0026-895X/95/030407-10$3.00/0

MOLECULAR PHARMACOLOGY, 48:407–416 (1995).

Molecular Cloning of Human 5-Hydroxytryptamine$_3$ Receptor: Heterogeneity in Distribution and Function among Species

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Received May 16, 1995; Accepted June 20, 1995

SUMMARY

The 5-hydroxytryptamine$_3$ receptor 5-HT$_3$R has been implicated in gut and cardiac motility and in behavioral disorders. Characteristics of 5-HT$_3$Rs appear to be heterogeneous among species, but human 5-HT$_3$R cDNA has not been identified. We isolated a cDNA encoding 5-HT$_3$R from human hippocampus. The mouse 5-HT$_3$R gene has been reported to generate two alternative splicing isoforms that differ by six amino acids. All of our isolated human clones corresponded to the shorter isoform. Amino acid identities with mouse neuroblastoma N1E-115 and rat brain 5-HT$_3$Rs were 84% for each. Southern blot analysis of human genomic DNA suggested that our cloned transcript encoded a human counterpart for the rodent 5-HT$_3$Rs. This gene was assigned to chromosome 11 using polymerase chain reaction analysis of a human/rodent somatic cell hybrid panel. With the use of Northern blot analysis, 5-HT$_3$R transcripts were identified in human small intestine, colon, and brain regions including hippocampus, amygdala, and striatum. In human heart, 5-HT$_3$R expression was not detectable even with reverse transcriptase-polymerase chain reaction analysis, although it was detectable in mouse heart. Transfection of COS-1 with human 5-HT$_3$R cDNA induced specific binding of the 5-HT$_3$R-selective radioligand [^{3}H]YM060. Human 5-HT$_3$R showed typical characteristics of the 5-HT$_3$R, but its affinity for the 5-HT$_3$R agonist *m*-chlorophenylbiguanide was much lower than that of rat 5-HT$_3$R. When injected with human 5-HT$_3$R cRNA, the oocytes responded to 5-HT$_3$R agonists with a rapidly developing inward current. The potency of the agonists to induce inward current paralleled that to compete with the radioligand binding, and 2-methyl-5-hydroxytryptamine, a partial agonist for mouse 5-HT$_3$R, was a full agonist for human 5-HT$_3$R. Our data revealed that the 5-HT$_3$R molecule has interspecies differences in both tissue distribution and functional profile.

5-HT is an important neurotransmitter in the central and peripheral nervous systems. Receptors mediating the actions of 5-HT are pharmacologically classified into four major groups: 5-HT$_1$, 5-HT$_2$, 5-HT$_3$ and 5-HT$_4$ (1). The 5-HT$_3$R is a ligand-gated ion channel, whereas all other subtypes are G protein-coupled receptors. The characterization of 5-HT$_3$Rs has been facilitated by the development of selective ligands for this subtype. In animal models, 5-HT$_3$R-selective antagonists have a variety of potential therapeutic actions for behavioral disorders, such as anxiolytic and antipsychotic actions, and facilitation of withdrawal from drug abuse (2). However, the distribution of 5-HT$_3$R radioligand binding sites within the human brain is unlike that of rodents (3, 4). In the periphery, 5-HT$_3$Rs have been characterized on the basis of such physiological fields as depolarization of the vagus nerve, intestinal contraction, and tachycardia and bradycardia in heart (1). Bradycardia induced by 5-HT has been reported to be regulated by 5-HT$_3$R in most species, whereas 5-HT-induced cardiac stimulation, including tachycardia, appears to be mediated by different receptors in different species (5). Thus, in both the central and peripheral nervous systems, the responses mediated by 5-HT$_3$Rs are likely to be heterogeneous among species. Considering the various potential therapeutic indications of 5-HT$_3$Rs, it is of interest to determine the differences between humans and animals. In addition to heterogeneous distribution among species, previous findings suggest the possibility that 5-HT$_3$Rs exist as multiple subtypes within a single species (1, 6). Thus, the 5-HT$_3$R-selective radioligands that have been identified may not recognize single populations of 5-HT$_3$Rs. Furthermore, the pharmacological heterogeneity of 5-HT$_3$Rs among species (1) confuses elucidation of characteristics of 5-HT$_3$Rs through binding analysis only. Molecular cloning of 5-HT$_3$Rs will allow the elucidation of the characteristics of the single populations of 5-HT$_3$R.

The nucleotide sequences of human and rat 5-HT$_3$R cDNAs have been submitted to the GenBank with accession numbers D49394 and D49395, respectively.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-HT$_3$R, 5-HT$_3$ receptor; mCPBG, *m*-chlorophenylbiguanide; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; RT-PCR, reverse-transcriptase polymerase chain reaction; RACE, rapid amplification of cDNA ends; nAchR, nicotinic acetylcholine receptor; SSC, standard saline citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Isolation of 5-HT_3R cDNA was initially reported in the mouse neuroblastoma cell line NCB20 (7). Its splice variant has since been isolated from the other mouse neuroblastoma lines N1E-115 (8) and NG108–15 (9) and from mouse brain (10), which encodes a shorter isoform with a six-amino acid deletion. Rat 5-HT_3R cDNA has also been isolated from superior cervical ganglion (11), although the coding region was not completely included. In the present study, we isolated human 5-HT_3R cDNA from hippocampus and determined the interspecies heterogeneity in tissue distribution of 5-HT_3R. Furthermore, we compared the functional profile of recombinant human 5-HT_3R with that of recombinant rat 5-HT_3R after isolating the cDNA with the entire coding region from rat brain.

Experimental Procedures

Materials. 5-HT, mCPBG, 2-Me-5-HT, and methysergide were purchased from Research Biochemical (Natick, MA). YM060 (12), YM114 (KAE-393) (13), ondansetron, tropisetron, and granisetron were synthesized in our institute. [^{3}H]YM060 (78 Ci/mmol) was synthesized by Amersham. The oligonucleotide primers used for RT-PCR were as follows: MS1, 5′tca acg tgg atg aga aga acc3′; MS2, 5′aat cct gct tat gca cca gc3′; HS11, 5′cgg agg cac tcc tat gct tg3′; HS12, 5′aag cca tgg tga gct gct g3′; S1, 5′cat ctg gga agc ttg cca tg3′; S6, 5′agg atg cag gac tca cat cc3′; S8, 5′aag cca tga tag cga agt cg3′; G1, 5′cca tca cca tct tcc agg ag3′; G2, 5′cct gct tca cca cct tct tg3′; C1, 5′gac agc atc tgg gtc cc3′; and C2, 5′agc aga gcc atg cac ac3′.

