Human serotonin 5-HT$_7$ receptor: cloning and pharmacological characterisation of two receptor variants

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Abstract Two splice variants of the 5-HT$_7$ receptor were identified in human brain that differ in the lengths of their intracellular carboxy terminal tail. Identification of the variants of this receptor is of particular interest since the 5-HT$_7$ receptor is known to have a high affinity for a number of antidepressants and is localized in brain regions thought to be implicated in depression. The two isoforms are expressed in roughly equal amounts in various regions of the human brain. When expressed in NIH-3T3 cells, both variants encode functional 5-HT$_7$ receptors, positively coupled to adenylyl cyclase. We suggest that both variants are derived from a single gene by alternative mRNA splicing. Furthermore, our results from Southern blot analysis studies suggest that additional 5-HT$_7$ receptor genes may exist in human.

Key words: G-protein-coupled receptor; Receptor variant; 5-HT; Alternative splicing

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that has been implicated in a wide diversity of behavioural and physiological processes [1]. Molecular cloning studies have indicated the existence of 14 different genes, each encoding a distinct 5-HT receptor subtype (reviewed in [2]). All of these, with the exception of the 5-HT$_3$ receptor belong to the family of G-protein-coupled receptors. These receptor subtypes differ with respect to tissue distribution, ligand-binding characteristics, and coupling to intracellular second-messenger systems. On the basis of molecular and pharmacological criteria, these subtypes have been classified into seven main classes: 5-HT$_1$ to 5-HT$_7$ [2].

For many neurotransmitter receptors, the molecular diversity is further increased by the occurrence of splice variants and receptor isoforms of a particular receptor subtype. In case of the 5-HT$_4$ receptor, for example, two different mRNA splice variants have been identified encoding proteins which differ at their C-termini [3]. These splice variants vary both in tissue distribution and in cAMP coupling [3]. Furthermore, for the prostaglandin EP$_3$ receptor and the somatostatin S$_2$ receptor isoforms have been identified that differ at their C-terminal tails due to alternative splicing, activating different G-proteins and intracellular second-messenger systems [4,5]. Among the dopamine receptors, the D4 receptor subtype is unique in that a large number of polymorphic variants of this receptor has been identified in the human population [6,7]. The most extensive polymorphism is a 48-bp tandem repeat located within the third cytoplasmic loop of the receptor [6,7].

In this study we report the cloning of two cDNAs for 5-HT$_7$ receptor variants from human brain. The identification of variants of the 5-HT$_7$ receptor subtype is of particular interest since this receptor is thought to be localized to limbic and cortical regions of the brain [8–12]. Moreover, this receptor shows a high affinity for serotonin as well as for a number of tricyclic antipsychotic and antidepressant drugs [8–12]. The existence of a variant of this receptor in human brain may have therapeutical implications for the treatment of psychiatric diseases. We show that the two isoforms are co-expressed at similar levels in various brain regions. Furthermore, using ligand-binding and functional studies we show that both versions of the receptor encode functional receptors that increase adenylyl cyclase activation.

2. Materials and methods

2.1. General methods

All general recombinant DNA procedures were performed as described by Sambrook et al. [13]. Restriction endonucleases were purchased from Pharmacia, radiolabeled nucleotides were from Du Pont (NEN). DNA sequencing was carried out with the Auto Read sequencing kit (Pharmacia) or with the Cycle Sequencing with Fluorescent labelled primer kit (Amersham) and either M13 forward or reverse oligonucleotide primers or specific primers.

2.2. Oligonucleotides and probes

Sequence primers and oligonucleotides for polymerase chain reaction (PCR) experiments were synthesised on the basis of the published sequence of the human *5-HT$_7$* gene [8]. The nucleotides corresponding to the 5-HT$_7$-specific primers are indicated below. Org 2518: 5′-GACCCTGTGCGTGATCAGCATCGACAGGTA (sense, 513–542); Org 2901: 5′-CATGACGGATCCTTCTGCTTTCAATCATGAATCATGACCTT (antisense, 1322–1350); Org 3077: 5′-GGCGCGATGATGGACGTTAA (sense, −6 to 14); Org 3078: 5′-TCACTATGCCATTCAGGGCG (antisense, 861–880); Org 3588: 5′-GCTCCAGTGCCAGTACCGGA (sense 1194–1213).

