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Organisation of the murine 5-HT_3 receptor gene and assignment to human chromosome 11

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Abstract

We have isolated the murine gene encoding the 5-HT_3 receptor (5-HT_3R), a member of the ligand-gated ion channels, that mediates a variety of physiological effects in central and peripheral neurons. DNA sequence analysis of the 5-HT_3R gene revealed its organisation in 9 exons distributed over approximately 12 kbp of DNA. Alternative use of exon 9 splice acceptor sites generated two 5-HT_3R variants. The 5-HT_3R gene, whose structure is closely related to neuronal and muscle AChRα genes, as demonstrated by four common splice junctions, was localised on human chromosome 11.

Key words: Serotonin; Gene structure; Alternative splicing; Mouse brain; Exon alignment

1. Introduction

5-Hydroxytryptamin (5-HT), a neurotransmitter involved in various physiological functions exerts its effects by binding to a variety of neuroreceptors. In contrast to the G-protein coupled serotonin receptors, the 5-HT_3 receptor (5-HT_3R) belongs to the family of ligand-gated ion channels [1,2]. The 5-HT_3 receptor is widely distributed in the peripheral [3] and central nervous system (CNS) [4,5] and is believed to play an important role in anxiety [6], cytotoxic drug evoked emesis [7], nociception [8] and may contribute to psychiatric disorders [9]. Recently a cDNA coding for a 5-HT_3R subunit was isolated from the mouse cell line NCB-20 by means of expression cloning [10]. The deduced 5-HT_3R protein revealed many structural characteristics common to other members of the ligand-gated ion channel superfamily, including four putative transmembrane regions (TM1-TM4), a cystein loop and a large intracellular loop separating TM3 and TM4. Pharmacological and physiological data have suggested the presence of interspecies variation of the 5-HT_3R, but no receptor variability within a species [11–15] which is in contrast to other members of the ligand-gated ion channel superfamily [16–19]. In this report we describe the isolation and sequencing of genomic DNA carrying the murine 5-HT_3R gene as a first step to examine mechanisms generating variable 5-HT_3R transcripts and to analyse the efficacy of DNA sequences in regulation of the 5-HT_3R gene activity. Moreover, the chromosomal localisation of the human homologue of the mouse 5-HT_3R gene was investigated.

2. Materials and methods

2.1. Screening of libraries

A 5-HT_3R cDNA clone was isolated by screening a mouse brain cDNA library constructed in λ-ZAPII (gift of K. Nave) using the end-labelled synthetic oligonucleotides 5HT7as (5′-GTCCCTTGCCACCTCCCGCATCTCATCCCGCTTCTCCAGGAAGTGGCGGAT-3′) and 5HT8as (5′-CTGAACTTCACCTCGATGATGCACGTACACATAAGGAATGTTCGGAGA-3′) corresponding to the NCB-20 derived cDNA sequence position 1360–1410 and 463–510 taken from the EMBL data library (Accession No. M74425) [10]. Recombinant λ-phages carrying the mouse 5-HT_3R gene were isolated by screening 5×10^5 PFU of a genomic DNA library constructed in λ-Fix [20] using the same oligonucleotides. Hybridisation was carried out at reduced stringency (35% formamide; 5 × SSC; 5 × Denhardt's solution; 20 mM Na-phosphate, pH 7.0; 100 μg sonicated salmon sperm DNA/ml) over night at 42°C and filters were washed subsequently in 5 × SSC; 2 × SSC; 1 × SSC (30 min each) at 45°C [21] and exposed to X-ray film (Amersham) overnight at −70°C.

2.2. Nucleotide sequence analysis

Plasmids carrying the cDNA were excised from the recombinant λ-ZapII phages according to Stratagenes protocol and additional deleted subclones useful in DNA sequence analysis were selected after plasmid preparation and restriction analysis on agarose gels. Similarily, overlapping restriciton fragments of genomic DNA from recombinant

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λ-Fix phages were clones in pBKS (Stratagene). DNA sequence analysis by the dideoxynucleotide chain termination method [22] was performed using the Sequenase kit version 2.0 (United States Biochemical Corporation) following the conditions recommended by the supplier.

