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Molecular cloning and pharmacological characterization of the guinea pig 5-HT_{1E} receptor

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Abstract

The human 5-HT_{1E} receptor gene was cloned more than a decade ago. Little is known about its function, and there have been no reports of its existence in the genome of small laboratory animals. In this study, attempts to clone the 5-HT_{1E} gene from the rat and mouse were unsuccessful. In fact, a search of the mouse genome database revealed that the 5-HT_{1E} receptor gene is missing from the mouse genome. However, the 5-HT_{1E} gene was cloned from guinea pig genomic DNA and was characterized. The guinea pig 5-HT_{1E} receptor gene encodes a protein of 365 amino acids. It shares 88% (nucleic acid) and 95% (amino acid) homology with the human receptor. The guinea pig 5-HT_{1E} receptor showed similar pharmacology to the human 5-HT_{1E} receptor in radioligand binding assays. Serotonin (5-hydroxytryptamine, 5-HT) dose-dependently stimulated [^{35}S]GTPγS binding to the guinea pig 5-HT_{1E} receptor with an EC_{50} of 13.6 ± 1.92 nM, similar to that of the human 5-HT_{1E} receptor (13.7 ± 1.78 nM). Activation of the guinea pig 5-HT_{1E} receptor was also achieved by ergonovine, α-methyl-5-HT, 1-naphthylpiperazine, methysergide, tryptamine, and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). Methiothepin exhibited antagonist activity. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that 5-HT_{1E} mRNA was present in the guinea pig brain with the greatest abundance in the hippocampus, followed by the olfactory bulb. Lower levels were detected in the cortex, thalamus, pons, hypothalamus, midbrain, striatum, and cerebellum. Our current study marks the first identification of the 5-HT_{1E} receptor gene in a commonly used laboratory animal species. This finding should allow the elucidation of the receptor's role(s) in the complex coordination of central serotonergic effects.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{1E} receptor; Cloning; Phylogenetic analysis; (Guinea pig)

1. Introduction

To date, five subtypes of serotonin receptors have been identified in the 5-HT_1 receptor family (5-$HT_{1A, 1B, 1D, 1E, 1F}$) (Barnes and Sharp, 1999). The 5-HT_{1E} receptor is a member of this family which represents a group of G protein-coupled receptors that activate the $G_{i/o}$ types of G proteins. The 5-HT_{1E} receptor was first identified by Leonhardt et al. (1989) in human cortical tissue using radioligand binding and demonstrated the characteristic pharmacologic feature of having high affinity for 5-HT and low affinity for 5-carboxyamidotryptamine (5-CT). Later, the 5-HT_{1E} gene was cloned by several independent laboratories from human genomic libraries (Levy et al., 1992; Zgombick et al., 1992) and a human cDNA library (McAllister et al., 1992). Further functional studies by these groups showed unanimously that the cloned receptors were able to mediate inhibition of adenylyl cyclase when activated by serotonin. Binding studies performed using transient or stable transfected cell lines revealed that the pharmacology of this newly cloned receptor was similar to that described by Leonhardt et al. (1989).

The human 5-HT_{1E} receptor gene is intronless and encodes a protein of 365 amino acids (McAllister et al., 1992; Zgombick et al., 1992; Gudermann et al., 1993). Sequence comparison demonstrates that it has the highest homology with the human 5-HT_{1F} receptor, followed by the

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5-HT_{1B} and the 5-HT_{1D} receptors (McAllister et al., 1992; Zgombick et al., 1992; Barnes and Sharp, 1999). In situ hybridization of human metaphase chromosomes using the cloned human 5-HT_{1E} gene shows that human 5-HT_{1E} receptor gene is localized in human chromosome 6q14–q15 (Levy et al., 1994).

The protein distribution of 5-HT_{1E} receptor in the brain is inconclusive because no selective ligands or antibodies are available to perform either radioligand autoradiographic-based or immunohistochemical-based mapping. Most distribution studies using [^{3}H]5-HT in the presence of 5-CT and mesulergine (to mask 5-$HT_{1A,}$ 5-$HT_{1B,}$ 5-HT_{1D} and 5-HT_{2C} sites) depict a combined localization of 5-$HT_{1E/1F}$ sites. The combined 5-$HT_{1E/1F}$ sites have been reported in human, guinea pig, rat, and mouse brains (Leonhardt et al., 1989; Miller and Teitler, 1992; Barone et al., 1994; Palacios et al., 1996; Fugelli et al., 1997). In human, these 5-$HT_{1E/1F}$ sites are detected in the cortex, caudate putamen, claustrum, hippocampus, and amygdala (Leonhardt et al., 1989; Miller and Teitler, 1992; Barone et al., 1994; Palacios et al., 1996; Fugelli et al., 1997). It is now known that the 5-HT_{1E} binding site has lower affinity for sumatriptan, a characteristic that makes it distinguishable from the 5-HT_{1F} binding sites. However, there is no report on the brain distribution of pure 5-HT_{1E} receptor after masking the 5-HT_{1F} binding sites. Nevertheless, the distributions of 5-HT_{1E} mRNA in both human and monkey brains were determined by in situ hybridization analysis, showing the presence of 5-HT_{1E} mRNA in the brain areas of caudate, putamen, hypothalamus, and cortex (Bruinvels et al., 1994).

While the 5-HT_{1E} receptor gene has been cloned from the human genome for more than a decade, there has been no report on the isolation of this gene from any commonly used laboratory species. The unknown nature of the presence of the 5-HT_{1E} receptor in species other than the human makes it difficult to establish an ideal experimental animal model to study the physiological function of this receptor. In the present study, the 5-HT_{1E} receptor gene was cloned from genomic DNA isolated from guinea pig. The cloned guinea pig 5-HT_{1E} receptor was then pharmacologically and functionally characterized. The results of this study revealed that the guinea pig 5-HT_{1E} receptor gene has a great homology with the human 5-HT_{1E} receptor. It also shared similar pharmacologic profiles as the human 5-HT_{1E} receptor when expressed in the same cell line. These findings represent the first identification of the 5-HT_{1E} receptor in a commonly used laboratory animal species.

2. Materials and methods

2.1. Animal tissues, chemicals and radioactive ligands

Brain tissue from monkey, dog, rabbit, guinea pig, chicken, gerbil, and hamster and choriod plexus from pig were obtained from the Neuroscience and Toxicology Divisions of Lilly Research Laboratories (Indianapolis, IN). All animal use procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to reduce the number of animals used and to minimize animal suffering. Protocols were approved by the Lilly Research Laboratories Animal Care and Use Committee. Serotonin (creatinine sulfate), 5-methoxytryptamine (HCl), 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT, HBr), α-methyl-5-HT (maleate), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (HCl), metergoline (free base), methiothepin (mesylate), 1-(3-chlorophenyl)piperazine (*m*-CPP, HCl), and 1-[3-(trifluoro-methyl)phenyl]piperazine (TFMPP, HCl) were purchased from RBI Biochemicals (Natick, MA). Tryptamine (free base), 5-carboxamidotryptamine (5-CT, maleate), 5-fluorotryptamine (HCl), methysergide (maleate), rauwolscine (HCl), ergonovine (maleate), pargyline (HCl), L-ascorbic acid (sodium salt), and GDP (sodium salt) were obtained from Sigma (St. Louis, MO). 1-Naphthylpiperazine (HCl) and sumatriptan (free base) were obtained from Lilly Research Laboratory. Tris base and $MgCl_2$ (hexahydrate) were from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ). [^{3}H]5-HT (trifluoroacetate) and [^{35}S]GTPγS were acquired from Amersham (Piscataway, NJ) and NEN (Boston, MA) respectively.

