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Cloning, expression, and characterization of ferret 5-HT_3 receptor subunit

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Abstract

Ferrets (*Mustela putorius furo*) are useful animals for determining anti-emetic activity via 5-HT_3 receptors in vivo. We isolated a cDNA encoding the 5-hydroxytryptamine (5-HT) type 3A receptor subunit (5-HT_{3A}) from ferret colon, expressed it in a human embryonic kidney cell line and determined its pharmacological properties. The open reading frame of the isolated cDNA encoded a 483-amino acid protein, corresponding to the shorter splice variant of 5-HT_{3A} receptors. Splice variants were no longer detected by reverse transcriptase-polymerase chain reaction. The ferret 5-HT_{3A} receptor exhibits a high degree of amino acid sequence identity ($\geq$ 80%) to that of other species. Binding studies demonstrated the following rank order of potency for agonists: *meta*-chlorophenylbiguanide (*m*CPBG) > 2-methyl-5-hydroxytryptamine (2-Me-5-HT) = 5-HT, and for antagonists: ondansetron = tropisetron > (+)-tubocurarine > metoclopramide. Electrophysiological studies revealed that *m*CPBG was a partial agonist and 2-Me-5-HT was an almost fully effective agonist compared to 5-HT.

Keywords: (Ferret); 5-HT_3 receptor; 5-HT_3 receptor agonist; 5-HT_3 receptor antagonist; Ligand-gated ion channel; HEK 293 cell

1. Introduction

5-HT_3 receptors are ligand-gated ion channels which cause fast, depolarizing responses in the central and peripheral nervous systems, and therefore mediate many physiological and behavioral responses, such as emesis (Fozard, 1987), anxiety (Jones et al., 1988), cognition (Barnes et al., 1990) and stress-induced defecation (Miyata et al., 1992). Pharmacological studies to define the roles of 5-HT_3 receptors have been greatly facilitated by the development of selective ligands (Greenshaw, 1993).

Therapeutically, selective 5-HT_3 receptor antagonists are useful in the treatment of emesis induced by cytotoxic chemotherapy or radiation therapy (Bunce et al., 1991). To assess the effects of these 5-HT_3 receptor-selective drugs, various animal models have been used, including mice, rats, guinea pigs, ferrets (*Mustela putorius furo*), dogs and monkeys. In these models, ferrets were found to be the most useful animals to determine anti-emetic activity in vivo (Andrews et al., 1990). However, there are pharmacological differences among species (Peters et al., 1992) and these differences can lead to problems during the clinical development of drugs. To overcome these problems, the molecular pharmacological characterization of the 5-HT_3 receptors in each species is essential.

cDNAs encoding the 5-HT_{3A} receptor subunit have been cloned from many mammalian species including mouse, rat, guinea pig and human (Maricq et al., 1991; Johnson and Heinemann, 1992; Isenberg et al., 1993; Belelli et al., 1995; Miyake et al., 1995; Lankiewicz et al., 1998). These cloned 5-HT_{3A} receptors form functional homo-oligomers when expressed in *Xenopus* oocytes or mammalian cells. Our previous study demonstrated that the binding affinity of the 5-HT_3 receptor-selective agonist, *meta*-chlorophenylbiguanide (*m*CPBG), for rat 5-HT_{3A} receptors was about 100 times higher than for human 5-HT_{3A} receptors, whereas the affinity of other ligands was almost identical in both species (Miyake et al., 1995). In addition, 2-methyl-5-hydroxytryptamine (2-Me-5-HT), a partial agonist at mouse 5-HT_{3A} receptors, was found to be a full agonist at human 5-HT_{3A} receptors (Miyake et al., 1995).

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Human 5-HT_{3A} receptor mRNA is expressed in several tissues, including colon (Miyake et al., 1995). In guinea pigs, contraction mediated via 5-HT_3 receptors has been demonstrated in isolated distal colon (Miyata et al., 1991). Therefore, we isolated 5-HT_{3A} receptors from ferret colon and expressed the homo-oligomeric receptors in a human embryonic kidney cell line (HEK 293).

2. Materials and methods

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

Total RNA was isolated from adult ferret colon using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Poly(A)$^+$ RNA was prepared using Oligotex dT30 super (Takara, Shiga, Japan). A cDNA library was prepared from poly(A)$^+$ RNA with a λZAPII cDNA synthesis kit (Stratagene, CA). Nylon membranes (NEN Dupont, MA) were prepared from a plated cDNA library, and prehybridized for 1 h at 42°C in 50% formamide, 4× Denhardt's solution, 5× standard saline citrate (SSC, 1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.4), 0.1% sodium dodecyl sulfate, 25 mM Na_3PO_4, and 100 μg/ml denatured salmon sperm DNA. The membranes were hybridized for 18 h at 42°C in the same medium with ^{32}P-labelled probes. Post-hybridization washes were performed at 65°C twice in 2× SSC for 30 min and then twice in 1× SSC for 30 min. X-ray films were exposed to the membranes at −80°C overnight and analyzed. Using ^{32}P-labelled human and rat 5-HT_{3A} receptor cDNA as probes, a total of 3×10^5 phages were screened for new clones. Two positive clones (3.5 and 3.1 kb) were isolated and the longer clone was sequenced by the chain termination method (BigDye Terminator Cycle Sequencing, Applied Biosystem, CA). The nucleotide sequence of ferret 5-HT_{3A} receptor cDNA has been submitted to the GenBank with accession number AB029898.

