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Molecular Cloning, Functional Expression, and mRNA Tissue Distribution of the Human 5-Hydroxytryptamine$_{2B}$ Receptor

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SUMMARY

Clones encoding a portion of the human 5-hydroxytryptamine (5-HT)$_{2B}$ receptor gene were isolated from a human placental genomic library. Based on distribution studies of 5-HT$_{2B}$ receptor mRNA, human uterus cDNA libraries were constructed and screened, resulting in the isolation of several full-length cDNA clones. These clones harbored a common single open reading frame encoding a protein of 481 amino acids. The deduced amino acid sequence of the human 5-HT$_{2B}$ receptor displayed 91.5% identity within the transmembrane domains and 82% identity overall with the rat 5-HT$_{2B}$ receptor. The human 5-HT$_{2B}$ receptor stably expressed in AV12-664 cells demonstrated high affinity (K_d = 10.18 ± 1.60 nM), saturable [^{3}H]serotonin binding, similar to that previously described for the rat 5-HT$_{2B}$ receptor. The pharmacological profile of the human 5-HT$_{2B}$ receptor was virtually identical to that of the rat 5-HT$_{2B}$ receptor, with the exceptions of the 5-HT$_{2A}$ receptor antagonists ketanserin and spiperone. Both compounds exhibited higher affinity at the human 5-HT$_{2B}$ receptor (ketanserin, K_i = 376 ± 58 nM; spiperone, K_i = 697 ± 54 nM) than at the rat 5-HT$_{2B}$ receptor (ketanserin, K_i = 3559 ± 175 nM; spiperone, K_i = 3278 ± 92 nM). Functional coupling of the human 5-HT$_{2B}$ receptor was also demonstrated in AV12-664 cells, where 5-HT produced a dose-dependent increase in phosphatidylinositol hydrolysis (EC_{50} = 27 ± 12 nM) analogous to that seen with the rat 5-HT$_{2B}$ receptor. Reverse transcription-polymerase chain reaction studies revealed human 5-HT$_{2B}$ receptor mRNA to be expressed in many tissues, including the central nervous system. The presence of 5-HT$_{2B}$ receptor mRNA in human brain and not in rat brain raises the possibility that the 5-HT$_{2B}$ receptor may be of significance in higher brain function.

Serotonin, a biogenic amine, mediates multiple physiological and behavioral functions in humans. These functions range from the regulation of basal tone of blood vessels to involvement in a number of central nervous system functions and disorders, including anxiety, depression, aggressive behavior, sleep, and sexual behavior (1). Serotonin exerts its effects on these various functions by specifically binding to cell surface receptors. To date, 13 5-HT receptor subtypes, representing 13 distinct gene products, have been cloned from mammalian sources, and a number of others have been characterized pharmacologically. Four subtypes have been cloned from invertebrate sources, but an evolutionary linkage to the existing mammalian subtypes has yet to be established. Fourteen mammalian subtypes can be grouped into seven distinct subfamilies (designated 5-HT$_1$ to 5-HT$_7$) on the basis of their relative degree of amino acid homology, second messenger coupling, affinity for 5-HT and related compounds, and possible genomic organization or evolutionary origin (2). All but one of these subfamilies (5-HT$_3$) and the four invertebrate receptor subtypes have been found to be members of the G protein-coupled receptor superfamily. Recently, we reported the cloning and pharmacological characterization of a novel member of the 5-HT$_2$ receptor subfamily, the 5-HT$_{2B(2F)}$ receptor from the rat stomach fundus (3). Furthermore, agonist affinity at the cloned rat 5-HT$_{2B}$ receptor correlated well with agonist activity at the classically described contractile receptor in the rat stomach fundus. Although Foguet *et al.* (4) have reported the 5-HT$_{2B}$ gene to be present in the human genome, as demonstrated by PCR, no data reporting a human 5-HT receptor subtype with molecular or pharmacological properties similar to those of the rat 5-HT$_{2B}$ receptor have been published.

Here we describe the cloning and initial pharmacological characterization of the human homologue of the rat 5-HT$_{2B}$ receptor cDNA. Using a reverse transcription-PCR assay, mRNA encoding the 5-HT$_{2B}$ receptor was found to be expressed in all human tissues examined, including the brain. This human brain distribution of the 5-HT$_{2B}$ receptor mRNA represents a marked contrast to the rat brain, where 5-HT$_{2B}$ receptor mRNA has yet to be found by any of several techniques. This finding suggests the possibility that the 5-HT$_{2B}$ receptor may mediate an important physiological role in higher cognitive brain function.

[1] D. A. Cox and M. L. Cohen. 5-Hydroxytryptamine (5-HT)$_{2B}$ receptor signaling in rat stomach fundus: role of voltage-dependent calcium channels, intracellular calcium release, and protein kinase C. Submitted for publication.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; kb, kilobase(s); PI, phosphatidylinositol; bp, base pair(s); TM, transmembrane; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane.

Materials and Methods

Chemicals. DOI, 5-carboxamidotryptamine, α-methyl-5-HT, ketanserin, spiperone, and yohimbine were purchased from Research Biochemicals, Inc. (Natick, MA). Serotonin creatinine sulfate complex and most other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Serotonin (27.4–38.1 Ci/mmol) and all other radionuclides were obtained from DuPont-NEN (Wilmington, DE).

PCR amplification and labeling of the rat 5-HT$_{2B}$ receptor DNA probe. Two oligonucleotide primers (5′-GCCTGGTTATTCCTTGATGTTCTCTT-3′ and 5′-AAAGTAGGTGACTATCATGATGGTGA-3′) were used to amplify and simultaneously label a 326-bp DNA fragment from a plasmid template harboring the rat 5-HT$_{2B}$ receptor cDNA. [^{32}P]dCTP (200 μCi) was lyophilized and resuspended in a 20-μl reaction mixture consisting of the other three deoxynucleoside triphosphates at 50 μM each, 0.5 unit of *Thermus aquatics* polymerase and 1× Taq reaction buffer (10 mM Tms-HCl, pH 8.3; 50 mM KCl; 150 μM $MgCl_2$; 0.0019 w/v gelatin; Perkin-Elmer-Cetus, Norwalk, CT), 4 pmol of each primer, and 10–20 ng of plasmid DNA template, as described by Showalter and Sommer (5). Thermocycling parameters were as follows: 95° for 1 min, 52° for 1 min, and 72° for 2 min, for 30 cycles. Unincorporated nucleotides were removed by G-25 Sephadex column spin-chromatography (Boehringer-Mannheim, Indianapolis, IN).

