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# Characterization of the Novel Human Serotonin Receptor Subunits 5-$HT_{3C}$, 5- $HT_{3D}$ and 5-$HT_{3E}$

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# Running title page

a) Human 5-$HT_3$R Subunits 5-$HT_{3C}$, 5- $HT_{3D}$ , 5-$HT_{3E}$

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

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# Abstract

Within the family of serotonin receptors, the 5-$HT_3$ receptor is the only ligand-gated ion channel. It is composed of five subunits, of which the 5-$HT_{3A}$ and 5-$HT_{3B}$ subunits are best characterized. Several studies, however, have reported on the functional diversity of native 5-$HT_3$ receptors which cannot solely be explained on the basis of the 5-$HT_{3A}$ and 5-$HT_{3B}$ subunits. Following our discovery of further putative 5-$HT_3$ serotonin receptor encoding genes, *HTR3C*, *HTR3D* and *HTR3E*, we investigated wether these novel candidates and the isoform 5-$HT_{3Ea}$ are able to form functional 5-$HT_3$ receptor complexes. Using immunofluorescence and immunoprecipitation studies of heterologous expressed proteins, we found that each of the respective candidates co-assembles with 5-$HT_{3A}$. To investigate if the novel subunits modulate 5-$HT_3$ receptor function, we performed radioligand-binding assays and calcium-influx studies in HEK293 cells. Our experiments revealed that the 5-$HT_{3C, D, E}$ and $_{Ea}$ subunit alone cannot form functional receptors. Co-expression with 5-$HT_{3A}$, 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-$HT_{3A}$ and heteromeric complexes. However, 5-HT showed increased efficacy with respect to 5-$HT_{3A/D}$ and 5-$HT_{3A/E}$ receptors, which is consistent with the increased surface expression compared to 5-$HT_{3A}$ receptors. In contrast, 5-$HT_{3A/C}$ and 5-$HT_{3A/Ea}$ receptors exhibited decreased 5-HT efficacy. This data shows for the first time that the novel 5-$HT_3$ subunits are able to form heteromeric 5-$HT_3$ receptors which exhibit quantitatively different functional properties compared to homomeric 5-$HT_{3A}$ receptors.

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The 5-$HT_3$ 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 acetycholine receptor and the 5-$HT_3$ receptor are most closely related. Both are cation channels. The 5-$HT_3$ receptor is formed by a pentameric complex and is permeable to $Na^+$, $K^+$ and $Ca^{2+}$. Binding of serotonin to the 5-$HT_3$ 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-$HT_3$ 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-$HT_{3A}$ subunits are able to form functional homo-oligomeric receptors after expression in *Xenopus* oocytes or HEK293 cells, whereas 5-$HT_{3B}$ 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-$HT_{3A}$ receptors show extremely reduced channel conductance compared to native receptors, whereas heteromeric complexes composed of both, 5-$HT_{3A}$ and 5-$HT_{3B,}$ subunits more closely resemble the properties of the native receptors (Davies et al., 1999). Since the complexity within the 5-$HT_3$ receptor system can still not solely be explained by differences in 5-$HT_{3A}$ and 5-$HT_{3B}$ subunit composition, we have investigated whether additional 5-$HT_3$ 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-$HT_{3C}$, 5-$HT_{3D}$ and 5-$HT_{3E}$ subunits share key features with common 5-$HT_3$ subunits and are most closely related to the 5-$HT_3$ receptor family (Reeves and Lummis, 2002; Niesler et al., 2003). 5-$HT_{3C}$ and 5-$HT_{3E}$ 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

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contrast, the architecture of the 5-$HT_{3D}$ 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-$HT_{3D}$ 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-$HT_3$ subunit. Unlike the other known *HTR3* genes which are almost ubiquitously expressed in the CNS as well as in the periphery, *HTR3D* is predominantly and *HTR3E* exclusively expressed in the gastrointestinal (GI) tract (Niesler et al., 2003). This has given rise to the hypothesis that the novel 5-$HT_{3D}$ and 5-$HT_{3E}$ subunits may act as modulators of 5-$HT_3$ function in the gastrointestinal tract (Karnovsky et al., 2003; Niesler et al., 2003).

5-$HT_3$ receptors are of particular interest due to their therapeutic potential and pathophysiological implications. 5-$HT_3$ 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 *HTR3* genes encode functional 5-$HT_3$ subunits. Using immunofluorescence and immunoprecipitation of recombinantly expressed proteins, we explored if they are able to form 5-$HT_3$ receptors. In addition, ligand-binding studies as well as calcium-influx analyses were performed to reveal if they modulate 5-$HT_3$ receptor function.

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# 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-$HT_{3A}$ 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-$HT_{3A}$ 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-$HT_{3A}$ 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 % $CO_2$.

## Expression constructs

All human 5-$HT_3$ 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-$HT_{3A}$ subunit at position

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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 *Hin*dIII/*Xba*I-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).



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## Membrane preparation and radioligand binding assay

For preparation of crude membranes, transiently transfected cells of a 175 $cm^2$ cell culture flask were harvested 48 h post transfection and washed once with binding assay buffer (NaCl 150 mM; $CaCl_2$ 1.8 mM; $MgCl_2$ 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 [$^3$H]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 [$^3$H]GR65630 which could be displaced by 100 µM MDL72222.

In one series of experiments [$^3$H]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 [$^3$H]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.

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## 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; $CaCl_2$ 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 $10^{-6}$ 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); $CaCl_2$ 100 mM) and recording luminescence at 0.5 s intervals for 15 s in the case of 5-HT maximal responses.