2.2. PCR detection of alternative splicing

A reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of ferret 5-HT_{3A} receptor transcript was performed using the primers 5′-TTCTCGAAGGCCCCT-TGTCATC-3′ and 5′-CCAAATGGCTGCAGTGGTTTCC-3′. Polymerase chain reaction (PCR) with the primers amplified an 80-bp fragment, including the splicing junction, in which mouse, rat and guinea pig 5-HT_{3A} receptor genes were alternatively spliced. Total RNA (1 μg) from ferret colon or hippocampus was converted into the first-strand cDNA using an oligo(dT) primer (Advantage RT-for-PCR kit, Clontech, CA). PCR was performed in 50 μl containing the cDNA (1/25 volume of cDNA synthesis reaction mixture), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 μg/ml gelatin, all four dNTPs (each at 0.2 mM), primers (each at 0.25 μM), and 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer, CT). The thermal cycle was as follows: initial denaturation at 94°C for 1 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 15 s, and extension at 72°C for 15 s. Aliquots (15 μl) of PCR products were separated by electrophoresis on a 4% NuSieve 3:1 agarose gel (FMC BioProducts, ME), including 0.5 μg/ml ethidium bromide. As a control experiment, rat 5-HT_{3A} receptor transcripts were analyzed by RT-PCR using the primers 5′-CCACCTTCCAAGCCAACAAGA-3′ and 5′-AAG-TCCTGAGGGCTTCCGACAT-3′, to detect the expression of short and long forms of rat 5-HT_{3A} receptor through the amplification of two fragments with 79 and 94 bp. Amplified fragments were directly cloned into the plasmid pCR2.1 (Invitrogen, CA) for sequence analysis.

2.3. Cell culture and stable transfection of HEK 293 cells

Culture and transfection of HEK 293 cells (ATCC CRL 1573) were performed as described previously (Mochizuki et al., 1999b). Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were transfected using a modified calcium phosphate precipitation method (Chen and Okayama, 1987), with a combination of the mammalian expression vector pME18S (Maruyama and Takebe, 1990) containing the ferret 5-HT_{3A} receptor cDNA and the neomycin resistance gene expression vector pMC1neo PolyA (Stratagene). Single colonies of G418 (1 mg/ml)-resistant cells were isolated individually and screened for receptor expression using the patch–clamp technique. The single clone, which elicited the highest current response to 5-hydroxytryptamine (5-HT, 100 μM), was selected for further studies.

2.4. Radioligand binding studies

HEK 293 cells stably expressing the ferret 5-HT_{3A} receptor were harvested and homogenized in HEPES buffer (50 mM, pH 7.4). The homogenate was centrifuged at $40{,}000\times g$ for 30 min. The resulting pellet was resuspended in HEPES buffer and stored at −80°C until further use.

For saturation studies, membranes (8–25 μg protein) were incubated with increasing concentrations of [^{3}H]GR65630 in a final volume of 500 μl for 1 h at 25°C. For competition studies, membranes (12 μg protein) were incubated with a single concentration (170 pM) of [^{3}H]GR65630 in the absence or in the presence of increasing concentrations of competing drugs. Incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandel Cell Harvester (Brandel, MD),

followed by washing with ice-cold HEPES buffer. Radioactivity retained on the filter was measured with a liquid scintillation counter (Packard 2100TR). Non-specific binding was determined in the presence of 1–10 μM tropisetron (saturation studies, 1–10 μM; competition studies, 1 μM). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad, CA).

2.5. Electrophysiological studies

Voltage clamp measurements were performed as described previously (Hamill et al., 1981). Currents were measured using whole-cell voltage-clamp configurations with an Axopatch ID patch–clamp amplifier (Axon Instruments, CA) using pClamp 6 and AxoGraph software (Axon Instruments). Transfected HEK 293 cells were voltage-clamped at a holding potential of −64 mV. The external solution contained (in mM): NaCl 145, KCl 5, $CaCl_2$ 2.4, HEPES 10, glucose 10, pH 7.4 with NaOH. Patch pipettes (2–4 MΩ) were filled with the internal solution containing (in mM): CsCl 150, EGTA 5, HEPES 10, pH 7.2 with CsOH. Series resistance was compensated for at least 80%. All recordings were done at room temperature (25°C).

5-HT_3 receptor agonists and antagonists were applied to the cell using a rapid perfusion system as described previously (Mochizuki et al., 1999b). Drug solutions were prepared in the external solution and applied from a tapered-tip glass pipette (600 μm i.d.), positioned 2 mm from the cell being measured. The pipette was connected to solenoid valves followed by reservoirs which contained various solutions. Only one valve was open at any one time, and the external solution flowed continuously between drug applications. At a flow rate of approximately 1 ml/min, the 10–90% exchange time (determined from current response obtained when switching an open patch electrode between solutions of different ionic composition) was less than 10 ms. Constant bath perfusion was also maintained at a flow rate of approximately 0.5 ml/min. Cells were repetitively exposed to the agonists at intervals of at least 90 s in order to allow complete recovery from desensitization.

2.6. Data analysis

Sequence motifs of 5-HT_{3A} receptors were analyzed using the PROSITE motif dictionary (release 15.0).

Radioligand binding and electrophysiological data were analyzed with a nonlinear curve fitting program (SAS Institute, Japan). The maximal binding of [^{3}H]GR65630 at equilibrium (B_{max}) and the equilibrium dissociation constant (K_d) were derived directly from saturation curves fitted with the one-site ligand binding model. IC_{50} values, the concentration required to inhibit specific binding by 50%, were calculated using the Hill equation. The inhibition constant (K_i) of the competing drug was calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$

where L is the radioligand concentration.

Concentration–response curves in electrophysiological studies were also fitted to the Hill equation. The antagonist dissociation constant (K_b) was calculated using the following equation (Craig, 1993):

$$K_b = \frac{IC_{50}}{1 + [A]/EC_{50}}$$

where IC_{50} is the antagonist concentration required to inhibit the agonist response by 50%, A is the agonist concentration used to induce the response, and EC_{50} is the agonist concentration evoking a half-maximal response.

