Molecular Cloning and Pharmacology of Porcine 5-Hydroxytryptamine Receptors Relevant to the Study of Antimigraine Drugs

Pankaj Bhalla

Molecular Cloning and Pharmacology of Porcine 5-Hydroxytryptamine Receptors Relevant to the Study of Antimigraine Drugs

Thesis Erasmus University Rotterdam. With summary in Dutch

ISBN 90-56772643
NUGI 741/746

Printed by Optima Grafische Communicatie, Rotterdam

Molecular Cloning and Pharmacology of Porcine 5-Hydroxytryptamine Receptors Relevant to the Study of Antimigraine Drugs

Moleculaire klonering en farmacologie van varken 5-hydroxytryptamine receptoren die relevant zijn voor geneesmiddelen-onderzoek voor migraine therapie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof. dr. ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 30 januari 2002 om 11.45 uur

door

Pankaj Bhalla

geboren te Lucknow, India

Promotiecommissie

Promotor	:	Prof. dr. P.R. Saxena
Overige leden	:	Prof. dr. J.M.J. Lamers Prof. dr. C.I. de Zeeuw Prof. dr. R. Leurs
Copromotor	:	Dr. H.S. Sharma

Financial support of the following institutions and companies is gratefully acknowledged:
Novartis Pharma AG. (Basel, Switzerland), Almirall Prodesfarma (Breda, The Netherlands), Nederlandse Hoofdpijn Vereniging and Erasmus Universiteit Rotterdam (Rotterdam, The Netherlands).

Dedicated to my parents

Table of Contents

Page

Chapter 1

General introduction

At the beginning of last century, Brodie (1900) described in an extensive study that injection of blood serum causes vasoconstriction and a vagally-mediated reflex resulting in a reversible bradycardia, hypotension and arrest of the respiration, while injection of blood plasma was devoid of these effects. About 50 years ago, the hormone and neurotransmitter *serotonin* (5-hydroxytryptamine; 5-HT) was isolated from blood serum and named due to its origin and vascular action (*sero*=serum and *tonin*=vasoconstriction) (Rapport *et al*., 1948). Similarly, another endogenous substance from the enterochromaffin cells (present in gastrointestinal mucosa) was isolated, functionally characterised and named *enteramine* (Erspamer & Asero, 1952). The functional properties of enteramine were mainly smooth muscle contraction and further investigations revealed that enteramine and serotonin were chemically and pharmacologically similar (Erspamer, 1954).

Classification and nomenclature of 5-HT receptors

At the end of 1950's, it was reported that serotonin produces smooth muscle contraction in guinea pig ileum that was mediated by two different receptors, one sensitive to morphine (5-HT-M) and another to dibenzyline (phenoxybenzamine; 5-HT-D); this was the first evidence for the existence of multiple receptors for serotonin (Gaddum & Picarelli, 1957). For nearly three decades, the further characterisation of these receptor types was hampered, although some report stated that non-5-HT-M/D receptors were involved in canine carotid vasoconstriction (Saxena, 1972).

The discovery of radioligand binding techniques provided important information for further characterisation of 5-HT receptors. Thus, the presence of two different receptor subtypes (5-HT_1 and 5-HT_2), preferentially labelled by [^{3}H]5-HT and [^{3}H]spiperone, respectively, were reported in the brain homogenate (Peroutka & Snyder, 1979).

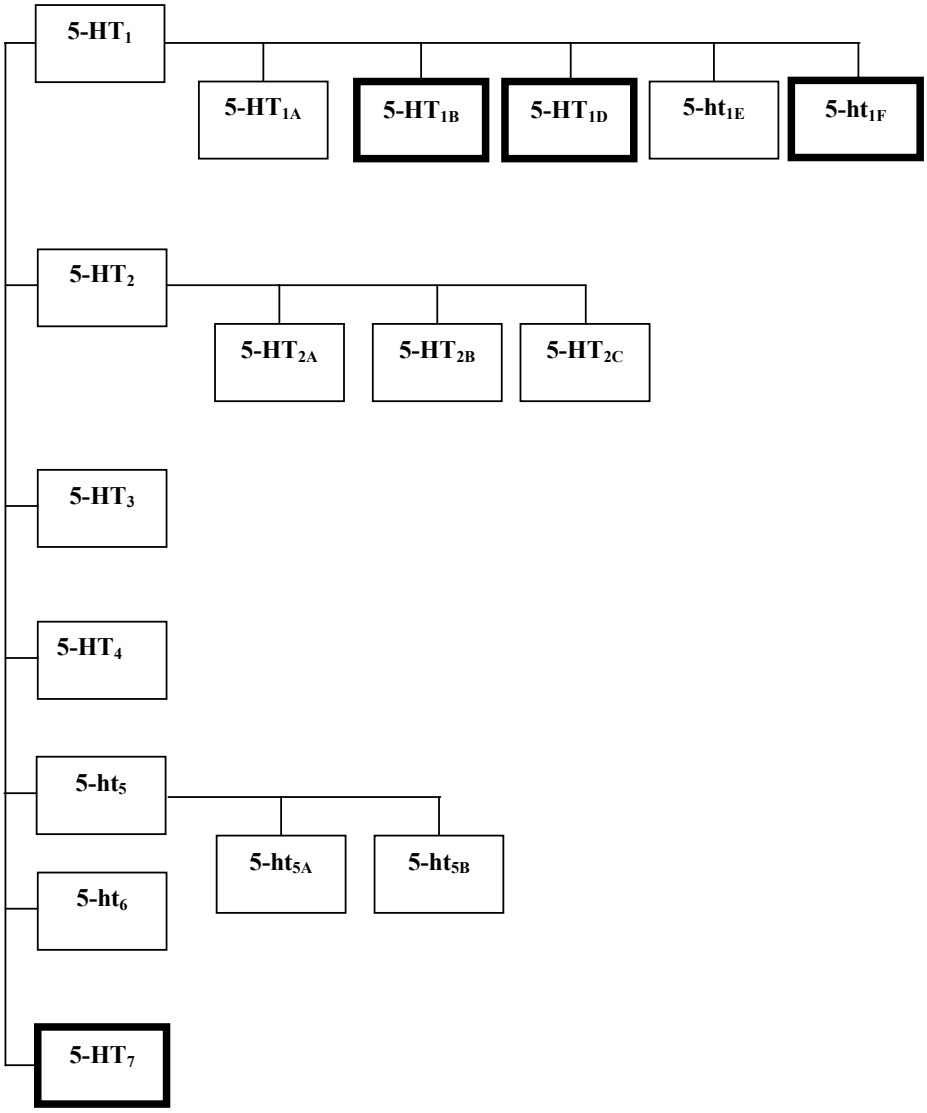


Figure 1.1. NC-IUPHAR serotonin (5-HT) receptor classification (Alexander & Peter, 1999). The highlighted boxes are discussed in more detail (see text). Lower case letters represent recombinant receptors without well-known functional characterisation, whereas upper case letters denote well-characterised receptors (Hoyer *et al.*, 1994).

In 1986, Bradley *et al.* proposed a different classification and nomenclature for 5-HT receptors, where three different 5-HT receptor subtypes were identified, namely: **1**) *'5-HT$_1$-like' receptors*, which displayed high affinity for 5-carboxamidotryptamine and could be labelled with [^{3}H]5-HT; **2**) *5-HT$_2$ receptor*s, being identical to the 5-HT-D receptor and displayed high affinity for ketanserin; and

3) *5-HT_3 receptors*, which are ion channels and identical to the 5-HT-M receptor (Bradley *et al*., 1986). However, with the introduction of several molecular biological tools and their application together with biochemical and functional techniques in the last decade led to an integrated approach for characterising receptors on the basis of amino acid composition, linkage to second messengers as well as functional responses. Several novel 5-HT receptors were identified, characterised and even sometime named differently in different laboratories (e.g. 5-HT_{1B} receptors were also known as 5-$HT_{1D\beta}$ or S12) (Levy *et al*., 1992; Weinshank *et al*., 1992). To overcome this ambiguity, the Serotonin Receptor Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) reclassified 5-HT receptors into seven subclasses, based on the modern criteria (Figure 1.1, Hoyer *et al*., 1994).

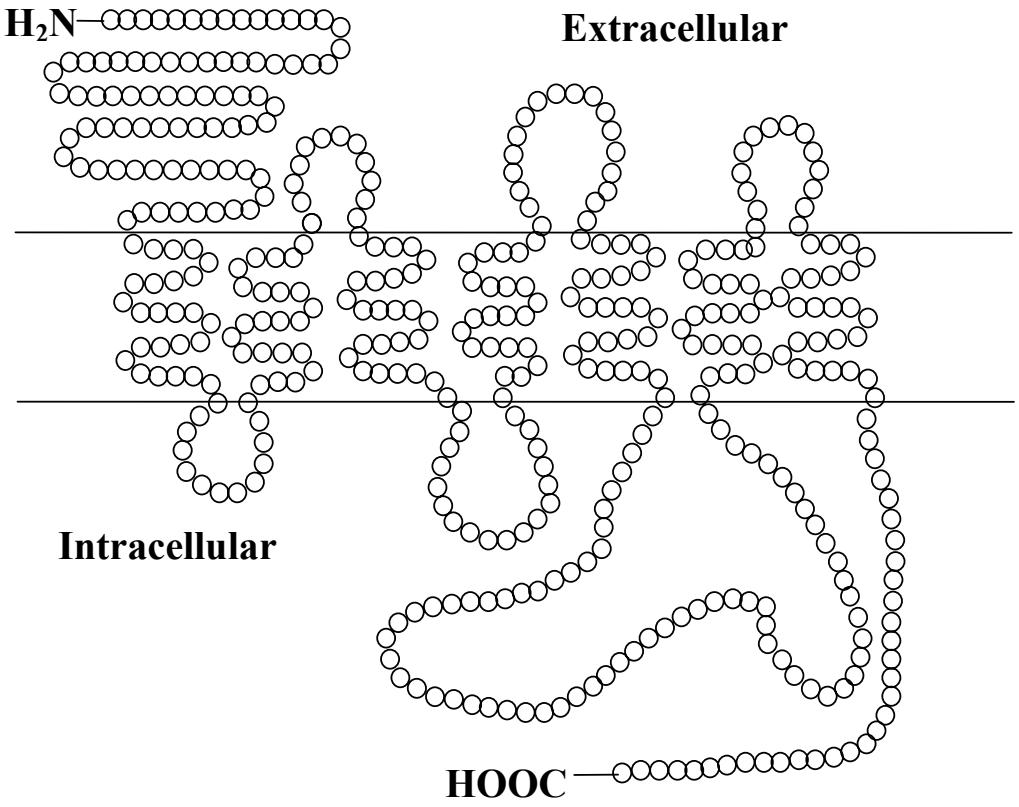


Figure 1.2. Schematic representation of a GPCR. The circles represent amino acids and both N- and C-terminals are given as NH_2- and COOH-group, respectively.

Basically on the basis of their molecular structure (see Figure 1.2), all 5-HT receptors (except 5-HT_3 receptor) are members of the G-protein-coupled receptor

superfamily (GPCRs), which consist of integral membrane proteins and interact with a large variety of hormones and neurotransmitters (Iismaa *et al*., 1995). A common feature of GPCRs is the seven transmembrane domains spanning the cell membrane, having the N-terminal on the extracellular side, while the C-terminal is present on the intracellular side. Mainly the C-terminal cytoplasmatic tail appears to be important as phoshorylation site where specific kinase enzyme catalyse the coupling of phosphate groups (for details, see Iismaa *et al*., 1995). Furthermore, it has become clear that the active binding site for ligands (agonists and antagonists) in these proteins are mainly located in the transmembrane regions (called α-helices) of the protein, as observed by site directed mutagenesis and chimeric receptors, e.g. in 5-HT_1 receptors (Adham *et al*., 1994; Oksenberg *et al*., 1992; Wurch *et al*., 1998).

After receptor activation and G-protein coupling, a number of possible signal transduction pathways have been described for GPCRs (for details, see Iismaa *et al*., 1995). The 5-HT receptors couple to the enzymes adenylyl cyclase (which promotes the production of cyclic adenosine monophosphate) and phospholipase C (which promotes the production of inositol triphosphate and increases intracellular calcium) or ion channels (e.g. potassium channels). Since these effector systems are present in every cell, GPCRs play a major important role in the regulation of physiological responses and actions of around 80% of all neurotransmitters and hormones (Birnbaumer *et al*., 1990).

Physiological responses produced by serotonin

5-HT is synthesised both in the intestines and brain from the essential amino acid L-tryptophan. Interestingly, since 5-HT cannot cross the blood-brain barrier, there is a clear distinction between its central and its peripheral functions. In the central nervous system, 5-HT plays an important role as neurotransmitter (Saxena, 1995) and is involved in appetite, sleep, memory, thermoregulation, sexual behaviour, hallucinations, anxiety and depression. In the periphery, 5-HT plays a role in the aggregations of thrombocytes, smooth muscle contraction, presynaptic transmitter release (neurotransmitter and neuropeptide) and stimulation of nerve fibres (Martin, 1994). As discussed later, it may be important to note that certain 5-HT_1 receptor subtypes mediate (among others) cranial vasoconstriction and inhibition of

neuropeptides release (e.g. substance P, CGRP, neurokinin A), which may be important for their role in migraine therapy (De Vries *et al*., 1999).

In the last decades, it has become clear that the cardiovascular effects of 5-HT are complex and they consist of bradycardia or tachycardia, hypotension or hypertension, vasodilatation and vasoconstriction (Saxena & Villalon, 1990; 1991). In most species, 5-HT-induced bradycardia involves 5-HT_3 receptors, via the so-called von Bezold-Jarisch reflex (Paintal, 1973). On the other hand, tachycardia produced by 5-HT is species-dependent, including a direct or indirect action at 5-HT_2 (rat, dog), 5-HT_3 (rabbit, dog), 5-HT_4 (pig, human) and 5-HT_7 (cat) receptors or by *tyramine-like* (guinea pig) and unidentified mechanisms (Saxena & Villalon, 1990; Villalón *et al*., 1997). With respect to the blood pressure changes, intravenous administration of 5-HT causes a triphasic response in anaesthetised animals (with intact vagal nerve), which comprises of: (*i*) an initial 5-HT_{1A} receptor-mediated hypotension, also due to the bradycardia; (*ii*) a 5-HT_{2A} receptor-mediated hypertension; and (*iii*) a long-lasting 5-HT_7 receptor-mediated hypotension (De Vries *et al*., 1997; De Vries *et al*., 1999; Kalkman *et al*., 1984; 1991; Saxena & Villalon, 1990). To date, it is known that 5-HT can act at a wide range of receptor, 5-$HT_{1A/B/D/F}$, 5-$HT_{2A/B}$, 5-HT_3, 5-HT_4, 5-$ht_{5A/B}$, 5-ht_6 and 5-HT_7 receptors (Figure 1.1), by which it can influence different physiological responses. Furthermore, because these receptor subtypes are widely distributed throughout the body, 5-HT has been implicated in the (patho)physiology of several diseases, including depression, migraine, schizophrenia or eating disorders (Hoyer *et al*., 1994; Saxena, 1995; Saxena & Villalon, 1990). With the development of more selective ligands at these receptors (agonists and antagonists) and with the application of several molecular biological and pharmacological assays, it may hopefully be possible to elucidate the importance of 5-HT receptor subtypes as potential therapeutic target in the near future. In the following sections, the possible role of specific 5-HT receptors as potential targets for the development of antimigraine agents has been explored in more detail.

Serotonin and migraine

Migraine is a syndrome that affects a substantial fraction of the world's population, with a higher prevalence in women (15-18%) than in men (6%) (Lipton & Stewart, 1997). According to the diagnostic criteria proposed by the Headache Classification

of the International Headache Society (IHS, 1988), a migraine attack can be divided into distinct phases (predromitory phase, aura phase, headache phase, resolution and recovery phase) and is characterised by an intense, pulsating and throbbing headache, nausea, vomiting and photo- and/or phonopobia.

Based on different lines of experimental evidence, migraine has been regarded as a "*low-serotonin syndrome*", indicating that serotonin may play an important role in its pathophysiology and/or treatment, such as:

- Reduction of urinary serotonin and elevation of its major metabolite 5-hydroxyindole acetic acid during a migraine attack (Curran *et al*., 1965).
- Platelet serotonin levels were found to drop rapidly during the onset of a migraine attack (Anthony *et al*., 1967).
- Potent centrally active 5-HT-depleting agents (e.g. reserpine) can precipitate a migraine attack (Carroll & Hilton, 1974).
- Intravenous injection of serotonin has been shown to lower headache in migraineurs, as demonstrated in both prophylactic and acute clinical trials (Kimball *et al*., 1960).

The above-mentioned observations provided the basis for the hypothesis that specific 5-HT receptors could be targets for antimigraine agents. The development of the acute antimigraine drug sumatriptan, a 5-HT_1 receptor agonist, further identified the important role of serotonin receptors in migraine therapy (Humphrey *et al*., 1990).

Pathophysiology of migraine

Currently available acute antimigraine agents, besides the ergot alkaloids (ergotamine and dihydroergotamine) include the triptans (sumatriptan, naratriptan, rizatriptan, zolmitriptan, eletriptan, almotriptan). In order to explain their clinical antimigraine efficacy in an appreciable manner, the vascular and neurogenic hypothesis have been proposed (see below and Figure 1.3).

Vascular hypothesis

Although, exact mechanisms of the initiation of a migraine attack are still not clear, the pulsatile and throbbing nature of migraine pain suggests the involvement of extra- and intracranial blood vessels (Goadsby, 1999; Humphrey *et al*., 1990; May & Goadsby, 2001; Saxena & Tfelt-Hansen, 2000). Earlier, Graham & Wolff (1938)

postulated that vasodilatation of cranial blood vessels may play an important role in the pathophysiology of migraine. These pioneers in migraine research have shown that relief of migraine headache was accomplished by ergotamine-induced vasoconstriction of the temporal artery. Further studies, performed by Heyck (1969), indicated that primarily carotid arteriovenous anastomoses are involved. Furthermore, other observations confirming the vascular hypothesis have been, for example, that mechanical distension of cranial arteries or administration of potent vasodilators, such as organic nitrates, cause headache (Dahl *et al*., 1990; Nichols *et al*., 1990). However, these organic nitrates not only dilate blood vessels but also release neuropeptides, probably via neuronal events raising the possibility that a part from vasodilatation some other mechanisms can also be involved in migraine pain (Goadsby & Edvinsson, 1993). Nevertheless, independent of underlying mechanisms of action, a general feature of all acute antimigraine agents (e.g. triptans and ergot alkaloids) is their vasoconstrictor property. In this sense, the discovery of acute antimigraine drug sumatriptan and its therapeutic effect was proposed to be due to constriction of dilated (cranial) blood vessels (Humphrey & Feniuk, 1991; Saxena & Tfelt-Hansen, 2000). Indeed, both ergot alkaloids and triptans produce similar vasoconstriction in the carotid circulation of different species, including the pig and dog. Using this experimental animal model predictive for antimigraine activity (De Vries *et al*., 1999; Saxena, 1995) and other *in vitro* studies (Bouchelet *et al*., 2000; Bouchelet *et al*., 1996; Longmore *et al*., 1997; Verheggen *et al*., 1998), it has been clearly demonstrated that 5-HT_{1B} receptors exclusively mediate the vasoconstrictor actions of sumatriptan (De Vries *et al*., 1998; Saxena & Tfelt-Hansen, 2000; Willems *et al*., 1998). Additionally, the 5-HT_{1B} receptor has been localised (Longmore *et al*., 1997) and functionally characterised to produce contraction of peripheral blood vessels, such as human isolated coronary artery (Longmore *et al*., 1997; MaassenVanDenBrink *et al*., 1998). It may be noted that contraction of human isolated arteries by the triptans may explain the associated chest pain in clinical migraine trials (Saxena & Tfelt-Hansen, 2000).

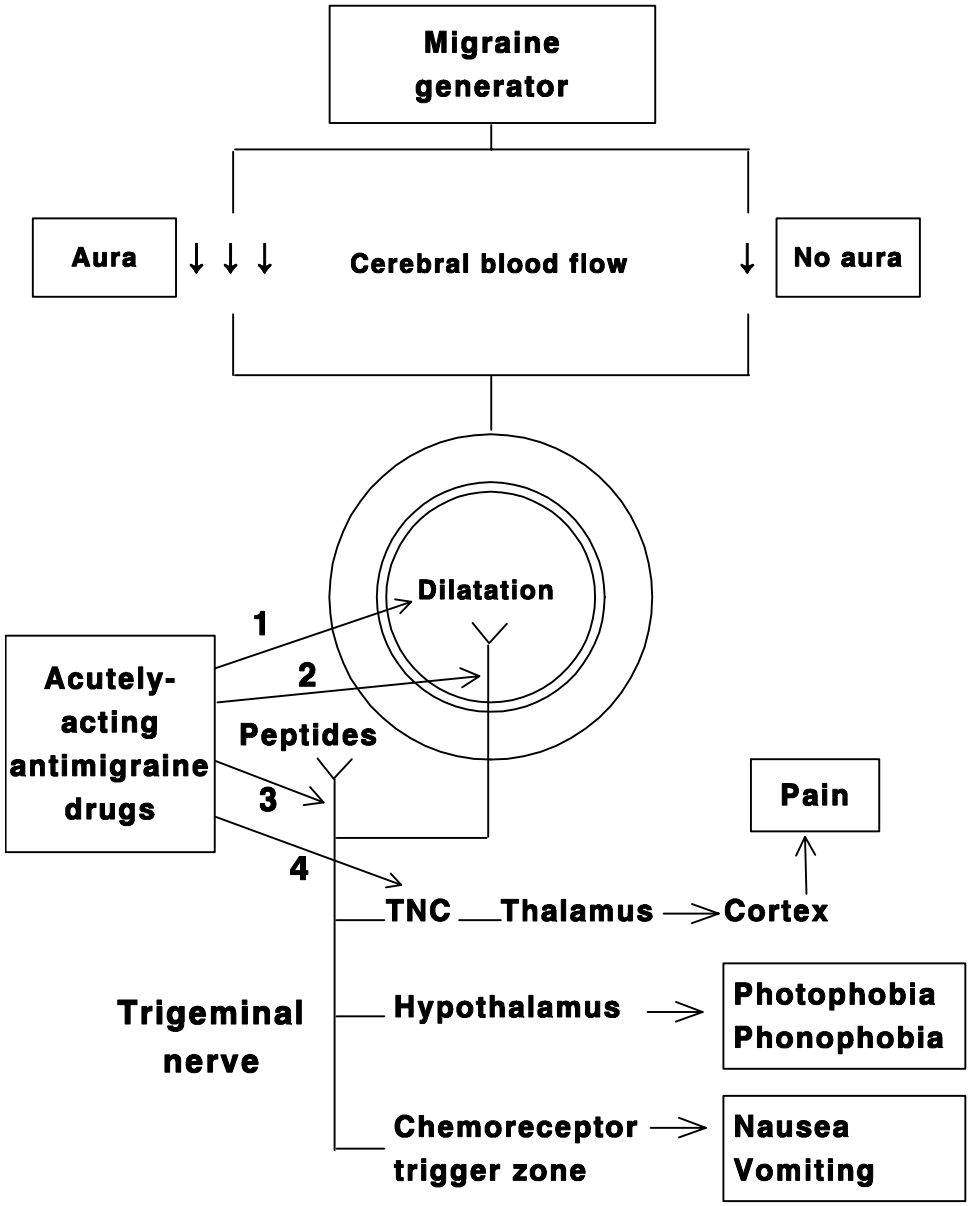


Figure 1.3. Diagrammatic representation of the possible site of acutely acting antimigraine drugs, based on De Vries *et al*. (1999). Site of action of antimigraine agents are believed to antimigraine action by direct vasoconstriction of dilated cranial blood vessels (**1**), inhibition of trigeminal stimulation induced cranial vasodilatation (**2**), plasma protein extravasation (**3**) and/or central neuronal activity (**4**). It may be mentioned that the plasma protein extravasation model has been questioned as being predictive for antimigraine activity (De Vries *et al.*, 1999). TNC represents trigeminal nucleus caudalis.

Neurogenic hypothesis

An alternate mechanism to explain the genesis of a migraine attack involves the central nervous system, in particular the trigeminovascular system (Goadsby & Edvinsson, 1993; Moskowitz, 1992). According to this hypothesis, migraine arises due to abnormal neuronal firing and neurotransmitter release in the brain neurones, possibly leading to cortical spreading depression and neurogenic inflammation and after subsequent cascades producing headache and other accompanying symptoms (as described above); while vasodilatation is an epiphenomenon. During neurogenic inflammation, the trigeminal ganglion is stimulated and induces neurogenic protein extravasation, which involves the release of the neuropeptide calcitonin gene-related peptide (CGRP), substance P and neurokinin A (Goadsby, 1999). Several animal studies have demonstrated that inhibition of neurogenic protein extravasation by acute antimigraine agents, such as sumatriptan, is mainly mediated by 5-HT_{1D} and/or 5-ht_{1F} receptors (Bouchelet *et al*., 2000; Goadsby & Edvinsson, 1993). However, of several possible neuropeptides involved, only circulating CGRP levels are elevated after trigeminal ganglion stimulation in cats, indicating that CGRP may play a major role in migraine headache (Goadsby & Edvinsson, 1993). Additionally, CGRP produces potent neurogenic dural vasodilatation in guinea pigs, a response that can be markedly attenuated by stimulation of presynaptic 5-HT_{1D} and/or 5-ht_{1F} receptor agonists (Bouchelet *et al*., 2000; Goadsby & Edvinsson, 1993). It may be important to note that 5-HT_{1D} and/or 5-ht_{1F} receptor agonists are devoid of vasoconstrictor actions (Bouchelet *et al*., 2000; Villalón *et al*., 1999), however, it also remains unclear whether these compounds are efficacious in migraine treatment (McCall, 1999). Considering both above-mentioned hypotheses (see Figure 1.3), it appears that both vascular and neuronal effects are important to explain the therapeutic action of antimigraine drugs; future studies may hopefully elucidate whether individual mechanisms are sufficient for antimigraine efficacy.

Role of 5-HT_1 and 5-HT_7 receptors in migraine therapy

As mentioned above, a combination of vascular and neurogenic hypotheses explains pathophysiology of migraine in a reasonable manner and the possible mechanisms of actions of acutely acting antimigraine drugs within the trigeminovascular system have been summarised in Figure 1.3. Due to the clinical antimigraine efficacy of triptans,

it is evident that the 5-HT_1 receptor subfamily is a general target for antimigraine activity, with special emphasis on the 5-HT_{1B}, 5-HT_{1D} and/or 5-ht_{1F} receptor. Furthermore, selective 5-HT_7 receptor antagonists may be efficacious in prophylactic migraine therapy. The molecular and pharmacological aspects of these receptors have been discussed in more detail in the following sections, whereas Table 1.1 provides an overall summary.

Serotonin 5-HT_{1B} receptor

Until two decades ago, 5-HT_{1B} receptors were observed only in the rat brain where high affinity binding sites were found for [^{3}H]5-HT, while spiperone was used to discriminate 5-HT_{1A} binding sites (Pedigo *et al*., 1981). Much later, it was established that the rat 5-HT_{1B} receptor (Adham *et al*., 1992; Voigt *et al*., 1991) displayed a high affinity for β-adrenoceptor antagonists (cyanopindolol, pindolol and propranolol).

A similar pharmacological profile was observed in mice (Maroteaux *et al*., 1992) and opossum (Cerutis *et al*., 1994). In contrast, the 5-HT_{1B} receptor in other species (human, dog and guinea pig) did not recognise β-adrenoceptor antagonists (Hoyer & Middlemiss, 1989). At that time, it was thought that both receptors, which shared comparable pharmacological properties, signal-transduction mechanisms and tissue distribution, may be species homologous of the same single gene product (Hoyer & Middlemiss, 1989). This possibility was negated with the presence of both 5-HT_{1B} and 5-HT_{1D} receptors in same species, e.g. human (Weinshank *et al*., 1992), rat (Adham *et al*., 1992; Voigt *et al*., 1991), rabbit (Bard *et al*., 1996; Harwood *et al*., 1995), guinea pig (Zgombick *et al*., 1997) and dog (Branchek *et al*., 1995).

Molecular aspects of 5-HT_{1B} receptor

During the early nineties of last century, cloning of human 5-HT_{1B} receptor was reported from several laboratories and was named the 5-$HT_{1D\beta}$ receptor (Hamblin & Metcalf, 1991; Weinshank *et al*., 1992). The human 5-HT_{1B} receptor is localised on chromosome 6, region 6q13 and consists of a 390 amino acid protein (Jin *et al*., 1992). At around the same time, the 5-HT_{1B} receptor was also cloned from rat, mouse and opossum and, interestingly, showed three amino acids missing in its receptor structure (Maroteaux *et al*., 1992; Voigt *et al*., 1991). The 5-HT_{1B} receptor gene has no introns and consists of seven transmembrane domains that are important for ligand binding and activation (Jin *et al*., 1992; Weinshank *et al*., 1992). The receptor

contains two sites for N-linked glycosylation on the N-terminal region and three protein kinase sites in different positions.

Table 1.1. Characteristic pharmacological properties of some important serotonin (5-HT) receptors that may be relevant for the development of antimigraine drugs

Receptor	**5-HT$_{1B}$**	**5-HT$_{1D}$**	**5-ht$_{1F}$**	**5-HT$_{7}$**
Receptor code	2.1.5HT.01B	2.1.5HT.01D	2.1.5HT.01F	2.1.5HT.07
Previous names	5-HT$_{1\text{-like}}$, 5-HT$_{1X}$, 5-HT$_{1D\beta}$	5-HT$_{1\text{-like}}$, 5-HT$_{1X}$, 5-HT$_{1D\alpha}$	5-HT$_{1E\beta}$, 5-HT$_{6}$	5-HT$_{1\text{-like}}$, 5-HT$_{1Y}$, orphan
Structural information	7TM h390aa, chr.6q13 r386aa	7TM h377aa, chr.1p34.3-36.3 r374aa	7TM h366aa, chr.3p11 r366aa	7TM h445aa, chr.10q23.3-24.3 r448aa
Signal transduction mechanism	G_i/G_o, inhibit cAMP formation, elevated $[Ca^{2+}]_i$	G_i/G_o, inhibit cAMP formation, elevated $[Ca^{2+}]_i$	G_i/G_o, inhibit cAMP formation.	G_s, increases cAMP formation.
Selective agonists	Sumatriptan CP93129 (rodents)	Sumatriptan PNU109291	LY344864 LY344370	None
Selective antagonists	GR127935* SB224289	GR127935* BRL15572	none	SB258719
Receptor Distribution	Vascular smooth muscle, substantia nigra, autonomic terminals, raphe nuclei	Trigeminal ganglion, dorsal raphe, autonomic and trigeminal nerve terminals	Cortex, hippocampus, claustrum, spinal cord, uterus, mesentery	Hypothalamus, raphe nuclei, gastrointestinal and vascular smooth muscle, sympathetic ganglion
Tissue Functions	Vasocontraction, autoreceptor in hippocampus, raphe nuclei	Autoreceptor in hippocampus, raphe nuclei; heteroreceptor in trigeminal ganglion	Trigeminal neuro-inhibition	Smooth muscle relaxation, circadian phase shifts

*, GR127935 is considered to be a selective 5-HT$_{1B/1D}$ receptor antagonist. Please see the list of abbreviations for further details.

The alignment of amino acid sequences of different mammalian species showed high overall homology (88-94%), which was even higher in the transmembrane domains (Adham *et al*., 1992; Hamblin *et al*., 1992; Jin *et al*., 1992; Maroteaux *et al*., 1992; Voigt *et al*., 1991; Weinshank *et al*., 1992). The presence of a threonine residue in the seventh transmembrane domain (at position 355) was observed in human, rabbit, dog and guinea pig 5-HT$_{1B}$ receptor and proved to be an important feature for the

pharmacological profile (Adham *et al*., 1992; Oksenberg *et al*., 1992). For example, the 5-HT_{1B} receptor cloned from other species (e.g. rat, mouse and opossum) showed an asparagine residue at this position, which drastically changed their pharmacological profile compared to that observed with those in mammalian homologues (Maroteaux *et al*., 1992; Voigt *et al*., 1991). For several years, it has been questioned whether the 5-HT_{1B} receptor in rodents and mammalian species are different. According to recent classification of 5-HT receptors (NC-IUPHAR), the 5-HT_{1B} receptor in rodents is a species homologue for that in human and have been termed **r**5-HT_{1B} (formerly 5-$HT_{1D\alpha}$) and **h**5-HT_{1B} (formerly 5-$HT_{1D\beta}$) receptor, respectively (Hoyer *et al*., 1994). The latter nomenclature of 5-HT receptors will be used in the following sections of present dissertation.

Binding profile of 5-HT_{1B} receptor ligands

The 5-HT_{1B} receptor was originally described in rodents by its high affinity for β-adrenoceptor antagonists (e.g. propranolol or pindolol) with the presumption for its absence in other species (Adham *et al*., 1992; Voigt *et al*., 1991). However, this latter assumption proved to be wrong, since the 5-HT_{1B} receptor was also cloned from other mammalian homologues (e.g. rat, mouse, dog, rabbit or human) (Branchek *et al*., 1995; Hamblin *et al*., 1992; Harwood *et al*., 1995; Maroteaux *et al*., 1992; Voigt *et al*., 1991; Weinshank *et al*., 1992). Competitive binding assays revealed a binding affinity profile for 5-HT_{1B} receptor as follows: 5-hydroxytryptamine > serotonin > sumatriptan > 8-OH-DPAT > pindolol, which was similar to that described for 5-HT_{1D} receptors (Weinshank *et al*., 1992). Subsequently, the pharmacological profiles of the 5-HT_{1B} receptor in rodents and human were found to be considerably different with regards to their interaction with above-mentioned β-adrenergic antagonists (Hoyer *et al*., 1994; Metcalf *et al*., 1992; Oksenberg *et al*., 1992; Parker *et al*., 1993). Interestingly, this difference in pharmacological profiles between human and rodent 5-HT_{1B} receptor has been proved to be due to a single amino acid difference (threonine or asparagine at residue 355) in the seventh transmembrane domain (Oksenberg *et al*., 1992).

Even though, recombinant human 5-HT_{1B} and 5-HT_{1D} receptors display nearly indistinguishable pharmacological properties (Weinshank *et al*., 1992), some compounds (e.g. ketanserin or methysergide) are more potent at human 5-HT_{1D} than

5-HT_{1B} receptors (Peroutka, 1994); see Table 1.2). Later, on the basis of both their molecular entities and individual pharmacological profiles these receptors were identified as independent targets for the drug development of antimigraine agents (see below).

Table 1.2. Binding affinity constants (pK_i values) of several compounds at recombinant 5-HT_{1B} receptor from different species

Ligand	Human[a]	Guinea pig[b]	Rabbit[c]	Rat[d]	Mouse[e]	Dog[d]
Agonists						
5-HT	7.94	8.28	8.52	8.56	7.40	8.42
5-CT	8.06	8.54	9.24	8.94	8.00	8.90
L694247	9.50[d]	-	-	10.02	-	9.71
Ergotamine	8.12	-	-	-	-	-
DHE	8.09	9.08	8.64[i]	10.56[g]	-	8.82[h]
CP122288	8.31[d]	-	-	6.81	-	8.13
Zolmitriptan	7.61	9.18	8.28	7.29	-	8.02
Sumatriptan	7.14	6.87	7.54	7.35	-	7.29
8-OH-DPAT	6.63[d]	6.59	7.01	5.12	4.50	6.42
Antagonists						
GR127935	8.53	8.53	9.74	8.78	-	8.71
Methiothepin	8.44	8.14	9.32	7.21	-	7.67
SB224289	8.22	-	-	-	-	-
Ketanserin	6.85	6.15	6.91	<5.1	5.20	5.28[h]
Ritanserin	5.76	6.92	-	-	-	-
BRL15572	5.51	-	-	-	-	-
Metergoline	7.82[c]	7.97	7.71[i]	9.05[g]	-	7.45[h]
(-)-Propranolol	5.64[d]	-	-	7.71		5.36
(-)-Pindolol	5.31[f]	5.27	7.19	7.57	7.20	5.18

Data are means from radioligand displacement experiments, using recombinant receptors expressed in different cell lines: [a], (Pauwels et al., 1996); [b], (Zgombick et al., 1997); [c], (Wurch et al., 1997) ; [d], (Beer et al., 1998); [e], (Maroteaux et al., 1992); [f], (Weinshank et al., 1992); [g], (Hamblin et al., 1992); [h], (Branchek et al., 1995); [i], (Bard et al., 1996).

Recently, the potent and selective antagonist (SB224289) of human 5-HT_{1B} receptor revealed that sumatriptan induced contraction of vascular smooth muscle is mainly mediated mainly by 5-HT_{1B}, rather than 5-HT_{1D}, receptors (De Vries *et al*., 1999; Verheggen *et al*., 1998).

Signal transduction mechanisms of 5-HT_{1B} receptor

After activation of 5-HT_{1B} receptors, their functions are mediated by a variety of G-proteins, including G_i and G_s (Hoyer *et al*., 1994). It has been shown that one of the signal transduction pathways, mediated by these receptors, involves negative coupling to adenylate cyclase, attenuating cAMP production in target cells (Hoyer *et al*., 1994). In this sense, inhibition of forskolin-stimulated adenylate cyclase by 5-HT was reported in Chinese Hamster Ovary (CHO) or HeLa cells expressing human 5-HT_{1B} receptors (Hamblin *et al*., 1992; Veldman & Bienkowski, 1992). Similarly, activation of 5-HT_{1B} receptors by 5-HT, 5-carboxytryptamine and sumatriptan decreases forskolin-induced accumulation of cAMP in LM(tK$^-$) cells (Levy *et al*., 1992; Weinshank *et al*., 1992). Apart from inhibition of adenylate cyclase, other studies also show that 5-HT_{1B} receptor activation (*via* G_s-proteins) also stimulates phospholipase C (Hoyer *et al*., 1994). Activation of this enzyme will result in the generation of inositol (1,4,5)-triphosphate (IP_3), which acts on the IP_3 receptor in the endoplasmatic reticulum to release stored Ca^{2+} and diacylglycerol that (together with Ca^{2+}) can activate protein kinase C. For example, Zgombick *et. al.* have demonstrated that stimulation of human 5-HT_{1B} receptor in stable expressed LM(tK$^-$) cells or in fibroblasts of mice elevates the inositol phosphate level via stimulation of phospholipase C (Zgombick *et al*., 1993). On the contrary, no changes in intracellular Ca^{2+} levels were observed after activation of human 5-HT_{1B} receptors expressed in stable LM(tK-) cells (Levy *et al*., 1992). Production of these second messengers results in smooth muscle contraction in particular vascular and non-vascular tissues. Besides investigating the production of these second messengers, it is also possible to study the effects of 5-HT_{1B} receptor activation at the G-protein level, by means of quantifying G-protein binding, using the [^{35}S]GTPγS binding assay (Beer *et al*., 1998; Pauwels *et al*., 1997); described in Chapter 3, we have used [^{35}S]GTPγS binding assay for functional receptor characterisation.

Tissue distribution of 5-HT$_{1B}$ receptor

As described by Hoyer (1994) and Bouchelet (1996), 5-HT$_{1B}$ receptors are found throughout central as well as peripheral tissues. Regarding its central distribution, Northern blot analysis revealed high expression of 5-HT$_{1B}$ receptor mRNA in striatum, moderate in hippocampus and frontal cortex and barely detectable expression was found in cerebellum (Jin *et al*., 1992). In the same study, *in situ* hybridisation experiments demonstrated a widespread expression of 5-HT$_{1B}$ receptors in human and rat brain tissue, mainly in striatum (nucleus caudatus), putamen and purkinje cells of the cerebellum. Other studies showed that the distribution of 5-HT$_{1B}$ receptor mRNA in rat brain tissue was profoundly observed in pyramidal cells of hippocampus as well as in subiculum and parasubiculum (Voigt *et al*., 1991). Earlier, autoradiographical studies, using radiolabelled [^{3}H]5-HT in rat brain tissues, showed high density of 5-HT$_{1B}$ receptors in the basal ganglion and cortical areas (Pazos & Palacios, 1985). In peripheral tissue, the presence of 5-HT$_{1B}$ receptor mRNA was demonstrated by the RT-PCR technique in various blood vessels of rat (aorta, renal artery, vena cava, portal, femoral and jugular veins), porcine (pulmonary, coronary and cerebral arteries and cerebral vein) and human (cerebral and coronary arteries) (Bouchelet *et al*., 1996; Nilsson *et al*., 1999; Ullmer *et al*., 1995). The use of selective antibodies showed a more precise localisation of 5-HT$_{1B}$ receptor proteins with very profound signals in smooth muscle layer and faint signals in the endothelium of human coronary and cerebral arteries (Nilsson *et al*., 1999; Nilsson *et al*., 1999). These findings were in agreement with functional studies using selective 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor antagonists, both *in vitro* and *in vivo* experiments (De Vries *et al*., 1998; De Vries *et al*., 1999; Kaumann *et al*., 1994; Verheggen *et al*., 1998).

Physiological functions of 5-HT$_{1B}$ receptor

Since a decade, mainly molecular and pharmacological studies (see above) have provided important information regarding the physiological role of 5-HT$_{1B}$ receptor. The exact role of 5-HT$_{1B}$ receptors in animals and human was hampered mainly due to the fact that recombinant clones as well as selective compounds were not available. Nevertheless, it has been demonstrated that activation of central 5-HT$_{1B}$ receptors can serve as an sympatho-regulatory role (inhibiting noradrenaline release) in both rat vena cava (Göthert *et al*., 1986) as well as within the carotid circulation of

anaesthetised dogs (Villalón *et al*., 2001). Moreover, activation of postsynaptic 5-HT_{1B} receptors produces vasoconstriction of rat isolated caudal artery (Craig & Martin, 1993) or carotid circulation (De Vries *et al*., 1998; Villalón *et al*., 1999). It is important to mention that the rat 5-HT_{1B} receptor displays a different molecular structure and pharmacological profile as compared to its human homologue (see above). According to the nomenclature and classification assigned by Bradley *et al.,* (1986), 5-HT_{1B} receptors mediating certain responses (e.g. contraction of cranial blood vessels, prejunctional inhibition of neuronal transmitter release, smooth muscle relaxation and tachycardia in the cat) belonged for a long time to the 5-$HT_{1\text{-like}}$ receptors category (Bradley *et al*., 1986). Subsequently, with the use of selective 5-$HT_{1B/1D}$ receptor antagonists, e.g. GR127935, it became apparent that 5-$HT_{1B/1D}$ receptors mediate some of these responses (vasoconstriction of cranial blood vessels and neuronal release), while others are mediated by 5-HT_7 receptors (see later) (Saxena *et al*., 1998). Using the selective 5-HT_{1B} receptor antagonist SB224289, it became clear that mainly 5-HT_{1B} receptors can mediate constriction in the cranial (e.g. carotid arteriovenous anastomoses, temporal or middle meningeal artery) as well as in the peripheral circulation (e.g. coronary artery, saphenous vein) (De Vries *et al*., 1998; De Vries *et al*., 1999; MaassenVanDenBrink *et al*., 1998; Saxena & Tfelt-Hansen, 2000; Verheggen *et al*., 1998). Interestingly, the latter observations were in agreement with those obtained from molecular and immunohistochemical studies (Bouchelet *et al*., 2000). On the other hand, potent and selective 5-HT_{1D} receptor agonists (e.g. PNU109291, L-775,606) are devoid of vasoconstrictor activity in human and bovine isolated blood vessels (Bouchelet *et al*., 2000). A vasodilator role of 5-HT_{1B} receptors has also been suggested, mainly based on the presence of its mRNA in cultured endothelium cells of human coronary artery (Ullmer *et al*., 1995) and its role in endothelium-dependent relaxation in pig coronary arteries (Schoeffter & Hoyer, 1990).

Serotonin 5-HT_{1D} receptor

The 5-HT_{1D} receptor was first identified in bovine caudate, using the radioligand [^{3}H]5-HT and different ligands at 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} (now called 5-HT_{2C}) receptors (Herrick-Davis & Titeler, 1989; Heuring & Peroutka, 1987). The first cDNA sequence of 5-HT_{1D} receptor was identified in canine thyroid cDNA library

(Libert *et al*., 1989) and later this receptor was pharmacologically characterised by ligand binding in transfected Cos-7 cells and LM (tK^- cells) (Maenhaut *et al*., 1991; Zgombick *et al*., 1991). As described later, an important physiological feature of 5-HT_{1D} receptors is their regulatory role in the release of several transmitters (e.g. neuropeptides) and may be involved in the clinical efficacy of several antimigraine agents.

Molecular aspect of 5-HT_{1D} receptor

The molecular cloning of dog 5-HT_{1D} receptor (RDC4) showed an intronless gene consist of a single open reading frame (1131bp sequence) encoding a protein of 377 amino acids (Libert *et al*., 1989; Zgombick *et al*., 1991). The human 5-HT_{1D} (formerly called 5-$HT_{1D\alpha}$) receptor consisted also of 377 amino acids and was localised on the chromosome 1, region 1p34.3-36.3 (Jin *et al*., 1992; Weinshank *et al*., 1992). This receptor showed a typical G-protein couple structure with seven transmembrane domains along with putative N-glycosylation, protein kinase A and C phoshorylation sites. At a later stage, the 5-HT_{1D} receptor was also cloned from other mammalian homologues, such as rabbit (Harwood *et al*., 1995), guinea pig (Wurch *et al*., 1997; Zgombick *et al*., 1997), rat and mouse (Hamblin *et al*., 1992; Wurch *et al*., 1997). Even though the differences in amino acid sequence (three amino acids are missing near the N-terminal extracellular region), as observed in the rat and mouse 5-HT_{1D} receptor, overall this receptor displays a high homology across other species (Wurch *et al*., 1997).

Binding profile of 5-HT_{1D} receptor ligands

Recombinant 5-HT_{1D} receptor showed high affinity for the radioligands [^{3}H]5-HT and [^{3}H]LSD in mammalian Cos-7 or Chinese hamster ovary (CHO) cell lines (Weinshank *et al*., 1992). Competitive binding assay revealed a binding affinity profile for 5-HT_{1D} receptor, as follows: 5-hydroxytryptamine > serotonin > sumatriptan > 8-OH-DPAT > pindolol, which was similar to that described for 5-HT_{1B} receptors (Weinshank *et al*., 1992). The pharmacological characterisation of 5-HT_{1D} receptors was largely hampered due to the unavailability of selective agonist and/or agonists at these receptors. To overcome this problem, different compounds were used that can discriminate between these receptors, e.g. the 5-HT_2 receptor antagonists ketanserin and ritanserin, which showed higher affinity at 5-HT_{1D} as

compared to 5-HT_{1B} receptors (Hoyer *et al*., 1994). Nevertheless, the latter property of ketanserin is not so apparent in dogs (Table 1.3) (Branchek *et al*., 1995). Additionally, dihydroergotamine (DHE) also shows high affinity for 5-HT_{1D} receptor as compare to 5-HT_{1B} receptor (Bard *et al*., 1996; Peroutka, 1994).

Table 1.3. Binding affinity constants (pK_i values) of several compounds at recombinant 5-HT_{1D} receptor from different species

Ligand	Human[a]	Guinea pig[b]	Rabbit[c]	Rat[d]	Dog[e]
Agonists					
5-HT	8.33	8.02	7.88	8.60	8.30
5-CT	8.91	8.55	8.53	9.43[h]	9.04
L694247	8.98	-	-	-	-
Ergotamine	8.01	-	-	-	-
DHE	8.09	9.21	9.22	10.19[h]	-
CP122288	8.10	-	-	-	-
Zolmitriptan	9.01	9.18[h]	-	9.10	-
Sumatriptan	8.24	7.26	7.12	7.84	8.79
8-OH-DPAT	6.92[f]	6.73	6.45	7.18	7.07
Antagonists					
GR127935	8.20	8.24	-	8.88	-
SB224289	6.63[k]	-	-	-	-
BRL15572	7.55[l]	-	-	-	-
Methiothepin	9.00	7.47	6.64	8.97	-
Ketanserin	7.66	6.86	7.66	7.98	5.52[j]
Ritanserin	7.82	6.81	-	7.96	-
Metergoline	8.32[g]	7.82	7.76	8.86	-
(-)-Propranolol		-	-	7.71[i]	<5.00
(-)-Pindolol	5.39[f]	5.26	-	-	<5.00

Data are means from radioligand displacement experiments using recombinant receptors expressed in different cell lines: [a], (Pauwels et al., 1996); [b], (Zgombick et al., 1997); [c], (Bard et al., 1996); [d], (Wurch et al., 1997); [e], (Zgombick et al., 1991); [f], (Weinshank et al., 1992); [g], (Zgombick et al., 1996); [h], (Hamblin et al., 1992); [i], (Bach et al., 1993); [j], (Branchek et al., 1995); [k], (Hagan et al., 1997); [l], (Price et al., 1997).

However, although methiothepin did not distinguish between the human 5-HT_{1B} and 5-HT_{1D} receptor, it showed several fold less affinity for rabbit 5-HT_{1D} as compare to 5-HT_{1B} receptor (Bard *et al*., 1996). Such species difference of the same receptor makes it very important to carefully extrapolate the drug receptor interaction from animal to human.

Recently, potent and selective antagonists at 5-HT_{1B} (SB224289) and 5-HT_{1D} (BRL15572) receptors have become available (Hagan *et al*., 1997; Price *et al*., 1997) and have been used in several studies to reveal their individual functional properties (see below); see Tables 1.2 and 1.3 for their affinity constants at 5-HT_{1B} and 5-HT_{1D} receptors.

Signal transduction mechanisms of 5-HT_{1D} receptor

In a similar manner as described for the 5-HT_{1B} receptor, 5-HT_{1D} receptors are part of the G-protein-coupled receptor superfamily and mediate its responses largely through actions of G_i proteins (Pauwels *et al*., 1996). It has been shown that serotonin (5-HT) as well as the 5-$HT_{1B/1D}$ receptor agonist sumatriptan produced a pertussin toxin-sensitive attenuation of *c*AMP production in different transfected cell lines, e.g. Chinese hamster ovary or LM (tK-), rat C6-glial cells or dog y1 adrenal (y1 Kin-8) cells (Hamblin & Metcalf, 1991; Pauwels *et al*., 1996; Weinshank *et al*., 1992). Interestingly, other reports have mentioned that 5-HT produces an increase, rather than a decrease, in cAMP production in Cos-7 cells and *Xenopus oocytes* expressing the 5-HT_{1D} receptor (Maenhaut *et al*., 1991). The latter opposite effect of 5-HT may be explained by either a difference in receptor coupling or by the different cell line used. Most of the studies using recombinant 5-HT_{1D} receptor cell lines were based on inhibition of adenylate cyclase activity as a functional assay for the receptor. However, the GTPγS binding assay has also been used to deduce the intrinsic activity and functional properties of serotonergic drugs at recombinant human 5-HT_{1D} receptor expressed in the stable transfected rat C6-glial cell lines (Pauwels *et al*., 1997). Besides inhibition of adenylyl cyclase, another well known effector that couples to the G-protein-coupled-receptor complex is activation of phospholipase C, as reported in LM(tk-) cells (Zgombick *et al*., 1993). Activation of this particular enzyme, e.g. by 5-HT, results in an elevated production of inositol triphosphate and intracellular Ca^{2+} release (Hoyer *et al*., 1994). It has been suggested that elevation of

the intracellular Ca^{2+}, rather than inhibition of cAMP production, is responsible for the contraction of vascular smooth muscle cells in the presence of a 5-HT_{1D} receptor agonist (Sumner *et al*., 1992).

Tissue distribution of 5-HT_{1D} receptor

Compare to the 5-HT_{1B} receptor, less expression of 5-HT_{1D} receptor mRNA has been detected both in the central nervous system as well as in the periphery (Bruinvels *et al*., 1994; Longmore *et al*., 1997; Nilsson *et al*., 1999). Earlier, weak mRNA signals of 5-HT_{1D} receptor were detected in rat brain parts, e.g. primary olfactory cortex, accumbens nucleus, caudate putamen, dorsal raphe nucleus and medial vestibular nucleus (Bach *et al*., 1993; Bruinvels *et al*., 1994). Similarly, these signals were also found in guinea pig brain, trigeminal ganglion and dorsal raphe (Bonaventure *et al*., 1998; Bonaventure *et al*., 1998) and in human trigeminal ganglion (Bouchelet *et al*., 1996). In the periphery, immunohistochemical studies failed to show the presence of 5-HT_{1D} receptor protein in the human coronary artery, neither on its smooth muscles nor in the endothelium (Nilsson *et al*., 1999). Additionally, the RT-PCR technique did not show 5-HT_{1D} receptor signals in a variety of blood vessels in different species, including the dog (large coronary arteries, saphenous vein), rat (aorta, renal artery, vena cava, portal, femoral and jugular vein) and pig (pulmonary, coronary and cerebral artery and cerebral vein), in human endothelial or smooth muscle cell cultures (Sgard *et al*., 1996; Ullmer *et al*., 1995). Some reports, however, indicate a minor presence of 5-HT_{1D} receptor *m*RNA in a few samples of human coronary artery (Ishida *et al*., 1999).

Physiological function of 5-HT_{1D} receptor

Sumatriptan as well as the second generation of triptans display high affinity for 5-HT_{1D} receptor, besides at 5-HT_{1B} and 5-ht_{1F} receptors. Earlier, the functional properties of 5-$HT_{1B/1D}$ receptors were deduced by using the non-selective antagonist GR127935 that antagonises sumatriptan-induced contraction in dog isolated basilar artery and saphenous vein (Skingle *et al*., 1996) and sumatriptan-induced constriction within porcine and canine carotid circulation (De Vries *et al*., 1998; De Vries *et al*., 1999). However, due to the non-discriminating nature of GR127935 and the almost similar pharmacological profiles of these receptors, selective antagonists at either 5-HT_{1B} or 5-HT_{1D} receptors turned out to be necessary to elucidate their physiological

roles. With the use of selective 5-HT_{1B} (SB224289, Hagan *et al*., 1997) and 5-HT_{1D} receptor antagonists (BRL15572, Price *et al*., 1997), it has been demonstrated that the vascular responses of sumatriptan are primarily mediated by 5-HT_{1B} receptors in different vascular beds, including human isolated arteries (Bouchelet *et al*., 2000; Morecroft *et al*., 1999; Verheggen *et al*., 1998) as well as porcine (De Vries *et al*., 1999; Saxena & Tfelt-Hansen, 2000) and canine carotid circulations (Centurion *et al*., 2001; Villalón *et al*., 2001). In agreement with the latter, 5-HT_{1D} receptor agonists (e.g. PNU109291 (Ennis *et al*., 1998) or L775606 (MacLeod *et al*., 1997)) are devoid of vasoconstrictor responses in both bovine and human isolated blood vessels (Bouchelet *et al*., 2000) as well as the canine carotid circulation (Centurion *et al*., 2001). Moreover, 5-HT_{1D} receptor mRNA or protein localisation confirmed the presence of this receptor preferably in neural rather than vascular tissues (Bouchelet *et al*., 2000; Nilsson *et al*., 1999). The neuronal effect of triptans possibly involves inhibition of trigeminovascular system (Moskowitz, 1992). It has been shown that the triptans inhibit dural plasma protein extravasation following electrical stimulation of the trigeminal ganglion in guinea pig (Ennis *et al*., 1998). Other studies using experimental animal models predictive for antimigraine activity showed that stimulation of trigeminal ganglion leads to elevated levels of several neuropeptides, including calcitonin gene related peptide (CGRP) (Goadsby & Edvinsson, 1994). The latter finding was confirmed in humans, since it has been demonstrated in migraineurs that only CGRP levels are elevated during a migraine attack (Goadsby *et al*., 1990). Additionally, in the same study it was shown that treatment of these patients with sumatriptan or dihydroergotamine reduced CGRP concentration to physiological level (Goadsby *et al*., 1990). Interestingly, the selective CGRP receptor antagonist, h-CGRP(8-37) markedly reduced trigeminal-evoked cerebral vasodilator response (Goadsby, 1993), showing the possible importance of CGRP receptor antagonists as a potential antimigraine agents. The clinical results of the recently developed, stable CGRP receptor antagonist BIBN4096BS (Doods *et al*., 2000; Wu *et al*., 2000) are awaited with great interest. Thus, it seems likely that the proposed acute antimigraine properties of 5-HT_{1D} receptor agonists are due to inhibition of neuropeptides (Goadsby, 1999), rather than cranial vasoconstriction.

Serotonin 5-ht$_{1F}$ receptor

The 5-ht$_{1F}$ receptor, the 'last' classified member of the 5-HT$_1$ receptor subfamily, has been identified only less than a decade ago (Adham *et al*., 1993). Several interesting papers have submerged on its pharmacological properties and, possible therapeutic applications (Cohen & Schenck, 1999; Phebus *et al*., 1997). In this sense, the acute antimigraine agent sumatriptan displays appreciable affinity at these receptors (see above and Table 1.4), besides at other members of this subfamily, as discussed in previous sections. Because it is still not completely clear which 5-HT$_1$ receptor subtype (5-HT$_{1B}$, 5-HT$_{1D}$ and/or 5-ht$_{1F}$) mediates underlying mechanisms of its antimigraine efficacy, it may be possible that sumatriptan (and other antimigraine drugs) acts *via* 5-ht$_{1F}$ receptors. With the current developments and understanding of this 5-HT$_1$ receptor subtype, other therapeutic applications may be possible in the near future (Saxena & Tfelt-Hansen, 2000). It may be important to mention that upper case letters are used for fully characterised (e.g. 5-HT$_{1B}$ and 5-HT$_{1D}$) receptors, while others (e.g. 5-ht$_{1F}$) that await complete characterisation have been designated by lower case letters. Unfortunately, selective 5-ht$_{1F}$ receptor antagonists are still not available.

Molecular aspects of 5-ht$_{1F}$ receptor

As described by Amlaiky *et. al.* (1992), the 5-ht$_{1F}$ receptor was first identified in mouse brain cDNA library, using a 5-HT$_{1B}$ receptor probe under low stringency screening (Amlaiky *et al*., 1992). It was reported that the 5-ht$_{1F}$ receptor showed a higher amino acid homology with the 5-HT$_{1E}$ (76%), than with 5-HT$_{1B}$ (63%) and 5-HT$_{1D}$ (60%) receptors. Subsequently, the 5-ht$_{1F}$ receptor was cloned and pharmacologically characterised from human, rat and guinea pig (Adham *et al*., 1997; Adham *et al*., 1993; Lovenberg *et al*., 1993). The human 5-ht$_{1F}$ receptor gene encodes for a protein of 366 amino acids. The receptor gene did not show any introns in its molecular structure same as observed in other 5-HT$_1$ receptor subtypes (see above). Similar to the 5-HT$_{1B}$ or 5-HT$_{1D}$ receptor, the presence of asparagine linked glycosylation sites and putative phosphorylation sites for protein kinase C were also observed in the 5-ht$_{1F}$ receptor.

Binding profile of 5-ht$_{1F}$ receptor ligands

As shown in Table 1.4, the endogenous neurotransmitter serotonin (5-HT) displays high affinity at human 5-ht$_{1F}$ receptors, while that of 5-carboxytryptamine was

expectedly lower (from IUPHAR guidelines, see Hoyer *et al*., 1994). Competition binding studies in human 5-ht_{1F} receptor revealed the following rank order of potencies for serotonergic ligands: 5-HT > sumatriptan >> 5-CT > 8-OH-DAPT > spiperone. (Adham *et al*., 1993). Interestingly, the rather high affinity of sumatriptan at these receptors was comparable to that observed at 5-HT_{1B} and 5-HT_{1D} receptor. The latter observation, together with the fact that 5-ht_{1F} receptor mRNA is present in the trigeminal ganglion (where it may regulate plasma protein extravasation and neurogenic inflammation), strongly indicates the above-mentioned possibility for 5-ht_{1F} receptors as therapeutic target in acute migraine therapy.

