Characterization of the Novel Human Serotonin Receptor Subunits

5-HT$_3$C, 5-HT$_3$D and 5-HT$_3$E

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Running title page

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Non-standard abbreviations: HEK293: human embryonic kidney; 5-HT: 5-hydroxytryptamine, serotonin; LGIC: ligand-gated ion channel; mCPBG: meta-chlorophenylbiguanide
Abstract

Within the family of serotonin receptors, the 5-HT₃ receptor is the only ligand-gated ion channel. It is composed of five subunits, of which the 5-HT₃A and 5-HT₃B subunits are best characterized. Several studies, however, have reported on the functional diversity of native 5-HT₃ receptors which cannot solely be explained on the basis of the 5-HT₃A and 5-HT₃B subunits. Following our discovery of further putative 5-HT₃ serotonin receptor encoding genes, HTR3C, HTR3D, and HTR3E, we investigated whether these novel candidates and the isoform 5-HT₃Ea are able to form functional 5-HT₃ receptor complexes. Using immunofluorescence and immunoprecipitation studies of heterologous expressed proteins, we found that each of the respective candidates co-assembles with 5-HT₃A. To investigate if the novel subunits modulate 5-HT₃ receptor function, we performed radioligand-binding assays and calcium-influx studies in HEK293 cells. Our experiments revealed that the 5-HT₃C, D, E and Ea subunit alone cannot form functional receptors. Co-expression with 5-HT₃A, however, result in the formation of functional heteromeric complexes with different serotonin efficacies. Potencies of two agonists and antagonists were nearly identical with respect to homomeric 5-HT₃A and heteromeric complexes. However, 5-HT showed increased efficacy with respect to 5-HT₃A/D and 5-HT₃A/E receptors, which is consistent with the increased surface expression compared to 5-HT₃A receptors. In contrast, 5-HT₃A/C and 5-HT₃A/Ea receptors exhibited decreased 5-HT efficacy. This data shows for the first time that the novel 5-HT₃ subunits are able to form heteromeric 5-HT₃ receptors which exhibit quantitatively different functional properties compared to homomeric 5-HT₃A receptors.
The 5-HT₃ receptor is the only ligand-gated ion channel (LGIC) within the family of serotonin (5-hydroxytryptamine, 5-HT) receptors (Hoyer et al., 2002). Based on structural and functional homologies, the nicotinic acetylcholine receptor and the 5-HT₃ receptor are most closely related. Both are cation channels. The 5-HT₃ receptor is formed by a pentameric complex and is permeable to Na⁺, K⁺ and Ca²⁺. Binding of serotonin to the 5-HT₃ receptor leads to a fast excitatory response of the neuron.

After cloning of the human HTR3A gene (Belelli et al., 1995; Miyake et al., 1995), findings concerning variable receptor compositions and properties led to the hypothesis that further 5-HT₃ receptor subunits and isoforms should exist (Hussy et al., 1994; Jackson and Yakel, 1995; Fletcher and Barnes, 1998). This hypothesis was confirmed by the cloning of the human HTR3B gene (Davies et al., 1999) and of two different human splice variants of the HTR3A gene (Bruss et al., 2000). To date, HTR3A and HTR3B (Belelli et al., 1995; Miyake et al., 1995; Davies et al., 1999) are well characterized. 5-HT₃A subunits are able to form functional homo-oligomeric receptors after expression in Xenopus oocytes or HEK293 cells, whereas 5-HT₃B subunits cannot build a functional homopentameric receptor on their own (Belelli et al., 1995; Miyake et al., 1995; Davies et al., 1999; Dubin et al., 1999). Recombinant homo-oligomeric 5-HT₃A receptors show extremely reduced channel conductance compared to native receptors, whereas heteromeric complexes composed of both, 5-HT₃A and 5-HT₃B, subunits more closely resemble the properties of the native receptors (Davies et al., 1999). Since the complexity within the 5-HT₃ receptor system can still not solely be explained by differences in 5-HT₃A and 5-HT₃B subunit composition, we have investigated whether additional 5-HT₃ receptor genes reside in the human genome. We isolated novel HTR3 homologous genes termed ‘HTR3-like genes’ HTR3C, HTR3D and HTR3E (Niesler et al., 2003) based on comparative sequence analysis. The putative 5-HT₃C, 5-HT₃D and 5-HT₃E subunits share key features with common 5-HT₃ subunits and are most closely related to the 5-HT₃ receptor family (Reeves and Lummis, 2002; Niesler et al., 2003). 5-HT₃C and 5-HT₃E present a huge N-terminal extracellular segment containing a cysteine loop, four hydrophobic transmembrane regions, a large intracellular loop between the third and fourth transmembrane region and an extracellular C-terminus. In
contrast, the architecture of the 5-HT\textsubscript{3D} subunit is rather different. It lacks the signal sequence and the large N-terminal region including the ligand binding site. This raises the question whether the 5-HT\textsubscript{3D} subunit itself is able to form a functional ion channel or is part of a ligand-gated ion channel showing only some of the crucial elements of a 5-HT\textsubscript{3} subunit. Unlike the other known \textit{HTR3} genes which are almost ubiquitously expressed in the CNS as well as in the periphery, \textit{HTR3D} is predominantly and \textit{HTR3E} exclusively expressed in the gastrointestinal (GI) tract (Niesler et al., 2003). This has given rise to the hypothesis that the novel 5-HT\textsubscript{3D} and 5-HT\textsubscript{3E} subunits may act as modulators of 5-HT\textsubscript{3} function in the gastrointestinal tract (Karnovsky et al., 2003; Niesler et al., 2003).