Southern blot analysis. A zoo blot containing 8 μg of *Eco*RI-digested genomic DNA from nine different species was purchased from Clonetech (Palo Alto, CA). Samples (10 μg) of human genomic DNA were digested with various restriction enzymes and blotted to nylon membranes using standard techniques (6). These blots were probed with the PCR fragment described above, under low stringency conditions (35°, 30% formamide, 0.1 μg/ml salmon sperm DNA, 0.1% sodium dodecyl sulfate, 5× standard saline phosphate EDTA (0.15 M NaCl; 0.015 M sodium citrate, pH 7.0; 0.01 M $NaH_2PO_4 \cdot H_2O$; 0.001 M EDTA), 5× Denhardt's solution). The filters were washed at 35° and exposed to film at −80° with an intensifying screen.

Genomic library screening. A human placental genomic library cloned into the λ vector EMBL3 was screened with the PCR probe described above, according to the methods of Hamilton *et al.* (7). Positively hybridizing plaques were purified to homogeneity according to standard techniques (6).

PCR amplification of DNA fragments from genomic phage clones. Oligonucleotide primers were designed from the end of the putative TM-2 region (sense, 5′-AGGCTACATGGCCCCTCCCACTGGC-3′), from midway into the putative TM-4 region (antisense, 5′-CTATTGAAATTAACCATACCACCGT-3′, sense, 5′-GCATCGCCATCCCAGTCCCATAAAA-3′), from the putative TM-5 region (sense, 5′-CCTCTCACCATCATGATAGTCACCTA-3′), and from the putative TM-7 region (antisense, 5′-GGATTCACTCCCGAGGAAACGTAGCCTACCCA-3′). The primers designed from the putative TM-2 and -4 regions started or ended at the intron-exon junctions of the mouse 5-HT$_{2B}$ receptor gene, as described by Foguet *et al.* (8). The primers from the putative TM-5 and -7 regions were very highly conserved sequences for all members of the 5-HT$_2$ receptor gene subfamily. Where no consensus occurred, preference was given to the nucleotide present in the rat 5-HT$_{2B}$ receptor cDNA sequence. A high titer phage preparation (10 μl) was boiled for 10 min and used as the template for the subsequent PCR reactions. DNA fragments were amplified using the GENEAMP KIT (Perkin-Elmer-Cetus), according to the vendor's instructions. DNA fragments were cloned into the pCR-II vector (Invitrogen, San Diego, CA) and sequenced as described previously (3).

PCR distribution studies of human 5-HT$_{2B}$ receptor mRNA expression. cDNAs prepared from various human tissues, both peripheral and within the central nervous system, were purchased from Clonetech (Palo Alto, CA). Two human 5-HT$_{2B}$ receptor-specific oligonucleotide primers were designed from the second (sense, 5′-ATATAACTCACGGGCTACAGCATTCA-3′) and third (antisense, 5′-CGGTGACGAGCAAGGTGTATCA-3′) (intracellular loops). DNA fragments were amplified from the various cDNAs, as described above. Samples were gel fractionated, transferred to nylon membranes, and probed with an internal human 5-HT$_{2B}$ receptor sequence-specific primer (5′-AACCCAAACAATATCACT3′ 5-TGTGTGCTGACAAAGGAACGTTTT-3′), following conventional techniques (6). Whole brains were dissected from Sprague-Dawley rats (Harland Sprague-Dawley, Inc., Indianapolis, IN) and various regions were subdivided. RNA extractions, cDNA syntheses, and PCR amplification were exactly as described (3). Two rat 5-HT$_{2B}$ receptor sequence-specific oligonucleotide primers (sense, 5′-CCTGTGAGCTGACAAAGGACCGCTTTGGCA-3′, antisense, 5′-GTGAGAGCCATCCAGCATCGCCATCTTTTCTGG-3′) were used to amplify a 248-bp DNA fragment from cDNAs derived from several rat brain subregions. An internal oligonucleotide primer (5′-AACGCCTAACACGGTGGACTGTGTCCACAGTTCTCCAAAGGG-3′) was used as a hybridization probe to confirm the specificity for the rat 5-HT$_{2B}$ receptor sequence.

Characterization of human 5-HT$_{2B}$ receptor cDNAs. mRNA purification, cDNA library construction in the λ cloning vector gt22 and screening, phage isolation, phage cDNA insert length determination, insert subcloning, DNA sequencing, and sequence analysis were all performed exactly as described previously (3).

Functional characterization of human 5-HT$_{2B}$ receptor cDNAs. A full-length cDNA encoding the human 5-HT$_{2B}$ receptor was subcloned into the PhD vector and transfected into AV12-664 cells (CRL 9595; American Type Culture Collection), and positive clones were selected with the folate antagonist methotrexate as described previously (3). Clonal isolates with the highest receptor levels were used for PI studies exactly as described (3).

Pharmacological characterization of the human 5-HT$_{2B}$ receptor. Membranes from cells expressing the human 5-HT$_{2B}$ receptor were purified as described (9). Binding assays and data analysis were also essentially as described previously (9). All assays were incubated at 37° using 90–200 μg of protein/assay tube. The IC_{50} values from the competition assays were determined by nonlinear regression analysis of four-parameter logistic equations (10) (Systat; Systat Inc., Evanston, IL). The IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (11). For the saturation assays, free [^{3}H]5-HT concentrations ranged from 0.7 to 37 nM. Two-way analysis of variance was performed on the pK_i values (−log K_i; in molar), followed by contrast analysis (JMP; SAS Institute, Inc., Cary, NC).