Sequence primers: Org 3579: 5′-AGGGTTAATGAGAGAGTTTG (antisense, 1124–1143); Org 3580: 5′-CGACAGCCAGACCCTTCATC (sense, 1040–1059); Org 3581: 5′-GAGGTAAGGTGATGGAGGCG (antisense, 630–649); Org 3582: 5′-TCCGCCAGCCCTCCAACTAC (sense, 335–354); Org 3594: 5′-GTGGCTGCTTTCTGTTCTCG (antisense, 961–980).

The probes used for Southern blot analysis were generated by restriction enzyme digestions. Probe 1 corresponds to nucleotides 1–836. Probe 2 corresponds to nucleotides 836–1258.

2.3. PCR amplification and cloning of the 5-HT$_7$ gene

First-strand cDNA was synthesized from 5 μg Poly(A$^+$) hippocampal RNA in a 20 μl reaction volume as described elsewhere [14]. The coding sequence of the 5-HT$_7$ receptor was amplified in two parts from human hypothalamic cDNA with the polymerase chain reaction

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technique [8]. For the 5′ part of the gene the primer set Org 3077/3078 was used and for the 3′ part Org 2518/2901. The sequence of the primers is given above. The PCR reaction (35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min with a Perkin Elmer Cetus 9600 thermal cycler) was performed using human hypothalamic cDNA as a template. The PCR amplification of the 5′ part of the gene was carried out under the following conditions: 20 mM Tris-HCl, pH 8.8; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% Triton X-100; 0.1 mg/ml bovine serum albumin (BSA); 3% DMSO; 0.2 mM of each of the deoxynucleotide triphosphates and 2.5 U of cloned Pfu DNA polymerase (Stratagene) in a total volume of 100 μl.

For the 3′ part of the gene the Advantage cDNA PCR kit (Clontech) was used. The PCR products were analysed by gel electrophoresis on a 1% agarose gel and ethidium bromide staining. The bands were recovered from the gel with the Qiaex II gel extraction kit (Qiagen) and cloned into the pCRII vector (Invitrogen) for sequencing.

2.4. Subcloning of the 5-HT$_7$ gene into the eukaryotic expression vector pKCRE

To facilitate subcloning of the intact coding sequence of the 5-HT$_7$ receptor into the *Bam*HI site of the eukaryotic expression vector pKCRE, the PCR amplified 5′ and 3′ ends of the coding sequence were ligated and subcloned into the vector pGEM4Z. In case of the 5′ end, the *Eco*RI site of the pCRII vector and the unique internal *Xho*I site were used. For the 3′ part, the same internal *Xho*I site and a *Bam*HI site that was introduced at the 3′ end by PCR were used. Subsequently, the construct p1 was digested with *Eco*RI and *Acc*I, the 5-HT$_7$ insert was blunt ended with T$_4$-DNA polymerase and li-

