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The 5-HT_4 receptor: molecular cloning and pharmacological characterization of two splice variants

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Molecular cloning efforts have provided primary amino acid sequence and signal transduction data for a large collection of serotonin receptor subtypes. These include five 5-HT_1-like receptors, three 5-HT_2 receptors, one 5-HT_3 receptor, two 5-HT_5 receptors, one 5-HT_6 receptor and one 5-HT_7 receptor. Molecular biological information on the 5-HT_4 receptor is notably absent from this list. We now report the cloning of the pharmacologically defined 5-HT_4 receptor. Using degenerate oligonucleotide primers, we identified a rat brain PCR fragment which encoded a '5-HT receptor-like' amino acid sequence. The corresponding full length cDNA was isolated from a rat brain cDNA library. Transiently expressed in COS-7 cells, this receptor stimulates adenylyl cyclase activity and is sensitive to the benzamide derivative cisapride. The response is also blocked by ICS-205930. Interestingly, we isolated two splice variants of the receptor, 5-HT_{4L} and 5-HT_{4S}, differing in the length and sequence of their C-termini. In rat brain, the 5-HT_{4S} transcripts are restricted to the striatum, but the 5-HT_{4L} transcripts are expressed throughout the brain, except in the cerebellum where it was barely detectable. In peripheral tissues, differential expression was also observed in the atrium of the heart where only the 5-HT_{4S} isoform was detectable.

Key words: G protein-coupled receptor/5-HT_4 receptor/ splice variants/stimulation of cAMP

Introduction

Serotonin (5-HT) is a neurotransmitter involved in a wide variety of physiological functions exerted through its interaction with multiple receptor subtypes, mainly in the central and peripheral nervous systems and in the gastrointestinal tract. Traditional pharmacological approaches have defined four receptor classes: 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 (Humphrey *et al.*, 1993). More recently, molecular biological studies have provided primary amino acid sequence and signal transduction data for a much larger than anticipated collection of serotonin receptor subtypes. These include five 5-HT_1-like, three 5-HT_2, one 5-HT_3, two 5-HT_5, one 5-HT_6 and one 5-HT_7 receptor (for a review, see Hoyer *et al.*, 1994). The 5-HT_1 subfamily includes: 5-HT_{1A} (Fargin *et al.*, 1988; Kobilka *et al.*, 1989), 5-HT_{1B}/5-$HT_{1D\beta}$ (Voigt *et al.*, 1991; Adham *et al.*, 1992; Demchyshyn *et al.*, 1992; Jin *et al.*, 1992; Maroteaux *et al.*, 1992; Weinshank *et al.*, 1992), 5-$HT_{1D\alpha}$ (Branchek *et al.*,1991; Hamblin and Metcalf, 1991; Weinshank *et al.*, 1992), 5-HT_{1E} (Levy *et al.*, 1992; McAllister *et al.*, 1992; Zgombick *et al.*, 1992) and 5-HT_{1F} (Amlaiky *et al.*, 1992; Adham *et al.*, 1993; Lovenberg *et al.*, 1993b). All five have been shown to couple to the inhibition of adenylyl cyclase activity. The 5-HT_2 family includes the 5-HT_{2A} receptor (Pritchett *et al.*, 1988), 5-HT_{2C} (Julius *et al.*, 1988) and 5-HT_{2B} (rat stomach fundus; Foguet *et al.*, 1992; Kursar *et al.*, 1992). These receptors all couple to phosphoinositide hydrolysis. The 5-HT_3 receptor is a ligand-gated ion channel (Maricq *et al.*, 1991). A signal transduction pathway has yet to be reported for the 5-HT_{5A} and 5-HT_{5B} receptors (Erlander *et al.*, 1993; Matthes *et al.*, 1993). Both 5-HT_6 (Monsma *et al.*, 1993) and 5-HT_7 receptors (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a; Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993) are coupled to stimulation of adenylyl cyclase.

