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Molecular cloning and expression of the porcine trigeminal ganglion cDNA encoding a 5-ht$_{1F}$ receptor

Pankaj Bhalla [a], Hari S. Sharma [a], Thierry Wurch [b], Petrus J. Pauwels [b], Pramod R. Saxena [a,*]

[a] *Department of Pharmacology, Erasmus University Medical Centre Rotterdam, Post Box 1738, 3000 DR Rotterdam, The Netherlands*
[b] *Department of Cellular and Molecular Biology, Centre de Recherche, Pierre Fabre, Castres Cédex, France*

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Abstract

Using a combination of reverse transcription polymerase chain reaction (RT-PCR) and inverse-PCR techniques, we amplified, cloned and sequenced a full-length porcine 5-hydroxytryptamine 1F (5-ht$_{1F}$) receptor complementary DNA (cDNA) derived from porcine trigeminal ganglion. Sequence analysis revealed 1101 base pairs (bp) encoding an open reading frame of 366 amino acids showing a high similarity (>90%) with the 5-ht$_{1F}$ receptor sequences from other species, including human. The recombinant porcine 5-ht$_{1F}$ receptor was expressed in African green monkey kidney cell lines (COS-7 cells) and its ligand binding profile was determined using [^{3}H]5-HT. The affinities of several agonists (LY334370 (5-(4-fluorobenzoyl)amino-3-(1-methylpiperidin-4-yl)-1H-indole fumarate)>CP122638 (*N*-methyl-3 [pyrrolidin 2(*R*)-yl methyl]-1H-indol-5-ylmethyl sulphonamide) = naratriptan = 5-HT>eletriptan>sumatriptan>frovatriptan = avitriptan>dihydroergotamine>zolmitriptan>5-carboxamidotryptamine>rizatriptan>alniditan = donitriptan>L694247 (2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl] ethylamine) and putative antagonists (methiothepin>GR127935 (*N*-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2′ -methyl 4′ -(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride)>ritanserin>SB224289 (2,3,6,7-tetrahydro-1′ -methyl-5-[2′ -methyl-4′ (5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carbonyl] furo [2,3-f] indole-3-spiro-4′ -piperidine hydrochloride)>BRL155572 ([1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(*S*,*R*) hydroxypropanyl)piperazine] hydrochloride)>ketanserin = pindolol) correlated highly with those described for the recombinant human 5-ht$_{1F}$ receptor (Spearman correlation coefficient; $r_s = 0.942$). Nevertheless, as compared to the human homologue, some triptans (i.e. sumatriptan, zolmitriptan and rizatriptan) displayed a 10- to 15-fold lower affinity for the porcine 5-ht$_{1F}$ receptor. Using RT-PCR technique, the expression of porcine 5-ht$_{1F}$ receptor mRNA was observed in cerebral cortex, trigeminal ganglion and several blood vessels, but not in skeletal muscles. In conclusion, we have cloned and established the amino acid sequence and ligand binding profile of the porcine 5-ht$_{1F}$ receptor as well as the distribution of its mRNA. This information may be helpful in exploring the role of 5-ht$_{1F}$ receptor in physiological processes and diseases, such as migraine.

Keywords: 5-ht$_{1F}$ receptor; Cloning; 5-HT (5-hydroxytryptamine, serotonin); Ligand binding; Migraine, (Pig); Sequencing; Trigeminal ganglion

1. Introduction

The physiological actions of serotonin (5-hydroxytryptamine, 5-HT) are mediated by multiple types of serotonin receptors and the characterisation (molecular, pharmacological and operational) of these receptors helps to recognise their importance as therapeutic targets (Hoyer et al., 1994; Saxena, 1995; Barnes and Sharp, 1999). Since the acute antimigraine agents (ergot alkaloids and triptans) display high affinity at 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-ht$_{1F}$ receptors, it became apparent that these receptors may be important targets for antimigraine drugs (Adham et al., 1993; Leysen et al., 1996; Johnson et al., 1997; Saxena and Tfelt-Hansen, 2000). An important feature of these antimigraine drugs is cranial vasoconstriction, which is mediated by the 5-HT$_{1B}$, rather than the 5-HT$_{1D}$ or 5-ht$_{1F}$ receptor (De Vries et al., 1999; Razzaque et al., 1999; Bouchelet et al., 2000). On the other hand, 5-HT$_{1D}$ and 5-ht$_{1F}$ receptors have been shown to be primarily involved in triptan-induced inhibition of neurogenic dural plasma protein extravasation (Humphrey, 1991; Moskowitz, 1992; Phebus et al., 1997; Goadsby, 1999). The presence of 5-HT$_{1D}$ and 5-ht$_{1F}$ receptors has been shown in the trigeminal ganglion (Bruinvels et al., 1994; Waeber and Moskowitz, 1995) and that agonists at these receptors, for example PNU109291 ([(*S*)-(-)-1-[2-[4-(4-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-methyl-isochroman-6-carboxamide]) and LY344864 ([(*R*)-(+)-*N*-[3-(*N*,*N*-dimethylamino)-1,2,3,4-tetrahydrocarbazol-6-yl]-4-fluoro-benzamide]),

* Corresponding author. Tel.: +31-10-408-7537; fax: +31-10-408-9458.
E-mail address: saxena@farma.fgg.eur.nl (P.R. Saxena).
URL: http://www-fgg.eur.nl/pharm/.

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respectively, inhibit neurogenic dural inflammation (Johnson et al., 1997; Phebus et al., 1997; Ennis et al., 1998). Moreover, some recent reports also showed the role of 5-ht_{1F} receptor in modulating c-*fos* protein expression and glutamate release in trigeminal ganglion and trigeminal nucleus caudalis in rats (Mitsikostas et al., 1999; Ma, 2001). Even though a direct role of the 5-ht_{1F} receptor in vasoconstriction has recently been ruled out (Razzaque et al., 1999; Villalón et al., 1999; Bouchelet et al., 2000), it is conceivable that the 5-ht_{1F} receptor may indirectly affect vasomotor activity via the trigeminovascular system.

Recently, we have cloned and sequenced the recombinant porcine 5-HT_{1B} (Bhalla et al., 2001) and 5-HT_{1D} (Bhalla et al., 2000) receptors, which possess a pharmacological profile very similar to those described for their human homologues. A notable exception, however, was BRL15572, a selective antagonist at the human 5-HT_{1D} receptor (Price et al., 1997); BRL15572 poorly recognised the recombinant porcine 5-HT_{1D} receptor (Bhalla et al., 2000). In the present study, we have cloned, sequenced the porcine 5-ht_{1F} receptor (p5-ht_{1F}; R.C.: 2.1.5HT.01F) and studied its ligand-binding profile and tissue distribution.

