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Cloning and pharmacological characterisation of the guinea pig 5-ht$_{5A}$ receptor

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Abstract

The guinea pig 5-hydroxytryptamine$_{5A}$ (gp5-ht$_{5A}$) receptor was cloned from guinea pig brain using degenerate polymerase chain reaction (PCR) and shows 88%, 85% and 84% amino acid sequence identity versus the human, rat and mouse 5-ht$_{5A}$ receptors, respectively. The receptor was transiently expressed in human embryonic kidney (HEK) 293 cells. [^{3}H]-Lysergic acid diethylamide (LSD) bound saturably to gp5-ht$_{5A}$/HEK293 membranes with a K_d of 2.3 ± 0.1 nM and B_{max} of 15.7 ± 3.4 pmol/mg protein. The receptor binding profile, determined by competition with [^{3}H]LSD, correlated well with that for the human 5-ht$_{5A}$ receptor. 5-HT stimulated [^{35}S]GTPγS binding to gp5-ht$_{5A}$/HEK293 membranes (pEC$_{50}$ 8.1 ± 0.2), and the response was surmountably antagonised by methiothepin and ritanserin, giving apparent pK_B values of 8.0 and 7.2, respectively. The 5-HT response was absent using membranes prepared from gp5-ht$_{5A}$/HEK293 cells pretreated with pertussis toxin (PTX). These data suggest that the gp5-ht$_{5A}$ receptor couples to G$_i$-proteins in this expression system and shows a similar pharmacological profile to that for the human 5-ht$_{5A}$ receptor.

Keywords: 5-ht$_{5A}$ Receptor; Guinea pig; Cloning; Transient expression

1. Introduction

5-Hydroxytryptamine (5-HT) receptors have been subdivided into seven major classes (5-HT$_{1-7}$), based on structural, functional and pharmacological criteria (Hoyer et al., 1994), and a total of 14 different genes encoding structurally distinct 5-HT subtypes have been identified (Barnes and Sharp, 1999). For the 5-ht$_5$ receptor class, two subtypes, 5-ht$_{5A}$ and 5-ht$_{5B}$, have been identified in the mouse (Plassat et al., 1992; Matthes et al., 1993) and rat (Erlander et al., 1993; Wisden et al., 1993). These paralogues share 71% and 73% sequence identity in the mouse and rat, respectively. Subsequently the human 5-ht$_{5A}$ receptor was cloned (Rees et al., 1994), and a number of studies have reported the human receptor couples negatively (via G$_i$) to adenylyl cyclase when stably expressed in human embryonic kidney (HEK) 293 cells. In contrast, the human 5-ht$_{5B}$ receptor gene has been reported to be a pseudogene and the human 5-ht$_{5B}$ receptor is not functionally expressed (Grailhe et al., 2001).

mRNA and protein localisation studies in a number of species including rat (Oliver et al., 2000) and human (Pasqualetti et al., 1998) have shown that the 5-ht$_{5A}$ receptor is localised predominantly in brain although localisation in the carotid body chemoreception pathway has also been reported (Wang et al., 2000). In brain, the receptor is widely localised in regions implicated in higher brain function, including cortical and limbic areas such as the hippocampus and amygdala (Oliver et al., 2000). Localisation in these regions appears to be mainly neuronal, for example, on pyramidal cells in cerebral cortex and hippocampus in the rat (Oliver et al., 2000). The receptor has also been reported to be localised on ascending 5-HT neurones in the dorsal

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and median raphe of hamster (Duncan et al., 2000). This raphe localisation pattern suggests a potential autoreceptor function for the 5-ht$_{5A}$ receptor to control raphe 5-HT neuronal activity.

The receptor has also been reported to be discretely localised in the suprachiasmatic nucleus of the hypothalamus (Oliver et al., 2000) potentially implicating the receptor in circadian rhythm and sleep control. In spite of such localisation studies, evidence for a role for the receptor in brain function is lacking, and these studies have been hindered by a lack of 5-ht$_{5A}$ receptor-selective ligands. 5-ht$_{5A}$ receptor homozygous knockout mice have been reported to show increased exploratory behaviour in a novel environment, compared to wild type animals (Grailhe et al., 1999), suggesting a possible role for the receptor in the control of exploratory behaviour. The 5-ht$_{5A}$ receptor has been reported to show a similar pharmacological profile across human, mouse and rat species, although minor differences have been noted. For example, methiothepin has been reported to show a higher affinity for the human compared to the mouse 5-ht$_{5A}$ receptor (Rees et al., 1994). In the present study, we report the cloning and pharmacological characterisation of the guinea pig 5-ht$_{5A}$ receptor and compare the profile in receptor binding studies with that for the 5-ht$_{5A}$ receptor from mouse, rat and human.

