Cloning and characterisation of the rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors

Gail Harwood[a], Michael Lockyer[a], Heather Giles[a], Neil Fairweather[b,*]

[a]*Receptor Pharmacology Research Groups, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK*
[b]*Molecular Pharmacology Research Groups, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK*

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Abstract The genes encoding the rabbit $5HT_{1D\alpha}$ and $5HT_{1D\beta}$ receptors have been cloned. The deduced amino acid sequence of these receptors shows 91–92% amino acid sequence identity with their human homologues, and similar high sequence identity with homologues from other species. The receptors were transiently expressed in COS-7 cells and exhibit a pharmacological profile closely resembling their human homologues, including a higher affinity of ketanserin for the 5-$HT_{1D\alpha}$ subtype. However, sumatriptan had a lower affinity for both the rabbit receptors compared to their human counterparts. This may be accounted for by differences between the primary amino acid sequences of these species homologues.

Key words: G-protein coupled receptor; Serotonin; 5-Hydroxytryptamine; Rabbit

1. Introduction

5-HT (serotonin) is a biogenic amine neurotransmitter which has diverse physiological actions in both the central and peripheral nervous systems. Multiple 5-HT receptors exist, which can be characterised by their amino acid sequence, pharmacology and functional coupling to second messengers. Five members of the human 5-HT_1 subfamily of receptors have been cloned: 5-HT_{1A}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1E} and 5-HT_{1F} [1,2]. The human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors have similar pharmacological profiles, but only 65% identity in their amino acid sequence. The rat 5-HT_{1B} receptor is the species homologue of the human 5-$HT_{1D\beta}$ receptor. These proteins have 93% amino acid sequence identity but distinct pharmacological profiles [3]. All members of the 5-HT_1 receptor subfamily have been shown to couple negatively to adenylate cyclase demonstrated by functional analysis in cell lines and can be characterised by distinct pharmacological profiles [1,2].

Early studies demonstrated the therapeutic utility of 5-HT in the treatment of migraine [4]. Sumatriptan, a treatment for acute migraine, is a selective 5-HT_1 agonist and mediates vasoconstriction of cranial blood vessels [5]. Sumatriptan binds with high affinity to cloned human 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$ and 5-HT_{1F} receptors expressed in cell lines [6,7], implicating these receptors as possible targets for migraine therapy.

The pharmacology of 5-HT_1-like receptors that mediate vasoconstriction has been studied in a variety of tissues from several species, including man, dog, and rabbit (for a review see [8]). In the rabbit saphenous vein, 5-HT_1-like receptors alone appear to mediate vasoconstriction, and in contrast to other species, ketanserin has a relatively high affinity for this receptor [9]. In order to begin to explore the molecular basis for any differences in pharmacology between the human and rabbit 5-HT_{1D} receptors, we report here the molecular cloning of rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors and their transient expression in COS-7 cells.

2. Experimental

2.1. Isolation and characterisation of genomic clones

A genomic DNA library constructed in λEMBL3 SP6/T7 from rabbit liver (Clontech) was screened using as probes fragments containing the entire cloned human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ genes. Probes were labelled by random priming with [α-^{32}P]d-ATP (Amersham) and hybridised at 65°C for 16 h in 4 × SSC, 4 × Denhardts, 0.1% SDS, 50 μg/ml salmon sperm DNA with library filter lifts made onto Colony/Plaque screen membranes (NEN/DuPont). Filters were washed at 65°C in 0.7 × SSC, 0.1% SDS. Positive plaques were purified, and characterised by restriction mapping and Southern blotting of bacteriophage DNA. Hybridising fragments were subcloned into pUC18 (Pharmacia), and DNA was directly sequenced using the Sequenase version 2.0 DNA sequencing kit (USB).

2.2. Cell culture and membrane preparation

COS-7 cells were maintained at 37°C with 5% CO_2 in Iscove's modified Dulbecco's medium with glutamine supplemented with 10% foetal calf serum and 1% non-essential amino acids (all from Gibco). The expression constructs pSVL/rab5-$HT_{1D\alpha}$ or pSVL/rab5-$HT_{1D\beta}$ were transfected into the cells using 0.5 mg/ml DEAE Dextran with an additional 2.5 h treatment with 100 μM chloroquine followed by a 2 min shock with 10% DMSO [10]. The cells were incubated for 72 h and were scraped into preparation buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 6 mM $MgCl_2$), homogenised (Ultraturax) and centrifuged at 135,000 × *g* for 60 min. The membrane pellet was resuspended in assay buffer (50 mM Tris-HCl pH 7.4, 4 mM $CaCl_2 \cdot 2H_2O$, 1% ascorbic acid, 10 μM pargyline), rehomogenised in a Dounce homogeniser and protein concentration assayed (Bio-Rad).

