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Human 5-HT$_5$ receptors: the 5-HT$_{5A}$ receptor is functional but the 5-HT$_{5B}$ receptor was lost during mammalian evolution

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Abstract

We have isolated from a human genomic library the human 5-hydroxytryptamine 5-HT$_{5A}$ and 5-HT$_{5B}$ genes. The human 5-HT$_{5A}$ gene encodes a protein with similar characteristics to its mouse homologue. When expressed in monkey COS-7 cells, the human 5-HT$_{5A}$ receptor displayed a high affinity for tritiated 5-carbamidotryptamine ([^{3}H]5-CT; $K_D = 2.8$ nM) and iodinated lysergic acid diethylamide ([^{125}I]LSD; $K_D = 187$ pM). These binding sites displayed the following displacement profile: Ergotamine > Methiothepin > 5-CT, Ritanserin > 5-HT. Reverse transcriptase polymerase chain reaction (RT-PCR) experiments revealed the presence of human 5-HT$_{5A}$ mRNA in the central nervous system but not in peripheral organs. When expressed in *Xenopus* oocytes, the 5-HT$_{5A}$ receptor was able to couple to the inwardly rectifying K$^+$ channel, GIRK$_1$. In contrast to the human 5-HT$_{5A}$ gene and the mouse 5-HT$_{5B}$ gene, the human 5-HT$_{5B}$ gene does not encode a functional protein because its coding sequence is interrupted by stop codons. Our results suggest, therefore, that the 5-HT$_{5B}$ receptor has been lost during evolution after the divergence between rodents and primates. The 5-HT$_{5B}$ receptor is the first example of a brain-specific protein that is absent in human.

Keywords: 5-HT$_{5A}$ receptor; G protein-coupled receptor; Evolution; Null; (Human)

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic monoamine found in a variety of tissues in the central and peripheral nervous systems. Pharmacological studies as well as molecular cloning of serotonin receptors have revealed a multiplicity of receptor subtypes which can even be found in species with primitive nervous system, such as mollusks, arthropods and Caenorhabditis elegans (Olde and McCombie, 1997; Venter et al., 1988).

Among the fourteen 5-HT receptors identified in mice and rats (Hoyer et al., 1994; Lucas and Hen, 1995), the 5-HT$_5$ receptor family comprises two receptors named 5-HT$_{5A}$ and 5-HT$_{5B}$ which share 69% amino acid identity and have 23–34% homology with the other 5-HT receptors (Erlander et al., 1993; Matthes et al., 1993; Plassat et al., 1992; Wisden et al., 1993). Recently, the distribution of mouse 5-HT$_5$ receptors was made possible by the availability of knockout mice lacking the 5-HT$_{5A}$ receptor (Grailhe et al., 1999; Waeber et al., 1998). 5-HT$_{5A}$ specific binding sites were identified in the olfactory bulb, neocortex, hippocampus, medial habenula, and caudate putamen. Furthermore, 5-HT$_{5B}$ binding sites were found in the medial habenula. The distribution of these binding sites is in good agreement with the previously published localization of the mRNAs encoding for the mouse 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors (Matthes et al., 1993; Plassat et al., 1992). In mouse brain, the 5-HT$_{5A}$ and 5-HT$_{5B}$ specific binding sites were displaced by guanine nucleotide analogs, Gpp(NH)p, suggesting a functional coupling of these receptors with G proteins. In cell culture, the situation is less clear. While a number of reports did not find any functional coupling of the 5-HT$_{5A}$ receptor in fibroblasts, there are two recent publications indicating that the activation of 5-HT$_{5A}$ receptors can result in a small inhibition of adenylate cyclase (Francken et al., 1998; Hurley et al., 1998).

In this paper, we report the cloning and pharmacological characterization of the human 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors. The human 5-HT$_{5A}$ receptor encodes a protein with similar pharmacological characteristics as the mouse 5-HT$_{5A}$ receptor. In addition, we show that the human 5-HT$_{5A}$ can couple to the inwardly rectifying potassium channel GIRK$_1$ in *Xenopus* oocytes. In contrast, the hu-

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man 5-HT_{5B} gene does not encode a functional protein because its coding sequence is interrupted by stop codons. Our results suggest that the 5-HT_{5B} receptor has been lost in evolution sometime after the divergence between rodents and human.

2. Materials and methods

2.1. Isolation and sequence of the human 5-HT_{5A} and 5-HT_{5B} genomic DNA

Using polymerase chain reaction, we amplified fragments from the mouse 5-HT_{5A} gene exon I (375–1224) and exon II (1305–1645) (Plassat et al., 1992) and from the mouse 5-HT_{5B} gene exon I (397–1088) and exon II (1089–1325) (Matthes et al., 1993). PCR products were labeled with [^{32}P]dCTP by random priming and used to screen a human placenta genomic library. Filter lifts were made onto Hybond-N^+ (Amersham) and hybridization performed at low stringency (O/N at 42°C in 30% formamide, 5× SSC, 1× Denhardt's, 1% sodium dodecyl sulfate (SDS), 20 mM sodium phosphate buffer pH = 6.5, 100 μg/ml Salmon Sperm DNA). Filters were then washed for 1 h at 42°C in 0.2× SSC, 0.1% SDS. An average of 10 positive phages were isolated from 5×10^6 independent plaques. The DNA inserts were characterized by restriction analysis followed by Southern blotting and small hybridizing fragments were subcloned in Bluescript SK(+) plasmid (Stratagene) and sequenced on both strands by the dideoxynucleotide technique using successive synthetic oligonucleotides.

2.2. Construction of the full-length human 5-HT_{5A} gene

Exon I was found to lie on a 3.0-kb *Sac*I restriction fragment and exon II on a 1.0-kb *Eco*RI fragment. Phages containing both exons were isolated. To verify that the two exons represent the same gene, reverse transcriptional PCR products (Fig. 2) of the cDNA sequence containing each exons was sequenced. Sequence analysis confirms that the intron–exon boundary is the same as for the mouse 5-HT_{5A} receptor gene. In order to join the two exons, exon I was amplified from genomic DNA phage by using the primers 5′-GCATGCGCGCGGCCGCGGCACCATGGATTTAC-CTGTGAACCTA-3′ and 5′-TTCGGATATCGGTGA-GACGC-3′. The resulting PCR product was digested with the restriction enzymes *Not*I and *Eco*RV. Exon II was similarly amplified using primers 5′-CCGATATC-CGAAGCTGTGGAGGTGAAGGACTCTGCCAAACA-3′ and 5′-AAGAATTCCTCGAGTCAGTGTTGCCTA-GAAAAGAAGT-3′. The resulting PCR product was digested with restriction enzymes *Eco*RV and *Xho*I. The two digested products were ligated and recovered into the *Not*I and *Xho*I sites of the expression vector p514, which is a derivative of pSG5 (Green et al., 1988). The construction p514/h5-HT_{5A} was sequenced on both strands.