Isolation of human 5-HT_3R cDNA. For cloning of a partial fragment of human 5-HT_3R cDNA, first strand cDNA was synthesized from human brain poly(A)$^+$ RNA (1 μg; Clontech) and applied to PCR amplification with the primers MS1 and MS2. PCR was performed in a 50-μl mixture with 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 μg/ml gelatin, all four dNTPs (each at 0.2 mM), each primer at 0.25 μM, and 1.25 units of recombinant *Taq* DNA polymerase (Takara Shuzo, Japan) using a Perkin-Elmer GeneAmp PCR System 9600. Cycles were as follows: initial denaturation at 94° for 1 min; 40 cycles of denaturation at 94° for 1 min, annealing at 59° for 1 min, and extension at 72° for 2 min; and a final hold at 72° for 10 min.

RACE was performed with human hippocampus poly(A)$^+$ RNA (2 μg; Clontech) according to the manufacturer's instructions with a 5′- or 3′-AmpliFINDER RACE Kit (Clontech). For 5′-RACE, the anchor oligonucleotide was ligated to the 3′ end of first strand cDNA primed by a gene-specific primer (antisense 638–656). The anchor-ligated cDNA was used as a template for PCR amplification with a primer complementary to the anchor and a nested primer (antisense 605–629). An aliquot (1/50 vol) of the PCR products was applied to a secondary PCR with the anchor primer and another nested primer (antisense 580–603). For 3′-RACE, cDNA synthesis was primed by a modified oligo(dT) primer containing an anchor sequence. A primary PCR was performed with a gene-specific primer (sense 1043–1066) and a primer complementary to the anchor sequence, followed by a secondary PCR with a nested primer (sense 1075–1098) and the anchor primer. Each PCR was performed as described above, except for 35 cycles at 94° for 45 sec, 60° for 45 sec, and 72° for 2 or 3 min.

To obtain full-length coding region, oligo(dT)-primed cDNA of human hippocampus was used as a template for PCR with the gene-specific primers HS11 and HS12. PCR was performed in a 50-μl mixture with 20 mM Tris·HCl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml bovine serum albumin, all four dNTPs (each at 0.2 mM), each primer at 0.25 μM, and 2.5 units of *pfu* DNA polymerase (Stratagene). Thirty-five cycles were performed as follows: 94° for 1 min, 66° for 1 min, and 72° for 3 min.

All PCR amplifications were done according to the Hot Start method (14) with AmpliWax (Perkin-Elmer). Amplified fragments using *Taq* and *pfu* were cloned into the plasmid pCRII (Invitrogen) and pCR-Script SK(+) (Stratagene), respectively.

Isolation of rat 5-HT_3R cDNA. Rat 5-HT_3R cDNA was amplified from random-primed cDNA of rat brain by PCR amplification. Because the cDNA sequence corresponding to the putative signal peptide of rat 5-HT_3R had not been published, the forward primer S1 was designed on the basis of the sequence adjacent to the translation initiation codon of mouse 5-HT_3R cDNA. The reverse primers S6 and S8 were based on the published sequence of rat 5-HT_3R cDNA. The deduced amino acid sequence of an amplified cDNA (1.5 kbp) was identical to that reported by Isenberg *et al.* (11), except Arg^{283} (CGC) was replaced by Gly (GGC) in the mature polypeptide.

Nucleotide sequence determination and analysis. Both strands of each fragment were sequenced with the use of *Taq* Dye Deoxy Terminator Cycle Sequencing Kit and an ABI 373A DNA Sequencer (Applied Biosystems). Nucleotide and amino acid sequences were analyzed with GeneWorks software (IntelliGenetics, Mountain View, CA). Fig. 1 shows consensus sequences involving no base misincorporated by PCR.

Southern blot analysis of human genomic DNA. Ten micrograms of human genomic DNA were digested with either *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Nco*I, or *Spe*I; separated by electrophoresis on a 0.7% agarose gel; and transferred to Biodyne A (Pall BioSupport). *Nco*I-digested DNA fragments (nucleotide 1358–1657) of PCR products generated with the primers HS17 and HS20 were radiolabeled with [α-^{32}P]dCTP by priming with random primers and used as a probe. The membrane was hybridized with the ^{32}P-labeled probe overnight at 65° with 5 × SSC (1 × SSC = 150 mM NaCl, 15mM sodium citrate, pH 7.4), 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml sheared denatured salmon sperm DNA and then washed twice for 20 min in 0.5 × SSC, 0.1% SDS at 60°. The blot was exposed to an X-ray film with two intensifying screens at −80° for 4 days.

Somatic cell hybrid analysis. Human 5-HT_3R gene was assigned by PCR analysis of the BIOMAPS Somatic Cell Hybrid Products (BIOS Laboratories, New Haven, CT). The PCR amplification was performed with specific forward primer (sense 1708–1727) and reverse primer (antisense 1884–1902) as follows: initial denaturation at 94° for 1 min; 30 cycles of 94° for 30 sec, 58° for 30 sec, and 72° for 30 sec; and a final hold at 72° for 10 min. Chromosomal assignment was performed with the use of the discordance table.

Northern blot and RT-PCR analysis of mRNA. Poly(A)$^+$ RNAs of human and mouse tissue were purchased from Clontech. Rat tissue poly(A)$^+$ RNAs were prepared by guanidine thiocyanate extraction followed by oligo(dT)-cellulose chromatography (15).

Northern blot analysis of mRNA was performed with membranes of Human Multiple Tissue Northern Blot (Clontech), on which ~2 μg of poly(A)$^+$ RNA per lane had been immobilized. The membranes were hybridized overnight with the probe as described at 42° with 50% formamide, 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 10 × Denhardt's solution, 2% SDS, and 100 μg/ml sheared denatured salmon sperm DNA and then washed twice for 20 min in 0.2 × SSC and 0.1% SDS at 55°. The blots were exposed to X-ray films with two intensifying screens at −80° for 12 days.

RT-PCR analysis of mRNA was performed with random-primed cDNA synthesized from each poly(A)$^+$ RNA (1 μg). The efficiency of poly(A)$^+$ RNA isolation and cDNA synthesis was estimated by PCR (25 cycles) with the primers G1 and G2 based on common sequence among human, rat, and mouse glyceraldehyde 3-phosphate dehydrogenase cDNAs. To detect 5-HT_3R transcripts, the primers C1 and C2 were derived from common sequences among human, rat, and mouse 5-HT_3R cDNAs. Twenty cycles were performed (94°, 62°, and 72° every 1 min). Ten-microliter aliquots of PCR products were separated by electrophoresis on a 1.5% agarose gel and transferred to Biodyne A. Full-length human 5-HT_3R cDNA was used as a probe. Hybridization and washing were done under the same conditions as genomic Southern analysis. The blot was exposed to an X-ray film with two intensifying screens at −80° for 1 days.