2. Methods

2.1. PCR amplification and cloning of 5-ht_{1F} receptor complementary DNA (cDNA)

Porcine specific 5-ht_{1F} receptor cDNA was amplified using a combination of reverse transcription polymerase chain reaction (RT-PCR) and inverse-PCR techniques; for details see Bhalla et al. (2000, 2001). cDNA was synthesised from RNA, extracted from trigeminal ganglia, obtained from a pig (Yorkshire × Landrace, female, 12 kg). The quality of RNA and cDNA preparations was checked by PCR amplification of β-actin (Ponte et al., 1984).

As depicted in Fig. 1, a PCR product containing a partial sequence of porcine 5-ht_{1F} receptor cDNA was amplified using a forward (5′-ATGGATTTCTTAAACTCATCT-3′, nucleotides 1–21) and a reverse (5′-CTAATATCGA CATCGTACAAG-3; nucleotides 1081–1101) oligonucleotide primers (Fig. 1A, a and b), designed on the basis of consensus sequence of 5-ht_{1F} receptors from other species (Amlaiky et al., 1992; Adham et al., 1993, 1997; Lovenberg et al., 1993). The amplified PCR products were separated on 1% agarose gel in TBE (100 mM Tris, 90 mM boric acid and 1 mM ethylenediaminetetraacetic acid (EDTA)) buffer containing ethidium bromide, visualised under UV light and photographed. The purified PCR products were then ligated into the pGEMT-Easy vector and transformed into competent JM109 cells. Four insert positive clones were processed for isolation of the plasmid DNA and sequencing.

Since the forward and reverse oligonucleotide primers used in RT-PCR for amplification were based on a consensus sequence derived from other species, we identified the 5′ and 3′ ends of recombinant porcine cDNA by inverse-PCR (Ochman et al., 1988). Porcine genomic DNA was digested with BamH1 restriction enzyme, as the cloned 5-ht_{1F} receptor cDNA did not show any restriction site for BamH1. After purification, the restricted DNA was ligated overnight at 16 °C in the presence of T_4-DNA ligase in order to obtain DNA circles. Using inverse primers based on the derived porcine cDNA sequence (5′-GCAGCTTTCGGGTCACAATAA-3′ for 5′ end and 5′-TTGCCAAGGAGGAACTGAATG-3′ for 3′ end; Fig. 1B, c and d), the ligated DNA fragments were subjected to PCR amplification. The PCR products were separated on a 1% agarose gel, purified, cloned and sequenced. Finally, porcine specific forward (5′-ATG-GATTTCTCAAACTCATCT-3′) and reverse (5′-CTAA-CACCGACACATCGCACA-3′) oligonucleotide primers, designed from the sequences generated from inverse-PCR (Fig. 1C, e and f), were used to amplify the full-length 5-ht_{1F} receptor cDNA from the porcine trigeminal ganglion (Fig. 1D).

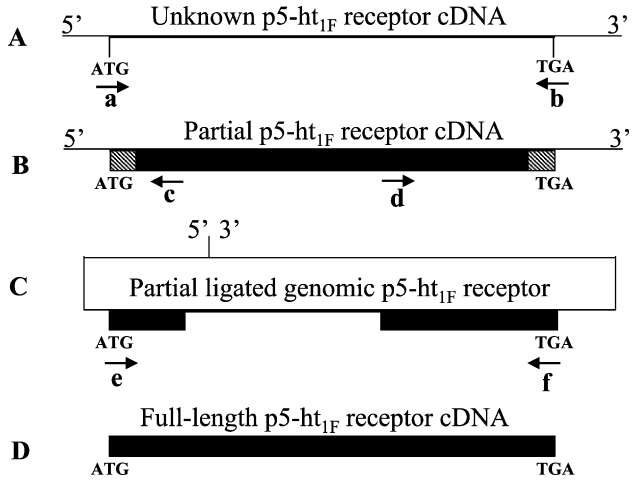

Fig. 1. Diagrammatic representation showing the methodology for cloning of the full-length porcine 5-ht_{1F} receptor and the positions of various primers used for PCR amplification. (A) Unknown full-length porcine 5-ht_{1F} receptor cDNA with 5′ (a) and 3′ (b) end primers designed from known sequence from other species. (B) The amplified full-length product containing a partial sequence of porcine 5-ht_{1F} receptor and the 5′ and 3′ end sequences derived from other species (hatched rectangles). The partial sequence of porcine 5-ht_{1F} receptor was used to design porcine-specific inverse primers (c) and (d). (C) The inverse-PCR amplified partial genomic sequence was used to design porcine specific primers (e) and (f) for amplifying the full-length porcine 5-ht_{1F} receptor cDNA. (D) The full-length amplified product of porcine 5-ht_{1F} receptor cDNA.

The full-length cDNA sequence of porcine 5-ht_{1F} receptor was derived from at least two independent PCR amplified products and further verified by multiple partial sequences derived from genomic DNA amplified products (inverse-PCR). In sporadic cases showing nucleotide discrepancy in the sequence, the nucleotide having a clear majority in clones was preferred for establishing the final full-length cDNA sequence, using the DNAMAN sequence analysis program (Version 3.2, Lynnon Biosoft© 1994–1997). The final sequence was translated as a peptide

sequence and compared with those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, USA; web site: http://www.ncbi.nlm.nih.gov/BLAST/). The hydrophobic regions indicating putative transmembrane domains and sequence homology with known 5-ht$_{1F}$ receptors from other species were established.

2.2. Transient transfection and radioligand binding assay

The purified full-length pig 5-ht$_{1F}$ receptor cDNA insert was subcloned into dephosphorylated eukaryotic expression vector, pcDNA3 (Invitrogen, San Diego, CA, USA). Monkey COS-7 cells were transiently transfected with the recombinant plasmid and membranes were prepared for radioligand binding assays using 8.0 nM [^{3}H]5-HT (Pauwels et al., 1996). Incubation mixtures consisted of 0.40 ml of cell membrane preparation (30–50 μg of protein), 0.05 ml of [^{3}H]5-HT and 0.05 ml of compounds for inhibition or 10 μM 5-HT to determine non-specific binding. The reaction was terminated by filtration over a Whatman GF/B glass-fibre filter with ice-cold Tris-buffer and the radioactivity on the filter paper was measured by using a liquid scintillation counter. Data were analysed graphically with inhibition curves and IC_{50} values were derived. Binding affinity constants (K_i values) were calculated according to the equation $K_i = IC_{50}/(1 + C/K_D)$, where C is the concentration and K_D is the equilibrium dissociation constant of the radioligand. Radioligand saturation binding curves were analysed by a non-linear least square curve-fitting programme to determine equilibrium dissociation constant (K_D) and maximum binding site density (B_{max}) values (Munson and Rodbard, 1980). Control binding experiments were run with non-transfected COS-7 cells and they did not display detectable specific [^{3}H]5-HT binding.

