Noninvasive Imaging of 5-HT₃ Receptor Trafficking in Live Cells

FROM BIOSYNTHESIS TO ENDOCYTOSIS

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Sequential stages in the life cycle of the ionotropic 5-HT₃ receptor (5-HT₃R) were resolved temporally and spatially in live cells by multicolor fluorescence confocal microscopy. The insertion of the enhanced cyan fluorescent protein into the large intracellular loop delivered a fluorescent 5-HT₃R fully functional in terms of ligand binding specificity and channel activity, which allowed for the first time a complete real-time visualization and documentation of intracellular biogenesis, membrane targeting, and ligand-mediated internalization of a receptor belonging to the ligand-gated ion channel superfamily. Fluorescence signals of newly expressed receptors were detectable in the endoplasmic reticulum about 3 h after transfection onset. At this stage receptor subunits assembled to form active ligand binding sites as demonstrated in situ by binding of a fluorescent 5-HT₃R-specific antagonist. After novel protein synthesis was chemically blocked, the 5-HT₃R populations in the endoplasmic reticulum and Golgi cisternae moved virtually quantitatively to the cell surface, indicating efficient receptor folding and assembly. Intracellular 5-HT₃ receptors were trafficking in vesicle-like structures along microtubules to the cell surface at a velocity generally below 1 μm/s and were inserted into the plasma membrane in a characteristic cluster distribution overlapping with actin-rich domains. Internalization of cell surface 5-HT₃ receptors was observed within minutes after exposure to an extracellular agonist. Our orchestrated use of spectrally distinguishable fluorescent labels for the receptor, its cognate ligand, and specific organelle markers can be regarded as a general approach allowing subcellular insights into dynamic processes of membrane receptor trafficking.

A fundamental concern in neurobiology is the study of processes involved in expression, assembly and subcellular trafficking of neuroreceptors. Of particular interest are members of the large family of ligand-gated ion channels (LGIC) that include nicotinic acetylcholine (nAChR), serotonin (5-HT₃R), γ-aminobutyric acid (GABA₃), glycine, and ionotropic glutamate receptors (1, 2). All of them are oligomeric membrane proteins composed of subunits, which surround an ion channel that opens upon neurotransmitter binding to the receptor. The structural relationship between the different LGICs suggests that their assembly and trafficking involves similar molecular events (3). In general, it is thought that subunit assembly and ligand binding site formation occurs shortly after biosynthesis in the endoplasmic reticulum (ER) followed by trafficking to the plasma membrane (4–8). Methodologies allowing the dynamic observation of these processes as well as a direct probing of receptor functions in intracellular compartments have still to be elaborated. In the present study, we focus on the ionotropic 5-HT₃R as a representative member of the LGICs and explore new strategies to monitor receptor biogenesis in real time starting with the delivery of their coding DNA into living cells. We will resolve events, occurring after the receptors “birth” from those leading to cell membrane insertion of mature receptors and finally to ligand-induced re-absorption of receptors into the cell reflecting the end point of the receptors lifespan. Improved understanding of these processes can provide valuable information for the therapeutic targeting of LGICs at specific stages in their life cycle.