5-HT\textsubscript{3} receptors are of particular interest due to their therapeutic potential and pathophysiological implications. 5-HT\textsubscript{3} receptor antagonists are used to treat emesis and nausea caused by radiotherapy or chemotherapy in patients with cancer and are the only effective treatment of diarrhea predominant irritable bowel syndrome (Gregory and Ettinger, 1998; Gershon, 2005; Mawe et al., 2006).

In this study we have investigated whether the novel putative \textit{HTR3} genes encode functional 5-HT\textsubscript{3} subunits. Using immunofluorescence and immunoprecipitation of recombinantly expressed proteins, we explored if they are able to form 5-HT\textsubscript{3} receptors. In addition, ligand-binding studies as well as calcium-influx analyses were performed to reveal if they modulate 5-HT\textsubscript{3} receptor function.
Materials and Methods

Cell culture and transfection

Human embryonic kidney (HEK293) cells were cultured as recommended and were grown to a relative density of 40-70%. For immunofluorescence and immunoprecipitation analysis, cells were transfected by Fugene (Roche, Mannheim, Germany): 0.250 µg (per well of a 12 well plate) and 10 µg (per 6 cm dish) plasmid DNA. Equal amounts of constructs were used in all transfections. The DNA mixtures were as follows: a) for the particular subunits: 1/2 subunit cDNA and 1/2 salmon sperm DNA; b) combination of 5-HT₃A with another subunit: 1/2 of each subunit cDNA c) negative control: pcDNA3 (mock transfection). For radioligand binding assays transfections were performed by TransIT®-293 (Mobitec, Goettingen, Germany) using 35 µg of DNA. The mixtures of cDNAs were the following: a) for the particular subunits: ½ subunit cDNA and ½ salmon sperm DNA; b) combination of 5-HT₃A with another subunit: ½ of each subunit cDNA. For aequorin assays 15 µg plasmid DNA were transfected. The DNA mixtures were as follows: a) for the particular subunits: 1/3 apoaequorin cDNA, 1/3 subunit cDNA and 1/3 salmon sperm DNA; b) combination of 5-HT₃A with another subunit: 1/3 apoaequorin cDNA and 1/3 of each subunit cDNA. Cells were analyzed 48 h post transfection. During cultivation cells were maintained in a humidified atmosphere at 37°C and 5% CO₂.

Expression constructs

All human 5-HT₃ subunit encoding cDNAs from HTR3A, HTR3B as well as the HTR3-like genes HTR3C, HTR3D, HTR3E and HTR3Ea (GenBank accession numbers: D49394, AF080582, AF459285, AY159812, AY159813, DQ644022), were cloned into the expression vector pcDNA3 (Invitrogen, Karlsruhe, Germany). To allow detection of the encoded protein by Western blot analysis as well as in immunofluorescence experiments, Myc (EQKLISEEDL) - and HA (YPYDVPDYA)- epitope tags were introduced after the signal sequence: HA- and Myc tag was introduced into the 5-HT₃A subunit at position...
29 and 30 (TT, as described before (Boyd et al., 2002)). The 5-HT$_{3B}$, 5-HT$_{3E}$ and Ea subunits were tagged by the Myc-epitope between amino acids 5/6 (P/Q, as described before (Boyd et al., 2002)), 120/121 and amino acids 105/106 (EE), respectively. In case of the 5-HT$_{3C}$ and D subunits the HA-epitope was inserted between amino acids 105/106 (KE) and 37/38 (ER), respectively (Figure 1 supplemental data).

A mutant of the 5-HT$_{3A}$ subunit was constructed by site-directed mutagenesis in which the tryptophan residue 178 was replaced by serine using the "Quick change" site-directed mutagenesis system (Stratagene, La Jolla, California, USA). This mutant was HA- and Myc-tagged at the same site as the 5-HT$_{3A}$ wild type subunit for immunofluorescence experiments. After cloning the fidelity of the cDNA sequences was verified by sequence analysis using the MEGABACE system from GE Healthcare (Munich, Germany) as indicated by the manufacturer. We carried out aequorin assays for the respective subunits to test the functional consequences of the epitope. Epitope-tagged subunits also formed LGICs together with the tagged 5-HT$_{3A}$ subunit with analogous properties compared to the untagged subunits (data not shown).

The aequorin cDNA (GenBank accession number L29571) encoding a jellyfish photoprotein was originally derived from cytAEQ/pcDNA1 (Invitrogen, Karlsruhe, Germany) and subcloned into HindIII/XbaI-digested pcDNA 3.1/Zeo (+) (Invitrogen, Karlsruhe, Germany).

**Drugs**

5-Hydroxytryptamine creatinine sulfate (5-HT), meta-chlorophenylbiguanide (mCPBG) and ondansetron hydrochloride were from Sigma (Munich, Germany). 3-Tropanyl-3,5-dichlorobenzoate (MDL72222) was from RBI (Nattick, USA). Azasetron (Y-25130) was obtained from Biotrend (Cologne, Germany). [$^3$H]-3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone ([*$^3$H]GR65630, specific activity 77.2 Ci/mmol resp. 86 Ci/mmol) was from Perkin Elmer Life Sciences (Boston, MA, USA).

**Solutions**

MDL72222 solution was prepared daily from a 10 mM DMSO stock (stored at –20°C). The other drug solutions were prepared daily from aqueous stocks (stored at –20°C).
Membrane preparation and radioligand binding assay

For preparation of crude membranes, transiently transfected cells of a 175 cm² cell culture flask were harvested 48 h post transfection and washed once with binding assay buffer (NaCl 150 mM; CaCl₂ 1.8 mM; MgCl₂ 1 mM; HEPES 10 mM; KCl 5.4 mM; pH 7.4). All steps were carried out on ice. After resuspension of the cells in 2 ml buffer, they were homogenized with a glass potter and homogenates were pelleted by centrifugation (1000 x g, 4 min, 4°C). Supernatant was recentrifuged in an ultracentrifuge (40.000 x g, 25 min, 4°C). After the pellet was washed once with buffer, an additional centrifugation step followed (40.000 x g, 25 min, 4°C). The final pellet was resuspended in 0.5 ml buffer and homogenized by pipetting through a 0.4 x 20 mm-gauge needle. Protein measurement of 10 µl membrane suspension was done by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumine as standard. Membranes were diluted to a protein concentration of 0.33 µg/µl and stored at –80°C until usage.

