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New sorting nexin (SNX27) and NHERF specifically interact with the 5-HT_{4(a)} receptor splice variant: roles in receptor targeting

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Summary

The 5-hydroxytryptamine type 4 receptor (5-HT₄R) is involved in learning, feeding, respiratory control and gastrointestinal transit. This receptor is one of the G-protein-coupled receptors for which alternative mRNA splicing generates the most variants that differ in their C-terminal extremities. Some 5-HT₄R variants (a, e and f) express canonical PDZ ligands at their C-termini. Here, we have examined whether some mouse 5-HT₄R variants associate with specific sets of proteins, using a proteomic approach based on peptide-affinity chromatography, two-dimensional electrophoresis and mass spectrometry. We have identified ten proteins that interact specifically with the 5-HT_{4(a)}R and three that only associate with the 5-HT_{4(e)}R. Most of them are PDZ proteins. Among the proteins that associated specifically with the 5-HT_{4(a)}R

variant, NHERF greatly modified its subcellular localization. Moreover, NHERF recruited the 5-HT_{4(a)}R to microvilli, where it localized with activated ezrin, consistent with the role of 5-HT_{4(a)}R in cytoskeleton remodelling. The 5-HT_{4(a)}R also interacted with both the constitutive and inducible (upon methamphetamine treatment) forms of the recently cloned sorting nexin 27 (SNX27a and b, respectively). We found that SNX27a redirected part of 5-HT_{4(a)}R to early endosomes. The interaction of the 5-HT₄R splice variants with distinct sets of PDZ proteins might specify their cellular localization as well as their signal transduction properties.

Key words: 5-HT₄ Receptor splice variants, PDZ domains, Proteomics, NHERF, Sorting nexin

Introduction

The role of the C-terminal domain of G-protein-coupled receptors (GPCRs) in their signal transduction has recently been recognized. Indeed, two-hybrid screens have identified no fewer than 50 proteins as binding partners of various GPCR C-termini (Bockaert et al., 2003; Hall and Lefkowitz, 2002; Kreienkamp, 2002). Most of them are scaffolding proteins that contain several structural interaction domains such as Src homology 2 (SH2) or 3 (SH3) domains, post-synaptic-density-95/disc-large/zonula-occludens-1 (PDZ) domains and *Drosophila* enabled and vasodilator-stimulated phosphoprotein homologous (EVH) domains. These proteins participate in the building of large submembrane protein signaling networks. We have recently isolated a complex of at least 15 proteins interacting with the C-terminal tail of the 5-HT_{2C} receptor, using a proteomic approach combining peptide-affinity chromatography and mass spectrometry. This further supports the interaction of the GPCR C-terminus with large protein networks. Moreover, functional studies have established that the proteins that interact with the GPCR C-terminus are implicated in various GPCR functions that do not involve G-proteins. These functions include trafficking, targeting to specific subcellular compartments, clustering with effectors, fine tuning of G-protein activation and desensitization.

Many GPCR mRNAs undergo alternative splicing, which gives rise to a range of isoforms that often differ in their C-termini. These variants express specific recognition motifs for scaffolding/signaling proteins and can thus recruit distinct protein complexes. For example, of the three C-terminal splice variants of the mGlu1 receptor (mGluR1a, mGluR1b and mGluR1c), only the long variant (mGluR1a) contains a canonical polyproline sequence (PPXFX) that binds to the EVH domain of Homer proteins (Brakeman et al., 1997). Moreover, only mGluR1a is part of a huge multiprotein complex including Homer, Shank, post-synaptic-density 95 (PSD-95), NMDA, inositol triphosphate and ryanodine receptors, as well as many other post-synaptic-density proteins (Ango et al., 2002; Husi and Grant, 2001). Furthermore, only mGluR1a exhibits constitutive activity, which is modulated by its interaction with Homer proteins (Ango et al., 2001).

The 5-HT₄R is certainly one of the GPCRs for which alternative mRNA splicing generates the most variants. To date, eight C-terminus splice variants have been cloned in humans (a-g,n) (Bender et al., 2000; Blondel et al., 1998; Claeysen et al., 1999; Vilaro et al., 2002), four in mouse (a,b,e,f) (Claeysen et al., 1999) and three in the rat (a,b,e) (Claeysen et al., 1999; Gerald et al., 1995). All these variants differ in their C-termini after a single position (L358, Fig.

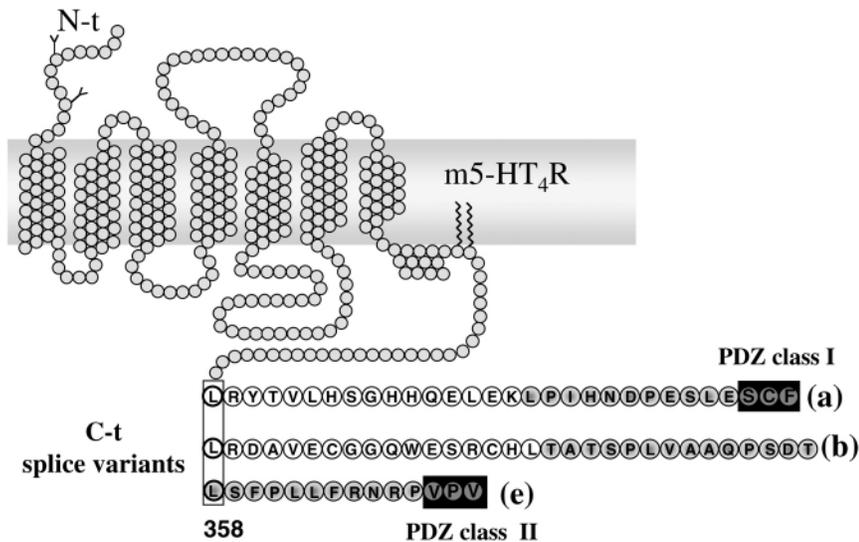


Fig. 1. C-Terminal sequences of the mouse 5-HT_{4(a)}, 5-HT_{4(b)} and 5-HT_{4(e)} receptor splice variants. Residues in grey represent the peptide baits used in the affinity-chromatography step of the proteomic screen [C-t(a), C-t(b) and C-t(e) for the 5-HT_{4(a)}R, 5-HT_{4(b)}R and 5-HT_{4(e)}R variant, respectively]. The boxes show the canonical PDZ ligands expressed in the C-termini of 5-HT_{4(a)}R and 5-HT_{4(e)}R.

1). All but the 5-HT_{4(d)} variant are expressed at various densities in the brain (Claeyens et al., 1999; Medhurst et al., 2001). Moreover, the rat and human 5-HT_{4(e)} mRNAs have only been detected in brain tissue. The 5-HT₄R variants also share identical pharmacological profiles and activate common signaling pathways, i.e. G_{αs} in cultured neurons (Dumuis et al., 1988) and G_{α13} in heterologous systems (Ponimaskin et al., 2002b). However, some differences in the signal-transduction characteristics of these variants have been reported. Short splice variants (e and f) exhibit a higher constitutive activity than do the long variants (Claeyens et al., 1999). In addition, truncation of the C-terminus of the 5-HT_{4(a)}R variant or mutation of the three C-terminal palmitoylated cysteines regulates the agonist-independent activity of the 5-HT_{4(a)}R (Claeyens et al., 2001; Ponimaskin et al., 2002a). This suggests that the C-terminus of 5-HT_{4(a)}R variants, which result from alternative splicing, modulate the allosteric transition from the inactive (R) to the active (R*) conformation of the receptor (Claeyens et al., 1999; Claeyens et al., 2001). However, the involvement of each splice variant in specific physiological processes remains to be elucidated.

Three mouse 5-HT₄R splice variants express a canonical recognition motif for PDZ domains (PDZ ligand) at their extreme C-termini. The PDZ ligand of the 5-HT_{4(a)}R variant (SCF) belongs to class I (S/TXϕ, where ϕ represents a hydrophobic residue), whereas that of the 5-HT_{4(e)}R and 5-HT_{4(f)}R variants (VPV) belongs to class II (ϕXϕ). This suggests that these receptor variants might interact with specific multiprotein complexes organized, at least in part, by PDZ-based interactions. By contrast, the 5-HT_{4(b)}R variant shows no consensus interaction motif at its C-terminus. The present study was performed to provide a unbiased identification of the proteins that interact with the PDZ ligand of the mouse 5-HT_{4(a)}R and 5-HT_{4(e)}R splice variants, using a proteomic approach based on peptide-affinity chromatography, 2D electrophoresis and mass spectrometry. We identified ten proteins that interact specifically with the 5-HT_{4(a)}R variant and three that associate with the 5-HT_{4(e)}R variant. Most of them are PDZ-domain-containing proteins.

Additional experiments were performed to determine whether these specific interactions occur in living cells. Particular attention was paid to the role of Na⁺/H⁺ exchanger regulatory factor (NHERF) and sorting nexin 27 (SNX27), two PDZ proteins identified as specific binding partners of the 5-HT_{4(a)}R variant, in the trafficking and targeting of the receptor.

