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Molecular cloning and pharmacological characterization of serotonin 5-HT_{3A} receptor subtype in dog

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Abstract

In order to establish if the canine 5-hydroxytryptamine type 3A (5-HT_{3A}) receptors share the pharmacological profile with human 5-HT_{3A} receptors, we cloned and performed a molecular pharmacological characterization of the canine 5-HT_{3A} receptor. The 5-HT_{3A} cDNA was cloned from canine brain by polymerase chain reaction amplification. It encodes a 483 amino acid peptide that exhibits from 80% (mice) to 90% (ferrets) identity to other sequenced mammalian 5-HT_{3A} receptors. The receptor agonists 5-hydroxytryptamine (5-HT) and *meta*-chlorophenylbiguanide (mCPBG) showed little differences between the two species, whereas 2-methyl-5-hydroxytryptamine (2-Me-5-HT) was ten times weaker at canine receptors than at human receptors. The potencies at the canine 5-HT_3 receptors were 9.9 μM (5-HT), 79 μM (2-Me-5-HT) and 0.8 μM (mCPBG). The selective, competitive receptor antagonist ondansetron was ten times more potent at human receptors compared to canine receptors (K_b=0.9 nM), while (+)-tubocurarine was 1000-fold more potent at canine receptors (K_b=3.0 nM) than at human receptors. Examination of the presumed ligand binding extracellular domain revealed one residue, where the canine receptor differs from all previously characterized 5-HT_{3A} receptors, i.e. other species contain a conserved Trp^{195}, whereas the canine orthologue contains a Leu^{195}. To address the differences in potencies at the human and canine 5-HT_{3A} receptors seen in this study, we introduced a L195W point mutation in the canine orthologue. Data showed that the 195 residue can affect receptor agonist potency and efficacy as well as antagonist potency, but did produce a pharmacological profile identical to the human orthologue. We therefore conclude that position 195 is strongly involved in the receptor–ligand interaction, but additional residues must contribute to the overall pharmacological profile.

Keywords: 5-HT_3 receptor; 5-HT_3 receptor agonist; 5-HT_3 receptor receptor antagonist; 5-HT_{3A}; (Canine); (Dog)

1. Introduction

5-hydroxytryptamine (5-HT) mediates its pharmacological actions via activation of both G-protein-coupled receptors and ionotropic receptors. Whereas much attention has been directed towards the G-protein-coupled 5-HT receptors, which subsequently has resulted in the development of tool compounds and drugs, the detailed understanding of the ionotropic 5-HT_3 receptor is still under rapid expansion (Hoyer et al., 2002; Panicker et al., 2004).

5-HT_3 receptors are formed as pentameric assemblies of subunits. Homomeric 5-HT_{3A} receptor orthologues, from mouse, rat, guinea pig, ferret and human, can be surface expressed and result in functional receptors in heterologous expression systems (Maricq et al., 1991; Mair et al., 1998; Lankiewicz et al., 1998; Mochizuki et al., 2000). The 5-HT_{3B} receptor subunits are not able to form functional receptors on their own and are not expressed on the plasma membrane. However, evidence suggests that at least some native 5-HT_{3A} receptors in both human and rodents, are co-expressed with 5-HT_{3B} receptor subunits (Maricq et al., 1991; Hapfelmeier et al., 2003; Reeves and Lummis, 2002). Heteromeric assemblies of human 5-HT_{3A} receptor subunits and 5-HT_{3B} receptor subunits, of unknown stoichiometry, form functional receptors which display a large single channel conductance, low permeability to calcium ions, and a current–voltage relationship, which resembles that of characterized native 5-HT_3 channels (Davies et al.,

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1999; Kelley et al., 2003). The heteromeric receptors also display distinctive pharmacological properties compared to the homomeric receptors (Das and Dillon, 2003; Stevens et al., 2005).

Several other 5-HT_3 receptor subunits have recently been identified (C through E), but an understanding of the functional significance of these subunits and the assembly with other 5-HT_3 receptor subunits is still lacking (Niesler et al., 2003; Karnovsky et al., 2003).

Species differences in receptor pharmacology in recombinantly expressed 5-HT_{3A} receptors have previously been documented (Jackson and Yakel, 1995). The most dramatic example is (+)-tubocurarine ((+)-Tc), which demonstrated an ~1800-fold difference in antagonist potency at human and mouse 5-HT_3 receptor subunit orthologues (Belelli et al., 1995). The structural basis of the selective action of (+)-Tc was addressed in 1999 using mouse and human 5-HT_3 receptor chimaeras. By construction of subunit chimaeras, wherein the N-terminal domains of the human and mouse 5-HT_3 receptor subunits were exchanged reciprocally it was shown that the structural determinants of the discriminatory potency of (+)-Tc were located entirely within the extracellular N-terminal domain (Hope et al., 1999).