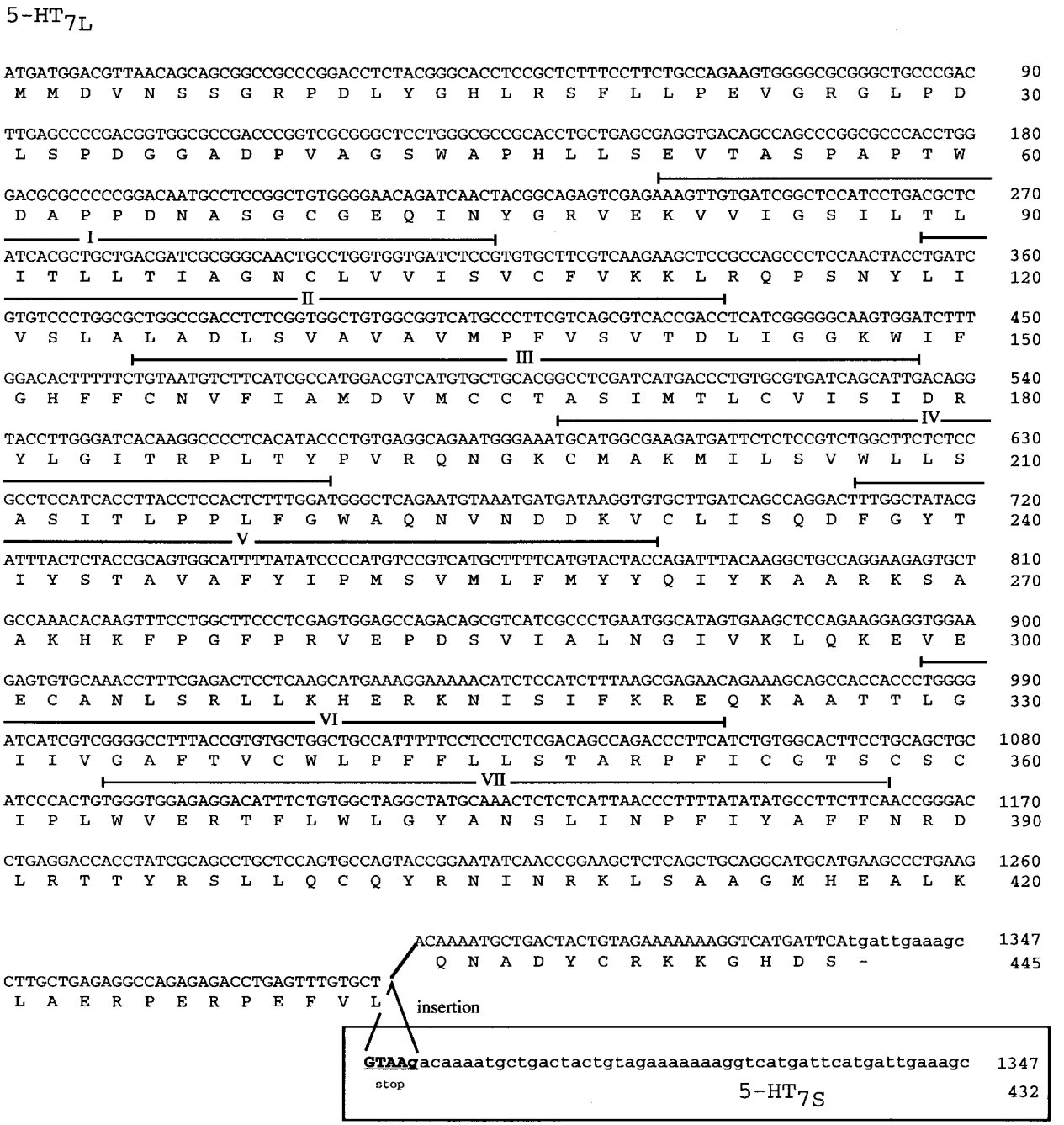

Fig. 1. Nucleotide and deduced amino acid sequences of the cDNAs for 5-HT$_{7L}$ and 5-HT$_{7S}$ isoforms. The complete nucleotide sequence of the 5-HT$_{7L}$ isoform is shown, followed by the 3′ end of the 5-HT$_{7S}$ variant (boxed). Deduced amino acids are shown in single letter code below the nucleotide sequence. The seven putative transmembrane domains are indicated by I–VII.

