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THE PDZ-BINDING DOMAIN IS ESSENTIAL FOR THE DENDRITIC TARGETING OF 5-HT_{2A} SEROTONIN RECEPTORS IN CORTICAL PYRAMIDAL NEURONS *IN VITRO*

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Abstract—The 5-HT_{2A} serotonin receptor represents an important molecular target for atypical antipsychotic drugs and for most hallucinogens. In the mammalian cerebral cortex, 5-HT_{2A} receptors are enriched in pyramidal neurons, within which 5-HT_{2A} receptors are preferentially sorted to the apical dendrites. In primary cortical cultures, 5-HT_{2A} receptors are sorted to dendrites and not found in the axons of pyramidal neurons. We identified a sorting motif that mediates the preferential targeting of 5-HT_{2A} receptors to the dendrites of cortical pyramidal neurons *in vitro*. We constructed green fluorescent protein-tagged 5-HT_{2A} receptors wherein potential sorting motifs were disrupted, and subsequently employed either the Semliki Forest virus or calcium phosphate for the transient expression of recombinant 5-HT_{2A} receptors in cultured cortical pyramidal neurons. Using dual-labeling immunofluorescent confocal microscopy, we quantified the axonal and dendritic sorting patterns of endogenous and recombinant 5-HT_{2A} receptors. We discovered that disruption of the PDZ-binding domain of the 5-HT_{2A} receptor greatly attenuates the dendritic targeting of 5-HT_{2A} receptors without inappropriately sorting 5-HT_{2A} receptors to axons. The PDZ-binding domain is therefore a necessary signal for the preferential targeting of the 5-HT_{2A} receptor to the dendritic compartment of cultured cortical pyramidal neurons, the first such role ascribed to this protein–protein interaction motif of any G protein-coupled receptor. **© 2003 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neuronal polarity, polarized protein sorting, G protein-coupled receptor, GPCR, axon, dendrite.

The serotonin (5-hydroxytryptamine) 2A receptor (5-HT_{2A}) serves as the principal molecular target for atypical antipsychotic drugs (Meltzer et al., 1989) and for most (Glennon et al., 1984) but not all hallucinogens (Roth et al., 2002; Sheffler and Roth, 2003). 5-HT_{2A}, the most abundant serotonin receptor in the cerebral cortex, is enriched in apical dendrites, and to a lesser extent, asymmetric synapses and dendritic spines of pyramidal neurons (Willins et al., 1997; Jakab and Goldman-Rakic, 1998; Cornea-Hebert et al., 1999, 2002; Miner et al., 2003). The apical dendrite is thought to function as the "information gate" for pyramidal neurons, wherein information from the extensive dendritic tree integrates before descending onto the soma and into the axon (Whitford et al., 2002).

The intraneuronal distribution of 5-HT_{2A} receptors, which coincides in part with the location of the "information gate," provides insights into its function in the CNS. Activation of 5-HT_{2A} receptors by certain hallucinogens disrupts the integrative function of pyramidal neurons thereby engendering perceptual and cognitive alterations, whereas antagonism of 5-HT_{2A} receptors by atypical antipsychotic agents may diminish psychosis and ameliorate cognitive deficits (Meltzer, 1999; Roth et al., 1999). At least one group has suggested that the inability of pyramidal neurons to attenuate firing in the absence of stimuli, due to either hyperactive 5-HT_{2A} receptors or abnormally high levels of 5-HT_{2A} receptors in apical dendrites, is responsible for some of the psychotic symptoms in schizophrenia (Jakab and Goldman-Rakic, 1998). Atypical antipsychotic agents that have revolutionized the treatment of schizophrenia (e.g. clozapine) mediate their therapeutic actions in part by antagonizing 5-HT_{2A} receptors (Meltzer et al., 1989) and by diminishing the dendritic targeting of 5-HT_{2A} receptors in pyramidal neurons of the prefrontal cortex (Willins et al., 1999). Hence, understanding the molecular and cellular mechanisms responsible for the dendritic targeting of 5-HT_{2A} receptors is important for elucidating atypical antipsychotic drug actions.

In neurons with distinct subcellular compartments, the intracellular carboxyl termini of numerous differentially targeted proteins, including G protein-coupled receptors (GPCRs), contain structural motifs responsible for appropriate sorting (El Far and Betz, 2002; Hung and Sheng, 2002) and intracellular trafficking (Gray and Roth, 2002). For some GPCRs, particularly metabotropic glutamate receptors, several studies have identified the distal end of the carboxyl terminus as the sorting motif (Stowell and Craig, 1999; Boudin et al., 2000; Jolimay et al., 2000). Interestingly, the last four amino acids (VSCV) of the carboxyl terminus of the 5-HT_{2A} receptor constitute a canonical Type I PDZ-binding motif (X-S/T-X-ϕ), a protein–pro-

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Abbreviations: BHK, baby hamster kidney; FBS, fetal bovine serum; FL-WGA, BODIPY-FL–wheat germ agglutinin; GAD65/67, glutamic acid decarboxylase 65 and 67; GFAP, glial fibrillary acid protein; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; MAP2, microtubule-associated protein-2; NF200k, neurofilament 200k; PBS, phosphate buffer saline; SFV, Semliki Forest virus; 5-HT_{2A}, 5-hydroxytryptamine 2A receptor.

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tein interaction motif that could potentially serve as a targeting signal (Kornau et al., 1995; Chung et al., 2002).

We hypothesized that the neuronal sorting apparatus targets 5-HT_{2A} receptors to the dendritic compartment by recognizing specific sorting motifs on the receptor protein. We thus investigated whether the PDZ-binding domain is necessary for the polarized targeting of 5-HT_{2A} receptors to dendrites of cortical pyramidal neurons *in vitro*. We also investigated whether this protein-protein interaction motif is responsible for the absence of 5-HT_{2A} receptors in axons.

EXPERIMENTAL PROCEDURES

DNA constructs

5-HT_{2A}-Δ CT was constructed as a truncation mutant of the rat 5-HT_{2A} receptor with a premature stop codon at amino acid 453. The construction of 5-HT_{2A}-Δ CT-green fluorescent protein (GFP)-CT was a multi-step process: (1) amplification of 5-HT_{2A}-Δ CT-*BamH1* by PCR (5′-primer: AAAGCGGCCGCGCCACCATG-GAAATTCTTTGTGAAGACAAT, 3′-primer: AAAGGATCCCTGT-TGTTTCCCCAGTGT), (2) amplification of *Bam*HI-GFP-*Hind*III from the GFP coding sequence of EGFP-C2 vector (BD Biosciences Clontech, Palo Alto, CA, USA) by PCR (5′-primer: AAAGGATCCATGGTGAGCAAGGGCGAG; 3′-primer: AAAGC GGCCGCTCAAAGCTTCTTGTACAGCTCGTCCAT), (3) amplification of *Hind*III-CT from the carboxyl-terminus of the rat 5-HT_{2A} receptor by PCR (5′-primer: AAAAAGCTTTCGGAAGAGAATTG-TACA; 3′-primer: AAAGCGGCCGCTCACACACAGCTAACCTT TTC), (4) 5-HT_{2A}-Δ CT-GFP-*Bam*HI was constructed by ligation of 5-HT_{2A}-Δ CT-*Bam*HI and *Bam*HI-GFP-*Hind*III and then by linear amplification by PCR, (5) *Bam*HI-GFP-CT was constructed by ligation of *Bam*HI-GFP-*Hind*III and carboxyl-terminus of the 5-HT_{2A} (*Hind*III-CT) and then by linear amplification by PCR, (6) 5-HT_{2A}-Δ CT-GFP-CT was constructed by ligation of 5-HT_{2A}-Δ CT-GFP-*Bam*HI and *Bam*HI-GFP-CT and then by linear amplification by PCR. 5-HT_{2A}-GFP has been described earlier (Bhatnagar et al., 2001). 5-HT_{2A}-Δ CT-GFP-CT-AAA was constructed using 5-HT_{2A}-Δ CT-GFP-CT as the template and substituting the last three amino acids of the carboxyl-terminus (469–471) with triple alanine residues by site-directed mutagenesis (5′-primer: CCGTGAATGAAAAGGTTGCCGCTGCGTGAGCGGCCGCTC-GAGC, 3′-primer: GCTCGAGCGGCCGCTCACGCAGCG-GCAACCTTTTCATTCACGG) (QuickChange; Stratagene, La Jolla, CA, USA). GFP-ct was constructed by amplifying the carboxyl terminus of the rat 5-HT_{2A} receptor by PCR (5′-primer: AAAGAATTCAAAACTTATAGGTCCGCC; 3′-primer: TTTGAAT-TCTCACACACAGCTAACCTTTTC) and cloning into EGFP-C2.