The absence of molecular biological information on the 5-HT_4 receptors, which have been shown in native tissues to couple to the activation of adenylyl cyclase as a primary mode of signal transduction (Dumuis *et al.*, 1988; Bockaert *et al.*, 1990), is apparent. The 5-HT_4 responses have been described in the heart, adrenal, bladder and alimentary canal of numerous species including man, indicating a potential role in diseases of the gastrointestinal tract, as well as in cardiac, urinary and endocrine function (Ford and Clarke, 1993). In addition, biochemical and electrophysiological responses attributed to the activation of 5-HT_4 receptors have been observed in the brain, particularly in areas such as the hippocampus, indicating a potential role in cognitive enhancement (Dumuis *et al.*, 1988).

Using a strategy based on nucleotide homology between transmembrane domains III and V of 5-HT receptors, we now report the cloning of the pharmacologically defined rat 5-HT_4 receptor. We show that this receptor stimulates adenylyl cyclase activity, is sensitive to the benzamide derivative cisapride and that this response is blocked by tropisetron (ICS-205930). Interestingly, we isolated what appear to be two splice variants of the receptor, 5-HT_{4L} and 5-HT_{4S}, differing in the length and sequence of their

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C-termini. Our data indicate that, in CD rats, the two isoforms are differentially expressed in the brain and the atrium of the heart.

Results

Isolation of two rat cDNA clones encoding the 5-HT_4 receptor

Based on a strategy recently described for the isolation of the 5-HT_{1F} receptor (Adham *et al.*, 1993), a 270 bp DNA fragment (S10) was identified when rat brain cDNA was used as template in a PCR amplification with two degenerate oligonucleotide primers derived from well conserved regions among several serotonin receptors, in the third and fifth putative transmembrane domains. The peptide sequence corresponding to the S10 PCR fragment contained a 'transmembrane IV-like' domain. Since we used 'serotonin receptor-specific' PCR primers, S10 represented a potentially new serotonin receptor, although it showed a low level of homology with all previously isolated receptors within this subfamily. The corresponding full length cDNA was isolated from a rat brain cDNA library. Since five amplified commercial phage cDNA libraries were found to be negative in our screening, we divided the plasmid cDNA library into small pools of $2.5–5\times10^4$ independent clones before amplification to avoid a potential growth bias against the S10 cDNA clone. By direct PCR analysis of bacterial pools, subsequent sib selection and standard filter hybridization, two cDNA clones were identified, named 5-HT_{4S} (5.5 kb) and 5-HT_{4L} (4.5 kb). The deduced amino acid sequences are shown in Figure 1A and B. Surprisingly, the peptide sequences between those two clones are only 96.1% identical, diverging in the second half of the C-terminus tails, at position 360. The 5-HT_{4S} and 5-HT_{4L} cDNAs encode 387 and 406 amino acid proteins, respectively. The cytoplasmic domain heterogeneity of the 5-HT_{4S} and 5-HT_{4L} receptors (Figure 1C) most likely arises by alternative splicing of pre-mRNA, as has been described for the EP3 subtype of the prostaglandin E receptor (Namba *et al.*, 1993) or the endothelial thromboxane A2 receptor (Raychowdhury *et al.*, 1994). The hydrophobicity plot displayed seven hydrophobic putative membrane-spanning regions (numbered I–VII in Figure 1A). Both 5-HT_{4S} and 5-HT_{4L} receptors carry a potential *N*-linked glycosylation site in their amino-terminal end, three potential protein kinase C phosphorylation sites and a potential palmitoylation site at the cysteine found in position 329 (see Figure 1A). A large number of G protein-coupled receptors carry a cysteine at the same position in their cytoplasmic tail and it has been speculated that it may play a role in the functional coupling of the human β_2-adrenergic and the human 5-HT_{1B} receptors to G proteins (O'Dowd *et al.*, 1989; Ng *et al.*, 1993). Moreover, the 5-HT_{4L} receptor carries an additional potential phosphorylation site for protein kinase C at position 400. In the transmembrane domains, amino acid sequence comparisons revealed low levels of identities between the 5-HT_4 and all other cloned rat serotonin receptors (Figure 2A), confirming that the 5-HT_4 receptor is a member of a distinct serotonin receptor family. This observation is further supported by the profile of a dendrogram constructed using amino acid homologies within transmembrane domains (Figure 2B). Again, the rat 5-HT_4 receptor shows little amino acid sequence relationship to all other known rat serotonin receptors, with the exception of a weak but significant clustering with the 5-HT_2 subfamily.