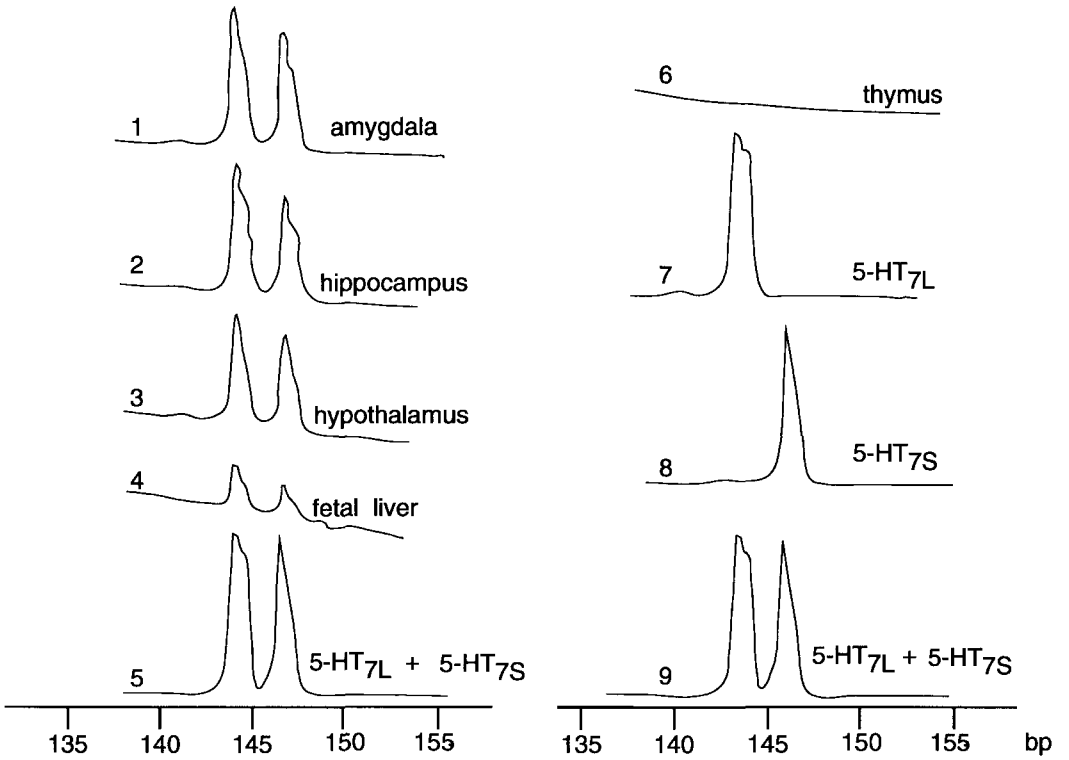

Fig. 2. Analysis of the relative expression patterns of the 5-HT_{7L} and 5-HT_{7S} variants in various human tissues. Poly(A^+) RNA prepared from the tissues indicated was copied with reverse transcriptase and submitted to PCR. The primers were directed against two specific regions of the 5-HT_7 receptor sequence that are conserved between the two isoforms. Samples 7 and 8 represent controls with plasmid DNA containing individual 5-HT_{7L} and 5-HT_{7S} receptor DNAs, respectively. Samples 5 and 9 contain plasmid DNA of the long and short variants in a 1:1 molecular ratio.

gated into the blunted *Bam*HI site of pKCRE. The *5-HT*$_7$ gene splice variant with five additional nucleotides in the C-terminus (5-HT_{7S}) was made by replacing an internal *Hin*dIII–*Bam*HI fragment with the corresponding fragment derived from another PCR product (Fig. 2).

The correct sequence and orientation of the *5-HT*$_7$ gene were confirmed by sequencing across the junctions, using as primers synthetic oligonucleotides made to anneal close to the ends of the insert and internal primers.

2.5. RT-PCR to determine the tissue distribution of the 5-HT$_7$ *variants*

cDNA was made with the Time Saver Kit (Pharmacia) from poly(A^+) RNA (Clontech). With PCR, part of the *5-HT*$_7$ gene was amplified using primers that span the variable region of the gene. As 5′ primer FITC-labelled Org 3588 was used and as 3′ primer Org 2901. The FITC-labelled PCR products were separated on a polyacrylamide sequencing gel and analysed with the ALF DNA sequencer (Pharmacia Biotech).