Materials and Methods

Plasmids

The RhoTag-5HT_{4(a)}/pRK5, RhoTag-5HT_{4(b)}/pRK5 and RhoTag-5HT_{4(e)}/pRK5 constructs were generated by fusing a sequence from the N-terminus of rhodopsin (MNGTEGPNFYVFPNSKTVGVV) (Adamus et al., 1991) to a cleavable signal peptide (MRPADLLQLVLLLDLPRDLGG) derived from the thyroid stimulating hormone (TSH) receptor. This sequence was inserted in a PRK5 vector using *Xba*I and *Bsr*GI restriction sites. Then, full-length mouse 5-HT_{4(a)}R, 5-HT_{4(b)}R and 5-HT_{4(e)}R cDNAs were subcloned in frame using *Bsr*GI and *Hind*III. Deletion of the PDZ ligand in the Rho-tagged 5-HT_{4(a)}R variant was generated by inserting a stop codon after residue 384, using the Quikchange TM site-directed mutagenesis kit (Stratagene).

The expression vector encoding rabbit NHERF fused to an N-terminal haemagglutinin (HA) tag (HA-NHERF/pBK) was generously provided by R. A. Hall (Emory University School of Medicine, Atlanta, GA). The coding sequence corresponding to CIPP fused to N-terminal FLAG tag (Flag-CIPP pCI) was a gift from M. Lazdunski (CNRS-UMR 6097, Valbonne, France). The coding region of both isoforms of SNX27 was amplified by PCR from a human Universal Quick Clone cDNA library (Clontech) using Advantage 2 polymerase (Clontech) and specific primers for the common 5' end (ATGGCGACGAGGACGGGGAAGGATT) and the specific 3' end of SNX27a (CTAGGTGGCCACATCTCTCTGCTGTGACCT) and SNX27b (CTAATATTCCTCTTTTCTCCACTTGAGCTC). A 5' *Xho*I site and a 3' *Not*I site were introduced during PCR amplification. Both SNX27a and SNX27b were subcloned into pMYC and pDHA using *Xho*I and *Not*I. Deletion mutants were generated using PCR and cloned as mentioned above. The c-Myc-tagged DLGH3 construct was kindly provided by B. Margolis (University of Michigan Medical School, Ann Arto, MI). The Veli-3 (Veli-3/PRK7) construct was previously described (Becamel et al., 2002a).

Construct HA-NHERF-RhoTag5HT₄ in pIRES2 was obtained by

replacing the green fluorescent protein (GFP) sequence of the pIRE5 vector by the PS-RhoTag-5HT₄ sequences [variants (a), (b) and (e), respectively] and subcloning the HA-NHERF sequence in the polylinker of each vector (Vlaeminck-Guillem et al., 2002).

Antibodies

Rabbit anti-HA and anti-Vel13 antibodies were purchased from Zymed Laboratories. Mouse anti-c-Myc was a gift from B. Mouillac (INSERM-U469, Montpellier, France), and rabbit anti-ezrin was generously provided by P. Mangeat (CRBM-CNRS, Montpellier, France). Rabbit anti-c-Myc and rabbit anti-Flag antibodies were from Sigma. Mouse anti-RhoTag antibody was provided by S. Costagliola (IRIBHN, Brussels, Belgium) (Adamus et al., 1991). The monoclonal antibody against EEA1 was purchased from BD Transduction Laboratories. The secondary antibodies labelled with Alexa 594 red, Alexa 488 green or Alexa 647 blue were purchased from Molecular Probes (Eugene, OR). For triple labelling of A431 cells, the fluorescein isothiocyanate (FITC) conjugated secondary antibody was from Jackson ImmunoResearch. Texas-red-labelled streptavidin was from Zymed Laboratories.

Peptide-affinity chromatography

Synthetic peptides (>95% purity; Eurogentec, Seraing, Belgium) encompassing the 14 C-terminal amino acids of the 5-HT_{4(a)}, the 5-HT_{4(b)} and the 5-HT_{4(c)} receptor variants were coupled via their N-terminal extremity to activated CH-Sepharose 4B (Amersham Biosciences, Upsalla, Sweden), according to the manufacturer's instructions (peptide sequences were LPIHNDPESLESCF for the 5-HT_{4(a)} variant, TATSPLVAAQPSDT for the 5-HT_{4(b)} variant, LSFPLLFRNRPVPV for the 5-HT_{4(c)} variant, LPIHNDPESLE for the 5-HT_{4(a)}ΔSCF, LSFPLLFRNR for the 5-HT_{4(c)}ΔPVPV and TATSPLVAAQP for the 5-HT_{4(b)}ΔSDT). Immobilized peptides were stored in 50 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol (DTT) to prevent the oxidation of the cysteine residue located in the PDZ ligand of the 5-HT_{4(a)} variant. Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry analysis indicated that the coupling efficacy was higher than 95% for each peptide.

Proteins solubilized from Swiss mouse brains (Janvier, France) (10 mg per condition) or cultured colliculi neurons (2 mg per condition) were incubated with immobilized peptides (2 μg each). Proteins retained by affinity were eluted with either 350 μl isoelectrofocusing medium containing 7 M urea, 2 M thiourea, 4% CHAPS, ampholines (preblended, pI 3.5-9.5, 8 mg ml⁻¹; Amersham Biosciences, Upsalla, Sweden), 100 mM DTT, 0.2% tergitol NP7 (Sigma) and traces of bromophenol blue for 2D electrophoresis analysis or 0.1 ml sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 30% glycerol, 100 mM DTT and bromophenol blue) for immunoblotting.

2D electrophoresis and identification of proteins by MALDI-TOF mass spectrometry

Proteins separated by 2D electrophoresis were stained with silver as previously described (Becamel et al., 2002b). Gels to be compared were always processed and stained in parallel. Gels were scanned using a computer-assisted densitometer (Amersham Biosciences).

Proteins of interest were excised and digested in gel using trypsin (Gold, Promega). Digest products were loaded onto the target of an Ultraflex MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) and mixed with the same volume of cyano-4-hydroxy-*trans*-cinnamic acid (Sigma; 10 mg ml⁻¹ in acetonitrile-TFA, 50-0.1%). Analysis was performed in reflectron mode with an accelerating voltage of 20 kV and a delayed extraction of 400 nanoseconds. Spectra were analysed using XTOF software

(Bruker-Franzen Analytik) and autoprolysis products of trypsin (*M_r* 842.51, 1045.56 and 2211.10) were used as internal calibrates. Identification of proteins was performed using both Mascot and PeptIdent software (<http://www.matrixscience.com/> and <http://www.expasy.org/tools/peptide.html>), as previously described (Becamel et al., 2002b).

Cell cultures and transfection

Primary cultures of colliculi neurons were prepared as previously described (Weiss et al., 1986). Briefly, cells dissociated from colliculi of 14-15-day-old Swiss mouse embryos were plated in serum-free medium in 100-mm culture dishes (1.5×10⁶ cells ml⁻¹, 10 ml per dish). Cultures were maintained for 9-11 days at 37°C in a humidified atmosphere in 6% CO₂, 94% air. Under these conditions, at least 95% of the cultured cells were neurons (Weiss et al., 1986).

COS-7, NIH-3T3, A431 and HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and antibiotics. They were transfected at 60-70% confluence either by electroporation, as previously described (Claeyens et al., 1999), or using Lipofectamine™ 2000 (Invitrogen) or EFFECTENE (Qiagen), depending on the cell type and the cDNA, as indicated in the figure legends. Cells were processed for immunofluorescence, western blot or immunoprecipitation 24 hours after transfection.

Immunofluorescence and confocal microscopy

COS-7 and NIH-3T3 cells, grown on coverslips, were fixed with paraformaldehyde (4% in PBS) for 20 minutes at room temperature. They were then washed three times with 0.1 M glycine, permeabilized with Triton X-100 [0.1% in PBS containing 2% gelatin (PBS-gelatin)] and incubated overnight at 4°C with the primary antibody diluted in PBS-gelatin. Cells were washed in PBS-gelatin and incubated with the secondary antibody (Alexa-594-red-labelled anti-rabbit antibody or Alexa-488-green-labelled anti-mouse antibody in PBS-gelatin) for 1 hour at room temperature. Cells were washed and mounted on glass slides using gel mount (Biomed, Foster City, USA). Image acquisition was made using an MRC 1024 confocal microscope (Bio-Rad Laboratories, Hercules, CA). Series of optical sections were collected with a step of 0.30 μm.

A431 cells were permeabilized with 0.1% (w/v) saponin in PBSCM containing 5% bovine serum albumin (BSA) for 30 minutes at room temperature before incubation with the indicated primary antibodies in fluorescence dilution buffer [5% foetal calf serum, 5% goat serum, 2% BSA in 1 mM PBS-Ca²⁺, 5 mM PBS (PBSCM)].

Immunoprecipitation

Cells grown in 100-mm culture dishes and transiently transfected with the indicated plasmids were lysed in 500 μl lysis buffer [25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail (Roche)]. After dilution with an equal quantity of lysis buffer, soluble proteins were immunoprecipitated at 4°C for 4 hours with 25 μl anti-Myc monoclonal antibody cross-linked to protein-A/Sepharose (Clontech). Immunoprecipitated proteins were eluted with SDS sample buffer and resolved by SDS-PAGE.

Immunoblotting

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (Hybond-C; Amersham Biosciences). Membranes were incubated overnight at 4°C with the primary antibody. Membranes were washed three times for 10 minutes each in blocking buffer and exposed for 1 hour at room temperature with secondary antibodies. Proteins were detected using the Chemiluminescence Reagent Plus kit (Perkin Elmer).

