Available online at www.sciencedirect.com
SCIENCE DIRECT®
Gene 310 (2003) 101–111
GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES
www.elsevier.com/locate/gene

Cloning, physical mapping and expression analysis of the human 5-HT$_3$ serotonin receptor-like genes *HTR3C*, *HTR3D* and *HTR3E*☆

Beate Niesler, Bernd Frank, Johannes Kapeller, Gudrun A. Rappold*

Institute of Human Genetics, University of Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

Received 8 November 2002; received in revised form 29 January 2003; accepted 6 March 2003
Received by M. D'Urso

Abstract

For more than 50 years the serotonin system has been the subject of intense research. This has provided an exciting insight and led to the discovery of multiple drugs targeting serotonin receptors, metabolising enzymes and re-uptake sites. During the past few years researchers focussed especially on elucidating the complexity of different physiological actions in the serotonergic network. We have identified two novel human serotonin 5-hydroxytryptamine type 3 receptor-like genes, *HTR3D* and *HTR3E*, by performing homology searches using the public human sequence databases and subsequently cloned the full length cDNAs by 5′ and 3′ rapid amplification of complementary DNA ends. Mapping of *HTR3D* and *HTR3E* by hybridisation, polymerase chain reaction and fluorescence *in situ* hybridisation revealed that both genes together with *HTR3C* are clustered in a subinterval of less than 100 kb on chromosome 3q27. Comparative expression analysis of all *HTR3* genes, namely *HTR3A, B, C, D* and *E* showed *HTR3D* expression to be restricted to kidney, colon and liver and *HTR3E* expression to colon and intestine, whereas all other genes are widely expressed in many tissues including brain.

Keywords: Serotonin receptor genes; 5-Hydroxytryptamine; Ligand-gated ion channel; *HTR3C*; *HTR3D*; *HTR3E*; Chromosome 3q27

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) controls a variety of physiological functions in the central and peripheral nervous systems. The serotonergic system is organised in a highly complex manner, as serotonin action is mediated by a multitude of 5-HT receptor subtypes. These subtypes can be divided into seven main classes (5-HT$_1$R–5-HT$_7$R) based on their structural and functional features (Hoyer et al., 2002). This high degree of receptor variability clearly emphasises the physiological importance of serotonin and points to an extraordinary diversity of functions. The unravelling of the properties of the system leading to this complexity is one of the major goals of serotonin research.

Except for the 5-HT$_3$ receptor, which is a ligand-gated ion channel, all serotonin receptors represent G-protein coupled binding proteins. However, the 5-HT$_3$ receptor shares characteristic features with the other members of this ion channel family: a large extracellular domain containing a conserved cysteine loop, four hydrophobic transmembrane segments, a large intracellular loop between the third and fourth transmembrane region and an extracellular C-terminus (Reeves and Lummis, 2002). The ion channel itself is an oligomeric complex composed of five subunits. During the last few years two different human 5-HT$_3$ receptor subunit genes, *HTR3A* and *HTR3B*, have been isolated (Miyake et al., 1995; Davies et al., 1999).

Several studies pointed out the complexity within the 5-HT$_3$ receptor system based on indications of pharmacological and biophysical variations between tissues and

☆ After submission of our paper similar work has been presented at the Neuroscience Meeting; Gotow et al. (2002): a cluster of novel 5-HT$_3$ receptor-like genes on chromosome 3; Poster 38.1.

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT$_3$ receptor, 5-hydroxytryptamine receptor type 3; cDNA, complementary DNA; BAC, bacterial artificial chromosome; EST, expressed sequence tag; FISH, fluorescence *in situ* hybridisation; FITC, fluorescein isothiocyanate; HEK293, human embryonic kidney 293; *HTR3*, 5-hydroxytryptamine receptor type 3 gene; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

* Corresponding author. Tel.: +49-6221-565-059; fax: +49-6221-565-332.
E-mail address: gudrun_rappold@med.uni-heidelberg.de (G.A. Rappold).

0378-1119/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-1119(03)00503-1

species. Different ligand binding affinities and ion channel conductances of diverse tissues and cell lines have been reported and pharmacologic profiles of homomeric and heteromeric receptors showed differences in the properties of the respective receptors. Only 5-HT_{3A} subunits can form functional homo-oligomeric complexes after expression in *Xenopus* oocytes or HEK293 cells, whereas the 5-HT_{3B} subunits are not able to build a functional receptor on their own (Miyake et al., 1995; Davies et al., 1999; Dubin et al., 1999). Recombinant homo-oligomeric 5-HT_{3A} receptors show extremely reduced channel conductances compared to native receptors, whereas heteromeric complexes composed of both 5-HT_{3A} and 5-HT_{3B} subunits resemble closely the properties of the native receptors (Davies et al., 1999). Variable receptor compositions have also been shown by Western blot experiments which led to the hypothesis that further 5-HT_3 receptor subunits actually should exist (McKernan, 1992; Hussy et al., 1994; Jackson and Yakel, 1995; Fletcher and Barnes, 1998). To verify this hypothesis we have established a 5-HT_3 receptor consensus sequence to screen databases and identified a human BAC clone (RP11-315J22) harbouring additional subunit sequences. We established primers and cloned the entire coding region of two novel *HTR3*-homologous genes by 5′ and 3′ RACE: *HTR3D* and *HTR3E*. Mapping data of *HTR3D* and *HTR3E* and of the recently cloned *HTR3C* gene (accession number AF459285) revealed that they all reside in close vicinity on chromosome 3q27. To get a better insight into the putative role of *HTR3D* and *HTR3E*, we carried out expression analysis and compared the expression profiles to those of *HTR3A*, *B* and *C*.

2. Material and methods

2.1. Establishment of the 5-HT_3 receptor consensus sequence

Multiple sequence alignment with the protein sequences of the human 5-HT_{3A} and 5-HT_{3B} subunits, as well as the 5-HT_{3A} sequences of rat, mouse and guinea pig (Uetz et al., 1994; Isenberg et al., 1993; Miyake et al., 1995; Lankiewicz et al., 1998; Davies et al., 1999) was carried out by the MALIGN algorithm of the Biocomputing home page (http://genius.embnet.dkfz-heidelberg.de/). Using this alignment we created a 5-HT_3 consensus sequence by the Profilemake algorithm. This 5-HT_3 consensus sequence (Fig. 1A) was subsequently used for searching the human genome database (http://www.ncbi.nlm.nih.gov/BLAST/), the Celera database (http://publication.celera.com/cds/login.cfm) as well as EST databases by the tblastn algorithm (http://www.ncbi.nlm.nih.gov/BLAST/: protein query – Translated db [tblastn]).

2.2. PCR analysis

PCRs were performed in 50 μl volumes containing 10–100 ng template, 25 pmole of each primer, 200 μM dNTPs (MBI Fermentas), 1.5 mM $MgCl_2$, 1 × PCR buffer and 2 U HotStarTaq DNA Polymerase (Qiagen). Thermal cycling was carried out in a Thermocycler PTC-200 (MJ Research) under the following conditions: initial denaturation at 94°C for 15 min followed by 35–40 cycles of 94°C for 30 s, annealing temperature (T_A) for 30 s and 72°C for 2 min. Final extension was carried out at 72°C for 5 min. Primer sequences and annealing temperatures are given in Table 1.