2.3. Functional expression of 5-HT$_3$R in Oocytes

A 5-HT$_3$R cDNA fragment of 1546 bp was amplified by PCR [23] using a sense primer (5-HT$_3$R-ATG: 5′-CTCTCTAGATCTGCCACCATGCGGCTCTGCATCCCGCAGGTG-3′) covering the translational start site (underlined) and an antisense primer (5-HT$_3$-TER: 5′-TCTCTCAGATCTAGTTCCATAGCCCCTCCCTGCC-3′) localized 35 nucleotides downstream of the translation termination codon. Furthermore, to increase translational efficiency of the in vitro synthesised mRNA, the translation start site CTTGCCATG was replaced by GCCACCATG which is more related to the Kozak consensus sequence [24]. PCR was performed in 50 μl using 10 ng of the supercoiled cDNA clone and 15 cycles of 1 min at 94°C, 1 min at 37°C and 1.5 min at 72°C were sufficient to amplify the cDNA fragment according to [25]. The PCR product was digested with *Bgl*II, purified and cloned into the *Bgl*II site of pSPOoD a derivativ of pSP64T [26,27]. Site-directed mutagenesis [25,28] was performed to replace amino acid differences of the extracellular N-terminal region corresponding to the NCB-20 cell line derived cDNA [10]. Finally, two differences remained, an additional alanine between TM2 and TM3 and a deletion of 6 amino acids (aa) within the large cytoplasmic loop [29]. To evaluate the relative contribution of these 6 aa to 5-HT$_3$R behaviour a receptor carrying these 6 aa was constructed. Specific mRNAs were synthesized from *Sal*I linearized plasmids in vitro with SP6-RNA polymerase (Promega). *Xenopus laevis* oocytes were injected with mRNA of a single 5-HT$_3$R variant and incubated for 2–5 days at 19°C [27]. The channel expression was determined by measuring the whole-cell current elicted in response to 3 μM 5-HT in normal frog Ringer solution and membrane potential held at −30mV.

2.4 Chromosomal mapping

DNA for genomic Southerns was prepared from cell lines, according to standard techniques. Aliquots (10 μg) of genomic DNA were digested with *Eco*RI, electrophoresed on 0.8% agarose gels, and transferred onto nylon membranes (GeneScreen, NEN) followed by UV crosslinking. GeneScreen filters were prehybridised and hybridised in 0.5 M NaPi; pH 7.2; 7% SDS; 1 mM EDTA at 65°C, essentially as described [30]. Hybridisation probes were radiolabeled to a specific activity of 1×10^9 cpm/μg by random priming and included in hybridisation at 2×10^7 cpm/ml. After hybridisation, filters were washed extensively in 40 mM NaPi, 1% SDS at 65°C prior to autoradiography.

3. Results

3.1. Isolation of a mouse brain 5-HT$_3$R cDNA

In order to characterise the 5-HT$_3$R gene, we initially cloned a mouse brain 5-HT$_3$R cDNA. For this purpose, a cDNA library was screened with the synthetic oligonucleotides 5HT7as and 5HT8as. Among 10^6 plaques one hybridizable recombinant phage was isolated and characterised by DNA sequence analysis. The mouse brain 5-HT$_3$R cDNA sequence resembled – with the exception of a 51 nucleotide (nt) extension at the 5′ end and two deletions of 4 bp and 5 bp apparently produced by erroneus splicing at the junctions of exon1/2 and 5/6 – the cDNA sequence of the N1E-115 clone [29] (data not shown). Both cDNA's represent a 5-HT$_3$R variant, where, in contrast to the NCB-20 cDNA [10], 18 nucleotides encoding 6 aa (GSDLLP) of the large cytoplasmic loop have been deleted. Similarily, the same deletion of 18 bp was identified in a rat brain 5-HT$_3$R cDNA (data not shown).