Results

Isolation and identification of proteins interacting with the PDZ ligand of the mouse 5-HT_{4(a)} and 5-HT_{4(e)} receptor splice variants

Synthetic peptides corresponding to the last 14 C-terminal residues of the 5-HT₄R variants (a) and (e) [C-t(a)wt and C-t(e)wt, respectively (Fig. 1, grey residues)], were immobilized on Sepharose beads and used as bait to fish out proteins that interact with the C-termini of these receptors. Truncated peptides lacking the three C-terminal amino acids required to interact with PDZ domain proteins were used as negative controls [C-t(a) Δ SCF and C-t(e) Δ PVPV, respectively] to identify proteins specifically recruited by the PDZ ligand of each 5-HT₄R variant. The peptides were incubated with whole-brain extracts, because the 5-HT₄R splice variants examined are widely distributed in the mouse central nervous system. Proteins retained during the affinity chromatography step were eluted, separated on 2D gels and stained with silver. The different 2D protein patterns obtained with the wild-type peptides and their truncated counterparts revealed that the PDZ ligands of both variants recruit distinct sets of proteins. Indeed, we detected 13 spots or groups of spots in the gels obtained with the C-terminal peptide of the 5-HT_{4(a)}R variant. These spots were not apparent in the gels obtained with C-t(a) Δ SCF (Fig. 2A, left, Fig. 2B, top). Three different protein spots were recruited in affinity chromatography performed with C-t(e)wt but not C-t(e) Δ PVPV were also detected (Fig. 2A, right, Fig. 2C, top). None of these proteins bound to a peptide encompassing the 14 C-terminal residues of the 5-HT_{4(b)}R, consistent with the lack of class I or II PDZ ligand in the C-terminus of this variant (Fig. 2B,C bottom). Moreover, we did not detect any protein spot that was recruited by the C-t(b)wt peptide but not the C-t(a)wt or C-t(e)wt peptides (not shown).

All protein spots that specifically bound to the 5-HT_{4(a)}R and 5-HT_{4(e)}R PDZ ligands were unambiguously identified by MALDI-TOF mass spectrometry (Table 1). Among the proteins identified as binding partners of the 5-HT_{4(a)}R, seven out of ten encompass one or several PDZ domains. These include the activin receptor-interacting protein 1 (ARIP-1, spot 1), now renamed membrane-associated guanylate-kinase inverted-2 (MAGI-2). MAGI-2 is a prototypic scaffolding protein that contains six PDZ domains and two WW domains, and has previously been shown to interact with the β_1 -adrenergic receptor PDZ ligand (Xu et al., 2001a). The membrane protein palmitoylated 3 (MPP3) (train of spots 2), a member of the P55 membrane-associated guanylate-kinase (MAGUK) family, was also recruited by the 5-HT_{4(a)}R C-terminus. MPP3 contains a single PDZ domain plus a SH3 domain and two vertebrate homologues of *Caenorhabditis elegans* LIN-7 (Veli) binding domains (L27 domain) (Karnak et al., 2002). This protein was previously identified in the complex interacting with the C-terminus of the 5-HT_{2c}R, which also includes a class I PDZ ligand. Another protein recruited by this 5-HT₄R variant is SNX27a (also called Mrt1a, train of spots 4). SNX27a contains one PDZ domain and one Phox-homology domain (PX domain). This protein belongs to the huge sorting nexin protein family, which is implicated in the regulation of membrane-protein trafficking (Kajii et al., 2003). NHERF, also designated as

ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50) is another PDZ protein fished out with the C-t(a)wt peptide. NHERF has previously been identified as a binding partner of several GPCRs including the β_2 -adrenergic receptor and the parathormone receptor1 (Hall and Lefkowitz, 2002). Several isoforms of NHERF with different p/s (ranging from 4.6 to 5.0) and molecular weights (36-50 kDa) were detected on 2D gels (trains of spots 5, 6 and 8). Surprisingly, trypsin digestion of these isoforms yielded a similar peptide mass fingerprint (12 peptides representing up to 40% sequence coverage), indicating that they differ in regions that are not covered by our mass-spectrometry analyses. Finally, we identified Veli1-Veli3 (spots 9-11, respectively, Fig. 2) as binding partners of the 5-HT_{4(a)}R C-terminus. These proteins contain a PDZ domain and a L27 domain that can interact with the L27 domain of MPP3 (Karnak et al., 2002). Veli proteins occur discretely in specific neuronal populations throughout the brain and are associated with both presynaptic and postsynaptic complexes (Butz et al., 1998). The 5-HT_{4(a)}R C-terminus also specifically recruited guanine aminase (spot 7) and peroxiredoxin 5 (spot 12). These proteins do not include any protein-protein interaction domain but their C-termini can be considered to be class-I PDZ ligands (SSV and SQL, respectively). This suggests that these proteins might be recruited indirectly by the 5-HT_{4(a)}R C-terminus via the PDZ proteins identified in the complex purified by affinity chromatography. The last protein recruited by the 5-HT_{4(a)}R PDZ ligand was identified as Ulip2 (train of spots 3, Fig. 2). This protein, first described as collapsin-response-mediator protein 2 (CRMP-2), is a cytosolic phosphoprotein only expressed in the nervous system (Inagaki et al., 2001) that is involved in the regulation of axonal growth. The mechanism by which the 5-HT_{4(a)}R C-terminus binds to this protein remains to be elucidated, because its sequence lacks any obvious PDZ domain and/or PDZ ligand.

The proteins that associate with the PDZ ligand of the 5-HT_{4(e)}R variant include: (1) the neuronal isoform of nitric oxide synthase (nNOS; Fig. 2, spot 13), which contains one PDZ domain; (2) channel-interacting PDZ protein (CIPP; Fig. 2, spot 14), which contains four PDZ domains and has been identified as a partner of the potassium channel Kir4.0 family, NMDA receptor NR2, neuroligin, neuroligins and acid-sensing ionic channel 3 (Kurschner et al., 1998); (3) Sec23 (Fig. 2, spot 15). This protein, which lacks any obvious PDZ domain, is a member of a protein complex involved in the budding of vesicles from the endoplasmic reticulum (ER) and ER-to-Golgi transport of proteins (Tang et al., 1999).

We next performed immunoblotting when specific antibodies were available to provide direct biochemical evidence for a physical association of these proteins with the 5-HT₄R C-terminus. We pulled down the receptor-associated complexes from whole-brain extracts and extracts originating from cultured colliculi neurons, which express a high density of all 5-HT₄R variants. Moreover, these cultures constitute the first native system in which functional 5-HT₄R has been described (Dumuis et al., 1988). These experiments confirmed that Ulip 2, NHERF and Veli proteins interact specifically with the C-terminus of the 5-HT_{4(a)}R but not the 5-HT_{4(b)}R and the 5-HT_{4(e)}R, whereas nNOS associated specifically with the 5-HT_{4(e)}R variant (Fig. 3).

Altogether, these experiments indicate that the PDZ ligands of 5-HT_{4(a)}R and 5-HT_{4(e)}R variants interact with distinct sets of proteins in vitro and that most of these

proteins are modular proteins that contain one or several PDZ domains and additional protein-protein interaction domains.