2.7. Drugs

2-Me-5-HT maleate, *m*CPBG hydrochloride, *endo*-8-methyl-8-azabicyclo[3.2.1]oct-3-yl-1*H*-indol-3-carboxylate hydrochloride (tropisetron), 4-amino-5-chloro-*N*-[(2-diethylamino)ethyl]-2-methoxy-bemzamide hydrochloride (metoclopramide hydrochloride) and (−)-9,10-didehydro-*N*-(1-(hydroxymethyl)propyl)-1,6-dimethyl-ergoline-8-β-carboxyamide maleate (methysergide maleate) were purchased from Research Biochemicals (Natick, MA); 5-HT creatinine sulfate was from Merck; (+)-tubocurarine was from Nakarai Tesque (Kyoto, Japan); and 3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-[^{3}H]-1*H*-indol-3-yl)-1-propanone, 2331 GBq/mmol ([^{3}H]GR65630) was from NEN Dupont. 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazole-1-yl)methy]-4*H*-carbazole-4-one hydrochloride (ondansetron) was synthesized at the Yamanouchi Institute for Drug Discovery Research.

3. Results

3.1. Structure of ferret 5-HT_{3A} receptor

A 3597-nucleotide cDNA sequence of the ferret 5-HT_{3A} receptor has an open reading frame, encoding a predicted 483-amino acid protein, with a potential signal peptide. Hydrophobicity analysis of the deduced amino acid sequence revealed features typical of ligand-gated ion channels, with four putative transmembrane domains (M1–M4), a large N-terminal extracellular domain, and a large cytoplasmic loop (Maricq et al., 1991). Also, the N-terminal extracellular domain contained the Cys–Cys loop (Cys^{162} and Cys^{176}), typical of nicotinic, glycine and $GABA_A$ receptor channels (Maricq et al., 1991). Potential sites for *N*-glycosylation (Asn^{33}, Asn^{109}, Asn^{175} and Asn^{191}) and protein kinase C phosphorylation (Ser^{368}, Thr^{402} and

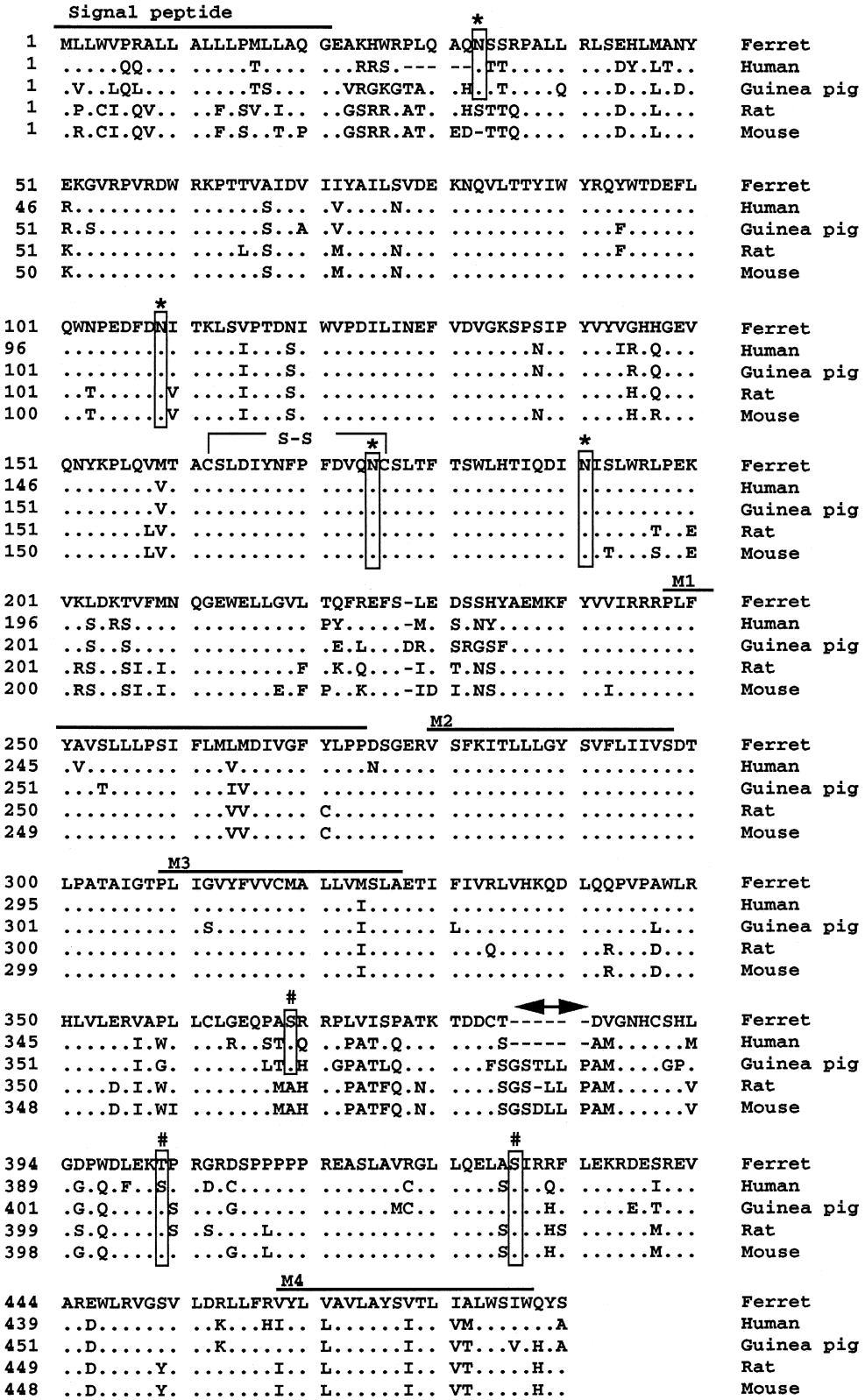

Fig. 1. Alignment of amino acid sequences of 5-HT_{3A} receptors. Ferret, human, and longer splice variants of guinea pig, rat and mouse 5-HT_{3A} receptors were aligned. Lines: a putative signal peptide or putative M1–M4 domains. Boxes: consensus sites of *N*-glycosylation (*) or protein kinase C phosphorylation (#). S–S: putative Cys–Cys loop. Double headed arrow: 5- or 6-amino acid insert in a longer splice variant of guinea pig, rat and mouse 5-HT_{3A} receptors.