2. Materials and methods

2.1. Cloning of the 5-ht$_{5A}$ receptor from guinea pig and other species

Poly-A RNA was purified from guinea pig brain, dorsal root ganglion and testis using the FastTrack RNA isolation kit (Invitrogen, Paisley, UK) and cDNA prepared using SuperScript II reverse transcriptase (Invitrogen) and the SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (BD Clontech, Oxford, UK). Degenerate polymerase chain reaction (PCR) primers were designed based on highly conserved regions of human, mouse, and rat 5-ht$_{5A}$ (forward 5′-TGGTGCTGGC(C/T/G) ACCATCCTC-3′, reverse 5′-GC(A/C)GCCTTGTA(G/A)AT(T/C) TTCCAGTA(T/C)AC-3′) and used in PCR to amplify a 460-bp fragment of the 5-ht$_{5A}$ gene from guinea pig genomic DNA. The remainder of the 1071-bp coding region was determined by 5′ and 3′ RACE using primers based on sequence data obtained from the degenerate PCR fragment, paired with the SMART RACE cDNA amplification kit primers, in nested PCR according to the manufacturer's protocol (BD Clontech). To make a full-length expression clone, a 1133-bp fragment of guinea pig 5-ht$_{5A}$ cDNA containing the complete coding region was amplified from brain cDNA using the proof-reading polymerase *pfu*turbo (Stratagene, Cambridge, UK) and the following primers: forward 5′-CACCATGGATCTGCTTGTGAACTTC-3′, reverse forward 5′-AGGGTGCAGAGTCCAAACTTC-3′. PCR products were purified from agarose gels using a Minelute kit (Qiagen, Hilden, Germany) and subcloned into either pCR2.1-TOPO vector (Invitrogen) or, for the expression clone of the complete coding region, into pcDNA3.1D/v5/His-TOPO vector (Invitrogen). Plasmid DNA was prepared using a Qiagen miniprep kit (Qiagen). Clones were sequenced on both strands from standard vector primers and, where necessary, internal gene-specific primers, using an ABI automated sequencer. Sequences were assembled with a software package, Seqman (DNASTAR, Madison, WI), and the alignments optimised manually to give an overall consensus. Clones from independent amplifications were compared to rule out PCR errors. Human, mouse and rat receptors were cloned by PCR with PfuTurbo DNA polymerase from respective brain cDNAs, using primers designed according to published sequences for human (Rees et al., 1994; forward primer: 5′-ACCATGGATTTACCAG TCAACCTAACC -3′; reverse primer: - 5′-GATCAGTGTTGCCTAGAAAA GAAGTTC-3′), mouse (Plassat et al., 1992; forward primer: 5′-CATTGGC CATCGGTCGCAAAC -3′; reverse primer: 5′ GGCAGCCTGCAAAGGTTC -3′) and rat (Erlander et al., 1993; forward primer 5′-ACCATGGATCT GCCTATAAACTTG -3′; reverse primer 5′-ATGTGGCCTCTCACTGCTG -3′).

2.2. Expression of the guinea pig, mouse, rat and human 5-ht$_{5A}$ receptors

For transient expression of the guinea pig, mouse or rat 5-ht$_{5A}$ receptor, HEK 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (FBS) and 1% non-essential amino acids. Cells were grown in 80-cm^2 flasks to approximately 90% confluence and transient expression was carried out by incubating with the guinea pig, mouse or rat 5-ht$_{5A}$/pcDNA3.1 vector (15 μg DNA per flask) using LIPOFECTAMINE PLUS™ reagent as per the protocol supplied by the manufacturer (Invitrogen). Cells were harvested 48 h following transfection. In some experiments, cells were treated with pertussis toxin (PTX) (100 ng/ml) for 18 h prior to harvesting. Untreated cells were grown in parallel. The human 5-ht$_{5A}$ receptor was stably expressed in Chinese hamster ovary (CHO-DG44) cells grown in alpha modified Eagle's medium (MEM) supplemented with 5% FBS and 0.8 mg/ml Geneticin. Transfection of pcDNA 3.1/human 5ht$_{5A}$-V5-His-Topo into CHO-DG44 cells was performed using LIPOFECTAMINE PLUS™ reagent as per the protocol supplied by the manufacturer. Selection against geneticin sulphate commenced 48 h post-transfection producing a stable mass culture. Single-cell clones were selected by dilution cloning. A high expression stable cell line (human 5-ht$_{5A}$/CHO-DG44) was selected on the basis of mRNA level by quantitative analysis by TAQMAN followed by saturation analysis using [^{3}H]-lysergic acid diethylamide (LSD) binding (see Table 1).

2.3. Radioligand binding

HEK 293 cells transiently expressing the guinea pig, rat or mouse 5-ht$_{5A}$ receptor or CHO cells stably expressing the human 5-ht$_{5A}$ receptor were harvested 48 h following transfection, washed twice by centrifugation and resuspension in phosphate buffered saline and the final pellet frozen (− 80 °C). For preparation of membranes, cell pellets were washed by homogenisation (Polytron, 15 s, setting 5) and centrifugation (50,000 × g, 15 min, 4 °C) in 20 volumes of Tris–HCl (25 mM pH 7.4) containing EDTA (0.1 mM). Membranes were then resuspended in buffer and incubated (37 °C, 20 min). Following centrifugation and a further two washing steps at 4 °C, the membranes were finally resuspended at a membrane concentration equivalent to 2×10^7 cells/ml (∼ 1 mg protein/ml) and stored at − 80 °C prior to use.

Membranes (10–50 μg protein/well) were incubated in Tris–HCl buffer (50 mM, pH 7.4 at 37 °C) containing $MgCl_2$ (10 mM), EDTA (0.1 mM) and ascorbic acid (0.5 mM). For saturation experiments, guinea pig 5-ht$_{5A}$/HEK293 membranes were incubated (60 min, 37 °C) with eight concentrations of [^{3}H]LSD (0.1–10 nM) or [^{3}H]-5-carboxamidotryptamine (5-CT) (0.3–25 nM). For competition studies, membranes were incubated (60 min, 37 °C) with 1–2 nM [^{3}H]LSD in the absence or presence of test compounds. For both saturation and competition experiments, non-specific binding was defined in the presence of 100 μM 5-HT. Incubation was stopped by rapid filtration through Whatman GF/B grade filters (pre-soaked with 0.3% polyethyleneimine) followed by 5 × 1 ml ice-cold buffer washes. Bound radioactivity was determined by liquid scintillation counting.

2.4. [^{35}S]GTPγS binding to guinea pig 5-ht$_{5A}$/HEK293 membranes

HEK 293 cells transiently expressing the guinea pig 5-ht$_{5A}$ receptor were harvested 48 h following transfection, washed twice by centrifugation and resuspension in phosphate-buffered saline and the final pellet homogenised (Ultra-Turrax, 20,500 rpm for 20 s) in 30 volumes of 20 mM HEPES/10 mM EDTA buffer (pH 7.4, 4 °C) and centrifuged (50,000 × g, 15 min, 4 °C). The resultant pellet was resuspended in 30 volumes of 20 mM HEPES/0.1 mM EDTA buffer and recentrifuged. Finally, the membranes were resuspended in 10 volumes of 20 mM HEPES/0.1 mM EDTA buffer and stored at − 80 °C prior to use.