2.3. Radioligand binding assay

For radioligand binding 30–120 μg of protein, 1.5 nM [^{3}H]5-HT and increasing concentrations of competing drug or assay buffer in a total assay volume of 1 ml were incubated at 27°C for 30 min. Incubations were terminated by rapid filtration (Brandel 24 well harvester) through GFB glassfibre filters (Whatman). The filters were washed twice with 5 ml ice cold 50 mM Tris-HCl pH 7.4, and filter discs counted in Optiphase 'Hisafe' (Wallac). Competition binding data were analysed by fitting to a three parameter logistic function to provide pIC_{50} estimates. Saturation binding analysis used doubling concentrations of [^{3}H]5-HT from 0.025–50 nM in the absence or presence of 10 μM cold 5-HT to measure non-specific binding. Total binding data were fitted to a hyperbola plus a straight line function to obtain estimates of K_d, Hill slope parameters and B_{max}. Non-specific binding data were fitted to a straight line function.

2.4. Drugs used

5-Hydroxytryptamine hydrochloride (Sigma), 5-carboxyamidotryptamine maleate (Tocris Cookson), ketanserin (RBI), pindolol (Sigma), metergoline (Farmitalia Carlo ERBA). Sumatriptan was synthesised by the Medicinal Chemistry Department at the Wellcome Research Laboratories. All drugs were dissolved in 2 mM HCl. Further dilutions were made in assay buffer.

*Corresponding author. Fax: (44) (181) 663-3645.

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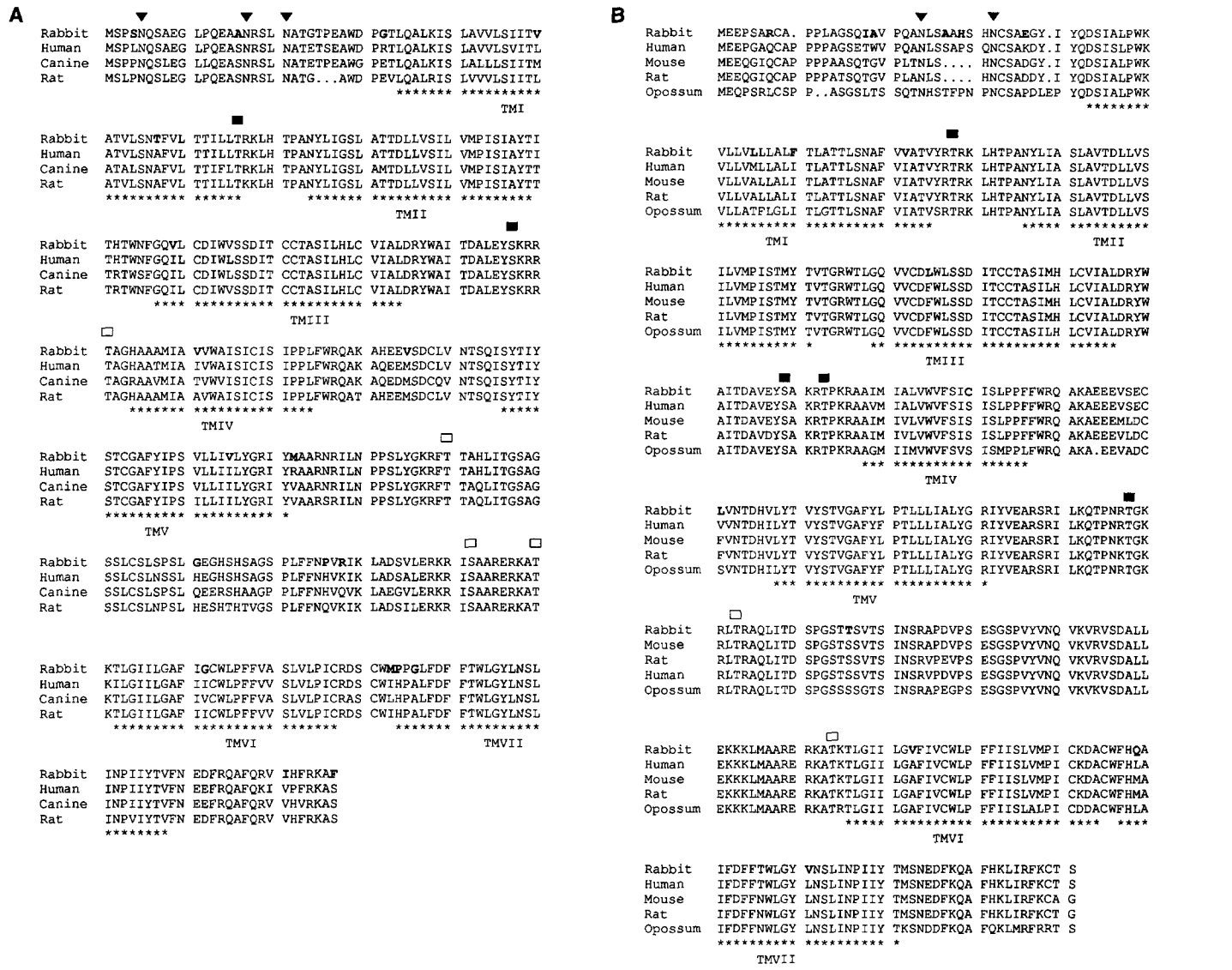