Ser[429]) were located in the N-terminal extracellular domain and cytoplasmic region between the M3 and M4 domains, respectively.

The amino acid sequence of the ferret 5-HT_{3A} receptor, corresponded to the shorter splice variant of 5-HT_{3A} receptors in other species. Cloned mouse, rat and guinea pig 5-HT_{3A} receptors, unlike human 5-HT_{3A} receptors, exist as two splice variants, where the longer splice variant has five or six amino acid insertions in the intracellular loop between the M3 and M4 domains. Fig. 1 shows the alignment of the amino acid sequences in ferret and human 5-HT_{3A} receptors (Miyake et al., 1995), and the longer splice variant of guinea pig (Lankiewicz et al., 1998), rat (Miyake et al., 1995) and mouse (Maricq et al., 1991) 5-HT_{3A} receptors. The ferret 5-HT_{3A} receptor exhibits amino acid sequence identity to that of other species as follows: human 85%, guinea pig 82%, rat 80% and mouse 80%. The M2 domain, which is believed to constitute the channel pore, is completely conserved. Glu[128] of the mouse sequence in Fig. 1, which affects ligand binding (Boess et al., 1997), was strictly conserved in all other species. In contrast, another putative ligand-binding domain of 5-HT_{3A} receptors, corresponding to Leu[215]–Tyr[228] of the human sequence in Fig. 1 (Mochizuki et al., 1999a), was not strictly conserved among species, although elements within the region were conserved.

3.2. Alternative splicing

To analyze alternative splicing of ferret 5-HT_{3A} receptors, we performed RT-PCR analysis with primers flanking the putative position of the insertions. cDNAs, synthesized from colon and hippocampus RNA from ferrets and rats, were used as templates for PCR reactions. Predicted products from the nucleotide sequence of these receptors were: ferret, 80 bp; rat short form, 79 bp, and rat long form, 94 bp. Gel electrophoresis of the PCR products demonstrated that only a single, predicted 80-bp band was synthesized from ferret colon and hippocampus cDNAs (Fig. 2). In contrast, short and long forms of the rat 5-HT_{3A} receptor

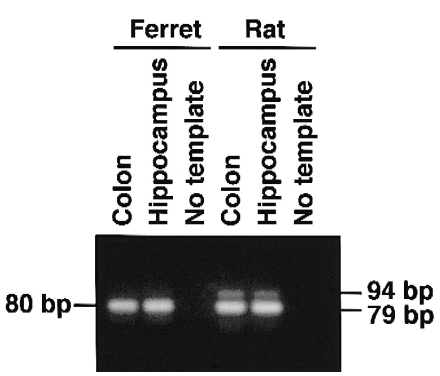

Fig. 2. RT-PCR analysis of 5-HT_{3A} receptor alternative splicing in ferret and rat tissues. PCR was performed with oligo (dT)-primed cDNAs. PCR products were separated by electrophoresis on 4% NuSieve 3:1 agarose gel including 0.5 μg/ml ethidium bromide.

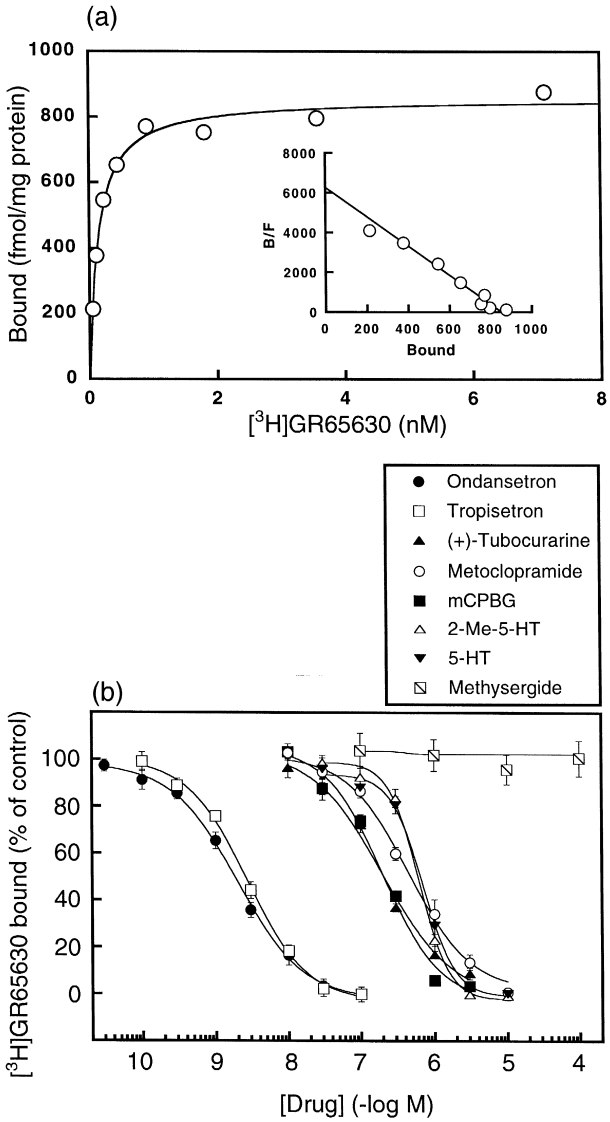

Fig. 3. Radioligand binding characteristics of the ferret 5-HT_{3A} receptor. (a) Binding of [^{3}H]GR65630 to membranes from HEK 293 cells stably expressing the ferret 5-HT_{3A} receptor. Membranes were incubated with indicated concentrations of [^{3}H]GR65630 in the presence or absence of 1–10 μM tropisetron. The difference is represented as specific binding. Inset: Scatchard plot of data. Values are means of duplicate determinations from a typical experiment. (b) Competition by drugs for [^{3}H]GR65630 binding to the membranes. Each data point is the mean ± S.E.M. ($n = 4$). Absence of error bars indicates that the S.E.M. is smaller than the symbol size. Curves were fitted to the points as described in Section 2.

cDNAs were found in both tissues (Fig. 2). Amplified fragments were confirmed as the predicted products by sequencing analysis. These results suggest that ferret 5-HT_{3A} receptor mRNA exists solely as a shorter splice variant, as in human 5-HT_{3A} receptor mRNA (Werner et al., 1994).