[^{35}S]GTPγS binding was carried out essentially as described by Thomas et al. (1995) with some minor modifications. Membranes (25–50 μg protein), expressing guinea pig or human 5-ht$_{5A}$ receptors, were initially preincubated on ice (10 min) in 20 mM HEPES buffer (pH 7.4) in the presence of 0.3 μM GDP and then at 30 °C for 30 min in the presence of 0.3 μM GDP, 5 mM $MgCl_2$, 75 mM NaCl, 0.2 mM ascorbate and in the presence or absence of test drugs. The incubation was started by addition of 50 μl of [^{35}S]GTPγS (300 pM) followed by mixing using an incubator/shaker (Wesbart IS89, 30 s, maximum shaker setting at 30 °C). The total assay volume was 500 μl. Incubated was carried out for 30 min at 30 °C and the reaction stopped by rapid filtration using Whatman GF/B grade filters followed by five 1-ml washes with ice-cold 20 mM HEPES/3 mM $MgCl_2$ buffer (Brandel 48 place cell harvester). All determinations were performed in triplicate. Non-specific binding was determined in the presence of 10 μM GTPγS. Radioactivity on the filters was counted using liquid scintillation spectrometry.

2.5. Data analysis

Analysis of saturation binding data was performed by non-linear curve fitting (Thomas et al., 2000) using KELL (Biosoft). This curve-fitting procedure provided estimates for K_d (equilibrium dissociation constant) and B_{max} (maximal binding density) for each radioligand. The concentration of drug inhibiting specific [^{3}H]LSD binding by 50% (IC_{50}) and Hill coefficient (n_H) was determined by iterative curve fitting (Bowen and Jerman, 1995). pK_i values (− log of the inhibition constant) were then calculated from the IC_{50} values (Cheng and Prusoff, 1973). Drug concentration–response curves from [^{35}S]GTPγS binding assays were fitted to a four-parameter logistic equation (GraphPad Prism, GraphPad Software). Agonist potency was expressed as the pEC_{50} (− log EC_{50}). Apparent pK_B values (− $\log_{10}$ of the antagonist equilibrium dissociation constant) for antagonism were determined using the equation: pK_B=(− log ([antagonist]/(concentration ratio − 1))) where concentration ratio = ratio of the agonist EC_{50} values in the presence and absence of antagonist. Data represent the mean ± S.E.M. of at least three separate experiments each performed using triplicate determinations.

2.6. Drugs

5-Hydroxytryptamine HCl (5-HT), 5-carboxamidotryptamine (5-CT), 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), lysergic acid diethylamide (LSD), methiothepin mesylate, clozapine, ritanserin, pindolol, *n*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*n*-2-pyridinylcyclohexanecarboxamide maleate (WAY-100635) and pertussis toxin were obtained from Sigma-Aldrich (Poole, UK). Ergotamine tartrate was obtained from Tocris Cookson, Avonmouth, UK. 1′-Methyl-5-([2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-biphenyl-4-yl]carbonyl)-2,3,6,7-tetrahydrospiro(furo[2,3-*f*]indole-3,4′-piperidine) (SB-224289), (*R*)-3-(2-(2-(4-methyl-piperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol (SB-269970-A) and *N*-[4-methoxy-3-(4-methyl-1-piperazinyl) were synthesised at GlaxoSmithKline (Harlow, UK). [^{3}H]LSD and [^{3}H]5-CT were obtained from Amersham (UK). Stock drug solutions were prepared in dimethylsulphoxide (DMSO) (the final assay concentration of

DMSO not exceeding 0.4%). Subsequent drug dilutions were prepared using 0.5 mM ascorbic acid.

3. Results

3.1. Cloning of the guinea pig 5-ht$_{5A}$ receptor

A 460-bp fragment of 5-ht$_{5A}$ exon 1 DNA was isolated from guinea pig genomic DNA by degenerate PCR, using primers derived from published mammalian 5-ht$_{5A}$ nucleotide sequences: human (Rees et al., 1994), rat (Erlander et al., 1993) and mouse (Plassat et al., 1992). Primers derived from the fragment were then used to generate 5′ and 3′ RACE clones from guinea pig brain, dorsal root ganglion and testis cDNAs. In composite, the clones contained a 2059-bp cDNA sequence which included a 1071-bp open reading frame and terminated in a poly-A tail. Comparison with the other mammalian 5-ht$_{5A}$ sequences, and the presence of termination codons in all three reading frames in the 587 nucleotides immediately 5′ to the putative initiation codon, indicated that the entire coding region had been found. The complete 5-ht$_{5A}$ coding region was then amplified from guinea pig brain cDNA. The encoded guinea pig 5-ht$_{5A}$ receptor is a protein of 356 amino acids with seven putative hydrophobic transmembrane domains. A sequence alignment showed that the guinea pig 5-ht$_{5A}$ protein has 88% amino acid identity to human, 85% to rat and 84% to mouse 5-ht$_{5A}$ proteins (Fig. 1). Fig. 2 shows a phylogenetic

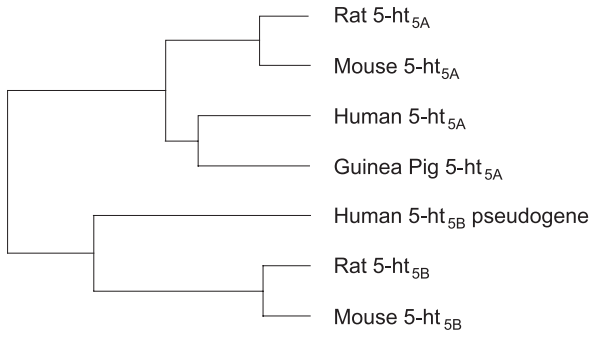

Fig. 2. An average distance tree representing the 5-ht$_{5A}$ and 5-ht$_{5B}$ inter-relationships was generated within Jalview (http://acer.gen.tcd.ie/embnet.news/vol5_4/embnet/body_jalview.html) using UPGMA and a distance matrix generated from percentage pairwise identity of aligned 5-ht$_{5A}$ and 5-ht$_{5B}$ nucleotide sequences from a variety of species. The sequences were aligned using CLUSTALW Higgins et al. (1991). Accession numbers of sequences aligned rat 5-ht$_{5A}$ (L10072), mouse 5-ht$_{5A}$ (Z18278), human 5-ht$_{5A}$ (AF498985), guinea pig 5-ht$_{5A}$ (AJ534845), human 5-ht$_{5B}$ Pseudogene (AJ308679), rat 5-ht$_{5B}$ (L10073) and mouse 5-ht$_{5B}$ (X69867).