The ionotropic 5-HT₃R is known to mediate fast signal transduction across synapses in the nervous system. Homopentameric complexes of recombinant 5-HT₃ receptors exhibit high cell surface expression levels in heterologous systems (9) and share substantial pharmacological and functional properties with native neuronal 5-HT₃R (10–12). To date the direct intracellular analysis of 5-HT₃ receptors has been limited to antibody labeling methods (8). However, these technologies are not adequate for visualizing receptors in live cells and cannot be used to label intracellular receptors in non-permeabilized cells. Here we fuse the enhanced cyan fluorescent protein (ECFP) to the 5-HT₃R to visualize the entire sequence of stages in the life of the receptor. The green fluorescent protein and its spectral variants have been widely used as molecular reporters to monitor gene expression, localization, and trafficking of proteins in living cells (13–15). GFP-tagged proteins often retain their biological activity and have the same trafficking pattern as native proteins (16–20). The combination of tagging 5-HT₃ receptors with...
autofluorescent proteins and the parallel use of fluorescent 5-HT₃R specific ligands (21) will be demonstrated as a powerful tool allowing the multicolor analysis of receptor biosynthesis, trafficking, and ligand-dependent receptor internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany), kits for plasmid and DNA fragment purification were from Qiagen GmbH (Hilden, Germany), restriction endonucleases (Clal, HindIII and NotI) were from New England Biolabs. The radioligand 3-([5-methyl-1H-imidazol-4-yl]-1(1H)ethenyl-1H-indol-3-yl)propanone ([^1]H)GR65630, 85.5 Ci/mmol) was from PerkinElmer Life Sciences; the agonist serotonin (5-HT) was obtained from Sigma (Buchs, Switzerland), and quipazine and 1-(m-chlorophenyl)-biguanide (mCPBG) were from Toecris-Cookson (Langford, UK). The fluorescent ligands GR-Cy5 (1,2,3,9-tetrahydro-3-[5-[methyl-1H-imidazol-4-yl]-methyl]-9-[3-amino-N-(N-cytosinidomethylpropyl)-4H-carbazol-4-one], GR-Fu (1,2,3,9-tetrahydro-3-[5-[methyl-1H-imidazol-4-yl]-methyl]-9-[3-amino-N-fluorescein-thioacamomylpropyl]-4H-carbazol-4-one) and GR-Rho (1,2,3,9-tetrahydro-3-[5-[methyl-1H-imidazol-4-yl]-methyl]-9-[3-amino-N-[rhodamine B-thioacamomylpropyl]-4H-carbazol-4-one) were prepared as described elsewhere (21, 22). Triton-X100 was from Fluka (Buchs, Switzerland). The plasmids pEYFP-ER, pEYFP-GolgI, pEYFP-Tub, and pEYFP-Actin were purchased from Clontech. Other chemicals were from Sigma.

**DNA Constructs**—All constructs are based on a vector containing the short splicing variant of the murine 5-hydroxytryptamine type 3A subunit cDNA (23) preceded by the human cytomegalovirus gene promoter, as described before (9). The vector pShT3R-ECFP, containing the ECFP-labeled receptor, was obtained as follows: the original vector was first mutated using the oligonucleotides 5'-CGT ATG CCA TGG GAA ACC-3' and 5'-CCA TCG ATA TGG TGA-3', adding a ClaI restriction site in the mature sequence numbering) to give 5-HT₃R-ECFP. The ECFP insert was obtained by PCR amplification on the template pECFP-N1 (Clontech) using the synthetic oligonucleotides 5'-CCA TGG ATC TAA GCT TCA CCA TGC GGC TCT GCA TCC CGC-3' and 5'-ATG CCA TCG ATA TGG TGA-3' which were prepared as described elsewhere (21, 22). Triton-X100 was from Fluka (Buchs, Switzerland). The plasmids pEYFP-ER, pEYFP-GolgI, pEYFP-Tub, and pEYFP-Actin were purchased from Clontech. Other chemicals were from Sigma.

**Cell Culture, Transfections, and Permeabilization**—Adherent human embryonic kidney (HEK293) and N1E-115 cells were grown in Dulbecco's modified Eagle medium/F-12 (Invitrogen) supplemented with 2.2 and 10% fetal calf serum (Invitrogen, respectively), using plastic flasks from TEP AG (Traasadingen, Switzerland). The cultures were split regularly and kept at 37 °C in a humidified atmosphere with 5% CO₂. For electrophysiology and confocal microscopy measurements, respectively, cells were seeded in 35-mm cell culture dishes and 6-well plates containing 22-mm diameter glass coverslips at a density of 150,000 cells/ml and transfected using LipofectAMINE 2000 reagent (Invitrogen).

**Transfection Efficiencies**—Confocal fluorescence microscopy on cell samples which were cotransfected with an enhanced GFP (pEGFP-N1; Clontech) reporter DNA. Fixation of cells was achieved by 10-min incubation at room temperature in a solution containing 3.7% formaldehyde in PBS. Subsequent permeabilization was performed by 5-min incubation in the presence of 0.1% Triton X-100 in PBS. Triton X-100 was used for cell permeabilization. This detergent was shown not to interfere with ligand binding affinity (12, 24). Cells were washed three times with PBS between incubations.

