Role of the C-terminal di-leucine motif of 5-HT$_{1A}$ and 5-HT$_{1B}$ serotonin receptors in plasma membrane targeting

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Introduction

The 5-HT$_{1A}$ and 5-HT$_{1B}$ serotonin receptors exhibit different subcellular localizations in neurons. Evidence has been reported that the C-terminal domain is involved in the somato-dendritic and axonal targeting of 5-HT$_{1A}$R and 5-HT$_{1B}$R, respectively. Here we analyzed the consequences of the mutation of a di-leucine motif and palmitoylated cysteines within this domain. Replacement of I414-I415 by a di-alanine in 5-HT$_{1A}$R led to endoplasmic reticulum (ER) sequestration of the corresponding mutant expressed in cell lines as well as in hippocampal neurons in culture. Furthermore, di-leucine-mutated receptors were unable to bind 5-HT$_{1A}$ agonists and presented a major deficit in their glycosylation state, suggesting that they are misfolded. By contrast, mutation of the di-leucine motif in the C-terminal domain of 5-HT$_{1B}$R had no major consequence on its subcellular targeting. However, in the case of the 1ActB chimera (substitution of the C-terminal domain of the 5-HT$_{1B}$R into 5-HT$_{1A}$R), this mutation was also found to cause sequestration within the ER. Replacement of palmitoylated cysteines by serines had no consequence on either receptor type. These data indicate that the di-leucine motif of the 5-HT$_{1A}$R and 5-HT$_{1B}$R tails is implicated in proper folding of these receptors, which is necessary for their ER export.

Key words: Di-leucine motif, Palmitoylation, Endoplasmic reticulum, Folding, 5-HT$_{1A}$, 5-HT$_{1B}$
terminal domains (Jolimay et al., 2000). All constructs were used for the transfection of two cell lines (COS-7 and LLC-PK1) and neurons and the localization of expressed proteins was analyzed by immunofluorescence and confocal microscopy. The intracellular localization of the di-leucine mutants was further characterized by double-labeling experiments with ER and Golgi markers. Finally, the characteristics of wild-type and mutated receptors expressed in transfected cells were further investigated by analyzing their glycosylation state, agonist binding properties and coupling to G proteins. Taken together, the data presented here indicate that the di-leucine motif of 5-HT1AR and 5-HT1BR tail is implicated in proper folding of the receptor, which is necessary for their ER export.

Results
Di-leucine motif and palmitoylated cysteines contained in the C-terminus of both receptors (Fig. 1) were replaced by alanine and serine residues, respectively, using site-directed mutagenesis. All constructs were tagged by addition of a Flag epitope at their extracellular N-terminus to analyze their subcellular localization.

Di-leucine-mutated 5-HT1AR is localized in the ER
Flag-tagged 5-HT1AR as well as I414/415A and C417/420S mutants were used to transfect COS-7 (Fig. 2A,C) or LLC-PK1 (Fig. 2C) cell lines or primary cultures of hippocampal neurons (Fig. 2B,C). Surface labeling was performed by incubating living cells with monoclonal mouse anti-Flag M2 antibody. Transfected cells were then fixed and permeabilized for the subsequent detection of the intracellular receptors using polyclonal rabbit anti-Flag antibody.

As expected, 5-HT1AR was mostly found at the plasma membrane of each cell type (~55-65% of surface labeling depending on cell type, Fig. 2C). By contrast, the I414/415A mutant was detected only at low levels at the plasma membrane (~10-24% of surface labeling), whereas the amounts of C417/420S mutant at the plasma membrane were very close to those of the wild-type 5-HT1AR.

Intracellular staining of di-leucine-mutated-5-HT1AR was distributed in perinuclear ER-like structures. Moreover, in transfected COS-7 cells, we observed a strong co-localization of this I414/415A mutant with the ER luminal marker calregulin but not with the cis and median Golgi marker giantin (Fig. 3A). For these co-localization experiments, cells were treated with the protein synthesis inhibitor cycloheximide before fixation, to lower as much as possible the presence of newly synthesized receptors in ER or Golgi apparatus. Under these conditions, wild-type and C417/420S 5-HT1AR did not co-localize with calregulin and giantin (Fig. 3A). This result indicates that the I414-I415 motif, but not the palmitoylated cysteines, is necessary for 5-HT1AR exit from the ER.