2.2. Polymerase chain reaction cloning and sequence analysis

Genomic DNA was extracted from monkey, pig, dog, rabbit, guinea pig, chicken, gerbil, and hamster tissues using genomic DNA isolation kits (Lamda Biotech, St. Louis, MO) according the manufacturer's instructions. Samples of

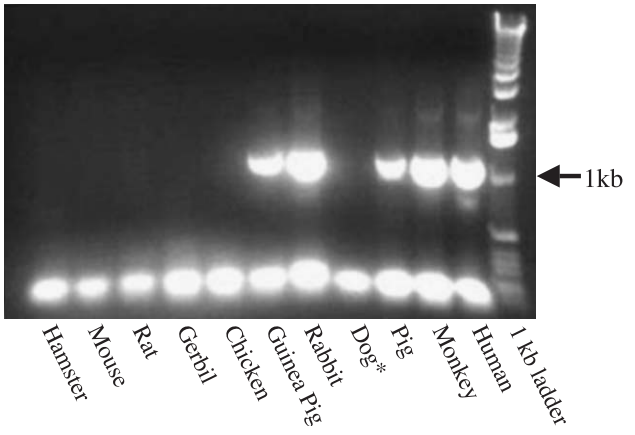

Fig. 1. PCR identification of the presence of a genomic fragment of ~ 1.1-kb size in the genome of guinea pig, rabbit, pig, monkey, and human. Degenerate primers were designed in correspondence to the sequence of the N- and C-terminal regions of the human 5-HT_{1E} receptor gene. The genomic DNA samples were used as templates. *Genomic DNA samples from two different dogs were used in the PCR reaction. No PCR product was observed in either of the samples.

genomic DNA isolated from human, rat, and mouse tissue were purchased from Clontech (Palo Alto, CA). These genomic DNA samples were used as templates for the PCR. Oligonucleotides of degenerate sequence were designed corresponding to the N-terminal and the C-terminal sequence of the human 5-HT$_{1E}$ receptor gene. The degenerate oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA) and were used as the primers for the PCR reaction. Nucleotide sequences of sense and antisense primers were as follows (with D = A or G or T; N = A or C or G or T; Y = C or T): 5′-GGGGTACCA TGAACATCACAAACTGTYACCNAC-3′; 5′-CCCT-CGAGCTAAGTATGCTCTCGG CAYCTDAT-3′. A sequence recognized by *Kpn* I was added to the 5′ end of sense primer and a sequence recognized by *Xho* I was added to the 5′ end of antisense primer. The PCR reaction was performed with a Peltier thermal cycler (PTC-200) for 40 cycles followed by an end cycle of 72 °C for 5 min. Each cycle consisted of 1 min at 94 °C, 45 s at 50 °C, and 1 min at 72 °C. The band of interest (~ 1.1 kb in size) from guinea pig was purified from 1% agarose gel following gel electrophoresis and was inserted between the *Kpn* I and *Xho* sites of expression vector pcDNA3.1+. The resulting recombinant was used to transform DH5α bacteria (Invitrogen, Carlsbad, CA). After amplification, the cloned DNA inserts were sequenced for both strands.

2.3. Evolutionary genetics analysis

The phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis software; MEGA version 2.1 (Kumar et al., 2001) available at http://www.megasoftware.net. The following amino acid sequences were included in the phylogenetic tree reconstruction: NP_000856 (human 5-HT$_{1E}$), NP_000857 (human 5-HT$_{1F}$), NP_000854 (human 5-HT$_{1B}$), NP_000855 (human 5-HT$_{1D}$), NP_000515 (human 5-HT$_{1A}$), NP_062873 (human 5-HT$_7$) and guinea pig 5-HT$_{1E}$ (Fig. 3).

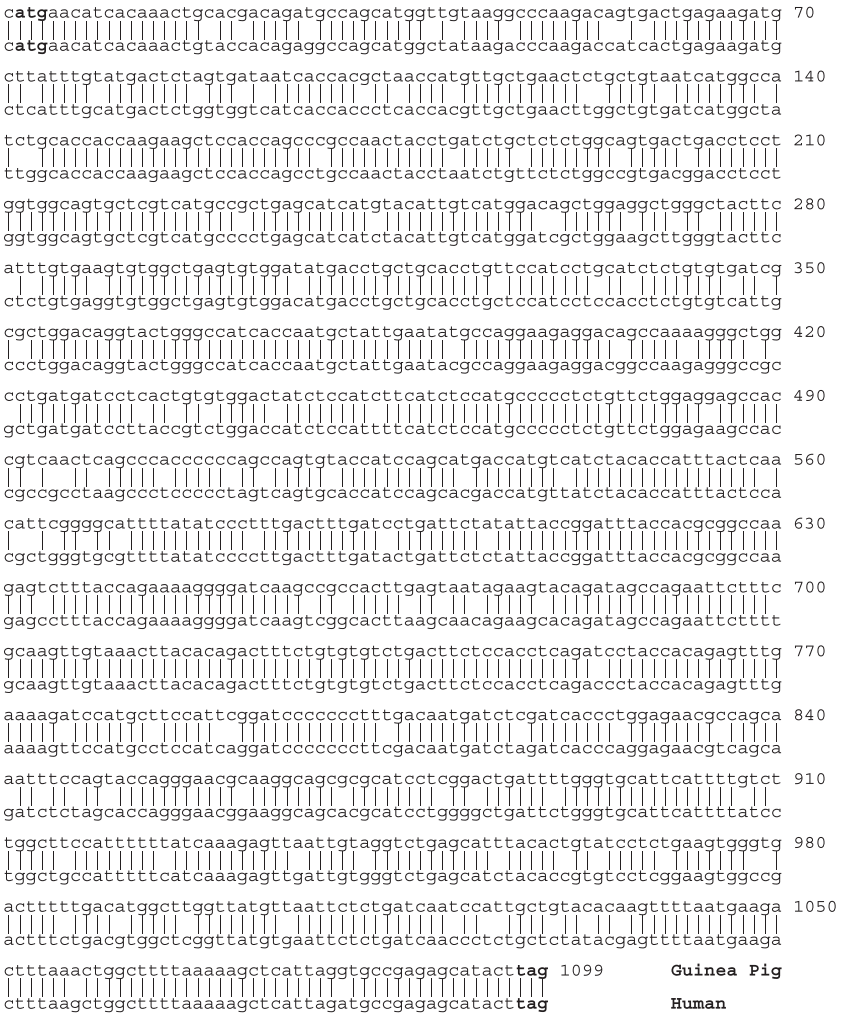

Fig. 2. Nucleotide sequence of the cloned guinea pig genomic fragment (Genbank accession number: AY344643) and its alignment with the human 5-HT$_{1E}$ gene. Guinea pig sequence is on the top, and the human sequence is at the bottom.

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MNITNCTTDASMVVRPKTVTEKMLICMTLVIITTLTMLLNSAVIMAICTTKKLHQPANYL
MNITNCTTEASMAIRPKTITEKMLICMTLVVITTLTTLLNLAVIMAIGTTKKLHQPANYL 60

ICSLAVTDLLVAVLVMPLSIMYIVMDSWRLGYFICEVWLSVDMTCCTCSILHLCVIALDR
ICSLAVTDLLVAVLVMPLSIIYIVMDRWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDR 120

YWAITNAIEYARKRTAKRAGLMILTVWTISIFISMPPLFWRSHRQLSPPPSQCTIQHDHV
YWAITNAIEYARKRTAKRAALMILTVWTISIFISMPPLFWRSHRRLSPPPSQCTIQHDHV 180

IYTIYSTFGAFYIPLTLILILYYRIYHAAKSLYQKRGSSRHLSNRSTDSQNSFASCKLTQ
IYTIYSTLGAFYIPLTLILILYYRIYHAAKSLYQKRGSSRHLSNRSTDSQNSFASCKLTQ 240

TFCVSDFSTSDPTTEFEKIHASIRIPPFDNDLDHPGERQQISSTRERKAARILGLILGAF
TFCVSDFSTSDPTTEFEKFHASIRIPPFDNDLDHPGERQQISSTRERKAARILGLILGAF 300

ILSWLPFFIKELIVGLSIYTVSSEVGDFLTWLGYVNSLINPLLYTSFNEDFKLAFKKLIR
ILSWLPFFIKELIVGLSIYTVSSEVADFLTWLGYVNSLINPLLYTSFNEDFKLAFKKLIR 360

CREHT      Guinea Pig
CREHT 365  Human
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Fig. 3. Amino acid sequence deduced from the nucleotide sequence of the cloned guinea pig genomic fragment. The guinea pig sequence (top) is aligned with the amino acid sequence of the human 5-HT_{1E} receptor (bottom). The amino acids that are different between the guinea pig and the human receptors are in bold. The putative transmembrane domains are underlined.