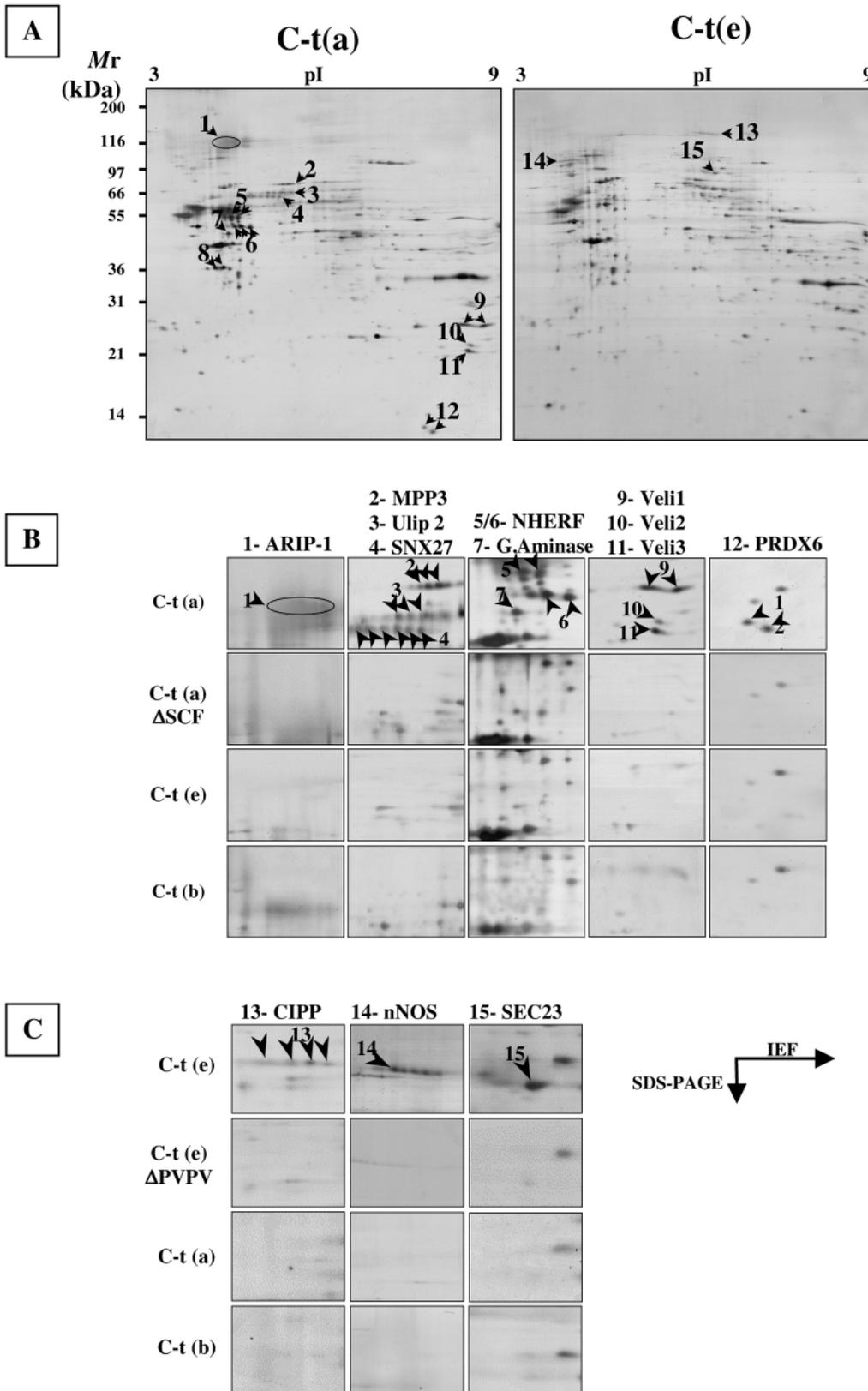


Fig. 2. Two-dimensional analysis of the proteins interacting with the C-termini of mouse 5-HT₄R splice variants. Proteins that bind to the C-terminus of the 5-HT_{4(a)}R and 5-HT_{4(e)}R variants were purified by affinity chromatography, separated by 2D electrophoresis and stained with silver. (A) 2D gels obtained with the C-t(a)wt and the C-t(e)wt peptides are illustrated. Arrows indicate the position of spots or trains of spots that were specifically retained by the C-t(a)wt and the C-t(b)wt peptides but not truncated peptides lacking their PDZ ligand [C-t(a)ΔSCF and C-t(e)ΔPVPV, respectively]. (B,C) Proteins recruited by affinity chromatography using the C-t(a)wt, C-t(b)wt, C-t(e)wt, C-t(a)ΔSCF or C-t(e)ΔPVPV peptides were separated by 2D electrophoresis and stained with silver. (B, top) Areas of interest of 2D gels showing the specific recruitment of ten spots by the 5-HT_{4(a)}R C-terminus [C-t(a)wt peptide] via a PDZ-based mechanism. (C, top) Areas of interest of 2D gels showing the specific recruitment of three spots by the 5-HT_{4(e)}R C-terminus [C-t(e)wt peptide] via a PDZ-based mechanism. The data are representative of four experiments performed independently.

Interaction of the 5-HT_{4(a)} receptor with both SNX27a and SNX27b in transfected HEK-293 cells

The *Mrt1* gene was recently identified as encoding two PDZ- and PX-domain proteins: SNX27a (Mrt1a) and SNX27b (Mrt1b) (Kajii et al., 2003). Both isoforms are generated by alternative splicing and only differ by the length of their C-termini. SNX27a is constitutively expressed in the brain and

testis, whereas SNX27b is specifically induced in adult brain by methamphetamine treatment. Our proteomic screen only identified the constitutive SNX27a form as a binding partner of the 5-HT_{4(a)}R PDZ ligand. We next examined whether the 5-HT_{4(a)}R interacts with both SNX27 isoforms in transfected HEK-293 cells co-expressing the Myc-tagged 5-HT_{4(a)}R and one of the HA-tagged SNX27 isoforms. Co-

Table 1. MALDI-TOF mass spectrometry identification of proteins interacting with the C-termini of 5-HT₄R splice variants

Spots*	Protein	Accession no. [†]	MALDI-TOF [‡]		PDZ [§]	Others [¶]	Interaction ^{**}			Functions
			Peptides	Coverage (%)			C-t(a)	C-t(b)	C-t(e)	
1	ARIP 1 (MAGI-2)	Q9WVQ1	18	21	6	2 WW, 1 GuK	+	-	-	Scaffolding, membrane targeting
2	MPP3 (Dlgh3)	O88910	21	45	1	L27, SH3, GuKc	+	-	-	Veli-interacting protein
3	Ulip2 (CRMP-2)	P47942	19	50	-	-	+	-	-	Axon growth
4	SNX27	Q8K4V4	18	36	1	PX	+	-	-	Intracellular trafficking
5,6,8	NHERF (EBP50)	P70441, Q9JJ19, Q9R1A1	40	2	ERM	+	-	-	-	Signalling, cytoskeleton modification
7	Guanine aminase	Q9R111	13	26	††	-	+	-	-	Purine-nucleotide metabolism
9	Veli1(Lin-7-Bb)	Q9Z250	8	36	1	L27	+	-	-	Targeting and trafficking
10	Veli2 (Lin-7B)	O88951	9	50	1	L27	+	-	-	Targeting and trafficking
11	Veli3 (Lin-7C)	O88952	9	52	1	L27	+	-	-	Targeting and trafficking
12	Peroxiredoxin 5	P99029	11	74	‡‡	-	+	-	-	Intracellular redox signalling
13	nNOS	Q9Z0J4	29	25	1	-	-	-	+	Signalling
14	CIPP	O70471	17	32	4	-	-	-	+	Scaffolding
15	SEC23	Q01405	19	34	-	-	-	-	+	Trafficking from ER to the Golgi apparatus

*The numbers correspond to those indicated in Fig. 2.

[†]Accession numbers are listed from SWISS-PROT and TrEMBL databanks.

[‡]When several protein spots were identified as a single protein, the results of the spot for which MALDI-TOF analysis yielded the largest sequence coverage are indicated.

[§]The number of PDZ domains encompassed in the protein sequence or the presence of a putative PDZ ligand.

[¶]Other protein-protein interaction domains.

^{**}Interaction of the protein with C-terminal peptides.

^{††}C-terminus sequence is SSV.

^{‡‡}C-terminus sequence is SQL.

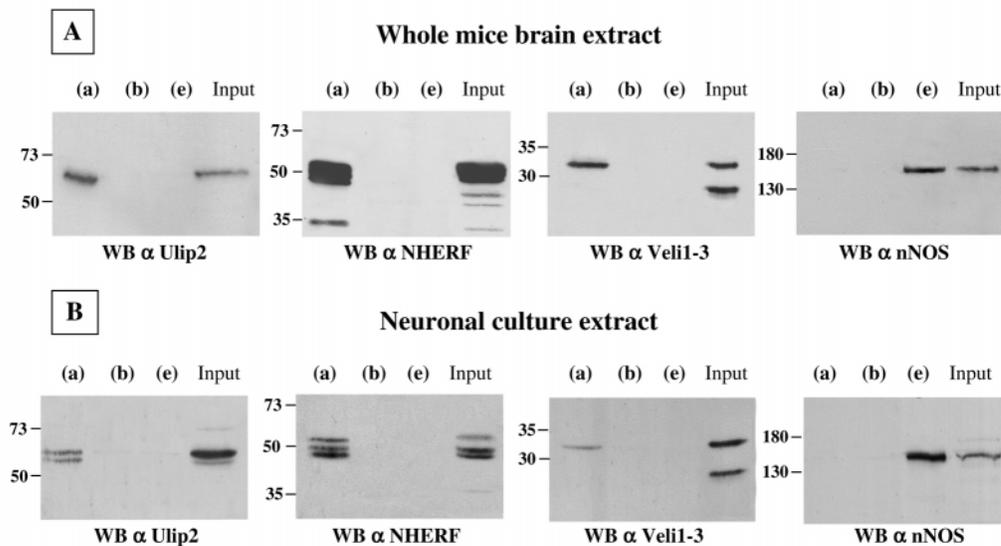


Fig. 3. Analysis of the interaction of 5-HT₄R C-termini with specific sets of proteins by immunoblotting. C-t(a), C-t(b) and C-t(e) peptide baits were incubated with protein extracts from either whole mouse brain (A) or mouse colliculi neurons in primary culture (B). Proteins retained by affinity were separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose sheets. Immunoblotting was performed with antibodies raised against the indicated proteins (anti-Ulip2, 1:2,000; anti-NHERF, 1:500; anti-Veli1-3, 1:200; anti-nNOS, 1:500). For each protein, the immunoreactive signals were found at molecular weights identical to those observed in silver-stained 2D gels. Input represents 5% of the total protein amount used in pull-down experiments. The data illustrated are representative of three experiments.

immunoprecipitation experiments performed with an anti-Myc antibody revealed that the 5-HT_{4(a)}R interacts with SNX27a and b isoforms (Fig. 4, lanes 1 and 6). By contrast, SNX27a and SNX27b were not co-immunoprecipitated with either the 5-HT_{4(b)}R (Fig. 4, lanes 5 and 10) or a PDZ-ligand-deleted 5-HT_{4(a)}R (Δ SCF, Fig. 4, lanes 4 and 9), consistent with the results obtained in the proteomic study. Further supporting the interaction of the 5-HT_{4(a)}R C-terminus with the PDZ domain of both SNX27 isoforms, PX-domain-deleted SNX27 (SNX27 Δ PX) [lanes 3 (SNX27a) and 8 (SNX27b)] but not PDZ domain-deleted SNX27 (SNX27 Δ PDZ) [lanes 2 (SNX27a) and 7 (SNX27b)] co-immunoprecipitated with the 5-HT_{4(a)}R. Altogether, these results indicate that the 5-HT_{4(a)}R PDZ ligand interacts with the PDZ domain of both the constitutive and inducible isoforms of SNX27 in HEK-293 cells.

