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New mouse 5-HT2-like receptor

Expression in brain, heart and intestine

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A novel member of the family of G protein-coupled receptors has been isolated from a mouse brain cDNA library by screening with polymerase chain reaction (PCR) generated fragment of mouse genomic DNA amplified using degenerated primers. Sequence comparison demonstrates that the encoded protein sequence shows the highest homology to the 5-HT2 family of receptors. The pharmacological profile of membranes from COS cells transfected with this cDNA, corresponds to a new 5-HT2-like receptor that we propose to call 5-HT2C. Its major sites of expression are in the mouse intestine and heart, also with detectable expression in brain and kidney. We speculate that it could account at least in part for the 'atypical' functions attributed to the 5-HT1C/5-HT2 receptors.

G protein-coupled receptor; cDNA cloning; Polymerase chain reaction; Smooth muscle; Ritanserin

1. INTRODUCTION

The aminergic neurotransmitter serotonin is believed to play an important role in a multitude of cognitive and behavioral functions and dysfunctions including motor control, feeding, anxiety, depression, and sexual activity [1]. This large diversity of functions is paralleled by the pharmacological complexity of serotonin receptors. At least four classes have been distinguished pharmacologically: 5-HT1, 5-HT2, 5-HT3, and 5-HT4. These categories are defined by both binding and effector coupling properties of the receptors [2,3]. More recently, molecular biological data have confirmed the existence of multiple serotonin receptors, including both ligand-gated receptors (5-HT3) and G protein-coupled receptors (GPRs) [4]. This latter class can be split into two classes [5], reflecting the second messenger system to which the receptor is coupled: the 5-HT1C and the 5-HT2 receptors which are coupled to the activation of the phospholipase C, and the 5-HT1A, B and D family, which interacts with the adenylyl cyclase; it is possible that 5-HT4 belongs to this class, although it has not yet been characterized molecularly. To date the adenylyl cyclase 5-HT1 subclass has been the most extensively studied, with more than five subtypes already described [6]. By contrast only two members of the 5-HT2 subfamily have been characterized, 5-HT1C and 5-HT2.

5-HT2 receptors mediate many of central and peripheral physiological functions of serotonin. Cardiovascular effects include contraction of blood vessels and shape change in platelets; central nervous system effects include neuronal sensitization to tactile stimuli and mediation of hallucinogenic effects of lysergic acid diethylamide and related phenylisopropylamine hallucinogens. Many investigators have observed that 5-HT1C and 5-HT2 receptors do not reflect all the properties attributed to them. For example some 5-HT2-like effects of serotonin on peripheral smooth muscles are classified as 'atypical' [7], leading to the hypothesis that other 5-HT2 receptor subtypes might exist.

Therefore, we have undertaken the cloning and recombinant expression of new 5-HT2 receptors in the mouse in order to study their functional properties. Previous molecular cloning experiments have demonstrated that the 5-HT1 and 5-HT2 receptor subclasses are encoded by members of the seven transmembrane domain containing gene superfamily. Taking advantage of the sequence conservation between related members of this family we have screened for new members of the 5-HT2 class. We describe here the cloning and functional characterization of a new 5-HT2-like receptor.

2. MATERIALS AND METHODS

2.1. *Drugs and chemicals*

Restriction endonucleases, AMV Reverse transcriptase, DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase, T3 or T7

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RNA polymerase, were purchased from Bethesda Research Laboratories, New England Biolabs, Boehringer-Mannheim, and Stratagene. Sequenase and sequencing reagents were purchased from United States Biochemical Corporation. Taq polymerase for PCR was purchased from Perkin-Elmer-Cetus and reactions performed in a Perkin-Elmer-Cetus cycler. Ketanserin, ritanserin and setoperone were kindly provided by Janssen (Beerse, Belgium). ICS 205-930 and MDL 72 222 were gifts from Sandoz (Basel, Switzerland) and Merell-Dow (Strasbourg, France), respectively. Other neurochemicals were from the RBI, or Sigma. ^{32}P-, and ^{35}S-labeled nucleotides as well as [^{125}I]DOI (2200 Ci/mmol), were from New England Nuclear.