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## 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 efficicency 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

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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 (catched 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. Immunoprecipiation was carried out as decribed previously (Boyd et al., 2002). For the immunoprecipitation of the 5-$HT_{3A}$ subunit the epitope-specific anti-5-$HT_{3A}$ (KGVRPVRDWRKPTTV), for the 5-$HT_{3C}$ subunit the anti-5-$HT_{3C}$ (GELAGKKLGPRETEPD) and for the 5-$HT_{3D}$ and 5-$HT_{3E}$ subunits the anti-5-$HT_{3DE}$ (WTRAQREHEAQKQHS) 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

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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-$HT_3$ receptors strongly suggest that in addition to the 5-$HT_{3A}$ and 5-$HT_{3B}$ 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-$HT_3$ receptor genes and here address the question if the novel candidates represent functional components of the 5-$HT_3$ receptor system.

### 5-$HT_{3C,D,E,Ea}$ constitute novel subunits of 5-$HT_3$ receptor complexes

To answer the question if the novel 5-$HT_3$ receptor candidates are able to form 5-$HT_3$ receptor complexes we carried out immunofluorescence as well as immunoprecipitation anaylsis. For this purpose, we generated HA-/Myc-epitope-tagged subunits of 5-$HT_{3A}$, 5-$HT_{3C}$, 5-$HT_{3D}$, 5-$HT_{3E}$ and the recently identified isoform 5-$HT_{3Ea}$ (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-$HT_{3A,}$ revealed membrane staining in all cases (Figure 1). Therefore, we propose that hetero-oligomerization of the novel subunits with the 5-$HT_{3A}$ 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-$HT_{3A}$ with the candidate subunits strongly suggests the assembly of heteromeric complexes (Figure 1).

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**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.

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**The novel 5-$HT_3$ receptor subunits modulate 5-$HT_3$ receptor function**

To investigate the pharmacological and functional properties of the novel 5-$HT_3$ receptors, we expressed the 5-$HT_{3C,\ D,\ E,}$ and $_{Ea}$ subunits in HEK293 cells and performed radioligand saturation binding studies as well as calcium influx analyses.

**Pharmacological properties of the novel 5-$HT_3$ receptor complexes**

The novel subunits expressed on their own showed no specific binding to the 5-$HT_3$ receptor antagonist [$^3$H]GR65630 (data not shown). To examine whether the novel subunits are able to bind the 5-$HT_3$ receptor antagonist [$^3$H]GR65630, HEK293 cells were co-transfected with a defined subunit cDNA and the 5-$HT_{3A}W^{178}S$ mutant cDNA. The latter has previously been shown to be non-functional and unable to bind the 5-$HT_3$ receptor antagonist [$^3$H]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 [$^3$H]GR65630 (data not shown). Using the 5-$HT_{3A}W^{178}S$ mutant, we were able to confirm this data by immunofluorescence experiments (Figure 5, supplemental data). Although the mutant was capable of transporting the 5-$HT_{3B}$ as well as the novel subunits (5-$HT_{3C,D,E,Ea}$) to the cell surface, the heteromeric 5-$HT_3$ receptor composed of one of these subunits and the mutant 5-$HT_{3A}$ subunit exhibited no specific binding of [$^3$H]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-$HT_{3A}$ subunit is crucial for ligand recognition. Analysis of the 5-$HT_{3A}$ subunit alone or co-expressed with 5-$HT_{3C,\ D,\ E}$ or $_{Ea}$ revealed a single binding site (Figure 4). The $K_d$ values did not significantly differ from each other (Table 1). All subunits but the 5-$HT_{3C}$ subunit led to a significant change of $B_{max}$ of the resulting receptor complex when co-expressed with 5-$HT_{3A}$. Upon co-expression with 5-$HT_{3D}$ and $_E$, the $B_{max}$ increased to 143.6 % and 147.4 % ($p < 0.01$) compared to the $B_{max}$ of the homopentameric 5-$HT_{3A}$ receptor, respectively. Conversely, co-expression of the splice variant 5-$HT_{3Ea}$ led to a decreased $B_{max}$ of 81.0 % ($p < 0.05$) (Table 1).

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**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-$HT_3$ subunit candidates: the aequorin assay. Based on the calcium permeability of the 5-$HT_3$ 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-$HT_3$ subunits expressed on their own (data not shown). Concentration-response curves at heteromeric complexes composed of the 5-$HT_{3A}$ 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-$HT_{3A}$ receptors the concentration-response curve for 5-HT at heteromeric 5-$HT_{3A/B}$ 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-$HT_3$ 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-$HT_{3A}$ receptor (Figure 6). The $E_{max}$ at the 5-$HT_{3A/C}$ receptor and the 5-$HT_{3A/Ea}$ 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-$HT_{3A/D}$ receptor (145.8 ± 10.2 % ($p < 0.01$)) and the 5-$HT_{3A/E}$ receptor (144.3 ± 22.8 % ($p < 0.05$)).

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# 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

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maturational, structural and functional diversity of the 5-$HT_3$ receptor system. Analogous data from the most closely related LGIC, the nicotinic acetylcholine receptor, revealed various stoichiometries of $\alpha,\beta,\gamma,\delta,\varepsilon$ 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 $Ca^{2+}$ levels (Brini et al., 1995). We have used this method for the first time for the characterization of 5-$HT_3$ 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-$HT_3$ receptor are similar to those reported using other functional methods. More established in the analysis of $Ca^{2+}$ permeable channels is the fluorescence imaging plate reader (FLIPR) assay using $Ca^{2+}$ sensitive dyes (Fitch et al., 2003). Recently, the 5-$HT_3$ receptor analysis has been described using $Ca^{2+}$ 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-$HT_3$ receptors containing the novel subunits exhibited similar pharmacological properties as homomeric 5-$HT_{3A}$ receptors for the agonists 5-HT and mCPBG and for inhibition of the 5-HT response by the 5-$HT_3$ receptor antagonists ondansetron and azasetron (Y-25139). Although heteromeric 5-$HT_{3A/B}$ receptors show some distinct differences compared to homomeric 5-$HT_{3A}$ receptors (e.g. for 5-HT, see also present study) the overall pharmacological profiles are very similar. The 5-$HT_{3B}$ receptor subunit was reported to predominantly alter the biophysical rather than the pharmacological properties of the 5-$HT_3$ receptor (Brady et al., 2001). Yet distinct pharmacological properties using structurally different compounds and 5-$HT_3$ 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-$HT_3$ 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-$HT_{3A}W^{178}S$ mutant did not show any binding of [$^3$H]GR65630.

