exposed or not to 1 mM TAT-5-HT_{2C}-Ct peptide. (D) The histogram represents mean values of ratios between the second and the first Ca^{2+} peak response in neurons treated with the indicated TAT peptides (1 mM each), calculated from at least 30 cells originating from three sets of cultured neurons. *$p < 0.05$, versus untreated neurons.

rons was then analyzed by immunocytochemistry (Figure 9C). We found that PSD-95 and MPP3 expression was markedly decreased in neurons transfected with the corresponding siRNA. siRNA knockdown of PSD-95 decreased the desensitization of the 5-HT_{2C} receptor Ca^{2+} response, whereas knockdown of MPP3 resulted in a slight but significant increase in its desensitization rate (Figure 9D). Note that the control siRNA did not alter the receptor desensitization status (Figure 9, A and D). These results are consistent with those obtained in overexpression experiments and indicate that the regulation of 5-HT_{2C} receptor function in neurons depends to the nature of the PDZ protein to which it binds.

The differential effects of PSD-95 and MPP3 led us to examine the role of receptor-associated PDZ proteins in a more global manner. For that purpose, neuronal cultures were transduced with a peptide made up of the nine C-terminal residues of the 5-HT_{2C} receptor to competitively occlude the interaction between the receptor's PDZ binding motif and target PDZ proteins (Aarts *et al.*, 2002). This peptide was N-terminally fused to the transduction domain of the Tat protein from the human immunodeficiency virus type-1 (TAT-5-HT_{2C}-Ct) to allow its intracellular delivery. First, pull-down experiments showed that the TAT-5-HT_{2C}-Cter peptide disrupted coupling of the 5-HT_{2C} receptor to its major PDZ binding partners (Figure 10A). In contrast, a mutant peptide in which the C-terminal valine was mutated into an alanine (TAT-5-HT_{2CSSA}-Ct) did not prevent the interaction between the receptor and PDZ proteins (Figure 10A). This peptide will be further used as control in Ca^{2+} imaging experiments. We also verified that such peptides were capable of transducing into cultured cortical neurons, by using a fluorescent derivative (conjugated via the N terminus to the MTS-4-fluorescein fluorophore Fluo TAT-5-HT_{2C}-Ct). Cultured neurons treated with the Fluo TAT-5-HT_{2C}-Ct peptide exhibited fluorescence within both cell bodies and processes (Figure 10B). Consistent with intracellular peptide location, neurons exposed to the corresponding peptide lacking the Tat transduction domain (Fluo TAT-empty-5-HT_{2C}-Ct) did not exhibit any detectable somatic nor dendritic fluorescence (Figure 10B). It is worth noting that all neurons present in the culture were labeled with the fluorescent peptide, as assessed by counterstaining cell nuclei with Hoechst 33,258. MTS-4-fluorescein labeling of neurons remained stable for at least 30 min after the peptide washout, strongly suggesting that Tat-conjugated peptides remained accumulated in neurons within the time frame of our Ca^{2+} imaging experiments.

Transducing the TAT-5-HT_{2C}-Ct peptide into neurons did not significantly change the mean amplitude or the decay rate of the Ca^{2+} mobilization induced by an initial serotonin exposure. In contrast, this peptide markedly decreased the response evoked by a second serotonin exposure (Figure 10, C and D). To assess the specificity of the TAT-5-HT_{2C}-Cter peptide effect, neurons were treated with various control peptides: a peptide including only the Tat transduction domain (TAT), the TAT-5-HT_{2CSSA}-Cter peptide, and a peptide corresponding to the C terminus of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor GluR2 subunit fused to Tat (TAT-GluR2-Cter). Indeed, the C terminus of GluR2 exhibits a canonical PDZ binding motif of class II (VKI), which recruits different sets of PDZ proteins, compared with class I PDZ ligands such as the one of the 5-HT_{2C} receptor (Sheng and Sala, 2001; Nourry *et al.*, 2003). As expected, none of these peptides significantly altered desensitization of the 5-HT_{2C} receptor-mediated Ca^{2+} response in cortical neurons (Figure 10D), highlighting the specificity of this peptidyl mimetic approach to target PDZ-based inter-

actions. Together, these results indicate that overall, interaction of 5-HT$_{2C}$ receptors with PDZ binding partners decreases receptor desensitization rate in neurons.