2.3. Expression of the human 5-HT_{5A} receptor in cell lines

For transient expression of the human 5-HT_{5A} receptor, COS-7 cells were grown in 175 cm^2 culture dish in 25 ml of Dulbecco's Modified Eagle's Medium maintained at 37°C with 5% CO_2. Medium was supplemented with 5% fetal calf serum. The p514/h5-HT_{5A} vector was introduced into COS-7 cells by calcium phosphate-mediated transfection (40 μg per 175 cm^2 dish) and harvested 48 h after transfection.

For stable expression of the human 5-HT_{5A} receptor, the p514/h5-HT_{5A} vector was transfected in human epithelial kidney cells (HEK-293) and mouse NIH-3T3 cells together with the recombinant pRSVneo, which encodes resistance to geneticin (G418). Transformed clones were isolated in presence of 0.5 mg/ml of geneticin. Isolated foci were amplified and membranes were prepared and analyzed for specific expression of human 5-HT_{5A} receptors. Two cell lines expressing the highest level of the human 5-HT_{5A} receptor, named HEK-5A and NIH-5A, respectively, were used for the functional assays.

2.4. Radioligand binding assay

Membranes from transiently transfected COS-7 cells were prepared as described (De Lean et al., 1982). [^{125}I]LSD saturation and competition binding experiments were performed respectively with 1 μg protein and 5 μg protein per sample in a final volume of 250 μl in 50 mM Tris–HCl (pH 7.4) at 37°C for 10 min. Competition binding experiments were performed using 25 pM [^{125}I]LSD. Reactions were terminated by filtration under vacuum over Whatman GF/C glass fiber filters and rinsed three times with 5 ml of 50 mM Tris–HCl (pH 7.4). Non-specific binding was defined with 10 μM 5-HT.

[^{3}H]5-CT saturation was carried out in 250 μl TME buffer [Tris–HCl (pH 7.4 at 22°C), 12.5 mM $MgCl_2$ and 1 μM EDTA] containing 25 μg transfected COS-7 membrane preparation, for 1 h at 22°C. Reactions were terminated by filtration under vacuum over Whatman GF/B glass fiber filters and rinsed three times with 5 ml of ice-cold wash buffer [20 mM Tris–HCl (pH = 7.4 at 4°C) and 2 mM $MgCl_2$]. The effect of the non-hydrolyzable GTP analogue Gpp(NH)p was measured with 1nM [^{3}H]5-CT. At this concentration the [^{3}H]5-CT should label almost exclusively the high affinity state of 5-HT_5 receptor (Wisden et al., 1993). Data were analyzed by the non-linear regression software EBDA/LIGAND (Munson and Rodbard, 1980) (Biosoft, MO, USA).

2.5. Adenylate cylase assay

Human HEK-5A and mouse NIH-5A cells stably expressing the human 5-HT_{5A} receptor were seeded into 24

well plates at a density of 250,000 cells/well. Cells were washed once with PBS 1× and incubated for 15 min at 37°C with 100 mM isobutylmethylxanthine and test agents in PBS. The reaction was stopped by aspiration of the medium, followed by the addition of 500 ml of ice cold ethanol. After 2 h at room temperature, the ethanol fraction was collected and lyophilized in a speed-vacuum. The pellet was reconstituted and cAMP was quantified by radioimmunoassay (Immunotech, Marseille, France).

2.6. Phospholipase C assay

HEK-5A and NIH-5A cells were labeled with [^{3}H]myoinositol (5 μCi/ml) for 48 h. Cells were washed in PBS and aliquots of 5×10^5 cells were treated with the test agents in PBS and 10 mM LiCl for 30 min at room temperature. The reaction was stopped by adding 10% trichloroacetate and the supernatant was further purified by a three-time ether extraction. Borax (3 mM) was added to adjust the pH and the sample was loaded to column prepacked with 2 ml AG1X8:H_2O (1:1). The columns were successively washed with water and 5 mM Borax/60 mM NaFormate. IP_1 and IP_2 were collected with 10 ml of 0.1 M Formic Acid/0.4 M NH_4-Formate. IP_3 was eluted with 10 ml of 0.1 M Formic acid/1 M NH_4-Formate. The two fractions were combined and subjected to scintillation counting.

2.7. Oocytes culture and electrophysiology

Xenopus oocytes were collected and incubated in 2 mg/ml collagenase type I (Sigma) in ND96 (Specialty Media, NJ, USA) for 3–4 h at room temperature. Oocytes were then washed four times with Barth's medium, transferred to L-15 medium and allow recovering at 18°C overnight before cRNA injection. Oocytes were maintained in L-15 at 18°C after cRNA injection, and experiments were performed between 1 and 7 days after injection. Full length 5-HT_{5A} and $GIRK_1$ coding regions were subcloned into a *Not*I *Xho*I site of the pSD64TR plasmid (Krieg and Melton, 1984). Linearized plasmids were used as a template for synthesis of cRNAs transcript (Promega, WI, USA). *Xenopus* oocytes were injected with the following cRNAs: rat $GIRK_1$, 1–2 ng; human 5-HT_{5A} receptor, 5–30 ng ; human 5-HT_{1A} receptor ; 5–30 ng.

Electrophysiological recordings were performed with a GeneClamp 500 amplifier (Axon Instruments, CA, USA) using a two-electrode voltage clamp with active ground configuration, as described (Miwa et al., 1999). The oocyte was placed in a chamber perfused with ND96, the holding potential was set at −80 mV, and the solution was changed to hK (96 mM KCl/2 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM HEPES pH = 7.6). Macroscopic currents were recorded and analyzed using Pclamp6 (Axon Instruments).