Results

Southern blot analysis of genomic DNA and genomic cloning. Southern blot analysis was performed on human genomic DNA, as well as DNA from other species, to establish the presence within these genomes of a gene homologous to that encoding the rat 5-HT$_{2B}$ receptor. A 326-bp DNA fragment extending from the beginning of the putative TM-3 region to the end of the putative TM-5 region of the rat 5-HT$_{2B}$ receptor cDNA was used as a probe. This fragment was amplified and simultaneously labeled with [^{32}P]dCTP by PCR, using oligonucleotide primers corresponding to the last 25 nucleotides at each end of the region defined above and the rat 5-HT$_{2B}$ receptor cDNA as a template. Nucleic acid sequence analysis, using the GAP program of the University of Wisconsin Genetics Core Group software package, of all cloned human 5-HT receptors and their corresponding rat and mouse homologues (dog homologue in the case of the 5-HT$_{1D}$ receptor) revealed this to be the best region of the rat 5-HT$_{2B}$ cDNA from which to derive a probe. This TM-3 to TM-5 region was found to be the longest, highly conserved region (>90%) within all pairs of receptor sequences (human versus rat, mouse, or dog). As a result of this analysis, we reasoned that this region might also be well conserved across species for the 5-HT$_{2B}$ receptor gene and thus serve as an excellent PCR template from which to

amplify the optimal probe for exhaustively screening for the presence of this gene in human DNA. The probe derived from this region was also assessed for its ability to recognize the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor genes. GAP analysis revealed <60% homology between the probe and either receptor gene. Low stringency hybridization conditions were chosen, which would allow hybridization to any putative human 5-HT$_{2B}$ receptor gene sequence but would exclude the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor gene sequences. Southern blot analysis of *Eco*RI-digested genomic DNA from a number of species resulted in banding patterns suggestive of a single copy gene for each species (Fig. 1A). The probe recognized only the 5-HT$_{2B}$ receptor gene and not the 5-HT$_{2A}$ or 5-HT$_{2C}$ genes in Fig. 1A, rat lane, as indicated by the presence of a single band in that lane. The same band was previously observed in an *Eco*RI digest of rat genomic DNA hybridized to a rat-specific probe using high stringency conditions (3). This argued for those signals present in the lanes for other species representing their homologues to the 5-HT$_{2B}$ gene and not cross-hybridization to their 5-HT$_{2A}$ or 5-HT$_{2C}$ receptor genes.

The probe was approximately 65% homologous to both the rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor genes but did not recognize these genes in rat genomic DNA. As a consequence, these genes would likely not be recognized in the lanes for other species, where the homology of the rat probe to the corresponding 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor genes would likely be <65%. Thus, the signal present in the human lane (see Lane 2, Fig. 1) and the banding pattern observed by Southern blot analysis of human genomic DNA (Fig. 1B) most likely represented the homologue of the rat 5-HT$_{2B}$ receptor gene and not the human homologues of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor genes.

We then screened a human placental genomic library with the same probe under conditions of low stringency, resulting in the isolation of 16 phage clones. These clones were purified to homogeneity, and selected regions of phage DNA were amplified by PCR using oligonucleotide primers derived from the putative TM-2, -4, -5, and -7 regions of the rat 5-HT$_{2B}$ receptor cDNA sequence. The inserts of these clones averaged approximately 23 kb of DNA in length; thus, we used PCR amplification to obtain nucleic acid sequence information from the coding region of these clones, rather than using standard genomic subcloning and mapping techniques. The primers designed from the putative TM-5 and -7 domains represented the most highly conserved sequence of these two TM regions for all members of the 5-HT$_2$ receptor subfamily and their species homologues. We then assumed that the genomic organization of the mouse 5-HT$_{2B}$ receptor gene described by Foguet *et al.* (8) was conserved in humans. Consequently, we designed the primers for the putative TM-2 and -4 regions at the analogous intron-exon junctions of the mouse 5-HT$_{2B}$ gene, to obtain as much of the human coding sequence as possible while excluding any intron sequences. Using three primer combinations, we successfully amplified from these genomic clones a 202-bp DNA fragment from the end of the TM-2 region to the middle of the TM-4 region, a 446-bp DNA fragment from the middle of the TM-5 region to the middle of the TM-7 region; and a 576-bp DNA fragment from the middle of the TM-4 region to the middle of the TM-7 region. A contiguous nucleic acid sequence of 778 bp was obtained from these three fragments upon subsequent cloning and sequencing. Translation of this composite sequence, using the UW/GCG software package, revealed a single open reading frame whose amino acid sequence shared highest homology with the rat 5-HT$_{2B}$ receptor sequence (Fig. 2, *underlined sequence*), thus confirming its identity as the human homologue of the rat 5-HT$_{2B}$ receptor gene.

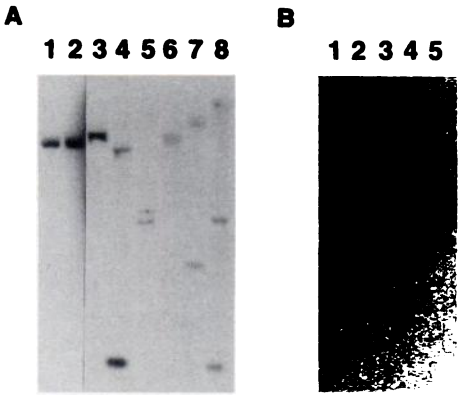

Fig. 1. Southern blot analyses of genomic DNA from several species. A, Samples (8 μg) of genomic DNA from monkey (*lane 1*), human (*lane 2*), rat (*lane 3*), mouse (*lane 4*), dog (*lane 5*), cow (*lane 6*), rabbit (*lane 7*), and chicken (*lane 8*) were digested with the restriction enzyme *Eco*RI. B, Samples (10 μg) of human genomic DNA were digested with the following restriction enzymes: *Eco*RI (*lane 1*), *Hind*III (*lane 2*), *Bam*HI (*lane 3*), *Pst*I (*lane 4*), and *Bg*/II (*lane 5*). Both blots were probed with the 326-bp ^{32}P-labeled PCR fragment described in Materials and Methods.

Another PCR experiment, using a TM-2 and TM-7 region primer combination, was unsuccessful in amplifying a contiguous 778-bp DNA fragment, suggesting the presence of introns in these genomic clones. Using different oligonucleotide primers, we demonstrated both the numbers and positions of the introns in these genomic clones to be consistent with those found in the mouse 5-HT$_{2B}$ gene (data not shown). Attempts to amplify the remainder of the coding sequence from these genomic clones, using primers based on the amino- and carboxyl-terminal sequences of the rat 5-HT$_{2B}$ receptor cDNA sequence, proved unsuccessful. These results suggested either that these clones lacked the entire coding sequence or that the homology between the rat and human sequences in these regions was too low for the primers to properly anneal.