2.2. *Standard molecular biology techniques*

Classical published procedures were used for library screening plasmid subcloning, RNA extraction, and COS cell transfections [8].

2.3. *PCR experiments*

We synthesized degenerated oligonucleotides coding for the conserved VIth (a) and VIIth (b and c) transmembrane domains sequences [9].

(a) TACCTCGAGGTCGACGGTIATGTGGTG{C,T}CCITT-{C,T}TT{C,T}AT

(b) AGAACTAGTGGTACCCA{G,A}IGT{G,A}TAIACIA{G,A}-IGG{G,A}TT

(c) AGAACTAGTGGTACCC{G,C}{A,T}{G,A}CAIAC{G,A}TA-ICC{G,A,T}ATCCA

A polylinker has been added at the 5′ end for subcloning. 1 μg of mouse genomic DNA was denatured 1 min at 94°C, annealed 2 min at 55°C, and amplified 3 min at 72°C in the presence of 3 mM $MgCl_2$ for 20 cycles for the first two sets of primers (a and b, 1 μg each). 1/10th of the reaction was reamplified with the first and third set of primers (a and c, 1 μg each) for 20 more cycles under the same conditions. Products were subcloned in pBluescript and sequenced. Oligonucleotides corresponding to the characterized products were synthesized and then used as probes.

For quantitative RT-PCR experiments, standard PCR reaction buffer was used in the presence of 10 μg of total RNA. After denaturation, AMV reverse transcriptase (13 U) and Taq polymerase (5 U) were added, and extension was done at 50°C for 15 min and then a standard PCR amplification protocol [8] was used. Samples were taken after 20, 25, and 30 cycles to ensure that the reaction was in the exponential phase of synthesis. We used, as an internal standard, primers corresponding to the mRNA of the ribosomal elongation factor, EF1A, amplified in the same reaction as the NP75 primers.

2.4. *[^{125}I]DOI binding assays*

[^{125}I]DOI [10] was used as the radioligand to detect expression of the NP75 gene product in membrane fractions isolated from COS-7 cells 48 h after DNA transfection. Briefly, the incubation medium (200 μl) contained 50 μl of radioligand, 50 μl of buffer or of competing drug, and 100 μl of membrane suspension (protein concentration, 50 μg/ml). The mixture was incubated at 30°C for 30 min. The assay was terminated by addition of ice-cold iso-osmotic solution, and rapid filtration through GF/B filters, followed by 4 washes of 5 ml of ice-cold buffer. Filters were dried rapidly and their radioactivity determined by liquid scintillation counting. Non-specific binding, determined in the presence of 10 μM unlabeled DOI, represented about 30% of total binding.

Competition studies for [^{125}I]DOI-binding were performed by adding increasing concentrations of test drug to the reaction.

Data were analyzed using the iterative non-linear regression fitting program LIGAND (Version 3.0, McPershon 1985) and RS1 (Release 4.0).

3. RESULTS AND DISCUSSION

G protein-coupled receptors share extensive homology at the amino acid level, especially within the transmembrane region. The sixth and seventh transmembrane domains present the highest score of homology between several members of this family of receptors [9]. We therefore designed degenerated oligonucleotides, to perform PCR reactions on mouse genomic DNA. Several genomic fragments whose sequences were similar to known GPRs were obtained, and subcloned. Some of these products were identical to the already cloned mouse serotonin receptors [11]. Among these products a novel sequence, NP75, was identified as the most similar to the 5-HT2 sequence. This we used to screen a mouse brain cDNA library.