We also analyzed the glycosylation state of 5-HT1AR and related mutants. Western blotting of membrane proteins from transfected LLC-PK1 cells showed that both wild-type and C417/420S 5-HT1AR migrated mainly as a broad band of ~65 kDa and, to a lesser extent, a thinner band of ~50 kDa (Fig. 3B). By contrast, the I414/415A mutant migrated only as a band of ~50 kDa, which suggests that this construction was not correctly glycosylated. We thus treated membranes with endoglycosidase H (Endo H) to remove high-mannose N-glycosylation or peptide N-glycosidase F (PNGase F) to remove both core and complex
N-glycosylation. The band observed after treatment with PNGase F should correspond to fully deglycosylated receptors. By contrast Endo H is active only on partially glycosylated proteins. After treatment of wild-type and C417/420S 5-HT1AR with PNGase F, both 50 kDa and 65 kDa bands shifted to a band of ~44 kDa, corresponding to the molecular mass calculated for non-glycosylated Flag-tagged 5-HT1AR. In the case of digestion with Endo H, the broad band of 65 kDa was still visible, confirming that this band corresponded to a fully glycosylated receptor. Only the thin 50 kDa band (partially glycosylated) was eliminated and converted to the ~44 kDa band. These results showed that the majority of wild-type and C417/420S 5-HT1AR was completely glycosylated. Concerning the I414/415A mutant, both treatments with Endo H and PNGase F converted the ~50 kDa band into the ~44 kDa band, suggesting that this mutant was only partially glycosylated (core glycosylated). These data are consistent with ER retention of the latter mutant, as complex glycosylation occurs only in the Golgi apparatus (Kornfeld and Kornfeld, 1985).

The di-leucine motif is necessary for ligand-binding capacity of 5-HT1AR

We compared the ligand binding capacity of 5-HT1AR and mutants using the mixed 5-HT1A-5-HT1B agonist radioligand, [3H]LSD (Darmon et al., 1998). Membranes of LLC-PK1 cells transfected with wild-type or C417/420S-5-HT1AR specifically bound equivalent amounts of tritium after incubation with 1.6 nM [3H]LSD (~1 pmol/mg of protein, depending on transfection efficiency). By contrast, membranes of cells transfected with I414/415A mutant specifically bound only a very low amount of the radioligand (Fig. 4A).

Interestingly, deglycosylation of wild-type 5-HT1AR with PNGase F did not affect its binding capacity under the same assay conditions (not shown). Thus, the reduced [3H]LSD binding capacity of the I414/415A mutant was very probably not caused by its incomplete glycosylation state. Because ligand binding requires correct folding, it can be inferred that the I414-I415 motif is necessary for correct folding of 5-HT1AR.

Role of the di-leucine motif and palmitoylated cysteines in 5-HT1AR coupling to G proteins

We first analyzed the interaction of I414/415A and C417/420S 5-HT1AR with α subunits of G proteins. As illustrated in Fig. 4B, 5-CT-stimulated [35S]GTPγS binding onto membranes from LLC-PK1 cells did not statistically differ whether cells were transfected with wild-type or C417/420S 5-HT1AR. However, this binding was significantly impaired in the case of membranes transfected with I414/415A mutant.

Furthermore, it was shown that 5-HT1ARs also interact with G protein βγ subunits to modulate the activity of ERK1/2 (Garnovskaya et al., 1996). We thus tested the ability of 5-HT1AR and related mutants to activate ERK in transfected LLC-PK1 cells. After treatment with the agonist 8-OH-DPAT for 5 minutes, a ~sixfold increase in ERK2 phosphorylation was observed in cells expressing wild-type or C417/420S 5-HT1AR (Fig. 4C,D). These results demonstrate that wild-type and C417/420S 5-HT1AR activate ERK in our experimental conditions. By contrast, 8-OH-DPAT treatment of cells transfected with I414/415A mutant only induced a ~2.7-fold increase in ERK phosphorylation.

Mutations in the C-terminus of 5-HT1BR have only minor effects on its subcellular localization

The percentages of 5-HT1BR and related mutants at the plasma membrane were also examined. In COS-7 (Fig. 5A,C) and LLC-PK1 cells (Fig. 5C), wild-type 5-HT1BR displayed a lower level of surface staining than 5-HT1AR (COS-7, 22.7±2.7%; LLC-PK1, 30.7±6.0%; P<0.001 compared with data in Fig. 2C for 5-HT1AR). However, this difference between 5-HT1AR and 5-HT1BR did not reach statistical significance in neurons. Replacement of the di-leucine motif in the C-terminus of 5-HT1BR by alanines (L379/380A) significantly reduced its amount at the plasma membrane in LLC-PK1 cells but not in COS-7 cells and neurons (Fig. 5A-C).

As observed in the case of 5-HT1AR (Fig. 2C), mutation of the palmitoylated cysteine into a serine (C384S) had no significant effect on the subcellular localization of 5-HT1BR in all cell types analyzed (Fig. 5A-C).

1ActB chimera reveals the role of the 5-HT1BR di-leucine motif

In a previous study, we substituted the cytosolic tail of 5-HT1BR into the 5-HT1AR and vice versa (Jolimay et al., 2000). Analysis of these chimeras expressed in LLC-PK1 cells and in
neurons showed that an apical/axonal targeting signal is located in the C-terminus of 5-HT1BR. The resulting chimeric receptors, 1ActB (5-HT1AR with C terminus of 5-HT1BR) and 1BctA (5-HT1BR with the C-terminus of 5-HT1AR), were tagged with the Flag epitope and mutants were constructed for both.

In transient transfection experiments, the 1BctA chimera showed a mostly intracellular localization (Fig. 6A,C), and the same observation was made for di-leucine-mutated-1BctA (I379/380A) and for cysteine-mutated-1BctA (C382/385S) in all cell types examined. On the other hand, 1ActB chimera was mostly localized at the plasma membrane (Fig. 6D-F), like the 5-HT1AR (Fig. 2).