All GFP-tagged rat recombinant 5-HT_{2A} constructs were subsequently subcloned into the pSFV1 vector (Life Technologies, Rockville, MD, USA). SFV-GFP (GFP) contained only the GFP coding sequence. SFV-5-HT_{2A}-Δ CT-GFP-CT (2A-GFP-CT) was the reconstituted rat 5-HT_{2A} receptor with an internal GFP epitope tag positioned between amino acid 452 and 453, serving as a positive viral expression control. SFV-5-HT_{2A}-GFP (2A-GFP) contained an intact, full length rat 5-HT_{2A} receptor with a carboxyl-terminal GFP tag that effectively prevented the PDZ-binding domain of the 5-HT_{2A} receptor from associating with any PDZ domain-containing proteins. SFV-5-HT_{2A}-Δ CT-GFP-CT-AAA (2A-GFP-CT-AAA) was constructed in the same background as SFV-5-HT_{2A}-Δ CT-GFP-CT except that the last three amino acids of the carboxyl-terminus (469–471) were altered from Ser-Cys-Val (SCV) to Ala-Ala-Ala (AAA), thus having the PDZ-binding domain physically disrupted. SFV-GFP-5-HT_{2A}-ct (GFP-ct) contained the carboxyl-terminus of the 5-HT_{2A} receptor with an amino-terminal GFP tag. All constructs containing inserts in the appropriate orientation were verified by automated sequencing (Cleveland Genomics, Cleveland, OH, USA).

Cell culture

Human embryonic kidney (HEK)-293 cells were maintained in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 mg/ml; GibcoBRL, Rockville, MD, USA) at 37 °C and 5% CO_2. Baby hamster kidney (BHK)-21 cells (a generous gift from S. Herlitze, Case Western Reserve University, Cleveland, OH, USA) were grown in 6-well plates using Glasgow minimum essential medium with L-glutamine (Gibco BRL) supplemented with 10% tryptose phosphate (ICN Biochemicals, Aurora, OH, USA), 5% FBS, 20 mM HEPES, penicillin (100 U/ml) and streptomycin (100 mg/ml). Frontal cortices from embryonic day 17 or 18 Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were dissected, dissociated with 0.2% trypsin and trituration, and then plated on poly-D-lysine- (Sigma, St. Louis, MO, USA) coated coverslips in 24-well plates (0.15 ml/cm^2). Poly-D-lysine was dissolved in 50 mM Tris–HCl, pH 6.9, and supplemented with 1 μg/ml laminin (Sigma). During the initial 4 days following plating, neurons were maintained at 37 °C and 5% CO_2 in complete neural basal medium supplemented with 2% B27 (Gibco BRL), 2 mM L-GlutaMAX (Gibco BRL), 25 μM L-glutamate (Sigma), penicillin (100 U/ml) and streptomycin (100 mg/ml). Neurons were subsequently maintained for up to 14 days in essentially the same media except for the removal of L-glutamate. Fresh media was added every 4 days thereafter. Since the expression of endogenous 5-HT_{2A} receptors declines after 7 days in culture (unpublished observation), cortical cultures were used for experiments on day 7 for optimal detection of endogenous 5-HT_{2A} receptors.

In vitro transcription

Recombinant and helper SFV constructs (Life Technologies) were linearized using *Spe*I and *in vitro* transcribed using SP6 RNA polymerase according to manufacturer's suggested protocol (Life Technologies). Each reaction yielded 12–15 μg RNA, saving 3 μl of the product for running on a 0.5% agarose gel for verification. The rest of the RNA product was aliquoted and stored at −80 °C for further use.

Transient transfection and SFV infection

Transient transfection of native 5-HT_{2A} receptors into HEK-293 cells with Fugene6 (Roche, Indianapolis, IN, USA) using a Fugene to DNA ratio of 5:1 was performed according to the manufacturer's recommendations. Recombinant and helper SFV RNA products were co-transfected into 80% confluent BHK-21 cells using the cationic lipid reagent DMRIE-C (Gibco BRL). Supernatants containing the viral particles were collected within 20 h, aliquoted, frozen at −80 °C and used subsequently to infect cultured neurons. Optimal efficiency of infection for immunofluorescent studies (approximately 5–10% infection) was reached by diluting the viral supernatant in a 1:5 ratio and then incubating for 12–16 h. For each construct, SFV-mediated transient expression was performed for at least three times. Neurons were also transfected with 2A-GFP-CT and 2A-GFP using a calcium phosphate-mediated protocol (Xia et al., 1996) for comparison with the viral method. In our hands, we were able to achieve less than 0.1% transfection efficiency using the calcium phosphate-mediated method.

Immunocytochemistry

A mouse monoclonal microtubule associated protein (MAP2; 1:100,000) antibody (Sigma) served as a marker for somas and dendrites as well as for pyramidal neurons, whereas a mouse monoclonal neurofilament 200k (NF200k, 1:1500) antibody

(Chemicon, Temecula, CA, USA) served as a marker for axons. Endogenous 5-HT_{2A} receptors were detected by either a rabbit polyclonal antibody (1:2000; Berry et al., 1996; Willins et al., 1997) or a mouse monoclonal antibody (1:1000; BD Biosciences–PharMingen, San Diego, CA, USA). GFP-containing constructs were visualized with either a rabbit polyclonal GFP antibody (1:2000; Abcam, Cambridge, UK) or a monoclonal GFP antibody (1:2000; BD Biosciences Clontech). A mouse monoclonal glutamic acid decarboxylase (GAD65/67; 1:1000) antibody (gift from A. Hall, Case Western Reserve University) and a rabbit glial fibrillary acid protein (GFAP; 1:1000) antibody (Accurate Chemical, Westbury, NY, USA) were used to detect GABAergic interneurons and astrocytes, respectively.

Dual-labeling immunocytochemistry was performed essentially as described previously (Bhatnagar et al., 2001). In short, cultured neurons were subject to the following procedure: washed with phosphate buffer saline (PBS), fixed with 4% paraformaldehyde, washed with PBS, permeabilized in 0.3% Triton X-100 at 4 °C, blocked with 5% milk, incubated with primary antibodies overnight at 4 °C, washed with PBS, incubated with secondary antibodies conjugated to the appropriate Alexa-Fluor dyes (Molecular Probes, Eugene, OR, USA), washed again with PBS, and finally mounted on glass slides.

For selective visualization of surface proteins, intact neurons were cooled to 4 °C and incubated for 20 min at 4 °C with 1 μg/ml BODIPY-FL–wheat germ agglutinin (FL-WGA; Molecular Probes). To terminate the reaction, neurons were extensively washed with ice-cold PBS, fixed with 4% paraformaldehyde, and then subject to immunocytochemistry as described above.

Confocal microscopy and quantification

Images were acquired digitally from a Zeiss 410 confocal microscope without saturation of pixel intensities and subsequently analyzed using MetaView (Universal Imaging, Downingtown, PA, USA). At least 25 neurons from three independent experiments were analyzed. Axons were defined as a thin process extending from the soma and not immunoreactive for MAP2, but immunoreactive for NF200k. Dendrites were defined as processes extending from the soma and immunoreactive for MAP2. For each neuron, the mean average pixel intensity was determined from three different sections of axon, three different dendritic processes and three different sections of the soma. Normalization of axonal sorting and dendritic sorting accounted for background and variation in expression and contrast level:

Normalized axonal sorting

$$=[\text{mean average intensity (axon)} - \text{intensity (background)}]/[\text{mean average intensity (soma)} - \text{intensity (background)}]\times 100$$

Normalized dendritic sorting

$$=[\text{mean average intensity (dendrite)} - \text{intensity (background)}]/[\text{mean average intensity (soma)} - \text{intensity (background)}]\times 100$$

Statistical analysis

Normalized dendritic sorting values for recombinant 5-HT_{2A} receptor constructs were compared with controls by *t*-test (SigmaPlot). The same comparison was performed for normalized axonal values. The quotient of dendritic sorting over axonal sorting was calculated using the following formula: dendritic sorting/axonal sorting = mean ± 95% confidence intervals. Since we were comparing axonal and dendritic measurements obtained from different neurons, we employed a statistical test that permitted the analysis of two unpaired variables. Upper and lower limits at the 95% confidence intervals were determined by obtaining the quotient of two means, that is, the normalized dendritic sorting pattern and the normalized axonal sorting pattern (courtesy of GraphPad-Prism.com: http://graphpad.com/quickcalcs/index.cfm → continuous data set). S.E.s were calculated as the difference between the quotient (i.e. the mean from dendritic over axonal sorting) and the upper and lower limit at the 95% confidence interval, respectively. Horizontal bars above vertical columns in the bar graph organized the data sets into negative control, positive controls, and experimental groups. An experimental group that manifested a significant statistical difference (defined as $P<0.05$) from both the positive controls and the negative control born a star (*). The absence of a star above the horizontal bar denoted the lack of statistical difference from either positive or negative control.

Table 1. Cell types represented in the cortical culture and their relative abundance[a]

Cell type	Immunoreactivity	Relative abundance
Pyramidal neurons	MAP2	78.8%
GABAergic interneurons	GAD65/67	11.9%
Astrocytes	GFAP	6.3%

[a] We counted the total number of cells in each field using phase-contrast light microscope, and then switched to confocal microscope to determine the number of cells for a cell type in the same field. We counted the following number of cells in the cortical culture: pyramidal neurons (197 MAP2-immunoreactive cells out of 250 cells), GABAergic interneurons (33 GAD65/67-immunoreactive cells out of 278 cells), Astrocytes (18 GFAP-immunoreactive cells out of 287 cells).