Pharmacological profile of the rat 5-HT_4 receptor

The cDNAs encoding the rat 5-HT_{4S} and 5-HT_{4L} receptors were transiently expressed in COS-7 cells for pharmacological evaluation. Saturation analysis revealed a single saturable site of high affinity for both receptors using [^{3}H]GR113808 (see Figure 3). For each receptor, non-specific binding increased linearly with increasing ligand concentration. The degree of specific binding at concentrations of [^{3}H]GR113808 (0.4–0.5 nM) ranged from 80 to 90%. The 5-HT_{4S} displayed an ~1.5-fold higher B_{max} than the 5-HT_{4L} receptor, although they had comparable K_d values for [^{3}H]GR113808 (Figure 3). The pharmacological binding profile of 5-HT_{4S} and 5-HT_{4L}, obtained from displacement studies using [^{3}H]GR113808, was very similar. Since the amino acid sequences of these two clones are identical in the transmembrane regions, it was anticipated that there would be no pharmacological differences between the receptors at the level of binding. This was verified for a set of compounds and effort was focused on the 5-HT_{4L} receptor. The data summarized in Table I demonstrate that the cloned 5-HT_4 receptors are similar to the 5-HT_4 receptor as defined in the literature by functional assays (Bockaert *et al.*, 1992) and radioligand binding assays using [^{3}H]GR113808 (Grossman *et al.*, 1993; Waeber *et al.*, 1993).

A range of 5-HT_4 receptor agonists and antagonists completely inhibited the specific binding of [^{3}H]GR113808 to both cloned 5-HT_4 receptors (Figure 4; Table I). Of the agonists tested, only those active in 5-HT_4-containing preparations (5-HT and 5-MeOT) potently inhibited [^{3}H]GR113808 binding. By contrast, agonists for other 5-HT receptors, for example the 5-HT_{1A} receptor agonist, 8-OH-DPAT, the 5-HT_{1D} receptor agonist, sumatriptan, the 5-HT_2 receptor antagonist, ketanserin, and the ergot derivative, D-LSD, had no effect on [^{3}H]GR113808 binding at concentrations up to 1 μM (Table I). The substituted benzamides (cisapride, BRL-24924 and zacopride), partial agonists at the 5-HT_4 receptors in native systems, all potently inhibited [^{3}H]GR113808 binding (Table I). Specific [^{3}H]GR113808 binding was also inhibited by the 5-HT_4 receptor antagonist ICS-205930, a tropanyl-indole derivative, which has been reported to be an antagonist at both 5-HT_3 and 5-HT_4 receptors (Bockaert *et al.*, 1992). Taken together, the pharmacological profile, as well as the amino acid sequence, differentiate the 5-HT_4 clones from the other adenylyl cyclase stimulatory subtypes, 5-HT_6 and 5-HT_7, that have been described recently by several groups (see Introduction). For both the rat 5-HT_{4S} and 5-HT_{4L} clones, Hill slopes for the inhibition of [^{3}H]GR113808 binding by 5-HT_4 receptor agonists, but not the antagonist ICS-205930, were shallow in the absence of Gpp(NH)p, with the exceptions of 5-CT and α-Me-5-HT. The effect of the non-hydrolyzable analog of GTP, GTPγS, on 5-HT affinity was determined for both 5-HT_{4S} and 5-HT_{4L} receptors. In COS-7 cells transfected with 5-HT_{4S} receptor gene, GTPγS (100 μM) produced little effect on the ability of 5-HT to displace [^{3}H]GR113808 binding, decreasing its affinity only mar-