3.3. Radioligand binding studies

Radioligand binding studies were performed with membranes obtained from HEK 293 cells stably expressing

Table 1
Binding parameters of ferret 5-HT_{3A} receptor expressed in HEK 293 cells

Drug	IC_{50} (M)	K_i (M)	n_H
[^{3}H]GR65630 (K_d)	–	$1.4 \pm 0.1 \times 10^{-10}$	–
5-HT	$7.9 \pm 1.2 \times 10^{-7}$	–	2.3 ± 0.3
2-Me-5-HT	$5.6 \pm 0.8 \times 10^{-7}$	–	2.1 ± 0.2
*m*CPBG	$1.9 \pm 0.2 \times 10^{-7}$	$9.5 \pm 0.4 \times 10^{-8}$	1.3 ± 0.1
Ondansetron	$2.0 \pm 0.2 \times 10^{-9}$	$9.5 \pm 0.7 \times 10^{-10}$	1.0 ± 0.1
Tropisetron	$3.0 \pm 0.6 \times 10^{-9}$	$1.5 \pm 0.2 \times 10^{-9}$	1.1 ± 0.1
Metoclopramide	$4.4 \pm 0.6 \times 10^{-7}$	$2.2 \pm 0.1 \times 10^{-7}$	1.1 ± 0.1
(+)-Tubocurarine	$2.0 \pm 0.5 \times 10^{-7}$	$1.1 \pm 0.2 \times 10^{-7}$	1.0 ± 0.1
Methysergide	$> 1 \times 10^{-4}$	–	–

Data are presented as mean ± S.E.M., $n = 5$–6.

ferret 5-HT_{3A} receptors. The 5-HT_3 receptor-selective radioligand [^{3}H]GR65630 (Kilpatrick et al., 1987) specifically bound to these membranes with a K_d value of 140 ± 10 pM and a B_{max} value of 990 ± 110 fmol/mg protein (mean ± S.E.M., $n = 5$; Fig. 3a). Only non-specific binding was detected in membranes prepared from untransfected HEK 293 cells (data not shown). To assess the binding potency of 5-HT_3 receptor agonists and antagonists, we performed competition studies using 170 pM [^{3}H]GR65630 (which is close to the K_d value). All the drugs tested showed a concentration-dependent inhibition of [^{3}H]GR65630 binding to the ferret 5-HT_{3A} receptor, while the 5-HT_1 and 5-HT_2 receptor antagonist methysergide did not significantly inhibit radioligand binding (Fig. 3b). The IC_{50} and n_H values derived from curve fitting to the Hill equation, and the K_i values derived from the Cheng–Prusoff equation, are summarized in Table 1. The rank order of potency was for agonists: *m*CPBG > 2-Me-5-HT = 5-HT, and for antagonists: ondansetron = tropisetron > (+)-tubocurarine > metoclopramide.