Table 1.4. Binding affinity constants (pK_i values) of several compounds at recombinant 5-ht_{1F} receptor from different species

Ligands	Human[a]	Guinea pig[b]	Rat[c]	Mouse[d]
Agonists				
5-HT	8.00	7.40	7.17	6.90
5-CT	6.14	5.63	5.61	5.50
Sumatriptan	7.64	7.27	7.19	7.10
LY334370	8.70[e]	-	-	-
Ergotamine	6.77	-	7.28	7.30
DHE	6.56	6.96	-	-
8-OH-DPAT	5.75	-	<6.00	5.80
Antagonists				
Methysergide	7.47	7.64	8.20	7.87
Methiothepin	6.19	6.43	-	-
Yohimbine	7.04	-	6.22	7.20
Metergoline	6.47	6.62	6.27	-

Data are means from radioligand displacement experiments using recombinant receptors expressed cell lines: [a], (Adham et al., 1993); [b], (Adham et al., 1997); [c], (Lovenberg et al., 1993); [d], (Amlaiky et al., 1992); [e], Pauwels P.J. (personal communication).

Recently, the development of the potent 5-ht_{1F} receptor agonists LY344864 and LY334370 may help to elucidate the functional properties of 5-ht_{1F} receptors as well

as to distinguish these from 5-HT_{1B} and 5-HT_{1D} receptor in the near future (Phebus *et al*., 1997).

Signal transduction mechanisms of 5-ht$_{1F}$ receptor

It has been shown that one of signal transduction mechanisms of 5-ht_{1F} receptors involves a negative coupling to adenylyl cyclase, as observed in two different cell lines (NIH 3T3 and LM (tK^-) fibroblasts) (Adham *et al*., 1993). Moreover, in comparison to LM (tK^-) cells, the NIH 3T3 cells expressing 5-ht_{1F} receptor showed higher inhibition of adenylyl cyclase activity in presence of 5-HT; thus, revealing cell-type dependent coupling. In the case of mouse or rat 5-ht_{1F} receptor, similar (negative) coupling to adenylyl cyclase activity was observed in stable NIH-3T3 or HeLa cells lines expressing this recombinant receptor (Amlaiky *et al*., 1992; Lovenberg *et al*., 1993). In addition, above-mentioned signal transduction mechanisms involve G_i-proteins, since their actions were found to be sensitive to pertussin toxin (Adham *et al*., 1993). As also described for 5-HT_{1B} and 5-HT_{1D} receptors, an additional signalling pathway is stimulation of phospholipase C, as measured by an increase of inositol phosphates accumulation and increase in intracellular Ca^{2+} ions concentration (Adham *et al*., 1993).

Tissue distribution of 5-ht$_{1F}$ receptor

The tissue distribution of 5-ht_{1F} receptor has been studied at *m*RNA level by Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridisation studies. Using the RT-PCR technique, human, mouse and rat 5-ht_{1F} receptor *m*RNA signals were detected in the brain (cortex, striatum, hippocampus, thalamus, pons, hypothalamus, cerebellum), uterus (endometrium and myometrium) and mesentery, but were absent in kidney, liver, lung, spleen, heart, pancreas or testes (Adham *et al*., 1993; Amlaiky *et al*., 1992; Lovenberg *et al*., 1993). Northern blot analysis also confirmed these findings in mice by demonstrating that mRNA signals for 5-ht_{1F} receptor were not detected in the brain, liver, kidney, lung, heart, spleen, and intestine tissue (Amlaiky *et al*., 1992; Lovenberg *et al*., 1993). Bouchelet and colleagues have shown that mRNA signals for 5-ht_{1F} receptor were detected in both neural as well as in vascular tissues, suggesting the possible physiological role of this receptor in central nervous system and in the periphery (Bouchelet *et al*., 1996). On the contrary, microvascular cultures from human cerebral blood vessels did not show

any signals for 5-ht_{1F} receptors (Cohen *et al*., 1999). In contrast, *in situ* hybridisation studies have revealed the presence of 5-ht_{1F} receptor mRNA signals in trigeminal and dorsal root ganglions, cerebral cortex and hippocampus in guinea pigs (Adham *et al*., 1997; Adham *et al*., 1993). Autoradiography studies using [^{3}H]5-HT in human brain showed high signal of the receptor in globus pallidus = substantia nigra > cortex > putamen > hippocampus (Pascual *et al*., 1996). Although 5-ht_{1F} receptor has been localised both in the brain and peripheral tissues, limited amount of studies have demonstrated its role in peripheral tissues. Nevertheless, it seems likely that 5-ht_{1F} receptors are involved in the regulation of neurogenic plasma extravasation and neurogenic inflammation (Johnson *et al*., 1997).

Physiological function of 5-ht_{1F} receptor

The antimigraine drug sumatriptan, launched as a potent and selective 5-$HT_{1B/1D}$ receptor agonist, also displays appreciable affinity 5-ht_{1F} receptors (see Table 1.4). As mentioned before, this feature may indicate the possibility of 5-ht_{1F} receptor agonist as an acute antimigraine drug for the treatment of migraine disease (Johnson *et al*., 1997; Leysen *et al*., 1996). Furthermore, the presence of 5-ht_{1F} receptor in the trigeminal ganglion of human and guinea pig predicts its presynaptic involvement in the regulation of neuropeptide release at the level of the cranial vasculature, thereby blocking the development of neurogenic inflammation (Adham *et al*., 1997; Bouchelet *et al*., 1996). The pharmacological profile of human 5-ht_{1F} receptor, using the [^{35}S]GTPγS binding assay, showed a significant correlation with its inhibitory role on dural plasma extravasation produced by trigeminal nerve stimulation in guinea pigs (Wainscott *et al*., 1998). This finding was supported by potent inhibitory effects of the 5-ht_{1F} receptor agonist, LY344864, on dural plasma extravasation as well as its attenuation of *c*-fos expression in rat trigeminal nucleus caudalis (Mitsikostas *et al*., 1999; Phebus *et al*., 1997). Interestingly, while 5-ht_{1F} receptor agonists (LY344864 and LY334370) failed to induce vasoconstriction in rabbit saphenous vein (Cohen & Schenck, 1999) as well as in human and bovine cerebral arteries (Bouchelet *et al*., 2000), the mRNA signals for these receptors were detected in these tissues. Most triptans show high affinity for 5-ht_{1F} receptors, some of them, such as rizatriptan (Wainscott *et al*., 1998) and alniditan (Leysen *et al*., 1996), display low affinity at these receptors, while still being effective in migraine treatment. On this basis, it may

be questioned whether the role of 5-ht_{1F} receptors is important for the acute treatment of migraine of the triptans. Nevertheless, the studies mentioned above may suggest the possibility that selective 5-ht_{1F} receptor agonists may represent a new class of effective acute antimigraine drugs, potentially, without cardiovascular side effects (Cohen & Schenck, 1999).

Serotonin 5-HT_7 receptor

The 5-HT_7 receptor is the most recently identified member of 5-HT receptor family and now fully characterised on the basis of structure, operational and expression studies (Vanhoenacker *et al*., 2000). Earlier, the cDNA encoding for this receptor has been isolated from different species by either PCR amplification with degenerate primers or by screening the libraries with probes derived from sequence from other species (Bard *et al*., 1993; Lovenberg *et al*., 1993; To *et al*., 1995). Previously, this receptor was known as a member of 5-HT_1-like receptor family, but under the category of 'orphan' receptor (Hoyer *et al*., 1994; Saxena *et al*., 1998). Interestingly, while 5-HT_7 receptors show high affinity for 5-HT, these receptors are insensitive to sumatriptan. It has been demonstrated that activation of 5-HT_7 receptors produces smooth muscle relaxation, e.g. in the canine carotid circulation (Villalón *et al*., 1997; 2001); however, also other physiological effects in mammalian brain and periphery have been proposed (Vanhoenacker *et al*., 2000).

Molecular aspects of 5-HT_7 receptor

Initially, the 5-HT_7 receptor was identified in the rat and mouse brain cDNA library by using degenerate oligonucleotide primers present in the conserve region of the 5-HT receptor (Plassat *et al*., 1993; Ruat *et al*., 1993). The full-length sequence of the rat and mouse cDNA encodes a protein of 448 amino acids, showing less than 50% homology with other 5-HT receptors. Later, the guinea pig 5-HT_7 receptor was also reported from the hippocampal library (Tsou *et al*., 1994). The molecular structure of human 5-HT_7 receptor cDNA contains an open reading frame of 1335 bp and encodes for a predicted protein of 445 amino acids (Bard *et al*., 1993). The human and rat 5-HT_7 receptor gene consist of two introns interrupting the coding sequences; the first between transmembrane domain 3 and domain 4, while the second is located near the end of C terminal region. Four isoforms of 5-HT_7 receptor gene has been identified by alternate splicing near the C terminal intron in both recombinant human and rat

5-HT_7 receptor (Heidmann *et al*., 1997; Heidmann *et al*., 1998). The alternate receptor isoforms differ in their predicted C-terminal intracellular tails and may contribute to the diversity in physiological actions mediated by 5-HT_7 receptors. Interestingly and similar to the above-mentioned 5-HT_1 receptor subtypes, the molecular structure of 5-HT_7 receptor shows seven transmembrane domains. The presence of two putative N-linked glycosylation sites as well as one putative recognition site for protein kinase C were observed in the amino terminal region and may be important for cAMP dependent protein kinase and calmodulin dependent protein kinase at the C terminal region (Bard *et al*., 1993; Lovenberg *et al*., 1993; Plassat *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993; Tsou *et al*., 1994). The major gene structural differences between the 5-HT_1 and 5-HT_7 receptors are that the latter consist of two introns, while the former family of receptors is intronless.

Binding profile of 5-HT_7 receptor ligands

For a long period of time, several compounds (see Table 1.5) were used to pharmacologically characterise 5-HT_7 receptors, mainly based on their rank-order of potencies. Recently, selective 5-HT_7 receptor antagonists (e.g. SB 258719) have been developed, showing at least 100-fold more selectivity for 5-HT_7 receptor as compared to other 5-HT receptor subtypes in recombinant human 5-HT_7 receptor cell lines (Forbes *et al*., 1998; Thomas *et al*., 1998). Suitable, but rather non-selective, radiolabelled agonists ([^{3}H]5-HT and [^{3}H]5-CT) and antagonists ([^{3}H]risperidone or [^{125}I]D-lysergic acid diethylamide [^{125}I]LSD) were used in receptor binding assays (Adham *et al*., 1998; Jasper *et al*., 1997). The pharmacological profile in affinity for various 5-HT receptor ligands showed the following rank of potency as 5-CT > 5-HT > methiothepin > metergoline > 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) > sumatriptan > ketanserin. This unambiguous unique pharmacological profile for 5-HT_7 receptors was observed to be similar across several species (Eglen *et al*., 1997).

Signal transduction mechanisms of 5-HT_7 receptor

5-HT_7 receptors are positively coupled to adenylate cyclase activity via G_s proteins (Adham *et al*., 1998), as observed for recombinant human (Bard *et al*., 1993), rat (Ruat *et al*., 1993) and guinea pig (Tsou *et al*., 1994) receptor. For this reason, the major signal transduction mechanism for 5-HT_7 receptors is the opposite of that

Table 1.5. Affinities (pK_i) of 5-HT receptor ligands for recombinant 5-HT_7 receptor from different species

Ligand	Human[a]	Guinea pig[b]	Rat[c]	Mouse[d]
Agonists				
5-HT	8.09	9.00	8.74[f]	8.30
5-CT	9.03	9.40	9.92	9.00
5-MeOT	8.30	9.00	8.75[f]	8.20
Sumatriptan	6.02	6.60	6.30	5.70
8-OH-DPAT	6.33	7.30	7.28	6.60
Antagonists				
Spiperone	6.96	7.00	7.70	7.20
Ergotamine	-	-	7.76[g]	7.30
Clozapine	-	-	7.87[f]	7.40
Ketanserin	5.87	6.20[e]	5.12	6.40
Metergoline	8.19	8.16[e]	8.69[f]	7.50
Methiothepin	8.43	8.00	8.99[f]	8.20
Mesulergine	7.74	7.81[e]	8.15[f]	7.60
Methysergide	7.08	7.40	7.90[f]	7.90
Ritanserin	7.35	7.34[e]	-	-
Cyproheptadine	6.91	6.90[e]	7.11	-

Data are the means from radioligand displacement experiments using recombinant receptors expressed in mammalian cell lines. Data from: [a], (Bard et al., 1993); [b], (Tsou et al., 1994); [c], (Ruat et al., 1993); [d], (Plassat et al., 1993); [e], (To et al., 1995); [f], (Shen et al., 1993); [g], (Lovenberg et al., 1993).

observed for the 5-HT_1 receptor family (Lovenberg *et al*., 1993; Plassat *et al*., 1993; Tsou *et al*., 1994). Interestingly, it has been demonstrated that different splice variants of the human 5-HT_7 receptor display different coupling to adenylyl cyclase (Krobert *et al*., 2001). However, no inhibition of adenylate activity or coupling to phospholipase C has been reported for the 5-HT_7 receptor.

Tissue distribution of 5-HT$_7$ receptor

The tissue distribution of 5-HT$_7$ receptors has been investigated using RT-PCR, Northern blot and *in situ* hybridisation studies in both central and peripheral tissues (Bard *et al*., 1993; Lovenberg *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993). In guinea pig brain tissue, the receptor has predominantly been observed in the pyramidal and granular cell layers of the hippocampus, periventricular thalamus and superficial cortex (Tsou *et al*., 1994). Moreover, Northern blot analysis of different brain tissues of rat and guinea pig showed two mRNAs of approximately 3.9 and 3.1 kb size (in rat) and 3.8 and 3.1 kb (in guinea pig) (Ruat *et al*., 1993). In the same study, *in situ* hybridisation analyses showed the expression of 5-HT$_7$ receptor *m*RNA in discrete areas of brain areas (pyramidal hippocampus cells, tenia tecta, amygdaloid or mammillary nucelei), indicating that 5-HT$_7$ receptor may be involved in the regulation of mood, learning, neuroendocrine, vegetative behaviours (Ruat *et al*., 1993) and in circadian phase shifts (Meyerhof *et al*., 1993). Bard *et al*. (1993) have shown that 5-HT$_7$ receptor *m*RNA signals were also found in peripheral human isolated tissues, such as coronary artery and the gastrointestinal tract. Interestingly, a major role of 5-HT$_7$ receptor has been proposed in the regulation of vascular tone in a variety of blood vessels (Ullmer *et al*., 1995). In this sense, a detailed study on tissue distribution of 5-HT$_7$ receptor has been performed in rat (aorta, renal artery, vena cava, portal vein, femoral vein and jugular vein) and porcine (pulmonary, coronary, cerebral arteries and cerebral vein) blood vessels, which showed the expression of this receptor in all the blood vessel except in rat jugular vein (Ullmer *et al*., 1995). On the other hand, using Northern blot analysis in peripheral tissues, 5-HT$_7$ receptor signals were not detected in most (except spleen) rat tissues, including lung, liver, kidney, gut, skeleton muscle, ovary, testis, pituitary, prostate and retina (Shen *et al*., 1993).

Physiological function of 5-HT$_7$ receptor

It has been shown that 5-HT$_7$ receptors are present in both the brain and peripheral tissues. In the central nervous system, the role of 5-HT$_7$ receptor was reported in circadian rhythm and depression. Earlier, it has been shown that the suprachiasmatic nuclei of the hypothalamus are the primary sites involved in the regulation of phase shift circadian rhythms produced by serotonin in mammals (Lovenberg *et al*., 1993). Both the presence of 5-HT$_7$ receptor *m*RNA in the suprachiasmatic nuclei of the

hypothalamus as well as the changes in the photic responses to 5-HT_7 receptor agonists in this structure of hamsters, provides the evidence for its role in this physiological response (Duncan *et al*., 1999; Lovenberg *et al*., 1993). Furthermore, different studies have demonstrated that activation of 5-HT_7 receptors produces direct smooth muscle relaxation, as observed for example in rabbit pulmonary artery (Morecroft & MacLean, 1998), dog basilar artery (Terrón & Falcón-Neri, 1999), monkey jugular artery (Leung *et al*., 1996), dog coronary artery (Terrón, 1996), canine carotid circulation (Villalón *et al*., 1999) and guinea pig ileum (Carter *et al*., 1995). The regulatory role of 5-HT_7 receptors in these blood vessels and tissues may hopefully open novel therapeutic application of this new class of 5-HT receptors, e.g. in hypertension or bowel syndrome. Also, since dilatation of cranial blood vessels has been proposed to play an important role in the pathogenesis of a migraine attack (De Vries *et al*., 1999; Saxena & Tfelt-Hansen, 2000), it may be possible that selective 5-HT_7 receptor antagonists are efficacious in prophylactic migraine therapy.

Porcine model to study antimigraine drugs

The pathophysiology of migraine headache is still not clear. However, on the basis of the neurovascular hypothesis several animal models are proposed for migraine. According to the vascular hypothesis, the alterations in the cranial extracerebral vascular beds may be the primary cause of the migraine. Earlier, Wolff (1963) has proposed that the vasodilatation of extracranial arteries supplied by the external carotid artery is the primary cause of the migraine headache. Heyck, (1969) performed preliminary experiments in migraine patients and suggested that arterial blood is being shunted to the venous blood flow due to dilatation of the cephalic arteriovenous anastomoses (AVA; see Figure 1.4) during migraine. Under physiological conditions, carotid AVAs are under a sympathetic constrictor tone, shunting only <5% of arterial blood back to the right side of the heart; resulting in a difference in oxygen saturation at the arterial and venous side ($AVSO_2$ difference). However, in migraine patients it was shown that this $AVSO_2$ difference was lower (Heyck, 1969).

In recent years, several animal models (for example pig, dog and cat) have been developed and used to study the clinical efficacy and possible mechanisms of action of current and novel antimigraine agents (De Vries *et al*., 1999).

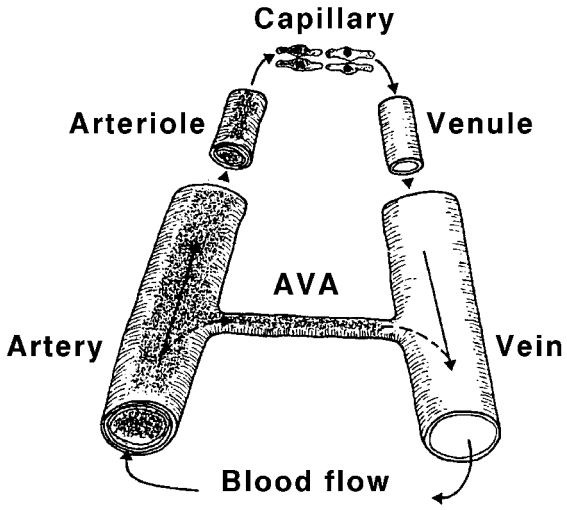


Figure 1.4. Schematic representation of an arteriovenous anastomosis (AVA) shunt model

In recent years we have shown that several acute antimigraine drugs, including the triptans and ergots, decrease total carotid blood flow in anaesthetised pigs, without producing major systemic haemodynamic changes (De Vries *et al*., 1999). Using the microsphere method (Saxena, 1995), it was shown that this response was exclusively caused by constriction of carotid AVAs. Additionally, consistent with the closure of carotid AVAs, all these agents increased $AVSO_2$ difference in these animals.

In summary, the cranial extracerebral vasodilatation may be an integral part of the pathophysiology of migraine and acute antimigraine drugs (e.g. sumatriptan and second generation of triptans) primarily constrict the dilated blood vessels to abort migraine attacks.

Aims of the thesis

1. To clone porcine 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptors.
2. To express the porcine 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptors in cell-lines for pharmacological characterisation.
3. To identify the tissue-distribution of porcine 5-HT_{1B}, 5-ht_{1F} and 5-HT_7 receptors.
4. To characterise 5-HT_{1B} and 5-HT_{1D} receptor in human blood vessels, functional assays and *in situ* hybridisation were performed.

References

Adham, N., Bard, J.A., Zgombick, J.M., Durkin, M.M., Kucharewicz, S., Weinshank, R.L. & Branchek, T.A. (1997). Cloning and characterization of the guinea pig 5-HT_{1F} receptor subtype: a comparison of the pharmacological profile to the human species homolog. Neuropharmacology, 36, 569-576.

Adham, N., Borden, L.A., Schechter, L.E., Gustafson, E.L., Cochran, T.L., Vaysse, P.J., Weinshank, R.L. & Branchek, T.A. (1993). Cell-specific coupling of the cloned human 5-HT_{1F} receptor to multiple signal transduction pathways. Naunyn-Schmiedeberg's Arch. Pharmacol., 348, 566-575.

Adham, N., Kao, H.T., Schecter, L.E., Bard, J., Olsen, M., Urquhart, D., Durkin, M., Hartig, P.R., Weinshank, R.L. & Branchek, T.A. (1993). Cloning of another human serotonin receptor (5-HT_{1F}): a fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. Proc. Natl. Acad. Sci., 90, 408-412.

Adham, N., Romanienko, P., Hartig, P., Weinshank, R.L. & Branchek, T. (1992). The rat 5-hydroxytryptamine$_{1B}$ receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. Mol. Pharmacol., 41, 1-7.

Adham, N., Tamm, J.A., Salon, J.A., Vaysse, P.J.-J., Weinshank, R.L. & Branchek, T.A. (1994). A single point mutation increases the affinity of serotonin 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-$HT_{1E}$and 5-HT_{1F} receptors for a-adrenergic antagonists. Neuropharmacology, 33, 387-391.

Adham, N., Zgombick, J.M., Bard, J. & Branchek, T.A. (1998). Functional characterization of the recombinant human 5-hydroxytryptamine$_{7(a)}$ receptor isoform coupled to adenylate cyclase stimulation. J. Pharmacol. Exp. Ther., 287, 508-514.

Amlaiky, N., Ramboz, S., Boschert, U., Plassat, J.L. & Hen, R. (1992). Isolation of a mouse "5HT_{1E}-like" serotonin receptor expressed predominantly in hippocampus. J. Biol. Chem., 267, 19761-19764.

Anthony, M., Hinterberger, H. & Lance, J.W. (1967). Plasma serotonin in migraine and stress. Arch. Neurol., 16, 544-552.

Bach, A.W., Unger, L., Sprengel, R., Mengod, G., Palacios, J., Seeburg, P.H. & Voigt, M.M. (1993). Structure functional expression and spatial distribution of a cloned cDNA encoding a rat 5-HT_{1D}-like receptor. J. Recept. Res., 13, 479-502.

Bard, J.A., Kucharewicz, S.A., Zgombick, J.M., Weinshank, R.L., Branchek, T.A. & Cohen, M.L. (1996). Differences in ligand binding profiles between cloned rabbit and human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors: ketanserin and methiothepin distinguish rabbit 5-HT_{1D} receptor subtypes. Naunyn-Schmiedeberg's Arch. Pharmacol., 354, 237-244.

Bard, J.A., Zgombick, J., Adham, N., Vaysse, P., Branchek, T.A. & Weinshank, R.L. (1993). Cloning of a novel human serotonin receptor (5-HT_7) positively linked to adenylate cyclase. J. Biol. Chem., 268, 23422-23426.

Beer, M.S., Heald, M.A., McAllister, G. & Stanton, J.A. (1998). Pharmacological characterisation of a cloned dog 5-HT_{1B} receptor cell line. Eur. J. Pharmacol., 360, 117-121.

Birnbaumer, L., Abramowitz, J. & Brown, A.M. (1990). Receptor-effector coupling by G proteins. Biochim. Biophys. Acta, 1031, 163-224.

Bonaventure, P., Langlois, X. & Leysen, J.E. (1998). Co-localization of 5-HT_{1B}- and 5-HT_{1D} receptor mRNA in serotonergic cell bodies in guinea pig dorsal raphe nucleus: a double labeling in situ hybridization histochemistry study. Neurosci Lett, 254, 113-116.

Bonaventure, P., Voorn, P., Luyten, W.H., Jurzak, M., Schotte, A. & Leysen, J.E. (1998). Detailed mapping of serotonin 5-HT_{1B} and 5-HT_{1D} receptor messenger RNA and ligand binding sites in guinea-pig brain and trigeminal ganglion: clues for function. Neuroscience, 82, 469-484.

Bouchelet, I., Case, B., Olivier, A. & Hamel, E. (2000). No contractile effect for 5-HT_{1D} and 5-HT_{1F} receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. Br. J. Pharmacol., 129, 501-508.

Bouchelet, I., Cohen, Z., Case, B., Seguela, P. & Hamel, E. (1996). Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. Mol. Pharmacol., 50, 219-223.

Bradley, P.B., Engel, G., Feniuk, W., Fozard, J.R., Humphrey, P.P., Middlemiss, D.N., Mylecharane, E.J., Richardson, B.P. & Saxena, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology, 25, 563-576.

Branchek, T.A., Bard, J.A., Kucharewicz, S.A., Zgombick, J.M., Weinshank, R.L. & Cohen, M.L. (1995). Migraine: relationship to cloned canine and human 5-HT_{1D} receptors. In Experimental headache models. ed. Olesen, J. & Moskowitz, M. pp. 123-134. New York: Raven Press.

Brodie, T.G. (1900). The immediate action of an intravenous injection of blood-serum. J. Physiol., 26, 48-71.

Bruinvels, A.T., Landwehrmeyer, B., Gustafson, E.L., Durkin, M.M., Mengod, G., Branchek, T.A., Hoyer, D. & Palacios, J.M. (1994). Localization of 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptor messenger RNA in rodent and primate brain. Neuropharmacology, 33, 367-386.

Carroll, J.D. & Hilton, B.P. (1974). The effects of reserpine injection on methysergide treated control and migrainous subjects. Headache, 14, 149-156.

Carter, D., Champney, M., Hwang, B. & Eglen, R.M. (1995). Characterization of a postjunctional 5-HT receptor mediating relaxation of guinea-pig isolated ileum. Eur. J. Pharmacol., 280, 243-250.

Centurion, D., Sanchez-Lopez, A., De Vries, P., Saxena, P.R. & Villalon, C.M. (2001). The GR127935-sensitive 5-$HT_{(1)}$ receptors mediating canine internal carotid vasoconstriction: resemblance to the 5-$HT_{(1B)}$, but not to the 5-$HT_{(1D)}$ or 5-$ht_{(1F)}$, receptor subtype. Br. J. Pharmacol., 132, 991-998.

Cerutis, D.R., Hass, N.A., Iversen, L.J. & Bylund, D.B. (1994). The cloning and expression of an OK cell cDNA encoding a 5- hydroxytryptamine$_{1B}$ receptor. Mol. Pharmacol., 45, 20-28.

Cohen, M.L. & Schenck, K. (1999). 5-Hydroxytryptamine$_{(1F)}$ receptors do not participate in vasoconstriction: lack of vasoconstriction to LY344864, a selective serotonin$_{(1F)}$ receptor agonist in rabbit saphenous vein. J. Pharmacol. Exp. Ther., 290, 935-939.

Cohen, Z., Bouchelet, I., Olivier, A., Villemure, J.G., Ball, R., Stanimirovic, D.B. & Hamel, E. (1999). Multiple microvascular and astroglial 5-hydroxytryptamine receptor subtypes in human brain: molecular and pharmacologic characterization. J. Cereb. Blood Flow Metab., 19, 908-917.

Craig, D.A. & Martin, G.R. (1993). 5-HT_{1B} receptors mediate potent contractile responses to 5-HT in rat caudal artery. Br. J. Pharmacol., 109, 609-611.

Curran, D.A., Hinterberger, H. & Lance, J.W. (1965). Total plasma serotonin, 5-hydroxyindoleacetic acid and p-hydroxy-m-methoxymandelic acid excretion in normal and migrainous subjects. Brain, 88, 997-1010.

Dahl, A., Russell, D., Nyberg-Hansen, R. & Rootwelt, K. (1990). Cluster headache: transcranial Doppler ultrasound and rCBF studies. Cephalalgia, 10, 87-94.

De Vries, P., Sanchez-Lopez, A., Centurion, D., Heiligers, J.P., Saxena, P.R. & Villalon, C.M. (1998). The canine external carotid vasoconstrictor 5-HT_1 receptor: blockade by 5-HT_{1B} (SB224289), but not by 5-HT_{1D} (BRL15572) receptor antagonists. Eur. J. Pharmacol., 362, 69-72.

De Vries, P., Villalón, C.M., Heiligers, J.P.C. & Saxena, P.R. (1998). Characterization of 5-HT receptors mediating constriction of porcine carotid arteriovenous anastomoses; involvement of 5-$HT_{1B/1D}$ and novel receptors. Br. J. Pharmacol., 123, 1561-1570.

De Vries, P., Villalón, C.M., Heiligers, J.P.C. & Saxena, P.R. (1997). Nature of 5-HT_1-like receptors mediating depressor responses in vagosympathectomized rats; close resemblance to the cloned 5-ht_7 receptor. Naunyn-Schmiedeberg's Arch. Pharmacol., 356, 90-99.

De Vries, P., Willems, E.W., Heiligers, J.P., Villalón, C.M. & Saxena, P.R. (1999). Investigation of the role of 5-HT_{1B} and 5-HT_{1D} receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. Br. J. Pharmacol., 127, 405-412.

De Vries, P., Villalón, C.M. & Saxena, P.R. (1999). Pharmacological aspects of experimental headache models in relation to acute antimigraine therapy. Eur. J. Pharmacol., 375, 61-74.

Doods, H., Hallermayer, G., Wu, D., Entzeroth, M., Rudolf, K., Engel, W. & Eberlein, W. (2000). Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. Br J Pharmacol, 129, 420-3.

Duncan, M.J., Short, J. & Wheeler, D.L. (1999). Comparison of the effects of aging on 5-HT_7 and 5-HT_{1A} receptors in discrete regions of the circadian timing system in hamsters. Brain Res., 829, 39-45.

Eglen, R.M., Jasper, J.R., Chang, D.J. & Martin, G.R. (1997). The 5-HT_7 receptor: orphan found. Trends Pharmacol. Sci., 18, 104-107.

Ennis, M.D., Ghazal, N.B., Hoffman, R.L., Smith, M.W., Schlachter, S.K., Lawson, C.F., Im, W.B., Pregenzer, J.F., Svensson, K.A., Lewis, R.A., Hall, E.D., Sutter, D.M., Harris, L.T. & McCall, R.B. (1998). Isochroman-6-carboxamides as highly selective 5-HT_{1D} agonists: potential new treatment for migraine without cardiovascular side effects. J. Med. Chem., 41, 2180-2183.

Erspamer, V. (1954). Pharmacology of indolealkylamines. Pharmacol. Rev., 6, 425-487.

Erspamer, V. & Asero, B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. Nature, 169, 800-801.

Forbes, I.T., Dabbs, S., Duckworth, D.M., Jennings, A.J., King, F.D., Lovell, P.J., Brown, A.M., Collin, L., Hagan, J.J., Middlemiss, D.N., Riley, G.J., Thomas, D.R. & Upton, N. (1998). (R)-3,N-dimethyl-N-[1-methyl-3-(4-methyl-piperidin-1-yl) propyl]benzenesulfonamide: the first selective 5-HT_7 receptor antagonist. J. Med. Chem., 41, 655-657.

Gaddum, J.H. & Picarelli, Z.P. (1957). Two kinds of tryptamine receptors. Br. J. Pharmacol., 12, 323-328.

Goadsby, P.J. (1999). Advances in the pharmacotherapy of migraine. How knowledge of pathophysiology is guiding drug development. Drugs R. D., 2, 361-374.

Goadsby, P.J. (1993). Inhibition of calcitonin gene-related peptide by h-CGRP(8-37) antagonizes the cerebral dilator response from nasociliary nerve stimulation in the cat. Neurosci. Lett., 151, 13-16.

Goadsby, P.J. & Edvinsson, L. (1994). Joint 1994 Wolff Award Presentation. Peripheral and central trigeminovascular activation in cat is blocked by the serotonin (5HT)-$_{1D}$ receptor agonist 311C90. Headache, 34, 394-349.

Goadsby, P.J. & Edvinsson, L. (1993). The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann. Neurol., 33, 48-56.

Goadsby, P.J., Edvinsson, L. & Ekman, R. (1990). Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann. Neurol., 28, 183-187.

Göthert, M., Schlicker, E. & Kollecker, P. (1986). Receptor-mediated effects of serotonin and 5-methoxytryptamine on noradrenaline release in the rat vena cava and in the heart of the pithed rat. Naunyn-Schmiedeberg's Arch. Pharmacol., 332, 124-130.

Graham, J. & Wolff, H. (1938). Mechanism of migraine headache and action of ergotamine tartrate. Archives of Neurology and Pschiatry, 39, 737-763.

Hagan, J.J., Slade, P.D., Gaster, L., Jeffrey, P., Hatcher, J.P. & Middlemiss, D.N. (1997). Stimulation of 5-HT_{1B} receptors causes hypothermia in the guinea pig. Eur. J. Pharmacol., 331, 169-174.

Hamblin, M.W. & Metcalf, M.A. (1991). Primary structure and functional characterization of a human 5-HT_{1D}- type serotonin receptor. Mol. Pharmacol., 40, 143-148.

Hamblin, M.W., Metcalf, M.A., McGuffin, R.W. & Karpells, S. (1992). Molecular cloning and functional characterization of a human 5-HT_{1B} serotonin receptor: a homologue of the rat 5-HT_{1B} receptor with 5-HT_{1D}- like pharmacological specificity. Biochem. Biophys. Res. Commun., 184, 752-759.

Harwood, G., Lockyer, M., Giles, H. & Fairweather, N. (1995). Cloning and characterisation of the rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. FEBS Lett., 377, 73-76.

Heidmann, D.E., Metcalf, M.A., Kohen, R. & Hamblin, M.W. (1997). Four 5-hydroxytryptamine7 (5-HT_7) receptor isoforms in human and rat produced by alternative splicing: species differences due to altered intron-exon organization. J. Neurochem., 68, 1372-13781.

Heidmann, D.E., Szot, P., Kohen, R. & Hamblin, M.W. (1998). Function and distribution of three rat 5-hydroxytryptamine7 (5-HT_7) receptor isoforms produced by alternative splicing. Neuropharmacology, 37, 1621-1632.

Herrick-Davis, K. & Titeler, M. (1989). Detection and characterization of a $5HT_{1D}$ serotonin receptor-GTP binding protein interaction in porcine and bovine brain. Synapse, 3, 325-330.

Heuring, R.E. & Peroutka, S.J. (1987). Characterization of a novel [^{3}H]-5-hydroxytryptamine binding site subtype in bovine brain membranes. J. Neurosci., 7, 894-903.

Heyck, H. (1969). Pathogenesis of migraine. Res. Clin. Stud. Headache, 2, 1-28.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R. & Humphrey, P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol. Rev., 46, 157-203.

Hoyer, D. & Middlemiss, D.N. (1989). Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. Trends Pharmacol. Sci., 10, 130-132.

Humphrey, P.P.A., Apperley, E., Feniuk, W. & Perren, M.J. (1990). A rational approach to identifying a fundamentally new drug for the treatment of migraine. In Cardiovascular Pharmacology of 5-Hydroxytryptamine: Prospective Therapeutic Applications. ed. Saxena, P.R., Wallis, D.I., Wouters, W. & Bevan, P. pp. 416-431. Dordrecht: Kluwer Academic Publishers.

Humphrey, P.P.A. & Feniuk, W. (1991). Mode of action of the anti-migraine drug sumatriptan. Trends Pharmacol. Sci., 12, 444-446.

IHS (1988). Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. Headache Classification Committee of the International Headache Society. Cephalalgia, 8, 1-96.

Iismaa, T.P., Biden, T.J. & Shine, J. (1995). G protein-coupled receptors. Heidelberg, Germany: Springer-Verlag.

Ishida, T., Hirata, K., Sakoda, T., Kawashima, S., Akita, H. & Yokoyama, M. (1999). Identification of mRNA for 5-HT_1 and 5-HT_2 receptor subtypes in human coronary arteries. Cardiovasc. Res., 41, 267-274.

Jasper, J.R., Kosaka, A., To, Z.P., Chang, D.J. & Eglen, R.M. (1997). Cloning, expression and pharmacology of a truncated splice variant of the human 5-HT_7 receptor (h5-HT_{7b}). Br. J. Pharmacol., 122, 126-132.

Jin, H., Oksenberg, D., Ashkenazi, A., Peroutka, S.J., Duncan, A.M., Rozmahel, R., Yang, Y., Mengod, G., Palacios, J.M. & O'Dowd, B.F. (1992). Characterization of the human 5-hydroxytryptamine$_{1B}$ receptor. J. Biol. Chem., 267, 5735-5738.

Johnson, K.W., Schaus, J.M., Durkin, M.M., Audia, J.E., Kaldor, S.W., Flaugh, M.E., Adham, N., Zgombick, J.M., Cohen, M.L., Branchek, T.A. & Phebus, L.A. (1997). 5-HT_{1F} receptor agonists inhibit neurogenic dural inflammation in guinea pigs. Neuroreport, 8, 2237-2240.

Kalkman, H.O., Engel, G. & Hoyer, D. (1984). Three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin in rats. J. Hypertens. Suppl., 2, S143-145.

Kaumann, A.J., Frenken, M., Posival, H. & Brown, A.M. (1994). Variable participation of 5-HT_1-like receptors and 5-HT_2 receptors in serotonin-induced contraction of human isolated coronary arteries. 5- HT_1-like receptors resemble cloned 5-$HT_{1D\beta}$ receptors. Circulation, 90, 1141-1153.

Kimball, R., Friedman, A. & Vallejo, E. (1960). Effect of serotonin in migraine patients. Neurology, 10, 107-111.

Krobert, K.A., Bach, T., Syversveen, T., Kvingedal, A.M. & Levy, F.O. (2001). The cloned human 5-HT_7 receptor splice variants: a comparative characterization of their pharmacology, function and distribution. Naunyn-Schmiedeberg's Arch. Pharmacol., 363, 620-632.

Leung, E., Walsh, L.K., Pulido-Rios, M.T. & Eglen, R.M. (1996). Characterization of putative 5-ht_7 receptors mediating direct relaxation in Cynomolgus monkey isolated jugular vein. Br. J. Pharmacol., 117, 926-930.

Levy, F.O., Gudermann, T., Perez-Reyes, E., Birnbaumer, M., Kaumann, A.J. & Birnbaumer, L. (1992). Molecular cloning of a human serotonin receptor (S12) with a pharmacological profile resembling that of the 5-HT_{1D} subtype. J. Biol. Chem, 267, 7553-7562.

Leysen, J.E., Gommeren, W., Heylen, L., Luyten, W.H., Van de Weyer, I., Vanhoenacker, P., Haegeman, G., Schotte, A., Van Gompel, P., Wouters, R. & Lesage, A.S. (1996). Alniditan, a new 5-hydroxytryptamine$_{1D}$ agonist and migraine-abortive agent: ligand-binding properties of human 5-hydroxytryptamine$_{1D\alpha}$, human 5-hydroxytryptamine$_{1D\beta}$, and calf 5-hydroxytryptamine$_{1D}$ receptors investigated with [^{3}H]5-hydroxytryptamine and [^{3}H]alniditan. Mol. Pharmacol., 50, 1567-1580.

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.J., Dumont, J.E. & Vassart, G. (1989). Selective amplification and cloning of four new members of the G protein-coupled receptor family. Science, 244, 569-572.

Lipton, R.B. & Stewart, W.F. (1997). Prevalence and impact of migraine. Neurol. Clin., 15, 1-13.

Longmore, J., Shaw, D., Smith, D., Hopkins, R., McAllister, G., Pickard, J.D., Sirinathsinghji, D.J., Butler, A.J. & Hill, R.G. (1997). Differential distribution of 5HT_{1D}- and 5HT_{1B}-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. Cephalalgia, 17, 833-842.

Lovenberg, T.W., Baron, B.M., de Lecea, L., Miller, J.D., Prosser, R.A., Rea, M.A., Foye, P.E., Racke, M., Slone, A.L., Siegel, B.W. & et al. (1993). A novel adenylyl cyclase-activating serotonin receptor (5-HT_7) implicated in the regulation of mammalian circadian rhythms. Neuron, 11, 449-458.

Lovenberg, T.W., Erlander, M.G., Baron, B.M., Racke, M., Slone, A.L., Siegel, B.W., Craft, C.M., Burns, J.E., Danielson, P.E. & Sutcliffe, J.G. (1993). Molecular cloning and functional expression of 5-HT_{1E}-like rat and human 5-hydroxytryptamine receptor genes. Proc. Natl. Acad. Sci. U S A, 90, 2184-2188.

MaassenVanDenBrink, A., Reekers, M., Bax, W.A., Ferrari, M.D. & Saxena, P.R. (1998). Coronary side-effect potential of current and prospective antimigraine drugs. Circulation, 98, 25-30.

MacLeod, A.M., Street, L.J., Reeve, A.J., Jelley, R.A., Sternfeld, F., Beer, M.S., Stanton, J.A., Watt, A.P., Rathbone, D. & Matassa, V.G. (1997). Selective, orally active 5-HT_{1D} receptor agonists as potential antimigraine agents. J. Med. Chem., 40, 3501-3503.

Maenhaut, C., Van Sande, J., Massart, C., Dinsart, C., Libert, F., Monferini, E., Giraldo, E., Ladinsky, H., Vassart, G. & Dumont, J.E. (1991). The orphan receptor cDNA RDC4 encodes a 5-HT_{1D} serotonin receptor. Biochem. Biophys. Res. Commun., 180, 1460-1468.

Maroteaux, L., Saudou, F., Amlaiky, N., Boschert, U., Plassat, J.L. & Hen, R. (1992). Mouse 5HT_{1B} serotonin receptor: cloning, functional expression, and localization in motor control centers. Proc. Natl. Acad. Sci. U S A, 89, 3020-3024.

Martin, G.R. (1994). Vascular receptors for 5-hydroxytryptamine: distribution, function and classification. Pharmacol Ther, 62, 283-324.

May, A. & Goadsby, P.J. (2001). Pharmacological opportunities and pitfalls in the therapy of migraine. Curr. Opin. Neurol., 14, 341-345.

McCall, R.B. (1999). Preclinical and clinical studies in migraine using the selective 5-HT_{1D} receptor agonist PNU-142633. In IBC's annual conference on migraine. Philadelphia, USA.

Metcalf, M.A., McGuffin, R.W. & Hamblin, M.W. (1992). Conversion of the human 5-HT_{1D} beta serotonin receptor to the rat 5- HT_{1B} ligand-binding phenotype by Thr355Asn site directed mutagenesis. Biochem. Pharmacol., 44, 1917-1920.

Meyerhof, W., Obermuller, F., Fehr, S. & Richter, D. (1993). A novel rat serotonin receptor: primary structure, pharmacology, and expression pattern in distinct brain regions. DNA Cell Biol., 12, 401-409.

Mitsikostas, D.D., Sanchez del Rio, M., Moskowitz, M.A. & Waeber, C. (1999). Both 5-HT_{1B} and 5-HT_{1F} receptors modulate c-fos expression within rat trigeminal nucleus caudalis. Eur. J. Pharmacol., 369, 271-277.

Morecroft, I., Heeley, R.P., Prentice, H.M., Kirk, A. & MacLean, M.R. (1999). 5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT_{1B} receptor. Br. J. Pharmacol., 128, 730-734.

Morecroft, I. & MacLean, M.R. (1998). 5-hydroxytryptamine receptors mediating vasoconstriction and vasodilation in perinatal and adult rabbit small pulmonary arteries. Br. J. Pharmacol., 125, 69-78.

Moskowitz, M.A. (1992). Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. Trends Pharmacol. Sci., 13, 307-311.

Nichols, F.T., 3rd, Mawad, M., Mohr, J.P., Stein, B., Hilal, S. & Michelsen, W.J. (1990). Focal headache during balloon inflation in the internal carotid and middle cerebral arteries. Stroke, 21, 555-559.

Nilsson, T., Longmore, J., Shaw, D., Olesen, I.J. & Edvinsson, L. (1999). Contractile 5-HT_{1B} receptors in human cerebral arteries: pharmacological characterization and localization with immunocytochemistry. Br. J. Pharmacol., 128, 1133-1140.

Nilsson, T., Longmore, J., Shaw, D., Pantev, E., Bard, J.A., Branchek, T. & Edvinsson, L. (1999). Characterisation of 5-HT receptors in human coronary arteries by molecular and pharmacological techniques. Eur. J. Pharmacol., 372, 49-56.

Oksenberg, D., Marsters, S.A., O'Dowd, B.F., Jin, H., Havlik, S., Peroutka, S.J. & Ashkenazi, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{1B} receptors. Nature, 360, 161-163.

Paintal, A.S. (1973). Sensory mechanisms involved in the Bezold-Jarisch effect. Aust. J. Exp. Biol. Med. Sci., 51, 3-15.

Parker, E.M., Grisel, D.A., Iben, L.G. & Shapiro, R.A. (1993). A single amino acid difference accounts for the pharmacological distinctions between the rat and human 5-hydroxytryptamine$_{1B}$ receptors. J. Neurochem., 60, 380-383.

Pascual, J., Del Arco, C., Romon, T., Del Olmo, E. & Pazos, A. (1996). [^{3}H]Sumatriptan binding sites in human brain: regional-dependent labelling of 5-HT_{1D} and 5-HT_{1F} receptors. Eur. J. Pharmacol., 295, 271-274.

Pauwels, P.J., Palmier, C., Wurch, T. & Colpaert, F.C. (1996). Pharmacology of cloned human 5-HT_{1D} receptor-mediated functional responses in stably transfected rat C6-glial cell lines: further evidence differentiating human 5-HT_{1D} and 5-HT_{1B} receptors. Naunyn-Schmiedeberg's Arch. Pharmacol., 353, 144-156.

Pauwels, P.J., Tardif, S., Palmier, C., Wurch, T. & Colpaert, F.C. (1997). How efficacious are 5-$HT_{1B/D}$ receptor ligands: an answer from GTP gamma S binding studies with stably transfected C6-glial cell lines. Neuropharmacology, 36, 499-512.

Pazos, A. & Palacios, J.M. (1985). Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Res, 346, 205-30.

Pedigo, N.W., Yamamura, H.I. & Nelson, D.L. (1981). Discrimination of multiple [^{3}H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. J. Neurochem., 36, 220-226.

Peroutka, S.J. (1994). Pharmacological differentiation of human 5-HT_{1B} and 5-HT_{1D} receptors. Biol. Signals, 3, 217-222.

Peroutka, S.J. & Snyder, S.H. (1979). Multiple serotonin receptors: differential binding of [^{3}H]5-hydroxytryptamine, [^{3}H]lysergic acid diethylamide and [^{3}H]spiroperidol. Mol. Pharmacol., 16, 687-699.

Phebus, L.A., Johnson, K.W., Zgombick, J.M., Gilbert, P.J., Van Belle, K., Mancuso, V., Nelson, D.L., Calligaro, D.O., Kiefer, A.D., Jr., Branchek, T.A. & Flaugh, M.E. (1997). Characterization of LY344864 as a pharmacological tool to study 5-HT_{1F} receptors: binding affinities, brain penetration and activity in the neurogenic dural inflammation model of migraine. Life Sci., 61, 2117-2126.

Plassat, J.L., Amlaiky, N. & Hen, R. (1993). Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Mol. Pharmacol., 44, 229-236.

Price, G.W., Burton, M.J., Collin, L.J., Duckworth, M., Gaster, L., Göthert, M., Jones, B.J., Roberts, C., Watson, J.M. & Middlemiss, D.N. (1997). SB-216641 and BRL-15572-compounds to pharmacologically discriminate h5-HT_{1B} and h5-HT_{1D} receptors. Naunyn-Schmiedeberg's Arch. Pharmacol., 356, 312-320.

Rapport, M.M., Green, A.A. & Page, I.H. (1948). Serum vasoconstrictor (serotonin). IV. Isolation and characterization. J. Biol. Chem., 176, 1243-1251.

Ruat, M., Traiffort, E., Leurs, R., Tardivel-Lacombe, J., Diaz, J., Arrang, J.M. & Schwartz, J.C. (1993). Molecular cloning, characterization, and localization of a high-affinity serotonin receptor (5-HT_7) activating cAMP formation. Proc. Natl. Acad. Sci. U S A, 90, 8547-8551.

Saxena, P.R. (1995). Cranial arteriovenous shunting, an in vivo animal model for migraine. In Experimental headache models. ed. Olesen, J. & Moskowitz, M.A. pp. 189-198. Philadelphia: Lippincott-Raven Publishers.

Saxena, P.R. (1972). The effects of antimigraine drugs on the vascular responses by 5-hydroxytryptamine and related biogenic substances on the external carotid bed of dogs: possible pharmacological implications to their antimigraine action. Headache, 12, 44-54.

Saxena, P.R. (1995). Serotonin receptors: subtypes, functional responses and therapeutic relevance. Pharmacol. Ther., 66, 339-368.

Saxena, P.R., De Vries, P. & Villalon, C.M. (1998). 5-HT_1-like receptors: a time to bid goodbye. Trends Pharmacol. Sci., 19, 311-316.

Saxena, P.R. & Tfelt-Hansen, P. (2000). Triptans, 5-$HT_{1B/1D}$ receptor agonists in the acute treatment of migraine. In The headaches. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 411-438. Philadelphia: Lippincott Williams & Wilkins.

Saxena, P.R. & Villalon, C.M. (1991). 5-Hydroxytryptamine: a chameleon in the heart. Trends Pharmacol. Sci., 12, 223-227.

Saxena, P.R. & Villalon, C.M. (1990). Cardiovascular effects of serotonin agonists and antagonists. J. Cardiovasc. Pharmacol., 15, S17-34.

Schoeffter, P. & Hoyer, D. (1990). 5-Hydroxytryptamine (5-HT)-induced endothelium-dependent relaxation of pig coronary arteries is mediated by 5-HT receptors similar to the 5-HT_{1D} receptor subtype. J. Pharmacol. Exp. Ther., 252, 387-395.

Sgard, F., Faure, C. & Graham, D. (1996). Evidence for 5-$HT_{1D\beta}$ but not 5-$HT_{1D\alpha}$ receptor subtype expression in canine large coronary arteries and saphenous vein. Cardiovasc. Res., 31, 793-799.

Shen, Y., Monsma, F.J., Jr., Metcalf, M.A., Jose, P.A., Hamblin, M.W. & Sibley, D.R. (1993). Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. J. Biol. Chem., 268, 18200-18204.

Skingle, M., Beattie, D.T., Scopes, D.I.T., Starkey, S.J., Connor, H.E., Feniuk, W. & Tyers, M.B. (1996). GR127935: a potent and selective 5-HT_{1D} receptor antagonist. Behav. Brain Res., 73, 157-161.

Sumner, M.J., Feniuk, W., McCormick, J.D. & Humphrey, P.P. (1992). Studies on the mechanism of 5-HT_1 receptor-induced smooth muscle contraction in dog saphenous vein. Br. J. Pharmacol., 105, 603-608.

Terrón, J.A. (1996). The relaxant 5-HT receptor in the dog coronary artery smooth muscle: pharmacological resemblance to the cloned 5-ht$_7$ receptor subtype. Br. J. Pharmacol., 118, 1421-1428.

Terrón, J.A. & Falcón-Neri, A. (1999). Pharmacological evidence for the 5-HT_7 receptor mediating smooth muscle relaxation in canine cerebral arteries. Br. J. Pharmacol., 127, 609-616.

Thomas, D.R., Gittins, S.A., Collin, L.L., Middlemiss, D.N., Riley, G., Hagan, J., Gloger, I., Ellis, C.E., Forbes, I.T. & Brown, A.M. (1998). Functional characterisation of the human cloned 5-HT_7 receptor (long form); antagonist profile of SB-258719. Br. J. Pharmacol., 124, 1300-1306.

To, Z.P., Bonhaus, D.W., Eglen, R.M. & Jakeman, L.B. (1995). Characterization and distribution of putative 5-ht_7 receptors in guinea-pig brain. Br. J. Pharmacol., 115, 107-116.

Tsou, A.P., Kosaka, A., Bach, C., Zuppan, P., Yee, C., Tom, L., Alvarez, R., Ramsey, S., Bonhaus, D.W., Stefanich, E. & et al. (1994). Cloning and expression of a 5-hydroxytryptamine$_7$ receptor positively coupled to adenylyl cyclase. J. Neurochem., 63, 456-464.

Ullmer, C., Schmuck, K., Kalkman, H.O. & Lubbert, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. FEBS Lett., 370, 215-221.

Vanhoenacker, P., Haegeman, G. & Leysen, J.E. (2000). 5-HT_7 receptors: current knowledge and future prospects. Trends Pharmacol. Sci., 21, 70-77.

Veldman, S.A. & Bienkowski, M.J. (1992). Cloning and pharmacological characterization of a novel human 5- hydroxytryptamine$_{1D}$ receptor subtype. Mol. Pharmacol., 42, 439-444.

Verheggen, R., Hundeshagen, A.G., Brown, A.M., Schindler, M. & Kaumann, A.J. (1998). 5-HT_{1B} receptor-mediated contractions in human temporal artery: evidence from selective antagonists and 5-HT receptor mRNA expression. Br. J. Pharmacol., 124, 1345-1354.

Villalón, C.M., Centurión, D., Luján-Estrada, M., Terrón, J.A. & Sánchez-López, A. (1997). Mediation of 5-HT-induced external carotid vasodilatation in GR127935-pretreated vagosympathectomized dogs by the putative 5-HT_7 receptor. Br. J. Pharmacol., 120, 1319-1327.

Villalón, C.M., Centurión, D., Sánchez-López, A., De Vries, P. & Saxena, P.R. (1999). 5-HT receptors mediating external carotid vasoconstriction in vagosympathectomised dogs. Acta Pharmacol. Sin., 20, 1057-1067.

Villalón, C.M., Sanchez-Lopez, A., Centurion, D. & Saxena, P.R. (2001). Unravelling the pharmacological profile of the canine external carotid vasodilator '5-HT_{1-like}' receptors: coexistence of sympatho-inhibitory 5-HT_{1B} and postjunctional 5-HT_7 receptors. Naunyn-Schmiedeberg's Arch. Pharmacol., 363, 73-80.

Voigt, M.M., Laurie, D.J., Seeburg, P.H. & Bach, A. (1991). Molecular cloning and characterization of a rat brain cDNA encoding a 5- hydroxytryptamine$_{1B}$ receptor. Embo J., 10, 4017-4023.

Wainscott, D.B., Johnson, K.W., Phebus, L.A., Schaus, J.M. & Nelson, D.L. (1998). Human 5-HT_{1F} receptor-stimulated [^{35}S]GTPgammaS binding: correlation with inhibition of guinea pig dural plasma protein extravasation. Eur. J. Pharmacol., 352, 117-124.

Weinshank, R.L., Zgombick, J.M., Macchi, M.J., Branchek, T.A. & Hartig, P.R. (1992). Human serotonin $_{1D}$ receptor is encoded by a subfamily of two distinct genes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. Proc. Natl. Acad. Sci. USA, 89, 3630-3634.

Willems, E., De Vries, P., Heiligers, J.P. & Saxena, P.R. (1998). Porcine carotid vascular effects of eletriptan (UK-116,044): a new 5-$HT_{1B/1D}$ receptor agonist with anti-migraine activity. Naunyn-Schmiedeberg's Arch. Pharmacol., 358, 212-219.

Wolff, H.G. (1963). Headache and other head pain. New York: Oxford university press.

Wu, D., Eberlein, W., Rudolf, K., Engel, W., Hallermayer, G. & Doods, H. (2000). Characterisation of calcitonin gene-related peptide receptors in rat atrium and vas deferens: evidence for a [Cys(Et)(2,7)]hCGRP-preferring receptor. Eur J Pharmacol, 400, 313-319.

Wurch, T., Colpaert, F.C. & Pauwels, P.J. (1998). Chimeric receptor analysis of the ketanserin binding site in the human 5-Hydroxytryptamine$_{1D}$ receptor: importance of the second extracellular loop and fifth transmembrane domain in antagonist binding. Mol. Pharmacol., 54, 1088-1096.

Wurch, T., Palmier, C., Colpaert, F.C. & Pauwels, P.J. (1997). Sequence and functional analysis of cloned guinea pig and rat serotonin 5-HT_{1D} receptors: common pharmacological features within the 5-HT_{1D} receptor subfamily. J. Neurochem., 68, 410-418.

Zgombick, J.M., Bard, J.A., Kucharewicz, S.A., Urquhart, D.A., Weinshank, R.L. & Branchek, T.A. (1997). Molecular cloning and pharmacological characterization of guinea pig 5- HT_{1B} and 5-HT_{1D} receptors. Neuropharmacology, 36, 513-524.

Zgombick, J.M., Borden, L.A., Cochran, T.L., Kucharewicz, S.A., Weinshank, R.L. & Branchek, T.A. (1993). Dual coupling of cloned human 5-hydroxytryptamine$_{1D\alpha}$ and 5-hydroxytryptamine$_{1D\beta}$ receptors stably expressed in murine fibroblasts: inhibition of adenylate cyclase and elevation of intracellular calcium concentrations via pertussis toxin-sensitive G protein(s). Mol. Pharmacol., 44, 575-582.

Zgombick, J.M., Weinshank, R.L., Macchi, M., Schechter, L.E., Branchek, T.A. & Hartig, P.R. (1991). Expression and pharmacological characterization of a canine 5- hydroxytryptamine$_{1D}$ receptor subtype. Mol. Pharmacol., 40, 1036-1042.

Chapter 2

Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT_{1B} receptor

Summary Using a combination of RT-PCR and inverse-PCR techniques, we amplified, cloned and sequenced a full-length porcine 5-HT_{1B} receptor cDNA derived from porcine cerebral cortex. Sequence analysis revealed 1170 bp encoding an open reading frame of 390 amino acids showing a 95% similarity with the human 5-HT_{1B} receptor. The recombinant porcine 5-HT_{1B} cDNA was expressed in monkey Cos-7 cells and its pharmacological profile was determined by radioligand binding assay using [^{3}H]GR125743. The affinities of several agonists (L694247 > ergotamine ≥ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan) and putative antagonists (GR127935 > methiothepin > SB224289 >> ritanserin > ketanserin ≥ BRL15572) correlated highly with those described for the recombinant human 5-HT_{1B} receptor. In membranes obtained from cells co-expressing the porcine 5-HT_{1B} receptor and a mutant GαoCys^{351}Ile protein, 5-HT and zolmitriptan increased, while the 5-HT_{1B} receptor antagonist SB224289 decreased basal [^{35}S]GTPγS binding, thus showing inverse agonism. The potency of zolmitriptan in the [^{35}S]GTPγS binding assay (pEC50: 7.64±0.04) agreed with its affinity in displacing the antagonist [^{3}H]GR125743 (pK_i: 7.36±0.07). The 5-HT_{1B} receptor mRNA was observed by RT-PCR in several blood vessels, cerebral cortex, cerebellum and trigeminal ganglion. *In situ* hybridisation performed in frontal cerebral cortex sections revealed the expression of 5-HT_{1B} receptor mRNA in pyramidal cells. In conclusion, we have cloned and established the amino acid sequence, ligand binding profile and location of the porcine 5-HT_{1B} receptor. This information may be useful in exploring the role of 5-HT_{1B} receptor in pathophysiological processes relevant for novel drug discovery in diseases such as migraine.