2.3. Rapid amplification of cDNA ends (5′ and 3′ RACE)

To clone the 5′ and 3′ ends of *HTR3D* and *HTR3E*, 5′ RACE was performed using 'Marathon cDNA libraries' constructed as described by the manufacturer (BD Bioscience Clontech). The following oligonucleotide primers (Table 1) were used: 5′RACE: HTR3D2rev/HTR3E1rev; 3′RACE: HTR3D4for/HTR3D5for and the adaptor primer AP1. PCR was carried out using the following parameters: 94°C for 15 min, followed by 25 cycles of 94°C for 30 s, 55/60°C for 30 s, 72° for 2 min, final extension at 72°C for 5 min. A second round of PCR was performed using 1/50 of first PCR product and the following nested oligonucleotide primers: 5′RACE: HTR3D2ev2/HTR3E1rev2; 3′RACE: HTR3D4for2/HTR3D5for2 and the adaptor primer AP2. PCR was carried out at an annealing temperature of 55°C for 35 cycles.

2.4. Sequencing

2.4.1. Cloning and sequencing of PCR products

PCR products from cDNAs showing positive hybridisation signals were cloned into the pCRII Topo vector (pTOPO kit, Invitrogen). Overnight cultures of single colonies were lysed in 100 μl H_2O by boiling for 10 min. The lysates were used as templates for PCRs with the respective vector primers. Both strands were sequenced with the *Cy5*-labelled vector primers *M13 universal* and *M13 reverse* according to manufacturer's protocols (Cycle Reader Kit, MBI Fermentas) on an ALF*Express* automated sequencer (Pharmacia) or using unlabelled receptor sequence specific primers (Table 1, sequencing primers indicated with the extension 'S' in the primer name) on a MEGABACE Sequencer (Pharmacia).

2.4.2. Sequencing of BAC clones

In order to identify missing exon-intron junctions which were not available in the public databases, we carried out partial sequencing of the BAC clones RP11-810O14 and CTD-2545A22 using *Cy5*-labelled primers (Table 1;

A

```
  1  MMLWIPQVLL ALFLPTLLAQ GEGRRRRATQ AHNTTQPALL RLSBHLLANY
 51  RKGVRPVRBW RKPTTVSIBV IVYAILNVBE KNQVLTTYIW YRQYWTBEFL
101  QWNPEBFBNI TKLSIPTBSI WVPBILINEF VBVGKSPNIP YVYVHHQGEV
151  QNYKPLQVVT ACSLBIYNFP FBVQNCSLTF TSWLHTIQBI NISLWRSPEE
201  VKSBKSVFMN QGEWELLGVF PQFKEFSBIE SSNSYAEMKF YVVIRRRPLF
251  YAVSLLLPSI FLMVVBIVGF YLPPBSGERV SFKITLLLGY SVFLIIVSBT
301  LPATAIGTPL IGVYFVVCMA LLVISLAETI FIVRLVHKQB LQQPVPBWLR
351  HLVLBRIAWL LCLGEQPTAH RPPATFQANK TBBCSGSBLL PAMGNHCSHV
401  GGPQBLEKTP RGRGSPPPPP REASLAVCGL LQELSSIRHF LEKRBEMREV
451  ARBWLRVGSV LBRLLFRIYL LAVLAYSITL VTLWSIWHYA
```

B

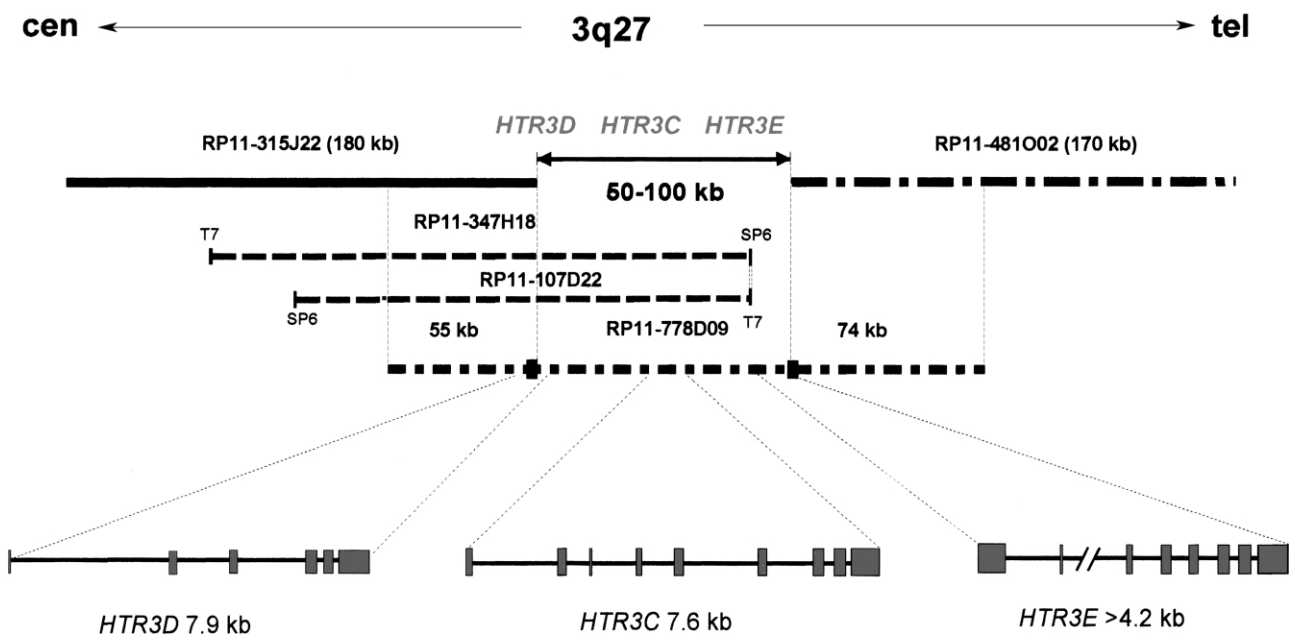


Fig. 1. (A) A 5-HT_3 receptor consensus sequence was created based on the amino acid sequence of the human 5-HT_{3A} and 5-HT_{3B} subunits, as well as the 5-HT_{3A} protein sequences of rat, mouse and guinea pig. (B) Genomic organisation and localisation of the *HTR3* genes *HTR3C, HTR3D* and *HTR3E* on chromosome 3q27. BAC clones RP11-347H18, RP11-107D22, RP11-778D09 (AC131235) and RP11-481O02 (AC048331) are not completely sequenced, which is indicated by dotted lines. RP11-347H18, RP11-107D22 are in the database as genomic survey sequences (GSS: AQ542473, AQ542471; AQ315444; AQ315441). RP11-315J22 (AC068644) is completely sequenced. The size of intron 2 in *HTR3E* is unknown.

