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Cloning and functional characterization of the human 5-HT_{2B} serotonin receptor

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Abstract

Recently, we have reported the cloning of the rat 5-HT_{2B} receptor cDNA. This receptor is particularly interesting since it may be involved in diseases such as migraine. Here, we describe the isolation of a human 5-HT_{2B} receptor clone from a cDNA library derived from SH-SY5Y cells. Although the receptor sequence was only 80% homologous to the rat sequence, the exon–intron distribution was conserved between the two species. In the human body, the receptor mRNA was detected in most peripheral organs. Only low expression levels were found in the brain. After expression in HEK 293 cells, activation of the receptor stimulated the production of phosphatidylinositol. The pharmacology of this functional response correlated well with that of the rodent receptor.

Key words: Phosphatidylinositol turnover; Cloning and expression; SH-SY5Y cell; HEK 293 cell

1. Introduction

Molecular cloning has revealed the existence of more than thirteen serotonin receptor types belonging to various subgroups [1]. With the exception of the 5-HT_3 receptors which are ligand-gated ion channels, all known serotonin receptors belong to the family of seven-helix receptors which couple to GTP binding proteins. The three receptor subtypes which are thought to stimulate phosphatidylinositol turnover [2,3] are called 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. The latter was called 5-HT_{1C} in the older literature [4]. In addition to their similar second messenger coupling, these three receptors can be distinguished from other serotonin receptors due to their similar pharmacology, high sequence homology, and, in the mouse genome, highly conserved intron-exon distribution [5]. Central effects of the 5-HT_{2A} receptors include an involvement in the serotonin induced wet-dog shake behavior [6] and behavioral excitation [7]. In the periphery, activation of this receptor causes vasoconstriction and platelet aggregation [8], and increased body temperature [9]. The 5-HT_{2C} receptor is expressed throughout the brain [10,11] and on the epithelial cell layer of the choroid plexus [12]. This receptor type has been reported to increase grooming, penile erection, oxytocin secretion [13], and transferrin levels in the choroid plexus [14].

Serotonin 5-HT_{2B} receptors were first functionally characterized in the rat stomach fundus where they trigger muscle contraction [15]. The human stomach does not contain a contractile tissue similar to the rat stomach fundus. Therefore, no function can unequivocally be ascribed to this receptor type in humans. There are, however, several 5-HT_2 receptor functions in which this receptor type may be involved. Usually it is not possible to distinguish between 5-HT_{2C} and 5-HT_{2B} receptor action based on the pharmacology reported in the literature. Therefore, several functions which were thought to be 5-HT_{2C} receptor mediated may involve the 5-HT_{2B} receptor. This includes the role of serotonin receptors in alcohol intake [16], in the reduction of aggressive behavior [17] or in neuroendocrine effects [18]. It is of particular interest that the involvement of serotonin receptors in the onset of migraine attacks, which has been speculated to be 5-HT_{2C} receptor mediated [19], may equally well be caused by 5-HT_{2B} receptors [20]. The cloning and characterization of the human receptor type described here therefore provides a tool to study the involvement of the receptor in these physiological and pathological processes.

2. Materials and methods

2.1. Oligonucleotides

ON 1–4, rat sequence; ON 5–13, human sequence.

ON 1: AGG CTA TAT GGC CCC TCC CAC T

ON 2: GAA ATT AAC CAT ACC ACT GTA ATC TTG

ON 3: GCA TCG CCA TCC CAG TCC CTA T

ON 4: AAA AGG GGC ACC ACA TAA GC

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ON 5: ACG TTC TCT TTT CAA CCG CA