Co-localization of the 5-HT_{4(a)}R and SNX27 in early endosomes of A431 cells

It is well documented that most members of the SNX protein family are localized to early endosomes, as assessed by their colocalization with the early endosome antigen 1 (EEA1) (Xu et al., 2001b). The next series of experiments was carried out to determine whether SNX27a contributes to the targeting of the 5-HT_{4(a)}R to early endosomes. This was examined in transiently transfected A431 cells, which display morphologically well-defined early endosomes. Co-immunofluorescence staining of A431 cells co-transfected with the Myc-tagged 5-HT_{4(a)}R and HA-tagged SNX27a revealed punctate labelling for both proteins, which strongly colocalized (Fig. 5Aa-d). Consistent

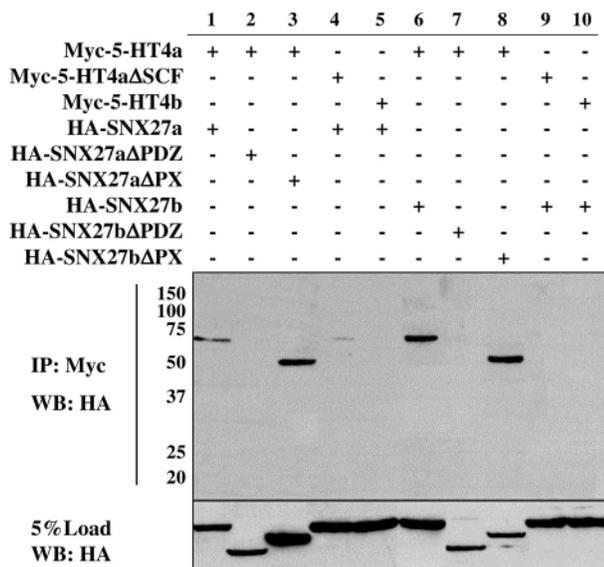


Fig. 4. Interaction of the 5-HT_{4(a)}R variant with both SNX27a and SNX27b in a PDZ-dependent manner. HEK-293 cells were co-transfected with the indicated cMyc-tagged-5-HT₄R, with or without HA-tagged-SNX27 constructs using Lipofectamine™ 2000. 24 hours after transfection, total cell lysates were immunoprecipitated with anti-Myc cross-linked protein-A/Sepharose. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by immunoblotting using the anti-HA antibody (top). Input (bottom) represents 5% of the total protein used in immunoprecipitation. The data illustrated are representative of three experiments.

with our biochemical observations, no colocalization was observed in cells co-expressing either the 5-HT_{4(a)}R and the SNX27a Δ PDZ (Fig. 5Ae-h) or SNX27a and 5-HT_{4(a)} Δ SCF (Fig. 5Ai-l). Triple-labelling experiments were then performed to determine the identity of the compartment to which SNX27a and the 5-HT_{4(a)}R are colocalized. Points identified by the presence of both SNX27a and 5-HT_{4(a)}R were also stained by an EEA1 antibody (Fig. 5Ba-d, white points), indicating that SNX27a and the 5-HT_{4(a)}R are colocalized to early endosomes. Moreover, disruption of the interaction between SNX27a and the 5-HT_{4(a)}R [by transfecting either SNX27a Δ PDZ (Fig. 5Be-h) or 5-HT_{4(a)} Δ SCF (Fig. 5Bi-l)], resulted in a loss of white punctates, which correspond to the triple colocalization. The targeting of the 5-HT_{4(a)}R to early endosomes was inhibited by the deletion of the PDZ ligand (Fig. 5Bi-l, white points). By contrast, SNX27a Δ PDZ still colocalized with EEA1 (Fig. 5Be-h). Taken together, these results suggest that interaction between SNX27a and the 5-HT_{4(a)}R occurs at early endosomes, and that SNX27a is responsible for the targeting of the 5-HT_{4(a)}R to early endosomes.

Colocalization of 5-HT_{4(a)}R with NHERF and ezrin in NIH-3T3 cells

We next examined whether NHERF, another PDZ protein identified as a binding partner of the 5-HT_{4(a)}R in our proteomic analysis, interacts specifically with this 5-HT₄R variant in living cells. This was evaluated by immunocytochemistry experiments in NIH-3T3 cells transiently co-transfected with NHERF and either the Myc-tagged 5-HT_{4(a)}R or the 5-HT_{4(b)}R variant, because this cell line lacks endogenous NHERF. Staining NIH-3T3 cells transfected with NHERF indicated a localization of the protein throughout the cytoplasm, with highest labelling at the submembrane level and in microvilli (Fig. 6A, left). In cells expressing either the 5-HT_{4(a)}R or the 5-HT_{4(b)}R, Myc immunostaining was mostly localized to the cell surface and at intracellular membrane structures (Fig. 6A, middle and right). No microvilli were detected in cells expressing the 5-HT_{4(a)}R or the 5-HT_{4(b)}R alone. In cells co-expressing NHERF and the 5-HT_{4(a)}R, the receptor was highly concentrated in microvilli and was strongly colocalized with NHERF (Fig. 6B). By contrast, in cells co-expressing the 5-HT_{4(b)}R with NHERF, we observed a distribution similar to that observed in cells expressing the receptor alone. Moreover, the 5-HT_{4(b)}R did not concentrate in microvilli and was not colocalized with NHERF (Fig. 6C).

NHERF interacts with the four-point-one/ezrin/radixin/moesin (FERM) domain of activated ERM proteins. We examined whether ezrin, a member of the ERM family, is recruited to a complex containing NHERF and the 5-HT_{4(a)}R in microvilli of NIH-3T3 cells. In non-transfected NIH-3T3 cells, endogenous ezrin showed a homogenous distribution throughout the cytoplasm (Fig. 7A, left, white arrows). The localization of ezrin remained unchanged in cells transfected with either the 5-HT_{4(a)}R or the 5-HT_{4(b)}R (Fig. 7A, left). By contrast, co-transfecting cells with the 5-HT_{4(a)}R variant and NHERF drastically changed the distribution of endogenous ezrin: the protein accumulated in microvilli, where it localized with the receptor (Fig. 7B, left and right). As expected, co-expression of NHERF with the 5-HT_{4(b)}R had no effect on ezrin distribution and no colocalization between NHERF and this 5-HT_{4(b)}R

variant was observed (Fig. 7C, left and right). These results indicate that the 5-HT_{4(a)}R interacts specifically with a protein complex including NHERF and ezrin that might participate in its targeting to specialized subcellular regions, such as microvilli.

Colocalization of 5-HT_{4(e)} but not 5-HT_{4(b)} receptor with CIPP in transfected COS-7 and NIH-3T3 cells

Experiments were also performed to determine whether CIPP,

a PDZ protein that was only recruited by the 5-HT_{4(e)}R C-terminus in our in vitro binding assay, specifically interacts with this receptor variant within intact cells. Staining COS-7 cells transiently transfected with Flag-CIPP revealed a diffuse localization of CIPP throughout the cytoplasm with some perinuclear concentration (Fig. 8A1). Cells transfected with either the Myc-5-HT_{4(e)}R or the Myc-5-HT_{4(b)}R exhibited labelling both at the cell surface and intracellular membrane-structures throughout the cytoplasm (Fig. 8A2-4). As shown