3.2. Organisation of the mouse 5-HT$_3$R gene

Functional characterisation of 5-HT$_3$ receptors in different tissues and species [11,12] have suggested the existence of various 5-HT$_3$R subtypes. To characterise the mammalian 5-HT$_3$R gene in more detail, a mouse genomic library constructed in λ-Fix [20] was screened with oligonucleotides 5HT7as and 5HT8as and two hybridizing recombinant phages λ2141 and λ2161 were isolated. Extensive digestion of the cloned genomic DNAs with different restriction enzymes revealed the identity of both recombinant clones. Hybridization experiments with 5-HT$_3$R cDNA fragments showed that phage λ2141 contained all coding sequences. Subsequent efforts were made to isolate overlapping clones spanning the complete coding region. However, during subcloning the *Xba*I DNA fragment carrying exon 5 and intron 5 (Fig. 1) parts of the intron sequences were repeatably deleted and nearly 1 kbp of intron 5 is lacking in the submitted genomic DNA sequence. The order of the overlapping DNA fragments was determined by hybridisation with cDNA fragments, and DNA sequence analysis was performed using the Sequenase system (USB). The mouse 5-HT$_3$R gene as defined herein is distributed over an area

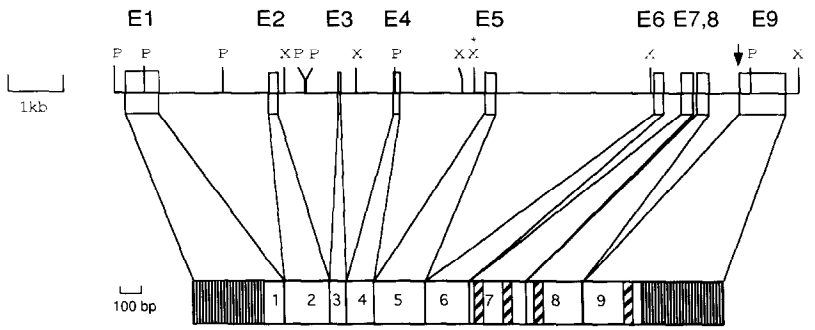

Fig. 1. The 5-HT$_3$R encoding region is distributed over an area of 12 kbp of DNA and organized in 9 exons numbered at the top (E1 to E9). The position of alternative splicing is indicated by an arrow. The mRNA is shown in 5′ to 3′ orientation subdivided by the exon borders. Vertical stripes in exon 1 and 9 indicate the 5′- and 3′-untranslated regions whereas the transmembrane segments are attributed by striped boxes in exon 7 (TM1, TM2), exon 8 (TM3) and exon 9 (TM4). Restriction endonuclease sites are indicated: X, *Xba*I; P, *Pst*I. An asterisk indicates the *Xba*I site (intron 4) that overlaps with a methylation site (Dam).

of 12 kbp of DNA (Fig. 1) and organized in 9 exons whose size are shown to range from 45 bp to 829 bp (Fig. 2). All splice junctions of the mouse 5-HT_3R gene are in agreement with the consensus sequence identified in vertebrates [31] and exon sequences and exon–intron borders are shown in Fig. 2. Alternative splicing of the mouse 5-HT_3R gene, which is obviously restricted to exon 9, deletes 18 bp by alternative use of splice acceptor signals (Fig. 2). In mouse brain as well as in mouse cell lines [10,29], two 5-HT_3R variants appear that differ in 6 aa of the large cytoplasmic loop. Northern blot analysis of mouse brain mRNA revealed a single low abundant 5-HT_3R transcript of about 2500 bp (Fig. 3).

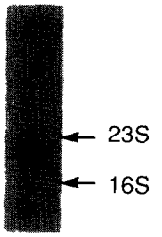

Fig. 3. Northern blot analysis with mouse brain poly(A)$^+$RNA (10μg) was performed as described [48]. The complete 5-HT_3R cDNA (specific activity of 1×10^9 cpm/μg) was used as probe and a single transcript of about 2.5 kbp was determined. The filter was exposed to X-ray film for 8 day at –70°C. Arrows indicate the positions of the *E. coli* ribosomal RNA's 16 S and 23 S.

3.3. Oocyte expression

To determine the importance of the 6 aa encoded by the alternatively spliced 18 nucleotides of exon 9, whole cell recordings of *Xenopus laevis* oocytes injected with RNA's of either of the variants were measured in the two-electrode voltage clamp configuration (27). Both 5-HT_3R variants elicited similar signals in response to 3 μM serotonin in normal frog Ringer's solution and are blocked by 3 nM D-Turbocurarine in similar way which suggests that channel properties are unaffected by these 6 aa (data not shown).