**Radioactive Binding Assays**—Receptor concentrations as well as ligand affinities were measured by radioligand binding assay. 100 μl containing ~1 × 10⁶ cells resuspended in 10 ml Hepes, pH 7.4, were incubated for 30 min at room temperature in solutions of 10 μM Hepes, pH 7.4, with varying concentrations of the specific antagonist [^1]HGR65630 in a final volume of 1 ml. A rapid filtration through Whatman GF/B filters (presoaked for 15 min in 0.5% (w/v) polyethyleneimine) followed by two washes with 3 ml of ice-cold 10 mM Hepes at pH 7.4 terminated the incubation. Filters were then transferred into scintillation vials and 4 ml of Ultima Gold (Packard, Meridian, CT) was added. The radioactivity was measured in a Tri-Carb 2200CA liquid scintillation counter (Packard). Nonspecific binding was determined in the presence of 1 μM quipazine.

Binding assay on permeabilized cells were processed as above, except that the cells were pre-treated with 0.1% saponin in 10 mM Hepes pH 7.4 for 5 min at room temperature before radioligand binding. All experiments were done in triplicate.

The dissociation constant Kᵦ of [^1]HGR65630 and the Hill coefficient n were calculated by fitting the experimental data to the following binding equation.

\[
\frac{[\text{H}]GR65630 \text{bound}}{[\text{H}]GR65630} = \frac{1}{1 + KL} \quad (\text{Eq. 1})
\]

**Electrophysiology**—We used the standard patch clamp technique in whole-cell voltage clamp to ~ 60 mV employing an EPC-9 patch clamp amplifier (HEKA Elektronik GmbH, Lambrecht, Germany). For data acquisition and storage the software PULSE 9.3 (HEKA Elektronik GmbH) was used. Borosilicate glass pipettes were heat polished and had resistances of 2~5 MΩ. Pipettes were filled with 140 mM KCl, 5 mM MgCl₂, 10 mM EGTA, 10 mM Hepes-KOH, pH 7.3. The external solution was 147 mM NaCl, 12 mM glucose, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes-NaOH, pH 7.4. Ligands dissolved in the external solution were supplied with the MSC-200 perfusion system (BioLogic, Claix, France). During experiments the cells were continuously perfused. All experiments were performed at room temperature.

**RESULTS**

We investigated the time course of 5-HT₃R plasma membrane expression by analyzing non-permeabilized HEK293 cell samples at different times after transfection via radioligand binding assay. 5-HT₃R cell surface expression increased over time and reached a maximum number of 2.6 × 10⁶ receptors per cell 34 h after transfection (Fig. 1). When these cells were permeabilized with saponin, it was possible to detect in addition to cell surface receptors also 5-HT₃R-specific ligand binding activity in the cytoplasm, which accounted for about 40% of the total ligand binding sites. In N1E-115 cells, which endogenously express 5-HT₃R receptors, about 60% of the ligand binding activity was localized inside the cell (Table I). These results indicate that a substantial fraction of intracellular receptors is capable of binding 5-HT₃R-specific ligands before plasma membrane integration. We then analyzed intracellular 5-HT₃R formation and trafficking in more detail. To observe in real-time 5-HT₃R biosynthesis after cell delivery of its coding DNA, we
which were in the order of 1
transfection. The values were obtained via radioligand binding assays
on the cellular membrane measured at different times after
bly of receptor subunits. Ligand binding to intracellular 5-HT3
specific ligand binding sites, which indicate the functional assem-
calculated receptor number was corrected taking into account cell divi-
line receptor (25) as well as by amino acid sequence homology
Information about the assembly of 5-HT3R subunits can be
primarily an indication of a folded ECFP marker protein, it
plasma membrane insertion occurred in about 30 min. The
receptors was first investigated by permeabilizing fixed HEK293
cells at different times after transfection and subsequent labeling
with fluorescent ligands. The binding specificity of those ligands
to 5-HT3 receptors in intracellular compartments was confirmed
by ligand displacement experiments (see supplemental data).