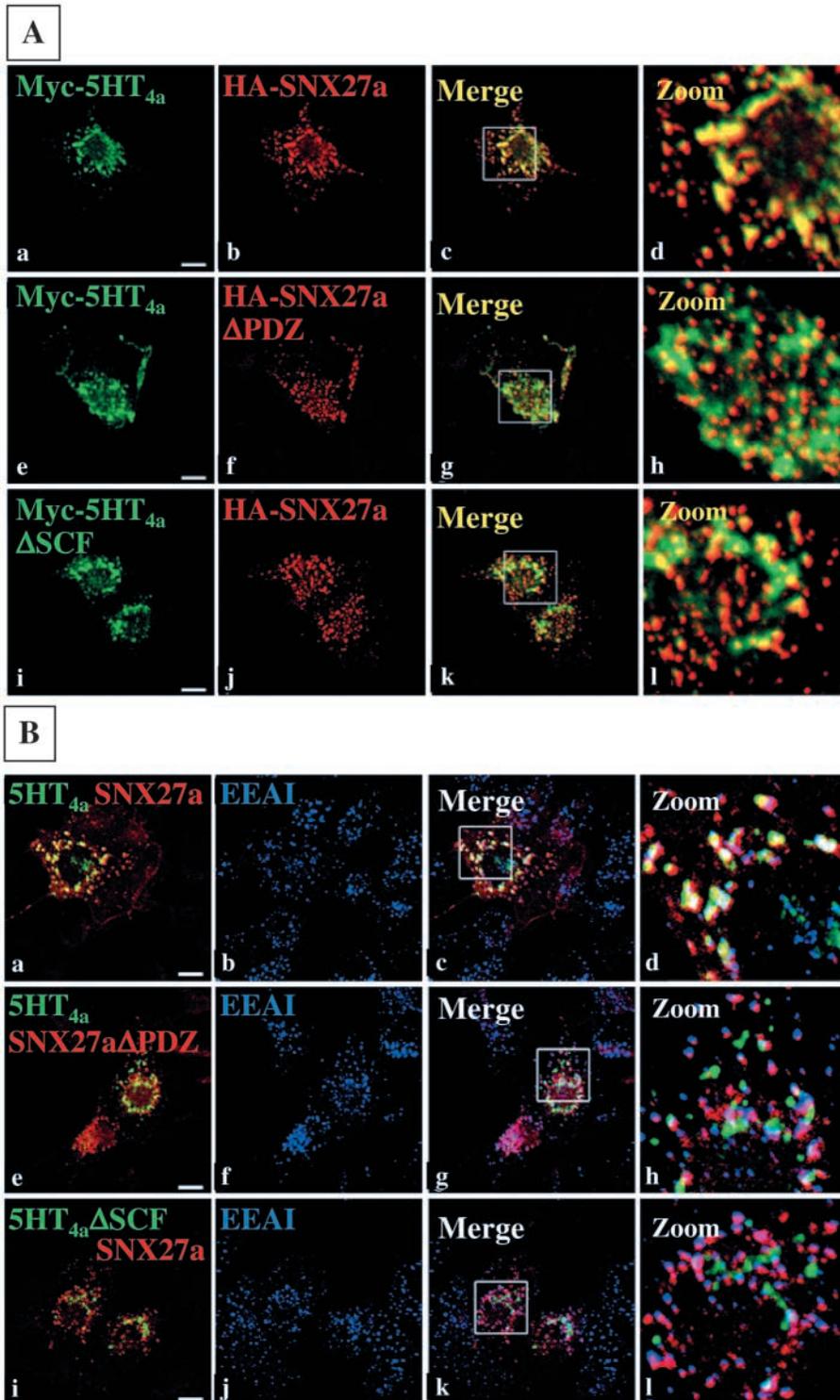


Fig. 5. Recruitment of the 5-HT_{4(a)}R by SNX27a to early endosomes in A431 cells. (A) A431 cells transiently transfected using EFECTENE with the cMyc-tagged-5-HT_{4(a)}R together with HA-tagged SNX27a (a-d) or HA-SNX27aΔPDZ (e-h) and the cMyc-tagged-5-HT_{4(a)}RΔSCF receptor together with HA-SNX27a (i-l) were processed for indirect immunofluorescence using the polyclonal anti-Myc antibody (visualized in green using goat anti-rabbit IgG conjugated to FITC) (a,e,i) and the monoclonal anti HA (visualized in red using goat anti-mouse IgG conjugated to AlexaFluor 555) (b,f,j). Merge images (c,g,k) were further magnified to show detail (d,h,l). Yellow staining highlights colocalization between 5-HT_{4(a)}R and SNX27a. (B) Cells transfected as described in A were processed for indirect immunofluorescence, initially using the polyclonal anti-Myc antibody visualized in green (a,e,i) and the monoclonal anti-EEA1 antibody (visualized in blue using goat anti-mouse IgG conjugated to AlexaFluor647) (b,f,j), followed by incubation with a biotin-labelled monoclonal anti-HA antibody (visualized in red using streptavidin conjugated to Texas Red) (a,e,i). Overlaid images (c,g,k) were further magnified to show detail (d,h,l). Yellow staining highlights colocalization of the 5-HT_{4(a)}R and SNX27a, whereas purple indicates colocalization of SNX27a and EEAI, and cyan indicates colocalization of 5-HT_{4(a)}R and EEAI. White staining indicates colocalization between the 5-HT_{4(a)}R, SNX27a and EEAI. Bars, 10 μm.

in Fig. 8A (Fig. 8A5-7), an extensive overlap of Flag and Myc immunostainings was observed within intracellular membrane structures of cells co-expressing the Myc-5-HT_{4(e)}R and Flag-CIPP constructs. By contrast, we detected no colocalization of the Myc-5-HT_{4(b)}R or the Myc-5-HT_{4(e)}ΔPVPV R and CIPP (Fig. 8A8-13). Experiments performed in NIH-3T3 cells (Fig. 8B) and HEK-293 cells (data not shown) yielded similar results. CIPP and the Myc-

5-HT_{4(e)}R were colocalized to cytoplasmic compartments (Fig. 8B, left), whereas CIPP did not localize with the Myc-5-HT_{4(b)}R or the Myc-5-HT_{4(e)}ΔPVPV R (Fig. 8B, middle and right).

These results suggest that CIPP interacts specifically in a PDZ-dependent manner with the 5-HT_{4(e)}R within living cells, consistent with the data obtained in our proteomic screen.

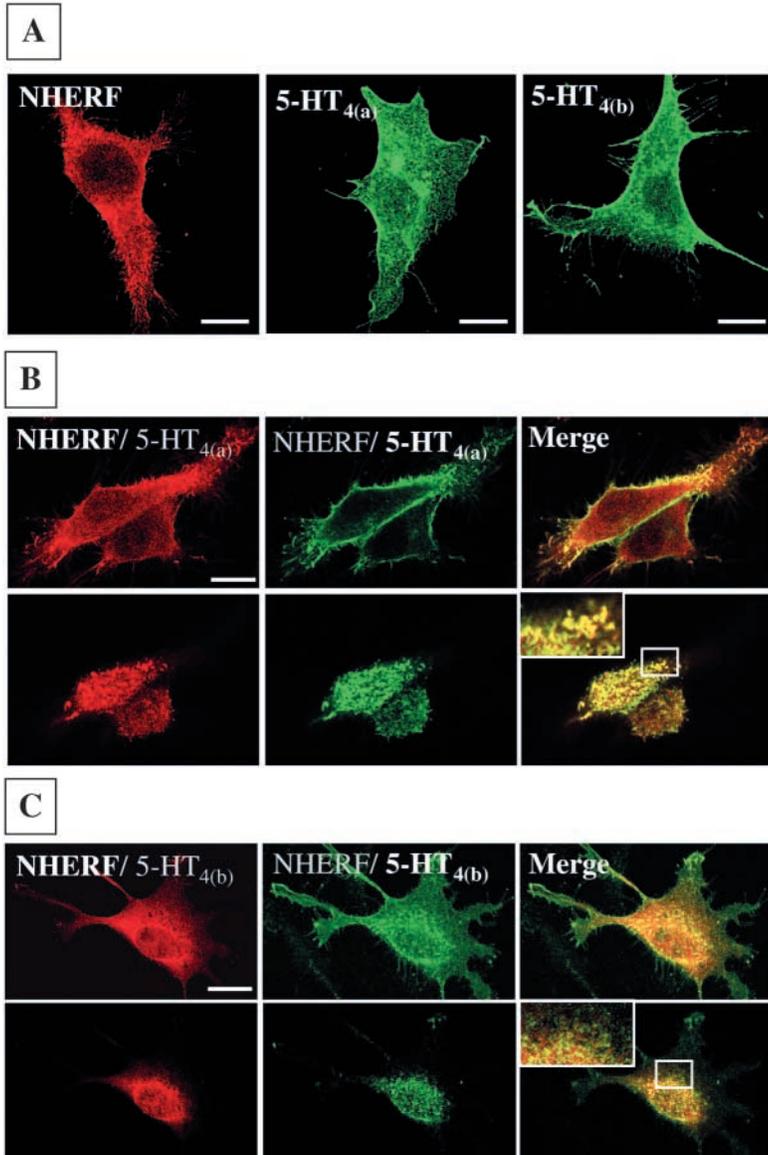


Fig. 6. Colocalization of NHERF with the 5-HT_{4(a)}R, but not the 5-HT_{4(b)}R splice variant in transiently transfected NIH-3T3 cells. (A) Cells were transfected by electroporation with constructs encoding HA-tagged NHERF (1), the Rho-tagged 5-HT_{4(a)}R (2) or the Rho-tagged 5-HT_{4(b)}R (3) alone. They were permeabilized, immunostained with the anti-HA (1:400) or the anti-RhoTag (1:100) antibody and analysed by confocal laser scanning microscopy. Mid-nuclear slices from representative cells are shown. (B,C) Cells were transfected with either the HA-NHERF-RhoTag 5-HT_{4(a)}R/pIRES2 (B) or the HA-NHERF-RhoTag-5-HT_{4(b)}R/pIRES2 (C) construct, which ensure efficient co-expression in the same cells. Confocal slices are shown from the mid-nuclear level (top) or close to the cell surface (bottom). Left, medium and right panels show HA staining, RhoTag staining and merged images, respectively. Bars, 10 μm.