3.4. Alignment of the 5-HT_3R gene with neuronal and muscle AChRα genes

The mouse 5-HT_3R gene intron–exon organisation showed striking similarities to muscular and neuronal nicotinic AChR genes [32–36], but is unrelated to other members of the ligand-gated ion channel family [20,37]. The exon–intron positions are compared with respect to the common structural features of the ligand-gated ion channel proteins, particularily the disulfide structure and the putative membrane spanning segments. The mouse 5-HT_3R cDNA revealed an overall homology of 45.9% with the chicken AChRα7 receptor sequence [32] corresponding to about 30% homology on protein level. In the mouse 5-HT_3R gene, as in the chicken genes AChRα7 [32] and AChRα [33], in the human and calf AChRα genes [34], and in the rat AChRβ4 gene [35] introns 2, 3 and 4 show identical positions and exon 3 (45 bp) as well as exon 4 (110 bp) show identical size. Furthermore, exon 3 encodes a considerably conserved peptide domain with striking homology to the corresponding region of neuronal and muscular AChR's [32–36] (chicken AChRα7 = 73.3%, chicken AChR α4 = 60%, chicken AChR α2 = 53%; rat AChR α2, 4, 5 = 60%, rat AChR α3, β4 = 46,6% and chicken AChr α = 46%, calf and human AChR α = 40%) and conservation is extended into exon 4 encoded sequences with peptide homologies of 48% = chicken AChRα and α2; 43% = rat AChRα2;

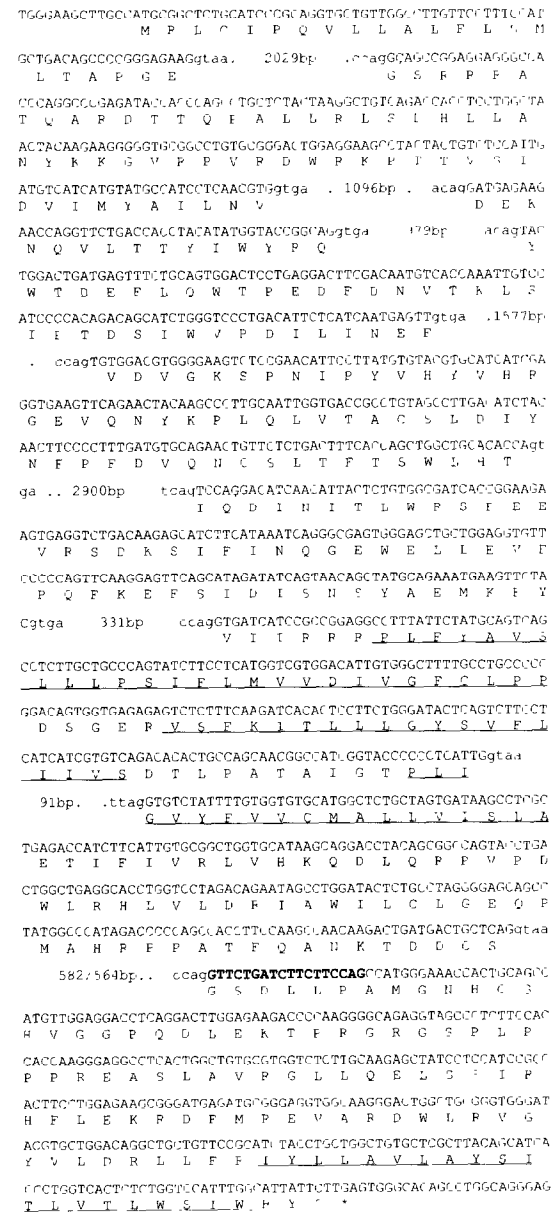

Fig. 2. The genomic sequence encoding the murine 5-HT_3R together with the deduced polypeptide sequence is shown in the one-letter code. Coding sequences are given in upper-case letters, the size of the introns (in lower-case letters) is indicated within the intronic gaps. Alternative spliced nucleotides are shown in bold letters, the transmembrane segments are underlined whereas the nucleotides lacking in the mouse brain cDNA are marked by a dotted line.

40% = chicken AChRα7 and α4; 38% = human AChRα; 32% = chicken AChRα3 and rat AChRβ4 [32–36]. Outside this region, only the cystein loop (60%) and the transmembrane segment TM2 (42%) showed an elevated level of homology compared to chicken AChα7 [32]. In addition, the splice acceptor position of exon 8 is conserved in chicken AChRα7, but not in other AChRα genes.