Peripheral tissue distribution of human 5-HT$_{2B}$ receptor mRNA. Although the aforementioned data verified the presence of a 5-HT$_{2B}$ receptor gene within the human genome, they did not demonstrate a transcriptionally active gene. To determine whether this gene was transcriptionally active and not simply a pseudogene or dormant genetic relic, a PCR assay was designed to investigate human tissue distribution of 5-HT$_{2B}$ receptor mRNA expression. Oligonucleotide primers, based on the composite nucleic acid sequence specific for the human 5-HT$_{2B}$ receptor gene, were designed for the second and third cytoplasmic loops. These primers were chosen both for their ability to impart specificity for the human 5-HT$_{2B}$ receptor sequences and because any potential DNA fragment they might amplify would have to cross a large intron if genomic DNA were present. These primers did not recognize the rat 5-HT$_{2B}$ receptor sequence or any other member of the 5-HT$_{2B}$ receptor subfamily, as determined by PCR experiments (data not shown). As a consequence, DNA fragments amplified in the reverse transcription-PCR assays could not be due to contamination either from rat 5-HT$_{2B}$ receptor cDNA or from our human genomic clones or human genomic DNA.

PCR assays using cDNAs from various human tissues revealed 5-HT$_{2B}$ receptor mRNA to be expressed in all tissues

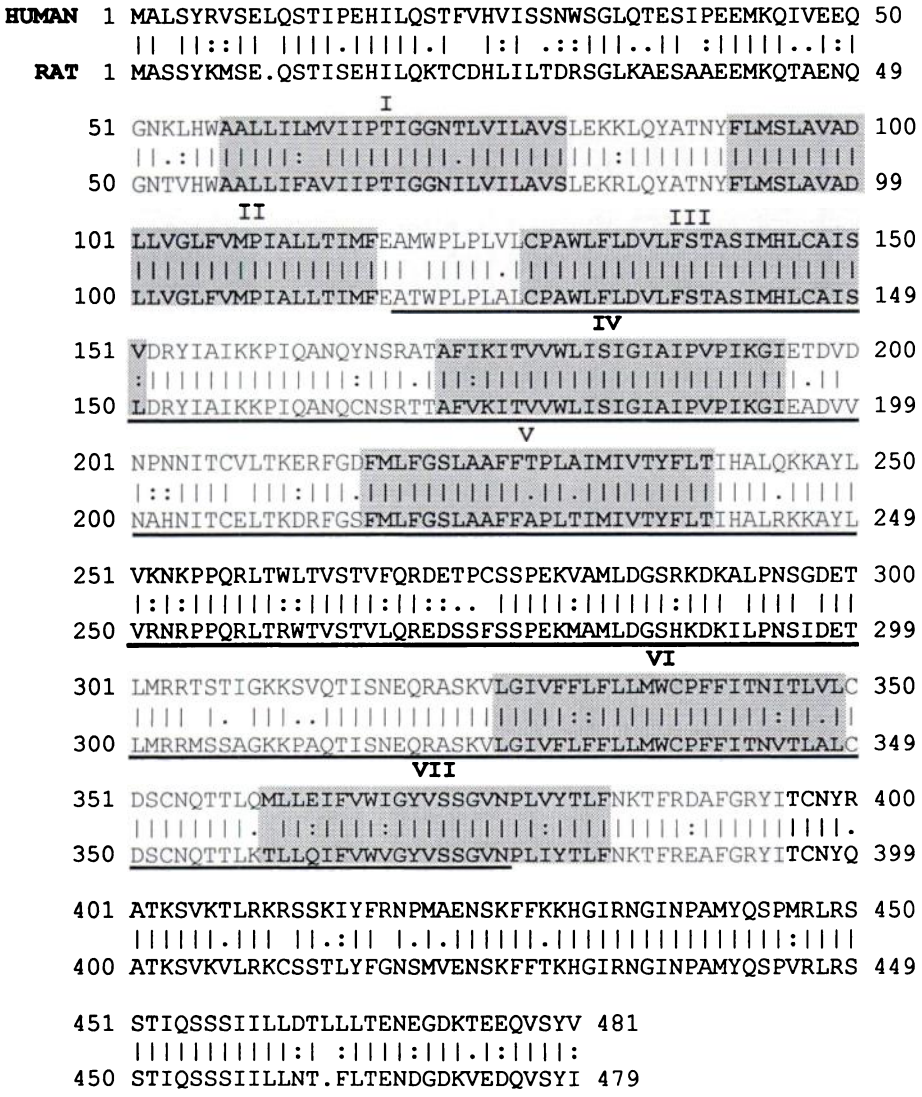

Fig. 2. Comparison of the amino acid sequences of the human and rat 5-HT$_{2B}$ receptors. The TM regions are *highlighted and labeled with Roman numerals*. The sequence obtained from the human placental genomic library clones is *underlined*. Symbols between sequence lines indicate amino acid identity (|), similarity (:), or low similarity (.). All analyses were performed with the UW/GCG software package.

examined (Fig. 3). Prominent signals were identified in the uterus, trachea, and small intestine samples. A third oligonucleotide primer specific for the human 5-HT$_{2B}$ receptor sequence, internal to the PCR primers, was used as a hybridization probe to verify that the amplified DNA fragments were indeed derived from the human 5-HT$_{2B}$ receptor mRNA (data not shown). Northern blot analysis also demonstrated 5-HT$_{2B}$ receptor mRNA to be present in these same tissues, with particularly strong signals once again being found in the uterus, trachea, and small intestine (data not shown).

Cloning of full length 5-HT$_{2B}$ receptor cDNAs. Based on the results described above, mRNA was purified from fresh human uterus tissue and used to construct a number of cDNA libraries. One of these libraries yielded 32 phage clones upon screening with the previously described 326-bp DNA probe. PCR amplification was used to determine the lengths of the cDNA inserts of these clones and revealed that a number of clones harbored cDNA inserts of approximately 2.3 kb. This was very similar to the size of the message obtained by Northern blot analysis, suggesting that these inserts contained the full coding sequence for the human 5-HT$_{2B}$ receptor and possibly even the full length message. The cDNA inserts from four clones were subcloned and sequenced for further characterization. Analysis of the nucleic acid sequence information from these clones revealed the human 5-HT$_{2B}$ receptor sequence to be 84.4% homologous to the rat 5-HT$_{2B}$ receptor sequence in the coding region. The 5′ and 3′ nontranslated regions of the human cDNAs were found to be 65% and 55% homologous to the rat 5-HT$_{2B}$ receptor mRNA nontranslated regions, respectively (see Fig. 2 for sequence comparison).