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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-$HT_{3A}$ receptors (Table 2, Figure 6). This may either be due to different $Ca^{2+}$ permeabilites, distinct biophysical properties (receptor kinetics or single channel conductance) or different receptor densities on the cell surface. In our experiments 5-$HT_{3A/D}$ and 5-$HT_{3A/E}$ receptors showed increased relative $E_{max}$ and $B_{max}$ values of about 145 % as compared to homomeric 5-$HT_{3A}$ 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-$HT_{3D}$ and the 5-$HT_{3E}$ subunit, seem to interact with the 5-$HT_{3A}$ subunit and influence the trafficking of functional 5-$HT_3$ receptors to the cell membrane. In addition, heteromeric 5-$HT_{3A/C}$ and 5-$HT_{3A/Ea}$ receptors exhibited a reduced relative maximum response of about 71 % and 44 % (Table 2). However, the relative $B_{max}$ values indicate no decrease in receptor densities of 5-$HT_{3A/C}$ receptors and only a reduction by 19 % of 5-$HT_{3A/Ea}$ receptors (Table 1). Consequently, the stronger reduction of the relative maximum effect is compatible with the view of reduced $Ca^{2+}$ permeabilites. For further characterization of the biophysical properties of heteromeric receptors composed of different 5-$HT_{3A,B,C,D,E}$ 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-$HT_3$ 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-$HT_3$ 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 tact is warranted to elucidate the role of the different 5-$HT_3$ receptors in the enteric nervous system.

Besides, 5-$HT_3$ receptors are attractive targets for the therapy of functional bowel disorders. To date, 5-$HT_3$ receptor antagonists are the only effective treatment for diarrhea-predominant irritable bowel



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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-$HT_3$ 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-$HT_3$ receptor complex are highly desirable.

In conclusion, we show for the first time that the novel 5-$HT_{3C,D,E}$ and $_{Ea}$ receptor subunits form serotonin receptors upon co-expression of the 5-$HT_{3A}$ subunit and modulate 5-$HT_3$ receptor function in HEK293 cells. Our results have significant implications since the novel 5-$HT_3$ receptor subunits may help to elucidate functional and regulatory processes in the serotonin receptor system.

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# Footnotes

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# Figures

## Figure 1

**Immunofluorescence analysis of transfected HEK293 cells co-expressing Myc-/HA- tagged 5-$HT_{3A}$ plus one of the novel subunits (5-$HT_{3C, D, E, Ea}$).** 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-$HT_{3A}$ subunit is stained in red, the novel 5-$HT_{3\ C, D, E}$ 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-$HT_{3A, C, D, E, Ea}$, co-expressed Myc-/HA-5-$HT_{3A}$ 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 chemiluminesence following standard protocols. Exposure times were 1 to 2 min. Immunoreactive bands were detectable for Myc-5-$HT_{3A}$ of ca 55 kDa (A, B) and of Myc-5-$HT_{3E}$ (C) and Myc-5-$HT_{3Ea}$ (D) of ca. 55/60 kDa.

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**Figure 3**

**Western Blot analysis of transfected and biotinylated HEK293 cells.** Shown are results obtained with co-expressed Myc-/HA-5-$HT_{3A}$ 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-$HT_{3A}$ (A, B), ca. 55 kDa for HA-5-$HT_{3C}$ (B), ca. 25 kDa in case of HA-$HT_{3D}$ (B) and of Myc-5-$HT_{3E}$ and Myc-5-$HT_{3Ea}$ (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 [$^3$H]GR65630 (0.03-3 nM) in membranes of HEK293 cells transiently expressing various 5-$HT_3$ 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-$HT_{3A}$ 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.

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## Figure 5

**Concentration-response curves for agonists and antagonists in the aequorin assay in HEK293 cells transiently expressing different 5-$HT_3$ 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-$HT_{3A/B}$ 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-$HT_3$ 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 $CaCl_2$ containing 0.1 % (vol/vol) Triton X-100). Data are expressed as percentage of the $E_{max}$ of the homopentameric 5-$HT_{3A}$ receptor (% of control). Bars represent means ± SEM of ten to twelve independent experiments. Significant differences (repeated

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ANOVA followed by Dunnett's post test) compared to the homopentameric 5-$HT_{3A}$ receptor are indicated with $p < 0.05$ (*) and $p < 0.01$ (**).