2.4. Expression of the guinea pig 5-HT_{1E} receptor in AV-12 cells

AV-12 cells (ATCC no. CRL9595) were transfected with recombinant DNA containing the cloned guinea pig 5-HT_{1E} gene insert using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). The guinea pig 5-HT_{1E} gene-positive AV-12 cells were selected in the presence of G418 (400 μg/ml). Cell lines were established from individual G418-resistant cell colonies and were screened for the presence of 5-HT_{1E} receptor by determination of their ability to bind [^{3}H]5-HT.

2.5. Screening of guinea pig 5-HT_{1E} receptor expressing AV-12 cell lines and membrane preparation from 5-HT_{1E} cell lines

Cell lines derived from G418-resistant cell colonies were seeded in a 96-well plate at a concentration of 3×10^4 cells/well. Each cell line was seeded in four individual wells in a square arrangement. A human 5-HT_{1E} receptor-expressing AV-12 cell line, which was established in the BioRTP Division of Lilly Research Laboratories, was cultured in the same plate and was used as a positive control. The cell monolayers were tested in duplicate for their ability to bind [^{3}H]5-HT. Two wells were used to define total binding and two wells to define nonspecific binding. The level of 5-HT_{1E} receptor expression, which is proportional to the specific binding, was calculated by the difference between total and nonspecific binding. Cell lines that showed high specific binding of [^{3}H]5-HT were cultured in monolayer and grown to confluence. They were harvested by scraping and resuspending in a hypotonic buffer, 50 mM Tris–HCl, pH 7.4. The membrane suspensions were subjected to a further quantitative [^{3}H]5-HT binding assay to identify a cell line which expressed cloned guinea pig 5-HT_{1E} receptor at a level similar to that of the known human 5-HT_{1E} receptor expressing AV-12 cell. The compatible guinea pig and human cell lines were then grown in suspension and harvested by centrifugation. The cells were resuspended in 50 mM Tris–HCl, pH 7.4 ($\sim 2.5 \times 10^8$ cells/ml), and stored at −70 °C until use. The membrane suspension was thawed on the day of the experiment and diluted to 30 ml with 50 mM Tris–HCl, pH 7.4. After centrifugation at 39,800 × *g*, 4 °C, the pellet was resuspended in 50 mM Tris–HCl, pH 7.4 and incubated at 37 °C for 10 min. The suspension was centrifuged at 39,800 × *g*, 4 °C, then washed again with 50 mM Tris–HCl, pH 7.4, followed by centrifugation at 39,800 × *g*, 4 °C. The final pellet was homogenized in binding buffer (67 mM Tris–HCl, 13.4 mM $MgCl_2$, 0.67 mM EDTA, pH 7.4) or [^{35}S]GTPγS assay buffer (67 mM Tris–HCl, 4 mM $MgCl_2$, 160 mM NaCl, 0.267 mM EDTA, pH 7.4), using a Tissuemizer (Tekmar, Cincinnati, OH) for 15 s at setting 65.

2.6. 5-HT_{1E} [^{3}H]5-HT binding assay

The guinea pig or human 5-HT_{1E} receptor binding assays using [^{3}H]5-HT were performed using a method that has been previously described with modifications to adapt to a scintillation proximity assay (SPA) (Wainscott et al., 1996). The assay was conducted in 96-well plates and was automated using a Biomek 1000 robotic workstation (Beckman Instruments, Fullerton, CA). Each cell line was tested in duplicate in assay mixtures of 200 μl. The assay mixtures included 50 μl of one of the drug dilutions in water, 50 μl of [^{3}H]5-HT in binding buffer (67 mM Tris–HCl, 13.4 mM $MgCl_2$, 0.67 mM EDTA, pH 7.4) containing pargyline (40 μM) and L-ascorbate acid (0.4%), 50 μl of Wheatgerm Agglutinin SPA beads (containing 1 mg beads, Amersham) in binding buffer, and 50 μl of membrane suspension(~ 2 μg protein). The assay plates were covered with sealing tape. The assay mixtures were mixed thoroughly and incubated at room temperature for 2 h. The plates were then centrifuged at ~ 200 × *g*. The amount of

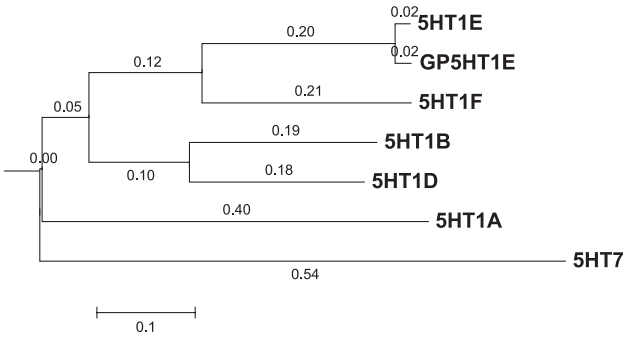

Fig. 4. A rooted phylogenetic tree of human and guinea pig 5-HT_1 receptors, using the human 5-HT_7 receptor as the out-group. The scale bar indicates a branch length of 0.1 inferred amino acid substitutions per site, and the percentage amino acid substitutions (*p* distances) are indicated on the corresponding tree branches. The guinea pig gene is indicated by "GP." All phylogenetic analyses were conducted by using MEGA version 2.1.

the [^{3}H]5-HT binding was counted on a Wallac Trilux Counter (PerkinElmer Wallac, Graithersburg, MD). The final [^{3}H]5-HT concentration was approximately 2 nM and was determined by sampling the supernatant of three total and three nonspecific wells after the plate was counted. The amount of [^{3}H]5-HT in the supernatant was determined by liquid scintillation spectrometry (Beckman LS6500, Beckman Coulter, Fullerton, CA). Nonspecific binding was determined in the presence of 10 μM serotonin. In the screening test for the guinea pig 5-HT$_{1E}$ receptor-expressing clones, the assay was performed 24 h after the cells were seeded. Because the cells were growing in monolayer in the plate, 50 μl of binding buffer instead of membrane suspension was used in the assay mixtures. A 5 nM sample of [^{3}H]5-HT was used as the final concentration for the ligand, and only total binding and nonspecific binding were determined.

2.7. [^{35}S]GTPγS assay

The [^{35}S]GTPγS assay was performed according to a previously reported procedure with modification to adapt to a SPA format (Thomas et al., 1995; Wainscott et al., 1998). The assay was also carried out in 96-well plates in an