Amino acid sequence analysis of the human 5-HT$_{2B}$ receptor. Translation of the nucleic acid sequences of these inserts revealed all four to contain a common single open reading frame encoding a protein of 481 amino acids. (The human sequence was two amino acids, both leucines, longer than the rat sequence. One leucine was found at position 10 in the amino terminus and the other at position 464 in the carboxyl terminus.) Comparison of the human 5-HT$_{2B}$ receptor amino acid sequence with the rat sequence revealed 91.5% homology within the putative TM domains and 82% homology overall. The initiator methionine for the human receptor corresponded to the first of two methionines present in the first seven residues of the rat receptor. All motifs associated with the rat 5-HT$_{2B}$ receptor were present, including the seven hydrophobic domains, the GN diresidue sequence in the putative TM-1 region, the DRY triresidue sequence at the end of

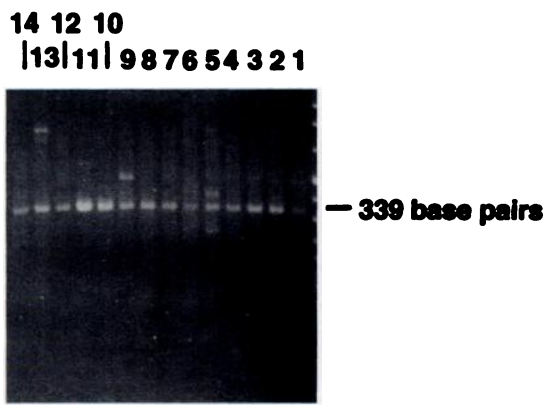

Fig. 3. Peripheral tissue distribution of human 5-HT$_{2B}$ receptor mRNA expression. Two human 5-HT$_{2B}$ receptor-specific oligonucleotide primers were used to amplify a 339-bp fragment from cDNAs derived from several human tissues, liver (*lane 1*), heart (*lane 2*), ovary (*lane 3*), lung (*lane 4*), skeletal muscle (*lane 5*), brain (*lane 6*), kidney (*lane 7*), trachea (*lane 8*), testis (*lane 9*), small intestine (*lane 10*), uterus (*lane 11*), placenta (*lane 12*), prostate (*lane 13*), and pancreas (*lane 14*). Aliquots (10 μl) of the amplified products were fractionated in agarose gels and stained with ethidium bromide. A third oligonucleotide, internal to the first two, was used as a probe to verify the specificity of these fragments for the 5-HT$_{2B}$ receptor.

the putative TM-3 region, conserved aspartate residues (Asp^{100} and Asp^{134}, in the putative TM-2 and -3, regions respectively), a conserved serine residue (Ser^{222}) in the putative TM-5 region, and a palmitoylation acceptor site at residue Cys^{397} in the carboxyl-5 terminus. Potential consensus *N*-linked glycosylation sites at residues Asn^{30}, Asn^{204}, and Asn^{354} and several consensus phosphorylation sites for cAMP-dependent protein kinase, protein kinase C, and tyrosine kinase were also identified using the UW/GCG DNA/protein sequence analysis software package (see Fig. 4). Phosphorylation sites identified in the third intracellular loop and the carboxyl terminus may be associated with regulatory functions such as desensitization and internalization, as described for other G protein-coupled receptors (12–15).

Eukaryotic expression and functional coupling of the human 5-HT$_{2B}$ receptor. To determine whether these clones encoded a functional receptor, a 2.3-kb cDNA insert was subcloned into the eukaryotic expression vector PhD and this construct was transfected into the AV12-664 cell line, as described previously (3). Those clones expressing the highest receptor levels were chosen for functional studies and ligand binding assays. Like all members of the 5-HT$_2$ receptor subfamily, the rat 5-HT$_{2B}$ receptor has been shown to couple to PI hydrolysis (3). We therefore examined the ability of the human 5-HT$_{2B}$ receptor to couple to PI hydrolysis, providing a model system for functional pharmacological characterization of this receptor. A concentration-dependent increase in PI hydrolysis (EC$_{50}$ = 27 ± 12 nM) was observed upon activation of the human 5-HT$_{2B}$ receptor with 5-HT (Fig. 5). The maximal response was observed to be approximately 10-fold higher than basal levels, analogous to that seen for the rat 5-HT$_{2B}$ receptor (3).

Ligand binding assays. Membranes purified from AV12-664 cells stably expressing the human 5-HT$_{2B}$ receptor demonstrated high affinity, saturable binding of [^{3}H]5-HT (Fig. 6). Significant binding to the AV12-664 parental line was not observed. Nonlinear regression analysis of saturation isotherms suggested a single class of bindings sites. The clonal isolate used for these assays expressed a B$_{max}$ of approximately 1 pmol of receptor/mg of protein. The pharmacological profile of the human 5-HT$_{2B}$ receptor was virtually identical to that of the rat 5-HT$_{2B}$ receptor, with the exceptions of the 5-HT$_{2A}$ antagonists ketanserin and spiperone (Table 1). Ten-fold higher affinity for ketanserin and 5-fold higher affinity for spiperone were found at the human 5-HT$_{2B}$ receptor than at the rat 5-HT$_{2B}$ receptor. The high affinity for [^{3}H]5-HT (K_d = 10.18 ± 1.60 nM) and the pharmacological profile described in Table 1 corroborated the molecular data verifying that this was indeed the homologue of the rat 5-HT$_{2B}$ receptor.

Brain distribution of 5-HT$_{2B}$ receptor mRNA. 5-HT$_{2B}$ receptor mRNA expression has yet to be demonstrated in rat brain, using several different assays. To evaluate whether this was also the case for human brain, a PCR assay was chosen to examine expression of 5-HT$_{2B}$ receptor transcripts in various regions of human brain. cDNAs from different human brain regions were examined by PCR using the human-specific oligonucleotide primers described for the peripheral tissue study. As with our peripheral tissue data, all examined regions of the human brain were found to express some level of 5-HT$_{2B}$ receptor mRNA (Fig. 7A). This was in marked contrast to the data for rat brain, where even with the extreme sensitivity afforded by PCR and Southern hybridization analysis no 5-HT$_{2B}$ receptor transcripts were identified in any region (Fig. 7B). These observations in rat brain data have been corroborated by a ribonuclease protection assay (data not shown), as well as by Northern blot analysis (3).