For saturation experiments 15 µg of membranes were incubated in triplicates with five increasing concentrations of [³H]GR65630 (0.03-3 nM) in a final reaction volume of 0.5 ml. After an incubation time of 60 min at room temperature, incubation mixes were filtered through GF/B-filters (Whatman, Kent, UK) using a Brandel cell harvester and washed three times with 2 ml of ice cold buffer. Measuring of radioactivity was carried out in a liquid scintillation counter (Beckman, Fullerton, CA, USA). Specific binding was determined as the fraction of [³H]GR65630 which could be displaced by 100 µM MDL72222.

In one series of experiments [³H]GR65630 binding was carried out using intact and transiently transfected HEK293 cells cultured in 24-well plates. After removal of the culture medium, cells were incubated (at room temperature for 120 min) in binding assay buffer (see above) containing 5.5 mM D-glucose and 1 nM [³H]GR65630. Non-specific binding was determined at mock transfected cells in the presence of 10 µM MDL72222. After three washes with ice-cold buffer, cells were solubilized with 0.1 % Triton X-100; protein and radioactivity was determined from the cell lysate as described above.
Aequorin luminescence assay

Cell preparation

HEK 293 cells were harvested by centrifugation (180 x g, 4 min) 48 h post transfection and resuspended in 1.5 ml of DMEM/Ham’s F12 + 10 % FCS. From now on all steps were performed under light protection. Cell suspension was supplemented with coelenterazine h (Nanolight, Pinetop, AZ, USA) at a final concentration of 5 µM and incubated for 3 h at room temperature. Following the coelenterazine incubation cells were harvested by centrifugation (45 x g, 3 min) and resuspended in aequorin assay buffer (NaCl 150 mM; CaCl₂ 1.8 mM; KCl 5.4 mM; HEPES 10 mM; Glucose 20 mM; pH 7.4) to obtain an approximate cell density of 2.5-3.5 x 10⁶ cells/ml. An incubation time of 10-20 min at room temperature followed.

Aequorin assay

For agonist concentration response curves, a white 96-well teflon plate with 80 µl of the cell suspension per well was placed into a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). Prior to injection of the agonist baseline luminescence was measured for 8 s at a sampling rate of 2 Hz. After injection of 20 µl agonist solution to the cells light emission was recorded for 35-70 s at the same sampling rate.

For antagonist concentration response curves 60 µl of the cell suspension were added per well of a white 96-well teflon plate. The wells were then supplemented with 20 µl of the different antagonist concentrations, mixed and incubated for 15 min at room temperature in order to reach an equilibrium. The following steps were identical to the agonist response curves.

Each drug concentration was measured in quadruplicate per transfection. After measurement of the agonist-induced light signal remaining aequorin luminescence was determined by injecting 100 µl of a ‘cell lysis solution’ (Triton X-100 0.2 % (vol/vol); CaCl₂ 100 mM) and recording luminescence at 0.5 s intervals for 15 s in the case of 5-HT maximal responses.
Data analysis

Peak values for the concentration response curves were obtained by subtraction of baseline luminescence from the agonist induced peak maximum luminescence. In the case of 5-HT maximal responses peak luminescence ($RLU_{peak}$) was normalized against total aequorin luminescence ($RLU_{max}$) after cell lysis in order to control for differences in transfection efficiency and cell number ($RLU_{peak}/RLU_{peak} + RLU_{max}$).

The concentration response curves and saturation binding curves as well as the corresponding constants $EC_{50}$, $IC_{50}$ and the binding constants $K_d$ and $B_{max}$ were calculated by means of GraphPad Prism® 4.0 (San Diego, California, USA). Data are represented as mean ± SEM. Statistical analysis was performed by using ANOVA followed by Dunnett’s post test. The unpaired Student’s test (t-test) was used in order to compare the results of only two groups. Differences were considered as significant with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

Immunofluorescence experiments

HEK293 cells were grown on poly-D-lysine coated coverslips. For subcellular localization of the respective subunits by immunofluorescence experiments we used the HA-/Myc epitope tagged subunit cDNAs. Cells were analyzed 48 h after transfection. Briefly, cells were washed twice using 1x PBS. Then they were fixed by incubation in 3.75 % paraformaldehyde for 15-20 min. Afterwards they were washed three times for 10 min in 1x PBS at room temperature. Then cells were blocked in 2 % BSA/PBS. In case of intracellular staining (permeabilized cells) blocking solution included 0.2 % Saponin. The first antibodies rabbit anti-Myc Tag (Upstate, Biomol, Hamburg, Germany) and rat anti-HA 3F10 (Roche, Mannheim, Germany) were diluted as recommended in blocking solution and applied for at least one hour at room temperature. Afterwards cells were washed 3 x 5 min in 1x PBS at room temperature and incubated in blocking solution containing the fluorochrome-labelled secondary antibodies (goat/sheep anti-mouse Cy3/FITC and goat/sheep anti-rabbit-Cy3/FITC (Sigma, Munich, Germany)) diluted as recommended for at least one hour. From now on every step was carried out light protected. Cells were
washed three times for 5 min in 1x PBS. A nuclear counterstain with DAPI (1:10.000) was carried out.