The complete NP75 cDNA contains an open reading frame of 1510 bp encoding a protein of 504 amino acids with a predicted molecular weight of 56,508 Da (Fig. 1). On the basis of the hydropathy plot (not shown), this protein displays seven hydrophobic domains, and has extensive homology with other members of the GPR family, including one potential *N*-glycosylation site at its amino terminus (Fig. 1), and consensus sequences for phosphorylation by different protein kinases in the cytoplasmic regions. In addition the presence of 19 Ser or Thr residues in the 121 carboxyterminal residues (Fig. 1) may indicate that this region is involved in the receptor desensitization by protein kinases as has been demonstrated for the β-adrenergic receptor [12].

When compared with other GPRs, the NP75 transmembrane region exhibits high degree of homology with the 5-HT2 and 5-HT1C receptors, especially within domains II and III where 12 and 20 amino acids, respectively, are identical. Alignment of the transmembrane region of various aminergic receptors suggests that the NP75 receptor belongs to the 5-HT2 subfamily [5], being slightly more divergent from 5-HT1C than from 5-HT2; homologies reach 60% with the mouse 5-HT2 receptor [11] and 57% with the mouse 5-HT1C [13]. Compared to the rat 5-HT1A receptor [14], or mouse 5-HT1B receptor [8] the homology is less than 30%. By comparison, the homologies between 5-HT2 and 5-HT1C receptor of the same species are around 70%, and the 5-HT2 receptors from different species have homologies over 90%. In addition, the NP75 gene includes two introns (not shown), which are present at equivalent positions to the introns in the 5-HT2 gene [11]. This confirms a probable common evolutionary origin, different from the other 5-HT1 genes which are devoid of introns. In order to confirm that this clone represents a new member of the 5-HT2 family we analysed it further by expression studies.

To determine the pharmacological properties of the predicted NP75 protein, the coding sequence of the cDNA was introduced into the eucaryotic vector pSG5 [15] and transfected into COS-7 cells, whose membranes were then tested for their binding properties. Steady-state binding assays with [^{125}I]DOI demonstrated that this ligand interacts specifically with the recombinant-transfected membrane preparation (and not to mock transfected cells) with high affinity and in a saturable