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**Table 1**

| | **5-HT$_{3A}$ (control)** | **5-HT$_{3A/C}$** | **5-HT$_{3A/D}$** | **5-HT$_{3A/E}$** | **5-HT$_{3A}$** | **5-HT$_{3A/Ea}$** |
|---|---|---|---|---|---|---|
| **$B_{max}$** [% of control] | 100.00 ± 9.30 | 96.58 ± 6.57 | 143.61 ± 10.14* | 147.44 ± 11.32* | 100.00 ± 2.63 | 81.02 ± 4.24** |
| $K_d$ [nM] | 0.39 ± 0.03 | 0.29 ± 0.03 | 0.46 ± 0.06 | 0.42 ± 0.05 | 0.30 ± 0.04 | 0.35 ± 0.09 |

**Relative $B_{max}$ values and $K_d$ values obtained from [$^3$H]GR65630 saturation binding to membranes of HEK293 cells transiently expressing either homopentameric 5-HT$_{3A}$ receptors or heteromeric receptors of 5-HT$_{3A}$ with one of the novel subunits**

Experiments were performed in two separate groups. $B_{max}$ values are expressed as the percentage fraction of the $B_{max}$ of the homopentameric 5-HT$_{3A}$ receptor (% of control). Significant differences (one way ANOVA followed by Dunnett's post test) compared to homopentameric 5-HT$_{3A}$ receptors: $p < 0.05$ (*) and $p < 0.01$ (**).



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**Table 2**

| Agonist $pEC_{50}$ (mean $EC_{50}$) [µM] | | | | | |
|---|---|---|---|---|---|
| | **5-$HT_{3A}$** | **5-$HT_{3A/C}$** | **5-$HT_{3A/D}$** | **5-$HT_{3A/E}$** | **5-$HT_{3A/Ea}$** |
| **5-HT** | 5.75 ± 0.03 (1.81) | 5.75 ± 0.04 (1.79) | 5.70 ± 0.03 (2.02) | 5.78 ± 0.03 (1.66) | 5.76 ± 0.03 (1.76) |
| **mCPBG** | 5.82 ± 0.03 (1.52) | 5.80 ± 0.03 (1.61) | 5.72 ± 0.03 (1.94) | 5.86 ± 0.04 (1.40) | 5.74 ± 0.04 (1.83) |

| Antagonist $pIC_{50}$ (mean $IC_{50}$) [nM] | | | | | | |
|---|---|---|---|---|---|---|
| | **5-$HT_{3A}$** | **5-$HT_{3A/C}$** | **5-$HT_{3A/E}$** | **5-$HT_{3A/Ea}$** | **5-$HT_{3A}$** | **5-$HT_{3A/D}$** |
| **ondansetron** | 9.23 ± 0.06 (0.63) | 9.34 ± 0.15 (0.52) | 9.22 ± 0.07 (0.63) | 9.35 ± 0.06 (0.46) | 9.00 ± 0.02 (1.01) | 8.88 ± 0.04 (1.33) |
| **azasetron** | 9.12 ± 0.01 (0.77) | 9.09 ± 0.03 (0.83) | 9.05 ± 0.04 (0.91) | 9.15 ± 0.07 (0.74) | 8.96 ± 0.03 (1.11) | 8.87 ± 0.06 (1.39) |

| | **5-$HT_{3A}$ (control)** | **5-$HT_{3A/C}$** | **5-$HT_{3A/E}$** | **5-$HT_{3A}$** | **5-$HT_{3A/D}$** | **5-$HT_{3A/Ea}$** |
|---|---|---|---|---|---|---|
| **$E_{max}$** [% of 5-$HT_{3A}$ + ss $E_{max}$] | 100.00 ± 15.20 | 70.72 ± 10.61* | 144.32 ± 22.82** | 100.00 ± 6.95 | 145.76 ± 10.22** | 43.87 ± 5.59** |

**$pEC_{50}$ values of agonists, $pIC_{50}$ 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-$HT_{3A}$ receptors or heteromeric receptors of 5-$HT_{3A}$ 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-$HT_{3A}$ receptors: $p < 0.05$ (*) and $p < 0.01$ (**); mCPBG, meta-chlorophenylbiguanide.