tree representing the 5-ht$_{5A}$ and 5-ht$_{5B}$ receptor inter-relationships. This comparison shows that the guinea pig sequence identified in the present study clusters with other 5-ht$_{5A}$ sequences supporting an assignment of this gene as the guinea pig 5-ht$_{5A}$ orthologue. Consistent with this, it shows a lower degree of homology to known 5-ht$_{5B}$ receptor proteins, having 70% amino acid identity to the rat and mouse 5-ht$_{5B}$ receptors. The guinea pig 5-ht$_{5A}$ receptor is more closely related to the human than the

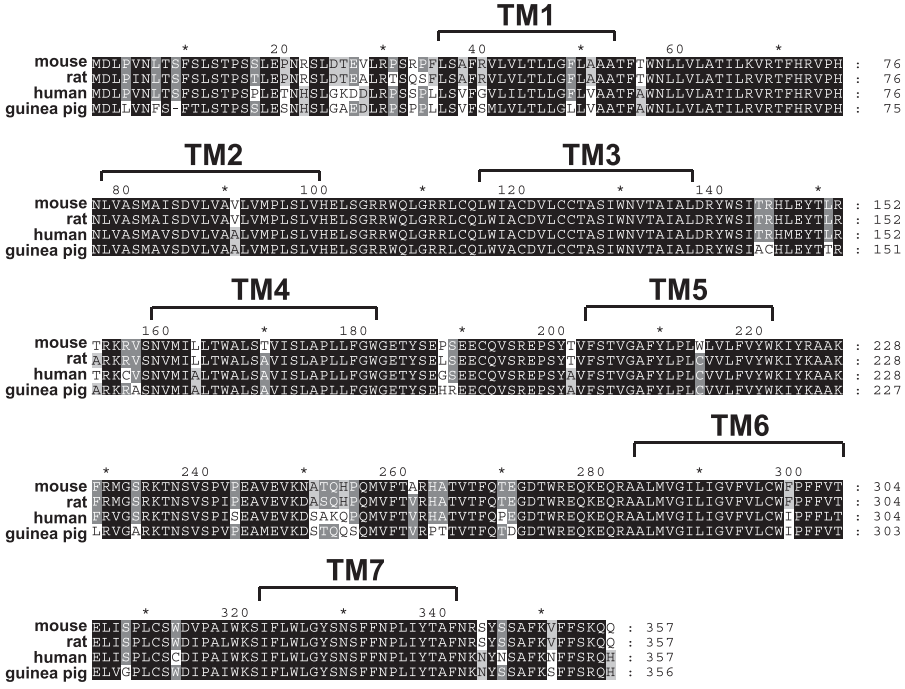

Fig. 1. Amino acid sequence alignment of mouse, rat, human and guinea pig 5-ht$_{5A}$ receptors. The alignment was generated using CLUSTALW version 1.8 (Thompson et al., 1994) and guinea pig, human (P47898), rat (P35364) and mouse (P30966) 5-ht$_{5A}$ protein translations. The transmembrane (TM) domains were predicted using TMPRED (Hoffman and Stoffel, 1993) and human 5-ht$_{5A}$ (P47898) protein sequence.

mouse or rat 5-ht$_{5A}$ sequences. The DNA sequence for the guinea pig 5-ht$_{5A}$ receptor has been submitted to EMBL/GenBank accession number: AJ534845.

3.2. Saturation binding analysis of [^{3}H]LSD and [^{3}H]5-CT binding to guinea pig 5-ht$_{5A}$/HEK293 membranes

[^{3}H]LSD, bound saturably and monophasically to membranes prepared from HEK293 cells transiently expressing the guinea pig 5-hydroxytryptamine$_{5A}$ receptor (gp5-ht$_{5A}$/HEK293 membranes). [^{3}H]LSD displayed a K_d of 2.29 ± 0.05 nM and the maximal binding density (B_{max}) was 15.7 ± 3.40 pmol/mg protein (Table 1). The agonist radioligand, [^{3}H]5-CT, also bound saturably and monophasically to gp5-ht$_{5A}$/HEK293 membranes with a K_d of 9.43 ± 3.01 nM and gave a B_{max} (4.52 ± 0.36 pmol/mg protein) significantly lower ($p < 0.05$, Student's t test) than that defined using [^{3}H]LSD (Table 1). In both cases, statistical analysis of the saturation binding data (using KELL) did not significantly favour a multi-site versus a single-site interaction consistent with both radioligands labelling a single population of binding sites. Figs. 3 and 4 show, respectively [^{3}H]LSD and [^{3}H]5-CT saturation binding data (and in each case Scatchard plots of the same data) from typical experiments which were repeated twice. Saturation binding of [^{3}H]LSD and [^{3}H]5-CT to human 5-ht$_{5A}$/CHO membranes also revealed a higher affinity of [^{3}H]LSD compared [^{3}H]5-CT. However, in contrast to the profile seen for the guinea pig 5-ht$_{5A}$ receptor, the calculated B_{max} was comparable for both radioligands (Table 1). [^{3}H]LSD also bound saturably and monophasically to membranes prepared from HEK293 cells transiently expressing the mouse or rat 5-ht$_{5A}$ receptor and with an affinity comparable to that at the guinea pig and human receptor (data not shown). No specific [^{3}H]-LSD binding was detected using membranes prepared from wild-type (non-transfected) HEK293 cells.