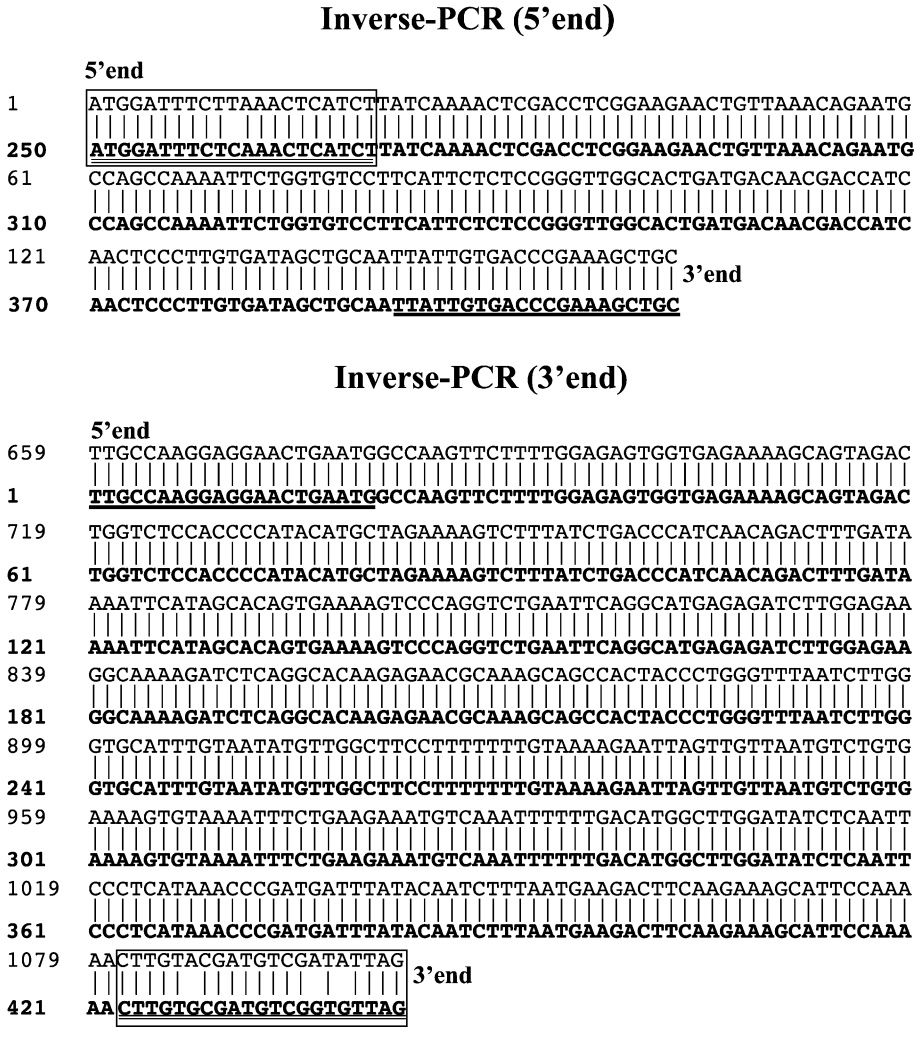

Fig. 2. Sequence of inverse-PCR amplified products from porcine genomic DNA circles (in bold letters) showing a high homology with porcine cDNA sequence derived from trigeminal ganglion (in normal letters). The deduced 5′ (N) and 3′ (C) terminal sequences of porcine 5-ht$_{1F}$ receptor are shown double underlined in boxes, while inverse-PCR primers are thick underlined. Please note that, except for a single mismatch at the 5′ end and three mismatches at the 3′ end (identified by missing vertical bar), there was a complete identity between the porcine cDNA and genomic DNA sequences.

2.3. RT-PCR for 5-ht$_{1F}$ receptor mRNA detection

The expression of 5-ht$_{1F}$ receptor mRNA was studied by RT-PCR technique in a number of porcine tissues (brain cortex, cerebellum, trigeminal ganglion, skeletal muscles, saphenous vein and mesenteric, coronary and pulmonary arteries) obtained from four pigs previously used in acute haemodynamic experiments, as described in detail earlier (Bhalla et al., 2001). The purified total RNA samples from the tissues were reverse transcribed into cDNA in the absence (control reaction to monitor DNA contamination) or the presence of reverse transcriptase enzyme. Porcine specific sense (5′-CCAAGCAGGCTGGCATTATG-3′, nucleotides 410–429 base pairs (bp)) and antisense (5′-GCTTTGCGTTCTCTTGTGCC-3′, nucleotides 853–872bp) primers were used for the amplification of partial porcine 5-ht$_{1F}$ receptor cDNA. The PCR amplified products were separated on 2% agarose gel by electrophoretic separation, stained with ethidium bromide, visualised under UV light and photographed.

2.4. Materials

All oligonucleotide primers were commercially procured from Life Technologies (Breda, The Netherlands). Various chemicals used in this study were of molecular biology and/or culture grade. pGEMT-Easy vector system, Wizard® PCR prep and mini-prep DNA purification systems were purchased from Promega Benelux (Leiden, The Netherlands). Oligotex mRNA purification kit was purchased from Qiagen (Hilden, Germany). AmpliTaqGold and dye terminator/cycle sequencing ready reaction kit were procured from Perkin-Elmer Applied Biosystem Benelux (Nieuwerkerk a/d Ijssel, The Netherlands).

The compounds used in pharmacological assays were: alniditan, avitriptan, BRL15572 (gift: Dr. A.A. Parsons, GlaxoSmithKline, Harlow, Essex, UK), 5-carboxamidotryptamine, CP122638 (*N*-methyl-3 [pyrrolidin 2(*R*)-yl methyl]-1H-indol-5-ylmethyl sulphonamide), dihydrergotamine, eletriptan, frovatriptan, GR127935 (*N*-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl 4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride, [^{3}H]5-HT (80–130 Ci mmol^{-1}, Amersham, Les Ulis, France), 5-HT creatinine sulphate (Sigma, St. Louis, MO, USA), ketanserin (Sigma), L694247 2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl] ethylamine, LY334370 (5-(4-fluorobenzoyl)amino-3-(1-methylpiperidin-4-yl)-1H-indole fumarate), methiothepin, naratriptan, pindolol, ritanserin, SB224289 (2,3,6,7-tetrahydro-1′-methyl-5-[2′-methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carbonyl] furo [2,3-f] indole-3-spiro-4′-piperidine hydrochloride), rizatriptan sumatriptan and zolmitriptan. Except those specified above, all other compounds were synthesised at Centre de Recherche Pierre Fabre (Castres, France).