Discussion

To date, no data describing a human 5-HT-like receptor with properties similar to those described for the rat 5-HT$_{2B}$ receptor have been published. Here we report the cloning, expression, basic pharmacological characterization, and mRNA tissue distribution of the human 5-HT$_{2B}$ receptor.

The lengths of the 5-HT$_{2B}$ receptor proteins (479 residues for the rat receptor versus 481 residues for the human receptor) and the high amino acid homology, both in the putative TM domains (91.5%) and overall (82%), leave no doubt as to the identity of this novel human receptor subtype (Fig. 2). Two introns were identified in our genomic clones, and the positional conservation of these introns in the human 5-HT$_{2B}$ receptor gene, relative to the mouse and rat 5-HT$_{2B}$ receptor genes and, in fact, all members of the 5-HT$_2$ subfamily and their various species homologues, is indicative of a common evolutionary origin for these receptors, independently of the other serotonin receptor subfamilies.

A mRNA transcript of 2.3 kb was identified by Northern blot analysis in all human tissues examined (data not shown). This is very similar in size to the transcript found in the rat stomach fundus (3), suggesting that there is also conservation of the transcriptional initiation site and its usage across species. A smaller transcript has been identified in all human tissues, but the exact nature of this transcript, whether a splicing variant, the product of an alternate promoter or a different polyadenylation site, or perhaps a related novel receptor subtype, has yet to be ascertained. Currently, experiments addressing some of these possibilities are in progress.

When comparisons of the amino acid sequences are made between humans and rats for particular 5-HT$_2$ receptor subtypes, the 5-HT$_{2B}$ receptor species pair displays the least degree of homology (82%) of all pairs. The rat and human 5-HT$_{2A}$ and

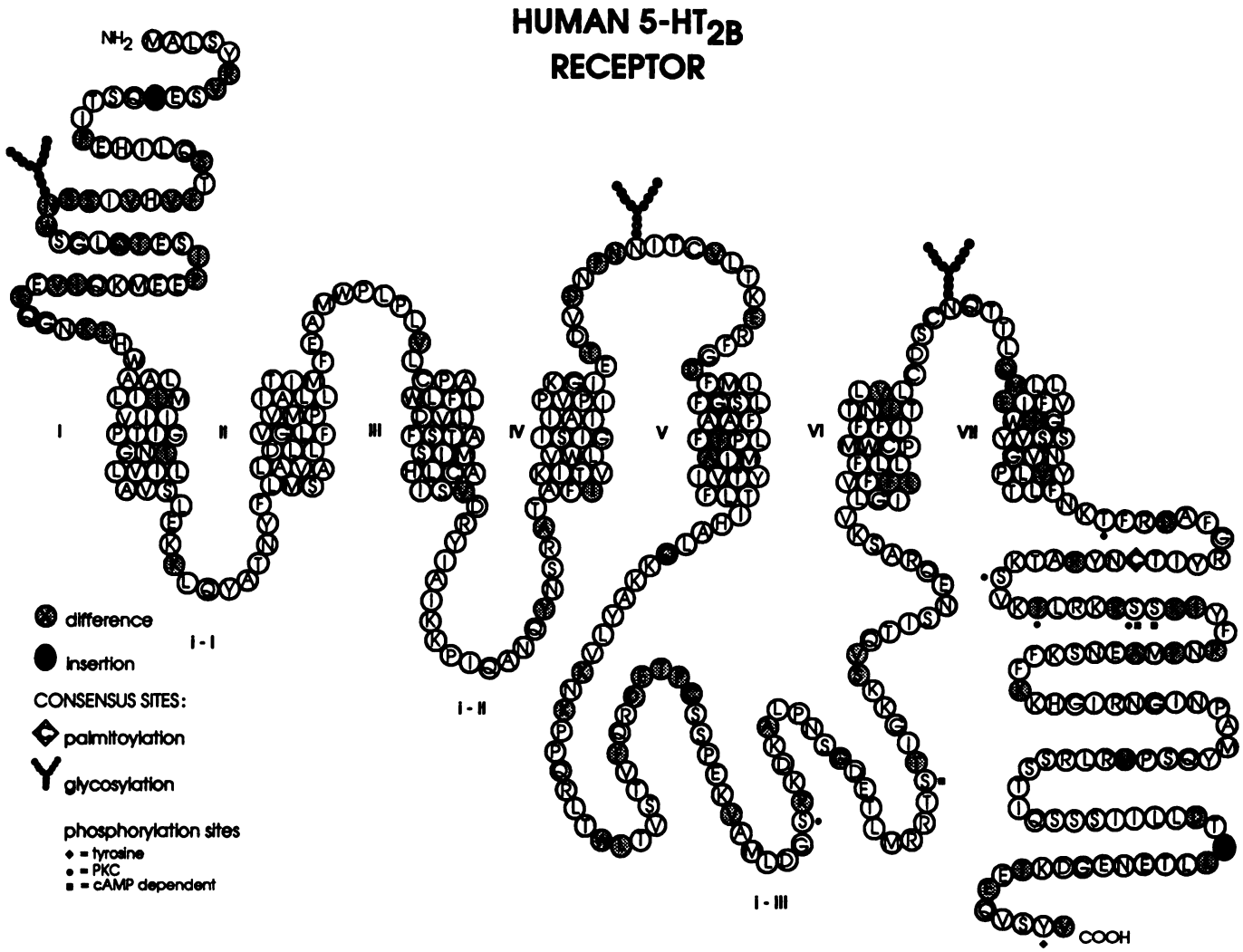

Fig. 4. Schematic representation of the human 5-HT_{2B} receptor. Differences from the rat 5-HT_{2B} receptor have been *highlighted*. Residues subject to potential modification have been indicated. Modifications have been deduced based on consensus motifs as defined by the UW/GCG software package. *PKC*, protein kinase C.

5-HT_{2C} receptors, on the other hand, are much more stringently conserved, with both pairs displaying 91% homology at the amino acid level. These observations suggest that there has been far less selective pressure to maintain the 5-HT_{2B} receptor sequences than either the 5-HT_{2A} or 5-HT_{2C} receptor sequences, although the physiological relevance of this is currently unknown.