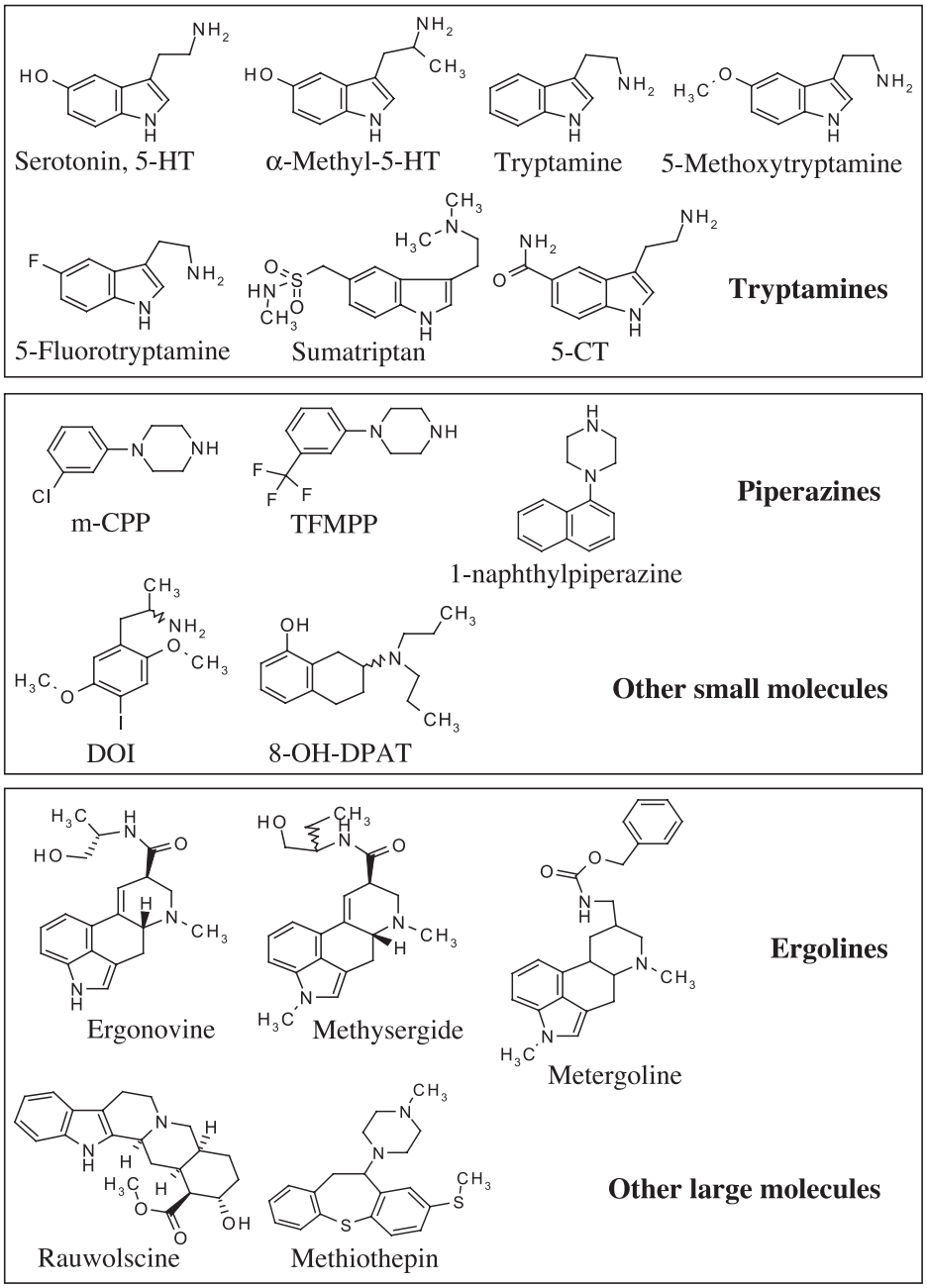

Fig. 5. Chemical structures of the serotonergic compounds used to characterize the guinea pig 5-HT$_{1E}$ receptor.

automated condition using a Biomek 1000. 5-HT_{1E} receptor expressing cell lines were tested in duplicate in assay mixtures of 200 μl. The assay mixtures consisted of 50 μl of l of one of the drug dilutions in water, 50 μl of [^{35}S]GTPγS in [^{35}S]GTPγS buffer (67 mM Tris–HCl, 4 mM $MgCl_2$, 160 mM NaCl, 0.267 mM EDTA, pH 7.4) containing 40 μM of GDP, 50 μl of Wheatgerm Agglutinin SPA beads (containing 1 mg beads) in [^{35}S]GTPγS buffer, and 50 μl of membrane suspension (approximately 5–10 μg of protein). The assay plates were covered with sealing tape. The assay mixtures were mixed thoroughly and incubated at room temperature for 2 h. The plates were then centrifuged at $\sim 200 \times g$. The amount of the [^{35}S]GTPγS binding was determined by counting on a Wallac Trilux Counter. The final [^{35}S]GTPγS concentration was approximately 0.3 nM. The maximal stimulation of [^{35}S]GTPγS binding was determined in the presence of 10 μM 5-HT, which was run as the standard with each curve. The stimulation of [^{35}S]GTPγS binding was calculated as the percentage of the effect achieved by 10 μM 5-HT. In the experiments, testing the antagonist activity of methiothepin, [^{35}S]GTPγS binding was stimulated by 100, 30, or 10 nM 5-HT, and inhibition was determined in the presence of serial dilutions of methiothepin.

2.8. qRT-PCR assay

Guinea pig brains were dissected to isolate the gross brain regions of olfactory bulb, cortex, hippocampus, striatum, thalamus, hypothalamus, midbrain, pons/medulla, and cerebellum. Total RNA was isolated from each of the above brain regions as well as from some guinea pig peripheral tissues, including liver, spleen, kidney, heart, lung, muscle, aorta, vena cava, and small intestine using RNeasy Mini Kit (Qiagen, Valencia, CA) according the manufacturer's instructions. RNA samples were treated with DNase I, Gibco, Gaithersburg, MD) and 3.5 μg of RNA was reverse-transcribed to cDNA using Super Script

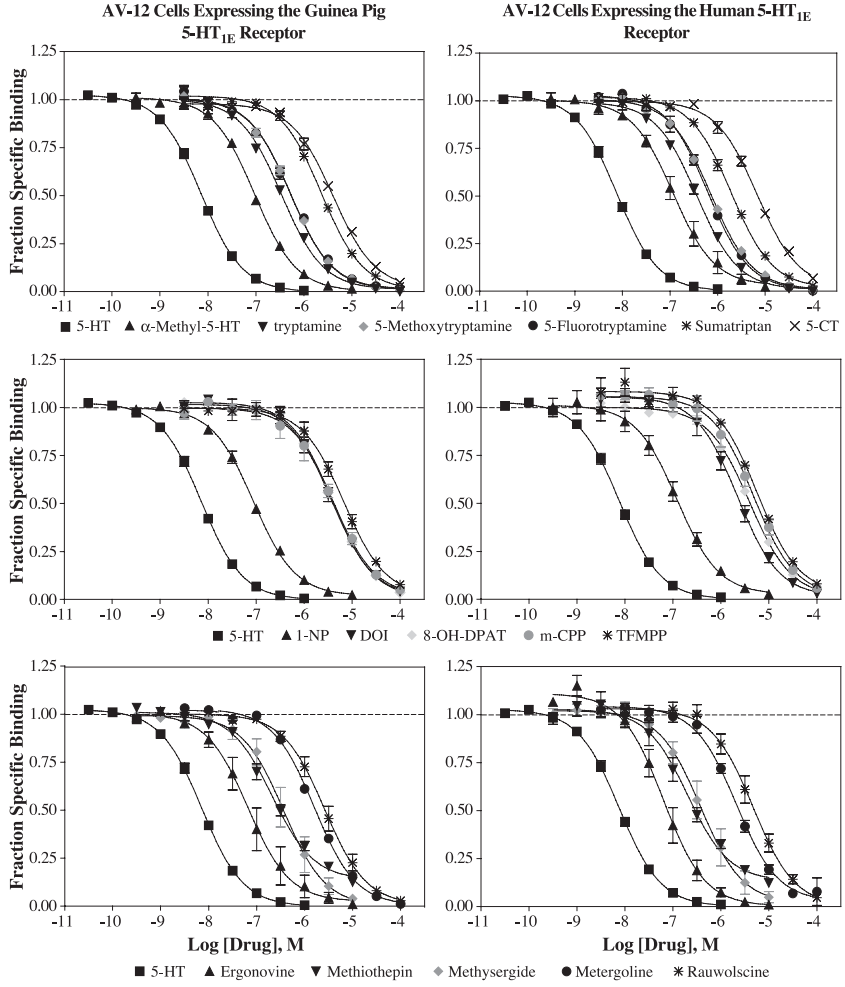

Fig. 6. Pharmacologic profile of the cloned guinea pig 5-HT_{1E} receptor compared to the human 5-HT_{1E} receptor. Ten-point inhibition curves were obtained for various serotonergic compounds in competition for [^{3}H]5-HT binding to the cloned guinea pig or cloned human 5-HT_{1E} receptor in AV-12 cell membranes. Nonspecific binding was determined in the presence of 10 μM 5-HT. Each data point is the mean of at least three separate experiments, each of which was conducted in duplicates. Mean pK_i values are given in Table 1. Refer to Fig. 5 for the chemical structures of the serotonergic compounds.