2.8. Tissue distribution of human 5-HT_{5A} mRNA

Total RNA was isolated from samples prepared from adult human brain (enthorinal cortex, septum, hippocampus, hypothalamus, striatum, raphe) and various organs (heart, kidney, liver, spleen, placenta) using a guanidine isothiocyanate/phenol extraction protocol. For the quantitative reverse transcriptase PCR analysis, we used the following oligonucleotides: (i) 5′-CCTGGCTCTCCCCTCAGTG-3′ and (ii) 5′-AGACCAATAGCGTCTCACC-3′. Respectively, 1 μg of total RNA from various tissues was reverse transcribed with 5 units of Avian Myeloblastosis Virus reverse transcriptase (AMV; Pharmacia, NJ, USA) and 50 ng of oligonucleotides (i) for 45 min at 42°C. One-twentieth of that reaction was amplified in the presence of 5 units of Taq Polymerase and 250 ng of oligonucleotides (i) and (ii) for 30 cycles. The PCR products after electrophoresis agarose gel migration were transferred to Hybond-N^+ membranes (Amersham) and hybridized with a ^{32}P-end-labeled oligoprobe (iii) 5′-AATGAGGATGCCCACCATGAG-3′. Hybridization was performed overnight at 42°C in hybridization buffer (5× SSC, 1× Denhardt's, 20 mM sodium phosphate buffer pH = 6.5, 0.1% SDS, 100 μg/ml tRNA). Southern blots were then washed 1 h at room temperature in 2× SSC, 1% SDS. Blots were exposed overnight to X-ray film (Kodak Biomax) with an intensifying screen.

2.9. Southern experiments

High-molecular-mass genomic DNA was isolated from placenta tissues by phenol chloroform extraction. Human genomic DNA was digested with five different restriction endonucleases *Sac*I, *Hin*dIII, *Asp*400, *Bam*hI, *Kpn*I and subject to Southern blot analysis. The Southern blots were hybridized successively with the random primed DNA labeled DNA probes such as a 691-bp mouse 5-HT_{5B} exon I (397–1088), a 735-bp human 5-HT_{5A} exon I and a 444-bp human 5-HT_{5B} exon I (*Spe*I–*Bgl*II). Two different hybridization conditions were used. A low stringency hybridization condition was applied (42°C overnight in 30% formamide, 5× SSC, 1× Denhardt's, 1% SDS, 20 mM sodium phosphate buffer pH = 6.5, 100 μg/ml tRNA) for the 691-bp 5-HT_{5B} mouse probe. A high stringency condition (42°C overnight in 50% formamide 5× SSC, 1× Denhardt's, 1% SDS, 20 mM sodium phosphate buffer pH = 6.5, 100 mg/μl tRNA) was performed for the human 5-HT_{5A} and 5-HT_{5B} probes.

2.10. PCR amplification on the human 5-HT_{5B}

High-molecular-mass genomic DNA was isolated from brain samples from different individuals (Caucasian, Asian and African-American). For the PCR amplification, we used the following oligonucleotides 5′-CCACCGAGC-

CTGGCGAGTGAG-3′ and 5′-GGGCGATGGCCGC-CACGTTCC-3′ surrounding the 35-bp repeat sequence of the human 5-HT_{5B} gene. The PCR products were separated by size on a 2% agarose gel, Southern blotted and hybridized with a ^{32}P-end-labeled human 5-HT_{5B} exon I oligoprobe 5′-CGTGGCACAGGCTCCGGCCCAGC-3′.

3. Results

3.1. Isolation of human genomic DNA encoding the 5-HT_{5A} receptor

In order to isolate the human 5-HT_{5A} gene, we generated two radio-labeled fragments of the mouse 5-HT_{5A} gene spanning exon I or II by polymerase chain reaction (PCR) and used them as probes to screen a human genomic library. Strongly hybridizing clones were isolated and characterized. We found several phages containing both exons of the 5-HT_{5A} gene. Partial sequence analysis revealed that the human 5-HT_{5A} gene contains an intron–exon boundary located in the middle of the sequence corresponding to the third cytoplasmic loop, at exactly the same position as in the mouse and rat 5-HT_{5A} genes (Fig. 1) (Matthes et al., 1993).

The two 5-HT_{5A} exons contain a 1071-bp open reading frame encoding a predicted 357 amino acid protein which is also the length of the mouse and rat 5-HT_{5A} receptors (Erlander et al., 1993; Plassat et al., 1992). This sequence