Based on: Bhalla, P., Sharma, H.S., Ma, X., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2001). Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT_{1B} receptor. *Br. J. Pharmacol.*, **133(6)**, 891-901

Introduction

Over a decade and a half ago, Bradley *et al.* (1986) provided a general framework for the classification and nomenclature of 5-hydroxytryptamine (5-HT; serotonin) receptors recognising three main types: '5-HT$_1$-like', 5-HT$_2$ and 5-HT$_3$ receptors. The term '5-HT$_1$-like' represented a heterogeneous group of receptors mediating a variety of responses, including the contraction of cranial blood vessels, prejunctional inhibition of neuronal transmitter release, smooth muscle relaxation and tachycardia in the cat. The antimigraine drug sumatriptan was described as a selective and potent agonist at 5-HT$_1$-like receptors (Humphrey *et al*., 1988; Humphrey *et al*., 1989). However, subsequent studies showed that sumatriptan had a poor affinity for receptors mediating smooth muscle relaxation and tachycardia in the cat (now called 5-HT$_7$ receptors, Villalón *et al*., 1997; Saxena *et al*., 1998; De Vries *et al*., 1999a), but possessed a high affinity for 5-HT$_{1D}$ recognition sites (Waeber *et al*., 1989; Hoyer *et al*., 1990). Molecular biological studies revealed that 5-HT$_{1D}$ recognition sites consisted of two distinct receptors, 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ (Weinshank *et al*., 1992) that, on alignment with the human genome, were renamed 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors, respectively (Hartig *et al*., 1996). Interestingly, the pharmacology of the human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors is much closer than that of the human and rat 5-HT$_{1B}$ receptors; the difference in the pharmacological profile of the two 5-HT$_{1B}$ receptors is due to just one amino acid in the seventh transmembrane domain (T355N, Metcalf *et al*., 1992; Oksenberg *et al*., 1992; Hoyer *et al*., 1994). In particular, some β-adrenoceptor antagonists are more potent, while sumatriptan is less potent at the rodent than at the human 5-HT$_{1B}$ receptor (Hoyer *et al*., 1994).

Sumatriptan as well as other triptans inhibit dural plasma protein extravasation, suppress action potentials in trigeminal nucleus caudalis, constrict isolated cranial blood vessels and decrease carotid arteriovenous anastomotic blood flow in anaesthetised animals (Moskowitz, 1992; Goadsby & Knight, 1997; Saxena & Tfelt-Hansen, 2000). Although the trigeminal neural effects of triptans, mediated mainly by the 5-HT$_{1D}$ receptor, may be involved to some extent in their antimigraine action (De Vries *et al*., 1999b; Hargreaves & Shepheard, 1999), the efficacy of triptans is primarily attributed to the 5-HT$_{1B}$ receptor-mediated cranial vasoconstriction (De Vries *et al*., 1999b; Saxena & Tfelt-Hansen, 2000). Previous

investigations from our laboratory have established that constriction of carotid arteriovenous anastomoses in the anaesthetised pig can serve as a predictive model for the antimigraine efficacy of 5-HT-based drugs (Saxena, 1995; De Vries *et al*., 1999b). To gain further insight into the mechanisms involved in drug actions as well as the disease, it is important to study the trigeminal neural control of porcine arteriovenous anastomoses and its potential modification by 5-HT receptor ligands. However, one of the difficulties in undertaking such studies is the limited knowledge of the molecular biology of porcine 5-HT receptors. We recently cloned the porcine 5-HT$_{1D}$ receptor and found that its amino acid sequence and ligand binding profile were very similar to that of the human 5-HT$_{1D}$ receptor, but a striking exception was the low affinity of BRL15572 ([1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl)piperazine] hydrochloride) at the porcine (Bhalla *et al*., 2000) compared to human (Price *et al*., 1997; Schlicker *et al*., 1997) receptor.

In the present investigation, we describe the molecular cloning and characterisation of the porcine 5-HT$_{1B}$ receptor (receptor code: 2.1.5HT.01B). Using the total RNA isolated from the pig cerebral cortex, a full-length cDNA encoding 5-HT$_{1B}$ receptor was PCR-amplified and the deduced amino acid sequence was compared with that in other species. The ligand binding profile of the porcine 5-HT$_{1B}$ receptor was evaluated after transient transfection in Cos-7 cells and its distribution in the porcine tissues was explored using reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridisation.

Methods

RNA isolation and RT-PCR

As described in detail previously (Bhalla *et al*., 2000), total RNA was extracted from a pig (Yorkshire x Landrace, female, 12 kg) cerebral cortex (Chomczynski & Sacchi, 1987; Sharma *et al*., 1996) and processed for the purification of poly(A^+) mRNA using an Oligotex mRNA purification Kit (Qiagen GmbH, Hilden, Germany). Poly(A^+) mRNA (0.5 µg) was denatured at 65 °C and the first strand of cDNA was synthesised in a reaction volume of 20 µl by adding sequentially the following reagents: reverse transcription buffer (25 mM Tris-HCl, pH 8.3; 50 mM KCl; 5.0 mM $MgCl_2$, 2.0 mM DTT), 1.0 mM dNTPs, ribonuclease inhibitor (1 U μl^{-1}), random hexamer (150 ng μg^{-1} mRNA) and, finally, AMV reverse transcriptase (14 U μg^{-1} of

mRNA; Pharmacia-LKB, Uppsala, Sweden). A parallel control without AMV reverse transcriptase was prepared to check the genomic contamination. The reactions were carried out for 90 min at 42 °C, extended for another 10 min at 75 °C and then cooled to 4 °C. The cDNA thus synthesised was diluted to 50 µl and stored at -20°C until used as a PCR template. The quality of cDNA was checked by PCR amplification of porcine β-actin using human specific oligonucleotide primers (Ponte *et al*., 1984).

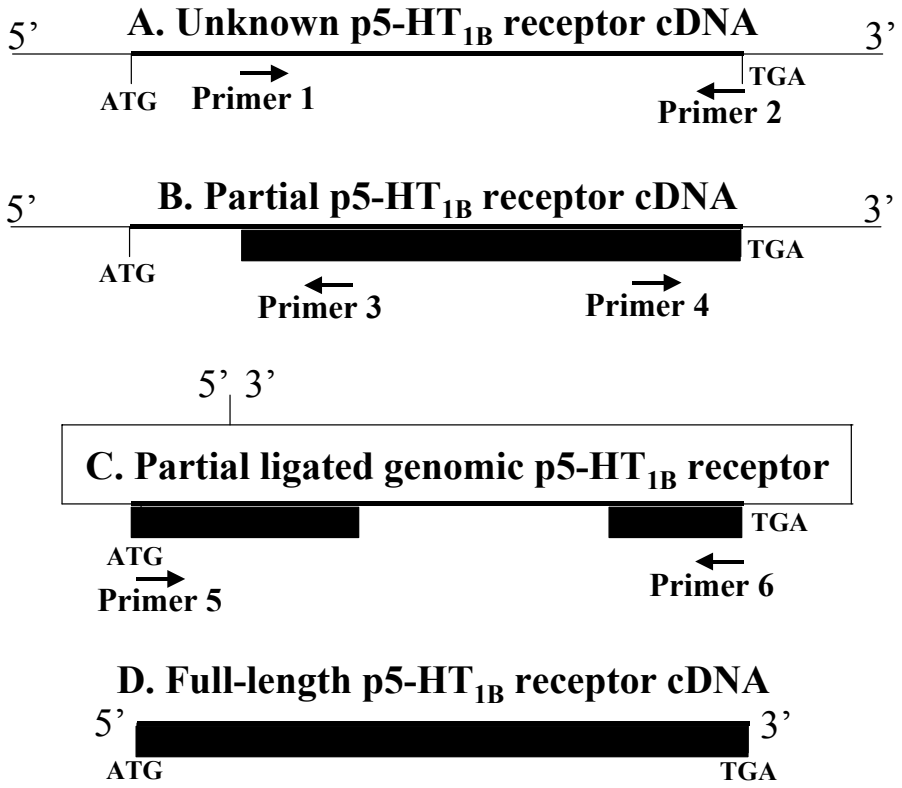


Figure 2.1. Strategy for cloning full-length porcine 5-HT$_{1B}$ receptor and position of various primers used for PCR amplification. **A**. Unknown full-length porcine 5-HT$_{1B}$ receptor cDNA with primers derived from porcine (**1**) and human (**2**) nucleotide sequences. **B**. The amplified partial sequence of porcine 5-HT$_{1B}$ receptor cDNA was used to design inverse porcine-specific primers (**3**) and (**4**). **C**. The inverse-PCR amplified partial genomic sequence enabled us to design full-length porcine specific primers (**5**) and (**6**). **D**. The full-length amplified product of porcine 5-HT$_{1B}$ receptor cDNA. Arrows and black rectangles denote the direction of the primers and amplified products, respectively.

Porcine specific 5-HT$_{1B}$ receptor cDNA was amplified using a combination of RT-PCR and inverse-PCR techniques. Initially, we attempted to obtain the full-length 5-HT$_{1B}$ receptor cDNA employing 5' and 3' end oligonucleotide primers designed

from the consensus sequences of other species (Demchyshyn *et al*., 1992; Maroteaux *et al*., 1992; Weinshank *et al*., 1992; Harwood *et al*., 1995; Zgombick *et al*., 1997). Unlike the cloning of porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000), we were unsuccessful in this case and, therefore, an alternative strategy, as depicted in Figure 2.1, was employed. New forward (5'-CCTGCCCTGGAAAGTAGTAC-3'; nucleotides 4-23; GenBank accession number Z47984) and reverse (5'-TCAACTTGTGCACTTAAAAC-3'; nucleotides 1772-1791; GenBank accession number M75128) oligonucleotide primers (Figure 2.1A, 1 and 2) were designed from the porcine (partial) and human 5-HT$_{1B}$ receptor sequence, respectively. For PCR amplification, a 20 µl reaction mixture containing the following components was prepared: 250 µM of each dNTP, 1.5 mM $MgCl_2$, PCR buffer (1xPCR buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl), Ampli Taq Gold^{TM} enzyme (0.5 U), 0.5 µM each of the forward and reverse primer and 5 µl of cDNA template. After brief centrifugation, the enzyme was first activated for 10 min at 94 °C in a PCR thermocycler (model PTC-100^{TM}, M.J. Research Inc, Watertown, USA). The timing of PCR was 1 min at 94 °C, 30 s at 55 °C and 90 s at 72 °C with total of 36 cycles. Finally, the reaction was extended for additional 10 min at 72 °C. The PCR amplified product of expected size was purified using a PCR purification kit (Promega Benelux b.v., Leiden, The Netherlands) and ligated into a pCR™ II vector using TA cloning kit (Invitrogen BV., Groningen, The Netherlands). The ligated vector was transformed into competent TOP10 cells and grown overnight on LB agar plates containing kanamycin (50 µg ml^{-1}) with IPTG and X-gal at 37 °C. White over blue colonies were selected to identify positive clones containing the DNA insert of expected size. Three insert positive clones (namely, pHTB-2, pHTB-4, pHTB-19) were further processed for the plasmid DNA isolation (mini-prep, Promega Benelux b.v., Leiden, The Netherlands) and sequenced by the dideoxy nucleotide chain termination method using an automated fluorescence based DNA sequencer (ABI Prism^{TM} 310 Genetic analyser, Perkin Elmer Applied Biosystem Benelux, Nieuwerkerk a/d IJssel, The Netherlands). The nucleotide sequences were compared and a consensus sequence was derived (DNAMAN sequence analysis program, Version 3.2, Lynnon Biosoft© 1994-1997). The final partial cDNA sequence (Figure 2.1B) was compared with

those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, USA).

Analysis of 5' and 3' ends of porcine 5-HT$_{1B}$ receptor by Inverse-PCR

Inverse-PCR was performed to establish the porcine specific sequence of 5' and 3' ends (Ochman *et al*., 1988). Porcine genomic DNA was digested with Bgl II restriction enzyme, because the cloned human 5-HT$_{1B}$ receptor cDNA did not show any restriction site for this enzyme. 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. The ligated DNA circles were subjected to inverse-PCR using primers specific for porcine 5-HT$_{1B}$ receptor (5'-GAGGCGATCAGGTAGTTGGC-3' for 5' end and 5'-GATGCCTGCTGGTTCCACC-3' for 3' end; Figure 2.1B, 3 and 4). The amplified products were separated on a 1% agarose gel, purified, cloned and sequenced, as described above.

Amplification and cloning of full-length porcine 5-HT$_{1B}$ receptor

For the amplification of full-length porcine 5-HT$_{1B}$ receptor cDNA, forward and reverse oligonucleotide primers (5'-ATGGAGGAAGCGGGCGCTCAG-3' and 5'-TCAGCTTGTGCACTTAAAGCG-3') were designed from the sequences generated from inverse-PCR (Figure 2.1C, 5 and 6). After PCR amplification (see above), a product of expected size was purified, ligated into the pGEMT-Easy vector (Promega Benelux b.v., Leiden, The Netherlands), transformed into competent JM 109 cells and plasmid DNA was isolated. Four insert positive clones (pHTB-11, pHTB-12, pHTB-14, pHTB-17) were further processed for the plasmid DNA purification and sequencing. The full-length cDNA sequence of porcine 5-HT$_{1B}$ receptor was derived from two independent PCR amplified products and further verified by multiple partial sequences derived from cDNA (RT-PCR) as well as genomic DNA (inverse-PCR) amplified products. 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 (Figure 2.1D) was translated as a peptide sequence and compared with those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, USA). The hydrophobic regions (indicating putative

transmembrane domains) and sequence homology with known 5-HT_{1B} receptors from other species were established.

Transient transfection and radioligand binding assay

The purified full-length 5-HT_{1B} receptor cDNA insert was subcloned into dephosphorylated eukaryotic expression vector, pcDNA3 and transformed into TOP10 competent cells. The plasmid DNA was purified according to maxi prep protocol using a commercially available kit (Qiagen SA, Courtaboeuf, France). Monkey Cos-7 cells were transiently transfected with the plasmid using a gene pulser transfection apparatus (Bio-Rad S.A., Ivry Sur Seine, France), as described earlier by Pauwels *et al*. (1996). After transfection, the cells were incubated for 48 h in Dulbecco modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal calf serum and antibiotics at 37 °C in a humidified chamber containing 5% CO_2.

The transfected cells were washed twice with phosphate buffer saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_4; pH 7.2) and kept at -80 °C for 10 min. The cells were scrapped from the petri-dish in ice-cold Tris-buffer (pH: 7.7) and homogenised. The homogenate was centrifuged at 1,000 rpm for 5 min at 4 °C and the supernatant was collected and centrifuged again at 13,000 rpm for 20 min. The membrane pellet was resuspended into 50 mM Tris-HCl buffer (pH: 7.7) containing 4 mM $CaCl_2$, 10 μM pargyline and 0.1 % ascorbic acid, as described before (Pauwels & Colpaert, 1996). The membrane protein concentration was measured by dye binding assay (Bradford, 1976) using the Bio-Rad Kit and bovine serum albumin was used as a standard.

Binding assays to membranes obtained from transfected Cos-7 cells were performed using 1.0 nM [^{3}H]GR125743 ([^{3}H]N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide) as radioligand. Incubation mixtures consisted of 0.4 ml of cell membrane preparation (30-50 μg of protein), 0.05 ml of the radioligand and 0.05 ml of compounds for inhibition or 10 μM 5-HT to determine non-specific binding. The reaction was terminated by filtration with ice-cold Tris-buffer and radioactivity on the filter paper was measured by using a liquid scintillation counter (Pauwels *et al*., 1996; Wurch *et al*., 1997). The filtration was performed over 0.2% polyethyleneimine-treated Whatman (Clifton, NJ, USA) GF/B glass fibre filters. Data were analysed graphically with inhibition curves, and IC_{50}

values were derived. 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. Ligand saturation binding curves were analysed by the nonlinear least square curve-fitting programme to determine K_d and B_{max} values (Munson & Rodbard, 1980). Control binding experiments were run with nontransfected cells and they did not display specific [^{3}H]GR125743 binding.

[^{35}S]GTPγS binding response

CHO-K1 cells transiently co-expressing the cloned porcine 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein (Dupuis *et al*., 1999) were collected in phosphate-buffered-saline (pH 7.4) and centrifuged for 20 min at 48,000 g and the pellet containing the membrane fraction was stored at -80 °C. [^{35}S]GTPγS binding was measured using the method previously described by Pauwels *et al*. (1997). Briefly, the pellet was thawed and diluted in 20 mM HEPES buffer (pH 7.4) containing 30 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ascorbic acid. Incubation mixtures were prepared in glass tubes and consisted of 0.4 ml of membrane preparation (containing 5 μg protein) with 5-HT (10 μM), 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; 1 μM) or zolmitriptan (0.1 nM -10 μM) in a volume of 0.05 ml. After an incubation period of 30 min at 25 °C, 0.05 ml [^{35}S]GTPγS (0.5 nM) was added for an additional period of 30 min. The reaction was stopped by adding 3 ml of ice-cold 20 mM HEPES (pH 7.4) containing 3 mM $MgCl_2$ and rapid filtration over Whatmann GF/B glass fibre filters with a Brandel harvester. The filters were rinsed three additional times with 3 ml HEPES buffer, placed in scintillation vials and the radioactivity was extracted in 4 ml of Emulsifier-Safe. Maximal stimulation of [^{35}S]GTPγS binding was defined in the presence of 10 μM 5-HT. E_{max} values were expressed as a percentage of the maximal response obtained with 10 μM 5-HT. EC_{50} values were defined as the concentration of compound at which 50% of its own maximal stimulation was obtained.

Detection of 5-HT$_{1B}$ receptor mRNA by RT-PCR

RT-PCR was used to detect 5-HT$_{1B}$ receptor mRNA in the following tissues obtained from pigs (Yorkshire x Landrace, female, 12-15 kg): brain cortex, cerebellum, trigeminal ganglion, left cardiac ventricle, left anterior descending coronary, pulmonary, common carotid, superior mesenteric and femoral arteries and saphenous vein. The tissue samples were dissected and cleaned and the total RNA was isolated as described above. The residual DNA contamination was removed by treatment with RNase-free DNase (10 U 6 μg^{-1} RNA) for 25 min at 37 °C as per instruction (Promega Benelux b.v., Leiden, The Netherlands). The purified total RNA samples were reverse transcribed into cDNA in presence of reverse transcriptase enzyme. A control reaction was always prepared in absence of reverse transcriptase to monitor the DNA contamination. For the PCR amplification of porcine 5-HT$_{1B}$ receptor, porcine specific sense (5'-CCTGCCCTGGAAAGTAGTAC-3'; nucleotides 135-154) and antisense (5'-TGATGGGCATCACCAGGATG-3'; nucleotides 297-316) primers were used along with other components of PCR exactly as described earlier, except that the annealing temperature was kept at 60 °C instead of 55 °C. The PCR amplified products (12 μl each) were separated on 3% agarose gel by electrophoretic separation and photographed.

Localisation of 5-HT$_{1B}$ receptor mRNA by in situ hybridisation

A piece of frontal cerebral cortex, obtained from a pig (Yorkshire x Landrace, female, 14 kg), was fixed using phosphate buffer saline containing 4% paraformaldehyde. After dehydration and embedding in paraffin, 5 μm thick sections were cut (Microtome, Microm Type HM325, Walldorf, Germany) and layered on superfrost plus® glass slides (Menzel-Glaser, Braunschweig, Germany). For *in situ* hybridisation, a digoxigenin-labelled cRNA probe was prepared from recombinant porcine 5-HT$_{1B}$ receptor cDNA containing 470 bp (nucleotides 135-695). The cloned plasmid DNA was linearised with either Bam HI or Xba I, purified and transcribed to synthesise sense and antisense cRNA probes, using T_7 or SP_6 RNA polymerase. The cRNA probes were quantified by dot blotting as per protocol described for DIG RNA labelling kit (Roche Diagnostics Nederland B.V., Almere, The Netherlands). *In situ* hybridisation was performed following the method described by de Boer *et al.* (1998). The purple coloured hybrids representing the 5-HT$_{1B}$ receptor mRNA in the tissue

sections was visualised under a light microscope (Leica DM RBE, GmbH, Wetzlar, Germany).

Drugs and other chemicals

All chemicals used in this study were of molecular biology and/or culture grade. The oligonucleotide primers were commercially procured from Life Technologies b.v. (Breda, The Netherlands) and the sources of the kits used have been identified in the text.

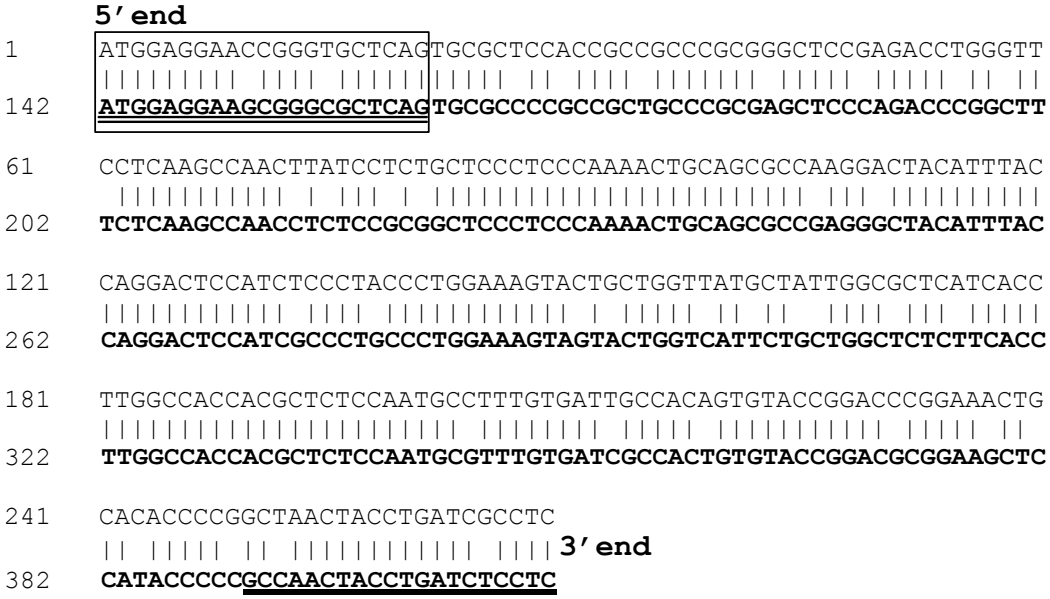
The compounds used in pharmacological assays were: 5-HT creatinine sulphate (Sigma Chemicals, St. Louis, MO, USA), BRL15572 (gift: Dr. A.A. Parsons, SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK), 5-carboxamidotryptamine, CP122638 (N-methyl-3-[pyrrolidin-2(R)-ylmethyl]-1H-indol-5-ylmethyl sulphonamide), [^{3}H]GR125743 (83.0 Ci mmol^{-1}; Amersham, Les Ulis, France), 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, [^{35}S]GTPγS (1000 Ci mmol^{-1}; Amersham, Les Ulis, France), ketanserin (Sigma Chemicals, St. Louis, MO, USA), L694247 (2-[5-[3-(4-methylsulphonylamino) benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl] ethylamine; Tocris Cookson, Bristol, UK), methiothepin, ritanserin, sumatriptan, SB224289 and zolmitriptan. Except those specified above, all other compounds were synthesised at Centre de Recherche Pierre Fabre (Castres, France).

Results

Cloning of 5-HT$_{1B}$ receptor cDNA derived from porcine cerebral cortex

As described in the Methods section (Figure 2.1), using RT-PCR technique, we amplified a partial fragment (1038 bp) from porcine brain cortex cDNA. Upon sequencing, this fragment revealed a high homology with the human 5-HT$_{1B}$ receptor (data not shown). On the basis of this porcine specific partial sequence of 5-HT$_{1B}$ receptor, inverse primers were designed and used on porcine ligated genomic DNA to establish the sequence of 5' and 3' ends of the porcine 5-HT$_{1B}$ receptor (Figure 2.2).

Inverse-PCR (5'end)



Inverse-PCR (3'end)

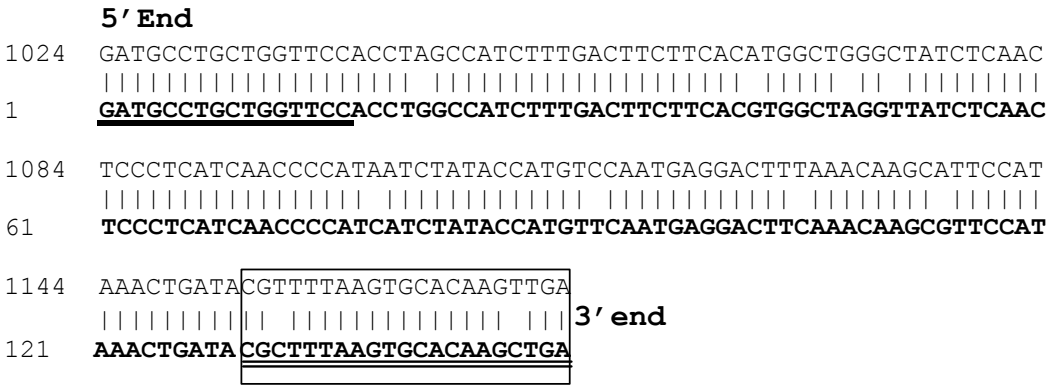


Figure 2.2. Sequence of inverse-PCR amplified product from porcine genomic DNA circles (in bold letters) showing high homology with the sequence of human 5-HT$_{1B}$ receptor (in normal letters). The deduced 5' (N) and 3' (C) terminal sequences of porcine 5-HT$_{1B}$ receptor are shown double underlined in boxes, while the inverse-PCR primers are thick underlined.

To amplify the full-length porcine 5-HT$_{1B}$ receptor, the cDNA template derived from poly(A$^+$) mRNA of porcine brain cortex was used with porcine specific 5' and 3' end primers. Agarose gel electrophoresis of RT-PCR products (Figure 2.3) shows amplification of the porcine β-actin cDNA (625 bp), which ensures that the quality of cDNA samples was adequate for amplification of other products. The presence of

genomic DNA contamination was excluded by performing a parallel reaction in the absence of reverse transcriptase. The expected band (~1200 bp) of porcine cDNA, observed in presence of porcine specific primers, was cloned (Figure 2.3).

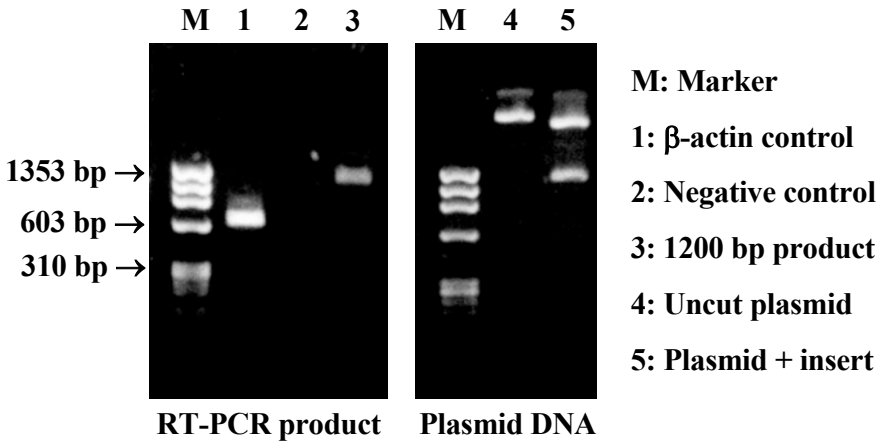


Figure 2.3. Agarose gel electrophoresis of RT-PCR products of cDNA synthesised from porcine cerebral cortex (left panel) and recombinant plasmid with insert cDNA (right panel). The different lanes marked on top denote: ϕx174 DNA/Hae III marker (M), positive control showing RT-PCR product of 625 bp using β-actin primers (1), negative control, i.e. a sample without reverse transcriptase to monitor genomic and/or PCR contamination (2), RT-PCR product of approximately 1200 bp obtained using forward and reverse primers of porcine specific 5-HT$_{1B}$ receptor (3), recombinant uncut plasmid DNA vector (4) and plasmid DNA vector restricted with EcoR1 enzyme and showing a DNA insert of approximately 1200 bp (5). The size of 3 marker bands is indicated in the left margin.

Sequence analysis of cloned porcine cDNA

Sequencing of the recombinant plasmid revealed 1170 bp, starting with ATG codon and ending with TGA codon. DNAMAN analysis showed that this full-length porcine cDNA encoded a 390 amino acid protein exhibiting features of a typical G-protein coupled receptor with predicted seven transmembrane domains and putative N-glycosylation and phosphorylation sites (Figure 2.4). A BLAST search at GenBank of the 1170 bp nucleotide sequence revealed resemblance with the sequence of 5-HT$_{1B}$ receptors from other species.

```
1    ATG GAG GAA GCG GGC GCT CAG TGC GCC CCG CCG CTG CCC GCG AGC TCC CAG ACC CGG CTT
1    MET Glu Glu Ala Gly Ala Gln Cys Ala Pro Pro Leu Pro Ala Ser Ser Gln Thr Arg Leu
                 ℗                                   ℗
61   TCT CAA GCC AAC CTC TCC GCG GCT CCC TCC CAA AAC TGC AGC GCC GAG GGC TAC ATT TAC
21   Ser Gln Ala Asn Leu Ser Ala Ala Pro Ser Gln Asn Cys Ser Ala Glu Gly Tyr Ile Tyr

121  CAG GAC TCC ATC GCC CTG CCC TGG AAA GTA GTA CTG GTC ATT CTG CTG GCT CTC TTC ACC
41   Gln Asp Ser Ile Ala Leu Pro Trp Lys Val Val Leu Val Ile Leu Leu Ala Leu Phe Thr   TM 1

181  TTG GCC ACC ACG CTC TCC AAT GCG TTT GTG ATC GCC ACT GTG TAC CGG ACG CGG AAG CTC
61   Leu Ala Thr Thr Leu Ser Asn Ala Phe Val Ile Ala Thr Val Tyr Arg Thr Arg Lys Leu

241  CAT ACC CCC GCC AAC TAC CTG ATC GCC TCC TTG GCG GTC ACC GAC CTG CTG GTA TCC ATC
81   His Thr Pro Ala Asn Tyr Leu Ile Ala Ser Leu Ala Val Thr Asp Leu Leu Val Ser Ile   TM 2

301  CTG GTG ATG CCC ATC AGC ACC ATG TAC ACG GTC ACC GGC CGC TGG ACG CTA GGC CAG GTG
101  Leu Val Met Pro Ile Ser Thr Met Tyr Thr Val Thr Gly Arg Trp Thr Leu Gly Gln Val

361  GTC TGC GAC TTC TGG CTG TCG TCG GAC ATC ACC TGT TGC ACT GCT TCC ATC TTG CAC CTC
121  Val Cys Asp Phe Trp Leu Ser Ser Asp Ile Thr Cys Cys Thr Ala Ser Ile Leu His Leu   TM 3

421  TGT GTC ATC GCT TTG GAC CGC TAC TGG GCC ATC ACG GAC GCC GTG GAG TAC TCG GCT AAA
141  Cys Val Ile Ala Leu Asp Arg Tyr Trp Ala Ile Thr Asp Ala Val Glu Tyr Ser Ala Lys
          ●
481  AGG ACT CCC AAG AGG GCG GCC GTC ATG ATC GCG CTG GTG TGG GTC TTC TCC ATT TCC ATC
161  Arg Thr Pro Lys Arg Ala Ala Val Met Ile Ala Leu Val Trp Val Phe Ser Ile Ser Ile   TM 4

541  TCG CTG CCG CCC TTC TTC TGG CGT CAG GCC AAA GCC GAG GAG GAG GTG TCG GAC TGC GTG
181  Ser Leu Pro Pro Phe Phe Trp Arg Gln Ala Lys Ala Glu Glu Glu Val Ser Asp Cys Val

601  GTG AAC ACG GAC CAC ATC CTC TAC ACT GTC TAC TCC ACG GTG GGC GCT TTC TAC TTC CCC
201  Val Asn Thr Asp His Ile Leu Tyr Thr Val Tyr Ser Thr Val Gly Ala Phe Tyr Phe Pro   TM 5

661  ACC CTG CTC CTC ATC GCC CTC TAT GGC CGC ATC TAT GTG GAA GCC CGC TCC CGG ATT TTG
221  Thr Leu Leu Leu Ile Ala Leu Tyr Gly Arg Ile Tyr Val Glu Ala Arg Ser Arg Ile Leu
                              ●                   ■
721  AAA CAG ACA CCT AAC AGA ACT GGC AAG CGC CTG ACC CGA GCC CAA CTG ATA ACT GAC TCC
241  Lys Gln Thr Pro Asn Arg Thr Gly Lys Arg Leu Thr Arg Ala Gln Leu Ile Thr Asp Ser

781  CCC GGG TCC ACA TCT TCG GTC ACC TCC ATT AAC TCA CGA GCT CCA GAC TTA CCC AGC GAG
261  Pro Gly Ser Thr Ser Ser Val Thr Ser Ile Asn Ser Arg Ala Pro Asp Leu Pro Ser Glu

841  TCA GGA TCT CCT GTG TAC GTG AAT CAA GTC AAA GTG CGA GTC TCA GAC GCC CTG CTG GAG
281  Ser Gly Ser Pro Val Tyr Val Asn Gln Val Lys Val Arg Val Ser Asp Ala Leu Leu Glu
                                                    ■
901  AAG AAG AAA CTC ATG GCC GCT AGG GAG CGC AAA GCG ACA AAG ACC CTG GGG ATC ATT TTG
301  Lys Lys Lys Leu Met Ala Ala Arg Glu Arg Lys Ala Thr Lys Thr Leu Gly Ile Ile Leu   TM 6

961  GGA GCA TTT ATT GTG TGT TGG CTG CCC TTC TTC ATC ATT TCT CTG GCC ATG CCT ATC TGC
321  Gly Ala Phe Ile Val Cys Trp Leu Pro Phe Phe Ile Ile Ser Leu Ala Met Pro Ile Cys

1021 AAG GAT GCC TGC TGG TTC CAC CTG GCC ATC TTT GAC TTC TTC.ACG TGG CTA GGT TAT CTC
341  Lys Asp Ala Cys Trp Phe His Leu Ala Ile Phe Asp Phe Phe Thr Trp Leu Gly Tyr Leu   TM 7

1081 AAC TCC CTC ATC AAC CCC ATC ATC TAT ACC ATG TTC AAT GAG GAC TTC AAA CAA GCG TTC
361  Asn Ser Leu Ile Asn Pro Ile Ile Tyr Thr Met Phe Asn Glu Asp Phe Lys Gln Ala Phe

1141 CAT AAA CTG ATA CGC TTT AAG TGC ACA AGC TGA
381  His Lys Leu Ile Arg Phe Lys Cys Thr Ser ***
```

℗ **N-glycosylation; • PKA-phosphorylation; ▪ PKC-phosphorylation**

Figure 2.4. Nucleotide and deduced amino acid (in bold) sequences of the recombinant cDNA derived from porcine cerebral cortex. Numbering of nucleotide and amino acids is shown on the left. Computer analysis (software DNAMAN, version 3.2, Lynnon Biosoft©) predicted a typical G-protein coupled receptor with transmembrane (TM) 1-7 domains (underlined) as well as the putative N-glycosylation and protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites.

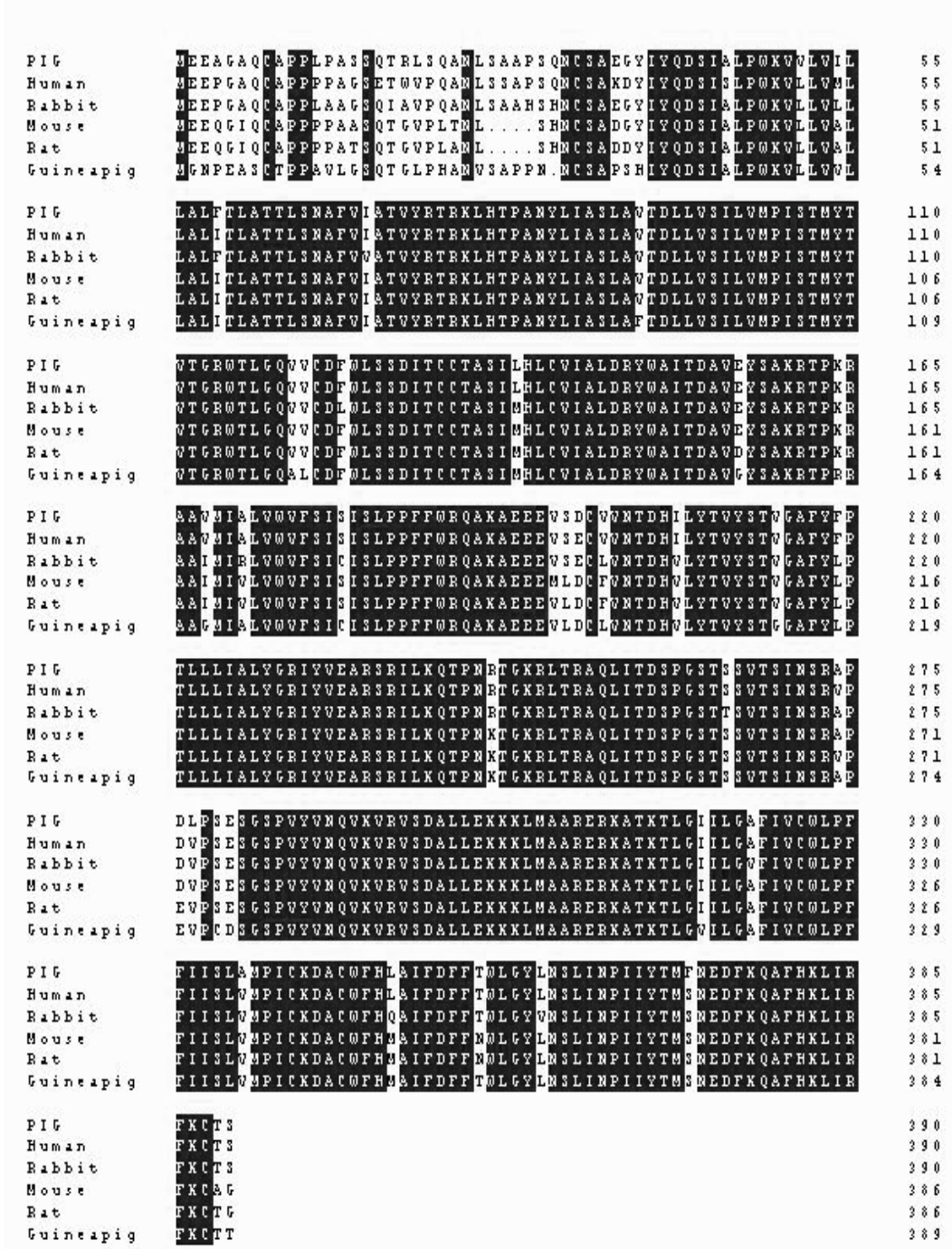


Figure 2.5. Comparison of amino acid sequences of the pig 5-HT$_{1B}$ receptor (Genbank accession number: AF188626) with the human (Swissprot accession number P28222), rabbit (P49144), mouse (P28334), rat (P28564) and guinea pig (O08892) 5-HT$_{1B}$ receptor. The areas shaded black show identity across the different species.

Figure 2.5 compares the amino acid sequence of the porcine 5-HT$_{1B}$ receptor with those of other mammalian species (human, rabbit, mouse, rat and guinea pig).

Across the species, there was an 88-95% similarity in the overall sequence and the transmembrane domains 2-5 in porcine and human receptors were identical. However, it may be noted that some amino acids were unique to the porcine 5-HT$_{1B}$ receptor: Ala^{4}, Ser^{15,21} and Arg^{19} towards the N-terminal end, Val^{51} and Ile^{54} in the first transmembrane domain, Leu^{277} in the third intracellular loop, Ala^{336} in the sixth transmembrane domain as well as Phe^{372} in the seventh transmembrane domain.

Pharmacological characterisation of recombinant porcine 5-HT$_{1B}$ receptor

Membrane preparations from COS-7 cells transfected with porcine 5-HT$_{1B}$ receptor showed a high affinity for [^{3}H]GR125743. The equilibrium dissociation constant (K_d) and total number of binding sites (B_{max}) for [^{3}H]GR125743 were found to be 0.66±0.05 nM and 2.70±0.36 pmol mg^{-1} protein (n=3 each), respectively.

The affinity constants (pK_i values) of 14 serotonergic compounds (8 agonists and 6 putative antagonists) for the displacement of [^{3}H]GR125743 from membranes obtained from COS-7 cells expressing porcine 5-HT$_{1B}$ receptor are shown in Table 2.1.

Table 2.1 Inhibition by serotonergic compounds of [^{3}H]GR125743 (1 nM) binding to membranes derived from Cos-7 cells transiently expressing the pig 5-HT$_{1B}$ receptor cDNA

Agonists	**pK_i**	**Putative antagonists**	**pK_i**
L694247	9.07 ± 0.02	GR127935	8.50 ± 0.03
Ergotamine	8.06 ± 0.00	Methiothepin	8.29 ± 0.04
5-Carboxamidotryptamine	8.06 ± 0.18	SB224289	7.88 ± 0.07
Dihydroergotamine	8.00 ± 0.10	Ritanserin	6.67 ± 0.01
5-HT	7.84 ± 0.07	Ketanserin	5.78 ± 0.12
CP122638	7.37 ± 0.05	BRL15572	5.58 ± 0.07
Zolmitriptan	7.36 ± 0.07		
Sumatriptan	7.25 ± 0.15		

Data are mean ± s.e.mean (n=3).

The rank order of potency of agonists was L694247 > ergotamine ≥ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan, while that of the putative antagonists was GR127935 > methiothepin > SB224289 >> ritanserin > ketanserin ≥ BRL15572.

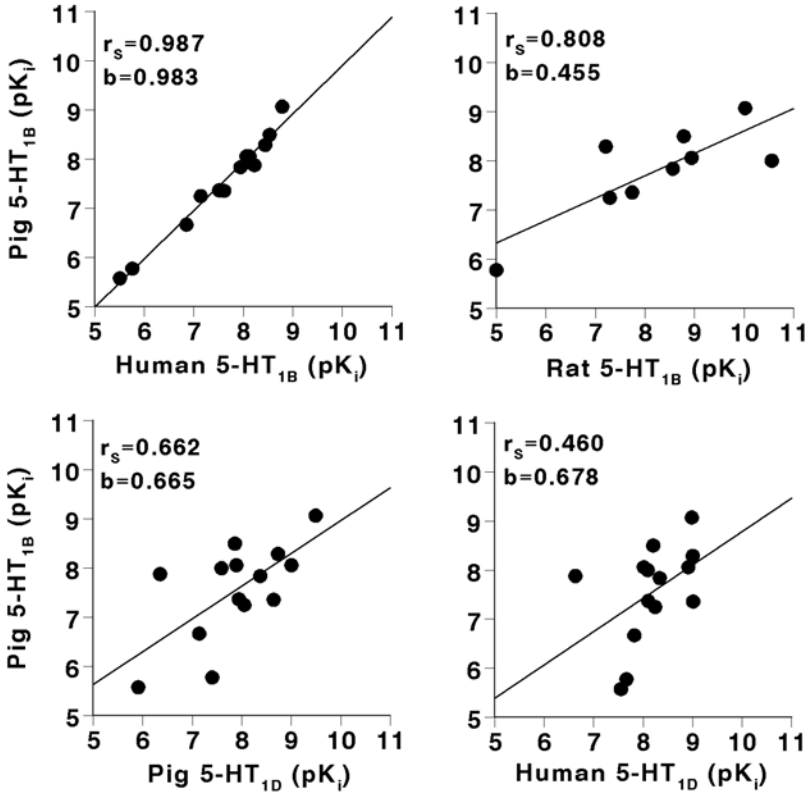


Figure 2.6. Regression analysis of pK_i values (affinity constants) of 5-HT receptor ligands obtained with the cloned pig 5-HT$_{1B}$ receptor (see Table 2.1) with those reported with cloned 5-HT$_{1B}$ (human and rat; upper panels) and 5-HT$_{1D}$ (pig and human; lower panels) receptors (for references, see text). In each case [^{3}H]GR125743 was used as radioligand, except for the rat 5-HT$_{1B}$ receptor, where [^{3}H]5-HT was used and pK_i values of some compounds included in Table 2.1 (ergotamine, CP122638, SB224289, ritanserin and BRL15572) were not available. 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.

In Figure 2.6, pK_i values of the above compounds obtained in the present experiments with membranes from cells expressing the cloned pig 5-HT$_{1B}$ receptor (Table 2.1) have been plotted against pK_i values obtained earlier with the same compounds using membranes from cells expressing the cloned human (Wurch *et al*., 1998 and unpublished) or rat (Beer *et al*., 1998) 5-HT$_{1B}$ receptor as well as the cloned porcine (Bhalla *et al*., 2000) or human (Wurch *et al*., 1998 and unpublished) 5-HT$_{1D}$ receptor. The affinity constants at the porcine 5-HT$_{1B}$ receptor showed the highest correlation with those at the human 5-HT$_{1B}$ receptor (r_s=0.988; b=0.985).This was closely followed by that at the rat 5-HT$_{1B}$ receptor (r_s=0.808), although the slope (b=0.455)

was conspicuously lower. The correlation at the porcine (r_s=0.656) or human (r_s=0.455) 5-HT$_{1D}$ receptors was much weaker. It may be noted that the selective 5-HT$_{1B}$ receptor antagonist SB224289 (Schlicker *et al*., 1997; Gaster *et al*., 1998; Selkirk *et al*., 1998) showed a high affinity (pK_i: 7.88±0.07, Table 2.1), while the selective 5-HT$_{1D}$ receptor antagonist BRL15572 (Schlicker *et al*., 1997; Gaster *et al*., 1998) a low affinity (pK_i: 5.58±0.07; Table 2.1) at the porcine 5-HT$_{1B}$ receptor.

[^{35}S]GTPγS binding response

The basal [^{35}S]GTPγS binding to membranes obtained from CHO-K1 cells transiently co-expressing the cloned porcine 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein was 181±34 fmol [^{35}S]GTPγS mg^{-1} protein (n=5).

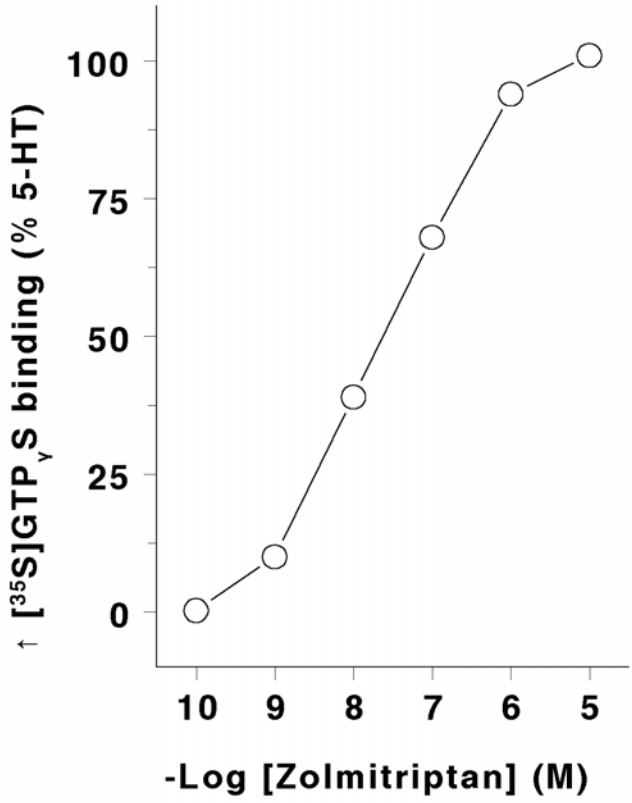
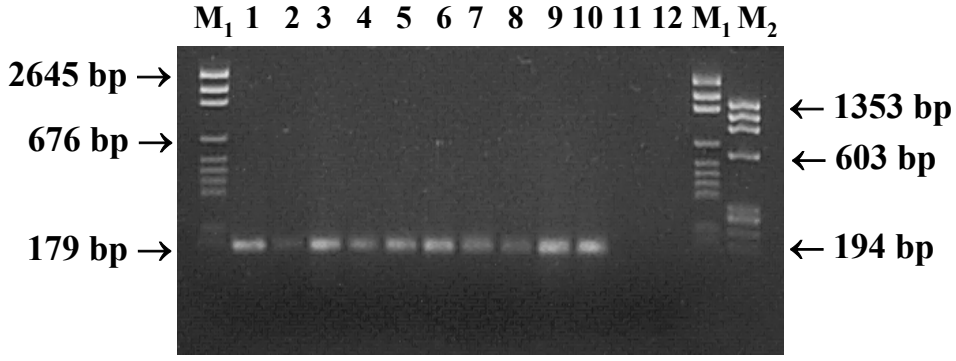


Figure 2.7. Increase in [^{35}S]GTPγS binding, as percentage of the response to 10 μM 5-HT, by zolmitriptan in CHO-K_1 cells transiently co-expressing the cloned pig 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein (pEC_{50} zolmitriptan: 7.64±0.04). Data are mean±s.e.mean (n=5); s.e.mean values fall within the symbol. The basal ^{35}S]GTPγS binding (181±34 fmol mg^{-1} protein; n=5) was increased by 10 μM 5-HT to 390±62 fmol mg^{-1} protein (123±10% of the basal value; n=5) and decreased by 1 μM SB224289 to 136±29 fmol mg^{-1} protein (-27±4% of the basal value; n=5).

5-HT (10 μM) increased [^{35}S]GTPγS binding by 123±10% (n=5) over the basal level; no effect was observed in membranes from cells transfected with empty plasmid. Zolmitriptan caused a concentration-dependent increase in [^{35}S]GTPγS binding and elicited a full agonist response at the receptor (100±2% increase as compared to 10 μM 5-HT; Figure 2.7). The potency (pEC$_{50}$ value) of zolmitriptan was 7.64±0.04 nM (n=5), which is close to its binding affinity at the porcine 5-HT$_{1B}$ receptor (pK$_i$: 7.36±0.07 nM, n=3; Table 2.1). The selective 5-HT$_{1B}$ receptor antagonist SB224289 (1 μM) decreased basal [^{35}S]GTPγS binding by -27±4% (n=5), showing that SB224289 exhibits a negative efficacy (inverse agonism).

5-HT$_{1B}$ receptor mRNA expression in various porcine tissues

RT-PCR technique was used to assess the expression of 5-HT$_{1B}$ receptor in various porcine tissues. As shown in Figure 2.8, a fragment of expected size (approximately 180 bp), representing porcine 5-HT$_{1B}$ receptor mRNA, was detected in the brain (cortex and cerebellum), trigeminal ganglion, heart (left ventricle) and blood vessels (left anterior descending coronary, pulmonary, common carotid, superior mesenteric and femoral arteries and saphenous vein).



M$_1$: pGEM marker
1: Brain cortex
2: Cerebellum
3. Trigeminal ganglion
4: Left ventricle
5: LAD coronary artery
6: Pulmonary artery
7: Carotid artery
8: Mesenteric artery
9: Femoral artery
10: Saphenous vein
11: Brain cortex without RT
12: Autoclaved water
M$_2$: ϕx174 DNA/Hae III marker

Figure 2.8. Agarose gel electophoresis of PCR amplified products derived from cDNA obtained from a number of porcine tissues. The size of marker bands is indicated in margins. LAD, left anterior descending; RT, reverse transcriptase. No such fragment was found in the negative controls (autoclaved water or brain cortex in the absence of reverse transcriptase step) run simultaneously during PCR, thus ruling out the possibility of genomic DNA and/or PCR contamination.

In situ hybridisation

In situ hybridisation technique was used to localise the expression pattern of 5-HT$_{1B}$ receptor mRNA in porcine frontal cerebral cortex.

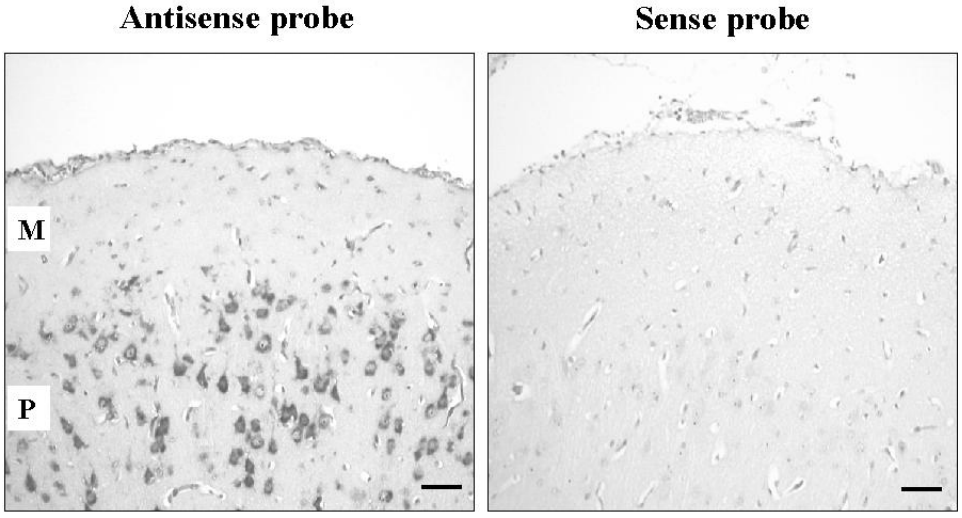


Figure 2.9. Localisation of 5-HT$_{1B}$ receptor mRNA in the frontal part of porcine cerebral cortex by *in situ* hybridisation. *Left panel*: photomicrograph showing the expression of 5-HT$_{1B}$ receptor mRNA with the antisense DIG-labelled cRNA probe (dark spots). *Right panel*: photomicrograph showing no hybridisation signals with the sense probe. M, Molecular layer; P, pyramidal layer. Scale bar = 50 μm.

Using the antisense DIG-labelled cRNA probe, the 5-HT$_{1B}$ receptor mRNA was clearly visualised in the pyramidal cells and weak signals were also observed in the outer molecular layer (Figure 2.9, *left panel*). The specificity of the mRNA signals was confirmed with the sense probe, which did not show any signal in the cerebral cortex (Figure 2.9, *right panel*).

Discussion

Sequence analysis of porcine 5-HT$_{1B}$ receptor

The specific primer sequences of the full-length porcine 5-HT$_{1B}$ receptor were identified by inverse PCR on ligated genomic DNA and used for amplification of the full-length porcine 5-HT$_{1B}$ receptor cDNA from cerebral cortex. The nucleotide sequence of porcine 5-HT$_{1B}$ receptor cDNA, which completely matched with a partial sequence submitted to GenBank by T. Wurch and colleagues (accession number: Y11867), revealed 1170 bp encoding an open reading frame of 390 amino acid peptide. This peptide showed a high homology (88-95%) with the sequence of 5-HT$_{1B}$ receptors in other species (Hamblin *et al*., 1992; Harwood *et al*., 1995; Jin *et al*., 1992; Maroteaux *et al*., 1992; Voigt *et al*., 1991; Zgombick *et al*., 1997). The homology between the cloned porcine and human 5-HT$_{1B}$ receptors (Hamblin *et al*., 1992; Jin *et al*., 1992) was the highest (95%) and there was a total identity in transmembrane domains 2-5. It is to be noted that the presence of the amino acid threonine at position 355 within the seventh transmembrane domain of the porcine 5-HT$_{1B}$ receptor, rather than asparagine at the corresponding position 351 in the mouse (Maroteaux *et al*., 1992) and rat (Voigt *et al*., 1991), was identical to the human (Hamblin *et al*., 1992; Jin *et al*., 1992), rabbit (Harwood *et al*., 1995) and guinea pig (Zgombick *et al*., 1997) 5-HT$_{1B}$ receptors. Furthermore, the presence of putative asparagine-linked glycosylation and protein kinase A and C phoshorylation sites were consistent with the previously cloned receptors from other species (Harwood *et al*., 1995; Jin *et al*., 1992).

As observed in the porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000), with which there was an overall 61% amino acid identity, the porcine 5-HT$_{1B}$ receptor showed some unique amino acids (Ala^{4}, Ser^{15,21} and Arg^{19} towards the N-terminal end, Val^{51} and Ile^{54} in the first transmembrane domain, Leu^{277} in the third intracellular loop, Ala^{336} in the sixth transmembrane domain as well as Phe^{372} in the seventh transmembrane domain). These divergent amino acids were reconfirmed in the inverse PCR sequence derived from genomic DNA. Furthermore, it was most likely the presence of Ala^{4} that interfered with the amplification of full-length porcine 5-HT$_{1B}$ receptor cDNA based on primers designed from other species and necessitated the alternative cloning strategy used here (see Methods section).

Ligand binding properties of porcine 5-HT$_{1B}$ receptor

It is well known that the pharmacological profile of the rodent and human 5-HT$_{1B}$ receptor differs substantially (Hoyer *et al*., 1994; Metcalf *et al*., 1992; Oksenberg *et al*., 1992). Our recent investigation also revealed that BRL15572, a selective antagonist at human 5-HT$_{1D}$ receptor (Price *et al*., 1997; Schlicker *et al*., 1997), unexpectedly exhibited a low affinity at the recombinant porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000). Thus, for a pathophysiological animal model to be of value in the evaluation of new drugs it is imperative that the pharmacology of the candidate receptor in the specific animal species must be comparable to that of the human receptor homologue.

Membranes prepared from COS-7 cells transfected with the porcine 5-HT$_{1B}$ receptor showed a high affinity and saturable binding for the 5-HT$_{1B/1D}$ receptor radioligand [^{3}H]GR125743. Ligand displacement studies established that the pharmacological profile of the porcine 5-HT$_{1B}$ receptor and the affinity rank order for agonists (L694247 > ergotamine ≥ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan) and putative antagonists (GR127935 > methiothepin > SB224289 >> ritanserin > ketanserin ≥ BRL15572) were close to those described for the recombinant human 5-HT$_{1B}$ receptor (Pauwels *et al*., 1996a). It may be emphasised that, unlike the low affinity of BRL15572 at the porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000), the selective 5-HT$_{1B}$ receptor antagonist SB224289 (Gaster *et al*., 1998; Hagan *et al*., 1997; Selkirk *et al*., 1998) did have a high affinity at the recombinant porcine 5-HT$_{1B}$ receptor. As expected, BRL15572 showed a low affinity for the porcine 5-HT$_{1B}$ receptor, but this compound cannot be used to differentiate between porcine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes. In addition, ketanserin differentiated between porcine 5-HT$_{1B}$ (pK_i: 5.78±0.12) and 5-HT$_{1D}$ (pK_i: 7.42±0.04) receptors showing a 40-fold selectivity for the 5-HT$_{1D}$ over 5-HT$_{1B}$ receptor (present results, Bhalla *et al*., 2000). It is known that ketanserin differentiates between recombinant human, guinea pig, rabbit and rat 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes (Bard *et al*., 1996; Beer *et al*., 1998; Harwood *et al*., 1995; Kaumann *et al*., 1994; Zgombick *et al*., 1997), but not the canine receptors (Wurch *et al*., 2000; Zgombick *et al*., 1991).

The correlation between the affinity constants (as well as the associated slope) for the porcine and human 5-HT$_{1B}$ receptors was higher than for the porcine and rat 5-HT$_{1B}$ receptors (Figure 2.5). Although we recognise that the affinity constants for the rat 5-HT$_{1B}$ receptor were obtained using [^{3}H]5-HT (not [^{3}H]GR125743 as in pig) and β-adrenoceptor antagonists were not evaluated, its molecular basis may be the presence of threonine at position 355 in the seventh transmembrane domain of porcine and human 5-HT$_{1B}$ receptors, rather than asparagine at the corresponding position in rodent receptors (Oksenberg *et al*., 1992). The correlation between the porcine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors for binding affinity of ligands was not so high, as observed earlier for the cloned human and guinea pig receptors (Weinshank *et al*., 1992; Zgombick *et al*., 1997).