3. Results

3.1. Cloning of porcine 5-ht$_{1F}$ receptor cDNA derived from trigeminal ganglion

Using RT-PCR technique, the trigeminal ganglion cDNA yielded a full-length clone of approximately 1150 bp containing a partial sequence of porcine 5-ht$_{1F}$ receptor (see Fig. 1B). The nucleotide sequence showed a high homology with 5-ht$_{1F}$ receptors from other species (data not shown). On the basis of this sequence, porcine specific inverse

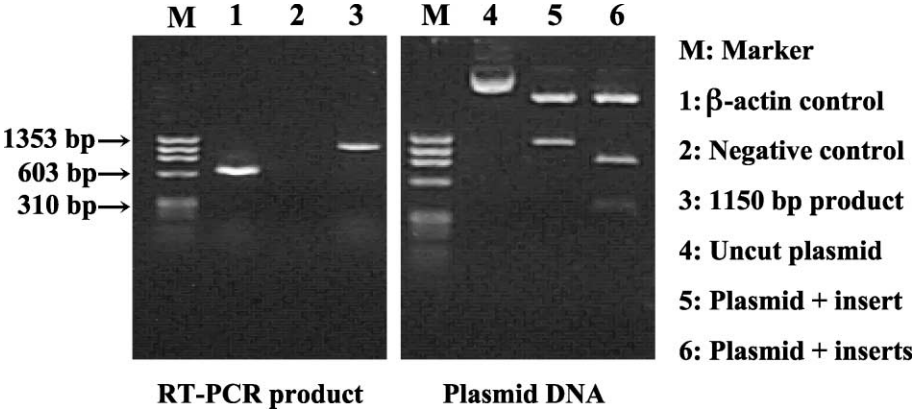

Fig. 3. Agarose gel electrophoresis of RT-PCR products of cDNA synthesised from porcine trigeminal ganglion (left panel) and recombinant plasmid with insert cDNA (right panel). The different lanes marked on the top denote: (M) ϕx174 DNA/Hae III marker; (1) Positive control showing a RT-PCR product of 625bp using β-actin primers; (2) Negative control, i.e. a sample without the reverse transcriptase enzyme to monitor genomic and/or PCR contamination; (3) A RT-PCR product of approximately 1150 bp obtained using porcine specific forward and reverse primers of 5-ht$_{1F}$ receptor; (4) Non-digested recombinant plasmid DNA; (5) Recombinant plasmid DNA restricted with Not1 enzyme and showing a DNA insert of approximately 1150 bp; (6) Recombinant plasmid DNA restricted with *Eco*RI enzyme and showing two DNA fragments of approximately 800 and 300 bp. The size of three marker bands is indicated in the left margin.

primers were designed and used on porcine genomic DNA circles to establish the sequence of 5′ and 3′ ends of the porcine 5-ht$_{1F}$ receptor by inverse-PCR (Fig. 1C). A PCR product of approximately 1200 bp was amplified, cloned and sequenced. Whereas sequence analysis revealed a single mismatch in the 5′ end and three mismatches in the 3′ end of the 5-ht$_{1F}$ receptor, there were no differences in the rest of the sequence derived from the porcine 5-ht$_{1F}$ receptor cDNA and genomic DNA (Fig. 2).

The full-length porcine 5-ht$_{1F}$ receptor cDNA was finally amplified by using cDNA templates derived from poly(A$^+$) mRNA of porcine trigeminal ganglion and porcine specific 5′ end (sense) and 3′ end (antisense) primers (Fig. 1D) and agarose gel electrophoresis of RT-PCR products is shown in Fig. 3. The amplification of the porcine β-actin cDNA (approximately 625 bp) ensured that the quality of cDNA samples was adequate for the amplification of other products, while any genomic contamination was ruled out by the