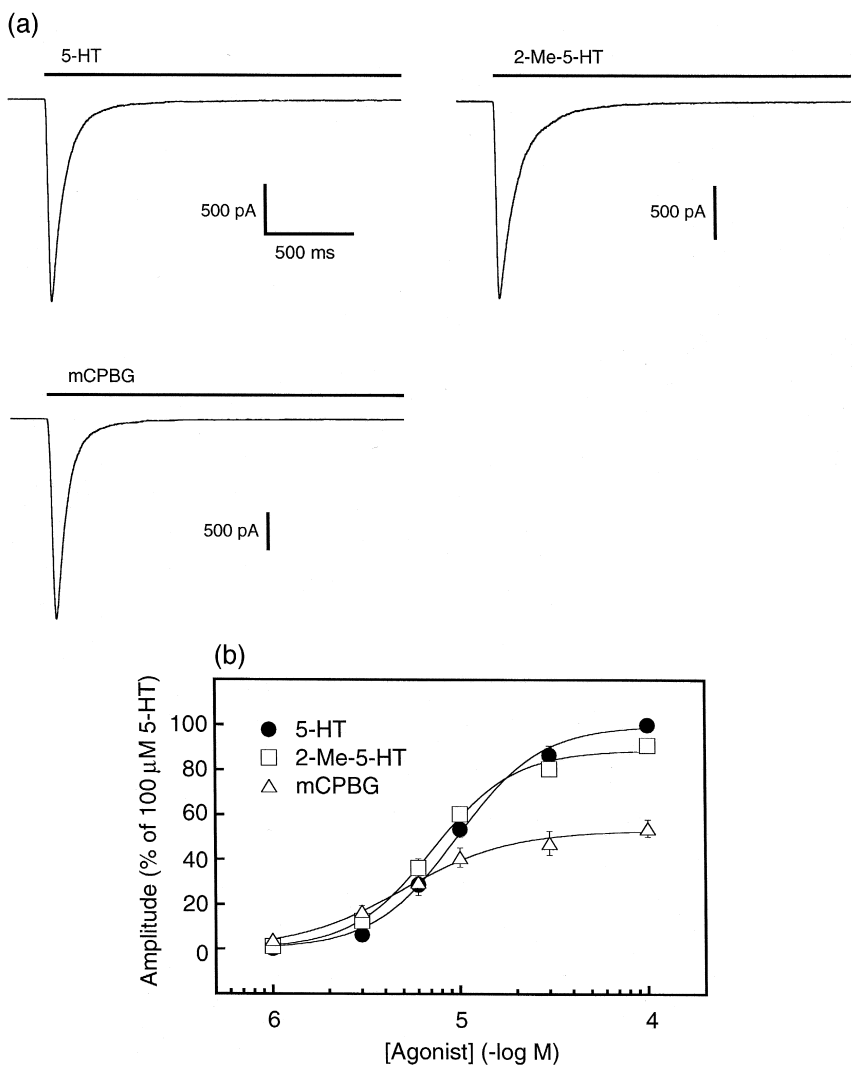

Fig. 4. Agonist pharmacology of the ferret 5-HT_{3A} receptor expressed in HEK 293 cells. (a) Typical responses elicited by 100 μM 5-HT, 2-Me-5-HT and *m*CPBG recorded at a holding potential of −64 mV. Agonist applications are indicated by horizontal bars above each trace. Each response was recorded from a different cell. (b) Concentration–response curves for 5-HT, 2-Me-5-HT and *m*CPBG at the ferret 5-HT_{3A} receptor. Peak current amplitudes were normalized to 100 μM 5-HT-induced current. Each data point is the mean ± S.E.M. ($n = 5$). Absence of error bars indicates that the S.E.M. is smaller than the symbol size. Curves were fitted to the points as described in Section 2.

Table 2
Pharmacological properties of ferret 5-HT_{3A} receptor expressed in HEK 293 cells

Drug	EC_{50} or IC_{50} (M)	n_H	K_b (M)	Maximal response[a] (%)
5-HT	$9.6\ (8.9–10.6) \times 10^{-6}$	1.9 (1.6–2.3)	–	100 (96–103)
2-Me-5-HT	$7.2\ (6.5–8.0) \times 10^{-6}$	2.0 (1.6–2.4)	–	89 (84–93)
*m*CPBG	$4.9\ (3.6–8.0) \times 10^{-6}$	1.5 (0.7–2.3)	–	53 (45–61)
Ondansetron	$2.9\ (2.5–3.3) \times 10^{-10}$	−1.6 (−2.0 − 1.3)	$1.3\ (1.1–1.5) \times 10^{-10}$	–
(+)-Tubocurarine	$2.7\ (2.1–3.5) \times 10^{-8}$	−0.8 (−1.0 − 0.6)	$1.2\ (0.9–1.6) \times 10^{-8}$	–

Antagonists were tested in the presence of 12 μM 5-HT. Values are means with 95% confidence intervals in parentheses, $n = 5$ (agonists) or 4 (antagonists).

[a]Peak amplitude of inward currents, elicited by the agonists, is expressed as a percentage of 100 μM 5-HT-evoked response, and fitted to the Hill equation to yield a maximal response.

3.4. Electrophysiological studies

We examined the potency and efficacy of 5-HT_3 receptor agonists (5-HT, 2-Me-5-HT and *m*CPBG). Ferret 5-HT_{3A} receptors expressing HEK 293 cells were recorded in the whole-cell voltage-clamp configuration. Application of agonists evoked a rapidly developing inward current, which was desensitized in the continued presence of the