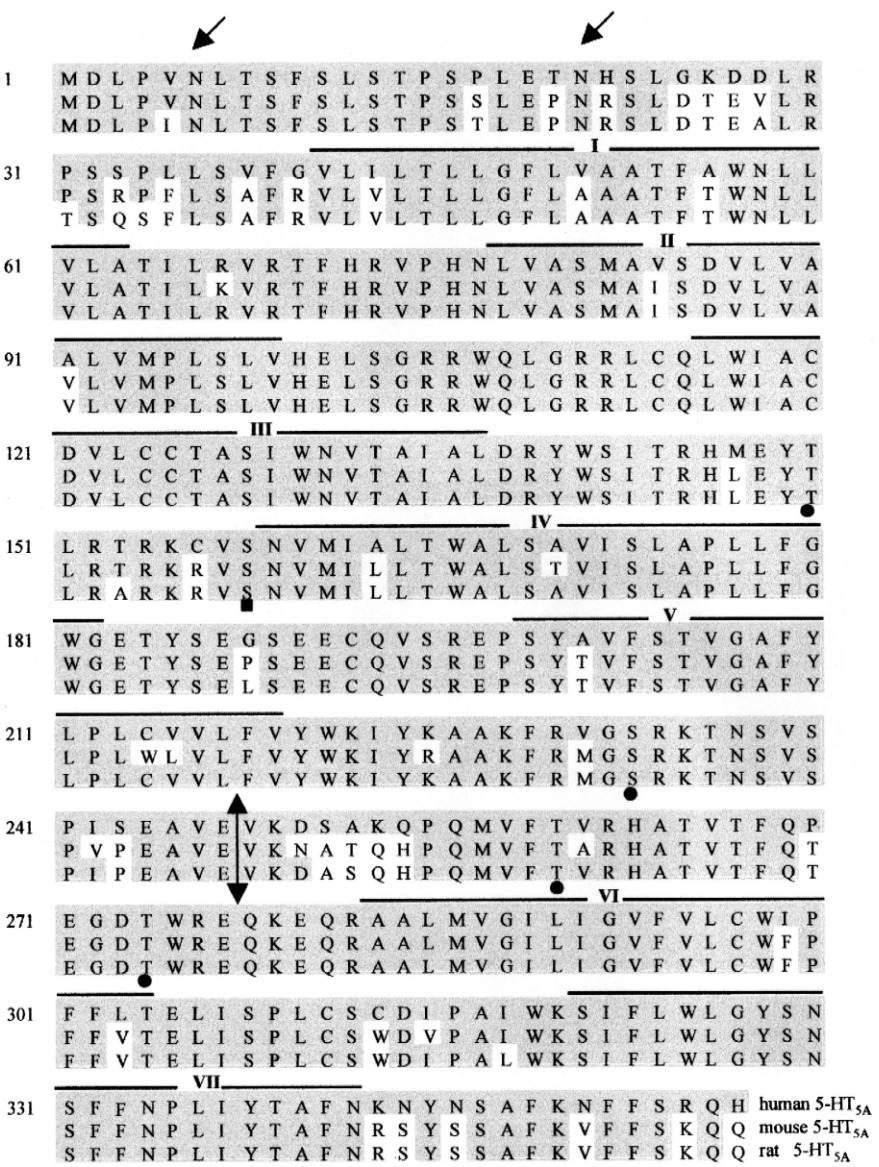

Fig. 1. Amino acid comparisons of the human, mouse and rat 5-HT_{5A} receptors. The putative seven transmembrane domains are indicated by lines at the top of the amino acid sequences and numbered (I–VII). The region of sequence homology shared by the 5-HT_{5A} homologous species is shaded. A simple arrow indicates potential glycosylation sites. A double-headed arrow between transmembrane domains V and VI indicates intron–exon boundary. Square and circle correspond to consensus sites for phosphorylation by protein kinases A and C, respectively.

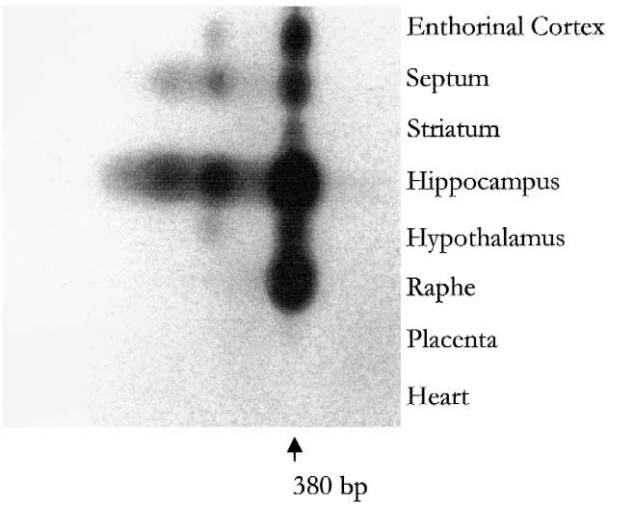

Fig. 2. Distribution of human 5-HT_{5A} transcripts by RT-PCR analysis performed with total RNA from various tissues. A 380-bp specific PCR product (arrow) is detected in all brain regions, but not in peripheral organs tested.

displays 92% amino acids and 82% nucleotide homologies with the mouse 5-HT_{5A} protein and gene, respectively. An identical genomic sequence was reported (Rees et al., 1994), which differed only by a silent polymorphism found at the fourth codon CCT → CCA. The amino-terminal end displayed two putative sites for N-linked glycosylation and the presumed cytoplasmic domains contained putative phosphorylation sites by protein kinase A and C. All sites are conserved in mice and rats (Fig. 1).

3.2. The 5-HT_{5A} receptor is expressed exclusively in the central nervous system

The expression profile of the 5-HT_{5A} receptor was analyzed by RT-PCR in RNA samples extracted from various adult human tissues. Exonic primers that flanked the intronic sequence were selected in order to circumvent the problem of contaminant genomic DNA. A 380-bp fragment corresponding to the expected length of the spliced mRNA was detected in the hippocampus, raphe, hypothalamus, enthorinal cortex, and striatum (Fig. 2). No signal was detected in placenta, heart (Fig. 2), kidney, liver and spleen (data not shown).

3.3. Functional expression of the human 5-HT_{5A} receptor

In order to express the human 5-HT_{5A} protein, both exons were joined using a PCR strategy and cloned into the expression vector, p514 (Green et al., 1988). The recombinant plasmid p514/h5-HT_{5A} was introduced into COS-7 cells by using a calcium phosphate transfection method. Membranes of transfected cells were then assayed for their ability to bind radio-labeled serotoninergic ligands. [^{125}I]LSD and [^{3}H]5-CT displayed a single saturable binding site (Fig. 3). The K_D for [^{125}I]LSD and [^{3}H]5-CT were 187 pM and 2.8 nM, respectively, and the B_{max} were 3.1 pmol and 3.5 pmol/mg of membrane protein, respectively. In the presence of Gpp(NH)p, specific [^{3}H]5-CT binding on COS-7 cell membranes expressing the human 5-HT_{5A} remained the same (data not shown). In contrast, under the same conditions, membranes of COS-7 cells expressing the mouse 5-HT_{1B} receptors showed a reduc-