Since dogs are used routinely in the safety evaluation of novel drugs, a detailed understanding of the correlation between the pharmacological profile of a drug in dogs and humans is important. 5-HT_3 receptors are abundantly expressed in both the human and canine central and peripheral nervous system, so eventual 5-HT_3 receptor mediated side effects in man may be predicted from dog studies. In order to establish if the overall pharmacological profile of canine 5-HT_{3A} receptors is comparable to human 5-HT_{3A} receptors, we isolated cDNA from canine brain and expressed the homo-oligomeric receptors in *Xenopus* oocytes. The electrophysiological and pharmacological properties of the recombinant receptor from canine were examined.

2. Materials and methods

2.1. Cloning of canine 5-HT_{3A} receptor

Total RNA was isolated from beagle dog brain with RNAeasy mini kit (Qiagen, Hilden, Germany). cDNA was prepared by oligo-dT primed reverse transcription using the Taqman kit (Applied Biosystems, Foster City, USA). The sequence around start and stop of the canine 5-HT_{3A} coding region was identified by comparison of the canine genomic sequences to ferret (Mochizuki et al., 2000) and human (Maricq et al., 1991) 5-HT_{3A} protein sequences using TBLASTN at NCBI. On the basis of that analysis, canine 5-HT_{3A} primers were made at either end of the coding region: forward primer: 5′-*ccggaatt*caccatgctgttgtgggt-3′ and reverse primer: 5′-*gagctctag*actcaagaatactgccagatgga-3′ (restriction site overhangs without homology to the canine sequence are in italics). These were used to amplify the gene by polymerase chain reaction (PCR) with Pfu polymerase (Stratagene, Heidelberg, Germany), and the resulting about 1500 bp product was extracted from the gel with Freeze'n Squeeze DNA (Biorad, Hercules, USA) and cloned into pGEMHE (Liman et al., 1992) using *Eco*R1 and *Xba*1. Six independent clones were sequenced — all were identical. The nucleic acid sequence has been submitted to Genbank with the accession number D2483094.

2.2. RNA

The pGEMHE DNA was linearised with *Nhe*1 and the linearised DNA was transcribed in vitro using the mMessage mMachine kit from Ambion (Austin, Texas). The quality of the RNA was evaluated by electrophoresis.

2.3. Expression and electrophysiological studies

Xenopus oocytes were isolated as previously described (Hope et al., 1993) and incubated for 24 h in Modified Barth's Saline buffer [88 mM NaCl, 1 mM KCl, 15 mM HEPES, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$] supplemented with 2 mM sodium pyruvate, 0.1 U/l penicillin and 0.1 μg/l streptomycin. Stage IV oocytes were identified and injected with 23–50 nl of nuclease free water containing 20–50 ng of cRNA. Oocytes were placed in a 60 μl bath and perfused with Ringer buffer [115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7.5]. Cells were impaled with agar plugged 0.5–1 MΩ electrodes containing 3 M KCl and voltage clamped at −70 mV by a GeneClamp 500B amplifier (Axon Instruments). The cells were continuously perfused with Ringer buffer at 4–6 ml/min, and the drugs were applied in the perfusate. Receptor agonist-solutions were applied until the peak of the response was observed, usually after 30 s or less. Five minutes of wash time between 5-HT applications were allowed in order to recover from receptor desensitization. The potencies of 5-HT_3 receptor antagonists were examined by measuring concentration–response curves with 5-HT in the absence and in the presence of antagonists, respectively. To ensure complete binding, receptor antagonists were pre-applied for 300 s before co-application with 5-HT. Current–voltage curves for receptor agonists induced currents were determined by the digital subtraction of sweeps consisting of voltage ramps (−90 to +40 mV; 0.05 mV ms^{-1}) applied at the peak of the current response in the presence and absence, respectively of 5-HT 1 μM (1 μM 5-HT did not evoke measurable desensitization during the period of receptor agonist application at any of the measured potentials).

2.4. Data analysis

Concentration–response curves were fitted by use of the non-linear, least-squares fitting program, GraFit 5.0.11 (Erithacus Software, Ltd.). 5-HT_3 receptor agonist responses were fitted to the Hill equation (present in GraFit 5.011 by Erithacus Software, Ltd.):

$$E = \frac{E_{max} \cdot [L]^{n_H}}{EC_{50}^{n_H} + [L]^{n_H}}$$

where E_{max} is the maximum response, [L] is the concentration of the ligand, EC_{50} is the concentration of the ligand evoking a half maximal response and n_H is the Hill coefficient. K_b values of competitive receptor antagonists were calculated by use of the Waud equation (Lazareno and Birdsall, 1993):

$$E = \frac{E_{max} \times [L]^{n_H}}{EC_{50}^{n_H} \cdot \left(1 + \frac{[I]}{K_b}\right) + [L]^{n_H}}$$

and the dissociation constants for the reversible noncompetitive receptor antagonists were calculated using the following equation (Krehan et al., 2006):

$$E = \frac{E_{max} \times [L]^{n_H}}{EC_{50}^{n_H} + [L]^{n_H}} \times \frac{1}{1 + \frac{[I]}{K_b}}$$

where I is the concentration of antagonist. Geometric means of the EC_{50} and K_b values obtained for the individual fits were calculated and the results presented as mean±S.E.M.