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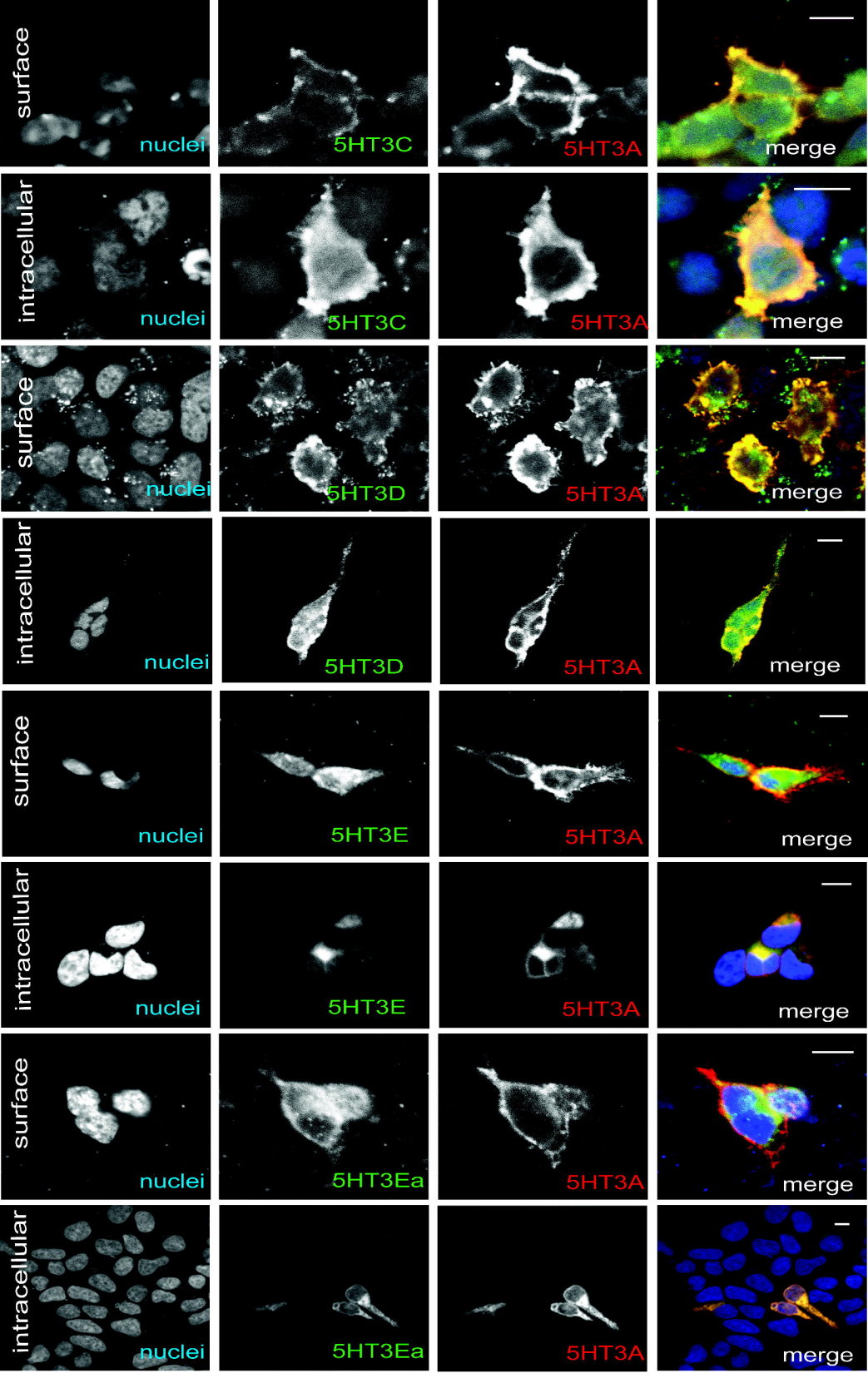
surface
nuclei
5HT3C
5HT3A
merge
intracellular
nuclei
5HT3C
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merge
surface
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merge
intracellular
nuclei
5HT3D
5HT3A
merge
surface
nuclei
5HT3E
5HT3A
merge
intracellular
nuclei
5HT3E
5HT3A
merge
surface
nuclei
5HT3Ea
5HT3A
merge
intracellular
nuclei
5HT3Ea
5HT3A
merge

Figure 1

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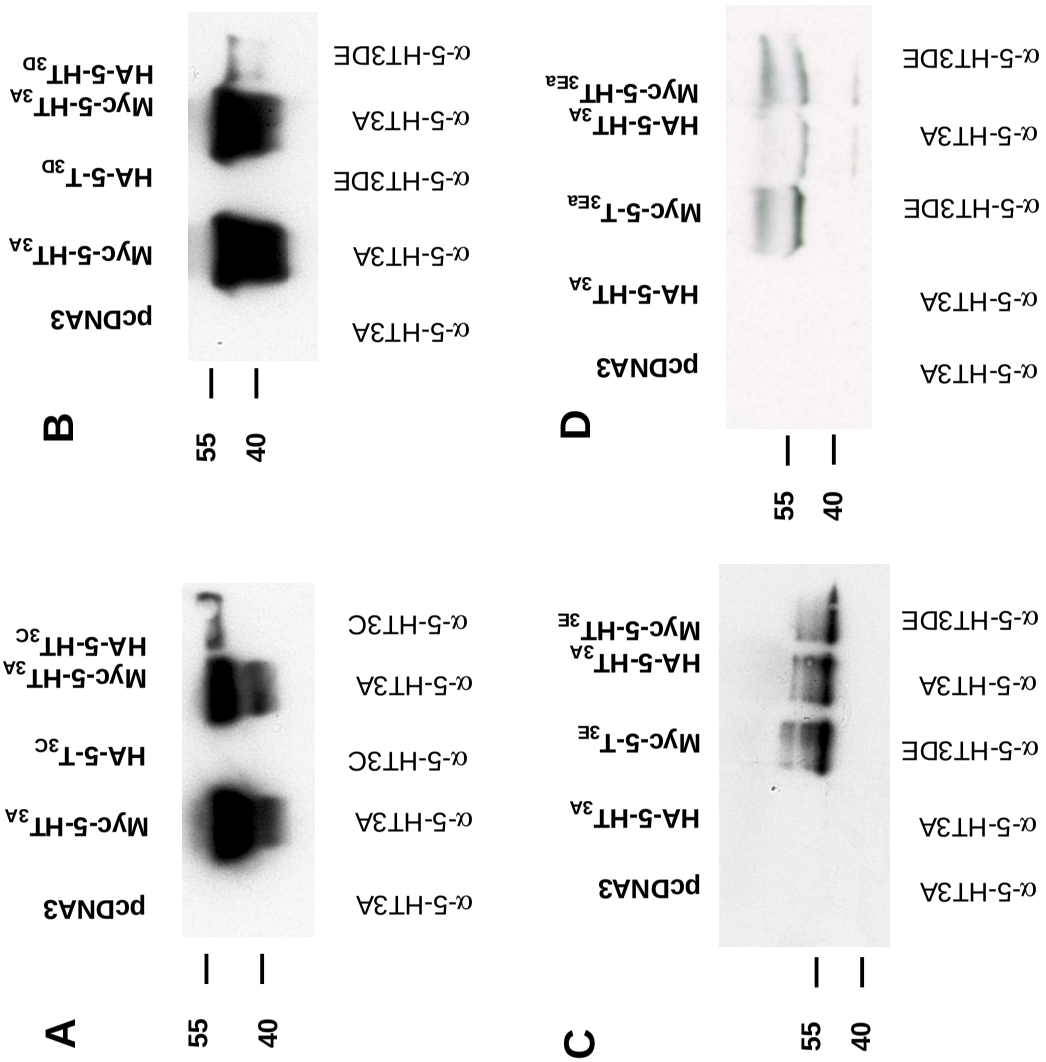