Table 1
HTRC, *HTR3D* and *HTR3E* specific primers

HTR3C	*HTR3D*	*HTR3E*	Primer name	Sequence (5′ → 3′)	Primer name	Sequence (5′ → 3′)	T_A (°C)
e1	–	–	HTR3C5′UTRfor	TCC CCA GAG AAG AGT CCA GA	HTR3CEx1rev2	CCC TCC AAG TGC TAG AGG TG	64
CDS	–	–	HTR3C5′UTRfor	TCC CCA GAG AAG AGT CCA GA	HTR3C3′UTRrev	GAC CAG CAG AAA CTC CAA GC	55
–	CDS	–	HTR3DEx1RACE	GGC TAG ATT CAG GCC CAG TTA AAG	HTR3D5rev2	CTG CCT AGG TGT TCC AGA GG	55
–	hcDNA	–	HTR3D1for	CTG CAG CCT TTC AAG CAG T	D5rev	CTG CCT AGG TGT TCC AGA GGC AT	55
–	5′UTR	–	HTR3D2rev	GGG AAA TAG AAG GTG TCC AGT TTG	AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC	55
–	5′UTR	–	HTR3D2rev2	GTG TCC AGT TTG CAG ATG CTG AC	AP2	ACT CAC TAT AGG GCT CGA GCG GC	55
–	3′UTR	–	HTR3D5for	AGC TGT GGG TGC AGT TCA G	AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC	60
–	3′UTR	–	HTR3D5for2	CTC CAT CAT CAC CGT CAT ATG	AP2	ACT CAC TAT AGG GCT CGA GCG GC	60
–	he1	–	HTR3D1for	CTG CAG CCT TTC AAG CAG T	HTR3D1rev	ATG GCA GAC AAG GTG AAG GA	60
–	e3	–	HTR3D2for	ATA AGC CAA TGT GGG TGG TC	HTR3D2rev	GGG AAA TAG AAG GTG TCC AGT TTG	60
–	e4	–	HTR3D3for	TGG TAA ACT TTC TGG TGC CC	HTR3D3rev	TGG GAG CAA GTC ATT CAT CA	60
–	e5	–	HTR3D4for	GTG TCT ACT TCG CCC TGT GC	HTR3D4rev	GGC CCT TAT TTC CCT TCT GG	60
–	e6	–	HTR3D5for	AGC TGT GGG TGC AGT TCA G	HTR3D5rev	CTG CCT AGG TGT TCC AGA GGC AT	60
–	–	3′UTR	HTR3D4for	GTG TCT ACT TCG CCC TGT GC	AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC	55
–	–	3′UTR	HTR3D4for2	TGC CTG TCC CTG ATG GTG GG	AP2	ACT CAC TAT AGG GCT CGA GCG GC	55
–	–	CDS	5′HTR3Dfor2	ATG TTA GCT TTC ATT TTA TCA CGG GC	HTR3D5rev	CTG CCT AGG TGT TCC AGA GGC AT	55
–	–	3′UTR	3′UTR_HTR3Efor	GCT TCT CTT GCC TCC AGG G	3′UTR_HTR3Erev(S)	AAG AGG TAT AGT CTG CTA TGC	60
–	–	i1	HTR3E1inFOR(S)	TAC CTG ACA CAC AGC CAG TGC	HTR3E1inREV(S)	TAA GGA CAC CAA GGA GGG CTA	64
–	–	5′UTR	HTR3EE1rev	GCG AAG GAG ATG TTG ACT TGG G	AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC	55
–	–	5′UTR	HTR3EE1rev2	GTT GGT GAC CGG ACG GAA GG	AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC	55
–	–	e3	HTR3EE2revS	CAA TGA TGA AAA TGT CTG GG	HTR3EE2for2S	GGA ACC CAG AGG AAT GTG AG	56
–	–	i2	HTR3EE2aINrev1(S)	TGA GAG AAT AAA GTG TTG TCC A	–	–	60
–	–	i2	HTR3EE2aINrev2(S)	TGT TTC AAA CCA ATG TTA GGG A	–	–	60
–	–	e5	HTR3EE3inFOR	GGA TGG AAA AAG AGT GCA GT	HTR3EE3inREV	CTT GGG AGA TAC ATA TTT GAT G	60
–	–	e6	HTR3E5inFOR(S)	GGT TCC TCT GAC CCC ATA ACT	HTR3E5inREV(S)	CCC ACC CTT CTC TTC CAA AA	62
–	–	e7	HTR3EE6inFOR	CCT CTG GCC CTC ACT AGG C	HTR3D4rev	GGC CCT TAT TTC CCT TCT GG	62

Sequencing primers are indicated by an (S) and were labelled by Cy5. As *HTR3C*, *D* and *E* are highly homologous within their 3' part, *HTR3D* specific primers could be used for RACE, RT-PCR and PCR experiments amplifying *HTR3D* and *HTR3E*, respectively. CDS: coding sequence, e: exon, i: intron, hcDNA: hypothetical predicted *HTR3D* cDNA, UTR: untranslated region, T_A: annealing temperature.

sequencing primers indicated with the extension 'S' in the primer name) according to manufacturer's protocols (Cycle Reader Kit, MBI Fermentas) on an ALF*Express* automated sequencer (Pharmacia).

2.5. Expression analysis

Fifty ng Poly(A)$^+$ RNAs 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 Gibco BRL as described by the manufacturer. PCR analysis was performed using different gene specific primers (Table 1) as described above.

2.6. Genomic clones and physical mapping

BACs were derived from the Resource Center, Oakland, USA (RPCI-11, Roswell Park Cancer Institute Human BAC Library) or from Research Genetics, Invitrogen Corporation, Breda, The Netherlands (CTD). Bacteria bearing the respective BAC clones were grown overnight at 37°C in LB medium (25 μg/ml chloramphenicol). BAC DNA was isolated using the Nucleobond Maxi Preparation Kit from Macherey and Nagel as recommended by the manufacturer. Clones were digested with 200 U *Eco* RI in the appropriate buffer in a 100 μl volume. Digested BACs were run on a 0.8% agarose gel. Overlapping clones were confirmed by PCR and hybridisation analysis.

2.7. Southern blot hybridisation

Southern blot hybridisations were carried out using respective probes containing the conserved regions of *HTR3C, D* and *E*: an exon 1 probe of *HTR3C*, 3′UTRs of *HTR3D* and *E* in hybridisation buffer (5 × SSPE, 10 × Denhardt's, 2% SDS, 20 ng/ml herring sperm DNA) at 65°C overnight and washed twice in 2 × SSC, 0.5% SDS and once in 0.2 × SSC, 0.1% SDS at 65°C. Filters were exposed to Kodak X-omat films at −80°C or room temperature for several minutes/hours.

2.8. Fluorescence in situ Hybridisation (FISH) analysis

Biotinylated BAC DNAs of the clones RP11-315J22, RP11-778D09, RP11-810O14, RP11-107D22, RP11-347H18 and CTD-2545A22 were hybridised to metaphase chromosomes from control individuals under conditions as described before (Lichter and Cremer, 1992). To confirm the localisation of the BAC clones on chromosome 3, a control BAC probe (CIT-B 159N23) partially harbouring the MEGAP gene residing on chromosome 3p25 was used (Endris et al., 2002). The hybridised probes were detected via avidin-conjugated FITC and anti-digoxygenin-conjugated Cy3.

2.9. Bioinformatics

Multiple sequence alignments were performed using MALIGN and CLUSTAL from the HUSAR program package on the Biocomputing home page (http://genius.embnet.dkfz-heidelberg.de/). Sequence comparisons and homology searches were carried out using GAP and BESTFIT from the HUSAR program package and different BLAST algorithms at the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic trees were created using the evolutionary analysis software at the Biocomputing home page. Sequence analysis of genomic sequences was carried out with the NIX analysis software (http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/).

Pattern and profile searches as well as hydropathy analysis for the transmembrane topology were done by Prosite and TMHMM at the Biocomputing home page. Signal peptide cleavage sites and theoretical peptide mass were predicted by the Peptide characterisation software on the Expasy server (http://www.expasy.ch) and by using the Sigfind Software (Signal Peptide Prediction Server: http://www.stepc.gr/~synaptic/sigfind.html).