ON 6: CCG GTG ACG AGC AAG GTG TT

ON 7: TTA TCA CCA TGA GTA TCA GA

ON 8: GCT GTT TCA CTG GAG AAG AA

ON 9: TGC AGT TTA TTT CCC TGT TC

ON 10: GAA GCT GCA GTA TGC TAC TAA T

ON 11: GAA ATA ACC AGG CAG GAC AT

ON 12: ACC AGG CAG GAC ATA GAA CA

ON 13: CAA ATC CAG GCC AAT CAA TA

2.2. Cloning, sequencing and sequence analysis

Two sets of primers were synthesized coding for transmembrane domain III/IV (ON 1 and 2) and IV/VI (ON 3 and 4) of the rat 5-HT_{2B} sequence. PCR reactions with primer pairs ON 1 and 2 or ON 3 and 4, respectively, were performed using 1 μg of HeLa cell DNA as a template. The reaction was performed for 32 cycles in a total volume of 50 μl. Both PCR products were reamplified in a second PCR using 2 μl of the first PCR with the same primer pairs for another 32 cycles. Products were isolated by agarose gel electrophoresis and sequenced directly using the Hot tub DNA sequencing kit (Amersham). From the resulting human sequence specific oligonucleotides ON 5 and 6 were designed for the cDNA library screening. Poly A^+ RNA was isolated from SH-SY5Y cells and a cDNA library was prepared in a modified bluescript vector as described previously [2]. The library was transformed into *E. coli* MC 1061 by electroporation (Gene Pulser, BioRad). It contained 2×10^7 independent transformants with an average insert size of 2 kb. Each of 25 pools with 5×10^4 clones were grown overnight and plasmid DNA was isolated. Of these DNAs, 0.5 μg were linearized and PCR was performed using the human primers ON 5 and 6 as described above. Four pools gave rise to the expected PCR product. One of them was transformed into *E. coli* DH5α, plated on agar and screened by standard filter hybridizations [21] using a PCR ssDNA probe [22]. DNA sequencing was performed with the T7 sequencing kit from Pharmacia. The DNASIS and PROSIS programs (Hitachi) were used for the sequence analysis.

2.3. Determination of exon–intron boundaries

The location of introns within the 5-HT_{2B} receptor gene was determined using inverse PCR [23] and direct sequencing of the PCR products. To determine the exon–intron boundaries within the coding region, 5 μg of HeLa cell DNA was digested with *Rsa*I. After circularization of 200 ng DNA with T4 DNA-ligase in a final volume of 100 μl, 30 cycles of PCR were performed with 40 ng of this DNA and 10 pmol each of the primers ON 7 and 8 or ON 11 and 5. Of these PCRs 2 μl were further amplified using the nested primer pairs ON 9 and 10 or ON 12 and 13 under identical conditions. PCR products were isolated by agarose gel electrophoresis and sequenced directly as described.

2.4. Distribution of receptor expression in human RNA's

The receptor RNA was identified by RT-PCR. RNAs from human tissues were obtained from Clontech. For the RT-PCR 1 μg of each RNA was reverse transcribed in a final volume of 20 μl using M-MLV reverse transcriptase (BRL) in PCR-Buffer (Boehringer) according to supplier's instructions. The PCR contained 2 μCi [α-^{32}P]dCTP and 10 pmol each of the primers ON 5 and 6 in a final volume of 50 μl. The ^{32}P-labeled PCR-products were separated on 4% agarose gels (NuSieve, FML). The gels were dried and exposed to X-ray films.

2.5. Functional characterization of the receptor

The human 5-HT_{2B} receptor cDNA was subcloned into the *Sal*I restriction site of the mammalian expression vector pXMD1 [24]. HEK 293 (Human Embryonic Kidney) cells were grown in MEM (Earl's salts, L-glutamine) supplemented with 10% fetal calf serum. For transfection, the cells were plated at 1.5×10^6 cells per 10 cm plate and used for transfection 24 h later. Plasmid DNA (6 μg per 10 cm plate) was transfected with the $CaPO_4$ method as described [25].