Statistical significance, using SigmaStat 3.0.1 (SPSS, inc.), was determined by the use of Student's *t*-test or Mann–Whitney Rank Sum Test, as appropriate.

2.5. Drugs

5-HT creatinine sulphate, 2-methyl-5-HT maleate (2-Me-5-HT), *meta*-chlorophenylbiguanide (mCPBG), (+)-tubocurarine chloride hydrate ((+)-Tc) and ondansetron hydrochloride, were obtained from Sigma-Aldrich. All drugs were freshly dissolved as concentrated stock solutions in either double-distilled de-ionised water or Modified Barth's solution and diluted into Modified Barth's solution. To prevent degradation, 10 mM ascorbic acid was added to the 10 μM 5-HT stock solution.

3. Results

3.1. Cloning of canine 5-HT_{3A} receptor cDNA

Using primers designed from comparisons between known 5-HT_{3A} sequences and canine genomic sequences, the 5-HT_{3A} cDNA was cloned by RT-PCR from canine brain RNA. The canine 5-HT_{3A} receptor cDNA clones encode a peptide of 483 amino acids that is very similar in structure to the other known 5-HT_{3A} receptors, with only minor or no deletion/insertion events. Fig. 1 shows an alignment of the canine sequence with

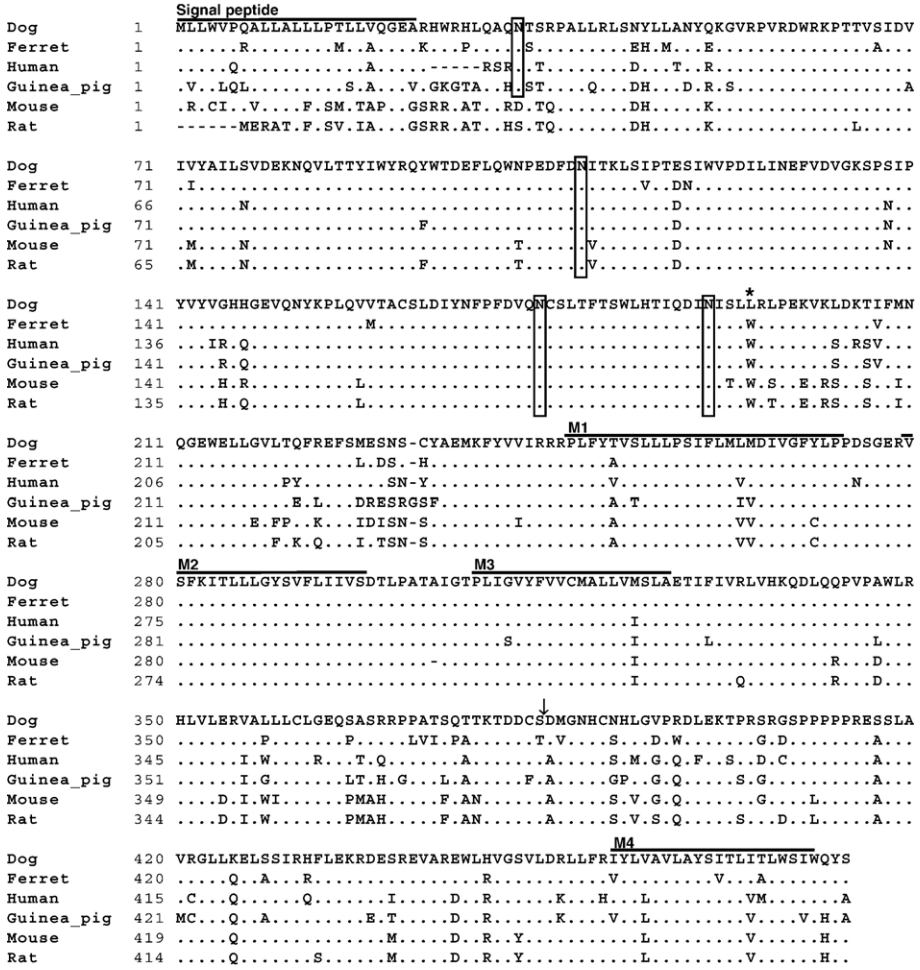

Fig. 1. Amino acid sequence alignment of sequenced mammalian 5-HT_{3A} receptors. Sequences from dog, ferret, human and the short splice variants from guinea pig, mouse and rat were aligned. Putative signal peptide and M1–M4 domains are marked with lines. (↓) Indicates the position of the extra 5–6 amino acids in the long murine splice variant. (*) marks L195 — this position is a tryptophan in all other sequenced 5-HT_{3A} receptors. Consensus *N*-glycosylation sites are marked with boxes.