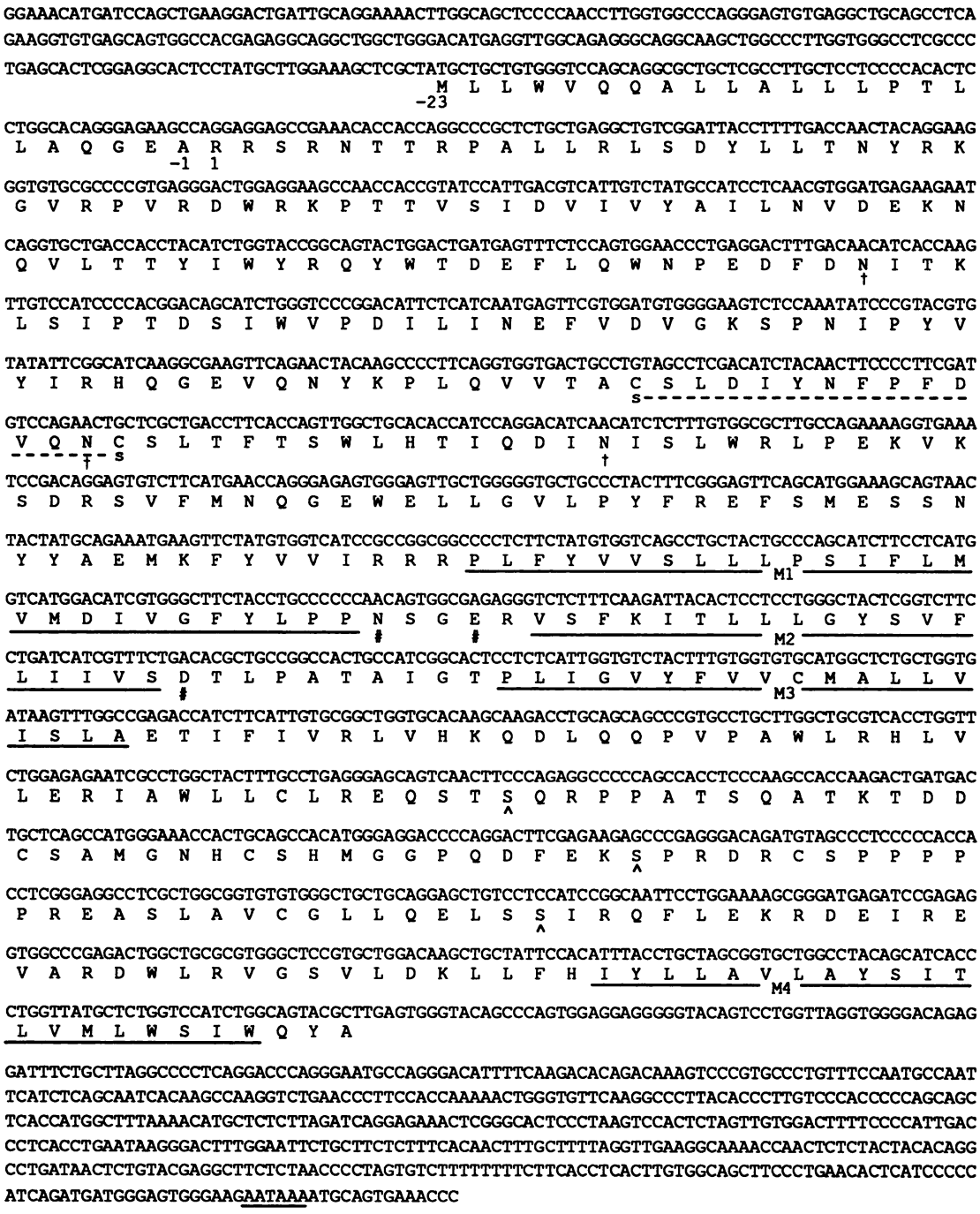

Fig. 1. Structure of human 5-HT$_3$R transcript. *Solid lines labeled M1–M4*, the putative transmembrane domains predicted by Kyte-Doolittle criteria and similarity with rodent 5-HT$_3$Rs. Numbering of amino acid corresponds to the putative mature polypeptide. *Underline*, a putative polyadenylation signal addition site; *S–S*, Cys-Cys loop; *, potential *N*-glycosylation sites; *caret*, protein kinase C phosphorylation sites; #, amino acids corresponding to three rings adjacent to the M2 segment.

Radioligand binding studies. A cDNA fragment containing the entire coding region of human 5-HT$_3$R (1.7 kbp) or rat 5-HT$_3$R (1.5 kbp) was subcloned into the mammalian expression vector pEF-BOS (16). COS-1 cells were transfected with each plasmid as described previously (17). COS-1 (1–2 × 10^6 cells) were incubated overnight, exposed to the plasmid DNA (15 μg) with DEAE-dextran (0.25 mg/ml) for 14 hr, and then exposed to 0.1 mM chloroquine for 2.5 hr. After a 3-day culture, the transfected cells were homogenized in 50 mM HEPES, pH 7.4, and centrifuged at 48,000 × *g* for 10 min. Membranes (5–50 μg protein) were incubated with the indicated concentrations of [^{3}H]YM060 for 30 min at 25°. The reactions were terminated by rapid filtration and washing with ice-cold HEPES buffer through Whatman GF/B filters using a Brandel Cell Harvester. Radioactivity of the filter was counted with a Packard 2000CA liquid scintillation counter. Nonspecific binding was determined in the presence of 1 μM tropisetron. Data were analyzed with a nonlinear curve fitting to the Hill equation. K_i values were calculated with the following equation: $K_i = IC_{50}/(1 + [L]/K_d)$.

Elecrophysiological studies. Currents were measured in *Xenopus* oocytes expressing human 5-HT$_3$R as previously described (18). For *in vitro* transcription, pCR-Script SK(+) containing human 5-HT$_3$R cDNA was linearized with *Bam*HI and transcribed with T3

RNA polymerase (Stratagene) in the presence of the cap analog $m^7G(5')ppp(5')G$. The synthesized cRNA was purified with a Sephadex G-50 spin column. Oocytes were injected with 0.9–21 ng of the cRNA and incubated in a modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 15 mM HEPES, pH 7.6), at 19° for 2–7 days. Electrophysiological experiments were performed with a conventional two-microelectrode voltage clamp technique. The microelectrodes were filled with 3 M KCl, which had resistances between 1 and 4 MΩ. Defolliculated oocytes were continuously perfused at the rate of 5 ml/min in bathing solution (112 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.2), and voltage clamped at −60 mV. Drugs were incubated in the bathing solution during the indicated periods. Dose-response curves for agonists were fitted to the Hill equation.

Results

Structure of human 5-HT_3R. We first isolated a partial fragment of human 5-HT_3R cDNA from human brain by RT-PCR cloning. Among mammalian 5-HT_3 (7–11) and nAchR α7 genes (19, 20) analyzed to date, the exonic sequences adjacent to intron 2 are extremely conserved; in particular, the 16-nucleotide sequence is identical. The forward primer MS1 was designed on the basis of this sequence. The reverse primer MS2 was based on a common sequence between mouse and rat 5-HT_3R cDNA encoding the putative large intracellular loop. RT-PCR amplified 0.8-kbp DNA fragments, which had significant similarity to rodent 5-HT_3R. Human hippocampus poly(A)$^+$ RNA was positive for RT-PCR analysis with the specific primers for the above fragment. To obtain both remaining cDNA ends, we performed RACE with this poly(A)$^+$ RNA. RACE amplified 0.6- and 1.2-kbp fragments as 5′ and 3′ end cDNAs, respectively. Full-length coding region was amplified from hippocampus poly(A)$^+$ RNA with the primers HS11 and HS12 based on the sequences of both RACE products. The amplified fragments contained an identical sequence to the fragment initially isolated and had an open reading frame with 83% identity to the coding region of the short isoform of mouse 5-HT_3R cDNA (8). All 14 of our isolated human clones corresponded to the shorter isoform of mouse 5-HT_3R.