For the measurement of the [^{3}H]inositol phosphate formation cells were split into 12 wells of a 24-well plate 24 h after transfection and labeled to equilibrium with [^{3}H]inositol (3 μCi/ml) for an additional 24 h in growth medium. Then, two min after applying Li^+ (20 mM) in HBS-buffer (130 mM NaCl, 900 μM NaH_2PO_4, 800 μM $MgSO_4$, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM glucose in 20 mM HEPES pH 7.4) cells were stimulated by the addition of the agonist. Antagonists were added 5 min prior to 10^{-7} M 5-HT. The measurement of total [^{3}H]inositol phosphate formation was performed as described [26].

3. Results

3.1. Isolation of a human 5-HT_{2B} receptor cDNA clone

To obtain some sequence information on the human 5-HT_{2B} receptor we amplified and sequenced Hela cell DNA from individual exons of the 5-HT_{2B} receptor gene. Assumptions about the exon–intron distribution were based on that in the mouse genome [5]. For the cloning of the human 5-HT_{2B} receptor cDNA we then searched for human cell lines expressing this receptor. Using RT-PCR with primers derived from the human sequence and direct sequencing of the PCR products, we found relatively high levels of 5-HT_{2B} receptor mRNA in the human neuroblastoma cell line SH-SY5Y (data not shown). We then prepared a cDNA library from these cells and isolated a 5-HT_{2B} receptor cDNA clone from this library. The sequence contained an open reading frame encoding a protein of 483 amino acids (Fig. 1A). The hydrophobicity plot displayed seven hydrophobic, putatively membrane-spanning domains (data not shown). The protein contained five consensus sequences for N-linked glycosylation [27], one of them located within the putatively extracellular N-terminal domain and two in the last and second-last extracellular loop (Fig. 1A). A potential site for palmitoylation was present in the carboxyterminal part of the molecule (Fig. 1A). Depalmitoylation at an analogous site of the β_2-adrenergic receptor has resulted in receptor uncoupling [28,29]. Although the sequence contained recognition motifs for various protein kinases [30], including cAMP- and cGMP-dependent protein kinases, casein kinase II and S6 kinase, there was no consensus sequence for phosphorylation by protein kinase C. Of particular interest may be the recognition sequences for cAMP-dependent protein kinases in the third intracellular loop and the carboxy-terminus.

3.2. Identification of exon–intron boundaries in the human 5-HT_{2B} receptor gene

The location of introns and the sequences of the exon–intron boundaries in the human 5-HT_{2B} receptor gene were determined using inverse PCR and direct sequencing of the PCR products (Fig. 1A and B). Two introns were found between nucleotides 407/408 and 608/609. In these same locations introns are present in the genes for all three mouse 5-HT_2 receptor subtypes [5]. Further PCR experiments with HeLa DNA confirmed the absence of additional introns within the coding region (data not shown).