Figure 2

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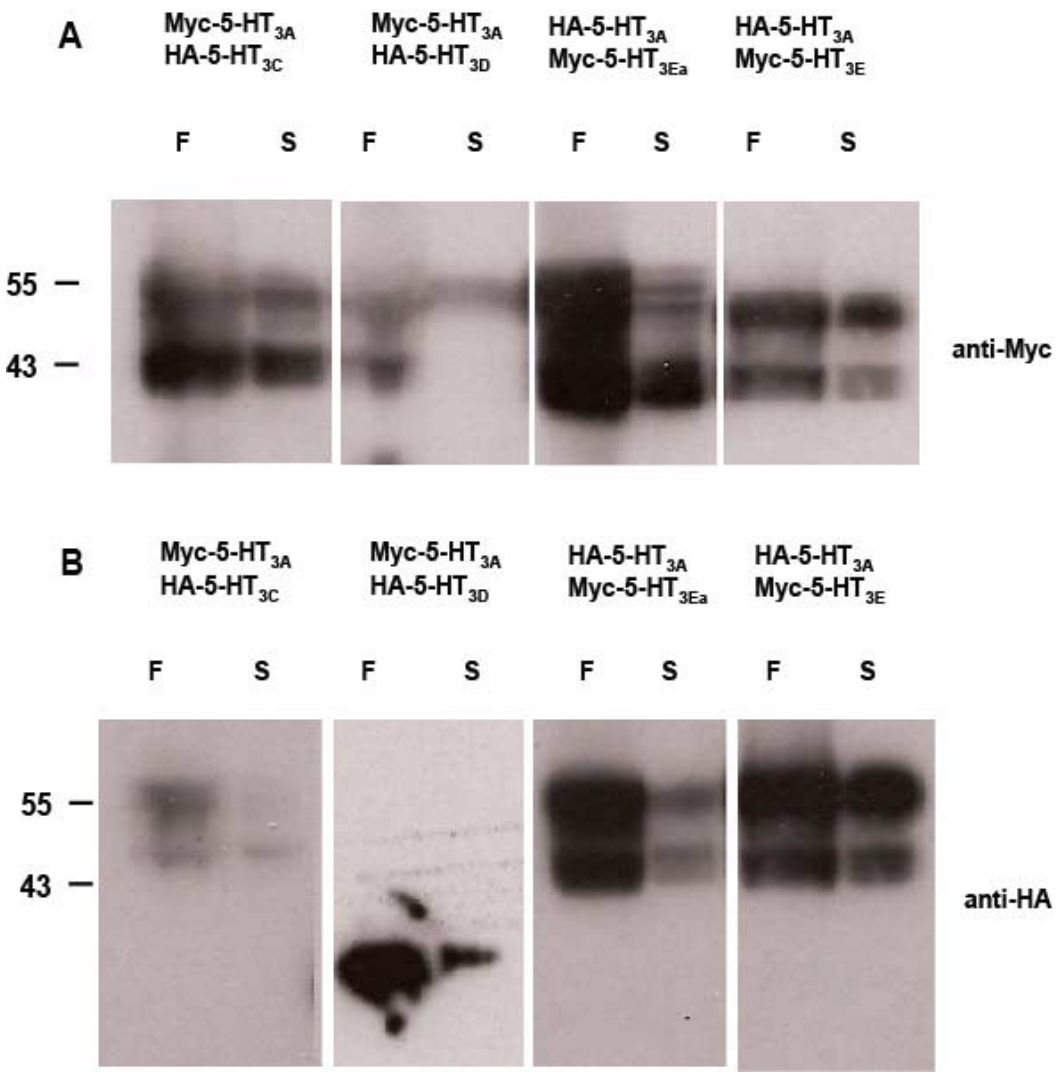


Figure 3

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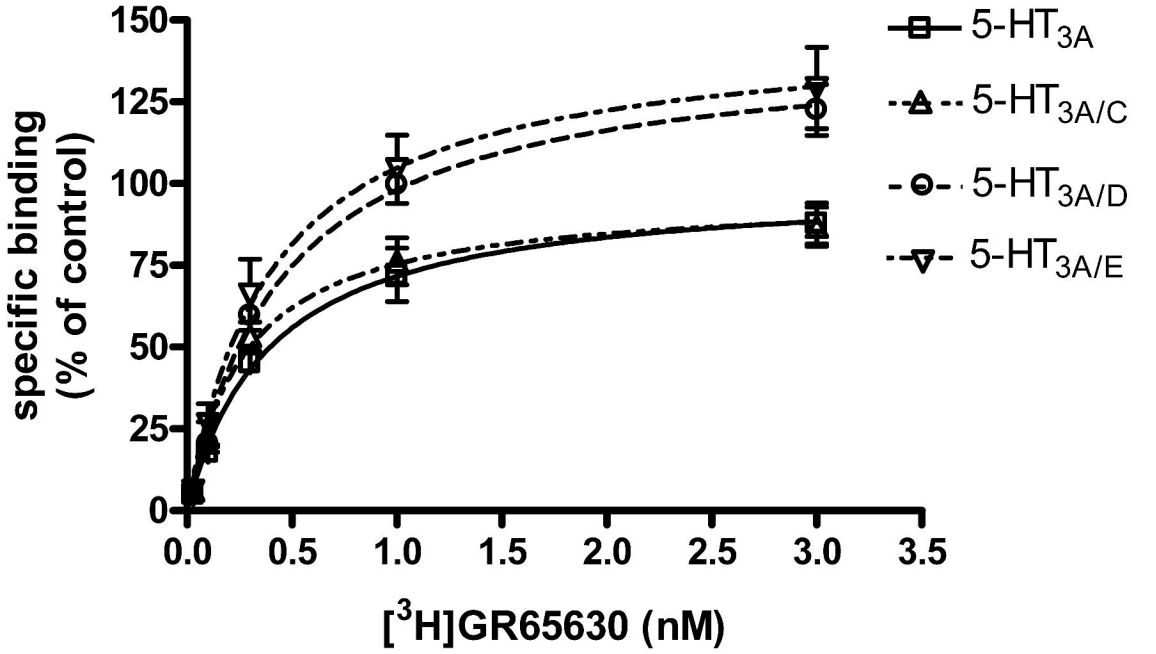
specific binding
(% of control)
150
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
[3H]GR65630 (nM)
5-HT3A
5-HT3A/C
5-HT3A/D
5-HT3A/E