3. Results

3.1. Identification of novel 5-HT$_3$-like receptor subunit genes

To investigate the existence of further 5-HT$_3$ receptor encoding genes in the human genome, we established a consensus sequence (Fig. 1A) taking advantage of amino acid sequences of the human 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits, as well as the 5-HT$_{3A}$ protein sequences of rat, mouse and guinea pig to search the public databases for homologous sequences (Uetz et al., 1994; Isenberg et al., 1993; Miyake et al., 1995; Lankiewicz et al., 1998; Davies et al., 1999). Using this consensus sequence, we were able to identify the human BAC clone RP11-315J22 located on chromosome 3q27 that enclosed short intervals encoding homologous protein stretches between 35 and 99 amino acids in length and similarities of 45–72%. Further sequence analysis of the RP11-315J22 genomic sequence by FGENES, Genescan and Genefinder with the NIX analysis software (http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/) predicted several exons of an *HTR3* homologous gene of excellent quality.

3.2. Cloning of full length cDNAs

Based on the genomic sequence we designed primers residing in the most upstream and downstream putative exons (HTR3D1for/HTR3D5rev). By RT-PCR we were able to amplify a 1.8 kb product from kidney. Using 5′ and 3′ RACE succeeded in cloning the entire coding region of not only one but two novel cDNAs. We named the new homologous sequences *HTR3D* and *HTR3E* (AY159812;

AY159813), since a gene termed *HTR3C* has recently become available in the database (accession number AF459285). The cDNA of *HTR3D* spans 1499 base pairs; the coding region of 840 bp (position 227–1066) encodes a predicted protein of 279 amino acids. The complete cDNA of *HTR3E* is 1925 base pairs in length; the coding region spans 1416 bp (position 195–1610) and encodes a predicted protein of 471 amino acids. In comparison, the complete cDNA of *HTR3C* is 1745 base pairs in length; its coding region spans 1344 bp (position 35–1378 of AF459285) and encodes a predicted protein of 447 amino acids.

To investigate the evolutionary relationship between *HTR3C, D* and *E*, we carried out sequence comparison on nucleotide and amino acid level. Comparison on the nucleotide level revealed overall homologies of 82.8–90.1% between the three cDNAs. Comparison of *HTR3C, D* and *E* to the previously known genes of the *HTR3* class, *HTR3A* and *HTR3B,* showed that similarity is much lower (Table 3). On protein level the 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits show identities between 64.8 and 74.3%, while their identity compared to 5-HT_{3A} and 5-HT_{3B} ranges from 26.8 to 39.8% (Table 3). It is very unlikely that the novel genes represent members of the nicotinic acetylcholine receptor (nAChR) family. The nAChR subunits most closely related are alpha 9 and alpha 10 revealing overall identities of only 27%. We have established dendrograms based on multiple sequence alignments using all known *HTR3* cDNAs of human and other species. These data strongly suggest that *HTR3C*, *D* and *E* share a common evolutionary history, whereas *HTR3A* and *HTR3B* are evolutionary more distant.

3.3. Analysis of the encoded protein sequences

Analysis of the 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits revealed a signal peptide sequence for 5-HT_{3C} and 5-HT_{3E} encompassing 27 and 16 amino acids. Both subunits contain a cysteine loop at the extracellular N-terminal part of the protein. In contrast, 5-HT_{3D} shows no signal peptide sequence and a very short extracellular N-terminus lacking the cysteine loop. All three subunits contain four transmembrane regions. The C-terminus of the protein is predicted to reside extracellular in the case of 5-HT_{3C} and 5-HT_{3E} and it would be located intracellular for the 5-HT_{3D} subunit. The analysis of the protein sequences using the Prosite program predicted several putative Asn-glycosylation, PKC, CKII, cAMP and tyrosine kinase phosphorylation sites (Fig. 4). The theoretical mass of the encoded subunits was calculated using the Peptide Mass Peptide Characterisation Software on the Expasy server as follows: 5-HT_{3C}: 48 kDa, 5-HT_{3D}: 30 kDa and 5-HT_{3E}: 52 kDa.

3.4. Physical mapping of HTR3C, HTR3D and HTR3E

In addition to searching the HUMANace-Human Genomic Physical Map Tracking Database (http://genome.wustl.

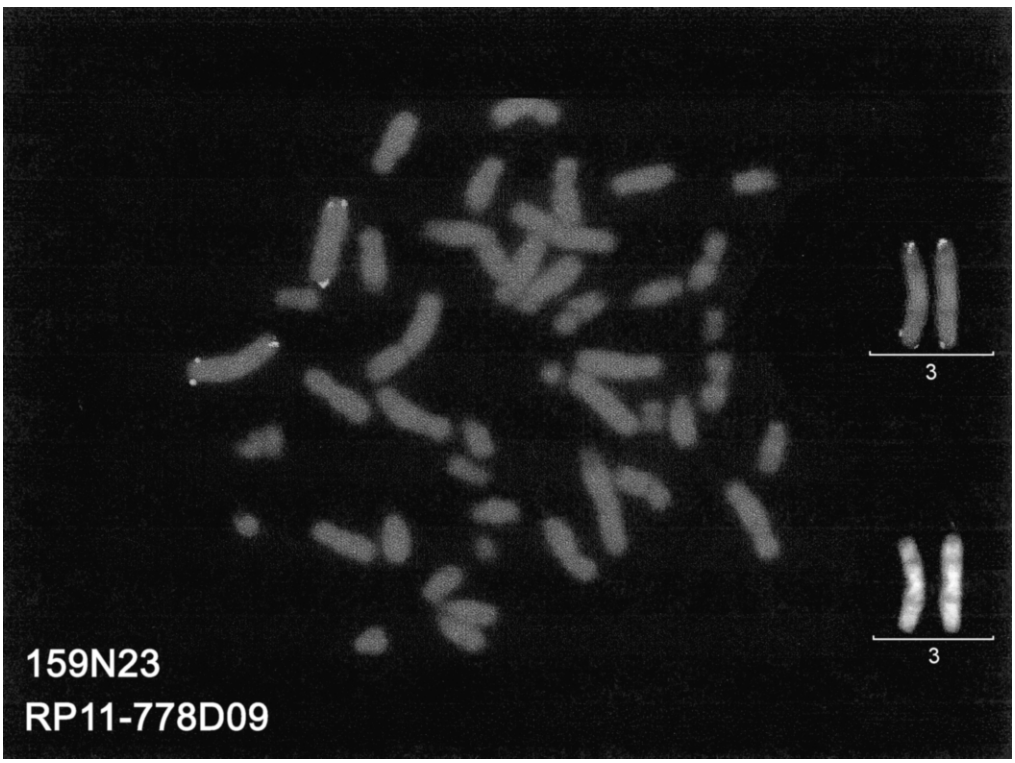


Fig. 2. Fluorescence *in situ* hybridisation on metaphase chromosomes of a human male using the BAC clone RP11-778D09 harbouring the *HTR3* genes *HTR3C, HTR3D* and *HTR3E*. The clone displayed signals on the long arm of chromosome 3 band 3q27. The localisation on chromosome 3 was verified by co-hybridisation with the BAC probe CIT-B 159N23, that partially encompasses the MEGAP gene on 3p25 (Endris et al., 2002).

edu/cgi-bin/ace), BLASTN analysis using sequence data of RP11-315J22 and the *HTR3D/E* cDNA sequences led to the identification of distally overlapping BAC clones (RP11-778D09, RP11-810O14, RP11-107D22, RP11-347H18, CTD-2545A22) which had all been pre-assigned to chromosome 3q27 (four of them are indicated in Fig. 1). FISH analysis of all six clones confirmed their chromosomal localisation to 3q27. Fig. 2 depicts the localisation of clone 778D09. Physical mapping of *HTR3C*, *HTR3D* and HTR3*E* by hybridisation and PCR analysis showed that all three genes map within an interval of less than 100 kb in the following order proximal to distal: *HTR3D*, *HTR3C*, *HTR3E* (Fig. 1).