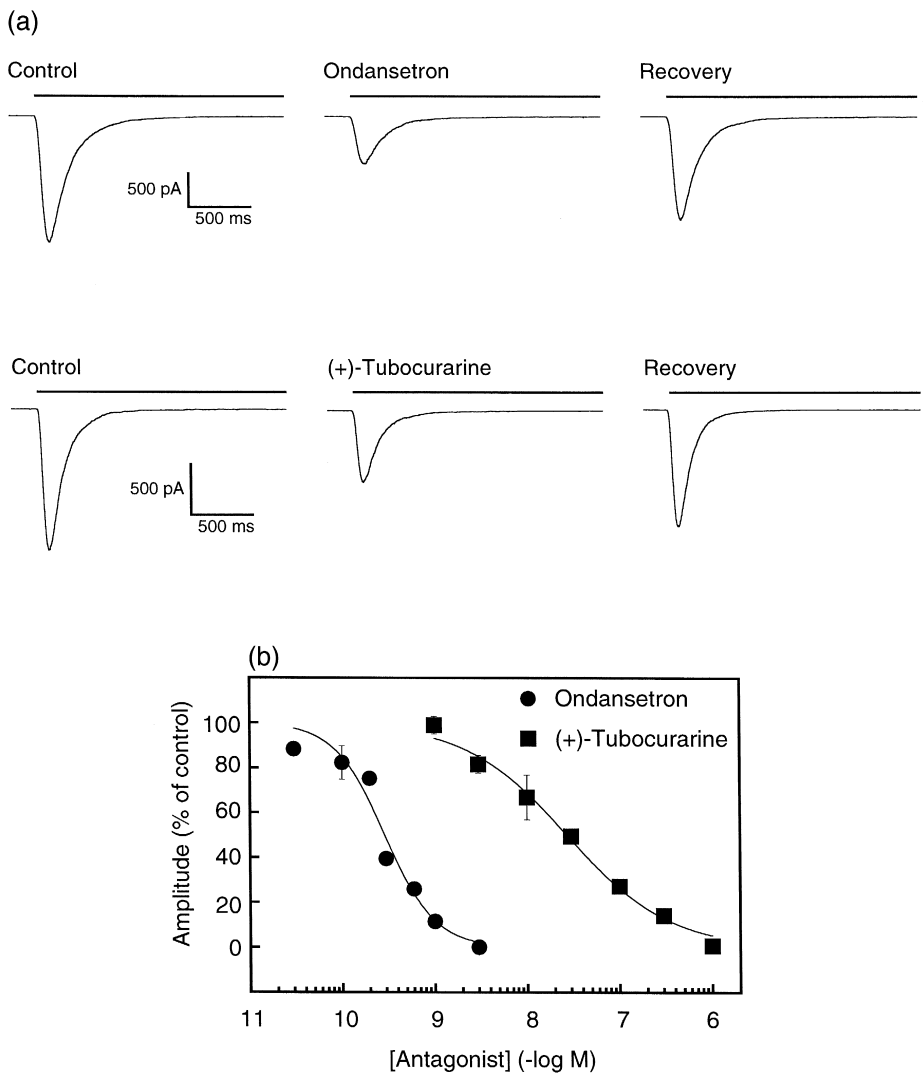

Fig. 5. Antagonist pharmacology of the ferret 5-HT_{3A} receptor expressed in HEK 293 cells. (a) Blockade of 12 μM 5-HT-induced currents by 300 pM ondansetron and 30 nM (+)-tubocurarine recorded at a holding potential of −64 mV. 5-HT applications are indicated by horizontal bars above each trace. Intervals between 5-HT applications were 90 s. Cells were pre-perfused for 30 s with either of antagonists, and then 5-HT was co-applied with the antagonist. Each antagonist was tested on a different cell. (b) Concentration–response curves for ondansetron and (+)-tubocurarine. Peak current amplitudes are expressed as a percentage of the control current response to 12 μM 5-HT. Each data point is the mean ± S.E.M. ($n = 4$). Absence of error bars indicates that the S.E.M. is smaller than the symbol size. Curves were fitted to the points as described in Section 2.

agonists (Fig. 4a). The rates of desensitization fitted well to two-exponential functions. *m*CPBG-elicited currents showed a rather fast desensitization (100 μM, time constant $\tau_{fast} = 59 \pm 4$ ms, $\tau_{slow} = 239 \pm 35$ ms, $n = 6$), compared with the responses elicited by 5-HT (100 μM, $\tau_{fast} = 87 \pm 7$ ms, $\tau_{slow} = 612 \pm 250$ ms, $n = 7$) and 2-Me-5-HT (100 μM, $\tau_{fast} = 140 \pm 41$ ms, $\tau_{slow} = 502 \pm 176$ ms, $n = 5$).

The concentration–response curves for the agonists, normalized to the 100 μM 5-HT-elicited current response (assigned a value of 100%), are shown in Fig. 4b. EC_{50} and n_H values derived from curve fitting to the Hill equation are summarized in Table 2. Although the rank order of potency of the agonists was *m*CPBG = 2-Me-5-HT > 5-HT, differences in potency among the agonists varied only twofold. This phenomenon, a small difference in agonist potency, was similar to that seen with cloned human 5-HT_{3A} receptors (Miyake et al., 1995; Davies et al., 1999).

The maximal current amplitudes, predicted from concentration–response curves (Fig. 4b), are shown in Table 2. *m*CPBG demonstrated partial agonistic responses and 2-Me-5-HT elicited almost full agonistic responses, compared with 5-HT at the ferret 5-HT_{3A} receptor.

To evaluate the antagonists pharmacologically, we focused on ondansetron, a potent and selective antagonist of 5-HT_3 receptors, and (+)-tubocurarine, a non-selective antagonist, which has a potency that is variable among species. Current responses elicited by 12 μM 5-HT (which is close to the EC_{50} value of 5-HT) were suppressed in a concentration-dependent manner by ondansetron (Fig. 5b, Table 2). The potency of ondansetron for the cloned ferret 5-HT_{3A} receptor ($IC_{50} = 290$ pM) was comparable to that for cloned mouse ($IC_{50} = 440$ pM; Gill et al., 1995), rat ($IC_{50} = 231$ pM; Mair et al., 1998), and human 5-HT_{3A} receptors ($IC_{50} = 103$ pM; Brown et al., 1998). Although (+)-tubocurarine also inhibited the 5-HT elicited responses (Fig. 5b, Table 2), the IC_{50} value (27 nM) was 90-fold higher than that for ondansetron. The antagonism of ondansetron and (+)-tubocurarine was readily reversed on washout (Fig. 5a).

4. Discussion

Isolated ferret 5-HT_{3A} receptor cDNA corresponded to the shorter splice variant of 5-HT_{3A} receptors. Although in rat, two splice variants were detected in colon and hippocampus, the longer splice variant of the ferret 5-HT_{3A} receptor was detected in neither of the tissues by RT-PCR analysis. 5-HT_{3A} receptors of mouse, rat and guinea pig exist as two splice variants (Maricq et al., 1991; Hope et al., 1993; Belelli et al., 1995; Miyake et al., 1995; Lankiewicz et al., 1998). In contrast, the cloned human 5-HT_{3A} receptor was found to be of the shorter splice variant (Belelli et al., 1995; Miyake et al., 1995), owing to the lack of splicing consensus sequence in human genomic DNA (Werner et al., 1994). The lack of detection of the longer splice variant of the ferret 5-HT_{3A} receptor by RT-PCR analysis might be caused by a very low expression level of the variant (Werner et al., 1994; Miquel et al., 1995; Emerit et al., 1995; Lankiewicz et al., 1998). Further genomic DNA analysis may still be required to conclude whether the ferret 5-HT_{3A} receptor lacks the longer splice variant, as does the human 5-HT_{3A} receptor.

The rank order of agonist binding potency at ferret 5-HT_{3A} receptors was *m*CPBG > 2-Me-5-HT = 5-HT with differences among IC_{50} values for [^{3}H]GR65630 binding within a fourfold range. This finding was comparable with the electrophysiological study results, which yielded the order of potency as *m*CPBG = 2-Me-5-HT > 5-HT with differences among EC_{50} values within a twofold range. *m*CPBG binding affinity is quite variable among species. At the rat 5-HT_{3A} receptor, *m*CPBG affinity is about 40–60-fold higher than that of 5-HT and 2-Me-5-HT, whereas at the human 5-HT_{3A} receptor, these drugs are almost equally potent (Miyake et al., 1995). Additionally, *m*CPBG demonstrates 35-fold higher affinity over 2-Me-5-HT at the mouse 5-HT_{3A} receptor (Boess et al., 1997). Therefore, the pharmacological response of the ferret 5-HT_{3A} receptor closely resembles that of the human 5-HT_{3A} receptor, with relatively small differences in affinity among these agonists.