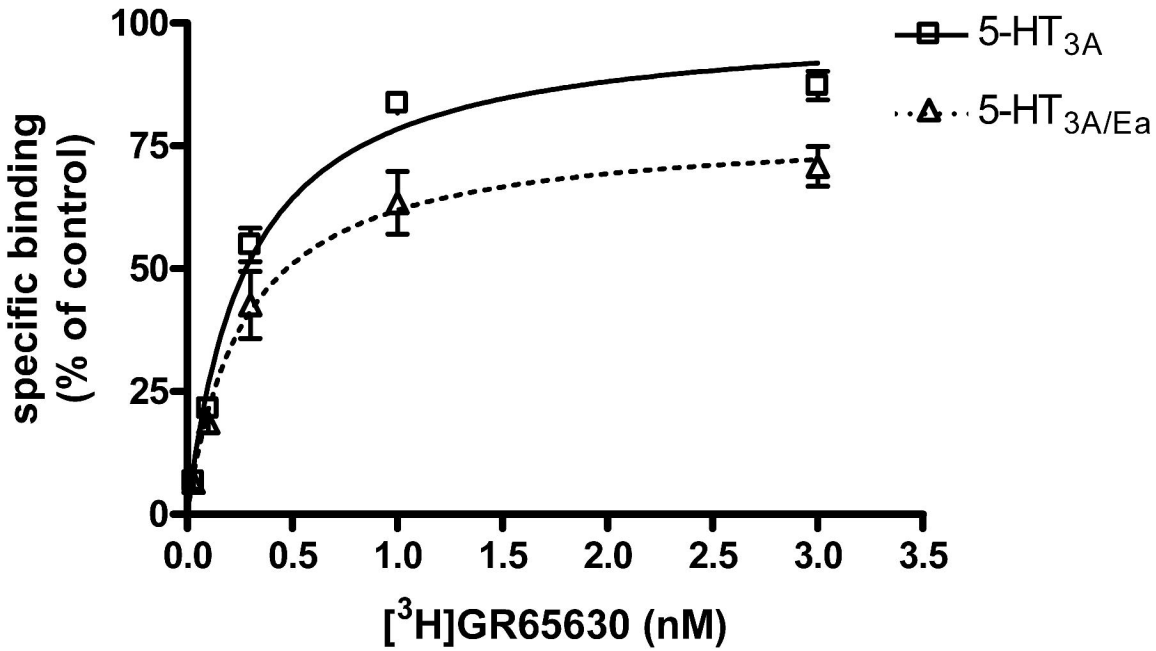
specific binding
(% of control)
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
[3H]GR65630 (nM)
5-HT3A
5-HT3A/Ea

Figure 4

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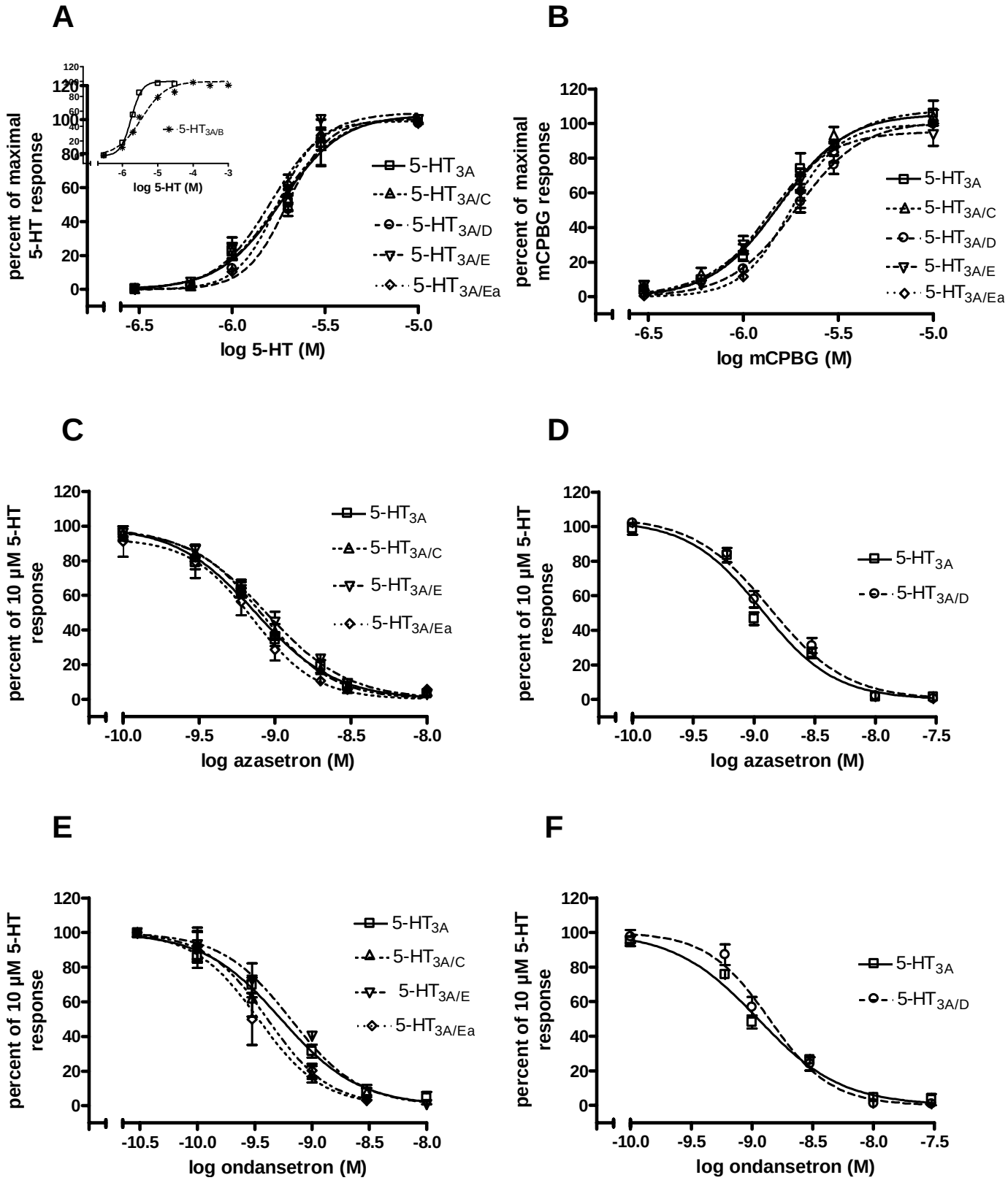