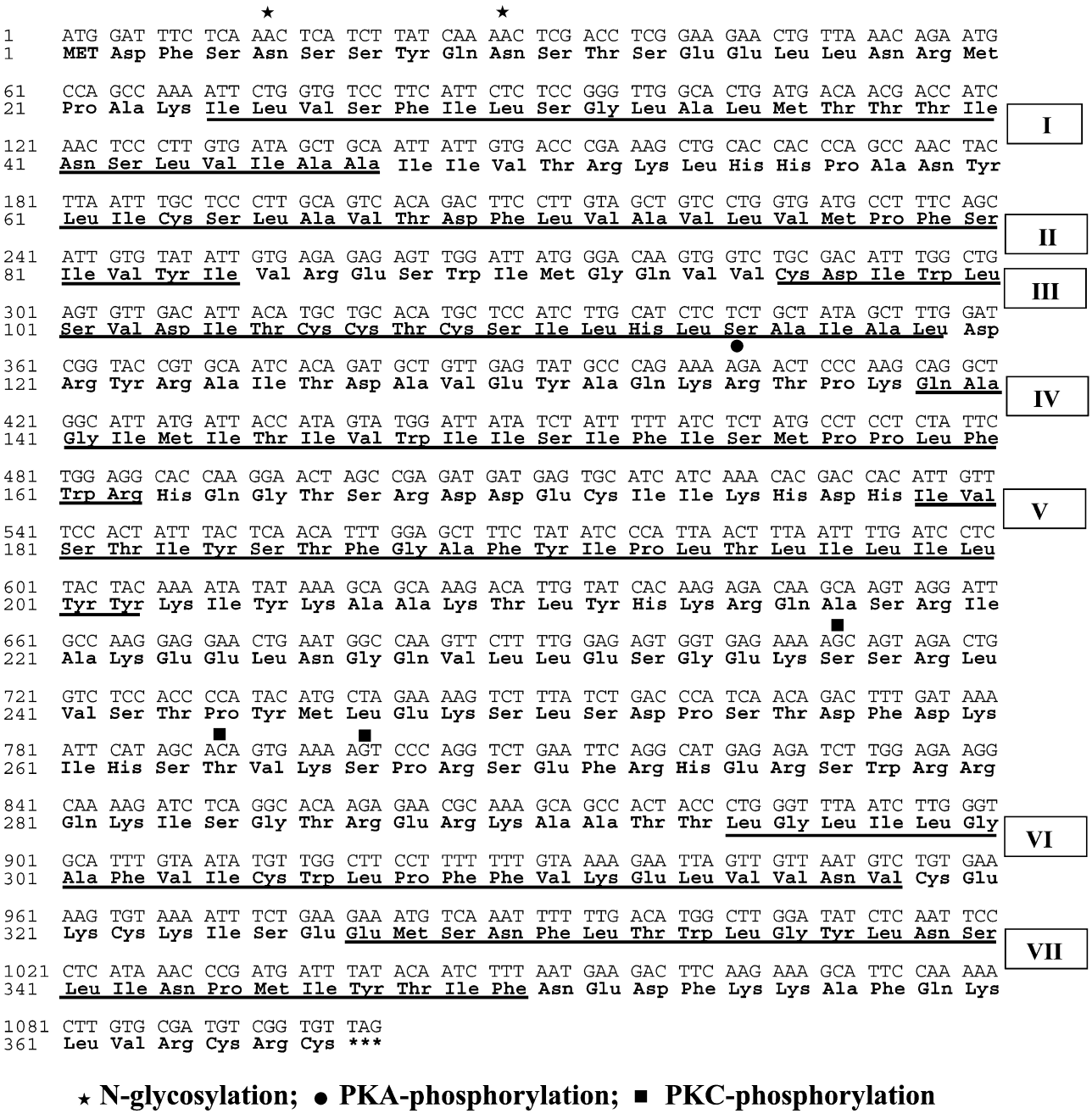

Fig. 4. Nucleotide and deduced amino acid (in bold) sequences of recombinant porcine 5-ht$_{1F}$ receptor cDNA, derived from trigeminal ganglion (GenBank accession number: AF 255663). Numbering of nucleotides and amino acids is shown on the left. Computer analysis (software DNAMAN, version 3.2, Lynnon Biosoft©) predicted a typical G-protein receptor structure with seven transmembrane domains I–VII (underlined) as well as putative *N*-glycosylation, protein kinase A phoshorylation and protein kinase C phoshorylation sites.

absence of any band in the RNA samples lacking reverse transcriptase. The trigeminal ganglion cDNA showed a PCR product band of approximately 1150 bp, which was cloned into pGEMT-Easy vector and sequenced. When the recombinant plasmid was checked by restriction analysis, it yielded a full-length cloned product with Not1, whereas with *Eco*RI used on the basis of a single restriction site in 5-ht_{1F} receptors of other species (Amlaiky et al., 1992; Adham et al., 1993, 1997; Lovenberg et al., 1993), resulted into two bands of 800 and 300 bp.

Sequencing of the recombinant plasmid revealed an open reading frame of 1101 bp (Fig. 4). DNAMAN analysis

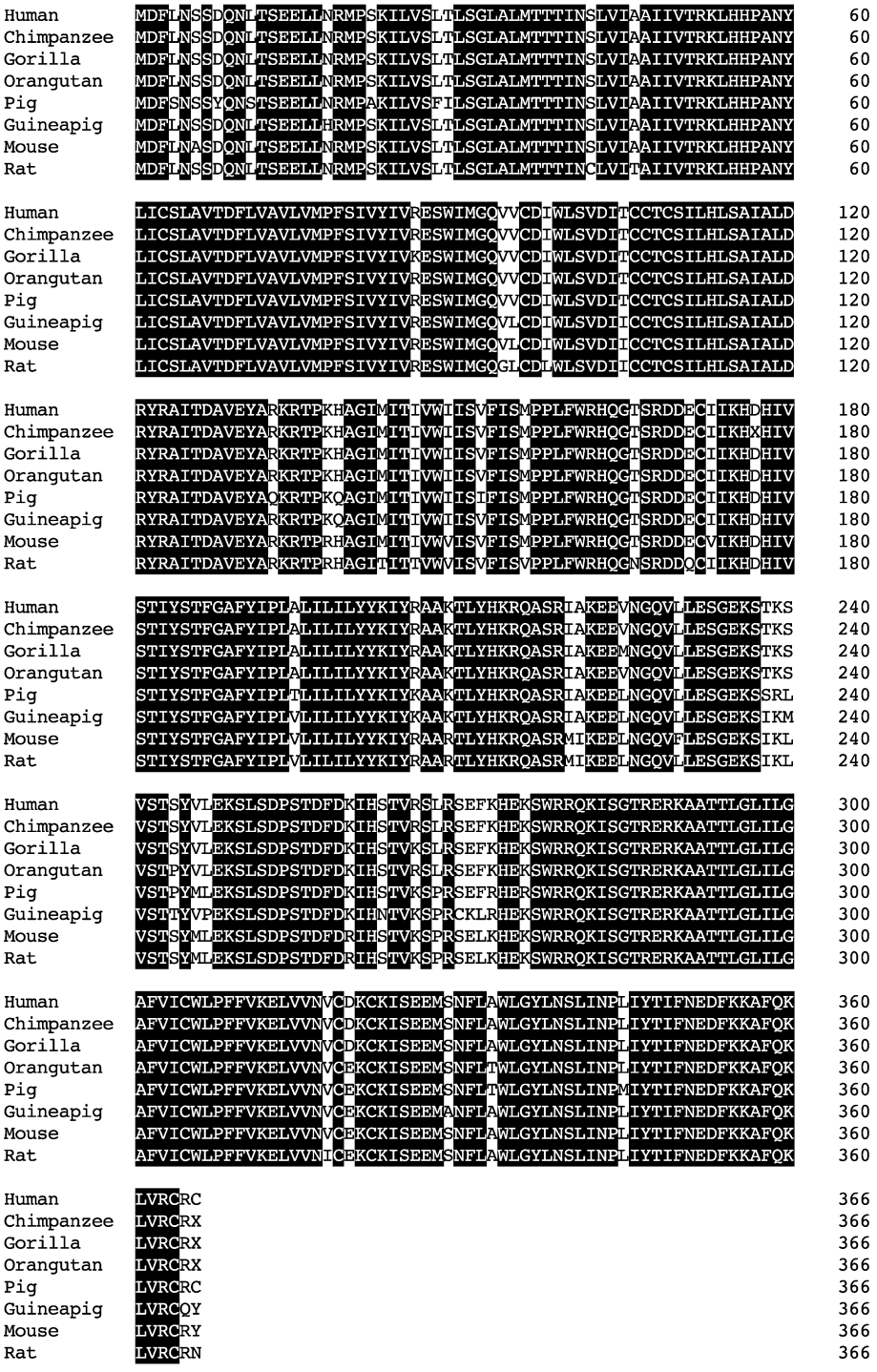

Fig. 5. Comparison of amino acid sequences of the porcine 5-ht_{1F} receptor (Swissprot accession number: AAG44634) with the human (P30939), chimpanzee (BAA90454), gorilla (BAA90455), orang-utan (BAA90456), guinea pig (O08890), mouse (Q02284) and rat (P30940) (software DNAMAN, version 3.2, Lynnon Biosoft©). Shaded boxes show identity across the different species.