3.5. Genomic organisation of HTR3C, D and E

Exon-intron organisation of all three *HTR3* genes was determined using sequence data available in the human genome and the Celera databases and the SIM4 algorithm (http://pbil.univ-lyon1.fr/sim4.html) (Table 2). In cases where exon-intron boundary sequences were missing in the database, we carried out partial genomic sequencing using the respective BAC clones. All of the determined exon-intron junctions are consistent with the consensus splice site sequences (Breathnach and Chambon, 1981).

A comparison of the cDNAs with the genomic sequence reveals that *HTR3C* has nine exons (45–522 bp), whereas *HTR3D* is composed of only six exons (62–557 bp). *HTR3E* contains eight exons (45–527 bp) (Table 2, Fig. 1). In all three cases the last three exons of the respective genes are separated by only very small introns of less than 200 bp (Table 2). Exon sizes as well as exon-intron junctions of *HTR3C* and *E* are almost identical. Both also contain a similar number of exons (9 and 8). In contrast, *HTR3D* shows a completely different genomic organisation with only six exons. All three genes are small, between at least 4 kb for *HTR3E* and about 8 kb in the cases of *HTR3C* and *HTR3D* (see Fig. 1).

3.6. Expression analysis

We performed comparative expression analysis by RT-PCR amplifying the complete coding regions of all *HTR3* genes using a variety of cDNAs prepared from human adult and fetal tissues (Fig. 3). Hybridisation probes specific for each *HTR3* gene were used to confirm the PCR products. Expression of *HTR3D* and *HTR3E* was shown to be confined to only three and two tissues, respectively (*HTR3D*: adult: kidney, colon, liver; fetal: colon and kidney; *HTR3E*: adult: colon, intestine). *HTR3C* was expressed in many more tissues (adult: brain, colon, intestine, lung, muscle, stomach; fetal: colon and kidney). Therefore, the expression

Table 2
Exon-intron boundaries of *HTR3C*, *HTR3D* and *HTR3E*

Exon	Intron	Exon	Intron	Exon size (bp)	Intron	Intron size (bp)
HTR3C						
1	…gggtttgggagctcc	TGGTGAATCC…CTGCTTCAAG	**gt**aagatgggacgagaacag…	101	1	1575
2	…ggagtctctgctctctat**ag**	GAAGAGGCGA…CCTGGGAGTG	**gt**gagacttagtccctgcat…	167	2	424
3	…agtggaaatttccttgtc**ag**	GATGCACAGC…GATGGATTTG	**gt**aaggcagattcaactatct	45	3	820
4	…ccctcactgccctgatgc**ag**	GTATGGGACA…TCGTGGAATC	**gt**gcgtatgcaggctgggga…	110	4	588
5	…tccctcccttccccaaac**ag**	CATGGATGTG…CTCTACACAG	**gt**aagtgtgacacattttgg…	170	5	1382
6	…ctgaccggcctcccttcc**ag**	TGGACAGCAT…CATGTTTTAT	**gt**gagtccaggggcccctgt…	161	6	848
7	…cttctccggtctctctcc**ag**	GTGGCCATCA…CCCCTCATCA	**gt**atggctcctcccactttc…	205	7	187
8	…ataatttgctctgccctc**ag**	GTGTCTACTT…CACCTGCCTG	**gt**gagggaagccagcactgt…	216	8	106
9	…agcctctgtcctctccac**ag**	GCCCAAAGGA…ACTTTCCAGG	aagcactggctcttc…	522		
HTR3D						
1	…cacatatttttgcttcctct	TCTCCAAACT…AAAGCACAGG	**gt**gagttattcctctgtgac…	28	1	3485
2	…tgatctacatcatttttc**ag**	TGGCTTAGAT…GCTAAATATG	**gt**atgacagactcagtttcc…	167	2	1146
3	…cctgtccttctcccacac**ag**	CATCAGTGTG…ACCTACACAG	**gt**aagtggggctcactaaag…	172	3	1480
4	…ctccttctcctccccacc**ag**	GTGGCCATCA…CAAAAGCGAG	**gt**gtgtgttggatggggaga…	253	4	135
5	…gcagaccccttgcctgc**ag**	GTGTCTACTT…CACCTGCCCG	**gt**gagggaagtcatacttcc…	216	5	121
6	…gagtctctgtctttctgt**ag**	GTGTGAAGGA…GATTTTCTCTT	acgtgtgtgtttttttaagt…	635		
HTR3E						
1	gcagacaaacctgggttca	GAACAAGTCC…CCTAGATGTG	**gt**gagtgctgacctctctag…	473	1	833
2	…ataggaaattcttttggc**ag**	AATGAACAGC…GCTGGAAATG	**gt**atggacaacactttattc…	45	2	?
3	…ctcctccacctgggctct**ag**	GTTTGGGATA…TCATTGAACT	**gt**gcgtatcaagggctggtc…	110	3	495
4	…ttgcgttatctctcctcc**ag**	CATGGATGTG…CTCTACACAG	**gt**aagttgcagtgaggtctc…	170	4	310
5	…cagatggtttctcatttc**ag**	TGGACAGCAT…CGTGTTCTAT	**gt**gagcttggaggctcttac…	161	5	337
6	…accctctcctaccccacc**ag**	GTGGCCATCA…CCCCTCATCG	**gt**atggctcctcccacctttt…	205	6	158
7	…taggcccccttcccctcc**ag**	GTGTCTACTT…CACCTGCCCG	**gt**gagggaagtcacattcct…	216	7	120
8	…gagtctctgtctttctgt**ag**	GTGTGAAGGA…AATTCTGCAG	agatttctggctctttgtca…	527		

Exon sequences are shown in capital letters, donor and acceptor splice sites are indicated in bold type. The size of intron 2 in *HTR3E* is unknown.

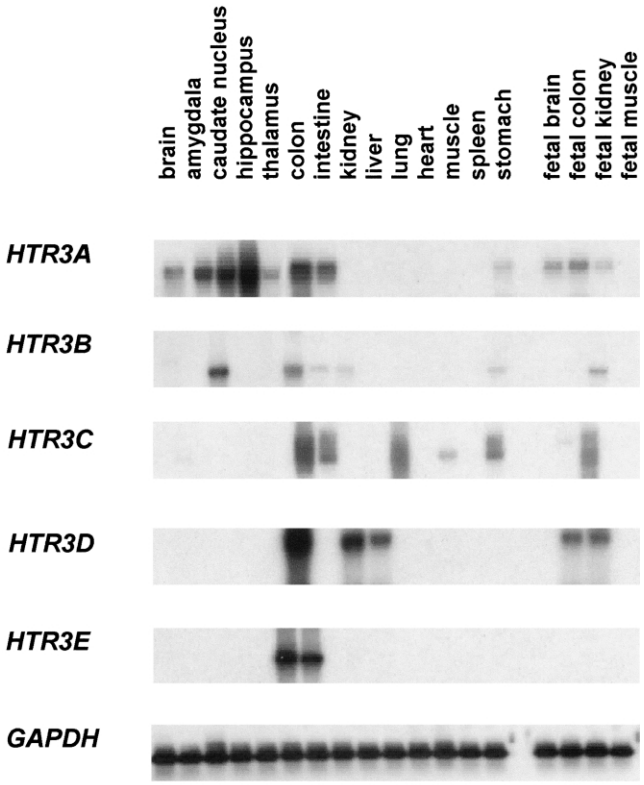


Fig. 3. Expression analysis. RT-PCR analysis of the novel genes *HTR3D* and *HTR3E* compared to *HTR3A*, *HTR3B* and *HTR3C* using cDNAs from 18 different human fetal and adult tissues. Sizes of PCR products were 1.5 kb for *HTR3A*, 1.4 kb for *HTR3B*, *HTR3C* and *HTR3E* and 1.1 kb for *HTR3D*. *GAPDH* expression was analysed as a control for cDNA integrity.

pattern of *HTR3C* resembles more closely the expression profiles of *HTR3A* and *HTR3B* (Miyake et al., 1995; Davies et al., 1999).