RESULTS

In the mammalian cerebral cortex, 5-HT_{2A} receptors are abundant in pyramidal neurons, but are also found to a lesser extent in interneurons (Willins et al., 1997; Jakab and Goldman-Rakic, 1998; Cornea-Hebert et al., 1999, 2002; Miner et al., 2003). In primary cultures prepared from dissociated frontal cortices of E17–18 rats, the majority of the cells were MAP2-immunoreactive pyramidal neurons, while the remainder comprised GAD65/67-immunoreactive GABAergic interneurons, GFAP-immunoreactive astrocytes, and other unidentified cell types (Table 1). Hence, we chose the cortical culture as the biological system for investigating the molecular and cellular mechanisms responsible for the preferential targeting of 5-HT_{2A} receptors in pyramidal neurons.

By 5 days in culture, pyramidal neurons have clearly established polarity in that axons and dendrites become distinguishable both morphologically and immunocytochemically (Silverman et al., 2001). An axon assumes the shape of a thin and elongated process, whereas a dendrite possesses a thicker and relatively shorter process. Within each pyramidal neuron, the MAP2 was localized primarily to the soma and dendrites, whereas the NF200k protein was distributed predominantly to the axon (not shown). Consistent with previous *in vivo* and *in vitro* reports (Bernhardt and Matus, 1984; Caceres et al., 1984; De Camilli et

al., 1984; Shaw et al., 1986; Jolimay et al., 2000), MAP2 and NF200k mark distinct subcellular compartments in cultured cortical pyramidal neurons.

Prior quantitative immuno-electron microscopic studies (Cornea-Hebert et al., 2002; Miner et al., 2003) have demonstrated that 5-HT_{2A} receptors are localized primarily to the dendritic compartment of cortical pyramidal neurons *in vivo*. Specifically, 5-HT_{2A} receptors are enriched in apical dendrites and, to a lesser extent, in the basilar dendrites of cortical pyramidal neurons (Willins et al., 1997; Jakab and Goldman-Rakic, 1998; Cornea-Hebert et al., 1999). Within the dendrites of cortical pyramidal neurons, the majority of the 5-HT_{2A} receptors are found intracellularly in close association with MAP-1A (Cornea-Hebert et al., 2002), with a small population of 5-HT_{2A} receptors also present in dendritic spines and post-synaptic densities (Miner et al., 2003). We thus investigated whether cortical pyramidal neurons *in vitro* sort 5-HT_{2A} receptors in a manner comparable to their counterparts *in vivo*.

For some of these studies, we pre-labeled surface proteins with BODIPY-FL-WGA and then prepared cultured cortical neurons for immunofluorescent confocal microscopy. A subpopulation of 5-HT_{2A} receptors co-localized with the cell surface marker WGA, which labels glycosylated proteins on the plasma membrane, suggesting that 5-HT_{2A} receptors in cultured neurons were found predominantly in the intracellular pool as they are *in vivo* (Fig. 1A–C and Fig. 1D–F). In contrast, PSD-95 was co-localized with BODIPY-FL-WGA (Fig. 1G–I), confirming that this major synaptic protein of the excitatory post-synaptic density was appropriately sorted to the plasma membrane of cortical pyramidal neurons in culture. Furthermore, PSD-95 is known to associate directly with NMDA ionotropic glutamate receptors (NMDA-R) and indirectly with AMPA ionotropic glutamate receptors (AMPA-R) via stargazin (Kornau et al., 1995; Chen et al., 2000; El-Husseini et al., 2000; Chetkovich et al., 2002; Choi et al., 2002; Schnell et al., 2002), and has been shown to directly interact with the 5-HT_{2A} receptor (Xia et al., 2003). As expected, PSD-95 in cultured neurons co-localized with NMDA receptors (data not shown) and 5-HT_{2A} receptors (Fig. 1J–L). Most important, endogenous 5-HT_{2A} receptors were targeted predominantly to dendrites (Fig. 2A–C) and were not found in axons (Fig. 2D–F). Finally, transiently expressing GFP proteins, which do not possess any polarizing signal, were uniformly distributed to both dendrites (Fig. 2G–I) and axons (Fig. 2J–L) in cultured pyramidal neurons as reported *in vivo* (Ehrengruber et al., 1999). Taken together, these findings strongly indicate that cortical pyramidal neurons in the dissociated cortical culture sort proteins, including 5-HT_{2A} receptors and major synaptic membrane proteins, in the same manner as seen *in vivo*.

To efficiently express the recombinant 5-HT_{2A} receptor constructs in cultured neurons, we resorted to the Semliki Forest virus (SFV) expression system (Lundstrom et al., 2001; Ehrengruber, 2002), as we found the calcium phosphate technique to be much less efficient in our hands than the SFV system. We thus examined whether the viral expression system interfered with the polarized distribution of one of the subcellular markers that would be used in subsequent studies. Expression of a recombinant SFV construct containing only the GFP coding sequence (SFV-GFP) did not alter the predominantly dendritic localization of MAP2 (Fig. 3A–E), suggesting that the viral expression system does not interfere with the sorting pattern of the somatodendritic marker. In addition, we examined whether recombinant proteins that were introduced into cultured cortical pyramidal neurons by the SFV expression system were targeted in the same manner as those transfected by the calcium phosphate method. We found no difference in the dendritic sorting pattern of the two recombinant 5-HT_{2A} receptor constructs (2A-GFP-CT and 2A-GFP) that were transiently expressed in neurons using either method (Fig. 3F). Taken together, these findings strongly suggest that the SFV expression system is comparable to another well-accepted, though less efficient, technique for transient expression in neurons. Unless noted otherwise, recombinant constructs were introduced into the cultured pyramidal neurons by the SFV expression system.

We initially examined the expression of recombinant 5-HT_{2A} receptors that we employed in ensuing studies in HEK-293 cells. Recombinant 5-HT_{2A} receptors, including 2A-GFP-CT, 2A-GFP, and 2A-GFP-CT-AAA, exhibited robust levels of expression similar to the native 5-HT_{2A} receptor and were appropriately inserted into the plasma membrane in HEK-293 cells (Fig. 4). Furthermore, it is worth noting that 2A-GFP-CT, the reconstituted 5-HT_{2A} receptor that served as the positive control for virally mediated transient expression, demonstrated an agonist potency (EC_{50}) and efficacy (E_{max}) similar to the native 5-HT_{2A} receptor (Table 2). Taken together, these control studies demonstrate that recombinant 5-HT_{2A} receptor constructs are expressed and translocated to the cell surface in a similar manner as native 5-HT_{2A} receptors and that altering the PDZ-binding domain does not affect the stability or the steady-state expression levels of the receptor constructs.

We then investigated the distribution pattern of all recombinant 5-HT_{2A} receptors in cortical pyramidal neurons in culture. The reconstituted GFP-tagged 5-HT_{2A} receptors with an intact and unhindered PDZ-binding domain (2A-GFP-CT, Fig. 8A) were targeted predominantly to dendrites in both SFV-infected (Fig. 5A–C) or calcium phosphate-transfected cortical pyramidal neurons (Fig. 3F), and excluded from axons (Fig. 6A–C). Importantly, the dendritic distribution of the reconstituted 5-HT_{2A} receptor appears intracellular, closely resembling the endogenous 5-HT_{2A} receptor in culture (compare Fig. 5A–C to Fig. 2A–C) and *in vivo*.

The PDZ-binding domain of the 5-HT_{2A} receptor comprises the last three amino acids of the carboxyl-terminus. We discovered that a downstream GFP tag, which effectively disrupts the PDZ-binding domain by steric hindrance (2A-GFP; Fig. 8A), greatly attenuated the dendritic targeting of 5-HT_{2A} receptors (Fig. 5D–F; also see Fig. 3F) without interfering with axonal exclusion (Fig. 6D–F). We subsequently abrogated the PDZ-binding domain by site-directed mutagenesis (2A-GFP-CT-AAA; Fig. 8A). The