2.6. Southern blot hybridization

Southern blots with human genomic DNA, digested with various restriction enzymes, were obtained from Clontech. The filters were prehybridized for 30 min at 60°C in Express Hyb Hybridization solution (Clontech). Hybridization was performed overnight at 60°C in the same buffer with probe 1 (nucleotides 1–836) or probe 2 (nucleotides 836–1258). The probe was labelled using Ready To Go DNA labelling beads (-dCTP) (Pharmacia Biotech) according to the manufacturer's protocol. The filter was washed 2 times for 30 min each in 2×SSC/0.1% SDS at 60°C and 2 times for 30 min each in 1×SSC/0.1% SDS.

2.7. Cell culture and expression of the 5-HT$_7$ *gene variants in NIH-3T3 cells*

NIH-3T3 cells were obtained from the ATCC and maintained at 37°C in a humidified atmosphere (5% CO_2) as a monolayer culture in Dulbecco's Modified Eagles Medium/Hams F12 (1:1 mix) supplemented with 10% fetal calf or fortified bovine calf serum (Hyclone). To express the cloned genes, the expression constructs of the *5-HT*$_7$ and *5-HT*$_{7S}$ genes were transfected into NIH-3T3 cells using Transfectam Reagent (Promega) together with the plasmid pAG60MT_{II}, which encodes resistance to Geneticin (Gibco, NY). Transformed clones were selected in the presence of 600 μg/ml Geneticin. Membranes prepared from pools of transfected cells were analysed for expression of 5-HT_7 receptors by a ligand-binding assay using the radiolabelled ligand [^{3}H]LSD. For both splice variants, single-cell clones were selected by the limiting dilution method.

2.8. Membrane preparation and radioligand-binding assay

Cells were grown to confluency and then harvested in phosphate-buffered saline containing 1 mM EDTA. The cells were centrifuged at 500×*g* for 10 min and the pellets were stored at −70°C until use. Immediately before the assay, the pellet was thawed, resuspended in assay buffer, and homogenised before addition to the reaction mixture. A small aliquot of the final homogenate was taken for determination of protein concentration using the method of Bradford (Bio-Rad reagent).

Assays were performed in a total volume of 500 μl which consisted of 100 μl of radioligand ([^{3}H]LSD or [^{3}H]5-HT), 300 μl of cell suspension and 100 μl of either assay buffer (total binding) or clozapine (10^{-5} M) (non-specific binding). Incubations were carried out at room temperature in the dark for 90 min. Assays were terminated by rapid vacuum filtration over glass-fiber filters. Filters were soaked in 0.1% polyethyleneimine before use. Filters were dried, transferred to 1.25 ml Microscint-20 and counted in a TopCount.

2.9. Measurement of cAMP formation

NIH-3T3 cells transfected with either the human 5-HT_{7L} or 5-HT_{7S} construct were seeded on poly-L-lysine-coated 12-well plates (5×10^4 cells per well). The cells were grown for 1 day in 1 ml of culture medium with 10% fortified calf serum, Hyclone). After removal of the medium the wells were washed twice with 1 ml of serum-free culture medium and incubated for 30 min with 900 μl of serum-free