Di-leucine mutation of the chimera (I414/415A) resulted in a very low level of plasma membrane localization. By contrast, the amounts of C419S-1ActB mutant at the plasma membrane were very close to those of non-mutated 1ActB (Fig. 6D-F).

Discussion
As with numerous other GPCRs, the cytosolic C-terminal region of 5-HT1AR and 5-HT1BR plays an important role in their subcellular localization. Indeed, previous studies showed that this region is necessary for receptor exit from the ER and also that the cytosolic C-terminal tail of 5-HT1AR contains a dominant axonal-targeting signal (Jolimay et al., 2000). Here, we investigated the potential role of a di-leucine motif and palmitoylated cysteines contained in this receptor domain using site-directed mutagenesis.

The data reported here clearly showed that the di-leucine motif contained in the C-terminal domain of 5-HT1AR is implicated in its targeting to the plasma membrane. More precisely, this motif plays a crucial role in the correct folding of the receptor, which is necessary for its exit from the ER towards the plasma membrane. The role of the C-terminal di-leucine motif of 5-HT1BR is less clear. Its mutation into a di-alanine motif did not modify the localization of the receptor in transfected neurons. However, in the 1ActB chimera, in which the C-terminal tail of 5-HT1AR has been replaced by the C-terminal tail of 5-HT1BR, the same di-leucine motif appeared to be implicated in receptor targeting to the plasma membrane. However, substitution of the palmitoylated cysteine residues with serines did not affect the subcellular localization of receptors as well as chimeras, and in the case of 5-HT1AR, did not affect receptor binding or coupling to G proteins.

Role of the di-leucine motif of 5-HT1AR and 5-HT1BR
In GPCRs, di-leucine motifs localized mainly in the C-terminal cytosolic tail were found to be important for targeting. Some of these motifs were shown to act as clathrin-dependent endocytosis signals (Fan et al., 2001; Gabilondo et al., 1997; Gaudreau et al., 2004; Orsini et al., 1999). In addition, a role in receptor transport from the ER to the plasma membrane was found for a di-leucine motif with an upstream acidic residue in the case of vasopressin V2 receptor (Schülein et al., 1998) and, more recently, for a di-leucine motif associated with an upstream phenylalanine residue in the case
of α2β-adrenergic and angiotensin II type 1A receptors (Duvernay et al., 2004) or surrounded by three hydrophobic residues in the case of vasopressin V3 receptor (Robert et al., 2005). Our results concerning the I414-I415 motif in the C-terminal tail of 5-HT1AR are consistent with these previous findings. Indeed, replacement of this motif by two alanines resulted in a 5-HT1AR mutant sequestrated within the ER. The loss of [3H]LSD binding capacity of this mutant (29% of wild-type binding) suggests that such sequestration might be caused by an incorrect folding. However, this binding assay was performed with purified membranes of cells expressing the receptors and does not allow distinction of functional characteristics of plasma membrane versus intracellular receptors because ligand could access both pools of receptors. By contrast, for ERK phosphorylation assays, the ligand 8-OH-DPAT was added to living cells, and only plasma-membrane-localized receptors could thus be activated in this protocol. Interestingly, 8-OH-DPAT-induced activation of ERK phosphorylation in cells transfected with I414/I415A mutant was still ~2.7-fold over basal levels, corresponding to ~45% of the increase observed for the wild-type receptor. We therefore propose that the observed decrease in ERK activation is most probably due to intracellular sequestration of a large amount of this mutant and that the residual plasma-membrane-localized I414/I415A 5-HT1ARs are functional. This would support the idea of an incorrect folding of sequestrated mutants, as receptors that can exit the ER and reach the plasma membrane seem to be functional, implying their correct folding. However, we cannot entirely exclude that the di-leucine motif of 5-HT1AR may also participate in receptor ER exit by interaction with COP-II-associated proteins (for a review, see Barlowe, 2003), and that defective interactions caused by the mutation were actually responsible for ER sequestration of the I414/I415A mutant.

As noted by Schülein et al. (Schülein et al., 1998) and Duvernay et al. (Duvernay et al., 2004), this di-leucine motif is highly conserved in the C-terminal tail of GPCRs, suggesting a general role in the exit from ER for most of these membrane proteins. However, in our study, replacement of the di-leucine motif by two alanines in the C-terminal tail of 5-HT1B only affected its subcellular localization in LLC-PK1 cells, as this mutant did not differ from the wild-type 5-HT1B regarding its targeting to the plasma membrane in both COS-7 cells and hippocampal neurons. This apparent discrepancy is not unique among receptors that can exit the ER and reach the plasma membrane seem to be functional, implying their correct folding. However, we cannot entirely exclude that the di-leucine motif of the C-terminal tail of 5-HT1AR allows correct folding of this receptor in addition to the di-leucine motif of the C-terminal domain of 5-HT1AR and 5-HT1BR. Therefore, the implication of the two leucines (or isoleucines) localized in the cytosolic C-terminal tail of GPCRs in the exit of the receptor from the ER may not be universal, but would depend on its environment or be associated with other signals. Concerning the 5-HT1BR, its predominant intracellular localization found in most cell types tested could also explain why substitution of the di-leucine motif did not generally produce further detectable intracellular sequestration.