A

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M D R L D A N V S S N E G F G S V E K V V L L T F F A M V I   30
            ●                                 I
L M A I L G N L L V M V A V C R D R Q L R K I K T N Y F I V   60

S L A F A D L L V S V L V N A F G A I E L V Q D I W F Y G E   90
              II
M F C L V R T S L D V L L T T A S I F H L C C I S L D R Y Y  120
                          III
A I C C Q P L V Y R N K M T P L R I A L M L G G C W V I P M  150
                                                  IV
F I S F L P I M Q G W N N I G I V D V I E K R K F N H N S N  180

S T F C V F M V N K P Y A I T C S V V A F Y I P F L L M V L  210
                                          V
A Y Y R I Y V T A K E H A Q Q I Q M L Q R A G A T S E S R P  240
              ▲
Q T A D Q H S T H R M R T E T K A A K T L C V I M G C F C F  270
              ▲
C W A P F F V T N I V D P F I D Y T V P E K V W T A F L W L  300
    VI
G Y I N S G L N P F L Y A F L N K S F R R A F L I I L C C D  330
              VII                  ▲                   ↑
D E R Y K R P P I L G Q T V P C S T T T I N G S T H V L R    359
D T V E C G G Q W E S R C H L T A T S P L V A A Q P V I R R  389

P Q D N D L E D S C S L K R S Q S                            406
                    ▲
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B

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Y T V L H S G Q H Q E L E K L P I H N D P E S L E S C F      387
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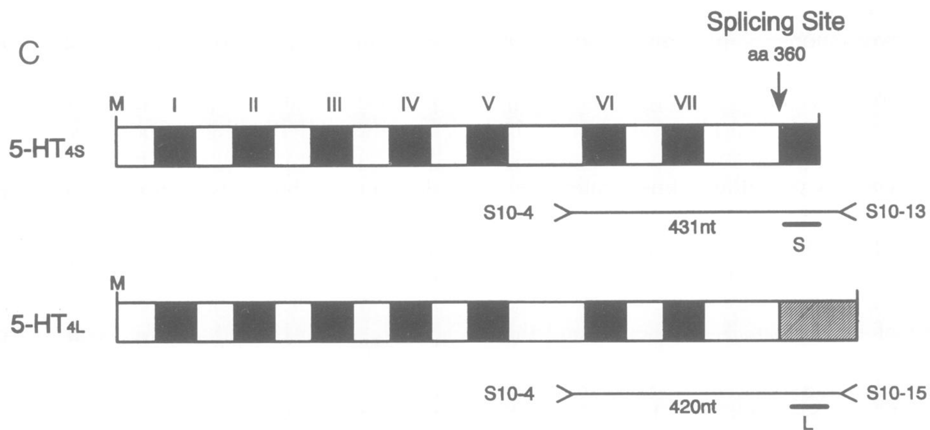

Fig. 1. (**A**) Amino acid sequence corresponding to the longest open reading frame in the rat 5-HT_{4L} cDNA. The seven putative transmembrane domains are underlined and numbered I–VII. A black circle indicates a potential *N*-linked glycosylation site. Triangles correspond to the protein kinase C consensus site. The arrow indicates a potential palmitoylation site. The second half of the carboxy tail diverging at position 360 between 5-HT_{4L} and 5-HT_{4S} is shaded. The GenBank accession number for 5-HT_{4L} is U20907 and for 5-HT_{4S} is U20906. (**B**) Amino acid sequence of the rat 5-HT_{4S} receptor. Only the divergent sequence in the carboxy tail is given in shaded letters, the sequence upstream of position 360 being 100% identical between the two receptor isoforms. (**C**) Schematic representation of the cytoplasmic domain heterogeneity of the 5-HT_{4L} and 5-HT_{4S} receptors. Transmembrane domains are shown as closed boxes and divergent C-terminal tails as hatched boxes. Also shown are the PCR primers, corresponding positions in each cDNA, the expected length of the PCR products and the position of the probes used in the CNS distribution.