Table 1
K_d and B_{max} values from saturation analysis of [^{3}H]LSD and [^{3}H]5-CT binding to guinea pig 5-ht$_{5A}$/HEK293 and human 5-ht$_{5A}$/CHO cell membranes

	K_d (nM)	B_{max} (pmol/mg protein)
Guinea pig		
[^{3}H]LSD	2.29 ± 0.05	15.7 ± 3.40
[^{3}H]5-CT	9.43 ± 3.01	4.52 ± 0.36[a]
Human		
[^{3}H]LSD	1.41 ± 0.21	4.03 ± 0.70
[^{3}H]5-CT	13.0 ± 2.61	4.15 ± 0.86

K_d (equilibrium dissociation constant) and B_{max} (maximal binding density in fmol/mg protein) values were calculated from Scatchard analysis (Bound versus Bound/Free) of saturation plots for [^{3}H]LSD (0.05–10 nM) and [^{3}H]5-CT (0.1 to 30 nM) binding to membranes from HEK293 cells transiently expressing the guinea pig 5-ht$_{5A}$ receptor or CHO cells stable expressing the human 5-ht$_{5A}$ receptor. Data represent the mean ± S.E.M. of at least three separate experiments each performed using duplicate determinations.

[a] B_{max} significantly different from that determined using [^{3}H]LSD ($p < 0.05$, Student's t test).

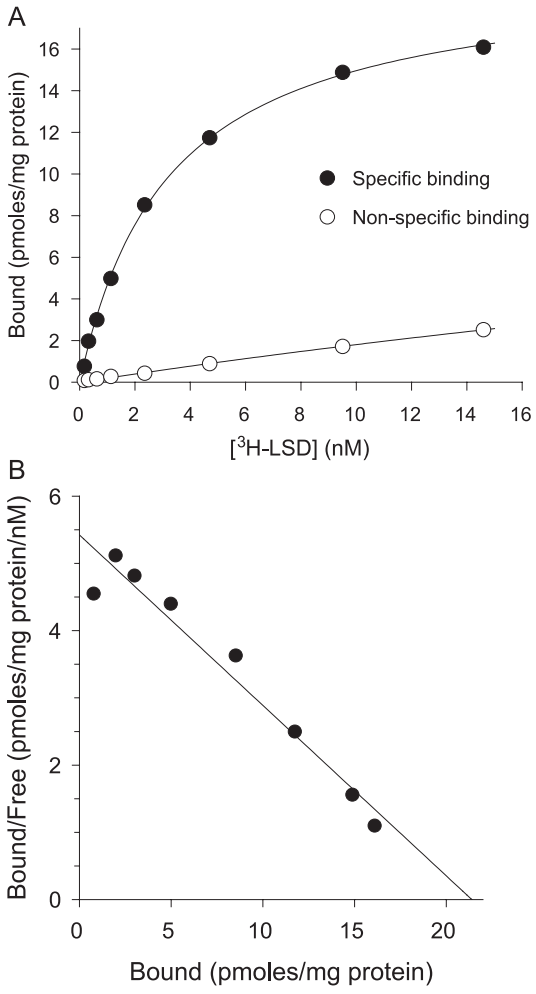

Fig. 3. (A) Saturation analysis of [^{3}H]LSD binding to guinea pig 5-ht$_{5A}$/HEK293 membranes. Data points represent specific binding, calculated by subtracting non-specific binding from total binding, and non-specific binding (defined in the presence of 100 μM 5-HT). Data are from a typical experiment performed using triplicate determinations and repeated twice ($n = 3$). (B) Scatchard plot (Bound in pmol/mg protein versus Bound/Free in pmol/mg protein/nM) of the specific binding data shown in (A).

3.3. Pharmacological characterisation of the guinea pig 5-ht$_{5A}$ receptor

The pharmacological profile of [^{3}H]LSD binding to gp5-ht$_{5A}$/HEK293 membranes was investigated using a range of 5-ht$_{5A}$ receptor agonists and antagonists and compared with the profile for the mouse, rat and human 5-ht$_{5A}$ receptors. Fig. 5 shows representative inhibition curves for displacement of [^{3}H]LSD binding to gp5-ht$_{5A}$/HEK293 membranes. Table 2 shows pK_i values (± S.E.M.) for inhibition of [^{3}H]LSD binding to the 5-ht$_{5A}$ receptor from guinea pig, mouse, rat and human. The test compounds displayed the following rank order of affinity: LSD > ergotamine > methiothepin > 5-CT ≥ 5-HT > ritanserin > SB-

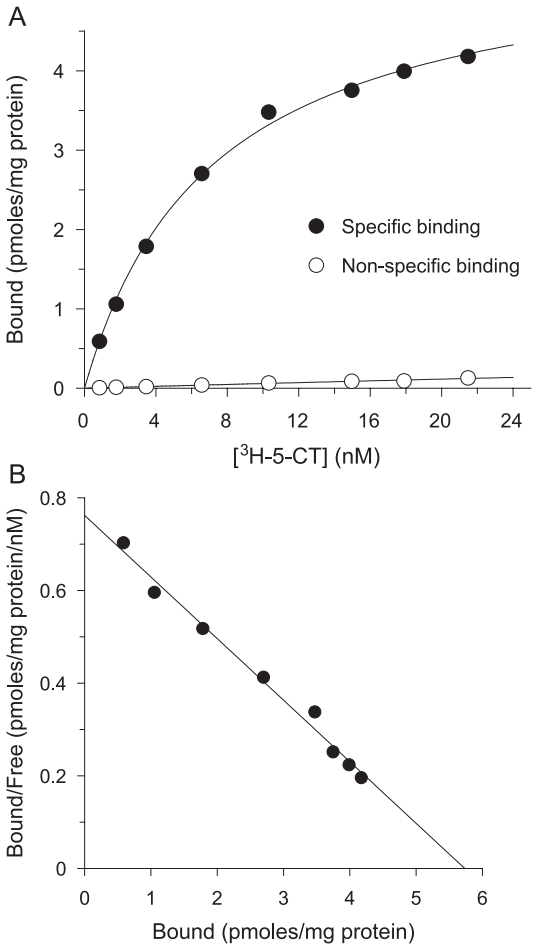

Fig. 4. (A) Saturation analysis of [^{3}H]5-CT binding to guinea pig 5-ht$_{5A}$/HEK293 membranes. Data points represent specific binding, calculated by subtracting non-specific binding from total binding, and non-specific binding (defined in the presence of 100 μM 5-HT). Data are from a typical experiment performed using triplicate determinations and repeated twice (n = 3). (B) Scatchard plot (Bound in pmol/mg protein versus Bound/Free in pmol/mg protein/nM) of the specific binding data shown in (A).