Fig. 1 shows the nucleotide sequence of complete cDNA. The 2202-nucleotide sequence had an open reading frame (nucleotide 220-1656) encoding a 478-amino acid protein with a potential signal peptide of 23 residues. The cDNA contained a putative polyadenylation signal addition site (AATAAA) followed by a poly(A) tail. The ATG at codon –23 was assigned as a translation initiation codon due to a close match with the Kozak consensus sequence (21) and similarity with the sequences of rodent 5-HT_3R. However, another in-frame ATG was present at position 202. The true initiation site will be determined by amino acid sequencing of the purified immature receptor protein. Also, two in-frame short open reading frames (nucleotides 7–27 and 133–183) preceded the initiation codon. The short open reading frame in the 5′-untranslated region has been found in transcripts encoding proto-oncoproteins, growth factors, and cell surface receptors and is well known to be involved in the translational regulation of gene expression (22). Thus, expression of human 5-HT_3R may be regulated posttranslationally.

Fig. 2 shows alignment of the amino acid sequences of human hippocampus 5-HT_3R, mouse N1E-115 5-HT_3R (8), and rat brain 5-HT_3R, whose cDNA was isolated with RT-PCR (see Experimental procedures). Amino acid identity of human 5-HT_3R with mouse or rat 5-HT_3R was 84%, less than that between mouse and rat receptors (94% identity). Deletion of five amino acids existed in the amino-terminal region of the predicted mature protein compared with mouse and rat receptors.

Hydrophobicity analysis of the deduced amino acid sequence showed the typical features of ligand-gated ion channels, with four putative transmembrane domains (M1-M4), a large amino-terminal extracellular domain, and a large cytoplasmic loop. Also, the amino-terminal extracellular domain contained the Cys-Cys loop typical of nicotinic, glycine, and $GABA_A$ receptor channels. Potential sites for *N*-glycosylation and protein kinase C phosphorylation were shared with rodent 5-HT_3Rs, except an additional site for protein kinase C phosphorylation. The M2 segment, which is believed to constitute the channel pore, was completely conserved. Adjacent to the M2 segment, Glu^{249} and Asp^{270} were shared with rodent 5-HT_3Rs, but Asn^{246} was not, although the positions of these amino acids were located in three charged rings that have been reported to be determinants of the ion channel properties of nAchRs.

Genomic Southern blot analysis. Our cloned cDNA from human hippocampus showed significant similarity with rodent 5-HT_3R but had several structural differences, as described. To determine whether our cloned cDNA encodes a human counterpart for previously cloned rodent 5-HT_3Rs, we performed Southern blot analysis of human genomic DNA with a partial fragment of cur cloned cDNA corresponding to coding region exon 9 in mouse 5-HT_3R gene as a probe. Hybridization of human genomic DNA with this probe indicated the existence of a single gene. Even under conditions that allow the human probe to hybridize to picogram quantities of rat 5-HT_3R cDNA, no second related gene was detected (Fig. 3). These results suggest that our cloned transcript is a human counterpart for rodent 5-HT_3R transcripts.

Chromosomal localization. To determine chromosomal localization of human 5-HT_3R gene, we screened DNA from human/rodent somatic cell hybrids using PCR analysis. A set of specific primers were designed on the basis of the sequence of the 3′-noncoding region of human 5-HT_3R cDNA (sense 1708–1727 and antisense 1884–1902), which was not conserved among species. The 195-bp fragment was amplified from human genomic DNA but not from rodent (mouse or hamster) genomic DNA. Among 20 somatic cell hybrids, the 195-bp PCR product was detected only in the hybrid (1049) containing chromosome 11. Human 5-HT_3R gene had 0% discordance only with chromosome 11, indicating that this gene is located in chromosome 11. Uetz *et al.* (10) reported obtaining the same result using cross-hybridization analysis with mouse 5-HT_3R probe. Their assignment is verified by the present study, in which we use specific sequence to human 5-HT_3R.

Tissue distribution. Tissue distribution of human 5-HT_3R transcripts was elucidated with Northern blot analysis. As shown in Fig. 4, ~2.4 kb of mRNA was detected in human small intestine, colon, and brain regions including the amygdala, hippocampus, and caudate nucleus. Slight signals were detected in spleen, thymus, and prostate. The existence of intestinal 5-HT_3R has been suggested through binding studies in guinea pigs, rabbits, and rats with the 5-HT_3R-selective radioligands [^{3}H]GR67330 (23) or [^{3}H]RS-