Fig. 1. (A) Comparison of amino acid sequences of 5-$HT_{1D\alpha}$ receptors. Alignment of the deduced amino acid sequences of the rabbit 5-$HT_{1D\alpha}$ receptor with the homologous human [6], rat [12] and canine [11] sequences. Putative transmembrane regions (TMI-TMVII) are denoted by asterisks. In the amino-terminal region, consensus sites for glycosylation are indicated (▼). Consensus sites for phosphorylation by protein kinases A (□) and C (■) are indicated. Amino acids in bold indicate residues in the rabbit sequence which differ from the corresponding residue in all other species. Sequences were aligned using the GCG suite of programmes. The nucleotide sequences were deposited with EMBL under accession numbers Z50162 and Z50163. (B) Comparison of amino acids sequences of 5-$HT_{1D\beta}$ receptors. Alignment of the deduced amino acid sequences of rabbit 5-$HT_{1D\beta}$ receptor with the homologous human [6], rat [3], mouse [13] and opossum (kidney cell line) [14] receptor sequences. See Fig. 1A for explanation of symbols.

3. Results

3.1. Cloning of rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors

A rabbit genomic library contructed in λEMBL3 SP6/T7 was screened by probing with a 1.4 kb *Ssp*I–*Eco*RI fragment containing the entire human 5-$HT_{1D\alpha}$ gene. Two positive clones were obtained and one of these, clone 10, chosen for further analysis was found to contain a 4.5 kb *Hin*dIII fragment which included the entire rabbit 5-$HT_{1D\alpha}$ gene. DNA sequence analysis revealed an open reading frame encoding 377 amino acids with high (91%) overall amino acid sequence identity to the human 5-$HT_{1D\alpha}$ receptor [6]. The homology to the human, canine and rat 5-$HT_{1D\alpha}$ recepeptors is shown in Fig. 1A. The same gene bank was screened for the the 5-$HT_{1D\beta}$ receptor gene using the human 5-$HT_{1D\beta}$ gene as a probe. Two partially overlapping clones were isolated and used to construct a 1.35 kb *Nco*I–*Bam*HI fragment encoding an open reading frame of 389 amino acids with 92% amino acid identity to the human 5- $HT_{1D\beta}$ receptor, [6] (Fig. 1B). Southern blot analysis using rabbit genomic DNA detected the presence of a single band of the predicted size with each probe (not shown).

Hydropathy analysis of both these sequences predicted seven hydrophobic transmembrane spanning regions with a large third intracellular loop. This, together with the high amino acid sequence homologies to the human receptors, suggests these sequences encode the rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. All putative N-linked glycosylation sites are conserved within the N-terminal extracellular domains of each receptor, despite the presence of several unique amino acid substitutions within this region (Fig. 1A and B).