4. Discussion

Neurons within the nervous system are organised in different neural networks through synaptic connections. Two fundamental components interact dynamically in these functional units: the neurons themselves and their synapses. Identifying cellular and synaptic properties is necessary to elucidate the links between neural network behaviour and physiological function and represents a useful step towards a better understanding of neurological diseases. Especially the serotonergic system has been in the centre of interest as it is implicated in a multitude of signalling processes and in the etiology of several diseases. One of the main goals of serotonin research is to enlighten the complexity of the system, in particular of receptor diversity and its underlying mechanisms. Complexity can be achieved on different levels: on the genomic level through different subtypes, on the expression level through different splice variants and on the protein level through post-translational modifications. Additionally, oligomerisation increases the possible composition of interacting molecules in the network as shown in different ligand-gated ion channels such as acetylcholine, GABA and 5-HT_3 receptors (Riordan, 1992; Colquhoun and Patrick, 1997; Morales et al., 2001; Pootanakit and Brunken, 2001; Morales and Wang, 2002).

We have investigated whether further 5-HT_3 receptor genes exist in the human genome. Two novel *HTR3* homologous genes, which we termed *HTR3D* and *HTR3E* (since *HTR3C* has been recently accessible through the database) were isolated. Analysis of the putatively encoded 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits show that all three subunits share key features with other 5-HT_3 subunits (Fig. 4) (Reeves and Lummis, 2002). All of them contain four transmembrane regions with a huge intracellular loop known to affect channel function (Niemeyer and Lummis, 1998). The transmembrane region 2 lines the ion pore which controls ion channel conductance. The ion channel is surrounded by three rings of negatively charged residues and a central ring of small polar residues. They are crucial determinants of ion currents. Unexpectedly, the TM2 region of the 5-HT_3 C, D as well as E subunits lack a polar residue within the central ring. Furthermore, the 5-HT_{3E} subunit bears a positively charged lysine residue in the anionic cytoplasmic ring. A comparable situation has also been reported in case of the 5-HT_{3B} subunit (Davies et al., 1999). However, the functional consequences have yet to be determined.

Several *N*-glycosylation and phosphorylation sites are predicted (Fig. 4). *N*-glycolsylation sites have also been reported in the extracellular domain of the known 5-$HT_{3A/3B}$ subunits (Uetz et al., 1994; Isenberg et al., 1993; Miyake et al., 1995; Lankiewicz et al., 1998; Davies et al., 1999) which are involved in receptor assembly (McKernan, 1992; Quirk and Siegel, 2000). Phosphorylation was reported to influence receptor conductance levels and desensitisation rates (van Hooft and Vijverberg, 1995; Hubbard et al., 2000). 5-HT_{3C} and 5-HT_{3E} present a large N-terminal extracellular portion with a cysteine loop with ligand binding capacity. In contrast, the architecture of the putative 5-HT_{3D} subunit is different. It lacks the signal leader sequence and the large N-terminal loop which includes the ligand binding site. This raises the question of 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. Gene identification programs genes and genescan predicted also a virtual *HTR3D* cDNA, which would encode a variant 5-HT_{3D} protein. Since we were not able to verify this hypothetical transcript in more than 20 analysed tissues the expression of the respective transcript remains questionable.

It has been proposed that evolutionary processes influence the dynamics of gene duplication and may in the end lead to genes with newly acquired functions. *HTR3C*, *HTR3D* and *HTR3E* map closely to each other in a region of less than 100 kb suggesting that they have arisen by gene duplication. This is reminiscent of *HTR3A* and *B* which also

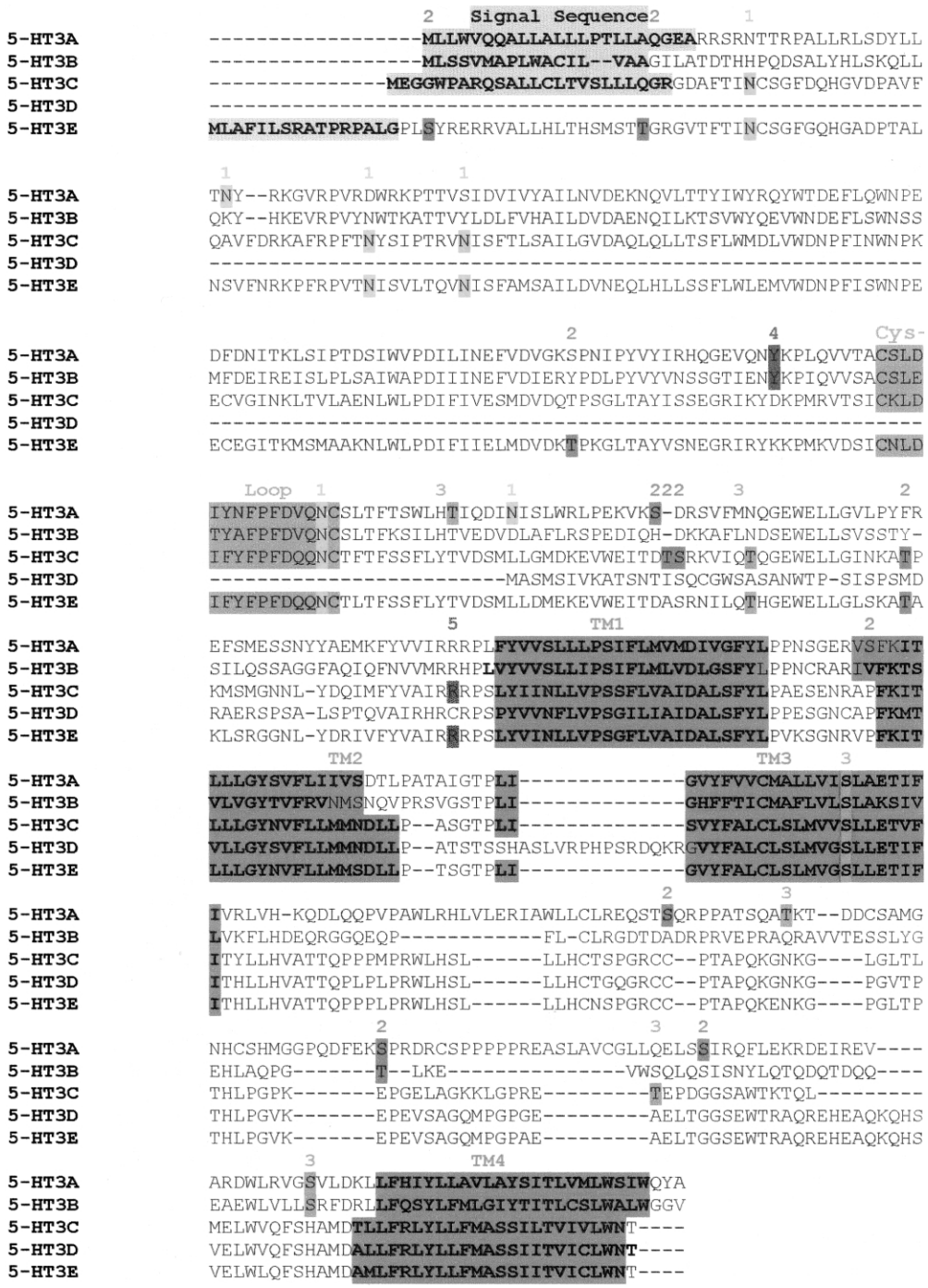
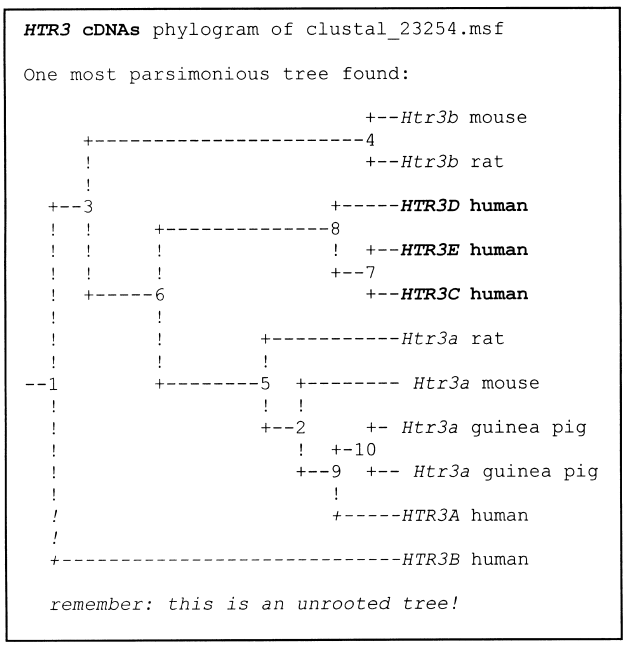