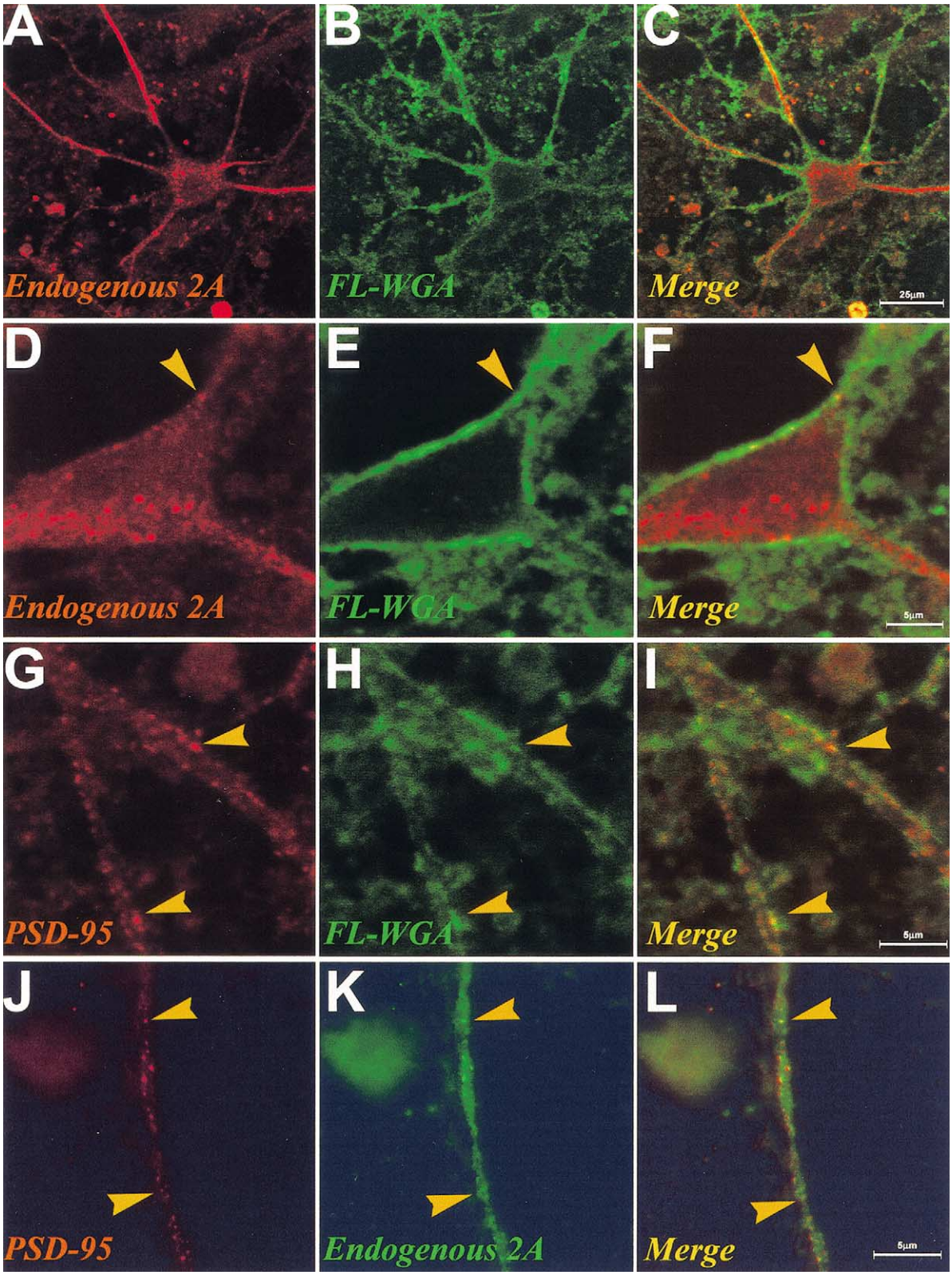

Fig. 1. Protein sorting in cultured cortical pyramidal neurons operates in a manner comparable to that *in vivo*. A–C (Low magnification) and D–F (High magnification), The majority of the endogenous 5-HT_{2A} receptors were not co-localized with the FL-WGA, which labels glycosylated proteins on the cell surface, but a small population of 5-HT_{2A} receptors was found on the plasma membrane (orange arrows). G–I, PSD-95 proteins were co-localized (orange arrows) with FL-WGA. J–L, A small population of the endogenous 5-HT_{2A} receptors was co-localized (orange arrows) with PSD-95. Scale bars are shown in the merged panels.

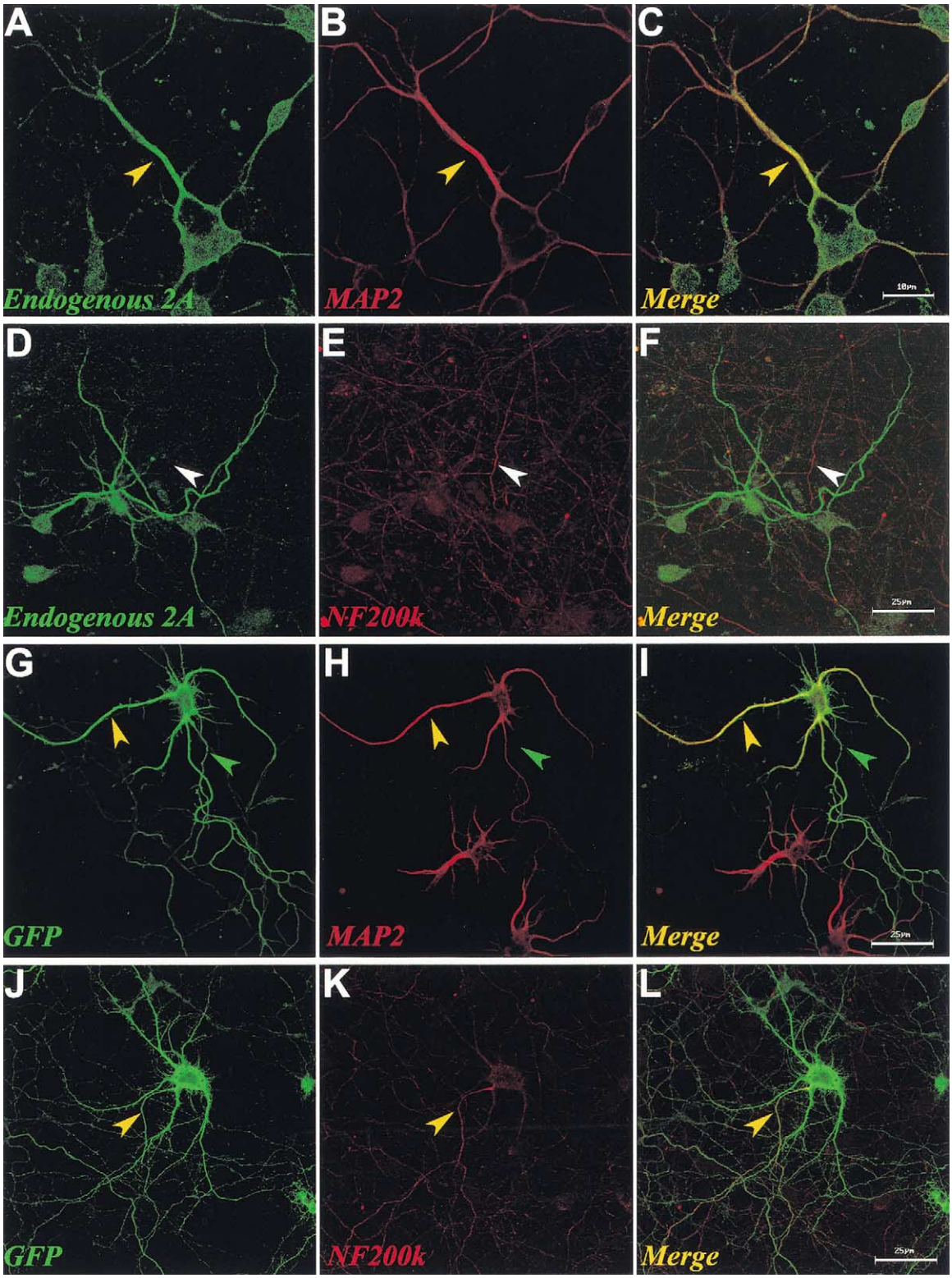

Fig. 2. Native 5-HT_{2A} receptors are selectively sorted to dendrites and absent in axons, whereas GFP proteins are uniformly distributed in cultured cortical pyramidal neurons. Endogenous 5-HT_{2A} receptors (A, D) and transiently expressing GFP proteins (G, J) are shown in the Green channel, and the somato-dendritic marker MAP2 (B, H) and the axonal marker NF200k (E, K) are shown in the Red channel. The merged images are shown in Panel C, F, I, and L. A–F, Endogenous 5-HT_{2A} receptors were targeted predominantly to dendrites and excluded from axons. Orange arrows denote co-localization with MAP2, whereas white arrows denote the lack of co-localization with NF200k. G–L, Transiently expressing GFP proteins were found in both dendrites and axons. Orange arrows denote co-localization with either MAP2 or NF200k. Green arrows denote an axon expressing GFP. Scale bars are shown in the merged panels.