ECFP-labeled 5-HT3Rs were localized in the endoplasmic reticulum or Golgi apparatus by comparing fluorescence im-
ages of the receptor with those of organelles labeled with EYFP
as described above. The diffusion of a Cy5-labeled 5-HT3R-
specific antagonist (GR-Cy5) into permeabilized cells allowed
us to detect ligand binding activity in ER and Golgi apparatus
(Fig. 3). This reports on receptor assembly at an early stage of
expression in the ER. The GR-Cy5 fluorescence signal located
in the Golgi was stronger than in the ER indicating substan-
tially higher amounts of assembled 5-HT3 receptors in the
Golgi apparatus.

The amount of 5-HT3R ligand binding sites on the cell sur-
face receptors as compared to those formed inside the cell was
then evaluated by double ligand binding experiments (Fig. 3,
G–I). Fluorescent rhodamine-labeled antagonist GR-Rho was
applied to non-permeabilized cells expressing 5-HT3R-ECFP to
label the receptors located on the plasma membrane. After
fixation and permeabilization of the cells, the antagonist GR-
Cy5 was applied to label the receptors inside the cell. Due to
the low off-rate of GR-Rho from 5-HT3R, it was not replaced by
GR-Cy5, as described before (9), and thus allowed us to visu-
alyze and to distinguish between extra- and intracellular ligand
binding sites. Analyzing images arising from the fluorescence
by cotransfecting EYFP-labeled tubulin
(pEYFP-Tub). To verify whether tubulin filaments are required
for 5-HT3 receptor transport to the plasma membrane, we
studied the influence of colchicine on receptor trafficking (Fig.
4, C and D). Colchicine is known to inhibit microtubule forma-
tion (28–30). 5 h after adding 100 µg/ml cycloheximide to cells,
which interferes with the translation machinery (31), the re-
ceptors were almost completely located on the cell membrane.
However, when the cells were treated 2 h before with 50 µg/ml
colloidine, the receptors did not completely localize at the mem-
brane, demonstrating that the tubulin filaments are required
for proper receptor trafficking and final membrane insertion.

Although the fluorescence emitted from the 5-HT3R-ECFP is
primarily an indication of a folded ECFP marker protein, it
allows also monitoring of 5-HT3 receptor biogenesis, since the
ECFP label is directly integrated into the receptor sequence.
Information about the assembly of 5-HT3R subunits can be
assessed via detection of the formation of the ligand binding
sites. It has been shown for the structurally related acetylcho-
line receptor (25) as well as by amino acid sequence homology
studies on the 5-HT3R (26, 27) that the ligand binding sites are
located between neighboring subunits; in consequence, ligand
binding can only be observed once the subunits are fully
assembled to a functional pentameric receptor.

After monitoring 5-HT3 biosynthesis in cytoplasmic compart-
ments we investigated the presence of intracellular 5-HT3-
specific ligand binding sites, which indicate the functional assem-
oney of receptor subunits. Ligand binding to intracellular 5-HT3
receptors was first investigated by permeabilizing fixed HEK293
cells at different times after transfection and subsequent labeling
with fluorescent ligands. The binding specificity of those ligands
to 5-HT3 receptors in intracellular compartments was confirmed
by ligand displacement experiments (see supplemental data).

Fig. 1. Average number per cell of 5-HT3R ligand binding sites
on the cellular membrane measured at different times after
transfection. The values were obtained via radioligand binding assays
on populations of HEK293 cells and divided by the number of cells,
which were in the order of 1 × 10⁶ per assay before transfection. The
calculated receptor number was corrected taking into account cell divi-
sion and transfection efficiency.

Using laser scanning confocal microscopy we found that the
ECFP-labeled receptors start to form in the endoplasmic retici-
ulum typically 3 h after transfection onset. The receptor-de-
ved fluorescence signal could be colocalized with a spectrally
distinguishable enhanced yellow fluorescent ER marker (Fig.
2B), which was transfected together with the ECFP-tagged
receptor DNA. In parallel experiments, we were able to overlap
fluorescence images arising from the receptor with EYFP tar-
geted to the Golgi apparatus at ~4 h after transfection (Fig.
2C). Receptor trafficking from the appearance in the Golgi to
plasma membrane insertion occurred in about 30 min. The
integration of mature receptors into the plasma membrane
could be confirmed by binding of a fluorescence-labeled
5-HT3R-specific antagonist (Fig. 2D).