Functional characterisation of porcine 5-HT$_{1B}$ receptor

Functional properties of recombinant 5-HT$_{1B}$ receptors have been established using predominantly cellular responses employing cAMP and [^{35}S]GTPγS binding assays (Pauwels *et al*., 1996b; Pauwels *et al*., 1997). Using membranes from cells transiently co-expressing the porcine 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein, we showed that 5-HT (10 μM) increased basal [^{35}S]GTPγS binding by 123% over the basal levels and that zolmitriptan behaved as a near full agonist in this respect. This result is in agreement with earlier observations in the recombinant human 5-HT$_{1B}$ receptor (Pauwels *et al*., 1997). Moreover, as can be expected, the potency of zolmitriptan in the [^{35}S]GTPγS binding assay (pEC_{50}: 7.64±0.04; Figure 2.7) closely agreed with its affinity in the ligand binding assay (pK_i: 7.36±0.07; Table 2.1). Finally, the 5-HT$_{1B}$ receptor antagonist SB224289 (Gaster *et al*., 1998; Hagan *et al*., 1997; Selkirk *et al*., 1998) inhibited basal [^{35}S]GTPγS binding, thus exhibiting a negative efficacy (inverse agonism), as noticed earlier using the recombinant human 5-HT$_{1B}$ receptor (Selkirk *et al*., 1998).

Localisation of porcine 5-HT$_{1B}$ receptor

Ullmer (1995) has previously reported that the 5-HT$_{1B}$ receptor mRNA is expressed in all blood vessels examined, both in the rat (aorta, renal, artery, vena cava and portal, femoral and jugular veins) and pig (coronary, cerebral and pulmonary arteries and cerebral vein). The present study also shows the ubiquitous presence of the 5-HT$_{1B}$ receptor mRNA in various porcine blood vessels. The presence of 5-HT$_{1B}$ receptor

mRNA indicates that the vasoconstrictor property of 5-HT$_{1B}$ receptor ligands, such as the triptans, is mediated by the 5-HT$_{1B}$ receptor. Indeed, sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses is antagonised by the selective 5-HT$_{1B}$ receptor antagonist SB224289 (De Vries *et al*., 1999). However, it may be pointed out that despite the expression of 5-HT$_{1B}$ receptor mRNA, sumatriptan fails to contract the porcine coronary artery (Humphrey *et al*., 1988; Humphrey *et al*., 1990). Interestingly, sumatriptan can reduce coronary blood flow in pigs fed a high cholesterol diet (Saxena, unpublished observations). It is therefore possible that that hypercholesterolaemia either increases the density of 5-HT$_{1B}$ receptors or improves their coupling with G-proteins.

The 5-HT$_{1B}$ receptor mRNA was also observed in porcine trigeminal ganglion, as is the case in the human trigeminal ganglion (Bouchelet *et al*., 1996; Bruinvels *et al*., 1992). Since no studies exploring neural effects of triptans have been performed in the pig, we do not know whether or not the neural effects of triptans (Goadsby *et al*., 1997; Saxena *et al*., 2000) are mediated by this receptor. Porcine frontal cortex and cerebellum also showed the presence of the 5-HT$_{1B}$ receptor mRNA and *in situ* hybridisation studies revealed mRNA expression mainly in the pyramidal neurones of the frontal cerebral cortex. The 5-HT$_{1B}$ receptor is widely distributed in the brain and it may serve as an autoreceptor inhibiting transmitter release (Bonaventure *et al*., 1998; Marcoli *et al*., 1999).

In conclusion, we have established the cDNA sequence of recombinant porcine 5-HT$_{1B}$ receptor, which shows a high homology with other species homologues. The pharmacological profile of the recombinant porcine 5-HT$_{1B}$ receptor is quite similar to that of the human 5-HT$_{1B}$ receptor. The cloned porcine 5-HT$_{1B}$ receptor is also functionally active as observed by [^{35}S]GTPγS binding and is ubiquitously expressed in blood vessels and brain tissues.

Acknowledgements

We thank Ms. Emine Yilmaz for her assistance in *in situ* hybridisation.

References

BARD, J.A., KUCHAREWICZ, S.A., ZGOMBICK, J.M., WEINSHANK, R.L., BRANCHEK, T.A. & COHEN, M.L. (1996). Differences in ligand binding profiles between cloned rabbit and human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors: ketanserin and methiothepin distinguish rabbit 5-HT$_{1D}$ receptor subtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 237-244.

BEER, M.S., HEALD, M.A., MCALLISTER, G. & STANTON, J.A. (1998). Pharmacological characterisation of a cloned dog 5-HT$_{1B}$ receptor cell line. *Eur. J. Pharmacol.*, **360,** 117-121.

BHALLA, P., SHARMA, H.S., WURCH, T., PAUWELS, P.J. & SAXENA, P.R. (2000). Molecular Cloning, Sequence analysis and pharmacological properties of the porcine 5-HT$_{1D}$ receptor. *Br. J. Pharmacol.*, **131,** 949-957.

BONAVENTURE, P., VOORN, P., LUYTEN, W.H., JURZAK, M., SCHOTTE, A. & LEYSEN, J.E. (1998). Detailed mapping of serotonin 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor messenger RNA and ligand binding sites in guinea-pig brain and trigeminal ganglion: clues for function. *Neuroscience*, **82,** 469-484.

BOUCHELET, I., COHEN, Z., CASE, B., SEGUELA, P. & HAMEL, E. (1996). Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. *Mol. Pharmacol.*, **50,** 219-223.

BRUINVELS, A.T., LANDWEHRMEYER, B., MOSKOWITZ, M.A. & HOYER, D. (1992). Evidence for the presence of 5-HT$_{1B}$ receptor messenger RNA in neurons of the rat trigeminal ganglia. *Eur. J. Pharmacol.*, **227,** 357-359.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P., VILLALÓN, C.M. & SAXENA, P.R. (1999). Investigation of the role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. *Br. J. Pharmacol.*, **127,** 405-412.

GASTER, L.M., BLANEY, F.E., DAVIES, S., DUCKWORTH, D.M., HAM, P., JENKINS, S., JENNINGS, A.J., JOINER, G.F., KING, F.D., MULHOLLAND, K.R., WYMAN, P.A., HAGAN, J.J., HATCHER, J., JONES, B.J., MIDDLEMISS, D.N., PRICE, G.W., RILEY, G., ROBERTS, C., ROUTLEDGE, C., SELKIRK, J. & SLADE, P.D. (1998). The selective 5-HT$_{1B}$ receptor inverse agonist 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydro-spiro[furo[2,3-f]indole-3,4'-piperidine] (SB-224289) potently blocks terminal 5-HT autoreceptor function both in vitro and in vivo. *J. Med. Chem.*, **41,** 1218-1235.

GOADSBY, P.J. & KNIGHT, Y. (1997). Inhibition of trigeminal neurones after intravenous administration of naratriptan through an action at 5-hydroxytryptamine 5-HT$_{1B/1D}$ receptors. *Br. J. Pharmacol.*, **122,** 918-922.

HAGAN, J.J., SLADE, P.D., GASTER, L., JEFFREY, P., HATCHER, J.P. & MIDDLEMISS, D.N. (1997). Stimulation of 5-HT$_{1B}$ receptors causes hypothermia in the guinea pig. *Eur. J. Pharmacol.*, **331,** 169-174.

HAMBLIN, M.W., METCALF, M.A., MCGUFFIN, R.W. & KARPELLS, S. (1992). Molecular cloning and functional characterization of a human 5-HT$_{1B}$ serotonin receptor: a homologue of the rat 5-HT$_{1B}$ receptor with 5-HT$_{1D}$-like pharmacological specificity. *Biochem. Biophys. Res. Commun.*, **184,** 752-759.

HARWOOD, G., LOCKYER, M., GILES, H. & FAIRWEATHER, N. (1995). Cloning and characterisation of the rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors. *FEBS Lett.*, **377,** 73-76.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 157-203.

HUMPHREY, P.P., FENIUK, W., PERREN, M.J., CONNOR, H.E., OXFORD, A.W., COATES, L.H. & BUTINA, D. (1988). GR43175, a selective agonist for the 5-HT$_1$-like receptor in dog isolated saphenous vein. *Br. J. Pharmacol.*, **94,** 1123-1132.

HUMPHREY, P.P.A., APPERLEY, E., FENIUK, W. & PERREN, M.J. (1990). A rational approach to identifying a fundamentally new drug for the treatment of migraine. In *Cardiovascular pharmacology of 5-hydroxytryptamine: prospective therapeutic applications*. ed. Saxena, P.R., Wallis, D.I., Wouters, W. & Bevan, P. pp. 416-431. Dordrecht: Kluwer academic publishers.

JIN, H., OKSENBERG, D., ASHKENAZI, A., PEROUTKA, S.J., DUNCAN, A.M., ROZMAHEL, R., YANG, Y., MENGOD, G., PALACIOS, J.M. & O'DOWD, B.F. (1992). Characterization of the human 5-hydroxytryptamine$_{1B}$ receptor. *J. Biol. Chem.*, **267,** 5735-5738.

KAUMANN, A.J., FRENKEN, M., POSIVAL, H. & BROWN, A.M. (1994). Variable participation of 5-HT$_1$-like receptors and 5-HT$_2$ receptors in serotonin-induced contraction of human isolated coronary arteries. 5-HT$_1$-like receptors resemble cloned 5-HT$_{1D\beta}$ receptors. *Circulation*, **90,** 1141-1153.

MARCOLI, M., MAURA, G., MUNARI, C., RUELLE, A. & RAITERI, M. (1999). Pharmacological diversity between native human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors sited on different neurons and involved in different functions. *Br. J. Pharmacol.*, **126,** 607-612.

MAROTEAUX, L., SAUDOU, F., AMLAIKY, N., BOSCHERT, U., PLASSAT, J.L. & HEN, R. (1992). Mouse 5HT$_{1B}$ serotonin receptor: cloning, functional expression, and localization in motor control centers. *Proc. Natl. Acad. Sci. USA*, **89,** 3020-3024.

METCALF, M.A., MCGUFFIN, R.W. & HAMBLIN, M.W. (1992). Conversion of the human 5-HT$_{1D\beta}$ serotonin receptor to the rat 5-HT$_{1B}$ ligand-binding phenotype by Thr^{355}Asn site directed mutagenesis. *Biochem. Pharmacol.*, **44,** 1917-1920.

OKSENBERG, D., MARSTERS, S.A., O'DOWD, B.F., JIN, H., HAVLIK, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT$_{1B}$ receptors. *Nature*, **360,** 161-163.

PAUWELS, P.J. & COLPAERT, F.C. (1996a). Selective antagonism of human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptor-mediated responses in stably transfected C6-glial cells by ketanserin and GR 127,935. *Eur. J. Pharmacol.*, **300,** 141-145.

PAUWELS, P.J., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1996b). Pharmacology of cloned human 5-HT$_{1D}$ receptor-mediated functional responses in stably transfected rat C6-glial cell lines: further evidence differentiating human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353,** 144-156.

PAUWELS, P.J., TARDIF, S., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1997). How efficacious are 5-HT$_{1B/D}$ receptor ligands: an answer from GTP gamma S binding studies with stably transfected C6-glial cell lines. *Neuropharmacology*, **36,** 499-512.

PRICE, G.W., BURTON, M.J., COLLIN, L.J., DUCKWORTH, M., GASTER, L., GOTHERT, M., JONES, B.J., ROBERTS, C., WATSON, J.M. & MIDDLEMISS, D.N. (1997). SB-216641 and BRL-15572 - compounds to pharmacologically discriminate h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 312-320.

SAXENA, P.R. & TFELT-HANSEN, P. (2000). Triptans, 5-HT$_{1B/1D}$ receptor agonists in the acute treatment of migraine. In *The headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 411-438. Philadelphia: Lippincott Williams & Wilkins.

SCHLICKER, E., FINK, K., MOLDERINGS, G.J., PRICE, G.W., DUCKWORTH, M., GASTER, L., MIDDLEMISS, D.N., ZENTNER, J., LIKUNGU, J. & GÖTHERT, M. (1997). Effects of selective h5-HT$_{1B}$ (SB-216641) and h5-HT$_{1D}$ (BRL-15572) receptor ligands on guinea-pig and human 5-HT auto- and heteroreceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 321-327.

SELKIRK, J.V., SCOTT, C., HO, M., BURTON, M.J., WATSON, J., GASTER, L.M., COLLIN, L., JONES, B.J., MIDDLEMISS, D.N. & PRICE, G.W. (1998). SB-224289 - a novel selective (human) 5-HT$_{1B}$ receptor antagonist with negative intrinsic activity. *Br. J. Pharmacol.*, **125,** 202-208.

ULLMER, C., SCHMUCK, K., KALKMAN, H.O. & LÜBBERT, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *FEBS Lett.*, **370,** 215-221.

VOIGT, M.M., LAURIE, D.J., SEEBURG, P.H. & BACH, A. (1991). Molecular cloning and characterization of a rat brain cDNA encoding a 5- hydroxytryptamine$_{1B}$ receptor. *Embo J.*, **10,** 4017-4023.

WEINSHANK, R.L., ZGOMBICK, J.M., MACCHI, M.J., BRANCHEK, T.A. & HARTIG, P.R. (1992). Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$. *Proc. Natl. Acad. Sci. USA*, **89,** 3630-3634.

WURCH, T. & PAUWELS, P.J. (2000). Coupling of canine serotonin 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes to the formation of inositol phosphates by dual interactions with endogenous G$_{i/o}$ and recombinant G$_{\alpha 15}$ proteins. *J. Neurochem.*, **75,** 1180-1189.

ZGOMBICK, J.M., BARD, J.A., KUCHAREWICZ, S.A., URQUHART, D.A., WEINSHANK, R.L. & BRANCHEK, T.A. (1997). Molecular cloning and pharmacological characterization of guinea pig 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. *Neuropharmacology*, **36,** 513-524.

ZGOMBICK, J.M., WEINSHANK, R.L., MACCHI, M., SCHECHTER, L.E., BRANCHEK, T.A. & HARTIG, P.R. (1991). Expression and pharmacological characterization of a canine 5-hydroxytryptamine$_{1D}$ receptor subtype. *Mol. Pharmacol.*, **40,** 1036-1042.

Chapter 3

Molecular cloning, sequence analysis and pharmacological properties of the porcine 5-HT_{1D} receptor

Summary A cDNA encoding the full-length 5-HT_{1D} receptor derived from porcine cerebral cortex was amplified, cloned and sequenced, using guinea pig 5-HT_{1D} receptor coding sequence oligonucleotide primers in reverse transcription-polymerase chain reaction (RT-PCR). The 5' and 3' ends of the porcine 5-HT_{1D} receptor cDNA were verified by inverse PCR. Sequence analysis of porcine 5-HT_{1D} receptor cDNA revealed an open reading frame of 1134 nucleotides encoding a polypeptide of 377 amino acids having 92% homology with the human 5-HT_{1D} receptor and 88-90% homology with other species homologues. The porcine 5-HT_{1D} receptor cDNA was further subcloned into a mammalian expression vector pcDNA3 and expressed in monkey Cos-7 cells. Radioligand binding assays using either [^{3}H]5-CT or [^{3}H]GR125743 on Cos-7 cell membranes showed that pK_i values of 14 serotonin ligands were highly correlated with those obtained with the human 5-HT_{1D} receptor. Nonetheless, a selective antagonist at the human 5-HT_{1D} receptor, BRL15572, only poorly recognised the porcine homologue. Using membranes from cells co-expressing the porcine 5-HT_{1D} receptor and rat $G_{\alpha i1}Cys^{351}Ile$ protein, it was shown that 5-HT and zolmitriptan increased, while ketanserin decreased basal [^{35}S]GTPγS binding. The potency of zolmitriptan in the [^{35}S]GTPγS binding assay (pEC_{50}: 8.46±0.08) agreed with its affinity in displacing the ligands [^{3}H]5-CT and [^{3}H]GR125743 (pK_i: 8.38±0.15 and 8.67±0.08, respectively). In conclusion, we have established the cDNA sequence and pharmacology of the cloned porcine 5-HT_{1D} receptor. This information would be useful in exploring the role of divergent amino acid residues in the receptor-ligand interaction as well as the role of 5-HT_{1D} receptor in pathophysiological processes relevant for novel drug discovery in diseases such as migraine.

Based on: Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2000). Molecular cloning, sequence analysis and pharmacological properties of the porcine 5-HT_{1D} receptor. *Br. J. Pharmacol.*, **131**, 949-957

Introduction

5-Hydroxytryptamine (5-HT; serotonin) has long been suspected to be involved in the pathophysiology of migraine, but the exact nature of its role is even today not clear (Hamel *et al*., 2000). Nevertheless, pharmacological characterisation of 5-HT receptors was instrumental in the design of the antimigraine drug sumatriptan (Humphrey *et al*., 1988; Humphrey *et al*., 1990; Saxena *et al*., 1992), an agonist at a novel, but heterogeneous group of 5-HT receptors, then called "5-HT$_1$-like" (Bradley *et al*., 1986). The term "5-HT$_1$-like" has since become redundant (Saxena *et al*., 1998), because this group has been shown to comprise of receptors where sumatriptan has either a high (5-HT$_{1B}$, 5-HT$_{1D}$ and 5-ht$_{1F}$) or low (5-HT$_7$) affinity (Hoyer *et al*., 1994; Peroutka *et al*., 1989; Waeber *et al*., 1990).

Sumatriptan as well as the second-generation triptans potently constrict isolated cranial blood vessels and decrease arteriovenous anastomotic fraction of carotid blood flow in anaesthetised animals. These drugs also inhibit dural plasma protein extravasation and action potentials in trigeminal nucleus caudalis following stimulation of the trigeminal ganglion and superior sagittal sinus, respectively (Edvinsson *et al*., 1998; Moskowitz, 1992; Saxena *et al*., 2000). Molecular and pharmacological studies have convincingly shown that the vasoconstrictor effect of triptans is mediated via 5-HT$_{1B}$, but not 5-HT$_{1D}$ or 5-ht$_{1F}$ receptors (Bouchelet *et al*., 2000; Bouchelet *et al*., 1996; Cohen *et al*., 1999; De Vries *et al*., 1998; De Vries *et al*., 1999b; Nilsson *et al*., 1999; Verheggen *et al*., 1998). On the other hand, the trigeminal neural effects of triptans seem to involve primarily the 5-HT$_{1D}$ receptor, although 5-HT$_{1B}$ and 5-ht$_{1F}$ receptors have also been implicated (De Vries *et al*., 1999a; Hargreaves *et al*., 1999; Hoyer *et al*., 1990; Longmore *et al*., 1997; McCall, 1997; Mitsikostas *et al*., 1999; Wainscott *et al*., 1998). The antimigraine efficacy of triptans has, therefore, been attributed to their ability to constrict large intracranial arteries and arteriovenous anastomoses via the 5-HT$_{1B}$ receptor and to the inhibition of peripheral trigeminal sensory nerve terminals in the meninges and central terminals in brain stem sensory nuclei via the 5-HT$_{1D}$ receptor (Hargreaves *et al*., 1999; Saxena *et al*., 2000).

Previous investigations from our laboratory have established that constriction of carotid arteriovenous anastomoses in the anaesthetised pig can serve as a predictive

model for the antimigraine efficacy of 5-HT-based drugs (De Vries *et al*., 1999a; De Vries *et al*., 1999c; Saxena, 1995). To gain further insight into the mechanisms involved in drug actions as well as the disease, it is important to study the trigeminal neural control of porcine arteriovenous anastomoses and its potential modification by 5-HT_{1D} receptor ligands. However, one of the difficulties in undertaking such studies is the lack of knowledge of molecular biology of porcine 5-HT_{1B} and 5-HT_{1D} receptors. Although we (De Vries *et al*., 1999b) have employed 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) and BRL15572 ([1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl) piperazine] hydrochloride), that have been shown to be selective antagonists for the human 5-HT_{1B} and 5-HT_{1D} receptors (Gaster *et al*., 1998; Price *et al*., 1997; Schlicker *et al*., 1997; Selkirk *et al*., 1998), respectively, it is difficult to be sure that these compounds also have a high and selective affinity at the respective porcine receptor.

In the present investigation, we describe the molecular cloning and characterisation of the porcine 5-HT_{1D} receptor (R.C.: 2.1.5HT.01D). Using the total RNA derived from the pig cerebral cortex, a full-length cDNA encoding 5-HT_{1D} receptor was amplified and the deduced amino acid sequence was compared with those in the other species. The pharmacological profile of the porcine 5-HT_{1D} receptor was evaluated after transient transfection in Cos-7 cells and compared with that of the recombinant human 5-HT_{1D} receptor. A part of these results has been presented to the British Pharmacological Society (Bhalla *et al*., 2000).

Methods

mRNA isolation and cDNA synthesis

Brain cortex obtained from a pig (Yorkshire x Landrace, female, 12 kg) killed after an acute haemodynamic experiment was snap frozen in liquid nitrogen and stored at -80°C. The frozen tissue was transferred to guanidium thiocyanate buffer, homogenised (Ultra-Turrax homogeniser, model T8, Janke & Kunkel GmbH, Staufen, Germany) and the total RNA was extracted as described earlier (Chomczynski *et al*., 1987; Sharma *et al*., 1996). The RNA concentration was measured by UV absorbance at 260 nm using a Gene Quant RNA/DNA calculator (Pharmacia-LKB, Biochrom, England) and the quality of RNA was assessed by OD_{260}/OD_{280} ratio of >1.8 as well

as by formaldehyde-agarose gel electrophoresis. Subsequently, poly(A^+) mRNA was purified from the total RNA using an Oligotex mRNA purification Kit (Qiagen GmbH, Hilden, Germany). Poly(A^+) mRNA (0.5 μg) was denatured at 65 °C and the first strand of cDNA was synthesised in a reaction volume of 20 μl by adding sequentially the following reagents: reverse transcription buffer (25 mM Tris-HCl, pH 8.3; 50 mM KCl; 5.0 mM $MgCl_2$, 2.0mM DTT), 1.0 mM dNTPs, ribonuclease inhibitor (1 U μl^{-1}), random hexamer (150 ng μg^{-1} mRNA) and, finally, AMV reverse transcriptase (14 U μg^{-1} mRNA). A control was similarly prepared, except that the AMV reverse transcriptase was omitted. The reactions were carried out for 90 min at 42 °C, extended for another 10 min at 75 °C and then cooled to 4°C. The cDNA thus synthesised was diluted to 50 μl and stored at –20°C until used as a PCR template. The quality of cDNA was checked by PCR amplification of β-actin using specific oligonucleotide primers (Ponte *et al*., 1984).

PCR amplification and cloning of 5-HT$_{1D}$ receptor cDNA

Oligonucleotide primers were designed according to the start and stop codon regions of the guinea pig 5-HT$_{1D}$ receptor gene (Wurch *et al*., 1997; Zgombick *et al*., 1997). The forward and reverse primers were 5'-ATGTCCCCGCCAAACCAGTC-3' and 5'-CTAGGAGGCTTTCCGGAAATG-3', respectively. The following components were added in a reaction volume of 20 μl: 250 μM of each dATP, dTTP, dGTP and dCTP, 1.5 mM $MgCl_2$, PCR buffer (1xPCR buffer: 10mM Tris-HCl, pH 8.3, 50 mM KCl), Ampli Taq Gold^{TM} (0.5 U), 0.5 μM each of the forward and reverse primer and 5 μl of cDNA template. After brief centrifugation, the enzyme was first activated for 10 min at 94 °C in a PCR thermocycler (model PTC-100^{TM}, M.J. Research Inc, Watertown, USA). cDNA was denatured for 1 min at 94 °C and annealed to the primers for 30 s at 60 °C with the reaction extended for 90 s at 72 °C and this procedure was repeated for 36 cycles. Finally, the reaction was extended for additional 10 min at 72 °C. Several independent PCR reactions were performed to exclude possible misincorporation of nucleotides by Ampli Taq for sequence analysis.

The amplified PCR products were separated on 1% agarose gel in TBE buffer (90 mM Tris-HCl, pH 8.0, 90 mM boric acid, 2 mM EDTA) containing ethidium bromide (0.5 μg ml^{-1}), visualised under UV light and photographed. Subsequently,

the PCR product of expected size was purified using a PCR purification kit (Promega Benelux b.v., Leiden, The Netherlands) and the amount of DNA was measured spectrophotometrically. An aliquot of purified PCR product was ligated into the pGEMT-Easy vector (Promega Benelux b.v., Leiden, The Netherlands). The ligated vector was transformed into competent JM 109 cells and grown on IPTG/X-gal-treated ampicillin resistant LB-Agar plates at 37 °C. White over blue colonies were selected to identify positive clones, which were further characterised by the presence of an expected size insert in them. Four insert positive clones (namely, pHTD-11, pHTD-13, pHTD-25, pHTD-27) were further processed for the plasmid DNA isolation and sequencing.

Plasmid purification and sequencing

Bacterial colonies harbouring recombinant plasmids were grown overnight at 37 °C in LB medium containing ampicillin (100 μg ml^{-1}). Bacteria were harvested by centrifugation and the plasmid DNA was isolated using a commercially available midi-prep plasmid isolation kit (Promega Benelux b.v., Leiden, The Netherlands). Purified plasmid DNA from all four clones (pHTD-11, pHTD-13, pHTD-25, pHTD-27) was sequenced by the dideoxy nucleotide chain termination method, using universal forward and reverse sequencing primers. Sequencing reactions were loaded on an automated fluorescence based DNA sequencer (ABI Prism™ 310 Genetic analyser, Perkin Elmer Applied Biosystem Benelux, Nieuwerkerk a/d Ijssel, The Netherlands) and the raw sequence data were processed and analysed. The nucleotide sequences thus obtained were compared and a consensus sequence was derived 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$_{1D}$ receptors from other species were established.

Inverse PCR

Since the forward and reverse oligonucleotide primers used in RT-PCR were designed from the guinea pig 5-HT$_{1D}$ receptor sequence, we identified the 5' and 3' ends of our cloned porcine cDNA by inverse PCR (Ochman *et al*., 1988). Porcine genomic DNA

was digested with EcoR1 restriction enzyme, as the cloned 5-HT_{1D} receptor cDNA did not show any restriction site for EcoR1. 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 porcine specific inverse primers, the ligated DNA fragments, as circles of different sizes in various dilutions, were subjected to PCR amplification. The internal oligonucleotide inverse primers, designed on the basis of porcine 5-HT_{1D} receptor cDNA sequence, were 5'-GCATTGGAAAGGACAGTGGC-3' (for 5' end) and 5'-TCATCTGCTGGTTGCCCTTC-3' (for 3' end). The PCR products were separated on a 1% agarose gel, purified, cloned and sequenced as described above.

Transient transfection and ligand receptor binding assay

The purified full-length pig 5-HT_{1D} receptor cDNA insert was subcloned into dephosphorylated eukaryotic expression vector, pcDNA3 (Invitrogen, San Diego, Ca, USA) and transformed into TOP10 competent cells. The clones containing the insert were selected and screened with restriction enzyme (HindIII and EcoR1) for appropriate orientation. The plasmid DNA was purified according to maxi prep protocol using a commercially available kit (Qiagen SA, Courtaboeuf, France). Monkey Cos-7 cells were transiently transfected with the plasmid using a gene pulser transfection apparatus (Bio-Rad S.A., Ivry Sur Seine, France), as described earlier by Pauwels *et al*. (1996). After transfection, the cells were incubated for 48 h in Dulbecco modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal bovine serum and antibiotics at 37 °C in a humidified chamber containing 5% CO_2.

The transfected cells were washed twice with phosphate buffer saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_4; pH 7.2) and kept at -80 °C for 10 min. The cells were scrapped from the petri-dish in ice-cold 50 nM Tris-HCl buffer (pH: 7.7) and homogenised. The homogenate was centrifuged at 1,000 rpm for 5 min at 4 °C and the supernatant was collected and centrifuged again at 13,000 rpm for 20 min. The membrane pellet was resuspended into 50 mM Tris-HCl buffer (pH: 7.7) containing 4 mM $CaCl_2$, 10 µM pargyline and 0.1 % ascorbic acid, as described before (Pauwels *et al*., 1996). The membrane protein concentration was measured by dye binding assay (Bradford, 1976) using a Bio-Rad Kit and bovine serum albumin as a standard.

Binding assays to membranes obtained from transfected Cos-7 cells were performed using either 3.0 nM [^{3}H]GR125743 ([^{3}H]N-[4-methoxy-3-(4-methyl piperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide) or 1.0 nM [^{3}H]5-carboxamidotryptamine ([^{3}H]5-CT) as radioligands. Incubation mixtures consisted of 0.40 ml of cell membrane preparation (30-50 μg of protein), 0.05 ml of one of the radioligands and 0.05 ml of compounds for inhibition or 10 μM 5-HT to determine non-specific binding. The reaction was terminated by filtration with ice-cold Tris-buffer and radioactivity on the filter paper was measured by using a liquid scintillation counter (Pauwels *et al*., 1996; Wurch *et al*., 1997). In case of [^{3}H]GR125743, the filtration was performed over 0.2% polyethyleneimine-treated Whatman (Clifton, NJ, USA) GF/B glass fibre filters. Data were analysed graphically with inhibition curves, and IC_{50} values were derived. 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. Ligand saturation binding curves were analysed by the nonlinear least square curve-fitting programme to determine K_d and B_{max} values (Munson *et al*., 1980). Control binding experiments were run with nontransfected cells and did not display specific [^{3}H]5-CT or [^{3}H]GR125743 binding.

[^{35}S]GTPγS binding

CHO-K1 cells co-expressing the porcine 5-HT$_{1D}$ receptor and mutant $G_{\alpha i1}Cys^{351}Ile$ protein (Dupuis *et al*., 1999) were collected in phosphate-buffered-saline (pH 7.4) and centrifuged for 20 min at 48,000 g and the pellet containing the membrane fraction was stored at -80 °C. [^{35}S]GTPγS binding was measured using the method previously described by Pauwels *et al*. (1997). Briefly, the pellet was thawed and diluted in 20 mM HEPES buffer (pH 7.4) containing 30 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ascorbic acid. Incubation mixtures were prepared in glass tubes and consisted of 0.4 ml of membrane preparation (containing 5 μg protein) with 5-HT (10 μM), ketanserin (10 μM) or zolmitriptan (0.1-10 μM) in a volume of 0.05 ml. After an incubation period of 30 min at 25 °C, 0.05 ml [^{35}S]GTPγS (0.5 nM) was added for an additional period of 30 min. The reaction was stopped by adding 3 ml of ice-cold 20 mM HEPES (pH 7.4) containing 3 mM $MgCl_2$ and rapid filtration over Whatmann GF/B glass fibre filters with a Brandel harvester. The filters were rinsed

three additional times with 3 ml HEPES buffer, placed in scintillation vials and the radioactivity was extracted in 4 ml of Emulsifier-Safe. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. Maximal stimulation of [^{35}S]GTPγS binding was defined in the presence of 10 μM 5-HT. E_{max} values were expressed as a percentage of the maximal response obtained with 10 μM 5-HT. EC_{50} values were defined as the concentration of compound at which 50% of its own maximal stimulation was obtained.

Materials

All oligonucleotide primers were commercially procured from Life Technologies b.v. (Breda, The Netherlands). pGEMT-Easy vector system, Wizard® PCR prep and mini-prep DNA purification systems were purchased from Promega Benelux b.v. (Leiden, The Netherlands). AmpliTaqGold and dye terminator/cycle sequencing ready reaction kit were procured from Perkin Elmer Applied Biosystem Benelux (Nieuwerkerk a/d Ijssel, The Netherlands). Oligotex mRNA purification kit was purchased from Qiagen GmbH (Hilden, Germany). Guanidinium thiocyanate was purchased from U.S. Biochemicals (Cleveland, OH, USA). AMV-Reverse transcriptase enzyme was obtained from Pharmacia-LKB (Uppsala, Sweden). All other chemicals used in this study were of molecular biology and/or tissue culture grade. The compounds used in pharmacological assays were: 5-HT creatinine sulphate (Sigma Chemicals, St. Louis, MO, USA), [^{3}H]5-CT (56.5 Ci mmol^{-1}, New England Nuclear, Les Ulis, France), BRL15572, CP122638 (N-methyl-3-[pyrrolidin-2(R)-yl-methyl]-1H-indol-5-ylmethyl sulphonamide), [^{3}H]GR125743 (83.0 Ci mmol^{-1}; Amersham, Les Ulis, France), 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, ketanserin (Sigma Chemicals, St. Louis, MO, USA), L694247 (2-[5-[3-(4-methylsulphonylamino) benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl] ethylamine), methiothepin, ritanserin, sumatriptan, SB224289 and zolmitriptan. Except BRL15572 (gift: Dr. A.A. Parsons (SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK) and L694247 (Tocris Cookson, Bristol, UK), all compounds were synthesised at Centre de Recherche Pierre Fabre (Castres, France).

Results

Cloning of 5-HT$_{1D}$ receptor cDNA

Figure 3.1 shows the gel electrophoresis of RT-PCR products of cDNA prepared from the poly(A^+) mRNA of porcine brain cortex used for the amplification of 5-HT$_{1D}$ receptor. The quality of cDNA was established by detecting a DNA fragment of expected size (625 bp) encoding β-actin cDNA.

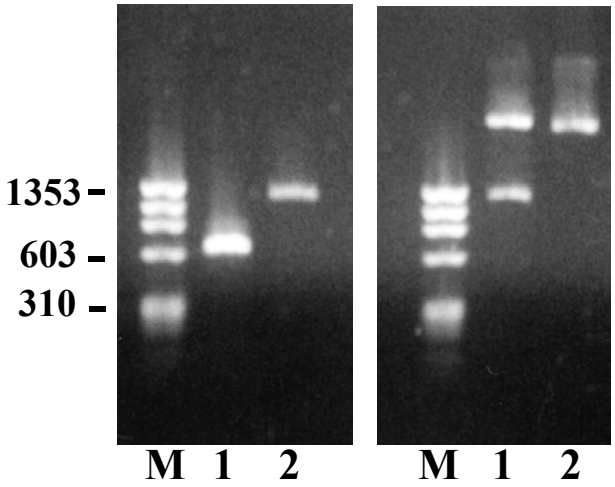


Figure 3.1. *Left panel.* Agarose gel electrophoresis of RT-PCR products of cDNA synthesised from porcine brain cortex (see Methods). M, ϕx174 DNA/Hae III marker; Lanes 1, RT-PCR product of 625 bp using β-actin primers; Lane 2, RT-PCR product of approximately 1200 bp obtained using forward and reverse primers of 5-HT$_{1D}$ receptors. A control without template cDNA (in absence of reverse transcriptase) did not show any signal (not included in the figure). *Right panel*. Agarose gel electrophoresis of recombinant plasmid with 5-HT$_{1D}$ receptor cDNA. M, ϕx174 DNA/Hae III marker; Lane 1, recombinant plasmid DNA restricted with EcoR1 enzyme and showing a DNA insert of approximately 1200 bp; Lane 2, non-digested recombinant plasmid DNA. The size (bp) of 3 markers is indicated in the left margin.

Employing this cDNA as a template and oligonucleotide primers designed from the known guinea pig 5-HT$_{1D}$ receptor cDNA sequence (Wurch *et al*., 1997; Zgombick *et al*., 1997), a DNA fragment of expected size (~1140 bp) was amplified by PCR (*left panel*). The PCR amplified product was cloned and the presence of the insert checked with EcoR1 (*right panel*).

Sequence analysis of 5-HT$_{1D}$ receptor

The full-length band disclosed a nucleotide sequence of 1134 bp encoding a 377 amino acid long protein. A BLAST search at the GenBank revealed that the identified

sequence most closely matched with the sequence of 5-HT$_{1D}$ receptor and the software DNAMAN analysis predicted seven hydrophobic transmembrane domains (Figure 3.2). Since porcine 5-HT$_{1D}$ receptor cDNA was amplified using primers based on 5' and 3' ends of guinea pig 5-HT$_{1D}$ receptor sequence (Wurch *et al*., 1997; Zgombick *et al*., 1997), the 5' and 3' terminals of the porcine cDNA were confirmed by inverse PCR.

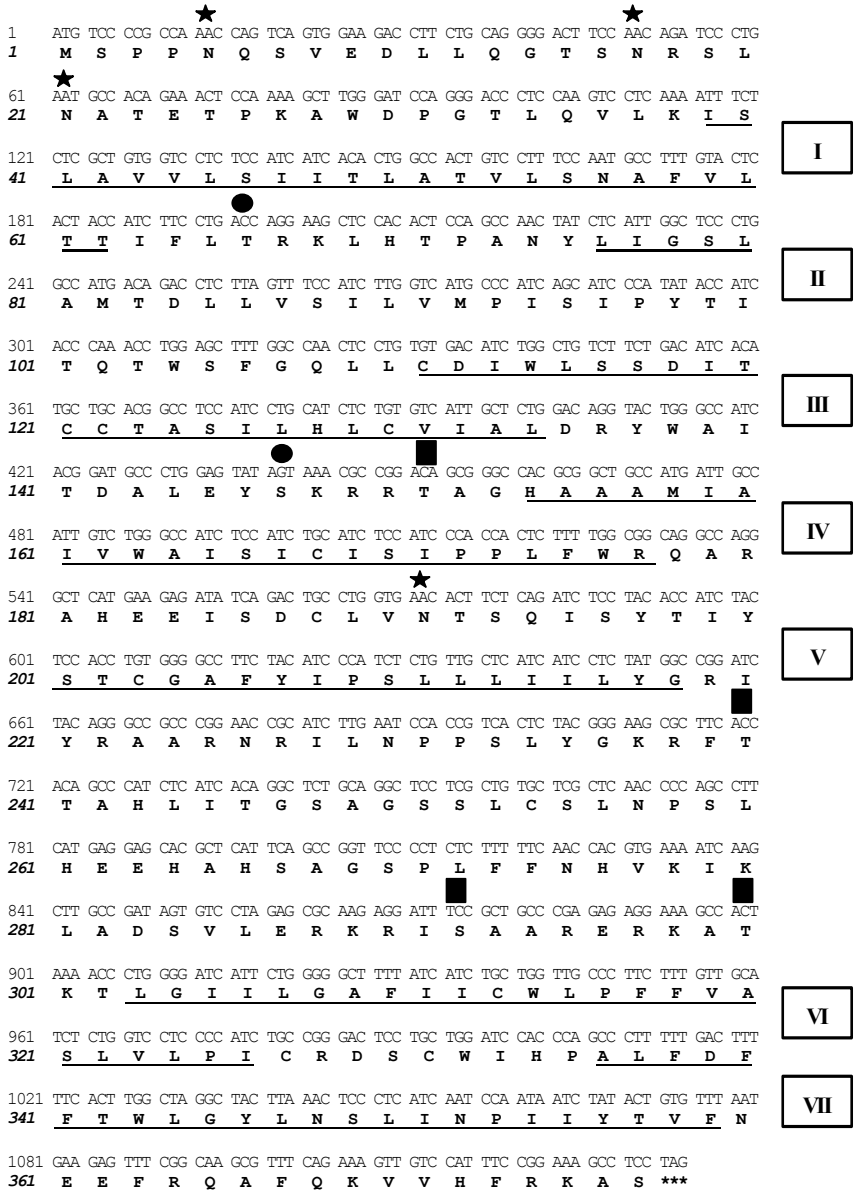


Figure 3.2. Nucleotide and deduced amino acid (in bold) sequences of the pig 5-HT$_{1D}$ receptor (GenBank accession number: AF 117655). The computer predicted (software DNAMAN, version 3.2, Lynnon Biosoft©) transmembrane domains I-VII (underlined) and putative N-glycosylation (★), protein kinase A phoshorylation (■) and protein kinase C phosphorylation (●) sites are indicated in the sequence.

Inverse-PCR (5' end)

5' end

```
1    ATGTCCCCGCCAAACCAGTCAGTGGAAGACCTTCTGCAGGGGACTTCCAACAGATCCCTG
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
21   ATGTCCCCGCCAAACCAGTCAGTGGAAGACCTTCTGCAGGGGACTTCCAACAGATCCCTG

61   AATGCCACAGAAACTCCAAAAGCTTGGGATCCAGGGACCCTCCAAGTCCTCAAAATTTCT
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
81   AATGCCACAGAAACTCCAAAAGCTTGGGATCCAGGGACCCTCCAAGTCCTCAAAATTTCT

121  CTCGCTGTGGTCCTCTCCATCATCACACTGGCCACTGTCCTTTCCAATGC
     ||||||||||||||||||||||||||||||||||||||||||||||||||  3' end
141  CTCGCTGTGGTCCTCTCCATCATCACACTGGCCACTGTCCTTTCCAATGC
```

Inverse-PCR (3' end)

5' end

```
932  TCATCTGCTGGTTGCCCTTCTTTGTTGCATCTCTGGTCCTCCCCATCTGCCGGGACTCCT
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1    TCATCTGCTGGTTGCCCTTCTTTGTTGCATCTCTGGTCCTCCCCATCTGCCGGGACTCCT

992  GCTGGATCCACCCAGCCCTTTTTGACTTTTTCACTTGGCTAGGCTACTTAAACTCCCTCA
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61   GCTGGATCCACCCAGCCCTTTTTGACTTTTTCACTTGGCTAGGCTACTTAAACTCCCTCA

1052 TCAATCCAATAATCTATACTGTGTTTAACGAAGAGTTTCGGCAAGCGTTTCAGAAAGTTG
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121  TCAATCCAATAATCTATACTGTGTTTAATGAAGAGTTTCGGCAAGCGTTTCAGAAAGTTG

1112 TCCATTTCCGGAAAGCCTCCTAG
     ||||||||||||||||||||||  3' end
181  TCCATTTCCGGAAAGCCTCCTAG
```

Figure 3.3. Sequence of inverse-PCR amplified products (in bold letters) showing a 100% homology at both 5' and 3' ends with the sequence of the cDNA identified from the pig brain cortex (in normal letters). The 5' and 3' terminal sequences are identified in shaded boxes, while the inverse PCR primers are underlined.

Using a set of porcine specific internal inverse primers (based on the derived sequence) and the porcine ligated genomic DNA fragments, a PCR product of approximately 400 bp was amplified, cloned and sequenced. Sequence analysis revealed that, compared to the derived porcine 5-HT$_{1D}$ receptor cDNA, there was a 100% identity with 170 nucleotides from the 5' end (Figure 3.3, *upper panel*) and 203 nucleotides from the 3' end (Figure 3.3, *lower panel*). The 5' (N) and 3' (C) terminals were identical to those obtained by cDNA sequencing (Figure 3.3, *shaded boxes*).

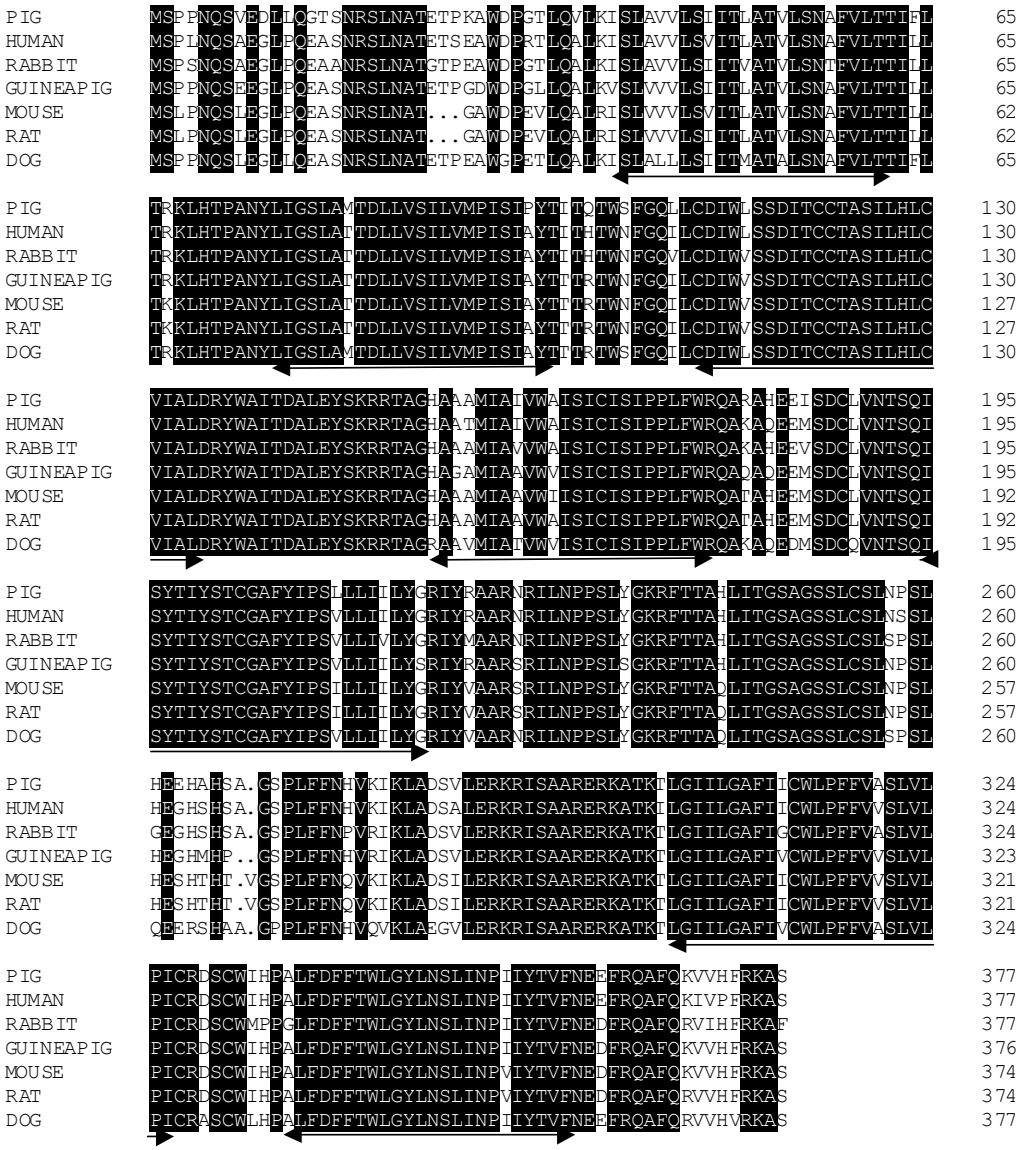


Figure 3.4. Comparison of amino acid sequences of the pig 5-HT$_{1D}$ receptor with human (GenBank accession number M89955), rabbit (Z50162), guinea pig (X94436), mouse (X94908), rat (M89953) and dog (X14049) 5-HT$_{1D}$ receptors (software DNAMAN, version 3.2, Lynnon Biosoft©). The arrows drawn across the amino acid sequence indicate the seven transmembrane regions. Shaded boxes show identity across the different species.

In the intervening portion (not shown in the figure), there were only 20 nucleotidesderived from the porcine genomic DNA, where the EcoR1 site was present 5 nucleotides away from 3' end and 15 nucleotides before 5' end.

Figure 3.4 compares the amino acid sequence of porcine 5-HT$_{1D}$ receptor with those of other mammalian species. It should be noted that towards the N-terminal of

porcine 5-HT$_{1D}$ receptor a number of amino acids (Val^{8,36}, Asp^{10}, Gly^{14}, Thr^{15}, Lys^{27}, Pro^{97} and Glu^{102}) were different from those in the other species. However, there was a high homology in the overall sequence of the receptor across the species (from 88% in dog to 92% in humans). Between the pig and human, the transmembrane-3 and transmembrane-7 regions showed an identical sequence while the remaining transmembrane regions showed a 92-96% homology.

Binding properties of recombinant porcine 5-HT$_{1D}$ Receptor

Membrane preparations from COS-7 cells transfected with porcine 5-HT$_{1D}$ receptor showed high affinity for the agonist [^{3}H]5-CT as well as the antagonist [^{3}H]GR125743. The equilibrium dissociation constant (K_d) of [^{3}H]5-CT and [^{3}H]GR125743 for the porcine 5-HT$_{1D}$ receptor was, respectively, 1.08±0.04 nM and 1.47±0.13 nM (both n=3). The B_{max} of [^{3}H]GR125743 binding (2.70±0.59 pmol mg^{-1} protein; n=3) was about one and a half times as high as that of [^{3}H]5-CT binding (1.74±0.04 pmol mg^{-1} protein; n=3), suggesting that part of the 5-HT$_{1D}$ receptor population was in the high affinity state.

Table 3.1 shows pK_i values of a number of 5-HT receptor agonists and antagonists for the displacement of [^{3}H]5-CT and [^{3}H]GR125743 from membranes obtained from COS-7 cells expressing the cloned porcine 5-HT$_{1D}$ receptor. There was an excellent correlation (r_S=0.984; p≤0.05) between values obtained with the two radioligands. The rank order of affinity of agonists was L694247 > 5-CT > zolmitriptan > 5-HT > sumatriptan = CP122638 = ergotamine > dihydroergotamine, while that of the antagonists was methiothepin > GR127935 > ketanserin > ritanserin >> SB224289 ≥ BRL 15572.

Table 3.1. pK_i values for a series of serotonergic compounds for the inhibition of [^{3}H]5-CT and [^{3}H]GR125743 binding to cloned pig 5-HT$_{1D}$ receptor expressed in Cos-7 cells

Compound	**[^{3}H]5-CT**	**[^{3}H]GR125743**
Agonists		
L694247	9.29±0.01	9.50±0.03
5-CT	8.79±0.06	9.00±0.05
Zolmitriptan	8.38±0.15	8.67±0.08
5-HT	8.22±0.12	8.40±0.09
Sumatriptan	7.97±0.04	8.06±0.04
CP122638	7.84±0.11	8.67±0.08
Ergotamine	8.00±0.04	7.89±0.03
Dihydroergotamine	7.87±0.12	7.73±0.24
Antagonists		
Methiothepin	8.74±0.06	8.81±0.14
GR127935	7.92±0.18	7.98±0.17
Ketanserin	7.17±0.06	7.42±0.04
Ritanserin	6.80±0.13	7.26±0.18
SB224289	6.15±0.22	6.39±0.08
BRL15572	5.94±0.09	5.93±0.09

Data are mean±s.e.mean (n=3-7). The pK_i values obtained with the two ligands correlated significantly (Spearman correlation coefficient r_S=0.984; $p \leq 0.05$, SlideWrite plus for Windows, Advanced Graphics Software, Encinitas, CA, USA).

In Figure 3.5, the affinity constants of the 14 compounds (8 agonists and 6 antagonists; see Table 3.1) investigated in the present experiments with the cloned pig 5-HT$_{1D}$ receptor have been plotted against values obtained earlier with the same compounds using membranes from cells expressing the human 5-HT$_{1B}$ or 5-HT$_{1D}$ receptor (Pauwels *et al*., 1996). The correlation between the affinity constants at the cloned porcine 5-HT$_{1D}$ receptor, particularly using [^{3}H]GR125743 as ligand, was clearly higher with the cloned human 5-HT$_{1D}$ than with 5-HT$_{1B}$ receptor (r_s=0.903 and 0.608, respectively; Figure 3.5, *right panels*). Admittedly, this difference was not as great using [^{3}H]5-CT as ligand (r_s=0.724 and 0.716, respectively; Figure 3.5, *left panels*).

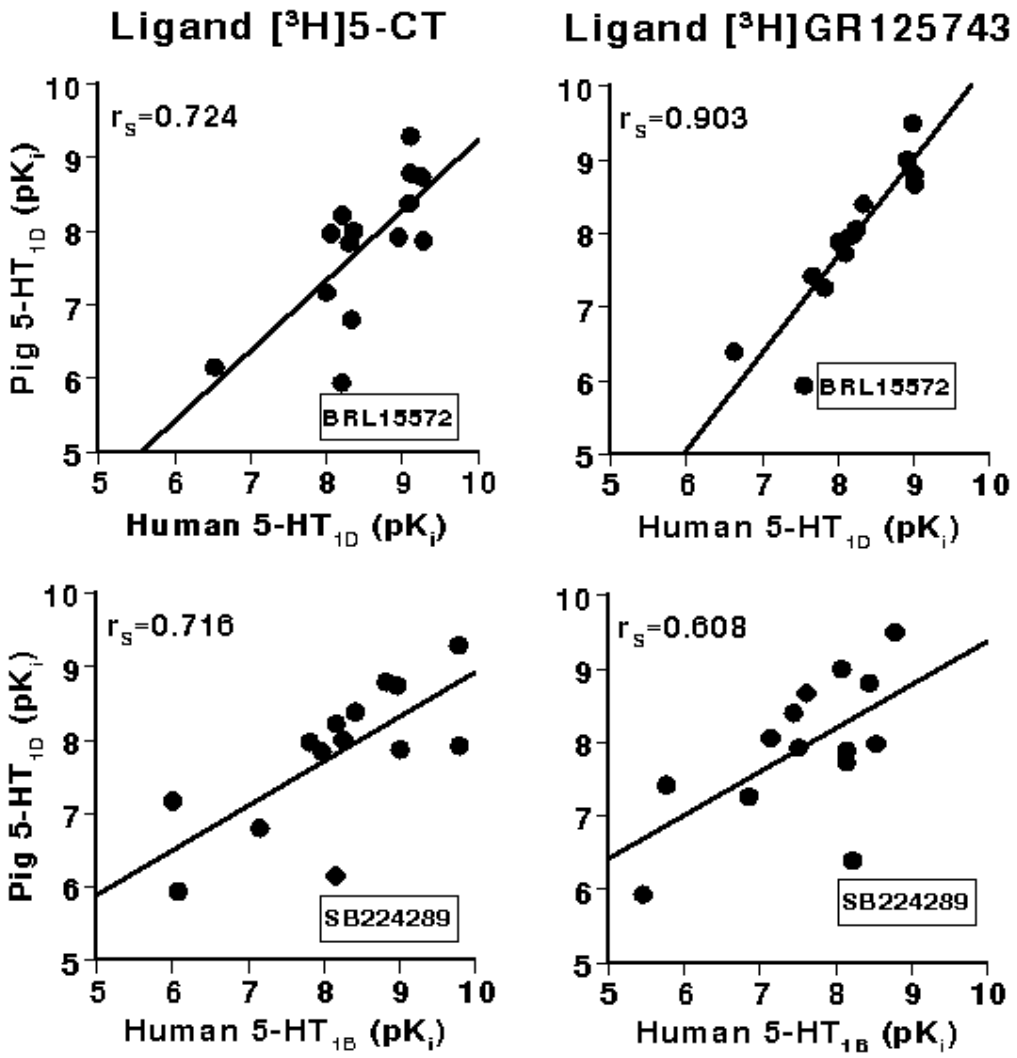


Figure 3.5. Regression analysis of pKi values (affinity constants) of 5-HT receptor ligands for the cloned pig 5-HT$_{1D}$ receptor (see Table 3.1 for ligands and data) against the cloned human 5-HT$_{1D}$ (*upper panels*) and 5-HT$_{1B}$ (*lower panels*) receptors (data from Pauwels *et al*., 1996; Wurch *et al*., 1998), using either [^{3}H]5-CT (*left panels*) or [^{3}H]GR125743 (right *panels*). The Spearman correlation coefficient r_S, calculated by using SlideWrite plus for Windows (Advanced Graphics Software, Encinitas, CA, USA), is listed in each panel. The compounds clearly falling outside the regression line (BRL15572 and SB224289) are identified in the graphs.

However, it is interesting to point out that the selective antagonist at the human 5-HT$_{1D}$ receptor, BRL15572 (Price *et al*., 1997; Schlicker *et al*., 1997), displayed much less affinity at the pig 5-HT$_{1D}$ receptor (Figure 3.5, *upper panels*). As can be expected, the selective antagonist at the human 5-HT$_{1B}$ receptor, SB224289 (Gaster *et al*., 1998; Selkirk *et al*., 1998), did not show a high affinity at the pig 5-HT$_{1D}$ receptor

(Figure 3.5, *lower panels*). When BRL15572 was excluded from analysis, the correlation between the pK_i values at pig and human 5-HT$_{1D}$ receptors was increased (r_s=0.798 and 0.957 using [^{3}H]5-CT and [^{3}H]GR125743, respectively), but that between pig 5-HT$_{1D}$ and human 5-HT$_{1B}$ receptors was decreased (r_s=0.602 and 0.386 using [^{3}H]5-CT and [^{3}H]GR125743, respectively).

[^{35}S]GTPγS binding

The basal [^{35}S]GTPγS binding in membranes obtained from CHO-K1 cells co-expressing the cloned porcine 5-HT$_{1D}$ receptor and the rat $G_{\alpha i1}Cys^{351}Ile$ protein was 277±37 fmol [^{35}S]GTPγS mg^{-1} protein.

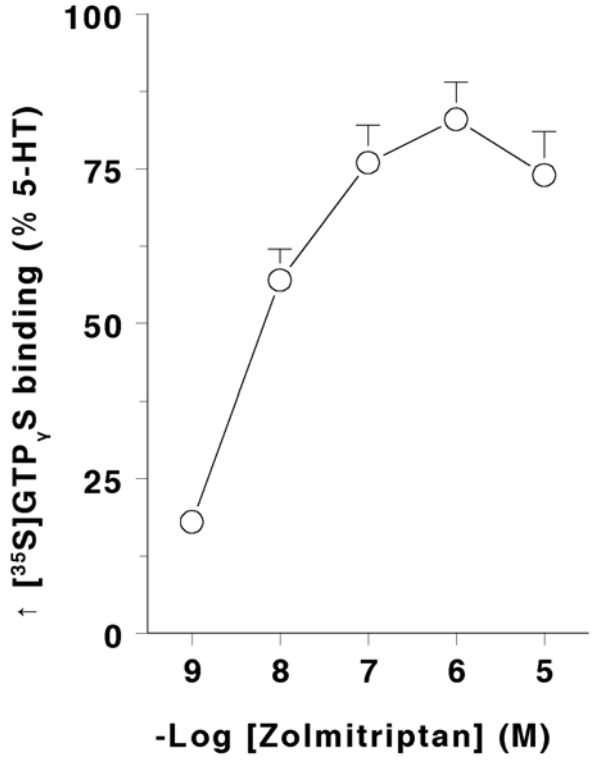


Figure 3.6. Increase in [^{35}S]GTPγS binding, as percentage of the response to 10 μM 5-HT, by zolmitriptan in CHO-K$_1$ cells co-transfected with the cloned pig 5-HT$_{1D}$ receptor and rat $G_{\alpha i1}Cys^{351}Ile$ protein. The basal [^{35}S]GTPγS binding (277±37 fmol mg^{-1} protein) was increased by 5-HT (10 μM) to 399±77 fmol mg^{-1} protein (44%) and decreased by ketanserin (10 μM) to 220±23 fmol mg^{-1} protein (-25%).

5-HT (10 μM) elicited an increase of 44% over the basal level. Zolmitriptan caused a concentration-dependent increase in [^{35}S]GTPγS binding and elicited a near full agonist response at the receptor (83±6% increase as compared to 10 μM 5-HT;

Figure 3.6). The potency of zolmitriptan (pEC_{50}: 8.46±0.08) was found to be close to the binding affinity at the porcine 5-HT$_{1D}$ receptor (pK_i 8.38 and 8.67, see Table 3.1). The putative 5-HT$_{1D}$ receptor antagonist, ketanserin (10 μM), decreased basal [^{35}S]GTPγS binding by about 25%, thus exhibiting a negative efficacy.