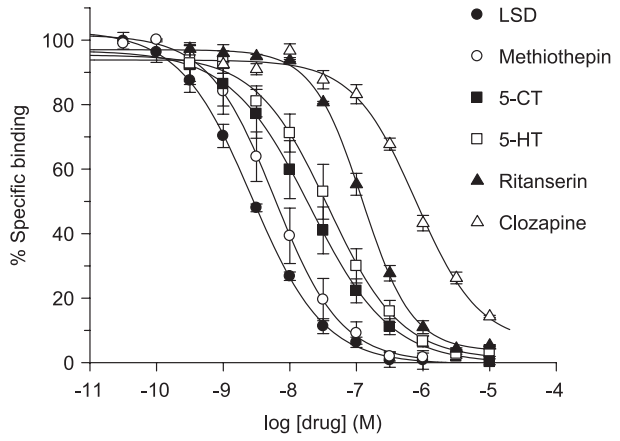

Fig. 5. Inhibition of 1 nM [^{3}H]LSD binding to guinea pig 5-ht$_{5A}$/HEK293 membranes by LSD, methiothepin, 5-CT, 5-HT, ritanserin and clozapine. Data points represent the mean ± S.E.M. of at least three separate experiments each performed using duplicate determinations. Results are expressed as % of specific binding where non-specific binding was defined using 100 μM 5-HT.

269970-A > clozapine ~ metergoline > 8-OH-DPAT > sumatriptan ~ pindolol ~ WAY-100635 ~ SB-224289. The pharmacological profile for the guinea pig receptor was similar to that seen for the human, mouse and rat receptors. Hill slope values (n_H) for compound inhibition of [^{3}H]LSD binding to gp5-ht$_{5A}$/HEK293 membranes were close to unity, ranging from 0.80 ± 0.13 (5-HT) to 1.05 ± 0.06 (8-OH-DPAT) and consistent with displacement of [^{3}H]LSD binding to a single population of binding sites (data not shown).

3.4. [^{35}S]GTPγS binding

5-HT (0.3 nM to 10 μM) produced a concentration-related stimulation of [^{35}S]GTPγS binding to gp5-ht$_{5A}$/HEK293 membranes with a pEC$_{50}$ of 8.1 ± 0.2 and a maximal stimulation of 31 ± 1.5% above basal (Fig. 6). In contrast, 5-HT did not stimulate [^{35}S]GTPγS binding in non-transfected HEK293 cells (data not shown). Non-specific [^{35}S]GTPγS binding to gp5-ht$_{5A}$/HEK293 membranes, defined in the presence of 10 μM GTPγS, was 14 ± 2.0% of total binding. The non-selective 5-ht$_{5A}$ receptor antagonists, methiothepin (1 μM) and ritanserin (10 μM), both produced a surmountable antagonism of the 5-HT-induced response, giving apparent pK_B values of 8.0 ± 0.2 and 7.2 ± 0.2, respectively. At the same concentrations tested, both methiothepin and ritanserin produced a small apparent reduction in basal binding in the absence of added 5-HT (Fig. 6).

In a subsequent study the effect of PTX treatment of HEK293 cells transfected with guinea pig 5-ht$_{5A}$ receptor cDNA was investigated. 5-HT (0.3 μM) stimulated [^{35}S]GTPγS binding to control (non-PTX treated)

Table 2
Compound affinities for cloned 5-ht$_{5A}$ receptors from different species

Compound	pK_i			
	Human	Guinea pig	Rat	Mouse
LSD	8.73 ± 0.13	9.01 ± 0.06	8.61 ± 0.10	9.10 ± 0.10
Ergotamine	8.34 ± 0.17	8.63 ± 0.14	8.40 ± 0.14	8.58 ± 0.10
Methiothepin	8.21 ± 0.21	8.45 ± 0.14	7.11 ± 0.10	8.40 ± 0.10
5-CT	8.04 ± 0.10	7.99 ± 0.21	7.70 ± 0.10	7.81 ± 0.10
5-HT	7.43 ± 0.10	7.75 ± 0.20	7.33 ± 0.03	7.12 ± 0.02
Ritanserin	7.90 ± 0.06	7.21 ± 0.04	7.01 ± 0.10	7.14 ± 0.09
SB-269970-A	7.20 ± 0.10	6.85 ± 0.05	7.81 ± 0.06	7.71 ± 0.06
Clozapine	6.01 ± 0.09	6.35 ± 0.04	5.56 ± 0.02	5.50 ± 0.01
Metergoline	6.20 ± 0.10	6.24 ± 0.12	6.34 ± 0.01	6.35 ± 0.01
8-OH-DPAT	6.07 ± 0.06	5.75 ± 0.08	6.23 ± 0.06	6.13 ± 0.07
Sumatriptan	5.80 ± 0.10	< 5 (2)	< 5 (2)	< 5 (2)
SB-224289	5.30 ± 0.10	< 5 (2)	–	–
Pindolol	< 5 (2)	< 5 (2)	–	–
WAY-100635	< 5 (2)	< 5 (2)	–	–

pK_i (− log inhibition constant) values from [^{3}H]LSD binding experiments. Data represent the mean ± S.E.M. from at least three separate experiments (except where shown (n)) each performed using duplicate determinations; (–) not determined.

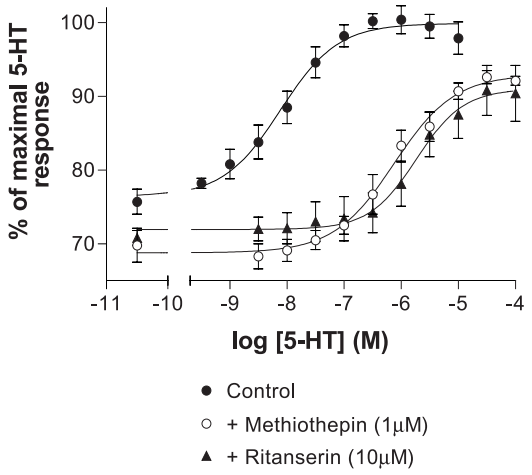

Fig. 6. 5-HT-induced stimulation of (0.3 nM) [^{35}S]GTPγS binding to guinea pig 5-ht$_{5A}$/HEK293 membranes in the absence and presence of methiothepin (1 μM) or ritanserin (10 μM). Data points represent the mean ± S.E.M. of at least three separate experiments each performed using triplicate determinations. Results are expressed as the % of the maximal 5-HT response.

gp5ht$_{5A}$/HEK293 membranes by ~23% above basal and this effect was reversed by methiothepin (1 μM) (Fig. 7A). In contrast, the 5-HT-induced stimulation of [^{35}S]GTPγS binding was absent using membranes prepared from PTX-pretreated gp5-ht$_{5A}$/HEK293 cells (Fig. 7B). Furthermore, PTX completely inhibited the 5-HT-induced stimulation of [^{35}S]GTPγS binding when investigated over a range of 5-HT concentrations (0.3 nM to 10 μM) (data not shown). These findings are consistent with the guinea pig 5ht$_{5A}$ receptor being G$_i$-coupled in this expression system.