The ferret 5-HT_{3A} receptor also resembles the human 5-HT_{3A} receptor in agonist efficacy. 2-Me-5-HT was an almost fully effective agonist, relative to 5-HT, at ferret 5-HT_{3A} receptors (89%), as it is at human 5-HT_{3A} receptors (87–97%; Belelli et al., 1995; Miyake et al., 1995), but different from that at mouse (23–28%; Niemeyer and Lummis, 1998; Werner et al., 1994) and rat 5-HT_{3A} receptors (66%; Mair et al., 1998). The efficacy of *m*CPBG (53%) at the ferret 5-HT_{3A} receptor was lower than at mouse (91%; Niemeyer and Lummis, 1998), rat (100%; Mair et al., 1998) and human 5-HT_{3A} receptors (79–99%; Davies et al., 1999; Miyake et al., 1995). Agonist efficacy is reported to be variable among expression and assay systems (Niemeyer and Lummis, 1998; Werner et al., 1994; Downie et al., 1994). Therefore, further studies carried out under the same experimental conditions may be warranted to clarify the inter-species differences.

(+)-Tubocurarine clearly discriminates between mouse, rat and human cloned 5-HT_{3A} receptors. In electrophysiological studies, the IC_{50} value for the ferret 5-HT_{3A} receptor ($IC_{50} = 27$ nM) was comparable to the value for rat receptors ($IC_{50} = 31.9$ nM; Mair et al., 1998), but was 100-fold lower than the IC_{50} for human receptors ($IC_{50} = 2.8$ μM; Brown et al., 1998), and was 15-fold higher than the IC_{50} for mouse receptors ($IC_{50} = 1.8–2.1$ nM; Gill et al., 1995; Hussy et al., 1994).

The N-terminal extracellular domain of the 5-HT_{3A} receptor contributes to (+)-tubocurarine potency (Hope et

al., 1999). In the mouse, Asp^{228} (Fig. 1) is thought to be one of the amino acids which contribute to the affinity of (+)-tubocurarine, because the substitution from Asp^{228} to Glu^{228} increases the IC_{50} value 10-fold compared with the wild type (Hope et al., 1999). The amino acid in the ferret 5-HT_{3A} receptor, which corresponds to Asp^{228} in the mouse receptor, is glutamic acid, a conserved amino acid in human and rat receptors. The higher IC_{50} values of the cloned receptors of the three species compared with the mouse can partly be explained by the presence of glutamic acid.

In our study, IC_{50}/K_i values from binding studies and EC_{50}/K_b values from electrophysiological studies were qualitatively similar, but quantitatively different. Similar observations have been made for mouse, rat and human 5-HT_{3A} receptors (Miyake et al., 1995; Boess et al., 1997; Mair et al., 1998). 5-HT_3 receptors are thought to exist as several conformational states: a closed state, a low agonist affinity activable state and a high agonist affinity desensitized state (Lummis et al., 1993; Sepúlveda et al., 1991). Therefore, the discrepancy between binding and electrophysiological studies might be due to methodological differences, because binding parameters were obtained from equilibrium reactions after prolonged exposure of the receptors to ligands (Sepúlveda et al., 1991).

Agonist-mediated competition binding curves with n_H values higher than unity have been observed with native and cloned 5-HT_3 receptors (Morain et al., 1994; Downie et al., 1995; Akuzawa et al., 1995, 1996). Our binding study with cloned ferret 5-HT_{3A} receptors demonstrated n_H values of 5-HT and 2-Me-5-HT close to 2. This result suggests a positive cooperativity of agonist binding, such as concerted switching of several binding sites between low and high affinity states (Bonhaus et al., 1995; Léna and Changeux, 1993).

The decay of current responses elicited to ferret 5-HT_{3A} receptors by prolonged application of 5-HT, 2-Me-5-HT and *m*CPBG fitted well to two-exponential functions. This phenomenon has also been demonstrated with cloned mouse and human 5-HT_{3A} receptors (Lankiewicz et al., 1998). It might mean that 5-HT_{3A} receptors have at least two desensitized states.

Newberry et al. (1992b) demonstrated the pharmacology of native 5-HT_3 receptors of ferret vagus nerve using an electrophysiological technique. The potency of agonists for the native ferret 5-HT_3 receptor (5-HT $_pEC_{50} = 4.89$, 2-Me-5-HT $_pEC_{50} = 5.18$) was comparable to that for the cloned ferret 5-HT_{3A} receptor. In addition, 2-Me-5-HT was a fully effective agonist relative to 5-HT at the native receptors. Although the potency of (+)-tubocurarine ($_pA_2 = 6.6$) was similar to that at the rat vagus 5-HT_3 receptor ($_pK_b = 7.2$, Newberry et al., 1992a), it was dissimilar to that at the cloned ferret 5-HT_{3A} receptor. This discrepancy might possibly be caused by the methodology or by the association of auxiliary subunits with the native 5-HT_3 receptor.

Recently, another subunit of the human 5-HT_3 receptor (5-HT_{3B}) has been isolated (Davies et al., 1999). The basic properties and pharmacological responses of the 5-HT_{3A} and 5-HT_{3B} subunits co-expressed in HEK 293 cells and *Xenopus* oocytes are reported to be different from those of the 5-HT_{3A} subunit alone. Although native 5-HT_3 receptors might exist as 5-HT_{3A} and 5-HT_{3B} hetero-oligomers, the 5-HT_{3B} subunits in other species have not been isolated and the pharmacological character of such hetero-oligomers is still not fully studied. The 5-HT_{3B} subunit is not functional as a homo-oligomer, and therefore, before the 5-HT_{3B} subunit functions are evaluated, it is essential to fully characterize the 5-HT_{3A} subunits themselves.

Our data show that 5-HT, 2-Me-5-HT and *m*CPBG were equipotent at cloned ferret and cloned human 5-HT_{3A} receptor subunits, although the efficacies were slightly different. These characteristics will be important for the development of receptor-selective drugs, especially agonists, in in vivo studies using ferrets.

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