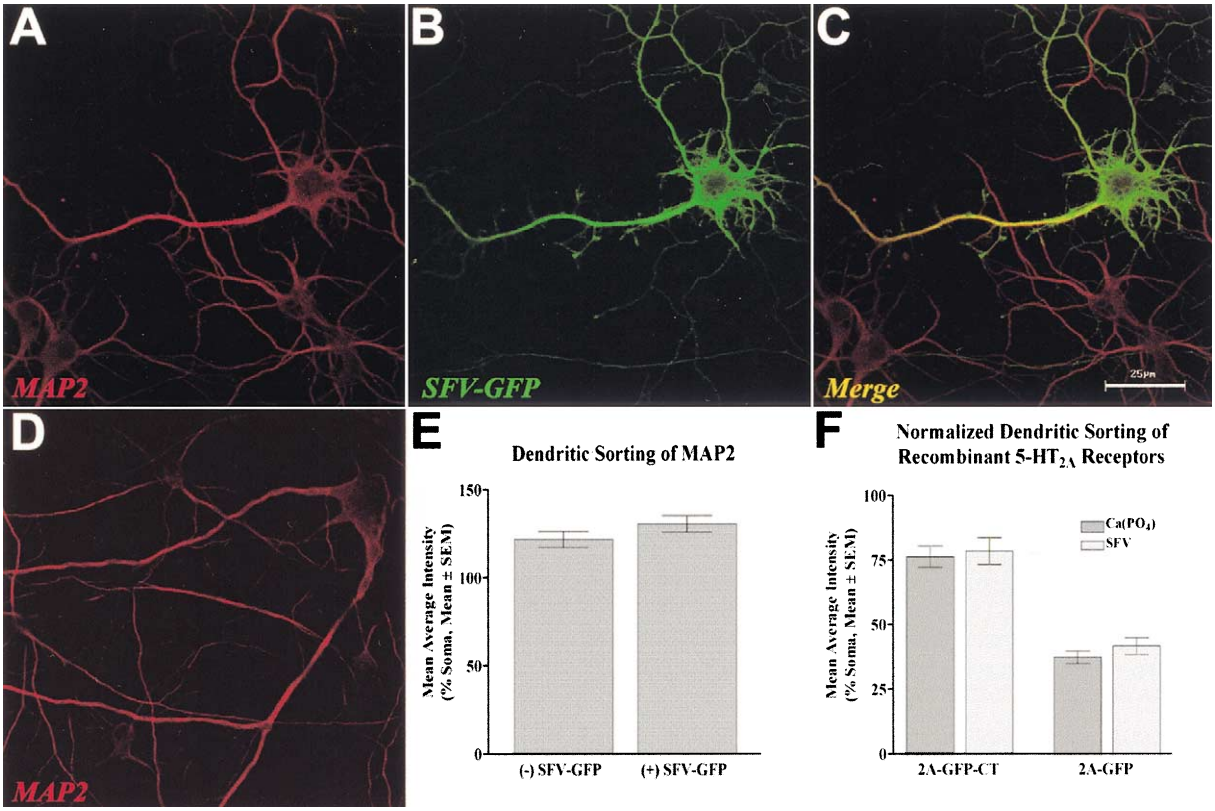

Fig. 3. The SFV expression system is an efficient and reliable method for the expression of 5-HT_{2A} receptor constructs in cultured cortical neurons. A–E, Transient expression with SFV constructs did not alter the sorting pattern of the somatodendritic marker MAP2. MAP2 (A, +SFV-GFP) sorting in the presence of SFV-GFP (B) was compared with MAP2 sorting in the absence of SFV infection (D, −SFV-GFP). Scale bar is shown in Panel C. The somatodendritic pattern of MAP2 was not altered by SFV infection (E). The number of neurons analyzed: (+)SFV-GFP (n=10), (−)SFV-GFP or uninfected (n=10). F, SFV-mediated infection and calcium phosphate [$Ca(PO_4)$]-mediated transient transfection of cultured pyramidal neurons resulted in no significant differences in 5-HT_{2A} receptor sorting. We compared the normalized dendritic sorting pattern of 2A-GFP-CT and 2A-GFP (See Experimental Procedures), expressed as the mean average pixel intensity (% Soma, mean±S.E.M.), achieved by both SFV-mediated infection and calcium phosphate-mediated transfection. The number of SFV-infected neurons was as the following: 2A-GFP-CT (n=36), 2A-GFP (n=31). The number of calcium phosphate-transfected neurons was: 2A-GFP-CT (n=10), 2A-GFP (n=22).

physical disruption of the PDZ-binding domain also diminished dendritic targeting (Fig. 5G–I) without affecting the axonal exclusion (Fig. 6G–I). In both 2A-GFP and 2A-GFP-AAA, the fluorescence signals in the soma are comparable to that of 2A-GFP-CT or the endogenous 5-HT_{2A} receptors (Figs. 2, 5 and 6).

Having established that the distal end of the carboxyl-terminus of the 5-HT_{2A} receptor is essential for dendritic targeting, we investigated whether the carboxyl-terminus alone would be sufficient for mediating the polarized sorting of molecules in pyramidal neurons. For these studies we examined the distribution pattern of the carboxyl-terminus of the 5-HT_{2A} receptor with an amino-terminally tagged GFP (GFP-ct; Fig. 8A). When over-expressed in cultured pyramidal neurons, the carboxyl-terminus of the 5-HT_{2A} receptor was targeted to dendrites albeit less efficiently (Fig. 7A–C) and excluded from axons (Fig. 7D–E).

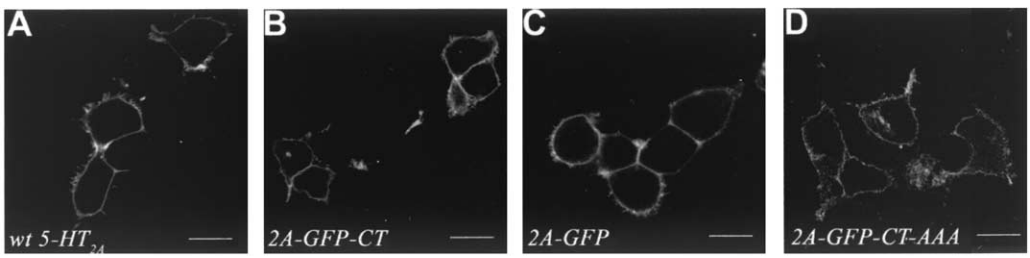

Fig. 4. Recombinant 5-HT_{2A} receptors are properly inserted into the plasma membrane in HEK-293 cells. Wild type 5-HT_{2A} receptors were transfected using Fugene and recombinant 5-HT_{2A} receptor constructs were expressed using the SFV system (see Experimental Procedures). Similar to the transiently expressing wild type 5-HT_{2A} receptors (A), recombinant 5-HT_{2A} receptor constructs, 2A-GFP-CT (B), 2A-GFP (C), and 2A-GFP-CT-AAA (D), exhibited robust levels of expression and are not hindered from insertion to the cell surface.

Table 2. Characterization of the reconstituted 5-HT_{2A} receptor in HEK-293 cells[a]

	Agonist potency EC_{50} ($pEC_{50}\pm$S.E.M.)	Relative agonist efficacy (normalized $E_{max}\pm$S.E.M.)
wt 5-HT_{2A}	68.7 (8.74±0.03)	1.00±0.02
2A-GFP-CT	42.8 (8.79±0.14)^{NS}	0.93±0.05^{NS}

[a] Agonist potency (EC_{50}) and relative agonist efficacy (normalized E_{max}) were determined from phosphoinositide hydrolysis assay as described previously (Gray et al., 2001). The results represented the average from three independent experiments. NS, no statistical significance.

We subsequently quantified the sorting patterns of endogenous and recombinant 5-HT_{2A} receptors in cultured cortical pyramidal neurons and subjected the data to statistical analysis (Fig. 8). We normalized the dendritic and axonal sorting patterns to account for both interneuronal variations in protein expression and variations in acquiring digital confocal images (e.g. brightness and contrast levels). Since we performed dual-labeling immunocytochemistry using only one subcellular marker (MAP2 or NF200k) at a given time, we examined dendritic distribution and axonal distribution in different neurons. We subsequently integrated these two unpaired variables by expressing them as a quotient of the normalized dendritic sorting pattern over the normalized axonal sorting pattern (D/A ratio) with the upper and lower limits at the 95% confidence interval as S.E.s (Fig. 8B).

As demonstrated by representative images (Figs. 5, 6 and 7), the PDZ-binding domain of the 5-HT_{2A} receptor is necessary but not sufficient for dendritic targeting in cultured cortical pyramidal neurons. Abrogating the PDZ-binding domain either sterically (2A-GFP: D/A=5.36, 95% confidence intervals from 4.16–7.09) or physically (2A-GFP-CT-AAA: D/A=5.44, 95% confidence intervals from 3.97–7.96) significantly attenuated the dendritic targeting of the 5-HT_{2A} receptor, when compared with both (1) endogenous 5-HT_{2A} receptors (endogenous 5-HT_{2A}: D/A=11.53, 95% confidence intervals from 8.71–15.40) and (2) reconstituted 5-HT_{2A} receptors with an unhindered PDZ-binding domain (2A-GFP-CT: D/A=11.78, 95% confidence intervals from 9.20–15.62; Fig. 8B). The PDZ-binding domain, however, does not appear to be required for excluding 5-HT_{2A} receptors from the axonal compartment since its disruption did not enhance 5-HT_{2A} receptor sorting to axons. Furthermore, the absence of a uniform distribution of 5-HT_{2A} receptors in mutants with disrupted PDZ-binding domains suggests that the preferential sorting of 5-HT_{2A} receptors to the dendritic compartment is an active process. We cannot exclude the existence of other dendritic targeting motifs within the 5-HT_{2A} receptor since disrupting the PDZ-binding domain did not reduce the dendritic targeting to the same level as GFP (D/A=2.44, 95% confidence intervals from 1.81–3.43; Fig. 8B).

The predominantly dendritic distribution of the carboxyl-terminus of the 5-HT_{2A} receptor (GFP-ct: D/A=6.91, 95% confidence intervals from 5.02–10.41) in cultured pyramidal neurons (Fig. 8B) corroborates the necessary role that the PDZ-binding domain plays in dendritic targeting. When compared with either the endogenous 5-HT_{2A} receptors (endogenous 5-HT_{2A}) or the reconstituted internal GFP-tagged 5-HT_{2A} receptor (2A-GFP-CT), the dendritic localization of GFP-ct was diminished but still marginally greater than that of the uniformly distributed GFP (Fig. 8B). It is worth noting the absence of any statistical difference between the dendritic distribution of GFP-ct and that of either 2A-GFP or 2A-GFP-AAA, suggesting that the c-terminus of the 5-HT_{2A} receptor is not sufficient for dendritic targeting (Fig. 8B).