After two washes in 1x PBS coverslips were mounted in Mowiol (Calbiochem, Darmstadt, Germany) and stored at 4°C until confocal microscopy.

**Confocal microscopy**

Confocal microscopy was performed on a Zeiss LSM510 Meta system (Zeiss, Oberkochen, Germany) equipped with a Zeiss PlanApochromat 63x/1.4NA objective, 405nm DPSS, Argon and green and red HeNe lasers. Pictures were analyzed by the Zeiss LSM Image Examiner software.

**Biotinylation of cell surface proteins**

Biotinylation of the cell surface proteins was carried out using the Pinpoint Cell Surface Protein Isolation Kit (Pierce, Rockford, IL, USA). Flow through and biotinylated cell surface proteins (caught by neutravidin) were separated on 4-12 % Bis-Tris NuPAGE Gels (Invitrogen, Karlsruhe, Germany). Gels were blotted onto PVDF membranes using the Xcell system from Invitrogen (Karlsruhe, Germany) and Western Blot analysis was carried out using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

**Western blot analysis**

Transfections of HEK293 cells were performed as described above. Immunoprecipitation was carried out as described previously (Boyd et al., 2002). For the immunoprecipitation of the 5-HT$_{3A}$ subunit the epitope-specific anti-5-HT$_{3A}$ (KGVRPVDRDKPTTV), for the 5-HT$_{3C}$ subunit the anti-5-HT$_{3C}$ (GELAGKKGPRETEPD) and for the 5-HT$_{3D}$ and 5-HT$_{3E}$ subunits the anti-5-HT$_{3DE}$ (WTRAQREHEAQPQKHS) antibodies, raised in rabbit and guinea pig, were used. Each cell lysate was split in half and immunoprecipitation was performed once using the anti-5-HT$_{3A}$ and once the respective anti-5-HT$_{3C}$/anti-5-HT$_{3DE}$ antibody. Immunoprecipitated proteins were separated on 4-12 % Bis-Tris NuPAGE Gels (Invitrogen, Karlsruhe, Germany). Gels were blotted onto PVDF membranes using the
Xcell system from Invitrogen (Karlsruhe, Germany). Western blotted membranes were blocked in milk buffer (1x PBS, 5% dry milk (w/v), 0.05% Tween 20) and incubated with the first antibody mouse anti-Myc Tag 9B11 (Cell Signalling, Frankfurt, Germany) in milk buffer over night. Then membranes were washed three times with 1x PBST (1x PBS, 0.05% Tween 20) for 10 min and incubated for at least 3 h with the second peroxidase conjugated goat anti-mouse antibody in milk buffer (Sigma, Munich, Germany). After a final washing step with 1x PBST for 10 min detection was carried out using enhanced chemiluminesence following standard protocols.
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Results

A plethora of data on the heterogeneity of native 5-HT₃ receptors strongly suggest that in addition to the 5-HT₃A and 5-HT₃B subunits other as yet unidentified subunits are part of native receptors and may contribute to this diversity. We recently reported on the identification of three novel putative 5-HT₃ receptor genes and here address the question if the novel candidates represent functional components of the 5-HT₃ receptor system.

5-HT₃C,D,E,Ea constitute novel subunits of 5-HT₃ receptor complexes

To answer the question if the novel 5-HT₃ receptor candidates are able to form 5-HT₃ receptor complexes we carried out immunofluorescence as well as immunoprecipitation analysis. For this purpose, we generated HA-/Myc-epitope-tagged subunits of 5-HT₃A, 5-HT₃C, 5-HT₃D, 5-HT₃E and the recently identified isoform 5-HT₃Ea (Figure 1, supplemental data; Figure 2, supplemental data, (Karnovsky et al., 2003)).

Immunofluorescence analysis

Immunofluorescence experiments of transfected HEK293 cells with and without prior permeabilization revealed that expression of neither candidate subunit alone results in membrane staining (Figure 3, supplemental data). In contrast, co-transfection experiments of the candidate subunits with epitope-tagged Myc-/HA-5-HT₃A, revealed membrane staining in all cases (Figure 1). Therefore, we propose that heterooligomerization of the novel subunits with the 5-HT₃A subunit is required to facilitate and/or stabilize the association of receptor complexes within the cell membrane In agreement with this finding, co-localization of the tagged 5-HT₃A with the candidate subunits strongly suggests the assembly of heteromeric complexes (Figure 1).
Immunoprecipitation experiments

Consequently, we were interested if the novel subunits are components of such respective heteromeric complexes. We carried out immunoprecipitation experiments of transfected and metabolically labelled HEK293 cells to test whether the tagged 5-HT$_{3C}$, D, E, Ea subunits co-precipitate with the tagged 5-HT$_{3A}$ subunit. Immunoreactive bands of the expected sizes were detectable: ca. 55 kDa for Myc-/HA-5-HT$_{3A}$, ca. 55 kDa for HA-5-HT$_{3C}$, ca. 25 kDa in case of HA-HT$_{3D}$ and approximately 60 kDa for Myc-5-HT$_{3E/Ea}$. In double transfected HEK293 cells, immunoreactive bands from both subunits were present indicating that these subunits must have been co-precipitated (Figure 4, supplemental data). In immunoprecipitation experiments using epitope specific anti-5-HT$_{3A}$, anti-5-HT$_{3C}$ and anti-5-HT$_{3DE}$ antibodies we were able to prove the co-precipitation of 5-HT$_{3C,D,E}$ and Ea with the 5-HT$_{3A}$ subunit (Figure 2). In conclusion, this data further supports the hypothesis that the novel subunits are part of the heteromeric 5-HT$_{3A/C}$, 5-HT$_{3A/D}$, 5-HT$_{3A/E}$, and 5-HT$_{3A/Ea}$ receptor complexes.