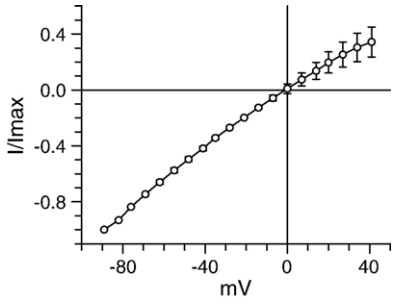

Fig. 2. Current/voltage relationship of the canine 5-HT_{3A} receptor. While applying 1 μM 5-HT (V_h −70 mV), voltage ramps (−90 to +40 mV; 0.05 mV/ms) were delivered. No pronounced inward rectification was seen. Reversal potentials were calculated from measurements in three different oocytes. Each point represents the mean±S.E.M. of 3 oocytes.

the other characterized 5-HT_{3A} receptors. The ferret 5-HT_{3A} receptor (Mochizuki et al., 2000) is most similar with 90% identity and no insertion and deletions, and the overall sequence identity to other cloned 5-HT_{3A} receptors are 87%, 83%, 82% and 80% for human, guinea pig, rat and mouse, respectively. No splice variants were observed in the canine orthologue, i.e. all clones correspond to the long murine splice variant.

3.2. Electrophysiological studies

In order to characterize the in vitro functional properties of the canine and human 5-HT_{3A} receptors, the subunits were expressed in *Xenopus* oocytes and concentration–response curves for receptor agonists and receptor antagonists were recorded using two-electrode voltage clamp. The current–voltage relationships of the canine (Fig. 2) and human (data not shown) 5-HT_{3A} receptors were obtained by applying a non-desensitizing concentration of 5-HT (1 μM) and delivering voltage ramps (−90 to +40 mV; 0.05 mV ms^{-1}) at the peak of the 5-HT induced response.

Voltage ramps from −90 to +40 mV (0.05 mV ms^{-1}) resulted in linear current–voltage relationships, with reversal potentials of −0.2±3.6 and −3.0±2.5 mV for the canine and human 5-HT_{3A} receptors, respectively, which is similar to findings of mouse (E_{rev}=+2.3 mV) and guinea pig (E_{rev}=−1.9 mV) receptors (Lankiewicz et al., 1998). A reversal potential of approximately 0 mV is consistent with the belief that the 5-HT_{3A} receptor is a relatively non-selective cationic channel that discriminates poorly among the monovalent inorganic cations (Jackson and Yakel, 1995).

We investigated the potencies and efficacies of the 5-HT_3 receptor agonists 5-HT, 2-Me-5-HT and mCPBG. Receptor agonist application to voltage-clamped *Xenopus* oocytes expressing canine or human 5-HT_{3A} receptors elicited inward desensitizing currents. Concentration–response curves were fitted to the Hill equation and derived parameters are presented in Table 1. The rank order of potencies of the 5-HT_3 receptor agonists at canine 5-HT_{3A} receptor was mCPBG>5-HT≫2-Me-5-HT (Fig. 3) and the corresponding order of efficacies was 5-HT>mCPBG>2-Me-5-HT.

Recordings at the human 5-HT_{3A} receptor yielded the order of potency as mCPBG≥5-HT≥2-Me-5-HT and efficacies were 5-HT≥2-Me-5-HT>mCPBG. The results obtained at the human 5-HT_{3A} receptor are consistent with previous findings (Davies et al., 1999; Miyake et al., 1995).

The results show that large differences in receptor agonist potency at the canine orthologue exist, where mCPBG is 10 times as potent as 5-HT, which again is eight times as potent as 2-Me-5-HT (see Table 1). Contrary to this, the characterized receptor agonist potencies at the human receptor are of the same order of magnitude. Considering both efficacy and potency the functional selectivity of 5-HT and mCPBG at the two orthologues is very small, whereas 2-Me-5-HT displays a large functional selectivity. Thus, as illustrated in Fig. 3, 5-HT and mCPBG at similar concentrations activate both human and canine receptors, whereas 2-Me-5-HT, at lower concentrations, primarily activates the human receptor.

Table 1
Summary of agonist and antagonist activities obtained by fitting dose response curves in *Xenopus* oocytes expressing human or canine 5-HT_{3A} receptors, n=4

Agonists species/drug	EC_{50} (μM)	pEC_{50} (±S.E.M.)	n_H (±S.E.M.)	Maximal response[‡] (±S.E.M.)
Canine				
5-HT	9.9[a]	5.00±0.03	1.78±0.13	100±1.5
2-Me-5-HT	79[c]	4.10±0.09	1.49±0.14	49±7.8[b]
mCPBG	0.8∗	6.09±0.06	1.91±0.27	69±10.4
Human				
5-HT	4.2	5.38±0.05	2.00±0.31	100±1.2
2-Me-5-HT	5.9	5.23±0.04	2.62±0.31	95±7.1
mCPBG	2.6	5.59±0.12	2.37±0.25	76±7.6

Antagonists species	Ondansetron		(+)-Tubocurarine	
	K_b (nM)	pK_b (±S.E.M.)	K_b (nM)	pK_b (±S.E.M.)
Canine	0.90[a]	9.05±0.08	3.0[c]	8.53±0.18
Human	0.086	10.07±0.13	2862	5.54±0.08

[a, b] and [c] indicate that a significant difference between the canine and human orthologue was found at $P<0.05$, $P<0.01$ and $P<0.001$, respectively. [‡]Maximal response is relative to 5-HT I_{max}.