Table 1
Affinities of serotonergic compounds to the cloned guinea pig and human 5-HT_{1E} receptors determined by their competition curves to [^{3}H]5-HT binding

Drugs	pK_i (− log[drug], M)[a]	
	Guinea pig	Human
5-HT	8.252 ± 0.036	8.216 ± 0.041
α-Methyl-5-HT	7.129 ± 0.025	7.017 ± 0.096
Tryptamine	6.573 ± 0.035	6.532 ± 0.045
5-Methoxytryptamine	6.379 ± 0.047	6.237 ± 0.026
5-Fluorotryptamine	6.387 ± 0.016	6.301 ± 0.044
Sumatriptan	5.742 ± 0.021	5.809 ± 0.030
5-CT	5.500 ± 0.029	5.268 ± 0.029
1-Naphthylpiperazine	7.158 ± 0.040	7.010 ± 0.045
DOI	5.535 ± 0.032	5.754 ± 0.043
8-OH-DPAT	5.516 ± 0.022	5.501 ± 0.012
m-CPP	5.513 ± 0.088	5.424 ± 0.089
TFMPP	5.277 ± 0.079	5.342 ± 0.060
Ergonovine	7.249 ± 0.209	7.287 ± 0.155
Methiothepin	6.780 ± 0.078	6.788 ± 0.046
Methysergide	6.520 ± 0.189	6.498 ± 0.154
Metergoline	5.904 ± 0.013	5.777 ± 0.033
Rauwolscine	5.674 ± 0.111	5.427 ± 0.092

All competition curves were best fit to one-site competition by nonlinear regression. K_d values for [^{3}H]5-HT binding were 5.691 ± 0.617 and 6.188 ± 0.700 nM for the guinea pig and the human receptors, respectively. B_{max} values were 36,917 ± 1774 and 44,631 ± 5176 fmol/mg protein for the guinea pig and the human receptors, respectively.

[a] Each value represents the mean ± S.E.M. of at least three separate experiments.

First-Strand Synthesis System for RT-PCR (Invitrogen). The same amount of RNA was used in a parallel reaction for the negative control, in which water instead of reverse transcriptase was added to the reaction mixture. Oligonucleotides were designed using the Primer Express software (Applied Biosystem, Foster City, CA) in correspondence to the nucleic acid sequence from position 827 to position 887 and were used as forward primer (5′-AGCAAATTC-CAGTACCAGGGA-3′), probe (5′-CGCAAGGCAGCGC-GCATC-3′) and reverse primer (5′-GCACCCAAAATC-AGTCCGA-3′). The quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a mixture of 25 μl containing cDNA (in equivalence to 0.8 μg mRNA), 20 pmol each of the forward and the reverse primers, 5 pmol of probe, and 12.5 μl of 2 × universal master mix (Applied Biosystem). qRT-PCR was performed on an AB1 Prism 7700 Sequence Detector (Applied Biosystem). The initial denaturation was at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 15 s) and annealing/elongation (60 °C for 1 min). The assay was performed in triplicate, and the data were normalized with the 18S ribosomal mRNA as an endogenous control.

2.9. Data analysis

All curves were fitted by nonlinear regression analysis. EC_{50}, IC_{50}, and E_{max} values were calculated using GraphPad PRISM. Affinity constants (K_i) were converted from the IC_{50} values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973), and pK_i values were calculated as $-\log[K_i]$, M. Similarly, pEC_{50} values were calculated as $-\log EC_{50}$, M. K_d and B_{max} values for [^{3}H]5-HT binding were calculated from the homologous competition curve using an equation rearranged from Swillens (1992). In detail, Bound=$((B_{max} \times L^*)/(K_d + L + L^*)) + \min$; where Bound = the amount of [^{3}H]5-HT bound, B_{max} = the maximum number of binding sites, L = the concentration of unlabeled 5-HT, L^* = the free concentration of [^{3}H]5-HT, and min = the amount of nonspecific [^{3}H]5-HT bound.

3. Results

3.1. Molecular cloning

PCR analysis using primers designed to correspond to the N-terminal and the C-terminal sequences of the human 5-HT_{1E} receptor gene yielded a product of ~ 1.1 kb from human genomic DNA. Genomic fragments of the same size were also found in the monkey, pig, rabbit, and guinea pig, but not in the dog, chicken, gerbil, rat, mouse, or hamster (Fig. 1). Although both the rabbit and the guinea pig are relatively small laboratory animals, subsequent work focused only on the guinea pig receptor. The guinea pig was selected because of local experience using this species in behavioral assays. In addition, in comparison to the rabbit, the guinea pig has a smaller body size, therefore requiring lesser amounts of compounds for future in vivo pharmacological studies. The guinea pig genomic fragment of ~ 1.1

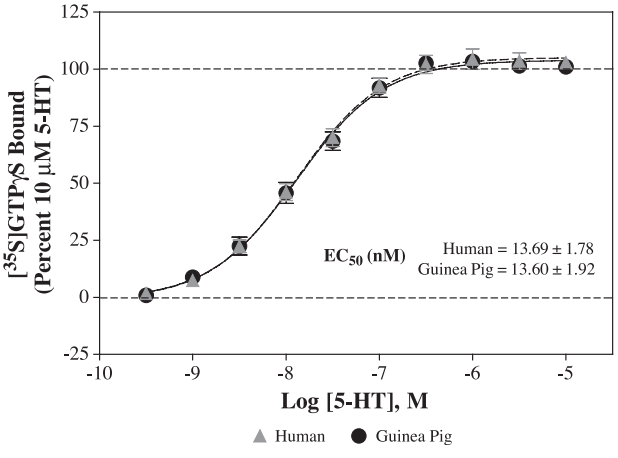

Fig. 7. Ten-point dose–response curves of 5-HT stimulated [^{35}S]GTPγS binding to the membranes of AV-12 cells expressing either the cloned guinea pig 5-HT_{1E} receptor or the cloned human 5-HT_{1E} receptor. The maximum stimulation of [^{35}S]GTPγS binding was determined in the presence of 10 μM 5-HT. The stimulation of [^{35}S]GTPγS binding was calculated as the percentage of the effect achieved by 10 μM 5-HT. EC_{50} values were calculated as described in Materials and methods. Each data point is the mean of at least three independent experiments, each of which was conducted in duplicate.

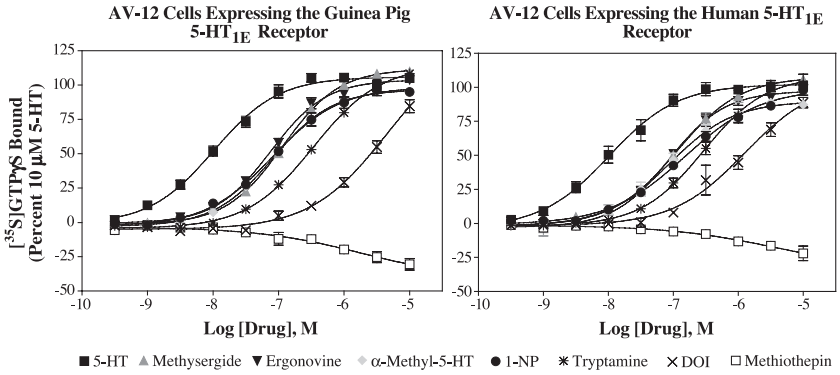

Fig. 8. Stimulation of [^{35}S]GTPγS binding to the membranes of AV-12 cells expressing either the cloned guinea pig 5-HT$_{1E}$ receptor or the cloned human 5-HT$_{1E}$ receptor by serotonergic compounds. The maximum stimulation of [^{35}S]GTPγS binding was determined in the presence of 10 μM 5-HT. The stimulation of [^{35}S]GTPγS binding was calculated as the percentage of the effect achieved by 10 μM 5-HT. Each data point is the mean of at least three independent experiments, each of which was conducted in duplicate. Mean EC_{50} and E_{max} values are given in Table 2. Refer to Fig. 5 for the chemical structures of the serotonergic compounds.

kb was isolated, cloned into the pcDNA3.1+ vector, and sequenced. Double-stranded sequencing revealed identical nucleic acid sequences from both strands (Genbank accession number: AY344643). Sequence analysis showed that the guinea pig genomic fragment encoded a protein of 365 amino acids. Sequence comparisons demonstrated an 88% nucleic acid and a 95% amino acid homology with the human 5-HT$_{1E}$ receptor (Figs. 2 and 3). The great similarity of this guinea pig genomic fragment with the human 5-HT$_{1E}$ receptor gene suggested that these are species homologues of the same receptor.