Biotinylation experiments

In order to additionally investigate if the novel subunits are part of heteromeric complexes on the cell surface, we carried out biotinylation experiments. Double transfected HEK293 cells were analysed whether the tagged 5-HT$_{3C}$, D, E, Ea subunits and the tagged 5-HT$_{3A}$ subunit are biotinylated. In this experiment immunoreactive bands from both subunits were present in the eluat (the intracellular, non-biotinylated fraction) and in the biotinylated cell surface fraction on the Western blot, indicating that these subunits must have been co-labelled on the cell surface (Figure 3). In summary, this data further confirms that the novel subunits must be part of heteromeric 5-HT$_{3A/C}$, 5-HT$_{3A/D}$, 5-HT$_{3A/E}$, and 5-HT$_{3A/Ea}$ receptor complexes at the cell surface.
The novel 5-HT₃ receptor subunits modulate 5-HT₃ receptor function

To investigate the pharmacological and functional properties of the novel 5-HT₃ receptors, we expressed the 5-HT₃C, D, E, and Ea subunits in HEK293 cells and performed radioligand saturation binding studies as well as calcium influx analyses.

Pharmacological properties of the novel 5-HT₃ receptor complexes

The novel subunits expressed on their own showed no specific binding to the 5-HT₃ receptor antagonist [³H]GR65630 (data not shown). To examine whether the novel subunits are able to bind the 5-HT₃ receptor antagonist [³H]GR65630, HEK293 cells were co-transfected with a defined subunit cDNA and the 5-HT₃ₐW¹⁷⁸S mutant cDNA. The latter has previously been shown to be non-functional and unable to bind the 5-HT₃ receptor antagonist [³H]granisetron although it can access the cell surface (Boyd et al., 2002). We could confirm that this mutant is expressed at the cell surface and shows no specific binding of [³H]GR65630 (data not shown). Using the 5-HT₃ₐW¹⁷⁸S mutant, we were able to confirm this data by immunofluorescence experiments (Figure 5, supplemental data). Although the mutant was capable of transporting the 5-HT₃B as well as the novel subunits (5-HT₃C,D,E,Ea) to the cell surface, the heteromeric 5-HT₃ receptor composed of one of these subunits and the mutant 5-HT₃ₐ subunit exhibited no specific binding of [³H]GR65630 (data not shown). This suggests that a tryptophan residue in this region of the ligand binding domain which is present only in the 5-HT₃ₐ subunit is crucial for ligand recognition. Analysis of the 5-HT₃ₐ subunit alone or co-expressed with 5-HT₃C,D,E or Ea revealed a single binding site (Figure 4). The Kᵅ values did not significantly differ from each other (Table 1). All subunits but the 5-HT₃₃C subunit led to a significant change of Bₘₐₓ of the resulting receptor complex when co-expressed with 5-HT₃ₐ. Upon co-expression with 5-HT₃D and E, the Bₘₐₓ increased to 143.6 % and 147.4 % (p < 0.01) compared to the Bₘₐₓ of the homopentameric 5-HT₃ₐ receptor, respectively. Conversely, co-expression of the splice variant 5-HT₃Ea led to a decreased Bₘₐₓ of 81.0 % (p < 0.05) (Table 1).
Functional characterization of receptors using the aequorin assay

Since patch clamp analysis is time consuming and requires specialized technical equipment and expertise for the characterization of multiple ion channel isoforms, we have established an alternative technique for fast and effective functional analysis of the 5-HT3 subunit candidates: the aequorin assay. Based on the calcium permeability of the 5-HT3 receptor (Hargreaves et al., 1994; Ronde and Nichols, 1997; Davies et al., 1999) agonist stimulation induces a concentration-dependent influx of calcium ions through the open channel pore, which leads to aequorin bioluminescence (Brini et al., 1995). Aequorin assays were performed using two agonists (5-HT, mCPBG) and two antagonists (azasetron, ondansetron). No response was measurable for 5-HT in case of the novel 5-HT3 subunits expressed on their own (data not shown). Concentration-response curves at heteromeric complexes composed of the 5-HT3A together with one of the novel subunits revealed no significant differences in potency of the agonists 5-HT and mCPBG (Fig. 5 A, B; Table 2) as well as the antagonists azasetron (Y-25139, Fig. 5 C,D) and ondansetron (Fig. 5 E,F). However, in comparison to homomeric 5-HT3A receptors the concentration-response curve for 5-HT at heteromeric 5-HT3A/B receptors (inset in Fig.5 A) showed the typical shift to the right together with a decrease of the Hill coefficient as described previously (Davies et al. 1999). This is a pharmacological piece of evidence for the surface expression of heteromeric 5-HT3 receptor complexes. Remarkably, the maximum response (E_max) of 5-HT at the heteromeric receptors containing one of the novel subunits significantly differed compared to the homopentameric 5-HT3A receptor (Figure 6). The E_max at the 5-HT3A/C receptor and the 5-HT3A/Ea receptor was significantly reduced to 70.7 ± 10.6 % (p < 0.05) and 43.9 ± 5.6 % (p < 0.01), whereas an increased 5-HT maximum response was detected at the 5-HT3A/D receptor (145.8 ± 10.2 % (p < 0.01)) and the 5-HT3A/E receptor (144.3 ± 22.8 % (p < 0.05)).
Discussion