The pharmacological profile of the human 5-HT_{2B} receptor is virtually identical to that of the rat 5-HT_{2B} receptor. The human 5-HT_{2B} receptor exhibits essentially the same high affinity for [^{3}H]5-HT (K_d = 10.18 ± 1.60 nM) as does the rat 5-HT_{2B} receptor. The only exceptions in our limited profile were the 5-HT_{2A} antagonists ketanserin and spiperone; higher affinity for both compounds was seen at the human 5-HT_{2B} receptor (see Table 1). Mutagenesis experiments will be helpful in defining the residues in the TM regions responsible for the species differences in affinity for these two compounds.

Functional coupling of the human 5-HT_{2B} receptor was demonstrated in AV12-664 cells, where 5-HT triggered a concentration-dependent increase in PI turnover. Although the 5-HT_{2B} receptor couples to PI hydrolysis in AV12-664 cells, presumably through phospholipase C, it remains a matter of debate whether this is the preferred transduction system for the 5-HT_{2B} receptor. Secrest *et al.* (16) have not demonstrated coupling to PI hydrolysis in the rat stomach fundus. The fact that other members of the 5-HT_2 receptor subfamily couple to PI hydrolysis in a number of tissues suggests that the 5-HT_{2B} receptor might couple to PI hydrolysis as well, and indeed it does in heterologous expression systems such as AV12-664 cells (3) and *Xenopus* oocytes (4). However, the fact is that no clearly consistent consensus sequence structural motifs in these receptors have been identified that confer the ability of 5-HT_2 subfamily members to interact with a particular set of G proteins that transduce PI hydrolysis (G_q, G_{11}, etc.). Furthermore, published literature has substantiated that coupling in a heterologous system is not a clear indication of what occurs in tissues, because numerous examples of promiscuous couplings have been found with receptors expressed in heterologous cell lines. Furthermore, coupling of a particular receptor subtype to a specific second messenger system in a given tissue is not absolute, because promiscuity has been observed there as well (17). The 5-HT_{2A} receptor, for instance, has been reported to couple to phospholipase A_2 in cultured hippocampal neurons (18).

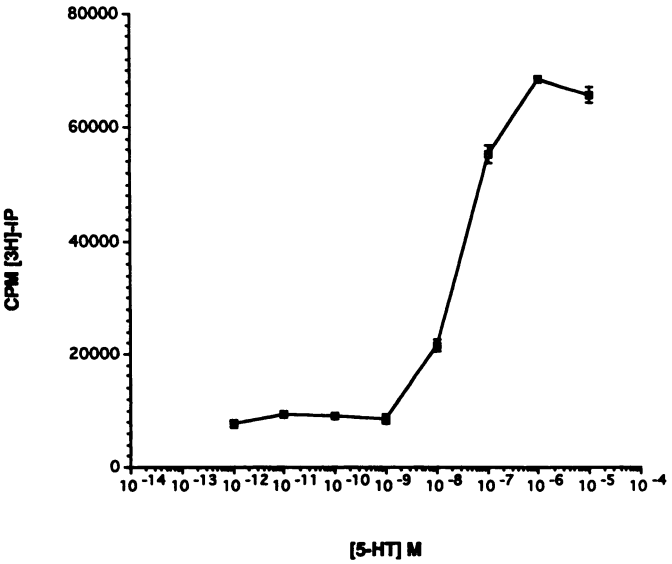

Fig. 5. Representative assay of PI turnover in the cell line stably expressing the human 5-HT$_{2B}$ receptor. Cells expressing the 5-HT$_{2B}$ receptor were labeled with *myo*-[^{3}H]inositol for 48 hr and stimulated with various concentrations of 5-HT for 1 hr at 37°. Inositol phosphates ([^{3}*H*]-*IP*) were extracted and quantified as described previously (3). Each concentration was assayed in triplicate.

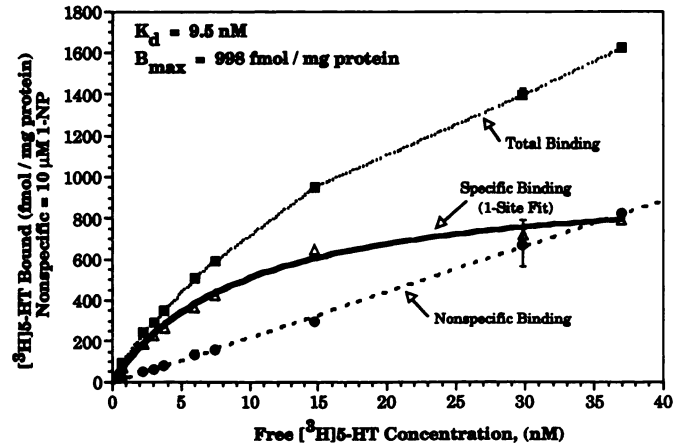

Fig. 6. Representative saturation isotherm for [^{3}H]5-HT binding to the cloned human 5-HT$_{2B}$ receptor. For total binding, *points* are the mean ± standard error of three data points. For nonspecific binding, *points* are the mean ± one half the range of two data points. The K_d was 10.18 ± 1.6 nM (mean ± standard error of three separate experiments). The *curve* for specific binding was modeled by nonlinear regression, and the *line* for nonspecific binding was fit by linear regression. *1-NP*, 1-naphthylpiperazine.

Recently, Wang *et al.* (19) have identified a novel $G_{\alpha z}$-like G protein that may be involved in 5-HT-mediated contractions of the rat stomach fundus. Although the effector coupled to this G protein is not known, $G_{\alpha z}$ has not been found to couple to the phospholipase $C_{\beta 1}$ isozyme in COS-7 cells (20). It may be that this G protein couples to a new second messenger system, as yet unidentified, that may be the preferred transduction system for the 5-HT$_{2B}$ receptor. In the rat stomach fundus, this transduction system apparently involves an influx of extracellular calcium through L-type voltage-dependent calcium channels, intracellular calcium release from the sarcoplasmic reticulum, and activation of protein kinase C, all of which are independent of PI turnover.[1] Except for the rat stomach fundus, there are no data available on the coupling

TABLE 1

Binding profiles for rat and human 5-HT$_{2B}$ receptors

Drug competition for binding was performed as described in Materials and Methods. K_i values are the mean ± standard error of the number of separate experiments given in parentheses.