Discussion

Sequence of Porcine 5-HT$_{1D}$ receptor

Using primers based on the nucleotide sequence of the guinea pig 5-HT$_{1D}$ receptor gene (Wurch *et al*., 1997; Zgombick *et al*., 1997) and cDNA derived from porcine cerebral cortex, a band of expected fragment size (~1140 bp) was amplified, cloned and sequenced. The BLAST search of the cloned porcine cDNA fragment revealed high resemblance with previously cloned 5-HT$_{1D}$ receptors. The full-length nucleotide sequence containing 1137 bp encoded a 377 amino acid peptide, which showed a close (88-92%) similarity with the human (Hamblin *et al*., 1991; Weinshank *et al*., 1992), rabbit (Harwood *et al*., 1995), guinea pig (Wurch *et al*., 1997; Zgombick *et al*., 1997), mouse (Wurch *et al*., 1997), rat (Hamblin *et al*., 1991; Wurch *et al*., 1997) and dog (Zgombick *et al*., 1991) 5-HT$_{1D}$ receptor. The similarity with the human 5-HT$_{1D}$ receptor in the predicted seven transmembrane regions was even higher (92-100%). Moreover, the full-length amino acid sequence completely matched the partial sequence of the porcine 5-HT$_{1D}$ receptor, previously submitted to the GenBank by T. Wurch and colleagues (accession number P79400). Thus, the nucleotide sequence of the mRNA obtained from the porcine cerebral cortex encodes the 5-HT$_{1D}$ receptor.

The computer-predicted hydrophobicity showed seven transmembrane spanning regions as well as putative N-linked glycosylation sites also observed in 5-HT$_{1D}$ receptors from the other species (Wurch *et al*., 1997). However, the N-terminal of porcine 5-HT$_{1D}$ receptor (see Figure 3.3) showed several unique amino acids not present in other species (Harwood *et al*., 1995; Wurch *et al*., 1997). The divergent nucleotides within the 5' (N) and 3' (C) ends were confirmed by inverse PCR using primers based on the derived sequence of the porcine 5-HT$_{1D}$ receptor. It may, however, be noted that the threonine residue, being the seventh amino acid in the transmembrane-7 region of thus far cloned 5-HT$_{1D}$ (Thr^{342}) as well as non-rodent 5-HT$_{1B}$ (Thr^{355}) receptors (see Harwood *et al*., 1995; Pregenzer *et al*., 1997; Wurch *et*

al., 1997; Zgombick *et al*., 1997; Zgombick *et al*., 1991), was conserved. The presence of threonine at this position seems to be important for the largely similar ligand binding properties of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Interestingly, a single amino acid change at the seventh residue (from Thr$^{354/355}$ in non-rodent species to Asp^{351} in the rat or mouse) in the 5-HT$_{1B}$ receptor is known to confer major pharmacological differences between the two groups of 5-HT$_{1B}$ receptors (Oksenberg *et al*., 1992; Zgombick *et al*., 1997).

Ligand binding properties of porcine 5-HT$_{1D}$ receptor

The cloned porcine 5-HT$_{1D}$ receptor expressed in COS-7 cells showed high affinity for the two 5-HT$_{1B/D}$ receptor radioligands ([^{3}H]5-CT and [^{3}H]GR125743) and the rank order of agonists (L694247 > 5-CT > zolmitriptan > 5-HT > sumatriptan = CP122638 = ergotamine > dihydroergotamine) as well as antagonists (methiothepin > GR127935 > ketanserin > ritanserin >> SB224289 ≥ BRL 15572) affinity was found to be the same using either radioligand. The affinity constants of these 14 compounds obtained in the present experiments with the cloned porcine 5-HT$_{1D}$ receptor showed a high correlation with those previously reported with the cloned human 5-HT$_{1D}$ receptor (Figure 3.5, Pauwels *et al*., 1996). However, the most salient finding in the present investigation was that BRL15572, which behaves as a selective antagonist at the human 5-HT$_{1D}$ receptor (Price *et al*., 1997; Schlicker *et al*., 1997), did not show high affinity at the porcine 5-HT$_{1D}$ receptor. The profound implications of this finding are obvious; a ligand selectively recognising a particular receptor may fail at other species homologues. Indeed, this is also the case with ketanserin, which has a moderate selectivity for the 5-HT$_{1D}$ over the 5-HT$_{1B}$ receptors in the human (Pauwels *et al*., 1996; Zgombick *et al*., 1995), rabbit (Bard *et al*., 1996; Harwood *et al*., 1995), rat (Bach *et al*., 1993; Weinshank *et al*., 1992) and guinea pig (Wurch *et al*., 1997), but not in the dog (Zgombick *et al*., 1991). Similarly, certain isochroman derivatives show a differential pharmacology at the guinea pig and gorilla 5-HT$_{1D}$ receptors (Pregenzer *et al*., 1997). Such species differences in the pharmacology of homologue receptors can be used to explore the role of divergent amino acid residues in the receptor-ligand interaction as well as the validation of animal models with respect to drug discovery for human diseases.

In view of the poor affinity of BRL15572 at the cloned porcine 5-HT$_{1D}$ receptor, we have to admit that the use of this compound by us to rule out the involvement of 5-HT$_{1D}$ receptor in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses (De Vries *et al*., 1999b) was not adequate. However, the conclusions that the response to sumatriptan is mediated by 5-HT$_{1B}$ receptor still seems valid, since both selective (SB224289, Gaster *et al*., 1998; Selkirk *et al*., 1998) as well as non-selective (GR127935, Pauwels *et al*., 1996; Skingle *et al*., 1996) 5-HT$_{1B}$ receptor antagonists potently antagonised the carotid arteriovenous anastomotic constriction by sumatriptan (De Vries *et al*., 1996; De Vries *et al*., 1999c).

Functional characterisation of porcine 5-HT$_{1D}$ receptor

Functional properties of recombinant 5-HT$_{1D}$ receptors have been established using predominantly cellular responses employing cAMP and [^{35}S]GTPγS binding assays (Pauwels *et al*., 1996; Pauwels *et al*., 1997; Thomas *et al*., 1995). Using membranes from cells co-expressing the porcine 5-HT$_{1D}$ receptor and rat $G_{\alpha i1}Cys^{351}Ile$ protein, we showed that 5-HT (10 μM) increased basal [^{35}S]GTPγS binding by 44% and that zolmitriptan behaved as a near full agonist in this respect. This result is in agreement with earlier observations on recombinant human 5-HT$_{1D}$ receptor (Pauwels *et al*., 1997). Moreover, as can be expected, the potency of zolmitriptan in the [^{35}S]GTPγS binding assay (pEC$_{50}$: 8.46±0.08, see Figure 3.6) closely agreed with its affinity in the ligand binding assay (pK$_i$ 8.38 and 8.67, see Table 3.1). Finally, the putative 5-HT$_{1D}$ receptor antagonist ketanserin (10 μM) inhibited basal [^{35}S]GTPγS binding, thus exhibiting negative efficacy (inverse agonism) as noticed earlier using the recombinant human 5-HT$_{1D}$ receptor (Pauwels *et al*., 1997; Thomas *et al*., 1995).

In conclusion, we have established the cDNA sequence of cloned porcine 5-HT$_{1D}$ receptor, which shows a similar ligand binding profile as the cloned human 5-HT$_{1D}$ receptor, except that BRL15572, a selective antagonist at the human 5-HT$_{1D}$ receptor, is not recognised by the porcine homologue. As shown by the increase in [^{35}S]GTPγS binding, the cloned porcine 5-HT$_{1D}$ receptor is also functionally active.

Acknowledgements

Authors thank Ms. Claudia Erpelinck and Mr. Gert Jan Botma for their assistance in DNA sequencing.

References

BACH, A.W., UNGER, L., SPRENGEL, R., MENGOD, G., PALACIOS, J., SEEBURG, P.H. & VOIGT, M.M. (1993). Structure functional expression and spatial distribution of a cloned cDNA encoding a rat 5-HT$_{1D}$-like receptor. *J. Recept. Res.*, **13,** 479-502.

BARD, J.A., KUCHAREWICZ, S.A., ZGOMBICK, J.M., WEINSHANK, R.L., BRANCHEK, T.A. & COHEN, M.L. (1996). Differences in ligand binding profiles between cloned rabbit and human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors: ketanserin and methiothepin distinguish rabbit 5-HT$_{1D}$ receptor subtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 237-244.

BHALLA, P., SHARMA, H.S., WURCH, T., PAUWELS, P.J. & SAXENA, P.R. (2000). Molecular cloning and pharmacological characterisation of porcine 5-HT$_{1D}$ receptor. *Br. J. Pharmacol.*, **129,** 189P.

BOUCHELET, I., CASE, B., OLIVIER, A. & HAMEL, E. (2000). No contractile effect for 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br. J. Pharmacol.*, **129,** 501-508.

BOUCHELET, I., COHEN, Z., CASE, B., SEGUELA, P. & HAMEL, E. (1996). Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. *Mol. Pharmacol.*, **50,** 219-223.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248-254.

BRADLEY, P.B., ENGEL, G., FENIUK, W., FOZARD, J.R., HUMPHREY, P.P., MIDDLEMISS, D.N., MYLECHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology*, **25,** 563-576.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal. Biochem.*, **162,** 156-159.

COHEN, M.L. & SCHENCK, K. (1999). 5-Hydroxytryptamine(1F) receptors do not participate in vasoconstriction: lack of vasoconstriction to LY344864, a selective serotonin(1F) receptor agonist in rabbit saphenous vein. *J. Pharmacol. Exp. Ther.*, **290,** 935-939.

DE VRIES, P., HEILIGERS, J.P.C., VILLALÓN, C.M. & SAXENA, P.R. (1996). Blockade of porcine carotid vascular response to sumatriptan by GR127935, a selective 5-HT$_{1D}$ receptor antagonist. *Br. J. Pharmacol.*, **118,** 85-92.

DE VRIES, P., SANCHEZ-LOPEZ, A., CENTURION, D., HEILIGERS, J.P., SAXENA, P.R. & VILLALON, C.M. (1998). The canine external carotid vasoconstrictor 5-HT$_1$ receptor: blockade by 5-HT$_{1B}$ (SB224289), but not by 5-HT$_{1D}$ (BRL15572) receptor antagonists. *Eur. J. Pharmacol.*, **362,** 69-72.

DE VRIES, P., VILLALON, C.M. & SAXENA, P.R. (1999a). Pharmacological aspects of experimental headache models in relation to acute antimigraine therapy. *Eur. J. Pharmacol.*, **375,** 61-74.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P., VILLALON, C.M. & SAXENA, P.R. (1999b). Investigation of the role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. *Br. J. Pharmacol.*, **127,** 405-412.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P.C., VILLALÓN, C.M. & SAXENA, P.R. (1999c). Constriction of porcine carotid arteriovenous anastomoses as indicator of antimigraine activity: the role of 5-HT$_{1B/1D}$ receptors as well as unidentified receptors. In *Migraine Pathophysiology*. ed. Edvinsson, L. pp. 119-132. London: Martin Dunitz Publishers.

DUPUIS, D.S., TARDIF, S., WURCH, T., COLPAERT, F.C. & PAUWELS, P.J. (1999). Modulation of 5-HT$_{1A}$ receptor signalling by point-mutation of cysteine351 in the rat Galpha(o) protein. *Neuropharmacology*, **38,** 1035-1041.

EDVINSSON, L. & GOADSBY, P.J. (1998). Neuropeptides in headache. *Eur. J. Neurol.*, **5,** 329-341.

GASTER, L.M., BLANEY, F.E., DAVIES, S., DUCKWORTH, D.M., HAM, P., JENKINS, S., JENNINGS, A.J., JOINER, G.F., KING, F.D., MULHOLLAND, K.R., WYMAN, P.A., HAGAN, J.J., HATCHER, J., JONES, B.J., MIDDLEMISS, D.N., PRICE, G.W., RILEY, G., ROBERTS, C., ROUTLEDGE, C., SELKIRK, J. & SLADE, P.D. (1998). The selective 5-HT$_{1B}$ receptor inverse agonist 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydro-spiro[furo[2,3-f]indole-3,4'-piperidine] (SB-224289) potently blocks terminal 5-HT autoreceptor function both in vitro and in vivo. *J. Med. Chem.*, **41,** 1218-1235.

HAMBLIN, M.W. & METCALF, M.A. (1991). Primary structure and functional characterization of a human 5-HT$_{1D}$- type serotonin receptor. *Mol. Pharmacol.*, **40,** 143-148.

HAMEL, E. & SAXENA, P.R. (2000). 5-Hydroxytryptamine involvement in migraine. In *The Headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 319-324. Philadelphia: Lippincott, Williams & Wilkins.

HARGREAVES, R.J. & SHEPHEARD, S.L. (1999). Pathophysiology of migraine -- new insights. *Can. J. Neurol. Sci.*, **26,** S12-9.

HARWOOD, G., LOCKYER, M., GILES, H. & FAIRWEATHER, N. (1995). Cloning and characterisation of the rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors. *FEBS Lett.*, **377,** 73-76.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 157-203.

HOYER, D., SCHOEFFTER, P., WAEBER, C. & PALACIOS, J.M. (1990). Serotonin 5-HT$_{1D}$ receptors. *Ann. N. Y. Acad. Sci.*, **600,** 168-81; discussion 181-2.

HUMPHREY, P.P., FENIUK, W., PERREN, M.J., CONNOR, H.E., OXFORD, A.W., COATES, L.H. & BUTINA, D. (1988). GR43175, a selective agonist for the 5-HT$_1$-like receptor in dog isolated saphenous vein. *Br. J. Pharmacol.*, **94,** 1123-1132.

HUMPHREY, P.P.A., APPERLEY, E., FENIUK, W. & PERREN, M.J. (1990). A rational approach to identifying a fundamentally new drug for the treatment of migraine. In *Cardiovascular pharmacology of 5-hydroxytryptamine: prospective therapeutic applications*. ed. Saxena, P.R., Wallis, D.I., Wouters, W. & Bevan, P. pp. 416-431. Dordrecht: Kluwer academic publishers.

LONGMORE, J., SHAW, D., SMITH, D., HOPKINS, R., MCALLISTER, G., PICKARD, J.D., SIRINATHSINGHJI, D.J., BUTLER, A.J. & HILL, R.G. (1997). Differential distribution of 5HT$_{1D}$- and 5HT$_{1B}$-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. *Cephalalgia*, **17,** 833-842.

MCCALL, R.B. (1997). Trigeminal ganglion elicited increases in nucleus trigeminal caudalis blood flow: a novel migraine model. *Brain Res.*, **775,** 189-192.

MITSIKOSTAS, D.D., SANCHEZ DEL RIO, M., MOSKOWITZ, M.A. & WAEBER, C. (1999). Both 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors modulate c-fos expression within rat trigeminal nucleus caudalis. *Eur. J. Pharmacol.*, **369,** 271-277.

MOSKOWITZ, M.A. (1992). Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. *Trends Pharmacol. Sci.*, **13,** 307-311.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107,** 220-239.

NILSSON, T., LONGMORE, J., SHAW, D., OLESEN, I.J. & EDVINSSON, L. (1999). Contractile 5-HT$_{1B}$ receptors in human cerebral arteries: pharmacological characterization and localization with immunocytochemistry. *Br. J. Pharmacol.*, **128,** 1133-1140.

OCHMAN, H., GERBER, A.S. & HARTL, D.L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics*, **120,** 621-623.

OKSENBERG, D., MARSTERS, S.A., O'DOWD, B.F., JIN, H., HAVLIK, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT$_{1B}$ receptors. *Nature*, **360,** 161-163.

PAUWELS, P.J., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1996). Pharmacology of cloned human 5-HT$_{1D}$ receptor-mediated functional responses in stably transfected rat C6-glial cell lines: further evidence differentiating human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353,** 144-156.

PAUWELS, P.J., TARDIF, S., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1997). How efficacious are 5-HT$_{1B/D}$ receptor ligands: an answer from GTP gamma S binding studies with stably transfected C6-glial cell lines. *Neuropharmacology*, **36,** 499-512.

PEROUTKA, S.J. & MCCARTHY, B.G. (1989). Sumatriptan (GR 43175) interacts selectively with 5-HT$_{1B}$ and 5-HT$_{1D}$ binding sites. *Eur. J. Pharmacol.*, **163,** 133-136.

PONTE, P., NG, S.Y., ENGEL, J., GUNNING, P. & KEDES, L. (1984). Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res*, **12,** 1687-1696.

PREGENZER, J.F., ALBERTS, G.L., BOCK, J.H., SLIGHTOM, J.L. & IM, W.B. (1997). Characterization of ligand binding properties of the 5-HT$_{1D}$ receptors cloned from chimpanzee, gorilla and rhesus monkey in comparison with those from the human and guinea pig receptors. *Neurosci. Lett.*, **235,** 117-120.

PRICE, G.W., BURTON, M.J., COLLIN, L.J., DUCKWORTH, M., GASTER, L., GÖTHERT, M., JONES, B.J., ROBERTS, C., WATSON, J.M. & MIDDLEMISS, D.N. (1997). SB-216641 and BRL-15572-compounds to pharmacologically discriminate h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 312-320.

SAXENA, P.R. (1995). Cranial arteriovenous shunting, an *in vivo* animal model for migraine. In *Experimental headache models*. ed. Olesen, J. & Moskowitz, M.A. pp. 189-198. Philadelphia: Lippincott-Raven Publishers.

SAXENA, P.R., DE VRIES, P. & VILLALON, C.M. (1998). 5-HT$_1$-like receptors: a time to bid goodbye. *Trends Pharmacol. Sci.*, **19,** 311-316.

SAXENA, P.R. & FERRARI, M.D. (1992). From serotonin receptor classification to the antimigraine drug sumatriptan. *Cephalalgia*, **12,** 187-196.

SAXENA, P.R. & TFELT-HANSEN, P. (2000). Triptans, 5-HT$_{1B/1D}$ receptor agonists in the acute treatment of migraine. In *The Headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 411-438. Philadelphia: Lippincott, Williams & Wilkins.

SCHLICKER, E., FINK, K., MOLDERINGS, G.J., PRICE, G.W., DUCKWORTH, M., GASTER, L., MIDDLEMISS, D.N., ZENTNER, J., LIKUNGU, J. & GÖTHERT, M. (1997). Effects of selective h5-HT$_{1B}$ (SB-216641) and h5-HT$_{1D}$ (BRL-15572) receptor ligands on guinea-pig and human 5-HT auto- and heteroreceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 321-327.

SELKIRK, J.V., SCOTT, C., HO, M., BURTON, M.J., WATSON, J., GASTER, L.M., COLLIN, L., JONES, B.J., MIDDLEMISS, D.N. & PRICE, G.W. (1998). SB-224289--a novel selective (human) 5-HT$_{1B}$ receptor antagonist with negative intrinsic activity. *Br. J. Pharmacol.*, **125,** 202-208.

SHARMA, H.S., MAULIK, N., GHO, B.C., DAS, D.K. & VERDOUW, P.D. (1996). Coordinated expression of heme oxygenase-1 and ubiquitin in the porcine heart subjected to ischemia and reperfusion. *Mol. Cell Biochem.*, **157,** 111-116.

SKINGLE, M., BEATTIE, D.T., SCOPES, D.I.T., STARKEY, S.J., CONNOR, H.E., FENIUK, W. & TYERS, M.B. (1996). GR127935: a potent and selective 5-HT$_{1D}$ receptor antagonist. *Behav. Brain Res.*, **73,** 157-61.

THOMAS, D.R., FARUQ, S.A., BALCAREK, J.M. & BROWN, A.M. (1995). Pharmacological characterisation of [^{35}S]-GTPgammaS binding to Chinese hamster ovary cell membranes stably expressing cloned human 5-HT$_{1D}$ receptor subtypes. *J. Recept. Signal Transduct. Res.*, **15,** 199-211.

VERHEGGEN, R., HUNDESHAGEN, A.G., BROWN, A.M., SCHINDLER, M. & KAUMANN, A.J. (1998). 5-HT$_{1B}$ receptor-mediated contractions in human temporal artery: evidence from selective antagonists and 5-HT receptor mRNA expression. *Br. J. Pharmacol.*, **124,** 1345-1354.

WAEBER, C., SCHOEFFTER, P., HOYER, D. & PALACIOS, J.M. (1990). The serotonin 5-HT$_{1D}$ receptor: a progress review. *Neurochem. Res.*, **15,** 567-582.

WAINSCOTT, D.B., JOHNSON, K.W., PHEBUS, L.A., SCHAUS, J.M. & NELSON, D.L. (1998). Human 5-HT$_{1F}$ receptor-stimulated [^{35}S]GTPgammaS binding: correlation with inhibition of guinea pig dural plasma protein extravasation. *Eur. J. Pharmacol.*, **352,** 117-124.

WEINSHANK, R.L., ZGOMBICK, J.M., MACCHI, M.J., BRANCHEK, T.A. & HARTIG, P.R. (1992). Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$. *Proc. Natl. Acad. Sci. USA*, **89,** 3630-3634.

WURCH, T., COLPAERT, F.C. & PAUWELS, P.J. (1998). Chimeric receptor analysis of the ketanserin binding site in the human 5-Hydroxytryptamine$_{1D}$ receptor: importance of the second extracellular loop and fifth transmembrane domain in antagonist binding. *Mol. Pharmacol.*, **54,** 1088-1096.

WURCH, T., PALMIER, C., COLPAERT, F.C. & PAUWELS, P.J. (1997). Sequence and functional analysis of cloned guinea pig and rat serotonin 5-HT$_{1D}$ receptors: common pharmacological features within the 5-HT$_{1D}$ receptor subfamily. *J. Neurochem.*, **68,** 410-418.

ZGOMBICK, J.M., BARD, J.A., KUCHAREWICZ, S.A., URQUHART, D.A., WEINSHANK, R.L. & BRANCHEK, T.A. (1997). Molecular cloning and pharmacological characterization of guinea pig 5- HT$_{1B}$ and 5-HT$_{1D}$ receptors. *Neuropharmacology*, **36,** 513-524.

ZGOMBICK, J.M., SCHECHTER, L.E., KUCHAREWICZ, S.A., WEINSHANK, R.L. & BRANCHEK, T.A. (1995). Ketanserin and ritanserin discriminate between recombinant human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor subtypes. *Eur. J. Pharmacol.*, **291,** 9-15.

ZGOMBICK, J.M., WEINSHANK, R.L., MACCHI, M., SCHECHTER, L.E., BRANCHEK, T.A. & HARTIG, P.R. (1991). Expression and pharmacological characterization of a canine 5- hydroxytryptamine$_{1D}$ receptor subtype. *Mol. Pharmacol.*, **40,** 1036-1042.

Chapter 4

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

Summary 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.

Based on: Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2001). Molecular cloning and expression of the porcine trigeminal ganglion cDNA encoding a 5-ht$_{1F}$ receptor. *Eur J Pharmacol:* **Submitted**

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 (Barnes & Sharp, 1999; Hoyer *et al*., 1994; Saxena, 1995). 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; Johnson *et al*., 1997; Leysen *et al*., 1996; Saxena & 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 (Bouchelet *et al*., 2000; De Vries *et al*., 1999; Razzaque *et al*., 1999). 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 (Goadsby, 1999; Humphrey, 1991; Moskowitz, 1992; Phebus *et al*., 1997). The presence of 5-HT$_{1D}$ and 5-ht$_{1F}$ receptor have been shown in the trigeminal ganglion (Bruinvels *et al*., 1994; Waeber & 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]) respectively, inhibit neurogenic dural inflammation (Ennis *et al*., 1998; Johnson *et al*., 1997; Phebus *et al*., 1997). 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 (Ma, 2001; Mitsikostas *et al*., 1999). Even though a direct role of the 5-ht$_{1F}$ receptor in vasoconstriction has recently been ruled out (Bouchelet *et al*., 2000; Razzaque *et al*., 1999; Villalón *et al*., 1999), 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.

Methods

PCR amplification and cloning of 5-ht$_{1F}$ receptor cDNA

Porcine specific 5-ht$_{1F}$ receptor cDNA was amplified using a combination of 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 x Landrace, female, 12 kg). The quality of RNA and cDNA preparations was checked by PCR amplification of β-actin (Ponte *et al*., 1984).

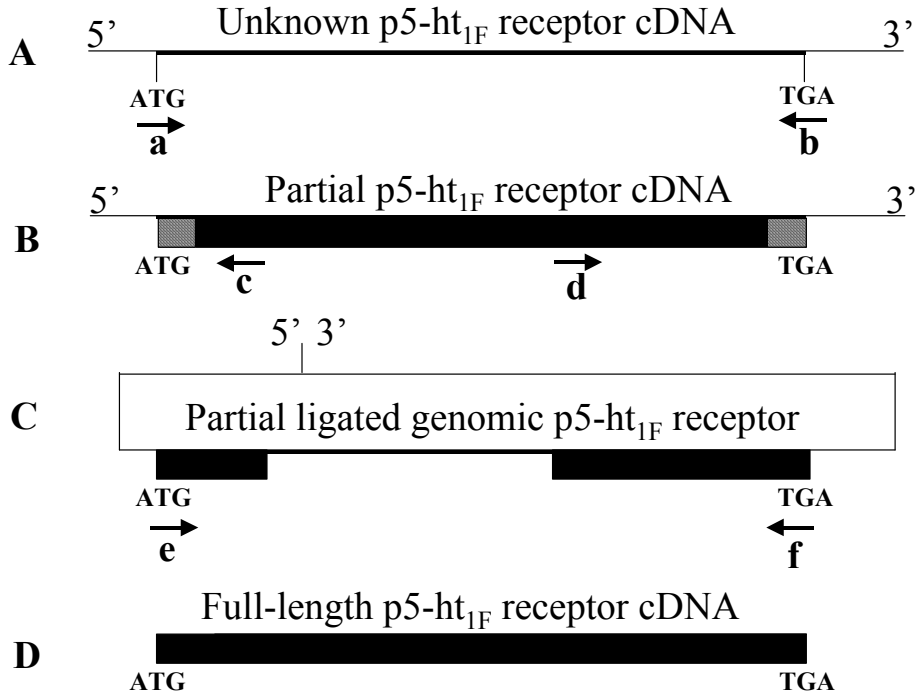


Figure 4.1. Diagrammatic representation showing the methodology for cloning of the full-length porcine 5-ht$_{1F}$ receptor and the positions of various primers used for PCR amplification. A. Unknown full-length porcine 5-ht$_{1F}$ receptor cDNA with 5' (a) and 3' (b) end primers designed from known 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.

As depicted in Figure 4.1, 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'-CTAATATCGACATCGTACAAG-3; nucleotides 1081-1101) oligonucleotide primers (Figure 4.1A, **a** and **b**), designed on the basis of consensus sequence of 5-ht$_{1F}$ receptors from other species (Adham *et al*., 1997; Adham *et al*., 1993; Amlaiky *et al*., 1992; Lovenberg *et al*., 1993). The amplified PCR products were separated on 1% agarose gel in TBE (100mM Tris, 90mM boric acid and 1mM 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; Figure 4.1B, **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'-ATGGATTTCTCAAACTCATCT-3') and reverse (5'-CTAACACCGACACATCGCACA-3') oligonucleotide primers, designed from the sequences generated from inverse-PCR (Figure 4.1C, **e** and **f**), were used to amplify the full-length 5-ht$_{1F}$ receptor cDNA from the porcine trigeminal ganglion (Figure 4.1D).

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.

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 & 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.

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 presence of reverse transcriptase enzyme. Porcine specific sense (5'-CCAAGCAGGCTGGCATTATG-3', nucleotides 410 bp-429 bp) and antisense (5'-GCTTTGCGTTCTCTTGTGCC-3', nucleotides 853 bp-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.

Materials

All oligonucleotide primers were commercially procured from Life Technologies b.v. (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 b.v. (Leiden, The Netherlands). Oligotex mRNA purification kit was purchased from Qiagen GmbH (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 Chemicals, St. Louis, MO, USA), ketanserin (Sigma Chemicals, St. Louis, MO, USA), 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).

Results

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 Figure 4.1B).

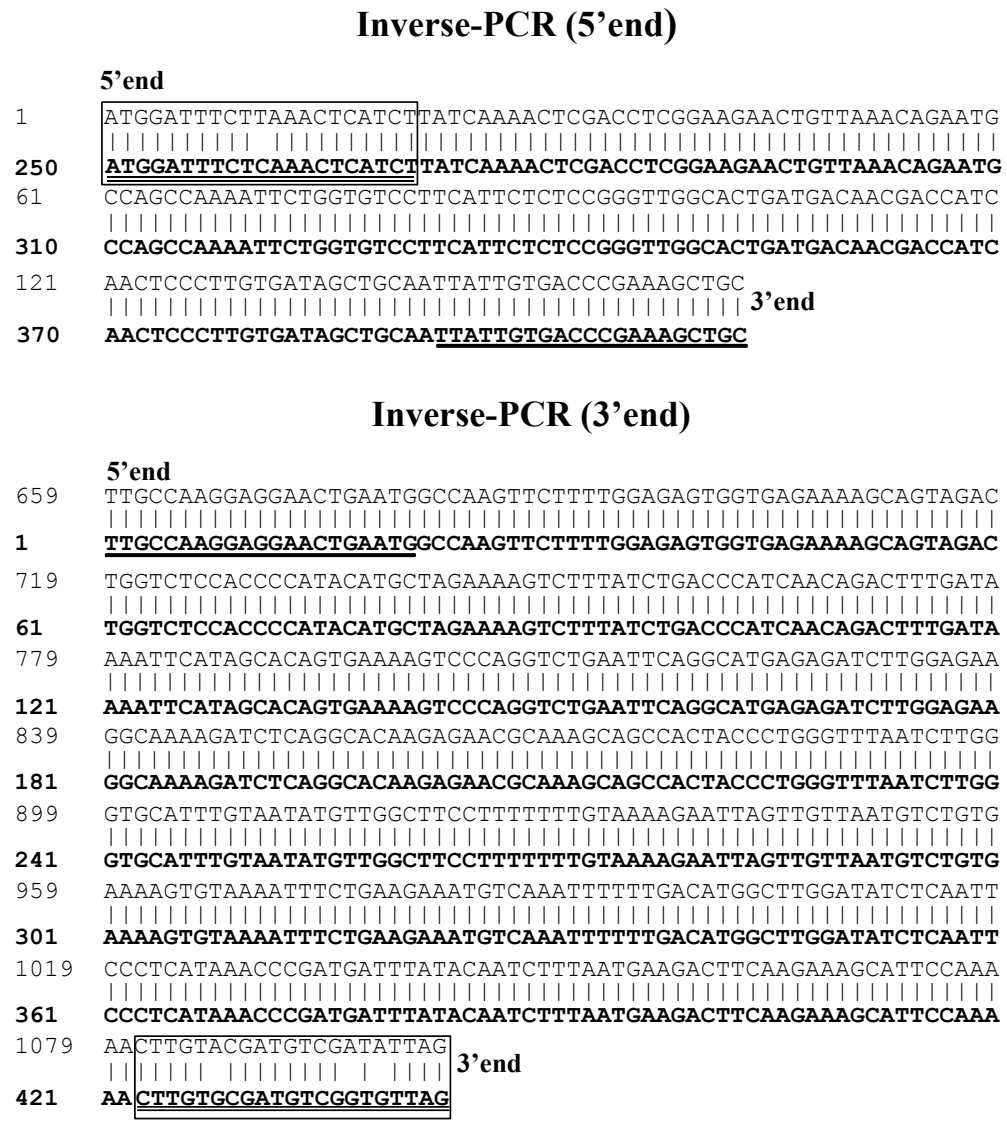


Figure 4.2. Sequence of inverse-PCR amplified products from porcine genomic DNA circles (in bold letters) showing a high homology with porcine cDNA sequence derived from trigeminal ganglion (in normal letters). The deduced 5' (N) and 3' (C) terminal sequences of porcine 5-ht$_{1F}$ receptor are shown 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.

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 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 (Figure 4.1C). 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 porcine 5-ht$_{1F}$ receptor cDNA and genomic DNA (Figure 4.2).

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 (Figure 4.1D) and agarose gel electrophoresis of RT-PCR products is shown in Figure 4.3.

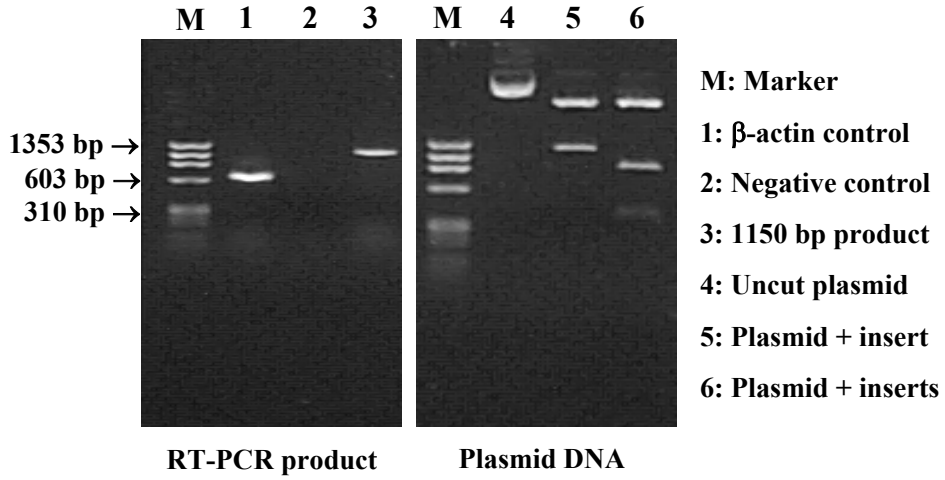


Figure 4.3. Agarose gel electrophoresis of RT-PCR products of cDNA synthesised from porcine trigeminal ganglion (left panel) and recombinant plasmid with insert cDNA (right panel). The different lanes marked on the top denote: M, ϕx174 DNA/Hae III marker; 1, Positive control showing a RT-PCR product 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 EcoR1 enzyme and showing two DNA fragments of approximately 800 bp and 300 bp. The size of 3 marker bands is indicated in the left margin.

The amplification of the porcine β-actin cDNA (approximately 625 bp) ensured that the quality of cDNA samples was adequate for amplification of other products,

while any genomic contamination was ruled out by the 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 EcoR1, used on the basis of a single restriction site in 5-ht$_{1F}$ receptors of other species (Adham *et al*., 1997; Adham *et al*., 1993; Amlaiky *et al*., 1992; Lovenberg *et al*., 1993), resulted into two bands of 800 bp and 300 bp.

```
                        ℗                   ℗
1    ATG GAT TTC TCA AAC TCA TCT TAT CAA AAC TCG ACC TCG GAA GAA CTG TTA AAC AGA ATG
1    MET Asp Phe Ser Asn Ser Ser Tyr Gln Asn Ser Thr Ser Glu Glu Leu Leu Asn Arg Met

61   CCA GCC AAA ATT CTG GTG TCC TTC ATT CTC TCC GGG TTG GCA CTG ATG ACA ACG ACC ATC
21   Pro Ala Lys Ile Leu Val Ser Phe Ile Leu Ser Gly Leu Ala Leu Met Thr Thr Thr Ile   I

121  AAC TCC CTT GTG ATA GCT GCA ATT ATT GTG ACC CGA AAG CTG CAC CAC CCA GCC AAC TAC
41   Asn Ser Leu Val Ile Ala Ala Ile Ile Val Thr Arg Lys Leu His His Pro Ala Asn Tyr

181  TTA ATT TGC TCC CTT GCA GTC ACA GAC TTC CTT GTA GCT GTC CTG GTG ATG CCT TTC AGC
61   Leu Ile Cys Ser Leu Ala Val Thr Asp Phe Leu Val Ala Val Leu Val Met Pro Phe Ser   II

241  ATT GTG TAT ATT GTG AGA GAG AGT TGG ATT ATG GGA CAA GTG GTC TGC GAC ATT TGG CTG
81   Ile Val Tyr Ile Val Arg Glu Ser Trp Ile Met Gly Gln Val Val Cys Asp Ile Trp Leu   III

301  AGT GTT GAC ATT ACA TGC TGC ACA TGC TCC ATC TTG CAT CTC TCT GCT ATA GCT TTG GAT
101  Ser Val Asp Ile Thr Cys Cys Thr Cys Ser Ile Leu His Leu Ser Ala Ile Ala Leu Asp
                                                  •

361  CGG TAC CGT GCA ATC ACA GAT GCT GTT GAG TAT GCC CAG AAA AGA ACT CCC AAG CAG GCT
121  Arg Tyr Arg Ala Ile Thr Asp Ala Val Glu Tyr Ala Gln Lys Arg Thr Pro Lys Gln Ala   IV

421  GGC ATT ATG ATT ACC ATA GTA TGG ATT ATA TCT ATT TTT ATC TCT ATG CCT CCT CTA TTC
141  Gly Ile Met Ile Thr Ile Val Trp Ile Ile Ser Ile Phe Ile Ser Met Pro Pro Leu Phe

481  TGG AGG CAC CAA GGA ACT AGC CGA GAT GAT GAG TGC ATC ATC AAA CAC GAC CAC ATT GTT
161  Trp Arg His Gln Gly Thr Ser Arg Asp Asp Glu Cys Ile Ile Lys His Asp His Ile Val   V

541  TCC ACT ATT TAC TCA ACA TTT GGA GCT TTC TAT ATC CCA TTA ACT TTA ATT TTG ATC CTC
181  Ser Thr Ile Tyr Ser Thr Phe Gly Ala Phe Tyr Ile Pro Leu Thr Leu Ile Leu Ile Leu

601  TAC TAC AAA ATA TAT AAA GCA GCA AAG ACA TTG TAT CAC AAG AGA CAA GCA AGT AGG ATT
201  Tyr Tyr Lys Ile Tyr Lys Ala Ala Lys Thr Leu Tyr His Lys Arg Gln Ala Ser Arg Ile
                                                                ■
661  GCC AAG GAG GAA CTG AAT GGC CAA GTT CTT TTG GAG AGT GGT GAG AAA AGC AGT AGA CTG
221  Ala Lys Glu Glu Leu Asn Gly Gln Val Leu Leu Glu Ser Gly Glu Lys Ser Ser Arg Leu

721  GTC TCC ACC CCA TAC ATG CTA GAA AAG TCT TTA TCT GAC CCA TCA ACA GAC TTT GAT AAA
241  Val Ser Thr Pro Tyr Met Leu Glu Lys Ser Leu Ser Asp Pro Ser Thr Asp Phe Asp Lys
           ■                   ■
781  ATT CAT AGC ACA GTG AAA AGT CCC AGG TCT GAA TTC AGG CAT GAG AGA TCT TGG AGA AGG
261  Ile His Ser Thr Val Lys Ser Pro Arg Ser Glu Phe Arg His Glu Arg Ser Trp Arg Arg

841  CAA AAG ATC TCA GGC ACA AGA GAA CGC AAA GCA GCC ACT ACC CTG GGT TTA ATC TTG GGT
281  Gln Lys Ile Ser Gly Thr Arg Glu Arg Lys Ala Ala Thr Thr Leu Gly Leu Ile Leu Gly   VI

901  GCA TTT GTA ATA TGT TGG CTT CCT TTT TTT GTA AAA GAA TTA GTT GTT AAT GTC TGT GAA
301  Ala Phe Val Ile Cys Trp Leu Pro Phe Phe Val Lys Glu Leu Val Val Asn Val Cys Glu

961  AAG TGT AAA ATT TCT GAA GAA ATG TCA AAT TTT TTG ACA TGG CTT GGA TAT CTC AAT TCC
321  Lys Cys Lys Ile Ser Glu Glu Met Ser Asn Phe Leu Thr Trp Leu Gly Tyr Leu Asn Ser   VII

1021 CTC ATA AAC CCG ATG ATT TAT ACA ATC TTT AAT GAA GAC TTC AAG AAA GCA TTC CAA AAA
341  Leu Ile Asn Pro Met Ile Tyr Thr Ile Phe Asn Glu Asp Phe Lys Lys Ala Phe Gln Lys

1081 CTT GTG CGA TGT CGG TGT TAG
361  Leu Val Arg Cys Arg Cys ***
```

℗ **N-glycosylation;** • **PKA-phosphorylation;** ■ **PKC-phosphorylation**

Figure 4.4. 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.

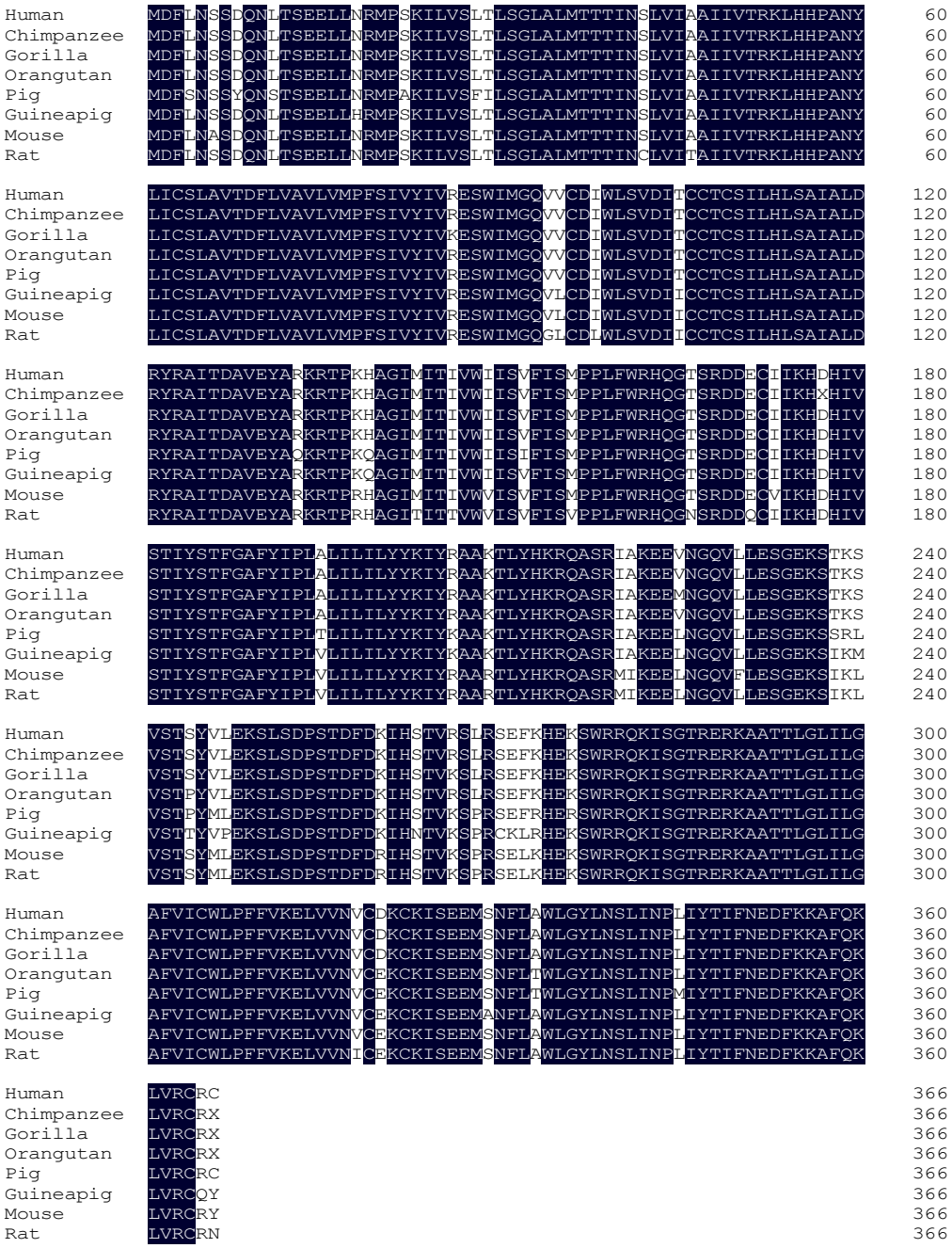


Figure 4.5. Comparison of amino acid sequences of the porcine 5-ht$_{1F}$ receptor (Swissprot accession number: AAG44634) with the human (P30939), chimpanzee (BAA90454), gorilla (BAA90455), orang-utan (BAA90456), guinea pig (O08890), mouse (Q02284) and rat (P30940) (software DNAMAN, version 3.2, Lynnon Biosoft©). Shaded boxes show identity across the different species.

Sequencing of the recombinant plasmid revealed an open reading frame of 1101 bp (Figure 4.4). DNAMAN analysis showed that this full-length porcine cDNA encoded a 366 amino acid protein (calculated molecular weight: 41.8 kDa) exhibiting features

of a typical G-protein-coupled receptor with predicted seven transmembrane domains and putative N-glycosylation and phosphorylation sites. In Figure 4.5, 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 the pig and orang-utan (Figure 4.5).

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 non transfected 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 (John *et al*., 1999 and Pauwels, unpublished; Pauwels *et al*., 1997) 5-ht$_{1F}$ receptor are shown in Table 4.1. Amongst 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.

Table 4.1. 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. Corresponding data for the human 5-ht$_{1F}$ receptor (John *et al*., 1999 and Pauwels, unpublished; Pauwels *et al*., 1997) is presented for comparison.

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-Carboxamidotryptamine	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	

Data are means ± S.E.M. (n=3-6). NA, Not available.

In Figure 4.6, 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$_{1F}$ (John *et al*., 1999 and Pauwels, unpublished; Pauwels *et al*., 1997) as well as human (Wurch *et al*., 1998 and unpublished) or porcine (Bhalla *et al*., 2001; Bhalla *et al*., 2000) 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors.

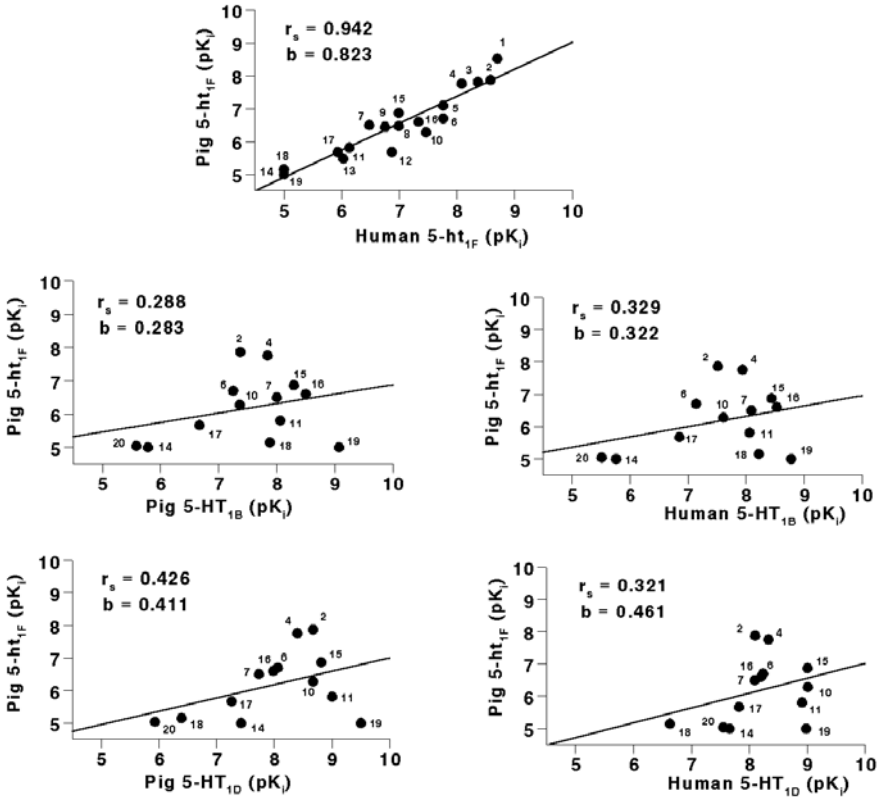
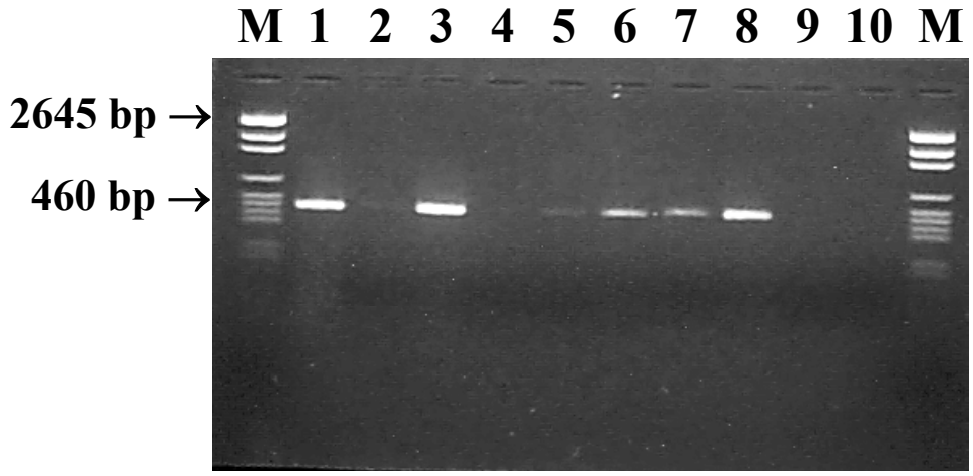


Figure 4.6. Regression analysis of binding affinity constants (pK_i values) of 5-HT receptor ligands at the cloned porcine 5-ht$_{1F}$ receptor (see Table 4.1) with those reported with cloned 5-ht$_{1F}$ (human; top panel), 5-HT$_{1B}$ (pig and human; middle panels) and 5-HT$_{1D}$ (pig and human; lower 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.

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 4.1).

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.



M: pGEM marker
1: Brain cortex
2: Cerebellum
3: Trigeminal ganglion
4: Skeletal muscles
5: Mesenteric artery
6: Coronary artery
7: Pulmonary artery
8: Saphenous vein
9: Brain cortex without RT
10: Autoclaved water

Figure 4.7. 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.

As shown in Figure 4.7, 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 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 absence of any band in negative controls (autoclaved water and brain cortex RNA in the absence of reverse transcription).

Discussion

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 (Adham *et al*., 1997; Adham *et al*., 1993; Amlaiky *et al*., 1992; 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 5' (Ser^{4,11}, Tyr^{8}, Phe^{28}, Ile^{29}) and 3' (Ser^{238}, Arg^{239,276}, Met^{345}) ends were also verified when the genomic DNA was used for inverse-PCR (see Figure 4.2), thus ruling out the possibility of sequencing error.

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 (KD: 9.2±0.99 nM, Adham *et al*., 1993), but is not dissimilar to that obtained with the guinea pig 5-ht$_{1F}$ receptor (KD: 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 4.1 and Figure 4.6). Also, LY334370 and CP122638, which are reasonably selective at the 5-ht$_{1F}$ receptor (Waeber & Moskowitz, 1995; Wainscott *et al*., 1998), were the two compounds with highest affinities at the porcine 5-ht$_{1F}$ receptor. However, it should be pointed out that generally the compounds investigated were less potent at the porcine compared to the human receptor (Table 4.1). 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 & 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 & Saxena, 2000).

Interestingly, like 5-HT$_{1D}$ (Thr^{342}) (Harwood *et al*., 1995; Weinshank *et al*., 1992; 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 Figure 4.5). 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}) (Adham *et al*., 1997; Adham *et al*., 1993; Amlaiky *et al*., 1992; 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 (Adham *et al*., 1994; Oksenberg *et al*., 1992). The presence of Thr^{333} in the porcine 5-ht$_{1F}$ receptor is in agreement with its low affinity for the β-adrenoceptors antagonist pindolol (K_i: >10,000 nM; Table 4.1). 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.

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 (Barnes & Sharp, 1999; Hoyer *et al*., 1994). Interestingly, despite the presence of the 5-ht$_{1F}$ receptor mRNA in some blood vessels (Bouchelet *et al*., 2000 and present results; Nilsson *et al*., 1999), it is practically ruled out that this receptor mediates vasoconstriction (Bouchelet *et al*., 2000; Cohen & Schenck, 2000; Shepheard *et al*., 1999). 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.

References

Adelman, J.U., Lipton, R.B., Ferrari, M.D., Diener, H.C., McCarroll, K.A., Vandormael, K. & Lines, C.R. (2001). Comparison of rizatriptan and other triptans on stringent measures of efficacy. *Neurology*, 57, 1377-83.

Adham, N., Bard, J.A., Zgombick, J.M., Durkin, M.M., Kucharewicz, S., Weinshank, R.L. & Branchek, T.A. (1997). Cloning and characterization of the guinea pig 5-HT$_{1F}$ receptor subtype: a comparison of the pharmacological profile to the human species homolog. *Neuropharmacology*, 36, 569-576.

Adham, N., Kao, H.T., Schecter, L.E., Bard, J., Olsen, M., Urquhart, D., Durkin, M., Hartig, P.R., Weinshank, R.L. & Branchek, T.A. (1993). Cloning of another human serotonin receptor (5-HT$_{1F}$): a fifth 5-HT$_1$ receptor subtype coupled to the inhibition of adenylate cyclase. *Proc. Natl. Acad. Sci. U S A*, 90, 408-412.

Adham, N., Tamm, J.A., Salon, J.A., Vaysse, P.J., Weinshank, R.L. & Branchek, T.A. (1994). A single point mutation increases the affinity of serotonin 5-HT$_{1Da}$, 5-HT$_{1Db}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ receptors for beta-adrenergic antagonists. *Neuropharmacology*, 33, 387-391.

Amlaiky, N., Ramboz, S., Boschert, U., Plassat, J.L. & Hen, R. (1992). Isolation of a mouse "5HT$_{1E}$-like" serotonin receptor expressed predominantly in hippocampus. *J. Biol. Chem.*, 267, 19761-19764.

Barnes, N.M. & Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology*, 38, 1083-152.

Bhalla, P., Sharma, H.S., Ma, X., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2001). Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT$_{1B}$ receptor. *Br. J. Pharmacol.*, 133, 891-901.

Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2000). Molecular cloning, sequence analysis and pharmacological properties of the porcine 5-HT$_{1D}$ receptor. *Br. J. Pharmacol.*, 131, 949-957.

Bouchelet, I., Case, B., Olivier, A. & Hamel, E. (2000). No contractile effect for 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br. J. Pharmacol.*, 129, 501-508.

Bruinvels, A.T., Landwehrmeyer, B., Gustafson, E.L., Durkin, M.M., Mengod, G., Branchek, T.A., Hoyer, D. & Palacios, J.M. (1994). Localization of 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ receptor messenger RNA in rodent and primate brain. *Neuropharmacology*, 33, 367-386.

Cohen, M.L. & Schenck, K. (2000). Contractile responses to sumatriptan and ergotamine in the rabbit saphenous vein: effect of selective 5-HT(1F) receptor agonists and PGF(2alpha). *Br. J. Pharmacol.*, 131, 562-568.

Dahlöf, C.G.H. & Saxena, P.R. (2000). Novel compounds in development for acute treatment of migraine. In *The Headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 439-443. Philadelphia: Lippincott, Williams and Wilkins.

De Vries, P., Willems, E.W., Heiligers, J.P., Villalon, C.M. & Saxena, P.R. (1999). Investigation of the role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. *Br. J. Pharmacol.*, 127, 405-412.

Diener, H.C., Tfelt-Hansen, P., de Beukelaar, F., Ferrari, M.D., Olesen, J., Dahlof, C. & Mathew, N. (2001). The efficacy and safety of sc alniditan vs. sc sumatriptan in the acute treatment of migraine: a randomized, double-blind, placebo-controlled trial. *Cephalalgia*, 21, 672-679.

Ennis, M.D., Ghazal, N.B., Hoffman, R.L., Smith, M.W., Schlachter, S.K., Lawson, C.F., Im, W.B., Pregenzer, J.F., Svensson, K.A., Lewis, R.A., Hall, E.D., Sutter, D.M., Harris, L.T. & McCall, R.B. (1998). Isochroman-6-carboxamides as highly selective 5-HT$_{1D}$ agonists: potential new treatment for migraine without cardiovascular side effects. *J. Med. Chem.*, 41, 2180-2183.

Goadsby, P.J. (1999). Advances in the pharmacotherapy of migraine. How knowledge of pathophysiology is guiding drug development. *Drugs R. D.*, 2, 361-374.

Goldstein, D.J., Roon, K.I., Offen, W.W., Ramadan, N.M., Phebus, L.A., Johnson, K.W., Schaus, J.M. & Ferrari, M.D. (2001). Selective serotonin 1F (5-HT$_{1F}$) receptor agonist LY334370 for acute migraine: a randomised controlled trial. *Lancet*, 358, 1230-4.

Harwood, G., Lockyer, M., Giles, H. & Fairweather, N. (1995). Cloning and characterisation of the rabbit 5-HT$_{1Da}$ and 5-HT$_{1Db}$ receptors. *FEBS Lett.*, 377, 73-76.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R. & Humphrey, P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, 46, 157-203.

Humphrey, P.P.A. (1991). Mode of action of the anti-migraine drug sumatriptan. *Trends Pharmacol. Sci.*, 12, 444-446.

Ishida, T., Hirata, K., Sakoda, T., Kawashima, S., Akita, H. & Yokoyama, M. (1999). Identification of mRNA for 5-HT$_1$ and 5-HT$_2$ receptor subtypes in human coronary arteries. *Cardiovasc Res*, 41, 267-74.

John, G.W., Pauwels, P.J., Perez, M., Halazy, S., Le Grand, B., Verscheure, Y., Valentin, J.P., Palmier, C., Wurch, T., Chopin, P., Marien, M., Kleven, M.S., Koek, W., Assi, M.B., Carilla-Durand, E., Tarayre, J.P. & Colpaert, F.C. (1999). F 11356, a novel 5-hydroxytryptamine (5-HT) derivative with potent, selective, and unique high intrinsic activity at 5-HT$_{1B/1D}$ receptors in models relevant to migraine. *J Pharmacol Exp Ther*, 290, 83-95.

Johnson, K.W., Schaus, J.M., Durkin, M.M., Audia, J.E., Kaldor, S.W., Flaugh, M.E., Adham, N., Zgombick, J.M., Cohen, M.L., Branchek, T.A. & Phebus, L.A. (1997). 5-HT$_{1F}$ receptor agonists inhibit neurogenic dural inflammation in guinea pigs. *Neuroreport*, 8, 2237-2240.

Leysen, J.E., Gommeren, W., Heylen, L., Luyten, W.H., Van de Weyer, I., Vanhoenacker, P., Haegeman, G., Schotte, A., Van Gompel, P., Wouters, R. & Lesage, A.S. (1996). Alniditan, a new 5-hydroxytryptamine$_{1D}$ agonist and migraine-abortive agent: ligand-binding properties of human 5-hydroxytryptamine$_{1D\alpha}$, human 5-hydroxytryptamine$_{1D\beta}$, and calf 5-hydroxytryptamine$_{1D}$ receptors investigated with [^{3}H]5-hydroxytryptamine and [^{3}H]alniditan. *Mol. Pharmacol.*, 50, 1567-1580.

Lovenberg, T.W., Erlander, M.G., Baron, B.M., Racke, M., Slone, A.L., Siegel, B.W., Craft, C.M., Burns, J.E., Danielson, P.E. & Sutcliffe, J.G. (1993). Molecular cloning and functional expression of 5-HT$_{1E}$-like rat and human 5-hydroxytryptamine receptor genes. *Proc. Natl. Acad. Sci. U S A*, 90, 2184-2188.

Ma, Q.P. (2001). Co-localization of 5-HT$_{1B/1D/1F}$ receptors and glutamate in trigeminal ganglia in rats. *Neuroreport*, 12, 1589-91.

Mitsikostas, D.D., Sanchez del Rio, M., Moskowitz, M.A. & Waeber, C. (1999). Both 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors modulate c-fos expression within rat trigeminal nucleus caudalis. *Eur. J. Pharmacol.*, 369, 271-277.

Moskowitz, M.A. (1992). Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. *Trends Pharmacol. Sci.*, 13, 307-311.

Munson, P.J. & Rodbard, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, 107, 220-239.