This study demonstrates for the first time that the novel putative HTR3 genes encode functional components of heteromeric 5-HT$_3$ receptor complexes. The 5-HT$_3$ C/D/E as well as Ea subunits are not able to form functional receptors on their own, but upon co-expression of the 5-HT$_{3A}$ subunit functional heteromeric receptors are assembled. A similar result was previously reported for the 5-HT$_{3B}$ subunit. Unlike the 5-HT$_{3A}$ subunit, the 5-HT$_{3B}$ subunit is not able to form functional homomeric receptors (Davies et al., 1999). After co-expression of the 5-HT$_{3A}$ and 5-HT$_{3B}$ subunit, heteromeric receptors are formed displaying properties of native neuronal receptors (Davies et al., 1999). Immunofluorescence and immunoprecipitation data clearly showed that the 5-HT$_{3A}$ plus the 5-HT$_{3C,D,E}$ and Ea subunit participate in the complex formation of the receptor. Similar results were obtained by analyzing HEK 293 cells co-expressing HA-5-HT$_{3A}$ together with HA-5-HT$_{3B}$ (Figure 6, supplemental data). Furthermore, biotinylation experiments confirmed the incorporation of the co-expressed subunits into the cell membrane at the cell surface. In addition, immunofluorescence data suggest that the 5-HT$_{3A}$ subunit plays a key role in 5-HT$_3$ receptor assembly and trafficking. We drew this conclusion since the 5-HT$_{3C,D,E}$ and Ea receptor subunits are intracellularly retained, presumably within the endoplasmic reticulum (ER) unless co-expressed with 5-HT$_{3A}$. The same phenomenon was reported for the heteromeric 5-HT$_{3A/B}$ receptors before (Boyd et al., 2002). In the case of the 5-HT$_{3B}$ subunit, the ER-retention signal has been identified (Boyd et al., 2003). This signal is not conserved in the novel subunits (Figure 1, supplemental data). Accordingly, future analysis of the novel subunits should lead to the identification of signal sequences contributing to ER retention.

Only recently atomic force microscopy identified the stoichiometry of 5-HT$_{3A/B}$ receptors as a complex of two 5-HT$_{3A}$ and three 5-HT$_{3B}$ subunits (Barrera et al., 2005). The exact stoichiometry of the individual native 5-HT$_3$ receptors will now be investigated by subsequent analysis of 5-HT$_3$ receptors composed of different mixtures of recombinant homo- and hetero-oligomeric 5-HT$_3$ receptor complexes using all five 5-HT$_3$ receptor subunits in different combinations. This will provide a far more detailed insight into the
maturational, structural and functional diversity of the 5-HT₃ receptor system. Analogous data from the most closely related LGIC, the nicotinic acetylcholine receptor, revealed various stoichiometries of α,β,γ,δ,ε subunits in native receptors with different properties and functions (Karlin, 2002; Le Novere et al., 2002).

The aequorin assay was previously used for measurement of intracellular Ca²⁺ levels (Brini et al., 1995). We have used this method for the first time for the characterization of 5-HT₃ receptors. Our results clearly show that this assay is a valuable tool for the fast analysis of serotonin type 3 receptors and demonstrate that pharmacological characteristics of the 5-HT₃ receptor are similar to those reported using other functional methods. More established in the analysis of Ca²⁺ permeable channels is the fluorescence imaging plate reader (FLIPR) assay using Ca²⁺ sensitive dyes (Fitch et al., 2003). Recently, the 5-HT₃ receptor analysis has been described using Ca²⁺ and membrane potential sensitive fluorescent dyes (Price and Lummis, 2005). The main advantage in using the aequorin method is that exclusively transfected cells are measured and background problems are largely avoided (Brini et al., 1995). In the aequorin assays all heteromeric 5-HT₃ receptors containing the novel subunits exhibited similar pharmacological properties as homomeric 5-HT₃A receptors for the agonists 5-HT and mCPBG and for inhibition of the 5-HT response by the 5-HT₃ receptor antagonists ondansetron and azasetron (Y-25139). Although heteromeric 5-HT₃₃B receptors show some distinct differences compared to homomeric 5-HT₃₃A receptors (e.g. for 5-HT, see also present study) the overall pharmacological profiles are very similar. The 5-HT₃₃B receptor subunit was reported to predominantly alter the biophysical rather than the pharmacological properties of the 5-HT₃ receptor (Brady et al., 2001). Yet distinct pharmacological properties using structurally different compounds and 5-HT₃ receptors of diverse composition of more than two different subunits cannot be excluded. However, we can rule out that the novel subunits contain a 5-HT₃ ligand binding site which may contribute to pharmacological effects. This is based on the fact that co-expression (at the cell surface) of the novel subunits together with the ligand binding incapable 5-HT₃₃AW₁₇₈S mutant did not show any binding of [³H]GR65630.
Although in the present study 5-HT had a similar potency at all examined receptor complexes containing the novel subunits, it exhibited different efficacies compared to homopentameric 5-HT₃A receptors (Table 2, Figure 6). This may either be due to different Ca²⁺ permeabilites, distinct biophysical properties (receptor kinetics or single channel conductance) or different receptor densities on the cell surface. In our experiments 5-HT₃A/D and 5-HT₃A/E receptors showed increased relative Eₘₐₓ and Bₘₐₓ values of about 145% as compared to homomeric 5-HT₃A receptors. Since the ligand-binding properties are not changed, the increased efficacies at these heteromeric receptors are obviously caused by an enhanced surface expression. Thus both, the 5-HT₃D and the 5-HT₃E subunit, seem to interact with the 5-HT₃A subunit and influence the trafficking of functional 5-HT₃ receptors to the cell membrane. In addition, heteromeric 5-HT₃A/C and 5-HT₃A/Ea receptors exhibited a reduced relative maximum response of about 71% and 44% (Table 2). However, the relative Bₘₐₓ values indicate no decrease in receptor densities of 5-HT₃A/C receptors and only a reduction by 19% of 5-HT₃A/Ea receptors (Table 1). Consequently, the stronger reduction of the relative maximum effect is compatible with the view of reduced Ca²⁺ permeabilites. For further characterization of the biophysical properties of heteromeric receptors composed of different 5-HT₃A,B,C,D,E subunits, patch-clamp analyses are warranted.