Discussion

There is accumulating evidence that the GPCR C-terminal domain is particularly important for their interaction with intracellular protein networks. These networks have become a well-recognized means of modulating GPCR localization, activation and signaling (Bockaert et al., 2003; Hall and Lefkowitz, 2002). It is important to relate this property to the observation that many GPCR splice variants differ in their C-termini. Therefore, understanding the specific roles of the different C-terminal GPCR splice variants certainly requires a detailed knowledge of proteins that specifically associate with each of them. In this regard, the 5-HT₄R, which is certainly one of the GPCRs for which alternative mRNA splicing generates the most variants differing in their C-termini, represents an interesting challenge. Indeed, some of these variants express PDZ ligands belonging to different subclasses at their extreme C-termini, suggesting that they interact with specific PDZ-based protein networks. These networks could assign a functional specificity to these 5-HT₄R.

In this study, we have addressed the question of whether different 5-HT₄R splice variants from the mouse interact with specific sets of proteins, using a proteomic approach based on peptide-affinity chromatography and mass spectrometry. We focused on the 5-HT_{4(a)}R and 5-HT_{4(e)}R variants because they express class-I and class-II PDZ ligands at their extreme C-termini, respectively. We found that the 5-HT_{4(a)}R interacts with a set of ten proteins, whereas the C-t(e)wt peptide recruits a different set of three proteins. Most of them are PDZ proteins. The observed interactions are PDZ dependent, and this is further confirmed by the fact that they were suppressed by the deletion of the three C-terminal residues of the receptor, which are crucial for the interaction with target PDZ domains. We also provide evidence that the C-terminus of the 5-HT_{4(b)}R variant (14 residues) recruits no specific proteins, in contrast to the 5-HT_{4(a)}R and 5-HT_{4(e)}R C-termini, consistent with the lack of recognition motifs for protein interaction domains in this sequence. This does not rule out that motifs located upstream of the peptide bait used for the fishing experiments interact with other specific proteins.

So far, most of the proteins that interact with GPCR C-termini have been identified using a two-hybrid screen. The present study is the third attempt to identify GPCR-associated protein networks using

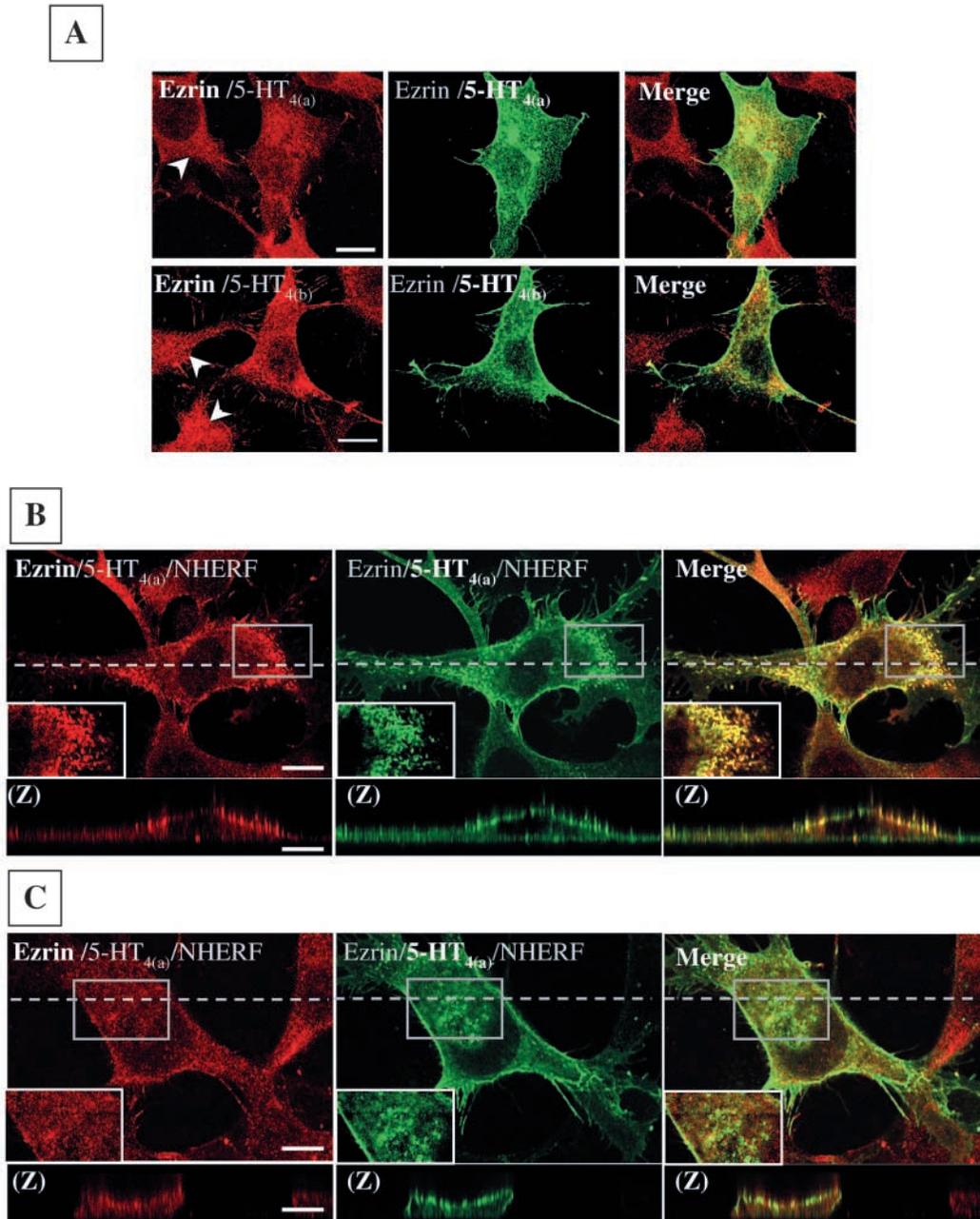


Fig. 7. Colocalization of endogenous ezrin with the 5-HT_{4(a)} but not the 5-HT_{4(b)} splice variant in the presence of NHERF in NIH-3T3 cells. Cells were transfected by electroporation with plasmids encoding either HA-NHERF-RhoTag-5-HT_{4(a)}R/pIRES2 (A, top, B) or the HA-NHERF-RhoTag-5-HT_{4(b)}R/pIRES2 (A, bottom, C) construct. They were permeabilized and examined by confocal microscopy. Left, medium and right panels illustrate endogenous ezrin staining (anti-ezrin antibody 1:1,000), RhoTag staining (anti-RhoTag antibody 1:100) and merged images, respectively. Mid-nuclear slices (inlay: zoom) and vertical scans in the *z* axis (*z*) (along the black lines in the *x-y* scans) are depicted. Representative cells are represented. Bars, 10 μ m.

a global proteomic approach. Indeed, we have recently used a similar strategy to characterize the multiprotein complex associated with the 5-HT_{2A} and the 5-HT_{2C} receptors (Becamel et al., 2002a; Becamel et al., 2004). The success of this third attempt proves that the method is reliable. One important question is the extent to which the different but often very similar PDZ ligands found in the C-termini of many GPCRs interact with specific sets of proteins. In other words, are these

'receptosomes' really specific? Our studies of the 5-HT₂ and 5-HT₄ receptors suggest that the protein components of GPCR-associated complexes are highly specific. For instance, the PDZ ligands of the 5-HT_{2A}, the 5-HT_{2C} and the 5-HT_{4(a)}R, which are quite similar (SCV, SSV and SCF, respectively), interact with different sets of PDZ proteins (Becamel et al., 2004). Indeed, both 5-HT₂ receptors but not the 5-HT_{4(a)}R interact with PSD-95 (Becamel et al., 2002a; Becamel et al.,