Nilsson, T., Longmore, J., Shaw, D., Pantev, E., Bard, J.A., Branchek, T. & Edvinsson, L. (1999). Characterisation of 5-HT receptors in human coronary arteries by molecular and pharmacological techniques. *Eur. J. Pharmacol.*, 372, 49-56.

Ochman, H., Gerber, A.S. & Hartl, D.L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics*, 120, 621-623.

Oksenberg, D., Marsters, S.A., O'Dowd, B.F., Jin, H., Havlik, S., Peroutka, S.J. & Ashkenazi, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT$_{1B}$ receptors. *Nature*, 360, 161-163.

Pauwels, P.J., Palmier, C., Wurch, T. & Colpaert, F.C. (1996). Pharmacology of cloned human 5-HT$_{1D}$ receptor-mediated functional responses in stably transfected rat C6-glial cell lines:

further evidence differentiating human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 353, 144-156.

Pauwels, P.J., Tardif, S., Palmier, C., Wurch, T. & Colpaert, F.C. (1997). How efficacious are 5-HT$_{1B/D}$ receptor ligands: an answer from GTP gamma S binding studies with stably transfected C6-glial cell lines. *Neuropharmacology*, 36, 499-512.

Phebus, L.A., Johnson, K.W., Zgombick, J.M., Gilbert, P.J., Van Belle, K., Mancuso, V., Nelson, D.L., Calligaro, D.O., Kiefer, A.D., Jr., Branchek, T.A. & Flaugh, M.E. (1997). Characterization of LY344864 as a pharmacological tool to study 5-HT$_{1F}$ receptors: binding affinities, brain penetration and activity in the neurogenic dural inflammation model of migraine. *Life Sci.*, 61, 2117-2126.

Ponte, P., Ng, S.Y., Engel, J., Gunning, P. & Kedes, L. (1984). Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res.*, 12, 1687-1696.

Price, G.W., Burton, M.J., Collin, L.J., Duckworth, M., Gaster, L., Göthert, M., Jones, B.J., Roberts, C., Watson, J.M. & Middlemiss, D.N. (1997). SB-216641 and BRL-15572-compounds to pharmacologically discriminate h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 356, 312-20.

Razzaque, Z., Heald, M.A., Pickard, J.D., Maskell, L., Beer, M.S., Hill, R.G. & Longmore, J. (1999). Vasoconstriction in human isolated middle meningeal arteries: determining the contribution of 5-HT$_{1B}$- and 5-HT$_{1F}$-receptor activation. *Br. J. Clin. Pharmacol.*, 47, 75-82.

Saxena, P.R. (1995). Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol. Ther.*, 66, 339-68.

Saxena, P.R. & Tfelt-Hansen, P. (2000). Triptans, 5-HT$_{1B/1D}$ receptor agonists in the acute treatment of migraine. In *The Headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 411-438. Philadelphia: Lippincott, Williams & Wilkins.

Shepheard, S., Edvinsson, L., Cumberbatch, M., Williamson, D., Mason, G., Webb, J., Boyce, S., Hill, R. & Hargreaves, R. (1999). Possible antimigraine mechanisms of action of the 5HT$_{1F}$ receptor agonist LY334370. *Cephalalgia*, 19, 851-858.

Villalón, C.M., Centurión, D., Sánchez-López, A., De Vries, P. & Saxena, P.R. (1999). 5-HT receptors mediating external carotid vasoconstriction in vagosympathectomised dogs. *Acta Pharmacol. Sin.*, 20, 1057-1067.

Waeber, C. & Moskowitz, M.A. (1995). [^{3}H]sumatriptan labels both 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor binding sites in the guinea pig brain: an autoradiographic study. *Naunyn- Schmiedeberg's Arch. Pharmacol.*, 352, 263-275.

Wainscott, D.B., Johnson, K.W., Phebus, L.A., Schaus, J.M. & Nelson, D.L. (1998). Human 5-HT$_{1F}$ receptor-stimulated [^{35}S]GTPgammaS binding: correlation with inhibition of guinea pig dural plasma protein extravasation. *Eur. J. Pharmacol.*, 352, 117-124.

Weinshank, R.L., Zgombick, J.M., Macchi, M.J., Branchek, T.A. & Hartig, P.R. (1992). Human seroton$_{1D}$ in receptor is encoded by a subfamily of two distinct genes: 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$. *Proc. Natl. Acad. Sci. U S A*, 89, 3630-3634.

Wurch, T., Colpaert, F.C. & Pauwels, P.J. (1998). Chimeric receptor analysis of the ketanserin binding site in the human 5-hydroxytryptamine$_{1D}$ receptor: importance of the second extracellular loop and fifth transmembrane domain in antagonist binding. *Mol. Pharmacol.*, 54, 1088-1096.

Wurch, T., Palmier, C., Colpaert, F.C. & Pauwels, P.J. (1997). Sequence and functional analysis of cloned guinea pig and rat serotonin 5-HT$_{1D}$ receptors: common pharmacological features within the 5-HT$_{1D}$ receptor subfamily. *J. Neurochem.*, 68, 410-418.

Chapter 5

Molecular cloning and tissue distribution of mRNA encoding porcine 5-HT_7 receptor and its comparison with the structure of other species

Summary The effects of 5-hydroxytriptamine (5-HT, serotonin) are mediated via five main receptor types of which the 5-HT_7 receptor is the most recently characterised member. The 5-HT_7 receptor has been shown to mediate cranial blood vessels dilatation that may result in migraine headache. We report here the cDNA cloning, sequencing and tissue distribution of porcine 5-HT_7 receptor and illustrate its comparison with corresponding receptor of known species. Employing a combination of reverse transcriptase and inverse polymerase chain reaction we amplified and sequenced a full length cDNA from the porcine cerebral cortex. The deduced amino acid sequence comparison confirmed that the cloned porcine receptor belongs to 5-HT_7 receptor as described for human and other species and showing overall homology of 92-96%. The expression of 5-HT_7 receptor mRNA was observed in porcine central (cerebral cortex, trigeminal ganglion and cerebellum) as well as in peripheral (pulmonary and coronary arteries, superior vena cava and saphenous vein) tissues. The established cDNA sequence and tissue distribution of porcine 5-HT_7 receptor will be helpful in exploring the role of this receptor in pathophysiological processes and to predict as a potential therapeutic target for antimigraine drug development.

Based on: Bhalla, P., Saxena, P.R. & Sharma, H.S. Molecular cloning, sequencing and tissue distribution of the porcine 5-HT_7 receptor. **Submitted**
S

Introduction

Devising a novel general framework featuring structural (peptide sequence), operational (ligands affinity rank-order and functional effects) and transductional (second messenger) criteria for receptor classification and nomenclature, Hoyer et al. (1994) recognised four main types 5-hydroxytryptamine (5-HT; serotonin) receptors (5-HT$_{1-4}$) as well as several 'orphan' receptors awaiting characterisation. One such 'orphan' category, termed '5-HT$_1$-like', was shown to be a heterogeneous group comprising four receptor types, namely 5-HT$_{1B}$, 5-HT$_{1D}$, 5-ht$_{1F}$ and 5-HT$_7$ (Saxena et al., 1998). The 5-HT$_7$ receptor (Receptor code: 2.1.5HT.07.000.00.00) is now recognised as a main class of 5-HT receptors mediating circadian phase shifts and gastrointestinal and vascular smooth muscle relaxation (Eglen et al., 1997; Martin, 1998; Saxena et al., 1998; Vanhoenacker et al., 2000), including dilatation of cranial blood vessels (Terrón et al., 1999; Villalón et al., 1997a; Villalón et al., 2001). The latter feature is a hallmark of migraine headache (Humphrey et al., 1991; Saxena, 1995b; Tfelt-Hansen et al., 2000a), a trigeminovascular disorder (May et al., 2001) involving 5-HT (Hamel et al., 2000). Moreover, prophylactic antimigraine drugs, particularly methysergide, behave as antagonists at the 5-HT$_7$ receptor (Hoyer et al., 1994; Tfelt-Hansen et al., 2000b), which is located not only on cerebral blood vessels (Shen et al., 1993) but also on trigeminal ganglion (Terrón et al., 2001).

The 5-HT$_7$ receptor is the recently identified member of 5-HT receptor family and has been characterised on the basis of structural, operational and transductional characteristics. The 5-HT$_7$ receptor has been cloned from human, rat, mouse and guinea pig, belonging to the G-protein-coupled receptor superfamily and is positively linked to adenylyl cyclase (Eglen *et al*., 1997; Vanhoenacker *et al*., 2000). Genomic structure of the human 5-HT$_7$ receptor showed two introns, first between the 3rd and 4th transmembrane domains coding regions and second near the C terminal region (Ruat *et al*., 1993) and the entire gene is localised on chromosome 10q23.3-q24.4 (Gelernter *et al*., 1995). The splice variants of 5-HT$_7$ receptor have been reported in rodents and human tissues (Heidmann *et al*., 1997). Recently, the three splice variants in case of human 5-HT$_7$ receptor were found to be indistinguishable both pharmacologically as well as coupling to adenylyl cyclase activity (Krobert *et al*.,

2001). However, these splice variants of 5-HT$_7$ receptor showed altered pattern of tissue distribution (Heidmann *et al*., 1997; Heidmann *et al*., 1998; Krobert *et al*., 2001).

Previous investigations from our laboratory have established that constriction of carotid arteriovenous anastomoses in the anaesthetised pig can serve as a predictive model for the antimigraine efficacy of 5-HT-based drugs (De Vries et al., 1999; Saxena, 1995b). To complement our continuing *in vivo* investigations, we set out to clone and sequence the porcine 5-HT$_7$ receptor cDNA from cerebral cortex and investigated its distribution in various central and peripheral tissues.

Materials and methods

PCR amplification and cloning of 5-HT$_7$ receptor cDNA

Porcine 5-HT$_7$ receptor cDNA was amplified using a combination of RT-PCR and inverse-PCR techniques; for details see Bhalla *et al.* (2001; 2000). cDNA was synthesised from RNA extracted from cerebral cortex obtained from a pig (Yorkshire x Landrace, female, 12 kg). The quality of RNA extraction and cDNA preparation was checked by PCR amplification of β-actin (Ponte et al., 1984).

Table 5.1. Primers used for RT-PCR and inverse PCR amplification of porcine 5-HT$_7$ receptor[1]

Primers		Sequence	Amplified fragment	
No.	Direction		Nucleotides	Amino acids
a	Forward	5'-GCAGTGGCGTTCTACATCCC-3'	739-993	247-331
b	Reverse	5'-CAAGGTGGTGGCTGCTTTCT-3'		
c	Forward	5'-GAGGGAAGCCTGGGAACTTG-3'	544-844;	182-281;
d	Reverse	5'-GGAGGAGTGTGCGAACCTTTC-3'	903-1302	302-434
e	Forward	ATGATGGGCGTTAACAGCAGC-3'	1-648	1-216
f	Reverse	5'-GTGATGGAGGCGGAGAGAAG-3'		
g	Forward	5'-CCTTGGGATCACGAGGCCGC-3'	549-1277	184-426
h	Reverse	5'-CTCTCAGCAAGCTTCAGGGCC-3'		
i	Forward	5'-GGAGGAGTGTGCGAACCTTTC-3'	903-1344	302-447
j	Reverse	5'-GGATCATGAATCATGACCTTT-3'		

[1]These primers were based either on PCR amplified sequences of porcine 5-HT$_7$ receptor (primers a-d, f-i) or consensus sequences of known recombinant 5-HT$_7$ receptors from other species (Bard et al., 1993; Plassat et al., 1993; Ruat et al., 1993; Tsou et al., 1994) (primers e and j).

As shown in Figure 5.1, porcine 5-HT$_7$ receptor was amplified using a combination of RT-PCR and inverse-PCR techniques (for details, see Bhalla et al., 2001). Based on the partial sequence of porcine 5-HT$_7$ receptor (Ullmer et al., 1995, GenBank accession number Z48177), primers a and b were used to amplify a product of approximately 250 base pairs (bp), which was used to design porcine-specific inverse primers c and d (Figure 5.1A, B; Table 5.1).

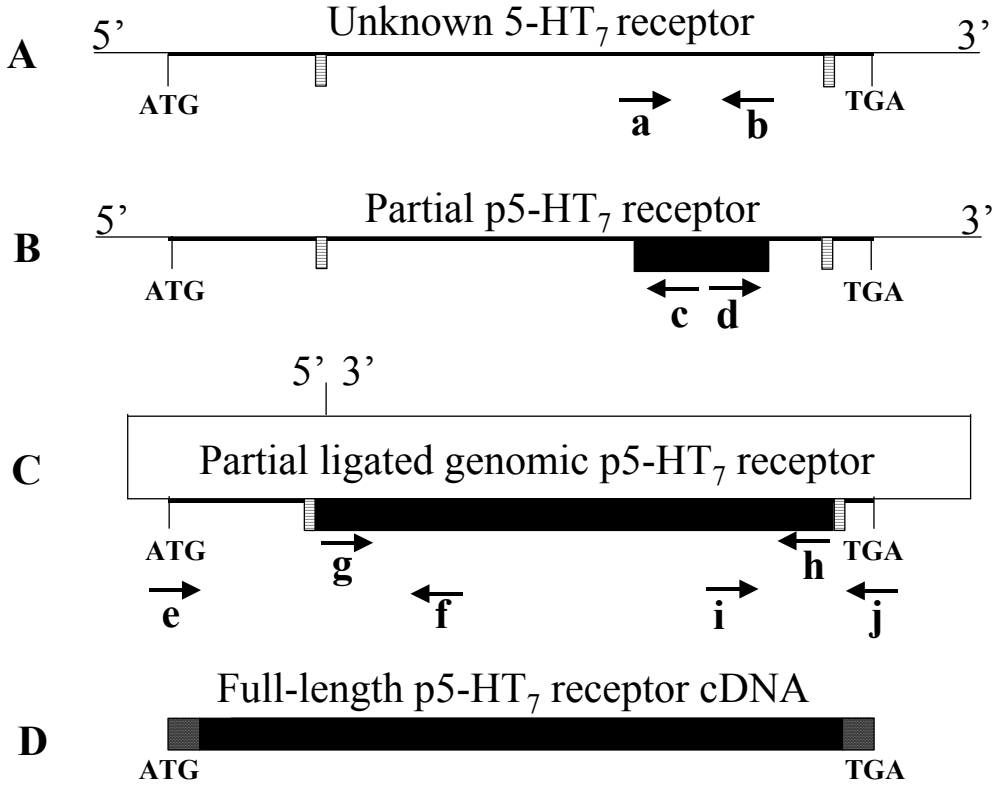


Figure 5.1. Strategy for cloning porcine 5-HT$_7$ receptor and position of various primers (a-j, with directions given by the arrows) used for PCR amplification. Horizontal bars show the position of introns, black bars denote the amplified products, while crossed bars indicate the sequence of primers e and j. A. Unknown full-length porcine 5-HT$_7$ receptor with primers (a and b) derived from a partial sequence of porcine 5-HT$_7$ receptor (GenBank accession number Z48177). B. The amplified partial sequence of porcine 5-HT$_7$ receptor cDNA was used to design inverse porcine specific primers (c and d). C. The inverse-PCR amplified partial genomic sequence enabled us to design further porcine specific primers between the introns (f-i) and 5'and 3' ends primers designed from other species (e and j). D. Three different sizes of amplified products showed the full-length sequence of porcine 5-HT$_7$ receptor cDNA.

These inverse primers were used with genomic DNA circles prepared after digesting the porcine genomic DNA with Bgl II restriction enzyme, selected on the basis of the absence of Bgl II-sensitive restriction site in the cDNA sequence of 5-HT$_7$ receptor from other species (DNAMAN sequence analysis program, Version 3.2, Lynnon Biosoft©, 1994-1997). The PCR amplified products were purified and ligated into pGEMT Easy vector (Promega Benelux b.v., Leiden, The Netherlands). The ligated products were transformed into competent JM 109 cells and grown overnight on LB agar (containing ampicillin, IPTG and X-gal) plates at 37°C. White over blue colonies were further processed for the plasmid DNA isolation (mini-prep, Promega Benelux b.v., Leiden, The Netherlands) and sequenced by the dideoxy nucleotide chain termination method using an automated fluorescence based DNA sequencer (ABI Prism™ 310 Genetic analyser, Perkin Elmer Applied Biosystem Benelux, Nieuwerkerk aan den IJssel, The Netherlands). The nucleotide sequences were compared and a consensus sequence was derived using the DNAMAN sequence analysis program software. The final partial genomic DNA sequence (Figure 5.1C) was compared with those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, USA).

For the amplification of full-length porcine 5-HT$_7$ receptor cDNA, three sets of primers (Figure 5.1C) were used with the cDNA template derived from porcine cerebral cortex: (i) primers e and f, (ii) primers g and h, and (iii) primers i and j (Figure 5.1C, D; Table 5.1). These primers were designed based either on the inverse PCR amplified sequence (primers f-i) of porcine 5-HT$_7$ receptor or consensus sequences (primers e and j) of recombinant 5-HT$_7$ receptors from the human (Bard et al., 1993), rat (Ruat et al., 1993) (now the 5-HT$_{7(a)}$ isoform in these two species (Vanhoenacker et al., 2000)), mouse (Plassat et al., 1993), and guinea pig (Tsou et al., 1994). Thus, the amplified three products were checked on a 1% agarose gel, purified, cloned and sequenced. The nucleotide sequences obtained from the amplified products were aligned together to derive a final sequence of the porcine 5-HT$_7$ receptor, using the DNAMAN sequence analysis program (Version 3.2, Lynnon Biosoft©, 1994-1997). The cDNA sequences of the cloned products were confirmed by at least two independent PCRs and further verified by genomic DNA (inverse-PCR) amplified products. 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.

The final sequence (Figure 5.1D) was translated as a peptide sequence and compared with those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, USA). The hydrophobic regions (indicating putative transmembrane domains) and sequence homology with known 5-HT$_7$ receptors from other species were identified.

Tissue distribution of the 5-HT$_7$ receptor mRNA

As described in detail before (Bhalla et al., 2001; Bhalla et al., 2000), RT-PCR technique was used to detect 5-HT$_7$ receptor mRNA in the following porcine tissues obtained from pigs (Yorkshire x Landrace, female, 12-15 kg): brain cortex, trigeminal ganglion, cerebellum, pulmonary and coronary arteries, left cardiac ventricle, superior vena cava and saphenous vein. The tissue samples were dissected and cleaned and total RNA was isolated. The purified total RNA samples were reverse transcribed into cDNA in the presence of reverse transcriptase enzyme. A control reaction was always prepared in the absence of reverse transcriptase to monitor possible DNA contamination. The porcine 5-HT$_7$ receptor was detected using porcine specific sense (5'-GCAGTGGCGTTCTACATCCC-3'; nucleotides 739-758) and antisense (5'-CAAGGTGGTGGCTGCTTTCT-3'; nucleotides 974-993) primers. The PCR amplified products were separated on 1.5% agarose gel by electrophoretic separation and photographed.

All oligonucleotide primers were commercially procured from Life Technologies b.v. (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 b.v. (Leiden, The Netherlands). Oligotex mRNA purification kit was purchased from Qiagen GmbH (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).

Results

Cloning and sequence analysis of porcine 5-HT$_7$ receptor cDNA

The various primers and the approximate length of products that were amplified using RT-PCR and inverse PCR are shown in Table 5.1. The first two primers (a and b) yielded a product of expected length from the porcine cDNA derived from brain cortex. This product corresponded to a fragment of porcine 5-HT$_7$ receptor cDNA reported earlier (Ullmer et al., 1995, GenBank accession number Z48177). Based on this fragment, we designed two porcine-specific inverse primers (c and d) and obtained a product of approximately 1100 bp from ligated porcine genomic DNA. Upon sequencing, this fragment revealed a high homology (>90%) with the human 5-HT$_7$ receptor (data not shown) and represented a part of porcine 5-HT$_7$ receptor between the two introns. Subsequently, we amplified and cloned three fragments of expected sizes from the cDNA template derived from porcine cerebral cortex, used with specially designed primer sets (Table 5.1; Figure 5.2, lanes 2, 3 and 4). Sequencing of these cloned fragments revealed the specific partial sequences of porcine 5-HT$_7$ receptor and these sequences were overlapped to obtain the full-length sequence of porcine 5-HT$_7$ receptor cDNA (Figure 5.3). It may, however, be pointed out that the 5' and 3' end primers were from different species (see Table 5.1).

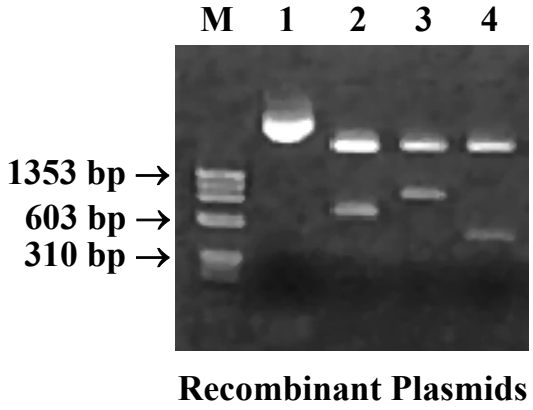


Figure 5.2. Agarose gel electrophoresis of recombinant plasmid with different sizes of insert cDNA (amplified from cDNA template of porcine cerebral cortex). The different lanes marked on the top denote: ϕx174 DNA/Hae III marker (M), recombinant uncut plasmid DNA vector (1) and plasmid DNA vector restricted with EcoR1 enzyme and showing different DNA insert of approximately 650, 750 and 450bp (2, 3 and 4). The size (base pairs; bp) of 3 marker bands is indicated on the left margin.

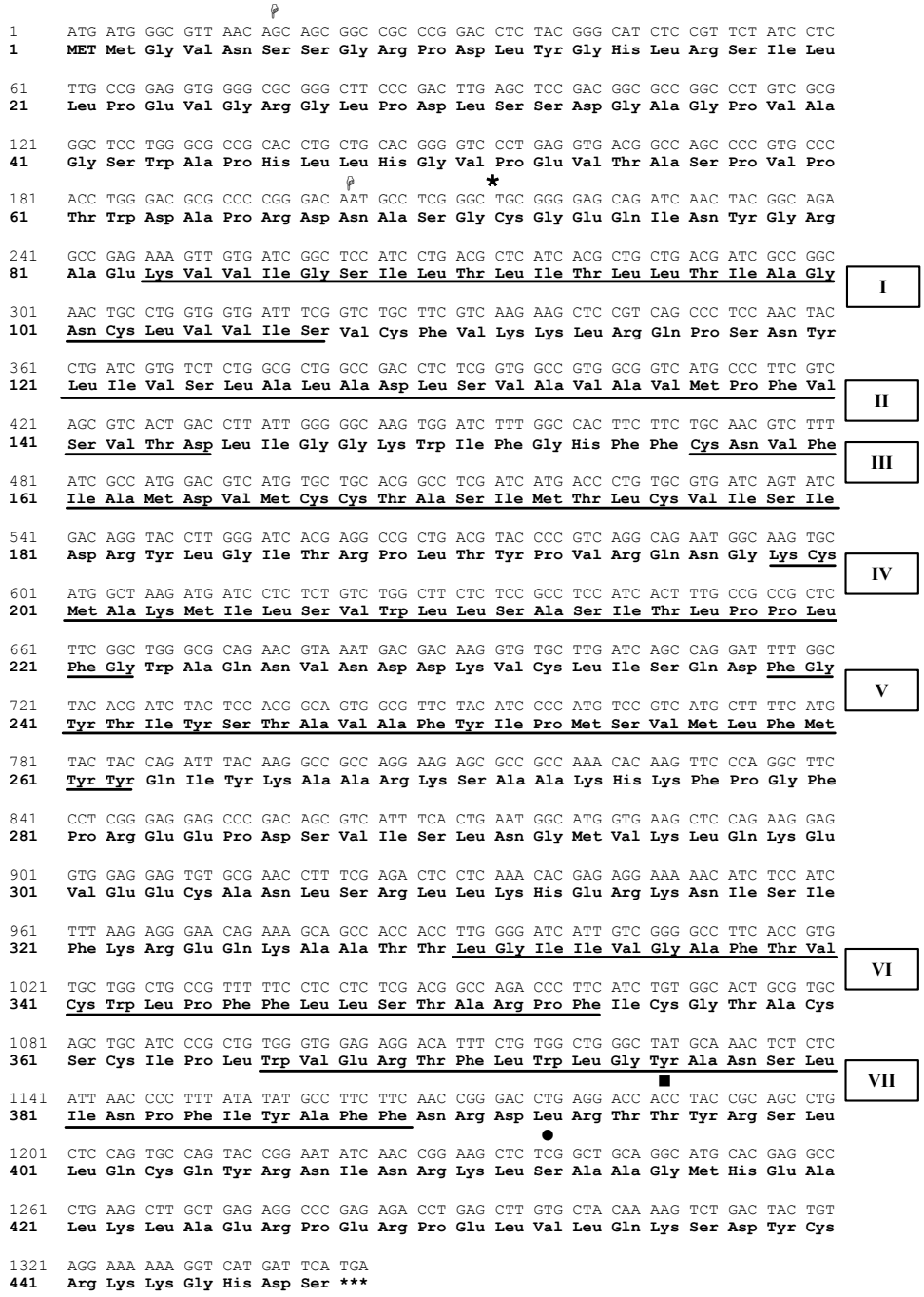


Figure 5.3. Nucleotide and deduced amino acid (in bold) sequences of the recombinant cDNA derived from porcine cerebral cortex. Numbering of nucleotide and amino acids is shown on the left. Computer analysis (software DNAMAN, version 3.2, Lynnon Biosoft©) predicted a typical G-protein coupled receptor with transmembrane domains I-VII (underlined) as well as the putative N-glycosylation, protein kinase A phoshorylation and protein kinase C phosphorylation sites.

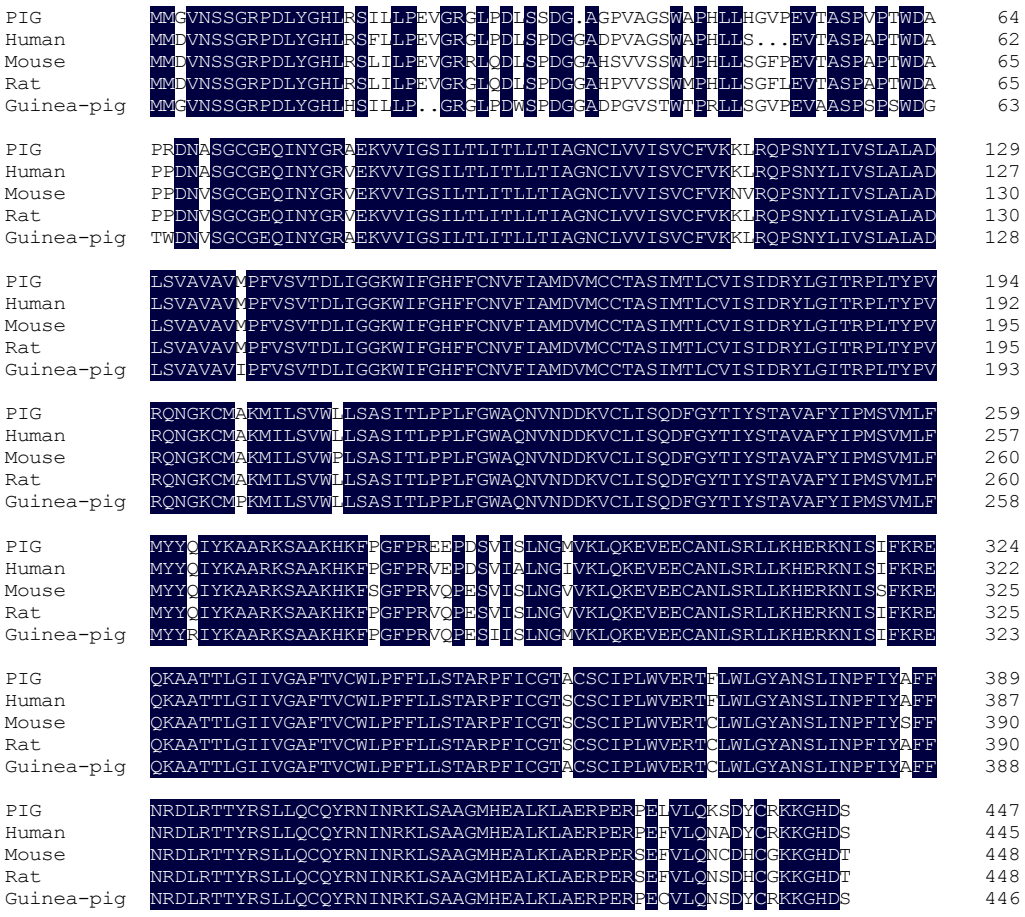


Figure 5.4. Comparison of amino acid sequences of the pig 5-HT$_7$ receptor with those of human (Swissprot accession number AAC37538), mouse (CAA80654), rat (AAA40617) and guinea pig (AAA83015) 5-HT$_7$ receptors. The areas shaded black show identity across the species.

The full-length porcine 5-HT$_7$ receptor cDNA consisted of 1344 bp, starting with ATG codon and ending with TGA codon. DNAMAN analysis showed that this porcine cDNA encoded an open reading frame of 447 amino acid peptide exhibiting features of a typical G-protein-coupled receptor with predicted seven transmembrane domains and putative N-glycosylation and phosphorylation sites (Figure 5.3). A BLAST search at GenBank of the 1344 bp nucleotide sequence revealed high homology (>90%) with the sequence of the 5-HT$_7$ receptor from other species.

Figure 5.4 compares the amino acid sequence of porcine 5-HT$_7$ receptors with that reported from other mammalian species (5-HT$_{7(a)}$ isoform in the case of human and rat). The amino acid homology between the cloned porcine 5-HT$_7$ and human 5-HT$_{7(a)}$ receptors was the highest (96%) and there was a 100% identity in

transmembrane domains, except in the IV and V domains (96%). Nevertheless, some unique amino acids were unique to the porcine 5-HT$_7$ receptor sequence, namely: Ser^{33}, Gly^{37}, His^{49}, Val^{59} and Arg^{66} (N-terminal extracellular region), Leu^{221} (IV transmembrane domain), Met^{248} (V transmembrane domain), Glu^{283} (III intracellular loop) and Leu^{433} and Lys^{436} (C-terminal intracellular region).

Tissue distribution of 5-HT$_7$ receptor

RT-PCR technique was used to assess the expression of 5-HT$_7$ receptor in several porcine tissues. As shown in Figure 5.5, a fragment of expected size (approximately 300 bp), representing porcine 5-HT$_7$ receptor mRNA, was detected in the brain cortex, trigeminal ganglion, cerebellum (weak signal), pulmonary and coronary arteries, superior vena cava and saphenous vein, but not in the left cardiac ventricle.

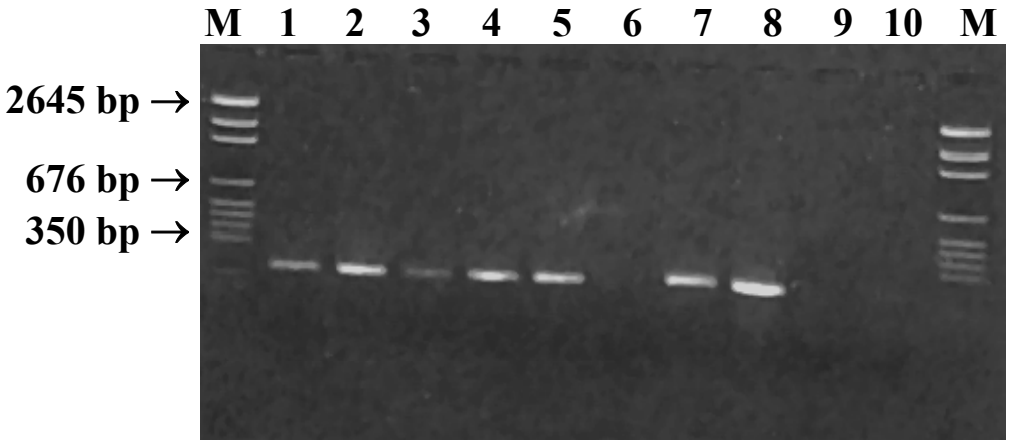


Figure 5.5. Agarose gel electophoresis of PCR amplified products derived from cDNA obtained from porcine tissues. The lanes marked on top denote: pGEM marker (M), brain cortex (1), trigeminal ganglion (2), cerebellum (3), pulmonary artery (4), coronary artery (5), left cardiac ventricle (6), superior vena cava (7), saphenous vein (8), brain cortex without reverse transcriptase (9), autoclaved water (10) and pGEM marker (M). The size of marker bands is indicated in the left margin.

Possible genomic DNA and/or PCR contamination in the present experiments was ruled out because no amplification was found in the negative controls (autoclaved water or brain cortex in the absence of reverse transcriptase step).

Discussion

Sequence analysis of porcine 5-HT$_7$ receptor

The primers derived from inverse PCR sequence of porcine genomic DNA as well as from consensus sequence of other species were used for the amplification of full-length porcine 5-HT$_7$ receptor cDNA from the cerebral cortex. The nucleotide sequence of porcine 5-HT$_7$ receptor encoded an open reading frame of 447 amino acid peptide, which showed a high homology (92-96%) with the sequence of human (Bard et al., 1993), mouse (Plassat et al., 1993), rat (Lovenberg et al., 1993; Ruat et al., 1993; Shen et al., 1993) and guinea pig (Tsou et al., 1994) homologues. Additionally, the partial sequence of porcine 5-HT$_7$ receptor, previously submitted to the GenBank by Ullmer et al. (1995) was identical to the amino acid sequence present in full-length porcine 5-HT$_7$ receptor. Thus, the nucleotide sequence of the mRNA obtained from porcine cerebral cortex encodes for the 5-HT$_7$ receptor.

It is important to point out that the porcine 5-HT$_7$ receptor gene, as is the case in other species (Bard et al., 1993; Ruat et al., 1993) contains two introns, the first between the 3rd and 4th transmembrane domains and the second near the C terminal region (Bard et al., 1993; Ruat et al., 1993). Three splice variants of the 5-HT$_7$ receptor have been reported in rats (5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(c)}$ with 448, 435 and 470 amino acids, respectively) as well as humans (5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ with 445, 432 and 479 amino acids, respectively) (Heidmann et al., 1997). Although these splice variants are pharmacologically indistinguishable and are all positively coupled to adenylyl cyclase, they do seem to differ in the pattern of tissue distribution (Heidmann et al., 1998; Krobert et al., 2001). We do not yet know if alternate splicing yielding splice variants occurs in the pig, but the number (447) and sequence of amino acids present suggest that the present porcine 5-HT$_7$ receptor may represent the 5-HT$_{7(a)}$ isoform.

Tissue distribution of 5-HT$_7$ receptor

The 5-HT$_7$ receptor mRNA signals were detected in the porcine brain cortex, trigeminal ganglion, cerebellum, pulmonary and coronary arteries, superior vena cava and saphenous vein. A similar distribution has been described in the human, guinea pig and rat (Bard et al., 1993; Heidmann et al., 1998; Terrón et al., 2001; To et

al., 1995) as well as in the pig. Although the full sequence of the porcine 5-HT$_7$ receptor had not been established, Ullmer et al. (1995) reported that the 5-HT$_7$ receptor mRNA was present in the coronary, cerebral and pulmonary arteries and cerebral veins. The ubiquitous presence of the 5-HT$_7$ receptor in blood vessels, coupled with the very high affinity for 5-HT (Hoyer et al., 1994), indicates that the 5-HT$_7$ receptor may be involved in the physiological regulation vascular tone.

Interestingly, the 5-HT$_7$ receptor mRNA was detected neither in the pig heart (present results) nor in the human heart (Bard et al., 1993). This is in agreement with functional characterisation of cardiac 5-HT receptors revealing that, while the 5-HT$_7$ receptor mediates tachycardia in the cat, that in the pig and human is mediated by the 5-HT$_4$ receptor (Saxena et al., 1991).

Clinical relevance

Being the latest member of the 5-HT receptor family, the 5-HT$_7$ receptor is an attractive therapeutic target to explore in depression, migraine and circadian rhythm, neuroendocrine, and affective behaviour disorders (Eglen et al., 1997; Vanhoenacker et al., 2000; Villalón et al., 1997b). This may be particularly the case with selective 5-HT$_7$ receptor antagonists, such as LY215840 (Cushing et al., 1996) and SB269970 (Lovell et al., 2000; Roberts et al., 2001), for use in migraine. Indeed, it is possible that cranial vasodilatation, which is an integral part of migraine pathophysiology (Humphrey et al., 1991; Saxena, 1995b; Tfelt-Hansen et al., 2000a), may be mediated by the 5-HT$_7$ receptor (Terrón et al., 1999; Villalón et al., 1997b; Villalón et al., 2001). Moreover, 5-HT$_7$ receptors may be involved in pain transmission, hyperalgesia and neurogenic inflammation (see Eglen et al., 1997; Terrón, 1998). The cloning of the porcine 5-HT$_7$ receptor may help initiate further studies in animal models predictive for antimigraine efficacy (De Vries et al., 1999; Saxena, 1995a).

References

Bard, J.A., Zgombick, J., Adham, N., Vaysse, P., Branchek, T.A. & Weinshank, R.L. (1993). Cloning of a novel human serotonin receptor (5-HT$_7$) positively linked to adenylate cyclase. *J Biol Chem*, 268, 23422-23426.

Bhalla, P., Sharma, H.S., Ma, X., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2001). Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT$_{1B}$ receptor. *Br J Pharmacol*, 133, 891-901.

Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2000). Molecular Cloning, Sequence analysis and pharmacological properties of the porcine 5-HT$_{1D}$ receptor. *Br J Pharmacol*, 131, 949-957.

Cushing, D.J., Zgombick, J.M., Nelson, D.L. & Cohen, M.L. (1996). LY215840, a high-affinity 5-HT$_7$ receptor ligand, blocks serotonin-induced relaxation in canine coronary artery. *J Pharmacol Exp Ther*, 277, 1560-1566.

De Vries, P., Villalón, C.M. & Saxena, P.R. (1999). Pharmacological aspects of experimental headache models in relation to acute antimigraine therapy. *Eur J Pharmacol*, 375, 61-74.

Eglen, R.M., Jasper, J.R., Chang, D.J. & Martin, G.R. (1997). The 5-HT$_7$ receptor: orphan found. *Trends Pharmacol Sci*, 18, 104-107.

Gelernter, J., Rao, P.A., Pauls, D.L., Hamblin, M.W., Sibley, D.R. & Kidd, K.K. (1995). Assignment of the 5HT$_7$ receptor gene (HTR$_7$) to chromosome 10q and exclusion of genetic linkage with Tourette syndrome. *Genomics*, 26, 207-209.

Hamel, E. & Saxena, P.R. (2000). 5-Hydroxytryptamine involvement in migraine. In *The Headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 319-24. Philadelphia: Lippincott, Williams & Wilkins.

Heidmann, D.E., Metcalf, M.A., Kohen, R. & Hamblin, M.W. (1997). Four 5-hydroxytryptamine$_7$ (5-HT$_7$) receptor isoforms in human and rat produced by alternative splicing: species differences due to altered intron-exon organization. *J Neurochem*, 68, 1372-1381.

Heidmann, D.E., Szot, P., Kohen, R. & Hamblin, M.W. (1998). Function and distribution of three rat 5-hydroxytryptamine$_7$ (5-HT$_7$) receptor isoforms produced by alternative splicing. *Neuropharmacology*, 37, 1621-1632.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R. & Humphrey, P.P.A. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol Rev*, 46, 157-203.

Humphrey, P.P.A. & Feniuk, W. (1991). Mode of action of the anti-migraine drug sumatriptan. *Trends Pharmacol Sci*, 12, 444-6.

Krobert, K.A., Bach, T., Syversveen, T., Kvingedal, A.M. & Levy, F.O. (2001). The cloned human 5-HT$_7$ receptor splice variants: a comparative characterization of their pharmacology, function and distribution. *Naunyn-Schmiedeberg's Arch Pharmacol*, 363, 620-632.

Lovell, P.J., Bromidge, S.M., Dabbs, S., Duckworth, D.M., Forbes, I.T., Jennings, A.J., King, F.D., Middlemiss, D.N., Rahman, S.K., Saunders, D.V., Collin, L.L., Hagan, J.J., Riley, G.J. & Thomas, D.R. (2000). A novel, potent, and selective 5-HT$_7$ antagonist: (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)pyrrolidine-1-sulfonyl) phen ol (SB-269970). *J Med Chem*, 43, 342-345.

Lovenberg, T.W., Baron, B.M., de Lecea, L., Miller, J.D., Prosser, R.A., Rea, M.A., Foye, P.E., Racke, M., Slone, A.L., Siegel, B.W. & et al. (1993). A novel adenylyl cyclase-activating serotonin receptor (5-HT$_7$) implicated in the regulation of mammalian circadian rhythms. *Neuron*, 11, 449-458.

Martin, G.R. (1998). 5-Hydroxytrptamine receptors. In *The International Union of Pharmacology Compendium of Receptor Characterization and Classification*. ed. IUPHAR Committee on Receptor Nomenclature and Drug Classification. pp. 167-185. London: IUPHAR Media.

May, A. & Goadsby, P.J. (2001). Pharmacological opportunities and pitfalls in the therapy of migraine. *Curr Opin Neurol*, 14, 341-5.

Plassat, J.L., Amlaiky, N. & Hen, R. (1993). Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. *Mol Pharmacol*, 44, 229-236.

Ponte, P., Ng, S.Y., Engel, J., Gunning, P. & Kedes, L. (1984). Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res*, 12, 1687-1696.

Roberts, C., Allen, L., Langmead, C.J., Hagan, J.J., Middlemiss, D.N. & Price, G.W. (2001). The effect of SB-269970, a 5-HT$_7$ receptor antagonist, on 5-HT release from serotonergic terminals and cell bodies. *Br J Pharmacol*, 132, 1574-1580.

Ruat, M., Traiffort, E., Leurs, R., Tardivel-Lacombe, J., Diaz, J., Arrang, J.M. & Schwartz, J.C. (1993). Molecular cloning, characterization, and localization of a high-affinity serotonin receptor (5-HT$_7$) activating cAMP formation. *Proc Natl Acad Sci U S A*, 90, 8547-8551.

Saxena, P., De Vries, P. & Villalon, C. (1998). 5-HT$_1$-like receptors: a time to bid goodbye. *Trends Pharmacol Sci*, 19, 311-319.

Saxena, P.R. (1995). Cranial arteriovenous shunting, an *in vivo* animal model for migraine. In *Experimental headache models*. ed. Olesen, J. & Moskowitz, M.A. pp. 189-198. Philadelphia, USA: Lippincott-Raven Publishers.

Saxena, P.R. (1995). Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol Ther*, 66, 339-368.

Saxena, P.R. & Villalón, C.M. (1991). 5-Hydroxytryptamine: a chameleon in the heart. *Trends Pharmacol Sci*, 12, 223-227.

Shen, Y., Monsma, F.J., Jr., Metcalf, M.A., Jose, P.A., Hamblin, M.W. & Sibley, D.R. (1993). Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. *J Biol Chem*, 268, 18200-18204.

Terrón, J.A. (1998). 5-HT$_7$ receptor: a target for novel therapeutic avenues? *Invest Drugs*, 1, 302-310.

Terrón, J.A., Bouchelet, I. & Hamel, E. (2001). 5-HT$_7$ receptor mRNA expression in human trigeminal ganglia. *Neurosci Lett*, 302, 9-12.

Terrón, J.A. & Falcón-Neri, A. (1999). Pharmacological evidence for the 5-HT$_7$ receptor mediating smooth muscle relaxation in canine cerebral arteries. *Br J Pharmacol*, 127, 609-616.

Tfelt-Hansen, P., De Vries, P. & Saxena, P.R. (2000). Triptans in migraine: a comparative review of pharmacology, pharmacokinetics and efficacy. *Drugs*, 60, 1259-87.

Tfelt-Hansen, P. & Saxena, P.R. (2000). Antiserotonin drugs in migraine prophylaxis. In *The Headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 467-76. Philadelphia: Lippincott, Williams & Wilkins.

To, Z.P., Bonhaus, D.W., Eglen, R.M. & Jakeman, L.B. (1995). Characterization and distribution of putative 5-ht$_7$ receptors in guinea-pig brain. *Br J Pharmacol*, 115, 107-116.

Tsou, A.P., Kosaka, A., Bach, C., Zuppan, P., Yee, C., Tom, L., Alvarez, R., Ramsey, S., Bonhaus, D.W. & Stefanich, E. (1994). Cloning and expression of a 5-hydroxytryptamine$_7$ receptor positively coupled to adenylyl cyclase. *J Neurochem*, 63, 456-464.

Ullmer, C., Schmuck, K., Kalkman, H.O. & Lubbert, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *FEBS Lett*, 370, 215-221.

Vanhoenacker, P., Haegeman, G. & Leysen, J.E. (2000). 5-HT$_7$ receptors: current knowledge and future prospects. *Trends Pharmacol Sci*, 21, 70-77.

Villalón, C.M., Centurión, D., Lujan-Estrada, M., Terrón, J.A. & Sánchez-López, A. (1997). Mediation of 5-HT-induced external carotid vasodilatation in GR 127935- pretreated vagosympathectomized dogs by the putative 5-HT$_7$ receptor. *Br J Pharmacol*, 120, 1319-27.

Villalón, C.M., DeVries, P. & Saxena, P.R. (1997). Serotonin receptors as cardiovascular targets. *Drug Discovery Today*, 2, 294-300.

Villalón, C.M., Sánchez-López, A., Centurión, D. & Saxena, P.R. (2001). Unravelling the pharmacological profile of the canine external carotid vasodilator '5-HT$_{1\text{-like}}$' receptors: coexistence of sympatho-inhibitory 5-HT$_{1B}$ and postjunctional 5-HT$_7$ receptors. *Naunyn-Schmiedeberg's Arch Pharmacol*, 363, 73-80.

Chapter 6

Characterisation of sumatriptan-induced contractions in human isolated blood vessels using selective 5-HT_{1B} and 5-HT_{1D} receptor antagonists and *in situ* hybridisation

Summary. The 5-$HT_{1B/1D}$ receptor agonist sumatriptan is effective in aborting acute attacks of migraine and is known to cause constriction of cranial arteries as well as some peripheral blood vessels. The present study set out to investigate whether 5-HT_{1B} and/or 5-HT_{1D} receptors mediate contractions of the human isolated middle meningeal and temporal arteries (models for antimigraine efficacy) and coronary artery and saphenous vein (models for side-effect potential). Concentration-response curves were made with sumatriptan (1 nM-100 μM) in blood vessels in the absence or presence of selective antagonists at 5-HT_{1B} (SB224289) and 5-HT_{1D} (BRL15572) receptors. SB224289 antagonised sumatriptan-induced contractions in all blood vessels, although the antagonism profile was different amongst these blood vessels. In the temporal artery, SB224289 abolished contraction to sumatriptan, whereas in the middle meningeal artery and saphenous vein, sumatriptan-induced contractions were blocked in an insurmountable fashion. Moreover, SB224289 acted as a weak surmountable antagonist in the coronary artery (pK_B: 6.4±0.2). In contrast, BRL15572 had little or no effect on sumatriptan-induced contractions in the four blood vessels investigated. *In situ* hybridisation revealed the expression of 5-HT_{1B} receptor mRNA in the smooth muscle as well as endothelial cells of the blood vessels, whereas the mRNA for the 5-HT_{1D} receptor was only very weakly expressed. These results show that the 5-HT_{1B} receptor is primarily involved in sumatriptan-induced contractions of human cranial as well as peripheral blood vessels.

Based on: Rémon W. M. van den Broek, Pankaj Bhalla, Antoinette MaassenVanDenBrink, René de Vries, Hari S. Sharma and Pramod R. Saxena (2001). Characterisation of sumatriptan-induced contractions in human isolated blood vessels using selective 5-HT_{1B} and 5-HT_{1D} receptor antagonists and *in situ* hybridisation. *Cephalalgia*; **In press**.

Introduction

Sumatriptan, the first of the 5-HT$_{1B/1D}$ receptor agonists, is highly effective in aborting migraine headaches (Goadsby, 1998; Tfelt-Hansen *et al*., 2000). It is believed that migraine headache results from dilation of extracerebral cranial blood vessels and that sumatriptan, as well as other triptans, constrict these dilated vessels (Humphrey *et al*., 1991; Tfelt-Hansen *et al*., 2000). Although the triptans are known to be cranioselective, they all have the propensity to constrict the coronary artery (Longmore *et al*., 1998; MaassenVanDenBrink *et al*., 2000b; Parsons *et al*., 1998; Van den Broek *et al*., 2000). Constriction of the coronary artery may lead to cardiovascular adverse events, including myocardial ischaemia and infarction in predisposed individuals (Ottervanger *et al*., 1997).

The triptans are high affinity agonists at 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, but their vasoconstrictor effect seems to be mediated via the 5-HT$_{1B}$ receptor (Tfelt-Hansen *et al*., 2000). Using RT-PCR techniques, it has also been shown that the 5-HT$_{1B}$ receptor mRNA is predominant over 5-HT$_{1D}$ receptor mRNA in the human middle cerebral (Bouchelet *et al*., 1996; Hamel *et al*., 1993), middle meningeal (Schmuck *et al*., 1996), temporal (Verheggen *et al*., 1998) and coronary (Bouchelet *et al*., 2000; Nilsson *et al*., 1999b) arteries. The 5-HT$_{1B}$ receptor protein has also been localised in the smooth muscle layer as well as endothelium of the human middle cerebral (Nilsson *et al*., 1999a), middle meningeal (Longmore *et al*., 1998; Longmore *et al*., 1997) and coronary (Longmore *et al*., 1998; Longmore *et al*., 1997; Nilsson *et al*., 1999b) arteries, where the 5-HT$_{1D}$ receptor protein is not, or poorly, expressed. Functional pharmacological *in vitro* studies suggest that sumatriptan behaves as a full agonist in blood vessels (Bax *et al*., 1992; Jansen *et al*., 1992; Kaumann *et al*., 1994; Kaumann *et al*., 1993; Verheggen *et al*., 1996), which do not contract in response to selective 5-HT$_{1D}$ receptor agonists (Bouchelet *et al*., 2000; Ennis *et al*., 1998).

A more direct evidence for a 5-HT$_{1B}$ receptor-mediated vasoconstriction to sumatriptan can be obtained from antagonist studies. Until now, studies in the human isolated middle meningeal (Jansen *et al*., 1992; Razzaque *et al*., 1999; Van den Broek *et al*., 2000) and coronary (Bax *et al*., 1993; Kaumann *et al*., 1994; MaassenVanDenBrink *et al*., 2000a; Van den Broek *et al*., 2000) arteries and saphenous vein (Bax *et al*., 1992), have used non-selective 5-HT$_{1B/1D}$ receptor

antagonists (GR127935, GR55562 and GR125743). Recently, SB224289 and BRL15572 have been introduced as selective 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor antagonists, respectively (Schlicker *et al*., 1997; Selkirk *et al*., 1998). The use of these compounds revealed that sumatriptan-induced contractions in human isolated temporal (Verheggen *et al*., 1998) and small pulmonary (Morecroft *et al*., 1999) arteries as well as canine (De Vries *et al*., 1998) and porcine (De Vries *et al*., 1999) carotid vascular beds are mediated via the 5-HT$_{1B}$ receptor. Using both functional *in vitro* and *in situ* hybridisation techniques, we investigated the role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in mediating contractions of the human isolated middle meningeal and temporal arteries (models for therapeutic efficacy in migraine) and coronary artery and saphenous vein (models for peripheral side-effect potential) (MaassenVanDenBrink *et al*., 1998; MaassenVanDenBrink *et al*., 2000b; Van den Broek *et al*., 2000).

Material and methods

Tissue collection

The middle meningeal (4 male, 5 female; age 30-72 years) and temporal (3 female; age 45-59 years) arteries and saphenous vein (8 male, 1 female; age 45-78 years) were obtained postoperatively from patients undergoing craniotomy (middle meningeal and temporal artery: 8 aneurysms; 4 meningiomas) or coronary artery bypass grafting (saphenous vein) at the Erasmus University Medical Centre, Rotterdam, The Netherlands. The blood vessels were placed in a propylene tube filled with ice-cold (0-4 °C) physiological saline, transported immediately to the laboratory and used within 2 hr of surgery.

The right epicardial coronary artery was obtained from 9 heart beating organ donors (3 male, 6 female; 37-64 years) who died of non-cardiac disorders (6 cerebrovascular accident, 2 cerebral infarction, 1 head trauma). The Rotterdam Heart Valve Bank, Rotterdam, The Netherlands provided the hearts, after donor mediation by Bio Implant Services Foundation/Eurostransplant Foundation, Leiden, The Netherlands. The vessel was stored overnight in a modified Krebs bicarbonate solution (see below) and used the next day.

Organ bath experiments

Measurement of vascular contractions. The methods used were similar to those described in detail earlier (MaassenVanDenBrink *et al*., 2000b; Van den Broek *et al*., 2000). Briefly, approximately 4-mm segments, obtained from pieces of the middle meningeal (n=6), temporal (n=3), coronary (n=6) arteries and saphenous vein (n=6), were mounted on metal prongs in organ baths, containing a modified Krebs bicarbonate solution (pH 7.4; 37 ^{o}C), aerated with 95% O_2 and 5% CO_2. The composition (mM) of the Krebs bicarbonate solution was NaCl 119, KCl 4.7, $CaCl_2$ 1.25 (or 2.5 for coronary artery and saphenous vein), $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1 (or 8.3 for coronary artery and saphenous vein). The cyclo-oxygenase inhibitor indomethacin (0.1 μM) was added to the Krebs solution to prevent prostaglandin synthesis. In addition, the Krebs solution was enriched with the muscarinic receptor antagonist, atropine, the histamine H_1 receptor antagonist mepyramine, the mixed 5-HT$_{2/7}$ receptor antagonist mesulergine, the α_1-adrenoceptor antagonist prazosin, the inhibitor of neuronal uptake$_1$, imipramine (all 1 μM) and the inhibitor of extra-neuronal uptake$_2$, corticosterone (10 μM) to exclude the putative involvement of these receptors/mechanisms. Changes in isometric tension were registered on recording set-ups from either EMKA Technology (Paris, France) for the middle meningeal and temporal arteries or Harvard Apparatus (South Natick, MA, USA) for the coronary artery and saphenous vein. The segments were allowed to equilibrate for at least 30 min, stretched to a passive tension of 4 mN (middle meningeal and temporal arteries), 15 mN (coronary artery) or 10 mN (saphenous vein). Since sumatriptan is metabolised by monoamine oxidase (Dixon *et al*., 1994), we then treated the segments with pargyline (100 μM) for 15 min to prevent its possible breakdown. After washing, all segments were exposed 2-3 times to either 0.1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; middle meningeal and temporal arteries) or 30 mM KCl (coronary artery and saphenous vein) to demonstrate the reproducibility of the evoked contractions. Subsequently, the relaxation response to 10 nM substance P (1 nM in case of coronary artery or 0.1 μM bradykinin in the case of saphenous vein) in vessel segments pre-contracted with $PGF_{2\alpha}$ (1 μM) was used to verify the functional integrity of the endothelium.

After washing, the segments were allowed to equilibrate for 60 min, with replacement of the Krebs solution every 15 min. The segments were then studied in a paired parallel experimental set-up (MaassenVanDenBrink *et al*., 2000b; Van den Broek *et al*., 2000), where a single concentration response curve to sumatriptan (1 nM-100 μM) was constructed in each segment incubated for 60 min with either the vehicle, SB224289, BRL15572 or both antagonists together. Since only 3 segments could be obtained from the human temporal artery, addition of both antagonists together was omitted. In view of the affinity of SB224289 and BRL15572 at recombinant h5-HT$_{1B}$ (pK$_i$: 8.0 and 6.1, respectively) and h5-HT$_{1D}$ receptors (pK$_i$: 6.2 and 7.9, respectively) (Schlicker *et al*., 1997; Selkirk *et al*., 1998), we initially employed the two antagonists in a concentration range of 10-30 μM in the coronary artery (without the cocktail of inhibitors as mentioned above) and saphenous vein. Since in these concentrations neither antagonist affected the concentration response curves of sumatriptan, all experiments were performed with 1 μM of SB224289 and BRL15572.

Data presentation. Contractile responses were expressed as percentage of the contractile response to 1 μM PGF$_{2\alpha}$ (temporal and middle meningeal arteries) or 100 mM KCl (coronary artery and saphenous vein). The occasional spontaneous phasic contractions, observed in some coronary artery and saphenous vein segments, were ignored where measuring contractions. When the concentration contraction curve to sumatriptan did not attain a plateau, the contraction with its highest concentration (100 μM) was considered as the apparent maximum contraction (E$_{max}$). Initially, the mean value of E$_{max}$ of sumatriptan observed in individual experiments was calculated. The mean concentration response curves were analysed with a non-linear regression fitting technique for sigmoidal functions with variable slope using Graphpad Prism 3.0 (Graphpad Software Inc., San Diego, CA, USA) to calculate potency (pEC$_{50}$) and Hill slopes for the agonists in the absence or presence of antagonists. When mean (apparent) E$_{max}$ and Hill slopes were not significantly different in control and antagonist experiments, a surmountable antagonism was assumed. The whole data set was then transformed using SPSS 7.5 non-linear regression statistics (SPSS Inc., Chicago, IL, USA) into a dependent fitting model, where the mean maximal-induced contraction was set to the respective agonist control

value. In case of a parallel rightward shift, a Schild regression analysis was performed with a slope set to unity to calculate the pK_B value. Due to the insurmountable behaviour of SB224289 in the middle meningeal artery and saphenous vein, pK_B values could not be calculated. Instead, we calculated the negative logarithm of the mean concentration of sumatriptan eliciting a contraction equivalent to 25% of E_{max} in the individual control experiments ($pEC_{25\%}$) in the absence or presence of antagonists. This parameter enabled us to compare the properties of the antagonists in these vascular preparations against low concentrations of sumatriptan.

Statistical analysis. Differences between the (apparent) E_{max} of sumatriptan in the absence or presence of antagonists were analysed with a paired t-test, using Graphpad Prism 3.0 (Graphpad Software Inc., San Diego, CA, USA). In case the E_{max} values in the absence of antagonists were similar to those in the presence of antagonist, the differences in Hill slopes and pEC_{50} values of mean concentration response curves between vehicle and antagonist groups were analysed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test. In case E_{max} values were different between the respective groups, we performed a two-way repeated measurement analysis of variance (RM-ANOVA) followed by Bonferroni's multiple comparison t-test to evaluate the effect of treatments (i.e. control or antagonist) at repetitive concentrations of sumatriptan. Differences between the $pEC_{25\%}$ values of sumatriptan in absence or presence of antagonists were calculated according to paired t-test. In all cases statistical significance was assumed when $P<0.05$. Data are presented as mean±s.e. mean.

Molecular biological experiments

In situ hybridisation. 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor mRNAs were localised employing non-radioactive *in situ* hybridisation on the human middle meningeal artery, coronary artery and saphenous vein (n=3 each). After cleaning the surrounding tissue, the blood vessels were fixed for 24 h in 4% paraformaldehyde dissolved in phosphate buffered saline. Chinese hamster ovary (CHO) cells expressing either the human recombinant 5-HT$_{1B}$ or 5-HT$_{1D}$ receptor served as positive controls. After dehydration with increasing percentage of ethanol in phosphate buffered saline, the blood vessels were embedded in paraffin and 5-µm thick sections were cut with a

microtome (model HM325, Microm GmbH, Walldorf, Germany). The sections were mounted on superfrost plus® glass slides (Menzel-Glaser, Braunschweig, Germany) and dried at 37 °C for 48 h.

