Supplemental Data

Materials and Methods

Expression analysis

Poly A+ RNAs (50 ng) from 18 different tissues (adult: brain, amygdala, caudate nucleus, hippocampus, thalamus, colon, intestine, kidney, liver, lung, heart, muscle, spleen, stomach; fetal: brain, colon, kidney, muscle) were reverse transcribed using the superscript system from Invitrogen as described by the manufacturer. PCR analysis was performed using different *HTR3E* gene splice variant specific primers (*HTR3E*: forward: ATG TTA GCT TTC ATT TTA TCA CGG GC; reverse: CTG CCT AGG TGT TCC AGA GGC AT; *HTR3Ea*: forward: ATG GAA GGA AGC TGG TTC CA; reverse: CTG CCT AGG TGT TCC AGA GGC AT; *GAPDH* forward: ACC ACA GTC CAT GCC ATC AC; reverse: TCC ACC ACC CTG TTG CTG TA). PCRs were performed in 50 µl volumes containing 10-100 ng template, 25 pmoles of each primer, 200 µM dNTPs (MBI Fermentas, St. Leon-Rot, Germany), 1.5 mM $MgCl_2$, 1x PCR buffer and 2 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Thermal cycling was carried out in a Thermocycler PTC-200 (MJ Research, Waltham, Mass, USA) under the following conditions: Initial denaturation at 94°C for 15 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min. Final extension was carried out at 72°C for 5 min. 5'RACE experiments were carried out as reported previously (Niesler et al., 2003) and led to the cloning of the full length *HTR3Ea* cDNA (accession number DQ644022).

Southern blot hybridization

To confirm the identity of RT-PCR products, Southern blot hybridizations were carried out using ^{32}P dCTP labeled cDNA probes containing exons 6-8 of *HTR3E* and exons 1a-4 of *HTR3Ea* (hybridization buffer: 5x SSPE, 10x Denhardt's, 2 % SDS, 20 ng/ml herring sperm DNA) at 65°C over night and washed twice in 2x SSC, 0.5 % SDS and once in 0.2x SSC, 0.1 % SDS at 65°C.

Filters were exposed to Kodak X-omat films (Kodak, Rochester, NY, USA) at room temperature for several minutes.

Metabolic labeling of cells and immunoprecipitation

Transfections of HEK293 cells were carried out as described in the material and methods section of the manuscript. Cells were metabolically labelled 24 h after transfection using Promix (^{35}S-methionin/^{35}S-cystein) from Amersham Pharmacia (Munich, Germany) and immunoprecipiation was performed as decribed previously (Connolly et al., 1996). Each cell lysate was split in half and immunoprecipitation was performed once by the anti-HA 3F10 (Roche, Mannheim, Germany) and once by the anti-Myc Tag 9E10 (Upstate: Biomol, Hamburg, Germany) antibody. Immunoprecipitated proteins were separated on 4-12 % Bis-Tris NuPAGE Gels (Invitrogen, Karlsruhe, Germany), gels vacuum dried and then exposed to a Kodak Biomax (Kodak, Rochester, NY, USA) film in a X-ray cassette over night at -80°C.

Figures

Figure 1 supplemental data

Multiple sequence alignment of the human 5-HT$_3$ receptor subunits 5-HT$_{3A, B, C, D, E}$ and $_{Ea}$. The signal peptide is marked in light grey and white letters, the cysteine loop is indicated in bold face and the four transmembrane regions are illustrated in grey. The epitope tags were introduced into the respective subunits between the black boxed amino acids (TT, PQ, EE or ER). The ER-retention signal of 5-HT$_{3B}$ (CRAR between transmembrane region 1 and 2 in bold face) is underlined (Boyd et al., 2003).