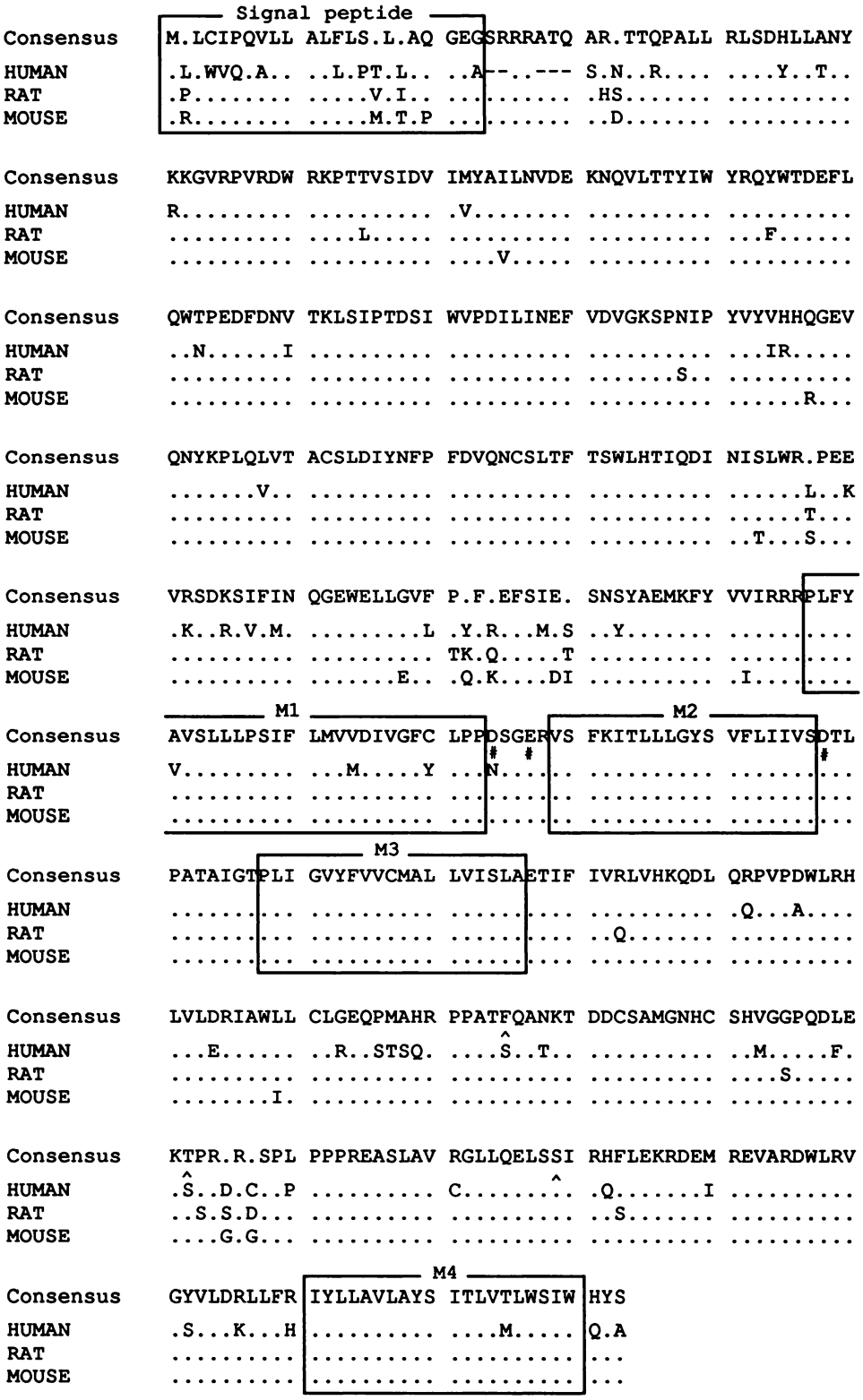

Fig. 2. Alignment of the amino acid sequences of 5-HT$_3$Rs. Human hippocampus 5-HT$_3$R, rat brain 5-HT$_3$R, and mouse N1E-115 5-HT$_3$R (8) were aligned. *Boxes*, putative signal peptide and M1–M4 segments; *caret*, protein kinase C phosphorylation sites; #, amino acids corresponding to three rings adjacent to the M2 segment.

42358–197 (24). In addition, electrophysiological study has indicated the existence of 5-HT$_3$R channels in ganglionic neurons of guinea pig intestine (25). Regarding the brain regions, expression of rodent 5-HT$_3$R has been determined in the limbic region, including amygdala and hippocampus, with not only 5-HT$_3$R-selective radioligand binding (26) but also *in situ* hybridization with mouse 5-HT$_3$R cRNA as a probe (27). Expression of 5-HT$_3$R in these regions has been

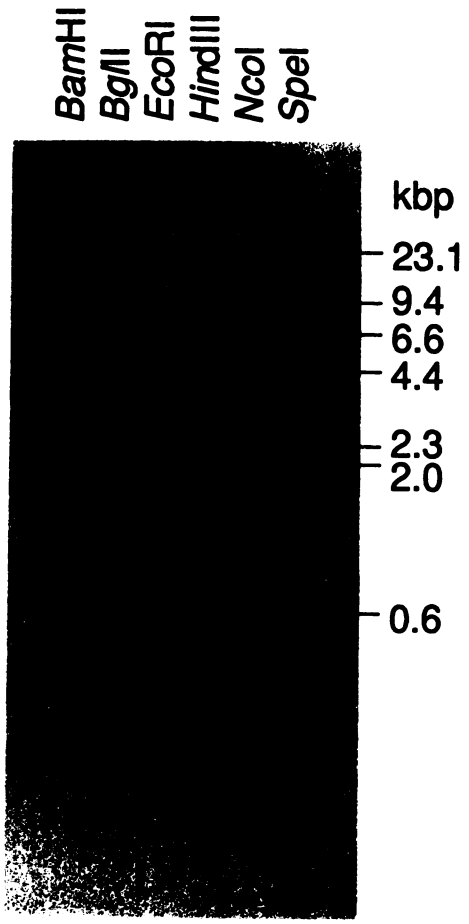

Fig. 3. Southern blot analysis of human genomic DNA. 10 μg of human genomic DNA were digested with either *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Nco*I, or *Spe*I, separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized (under conditions described in Experimental Procedures) with a probe corresponding to nucleotide 1358–1657 of human 5-HT_3R cDNA.

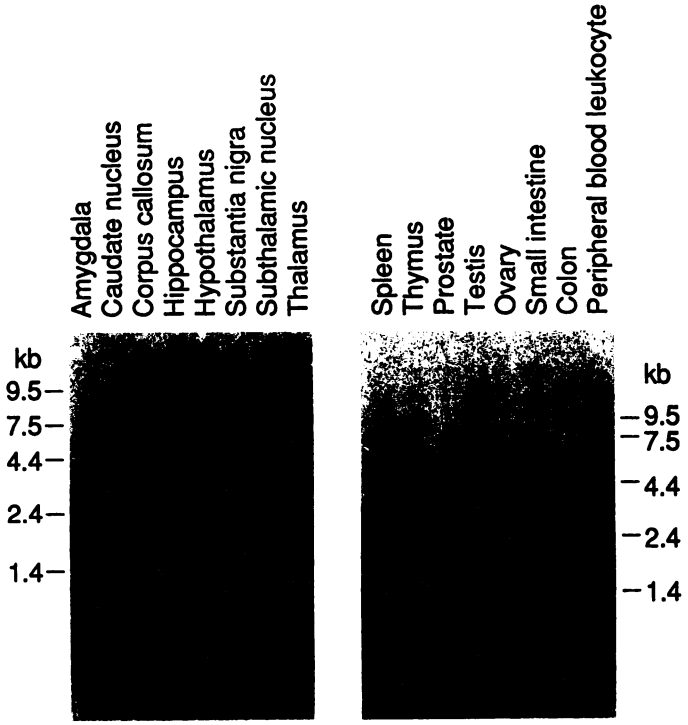

Fig. 4. Northern blot analysis of human 5-HT_3R mRNA. Membranes on which 2 μg of poly(A)$^+$ RNA had been immobilized were hybridized (under conditions described in Experimental Procedures) with a probe corresponding to nucleotide 1358–1657 of human 5-HT_3R cDNA.