Figure 5

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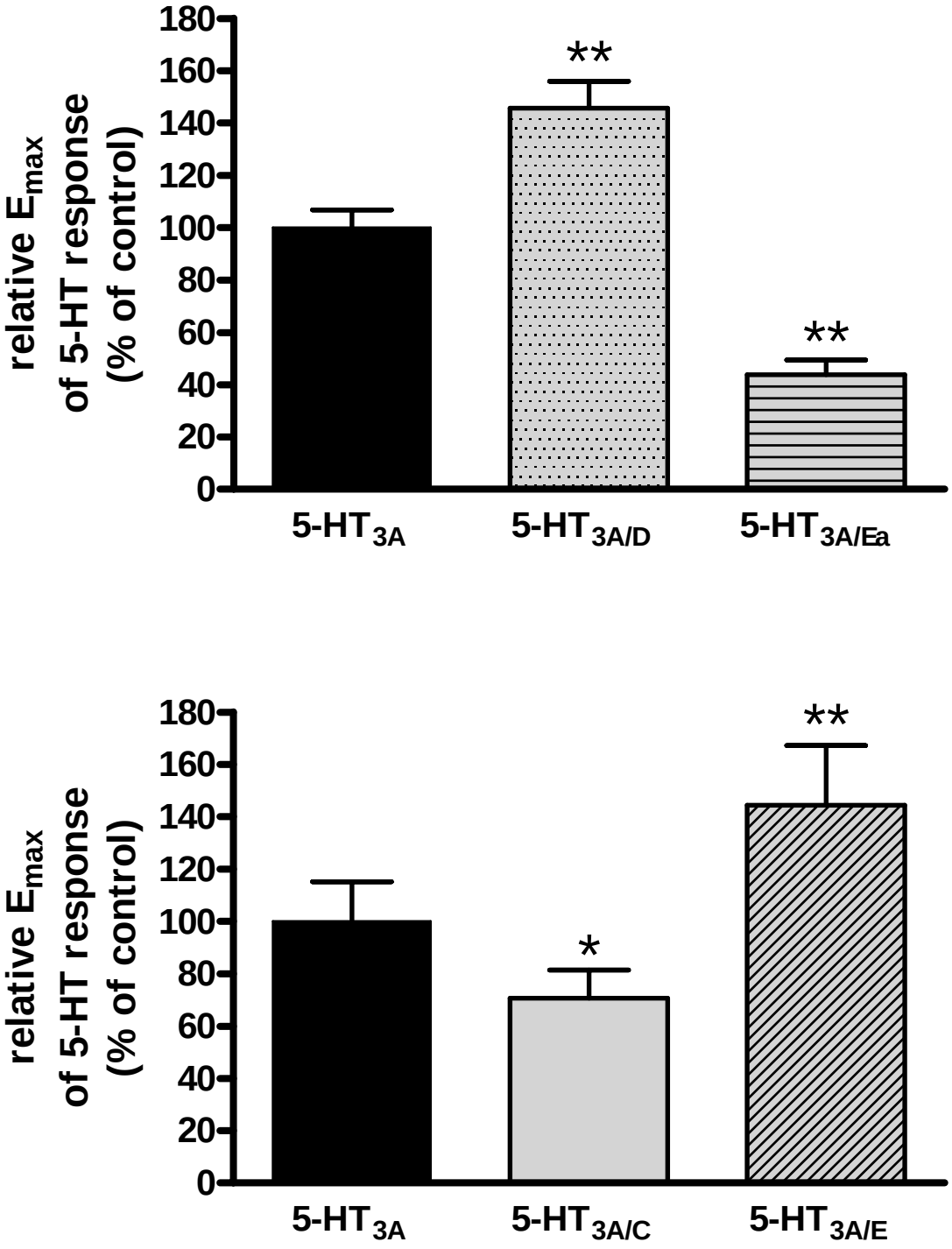


Figure 6

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