showed that this full-length porcine cDNA encoded a 366 amino acid protein (calculated molecular weight: 41.8 kDa) exhibiting the features of a typical G-protein-coupled receptor with predicted seven transmembrane domains and putative *N*-glycosylation and phosphorylation sites (Fig. 4). A BLAST search of the nucleotide sequence at GenBank revealed a high resemblance with the sequence of 5-ht$_{1F}$ receptors from other species. In Fig. 5, the amino acid sequence of the porcine 5-ht$_{1F}$ receptor has been compared with those of the human, chimpanzee, gorilla, orang-utan, guinea pig, mouse and rat. Across the species, there was a 90% (rat) to 93% (human) similarity in the overall sequence. Nevertheless, it may be noted that the porcine 5-ht$_{1F}$ receptor contains several amino acids that are unique: Ser^{4,11}, Tyr^{8}, Ala^{22} (N-terminal extracellular region), Phe^{28}, Ile^{29} (first transmembrane domain), Gln^{133} (second intracellular loop), Thr^{195} (fifth transmembrane domain), Ser^{238}, Arg^{239,276} (third intracellular loop) and Met^{345} (seventh transmembrane domain). Further, it may be noted that Pro^{244} (third intracellular loop) and Thr^{333} (seventh transmembrane domain) were present only in pig and orang-utan (Fig. 5).

3.2. Ligand binding profile of recombinant porcine 5-ht$_{1F}$ receptor

Saturation binding studies and Scatchard analysis, performed over 10 concentrations of 5-HT, demonstrated that membranes obtained from monkey COS-7 cells transiently transfected with the porcine 5-ht$_{1F}$ receptor showed a single population of high affinity binding sites for [^{3}H]5-HT. The equilibrium dissociation constant (K_D) and maximum receptor density (B_{max}) for [^{3}H]5-HT were, respectively, 20.8 ± 1.9 nM and 7.12 ± 1.85 pmol mg^{-1} of protein ($n = 3$ each). No detectable specific [^{3}H]5-HT binding was observed in nontransfected cell membranes.

The affinity constants (pK_i values) of 22 serotonergic compounds (15 putative agonists and 7 putative antagonists) for the displacement of [^{3}H]5-HT from membranes obtained from COS-7 cells expressing porcine (present results) and human (Pauwels et al., 1997; John et al., 1999 and Pauwels, unpublished) 5-ht$_{1F}$ receptor are shown in Table 1. Among the compounds tested, the potent and selective 5-ht$_{1F}$ receptor agonist LY334370 (Phebus et al., 1997) showed the highest affinity. The rank order of affinity of the putative agonists was LY334370>CP122638 = naratriptan = 5-HT>eletriptan>sumatriptan>frovatriptan = avitriptan>dihydroergotamine>zolmitriptan>5-carboxamidotryptamine>rizatriptan>alniditan = donitriptan>L694247, while that of putative antagonists was methiothepin>GR127935>ritanserin> SB224289>BRL15572>ketanserin = pindolol.

In Fig. 6, pK_i values of the above compounds obtained in the present experiments with membranes from cells expressing the cloned pig 5-ht$_{1F}$ receptor have been plotted against pK_i values obtained earlier with the same compounds using membranes from cells expressing the cloned human 5-ht$_{1F}$ (Pauwels et al., 1997; John et al., 1999 and Pauwels, unpublished) as well as human (Wurch et al., 1998 and unpublished) or porcine (Bhalla et al., 2000, 2001) 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. The affinity constants at the porcine 5-ht$_{1F}$ receptor showed the highest correlation with those at the human 5-ht$_{1F}$ receptor ($r_s = 0.944$; $b = 0.815$). In contrast, the correlation at the porcine or human 5-HT$_{1B}$ ($r_s = 0.287$ and 0.329, respectively) and 5-HT$_{1D}$ ($r_s = 0.424$ and 0.318, respectively) receptors was much weaker. However, it may be noted that, except dihydroergotamine, all compounds and, in particular, sumatriptan, zolmitriptan and rizatriptan, displayed 10- to 15-times less affinity at the porcine than at the human 5-ht$_{1F}$ receptors (see Table 1).

3.3. Expression of 5-ht$_{1F}$ receptor mRNA in porcine tissues

Based on the recombinant porcine specific 5-ht$_{1F}$ receptor sequence, two internal primers were designed and used on cDNA templates prepared from several porcine tissues. As shown in Fig. 7, a single band of PCR amplified product (approximately 460 bp) representing the presence of 5-ht$_{1F}$ receptor mRNA was clearly detected in the brain cortex, trigeminal ganglion and saphenous vein. Moderate signals

Table 1
Affinity constants (K_i, nM) of serotonergic ligands for inhibition of [^{3}H]5-HT binding to membranes derived from monkey COS-7 cells expressing recombinant porcine 5-ht$_{1F}$ receptor

Compound	Porcine 5-ht$_{1F}$	Human 5-ht$_{1F}$	Ratio porcine vs. human
Agonists			
LY334370	3.0 ± 0.7	2.0 ± 0.3	1.5
CP122638	13.6 ± 3.1	2.6 ± 0.7	5.2
Naratriptan	15.2 ± 2.1	4.3 ± 0.7	3.5
5-HT	17.4 ± 5.3	8.4 ± 1.2	2.1
Eletriptan	81.4 ± 26.5	17.6 ± 3.5	4.6
Sumatriptan	199 ± 37	17.3 ± 3.1	11.5
Frovatriptan	338 ± 78	102 ± 38	3.3
Avitriptan	360 ± 27	179 ± 37	2.0
Dihydroergotamine	431 ± 64	330 ± 38	0.96
Zolmitriptan	524 ± 93	34.4 ± 4.6	15.2
5-Carboxamido-tryptamine	1555 ± 49	738 ± 85	2.1
Rizatriptan	2100 ± 221	135 ± 21	15.6
Alniditan	3321 ± 477	933 ± 89	3.6
Donitriptan	4264 ± 990	3755 ± 1020	1.1
L 694247	>10000	>10000	
Antagonists			
Methiothepin	136 ± 23	102 ± 33	1.3
GR127935	250 ± 17	46.9 ± 6.2	5.3
Ritanserin	2151 ± 267	1178 ± 14	1.8
SB-224289	7050 ± 528	>10000	
BRL 15572	9138 ± 120	NA	
Ketanserin	>10000	>10000	
Pindolol	>10000	NA	

Corresponding data for the human 5-ht$_{1F}$ receptor (Pauwels et al., 1997; John et al., 1999 and Pauwels, unpublished) is presented for comparison. Data are means ± S.E.M. ($n = 3–6$). NA, Not available.