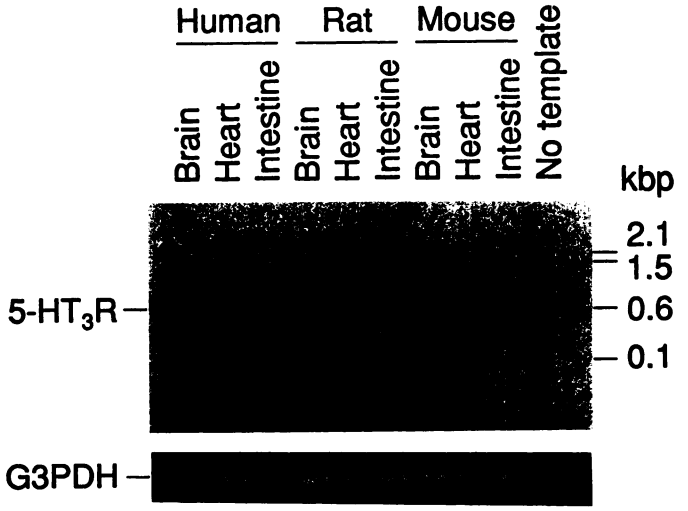

Fig. 5. RT-PCR analysis of 5-HT_3R transcripts in mouse, rat, and human tissue. Random-primed cDNAs were synthesized from mouse, rat. and human tissue poly(A)$^+$ RNAs (1 μg) and used as templates for PCR amplification. *Top*, For detection of 5-HT_3 transcripts, PCR (20 cycles) was performed with C1 and C2, consensus primers among these species. Ten-microliter aliquots of PCR products were separated by electrophoresis on a 1.5% agarose gel, transferred to a nylon membrane, and hybridized under conditions described in Experimental procedures with a probe corresponding to full-length human 5-HT_3R cDNA. *Bottom*, The efficiency of poly(A)$^+$ RNA isolation and cDNA synthesis was estimated by PCR (25 cycles) with G1 and G2, consensus primers for glyceraldehyde 3-phosphate dehydrogenase.

implicated in animal models of behavioral disorders such as anxiety, psychosis, and drug abuse (2). Our data support a similar implication in humans. Expression level in caudate nucleus of the striatal region was higher than in these limbic regions.

No signal was detected in whole brain and heart poly(A)$^+$ RNA (2 μg) by Northern blot analysis (data not shown). Transcripts of 5-HT_3R may exist at localized regions in human brain. Maricq *et al.* (7) also reported that 5-HT_3R transcripts were not detectable in mouse tissues, including brain, by Northern blot analysis but was detected in mouse brain and heart by RT-PCR analysis. With RT-PCR analysis, we also detected 5-HT_3R transcripts in mouse brain and heart. The 5-HT_3R transcripts were detected in human brain but not in human heart (Fig. 5). Furthermore, heart 5-HT_3R transcripts were not detected in rats. These results suggest that the tissue distribution of 5-HT_3R is heterogeneous among species.

Radioligand binding characteristics. Radioligand binding studies of recombinant human 5-HT_3R were performed with membrane preparations of COS-1 cells transfected with this receptor cDNA. The 5-HT_3R-selective radioligand [^{3}H]YM060 (28) specifically bound to these membranes with a K_d value of 0.16 ± 0.02 nM and a B_{max} value of 0.41 ± 0.03 pmol/mg protein (mean ± SE, n = 3; Fig. 6A). COS-1 cells transfected with the plasmid vector only showed no specific binding (data not shown). To assess the binding potency of 5-HT_3R agonists and antagonists, we performed competition studies. As shown in Fig. 6B, the rank

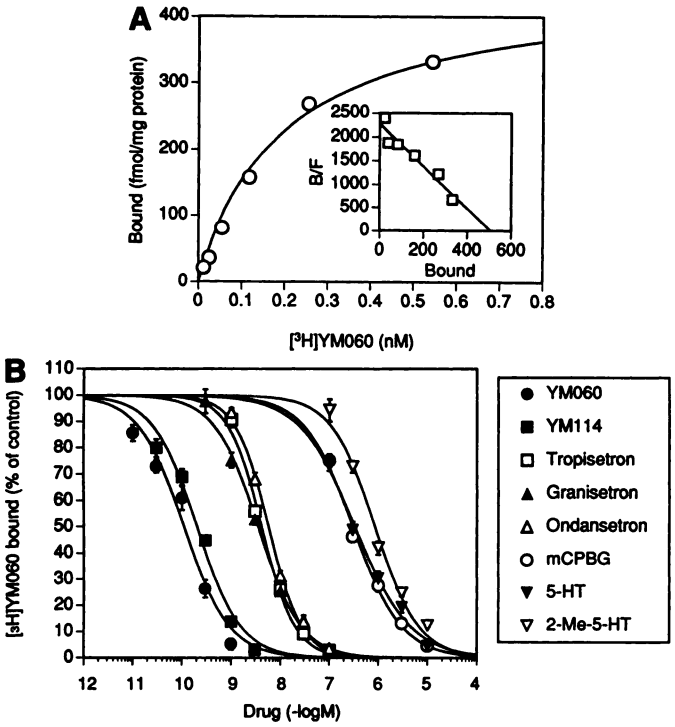

Fig. 6. Radioligand binding characteristics of 5-HT$_3$R. A, Binding of [^{3}H]YM060 to membranes from COS-1 cells transfected with human 5-HT$_3$R cDNA. Membranes were incubated with the indicated concentrations of [^{3}H]YM060 in the absence or presence of 1 μM tropisetron. The difference between them is represented as a specific binding. *Inset*, Scatchard plot of the data. B, Competition by drugs for [^{3}H]YM060 binding to membranes from COS-1 cells transfected with human 5-HT$_3$R cDNA. Membranes were incubated with the indicated concentrations of the drugs and 0.05 nM [^{3}H]YM060. Each point is mean ± SE of three different experiments performed in duplicate.

order of potency was YM060 > YM114 > granisetron = tropisetron = ondansetron > mCPBG = 5-HT ≥ 2-Me-5-HT. The 5-HT$_1$ and 5-HT$_2$ receptor antagonist methysergide (10 μM) did not inhibit [^{3}H]YM060 binding (data not shown). Then, to determine pharmacological differences among species, we characterized binding properties of recombinant rat 5-HT$_3$R using COS-1 cells transfected with rat brain 5-HT$_3$R cDNA, which we had cloned. As shown in Table 1, the affinity of the 5-HT$_3$R-selective agonist mCPBG was markedly different for the two species, whereas the affinities of other ligands were almost parallel.

TABLE 1

Comparison of radioligand binding characteristics between human and rat 5-HT$_3$R

The pK_i values were calculated from IC$_{50}$ values based on competition studies using [^{3}H]YM060 and membrane of COS-1 cells transfected with human or rat 5-HT$_3$R cDNA. Data for human receptor was derived from data in Fig. 6B.

Drug	pK_i	
	Human	Rat
5-HT	6.47 ± 0.17	7.39 ± 0.05
2-Me-5-HT	6.18 ± 0.09	7.22 ± 0.05
mCPBG	6.65 ± 0.07	8.98 ± 0.01
YM060	10.21 ± 0.05	10.89 ± 0.19
YM114	9.93 ± 0.07	9.95 ± 0.12
Granisetron	8.60 ± 0.10	8.92 ± 0.09
Tropisetron	8.48 ± 0.06	8.90 ± 0.07
Ondansetron	8.34 ± 0.04	8.52 ± 0.09

Elecrophysiological characteristics. Homo-oligomeric complex of mouse 5-HT$_3$R has been reported to be functional in *Xenopus* oocyte and mammalian cells. We examined electrophysiological responses to 5-HT$_3$R agonists in oocytes injected with human 5-HT$_3$R cRNA. As shown in Fig. 7A, bath application of 5-HT evoked a rapidly developing inward current that was desensitized in the continued presence of the agonist. Furthermore, the 5-HT$_3$R-selective agonist mCPBG or 2-Me-5-HT induced a similar response.