DISCUSSION

In this study, we found that the interaction of 5-HT$_{2C}$ receptors with their major PDZ protein partners did not affect efficacy of receptor signal transduction but that it did differentially modulate receptor desensitization and trafficking. PSD-95 increased desensitization of the 5-HT$_{2C}$ receptor Ca^{2+} response as well as constitutive and agonist-induced receptor internalization, whereas MPP3 stabilized the receptor at the plasma membrane and prevented desensitization of the receptor Ca^{2+} response. Opposite effects of PSD-95 and MPP3 on 5-HT$_{2C}$ receptor function may arise from their interaction with specific protein partners. These PDZ proteins contain either several PDZ domains and/or additional protein interaction domains, including L27, Src homology 3, and guanylate kinase domains (Figures 5A and 6A). In this regard, coexpressing the first two PDZ domains of PSD-95, which are involved in the binding of the whole protein to the 5-HT$_{2C}$ receptor C terminus, did not alter receptor desensitization. Only a truncation mutant containing the three PDZ domains reproduced the effect of PSD-95 on 5-HT$_{2C}$ receptor desensitization, suggesting that this effect may involve additional protein interactions mediated by the third PDZ domain of PSD-95. However, one cannot rule out that PDZ3 simply stabilizes this truncated protein in an active conformation capable of increasing the desensitization of 5-HT$_{2C}$ receptors. In addition to their possible different scaffolding functions, PSD-95 and MPP3 may also exert different allosteric actions on receptor conformation, affecting its plasma membrane stability. Alternatively, both PDZ proteins may differentially affect the recruitment of proteins involved in receptor desensitization and/or internalization such as GRKs and arrestins.

Such opposite effects of PDZ proteins on the trafficking and function of individual receptors or ionic channels have already been reported. For example, β_1-adrenergic receptor internalization is inhibited by its association with PSD-95, but promoted by interaction with the PDZ protein MAGI-2 (membrane-associated guanylate kinase inverted-2) (Hu *et al*., 2000; Xu *et al*., 2001). PSD-95 reduces the amplitude of the acid-sensing ion channel-3 (ASIC3) acid-evoked current, whereas another vertebrate homologue of Lin7, Veli2, increases current amplitude by decreasing or increasing, respectively, the amount of ASIC3 on the cell surface (Hruska-Hageman *et al*., 2004). These results also highlight how a single PDZ protein (PSD-95) can differently affect the trafficking in and out of the plasma membrane of distinct target proteins. In this regard, the trafficking of 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors is modulated in an opposite manner by PSD-95. Indeed, we found that coexpressing PSD-95 with the 5-HT$_{2A}$ receptor in COS-7 cells increased its expression at the cell surface (our unpublished results), consistent with recent findings indicating that PSD-95 augments 5-HT$_{2A}$ receptor signaling at least in part by inhibiting agonist-induced receptor internalization (Xia *et al*., 2003). Together, these results indicate that 5-HT$_2$ receptor trafficking can be finely and specifically regulated by PDZ-based interactions depending on both the receptor subtype and the PDZ protein to which it binds.