It is striking that HTR3D is predominantly and HTR3E and HTR3Ea exclusively expressed in the gastrointestinal (GI) tract. 5-HT₃ receptors are known to be key players in motor-sensory control of normal GI function and sensitivity (Gershon, 2005; Mawe et al., 2006). Therefore, we hypothesize that the novel subunits may play a crucial role in the enteric nervous system and may be involved in functional diseases of the GI tract including irritable bowel syndrome. Since the majority of data of 5-HT₃ receptors are extrapolated from animal studies in mice and rat (Gershon, 2004; Chameau and van Hooft, 2006) and the novel subunits apparently are absent in rodents (Karnovsky et al., 2003), the investigation of the human GI tract is warranted to elucidate the role of the different 5-HT₃ receptors in the enteric nervous system.

Besides, 5-HT₃ receptors are attractive targets for the therapy of functional bowel disorders. To date, 5-HT₃ receptor antagonists are the only effective treatment for diarrhea-predominant irritable bowel
syndrome (Andresen and Camilleri, 2006; Mawe et al., 2006). Additionally, chemotherapy and radiotherapy induced emesis and nausea of cancer patients is routinely treated by 5-HT\textsubscript{3} receptor antagonists (Gregory and Ettinger, 1998). Hereby side effects like constipation, dizziness, headache up to complications such as ischemic colitis present a major problem. New drugs with fewer side effects and a higher selectivity for a certain, as yet undefined, 5-HT\textsubscript{3} receptor complex are highly desirable.

In conclusion, we show for the first time that the novel 5-HT\textsubscript{3C,D,E} and \textsubscript{Ea} receptor subunits form serotonin receptors upon co-expression of the 5-HT\textsubscript{3A} subunit and modulate 5-HT\textsubscript{3} receptor function in HEK293 cells. Our results have significant implications since the novel 5-HT\textsubscript{3} receptor subunits may help to elucidate functional and regulatory processes in the serotonin receptor system.
Acknowledgements

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References


MOL #32144


Footnotes

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Figures

Figure 1

Immunofluorescence analysis of transfected HEK293 cells co-expressing Myc-/HA- tagged 5-HT₃A plus one of the novel subunits (5-HT₃C, D, E, Ea). Surface: not permeabilized; intracellular: permeabilized. Antibodies used were rat anti-HA (3F10) and rabbit anti-Myc. Secondary antibodies were either anti-mouse-CY3 or anti-mouse-FITC, anti-rabbit-FITC or anti-rabbit-CY3. The 5-HT₃A subunit is stained in red, the novel 5-HT₃ C, D, E and Ea subunits are visualized in green. All cells were DAPI counterstained to visualize nuclei in blue. Bar indicates 10 µm.

Figure 2

Western Blot analysis of transfected HEK293 cells. Shown are results obtained with Myc-or HA-tagged 5-HT₃A, C, D, E, Ea co-expressed Myc-/HA-5-HT₃A plus C, D, E, Ea and pcDNA3 (mock transfected) negative controls. Immunoprecipitated proteins (antibodies used are indicated below the blots) were separated on a 4-12 % Bis-Tris NuPAGE Gel (Invitrogen), after gel run X-Cell blotted onto PVDF membranes as recommended by the manufacturer and then probed using the anti-Myc (9B11) antibody (Upstate) after incubation over night. As secondary antibody the anti-mouse-HRP (Sigma) was used and bands were visualized using enhanced chemiluminescence following standard protocols. Exposure times were 1 to 2 min. Immunoreactive bands were detectable for Myc-5-HT₃A of ca 55 kDa (A, B) and of Myc-5-HT₃E (C) and Myc-5-HT₃Ea (D) of ca. 55/60 kDa.
Figure 3

**Western Blot analysis of transfected and biotinylated HEK293 cells.** Shown are results obtained with co-expressed Myc-/HA-5-HT₃A plus C, D, E, Ea. Intracellular proteins (flow through:F, non-biotinylated) and biotinylated cell surface proteins (S) were separated on a 4-12 % Bis-Tris-NuPAGE Gel (Invitrogen), after gel run X-Cell blotted onto PVDF membranes and then probed using the anti-Myc (9B11) antibody (Upstate) (A) or the anti-HA (3F10) antibody (Roche) (B) after incubation over night. As secondary antibody the anti-mouse-HRP or anti-rat-HRP (Sigma) was used and bands visualized using Western Pico Kit (Pierce) as recommended. Exposure times were 30 sec to 1 min. Immunoreactive bands of ca 55 kDa for HA/Myc-5-HT₃A (A, B), ca. 55 kDa for HA-5-HT₃C (B), ca. 25 kDa in case of HA-HT₃D (B) and of Myc-5-HT₃E and Myc-5-HT₃Ea (A) of ca. 55/60 kDa were detectable. The respective antibody used for immunoblot detection is indicated.