3.2. Evolutionary genetics analysis

In order to confirm the orthologous relationship between the human 5-HT$_{1E}$ receptor and the cloned guinea pig 5-HT$_{1E}$ receptor, we conducted a phylogenetic analysis using the amino acid sequences from different human 5-HT receptors and the cloned guinea pig gene. Here, we used a subset of human biogenic amine receptors, namely, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{1F}$, and 5-HT$_7$, because previous studies show that they form an evolutionary clade that includes 5-HT$_{1E}$ and its closest relatives (Joost and Methner, 2002). Our phylogenetic tree showed that the human 5-HT$_{1E}$ and the cloned guinea pig gene form a tight evolutionary cluster that is distinct from 5-HT$_{1F}$ and all other known 5-HT$_1$ receptors (Fig. 4). This result confirmed that the cloned guinea pig gene is indeed the ortholog of the human 5-HT$_{1E}$ receptor in the guinea pig species.

3.3. Stable expression of the cloned guinea pig 5-HT$_{1E}$ receptor

Forty five cell lines derived from G418 resistant cell colonies were established and were screened for the expression of the guinea pig 5-HT$_{1E}$ receptor by determination of their ability to bind with [^{3}H]5-HT. Among these cell lines, 37 lines showed [^{3}H]5-HT binding of various capacities. Five lines demonstrated specific binding values comparable to that of the human 5-HT$_{1E}$ receptor-expressing cells. Quantitative [^{3}H]5-HT binding curves using

Table 2
Potencies and efficacies of serotonergic compounds to stimulate [^{35}S]GTPγS binding to the cloned guinea pig and human 5-HT$_{1E}$ receptors determined by their dose–response curves

Drugs	Guinea pig[a]		Human[a]	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
5-HT	11.03 ± 2.121	106.3 ± 3.635	11.18 ± 2.103	102.6 ± 1.317
Tryptamine	343.1 ± 39.58	114.3 ± 5.216	298.2 ± 48.05	108.7 ± 7.190
1-Naphthylpiperazine	86.98 ± 11.68	99.12 ± 5.274	169.0 ± 39.56	100.2 ± 3.750
α-Methyl-5-HT	85.91 ± 15.42	96.95 ± 2.164	94.40 ± 21.46	89.92 ± 2.163
DOI	4638 ± 1022	133.4 ± 13.24	1090 ± 183.0	99.93 ± 3.683
Methysergide	114.1 ± 9.134	111.6 ± 2.736	122.7 ± 24.67	108.4 ± 1.571
Ergonovine	80.13 ± 12.29	104.7 ± 1.648	88.02 ± 13.32	97.39 ± 2.974
Methiothepin		− 37.06 ± 11.68[b]		− 34.94 ± 12.22[b]

All curves were best fit to sigmoidal dose–response (variable slope) by nonlinear regression.
[a] Each value represents the mean ± S.E.M. of at least three separate experiments.
[b] Values are E_{min} values.

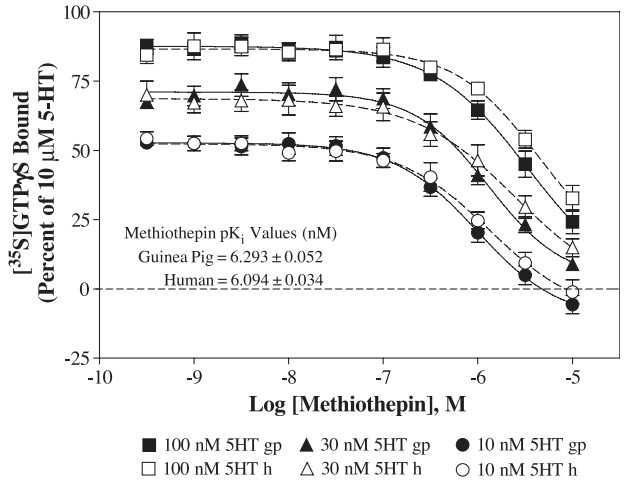

Fig. 9. Inhibition of 5-HT stimulated [^{35}S]GTPγS binding to the membranes of AV-12 cells expressing either the cloned guinea pig 5-HT$_{1E}$ receptor or the cloned human 5-HT$_{1E}$ receptor by methiothepin. The maximum stimulation of [^{35}S]GTPγS binding was determined in the presence of 10 μM 5-HT. The stimulation of [^{35}S]GTPγS binding was calculated by the percentage of the effect achieved by 10 μM 5 HT. Each data point is the mean of at least three independent experiments, each of which was conducted in duplicate.

homologous competition were determined in three of those guinea pig 5-HT$_{1E}$ receptor expressing cell lines, and 2 lines (clones #16 and #22) exhibited consistently high levels of guinea pig 5-HT$_{1E}$ receptor expression that was comparable with the human 5-HT$_{1E}$ receptor expressing cells (B_{max}>25,000 fmol/mg protein). Both the guinea pig 5-HT$_{1E}$ receptor high expressing cell lines and the human 5-HT$_{1E}$ receptor-expressing cells were grown in a large quantity in suspension, and cell membranes prepared from clone #22 guinea pig 5-HT$_{1E}$ cells and human 5-HT$_{1E}$ cells were used for further pharmacological and functional studies.

3.4. Pharmacological profiles of the cloned guinea pig 5-HT$_{1E}$ receptor

A variety of serotonergic compounds, encompassing both agonists and antagonists (see chemical structures of these compounds in Fig. 5), were tested for their affinity at the cloned guinea pig 5-HT$_{1E}$ receptor. The cloned human 5-HT$_{1E}$ receptor was examined in parallel for comparison. The cloned guinea pig 5-HT$_{1E}$ receptor, when stably expressed in AV-12 cells, showed a pharmacologic profile similar to that of the human 5-HT$_{1E}$ receptor expressed in the same cell line (Fig. 6 and Table 1). 5-HT binding was displaced by the serotonergic compounds in both cell lines with the following rank of order in affinity, (1) tryptamines: 5-HT>α-methyl-5-HT>tryptamine>5-fluorotryptamine>5-methoxytryptamine>sumatriptan>5-CT; (2) piperazines and other small molecules: 1-naphthylpiperazine>DOI>8-OH-DPAT>*m*-CPP>TFMPP; (3) ergolines and some large molecules: ergonovine>methiothepin>methysergide>metergoline>rauwolscine. Overall, the comparison of these compounds revealed that the cloned guinea pig 5-HT$_{1E}$ receptor exhibited highest affinity for 5-HT, followed by 1-naphthylpiperazine/ergonovine/α-methyl-5-HT. TFMPP, *m*-CPP, 8-OH-DPAT, 5-CT, and DOI were among the compounds with the lowest affinities for the guinea pig 5-HT$_{1E}$ receptor (Table 1). The compounds