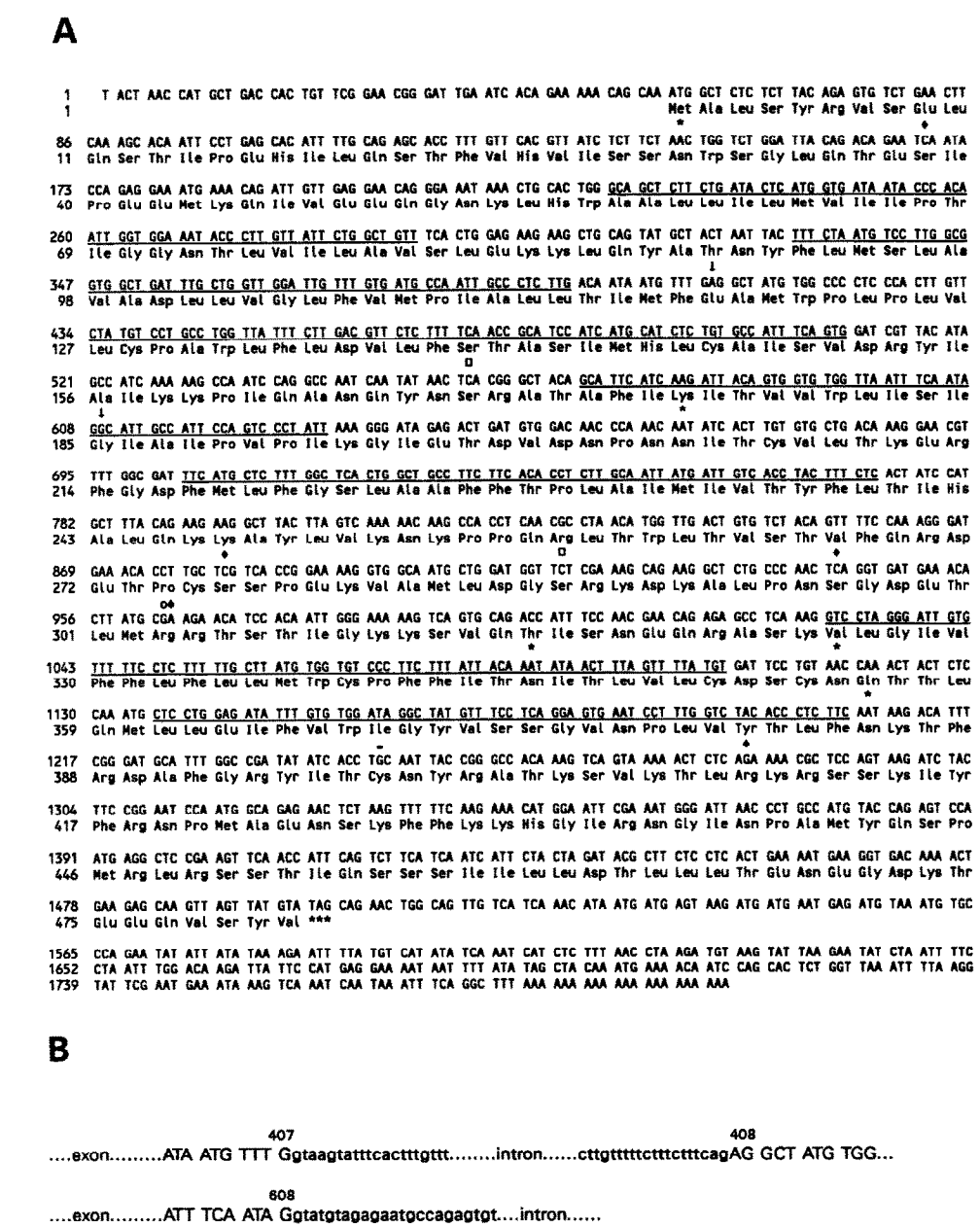


Fig. 1. (A) Nucleic acid and deduced amino acid sequence of the human 5-HT_{2B} cDNA and (B) sequences of the exon–intron boundaries. Underlined are the putative membrane spanning regions. Consensus sequences for N-linked glycosylation are marked by an asterisk, a bar marks the Cys which is possibly attaching the C-terminal region to the membrane via a palmitoyl anchor. Several recognition motifs for protein kinases are indicated (○ cAMP-dependent protein kinase, □ cGMP-dependent protein kinase, ◆ casein kinase II, ♠ S6 kinase). Splice sites are marked by an arrow. Nucleotides indicated by capital letters are located in exons, those in lower case letters in introns. The human 5-HT_{2B} receptor sequence has been deposited at the EMBL data library with the accession number X77307.