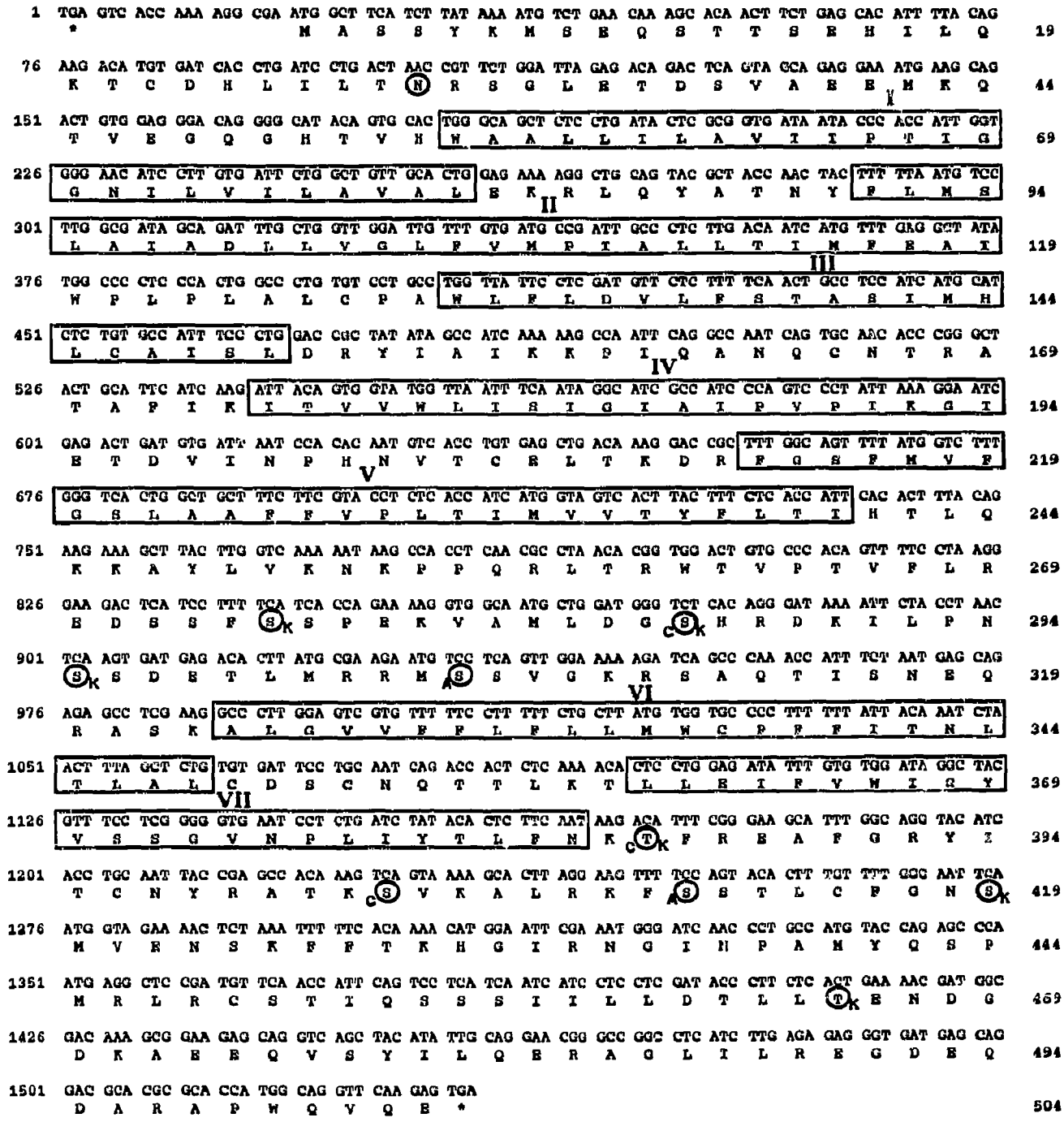

Fig. 1. Sequence of the NP75 cDNA. The NP75 cDNA and deduced protein sequences are displayed. Roman numbers over the boxes localize the transmembrane domains. The left numbering is for the DNA sequence, the right is for the protein. In frame stop codons are shown by *. The circled N indicates putative *N*-glycosylation sites. Circled serines or threonines represent consensus for phosphorylation by protein kinase A (A), protein kinase C (C), and casein kinase (K).

fashion (Fig. 2). The resulting saturation data exhibits a best-fit to a single site mode with an apparent K_d of 25.8 ± 0.54 nM (n = 3). The apparent B_{max} varied between 14.8 and 21.0 pmol/mg protein depending on the transfection efficiency. The K_d values of DOI for classical 5-HT2 receptor is 2.2 nM [10].

Competitive inhibition studies were performed in order to refine the pharmacological profile of this receptor. Table I shows the following rank order of potencies for selected drugs: Ritanserin > *N*-acetyl 5-HT > Methysergide > Setoperone = Cyproheptadine > Spiperone > Ketanserin > Tryptamine > 5-HT > 8-CH-DPAT. This rank order as well as the apparent K_d values for these compounds correlate with those of 5-

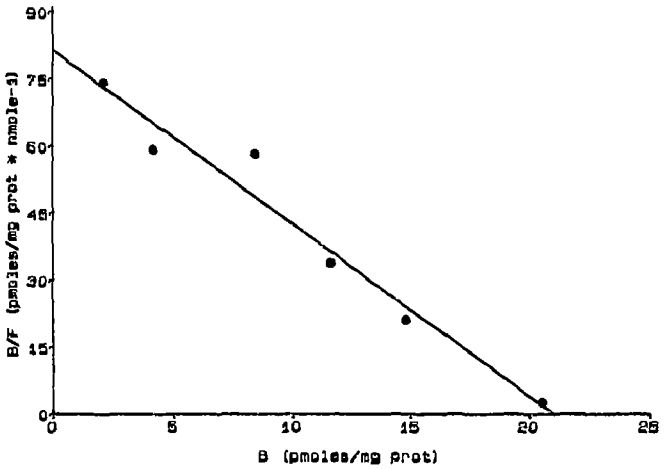

Fig. 2. Saturation binding of NP75 COS transfected membranes. The scatchard plot of the dose binding curves with [^{125}I]DOI as radioligand is represented with each value as the mean of at least 3 independent trials run in triplicate. The mean K_d value and the maximal binding capacity value are respectively 25.8 nM and 18.4 pmol/mg of protein.