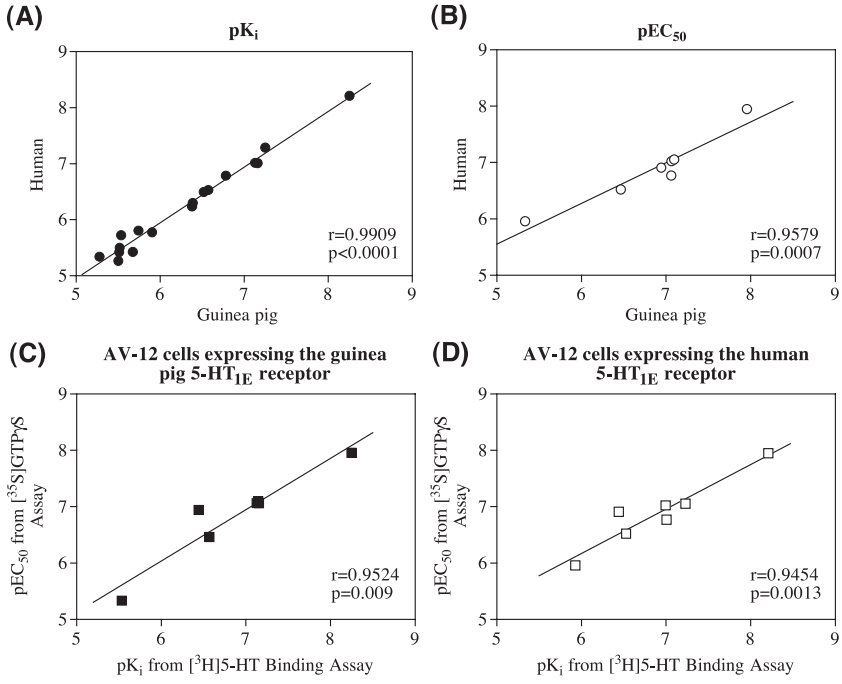

Fig. 10. Correlation of the pK_i and pEC$_{50}$ values for serotonergic compounds between the guinea pig 5-HT$_{1E}$ receptor and the human 5-HT$_{1E}$ receptor (A and B). Correlation between the pK_i values from the [^{3}H]5-HT binding assay and pEC$_{50}$ values from the [^{35}S]GTPγS assay (C and D).

showed significant correlation between the guinea pig and the human in the binding affinity at the 5-HT_{1E} receptor (Fig. 10A).

3.5. Functional activity of the cloned guinea pig 5-HT_{1E} receptor

When a G protein-coupled receptor is activated, the α subunit of the G protein heterotrimer dissociates from the β−γ complex and undergoes a conformation change when GDP is exchanged for GTP. Therefore, GTP binding to the receptor complex is an indicator of a functional receptor (Wainscott et al., 1998). To determine whether the cloned guinea pig 5-HT_{1E} receptor was a functional receptor, 5-HT_{1E} receptor-mediated stimulation of [^{35}S]GTPγS binding was determined. 5-HT dose-dependently activated the cloned guinea pig 5-HT_{1E} receptor with an EC_{50} of 13.6 ± 1.92 nM, almost identical to that of the human 5-HT_{1E} receptor (EC_{50} = 13.7 ± 1.78 nM) (Fig. 7). Functional activation of the cloned guinea pig 5-HT_{1E} receptor was also achieved with other serotonergic compounds. Among these compounds, ergonovine, α-methyl-5-HT, 1-naphthylpiperazine, methysergide, tryptamine, and DOI were shown to be agonists, with EC_{50}s (nM) of 80.1 ± 1.78, 85.9 ± 15.4, 87.0 ± 11.7, 114 ± 9.13, 343 ± 39.6, and 4638 ± 1022, respectively (Fig. 8 and Table 2). Methiothepin exhibited antagonist activity, demonstrated by its ability to inhibit 5-HT-stimulated [^{35}S]GTPγS binding. The antagonist effects of methiothepin were tested at different 5-HT concentrations (10, 30, and 100 nM), and the average pK_i (− log[drug], M) value for methiothepin was 6.29 ± 0.05 (Fig. 9). All the agonists showed significant correlation between the guinea pig and the human in the EC_{50} values in the 5-HT_{1E} receptor-mediated [^{35}S]GTPγS binding (Fig. 10B). The agonists also exhibited significant correlation between binding affinity and potency to stimulate [^{35}S]GTPγS binding (Fig. 10C and D).

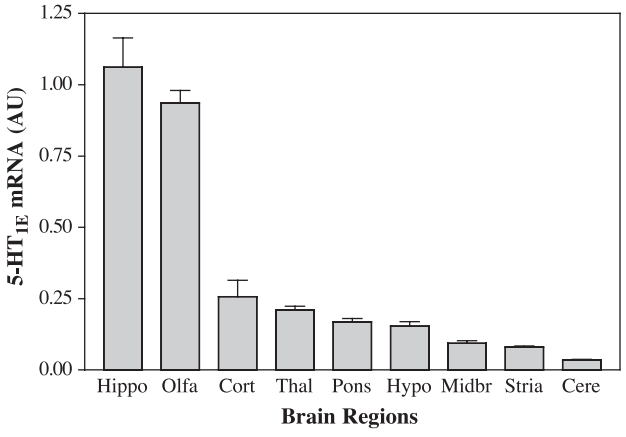

Fig. 11. Relative quantity of the 5-HT_{1E} receptor mRNA expression in the guinea pig brain. qRT-PCR was performed in the tissues of grossly dissected brain regions in guinea pig, using primers and a probe designed according to the guinea pig 5-HT_{1E} gene sequence. Data were normalized with the level of 18S ribosomal mRNA.

3.6. Distribution of 5-HT_{1E} receptor in the guinea pig brain

qRT-PCR analysis revealed that 5-HT_{1E} receptor mRNA was present in various regions of guinea pig brain (Fig. 11). The highest expression of 5-HT_{1E} mRNA was found in the hippocampus followed by olfactory bulb. Relatively lower expression was detected in the cortex, followed by the thalamus, pons, hypothalamus, midbrain, striatum, and cerebellum. Under the current conditions, no detectable levels of 5-HT_{1E} mRNA were identified in any of the tissues originating from the guinea pig peripheral organs, including liver, spleen, kidney, heart, lung, muscle, aorta, vena cava, and small intestine. No 5-HT_{1E} mRNA was detected in the samples using the product from the reactions without reverse transcriptase as template.

4. Discussion

In the present study, the 5-HT_{1E} receptor gene was successfully cloned from guinea pig genomic DNA. An AV-12 cell line, which stably expresses the cloned guinea pig 5-HT_{1E} receptor, was established and tested for pharmacology and function of the cloned receptor. In addition, the relative quantity of the 5-HT_{1E} mRNA was determined in various guinea pig brain regions. This represents the first identification, isolation, and characterization of the 5-HT_{1E} gene in a commonly used small laboratory animal species. Although the 5-HT_{1E} binding sites were discovered and the receptor gene was cloned from human more than a decade ago, there is no published information available about the complete sequence of this receptor in other species. Molecular cloning of "5-HT_{1E} -like" receptors from mouse and rat was reported in the early 1990s (Amlaiky et al., 1992; Lovenberg et al., 1993), but comparison of their nucleic acid and amino acid sequences indicates that they are all in fact the 5-HT_{1F} receptor. Pharmacological profiles of the rat "5-HT_{1E} -like" receptor also confirm that it shows high affinity for sumatriptan, a 5-HT_{1F} receptor characteristic (Lovenberg et al., 1993). Among the species examined, the current study has identified guinea pig and rabbit to be the only commonly used laboratory animals that carry the 5-HT_{1E} gene (Fig. 1). Our attempts to clone the 5-HT_{1E} gene from the rat and mouse were unsuccessful. To identify the putative mouse ortholog 5-HT_{1E} gene, we searched the mouse genome database (NCBI build 30; http://www.ensembl.org/Mus_musculus/)) using the human and guinea pig 5-HT_{1E} protein sequences as query, with the default TBLASTN parameters (Altschul et al., 1990). The most significant homologue to the human and the guinea pig 5-HT_{1E} proteins is the mouse 5-HT_{1F} receptor, indicating that the true ortholog 5-HT_{1E} is missing from the mouse genome. This is unlikely to be in the unsequenced portion

of the mouse genome, since its sequencing is now 93.7% complete (http://www.ensembl.org/Mus_musculus/stats/status.html).