Although multiple binding partners of PSD-95 have been identified and its action on ionic channel and receptor clustering, postsynaptic targeting, trafficking, and signaling are well documented, much less is known about the function of MPP3. The *MPP3* gene was originally identified by virtue of its genomic location to human chromosome 17q12–21 adjacent to the *BRCA1* tumor suppressor locus, which suggests potential involvement of MPP3 in the regulation of cell proliferation (Smith *et al*., 1996; Lin *et al*., 1998). Consistently, MPP3 associates with the PDZ binding motif of the lung tumor suppressor TSLC1 (Fukuhara *et al*., 2003). MPP3 has been identified as one of the major protein partners of inward rectifier potassium (Kir2.2) channels in the brain, in which highest levels of MPP3 transcripts were detected (Leonoudakis *et al*., 2004). However, the functionality of this interaction has not been elucidated. More recently, MPP3 was identified as a member of a protein scaffold at the retinal outer limiting membrane that may contribute to the maintenance of retinal integrity by regulation of cell adhesion between photoreceptors and Müller glial cells (Kantardzhieva *et al*., 2006). The present study, which shows that MPP3 increases cell surface expression of the 5-HT$_{2C}$ receptor and prevents its desensitization, describes for the first time a functional role of MPP3 on one of its associated proteins.

GPCR desensitization includes several events. The first event is uncoupling of the receptor from the G protein and is sometimes designated as the desensitization per se. The second is the internalization of the receptor, which is followed by either receptor recycling or down-regulation. Some GPCRs, including nonedited 5-HT$_{2C}$ receptors, undergo constitutive activity, which is accompanied by constitutive desensitization/internalization (Marion *et al*., 2004; Berg *et al*., 2005). Our results indicate that PSD-95 and MPP3 differentially affect membrane stability of 5-HT$_{2C}$ receptors. PSD-95 increased constitutive as well as ligand-induced internalization. The effect on agonist-induced internalization was certainly involved in the slower recovery of Ca^{2+} responses in the presence of PSD-95. However, we cannot rule out a possible effect of PSD-95 on receptor uncoupling. Contrasting to the effect of PSD-95, MPP3 clearly inhibited constitutive and agonist-induced internalization. In the presence of MPP3, the receptor density at the plasma membrane remained so high after a serotonin challenge that it is not surprising that subsequent 5-HT$_{2C}$ receptor Ca^{2+} responses were fully recovered after a 3-min washout. Our data thus show a striking correlation between the desensitization of Ca^{2+} responses and the degree of receptor internalization after agonist exposure. However, we must point out that the amplitude of Ca^{2+} signals were not directly related to cell surface receptor density. For example, there was some discrepancy between the magnitude of receptor desensitization and its expression level at the plasma membrane after serotonin treatment. Such a discrepancy may be caused by both receptor reserve and the process of signal amplification.

The differential regulation of 5-HT$_{2C}$ receptor desensitization and trafficking by two of its PDZ binding partners cannot predict how the interactions with PDZ proteins will globally affect the receptor's signaling properties in neurons. Modulation of 5-HT$_{2C}$ receptor function will depend on the combination of endogenous PDZ proteins that are coexpressed with the receptor and on the dynamics of these interactions. Most of the 5-HT$_{2C}$ receptor's PDZ binding partners have several PDZ domains and other protein interaction domains, which could generate additional interactions that could also regulate receptor signaling. Using a membrane-permeant peptide that competitively blocks interactions between the 5-HT$_{2C}$ receptor C terminus and its target PDZ proteins, we demonstrated that, overall, these interactions inhibit receptor desensitization in neurons. Although little information is available on the stoichiometry of such peptidyl mimetics within living cells, the specificity of

such an approach was assessed by the lack of effect of several control peptides including one carrying a mutation on the C-terminal residue that impairs its association with PDZ proteins. These results highlight how PDZ-based interactions can be specifically modulated by blocking agents and used as drug targets, providing small molecule inhibitors are developed instead of peptides to prevent undesired degradation by proteases (Dev, 2004). With regard to 5-HT_{2C} receptor function, several lines of evidence indicate that antidepressant effects of selective serotonin reuptake inhibitors depend, at least in part, upon the desensitization and/or down-regulation of 5-HT_{2C} receptors, which in turn promote the activity of the mesolimbic dopaminergic system (Giorgetti and Tecott, 2004; Esposito, 2006). Thus, targeting 5-HT_{2C} receptor–PDZ protein interactions constitutes an attractive strategy to develop new drugs aimed at reinforcing the effectiveness of antidepressant treatments with presumably less side effects than 5-HT_{2C} receptor antagonists.

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