A

%TM Amino Acid Identity of Serotonin Receptors

	r5HT$_4$	r5HT$_{1A}$	r5HT$_{1D\alpha}$	r5HT$_{1B}$	h5HT$_{1E}$	r5HT$_{1F}$	r5HT$_{2A}$	r5HT$_{2B}$	r5HT$_{2C}$	r5HT$_{5A}$	r5HT$_{5B}$	r5HT$_6$	r5HT$_7$
r5HT$_4$	100												
r5HT$_{1A}$	46	100											
r5HT$_{1D\alpha}$	41	58	100										
r5HT$_{1B}$	43	54	73	100									
h5HT$_{1E}$	42	53	64	63	100								
r5HT$_{1F}$	40	54	63	59	68	100							
r5HT$_{2A}$	43	39	38	40	42	42	100						
r5HT$_{2B}$	40	43	40	40	45	38	68	100					
r5HT$_{2C}$	43	39	43	40	44	42	80	71	100				
r5HT$_{5A}$	36	49	48	48	48	49	38	37	40	100			
r5HT$_{5B}$	38	48	49	49	49	45	37	39	41	84	100		
r5HT$_6$	35	40	40	38	37	36	43	41	42	39	37	100	
r5HT$_7$	46	50	49	52	53	49	40	37	39	44	44	39	100

B

Serotonin Receptor Relationships

Fig. 2. (**A**) Transmembrane domain (Probst *et al.*, 1992) amino acid identities between the rat 5-HT_4 and other known rat serotonin receptors. (**B**) Dendrogram. Known serotonin receptor sequences were compared and clustered according to sequence homology using the Pileup program (Genetics Computer Group, Inc., Madison, USA). The rat 5-HT_4 sequence was compared with the rat 5-HT_{1A} (70% similarity; Albert *et al.*, 1990), rat 5-$HT_{1D\alpha}$ (67%; Hamblin *et al.*, 1992), rat 5-HT_{1B} (69%; Adham *et al.*, 1992), human 5-HT_{1E} (69%; Zgombick *et al.*, 1992), rat 5-HT_{1F} (68%; Lovenberg *et al.*, 1993b), rat 5-HT_{2A} (73%; Julius *et al.*, 1990), rat 5-HT_{2B} (70%; Foguet *et al.*, 1992), rat 5-HT_{2C} (77%; Julius *et al.*, 1988), rat 5-$HT_{5A,B}$ (68%,69%; Erlander *et al.*, 1993), rat 5-HT_6 (65%; Monsma *et al.*, 1993), rat 5-HT_7 (67%; Shen *et al.*, 1993). The lengths of the horizontal lines are inversely proportional to the sequence homology (similarity, not identity).

Fig. 3. Saturation analysis of [^{3}H]GR113808 binding on (**A**) 5-HT_{4S} and (**B**) 5-HT_{4L} clones. Membranes harvested from transiently transfected COS-7 cells were incubated with 8–10 concentrations of [^{3}H]GR113808 (0.005–2.5 nM) for 30 min at 37°C. Non-specific binding was defined by 50 μM unlabeled 5-HT. Results are from a single experiment but are representative of two such experiments. K_d and B_{max} values were determined by computer-assisted non-linear regression analysis (Accufit; Lundon Software).

ginally. In contrast, the 5-HT_{4L} receptors showed a more pronounced, albeit small (2-fold), reduction in the affinity of 5-HT which was observed with a concomitant increase in the Hill coefficient (0.65–0.95, $n = 2$). However, this effect was very variable and could be demonstrated in half of the preparations studied (Figure 5). The source of this variability is not understood but is not unique to the 5-HT_4 receptor (e.g. human 5-HT_{2A} receptor, N.Adham, unpublished observations).