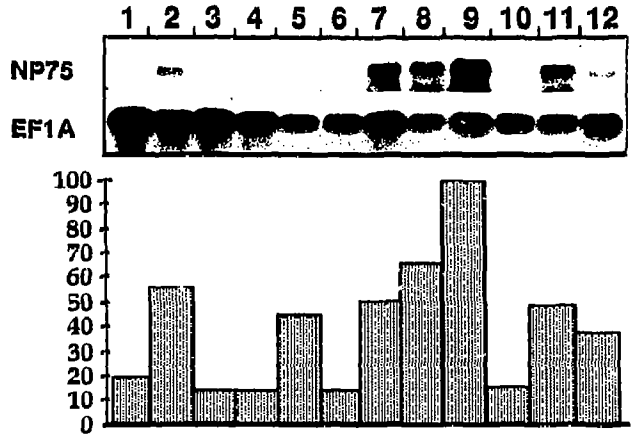

Fig. 3. RT-PCR analysis of NP75 in vivo expression. PCR experiments were done in the presence of 10 μg of total RNA, using NP75 and EFIA specific primers. PCR products were analyzed and revealed by hybridization to ^{32}P-labeled primers different from the amplimers, after 25 cycles for NP75 (row NP75) or after 20 cycles for EFIA (row EFIA). The resulting autoradiograms were scanned and the EFIA normalized intensity is displayed (lower panel). Lane 1 contains RNA from LMTK cell line; lane 2, from 10.5 days mouse embryo; lane 3, from mouse testis; lane 4, from 3T6 cell line; lane 5, from MBK cell line; lane 6, from mouse liver; lane 7, from mouse cerebellum; lane 8, from mouse heart; lane 9, from mouse intestine; lane 10, from mouse spleen; lane 11, from mouse kidney; and lane 12, from total mouse brain.

HT2 ($P = 0.0436$) or 5-HT1C ($P = 0.022$) (Kendall rank correlation), but not with other 5-HT2 receptor subtypes ($P > 0.05$): the low affinity of this receptor for serotonin would place it in the 5-HT2 family of receptors, that is confirmed by the low affinity for the 5-HT1A selective agonist 8-OH-DPAT. In addition the poor competition by the 5-HT3 selective compounds ICS 205-930, MDL 72222, and quipazine rule out the possibility that this belongs to the 5-HT3 family. The receptor affinity for ketanserin is too low for it to be a typical 5-HT2, although the high affinity that it has for the 5-HT2 antagonist ritanserin makes it difficult to classify NP75 within the 5-HT2/5-HT1C family. Furthermore, preliminary evidence shows that like 5-HT2 and 5-HT1C receptors, NP75 is coupled to the PLC second messenger system (not shown).

Table 1

Pharmacology of the mouse 5-HT receptor expressed in COS cells

Drug	COS cell binding	5-HT2	5-HT1C
Agonists			
5-HT	5.90	5.5	7.5
2-Me-5-HT	5.18	5.2	5.8
Tryptamine	6.70	6.0	7.2
1-Me-5-HT	5.60	6.3	8.4
NNdiMe-5-MeOT	5.13	6.2	7.0
8-OH-DPAT	5.19	5.0	5.2
Quipazine	5.18	6.2	6.7
Histamine	<4.0	<4.0	<4.0
Antagonists			
Ritanserin	8.44	9.3	8.6
Methysergide	7.90	8.6	8.6
Setoperone	7.61	8.6	7.3
Cyproheptadine	7.65	8.5	7.9
Spiperone	7.30	8.8	5.9
Ketanserin	6.69	8.9	7.0
ICS 205-930	5.30	5.3	4.6
MDL72222	4.62	6.7	<5.0
Chlorpromazine	<4.0	<4.0	<4.0

Competition experiment for [^{125}I]DOI labeled membrane from NP75 transfected COS cells as compared with published values for native rat 5-HT1C and 5-HT2 receptor taken from [22]. Each value, expressed as pK_d (−log mol/l) is the mean of at least 3 independent trial runs in triplicate.