DISCUSSION

The major finding of this paper is that the PDZ-binding domain is a necessary dendritic targeting signal for 5-HT_{2A} receptors in cortical pyramidal neurons *in vitro*. To our knowledge, this represents the first demonstration of the PDZ-binding domain in mediating the selective sorting of a GPCR to dendrites. The PDZ-binding domain, however, is not involved in excluding 5-HT_{2A} receptors from axons. The present work supports a mechanism of dendritic targeting in neurons whereby the protein sorting apparatus recognizes specific targeting signals on differentially distributed proteins and targets them to the appropriate subcellular compartment.

The biological context in which we investigated the polarized distribution of 5-HT_{2A} receptors was the primary culture from dissociated cerebral cortices of embryonic rats. Since the 5-HT_{2A} receptor is abundant in cortical pyramidal neurons, it is likely that targeting signals identified from studies that are conducted in cultured cortical pyramidal neurons bear more physiological relevance than those from polarized epithelial cells. Indeed, the “epithelial metaphor” (Colman, 1999), which posits that neuronal and polarized epithelial cells share similar subcellular sorting apparatuses (Dotti and Simons, 1990), has proven to be limited in that apical and basolateral surfaces of the plasma membrane in epithelial cells do not always correspond to the axonal and dendritic compartments of neurons (West et al., 1997; Jareb and Banker, 1998; Wozniak and Limbird, 1998; Ghavami et al., 1999; Stowell and Craig, 1999; Burack et al., 2000). The cortical culture, however, has its own intrinsic limitations in that we cannot differentiate apical from basilar dendrites in pyramidal neurons, and we accordingly frame our study within the boundary of axonal versus dendritic targeting.

Virus-mediated gene delivery systems, such as the SFV expression system, have greatly facilitated the expression of recombinant proteins in neurons that are otherwise recalcitrant to transfection. The SFV system has two additional advantages: (1) the viral genome replicates in the cytoplasm, thus obviating potential difficulties encountered with nuclear expression systems (e.g. mRNA splicing, capping and transport, and limitations in transcription factors); (2) SFV, despite its high efficiency of replication in neurons, induces cytotoxicity only after prolonged periods of infection (Lundstrom et al., 2001; Ehrengruber, 2002). The SFV expression system has thus enabled us to

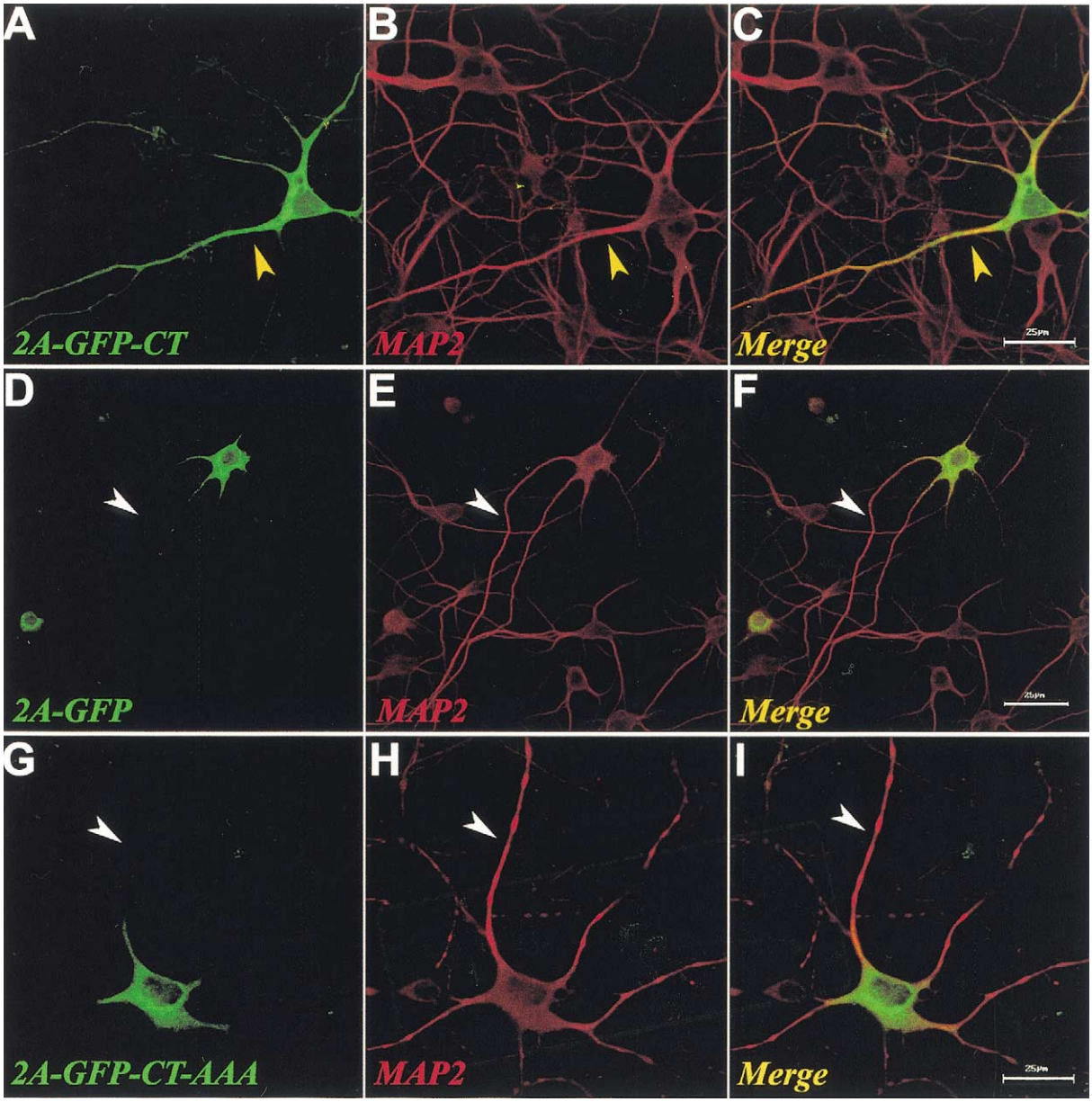

Fig. 5. The PDZ-binding domain of the 5-HT_{2A} receptor serves as a necessary dendritic targeting motif in cultured cortical pyramidal neurons. Transiently expressing recombinant 5-HT_{2A} constructs, including 2A-GFP-CT (A), 2A-GFP (D), and 2A-GFP-CT-AAA (G) are shown in the Green channel. The somatodendritic marker, MAP2 (B, E, H), is shown in the Red channel. The merged images are shown in Panel C, F, and I. Orange arrows denote dendrites. A–C, The reconstituted, internal GFP-tagged 5-HT_{2A} receptor (2A-GFP-CT) was appropriately targeted to the dendritic compartment. D–F, Transiently expressing 2A-GFP whose PDZ-binding domain was effectively blocked by a downstream GFP tag showed greatly attenuated targeting to dendrites. G–I, Transiently expressing 2A-GFP-CT-AAA, in which the PDZ-binding domain of the 5-HT_{2A} receptor was physically disrupted, showed greatly attenuated targeting to dendrites. Orange arrows denote co-localization with MAP2, whereas white arrows denote the absence of co-localization with MAP2. Scale bars are shown in the merged panels.

investigate the distribution of recombinant 5-HT_{2A} receptors in a large number of transfected neurons, a technical improvement that has made our data amenable to meaningful statistical analysis. Three lines of evidence suggest that SFV is a valid method of gene delivery for our purposes. First, neurons expressing recombinant constructs appeared morphologically intact and indistinguishable from uninfected neurons. Second, the expression of recombinant constructs had no apparent impact on the polarized targeting of subcellular markers used in dual-labeling immunofluorescent studies. Third and most important, transient expression of recombinant constructs using SFV or calcium phosphate-mediated transfection resulted in comparable intraneuronal distribution patterns. Taken together, these observations demonstrate that the SFV expression system, when suitably employed, does not overtly interfere with the subcellular protein sorting apparatus of cortical cultured pyramidal neurons.