4. Discussion

In the present study, we have cloned the guinea pig 5-ht$_{5A}$ receptor from brain DNA and characterised the pharmacological profile following transient expression of the receptor in HEK293 cells. The guinea pig 5-ht$_{5A}$ receptor is a protein of 356 amino acids with seven putative hydrophobic transmembrane domains. In phylogenetic terms the guinea pig 5-ht$_{5A}$ sequence clusters with other 5-ht$_{5A}$ sequences from human, mouse and rat supporting the assignment of this gene as the guinea pig 5-ht$_{5A}$ orthologue. The guinea pig receptor shows 88%, 85% and 84% amino acid sequence identity versus the human, rat and mouse 5-ht$_{5A}$ receptors, respectively. Therefore, the guinea pig 5-ht$_{5A}$ sequence is more closely related to the human than the mouse or rat 5-ht$_{5A}$ sequences.

The pharmacological profile for the guinea pig 5-ht$_{5A}$ receptor was characterised using radioligand binding. In the absence of suitable 5-ht$_{5A}$ receptor-selective radioligands, previous studies to characterise the 5-ht$_{5A}$ receptor from mouse, rat and human have utilised either the antagonist radioligands, [^{3}H]LSD (Francken et al., 1998) or [^{125}I]LSD (Plassat et al., 1992; Erlander et al., 1993; Grailhe et al., 2001) or the agonist radioligand, [^{3}H]5-CT (Rees et al., 1994). Although non-selective for the 5-ht$_{5A}$ receptor, such radioligands have proved useful for characterising 5-ht$_{5A}$ receptor binding sites in a number of recombinant systems. In the present study, [^{3}H]LSD bound saturably and with high affinity (K_d 2.3 nM) to the guinea pig 5-ht$_{5A}$ receptor. A high expression density was achieved following transient expression (B_{max} 15.7 pmol/mg protein) and the binding was monophasic, consistent with labelling of a single population of binding sites. [^{3}H]5-CT also showed saturable, high affinity binding to gp5-ht$_{5A}$/HEK293 membranes. However, the B_{max} defined using [^{3}H]5-CT (6.7 pmol/mg protein) was lower than that defined using [^{3}H]LSD. A lower B_{max} for [^{3}H]5-CT saturation binding compared to that for [^{3}H]LSD binding has been reported previously for the human 5-ht$_{5A}$ receptor stably expressed in HEK293 cells (Francken et al., 1998) and

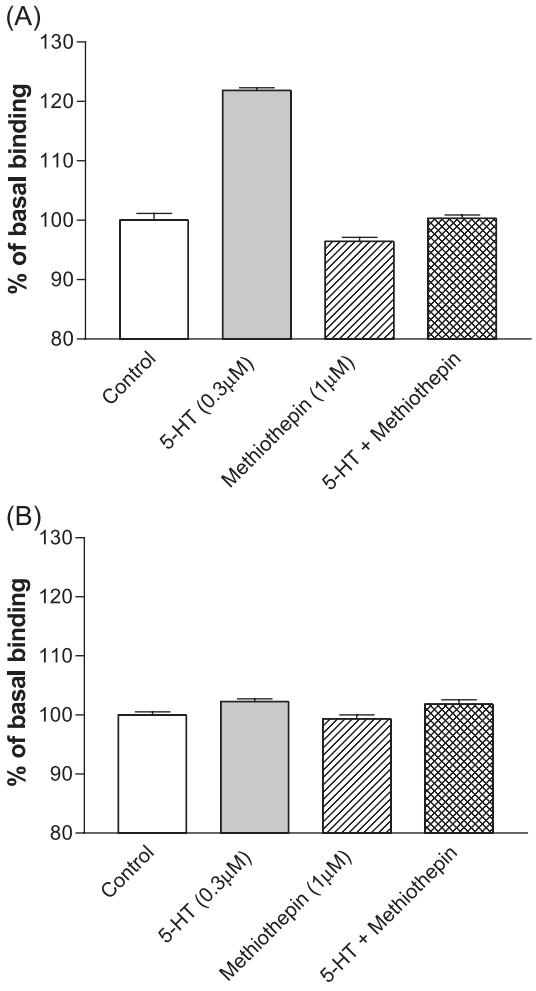

Fig. 7. [^{35}S]GTPγS binding to membranes prepared from HEK293 cells transfected with guinea pig 5-HT$_{5A}$ receptor cDNA and then grown (A) under control conditions, or (B) in the presence of pertussis toxin (100 ng/ml) for 18 h before cell harvesting. Binding was measured either in the absence of test drug (basal binding), or, in the presence of 5-HT (0.3 μM), methiothepin (1 μM) or 5-HT (1 μM) with methiothepin (1 μM). Data bars represent the mean ± S.E.M. of at least three separate experiments each performed using triplicate determinations.

these workers also reported that [^{3}H]5-CT labelled high and low affinity states of the receptor. Therefore, one possible explanation for the difference in B_{max} measured in the present study is the presence of more than one receptor affinity state with respect to [^{3}H]5-CT, which may display high affinity for a subpopulation of the binding sites labelled by [^{3}H]LSD, but a much lower affinity for the remainder of the sites which were not detected under the assay conditions used.