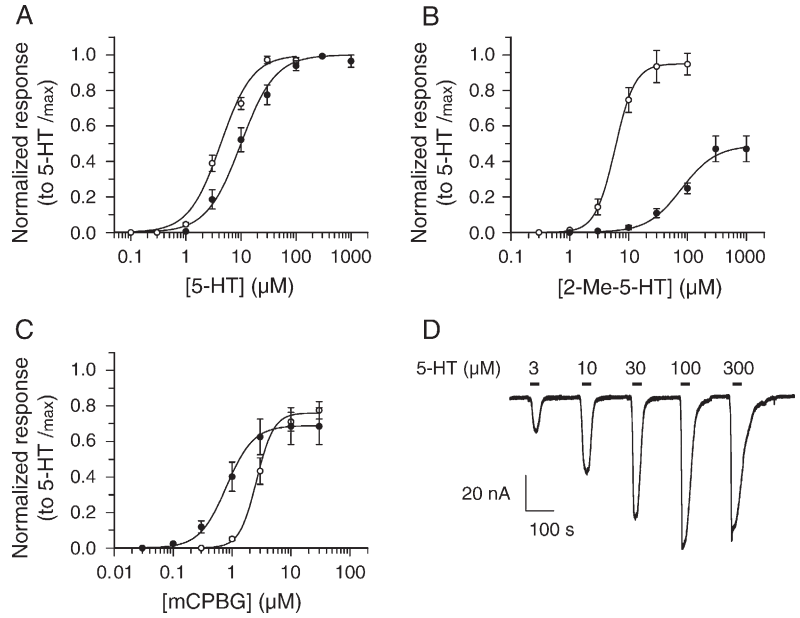

Fig. 3. Concentration–response curves for 5-HT (A), 2-Me-5-HT (B) and mCPBG (C) obtained at human 5-HT_{3A} (open circles) and canine 5-HT_{3A} receptors (closed circles) expressed in *Xenopus* oocytes. Notice that the *x*-axis for mCPBG in graph C is different from the other graphs. Currents were normalized to 5-HT I_{max}. Each point represents the mean±S.E.M. of 4 oocytes. Representative traces for 5-HT at canine 5-HT_{3A} receptors (D).

To elucidate the receptor antagonists pharmacology, we used ondansetron, a potent and selective inhibitor of 5-HT_3 receptors, and (+)-Tc, a structurally rigid, non-selective receptor antagonist, with a potency that has been shown to be highly variable among species (Mochizuki et al., 2000; Belelli et al., 1995). Concentration–response curves for 5-HT, in the absence of or with (+)-Tc or ondansetron were recorded (Fig. 4), and K_b values were calculated and are presented in Table 1.

As illustrated in Fig. 4, the antagonism by (+)-Tc, in lower concentrations at the canine orthologue, was competitive and readily reversible, whereas high concentrations of (+)-Tc at the human orthologue resulted in insurmountable antagonism,

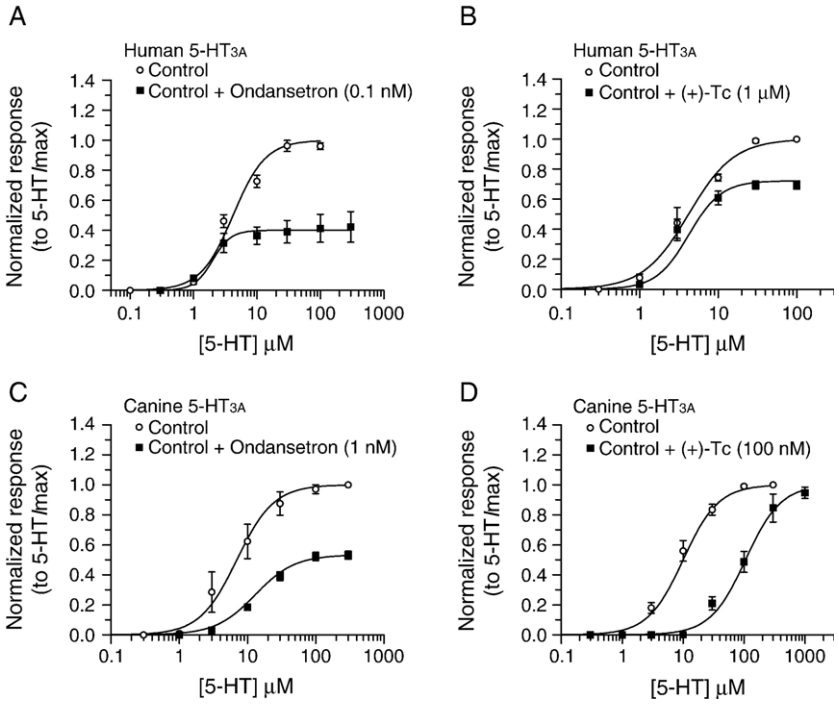

Fig. 4. Concentration–response curves for 5-HT alone (open circles) or in the presence of receptor antagonists (closed squares). A, B were recorded at human and C, D at canine 5-HT_{3A} receptors expressed in *Xenopus* oocytes. Each point represents the mean±S.E.M. of 4 oocytes.