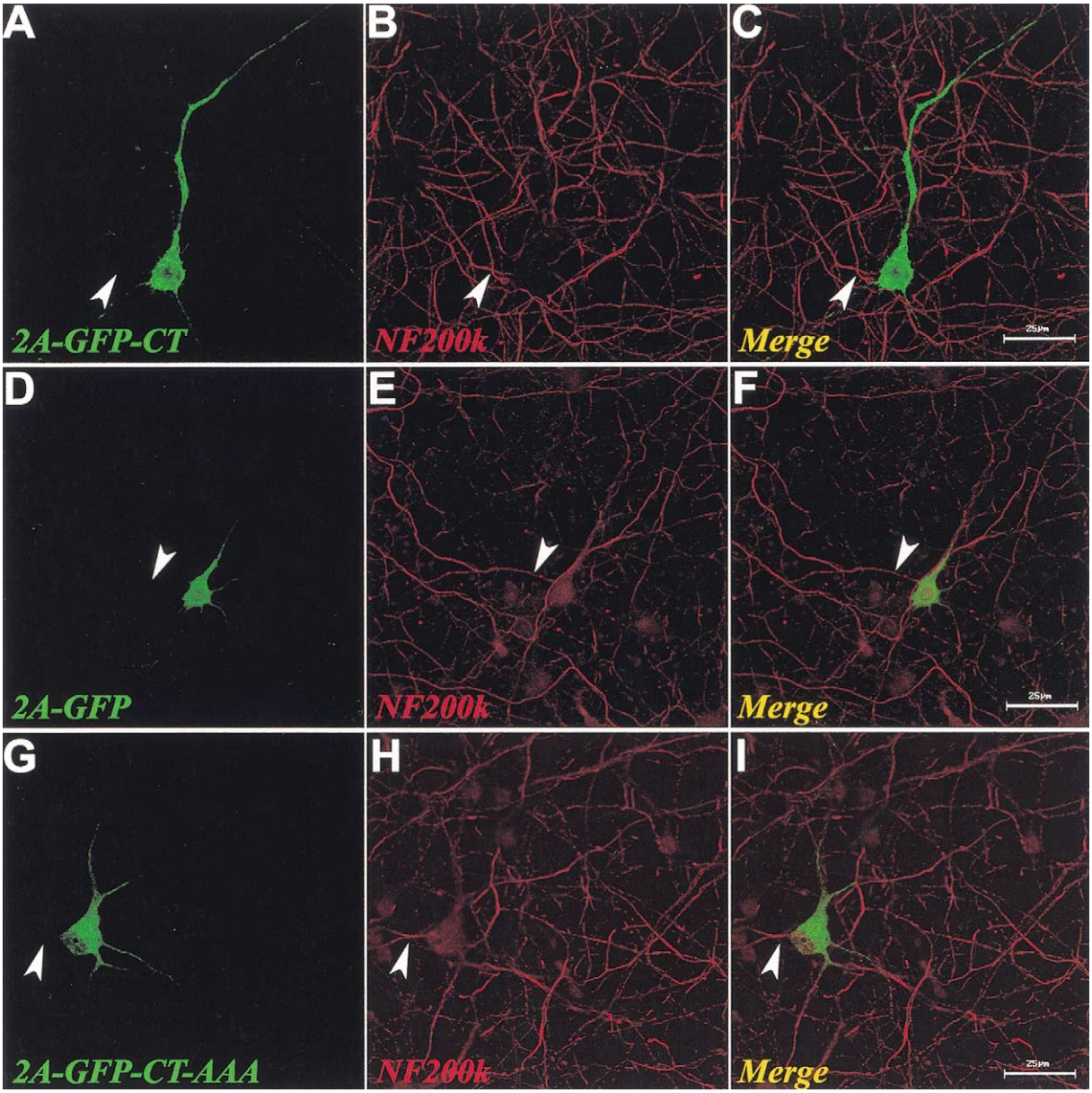

Fig. 6. The PDZ-binding domain is not essential for the absence of 5-HT_{2A} receptors in the axons of cultured cortical pyramidal neurons. Transiently expressing recombinant 5-HT_{2A} constructs, including 2A-GFP-CT (*A*), 2A-GFP (D), and 2A-GFP-CT-AAA (G) are shown in the Green channel. The axonal marker, NF200k (B, E, H), is shown in the Red channel. The merged images are shown in Panel C, F, I. Orange arrows denote axons. A–C, Transiently expressing 2A-GFP-CT was excluded from axons. D–F, Transiently expressing 2A-GFP was excluded from axons. G–I, Transiently expressing 2A-GFP-CT-AAA was excluded from axons. White arrows denote the absence of co-localization with NF200k. Scale bars are shown in the merged panels.

Proper neuronal function requires the selective targeting of proteins to the appropriate subcellular compartment, but the mechanisms that establish neuronal polarity are not well understood. It appears that axonal and dendritic targeting involve different components of the sorting apparatus (Foletti et al., 1999). For example, different populations of carrier vesicles are responsible for delivering dendritic and axonal proteins (Burack et al., 2000). Selective sorting to dendrites relies on microtubule-based transport, whereas preferential distribution to axons involves selective retention at the destination (Burack et al., 2000). Furthermore, intact lipid rafts, consisting of detergent-insoluble glycosphingolipid-cholesterol complexes, appear essential for the targeting of some axonal proteins (Ledesma et al., 1998), but no such requirement has been reported for dendritic proteins.

What emerges from these reports is that a signal-dependent targeting mechanism participates in both axon- and dendrite-selective transport. The cytosolic carboxyl-terminus of dendritically targeted proteins has long been suggested to contain subcellular sorting motifs (West et al., 1997; El Far and Betz, 2002). Likewise, necessary and

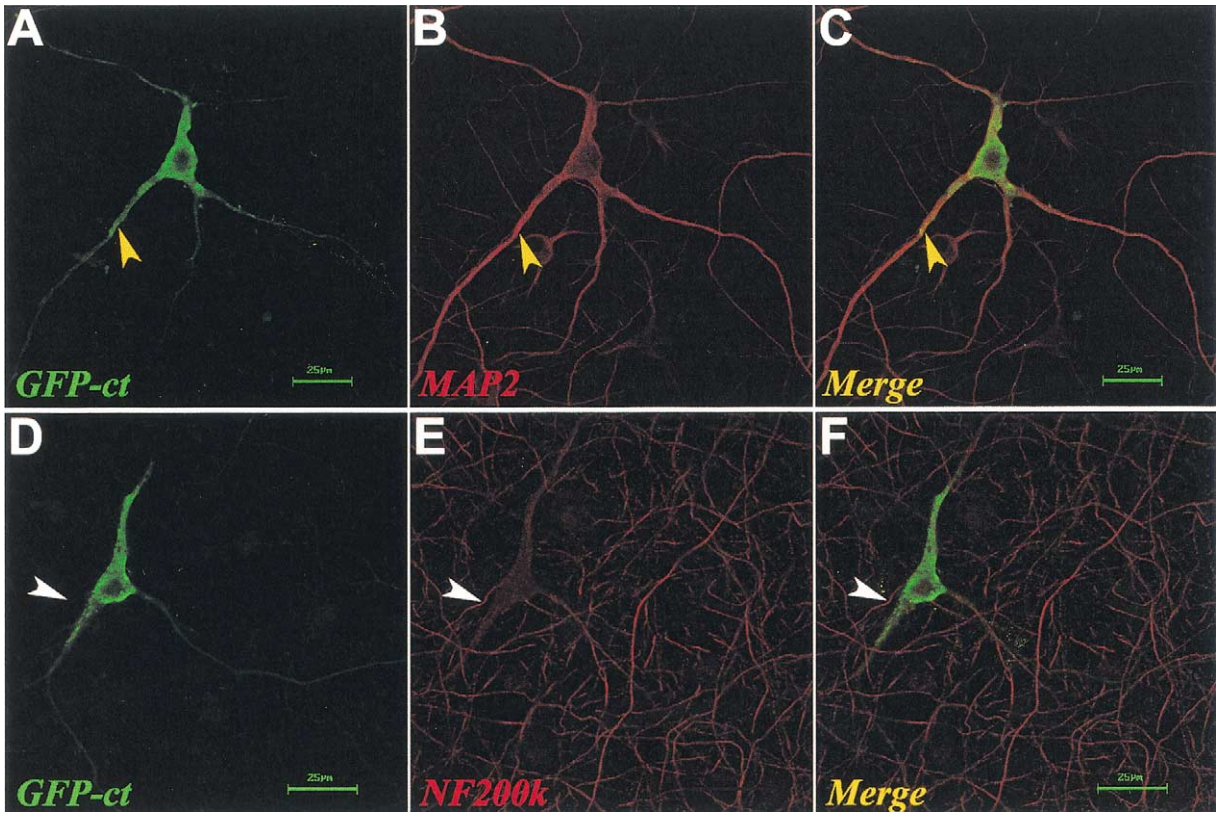

Fig. 7. The carboxyl-terminus of the 5-HT_{2A} receptor mediates the preferential sorting to the dendritic compartment in cultured cortical pyramidal neurons. GFP-tagged carboxyl-terminus of the 5-HT_{2A} receptor (GFP-ct) is shown in the Green channel (A, D). MAP2 (B) and NF200k (E) are shown in the Red channel. The merged images are shown in Panel C and F. A–C, GFP-ct was localized more to the dendritic compartment. D–F, Sorting to the axonal compartment was diminished. Orange arrows denote co-localization with MAP2, whereas white arrows denote the diminished co-localization with NF200k. Scale bars are shown in the merged panels.

sufficient axonal sorting signals have been discovered in the carboxyl-terminus of several metabotropic glutamate receptors (Stowell and Craig, 1999; Francesconi and Duvoisin, 2002).