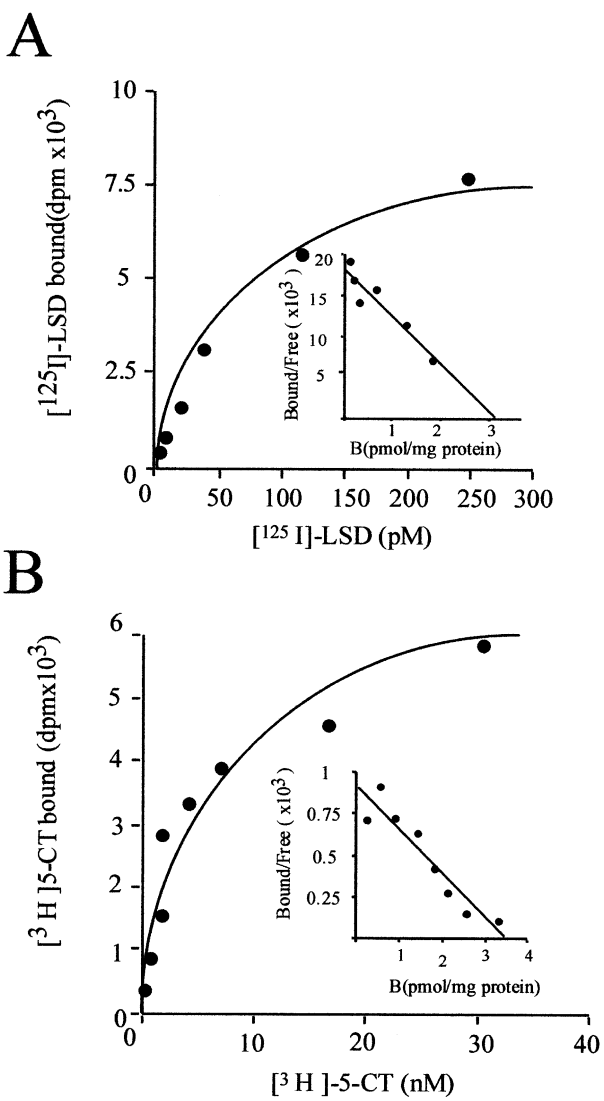

Fig. 3. Saturation isotherm of [^{125}I]LSD binding and [^{3}H]5-CT to membranes of COS-7 cells expressing the human 5-HT_{5A} receptor. (A) Membranes were incubated with concentrations of [^{125}I]LSD ranging from 5 to 250 pM, with or without 10 μM 5-HT. Specific binding is represented. Inset: Scatchard analysis of [^{125}I]LSD binding (K_D = 187 pM, B_{max} = 3.1 pmol receptor/mg of membrane protein). (B) Membranes were incubated with concentrations of [^{3}H]5-CT ranging from 1 to 30 pM with or without 10 μM 5-HT. Inset: Scatchard analysis of [^{3}H]5-CT binding (K_D = 2.8 nM, B_{max} = 3.5 pmol receptor/mg of membrane protein). Data are representative of three independent experiments, with each point being measured in triplicate.

Table 1
Pharmacological profile of the cloned human and mouse 5-HT_{5A} receptors
Binding data correspond to competition for [^{125}I]LSD binding to membranes of COS-7 cells expressing transiently the human 5-HT_{5A} receptor (1st column) and mouse 5-HT_{5A} receptor (2nd column). IC_{50} values required to displace 50% of [^{125}I]LSD were determined experimentally and converted to pK_i values according to the equation $K_i = IC_{50}/(1 + C/K_D)$, where C is the [^{125}I]LSD concentration (150 pM) and K_D is the equilibrium dissociation constant of [^{125}I]LSD. Individual K_i values differed by less than 20%.

	pK_i values	
Radioligand species	[^{125}I]LSD Human	[^{125}I]LSD Mouse
Lisuride	8.7	9.0
Ergotamine	8.0	8.4
5-CT	7.6	7.8
Ritanserin	7.6	7.4
Methiothepin	8.5	7.0
5-HT	6.7	6.6
Clozapine	6.5	5.3
8-OH-DPAT	5.7	5.9
TFMPP	5.7	5.6
Sumatriptan	5.3	4.8

tion of 95% of specific binding in the presence of Gpp(NH)p. These results indicate that in conditions where the 5-HT_{1B} receptor is readily coupled to G proteins, the human 5-HT_{5A} does not appear to be effectively coupled to G proteins in COS-7 cells.

To further characterize the human 5-HT_{5A}-specific binding sites, displacement studies were performed with various drugs. All competition curves were monophasic. The tested compounds displayed the following rank order of potencies: Lisuride > Methiothepin > Ergotamine > 5-CT, Ritanserin > 5-HT > Clozapine > 8-hydoxydipropylamino tetralin; 8-OH-DPAT, 1-(3-trifluoromethylphenyl)piperazine; TFMPP > Sumatriptan (Table 1). These results are in good agreement with affinity constants derived from previous studies performed on the cloned rat and mouse 5-HT_{5A} receptors, except for methiothepin and clozapine that have a higher affinity for the human receptor.

Stable cell lines were generated that express this receptor. HEK-293 and NIH-3T3 cells were chosen because they do not express any endogenous 5-HT receptors. The p514/h5-HT_{5A} expression vector was introduced in these cells together with the neomycin gene encoding resistance to G418. G418-resistant lines were isolated and membranes prepared from these cells were analyzed by binding experiments with [^{125}I]LSD. We selected two cell lines with high expression levels, HEK-5A and NIH-5A. A Scatchard analysis revealed that NIH-5A cells expressed 0.9 pmol/mg of membrane protein and HEK-5A expressed 3.6 pmol/mg of membrane protein (data not shown). HEK-5A and NIH-5A cells were used to investigate a possible effect of the 5-HT_{5A} receptor on second messenger levels. There was no detectable change in basal cAMP levels nor in forskolin induced cAMP levels in response to 5-HT (10 nM–10 μM). We also found no change in the accumulation of inositol phosphates in the HEK-5A and NIH-5A cell lines in response to 5-HT (10 nM–10 μM; data not shown).

In order to study the possible coupling of the 5-HT_{5A} receptor to K^+ channels, we co-expressed the 5-HT_{5A} receptor with the G protein-activated K^+ channel termed $GIRK_1$ in *Xenopus* oocytes (Dascal et al., 1993). The inwardly rectifying K^+ current was activated in the presence of 5-HT when both $GIRK_1$ and 5-HT_{5A} were coinjected, but not when 5-HT_{5A} or $GIRK_1$ alone was injected (Fig. 4). The 5-HT_{5A} receptor response was elicited by 100 μM 5-HT and was reversible upon washout of the drug. Similar inward currents were obtained from oocytes coinjected with 5-HT_{1A} and $GIRK_1$ cRNAs (data not shown).

3.4. Isolation of human genomic DNA encoding the 5-HT_{5B} gene

Two different fragments of mouse 5-HT_{5B} genes, spanning exon I and exon II, were amplified by PCR from mouse cDNA clones and used to screen a human genomic library. No single phage was isolated that hybridized to both exon DNA probes suggesting the existence of a large intron. Human 5-HT_{5B} exons I and II sequences have been deposited with EMBL Nucleotide Sequence Database, having accession numbers AJ308679 and AJ308680, respectively. Comparison of human 5-HT_{5B} exon sequences with homologous genomic sequences using BLAST 2.0 was used to identify human genomic fragment containing both exons. Analysis of the *Homo sapiens* bacterial artificial chromosome (BAC) clone, RP11-28H22 showed that the 5-HT_{5B} intron is 43-kbp long, located in the middle of the coding sequence corresponding to the third cytoplasmic loop. The intron–exon boundary is conserved and is at the same position as in the mouse 5-HT_{5B} gene (Matthes et al.,

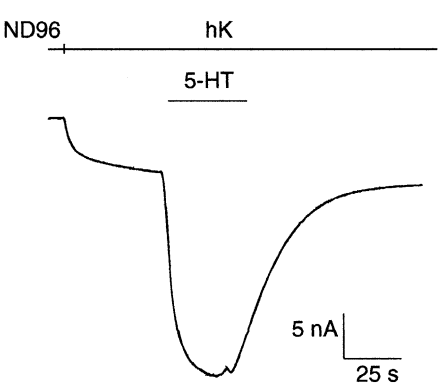

Fig. 4. K^+ currents induced by 5-HT in oocytes injected with RNAs encoding 5-HT_{5A} receptor and potassium channel subunits $GIRK_1$. Inward currents evoked by the perfusion of the hK solution and 100 nM 5-HT. The switch from ND96 to hK solution is indicated on the horizontal line above the current traces.