To further address this question, we constructed chimeras in which the C-terminal domains of 5-HT1AR and 5-HT1BR were switched (Jolimay et al., 2000). In transient transfection experiments, 1BctA chimera (5-HT1B core with 5-HT1AR C-tail) exhibited nearly exclusive perinuclear localization, indicating that this construct is probably not functional. On the other hand, a relatively high proportion (~50%) of 1ActB chimera was localized at the plasma membrane (Fig. 5F), like that observed with the wild-type 5-HT1AR (Fig. 2C). Accordingly, it can be inferred that the di-leucine motif of the C-terminal domain of 5-HT1BR allows correct folding of this 1ActB chimera and its exit from ER. This would suggest the need for another signal localized in a different domain of the receptor in addition to the di-leucine motif of the C-terminal tail, which would be present in the 5-HT1AR but not in the 5-HT1BR, thereby leading to plasma membrane targeting of the 1ActB but not the 1BctA chimera. Alternatively, it is possible...
that the high intracellular repartition of transfected 5-HT1bR masks the actual role of its di-leucine motif.

Role of palmitoylated cysteines
5-HT1aR and 5-HT1bR were shown to contain palmitoylated cysteines in their C-terminal domain (Ng et al., 1993; Papoucheva et al., 2004). Substitution of these residues with serines did not affect the subcellular localization of either receptors or chimeras. Furthermore, we found that the glycosylation state and the ligand-binding capacity of 5-HT1aR were not dependent on the presence of the palmitoylated cysteines. These data are consistent with results obtained by Papoucheva et al. (Papoucheva et al., 2004) who recently demonstrated the constitutive palmitoylation of cysteines 417 and 420 of this receptor. These authors also showed that palmitoylated cysteines 417 and 420 are necessary for the receptor coupling to Galpha3 subunit in transfected insect Sf9 cells, as well as for its capacity to inhibit adenyl cyclase activity in NIH3T3 cells and to activate ERK in CHO cells. By contrast, using [35S]GTPgammaS binding and ERK activation assays, we showed here that the mutation of both palmitoylated cysteines 417 and 420 did not significantly affect 5-HT1aR coupling with Galpha proteins and activation of ERK in LLC-PK1 cells. Such discrepancies might be explained by the use of different cell lines expressing different Galpha subunits and other signaling molecules, in our studies compared with those of Papoucheva et al. (Papoucheva et al., 2004). First, in Sf9 cells, the Galpha2 subunit was cotransfected with receptors. In LLC-PK1 cells, both Galpha2 and Galpha3 subunits are endogenously expressed but exhibit different subcellular compartmentations: Galpha2 is localized at the basolateral membrane, whereas Galpha3 is restricted to the Golgi apparatus (Ercolani et al., 1990). As we found that 5-HT1aR is mainly localized at the plasma membrane (Fig. 2), it should interact primarily with Galpha3 in LLC-PK1 cells. Thus, [35S]GTPgammaS binding results agreed with the hypothesis that the palmitoylated cysteines 417 and 420 are necessary for 5-HT1aR coupling with the Galpha3 but not the Galpha2 subunit. In the case of ERK phosphorylation, the differences between cell lines tested is less clear, because CHO cells express both Galpha2 and Galpha3 subunits. However, multiple signaling pathways can lead to the activation of ERK after stimulation of a particular GPCR, and some of these pathways may be activated independently of G proteins (for a review, see Werry et al., 2005). In the case of the 5-HT1aR, the intracellular...
signaling pathway leading to ERK activation has been shown to implicate Gsα subunits in CHO cells (Garnovskaya et al., 1996; Della Rocca et al., 1999). However, to date, it is not known whether the same pathway is involved in other cell lines, such as LLC-PK1 cells. It would thus be of interest to identify which intracellular signaling molecules contribute to ERK activation by 8-OH-DPAT in LLC-PK1 cells. In any case, these results suggest that palmitoylated cysteines play variable roles in 5-HT1AR functional characteristics, depending on the cell type and the signaling molecules available. Such differences were already reported for other GPCRs. Thus, substitution of palmitoylated cysteines by alanines within 5-HT1AR and 1BctA chimera; Oligo 5-HT1B, C384S: CTGATACGCTTTAAG

In conclusion, our data show that the di-leucine motif in the C-terminal domain of 5-HT1A and 5-HT1B receptors is necessary for their ER sorting through its implication in the proper folding of receptors. They also provide further support for the statement that 5-HT1A and 5-HT1B receptors are routed through distinct intracellular pathways towards their final targeting in neurons.