3.3. Protein sequence comparisons

The human 5-HT_{2B} aminoacid sequence displayed 79 and 82% overall sequence identity to the rat and mouse 5-HT_{2B} receptors, respectively [2,31], 58% homology to the human 5-HT_{2A} [32] and 51% to the human 5-HT_{2C} receptor [32]. The homology within the membrane spanning regions was 91.5, 92, 71, and 73% for the rat and mouse 5-HT_{2B}, the human 5-HT_{2A} or 5-HT_{2C} receptors, respectively. The usual amino acids are conserved in this member of the family of G-protein coupled receptors. An alignment of the human, rat and mouse 5-HT_{2B}, the human 5-HT_{2A}, and the human 5-HT_{2C} receptor sequences are depicted in Fig. 2.

3.4. Distribution of receptor RNA

Using RT-PCR with RNAs isolated from various human organs, we have analyzed the distribution of receptor expression (Fig. 3). Although this kind of analysis is, without using internal standards, only semiquantitative, we found relatively high levels of expression in most

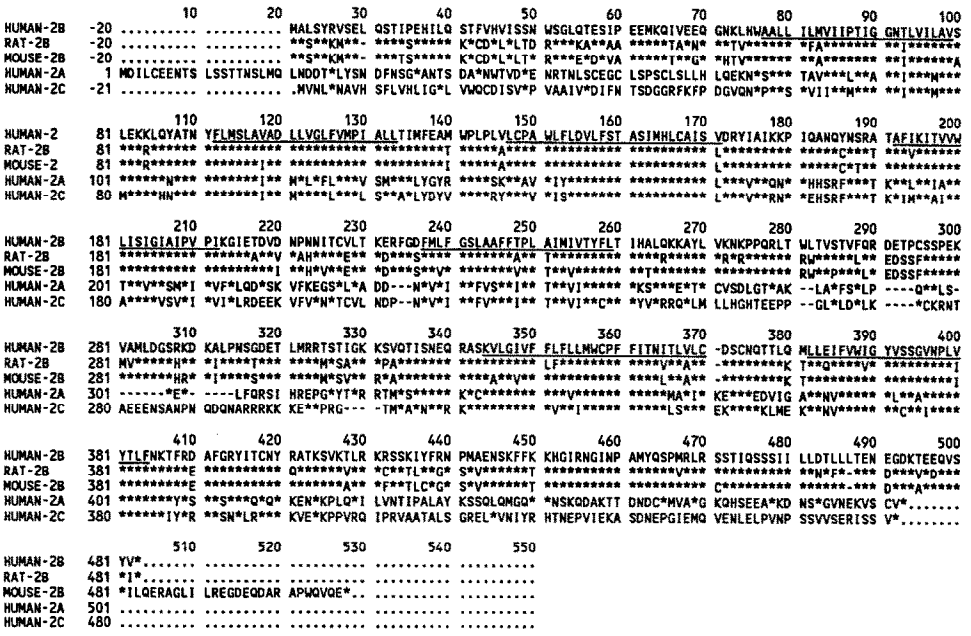
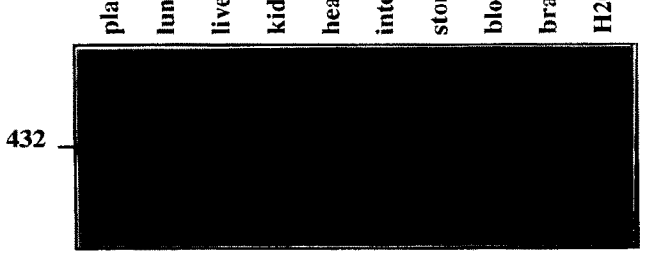


Fig. 2. Alignment of the human, rat and mouse 5-HT_{2B} [2,31] and the human 5-HT_{2A} and 5-HT_{2C} [32] receptor sequences. Bars represent the putative membrane spanning regions. Amino acid identities are illustrated by asterisks.

peripheral tissues. Strongest signals were found with kidney, heart and intestine cDNAs. Only very low expression levels were seen in the brain and in blood.