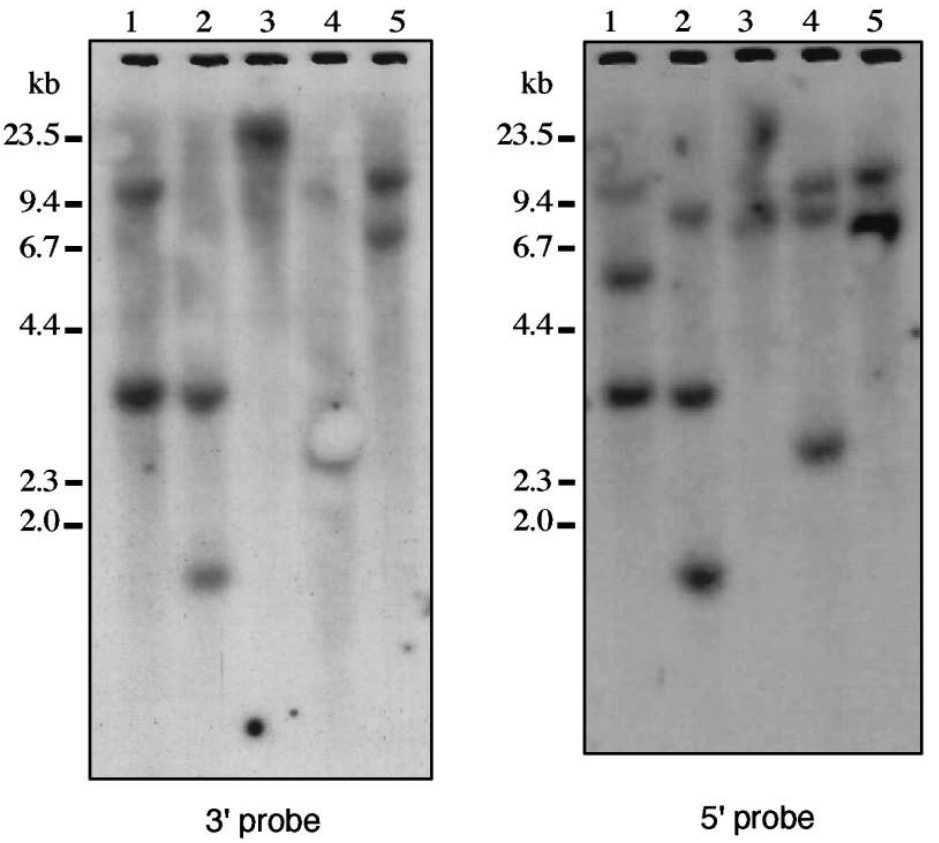

Fig. 3. Southern blot analysis of the human *5-HT$_7$* receptor gene. Human genomic DNA (10 μg) was digested with the various restriction enzymes indicated. Two different probes were used: (1) a 836-bp fragment derived from the 5′ -end (right panel), and (2) a 422-bp fragment corresponding to the 3′ end of the receptor (left panel). Lane numbers indicate the the restriction enzymes used to digest the DNA. Lane 1, *Eco*RI; lane 2, *Hin*dIII; lane 3, *Bam*HI; lane 4, *Pst*I; lane 5, *Bgl*I. Numbers on the left refer to DNA size markers.

medium that contained 0.5 mM IBMX. 5-HT (10× final concentration) was added in a volume of 100 μl of culture medium. The experiment was terminated by taking 500 μl of extracellular medium for assessment of cAMP formation using a cyclic AMP radioimmunoassay kit (Immunotech International). 100 μl of standards or samples was transferred to antibody-coated tubes and 500 μl of [^{125}I]cAMP tracer was subsequently added. All tubes were incubated for 18 h at 4°C. After an 18 h incubation the contents of all tubes were removed. The radioactivity left in the tubes was determined in a gamma counter.

3. Results and discussion

To identify 5-HT$_7$ receptor variants in human brain, reverse transcriptase (RT)-PCR was performed with several sets of 5-HT$_7$-specific primers and poly(A$^+$) mRNA extracted from human hypothalamus. The primers were designed on the basis of a published 5-HT$_7$ receptor sequence [8]. Two types of 5-HT$_7$ receptor sequences were amplified that were subcloned into the vector pCRII for sequencing.