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primarily an indication of a folded ECFP marker protein, it
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After monitoring 5-HT3 biosynthesis in cytoplasmic compartiments we investigated the presence of intracellular 5-HT3-specific ligand binding sites, which indicate the functional assembly of receptor subunits. Ligand binding to intracellular 5-HT3 receptors was first investigated by permeabilizing fixed HEK293 cells at different times after transfection and subsequent labeling with fluorescent ligands. The binding specificity of those ligands to 5-HT3 receptors in intracellular compartments was confirmed by ligand displacement experiments (see supplemental data).
Noninvasive Imaging of 5-HT<sub>3</sub> Receptor Trafficking

Radioactive binding of <sup>[3H]</sup>GR65630 to 5-HT<sub>3</sub>R on whole cells (1) and on saponin-permeabilized cells (2)
The values represent average number of 5-HT<sub>3</sub>Rs on populations of 1 × 10<sup>6</sup> cells as measured 34 h after transient transfection of HEK293 cells or on non-transfected N1E-115 cells.

|                        | N1E-115 cells | HEK293 cells |
|------------------------|---------------|--------------|
| 1) Receptor binding sites per cell on membrane      | 5.0 ± 0.6 × 10<sup>4</sup> | 2.6 ± 0.2 × 10<sup>6</sup> |
| 2) Total number of receptor binding sites per cell  | 1.2 ± 0.2 × 10<sup>5</sup> | 4.4 ± 0.2 × 10<sup>6</sup> |
| Percentage of receptor binding sites localized on membrane | 42 ± 12% | 59 ± 7% |

Fusion of ECFP to the 5-HT<sub>3</sub>R and cellular trafficking visualized by confocal fluorescence microscopy.

For antagonist-agonist competition experiments, cells were perfused with graniisetron-containing solutions for three minutes before the addition of 30 μM serotonin. The agonist was then quickly removed by three washes with PBS buffer. Subsequent labeling with GR-Cy5 allowed visualization of remaining 5-HT<sub>3</sub>Rs on the cell surface. We observed that more than half of the receptors were internalized after 5 min of constant exposure to the agonist (Fig. 6A). The internalized receptors progressively moved away from the cell surface after endocytosis, and after 2 h of constant exposure to the agonist, labeling of the cell membrane with R18 clearly revealed no overlap of the ECFP receptor fluorescence with the membrane dye indicating complete internalization of the receptor (Fig. 6, B–D).

During agonist-induced receptor internalization we observed a progressive disappearance of receptor clusters on the membrane to a more even distribution. By coexpression of 5-HT<sub>3</sub>R-ECFP together with the actin marker EYFP-actin, we could observe that the receptor clusters were colocalized with the actin marker, suggesting the involvement of actin filaments in the process of receptor internalization.
the membrane organization of the 5-HT₃R (Fig. 6, E and F). Analysis of images of cells expressing 5-HT₃R-ECFP over time indicated that the fraction of clustered receptors, which before agonist addition was originally almost 50% of total membrane receptors, decreased by a factor of two after 4 min incubation with 500 nM mCPBG (Fig. 6), a time period comparable with that of internalization as measured conjointly with the fluorescent antagonist.

DISCUSSION

Receptor labeling by genetic fusion with the enhanced version of the cyan fluorescent protein (ECFP) allowed monitoring of 5-HT₃R expression, trafficking, plasma membrane targeting, and ligand-induced endocytosis in real time in living cells. The fluorescent receptor retained ligand binding and channel activity and permitted in vivo observation of its “birth” after delivery of its coding DNA into HEK293 cells. These cells were used as host system for this trafficking study because they efficiently express 5-HT₃ receptors in a functional form (9).