Materials and Methods

Antibodies

Anti-rat 5-HT1AR antibody has been described previously (El Mestikawy et al., 1996; Della Rocca et al., 1999). However, to data, it is not known whether the same pathway is involved in other cell lines, such as LLC-PK1 cells. It would thus be of interest to identify which intracellular signaling molecules contribute to ERK activation by 8-OH-DPAT in LLC-PK1 cells. In any case, these results suggest that palmitoylated cysteines play variable roles in 5-HT1AR functional characteristics, depending on the cell type and the signaling molecules available. Such differences were already reported for other GPCRs. Thus, substitution of palmitoylated cysteines by alanines within 5-HT1AR and 1BctA chimera; Oligo 5-HT1B, C384S: CTGATACGCTTTAAG

DNA constructs

The complete coding sequences of 1AcB and 1BctA chimeras (Jolimay et al., 2000), rat 5-HT1AR (Albert et al., 1990) and rat 5-HT1AR (Hambli et al., 1992) were subcloned into pFlag-CMV-6c expression vector (Sigma) to obtain constructs tagged with Flag at their N-termini. Receptor mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Only the sense oligonucleotides are listed below, with the mutated nucleotides in bold letters. Oligo 5-HT1AR, A1414/A1550: GCCTTTAAGAGGCGGAAGCATTGGAAGCTGCC; for di-leucine motif substitution by alanines within 5-HT1AR and 1BctA chimera; Oligo 5-HT1AR, C384S: CTGATACGCTTTAAG

Cell cultures

LLC-PK1 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 4.5 g/l glucose, GlutaMAX I (Invitrogen, Cergy Pontoise, France), 10% fetal bovine serum, 10 U/ml penicillin G and 10 mg/ml streptomycin. COS-7 cells were grown in DMEM supplemented with 1 g/l glucose, 10% fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin G and 10 mg/ml streptomycin.

Neuronal cultures were made as described previously (Goslin et al., 1998) with some modifications. Hippocampi of rat embryos were dissected at day 17-18. After trypsinization, tissue dissociation was achieved with a Pasteur pipette. Cells were counted and plated on poly-L-lysine-coated 12-mm-diameter coverslips, at a density of 60,000-75,000 cells per 16-mm dish (300-375 cells per square millimeter), in complete Neurobasal medium supplemented with B27 (Invitrogen), containing 0.5 mM L-glutamine, 10 U/ml penicillin G, and 10 mg/ml streptomycin. Five hours after plating, coverslips were transferred to a 90-mm dish containing conditioned medium obtained by incubating glial cultures (70-80% confluency) for 24 hours in the complete medium described above. Experiments were performed in agreement with the institutional guidelines for use of animals and their care, in compliance with national and international laws and policies (Council directives no. 87-848, 1987, Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions nos. 75-116 to M.H. and 75-974 to M.D.).

Cell transfections

For immunofluorescence experiments, LLC-PK1 and COS-7 cells were transfected to 12-mm-diameter coverslips 16 hours before transfection to obtain 30-50% confluency cultures. LLC-PK1 cells were transfected using Lipofectin reagent (Invitrogen). For each coverslip, 1 μg plasmid DNA and 1-3 μl Lipofectin were both incubated separately in 125 μl serum-free DMEM. After a 30-45 minute incubation at room temperature, the two dilutions were combined and the resulting mix was left for another 10-15 minutes at room temperature. Cells were washed with 500 μl serum-free DMEM and mix was added for an overnight incubation at 37°C.

COS-7 cells were transfected using FuGENE reagent (Roche, Meylan, France). For each coverslip, 2 μl FuGENE were diluted in 50 μl D-PBS (Dulbecco’s phosphate-buffered saline, Invitrogen) and incubated for 5 minutes at room temperature. The dilution was then mixed with 1 μg plasmid DNA, and incubation proceeded for another 15 minutes. This mix was added to the growth medium (250 μl) overlaying the cells and transfection lasted 24 hours at 37°C.

Hippocampal neurons were transfected on the 7-8th day in vitro as follows: for each coverslip, plasmid DNA (0.8 μg) was mixed with 50 μl Neurobasal medium without B27 supplement. After 15 minutes at room temperature, 0.8 μl Lipofectamine 2000 (Invitrogen) in 50 μl Neurobasal medium were added and incubation continued for another 20 minutes. After the addition of 150 μl of complete Neurobasal medium containing B27 supplement, the mix was applied onto the neuronal culture, and transfection lasted for 3 hours at 37°C. Typically, 5-10% of neurons expressed the receptors after transfection.

For both cell lines and hippocampal neurons, receptor expression was allowed in growth medium for 24 hours after transfection.

For preparation of membranes and ERK phosphorylation assays (see below), LLC-PK1 cells were transfected by electroporation using Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA; 135 V, 1800 μF in 200 μl DMEM containing 5-10×104 cells, plasmid DNA; relaxation time: ~40 milliseconds).

Cells were then transferred to a 90-mm dish and grown for 3 days in LLC-PK1 growth medium.