One of the cDNAs (referred to as 5-HT$_{7L}$, 'L' for long) contained an open reading frame of 1335 bp coding for a 5-HT$_7$ receptor with an identical sequence as the 5-HT$_7$ sequence reported by Bard et al. [8]. The sequence of the second cDNA (termed 5-HT$_{7S}$, 'S' for short) was identical to 5-HT$_{7L}$, except for a 5-bp insertion (GTAAG) within the coding sequence at position 1296. This insertion results in a premature termination of the open reading frame at position 1297 (Fig. 1). Hence, this novel 5-HT$_7$ transcript encodes a truncated 5-HT$_7$ receptor that is virtually identical to the larger 5-HT$_7$ receptor (5-HT$_{7L}$), except that it lacks the last 13 residues of the carboxy terminus of the 5-HT$_{7L}$ receptor.

RT-PCR was used to examine the tissue distribution of the 5-HT$_{7L}$ and 5-HT$_{7S}$ receptor variants in various human brain regions. Using 5-HT$_7$-specific primers that do not discriminate

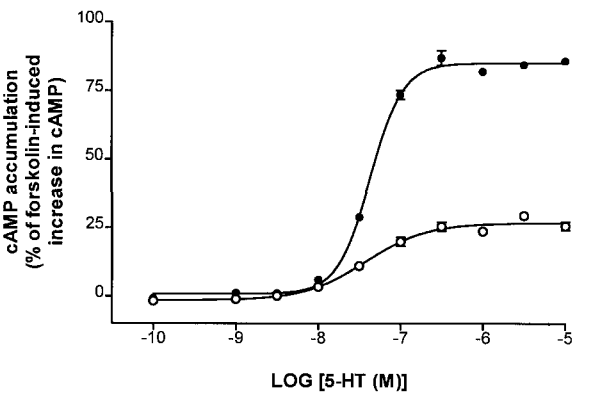

Fig. 4. The effect of 5-HT on cAMP accumulation in NIH-3T3 cells expressing the two isoforms of the 5-HT$_7$ receptor. Data are expressed as a percentage of the increase induced by forskolin. The increase induced by forskolin was 8.6-fold for the long variant (closed symbols) and 9.4-fold for the short variant (open symbols). Data are from a representative experiment that was repeated twice with similar results.

Table 1
Binding parameters for radioligand binding to the isoforms of the 5-HT_7 receptor expressed in NIH-3T3 cells

Receptor	[^{3}H]5-HT			[^{3}H]LSD		
	K_d (nM)	B_{max} (pmol/mg)	n	K_d (nM)	B_{max} (pmol/mg)	n
5-HT_{7L}	1.8	5.4	4	3.5	2.6	2
5-HT_{7S}	0.9	2.0	4	2.5	1.3	3

between the two mRNAs, the amplified DNA fragments were analysed on acrylamide gels and analysed with an ALF automated fragment analyser (Pharmacia). The primers do not discriminate between the two variants, and therefore both types of mRNA were expected to be amplified equally well. In all tissues in which the 5-HT_{7L} variant was expressed, the 5-HT_{7S} isoform was co-expressed. Although the absolute level at which the 5-HT_7 transcripts are present varied per tissue, the ratio in which the two types of mRNAs were expressed was roughly equal. In thymus, a tissue known not to express 5-HT_7 receptors, neither the 5-HT_{7L} nor 5-HT_{7S} variants, could be detected.