Figure 2 supplemental data

Analysis of the human *HTR3E* gene (A) Expression analysis by RT-PCR of the human *HTR3E* gene using cDNAs from 18 different human fetal and adult tissues. *HTR3E* and its alternatively spliced isoform *HTR3Ea* (Karnovsky et al., 2003), are exclusively expressed in the gastrointestinal tract (*HTR3E/Ea*:colon and intestine, *HTR3Ea*: stomach). Sizes of PCR products were 1.5 kb for *HTR3E* and 1.6 kb for *HTR3Ea*. *GAPDH* expression was analysed as a control for cDNA integrity. The identity of the RT-PCR products was confirmed by Southern Blot Hybridization of ^{32}P labeled cDNA probes at 65°C. (B) Genomic organisation of the *HTR3E*-gene on the top and its different splice variants *HTR3E* and *HTR3Ea* underneath. The alternative *HTR3Ea* transcript is composed of an additional first upstream exon (1a) fused to the 3'part of exon 1 of *HTR3E* (1b). Exons are indicated as boxes and numbered from 1a to 8. Grey: identical, black: partial identical, white: different between E and Ea. (C) Predicted signal sequences of the human 5-HT$_{3A}$, 5-HT$_{3B}$, 5-HT$_{3C}$, 5-HT$_{3D}$, 5-HT$_{3E}$ and 5-HT$_{3Ea}$ subunits at the N-termini of the subunits are grey shaded. The resulting 5-HT$_{3Ea}$ subunit differs at its N-terminal end due to a completely different signal sequence and 15 additional amino acids compared to 5-HT$_{3E.}$

Figure 3 supplemental data

Immunofluorescence analysis of transfected HEK293 cells expressing the novel Myc-/HA-tagged-$HT_{3C,D,E,Ea}$ subunits. Surface: not permeabilized; intracellular: permeabilized. Antibodies used were rat anti-HA (3F10) and mouse-Myc (9B11). Secondary antibody was anti-mouse-FITC. The novel 5-$HT_{3\ C,\ D,\ E}$ and $_{Ea}$ subunits are stained in green. All cells were DAPI counterstained to visualize nuclei in blue. Bar indicates 10 µm.

Figure 4 supplemental data

Immunoprecipitation of metabolically labelled HEK293 cells (A-D). 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. Each cell lysate was split in half and immunoprecipitation was performed once using the anti-HA tag and once the anti-Myc tag antibody. Immunoprecipitated proteins were separated on a 4-12 % Bis-Tris NuPAGE Gel (Invitrogen, Karlsruhe, Germany),gels vacuum dried and then exposed on Biomax film (Kodak, Rochester, NY, USA) in an X-ray cassette over night at -80°C. Antibodies used for immunoprecipitation are indicated underneath. No specific immunoreactive band was detectable with either antibody in mock transfected cells and with the anti-HA in case of a Myc-tagged epitope and vice versa. A faint non-specific band of ca. 40 kDa showed up in all transfections. Immunoreactive bands of HA-/Myc-5-HT_{3A} of ca. 55 kDa (A-D), ca. 55 kDa for HA-5-HT_{3C} (A), ca. 25 kDa in case of HA-HT_{3D} (B) and Myc-5-HT_{3Ea} (C) of ca. 60 kDa are marked by arrows. In double transfected HEK293 cells except for the 5-HT_{3E} subunit immunoreactive bands from both subunits in each immunoprecipitation experiment were present. This indicates that these subunits must have been co-precipitated (A-C). For the Myc-5- HT_{3E} subunit experiment no immunoreactive band was detectable (indicated by an arrow, (D)). In order to find out if there is a

specific detection problem in case of the 5-HT_{3E} subunit we carried out systematic immunoprecipitation experiments using 5-HT_{3E} specific antibodies recognizing different epitopes of the subunit. By using the anti-5-HT_{3DE} antibody, which recognizes an epitope within the large intracellular loop, we were able to identify protein bands of the expected size of roughly 60 kDa for Myc-5-HT_{3E} in the Western blot experiment (Figure 2C, manuscript). This confirms that the 5-HT_{3E} subunit is also part of a heteromeric 5-$HT_{3A/E}$ complex.

Figure 5 supplemental data

Immunofluorescence analysis of transfected HEK293 cells co-expressing Myc-/HA-tagged 5-$HT_{3A}W^{178}S$ mutant 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 6 supplemental data

Immunofluorescence analysis of transfected HEK293 cells co-expressing HA-tagged 5-HT_{3A} plus Myc-5-HT_{3B}. Surface: not permeabilized; intracellular: permeabilized. Antibodies used were rat anti-HA (3F10) and rabbit anti-Myc. Secondary antibodies were anti-mouse-CY3 and anti-rabbit-FITC. The 5-HT_{3A} subunit is stained in red, the 5-HT_{3B} subunit is visualized in green. All cells were DAPI counterstained to visualize nuclei in blue. Bar indicates 10 µm.

References

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