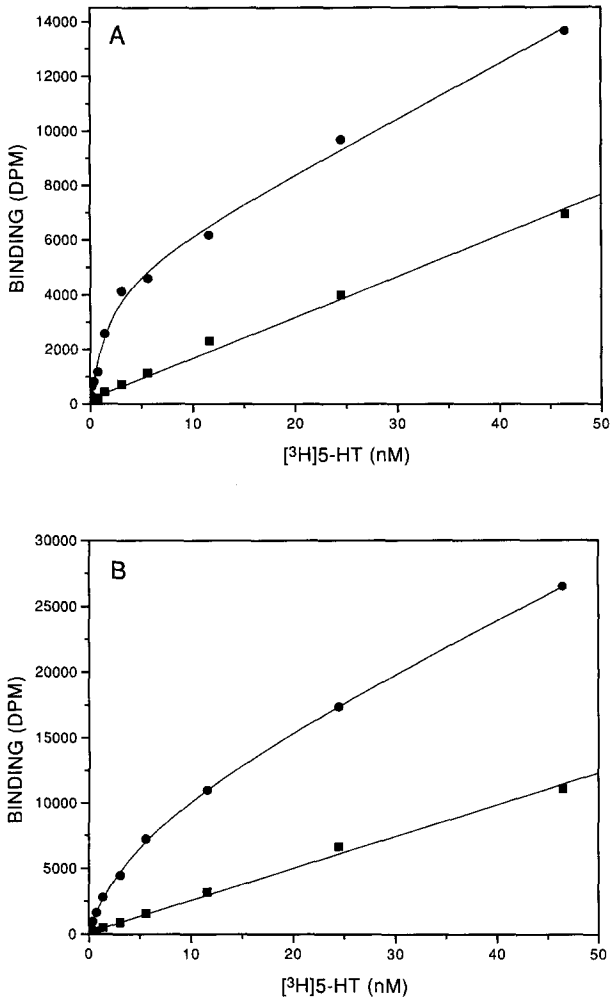

Fig. 2. Saturation binding of [^{3}H]5-HT to rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors. COS-7 cells were transfected with cloned 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptors, membranes prepared and binding was measured after incubation with ligand for 30 min. (A) 5-HT$_{1D\alpha}$. (B) 5-HT$_{1D\beta}$. ● = total binding; ■ = non-specific binding.

3.2. Pharmacological analysis

The rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ genes were subcloned into the expression vector pSVL (Pharmacia). The ligand binding properties of the receptors were investigated using [^{3}H]5-HT together with a range of ligands following transient transfection of 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ genes into COS-7 cells. [^{3}H]5-HT binding was measured over a range of ligand concentrations (0.025–50 nM) and non-specific binding was linear over this range. Representative 5-HT saturation binding curves for both receptors are shown in Fig. 2A and B. Hill slope parameters were not significantly different from unity. The pK_d estimates (n = 3) for 5-HT for the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors were -8.47 ± 0.15 (K_d = 3.8 nM) and -7.95 ± 0.05 (K_d = 11.2 nM) respectively, with B_{max} values of 0.37 ± 0.05 pmol/mg for 5-HT$_{1D\alpha}$, and 0.87 ± 0.12 for 5-HT$_{1D\beta}$. pIC$_{50}$ values and Hill slope parameters for the competition curves of the ligands are given in Table 1. In general Hill slope parameters were less than unity, therefore it is invalid to calculate K_i affinity estimates. These values show good agreement with the affinity values derived from studies on the cloned human receptors, with the exception of sumatriptan which has a higher affinity at the human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors than at their respective rabbit homologues. The rank order of affinity for the rabbit 5-HT$_{1D\alpha}$ receptor is: 5-CT>5-HT = metergoline > ketanserin = sumatriptan > pindolol. For the rabbit 5-HT$_{1D\beta}$ receptor the order is: 5-CT > 5-HT = metergoline > sumatriptan > ketanserin > pindolol. The low affinity of pindolol at the rabbit 5-HT$_{1D\beta}$ receptor is consistent with the amino acid sequence (see above) predicting a 5-HT$_{1D}$ pharmacology rather than a 5-HT$_{1B}$ pharmacology. Ketanserin displayed an 800-fold greater affinity for the rabbit 5-HT$_{1D\alpha}$ receptor over the 5-HT$_{1D\beta}$ receptor, as seen with the human receptors [15].

4. Discussion

We have cloned the rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor genes from genomic DNA using high stringency hybridisation with the corresponding human gene probes. The rabbit 5-HT$_{1D\alpha}$ receptor gene encodes an open reading frame of 377 amino acids with high amino acid sequence homology to the human, canine and rat receptors. The rabbit 5-HT$_{1D\beta}$ receptor encodes a 390 amino acid protein and, like the 5-HT$_{1D\alpha}$ gene, shows high amino acid homology to the human receptor.