	K_i for [^{3}H]5-HT binding	
	Human	Rat
5-HT	9.8 ± 2.3 (5)	10.2 ± 2.0[a] (6)
α-Methyl-5-HT	11.9 ± 1.1 (3)	10.5 ± 1.5[a] (4)
5-Carboxamidotryptamine	127 ± 16 (3)	147 ± 13 (7)
1-Naphthylpiperazine	3.8 ± 0.8 (8)	4.5 ± 0.5 (12)
DOI	19.9 ± 3.6 (4)	26.6 ± 1.6 (5)
Yohimbine	40.6 ± 4.7 (3)	53.1 ± 4.6[a] (4)
Ketanserin	376 ± 58[b] (3)	3559 ± 175[a] (3)
Spiperone	697 ± 54[b] (3)	3278 ± 92[a] (3)

[a] K_i values for the rat receptor that were taken from Ref. 9.
[b] Significant difference from the rat K_i value for the same compound ($p < 0.001$).

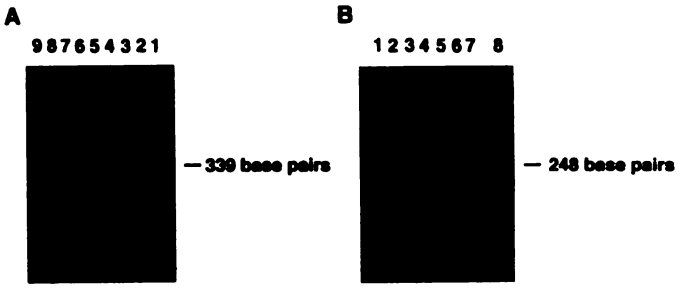

Fig. 7. Brain distribution of 5-HT$_{2B}$ mRNA expression. A, Two human 5-HT$_{2B}$ receptor-specific oligonucleotide primers, described in Materials and Methods, were used to amplify a 339-bp DNA fragment from the following regions of human brain: cerebellum (*lane 1*), cerebral cortex (*lane 2*), corpus callosum (*lane 3*), amygdala (*lane 4*), substantia nigra (*lane 5*), caudate (*lane 6*), thalamus (*lane 7*), hypothalamus (*lane 8*), and retina (*lane 9*). B, Two rat-specific oligonucleotide primers were used to amplify a 248-bp DNA fragment from the following regions of rat brain: brainstem (*lane 1*), caudate (*lane 2*), cerebellum (*lane 3*), cortex (*lane 4*), hippocampus (*lane 5*), hypothalamus (*lane 6*), and pituitary (*lane 7*), with AV12-664 cells expressing the rat 5-HT$_{2B}$ receptor as a positive control (*lane 8*). Amplified products were examined as described for Fig. 2. B is a Southern hybridization analysis of amplification reactions for the various rat brain regions and positive control cells.

mechanism of the 5-HT$_{2B}$ receptor or a 5-HT$_{2B}$-like receptor. Obviously, much work remains to be done in this area.

The fact that 5-HT$_{2B}$ receptor mRNA is present not only in human brain but also in peripheral tissues raises some possibilities regarding its potential physiological roles in these tissues, and one might begin to ask questions about the nature of these roles. For instance, serotonin receptors are known to mediate both contraction and relaxation in many blood vessels, and it may be that this receptor is present in a vascular structure common to all of these tissues, including the brain, such as venules, arterioles, or capillaries. In potential support of this idea, two groups have recently reported 5-HT$_2$-like receptors on endothelial cells with pharmacological properties similar to those of the rat 5-HT$_{2B}$ receptor. The relaxant 5-HT receptor found on the endothelium of porcine pulmonary arteries is similar to the rat 5-HT$_{2B}$ receptor described by Hoyer *et al.* (2). Other endothelial receptors mediating relaxation have been identified in rat (21) and rabbit (22) jugular veins, and these also have pharmacological properties similar to those of the rat 5-HT$_{2B}$ receptor. Bodelsson *et al.* (21) allude to the similarity of the rat jugular vein endothelial receptor to the contractile receptor of the rat stomach fundus, although the panel of compounds chosen to characterize the rat jugular vein

endothelial receptor was not extensive enough to definitively confirm this relationship.

The 5-HT_{2B} receptor is responsible for mediating contraction of the rat stomach fundus, so it is also possible that it may mediate contractions in human blood vessels, although this function is thought to be effected by the 5-HT_{2A} and 5-$HT_{1D\beta}$ receptors in the human vessels examined (23). It is important to note, however, as Kaumann *et al.* (23) point out, that animal models of 5-HT receptor action are often of no use in describing actions in humans, thus, great care must be taken in drawing analogies from one species to another. Still, these relationships may serve as a starting point for investigating potential functions in humans. In fact, a 5-HT-mediated relaxation response has been reported in human vessels, the exact nature of which is still very much open to further investigation (23). Experiments are currently in progress to look for the presence of the 5-HT_{2B} receptor in human endothelial and smooth muscle cells.

A different line of thought might suggest that the 5-HT_{2B} receptor mediates different physiological functions in different tissues. For example, the 5-HT_{2A} receptor is known to have multiple physiological roles within the same species. These include mechanical physiological functions (e.g., smooth muscle contraction of some blood vessels) (24) and cognitive functions of the brain (e.g., the 5-HT_{2A} receptor is thought to be the site at which lysergic acid diethylamide and other hallucinogens act) (25). Therefore, there is precedence for the idea that the 5-HT_{2B} receptor may serve different roles in different tissues. It may be that in peripheral tissues it serves one of the roles mentioned above or is involved in completely different roles (smooth muscle contraction in gut motility, neural transmission, etc.), while at the same time it might be associated with some form of higher cognitive function in the human brain. The presence of the 5-HT_{2B} receptor in human brain but not rat brain may lend some support to this hypothesis. This brain distribution difference is especially interesting because differences in microlocalization and regional expression of 5-HT receptor subtypes have been observed between species but the complete presence or absence of a receptor subtype has not been noted previously. Whether this has some important physiological significance for brain function remains to be seen. Microlocalization experiments using either *in situ* hybridization, immunohistochemistry, or radiolabeled ligand binding to tissue slices will be most helpful in sorting out the cell types expressing this receptor subtype, which may then help to define its function(s).

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Schmuck et al. have reported the cloning of the human 5-HT_{2B} receptor after submission of this manuscript, FEBS Lett. **342:** 85–90 (1994).

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