The rank order of potencies of the 5-HT$_3$R agonists was mCPBG = 5-HT ≥ 2-Me-5-HT (Fig. 7B). The EC$_{50}$ and Hill coefficient values were 1.7 ± 0.09 μM and 3.0 ± 0.33, 1.8 ± 0.12 μM and 2.3 ± 0.26, and 2.7 ± 0.18 μM and 2.7 ± 0.02 (mean ± SD, n = 3) for 5-HT, mCPBG, and 2-Me-5-HT, respectively. These results are consistent with the radioligand binding data described and show that the intrinsic potency of mCPBG for human 5-HT$_3$R is low. The Hill coefficient values of the 5-HT$_3$R agonists were >2, as seen in mouse recombinant and neuroblastoma 5-HT$_3$R. These results suggests the existence of positive cooperative interactions in agonist binding within the homo-oligomeric complex.

We compared efficacy of each agonist. To measure maximal responses, currents were elicited by 20 μM 5-HT, 20 μM mCPBG, or 30 μM 2-Me-5-HT. Responses to mCPBG and 2-Me-5-HT were similar to those to 5-HT at 99 ± 2% and 97 ± 3% (mean ± SD, n = 7) of 5-HT-induced responses, respectively. These results are in contrast to the previous findings that 2-Me-5-HT is a partial agonist for mouse neuroblastoma N1E-115 cells (29), rat neural crest-derived PC12 cells (30), and mouse hippocampal neurons (31). In addition, the response to 2-Me-5-HT has been reported to be partial in oocytes expressing the shorter isoform of mouse 5-HT$_3$R, at only ~10% of that for 5-HT (8). These findings suggest that the agonist efficacy of 2-Me-5-HT varies from partial to full among species.

Antagonist profiles were typical of the 5-HT$_3$R as shown in Fig. 7C. Ondansetron (5 nM) reversibly blocked current elicited by 5 μM 5-HT at 7.6 ± 3.4% of control (mean ± SD, n = 5). In contrast, the effect of methysergide was not appreciable at 5 μM (88 ± 4.3%, n = 5). The nAchR antagonist curare (5 μM) also inhibited 5-HT-induced current (29 ± 3.8%, n = 7). Inhibition by this antagonist has been reported in mouse recombinant 5-HT$_3$R (7, 8). However, our data suggest that human 5-HT$_3$R has lower sensitivity to curare than mouse 5-HT$_3$R.

Discussion

Results of the present study revealed the primary structure of human 5-HT$_3$R through cDNA cloning and clarified several heterogeneous characteristics of 5-HT$_3$R among species. Interspecies differences are seen in terms of not only the structure but also tissue distribution and functional profile.

The deduced amino acid sequence indicates several structural differences specific to humans: (a) charges of amino acids adjacent to the M2 segment are not conserved between humans and rodents, (b) a six-amino acid insertion in the large cytoplasmic loop has not been identified in human 5-HT$_3$R, and (c) human 5-HT$_3$R has a five-amino acid deletion in the amino-terminal of the mature protein compared with rodent 5-HT$_3$Rs.

Among ligand-gated ion channels, 5-HT$_3$R belongs to the nAchR

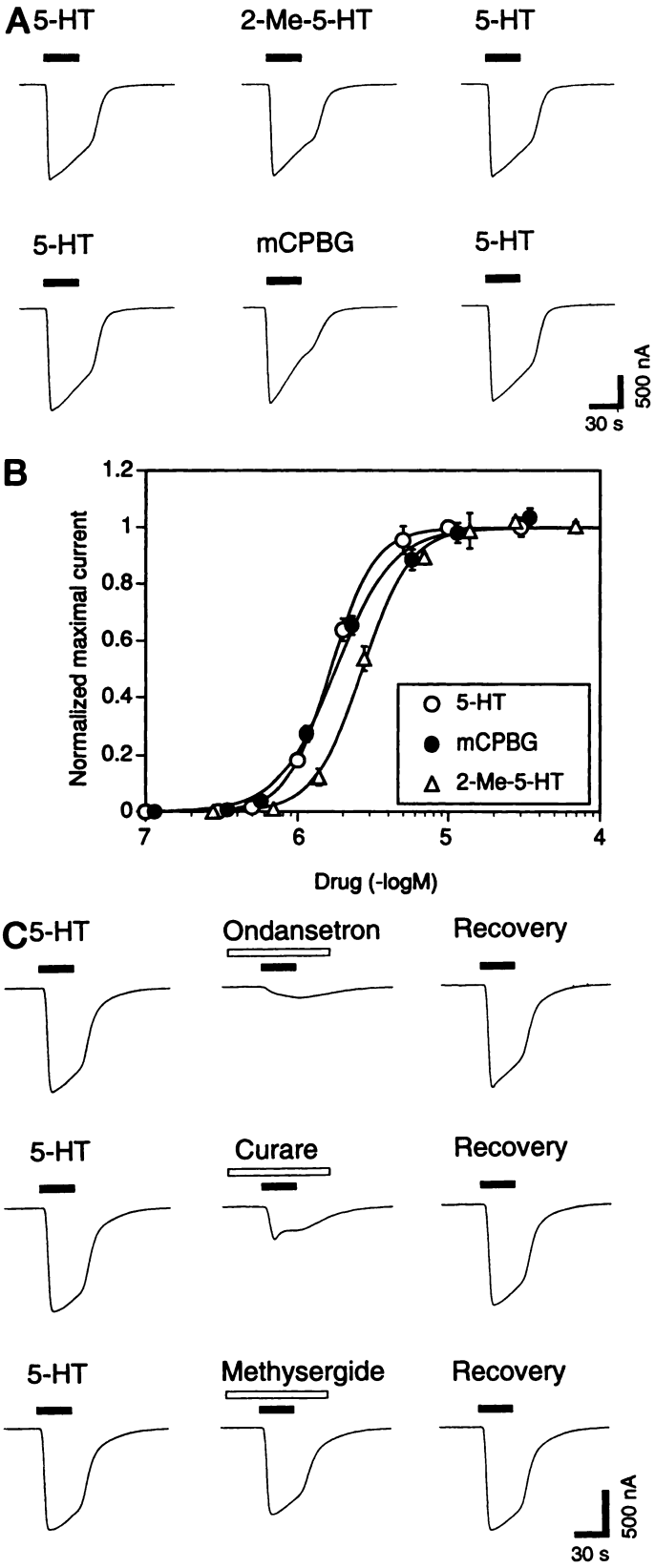

Fig. 7. Electrophysiological characterization of human 5-HT_3R. Oocytes were injected with human 5-HT_3R cRNA, incubated for 2–7 days, and voltage clamped at −60 mV. *A*, Current responses elicited by 5-HT (20 μM), mCPBG (20 μM), and 2-Me-5-HT (30 μM). Drugs were applied for 30 sec (*closed bar*). Recovery period was 5 min. All records were obtained from one oocyte. *B*, Dose-response curves for 5-HT, mCPBG, and 2-Me-5-HT. Recordings were performed as in *A*. Each point is mean ± SD of three oocytes. *C*, Effects of ondansetron (5 nM), curare (5 μM), or methysergide (5 μM) on 5-HT-induced current. Oocytes were bathed in a solution containing each drug (*open bar*). 5-HT (5 μM) was then applied for 30 sec (*closed bar*). Recovery time for the drugs was 5 min, except ondansetron required 25 min. All records were obtained from one oocyte.