The pharmacological profile for the guinea pig 5-ht$_{5A}$ receptor was characterised in terms of displacement of specific [^{3}H]LSD binding. A number of standard agonists and antagonists inhibited [^{3}H]LSD binding with a rank order of potency consistent with that for a 5-ht$_{5A}$ receptor. Furthermore, Hill slope values (n_H) for all compounds tested were close to unity, consistent with displacement of binding of [^{3}H]LSD from a single population of binding sites. The pharmacological profile for the guinea pig 5-ht$_{5A}$ receptor correlated well with that for the human receptor in terms of rank order of affinity for a number of standard ligands (correlation coefficient 0.91) and was also similar to that for the mouse 5-ht$_{5A}$ receptor (correlation coefficient 0.88) although the 5-HT$_7$ receptor antagonist, SB-269970-A, appeared to display a slightly higher affinity for the mouse receptor compared to guinea pig (pK_i values 7.7 and 6.9, respectively). Comparison between the guinea pig and the rat 5-ht$_{5A}$ receptor (correlation coefficient 0.78) revealed a number of differences in pharmacological profile. As noted for the mouse receptor, SB-269970-A appeared to display a higher affinity for the rat receptor compared to the guinea pig receptor (pK_i values 7.8 and 6.9, respectively). In addition, the non-selective 5-HT receptor antagonist, methiothepin, showed a lower affinity for the rat receptor (pK_i 7.1) compared to that at the guinea pig, mouse or human receptor (pK_i values 8.5, 8.4 and 8.2, respectively).

5-HT produced a concentration-related stimulation of [^{35}S]GTPγS binding to gp5-ht$_{5A}$/HEK293 membranes with a pEC$_{50}$ of 8.1 ± 0.2, comparable to the gp5-ht$_{5A}$ receptor affinity for 5-HT (pK_i 7.8) determined in [^{3}H]LSD binding studies. In contrast, 5-HT did not stimulate [^{35}S]GTPγS binding in non-transfected HEK293 cells. The non-selective 5-HT receptor antagonists, methiothepin and ritanserin, both produced a surmountable, apparently competitive, antagonism of the 5-HT response giving apparent pK_B values (8.0 and 7.2, respectively) comparable to their respective guinea pig 5-ht$_{5A}$ receptor affinities determined using [^{3}H]LSD binding. This functional profile is consistent with a 5-ht$_{5A}$ receptor-mediated stimulation of [^{35}S]GTPγS binding in this expression system.

In addition to antagonising the response to 5-HT, methiothepin and ritanserin also appeared to produce a small reduction in basal [^{35}S]GTPγS binding. The reason for this apparent inhibition of basal binding is unclear but might be explained by an inverse agonist action to inhibit constitutive receptor activity. Constitutive receptor activity has been reported for a number of other recombinant 5-HT receptors when using [^{35}S]GTPγS binding as an index of receptor-G-protein coupling (e.g., Thomas et al., 1995; Newman-Tancredi et al., 1997).

The 5-HT-induced stimulation of [^{35}S]GTPγS binding was absent using membranes prepared from pertussis toxin-treated 5-ht$_{5A}$/HEK293 cells, consistent with the guinea pig 5-ht$_{5A}$ receptor coupling to G$_i$ proteins in this expression system. The functional coupling of the guinea pig 5-ht$_{5A}$ receptor to G$_i$-proteins in the present study is consistent with studies reporting that the human 5-ht$_{5A}$ receptor is also negatively (G$_i$) coupled to adenylyl cyclase when expressed in HEK293 cells (e.g., Francken et al., 1998). Agonist-induced modulation of cAMP production in HEK293 cells transiently expressing the guinea pig 5-ht$_{5A}$ receptor has not been investigated to date.

The maximal degree of stimulation by 5-HT of [^{35}S]GTPγS binding to gp5-ht$_{5A}$/HEK293 membranes (23–30% above basal) was relatively modest compared to that reported for a number of other G$_i$-coupled 5-HT receptors (e.g., Thomas et al., 1995; Newman-Tancredi et al., 1997). This might suggest a low efficiency of coupling of the guinea pig 5-ht$_{5A}$ receptor to G$_i$-proteins in the HEK293 cell expression system in spite of the relatively high receptor expression level achieved. Consistent with this possibility, the human 5-ht$_{5A}$ receptor has previously been reported to show low coupling efficiency when expressed in HEK293 cells (Hurley et al., 1998; Grailhe et al., 2001). However, Francken et al. (1998), who stably expressed the human 5-ht$_{5A}$ receptor in HEK293 cells, reported a much larger degree of stimulation (~ 130% above basal) of [^{35}S]GTPγ binding by 5-HT compared to that seen for the guinea pig 5-ht$_{5A}$ receptor in the present study. This difference might, at least partly, be explained by the very high receptor expression level achieved (30–50 pmol/mg protein) in the study of Francken et al., although the possibility of species-related differences with respect to 5-ht$_{5A}$ receptor-G-protein coupling efficiency cannot be ruled out.

Cloning of species orthologues, as described in the present study, can be expected to provide insights into the amino acid changes between species responsible for the inter-species variation in pharmacology. In relation to this, several amino acid residues in the human 5-ht$_{5A}$ receptor are conserved in the guinea pig receptor but not in the rat or mouse receptor. This might explain the similarity in pharmacological profile between the guinea pig and human 5-ht$_{5A}$ receptors. Site-directed mutagenesis studies will be required to identify those residues that are important for determining the pharmacological profile seen across species.

The 5-ht$_{5A}$ receptor has been reported to be preferentially expressed in the CNS and localised in areas implicated in higher brain function (Oliver et al., 2000). To date, however, a role for the 5-ht$_{5A}$ receptor in brain function has not been established, and it is likely that such studies have been hindered by a lack of 5-ht$_{5A}$ receptor-selective ligands. Until such ligands are identified, pharmacological studies to investigate the role of the receptor in native tissue function must utililise non-selective ligands.

In conclusion, in the present study, we report the cloning and pharmacological characterisation, using non-selective ligands, of the guinea pig 5-ht$_{5A}$ receptor. This study should provide useful information for studies to investigate the potential role of the 5-ht$_{5A}$ receptor in guinea pig brain function.

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