By colocalization with EYFP-labeled subcellular markers, it was possible to monitor the receptor successive appearance in different organelles over time. Furthermore, the formation of ligand binding sites was observable in situ at an early step in 5-HT₃R synthesis in the endoplasmic reticulum as visualized by labeling with fluorescent antagonists on permeabilized cells. Recent studies on the ligand binding domain of nicotinic receptors indicated that the assembly of subunits was required for proper functioning of its binding site (25). Homology studies between acetylcholine receptor and 5-HT₃R amino acid sequences point to the fact that the ligand binding site might be located at the interface between two subunits (26, 27, 32) indicating that assembly of the 5-HT₃R subunits to a functional receptor is likely to occur in the endoplasmic reticulum, since the labeling with the 5-HT₃R-specific antagonist GR-Cy5 correlates in time and space with the fluorescence signal of the 5-HT₃R-ECFP in this subcellular compartment. Thus, we provide the first evidence obtained in

![Fig. 4. Spatial and temporal resolution in live HEK293 cells of post-Golgi vesicular carriers containing the 5-HT₃R-ECFP and influence of cycloheximide and colchicine on receptor localization imaged by confocal microscopy. A, average speed of vesicles as measured by consecutive image acquisition tracking 10 individual receptor carriers. B, cotransfection of HEK293 cells with ECFP-labeled 5-HT₃R and EYFP-labeled tubulin shows the movement of carrier vesicles along tubulin-rich domains (white dots in inlet enlargement). C, 100 μg/ml cycloheximide was applied to the cells 5 h prior to imaging. D, pretreatment of cells with 50 μg/ml colchicine for 2 h before the same cycloheximide administration did not lead to complete cell surface localization of the receptors 5 h later, indicating the importance of tubulin for proper receptor trafficking and final membrane insertion. Size bars represent 2 μm.](image)

![Fig. 5. Internalization of the 5-HT₃R visualized by fluorescent antagonist accessibility. A–E, the 5-HT₃R-ECFP chimera (cyan) was labeled with the fluorescent antagonist GR-Cy5 (red). The cells were then washed with PBS, and the agonist mCPBG was applied for 30 min, leading to the internalization of the receptors. F–J, incubation of internalized receptors with the fluorescent antagonist GR-Cy5 reflected the inaccessibility of the binding sites. A, F, D, and I, confocal imaging cross-sections; B, G, E, and J, tridimensional projection of confocal images; C and H, transmission channel; 5-HT₃R-ECFP and GR-Cy5 fluorescence signals are represented in cyan and red, respectively. Size bar represents 10 μm.](image)
chimera were exposed to 500 nM mCPBG for the indicated lengths of time. After washing with PBS, cells were incubated with 12 nM GR-Cy5; first incubated for 2 h in a solution of 500 nM agonist mCPBG, and then the cell membrane was stained by R18, a membrane-integrating dye. Experiments from our laboratory (9) showed a cluster distribution, in agreement with recent experiments (36–40). The first appearance of receptors on the plasma membrane occurred about 4–5 h after transfection onset and exhibited a more homogeneous distribution, presumably in endosomes. The localization of internalized receptors below the membrane diminished almost by a factor of three and in parallel their cluster organization was consistently overtaken by a more homogeneous distribution of the receptors below the cell membrane (G). Size bars represent 5 μm.

**Noninvasive Imaging of 5-HT₃ Receptor Trafficking**

**FIG. 6. Temporal resolution of agonist-induced internalization of 5-HT₃R binding sites and disappearance of 5-HT₃R clusters colocalized with actin filaments as revealed by confocal fluorescence microscopy.** A, live HEK293 cells expressing the 5-HT₃R-ECFP chimera were exposed to 500 nM mCPBG for the indicated lengths of time. After washing with PBS, cells were incubated with 12 nM GR-Cy5; comparing ECFP and Cy5 fluorescence intensities allowed for the quantification of non-internalized receptors. Each horizontal dash represents one experimental value measured on a cell and the line connects the corresponding mean values for one particular incubation time. B–D, confocal fluorescence microscopy images of one representative experiment on HEK293 cells expressing 5-HT₃R-ECFP 34 h after transfection. Cells were first incubated for 2 h in a solution of 500 nM agonist mCPBG, and then the cell membrane was stained by R18, a membrane-integrating dye. The tridimensional projection of confocal images of the ECFP label shows a partial clustered organization of the internalized receptors (B). A magnified image reveals that the ECFP receptor fluorescence does not overlap with the red fluorescence of the membrane-integrated dye (C). The red fluorescence dye, which yielded red images of membrane structures together with cyan-colored intracellular 5-HT₃R-ECFP (B). A magnified image reveals that the cyan receptor fluorescence does not overlap with the red fluorescence of the membrane-integrated dye (C). The tridimensional projection of confocal images of the ECYP label shows a partial clustered organization of the internalized receptors (D). The 5-HT₃R-ECFP has a cluster distribution on the plasma membrane of a HEK293 cell (E), which colocalizes with actin-rich domains visualized by coexpression of EYFP-actin (F). Arrows in E and F indicate regions of colocalization. After inducing receptor activation by applying 500 nM of the agonist mCPBG, the percentage of membrane receptors in cluster form is rapidly decreasing, leading to a more homogeneous distribution of the receptors below the cell membrane (G). Size bars represent 5 μm.