The present study demonstrates that the PDZ-binding domain of the 5-HT_{2A} receptor is a necessary dendritic targeting signal. To our knowledge, this is the first report that the PDZ-binding domain mediates the selective sorting of a GPCR to dendrites. There is one other account that ascribes to the PDZ-binding domain any role for the selective targeting of GPCRs. Specifically, the PDZ-binding domain of the mGluR7 receptor is recognized by PICK1, a PKCα-binding protein, and association with PICK1 serves to cluster mGluR7 to the presynaptic active zone in cultured hippocampal neurons (Boudin et al., 2000; Boudin and Craig, 2001). The PDZ-binding domain in the case of mGluR7 thus serves to target mGluR7 receptors to a *specialized* region within a subcellular compartment (i.e. the presynaptic terminal in axon). The signal that mediates selective sorting to axons was presumed to be upstream of the PDZ-binding domain in the central portion of the carboxyl-terminus of the mGluR7 receptor (Stowell and Craig, 1999; Boudin et al., 2000). In contrast, our studies directly implicate the PDZ-binding domain in targeting 5-HT_{2A} receptors to the dendritic compartment.

We also report that the PDZ-binding domain is not essential for the axonal exclusion of the 5-HT_{2A} receptor. The disruption of the PDZ-binding domain does not result in a uniform distribution of the recombinant 5-HT_{2A} receptors to both axons and dendrites. By contrast, the proximal carboxyl-terminus of the transferrin receptor contains necessary dendritic targeting motifs, the disruption of which leads to uniform distribution of the transferrin receptor (West et al., 1997). In the case of the 5-HT_{2A} receptor, selective targeting to dendrites involves at least two separate processes that could hypothetically operate in synergy: (1) active targeting to dendrites in a signal-dependent manner; (2) selective exclusion from axons. Our present study has established that the PDZ-binding domain of the 5-HT_{2A} receptor is involved in the first process but not in the second. A likely component of the neuronal sorting apparatus responsible for axonal exclusion is the membrane diffusion barrier composed of cytoskeletal elements at the proximal axonal segment (Winckler and Mellman, 1999). Such a barrier restricts lateral protein mobility and contributes to the maintenance of a polarized distribution of membrane proteins (Winckler et al., 1999). Alternatively, axonal exclusion simply results from the selective targeting of the 5-HT_{2A} receptor to dendrites and may not require a separate process.

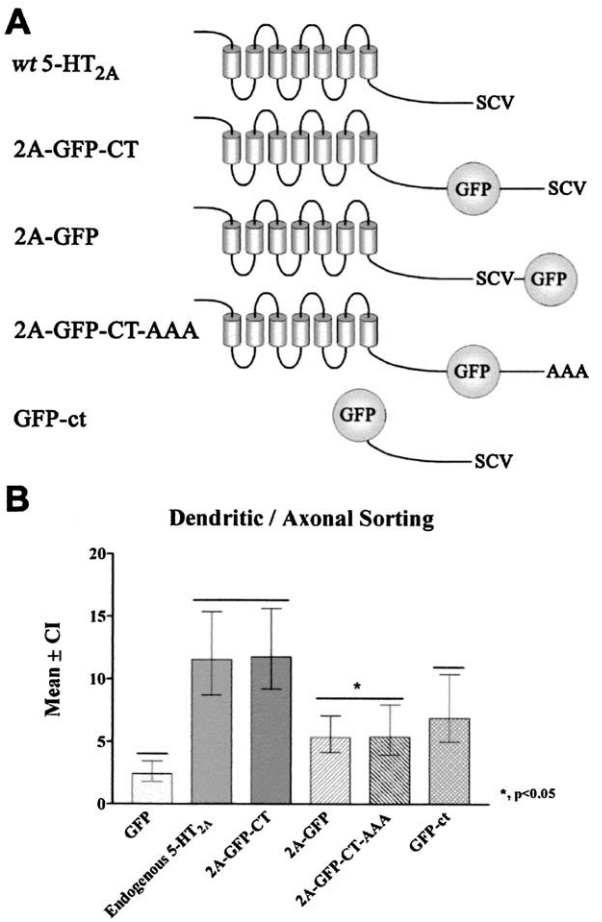

Fig. 8. Quantification and statistical analysis of dendritic and axonal sorting patterns of endogenous and recombinant 5-HT_{2A} receptors. See Experimental Procedures section for a more detailed explanation of quantification and statistical analysis. A, Schematic diagram demonstrates the structure of native and recombinant 5-HT_{2A} receptors. B, Dendritic sorting over axonal sorting (D/A ratio), expressed as mean±95% confidence intervals. Since the errors are expressed as the upper and lower limits at the 95% confidence interval, they are not always of the same magnitude. The sorting patterns of 2A-GFP and 2A-GFP-CT-AAA were significantly different (*, $P<0.05$) from endogenous 5-HT_{2A} receptors and 2A-GFP-CT, as well as GFP. The sorting pattern of GFP-ct was not significantly different from endogenous 5-HT_{2A} receptors and 2A-GFP-CT, but was significantly different from GFP. Number of neurons analyzed for normalized dendritic sorting was as the following: GFP (n=23), Endogenous 5-HT_{2A} (n=30), 2A-GFP-CT (n=36), 2A-GFP (n=31), 2A-GFP-CT-AAA (n=37), GFP-ct (n=20). Number of neurons analyzed for normalized axonal sorting was as the following: GFP (n=25), endogenous 5-HT_{2A} (n=27), 2A-GFP-CT (n=25), 2A-GFP (n=26), 2A-GFP-CT-AAA (n=33), GFP-ct (n=24).

The carboxyl-terminus alone is better targeted to dendrites than the uniformly distributed GFP and its dendritic distribution does not differ statistically from that of the endogenous or the reconstituted 5-HT_{2A} receptors. Importantly, the dendritic distribution of the carboxyl-terminus more closely resembles that of the mutant 5-HT_{2A} receptors lacking a functional PDZ-binding domain. Taken together, these findings suggest that although the carboxyl-terminus of the 5-HT_{2A} receptor contains a necessary dendritic targeting signal (i.e. the PDZ-binding domain), it is not sufficient to mediate the dendrite-selective targeting of the receptor. One possible explanation for the less efficient dendritic targeting of carboxyl-terminus is that the loss of proximity to the neuronal protein sorting apparatus due to the inability of GFP-ct to be inserted into the plasma membrane. We plan to test this possibility by examining the distribution pattern of the carboxyl-terminus of the 5-HT_{2A} receptor with an attached membrane targeting signal in future experiments. Alternatively, the 5-HT_{2A} receptor might contain additional dendritic targeting signals in regions other than the carboxyl-terminus such as the third intracellular loop. That abrogating the PDZ-binding domain failed to completely abolish the dendritic targeting of the 5-HT_{2A} receptor and that the carboxyl-terminus is not sufficient to mediate 5-HT_{2A} receptor targeting to dendrites both support this latter possibility.

We have not formally ruled out the possibility that 5-HT_{2A} receptors are selectively sorted at the mRNA level. Recent studies have identified dendritic targeting elements in the 3′-untranslated region of α-CaMKII and MAP2 as *cis*-acting sequences (Blichenberg et al., 1999; Miller et al., 2002), which associate with *trans*-acting factors that mediate the selective transport (Job and Eberwine, 2001; Rehbein et al., 2002). To our knowledge, there has been no report of the presence of dendritic targeting signal in the 5-HT_{2A} mRNA.

The discovery of a necessary dendritic targeting signal on the 5-HT_{2A} receptor is the initial step toward the elucidation of mechanisms underlying the selective targeting of this GPCR to the apical dendrite *in vivo*. 5-HT_{2A} receptors in apical dendrites mediate the 5-HT-induced enhancement of excitatory postsynaptic potential in pyramidal neurons and may be responsible for the psychotomimetic effects of some hallucinogens (Aghajanian and Marek, 1997, 1999). In addition, the 5-HT_{2A} receptor contributes to the formation of working memory in primates, and its dysfunction has been postulated to account for the cognitive deficits in schizophrenia (Williams et al., 2002).

We have recently obtained convincing evidence that PSD-95, a prototypic PDZ domain-containing protein and a key component of the excitatory post-synaptic density, directly associates with the PDZ-binding domain of the 5-HT_{2A} receptor and regulates the signaling and subcellular distribution of 5-HT_{2A} receptors (Xia et al., 2003). Interestingly, whereas the PDZ-binding domain of the mGluR7 receptor (Φ -X-Φ, where Φ is a hydrophobic residue) is recognized by class II PDZ domains, that of the 5-HT_{2A} receptor (S/T-X-V/I) is recognized by class I PDZ domains. In this regard, the third PDZ domain from PSD-95 prefers valine as the last amino acid of the PDZ-binding domain (Harris and Lim, 2001), a residue found in the 5-HT_{2A} receptor. Since mGluR7 receptors and 5-HT_{2A} receptors are targeted to axons and dendrites, respectively, it is tempting to speculate that selective sorting mediated by different types of PDZ-binding domains (i.e. Type I to dendrites and Type II to axons) is related to the different molecular targets that each targeting motif recognizes.

In summary, we have identified the PDZ-binding domain of the 5-HT_{2A} receptor as a necessary signal for the

selective targeting of 5-HT_{2A} receptors to dendrites in pyramidal neurons from dissociated cortical culture. To our knowledge, this is the first report demonstrating that a PDZ-binding domain is essential for the dendritic targeting of a GPCR. Because of the crucial role that 5-HT_{2A} receptors play in the actions of hallucinogens and atypical antipsychotic drugs, these findings are likely to enhance our understanding of how these drugs exert their profound effects on human perception, cognition and emotions.

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