3.5. *Functional characterization of the human 5-HT_{2B} receptor*

After transient expression of the receptor in HEK 293 cells, serotonin application to the cells stimulated the hydrolysis of phosphatidylinositol. The pK_D for serotonin in this assay was 8.04. Other agonists, DOI and MeOT, stimulated the second messenger system to similar levels. Their potencies were 7.95 and 7.63, respectively (Fig. 4A). The concentrations of the antagonists yohimbine, mianserin, pizotifen, and spiperone required for 50% inhibition of the stimulatory effect of serotonin were determined (Fig. 4B). Apparent K_i values were then estimated from these IC_{50} values, adapting the Cheng-Prusoff equation [33], $K_i = IC_{50}/(1 + [5\text{-HT}]/EC_{50})$, where [5-HT] is the serotonin concentration used (= 10^{-7} M) and EC_{50} the concentration of serotonin producing half-maximal stimulation (= 9×10^{-9} M). For yohimbine, mianserin, pizotifen, and spiperone the pK_i values were 7.61, 7.23, 8.18, and < 5, respectively.



Distribution of the human 5-HT2B-receptor mRNA

Fig. 3. Distribution of the 5-HT_{2B} receptor gene expression. After reverse transcription of the RNAs 32 cycles of PCR were performed. PCR products were separated by gel electrophoresis. The size of the PCR products are indicated in base pairs.

4. Discussion

We have isolated a cDNA clone for the human 5-HT_{2B} receptor from SH-SY5Y cells and determined the exon–intron distribution of the human gene. Although the overall sequence homology between the rodent and human receptors is relatively low (about 80%), it is significantly higher within the putative membrane-spanning regions (above 90%). Based on the homology and the pharmacological characteristics of the receptors, they are clearly species homologs of one another. The human receptor contains an N-linked glycosylation signal in its N-terminal extracellular domain. This signal is neither found in the rat nor in the mouse receptor. This observation may be of interest but it cannot yet be interpreted since functional importance of these glycosylation sites which occur in almost all G-protein coupled receptors could not be demonstrated [34].

The gene of the 5-HT_{2B} receptor contains at least two introns in positions where introns were also found in the mouse 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor genes [5]. This emphasizes the close relationship between these receptors which in evolution have obviously developed from a common ancestor.