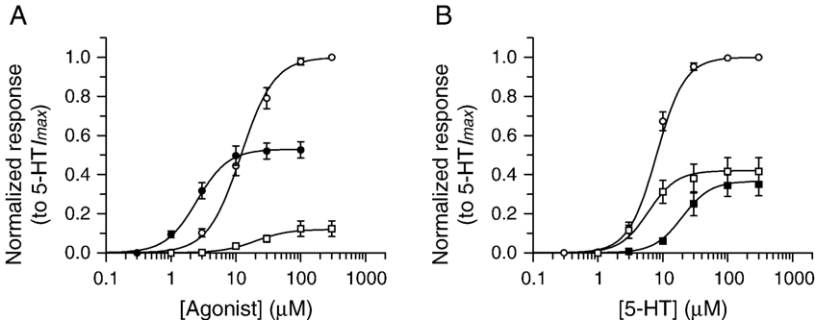

Fig. 5. (A) Concentration–response curves for 5-HT (open circles), 2-Me-5-HT (open squares) and mCPBG (closed circles) obtained at the canine L195W 5-HT_{3A} mutant receptors expressed in *Xenopus* oocytes. Currents were normalized to 5-HT I_{max}. Each point represents the mean±S.E.M. of 4 oocytes. (B) Concentration–response curves for 5-HT alone (open circles) or in the presence of 100 pM ondansetron (open squares) or 100 nM (+)-Tc (closed squares) recorded at the canine L195W 5-HT_{3A} mutant receptors expressed in *Xenopus* oocytes. Each point represents the mean±S.E.M. of 4 oocytes.

which is identical to the effect mediated by 5-HT_3 receptors endogenous to guinea pig neurons (Newberry et al., 1991). The effect of ondansetron was insurmountable, which is in agreement with findings reported at the rat 5-HT_{3A} receptor (Mair et al., 1998).

With a K_b value of 900 pM, at the canine 5-HT_{3A} receptor, ondansetron proved to be a potent inhibitor of 5-HT induced currents. However, the potency of ondansetron at the cloned canine 5-HT_{3A} receptor was lower than that for the cloned human 5-HT_{3A} receptor (K_b=86 pM) measured in this study and also the lowest compared to what has been reported in other species; mouse (IC_{50}=440 pM; (Gill et al., 1995)), rat (IC_{50}=231 pM; (Mair et al., 1998)) and ferret (K_b∼130 pM; (Mochizuki et al., 2000)).

(+)-Tc inhibited 5-HT induced currents, with K_b values of 3.0 nM at canine 5-HT_{3A} receptors and 2862 nM at human 5-HT_{3A} receptors. The potency at canine 5-HT_{3A} receptors is comparable to results obtained at mouse 5-HT_{3A} receptors and the potency found for human 5-HT_{3A} receptors is in line with previous results (Belelli et al., 1995).

All 5-HT_{3A} from previously cloned species, including ferret, human, guinea pig, mouse and rat contain a conserved Trp^{195} residue in the presumed ligand binding first extracellular domain. In the canine orthologue, however, cloned for the first time in this study, the aliphatic amino acid leucine takes the place of the aromatic tryptophan, possibly playing an important role in the distinct pharmacology of the canine 5-HT_{3A} receptor.

To investigate the functional role of the Leu^{195} residue in the canine receptor at the position of the conserved Trp^{195} in 5-HT_{3A} orthologues in other species, we changed the leucine residue to tryptophan using site-directed mutagenesis. The mutation of Leu^{195} resulted in functionally expressed receptors. The rank order of potencies of the 5-HT_3 receptor agonists at the mutant canine 5-HT_{3A} receptor was mCPBG>5-HT>2-Me-5-HT and the corresponding order of efficacies was 5-HT> mCPBG≫2-Me-5-HT (Fig. 5), which is similar to the wild type canine 5-HT_{3A} receptor. The efficacies and potencies of both 5-HT and mCPBG at the mutant receptor were also comparable to the wild type (Table 2). Interestingly, the mutation of Leu^{195} to Trp^{195} resulted in a 5-fold decrease in efficacy of 2-Me-5-HT and a 4-fold increase in potency.

The potencies of ondansetron and (+)-Tc were also affected by the mutation at position 195. The potency of ondansetron at the L195W mutant increased 5-fold compared with the wild type, whereas the potency of (+)-Tc decreased 12-fold compared to the wild type (Fig. 5 and Table 2).