1993). In contrast to the 5-HT_{5A} gene, the human 5-HT_{5B} open reading frame encodes a shorter protein, when compare to the mouse 5-HT_{5B} gene. The nucleotide sequence of human 5-HT_{5B} exon I was found to be only 75% homologous to the mouse 5-HT_{5B} gene and 58% homologous to the mouse 5-HT_{5A} gene. Exon I is interrupted by five stop codons, one 35-bp repeat sequence and one 7-bp insert (Fig. 5). Surprisingly, the nucleotide sequence of exon II is 90% homologous to the mouse exon II and does not contain any frame interruptions. As a result two open reading frames can be found: the first starts before the putative transmembrane domain V in exon I ; the second at the end of exon I. Both extend throughout exon II. The second putative open reading frame is preceded by a perfect Kozak sequence (Fig. 5) (Kozak, 1987). The translation product of such a transcript would be a protein containing the transmembrane domains V to VII. Such transcription products would likely result in a non-functional truncated protein (Heymann and Subramaniam, 1997). To determine whether the human 5-HT_{5B} gene is

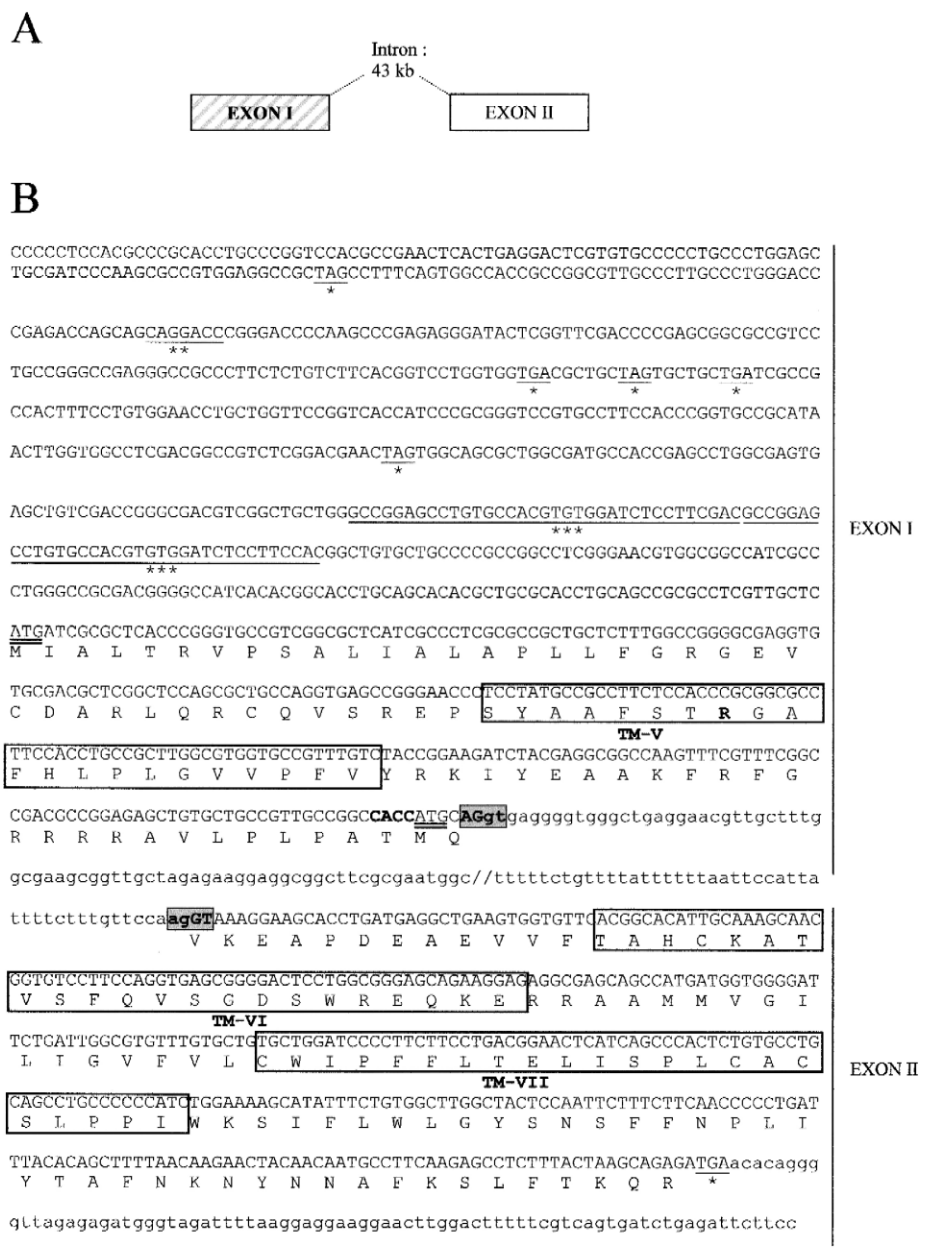

Fig. 5. Sequences of both exons and exon–intron boundary of the human 5-HT_{5B} gene. The sequences of exons are shown in capital letters and intron sequences in lowercase letters. The deduced amino acid sequences are listed underneath the DNA sequences. Regions disrupting the coding sequence, such as stop codons (*), a 7-bp insert (* *) and a 35-bp repeat sequence (* * *) are shown. The putative transmembrane domains are boxed and numbered (V to VII). Two putative start codons are double-underlined preceded by kozak sequence (in bold). Filled box indicates splicing sites.