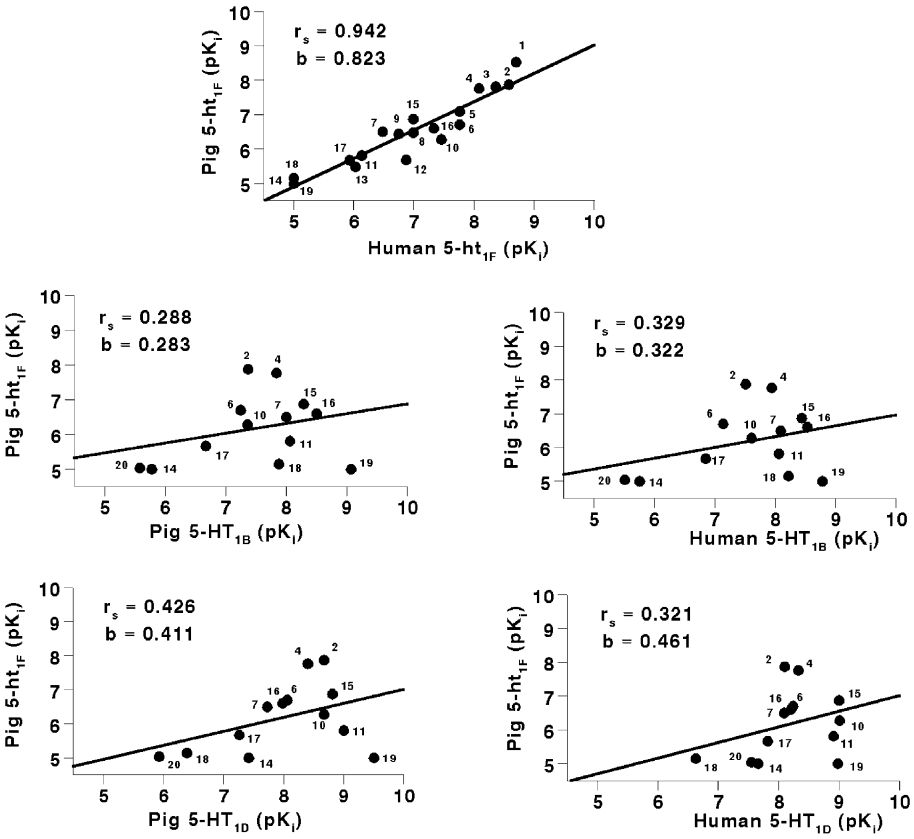

Fig. 6. Regression analysis of binding affinity constants (pK_i values) of 5-HT receptor ligands at the cloned porcine 5-ht$_{1F}$ receptor (see Table 1) with those reported with cloned 5-ht$_{1F}$ (human; top panel), 5-HT$_{1B}$ (pig and human; middle panels) and 5-HT$_{1D}$ (pig and human; lower panels) receptors, using [^{3}H]5-HT (5-ht$_{1F}$) and [^{3}H]GR125743 (5-HT$_{1B}$ and 5-HT$_{1D}$) as ligands (for references, see text). Please note that values for some compounds were not available for all receptors. The Spearman correlation coefficient (r_S) and the corresponding values of the slope (b), calculated with SlideWrite plus for Windows© (Advanced Graphics Software, Encinitas, CA, USA), are listed in each panel. The compounds included in the graphs are (1) LY334370, (2) CP122638, (3) naratriptan, (4) 5-HT, (5) eletriptan, (6) sumatriptan, (7) dihydroergotamine, (8) frovatriptan, (9) avitriptan, (10) zolmitriptan, (11) 5-carboxamidotryptamine, (12) rizatriptan, (13) alniditan, (14) ketanserin, (15) methiothepin, (16) GR127935, (17) ritanserin, (18) SB224289, (19) L694247, and (20) BRL15572.

were observed in the coronary and pulmonary artery, while only weak or no signals were noticed in the cerebellum, mesenteric artery and skeletal muscles. The possibility of genomic DNA and/or PCR contamination was ruled out on the basis of the absence of any band in negative controls (autoclaved water and brain cortex RNA in the absence of reverse transcription).

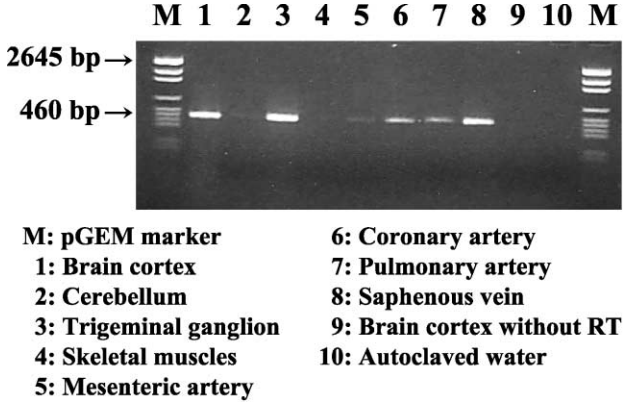

Fig. 7. Agarose gel electrophoresis of PCR amplified products derived from cDNA obtained from a number of porcine tissue samples. The size of marker bands is indicated in the left margin. RT, Reverse transcriptase enzyme.

4. Discussion

4.1. Cloning and sequence analysis

Specific primer sequences of the full-length porcine 5-ht$_{1F}$ receptor were identified by inverse-PCR on genomic DNA and used for amplification of full-length 5-ht$_{1F}$ receptor cDNA prepared from the porcine trigeminal ganglion. The nucleotide sequence of 1101 bp DNA fragment revealed an open reading frame of 366 amino acid protein of the porcine 5-ht$_{1F}$ receptor displaying a high homology (90–93%) with the human and several other mammalian species. The computer analysis predicted seven transmembrane domains as well as putative *N*-glycosylation and phosphorylation sites, similar to those observed in other species (Amlaiky et al., 1992; Adham et al., 1993, 1997; Lovenberg et al., 1993).