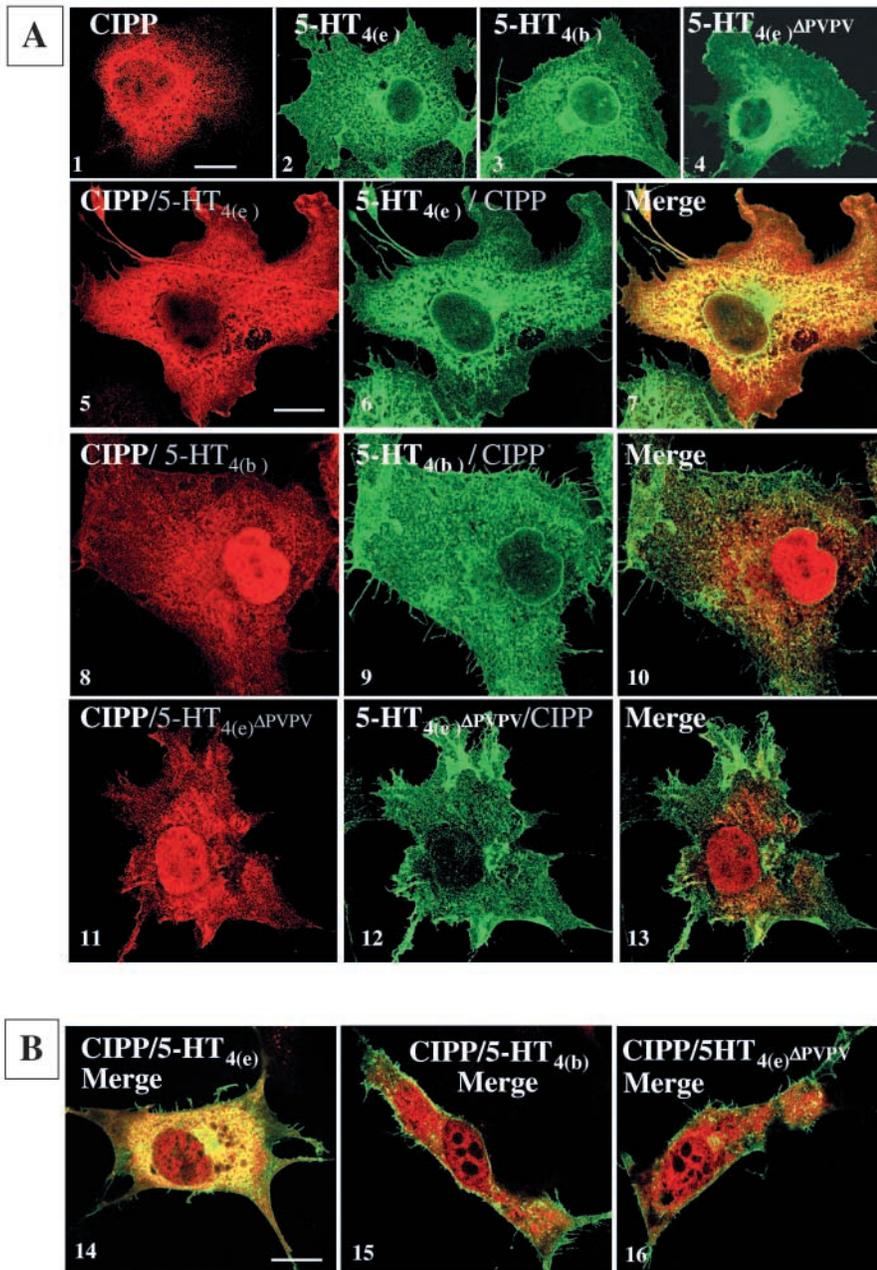


Fig. 8. Interaction of CIPP with the 5-HT_{4(e)}R in transiently transfected COS-7 and NIH-3T3 cells. (A) COS-7 cells were transfected with Flag-tagged CIPP (1), the cMyc-5-HT_{4(e)}R (2), the cMyc-5-HT_{4(b)}R (3), the cMyc-5-HT_{4(e)}ΔPVPV R (4) or Flag-tagged CIPP together with each 5-HT₄R construct (5-13) using Lipofectamine™ 2000. Cells were examined by confocal microscopy. (left) Flag staining (1:1,000); (middle) Myc staining (1:400); (right) merged images. (B) NIH-3T3 cells were transfected with the same constructs using Lipofectamine 2000. Merged images obtained after Flag and Myc staining of cells co-transfected with Flag-tagged CIPP and the cMyc-5-HT_{4(e)}R (14), the cMyc-5-HT_{4(b)}R (15) or the cMyc-5-HT_{4(e)}ΔPVPV R (16) are depicted. Bars, 10 μm.

2004; Xia et al., 2003). Moreover, Veli3 is only recruited by the 5-HT_{2c}R and the 5-HT_{4(a)}R, whereas only the 5-HT_{4(a)}R C-terminus interacts with SNX27 and NHERF. This suggests that the specificity of PDZ ligands for PDZ domains is not only based on three residues at the extreme C-terminus of the receptors. Accordingly, the tyrosine at -7 position in the ErbB2 C-terminus is involved in its interaction with the erbin PDZ domain (Birrane et al., 2003). Similarly, the last eight or nine residues at the C-termini of CRIPT and the Kv1.4 potassium channel subunit determines their binding specificities for PDZ3 and PDZ1/2 of PSD-95, and SAP102 and SAP97, respectively (Niethammer et al., 1998).

Another major advantage of the proteomic approach over the two-hybrid strategy is that it can isolate proteins that bind not only directly but also indirectly to the bait. In this regard, Veli1-

Veli3 and MPP3, which were identified as binding partners of the 5-HT_{4(a)}R variant, can associate through their L27 domain. Our approach does not indicate whether these proteins interact directly with the receptor PDZ ligand or whether some of them are indirectly recruited via a L27-domain-based scaffold. Moreover, like two-hybrid screens, proteomic approaches do not remove the task of investigating the physiological relevance of the observed interactions. Here, we have focused our attention on two proteins that are recruited in large amounts, compared with whole-brain extracts (data not shown), by the C-termini of 5-HT_{4(a)}R, NHERF and SNX27a.

Immunofluorescence and confocal microscopy performed in NIH-3T3 cells (this cell line was chosen because it lacks endogenous NHERF) indicated that the co-expression NHERF with the 5-HT_{4(a)}R greatly modified the cellular

localization of the receptor. Moreover, the receptor concentrated in microvilli, where it localized with NHERF. Further supporting the specificity of our proteomic approach, the 5-HT_{4(b)}R variant, which does not bind to NHERF, was not recruited to microvilli. Interestingly, ezrin (a ERM family member), which was homogeneously distributed throughout the cytoplasm in non-transfected cells, was mainly detected in microvilli of cells co-transfected with the 5-HT_{4(a)}R and NHERF. Ezrin redistribution in microvilli is known to result from its association with membrane phosphatidylinositol-(4,5)-bisphosphate (Barret et al., 2000) and its phosphorylation by either Rho kinase (Matsui et al., 1998) or protein kinase C θ (Pietromonaco et al., 1998). This process induces a conformational change of the protein, which unmask both its N-terminal FERM domain and its C-terminal ERMAD domain (active state). The unmasked FERM and ERMAD domains then bind to NHERF and F-actin, respectively. Thus, ezrin (and other ERM proteins) might constitute a key link between the cytoskeleton and membrane proteins, which is essential to regulate cell shape (such as microvilli) and also cell adhesion and motility (Bretscher et al., 2002). The molecular mechanisms that contribute to ezrin conformational change and microvillus formation in cells co-expressing NHERF and the 5-HT_{4(a)}R remain to be elucidated. This process might be related to the known ability of this receptor to activate the G α 13-Rho pathway in NIH-3T3 cells (Ponimaskin et al., 2002b). 5-HT_{4(a)}R activation by its agonist was obviously not required. This is certainly due to the relatively high constitutive activity of the receptor (Claeysen et al., 1999). In any case, our study strongly suggests that the interaction of the 5-HT_{4(a)}R with NHERF contributes to the targeting of the receptor to specialized subcellular regions and the regulation of cell shape.

Another promising protein identified as a binding partner of the 5-HT_{4(a)}R is SNX27. This was initially cloned from the human genome (TrEMBL accession number Q96L92). Two different forms of the protein (Mrt1a and Mrt1b) encoded by a single gene (*Mrt1*) and differing in their C-termini were further identified in the rat and are identical to the human SNX27a and SNX27b. Both proteins are predominantly expressed in the brain. Further analysis revealed that only *Mrt1b* mRNA is upregulated in the neocortex of adult rats after acute methamphetamine treatment or a daily treatment for 5 days. By contrast, the level of *Mrt1a* mRNA, which is constitutively expressed, was not affected by this treatment (Kajii et al., 2003).

SNX27a and SNX27b belong to a family of proteins that are implicated in endocytic targeting of cell-surface receptors. SNX1 was originally identified as enhancing the degradation of the epidermal growth factor receptor, probably by a facilitation of the endosome-to-lysosome targeting (Kurten et al., 1996). Further studies indicated that SNX1 participates in the sorting of the protease-activated-receptor-1 from early endosomes to lysosomes (Wang et al., 2002). Another sorting nexin, designated SNX3, drastically enhanced surface receptor transport from early to recycling endosomes (Xu et al., 2001b). The present study demonstrates that the 5-HT_{4(a)}R but not the 5-HT_{4(b)}R variant interacts with both SNX27a and SNX27b through a PDZ-dependent mechanism. Moreover, immunocytochemistry experiments indicated that overexpression of SNX27a redirects at least part of the 5-

HT_{4(a)}R from plasma membrane to early endosomes. This suggests that SNX27 plays a crucial role in the targeting of 5-HT_{4(a)}R. We have previously shown that the 5-HT₄R-mediated cAMP accumulation in cultured neurones rapidly becomes desensitized (Ansanay et al., 1992). This desensitization process is followed by a sequestration of the receptor. Internalized receptors are delivered via clathrin-coated vesicles to early endosomes (Shenoy and Lefkowitz, 2003). Our study indicates that SNX27 is responsible for the specific recruitment of the 5-HT_{4(a)}R to this compartment. In this way, SNX27 could assign specific signalling properties to this variant.

As previously mentioned, methamphetamine treatment repeated daily for 5 days induced a persistent (2-3 week) increase in *Mrt1b* mRNA expression (encoding SNX27b) in the adult rat neocortex (Kajii et al., 2003). This effect, which was blocked by D1 antagonists, is reminiscent of stimulant-induced behavioural sensitization. Furthermore, there is evidence to suggest a role for 5-HT₄R in drug sensitization: (1) antagonists of 5-HT₄R attenuate hyperactivity induced by cocaine (McMahon and Cunningham, 1999); (2) 3,4-methylenedioxymethamphetamine (MDMA)- or cocaine-induced anorexia is attenuated following the administration of the 5-HT₄ receptor antagonist RS 39604; (3) novelty-induced exploratory activity is decreased in the 5-HT₄R knockout mice (Compan et al., 2004). Further studies of the 5-HT₄R/SNX27b interaction will certainly be fruitful for understanding the mechanism underlying drug sensitisation and addiction processes.

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