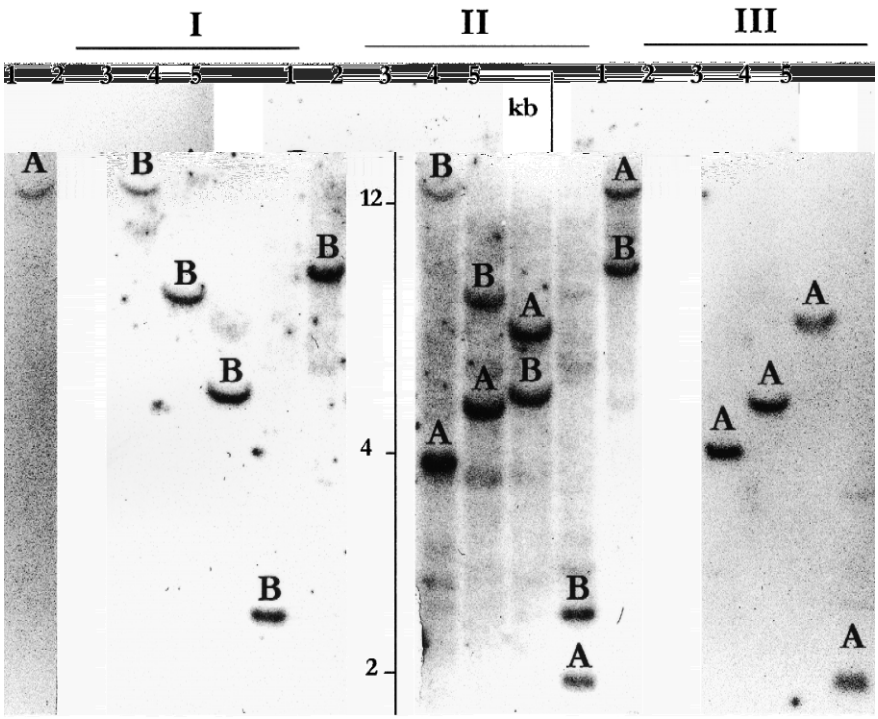

Fig. 6. Detection of 5-HT_{5A} and 5-HT_{5B} genes in human genome by Southern analysis. Human genomic DNA was digested with five different restriction enzymes, i.e *Sac*I(1), *Hin*dIII(2), *Asp*400(3), *Bam*hI(4), and *Kpn*I(5) and subjected to Southern blot analysis using the radio-labeled fragments of the exon I from the mouse 5-HT_{5B} (I), the human 5-HT_{5A} (II) and the human 5-HT_{5B} (III) genes. The letters A and B correspond to the DNA fragment belonging to the human 5-HT_{5A} exon I and the human 5-HT_{5B} exon I, respectively.

transcribed, we performed a RT-PCR experiment on adult brain RNA extracts. Using specific primers surrounding the intronic sequence, RT-PCR products were analyzed by Southern blot. A faint signal corresponding to the spliced human 5-HT_{5B} mRNA was found in human brain tissue suggesting a very low level of transcription of this gene (data not shown).

In order to demonstrate that the non-functional gene that we isolated is the only human 5-HT_{5B} gene, we screened the entire human genome by Southern analysis. Human genomic DNA was digested with five different restriction enzymes: *Sac*I, *Hin*dIII, *Asp*400, *Bam*hI, *Kpn*I and subjected to Southern blot analysis using probes corresponding to mouse 5-HT_{5B} exon I, human 5-HT_{5A} exon I, and human 5-HT_{5B} exon I (Fig. 6). Under low stringency hybridization condition, using a mouse 5-HT_{5B} exon I DNA probe (probe I), two genomic fragments were detected for each digest suggesting the existence of two genes with a strong homology for the mouse 5-HT_{5B}. Under stringent hybridization conditions, the same blots were probed successively with a human 5-HT_{5A} exon I DNA probe (probe II) and then with a human 5-HT_{5B} exon I DNA probe (probe III). Each probe revealed one of the two bands found previously. The sum of the patterns obtained with probe II and probe III is equal to the pattern found with probe I. This result indicates that the only

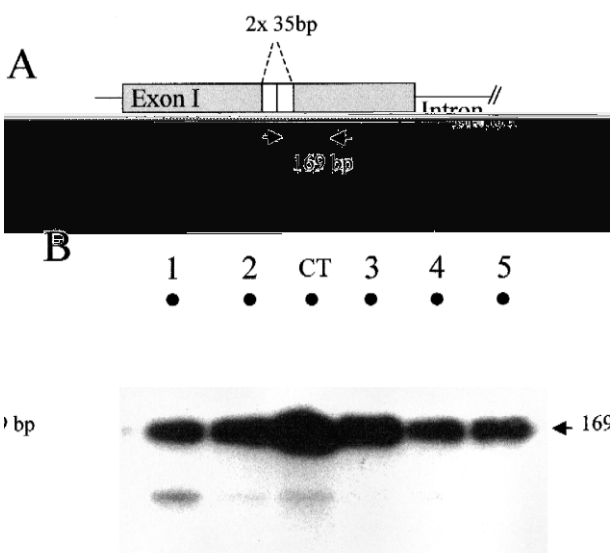

Fig. 7. PCR analysis of the non-functional 5-HT_{5B} gene in humans. (A) Schematic representation of the human 5-HT_{5B} exon I (filled box). Primers for PCR amplification are indicated by arrows. The 35-bp repeat sequence within exon I is indicated by the unfilled box. (B) DNA samples from different subjects with different ethnical backgrounds: Asian (1), African-American (2), Caucasian (3), Asian (4), and African-American (5) were used as templates for the PCR reaction. A 169-bp specific PCR product corresponding to the non-functional 5-HT_{5B} is detected for each subject. We used a template from the 5-HT_{5B} fragment isolated from the genomic library as a positive control (CT).

human DNA fragments homologous to the mouse 5-HT_{5B} probe are the functional 5-HT_{5A} gene and the non-functional human 5-HT_{5B} gene. These data are consistent with previous studies of chromosome in situ hybridization which have revealed a unique locus for the human 5-HT_{5B} gene (Danielson et al., 1994; Matthes et al., 1993).

To verify that the non-functional 5-HT_{5B} gene is present in a variety of individuals, PCR experiments were performed on five genomic DNA samples extracted from human tissues with various ethnic backgrounds (Fig. 7). PCR analyses were performed using specific primers located on each side of the 35-bp repeat sequence of the 5-HT_{5B} gene because this insert alone is sufficient to render the 5-HT_{5B} gene non-functional. PCR products were subjected to Southern blot analysis using a specific human 5-HT_{5B} oligo probe. As shown in Fig. 7, a specific 162 bp PCR product was found in each sample. This PCR fragment corresponds to the non-functional 5-HT_{5B} gene.