A comparison of porcine 5-ht_{1F} receptor amino acid sequence with previously characterised recombinant porcine 5-HT_{1B} (Bhalla et al., 2001) and 5-HT_{1D} (Bhalla et al., 2000) receptors showed 48% and 49% homology, respectively. Moreover, analogous to porcine 5-HT_{1B} (Ala^{4}, $Ser^{15,21}$ and Arg^{19}) and 5-HT_{1D} ($Val^{8,36}$, Asp^{10}, Gly^{14}, Thr^{15}, Lys^{27}, Pro^{97} and Glu^{102}) receptors, the porcine 5ht_{1F} receptor also showed some unique amino acids ($Ser^{4,11,238}$, Tyr^{8}, Phe^{28}, Ile^{29}, Gln^{133}, Thr^{195}, $Arg^{239,276}$, Met^{345}) as compared to other species. Interestingly, Pro^{244} and Thr^{333} were common to the pig and orang-utan 5ht_{1F} receptor. The encoding divergent amino acids at the N- ($Ser^{4,11}$, Tyr^{8}, Phe^{28}, Ile^{29}) and C- (Ser^{238}, $Arg^{239,276}$, Met^{345}) terminals were also verified when the genomic DNA was used for inverse-PCR (see Fig. 2), thus ruling out the possibility of sequencing error.

4.2. Ligand binding profile

Using [^{3}H]5-HT as a radioligand, membranes from Cos-7 cells expressing the porcine 5-ht_{1F} receptor showed a K_D value of 20.8 ± 1.9 nM, which agreed with the K_i value of 5-HT (17.4 ± 5.3 nM; Table 1). The present K_D value is somewhat higher than that obtained with the human 5-ht_{1F} receptor (K_D: 9.2 ± 0.99 nM, Adham et al., 1993), but is not dissimilar to that obtained with the guinea pig 5-ht_{1F} receptor (K_D: 14 ± 3 nM, Adham et al., 1997). Although in both cases [^{3}H]5-HT was used as radioligand, the guinea pig receptor was expressed, as in the present investigation, in Cos-7 cells, whereas the human receptor was expressed in LM(tK^{-}) cells.

Overall, the pharmacological profile of the porcine 5-ht_{1F} receptor was similar to that of the human homologue and the affinity constants of serotonergic drugs at the two 5-ht_{1F} receptors showed a very high correlation (Table 1 and Fig. 6). Also, LY334370 and CP122638, which are reasonably selective at the 5-ht_{1F} receptor (Waeber and Moskowitz, 1995; Wainscott et al., 1998), were the two compounds with the highest affinities at the porcine 5-ht_{1F} receptor. However, it should be pointed out that generally the compounds investigated showed a lower affinity at the porcine compared to the human receptor (Table 1). This was most marked for sumatriptan, zolmitriptan and rizatriptan, which had over 10-fold lower affinity at the porcine receptor. The lower affinity at the porcine 5-ht_{1F} receptor may be due to a single or multiple amino acid differences in its transmembrane domains. Indeed, as pointed out above, several amino acids in the porcine 5-ht_{1F} receptor are unique (Phe^{28} and Ile^{29} in the first, Thr^{195} in the fifth and Met^{345} in the seventh transmembrane domain). In any case, 5-ht_{1F} receptor agonism is not a requirement for antimigraine efficacy (Dahlöf and Saxena, 2000), since alniditan and rizatriptan, which do not have a particularly high affinity at the 5-ht_{1F} receptor (Table 1), effectively abort migraine attacks (Adelman et al., 2001; Diener et al., 2001). Moreover, it is still unclear if the efficacy of the 5-ht_{1F} receptor agonist LY33470 in migraine (Goldstein et al., 2001) is due to a selective action on this receptor (Dahlöf and Saxena, 2000).

Interestingly, like 5-HT_{1D} (Thr^{342}) (Weinshank et al., 1992; Harwood et al., 1995; Wurch et al., 1997) and non-rodent 5-HT_{1B} (Thr^{355}) (Adham et al., 1994) receptors, the porcine (and orang-utan) 5-ht_{1F} receptor has a polar threonine amino acid at the homologous position within the seventh transmembrane domain (Thr^{333}) (see Fig. 5). This is strikingly different from the human, guinea pig, mouse and rat 5-ht_{1F} receptors, which all have a non-polar alanine residue (Ala^{333}) (Amlaiky et al., 1992; Adham et al., 1993, 1997; Lovenberg et al., 1993) or from the rat and mouse 5-HT_{1B} receptors, which have a polar asparagine residue (Asn^{355}) (Oksenberg et al., 1992). It is known that a single point mutation of either threonine (5-HT_{1B}, 5-HT_{1D} and 5-ht_{1E}) or alanine (5-ht_{1F}) to asparagine considerably increases the affinity of these human receptors for β-adrenoceptors antagonists (Oksenberg et al., 1992; Adham et al., 1994). The presence of Thr^{333} in the porcine 5-ht_{1F} receptor is in agreement with its low affinity for the β-adrenoceptor antagonist pindolol (K_i: >10,000 nM; Table 1). However, despite Thr^{333}, the ligand binding profile of the porcine 5-ht_{1F} receptor resembles that of the human 5-ht_{1F} receptor having an Ala^{333} and not that of the 5-HT_{1B} (Thr^{355}) or 5-HT_{1D} (Thr^{342}) receptors. Whether or not Thr^{333} in the porcine 5-ht_{1F} receptor is responsible for the lower affinity of these drugs will be worth investigating.

4.3. Tissue distribution of mRNA

Expression of mRNA for 5-ht_{1F} receptor in the porcine trigeminal ganglion supports the possible central role of these receptors in inhibiting dural plasma protein extravasation (Johnson et al., 1997; Phebus et al., 1997), presumably by a presynaptic action. Furthermore, since the mRNA signals were found in other brain tissues (cortex, cerebellum), the 5-ht_{1F} receptors may function as auto- and/or heteroreceptor (Hoyer et al., 1994; Barnes and Sharp, 1999). Interestingly, despite the presence of the 5-ht_{1F} receptor mRNA in some blood vessels (Nilsson et al., 1999; Bouchelet et al., 2000 and present results), it is practically ruled out that this receptor mediates vasoconstriction (Shepheard et al., 1999; Bouchelet et al., 2000; Cohen and Schenck, 2000). In the case of porcine coronary artery, the mRNA signals of 5-ht_{1F} receptor were detected. Earlier reports in the human coronary artery, also employing the RT-PCR technique, have either denied (Ishida et al., 1999) or advocated (Nilsson et al., 1999) the presence of 5-ht_{1F} receptor mRNA. Even though, we do not have a clear explanation for the presence of mRNA signals in vascular beds, it may be that these receptors mediate some other (patho)physiological responses, for example plasma protein extravasation or mitosis leading to vascular remodelling.

In conclusion, we have cloned the porcine 5-ht_{1F} receptor cDNA from the trigeminal ganglion by RT-PCR technique. The ligand binding profile of porcine 5-ht_{1F} receptor was

consistent with the human 5-ht$_{1F}$ receptor, although some triptans exhibited a conspicuously lower affinity for the porcine receptor. This information may be helpful in exploring the role of 5-ht$_{1F}$ receptor in physiological processes and diseases, such as migraine.

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