Indirect immunofluorescence

Cells on coverslips were washed with D-PBS+ (D-PBS containing 0.1 mM CaCl2 and 0.1 mM MgCl2) 37°C, then fixed with paraformaldehyde (3%) containing 4% sucrose in 0.1% Triton X-100 in D-PBS+, and permeabilized with 0.5% saponin in 1×PBS (Sigma). Cells were washed in 1×PBS, and blocked in 10% normal goat serum in PBS. For antigen detection, anti-Flag M2 antibody (2.5 μg/ml; Sigma, St. Louis, MO) was incubated with living cells for 30 minutes at room temperature. The dilution was then mixed with 1:1600 anti-mouse IgG (Molecular Probes, Eugene, OR). The coverslips were washed three times in 1×PBS, and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1600 dilution, Eugene, OR) was incubated for 30 minutes in blocking solution. Coverslips were finally mounted in Fluoromount-G solution (Clinisciences, Montrouge, France). For ER and Golgi co-localization experiments, cells were treated with the protein synthesis inhibitor cycloheximide (70 μM) for 4 hours before fixation, to lower as much as possible the presence of newly synthesized receptors in ER or Golgi apparatus. ER and Golgi labeling was performed using anti-calreulin antibody (1:1000 dilution) and anti-giantin antibody (1:2000 dilution), respectively, and receptors were labeled using anti-Flag M2 monoclonal antibody. For surface detection, anti-Flag M2 antibody (2.5 μg/ml) was incubated with living cells for 20 minutes at room temperature. Cells were washed in D-PBS+, fixed with paraformaldehyde (5%) containing 4% sucrose, and incubated for 1 hour with Alexa Fluor 488-conjugated goat anti-mouse IgG in antibody buffer. After permeabilization with 0.1% Triton X-100 in D-PBS+, intracellular epitopes were detected using rabbit anti-Flag polyclonal antibody (0.85 μg/ml) subsequently revealed by Cy3-conjugated donkey anti-rabbit IgG (1:1600 dilution; Jackson ImmunoResearch, West Grove, PA). For quantitative analysis, four coverslips were analyzed per condition.

Immunofluorescence images were generated using a Leica laser-scanning confocal microscope. For relative surface label analysis, unsaturated acquisitions were made with the same exposure settings and laser gain for each condition. For each cell type, at least ten cells were analyzed. Quantification of surface and intracellular staining were performed using ImageJ software (NIH) according to Jaskolski et al. (Jaskolski et al., 2004) with modifications, and statistical analysis was carried out using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Background was lowered using Gaussian blur (radius 1 pixel) and an intensity threshold was fixed just above the background level to maximally reduce non-specific staining. Single cells were selected and carefully traced manually. Surface (S) and intracellular (I) areas with labeling above threshold were measured and the percentage of surface receptor labeling calculated as S/(S+I).*
Preparation of membranes

Transfected LLC-PK1 cells were washed with D-PBS, scraped into Tris buffer (50 mM Tris-HCl, pH 7.4), and homogenized with a Polytron. After each of four successive washings in Tris buffer, the membranes were collected by centrifugation at 31,000 g for 20 minutes at 4°C. An incubation for 10 minutes at 37°C was performed after the first washing to eliminate 5-HT (from the serum in the culture medium), and the final pellet was suspended in the same Tris buffer to be stored at -80°C until use. Protein concentration was measured using BCA protein assay kit (Pierce, Rockford, IL).

Deglycosylation assays

For deglycosylation assays, membranes were first denaturated by incubation for 10 minutes at 100°C in 0.5% (v/v) SDS, 1% (v/v) β-mercaptoethanol. Membranes of (40 µg of protein) were then incubated with or without 500 U Endo H or PNGase F (Ozyme, Saint-Quentin Fallavier, France) for 1 hour at 37°C, according to supplier’s recommendations. Proteins were separated by electrophoresis, transferred to nitrocellulose, and probed with anti-5-HT(3R) antibody (1:1000 dilution). After incubation with anti-rabbit antibodies coupled to horseshadish peroxidase (Sigma, 1:10,000 dilution), bands were detected with the ECL+ kit (Amerham Biosciences, Amersham, UK). The band corresponding to PNGase-F-resistant bands were detected with the ECL+ kit (Amersham Biosciences, Amersham, UK). The band corresponding to PNGase-F-resistant bands were detected with the ECL+ kit (Amersham Biosciences, Amersham, UK).

For membranes subsequently used for binding assays, deglycosylation was done without denaturation.

Radioligand binding assays

Binding assays were performed using 20-25 µg membrane proteins in 500 µl of 50 mM Tris-HCl buffer, pH 7.4, supplemented with 1.6 mM [H]Helysine acid diethylamide ([H]HLS: 79.2 Ci/mmol, Amerham Biosciences). Incubations were performed for 90 minutes at 25°C. Non-specific binding was determined in the presence of 10 µM 5-HT. Assays were stopped by rapid filtration through Whatman GF/B filters coated with polyethylenimine (0.5% v/v). Subsequent washing and counting of entrapped radioactivity were as described by Fabre et al. (Fabre et al., 1997). Specific binding is expressed as a percentage of total type specific binding. Data were corrected for individual variations in transfection efficiency by relative quantification of receptors as detailed in the deglycosylation procedures. Data analysis was done using GraphPad Prism 4.