4. Discussion

4.1. The human 5-HT_{5A} receptor is expressed and functional

mRNA encoding the human 5-HT_{5A} receptor was found exclusively in the central nervous system and predominantly in cortex, hippocampus and cerebellum. Our RT-PCR data are in good agreement with two previous studies that localized the human 5-HT_{5A} mRNA by RT-PCR and in-situ hybridization. This mRNA distribution is also similar to the distribution of the 5-HT_{5A} mRNA in rat and mouse.

While the localization of the 5-HT_{5A} receptor has not been reported in humans, the distribution of this protein was recently characterized in mice (Grailhe et al., 1999). The mouse 5-HT_{5A} receptor was found predominantly in cortex, hippocampus and olfactory bulb, which are also the areas that contains the highest levels of 5-HT_{5A} mRNA. It is therefore likely that the human 5-HT_{5A} receptor will also be localized in the same structures as the human 5-HT_{5A} mRNA. An earlier report had suggested that the 5-HT_{5A} receptor is also expressed in glial cells (Carson et al., 1996). However, we showed that the antibodies used in that study are not specific for the 5-HT_{5A} receptor (Grailhe et al., 1999).

Initial reports aimed at studying the coupling of 5-HT_{5A} receptor to second messengers were not successful (Erlander et al., 1993; Plassat et al., 1992). Specifically, the mouse and rat 5-HT_{5A} receptor did not elicit any change in cAMP or IP_3 levels when expressed in a number of different cell lines. In agreement with these studies, we did not find any coupling of the human 5-HT_{5A} with either the cAMP or the IP_3 pathways in both HEK-293 and NIH-3T3 cell lines.

However, in the mouse brain, the 5-HT_{5A} receptor was shown to be coupled with G protein (Grailhe et al., 1999; Waeber et al., 1998). In addition, two recent studies have reported a coupling of the 5-HT_{5A} receptor with a pertussis toxin-sensitive G protein and with a weak inhibition of adenylate cyclase activity (Francken et al., 1998; Hurley et al., 1998). Discrepancies between early studies and the more recent ones may be explained either by the recent use of a more sensitive cAMP assay or by the fact that the latest studies were performed in 5-HT-free medium. The latter explanation suggests that the 5-HT_{5A} receptor is rapidly desensitized by the serotonin present in the fetal calf serum.

Interestingly, while we found no coupling of the human 5-HT_{5A} receptor in two cell lines, we were able to detect a coupling with an inwardly rectifying K^+ channel in *Xenopus* oocytes. A number of Gi-coupled receptors have been shown to activate $GIRK_1$ channels in oocytes. Our results are therefore consistent with the negative coupling to adenylate cyclase reported in cell lines.

Concerning the function of the 5-HT_{5A} receptor, the only indication we have so far is that mice lacking this receptor exhibit increased exploratory behavior and altered locomotor response to LSD. It is not clear at present whether the phenotype of the knockout mice reflects the acute absence of the receptor or plastic changes that took place during the development of the knockout mice. However, it is worth noticing that the human 5-HT_{5A} receptor has a high affinity for LSD and that functional studies have revealed that LSD is a partial agonist of the 5-HT_{5A} receptor (Francken et al., 1998). It is therefore possible that activation of the 5-HT_{5A} receptor contributes to some of the psychotropic effects of LSD. Confirmation of such a hypothesis will have to await the availability of specific ligands (Teitler et al., 1998).

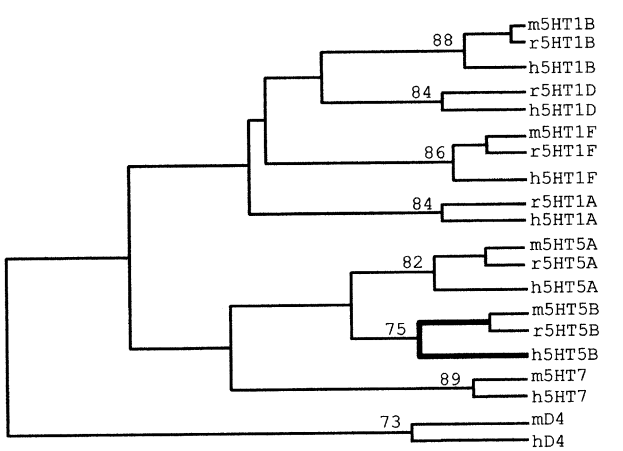

Fig. 8. Phylogenetic tree. Nucleotide sequence comparisons of the entire coding region revealed homologies within a family of seven transmembrane domain 5-HT and dopamine receptor genes. This representation illustrates the loss of homology of the 5-HT_{5B} from rodent to human species scoring 75% as compared to the 5-HT receptor gene scoring between 82% and 89% homologies.

4.2. The human 5-HT_{5B} is non-functional

We have shown that the human 5-HT_{5B} receptor does not encode a functional protein because the coding sequence is interrupted by stop codons. The 5-HT_{5B} receptor therefore belongs to a very small group of genes that have been lost following the divergence of rodents and humans. The loss of a gene during evolution may be due either to the loss of the corresponding function and/or to an evolutionary advantage that would be gained from this loss. In others words the 5-HT_{5B} receptor may either fulfill a function no longer needed or reduced in humans such as pheromone perception or alternatively the disappearance of the 5-HT_{5B} receptor may be advantageous. For example, a decrease in aggressive behavior may allow for more social interactions and favors certain species. The only hint we have about the function of the 5-HT_{5B} receptor in rodents comes from its pattern of expression, which is highly specific. In mice and rat species, the 5-HT_{5B} receptor is expressed exclusively in CA1 pyramidal neurons and in the habenula (Matthes et al., 1993; Wisden et al., 1993). This restricted pattern of expression may suggest a specific function. An additional example of gene lost between rodents and human is urate oxidase. Interestingly, while this gene is dispensable in human, it is essential for rodents since its ablation can be lethal (Wu et al., 1994). The D_4 dopamine receptor is an example of G protein-coupled receptor that although expressed in human has diverged significantly from its mouse counterpart. In fact among G protein-coupled receptors, D_4 displays the least homology between mice and humans (Fig. 8). In addition, within humans, the dopamine D_4 receptor displays more sequence variability than other receptors. Finally, null mutations have been identified in the human population (Nothen et al., 1994). It is therefore tempting to speculate that selective pressure in the sequence of the D_4 receptor is decreasing and that the D_4 receptor is about to disappear. In order to gain further insights into the reason for the disappearance of the 5-HT_{5B} receptor, it would be interesting to investigate its function in rodents and to determine in which species the 5-HT_{5B} receptor become mutated after the divergence between rodents and humans.

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