Recombinant plasmid DNAs encoding the human 5-HT$_{1B}$ (Genbank accession number D10995, nucleotide 40-390, length 350 bp) or 5-HT$_{1D}$ (Genbank accession number M81589; nucleotide 1-400, length 400 bp) receptor were employed for the preparation of non-radioactive cRNA probes. Linearised DNA templates were transcribed to synthesise the sense and antisense cRNAs probes, using T_7 or SP_6 RNA polymerase as per protocol described for the DIG-RNA labelling kit. DIG-labelled cRNA probes were quantified by dot blotting and using serial dilutions of standard DIG-labelled control RNA supplied in the kit. Treatment of tissue sections and cells and subsequent hybridisation was performed as described earlier (de Boer *et al*., 1998). The tissues were hybridised with 25 ng of cRNA probe per slide for 16 h at 55 °C and the DIG-labelled hybrids were detected by incubation with antidigoxigenin antibody (1:2000 dilution) conjugated to alkaline phosphatase for 2.5 h at room temperature. The immunodetection of DIG-labelled hybrids was done using 4-nitroblue tetrazolium chloride (NBT) as chromogen and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as coupling agent. Slides were counter-stained with nuclear red solution, dehydrated with ethanol gradients and mounted with Euparal (Chroma-Gesellschaft, Schmid GmbH, Köngen, Germany). Cells and tissue sections were visualised under a light microscope (model Leica DM RBE, Leica NL, BV, Rijswijk, The Netherlands) and photographed using a CCD video camera (Sony DXC-950, Sony Corporation, Japan). The sense riboprobes were included as negative controls, which showed no or little staining compared to antisense riboprobes.

Endothelial staining. Serial sections of 5-µm thickness were processed for immunohistochemical localisation of endothelial cell marker, CD31. Sections were deparaffinised, rehydrated and incubated with pronase (1 mg/ml) at 37 °C for 10 min prior to incubation with specific purified mouse monoclonal antibodies raised against human CD31 (Neomarkers, Union City, CA, USA). To block non-specific binding, sections were incubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (pH = 7.4). Subsequently, sections were incubated overnight at 4 °C with primary antibodies, CD31 (1:75 v/v). Incubation for

30 min with secondary biotinylated anti-immunoglobulins (Multilink®, 1:75 v/v, Biogenex, San Ramon, USA) and tertiary complex of streptavidin conjugated to Alkaline Phosphatase (Label® 1:50 v/v, Biogenex, San Ramon, USA) were used to enhance the detection sensitivity. Colour was developed using New Fuchsine as chromogen, while endogenous alkaline phosphatase activity was inhibited by 0.01 M levamisole (Sigma, St Louis, USA). Slides were counterstained with Mayer's hematoxylin. Positive controls consisted of human cardiac tissue where intense staining was seen in the endothelium of all blood vessels. The optimal dilutions of primary antibody were identified by examining the intensity of staining obtained with a series of dilutions, which gave specific and easily visible signal on paraffin sections of control tissues. Slides were mounted and visualised using light-microscopy. Negative controls consisted of omission of the primary antibody.

Ethical approval

The Medical Ethics Committee of the Erasmus University Medical Centre Rotterdam, dealing with the use of the human material for scientific experiments, approved the protocols for this investigation.

Compounds and kits

For pharmacological study, the following compounds were used: atropine sulphate, bradykinin acetate, corticosterone, 5-hydroxytryptamine creatinine sulphate (serotonin; 5-HT), imipramine hydrochloride, indomethacin hydrochloride, mepyramine maleate, pargyline hydrochloride, prostaglandin $F_{2\alpha}$ tris salt ($PGF_{2\alpha}$) and substance P acetate (all purchased from Sigma Chemical Co., St. Louis, MO, USA), mesulergine hydrochloride (gift: Novartis AG, Basel, Switzerland), prazosin hydrochloride (gift: Pfizer, Sandwich, UK), sumatriptan succinate, SB224289 (2,3,6,7- tetrahydro-1'-methyl-5-[2'-methyl-4' (5-methyl-1,2,4-oxadiazo l-3-yl) biphenyl-4-carbonyl] furo [2,3f]indole-3-spiro-4'-piperidine hydrochloride) and BRL15572 (1-3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl) piperazine] hydrochloride); both from GlaxoSmithKline, Harlow, Essex, UK; courtesy: Dr. A.A. Parsons. Stock solutions of corticosterone (100 mM), indomethacin, mesulergine, prazosin, SB224289 and BRL15572 (all 10 mM) were dissolved in 100% v/v dimethyl sulphoxide and further diluted in distilled water. All other compounds were dissolved in distilled water.

For molecular biology experiments, the materials used were: acetic anhydride, diethyl pyrocarbonate, levamisol, maleic acid, triethanolamine and xylene (Sigma Chemical Co., St. Louis, MO, USA), antidigoxigenin-AP Fab fragments, blocking reagent for nucleic hybridisation, DIG-RNA labelling kit, glycine and RNase T_1 (Boehringer Mannheim, Almere, The Netherlands), bovine serum albumin, dextran sulphate, ethylene diaminetetraacetic acid, ficoll, hering sperm DNA, phenol and Tris-HCl (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). BCIP, formamide, NBT, proteinase K, sodium citrate and yeast tRNA (Life Technologies, Breda, The Netherlands), DNase, RNase inhibitors and Triton-X-100 (Promega Benelux, Leiden, The Netherlands) and RNase away solution (Molecular Bio-products, San Diego, CA, USA).

Results

Relaxation responses to substance P and bradykinin

The relaxation to substance P (10 nM) amounted to 29% (range: 17-44%, n=6) and 42% (range: 9-86%, n=3) of precontraction with 1 μM $PGF_{2\alpha}$ in the middle meningeal and temporal arteries, respectively. In the coronary artery, relaxation to substance P (1 nM) was 46% (range: 30-66%, n=6) of precontraction to 1 μM $PGF_{2\alpha}$. In the saphenous vein, relaxation to bradykinin (1 μM) was 54% (range: 26-82%, n=6) of precontraction to 1 μM $PGF_{2\alpha}$.

Effects of sumatriptan on cranial arteries

Concentration response curves to sumatriptan in the middle meningeal and temporal arteries are depicted in Figure 6.1. In both blood vessels, sumatriptan elicited a concentration-dependent contraction. The E_{max} values (efficacy) of sumatriptan in the middle meningeal and temporal arteries were 83±15% and 68±28% of the contraction to 1 μM $PGF_{2\alpha}$, respectively and the pEC_{50} values (potency) amounted to 6.7±0.2 and 6.7±0.3, respectively (Table 6.1). The responses to sumatriptan in both cranial arteries were antagonised by the 5-HT_{1B} receptor antagonist SB224289 (1 μM) and the magnitude of antagonism did not correlate with the functional integrity of the endothelium (substance P response).

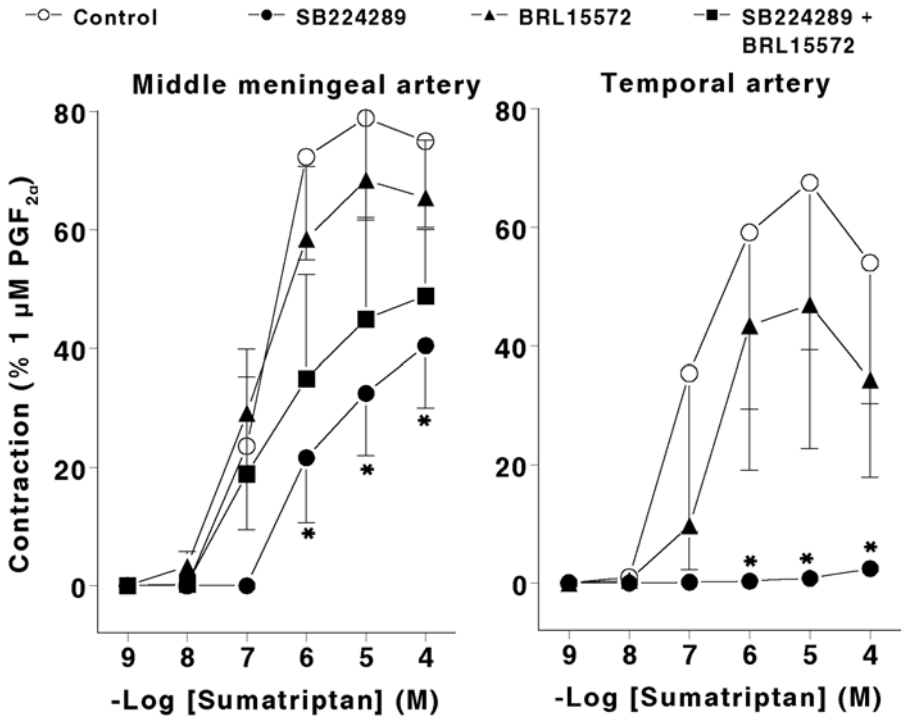


Figure 6.1. Cumulative concentration response curves to sumatriptan in the human isolated middle meningeal (n=5-6) and temporal (n=3) arteries in the absence (control) or presence of SB224289 (1 µM), BRL15572 (1 µM) or both antagonists (1 µM each). Symbols and vertical bars represent the means ± S.E.M. *, Significant difference from control contraction elicited by respective concentrations of sumatriptan (Bonferroni multiple comparison t-test, $P<0.05$).

Since SB224289 decreased the E_{max} of sumatriptan, the antagonism was apparently insurmountable and pEC_{50} values of sumatriptan were not determined (Figure 6.1, Table 6.1). The 5-HT_{1D} receptor antagonist BRL15572 (1 µM) had no effect on either the E_{max} or pEC_{50} values of sumatriptan. Also, no additional antagonism was observed with the combination of the two antagonists (Figure 6.1, Table 6.1).

Table 6.1. Apparent E_{max} and pEC_{50} values of sumatriptan in contracting human isolated blood vessels in the absence or presence of antagonists

Antagonist	Middle meningeal artery		Temporal artery		Coronary artery		Saphenous vein	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
None	83±15	6.7±0.2	68±28	6.7±0.3	13±2	5.7±0.1	62±4	6.1±0.1
SB224289 (1 μM)	40±11*	ND	2±1*	ND	11±3	5.0±0.1*	35±7*	ND
BRL15572 (1 μM)	72±10	6.8±0.2	47±24	6.5±0.2	14±2	5.7±0.1	44±7*	6.1±0.1
SB224289 (1 μM) + BRL15572 (1 μM)	53±14	ND	-	-	10±2	5.0±0.2*	32±4*	ND

Data are means±s.e.m. (n=3-6). Apparent E_{max} is presented as % of contraction elicited by either 1 μM $PGF_{2\alpha}$ (middle meningeal and temporal artery) or 100 mM KCl (coronary artery and saphenous vein). ND, Not determined because of insurmountable nature of the antagonism; -, not investigated. *, Significantly different from sumatriptan control (P<0.05).

Due to the insurmountable antagonism of sumatriptan by SB224289, pK_B values could not be estimated. Alternatively, we calculated the negative logarithm of sumatriptan concentration eliciting a response equivalent to 25% of sumatriptan control E_{max} ($pEC_{25\%}$) in the absence or presence of the antagonists in the middle meningeal artery; the temporal artery data was not further processed as SB224289 virtually abolished sumatriptan-induced contractions (Table 6.2).

Table 6.2. $pEC_{25\%}$ of sumatriptan in contracting the middle meningeal artery and saphenous vein in the absence or presence of antagonists

Antagonist	Middle meningeal artery	Coronary artery	Saphenous vein
None	6.8±0.2	6.3±0.1	6.4±0.1
SB224289 (1 μM)	5.6±0.4*	5.7±0.3	5.3±0.2*
BRL15572 (1 μM)	7.0±0.5	6.1±0.1	6.3±0.1
SB224289 (1 μM) + BRL15572 (1 μM)	6.0±0.5	5.7±0.2*	5.7±0.3*

Data are mean±s.e.m. (n=4-6). $pEC_{25\%}$ represents the negative logarithm of the concentrations of sumatriptan eliciting a contraction equivalent to 25% of individual control E_{max} (middle meningeal artery, 20.8±3.8% of the response to 1 μM $PGF_{2\alpha}$; coronary artery, 3.3±0.4% of the response to 100 mM KCl; saphenous vein, 15.2±0.9% of the response to 100 mM KCl). * Significantly different from sumatriptan control (P<0.05).

While BRL15572 was ineffective, the $pEC_{25\%}$ of sumatriptan was significantly decreased by SB224289. No additional antagonism was observed with the combination of the two antagonists (Table 6.2).

Effect of sumatriptan on peripheral vessels

Sumatriptan also contracted the human coronary artery and saphenous vein in a concentration-dependent manner. The pEC_{50} and (apparent) E_{max} of sumatriptan were, respectively, 5.7±0.1 and 13±2% of the response to 100 mM KCl in the coronary artery and 6.1±0.1 and 62±1% of the response to 100 mM KCl in the saphenous vein (Figure 6.2, Table 6.1).

In the coronary artery, SB224289 caused a small parallel rightward shift in the concentration response curve to sumatriptan, yielding a significant decrease in the pEC_{50} (5.0±0.1) with no change in the apparent E_{max} (11±3% of the response to 100 mM KCl) of sumatriptan. Schild regression analysis revealed a pK_B value of 6.4±0.2 for SB224289 against sumatriptan. The shift in the concentration response curve of

sumatriptan by SB224289 was independent of the functional integrity of the endothelium. BRL15572 did not have any effect on sumatriptan-induced contraction. Incubation with both antagonists together resulted in a small parallel rightward shift as was noticed with SB224289 alone (Figure 6.2, Table 6.1).

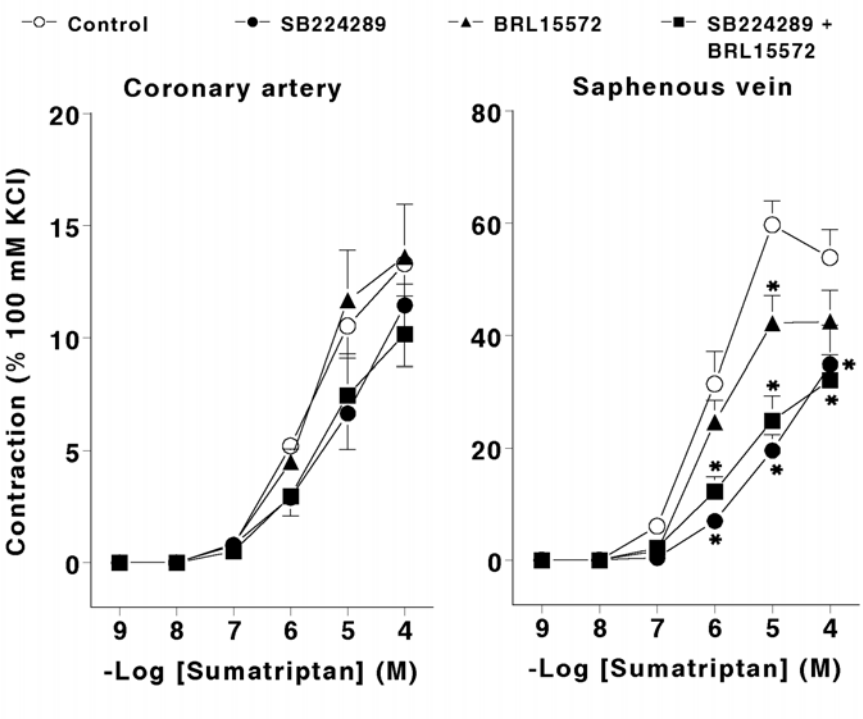


Figure 6.2. Cumulative concentration response curves to sumatriptan in the human isolated coronary artery (n=5-6) and saphenous vein (n=4-6) in the absence (control) or presence of SB224289 (1 μM), BRL15572 (1 μM) or both antagonists (1 μM each). Symbols and vertical bars represent the means ± S.E.M. *, Significant difference from control contraction elicited by respective concentrations of sumatriptan (Bonferroni multiple comparison t-test, $P<0.05$).

In the saphenous vein, the response to sumatriptan was antagonised by SB224289 (1 μM). Although the contraction to sumatriptan increased with concentration, the response did not reach a plateau with the highest concentration used and, therefore, pEC_{50} and pK_B values after SB224289 were not determined. As observed in the other blood vessels, the antagonism of SB224289 was independent of the functional integrity of the endothelium. Incubation with BRL15572 (1 μM) resulted in a slight attenuation of the response to 10 μM sumatriptan as well as its E_{max}, but no change was observed in the pEC_{50} value of sumatriptan. Also, no additional antagonism was observed with the combination of the two antagonists (Figure 6.2, Table 6.1). The

$pEC_{25\%}$ of sumatriptan was not affected by BRL15572, but was reduced after SB224289 alone or in combination with BRL15572 (Table 6.2).

In situ hybridisation

The specificity of mRNA signals and standardisation of the *in situ* hybridisation conditions were first established by the use of specific sense and antisense probes in CHO cells expressing either the human 5-HT$_{1B}$ or 5-HT$_{1D}$ receptor.

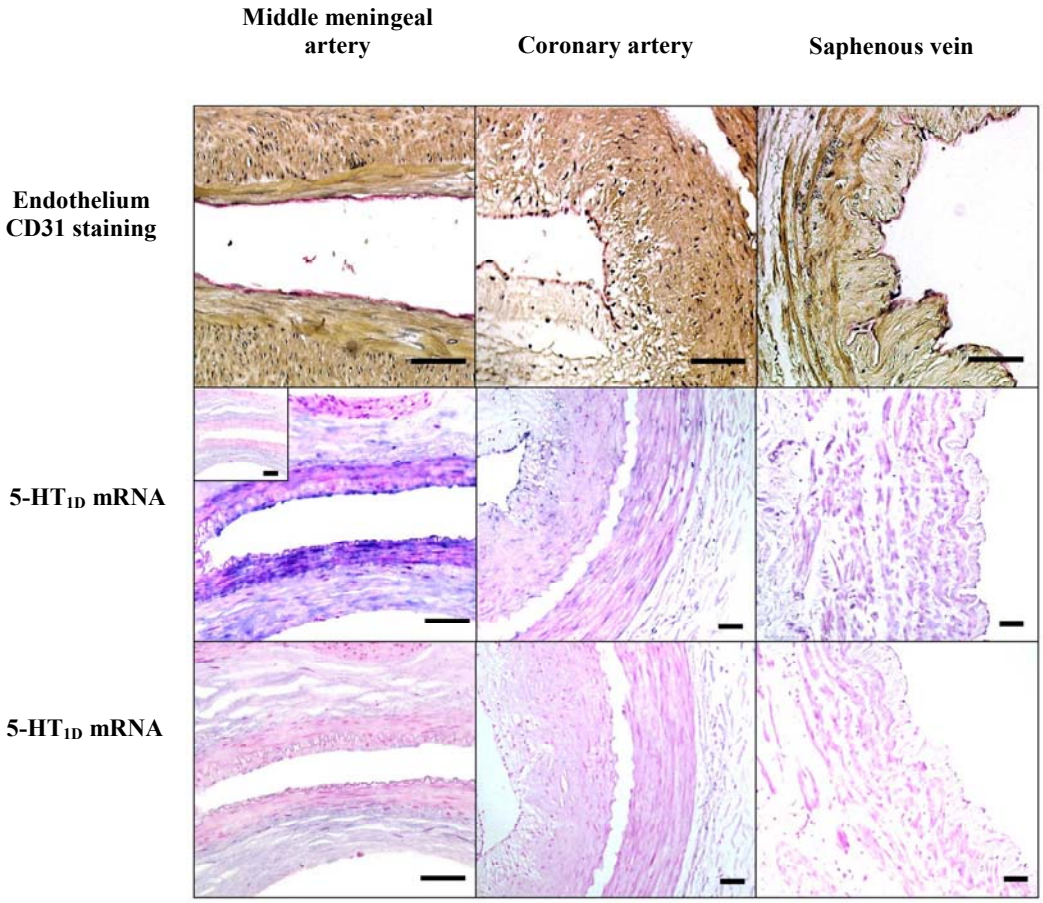


Figure 6.3. Photographs of immunohistochemical staining with CD31 endothelial marker (top panels) and *in situ* hybridisation with DIG-labelled cRNA probes for h5-HT$_{1B}$ (middle panels) and h5-HT$_{1D}$ (lower panels) receptor mRNAs in the human middle meningeal and coronary arteries and saphenous vein. The reddish colour in the top panels denotes endothelial cells, while the purple precipitates in the middle panels correspond to 5-HT$_{1B}$ receptor mRNA signals. The 5-HT$_{1D}$ receptor mRNA (lower panels) as well as the sense riboprobes, as exemplified by the left middle panel inset, did not show signals in the three blood vessels. Scale bar = 50 μm.

Strong cytoplasmic and nuclear mRNA expression as a dark purple/blue colour for respective antisense riboprobes was observed in these cells, whereas sense riboprobes depicted no staining. Furthermore, the sense riboprobes did not show any specific mRNA expression assessed as purple/blue staining in the tissue samples included in this study. If at all staining with sense riboprobes was detected, it was always far less than the staining with antisense probes (data not shown).

Cellular localisation of mRNAs for the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the human middle meningeal and coronary arteries and saphenous vein is depicted in Figure 6.3. In the middle meningeal artery, hybridisation with antisense riboprobe showed 5-HT$_{1B}$ receptor mRNA signals in the medial smooth muscle layer as well as in the endothelial cells, whereas adventitial cells showed only a faint staining. Although not so intense, the coronary artery and saphenous vein also expressed the 5-HT$_{1B}$ receptor mRNA in the vascular smooth muscle cells. Specific expression for the 5-HT$_{1B}$ receptor was seen in the luminal endothelial cells in the case of coronary artery. In order to verify the *in situ* hybridisation data for the endothelial cell expression of 5-HT$_{1B}$ receptor mRNA, CD31 immunohistochemistry was performed and it confirmed a cytoplasmic endothelial cell staining (Figure 6.3). In contrast to the 5-HT$_{1B}$ receptor mRNA, no signals were detected for the 5-HT$_{1D}$ receptor mRNA in both smooth muscle and endothelial cells in the middle meningeal artery and saphenous vein. However, a weak staining for the 5-HT$_{1D}$ receptor mRNA was noticed in some endothelial cells in the coronary artery and in adventitial cells in the case of middle meningeal as well as coronary arteries (Figure 6.3).

Discussion

Craniovascular selectivity of sumatriptan

Sumatriptan contracted in a concentration-dependent manner both cranial and peripheral blood vessels, used as models for antimigraine activity (middle meningeal and temporal artery) and possible peripheral side-effect potential (coronary artery and saphenous vein), respectively. The potency of sumatriptan was higher at the middle meningeal and temporal arteries (pEC$_{50}$: 6.7 each) than at the coronary artery (pEC$_{50}$: 5.7) and saphenous vein (pEC$_{50}$: 6.1). Compared to the other vessels, the efficacy (E$_{max}$) of sumatriptan in the coronary artery was clearly lower (see Table 6.1). These data, which confirm the cranioselectivity of sumatriptan, are in accordance with other

studies dealing with middle meningeal (Jansen *et al*., 1992; Longmore *et al*., 1998; MaassenVanDenBrink *et al*., 2000b; Razzaque *et al*., 1999), temporal (Jansen *et al*., 1992; Verheggen *et al*., 1998) and coronary (Connor *et al*., 1989; Kaumann *et al*., 1994; MaassenVanDenBrink *et al*., 1998; MaassenVanDenBrink *et al*., 2000b; Nilsson *et al*., 1999b; Van den Broek *et al*., 2000) arteries and saphenous vein (Bax *et al*., 1992; MaassenVanDenBrink *et al*., 2000b; Van den Broek *et al*., 2000).

Receptors mediating sumatriptan-induced contractions

The data obtained in *in vitro* functional studies show that the 5-HT$_{1B}$ receptor antagonist SB224289 clearly attenuated sumatriptan-induced contractions, while the 5-HT$_{1D}$ receptor antagonist BRL15572 had little effect on the concentration response curves to sumatriptan; the combination of SB224289 and BRL15572 did not reveal any additional antagonism. These results establish that the 5-HT$_{1B}$ receptor mediates the contractile effects of sumatriptan in both cranial and peripheral blood vessels, while the 5-HT$_{1D}$ receptor does not seem to play any role. The latter conclusion is also supported by the fact that selective 5-HT$_{1D}$ receptor agonists (PNU-109291 and L775,606) did not contract isolated blood vessels (Bouchelet *et al*., 2000; Ennis *et al*., 1998; Longmore *et al*., 2000) and, interestingly, one such compound (PNU-142633) was also found ineffective in migraine (Cutler *et al*., 2000). Based on the high affinity of sumatriptan for the 5-HT$_{1F}$ receptor (pKi: 7.9, Leysen *et al*., 1996), it has been argued that this receptor might play a role in the therapeutic action of sumatriptan (Johnson *et al*., 1997). Although we cannot rule this out, the 5-HT$_{1F}$ receptor is not involved in vasoconstriction, since selective 5-HT$_{1F}$ receptor agonists (LY344864 and LY334370) show no vasoconstrictor effect (Bouchelet *et al*., 2000; Cohen *et al*., 1999; Shepheard *et al*., 1999). In addition, Verheggen *et al*. (1998) have suggested that in the presence of SB224289 high concentrations of sumatriptan can elicit contractions of the human isolated temporal artery via the 5-HT$_{2A}$ receptor. This was not the case in the present studies, probably because we included the mixed 5-HT$_{2/7}$ receptor antagonist mesulergine (pKB: 9.1 and 8.2, respectively, Hoyer *et al*., 1994) in the Krebs solution. However, the 5-HT$_2$ receptor antagonist ketanserin was unable to block sumatriptan-induced contractions in the human middle meningeal (Jansen *et al*., 1992) and coronary (Connor *et al*., 1989; Kaumann *et al*., 1994) arteries as well as the saphenous vein (Bax *et al*., 1992).

It may be noted that SB224289 antagonised the responses to sumatriptan in an insurmountable manner in the middle meningeal artery, whereas in the temporal artery the contractions were virtually abolished. In the coronary artery, there was a weak competitive (surmountable) antagonism, whilst in the saphenous vein an intermediate antagonistic response was observed. The nature of the difference in the antagonistic behaviour of SB224289 in these blood vessels is not clear. A possible explanation (Schutz *et al*., 1992) could be that SB224289, which acts as an inverse agonist in cells expressing recombinant 5-HT$_{1B}$ receptors (Gaster *et al*., 1998), may also do so at constitutive 5-HT$_{1B}$ receptors. Another explanation for the observed differences could be the influence of receptor density and receptor reserve, but, in view of considerably higher 5-HT$_{1B}$ receptor density in meningeal artery as compared to coronary artery (Longmore *et al*., 1997), we should have observed a surmountable antagonism in the middle meningeal artery and insurmountable antagonism in the coronary artery. Finally, in view of the localisation of the 5-HT$_{1B}$ receptor on the endothelium (see below), the effect of SB224289 may be influenced to a different degree by endothelial factors (either contractile or relaxing) released upon activation of the 5-HT$_{1B}$ receptor. Whatever the mechanism, differences in the nature of antagonism have also been observed between sumatriptan and the non-selective 5-HT$_{1B/1D}$ receptor antagonist GR127935 (Bouchelet *et al*., 2000; Razzaque *et al*., 1999).

It is surprising that SB224289 proved to be a weak antagonist in our experiments as its pK$_B$ (6.4±0.2) against sumatriptan in the coronary artery resembled more its pK$_i$ (6.2) at the 5-HT$_{1D}$ receptor and was far less than its pK$_i$ at the 5-HT$_{1B}$ receptor (8.2) (Schlicker *et al*., 1997; Selkirk *et al*., 1998). However, if 5-HT$_{1D}$ receptors were involved, we would have found BRL15572 to be an even more potent antagonist than SB224289; this was obviously not the case. The involvement of 5-HT$_{1B}$ receptor in the sumatriptan-induced coronary artery contraction is also supported by previous investigations using non-selective 5-HT$_{1B/1D}$ receptor antagonists (Bax *et al*., 1993; Connor *et al*., 1989; MaassenVanDenBrink *et al*., 2000b; Nilsson *et al*., 1999b; Van den Broek *et al*., 2000). However, the weaker antagonism by SB224289 than these non-selective antagonists suggests that an additional mechanism is partly responsible for the coronary contraction induced by sumatriptan. The nature of this additional mechanism is not known, but it cannot be related to 5-HT$_2$ receptors in view of the presence of mesulergine in the organ bath. Certainly, this additional mechanism is of

interest in regard to the sumatriptan related cardiac side effects and may prove relevant in future antimigraine drug development.

Vascular localisation of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor mRNA

Although RT-PCR studies have shown the presence of 5-HT$_{1B}$ receptor mRNA in the human middle meningeal (Schmuck *et al*., 1996) and coronary (Bouchelet *et al*., 2000; Nilsson *et al*., 1999b) arteries, the cellular localisation of 5-HT receptor subtypes at mRNA level has not been demonstrated. Our results of *in situ* hybridisation showed the mRNA expression of 5-HT$_{1B}$ receptor in both smooth muscle and endothelium, whereas the expression of 5-HT$_{1D}$ receptor mRNA was weak, if any, in the three human blood vessels investigated (Figure 6.3). These results are in agreement with the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor protein localisation in coronary and middle meningeal arteries (Longmore *et al*., 1998; Longmore *et al*., 1997; Nilsson *et al*., 1999b). The localisation of 5-HT$_{1B}$ receptor mRNA within the smooth muscle cells of these blood vessels, and the lack of 5-HT$_{1D}$ receptor mRNA, strongly suggest that contraction to sumatriptan is mediated via the 5-HT$_{1B}$ receptor rather than the 5-HT$_{1D}$ receptor. Although the exact role of the endothelial 5-HT$_{1B}$ receptor in these vessels is not well understood, we cannot rule out the release of relaxing and/or contractile substances upon stimulation.

Conclusion

In conclusion, our data show that sumatriptan contracts the human middle meningeal, temporal and coronary arteries and saphenous vein via the 5-HT$_{1B}$ receptor, but not 5-HT$_{1D}$ receptor. In the human coronary artery, the contraction may also be mediated by an unknown, SB224289-resistant, mechanism.

Acknowledgements

The authors wish to express their gratitude to the staff of the Neurosurgery (Head: Prof. C.J.J. Avezaat), Cardiothoracic surgery (Head: Prof. A.J.J.C. Bogers), Erasmus University Medical Centre, Rotterdam as well as the Rotterdam Heart Valve Bank (Dr. A.P.A. Stegmann) for providing human blood vessels. We thank Dr. Mike Mosley (Pfizer Ltd, Sandwich, UK) for kindly providing us with recombinant plasmid DNAs encoding human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors and transfected CHO cells. Dr. H. Motulsky (Graphpad Software Inc., San Diego, CA,

USA) and Dr. P.G.M. Mulder (Department of Epidemiology and Biostatistics, Erasmus University Medical Centre, Rotterdam) helped us with statistical analyses of the data.

References

BAX, W.A., RENZENBRINK, G.J., VAN HEUVEN-NOLSEN, D., THIJSSEN, E.J., BOS, E. & SAXENA, P.R. (1993). 5-HT receptors mediating contractions of the isolated human coronary artery. *Eur. J. Pharmacol.*, **239,** 203-210.

BAX, W.A., VAN HEUVEN-NOLSEN, D., BOS, E., SIMOONS, M.L. & SAXENA, P.R. (1992). 5-Hydroxytryptamine-induced contractions of the human isolated saphenous vein: involvement of 5-HT$_2$ and 5-HT$_{1D}$-like receptors, and a comparison with grafted veins. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **345,** 500-508.

BOUCHELET, I., CASE, B., OLIVIER, A. & HAMEL, E. (2000). No contractile effect for 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br. J. Pharmacol.*, **129,** 501-508.

BOUCHELET, I., COHEN, Z., CASE, B., SEGUELA, P. & HAMEL, E. (1996). Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. *Mol. Pharmacol.*, **50,** 219-223.

COHEN, M.L. & SCHENCK, K. (1999). 5-Hydroxytryptamine$_{1F}$ receptors do not participate in vasoconstriction: lack of vasoconstriction to LY344864, a selective serotonin$_{1F}$ receptor agonist in rabbit saphenous vein. *J. Pharmacol. Exp. Ther.*, **290,** 935-939.

CONNOR, H.E., FENIUK, W. & HUMPHREY, P.P.A. (1989). 5-Hydroxytryptamine contracts human coronary arteries predominantly via 5-HT$_2$ receptor activation. *Eur. J. Pharmacol.*, **161,** 91-94.

CUTLER, N.R., GOMEZ-MANCILLA, B., LEIBOWITZ, M.T. & FLEISHAKER, J. (2000). A study of safety and efficacy in patients with acute migraine, using PNU-142633, a selective 5-HT$_{1D}$ agonist. *Cephalalgia*, **20,** 268.

DE BOER, W.I., VAN SCHADEWIJK, A., SONT, J.K., SHARMA, H.S., STOLK, J., HIEMSTRA, P.S. & VAN KRIEKEN, J.H. (1998). Transforming growth factor b$_1$ and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **158,** 1951-1957.

DE VRIES, P., SANCHEZ-LOPEZ, A., CENTURION, D., HEILIGERS, J.P., SAXENA, P.R. & VILLALÓN, C.M. (1998). The canine external carotid vasoconstrictor 5-HT$_1$ receptor: blockade by 5-HT$_{1B}$ (SB224289), but not by 5-HT$_{1D}$ (BRL15572) receptor antagonists. *Eur. J. Pharmacol.*, **362,** 69-72.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P.C., VILLALÓN, C.M. & SAXENA, P.R. (1999). Investigation of the role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. *Br. J. Pharmacol.*, **127,** 405-412.

DIXON, C.M., PARK, G.R. & TARBIT, M.H. (1994). Characterization of the enzyme responsible for the metabolism of sumatriptan in human liver. *Biochem. Pharmacol.*, **47,** 1253-1257.

ENNIS, M.D., GHAZAL, N.B., HOFFMAN, R.L., SMITH, M.W., SCHLACHTER, S.K., LAWSON, C.F., IM, W.B., PREGENZER, J.F., SVENSSON, K.A., LEWIS, R.A., HALL, E.D., SUTTER, D.M., HARRIS, L.T. & MCCALL, R.B. (1998). Isochroman-6-carboxamides as highly selective 5-HT$_{1D}$ agonists: potential new treatment for migraine without cardiovascular side effects. *J. Med. Chem.*, **41,** 2180-2183.

GASTER, L.M., HAM, P., JOINER, G.F., KING, F.D., MULHOLLAND, K.R., WYMAN, P.A., HAGAN, J.J., PRICE, G.W., ROBERTS, C., ROUTLEDGE, C., SELKIRK, J., SLADE, P.D. & MIDDLEMISS, D.N. (1998). The selective 5-HT$_{1B}$ receptor inverse agonist SB-224289, potently blocks terminal 5-HT autoreceptor function both *in vitro* and *in vivo*. *Ann. N. Y. Acad. Sci.*, **861,** 270-271.

GOADSBY, P.J. (1998). A triptan too far? *J. Neurol. Neurosurg. Psychiatry*, **64,** 143-147.

HAMEL, E., FAN, E., LINVILLE, D., TING, V., VILLEMURE, J.G. & CHIA, L.S. (1993). Expression of mRNA for the serotonin 5-hydroxytryptamine$_{1Dß}$ receptor subtype in human and bovine cerebral arteries. *Mol. Pharmacol.*, **44,** 242-246.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 157-203.

HUMPHREY, P.P.A. & FENIUK, W. (1991). Mode of action of the anti-migraine drug sumatriptan. *Trends. Pharmacol. Sci.*, **12,** 444-446.

JANSEN, I., EDVINSSON, L., MORTENSEN, A. & OLESEN, J. (1992). Sumatriptan is a potent vasoconstrictor of human dural arteries via a 5- HT$_1$-like receptor. *Cephalalgia*, **12,** 202-205.

JOHNSON, K.W., SCHAUS, J.M., DURKIN, M.M., AUDIA, J.E., KALDOR, S.W., FLAUGH, M.E., ADHAM, N., ZGOMBICK, J.M., COHEN, M.L., BRANCHEK, T.A. & PHEBUS, L.A. (1997). 5-HT$_{1F}$ receptor agonists inhibit neurogenic dural inflammation in guinea pigs. *Neuroreport*, **8,** 2237-2240.

KAUMANN, A.J., FRENKEN, M., POSIVAL, H. & BROWN, A.M. (1994). Variable participation of 5-HT$_1$-like receptors and 5-HT$_2$ receptors in serotonin-induced contraction of human isolated coronary arteries. 5- HT$_1$-like receptors resemble cloned 5-HT$_{1D\beta}$ receptors. *Circulation*, **90,** 1141-1153.

KAUMANN, A.J., PARSONS, A.A. & BROWN, A.M. (1993). Human arterial constrictor serotonin receptors. *Cardiovasc. Res.*, **27,** 2094-2103.

LEYSEN, J.E., GOMMEREN, W., HEYLEN, L., LUYTEN, W.H., VAN DE WEYER, I., VANHOENACKER, P., HAEGEMAN, G., SCHOTTE, A., VAN GOMPEL, P., WOUTERS, R. & LESAGE, A.S. (1996). Alniditan, a new 5-hydroxytryptamine$_{1D}$ agonist and migraine-abortive agent: ligand-binding properties of human 5-hydroxytryptamine$_{1D\alpha}$, human 5-hydroxytryptamine$_{1D\beta}$, and calf 5-hydroxytryptamine$_{1D}$ receptors investigated with [^{3}H]5-hydroxytryptamine and [^{3}H]alniditan. *Mol. Pharmacol.*, **50,** 1567-1580.

LONGMORE, J., MAGUIRE, J.J., MACLEOD, A., STREET, L., SCHOFIELD, W.N. & HILL, R.G. (2000). Comparison of the vasoconstrictor effects of the selective 5-HT$_{1D}$-receptor agonist L-775,606 with the mixed 5-HT$_{1B/1D}$-receptor agonist sumatriptan and 5-HT in human isolated coronary artery. *Br. J. Clin. Pharmacol.*, **49,** 126-131.

LONGMORE, J., RAZZAQUE, Z., SHAW, D., DAVENPORT, A.P., MAGUIRE, J., PICKARD, J.D., SCHOFIELD, W.N. & HILL, R.G. (1998). Comparison of the vasoconstrictor effects of rizatriptan and sumatriptan in human isolated cranial arteries: immunohistological demonstration of the involvement of 5-HT$_{1B}$-receptors. *Br. J. Clin. Pharmacol.*, **46,** 577-582.

LONGMORE, J., SHAW, D., SMITH, D., HOPKINS, R., MCALLISTER, G., PICKARD, J.D., SIRINATHSINGHJI, D.J., BUTLER, A.J. & HILL, R.G. (1997). Differential distribution of 5HT$_{1D}$- and 5HT$_{1B}$-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. *Cephalalgia*, **17,** 833-842.

MAASSENVANDENBRINK, A., REEKERS, M., BAX, W.A., FERRARI, M.D. & SAXENA, P.R. (1998). Coronary side-effect potential of current and prospective antimigraine drugs. *Circulation*, **98,** 25-30.

MAASSENVANDENBRINK, A., REEKERS, M., BAX, W.A. & SAXENA, P.R. (2000a). Human isolated coronary artery contraction to sumatriptan characterised by the selective 5-HT$_{1B/1D}$ receptor antagonist GR55562. *Pharmacol. Toxicol.*, **86,** 287-290.

MAASSENVANDENBRINK, A., VAN DEN BROEK, R.W.M., DE VRIES, R., BOGERS, A.J.J.C., AVEZAAT, C.J.J. & SAXENA, P.R. (2000b). Craniovascular selectivity of eletriptan and sumatriptan in human isolated blood vessels. *Neurology*, **55,** 1524-1530.

MORECROFT, I., HEELEY, R.P., PRENTICE, H.M., KIRK, A. & MACLEAN, M.R. (1999). 5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT$_{1B}$ receptor. *Br. J. Pharmacol.*, **128,** 730-734.

NILSSON, T., LONGMORE, J., SHAW, D., OLESEN, I.J. & EDVINSSON, L. (1999a). Contractile 5-HT$_{1B}$ receptors in human cerebral arteries: pharmacological characterization and localization with immunocytochemistry. *Br. J. Pharmacol.*, **128,** 1133-1140.

NILSSON, T., LONGMORE, J., SHAW, D., PANTEV, E., BARD, J.A., BRANCHEK, T. & EDVINSSON, L. (1999b). Characterisation of 5-HT receptors in human coronary arteries by molecular and pharmacological techniques. *Eur. J. Pharmacol.*, **372,** 49-56.

OTTERVANGER, J.P., WILSON, J.H. & STRICKER, B.H. (1997). Drug-induced chest pain and myocardial infarction. Reports to a national centre and review of the literature. *Eur. J. Clin. Pharmacol.*, **53,** 105-110.

PARSONS, A.A., RAVAL, P., SMITH, S., TILFORD, N., KING, F.D., KAUMANN, A.J. & HUNTER, J. (1998). Effects of the novel high-affinity 5-HT$_{1B/1D}$-receptor ligand frovatriptan in human isolated basilar and coronary arteries. *J. Cardiovasc. Pharmacol.*, **32,** 220-224.

RAZZAQUE, Z., HEALD, M.A., PICKARD, J.D., MASKELL, L., BEER, M.S., HILL, R.G. & LONGMORE, J. (1999). Vasoconstriction in human isolated middle meningeal arteries: determining the contribution of 5-HT$_{1B}$- and 5-HT$_{1F}$-receptor activation. *Br. J. Clin. Pharmacol.*, **47,** 75-82.

SCHLICKER, E., FINK, K., MOLDERINGS, G.J., PRICE, G.W., DUCKWORTH, M., GASTER, L., MIDDLEMISS, D.N., ZENTNER, J., LIKUNGU, J. & GOTHERT, M. (1997). Effects of selective h5-HT$_{1B}$ (SB-216641) and h5-HT$_{1D}$ (BRL-15572) receptor ligands on guinea-pig and human 5-HT auto- and heteroreceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 321-327.

SCHMUCK, K., ULLMER, C., KALKMAN, H.O., PROBST, A. & LUBBERT, H. (1996). Activation of meningeal 5-HT$_{2B}$ receptors: an early step in the generation of migraine headache? *Eur. J. Neurosci.*, **8,** 959-967.

SCHUTZ, W. & FREISSMUTH, M. (1992). Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol. Sci.*, **13,** 376-380.

SELKIRK, J.V., SCOTT, C., HO, M., BURTON, M.J., WATSON, J., GASTER, L.M., COLLIN, L., JONES, B.J., MIDDLEMISS, D.N. & PRICE, G.W. (1998). SB-224289 - a novel selective (human) 5-HT$_{1B}$ receptor antagonist with negative intrinsic activity. *Br. J. Pharmacol.*, **125,** 202-208.

SHEPHEARD, S., EDVINSSON, L., CUMBERBATCH, M., WILLIAMSON, D., MASON, G., WEBB, J., BOYCE, S., HILL, R. & HARGREAVES, R. (1999). Possible antimigraine mechanisms of action of the 5-HT$_{1F}$ receptor agonist LY334370. *Cephalalgia*, **19,** 851-858.

TFELT-HANSEN, P., DE VRIES, P. & SAXENA, P.R. (2000). Triptans in migraine: a comparative review of pharmacology, pharmacokinetics and efficacy. *Drugs*, **60,** 1259-1287.

VAN DEN BROEK, R.W.M., MAASSENVANDENBRINK, A., DE VRIES, R., BOGERS, A.J.J.C., STEGMANN, A.P.A., AVEZAAT, C.J. & SAXENA, P.R. (2000). Pharmacological analysis of contractile effects of eletriptan and sumatriptan on human isolated blood vessels. *Eur. J. Pharmacol.*, **407,** 165-173.

VERHEGGEN, R., FREUDENTHALER, S., MEYER-DULHEUER, F. & KAUMANN, A.J. (1996). Participation of 5-HT$_1$-like and 5-HT$_{2A}$ receptors in the contraction of human temporal artery by 5-hydroxytryptamine and related drugs. *Br. J. Pharmacol.*, **117,** 283-289.

VERHEGGEN, R., HUNDESHAGEN, A.G., BROWN, A.M., SCHINDLER, M. & KAUMANN, A.J. (1998). 5-HT$_{1B}$ receptor-mediated contractions in human temporal artery: evidence from selective antagonists and 5-HT receptor mRNA expression. *Br. J. Pharmacol.*, **124,** 1345-1354.

Chapter 7

General Discussion

The treatment and management of patients with migraine and headache still remains a major problem world-wide. Migraine is a neurovascular disorder of which the pathophysiology is not completely understood. However, more information is emerging on the underlying mechanisms for the initiation of a migraine attack, the subsequent events leading to the aura and headache (Ferrari et al., 1995; Saxena, 1995a; De Vries et al., 1999; 2000). In line with the proposed neurovascular hypothesis, several experimental animal models have been developed that may explain the efficacy of acutely acting antimigraine drugs, as reviewed by De Vries *et al*. (1999). The predictive value of these models for migraine therapy is based on: (*i*) the involvement of the trigeminovascular system, i.e. inhibition of plasma protein extravasation (Moskowitz, 1993; 1979); (*ii*) central trigeminal inhibition and, thereby, reducing the release of neuropeptides (e.g. calcitonin gene related peptide) (Goadsby, 1999; May et al., 2001); (*iii*) vasoconstriction of cranial extracerebral (e.g. within carotid) vascular beds or isolated blood vessels (Verheggen et al., 1998; De Vries et al., 1999b; 1999; Saxena et al.,1995a; 2000); or (*iv*) by a combination of these mechanisms. It is important to note, however, that irrespective of the mechanisms involved, selective vasoconstriction within the carotid circulation is an important property of acutely acting antimigraine drugs (De Vries et al., 1999; Dahlöf et al., 2000; May et al., 2001).

Several lines of evidence support the involvement of serotonin (5-hydroxytryptamine; 5-HT) and its receptors in migraine (see Introduction). Subsequently, it is evidenced by a general feature of commonly used acute antimigraine drugs (ergot alkaloids and triptans), which all have a high affinity at 5-HT_1 receptor subtypes, particularly at 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptors (see Table 1.2, 1.3 and 1.5) (Bhalla et al., 2000; Saxena et al., 2000; Bhalla et al., 2001). Pharmacological findings, supported by molecular and immunohistochemical investigations (Verheggen et al., 1998; De Vries et al., 1999a; Bouchelet et al., 1996; 2000), clearly revealed that mainly 5-HT_{1B} receptors mediate sumatriptan-induced cranial vasoconstriction and may, thereby, explain its therapeutic efficacy in migraine

therapy. On the other hand, 5-HT_{1D} and/or 5-ht_{1F} receptors may mediate the presynaptic inhibition of the trigeminovascular inflammatory responses implicated in headache (Bouchelet et al., 2000). Indeed, selective 5-HT_{1D} (e.g. PNU109291, PNU142633, L-775,606) as well as 5-ht_{1F} receptor (LY344864) agonists elicit the above effect and these compounds are devoid of vasoconstrictor effects in human and bovine isolated cerebral blood vessels (Bouchelet et al., 2000). However, PNU142633 proved to be ineffective in the treatment of acute migraine (Gomez-Mancilla et al., 2001; May et al., 2001) and LY344864, which did show some efficacy (Roon, 2000), was used in doses which provided plasma concentrations where 5-HT_{1B} receptor activity cannot be ruled out. The introduction of a potent and selective 5-HT_{1B} receptor agonist for the treatment of migraine is awaited with great interest.

Although, the triptans are clearly established as acutely acting antimigraine agents, some research groups are trying to develop other effective antimigraine agents, acting *via* different mechanisms and/or receptors, which will hopefully lead to less side-effects (MaassenVanDenBrink et al., 1998; Saxena et al., 2000). Examples of possible novel avenues for the development of acute antimigraine agents include CGRP receptor antagonists, neurokinin NK_1 receptor antagonists and drugs that affect nitric oxide biosynthesis (Dahlöf et al., 2000; May et al., 2001). Furthermore, apart from specific 5-HT_1 receptor subtypes, the 5-HT_7 receptor has also been proposed as a potential avenue for the development of antimigraine agents. To date, several studies have shown that stimulation of 5-HT_7 receptors causes relaxation of vascular smooth muscle in different species, including dog (Terrón et al., 1999; Centurion et al., 2000) and pig (De Vries et al., 1998b; Saxena et al., 1998). The effectiveness of selective 5-HT_7 receptor antagonists (e.g. SB258719) may prevent the aforementioned cranial vasodilatation and, therefore, could be very useful as prophylactic antimigraine agents in the near future (Thomas et al., 1998).

Molecular cloning of porcine 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptors

Molecular cloning of 5-HT receptors not only opened the path for the discovery of several novel 5-HT receptors but has also made it possible to elucidate their pharmacological profile, tissue distribution and functional characterisation in more detailed manner (Hoyer et al., 1994). To date, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7

receptors have been cloned and characterised from human and other mammalian species (Weinshank et al., 1992; Adham et al., 1993b; Lovenberg et al., 1993; Tsou et al., 1994; Zgombick et al., 1997). However, the divergence in pharmacological profile of a receptor across different species may vary considerably despite a high sequence homology (Hamblin et al., 1992). Interestingly, this was the case for human and rat 5-HT_{1B} receptors; a phenomenon that was most likely manifested due to a single amino acid difference in the seventh transmembrane region (Oksenberg et al., 1992). Thus, it seems evident that extending particular pharmacological characteristics of a compound targeted for a specific human receptor across mammalian species may have serious consequences across mammalian species for the functional outcome. Therefore, drug receptor interactions should not be extended as such from animal to human species without verification of their molecular structures and pharmacological profiles. Moreover, identification of a receptor subtype from a species closer in structure and pharmacological profile with its human homologue may be useful in the screening and development of novel therapeutic strategies. In this connection, previous investigations from our laboratory have established that vasoconstriction of carotid arteriovenous anastomoses in anaesthetised pigs can serve as a predictive model for acute antimigraine activity, produced by 5-HT receptor-based drugs (De Vries et al., 1999). However, the lack of information about the pharmacological profiles of recombinant porcine 5-HT receptors restricts extrapolation of the derived experimental *in vivo* results to their therapeutic value in migraineurs. For above-mentioned reasons, it is important to clone and pharmacologically characterise porcine receptors, thus facilitating the development of antimigraine drugs. Moreover, a precise comparison of recombinant expression studies with *in vivo* experiments may eventually lead to a better understanding in the physiological role(s) of a specific receptor in a particular tissue or vascular bed (Mialet et al., 2000). Hence, we set out to clone and characterise porcine specific 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptors cDNAs and attempted to characterise them pharmacologically. The data presented in chapter 2, 3, 4 and 5 describes about porcine 5-$HT_{1B,}$ 5-$HT_{1D,}$ 5-$ht_{1F,}$ and 5-HT_7 receptor respectively.

Several classical molecular techniques, i.e. reverse transcriptase (RT) and inverse polymerase chain reaction (PCR), enabled us to clone and sequence porcine 5-HT receptors (namely 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7). It may be mentioned that

the RT-PCR and inverse PCR techniques were employed on either cDNA or genomic DNA, which was prepared from porcine tissues and blood respectively. Porcine 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptors showed a nucleotide sequence of 1173bp, 1134bp and 1101bp, which encodes for 390, 377 and 366 amino acid protein respectively. Both the 5' and 3' ends of the three cDNAs were verified by inverse PCR. These recombinant porcine 5-HT_1 receptor subtypes (5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F}) showed a typical G-protein coupled receptor structure with predicted seven transmembrane domains as well as putative N-glycosylation and phoshorylation sites as observed in other species (Weinshank et al., 1992; Adham et al., 1993b; Harwood et al., 1995). Although some of the amino acids in porcine 5-HT_1 receptor subtypes (5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F}) were observed to be different from other species, but an overall high amino acid homology (88-95%) was observed across a variety of species, including human (Weinshank et al., 1992; Adham et al., 1993b; Harwood et al., 1995; Bard et al., 1996; Adham et al., 1997; Wurch et al., 1997; Zgombick et al., 1997). Moreover, the comparison between the deduced amino acid sequence of the porcine 5-HT_{1B} and 5-HT_{1D} receptor displayed an overall (61%) homology, which was considerably higher in the transmembrane domains (77%) similar as observed in case of human receptors (Weinshank et al., 1992). The porcine 5-HT_{1B} receptor has exactly the same number of amino acids (390) as found in human and rabbit (Weinshank et al., 1992; Harwood et al., 1995); the rat and mouse 5-HT_{1B} receptors contain 386 (Voigt et al., 1991), while the guinea pig 5-HT_{1B} receptor contains 389 (Zgombick et al., 1997) amino acids. Recombinant porcine 5-HT_{1B} receptor revealed a conserved threonine (amino acid) at an identical position in the seventh transmembrane domain (at position 355) as observed in human, rabbit and guinea pig (Weinshank et al., 1992; Harwood et al., 1995; Zgombick et al., 1997). However, in case of rodents 5-HT_{1B} receptor, an asparagine is found at this position (Voigt et al., 1991). As mentioned before, this amino acid mutation is of particular interest because it alters the pharmacological properties drastically in the rodent 5-HT_{1B} receptor as compared to human 5-HT_{1B} receptor (Oksenberg et al., 1992).

Similarly, the number of total amino acids in the porcine 5-HT_{1D} receptor protein were the same as found in other species, e.g. human, rabbit, dog and guinea pig, while three amino acids are less in rodents (Wurch et al., 1997). As discussed for the 5-HT_{1B} receptor, the presence of threonine at the seventh transmembrane domain

was also observed in porcine 5-HT_{1D} receptor, similar to the other mammalian species, such as human, guinea pig, rat, mouse and rabbit (Weinshank et al., 1992; Harwood et al., 1995; Zgombick et al., 1997). Unlike the 5-HT_{1B} receptor, the presence of this threonine in 5-HT_{1D} receptor across these mammalian species seems to be responsible for the similar pharmacological profile of 5-HT_{1D} receptor across different species.

The porcine 5-ht_{1F} receptor polypeptide showed exactly the same number of amino acid as observed in other mammalian species (Adham et al., 1993b; Lovenberg et al., 1993; Adham et al., 1997). However, a threonine at position 333 in place of alanine (as observed in human, chimpanzee, gorilla, guniea pig, mouse and rat) was found in porcine and orang-utan 5-ht_{1F} receptor. In order to verify this difference at position 333, we sequenced a large number of cDNA as well as genomic DNA derived PCR products and eventually established the polypeptide sequence of 5-ht_{1F} receptor. As discussed in the next section, this particular modification in amino acid at this position may be the reason for the observed low affinity of some serotonergic ligands for the porcine compared to the human receptor (see Table 4.4). This could also be addressed by using site directed mutation or chimeric receptor studies on porcine 5-ht_{1F} receptor. Interestingly, it has been shown for human 5-ht_{1F} receptor that the change of alanine to asparagine at position 333 also resulted in a drastic increase of affinity for β adrenoceptor antagonists (such as propranolol and pindolol), but not for 5-HT (Adham et al., 1994). The porcine 5-ht_{1F} receptor also showed a low affinity for pindolol in analogy to human, but, unlike the human receptor, some triptans (rizatriptan, sumatriptan and zolmitriptan) showed several folds less affinity for porcine 5-ht_{1F} receptor.

The porcine 5-HT_7 receptor protein consists of 447 amino acids. The BLAST search at GenBank revealed high resemblance (92-96%) with the sequence of 5-HT_7 receptors from other species, including human, mouse, rat and guinea pig. It may be important to note that the amino acid homology between the cloned porcine and human 5-HT_7 receptors was the highest (96%). Nevertheless, the porcine 5-HT_7 receptor showed some different amino acids as compare to other species. For example, at the N-terminal, Ser^{33}, Gly^{37}, His^{49}, Val^{59} and Arg^{66} were shown to be located, while a Leu^{433} and Lys^{436} were found towards the C-terminal ends.

Moreover, Leu^{221} and Met^{248} were found in the fourth and fifth transmembrane domains, while a Glu^{283} in the fifth and sixth transmembrane domains.

Pharmacological characterisation of porcine 5-HT$_1$ receptor subtypes

The characterisation of receptors is based on *structural* (molecular), *operational* (pharmacological profile) and *transductional* (receptor-effector coupling) criteria (Hoyer et al., 1994; Hoyer et al., 1997). Therefore, we studied the ligand binding profile of the different recombinant porcine 5-HT$_1$ receptor subtypes (5-HT$_{1B}$, 5-HT$_{1D}$ and 5-ht$_{1F}$) in membranes obtained from mammalian cells expressing these receptors. Unfortunately, the ligand binding profile of the porcine 5-HT$_7$ receptor has not yet been studied due to the unavailability of a full-length cDNA insert for expression in cells.

In case of recombinant porcine 5-HT$_{1B}$ receptors, the competitive binding assay using [^{3}H]GR125743 showed the following rank order: L694247 > ergotamine ≥ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan for agonists; GR127935 > methiothepin > SB224289 >> ritanserin > ketanserin ≥ BRL15572 for putative antagonists. The pK$_i$ values showed a high correlation with those reported for the recombinant human receptor (r_s=0.988). On the other hand, the comparison between the pK$_i$ values at porcine and rat 5-HT$_{1B}$ receptors showed a lower correlation (r_s =0.808). The human 5-HT$_{1B}$ receptor selective antagonist SB224289 (Selkirk et al., 1998) also displayed a high affinity for the recombinant porcine 5-HT$_{1B}$ receptor, but the human selective 5-HT$_{1D}$ receptor antagonist BRL15572 (Price et al., 1997) exhibited more than 100-fold lower affinity. Moreover, functional characterisation of porcine 5-HT$_{1B}$ receptor showed that both 5-HT and zolmitriptan potently increased [^{35}S]GTPγS binding and this was similar to the recombinant human 5-HT$_{1B}$ receptor (Pauwels et al., 1997). Interestingly, the 5-HT$_{1B}$-receptor antagonist SB224289 inhibited [^{35}S]GTPγS binding and acted as an inverse agonist at porcine 5-HT$_{1B}$ receptor, as noticed earlier using the recombinant human 5-HT$_{1B}$ receptor (Selkirk et al., 1998). Thus, the porcine 5-HT$_{1B}$ receptor closely resembles the human 5-HT$_{1B}$ receptor in its pharmacology and, therefore, it can be used for screening 5-HT$_{1B}$ receptor ligands likely to be of therapeutic value in humans.

The competitive binding assay using either [^{3}H]GR125743 or [^{3}H]5-carboxamidotryptamine at porcine 5-HT_{1D} receptor revealed the following potency rank order: L694247 > 5-CT > zolmitriptan > 5-HT > sumatriptan = CP122638 = ergotamine > dihydroergotamine for agonists and methiothepin > GR127935 > ketanserin > ritanserin >> SB224289 ≥ BRL 15572 for antagonists. The pK_i values showed a high correlation with human 5-HT_{1D} receptor (r_s=.903) and, as in the human, ketanserin was found to be 40-fold more potent at the porcine 5-HT_{1D} than at the porcine 5-HT_{1B} receptor. However, in contrast to a selective and high antagonist potency at the human 5-HT_{1D} receptor (Price et al., 1997), BRL15572 had a poor affinity at the porcine 5-HT_{1D} receptor. This example shows that one should be cautious in extrapolating the affinity and efficacy of compounds from one species to the other. As seen with the 5-HT_{1B} receptor, both 5-HT and zolmitriptan increased [^{35}S]-GTPγS binding in membranes obtained from cells expressing porcine 5-HT_{1D} receptor.