The heterogeneity of 5-HT2 receptors has already been detected on preparations of cortical membranes, where the agonist competition curves for [^{3}H]ketanserin are shallow, indicating the presence of two types of binding site. The observation that the high affinity sites represented only a small subfraction of the ketanserin sites in addition to the existence of tissues containing only low affinity binding sites for the amphetamine derivative 4-bromo-2,5-dimethoxyphenylisopropylamine (DOB) led Peroutka and coworkers to propose the hypothesis that the high affinity agonist DOB binding must represent a unique receptor subtype 5-HT2A, whereas the low-affinity site (labeled by ketanserin but not by DOB) 5-HT2B represents the classical 5-HT2 receptor [16]. The low affinity of the NP75 receptor for ketanserin makes it unlikely to correspond to any one of these subtypes. Therefore this new receptor appears distinct from the 5-HT1C, 5-HT2A, and 5-HT2B, so we propose to call it 5-HT2C.

In order to have more complete information concern-

ing the properties of this new serotonin receptor, we analyzed for its in vivo expression. NP75 transcripts were analyzed by Northern blot (not shown) and by quantitative RT-PCR experiments. Fig. 3 displays the pattern of expression of NP75 seen after 25 cycles of amplification using 10 μg of total RNA. The highest expression is detected in the mouse intestine and in the mouse heart as well as at a lower level in the brain and kidney. In contrast, no expression is seen in the liver and spleen. We used the ribosomal elongation factor EF1A as an internal standard and primers located in two different exons in order to distinguish the amplification of RNA from DNA templates. The NP75 mRNA is also detected in the mouse embryo at day 10.5, and in the kidney derived cell line BHK. In situ experiments in the mouse brain as well as in the heart and intestine are in progress in order to refine these results.

The localization of this receptor to peripheral as well as to the central nervous system is puzzling. However, several reports have provided evidence that the peripheral action of serotonin cannot be mediated by the classical 5-HT1C or 5-HT2 receptors. For example, in the rat, administration of 5-HT causes tachycardia. This effect is antagonized by ketanserin, cyproheptadine, methysergide and metiothepine. Since DOI which exhibits a partial agonist action with 5-HT2 receptors, neither mimics nor blocks this tachycardiac action, the 5-HT2 receptor involved has been classified as 'atypical' [7]. In addition coronary vasoconstriction has been shown in several species to be mediated by a 5-HT2-like receptor [17], effects which are potentiated in coronary artery disease [7]. Similarly, the contractions of the rat stomach are mediated by an unknown serotonin receptor [18]. Furthermore, the activation of the peripheral serotonin receptor in platelets and in vascular smooth muscle has been shown to stimulate PI turnover. Nevertheless, mRNA analysis from several peripheral tissues, including heart and intestine, has failed to detect specific transcripts for either the 5-HT2C or the 5-HT2 receptor [19]. Therefore, we propose that some of the peripheral actions of 5-HT, specifically on smooth muscles, are mediated by the NP75 receptor.

Development of specific drugs to NP75/5-HT2C receptor could be used therapeutically in some cases of human cardiac pathology. For example, the recent finding that serotonin, known to have a vasodilating effect on normal human coronary arteries, has a direct vasoconstricting effect via 5-HT2-like receptors when the endothelium is damaged, (as in coronary artery disease) [20], makes it urgent to characterize in more detail the receptors involved.

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Note added in proof

During the reviewing process of this paper, a similar receptor has been described in rat [21]. These authors described the restricted expression of this receptor in the rat stomach. Although both protein sequences are very similar, we don't know if the difference in expression is species specific.