References

Albert, P. R., Zhou, Q. Q., Van Tol, H. H., Bunzow, J. R. and Civelli, O. (1990). Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. J. Biol. Chem. 265, 5825-5832.

Alper, R. H. and Nelson, D. L. (1998). Characterization of 5-HT(A) receptor-mediated [35S]GTPgammaS binding in rat hippocampal membrane preparations. Eur. J. Pharmacol. 343, 303-312.

Barlowe, C. (2003). Signals for COPII-dependent export from the ER: what’s the ticket out? Trends Cell Biol. 13, 295-300.

Barnes, N. M. and Sharp, T. (1999). A review of central 5-HT receptors and their function. Neuropharmacology 38, 1083-1152.

Bermak, J. C., Li, M., Bullock, C. and Zhou, Q. Y. (2001). Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein. Nat. Cell Biol. 3, 492-498.

Darmon, M., Langlois, X., Suffissette, L., Fattaccini, C. M. and Hamon, M. (1998). Differential membrane targeting and pharmacological characterization of chimeras of rat serotonin 5-HT(A) and 5-HT(B) receptors expressed in epithelial LLC-PK1 cells. J. Neurochem. 71, 2294-2303.

Della Rocca, G. J., Mukhin, Y. V., Gavrovskaya, M. N., Daaka, Y., Clark, G. J., Luttrell, L. M., Lefkowitz, R. J. and Raymond, J. R. (1999). Serotonin 5-HT(A) receptor-mediated ERK activation requires calcium/calmodulin-dependent receptor endocytosis. J. Biol. Chem. 274, 4749-4753.

Doi, T., Sugimoto, H., Arimoto, I., Hiroaki, Y. and Fujimoto, Y. (1999). Interactions of endothelin receptor subtypes A and B with Gi, Go, and Gq in reconstituted phospholipid vesicles. Biochemistry 38, 3090-3099.

Duvournay, M., Zhou, P. and Wu, G. (2004). A conserved motif for the transport of G protein-coupled receptors from the endoplasmic reticulum to the cell surface. J. Biol. Chem. 279, 37041-37050.

El Mestikawy, S., Riad, M., Laporte, A. M., Vergé, D., Daval, G., Gozlan, H. and Hamon, M. (1999). Production of specific anti-rat 5-HT(A) receptor antibodies in rabbits injected with a synthetic peptide. Neurosci. Lett. 118, 189-192.

Ercolani, L., Stow, J. L., Boyle, J. F., Holtzman, E. J., Lin, H., Grove, J. R. and Ausiello, D. A. (1990). Membrane localization of the pertussis toxin-sensitive G- protein subunits alpha i-2 and alpha i-3 and expression of a metallothionein-alanine i-2 fusion gene in LLC-PK1 cells. Proc. Natl. Acad. Sci. USA 87, 4635-4639.

Fabre, V., Boni, C., Mocaer, E., Lesourd, M., Hamon, M. and Laporte, A. M. (1997). [3H]Acpizipramine: a novel specific radioligand of 5-HT(1A) receptors in the rat brain. Eur. J. Pharmacol. 337, 297-308.

Fabre, V., Beaufour, C., Evrard, A., Rious, A., Hanoun, L., Desch, K. P., Murphy, D. L., Lanfuney, L., Hamon, M. and Martres, M. P. (2000). Altered expression and functional properties of serotonin 5-HT(1A) and 5-HT(2A) receptors in knock-out mice lacking the 5-HT transporter. Eur. J. Neurosci. 12, 2299-2310.

Fan, G. H., Yang, W., Wang, X. J., Qian, Q. and Richmond, A. (2001). Identification of a motif in the carboxyl terminus of CXXC2 that is involved in adaptin 2 binding and receptor internalization. Biochemistry 40, 791-800.

Gablondo, A. M., Hegler, J., Krassel, C., Boivin-Jahns, V., Hein, L. and Lohse, M. J. (1997). A diculeine motif in the C terminus of the beta2-adrenergic receptor is involved in receptor internalization. Proc. Natl. Acad. Sci. USA 94, 12285-12290.

Gavrovskaya, M. N., van Biesen, T., Hawe, B., Casanas Ramos, S., Lefkowitz, R. J. and Raymond, J. R. (1999). Serotonin 5-HT(1A) receptor-mediated ERK activation requires calcium/calmodulin-dependent receptor endocytosis. J. Biol. Chem. 274, 4749-4753.

Gaudreau, R., Brailien, M. E., Chen, Z., Le Guinoll, C., Livagne, P., Stankova, J. and Rola-Pleszczynski, M. (2004). Structural determinants regulating expression of the high affinity leukotriene B4 receptor: involvement of diculeine motifs and alpha-helix VIII. J. Biol. Chem. 279, 10338-10345.

Godin, K., Asssummen, H. and Banker, G. (1998). Rat hippocampal neurons in low-density culture. In Culturing Nerve Cells (ed. G. Banker and K. Golson), pp. 339-370. Cambridge, MA: MIT Press.