Table 2
Summary of agonist and antagonist activities obtained by fitting dose response curves in *Xenopus* oocytes expressing the canine L195W 5-HT_{3A} mutant receptors, n=4

Drug	EC_{50} (μM)	PEC_{50} (±S.E.M.)	n_H (±S.E.M.)	K_b (nM)	pK_b (±S.E.M.)	Maximal response‡
Agonists						
5-HT	10.3	4.98±0.06	1.89±0.29			100±0.2
2-Me-5-HT	19.1[a]	4.72±0.08	1.73±0.10			9±1.7[c]
mCPBG	2.5	5.61±0.08	2.02±0.21			53±4.2
Antagonists						
Ondansetron				0.17[a]	9.77±0.13	
(+)-Tc				38[b]	7.42±0.12	

[a,b] and [c] indicate that a significant difference between the mutant and wild type canine orthologue was found at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.
‡Maximal response is relative to 5-HT I_{max}.

4. Discussion

The canine 5-HT_{3A} receptor cDNA encodes a peptide of 483 amino acids (see Fig. 1) that is very similar in structure to the other known 5-HT_{3A} receptors, with a predicted long first extracellular domain and four transmembrane domains. Similarity to other cloned 5-HT_{3A} receptors ranges from 80% to 90% amino acid identity.

The human and canine 5-HT_{3A} receptors show similar rank order in potencies of receptor agonists, where mCPBG>5-HT>2-Me-5-HT. However, whereas mCPBG increases 3-fold in potency from human to canine, 5-HT and 2-Me-5-HT lose 2 and 13-fold, respectively. In terms of relative efficacy the change from man to dog only affects 2-Me-5-HT, where the relative maximum response declines from 95% to 49%. As has been the case with 5-HT_{3A} receptors from other species, the receptor agonist pharmacology of the canine 5-HT_{3A} receptor seems to be distinctly different from other species. Thus, whereas potencies

for the 5-HT_{3A} receptor agonists in rank order may fit the observations for human, the absolute values are very different. Especially the values for 2-Me-5-HT (EC_{50}=79 μM) at the canine receptors, are very different from the other species, including ferret (EC_{50}=7.2 μM, (Mochizuki et al., 2000)), rat (EC_{50}=4.1 μM, (Mair et al., 1998)), guinea pig (EC_{50}=2.7 μM, (Lankiewicz et al., 1998)) and human (5.9 μM), suggesting that the interaction of 2-Me-5-HT with the canine 5-HT_{3A} receptor is hindered. In line with this idea are the efficacy data, where 2-Me-5-HT at the canine receptors has a significantly lower relative maximum response (to 5-HT I_{max}) than at the human 5-HT_{3A} receptors.

Ondansetron clearly differentiated between the canine (0.9 nM) and human (0.09 nM) 5-HT_{3A} receptors and proved to be more potent at the canine 5-HT_{3A} receptor than at ferret (K_b=12 nM, Mochizuki et al., 2000), mouse (IC_{50}=1.4 nM, (Hope et al., 1999)) and rat (IC_{50}=231 nM, (Mair et al., 1998)).

(+)-Tc has long been known as a highly effective discriminatory agent at 5-HT_{3A} receptors of different species. A potency difference of approximately 1800-fold between the human receptor (IC_{50}=2.8 μM, Brown et al., 1998) and the mouse receptor (IC_{50}=1.8–2.1 nM; (Gill et al., 1995; Hussy et al., 1994)) has previously been addressed and explained by differences in the N-terminal domains of the orthologue subunits (Hope et al., 1999). In the present study, it was shown that (+)-Tc is very potent at the canine 5-HT_{3A} receptors (K_b=3 nM) and 1000-fold less potent at the human 5-HT_{3A} receptors (K_b=2900 nM). The potency seen at the canine orthologue closely resembles that of the mouse receptor (1.8–2.1 nM; (Gill et al., 1995; Hussy et al., 1994)) and is within 10-fold of the rat (IC_{50}=31.9 nM; (Mair et al., 1998)) and ferret (IC_{50}=27 nM; (Mochizuki et al., 2000)) 5-HT_{3A} receptors. The receptor antagonists ondansetron and (+)-Tc showed a 5-fold increase and a 12-fold decrease in potency at the mutant compared to the wild type receptor.

In the $GABA_A$ and the ionotropic glutamate receptor systems, the potency of receptor antagonists remains constant between species and independent of subunit composition. It has been proposed that since the physical binding site remains intact, irrespective of the subunits, the molecular interaction between receptor antagonists and binding site remains constant and hence displays a constant K_b value (Ebert et al., 1997). In the 5-HT_{3A} receptors this is clearly not the case, since the potency of the receptor antagonists are highly dependent on species. Our interpretation of these data is that the compounds may bind to different sites, depending on the origin of the 5-HT_{3A} receptors. The interaction between receptor antagonists and 5-HT_{3A} receptors may therefore display a species dependent structure activity relationship, which may prevent extrapolations from one species to another. Even closely related species like rat and mouse display a very high variation in the potency of ondansetron and (+)-Tc.