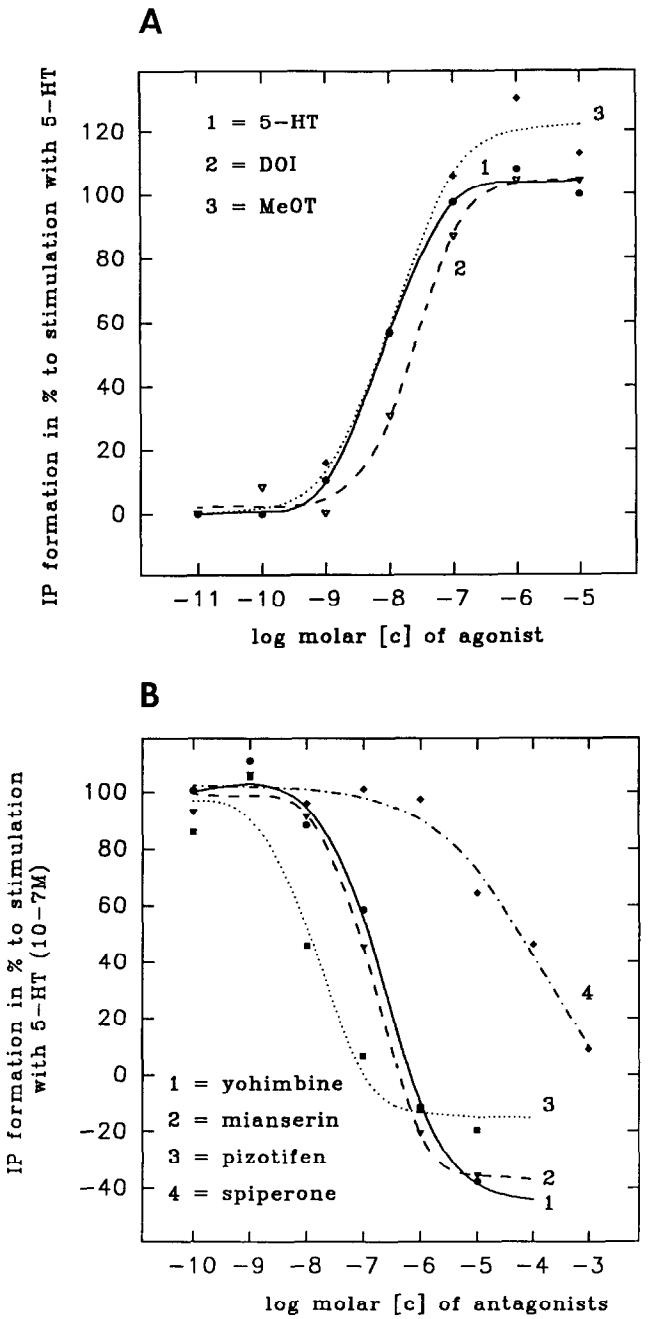


Fig. 4. Stimulation of phosphatidylinositol hydrolysis by the human 5-HT_{2B} receptor expressed in HEK 293 cells. (A) HEK 293 cells transiently expressing the receptor were stimulated with the indicated concentration of the agonists 5-HT, DOI, and MeOT. The accumulation of inositol phosphate was determined. Shown are the means of three independent determinations in a representative experiment. (B) Inhibition of the stimulation triggered with 10^{-7} M serotonin by increasing concentrations of various antagonists.

In humans the receptor is expressed in most peripheral organs. Only low expression levels are found in brain and blood. In a previous study where we analyzed the distribution of the rat 5-HT_{2B} receptor, we found by far the highest expression levels in the stomach fundus [2]. The low expression of this receptor in the brain is unusual since most other serotonin receptors display highest expression levels in this organ. The 5-HT_{2C} receptor which is most closely related to the 5-HT_{2B} receptor, is found almost exclusively in the brain and the choroid plexus [10–12]. It may be speculated that these two receptors are the central and peripheral counterparts of one another. The low level of 5-HT_{2B} receptor RNA found in brain tissue may then be attributed to the blood vessels. By comparison, the 5-HT_{2A} receptor is expressed in the brain and in the periphery [8,35,36]. Additional research will show if this distinction of 5-HT_{2B} as a peripheral and 5-HT_{2C} as a central receptor is valid.

After expression of the human 5-HT_{2B} receptor in HEK 293 cells, agonist-binding stimulates phosphatidylinositol hydrolysis. This had previously also been shown for the rat 5-HT_{2B} receptor [2,3]. The pharmacology of this response corresponds well with that of the rat 5-HT_{2B} receptor determined in membrane binding experiments and in a similar functional assay [2,3]. The high affinity of yohimbine and the low affinity of spiperone clearly distinguish the pharmacology of this receptor from those of the 5-HT_{2C} and 5-HT_{2A} receptors, respectively [2]. The efficiency of the serotonin-induced phosphatidylinositol hydrolysis is, however, significantly weaker in cells expressing the human 5-HT_{2B} than that observed in cells expressing other 5-HT_2 receptor types under the same conditions. In cells transiently transfected with a 5-HT_{2C} receptor clone, for example, the serotonin-induced stimulation of phosphatidylinositol breakdown is approximately three times stronger than that seen in cells transfected with the human 5-HT_{2B} receptor clone under the same conditions (data not shown). Previously, we demonstrated that the rat homologue of this receptor displays similar coupling in *Xenopus* oocytes which is also profoundly weaker than that observed with the other 5-HT_2 receptor types [2]. This raises the question, wether the 5-HT_{2B} receptor is able to couple to other signalling pathways as well.

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