Haj-Dahmane, S., Hamon, M. and Lanfuney, L. (1991). K+ channel and 5-hydroxytryptamine1A autoreceptor interactions in the rat dorsal raphe nucleus: an in vitro electrophysiological study. Neuroscience 41, 495-505.

Hamblin, M. W., McGuffin, R. W., Metcalfe, M. A., Dorsa, D. M. and Merchant, K. M. (1992). Distinct 5-HT(1A) and 5-HT(1B) serotonin receptors in rat: Structural and pharmacological comparison of the two cloned receptors. Mol. Cell. Neurosci. 4, 578-587.

Hunziker, W. and Fumey, C. (1994). A diculeine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. EMBO J. 13, 2969-2976.
Jaskolski, F., Coussen, F., Nagarajan, N., Normand, E., Rosenmund, C. and Mulle, C. (2004). Subunit composition and alternative splicing regulate membrane delivery of kainate receptors. J. Neurosci. 24, 2506-2515.

Jolimay, N., Louis, F., Langlois, X., Hamon, M. and Darmon, M. (2000). Dominant role of the cytosolic C-terminal domain of the rat 5-HT1B receptor in axonal-apical targeting. J. Neurosci. 20, 9111-9118.

Kia, H. K., Niquel, M. C., Briosguelli, M. J., Daval, G., Riad, M., El Mestikawy, S., Hamon, M. and Vergé, D. (1996). Immunocytochemical localization of serotonin1A receptors in the rat central nervous system. J. Comp. Neurol. 365, 289-305.

Kornfeld, R. and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 54, 631-664.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Letourneur, F. and Klausner, R. D. (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell 69, 1143-1157.

Ng, G. Y., George, S. R., Zastawny, R. L., Caron, M., Bouvier, M., Dennis, M. and O'Dowd, B. F. (1993). Human serotonin1B receptor expression in Sf9 cells: phosphorylation, palmitoylation, and adenylyl cyclase inhibition. Biochemistry 32, 11727-11733.

Oksche, A., Dehe, M., Schlülein, R., Wiesner, B. and Rosenthal, W. (1998). Folding and cell surface expression of the vasopressin V2 receptor: requirement of the intracellular C-terminus. FEBS Lett. 424, 57-62.

Orsini, M. J., Parent, J. L., Mundell, S. J., Benovic, J. L. and Marchese, A. (1999). Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization. J. Biol. Chem. 274, 11727-11733.

Pankevych, H., Korkov, V., Freissmuth, M. and Nanoff, C. (2003). Truncation of the A1 adenosine receptor reveals distinct roles of the membrane-proximal carboxyl terminus in receptor folding and G protein coupling. J. Biol. Chem. 278, 30283-30293.

Papoucheva, E., Duma, A., Sebben, M., Richter, D. W. and Ponimaskin, E. G. (2004). The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. J. Biol. Chem. 279, 3280-3291.

Qanbar, R. and Bouvier, M. (2003). Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. Pharmacol. Ther. 97, 1-33.

Riad, M., Garcia, S., Watkins, K. C., Jodoin, N., Doucet, E., Langlois, X., El Mestikawy, S., Hamon, M. and Descarries, L. (2000). Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. J. Comp. Neurol. 417, 181-194.

Robert, J., Clauser, E., Petit, P. X. and Ventura, M. A. (2005). A novel C-terminal motif is necessary for the export of the vasopressin V1b/V3 receptor to the plasma membrane. J. Biol. Chem. 280, 2300-2308.

Rodríguez, M. C., Xie, Y. B., Wang, H., Collison, K. and Segaloff, D. L. (1992). Effects of truncations of the cytoplasmic tail of the luteinizing hormone/chorionic gonadotropin receptor on receptor-mediated hormone internalization. Mol. Endocrinol. 6, 327-336.

Sari, Y. (2004). Serotonin1B receptors: from protein to physiological function and behavior. Neurosci. Biobehav. Rev. 28, 565-582.

Sari, Y., Miquel, M. C., Briosguelli, M. J., Ruiz, G., Doucet, E., Hamon, M. and Vergé, D. (1999). Cellular and subcellular localization of 5-hydroxytryptamine1B receptors in the rat central nervous system: immunocytochemical, autoradiographic and lesion studies. Neuroscience 88, 899-915.

Schülein, R., Hermosilla, R., Oksche, A., Dehe, M., Wiesner, B., Krause, G. and Rosenthal, W. (1998). A dileucine sequence and an upstream glutamate residue in the intracellular carboxyl-terminus of the vasopressin V2 receptor are essential for cell surface transport in COS.M6 cells. Mol. Pharmacol. 54, 525-535.

Tai, A. W., Chiang, J. Z., Hode, C., Wolfrum, U. and Sung, C. H. (1999). Rhodopsin’s carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynin light chain Tctex-1. Cell 97, 877-887.

Werry, T. D., Sexton, P. M. and Christopoulos, A. (2005). “Ins and outs” of seven-transmembrane receptor signalling to ERK. Trends Endocrinol. Metab. 16, 26-33.