Biochemical and mutagenesis studies have indicated that the ligand binding site of the 5-HT_3 receptor is formed by six loops, A–F, located in the extracellular N-terminal domain (Thompson et al., 2005). The conserved tryptophan residues, Trp^{90}, Trp^{183} and Trp^{195} have attracted attention, and have been suggested to play critical roles in ligand binding (Spier and Lummis, 2000), where it is assumed that the primary ammonium of serotonin makes a strong cation–π interaction with Trp^{183} (Mu et al., 2003). Site-directed mutagenesis studies in the mouse 5-HT_{3A} subunit have shown that mutation of Trp^{195} to serine results in functionally expressed receptors with a 9-fold reduced potency for 5-HT, a small 2–3-fold decrease in mCPBG potency and an 8-fold decreased affinity for [^{3}H] granisetron (Spier and Lummis, 2000). Comparison of the first extracellular domain from the sequenced 5-HT_{3A} receptors revealed that the canine receptor has a leucine at position 195, instead of the otherwise conserved tryptophan. Leucine is a more conservative substitution for tryptophan than the small polar residue serine, but leucine is smaller than tryptophan and aliphatic instead of aromatic. Interestingly, all sequenced 5-HT_{3B} receptors also have a leucine at the corresponding position.

To address the differences in potencies at the human and canine 5-HT_{3A} receptors seen in this study, we introduced a L195W point mutation. This exchange is associated with the incorporation of an aromatic group and concomitant increase in side chain volume in the canine orthologue. The electrophysiological effects of both 5-HT and mCPBG were largely unaltered, whereas 2-Me-5-HT showed a 5-fold decrease in efficacy and a 4-fold increase in potency at the L195W mutant compared to the wild type canine 5-HT_{3A} receptor. Assuming that the single-channel conductance, the desensitization rates and the efficiency of transfection remain unchanged (Colquhoun, 1998; Lynch, 2004), our data with 2-Me-5-HT are therefore in agreement with the notion that this compound must bind to the 5-HT_{3A} receptor in a manner different from that of both 5-HT and mCPBG. In agreement with proposals from the $GABA_A$ field (Mortensen et al., 2004) it is tempting to speculate that at the mutant receptor 2-Me-5-HT binds in a manner which prevents longer opening times. Since the potencies of both receptor antagonists also were affected by the singlepoint mutation, we conclude that position 195 is strongly involved in the receptor–ligand interaction.

It has been proposed that Trp^{195} affects a region of the binding pocket which plays a part in receptor antagonist and to a smaller extent receptor agonist binding (Spier and Lummis, 2000). Our data confirm that Trp^{195} is important for the potency of receptor antagonists, whereas 2-Me-5-HT was the only receptor agonist significantly affected by the mutation, with both an increase in potency and in particular a decrease in efficacy. However, since the overall pharmacological profile of the mutated canine receptor is different from the human orthologue, it is clear that other amino acid residues must contribute to the binding and conformational change involved in translating binding into channel opening.

The current–voltage relationship of the canine and human 5-HT_{3A} receptors was recorded using voltage ramps. The canine and human 5-HT_{3A} receptor had similar linear I/V curves, with little or no inward rectification, and reversal potentials around 0 mV, which corresponds well to a non-selective cationic channel. One of the prominent functional features of the 5-HT_3 receptor 5-HT current in hippocampal interneurons is a region of negative slope conductance in the I–V curve (Van Hooft and Wadman, 2003; Kawa, 1994). This region of negative slope conductance is due to voltage-

dependent block by calcium ions, analogous to the voltage-dependent block by magnesium ions of the *N*-methyl-D-aspartate (NMDA) receptor (Nowak et al., 1984). Except for two reports on the expression of homomeric 5-HT_{3A} receptor in *Xenopus* oocytes (Maricq et al., 1991; Eisele et al., 1993) the voltage-dependent block by Ca^{2+} ions, inducing the region of negative slope conductance in the *I–V* curve, has not been observed with 5-HT_3 receptor in clonal cell lines or heterologously expressed 5-HT_3 receptor (Van Hooft and Wadman, 2003). However, reports have shown that both 5-HT_3 receptor in N1E-115 neuroblastoma cells and cloned 5-HT_3 receptor receptors can be inhibited by physiological concentrations of Ca^{2+} (Peters et al., 1988; Gill et al., 1995). The inhibitory effect of Ca^{2+} and other divalent cations has been suggested to be a combined effect of a noncompetitive interaction of divalent cations within the pore (which very likely would be voltage-dependent) and via a site associated with the receptor agonist binding site, which appears to be competitive (Niemeyer and Lummis, 2001).

In conclusion, the present study provides the initial characterization of the pharmacological properties of the canine 5-HT_{3A} receptor subunit expressed in *Xenopus* oocytes. Both receptor agonist and receptor antagonist used were able to discriminate between the canine and human 5-HT_{3A} receptors, revealing a unique pharmacological profile of the canine 5-HT_{3A} receptors. The canine is frequently used to assess physiological or toxicological liabilities of early drug candidates, wherefore it is important to address the difference in pharmacology between the human and canine 5-HT_{3A} receptor.

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