To determine whether the 5-HT_7 receptor is a single copy gene, Southern blot analysis was performed on human genomic placenta DNA. The filters were hybridized with a probe specific for the 5′ end of the *5-HT$_7$* gene, and a duplicate filter was hybridized with a 3′ end specific probe. Since the 5′ end probe overlaps a large intron, and contains no sites for any of the restriction enzymes used, this probe was expected to hybridize with two fragments in each lane, for a single copy gene. For the 3′ end probe, one hybridizing band was expected in each lane in case of a single copy gene. As shown in Fig. 3, the 5′ end probe yielded three hybridizing bands in all lanes, whereas two hybridizing bands are present when hybridized with the 3′ end probe. These findings suggest that the human genome harbours at least two 5-HT_7-like genes, that show a great deal of structural homology. This, in combination with the existence of two types of 5-HT_7 transcripts in brain may suggest that the two 5-HT_7 mRNAs are derived from two different genes. On the other hand, it may also be the case that the two transcripts derive from the same gene by alternative mRNA splicing. This possibility is supported by: (1) the fact that both the human and rat *5-HT$_7$* receptor genes contain an intron at the position of the 5-bp insertion; (2) the presence of splice donor and splice acceptor sites at the junctions of the 5-bp insertion; and (3) the 100% sequence conservation between the short and long variants at the DNA level, even at all third base positions. If the two 5-HT_7 receptor transcripts indeed derive by alternative splicing from one of the two hybridizing genes in the human genome, the second gene may code for a novel 5-HT($_7$) subtype or a pseudogene. Elucidation of the genomic structure of both hybridizing DNA fragments from the human genome may shed some light on this matter.

To investigate whether the two 5-HT_7 cDNAs encode functional 5-HT_7 receptors, the cDNAs for the 5-HT_{7L} and 5-HT_{7S} receptors were subcloned into the mammalian expression vector pkCREH and stably transfected into NIH-3T3 cells. Using limiting dilution in combination with ligand-binding assays, a representative clone was selected for each of the two 5-HT_7 receptor variants. Both clones express roughly similar densities of receptors (the difference in B_{max} values is < 5). As shown in Fig. 4, the 5-HT_{7L} and 5-HT_{7S} receptor isoforms were found to display similar affinities for both the antagonist [^{3}H]LSD and the agonist [^{3}H]5-HT. In Table 1, the binding parameters of both clones are presented. Saturation experiments with LSD were less reliable than those with 5-HT. Therefore [^{3}H]5-HT was used routinely as radioligand. The affinity of 5-HT for the short variant of the receptor was consistently higher than that for the long variant ($n = 4$). K_d values are comparable to those obtained for the 5-HT_{7L} variant transiently transfected in COS-7 cells.

The occurrence of two types of 5-HT_7 receptors differing in their C-terminal tail is particularly interesting since studies with mutated and truncated receptors have shown that the intracellular C-terminal portion of G-protein-coupled receptors is involved in G-protein coupling [11,15–17]. In this respect, it is worth mentioning that for the 5-HT_4 receptor, two variants have been described that vary in their C-terminus as a result of alternative splicing and that differ in both their G-protein coupling and tissue distribution [3].

To determine whether the two 5-HT_7 receptor variants couple to G-proteins, both receptor clones were exposed to various concentrations of 5-HT after which intracellular cAMP levels were measured. As shown in Fig. 4, 5-HT induced a dose-dependent increase in cAMP accumulation in both clones with similar EC_{50} values, i.e. 38 and 43 nM for the short and long variants, respectively. We compared the maximal level of activation with that induced by forskolin. In this manner we could correct for differences between the single-cell clones in their ability to activate adenylyl cyclase. The 5-HT_{7S} receptor was clearly less effective in increasing cAMP. However, this can be explained by the lower level of receptor expression, since during the selection of the single-cell clones we observed that the effect on cAMP correlates well with the expression level (data not shown). Thus from the results from both radioligand-binding and functional experiments, we conclude that the 5-HT_{7L} and 5-HT_{7S} receptors do not differ significantly with respect to their interaction with their endogenous ligand, when expressed in NIH-3T3 cells. We do not know yet whether other drugs may discriminate between the two isoforms.

The identification of two 5-HT_7 variants that are equally well expressed in brain and that are conserved in evolution from rodents to man [8–10] makes it tempting to speculate that there is an advantage to have a 5-HT_{7L} and a 5-HT_{7S} variant. Although our experiments do not show marked pharmacological differences between the two receptors when expressed in NIH-3T3 cells, it should be noted that the two variants may behave differently with respect to pharmacological properties such as coupling or desensitisation, when expressed in a different cellular context, e.g. the brain.

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