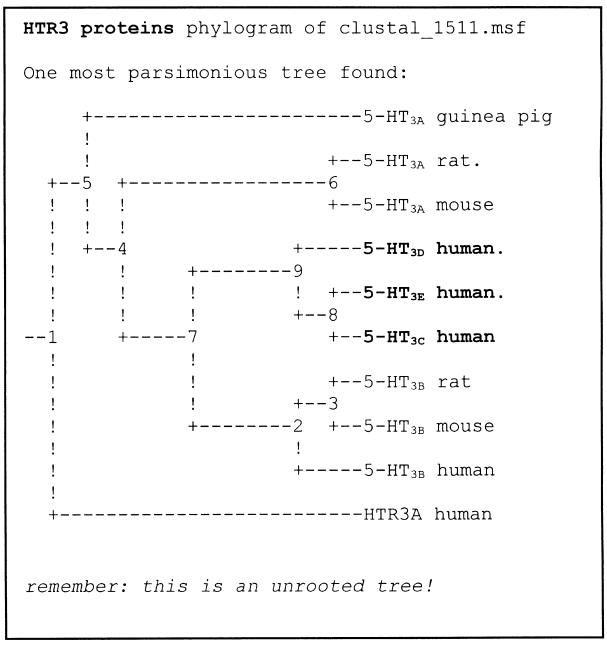
Fig. 4. Multiple sequence alignment of the human 5-HT_3 subunits A, B, C, D and E on protein level. Signal peptide (leader sequence) is marked in light grey, the cysteine loop is indicated in grey and the four transmembrane regions are coloured in dark grey. Putative phosphorylation and glycosylation sites are marked as follows: 1 Asn Glycosylation, 2 Protein Kinase C, 3 Casein Kinase, 4 Tyrosine Kinase, 5 CAMP Kinase.

map in close vicinity on chromosome 11q23. *HTR3A* and *B* are structurally very similar with exons almost identical in size and conserved splice sites. Identical exon-intron organisation is shared by *HTR3C* on 3q27, which by sequence comparison is the member most closely related to *HTR3A* and *HTR3B*. Among all the members of the *HTR3* class, *HTR3C*, *HTR3D* and *HTR3E* are the ones most closely related, suggesting that they diverged later in evolution. This is also predicted by the dendrogram (Table 3) which reveals three major evolutionary branches in this system: one for *HTR3A*, another for *HTR3B* and a third one for *HTR3C*, *HTR3D* and *HTR3E*. It is therefore likely that recent evolutionary processes have shaped these novel genes and that they have acquired novel ultimate functions.

Table 3
Homologies of *HTR3A, B, C, D* and *E* on nucleotide and protein level

cDNA homology (CDS only)	*HTR3A* (bestfit/gap; %)	*HTR3B* (bestfit/gap; %)	*HTR3C* (bestfit/gap; %)	*HTR3D* (bestfit/gap; %)
HTR3A	100			
HTR3B	61.2/55.3	100		
HTR3C	60.5/54.4	56.2/47	100	
HTR3D	66.9/47.7	65.7/47.4	84.9/83.3	100
HTR3E	63.4/54.1	55.5/47.8	80.2/82.8	88.6/90.1
Protein homology/identity	5-HT$_{3A}$	5-HT$_{3B}$	5-HT$_{3C}$	5-HT$_{3D}$
5-HT$_{3A}$	100			
5-HT$_{3B}$	56.4/45.9	100		
5-HT$_{3C}$	48.7/39.8	39.5/30.5	100	
5-HT$_{3D}$	40.8/35.5	35.0/26.8	69.6/64.8	100
5-HT$_{3E}$	46.9/39.5	40.8/32.2	79.6/74.3	76.9/73.8





Homologies on nucleotide level were determined by sequence comparison using the respective coding regions. Similarity and identity values were determined using the *bestfit* and *gap* tools of the HUSAR program package from DKFZ, Heidelberg.

To get an idea of the functional role of the different 5-HT$_3$ receptor subunits, we carried out comparative expression analysis of all *HTR3* genes by RT-PCR. Unlike the known *HTR3* genes which are almost ubiquitously expressed, expression of *HTR3D* is restricted to kidney, colon and liver. *HTR3E* expression was detected in colon and intestine, respectively (Fig. 3). We consider it likely that different subunit compositions of *HTR3C, D, E* as well as *HTR3A* and *B* in the 5-HT$_3$ receptor contribute to the complexity of the 5-HT$_3$ receptor system and therefore explain the varying properties of the respective proteins in different tissues. Whether the diverse properties of the receptor can also be explained by oligomerisation of different receptor subunits in the respective organs remains to be determined. The co-expression of 5-HT$_{3A/B}$ subunits leading to structurally different 5-HT$_3$ receptors has recently been shown in neurons of the rat central and peripheral nervous system as well as in mammalian retinae (Morales et al., 2001; Pootanakit and Brunken, 2001; Morales and Wang, 2002). With these new receptors available, expression and functional analysis of recombinant homo- and hetero-oligomeric 5-HT$_3$ receptor complexes will now provide a new insight into the maturational, structural and functional diversity of the 5-HT$_3$ receptor system.