Figure 4

**Radioligand binding studies** Saturation curves for specific (MDL72222-sensitive) binding of [³H]GR65630 (0.03-3 nM) in membranes of HEK293 cells transiently expressing various 5-HT₃ subunit combinations. For each subunit combination 3-5 independent experiments were performed in triplicates. Non-specific binding in the presence of 100 µM MDL72222 was about 10 % of over-all binding. Specific binding was expressed as percent of control i.e., as percentage of the B_max of the homopentameric 5-HT₃A receptor (means ± SEM of three to five experiments with separate membrane preparations). The absolute B_max values were 6.8 ± 0.6 pmol/mg protein and 10.5 ± 0.3 pmol/mg protein in part A and B of the figure, respectively. The difference is due to the fact that the experiments were performed in two separate groups.
Figure 5
Concentration-response curves for agonists and antagonists in the aequorin assay in HEK293 cells transiently expressing different 5-HT₃ subunit combinations. Bioluminescence signals in the aequorin assay due to calcium-influx induced by increasing concentrations of 5-HT was measured as described in Methods. Concentration-response curves for calcium-influx in the aequorin assay evoked by increasing concentrations of (A) 5-HT and (B) meta-chlorophenylbiguanide (mCPBG). Data are expressed as percentage of the agonist maximal peak response. The inset in part A additionally shows results obtained at heteromeric 5-HT₃A/B receptors (means of at least 4 experiments). (C - F) Inhibition of 5-HT (10 µM) induced calcium-influx in the aequorin assay by increasing concentrations of azasetron (C, D) and ondansetron (E, F). The antagonist was present 15 min before and during 5-HT application. Antagonist experiments were performed in two separate groups. Data are expressed as percentage of the 5-HT (10µM) response in the absence of the antagonist (means ± SEM of three to seven independent experiments).

Figure 6
Maximal peak responses (E_max) evoked by 5-HT (10 µM) induced calcium-influx in the aequorin assay of HEK293 cells transiently transfected with various 5-HT₃ subunit combinations. Experiments were performed in two separate groups. Responses were normalized by dividing the agonist induced peak luminescence (RLU_peak) by the over-all aequorin peak luminescence (RLU_max, agonist induced peak luminescence plus the peak luminescence after cell lysis by 50 mM CaCl₂ containing 0.1 % (vol/vol) Triton X-100). Data are expressed as percentage of the E_max of the homopentameric 5-HT₃A receptor (% of control). Bars represent means ± SEM of ten to twelve independent experiments. Significant differences (repeated
ANOVA followed by Dunnett’s post test) compared to the homopentameric 5-HT$_{3A}$ receptor are indicated with p < 0.05 (*) and p < 0.01 (**).
Table 1

<table>
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<tr>
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<th>5-HT₃ₐ (control)</th>
<th>5-HT₃ₐ/C</th>
<th>5-HT₃ₐ/D</th>
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<tr>
<td>Bₚmax [% of control]</td>
<td>100.00 ± 9.30</td>
<td>96.58 ± 6.57</td>
<td>143.61 ± 10.14*</td>
<td>147.44 ± 11.32*</td>
<td>100.00 ± 2.63</td>
<td>81.02 ± 4.24**</td>
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<td>Kₜd [nM]</td>
<td>0.39 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>0.46 ± 0.06</td>
<td>0.42 ± 0.05</td>
<td>0.30 ± 0.04</td>
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Relative Bₚmax values and Kₜd values obtained from [³H]GR65630 saturation binding to membranes of HEK293 cells transiently expressing either homopentameric 5-HT₃ₐ receptors or heteromeric receptors of 5-HT₃ₐ with one of the novel subunits.

Experiments were performed in two separate groups. Bₚmax values are expressed as the percentage fraction of the Bₚmax of the homopentameric 5-HT₃ₐ receptor (% of control). Significant differences (one way ANOVA followed by Dunnett’s post test) compared to homopentameric 5-HT₃ₐ receptors: p < 0.05 (*) and p < 0.01 (**).
Table 2

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<td>Agonist pEC&lt;sub&gt;50&lt;/sub&gt; (mean EC&lt;sub&gt;50&lt;/sub&gt;) [µM]</td>
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<td>5-HT</td>
<td>5.75 ± 0.03 (1.81)</td>
<td>5.75 ± 0.04 (1.79)</td>
<td>5.70 ± 0.03 (2.02)</td>
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<td>mCPBG</td>
<td>5.82 ± 0.03 (1.52)</td>
<td>5.80 ± 0.03 (1.61)</td>
<td>5.72 ± 0.03 (1.94)</td>
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<td>Antagonist pIC&lt;sub&gt;50&lt;/sub&gt; (mean IC&lt;sub&gt;50&lt;/sub&gt;) [nM]</td>
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<td>ondansetron</td>
<td>9.23 ± 0.06 (0.63)</td>
<td>9.34 ± 0.15 (0.52)</td>
<td>9.22 ± 0.07 (0.63)</td>
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<td>azasetron</td>
<td>9.12 ± 0.01 (0.77)</td>
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<td>8.96 ± 0.03 (1.11)</td>
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Emax [% of 5-HT<sub>3A</sub> + ss E<sub>max</sub>] | 100.00 ± 15.20 | 70.72 ± 10.61* | 144.32 ± 22.82** | 100.00 ± 6.95 | 145.76 ± 10.22** | 43.87 ± 5.59** |

pEC<sub>50</sub> values of agonists, pIC<sub>50</sub> values of antagonists (against the response to 10 µM 5-HT) and 5-HT maximum peak responses for calcium influx in the aequorin assay of HEK293 cells transiently expressing either homopentameric 5-HT<sub>3A</sub> receptors or heteromeric receptors of 5-HT<sub>3A</sub> with one of the novel subunits

Antagonist experiments and 5-HT maximum responses were performed in two separate groups. Significant differences (repeated ANOVA followed by Dunnett’s post test) compared to homopentameric 5-HT<sub>3A</sub> receptors: p < 0.05 (*) and p < 0.01 (**); mCPBG, meta-chlorophenylbiguanide.
Figure 3

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antι-Myc

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Figure 4

[Graph showing the specific binding of [3H]GR65630 to different 5-HT3 receptors (5-HT3A, 5-HT3A/C, 5-HT3A/D, and 5-HT3A/E) as a function of [3H]GR65630 concentration. The results are presented as the percentage of control binding.]
Figure 5
Figure 6