superfamily and shares functional properties with nAchRs such as cation selectivity and curare sensitivity. In nAchR, three clusters of negatively charged amino acids adjacent to the M2 segment, forming three anionic rings, have been reported to be major determinants of the rate of ion transport (32). These three amino acids are precisely conserved among species. However, in human 5-HT_3R, Asn^{246} corresponding to the inner ring was not charged, which is in contrast to Asp in mouse and rat 5-HT_3R. Only two neuronal nAchR subtypes have no charged amino acid at this position, namely, nAchR α5 (33, 34) and α9 (35). In particular, rat and chicken nAchR α5 have Asn as well as human 5-HT_3R, but their function as an ion channel has not been identified. In contrast, human 5-HT_3R is functional as a homo-oligomeric channel. These findings indicate that this species-specific difference in the three rings is unique among the nAchR/5-HT_3R superfamily.

Mouse 5-HT_3R gene is alternatively spliced at two adjacent splice acceptor sites in intron 8, generating two isoforms that differ by six amino acids (10). The shorter isoform has been demonstrated to be approximately fivefold more abundant than the longer isoform in mouse neuroblastoma cell lines and neuronal tissues, including hippocampus and superior cervical ganglion (9). All of our isolated human clones corresponded to the shorter isoform, suggesting that the shorter isoform is also predominant in humans. This finding is supported by a published partial sequence of human genomic DNA in which the splice consensus sequence for the longer isoform was not found (9). The previously isolated cDNA of rat 5-HT_3R corresponds to the short isoform (11). In rats, we isolated a cDNA encoding the longer isoform in addition to the shorter one. The longer isoform had a five-amino acid insertion (GSLLP), differing from the mouse isoform, which had a six-amino acid insertion (GSDLLP). The position of the splice acceptor sites for the longer isoform may vary among species.

Deletion of five amino acids in human 5-HT_3R was located just downstream of the putative signal peptide that is encoded by exon 1 of mouse 5-HT_3R gene, suggesting the possibility of alternative splicing. However, alignment of the nucleotide sequence of mouse, rat, and human 5-HT_3R did not clearly indicate that the position of the five-codon deletion was identical to that of the exon-intron junction of mouse 5-HT_3R gene. Cloning of human genomic 5-HT_3R DNA will determine whether five-amino acid deletion results from a change in the splicing site or in the genomic DNA sequence itself.

Tissue distribution of 5-HT_3R has been discussed based on radioligand binding and physiological studies. Apparent heterogeneity in tissue distribution has been suggested in the striatal region of the brain and in heart. Among the brain regions, the 5-HT_3R-selective radioligand [^{3}H]LY278584 labeled binding sites in human limbic and striatal regions (3), whereas binding sites of this ligand are virtually absent in rat striatum (4). *In situ* hybridization study on mouse brain sections has detected strong hybridization signals in the hip-

pocampus but has not accounted for the existence of 5-HT$_3$R transcripts in the striatum (27). In this report, we provide the first evidence of the existence of 5-HT$_3$R mRNA in the striatal region. Regarding the heart, in most species 5-HT is known to initiate a short-lasting bradycardia due to a Bezold-Jarisch-like reflex resulting from depolarization of vagal afferent nerves by stimulation of 5-HT$_3$Rs (5). Transcripts of 5-HT$_3$R detected only in mouse heart might not be associated with bradycardia. In contrast to the bradycardia, 5-HT-induced tachycardia appears to be mediated by different 5-HT receptors in different species – 5-HT$_3$Rs in rabbits and dogs, 5-HT$_4$ receptors in humans, and 5-HT$_2$ receptors in rats (5). Although the physiological functions of 5-HT$_3$R transcripts detected in heart remain unknown, our data support the possibility that the myocardial effects of 5-HT through 5-HT$_3$R may be heterogeneous among species.

Functional expression of human 5-HT$_3$R revealed two instances of pharmacological heterogeneity among species: mCPBG was not a potent agonist, and 2-Me-5-HT was a full agonist for human 5-HT$_3$R. Radioligand binding studies with native tissue membranes have indicated that mCPBG is less potent for mouse NG108–15 5-HT$_3$R than for rat brain 5-HT$_3$R (24). Thus, mCPBG is a 5-HT$_3$R agonist with high selectivity for rat 5-HT$_3$R. Although binding sites of 5-HT$_3$R agonists in 5-HT$_3$R have not been identified, acetylcholine binding sites of nAchRs were identified in the putative amino-terminal extracellular domain (36). The putative amino-terminal extracellular domain of 5-HT$_3$R was highly conserved in mice, rats, and humans, and only four amino acids were specific for rats (see Fig. 2). Identification of the residue contributing to these pharmacological differences may help in identifying agonist binding sites of 5-HT$_3$R. Alternatively, the efficacy change of 2-Me-5-HT from partial to full agonist among species is an important phenomenon in investigation of the association between agonist binding and channel opening. Recently, such an efficacy change was reported for nAchR α7 (19). However, the residue that accounts for this efficacy change has not been identified. Like nAchR α7, 5-HT$_3$R molecules will be a useful tool in studying the efficacy change by mutagenesis.

Finally, the present study reveals the characteristics of the human 5-HT$_3$R counterpart. It does not, however, account for the existence of other 5-HT$_3$R subtypes. Previous binding studies suggested the existence of a tissue-specific difference in 5-HT$_3$R characteristics between mouse brain and ileum (6). Because our data from Northern blot and RT-PCR analyses indicated the existence of our cloned transcript in both brain and intestine, additional subunits may modulate the binding characteristics of 5-HT$_3$R in brain or intestine. For example, neuronal nAchRs are constructed from two subunits, both of which contribute to agonist sensitivity (37) and desensitization properties (34). The possible existence of the additional subunits is also supported by previous electrophysiological studies in which native 5-HT$_3$R in mouse superior cervical ganglion showed higher conductance and different voltage dependence than the homo-oligomeric form of recombinant mouse 5-HT$_3$R (38). This possibility must be examined in future studies.

Acknowledgments

We thank Dr. Gensei Kon for helpful discussion.

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