Activation of the 5-HT$_3$ receptors subserves a variety of physiological effects in the central and peripheral neurons (Bloom and Morales, 1998) and it has been predicted that it probably plays a role in a number of human diseases such as anxiety, schizophrenia, depression, migraine, vasospasm, epilepsy, fibromyalgia, bulimia and irritable bowel

syndrome (Graeff, 1997). Since *HTR3D/E* have been shown to be specifically expressed in colon and intestine we hypothesise that these receptor genes are involved in the etiology of diseases of the gastrointestinal tract such as irritable bowel syndrome. Irritable bowel syndrome is associated with abdominal pain and abnormal bowel activity and may reflect hypersensitivity of the gastrointestinal tract to normal stimuli. The basis of the disease is not understood, but there is evidence that serotonin plays a crucial role since it is a major neurotransmitter in the gastrointestinal system. Furthermore, the fact that the 5-HT_3 receptor is involved in the control of gastrointestinal function and that 5-HT_3 receptor antagonists are used in the therapy of irritable bowel syndrome (Humphrey et al., 1999; Jones and Blackburn, 2002) predicts that *HTR3D* and *E* are interesting candidate genes for this disorder.

Acknowledgements

We would like to thank Dorothee Möller and Karin Schön for technical assistance, Rüdiger J. Blaschke, Nadja Muncke, Stefan Kirsch and Anne Jordan for helpful discussions and the Deutsche Forschungsgemeinschaft for support.

References

Bloom, F.E., Morales, M., 1998. The central 5-HT_3 receptor in CNS disorders. Neurochem. Res. 23, 653–659.

Breathnach, R., Chambon, P., 1981. Organisation of eukaryotic split genes coding for protein. Ann. Rev. Biochem. 50, 349–383.

Colquhoun, L.M., Patrick, J.W., 1997. Pharmacology of neuronal nicotinic acetylcholine receptor subtypes. Adv. Pharmacol. 39, 191–220.

Davies, P.A., Pistis, M., Hanna, M.C., Peters, J.A., Lambert, J.L., Hales, T.G., Kirkness, E.W., 1999. The 5-HT_{3B} subunit is a major determinant of serotonin receptor function. Nature 397, 359–363.

Dubin, A.E., Huvar, R., Dandrea, M.R., Pyati, J., Zhu, J.Y., Joy, K.C., Wilson, S.J., Galindo, J.E., Glass, C.A., Luo, L., Jackson, M.R., Lovenberg, T.W., Erlander, M.G., 1999. The pharmacological and functional characteristics of the serotonin 5-HT_{3A} receptor are specifically modified by 5-HT_{3B} receptor subunit. J. Biol. Chem. 274, 30799–30810.

Endris, V., Wogatzky, B., Leimer, U., Bartsch, D., Zatyka, M., Latif, F., Maher, E.R., Tariverdian, G., Kirsch, S., Karch, D., Rappold, G.A., 2002. The novel Rho-GTPase activating gene MEGAP/srGAP3 has a putative role in severe mental retardation. Proc. Natl. Acad. Sci. 99, 11754–11759.

Fletcher, S., Barnes, N.M., 1998. Desperately seeking subunits: are native 5-HT_3 receptors really homomeric complexes? Trends Pharmacol. Sci. 19, 212–215.

Graeff, F.G., 1997. Serotonergic systems. Psychiatr. Clin. N. Am. 20, 723–739.

Hoyer, D., Hannon, J.P., Martin, G.R., 2002. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol. Biochem. Behav. 71, 533–554.

Hubbard, P.C., Thompson, A.J., Lummis, S.C., 2000. Functional differences between splice variants of the murine 5-HT(3A) receptor: possible role for phosphorylation. Brain Res. Mol. Brain Res. 81, 101–108.

Humphrey, P.P.A., Bountra, C., Clayton, N., Kozlowski, K., 1999. The therapeutic potential of 5-HT_3 receptor antagonists in the treatment of irritable bowel syndrome. Aliment. Pharmacol. Ther. 13, 31–38.

Hussy, N., Lukas, W., Jones, K.A., 1994. Functional properties of a cloned 5-hydroxytryptamine ionotropic receptor subunit: comparison with native mouse receptors. J. Physiol. 481, 311–323.

Isenberg, K.E., Ukhun, I.A., Holstad, S.G., Jafri, S., Uchida, U., Zorumski, C.F., Yang, J., 1993. Partial cDNA cloning and NGF regulation of a rat 5-HT_3 receptor subunit. NeuroReport 5, 121–124.

Jackson, M.B., Yakel, J.L., 1995. The 5-HT_3 receptor channel. Annu. Rev. Physiol. 57, 447–468.

Jones, B.J., Blackburn, T.P., 2002. The medical benefit of 5-HT research. Pharmacol. Biochem. Behav. 71, 555–568.

Lankiewicz, S., Lobitz, N., Wetzel, C.H.R., Rupprecht, R., Gisselmann, G., Hatt, H., 1998. Molecular cloning, functional expression, and pharmacological characterisation of 5-hydroxytryptamine$_3$ receptor cDNA and its splice variants from guinea pig. Mol. Pharmacol. 53, 202–212.

Lichter, P., Cremer, T., 1992. Human Cytogenetics: a Practical Approach, IRL Press/Oxford Univ. Press, Oxford, New York, Tokyo.

McKernan, R.M., 1992. Biochemical properties of the 5-HT_3 receptor. In: Hamon, M., (Ed.), Central and Peripheral 5-HT_3 Receptors, Academic Press, London, pp. 89–102.

Miyake, A., Mochizuki, S., Takemoto, Y., Akuzawa, S., 1995. Molecular cloning of human 5-hydroxytryptamine$_3$ receptor: heterogeneity in distribution and function among species. Mol. Pharmacol. 48, 407–416.

Morales, M., Wang, S.D., 2002. Differential composition of 5-hydroxytryptamine$_3$ receptors synthesized in the rat CNS and peripheral nervous system. J. Neurosci. 22, 6732–6741.

Morales, M., McCollum, N., Kirkness, E.F., 2001. 5-HT_3-receptor subunits A and B are co-expressed in neurons of the dorsal root ganglion. J. Comp. Neurol. 438, 163–172.

Niemeyer, M.I., Lummis, S.C.R., 1998. Different efficacy of specific agonists at 5-HT_3 receptor splice variants: the role of the extra six amino acid segment. Br. J. Pharmacol. 123, 661–666.

Pootanakit, K., Brunken, W.J., 2001. Identification of 5-HT(3A) and 5-HT(3B) receptor subunits in mammalian retinae: potential presynaptic modulators of photoreceptors. Brain Res. 896, 77–85.

Quirk, P., Siegel, R., 2000. *N*-Glycosylation is necessary for surface expression of the 5-HT_3 receptor. Soc. Neurosci. Abstr. 811, 4.

Reeves, D.C., Lummis, S.C., 2002. The molecular basis of the structure and function of the 5-HT_3 receptor: a model ligand-gated ion channel. Mol. Membr. Biol. 19, 11–26.

Riordan, J.R., 1992. The molecular biology of chloride channels. Curr. Opin. Nephrol. Hypertens. 1, 34–42.

Uetz, P., Abdelatty, F., Villarroel, A., Rappold, G.A., Weiss, B., Koenen, M., 1994. Organisation of the murine 5-HT_3 receptor gene and assignment to human chromosome 11. FEBS Lett. 339, 302–306.

Van Hooft, J.A., Vijverberg, H.P., 1995. Phosphorylation controls conductance of 5-HT_3 receptor ligand-gated ion channels. Receptors Channels 3, 7–12.