As described in Chapter 4, the recombinant porcine 5-ht_{1F} receptor showed a high affinity for [^{3}H]5-HT. Competitive binding assay revealed that the selective 5-ht_{1F} receptor agonist LY 334370 displayed a high affinity for the porcine 5-ht_{1F} receptor. 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. The pK_i values obtained at the porcine 5-ht_{1F} receptor showed a high correlation (r_s=0.911) with those reported for the human 5-ht_{1F} receptor. Nevertheless, it may be important to mention that some triptans (sumatriptan, rizatriptan and zolmitriptan) exhibited more than 10 -fold less affinity for porcine 5-ht_{1F} receptor as compared to the human receptor. The molecular basis for the selectivity of these receptors among the species may be due to the differences in the transmembrane domain structure of the receptor and could be addressed by site directed mutation and chimeric receptor studies.

In conclusion, the porcine 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors revealed a high homology in amino acid sequence as well as a high correlation in pharmacological properties as compared to the human.

Expression of specific porcine 5-HT receptor subtype mRNA

By means of RT-PCR techniques, we have studied the presence of 5-HT_{1B}, 5-ht_{1F} and 5-HT_7 receptor *m*RNAs in various porcine blood vessels and brain tissues. This information may be of interest for the understanding of the pathophysiology and treatment of migraine.

As described in Chapter 2, porcine 5-HT_{1B} receptor *m*RNA was detected ubiquitously in brain (cortex and cerebellum), trigeminal ganglion, heart (left ventricle) and blood vessels (left anterior descending coronary, pulmonary, common carotid, superior mesenteric and femoral arteries and saphenous vein). Furthermore, other reports have also shown 5-HT_{1B} receptor *m*RNA expression in various cerebral arteries of rat, bovine and human (Hamel et al., 1993; Ullmer et al., 1995). The ubiquitous presence of 5-HT_{1B} receptor in various blood vessels suggests that peripheral vasoconstriction is mediated via 5-HT_{1B} receptor (Chapter 6, Van den Broek et al., 2001). On the other hand, the presence of 5-HT_{1B} receptor in brain cortex (also demonstrated by *in situ* hybridisation) and trigeminal ganglion indicates its role in inhibiting the release of neurotransmitters in the brain as well as peptides (CGRP, substance P, etc) from the trigeminal neurones.

As reported for the human trigeminal ganglion (Bouchelet et al., 1996), we also noticed the presence of the 5-ht_{1F} receptor in porcine trigeminal ganglion. The expression of the 5-ht_{1F} receptor in porcine trigeminal ganglion supports the notion that this receptor may mediate the inhibition of dural plasma protein extravasation by serotonergic ligands (Johnson et al., 1997; Phebus et al., 1997). The selective 5-ht_{1F} receptor agonist (LY334370) has been shown to block neurogenic plasma protein extravasation in the dura mater and reduce *c*-fos expression in the trigeminal nucleus caudalis without constricting cerebral or coronary arteries (Johnson et al., 1997; Phebus et al., 1997). Thus, a specific 5-ht_{1F}-receptor agonist may be useful as an antimigraine drug without causing vasoconstriction. However, in a recent study the selective 5-HT_{1D} receptor agonist (PNU-142633), but not LY334370, blocked neurogenic dural vasodilatation in anaesthetised guinea pig (Williamson et al., 2001), suggesting that 5-HT_{1D} rather than 5-HT_{1F} receptor may be the preferential target for the antimigraine action. The presence of mRNA signals for the 5-ht_{1F} receptor in brain cortex and cerebellum were similar to those reported by Lovenberg (1993) and point towards its function as an autoreceptor. While vasoconstrictor properties of

5-ht_{1F} receptors were ruled out in rabbit saphenous vein (Cohen et al., 1999), we found some mRNA signals in porcine saphenous vein and coronary artery; the latter was qualitatively similar with the recent findings in the human coronary artery (Bouchelet et al., 2000). The physiological implication of this finding is not clear as till date no functional role of the 5-ht_{1F} in blood vessels has been demonstrated. Since several triptans (e.g. rizatriptan and alniditan) display a poor affinity for the 5-HT_{1F} receptor, it would appear that an agonist action at the 5-HT_{1F} receptor is not a pre-requisite for the antimigraine efficacy (Saxena et al., 2000).

The 5-HT_7 receptor mRNA was observed in porcine brain (cortex, hemisphere, cerebellum) as well as in blood vessels (pulmonary and coronary artery and saphenous vein), but not in the heart. The 5-HT_7 receptor has also been observed in both brain and blood vessels of rat and human tissues (Ullmer et al., 1995; Heidmann et al., 1998). The distribution of the receptor in both central and peripheral tissues and vascular bed indicates that it may play an important physiological role. Expression of 5-HT_7 mRNA in brain tissues as well as high affinity of antipsychotic drugs (risperidone and clozapine) for this receptor suggests that the 5-HT_7 receptor may be a target for the treatment of psychotic disorders (Roth et al., 1994). Pharmacological studies have shown that 5-HT_7 receptor mediates smooth muscle relaxation in the guinea pig ileum (Carter et al., 1995), rabbit pulmonary artery (Morecroft et al., 1998), rabbit femoral vein (Martin et al., 1995), monkey jugular vein (Leung et al., 1996) and dog external carotid vasculature (Villalón et al., 1997) as well as tachycardia in the cat (Villalon et al., 1997). Interestingly, tachycardia in the pig and human heart is mediated by 5-HT_4 but not 5-HT_7 receptors (Saxena, 1995b) and this corresponds to the presence of 5-HT_4 (and not 5-HT_7) receptor mRNA in the human and porcine heart (Bard et al., 1993/, Chapter 5).

To correlate the porcine tissue distribution of 5-HT_{1B} and 5-HT_{1D} receptors in the view of clinical implication for humans, we performed *in vitro* functional and molecular biological studies using human isolated blood vessels relevant for the underlying mechanisms inducing a migraine attack (Chapter 6). For this reason, we used the middle meningeal and temporal arteries (to evaluate the therapeutic efficacy of current used antimigraine agents) and the coronary artery and saphenous vein (in order to evaluate potential side effects). Sumatriptan, a non-selective 5-$HT_{1B/1D}$ receptor agonist induces contraction of these blood vessels (De Vries et al., 1999;

Goadsby, 1999; Saxena et al., 2000). We found that the selective 5-HT_{1B} receptor antagonist SB224289 blocked these responses, while the selective 5-HT_{1D} receptor antagonist BRL15572 proved to be ineffective, thus showing that vasoconstriction was mediated by the 5-HT_{1B} but not 5-HT_{1D} receptor. As mentioned above, the localisation of these individual receptors in blood vessels was in agreement with this conclusion. Even though the protein distribution of 5-HT_{1B} and 5-HT_{1D} receptor in blood vessels has been reported by Longmore (1997), we feel that the precise localisation of *m*RNA provides more detailed information concerning the synthesis and expression of these receptors in a given cell type.

Limitations and precautions

In the present thesis, we have described the cloning (RT-PCR and inverse PCR), pharmacological characterisation (radioligand binding assays and functional assays) and localisation (non-radioactive *in situ* hybridisation) of porcine 5-HT receptors. The studies compiled in this thesis may have some limitations. Such limitations and precautions taken are itemised below:

1. Due to the very high sensitivity of the polymerase chain reaction (PCR) technique, any incorrect incorporation of a nucleotide will further be amplified in the cloned product. To rectify this, several full-length PCR amplified products were cloned and sequenced. Alternatively, one can screen the genomic or cDNA library derived from the porcine tissue to establishing the full-length sequence.

2. In some cases, two products were visible from inverse PCR technique on genomic DNA circles, even at a high (>60°C) annealing temperature. In such cases, the specific band of the larger product length was selected for further cloning and sequencing, as smaller bands showed only the partial sequence of the product.

3. For the localisation of 5-HT_1 receptors in various tissue, we performed non-radioactive *in situ* hybridisation technique, which is relatively less sensitive to detect mRNA. It would be better to complement our finding on localisation using radioactive *in situ* hybridisation also.

4. Pharmacological studies using membranes obtained from cells expressing the recombinant receptor provide useful data with respect to the affinity and efficacy, but one should keep in mind that drug activity may vary according to the level of expression of a particular receptor. For example, the arylkylamines propranolol and pindolol and the ergot derivative metergoline, which have been described as antagonists or partial agonists in *in vivo* or *in vitro* studies, were found to behave as full agonists in cells expressing the rat or human 5-HT_{1B} receptor (Miller et al., 1992; Adham et al., 1993a).

5. The *in vitro* studies performed with the isolated blood vessels in organ baths provide the possibility of a more detailed study than the *in vivo* studies on animals and/or patients. However, in organ bath studies, drugs are acting at both intraluminal and extraluminal sides of the blood vessels, while in *in vivo* experiments the intraluminal side of the blood vessels is only exposed.

Implication in future research

The results compiled in this thesis showing the amino acid sequence and pharmacological properties of porcine 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptors could be useful for the subsequent studies as outlined below:

1. To understand the potential role of these receptors in the pathophysiological processes leading to migraine attacks and, possibly, their usefulness in migraine therapy.

2. To investigate why the selective 5-HT_{1D} receptor antagonist BRL15572 showed a low affinity at porcine 5-HT_{1D} receptors. By means of site directed mutation / deletion experiments, it may be possible to recognise specific amino acid(s) in the transmembrane domain that may explain this interesting feature.

3. Similarly, by mutating a single amino acid (threonine at position 333) or multiple nucleotides in the porcine 5-ht_{1F} receptor may explain why the antimigraine triptans display lower affinity at this receptor in pigs as compared to that at the human 5-ht_{1F} receptor.

4. The recombinant porcine receptors (5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7) may be exploited to investigate possible signal transduction mechanisms involved in the vasomotor activity (constriction and dilatation) of blood vessels. In this regards, several mechanism(s) are still not clear, e.g.: (1) stimulation/inhibition of adenylyl cyclase activity; (2) increase/decrease of intracellular calcium concentration and the role of inositol triphosphates; and (3) opening/closing of calcium channels (e.g. Ca^{2+} dependent K^+, Ca^{2+} activated Cl^- channels) that couple after activation of the individual receptor.

5. Tissue localisation of 5-HT_7 receptor by using a specific probe derived from the recombinant receptor might be helpful to know the expression and functional relevance of this receptor in that particular tissue. Additionally, attempts should be made to clone a single full-length cDNA encoding porcine 5-HT_7 receptor that could further be used to express in mammalian cell lines to establish the pharmacological profile of porcine 5-HT_7 receptor. The distribution pattern, functional characterisation and pharmacological profile of various isoforms of porcine 5-HT_7 receptor (if present) would be helpful to know the differential properties of 5-HT_7 receptor family.

References

ADHAM, N., BARD, J.A., ZGOMBICK, J.M., DURKIN, M.M., KUCHAREWICZ, S., WEINSHANK, R.L. & BRANCHEK, T.A. (1997). Cloning and characterization of the guinea pig 5-HT_{1F} receptor subtype: a comparison of the pharmacological profile to the human species homolog. *Neuropharmacology*, **36,** 569-576.

ADHAM, N., ELLERBROCK, B., HARTIG, P., WEINSHANK, R.L. & BRANCHEK, T. (1993a). Receptor reserve masks partial agonist activity of drugs in a cloned rat 5-hydroxytryptamine1B receptor expression system. *Mol. Pharmacol.*, **43,** 427-433.

ADHAM, N., KAO, H.T., SCHECTER, L.E., BARD, J., OLSEN, M., URQUHART, D., DURKIN, M., HARTIG, P.R., WEINSHANK, R.L. & BRANCHEK, T.A. (1993b). Cloning of another human serotonin receptor (5-HT_{1F}): a fifth 5-HT_1 receptor subtype coupled to the inhibition of adenylate cyclase. *Proc. Natl. Acad. Sci. U S A*, **90,** 408-412.

ADHAM, N., TAMM, J.A., SALON, J.A., VAYSSE, P.J.-J., WEINSHANK, R.L. & BRANCHEK, T.A. (1994). A single point mutation increases the affinity of serotonin 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-$HT_{1E}$and 5-HT_{1F} receptors for α-adrenergic antagonists. *Neuropharmacology*, **33,** 387-391.

BARD, J.A., KUCHAREWICZ, S.A., ZGOMBICK, J.M., WEINSHANK, R.L., BRANCHEK, T.A. & COHEN, M.L. (1996). Differences in ligand binding profiles between cloned rabbit and human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors: ketanserin and methiothepin distinguish rabbit 5-HT_{1D} receptor subtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 237-244.

BARD, J.A., ZGOMBICK, J., ADHAM, N., VAYSSE, P., BRANCHEK, T.A. & WEINSHANK, R.L. (1993). Cloning of a novel human serotonin receptor (5-HT_7) positively linked to adenylate cyclase. *J. Biol. Chem.*, **268,** 23422-23426.

BHALLA, P., SHARMA, H.S., MA, X., WURCH, T., PAUWELS, P.J. & SAXENA, P.R. (2001). Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT_{1B} receptor. *Br. J. Pharmacol.*, **133,** 891-901.

BHALLA, P., SHARMA, H.S., WURCH, T., PAUWELS, P.J. & SAXENA, P.R. (2000). Molecular cloning, sequence analysis and pharmacological properties of the porcine 5-HT_{1D} receptor. *Br. J. Pharmacol.*, **131,** 949-957.

BOUCHELET, I., CASE, B., OLIVIER, A. & HAMEL, E. (2000). No contractile effect for 5-HT_{1D} and 5-HT_{1F} receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br. J. Pharmacol.*, **129,** 501-508.

BOUCHELET, I., COHEN, Z., CASE, B., SEGUELA, P. & HAMEL, E. (1996). Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. *Mol. Pharmacol.*, **50,** 219-223.

CARTER, D., CHAMPNEY, M., HWANG, B. & EGLEN, R.M. (1995). Characterization of a postjunctional 5-HT receptor mediating relaxation of guinea-pig isolated ileum. *Eur. J. Pharmacol.*, **280,** 243-250.

CENTURION, D., ARACELI SANCHEZ-LOPEZ, A., ORTIZ, M.I., DE VRIES, P., SAXENA, P.R. & VILLALON, C.M. (2000). Mediation of 5-HT-induced internal carotid vasoconstriction in GR127935- and ritanserin-pretreated dogs by 5-HT_7 receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **362,** 169-176.

COHEN, M.L. & SCHENCK, K. (1999). 5-Hydroxytryptamine$_{1F}$ receptors do not participate in vasoconstriction: lack of vasoconstriction to LY344864, a selective serotonin$_{1F}$ receptor agonist in rabbit saphenous vein. *J. Pharmacol. Exp. Ther.*, **290,** 935-939.

DAHLÖF, C.G.H. & SAXENA, P.R. (2000). Novel compounds in development for acute treatment of migraine. In *The Migraines (Second Eddition)*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 439-443. Philadelphia: Lippincott Wiliams & Wilkins.

DE VRIES, P., SANCHEZ-LOPEZ, A., CENTURION, D., HEILIGERS, J.P.C., SAXENA, P.R. & VILLALON, C.M. (1998a). The canine external carotid vasoconstrictor 5-HT1 receptor: blockade by 5-HT_{1B} (SB224289), but not by 5-HT_{1D} (BRL15572) receptor antagonists. *Eur. J. Pharmacol.*, **362,** 69-72.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P.C. & SAXENA, P.R. (1998b). Constriction of porcine arteriovenous anastomoses as indicator of antimigraine activity. Involvement of 5-$HT_{1B/1D}$ and novel receptors. *Br. J. Pharmacol.*, **123,** 1561-1570.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P.C., VILLALON, C.M. & SAXENA, P.R. (1999a). Investigations of the role of 5-HT_{1B} and 5-HT_{1D} receptors in the sumatriptan-induced constrction of porcine carotid arteriovenous anastomoses. *Br. J. Pharmacol.*, **127,** 405-412.

DE VRIES, P., WILLEMS, E.W., HEILIGERS, J.P.C., VILLALÓN, C.M. & SAXENA, P.R. (1999b). Constriction of porcine carotid arteriovenous anastomoses as indicator of antimigraine activity: the role of 5-$HT_{1B/1D}$, as well as unidentified receptors. In *Migraine & headache pathophysiology*. ed. Edvinsson, L. London: Martin Dunitz Ltd.

DE VRIES, P., VILLALÓN, C.M. & SAXENA, P.R. (1999). Pharmacological aspects of experimental headache models in relation to acute antimigraine therapy. *Eur. J. Pharmacol.*, **375,** 61-74.

FERRARI, M.D. & SAXENA, P.R. (1995). 5-HT_1 receptors in migraine pathophysiology and treatment. *European Journal of Neurology*, **2,** 5-21.

GOADSBY, P.J. (1999). Advances in the pharmacotherapy of migraine. How knowledge of pathophysiology is guiding drug development. *Drugs R D*, **2,** 361-374.

GOMEZ-MANCILLA, B., CUTLER, N., LEIBOWITZ, M., SPIERINGS, E., KLAPPER, J., DIAMOND, S., GOLDSTEIN, J., SMITH, T., COUCH, J., FLEISHAKER, J., AZIE, N. & BLUNT, D. (2001). Safety and efficacy of PNU-142633, a selective 5-HT_{1D} agonist, in patients with acute migraine. *Cephalalgia*, **21,** 727-732.

HAMBLIN, M.W., METCALF, M.A., MCGUFFIN, R.W. & KARPELLS, S. (1992). Molecular cloning and functional characterization of a human 5-HT_{1B} serotonin receptor: a homologue of the rat 5-HT_{1B} receptor with 5-HT_{1D}- like pharmacological specificity. *Biochem. Biophys. Res. Commun.*, **184,** 752-759.

HAMEL, E., FAN, E., LINVILLE, D., TING, V., VILLEMURE, J.G. & CHIA, L.S. (1993). Expression of mRNA for the serotonin 5-hydroxytryptamine$_{1D}$ beta receptor subtype in human and bovine cerebral arteries. *Mol. Pharmacol.*, **44,** 242-246.

HARWOOD, G., LOCKYER, M., GILES, H. & FAIRWEATHER, N. (1995). Cloning and characterisation of the rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. *FEBS Lett.*, **377,** 73-76.

HEIDMANN, D.E., SZOT, P., KOHEN, R. & HAMBLIN, M.W. (1998). Function and distribution of three rat 5-hydroxytryptamine7 (5-HT_7) receptor isoforms produced by alternative splicing. *Neuropharmacology*, **37,** 1621-1632.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 157-203.

HOYER, D. & HUMPHREY, P.P. (1997). Nomenclature and classification of transmitter receptors: an integrated approach. *J. Recept. Signal Transduct. Res.*, **17,** 551-568.

JOHNSON, K.W., SCHAUS, J.M., DURKIN, M.M., AUDIA, J.E., KALDOR, S.W., FLAUGH, M.E., ADHAM, N., ZGOMBICK, J.M., COHEN, M.L., BRANCHEK, T.A. & PHEBUS, L.A. (1997). 5-HT_{1F} receptor agonists inhibit neurogenic dural inflammation in guinea pigs. *Neuroreport*, **8,** 2237-2240.

LEUNG, E., WALSH, L.K.M., PULIDO-RIOS, M.T. & EGLEN, R.M. (1996). Characterization of putative 5-ht_7 receptors mediating direct relaxation in *Cynomolgus* monkey isolated jugular vein. *Br. J. Pharmacol.*, **117,** 926-930.

LONGMORE, J., SHAW, D., SMITH, D., HOPKINS, R., MCALLISTER, G., PICKARD, J.D., SIRINATHSINGHJI, D.J., BUTLER, A.J. & HILL, R.G. (1997). Differential distribution of 5HT_{1D}- and 5HT_{1B}-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. *Cephalalgia*, **17,** 833-842.

LOVENBERG, T.W., ERLANDER, M.G., BARON, B.M., RACKE, M., SLONE, A.L., SIEGEL, B.W., CRAFT, C.M., BURNS, J.E., DANIELSON, P.E. & SUTCLIFFE, J.G. (1993). Molecular cloning and functional expression of 5-HT_{1E}-like rat and human 5-hydroxytryptamine receptor genes. *Proc. Natl. Acad. Sci. U S A*, **90,** 2184-2188.

MAASSENVANDENBRINK, A., REEKERS, M., BAX, W.A., FERRARI, M.D. & SAXENA, P.R. (1998). Coronary side-effect potential of current and prospective antimigraine drugs. *Circulation*, **98,** 25-30.

MARTIN, G.R. & WILSON, R.J. (1995). Operational characteristics of a 5-HT receptor mediating direct vascular relaxation: identity with the 5-ht_7 receptor? *Br. J. Pharmacol.*, **114,** 383P.

MAY, A. & GOADSBY, P.J. (2001). Pharmacological opportunities and pitfalls in the therapy of migraine. *Curr. Opin. Neurol.*, **14,** 341-345.

MIALET, J., BERQUE-BESTEL, I., EFTEKHARI, P., GASTINEAU, M., GINER, M., DAHMOUNE, Y., DONZEAU-GOUGE, P., HOEBEKE, J., LANGLOIS, M., SICSIC, S., FISCHMEISTER, R. & LEZOUALC'H, F. (2000). Isolation of the serotoninergic 5-$HT_{4(e)}$ receptor from human heart and comparative analysis of its pharmacological profile in C6-glial and CHO cell lines. *Br. J. Pharmacol.*, **129,** 771-781.

MILLER, K.J., KING, A., DEMCHYSHYN, L., NIZNIK, H. & TEITLER, M. (1992). Agonist activity of sumatriptan and metergoline at the human 5-HT_{1D} beta receptor: further evidence for a role of the 5-HT1D receptor in the action of sumatriptan. *Eur. J. Pharmacol.*, **227,** 99-102.

MORECROFT, I. & MACLEAN, M.R. (1998). 5-hydroxytryptamine receptors mediating vasoconstriction and vasodilation in perinatal and adult rabbit small pulmonary arteries. *Br. J. Pharmacol.*, **125,** 69-78.

MOSKOWITZ, M.A. (1993). Neurogenic inflammation in the pathophysiology and treatment of migraine. *Neurology*, **43,** S16-20.

MOSKOWITZ, M.A., REINHARD, J.F., JR., ROMERO, J., MELAMED, E. & PETTIBONE, D.J. (1979). Neurotransmitters and the fifth cranial nerve: is there a relation to the headache phase of migraine? *Lancet*, **2,** 883-885.

OKSENBERG, D., MARSTERS, S.A., O'DOWD, B.F., JIN, H., HAVLIK, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{1B} receptors. *Nature*, **360,** 161-163.

PAUWELS, P.J., TARDIF, S., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1997). How efficacious are 5-$HT_{1B/D}$ receptor ligands: an answer from GTP gamma S binding studies with stably transfected C6-glial cell lines. *Neuropharmacology*, **36,** 499-512.

PHEBUS, L.A., JOHNSON, K.W., ZGOMBICK, J.M., GILBERT, P.J., VAN BELLE, K., MANCUSO, V., NELSON, D.L., CALLIGARO, D.O., KIEFER, A.D., JR., BRANCHEK, T.A. & FLAUGH, M.E. (1997). Characterization of LY344864 as a pharmacological tool to study 5-HT_{1F} receptors: binding affinities, brain penetration and activity in the neurogenic dural inflammation model of migraine. *Life Sci.*, **61,** 2117-2126.

PRICE, G.W., BURTON, M.J., COLLIN, L.J., DUCKWORTH, M., GASTER, L., GÖTHERT, M., JONES, B.J., ROBERTS, C., WATSON, J.M. & MIDDLEMISS, D.N. (1997). SB-216641 and BRL-15572-compounds to pharmacologically discriminate h5-HT_{1B} and h5-HT_{1D} receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **356,** 312-320.

ROON, K.I. (2000). New 5-HT_1 agonists in migraine, Clinical efficacy and mechanisms. In *Department of Neurology*. pp. 174. Leiden: Leiden University Medical Centre.

ROTH, B.L., CRAIGO, S.C., CHOUDHARY, M.S., ULUER, A., MONSMA, F.J., JR., SHEN, Y., MELTZER, H.Y. & SIBLEY, D.R. (1994). Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. *J. Pharmacol. Exp. Ther.*, **268,** 1403-1410.

SAXENA, P.R. (1995a). Cranial arteriovenous shunting, an *in vivo* animal model for migraine. In *Experimental headache models*. ed. Olesen, J. & Moskowitz, M.A. pp. 189-198. Philadelphia: Lippincott-Raven Publishers.

SAXENA, P.R. (1995b). Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol. Ther.*, **66,** 339-368.

SAXENA, P.R., DE VRIES, P. & VILLALON, C.M. (1998). 5-HT_1-like receptors: a time to bid goodbye. *Trends Pharmacol. Sci.*, **19,** 311-316.

SAXENA, P.R. & TFELT-HANSEN, P. (2000). Triptans, 5-$HT_{1B/1D}$ receptor agonists in the acute treatment of migraine. In *The headaches*. ed. Olesen, J., Tfelt-Hansen, P. & Welch, K.M.A. pp. 411-438. Philadelphia: Lippincott Williams & Wilkins.

SELKIRK, J.V., SCOTT, C., HO, M., BURTON, M.J., WATSON, J., GASTER, L.M., COLLIN, L., JONES, B.J., MIDDLEMISS, D.N. & PRICE, G.W. (1998). SB-224289 - a novel selective (human) 5-HT_{1B} receptor antagonist with negative intrinsic activity. *Br. J. Pharmacol.*, **125,** 202-208.

TERRÓN, J.A. & FALCÓN-NERI, A. (1999). Pharmacological evidence for the 5-HT_7 receptor mediating smooth muscle relaxation in canine cerebral arteries. *Br. J. Pharmacol.*, **127,** 609-616.

THOMAS, D.R., GITTINS, S.A., COLLIN, L.L., MIDDLEMISS, D.N., RILEY, G., HAGAN, J., GLOGER, I., ELLIS, C.E., FORBES, I.T. & BROWN, A.M. (1998). Functional characterisation of the human cloned 5-HT_7 receptor (long form); antagonist profile of SB-258719. *Br. J. Pharmacol.*, **124,** 1300-1306.

TSOU, A.P., KOSAKA, A., BACH, C., ZUPPAN, P., YEE, C., TOM, L., ALVAREZ, R., RAMSEY, S., BONHAUS, D.W., STEFANICH, E. & ET AL. (1994). Cloning and expression of a 5-hydroxytryptamine7 receptor positively coupled to adenylyl cyclase. *J. Neurochem.*, **63,** 456-464.

ULLMER, C., SCHMUCK, K., KALKMAN, H.O. & LUBBERT, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *FEBS Lett.*, **370,** 215-221.

VAN DEN BROEK, R.W.M., BHALLA, P., MAASSENVANDENBRINK, A., DE VRIES, R., SHARMA, H.S. & SAXENA, P.R. (2001). Characterisation of sumatriptan-induced contractions in human isolated blood vessels using selective 5-HT_{1B} and 5-HT_{1D} receptor antagonists and *in situ* hybridisation. *Cephalalgia***,** In press.

VERHEGGEN, R., HUNDESHAGEN, A.G., BROWN, A.M., SCHINDLER, M. & KAUMANN, A.J. (1998). 5-HT1B receptor-mediated contractions in human temporal artery: evidence from selective antagonists and 5-HT receptor mRNA expression. *Br. J. Pharmacol.*, **124,** 1345-1354.

VILLALÓN, C.M., CENTURIÓN, D., LUJÁN-ESTRADA, M., TERRÓN, J.A. & SÁNCHEZ-LÓPEZ, A. (1997). Mediation of 5-HT-induced external carotid vasodilatation in GR127935-pretreated vagosympathectomized dogs by the putative 5-HT_7 receptor. *Br. J. Pharmacol.*, **120,** 1319-1327.

VILLALON, C.M., HEILIGERS, J.P., CENTURION, D., DE VRIES, P. & SAXENA, P.R. (1997). Characterization of putative 5-HT7 receptors mediating tachycardia in the cat. *Br. J. Pharmacol.*, **121,** 1187-1195.

VOIGT, M.M., LAURIE, D.J., SEEBURG, P.H. & BACH, A. (1991). Molecular cloning and characterization of a rat brain cDNA encoding a 5- hydroxytryptamine$_{1B}$ receptor. *Embo J.*, **10,** 4017-4023.

WEINSHANK, R.L., ZGOMBICK, J.M., MACCHI, M.J., BRANCHEK, T.A. & HARTIG, P.R. (1992). Human serotonin $_{1D}$ receptor is encoded by a subfamily of two distinct genes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. *Proc. Natl. Acad. Sci. USA*, **89,** 3630-3634.

WILLIAMSON, D.J., HILL, R.G., SHEPHEARD, S.L. & HARGREAVES, R.J. (2001). The anti-migraine 5-$HT_{1B/1D}$ agonist rizatriptan inhibits neurogenic dural vasodilation in anaesthetized guinea-pigs. *Br. J. Pharmacol.*, **133,** 1029-1034.

WURCH, T., PALMIER, C., COLPAERT, F.C. & PAUWELS, P.J. (1997). Sequence and functional analysis of cloned guinea pig and rat serotonin 5-HT_{1D} receptors: common pharmacological features within the 5-HT_{1D} receptor subfamily. *J. Neurochem.*, **68,** 410-418.

ZGOMBICK, J.M., BARD, J.A., KUCHAREWICZ, S.A., URQUHART, D.A., WEINSHANK, R.L. & BRANCHEK, T.A. (1997). Molecular cloning and pharmacological characterization of guinea pig 5- HT_{1B} and 5-HT_{1D} receptors. *Neuropharmacology*, **36,** 513-524.

Chapter 8

Summaries

Summary in English

This thesis focuses on the molecular and pharmacological aspects of porcine 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptors, which seem to play important role in the pathogenesis and treatment of migraine headache. Constriction of porcine carotid arteriovenous anastomoses is a well-established model for evaluating antimigraine activity of 5-HT-based compounds. To complement this *in vivo* experimental model, we developed strategies for rapidly screening a number of potential antimigraine drugs. For this purpose, we have cloned, sequenced and pharmacologically characterised porcine specific 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptors. The result embodied in this thesis could be of importance in unravelling mechanisms underlying the pathogeneses of migraine and may help in developing effective therapeutic strategies.

Chapter 1 reviews the current knowledge about the role of 5-HT and its receptors in migraine as well as the rationale for the studies described in the present thesis. Since the pathophysiology of migraine is still not completely understood, a brief summary on the current hypotheses as well as possible mechanisms of action of current acute antimigraine drugs has been included.

To date, it is widely accepted that certain subtypes of the 5-HT_1 (i.e. 5-HT_{1B} and 5-HT_{1D}) receptor subfamily may be important targets for acutely acting antimigraine agents, like sumatriptan. In addition, there is some evidence suggesting the involvement of the 5-ht_{1F} receptor subtype. Regarding the prophylactic treatment of migraineurs, some studies have advocated that selective antagonists at 5-HT_7 receptors may also be clinically efficacious. Investigating the molecular pharmacological aspects of 5-HT_7 receptor is of particular interest due to the presence of different splice variants and the ubiquitous receptor distribution in both central and peripheral tissues. Thus, due to their importance, a detailed account has been

provided on the molecular biology of 5-HT_{1B}, 5-HT_{1D} 5-ht_{1F} and 5-HT_7 receptors and their potential relation with respect to migraine treatment.

It is interesting to note that even though human and rat 5-HT_{1B} receptor show high (>90%) similarity in molecular mRNA or amino acid sequence, their pharmacological profile differs markedly. On the other hand, human 5-HT_{1B} and 5-HT_{1D} receptors show relatively low homology (61%), but a similar pharmacological profile has been reported for these two receptors. From these reports it is evident that great care should be exercised when extrapolating results from experimental animal models to human. For several years, we have used anaesthetised pigs to investigate the ability of a number of compounds, including triptans, to produce constriction of carotid arteriovenous anastomoses as a predictive tool for antimigraine activity; the porcine shunt migraine model has been briefly discussed. For a more critical evaluation of current and potential novel antimigraine drugs that are being tested based on our porcine AVA model, we set out to elucidate the molecular and pharmacological aspects of the porcine 5-HT_1 receptor subtypes namely 5-HT_{1B} 5-HT_{1D} 5-ht_{1F} and 5-HT_7 receptors. Subsequently, molecular structures and pharmacological profiles of these receptors were compared across other species, including human.

At the end of this chapter, the aims of the present investigation have been listed.

Chapter 2 describes the molecular and pharmacological characteristics of recombinant porcine 5-HT_{1B} receptor. The main criteria for cloning this receptor were twofold: (i) the 5-HT_{1B} receptor has been shown to be an important molecular target for acute antimigraine drugs and (ii) the divergence in pharmacological properties of this receptor subtype when compared with human and rat. The full-length porcine 5-HT_{1B} receptor cDNA was derived from the pig cerebral cortex and the 5' and 3' ends of the cDNA were verified by inverse-PCR. The cDNA sequence of porcine 5-HT_{1B} receptor encoded a polypeptide of 390 amino acids and showed high homology (~90%) across other species, including human. Interestingly, the sequence of porcine 5-HT_{1B} receptor showed the conserved threonine at position 355 in the seventh transmembrane domain as observed in human, rabbit and guinea pig, rather than an aspargine at corresponding position 351 in the case of mouse and rat. Furthermore, competitive binding assays demonstrated that the pharmacological

profile of porcine 5-HT_{1B} receptor showed high correlation with the human 5-HT_{1B} receptor and weak correlation with rat 5-HT_{1B} receptor. Moreover, functional characterisation of porcine 5-HT_{1B} receptor employing [^{35}S]GTPγS binding showed that both 5-HT and the 5-$HT_{1B/1D}$ receptor agonist zolmitriptan potently increased [^{35}S]GTPγS binding, according to their affinity at 5-HT_{1B} receptors; since SB224289, a selective antagonist at 5-HT_{1B} receptor, decreased [^{35}S]GTPγS binding indicating that this compound may as an inverse agonist at this receptor. The 5-HT_{1B} receptor mRNA was expressed in various porcine blood vessels and in brain tissues. *In situ* localisation of the 5-HT_{1B} receptor mRNA was mainly observed in in pyramidal cells of porcine cerebral cortex.

Chapter 3 comprises data on the molecular cloning and pharmacological characterisation of the porcine 5-HT_{1D} receptor. Similar to the 5-HT_{1B} receptor, the 5-HT_{1D} receptor has also been implicated as a molecular target for acute antimigraine drugs. Thus, specific 5-HT_{1D} receptor agonists may be efficacious in acute migraine therapy, perhaps without causing vasoconstriction. Additionally, although the exact underlying mechanisms mediated by 5-HT_{1D} receptors in the pathophysiology of migraine are not yet clear, some agonists directed to this receptor have been demonstrated to reduce dural plasma extravasation and inflammation following trigeminal nerve stimulation. A cDNA encoding the full-length 5-HT_{1D} receptor was derived from porcine cerebral cortex and used for sequencing and pharmacological characterisation of this receptor. The 5' and 3' ends of the porcine 5-HT_{1D} receptor cDNA were confirmed by inverse PCR. The cDNA sequence of a PCR product of 1134 bp encoded a polypeptide of 377 amino acid with high homology to human as well as other species 5-HT_{1D} receptor. In contrast to the structure of recombinant human and rodent 5-HT_{1B} receptor (aspargine instead of threonine, as discussed above), the porcine 5-HT_{1D} receptor showed the presence of threonine at position 342 in the seventh transmembrane domain, as is the case in other species, including human and rodents. The presence of threonine at this position seems to be important largely for the similar ligand properties of 5-HT_{1B} and 5-HT_{1D} receptors. Pharmacological characterisation of the recombinant porcine 5-HT_{1D} receptor showed high affinity for a potent and selective agonist (5-carboxytryptamine; 5-CT) and antagonist

(GR125743) at this receptor. Subsequent competitive radioligand binding assay showed a typical 5-HT_{1D} receptor pharmacology and a high correlation with its human homologue. Ketanserin was found to discriminate between porcine 5-HT_{1B} and 5-HT_{1D} receptors displaying an approximately 40-fold higher affinity for the 5-HT_{1D} receptor. In contrast, the human selective 5-HT_{1D} receptor antagonist BRL15572 displayed several fold less affinity for the porcine 5-HT_{1D} receptor. As described for the 5-HT_{1B} receptor, both 5-HT and zolmitriptan increased, while ketanserin decreased the basal [^{35}S]GTPγS binding. The data presented in this chapter has provided further evidence on the differential binding mechanisms that explain the distinct pharmacological characteristics of BRL15572 at porcine in comparison to human 5-HT_{1D} receptor.

Chapter 4 demonstrates the molecular cloning and characterisation of porcine 5-ht_{1F} receptor. In addition to 5-HT_{1B} and 5-HT_{1D} receptors (see previous two chapters), the acute antimigraine drug sumatriptan also displays high affinity for 5-ht_{1F} receptor and, therefore, its antimigraine efficacy may possibly be due a direct action at the 5-ht_{1F} receptor receptor. In various animal models, the selective 5-ht_{1F} receptor agonist (LY334370) blocks neurogenic plasma protein extravasation in the dura mater, inhibits central c-fos expression in the trigeminal nucleus caudalis, without constricting the cerebral or coronary artery. Thus, a specific 5-ht_{1F} receptor agonist may be useful as an antimigraine drugs with less cardiovascular side effects. In this study, we firstly cloned and sequenced a cDNA derived from the trigeminal ganglion. The cDNA sequence analysis revealed a polypeptide of 366 amino acids, which showed a high homology with other species homologues, including human. Interestingly, in other species the amino acid alanine has been reported at position 333, while we found a threonine at this position in the recombinant porcine 5-ht_{1F} receptor. Even though the porcine 5-HT_{1F} receptor showed an expected pharmacological profile, quite similar to what is found in the human, some triptans showed a 10- to 15-fold less affinity. This may be due to few amino acid differences in the transmembrane domains (site of ligand binding) and could be addressed by site directed mutagenesis experiments and/or by chimeric receptor studies.

The tissue distribution of porcine 5-ht_{1F} receptor mRNA revealed that both brain and peripheral tissues (also blood vessels) contain the 5-ht_{1F} receptor mRNA.

Activation of 5-ht_{1F} receptor seemingly inhibits neuropeptide (e.g. CGRP, substance P) release from trigeminal nerves as well as dural neurogenic inflammation. Even though 5-ht_{1F} receptor mRNA has been found in blood vessels, activation of this receptor does not participate in vasoconstriction. The vascular expression of 5-ht_{1F} receptor still remains to be further evaluated for assigning a role to this receptor in the circulatory system.

Chapter 5 describes the molecular cloning of the porcine 5-HT_7 receptor. At present, the exact physiological role of 5-HT_7 receptors is still not clear. However, several reports have shown that activation of this receptor produces relaxation of vascular smooth muscles. Since dilatation of cranial blood vessels has been proposed in the pathophysiology of migraine, a potent and selective 5-HT_7 receptor antagonist may be efficacious for the treatment of migraine. Total mRNA was isolated from the porcine cerebral cortex and by using multiple set of internal primers the porcine 5-HT_7 receptor cDNA was amplified. Consensus aligned cDNA sequence revealed a polypeptide of 447 amino acids. The recombinant porcine 5-HT_7 showed high homology with other species homologues, including human, whereas all transmembrane domains were identical to porcine 5-HT_7 receptor except for numbers IV and V. The tissue distribution pattern of porcine 5-HT_7 receptor mRNA was observed in brain (cortex, hemisphere and cerebellum) and peripheral blood vessels (pulmonary and coronary arteries, superior vena cava and saphenous vein). The tissue distribution provides additional information in the (patho)physiological role of 5-HT_7 receptors, both in the central nervous system and peripheral system. In this sense, selective 5-HT_7 receptor antagonists with prophylactic antimigraine properties are awaited with great interest.

Chapter 6 shows the localisation of both human 5-HT_{1B} and 5-HT_{1D} receptors in human isolated blood vessels. As discussed in Chapter 1, we investigated the role of 5-HT_{1B} and 5-HT_{1D} receptors in mediating sumatriptan-induced contractions of human cranial (middle meningeal and temporal arteries) as well as peripheral (coronary artery and saphenous vein) blood vessels. Our results, using *in situ* hybridisation, show that 5-HT_{1B} receptor mRNA is mainly present in smooth muscle and endothelial cells of middle meningeal and coronary arteries as well as in

saphenous veins, whereas only a weak expression of 5-HT_{1D} receptor was observed in these blood vessels. In agreement with the mRNA localisation of these receptors, the use of selective antagonists at human 5-HT_{1B} (SB224289) and 5-HT_{1D} (BRL15572) receptors revealed that the 5-HT_{1B} receptor antagonist SB224289, but not the 5-HT_{1D} receptor antagonist BRL15572, attenuated sumatriptan-induced constrictions of these blood vessels. Even though SB224289 attenuated sumatriptan-induced contractions, thus demonstrating that the 5-HT_{1B} receptor primarily mediates vasoconstriction, other yet unidentified receptors/mechanisms may be involved in sumatriptan-induced responses in the human coronary artery.

Chapter 7 provides a general discussion, limitation and precautions and implications in future research.

In conclusion, the present studies revealed the molecular properties of porcine 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptors and their high homology with other species homologues, including human. Additionally, the pharmacological profiles of porcine 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptors showed a high correlation with that found for human recombinant receptors. In particular, the major findings of the present study were: (i) molecular cloning and pharmacological characterisation of porcine 5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F} receptor; (ii) the low affinity of the human selective 5-HT_{1D} receptor antagonist BRL15572 for the porcine 5-HT_{1D}; (iii) the presence of threonine at position 333 in the porcine 5-ht_{1F} receptor rather than alanine as found in other species; and (iv) the low affinity of some triptans for porcine 5-ht_{1F} receptor. It is anticipated that this knowledge may be useful in characterising 5-HT receptor ligands for therapeutic purposes.

Summary in Dutch; Samenvatting in het Nederlands

Dit proefschrift behandelt voornamelijk de moleculaire aspecten van een aantal serotonine (5-HT) receptoren, die mogelijkerwijs een belangrijke rol spelen in de pathogenese en behandeling van migraine. Verschillende studies hebben aangetoond dat de therapeutische waarde van een specifieke groep acuut werkende antimigraine medicijnen (voornamelijk triptanen, zoals sumatriptan) voornamelijk bepaald wordt door een directe werking op subtypen van de 5-HT_1 receptor sub-familie. Sinds de laatste jaren wordt er veel onderzoek gedaan om uit te zoeken welke van deze subtypen (5-HT_{1B}, 5-HT_{1D} en/of 5-ht_{1F} receptoren) verantwoordelijk is voor de therapeutische waarde, of voor de ongewilde bijwerkingen, van deze speciale klasse van antimigraine middelen. Naast de acute behandeling van migraine (bijvoorbeeld met de triptanen), is er mogelijkerwijs ook een belangrijke rol weggelegd voor 5-HT_7 receptoren in de profylactische behandeling van deze aandoening. Het is belangrijk om te vermelden dat constrictie van arterioveneuze anastomoses in het vaatbed van de arteria carotis (halsslagader) van genarcotiseerde varkens een voorspellend model is voor antimigraine activiteit. Zoals beschreven in onderstaande paragrafen, hebben we de 5-HT_{1B}, 5-HT_{1D} en 5-ht_{1F} receptoren gekloneerd, farmacologisch gekarakteriseerd in cellen van varkens en gelokaliseerd in weefsels van het varken van de mens. Voor een beter begrip is het nodig om te vermelden dat hoofdletters (5-HT) worden gebruikt om die receptoren aan te duiden waarvan de moleculaire structuur en farmacologische eigenschappen (affiniteit en activiteit) volledig bekend zijn (bijvoorbeeld 5-HT_{1B} receptoren) en kleine letters (5-ht) daarentegen worden gebruikt voor die receptoren die nog niet volledig farmacologisch zijn gekarakteriseerd (bijvoorbeeld 5-ht_{1F} receptoren).

De huidige resultaten bieden belangrijke informatie voor een betere ontwikkeling van verschillende stoffen die selectiever op deze receptoren werken, waarmee een belangrijke stap kan worden gezet voor betere migraine therapie in de nabije toekomst.

Hoofdstuk 1 geeft een overzicht over de huidige kennis van 5-HT receptoren alsmede de beweegredenen voor de studies, die beschreven zijn in de komende hoofdstukken. Omdat men nog steeds in het duister tast over de onderliggende biologische processen

die verantwoordelijk zijn voor het ontstaan van een migraine aanval, worden de huidige theorieën en de mogelijke werkingsmechanismen van enkele belangrijke antimigraine middelen in het kort beschreven. Zoals reeds vermeld, is er een aantal receptoren (5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} en 5-HT_7) dat mogelijkerwijs als aanknopingspunt fungeert voor antimigraine middelen. In de **hoofdstukken 2,3,4, en 5** beschrijven we de isolatie (klonering) en farmacologische karakterisering van deze receptoren in het varken en vergelijken deze met de moleculaire en farmacologische aspecten die beschreven zijn voor andere species, voornamelijk die in de mens (humaan). In de **hoofdstukken 2**, **3** en **4** wordt respectievelijk de 5-HT_{1B}, 5-HT_{1D} en 5-ht_{1F} receptor behandeld. Hoewel de moleculaire structuren van deze receptoren overeenkomstig zijn met die van humane receptoren (hoge mate van homologie), zijn enige specifieke aminozuren verschillend in sommige receptoren; deze verschillen kunnen grote gevolgen hebben voor de affiniteit van stoffen en de activiteit van de receptor. Bijvoorbeeld, in de varkens 5-HT_{1B} receptor (**Hoofdstuk 2**), bevindt het aminozuur asparagine zich in positie 355 (in het zevende transmembraan domein) zich bevindt in de muis en rat, terwijl er op deze positie in het varken, konijn, cavia en de mens het aminozuur threonine wordt gevonden. In tegenstelling tot de 5-HT_{1B} receptor in de rat, hebben we aangetoond dat verschillende stoffen een verwachte affiniteit (interactie met receptor) vertonen en een activatie van de receptor veroorzaken in varkens, vergelijkbaar met die beschreven in de mens. Ondanks de verwachte verschillen in receptorstructuur met die van de varkens 5-HT_{1B} receptor (een homologie van 61%), hebben we vergelijkbare resultaten voor de varkens 5-HT_{1D} (**Hoofdstuk 3**) en 5-ht_{1F} receptor (**Hoofdstuk 4**). Een interessante bevinding omtrent de varkens 5-ht_{1F} receptor is dat we op positie 333 het aminozuur threonine hebben gevonden in tegenstelling tot het aminozuur alanine in deze receptor in andere species (bijvoorbeeld de mens). Tevens waren de farmacologische profielen (directe en indirecte binding) van deze receptoren vergelijkbaar met die van humane receptoren. Om uit te zoeken welk 5-HT_1 receptor subtype (5-HT_{1B} en/of 5-HT_{1D}) betrokken is bij de vasculaire effecten (samentrekking van bloedvaten) effecten van de 5-$HT_{1B/1D}$ receptor agonist sumatriptan, hebben we met behulp van in situ hybridisatie het receptor messenger RNA (mRNA) gelokaliseerd in verschillende humane bloedvaten (coronair arterie, arteria meningea media en vena saphena). Deze lokalisatie hebben we getracht te correleren aan de effecten van selectieve 5-HT_{1B} (SB224289) en

5-HT_{1D} (BRL15572) receptor antagonisten op door sumatriptan geïnduceerde samentrekking van deze bloedvaten (zie **Hoofdstuk 6**). In alle bloedvaten werd het effect van sumatriptan alleen geblokkeerd door SB224289, wat aanduidt dat voornamelijk 5-HT_{1B} receptoren deze vasculaire effecten van sumatriptan mediëren; tevens is er aangetoond dat er ook andere receptoren meespelen in het geval van de coronair arterie. Daar de 5-HT_{1B}, 5-HT_{1D} en 5-ht_F receptoren in zowel centrale als perifere weefsels van varkens voorkomen, is het gebaseerd op deze resultaten moeilijk om een eenduidig antwoord te geven op de vraag welk 5-HT_1 receptor subtype de antimigraine effecten van sumatriptan mediëert.

In **Hoofdstuk 5** wordt de klonering van 5-HT_7 receptoren uit de cerebrale cortex van varkens beschreven. Ondanks het feit dat de onderliggende mechanismen nog niet volledig bekend zijn, produceert stimulatie van deze receptor een tal van biologische effecten, zoals bijvoorbeeld relaxatie van gladde spieren in verschillende weefsels, waaronder bloedvaten. Daar er wordt gedacht dat verwijding van craniële bloedvaten een belangrijke rol speelt in de pathofysiologie van een migraine aanval, zou een 5-HT_7 receptor antagonist effectief kunnen zijn in de profylactische behandeling van deze aandoening. We hebben in dit hoofdstuk laten zien dat de moleculaire structuur van de varkens 5-HT_7 receptor volledig (100%) is gecorreleerd aan die van de humane 5-HT_7 receptor. We hebben, weliswaar, nog niet het farmacologische profiel van deze receptor onderzocht. De beschreven verdeling van deze receptor in verschillende weefsels, zowel centraal als perifeer, geeft belangrijke informatie omtrent de mogelijke rol van de 5-HT_7 receptor in de behandeling van vasculaire aandoeningen zoals migraine. Eventuele resultaten van potente en selectieve 5-HT_7 receptor antagonisten in de behandeling van migraine worden met veel interesse tegemoet gezien.

In **Hoofdstuk 7** worden de resultaten van afgelopen hoofdstukken dichter onder de loep genomen en nauw met elkaar in verband gebracht. Tevens worden de tekortkomingen van de methoden en mogelijke (toekomstige) implicaties van de huidige resultaten beschreven.

In het kort samenvattend, hebben we in de huidige studie laten zien dat de moleculair structuren van de varkens 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1F} and 5-HT_7 receptoren een hoge mate van homologie vertonen met die in andere species, waaronder de mens.

Hiermee nauw samenhangend, hebben we aangetoond dat de farmacologische profielen van zowel de 5-HT_{1B} and 5-HT_{1D}, alsmede van de 5-ht_{1F} receptoren een hoge correlatie vertonen met die van de humane receptor. Naast deze observatie, zijn de drie meest interessante bevindingen dat: (i) de 5-HT_{1D} receptor antagonist BRL15572 een veel lagere affiniteit vertoonde in varkens in vergelijking met die in de mens; (ii) de varkens 5-ht_{1F} receptor het aminozuur threonine op positie 333 bevat, in tegenstelling tot alanine in andere species, dit kan belangrijke gevolgen hebben op het farmacologische profiel van de receptor; en (iii) de lage affiniteit van triptanen (acuut werkende antimigraine middelen) voor de varkens 5-ht_{1F} receptor.

Appendix

Acknowledgement

My gratitude to Prof. P.R. Saxena, my promotor for giving me the opportunity and support to work in the field of molecular pharmacology. Thank you for giving me the chance to interact in an international atmosphere, teaching me the minute points in the manuscripts and how to improve the text. Your personal support has been highly appreciating, especially during my initial days when I was first time away from my family.

I am equally thankful to Dr. H.S. Sharma for teaching me the molecular biology tools that helps me to characterise these receptors. Your personal support has been highly appreciating and facilitated me to learn how to organise my own things.

I would like to convey my sincere thanks to Prof. Dr. J.M.J. Lamers, Prof. Dr. C.I. de Zeeuw and Prof. Dr. R. Leurs for critictically reading my thesis.

The collaboration with Dr. P.J. Pauwels and Dr. T. Wurch from Pierre Fabre, France on ligand binding studies has been very fruitful and I thank you both for your support.

I also would like to extend my gratitude to Prof. Dr. P.C. Misra of Lucknow University who taught me the basics of biochemistry and its application in research.

Edwin, thank you for your co-operation and help particularly during the last phase of my thesis writing as well as for the nice times during the conferences, etc. I wish you all the best for the forthcoming postdoctoral period in Sydney, Australia.

My thanks go to all my colleagues (Sue, Marion, Mechteld, Erik, Emine, Sherif, Anna Andor, Antoinette, Peter, Beryl, Remon, Kapil, Jasper, Martin, Roeland and Uday) for their help and cooperation during this period of stay.

Jan (Heiligers), thank you for the co-operation to provide me the fresh pig tissues. The secretarial assistance of Mrs Magda Busscher-Lauw is also acknowledged.

I heartily thank to my father, Dr. T.N. Bhalla, who is the constant source of inspiration for me to work hard and achieve the goal and to my mother whose love and concern is difficult to express in words.

I am indebted to my wife (Savita) and sons (Ambuj and Aditya), without their moral support and cooperation I would have not been able to complete this thesis.

Lastly, my thanks are to all those people who have contributed to the completion of this thesis.

About the author

The author of this thesis was born at Lucknow, India on 23rd July 1966. He graduated in Chemistry, Botany and Zoology (1987) from Lucknow University and obtained his master's degree in Biochemistry (1989) from the same University. He initiated his research career by doing M.Phil in Biochemistry (1990) and later obtained his Ph.D. degree in Biochemistry (1996) on the work entitled "Relationship between trans-plasma membrane electron transport and nitrate reductase activity in Chick Pea". Thereafter, he switched to the field of molecular pharmacology and initiated another project entitled "Cloning and pharmacology of porcine serotonin receptors relevant to the study of antimigraine drugs" under the supervision of Prof. Dr. P.R. Saxena and Dr. H.S. Sharma. During this period, he also had the opportunity to work in the laboratory of Dr. P.J. Pauwels (Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, Castres Cedex, France) for the pharmacological and functional characterisation of these receptors.

Publications

Full Papers

Lawrence, K., Bhalla, P. and Misra, P.C. Changes in NAD(D)H-Dependent redox activities in plasmalemma enriched vesicles isolated from boron-deficient and zinc deficient chick pea roots. J. Plant Physiol. 1995; 146, 652-657.

Lawrence, K., Bhalla, P. and Misra, P.C. NADH-Dependent redox activities on the external face of plasma membrane vesciles of chick pea roots. J. Plant Physiol. 1995; 146, 763-765.

Bhalla, P., Gupta S.K., Lawrence, K., and Misra, P.C. Plasma membrane bound ferrocyanide oxidase activity in roots of chick pea (*Cicer arietinum*) seedlings. Ind. J. Exp. Biol. 1997; 35, 1229-1232.

Bhalla, P and Agrawal, D. Alterations in rat erythrocyte membrane due to hexachlorohexane (technical) exposure. Hum. Exp. Toxicol. 1998; 17, 638-642.

Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. and Saxena, P.R.. Molecular cloning, sequence analysis and pharmacological properties of the porcine 5-HT_{1D} receptor. Br. J. Pharmacol., 2000; 131, 949-957.

Bhalla, P., Sharma, H.S., Xiaqian, M., Wurch, T., Pauwels, P.J. and Saxena, P.R. Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT_{1B} receptor. Br. J. Pharmacol., 2001; 133, 891-901.

Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. and Saxena, P.R. Molecular Cloning and pharmacological characterisation of the porcine trigeminal ganglion cDNA encoding a 5-ht_{1F} receptor. Submitted.

Bhalla, P., Saxena, P.R and Sharma, H.S. Molecular Cloning and tissue distribution of the porcine 5-HT_7 receptor. Submitted.

Van den Broek, R., Bhalla, P., Maassenvandenbrink, M., Devries,R., Sharma,H.S. and Saxena, P.R. Characterisation of sumatriptan-induced contractions in human isolated blood vessels using selective 5-HT_{1B} and 5-HT_{1D} receptor antagonists and *in situ* hybridisation. Cephalalgia, In Press.

Book Chapter

Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. and Saxena, P.R.. Molecular cloning and pharmacological characterisation of porcine 5-HT_{1B} and 5-HT_{1D} receptor. In: Keyser A, MacGregor (eds) Headache and Migraine. Medeiagroep KUN/AZN, Nijmegen, 2000 Submitted

Abstracts published

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Structural and pharmacological characterization of cloned porcine 5-HT_{1D} receptor. Fund. Clin. Pharmacol., 1999; 13, 124.

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Molecular cloning and pharmacological characterisation of porcine 5-HT_{1D} receptor. Br. J. Pharmacol. 2000; 129, 189P.

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Molecular cloning and pharmacological characterisation of porcine 5-HT_{1B} receptor. Fund. Clin. Pharmacol., 2000; 14, 45.

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Molecular cloning and pharmacological characterisation of porcine 5-HT_{1B} and 5-HT_{1D} receptors. AngloDutch Migraine Association; 10th Annual meeting, Scotland, 2000.

Bhalla P: Cloning van relevante receptoren voor onderzoek naar antimigraine medicatie. 2nd Symposium over Hoofdpijnonderzoek, Zeist, The Netherlands, 2000; Abstract No. 8.

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Recombinant porcine 5-HT_1 receptors as tools to screen antimigraine drugs. Drug Discovery, Sandwich, UK 2000.

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Molecular cloning, sequencing and pharmacological characterisation of porcine 5-HT_1 receptor subtypes: Implication for screening antimigraine drugs. 10th International Headache Research Symposium, Copenhagen, Denmark 2000; Abstract No. S1.3

Van den Broek R.W., Bhalla P., Maassen vandenbrink, A., de Vreis R., Sharma H.S. and Saxena P.R.: Role of the 5-HT_{1B} and 5-HT_{1D} receptors in human isolated blood vessels important in migraine therapy. 10th International Headache Research Symposium, Copenhagen, Denmark, 2000; Abstract No. S2.11

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Molecular cloning and pharmacological characterisation of porcine trigeminal ganglion cDNA encoding 5-ht_{1F} receptor. Fund. Clin. Pharmacol., 2001; In Press.

Bhalla P., Sharma H.S., Wurch T., Pauwels P.J. and Saxena P.R.: Recombinant porcine 5-HT_1 receptor subtypes: Structural and pharmacological profile in relation to human 5-HT_1 receptor subtypes. International Headache Congress, June 28-July 3, New York, U.S.A. 2001; P1-H34.

Van den Broek R.W., Bhalla P., Maassenvandenbrink, A., de Vreis R., Sharma H.S. and Saxena P.R: Characterisation of sumatriptan-induced contraction in human isolated blood vessels using selective 5-HT_{1B} and 5-HT_{1D} receptor antagonists and *in situ* hybridisation. International Headache Congress, June 28-July 3, New York, U.S.A. 2001; P1-H18.

List of abbreviations

5-CT	: 5-carboxamidotriptamine
^{3}H	: Tritium
5-HT	: 5-Hydroxytryptamine
AMV	: Avian Myeloblastosis virus
AVA	: Arteriovenous anastomoses
BCIP	: 5-bromo-4-chloro-3-indolylphospate
bp	: base pair (s)
BRL15572	: [1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl)piperazine] hydrochloride
BSA	: Bovine serum albumin
cDNA	: complementary deoxyribonucleic acid
CGRP	: Calcitonin gene related peptide
CHO	: Chinese hamster ovary
CP122638	: N-methyl-3-[pyrrolidin-2(R)-ylmethyl]-1H-indol-5-ylmethyl sulphonamide
DNase	: Deoxyribonuclease
dNTP	: deoxynucleotide triphosphate
DTT	: Dithiothreitol
EC_{50}	: concentration of an agonist eliciting half the maximal effect
E_{max}	: maximal effect
GDP	: Guanosine diphosphate
GR125743	: N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide
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
GTP	: Guanosine triphosphate
HCA	: Human isolated coronary artery
HEPES	: (N-[2-Hydroxyethyl]piperazine-N'-[4-butanesulphonic acid])
HMA	: Human isolated middle meningeal artery
HSV	: Human isolated saphenous vein
IPTG	: β-D-isopropyl-thiogalactopyranoside
L694247	: 2-[5-[3-(4-methylsulphonylamino) benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine
mRNA	: messenger ribonucleic acid
NBT	: Nitro-blue tetrazolium
PCR	: Polymerase chain reaction
Rnase	: ribonuclease
RT-PCR	: Reverse transcription- polymerase chain reaction
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
SDS	: Sodium dodecyl sulphate
UV	: Ultraviolet
X-Gal	: 5-bromo-4-chloro-3-indolyl-β-galactopyranoside