situs on individual live cells that the first formation of functional 5-HT₃R specific binding sites occurs in the ER. Until now only indirect studies have been reported for 5-HT₃R subunit assembly by using cell fractionation to assay ER-retained 5-HT₃R receptors (12). In analogy to our findings, the assembly of nAChR subunits and thus the formation of α-bungarotoxin binding sites was also found to occur in the endoplasmic reticulum (33, 34). The recent discovery of an ER retention signal in 5-HT₃B subunits (35) and the fact that these subunits can only be “rescued” from the ER and assemble to functional receptors by coexpression of 5-HT₃ type A subunits in COS 7 cells (8), are complementary indirect indications in support of our observations. We were able to follow receptor trafficking from intracellular compartments up to the plasma membrane as a microtubule-based transport; receptor-containing vesicles leaving the Golgi apparatus could not reach the membrane when the microtubule formation was blocked. Another support for this hypothesis was provided by measuring the transport of receptor vesicles along microtubule inside living cells. We obtained values of v = 220–1850 nm/s by consecutive fluorescence imaging, which is similar as for the Golgi-to-plasma membrane transport via post-Golgi carriers on microtubules measured elsewhere (36–40). The first appearance of receptors on the plasma membrane occurred about 4–5 h after transfection onset and exhibited a cluster distribution, in agreement with recent experiments from our laboratory (9). Fluorescence images of an actin marker showed that these receptor clusters colocalized with actin-rich membrane domains, suggesting an actin-dependent membrane localization, supporting previous observations (41). Moreover, we could observe high receptor densities on cell-cell interfaces, corroborating with the large quantity of actin present in these domains (42). It has been shown for other ligand-gated channels such as nicotinic acetylcholine (43), GABA_A (44), and P2X (45) receptors that long time course desensitization provoke internalization by endocytosis. This property, which could furnish a pathway for receptor down-regulation in response to tonic levels of agonist, was so far not observed for the 5-HT₃R. We could monitor ligand-evoked endocytosis by combined imaging of 5-HT₃R-ECFP and the antagonist GR-Cy5 after application of the potent specific agonist mCPBG for different time lapses. We observed that after 5 min of incubation the number of receptors present on the plasma membrane diminished almost by a factor of three and in parallel that their cluster organization was consistently overtaken by a more homogeneous distribution, presumably in endosomes located below the cell membrane. The loss of cell surface binding of GR-Cy5 was the first indication for 5-HT₃R internalization. The localization of internalized receptors below the plasma membrane could be confirmed by employing a lipophilic membrane dye, which did not overlap with the bioluminescence signal of the receptor. In conclusion, the functionally silent insertion of ECFP into the 5-HT₃R proved to be a valuable tool
enabling spatial and temporal resolution of the receptor trafficking in single living cells. We have demonstrated that multicolor imaging could be used to study the receptor translocation to the plasma membrane after biosynthesis, appearing to follow the microtubule cytoskeleton in post-Golgi carriers whereas its localization and clustering on the plasma membrane would involve actin filaments. Furthermore, this is the first report to demonstrate in situ the ER-localized formation of the 5-HT₃R ligand binding sites. We could additionally track dynamic changes in the receptor localization by direct observation of ligand-induced internalization of the receptor in response to a 5-HT₃R-specific agonist. We believe the same methodology using multicolor dynamic tracking in living cells could be applied to a variety of other receptors to elucidate number of unraveled cellular mechanisms.

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