3.5. Chromosomal mapping

The localisation of the mouse 5-HT_3 receptor gene homologue on human chromosomes was determined by Southern analysis of DNA isolated from 39 different rodent–human hybrid cell lines. Filter with DNA of these cell lines containing defined overlapping subsets of 5–16 human chromosomes and 24 cell lines represent monochromosomal hybrids (NIGMS mapping panel #1 and #2 [38]). Chromosomal DNA from hybrid cells and parental lines were digested with *Eco*RI and examined by Southern analysis. The 5-HT_3R cDNA probe consistently revealed a hybridising human signal of 3.0 kbp in all cell lines containing chromosome 11 (GM/NA9934, GM/NA09936, GM/NA09938, GM/NA10927A; data not shown) and was easily distinguishable from the hamster and mouse signals, thus providing clear evidence that 5-HT_3R maps to human chromosome11 (data not shown).

4. Discussion

The results presented here provide the first description of the mouse gene encoding the 5-HT_3 receptor. A recombinant λ-phage (λ2141) carrying the complete coding region of the mouse 5-HT_3R was isolated and characterised by DNA sequence analysis. The mouse 5-HT_3R gene is organized in 9 exons spanning an area of approximately 12 kbp of DNA (Fig. 1). A remarkable structural conservation of exon 3 and exon 4 is observed in the mouse 5-HT_3R gene as well as in neuronal and muscular AChRα and β4 genes [32–36], but is absent in other ligand-gated ion channel encoding genes [20,37]. Moreover, exon 3 encoded amino acids are highly conserved between mouse 5-HT_3R gene and muscle and neuronal AChRα and AChRβ4 subunit genes [32–36], suggesting a possible similarity between the tertiary and quaternary structure of the agonist–antagonist binding domain of these receptors [39,40].

Functional diversity of ligand-gated ion channels is mainly generated by alternative splicing and variable combination of subunits [19,41]. For example, glutamate receptors exist in flip and flop versions which encode slightly different peptides of 38 amino acids (19,41). The presence of these exons (flip or flop) coincide with different pharmacological properties and different distribution in the mammalian brain [41]. Similarily, an alternative exon encoding 8 aa of the homologous region of the GABA-Aγ2 receptor subunit has been proposed to determine ethanol sensitivity in these receptors [42]. Such differences, however, are not observed for the 5-HT_3R gene, where, similar to the GABA-A-β4 [37] and the AChα5 subunit gene [35], receptor variants are generated by variable use of splice donor or acceptor signals. In mouse brain and mouse cell lines [10,29] two 5-HT_3R variants appeare by alternative use of two splice acceptor signals of exon 9 which remove 6 aa of the large cytoplasmic loop, however, whole cell currents revealed no difference in sensitivity to D-Turbocurarine between the 5-HT_3R and the splice variant lacking these 6 aa. T-PCR detected both 5-HT_3R variants in the mouse brain, but the variant lacking the 6 aa is seemingly predominant indicating a possible role of these amino acids in receptor distribution.

Using a series of somatic hybrids cell lines, we have localised the human homolog of the mouse 5-HT_3 receptor gene on human chromosome 11. The mapping of 5-HT_3R to chromosome 11 is interesting for a number of reasons. Recently, several families have been described where schizophrenia and manic depression segregate thighly with cytogenetic abnormalies involving either the short or the long arm of chromosome 11 [43–47]. In one of these families with schizophrenia, the observed translocation point involves a balanced t(11;1) translocation, which has been shown to reside between two genetic markers on 11q14-q21 [45]. Another family with manic depression carried a translocation t(9;11) on $11q22._3$ [46]. Positive linkage findings to maniac depression on 11p15 have also been reported [47]. Treatment of mental disorders with 5-HT_3R antagonists has been suggested to affect the course of the disease, e.g. in schizophrenia [9]. This potential correlation renders 5-HT_3R as an interesting candidate locus for linkage studies in major psychiatric disorders. Sublocalisation, e.g., by in situ hybridisation, of the 5-HT_3R sequence to either one of the mental disorder candidate regions on human chromosome 11 could be used to determine if defects in the human homolog of the mouse 5-HT_3R gene underly certain of the mental genetic disorders in man.

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