The cloned guinea pig genomic fragment encoded a protein of 365 amino acids. It has 88% (nucleic acid) and 95% (amino acid) homology with the human 5-HT_{1E} receptor. When compared with other 5-HT_1 receptors in the guinea pig, the amino acid sequence of the cloned receptor showed the highest homology with that of the 5-HT_{1F} receptor (53%), followed by the 5-HT_{1D} (43%) and 5-HT_{1B} (42%) receptors. When this cloned guinea pig genomic fragment was expressed in AV-12 cells, it encoded a receptor with a typical pharmacological character of the 5-HT_{1E} receptor. Serotonin bound to the receptor with high affinity ($K_i = 5.66 \pm 0.46$ nM), but 5-CT ($K_i = 3180 \pm 219$ nM) and sumatriptan ($K_i = 1816 \pm 90.2$ nM) showed low affinity, consistent with the profile shown for the cloned human receptor (Zgombick et al., 1992) and distinguishing the 5-HT_{1E} receptor from other 5-HT_1 receptor subtypes. The cloned guinea pig receptor also behaved as a functional G protein-coupled receptor in AV-12 cells, as demonstrated by its ability to stimulate [^{35}S]GTPγS binding. Serotonin, ergonovine, α-methyl-5-HT, 1-naphthylpiperazine, methysergide, tryptamine, and DOI acted as agonists at the guinea pig 5-HT_{1E} receptor. The agonists showed excellent correlation between binding affinity and potency to stimulate [^{35}S]GTPγS binding (Fig. 10C and D). Methiothepin was the only compound that exhibited antagonist activity. Methiothepin actually demonstrated a dose-dependent inhibition response in the [^{35}S]GTPγS binding assay (Fig. 8), indicating potential inverse agonism. The inhibition of [^{35}S]GTPγS binding by methiothepin started at 100 nM and reached a ~35% inhibition at 10 μM. Obviously, it would be desirable to find a neutral antagonist to determine whether the inhibition produced by methiothepin could be blocked.

The guinea pig 5-HT_{1E} receptor showed a great similarity to its human counterpart in structure, pharmacology, and function. First, as mentioned above, there was a great homology between the nucleic acid and amino acid sequences of the guinea pig and human forms of the receptor (Figs. 2 and 3). Second, a broad spectrum of compounds with various chemical structures bound to the guinea pig and the human receptors with similar affinities with significant correlation between the species (Table 1; Fig. 10A). Third, [^{35}S]GTPγS binding following 5-HT stimulation was achieved dose-dependently in AV-12 cells expressing either the guinea pig or the human receptor with almost identical curves. EC_{50} values were 13.6 ± 1.92 and 13.7 ± 1.78 nM for guinea pig and human receptors, respectively (Fig. 7). Fourth, the agonist profile at the guinea pig receptor matched that at the human receptor (Fig. 8). The potency of agonists to stimulate [^{35}S]GTPγS binding demonstrated a near perfect correlation between the guinea pig and the human receptors (Fig. 10B). Finally, methiothepin was an antagonist at both the guinea pig and the human receptors, showing dose-dependent inhibition of 5-HT stimulated [^{35}S]GTPγS binding with similar K_i values (Fig. 9). Furthermore, the pharmacological profile of the guinea pig 5-HT_{1E} receptor expressed in AV-12 cells was also consistent with a previous report on the human 5-HT_{1E} receptor expressed in HEK293 cells, in which very similar pK_i values were described for 5-HT, 5-CT, methysergide, sumatriptan, metergoline, and methiothepin in competition for [^{3}H]5-HT binding (McAllister et al., 1992).

qRT-PCR analysis detected the 5-HT_{1E} mRNA in various regions of the guinea pig brain (Fig. 11). The highest expression was found in the hippocampus, followed by the olfactory bulb. Relatively lower levels of 5-HT_{1E} mRNA were detected (in rank order) in the cortex, thalamus, pons, hypothalamus, midbrain, striatum, and cerebellum. In situ hybridization study in human and monkey brain has shown the presence of 5-HT_{1E} mRNA in the areas of striatum (caudate and putamen), hypothalamus, and cortex (Bruinvels et al., 1994). The present study confirmed that guinea pig expressed 5-HT_{1E} mRNA in the same brain regions as those of human and monkey. However, a relatively higher expression of 5-HT_{1E} mRNA was found in the hippocampus and olfactory bulb. Brain regions such as thalamus, pons, midbrain, and cerebellum, which have not been reported to contain the 5-HT_{1E} receptor in human and monkey, also displayed measurable levels of 5-HT_{1E} mRNA. The discrepancy in the 5-HT_{1E} mRNA expression and distribution between guinea pig and human/monkey may be attributed to several factors. Differences in the brain distribution of the 5-HT_{1E} receptor may exist among species. Limited availability of human brain tissues may have allowed the in situ hybridization to be performed only on sections containing certain brain structures, leading to a lack of information on other brain areas. The differences in methodology may also play a role. In the present study, qRT-PCR was performed on the tissues from whole brain areas, which might dilute those signals that tend to centralize at certain spots. This is particularly relevant to those brain structures with larger volumes, such as cortex, which exhibits a strong hybridization signal in occipital and entorhinal cortex in human, yet had a lower expression in guinea pig. While qRT-PCR provides an estimate of the relative quantity of 5-HT_{1E} mRNA expression, detailed mapping of 5-HT_{1E} receptor in the guinea pig brain will require radioligand or in situ hybridization-based autoradiography and immunohistochemistry.

Currently, nothing is known about the physiological function of the 5-HT_{1E} receptor. This is partly due to the unavailability of selective pharmacologic tools. The lack of an animal model also makes it hard to explore the function of this receptor. The present study has identified guinea pig and the rabbit as the only commonly used small laboratory species that carry the 5-HT_{1E} receptor, thus making it possible to perform behavioral or neurochemical tests to elucidate the physiological function of the 5-HT_{1E} receptor. The sequence of the guinea pig 5-HT_{1E} gene obtained from

this study will also be a valuable resource for the in situ genetic modification of this receptor (e.g., antisense), which can shed light on the function of the receptor. The present study has also demonstrated higher expression of the 5-HT_{1E} receptor mRNA in the hippocampus and olfactory bulb in comparison to other brain regions. Hippocampus and olfactory bulb have been shown to play an important role in the regulation of memory, learning, and emotion (Yamamoto, 1991; Squire, 1993). Since serotonergic systems are also known to be involved in these processes, it will be interesting to determine whether the 5-HT_{1E} receptor has a role in memory and/or emotion.

The presence or apparent absence of the 5-HT_{1E} receptor among the species examined is intriguing. The theory of concerted evolution suggests that the total number of genes in a genome remains constant. Gene duplication occurs, but many duplicate genes may die (e.g., as mutations or pseudogenes). The dead genes stay as pseudogenes in the genome or are eliminated by unequal crossover. This is called "evolution by the birth-and-death process" (Ota and Nei, 1994). Since the 5-HT_{1E} receptor is 61% homologous to the 5-HT_{1F} receptor and shares pharmacology with the 5-HT_{1F} receptor, it is possible that after gene duplication, there was no apparent need for duplicate functional receptors, and one was eliminated in rodents and certain other evolutionary lineages. Perhaps the conservation of the 5-HT_{1E} receptor may point to a separate function for 5-HT_{1E} in humans and the other species where it was maintained. Interestingly, a study has reported the results of an examination of possible structural variants of the human 5-HT_{1E} receptor (Shimron-Abarbanell et al., 1995), where the complete coding sequence of the 5-HT_{1E} receptor was screened for 157 unrelated individuals. Only a silent C to T transition at the third position of codon 177 was detected. Such low variability may indicate evolutionary pressure in humans to maintain the structure of this receptor, which in turn may suggest that this receptor plays a relatively important role in human physiology.

In summary, this study represents the first identification, isolation, and characterization of the 5-HT_{1E} receptor gene in guinea pig. The guinea pig 5-HT_{1E} receptor is very similar to the human 5-HT_{1E} receptor in its molecular biology and pharmacology. The 5-HT_{1E} receptor mRNA is expressed at a relatively higher level in the hippocampus and olfactory bulb in the guinea pig brain. The availability of a small laboratory animal in which to characterize the functions of the 5-HT_{1E} receptor will hopefully allow this receptor's role(s) to be established in the overall actions of central serotonergic effects.

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