The high degree of conservation of amino acid sequence between members of both the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ subtypes suggests evolutionary pressure to maintain structural features critical to the biological function of these receptors. For example the putative N-linked glycosylation sites, protein kinase A and C recognition sites (Fig. 1A and B) and residues known to be involved in monoamine ligand interaction [16] are conserved within these two groups of receptors.

Rodent 5-HT$_{1B}$ receptors share a high degree of amino acid

Table 1
pIC$_{50}$ values for cloned rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors

Compound	Rabbit 5-HT$_{1D\alpha}$		Human 5-HT$_{1D\alpha}$	Rabbit 5-HT$_{1D\beta}$		Human 5-HT$_{1D\beta}$
	pIC$_{50}$ ± SEM	n_H ± SEM	pIC$_{50}$ ± SEM	pIC$_{50}$ ± SEM	n_H ± SEM	pIC$_{50}$ ± SEM
5-HT	8.57 ± 0.01 (1)	0.86 ± 0.08	8.33 ± 0.05 (1)	8.33 ± 0.01 (1)	0.70 ± 0.04	8.00 ± 0.18 (1)
5-CT	9.08 ± 0.02 (0.31)	0.77 ± 0.06	9.26 ± 0.06 (0.12)	8.44 ± 0.01 (0.78)	0.68 ± 0.04	8.41 ± 0.01 (0.39)
Metergoline	8.51 ± 0.01 (1.15)	0.58 ± 0.06	8.64 ± 0.13 (0.49)	8.13 ± 0.12 (1.6)	0.68 ± 0.09	7.62 ± 0.01 (2.4)
Ketanserin	7.49 ± 0.25 (12)	0.56 ± 0.12	7.36 ± 0.06 (1.45)	5.40 ± 0.10 (831)	0.72 ± 0.10	5.27 ± 0.01 (537)
Sumatriptan	7.45 ± 0.12 (13)	1.08 ± 0.20	8.17 ± 0.03 (9.3)	6.52 ± 0.09 (65)	0.86 ± 0.11	7.42 ± 0.01 (3.8)
Pindolol	4.75 ± 0.12 (6606)	1.04 ± 0.06	4.96 ± 0.07 (2344)	4.30 ± 0.12 (10715)	1.43 ± 0.09	4.96 ± 0.04 (1096)

COS-7 cells were transfected with expression vectors encoding 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptors. Cell membranes were prepared and 1.5 nM [^{3}H]5-HT binding was measured in the presence of a variety of ligands. pIC$_{50}$ values represent the negative log$_{10}$ concentration of ligands at which 50% of the specific bound [^{3}H]5-HT could be displaced. pIC$_{50}$ values and Hill coefficients (n_H) are means ± S.E.M. from 4–10 determinations. The values for the human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ are taken from [15]. Affinity ratios are bracketed below the pIC$_{50}$ values.

sequence identity with human 5-$HT_{1D\beta}$ receptors [3,6,13] but have a distinct pharmacology exemplified by a high affinity for β-adrenergic antagonists, e.g. pindolol. The molecular basis for this difference has been localised to a threonine residue in TM7 of the human 5-$HT_{1D\beta}$ receptor. Mutation of this residue to asparagine confers a 5-HT_{1B} pharmacology with a high affinity for pindolol [17]. The presence of a threonine residue in the rabbit 5-$HT_{1D\beta}$ receptor at this position is consistent with the observed low affinity for pindolol at this receptor and restriction of the 5-HT_{1B} pharmacology to rodents. The rabbit represents the second species from which a 5-$HT_{1D\beta}$ subtype receptor has been characterised at the molecular level; the human is the only other species from which a 5-$HT_{1D\beta}$ receptor has been cloned.

The pharmacogical profile at the rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors were generally consistent with the data previously generated for the human receptors (see Table 1), including the considerably higher affinity of ketanserin for the 5-$HT_{1D\alpha}$ receptor compared with the 5-$HT_{1D\beta}$ receptor. However sumatriptan displayed a lower affinity for the rabbit receptors, particularly for the 5-$HT_{1D\beta}$ receptor where it had a 65-fold lower affinity than 5-HT, compared with an affinity ratio of only 4-fold at the human 5-$HT_{1D\beta}$ receptor.

The molecular basis for the higher affinity of sumatriptan for the human compared to the rabbit 5-$HT_{1D\beta}$ receptor could be addressed by examining the role of the amino acid changes between these receptors within the transmembrane domains. Construction of chimaeric human–rabbit receptors and site directed mutagenesis experiments may provide further insights into the molecular basis for this difference.

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