To examine the ability of 5-HT_{4S} and 5-HT_{4L} clones to couple to adenylyl cyclase, transiently transfected COS-7 cells were tested for the ability to exhibit an increase in basal cAMP release or a decrease in forskolin (FSK)-stimulated cAMP response. 5-HT (1 μM) had no effect on either basal or FSK-stimulated adenylyl cyclase activity in untransfected or mock-transfected COS-7 cells (data not shown), indicating that endogenous adenylyl cyclase-coupled serotonin receptors are not present in these cells. Preliminary studies were carried out by adding a single dose of various drugs in triplicate. Addition of 5-HT (1 μM) to this system produced stimulation of basal cAMP release (5-HT_{4S} = 0.055 ± 0.005; 5-HT_{4L} = 0.055 ± 0.007 pmol/ml/10 min) by ~20-fold for each clone. No inhibition of either the basal or FSK-stimulated cAMP release was observed. Addition of 10 μM FSK, together with 1 μM 5-HT, stimulated cAMP accumulation ~10-

Table I. Affinities of various compounds that compete for 0.2–0.4 nM [^{3}H]GR113808 binding to membranes of COS-7 cells transiently transfected with the rat 5-HT_{4L} gene

Compounds	K_i (nM)
Agonists	
5-HT	145 ± 30
5-MeOT	401 ± 69
α-Me-5-HT	1450 ± 406
5-CT	>10 000
Partial agonists	
Cisapride	122 ± 38
BRL-24924	243 ± 28
Zacopride	808 ± 79
Antagonist	
ICS-205930	544 ± 15
Inactive compounds	
8-OH-DPAT	>1000
Sumatriptan	>1000
Ketanserin	>1000
D-LSD	>1000

Affinity estimates are given as K_i values in nM and were determined from IC_{50} values obtained by computer-assisted non-linear curve analysis (Accucomp; Lundon Software), using the Cheng–Prussoff equation. K_i values are expressed as mean ± SEM from at least three determinations.

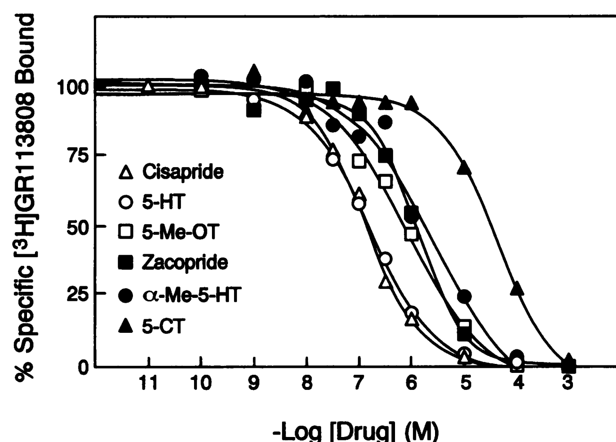

Fig. 4. Inhibition of specific [^{3}H]GR113808 binding to the rat cloned 5-HT_{4L} receptor. Membranes from transiently transfected COS-7 cells were incubated with 0.2–0.4 nM [^{3}H]GR113808. Non-specific binding was defined by 50 µM unlabeled 5-HT. Results are presented as a percentage of specific binding in the absence of a competing agent. Results are from a single experiment but are representative of three such experiments. Data were analyzed by computer-assisted non-linear regression analysis (Accucomp; Lundon Software).

fold more than either agent alone (data not shown). 5-HT caused a concentration-dependent stimulation of basal adenylyl cyclase activity (Figure 6). The mean EC_{50} values were significantly different [5-HT_{4S}, 26 ± 3 nM; 5-HT_{4L}, 51 ± 7 nM (n = 3), $P < 0.05$]. The measured EC_{50} values for some agonists such as 5-MeOT were lower than expected from the measured K_i values (Tables I and II). Furthermore, 5-HT produced a significantly higher maximal cAMP response for the 5-HT_{4L} clone as compared with the 5-HT_{4S} clone [E_{max} of 5-HT_{4S}, 2107 ± 52% and

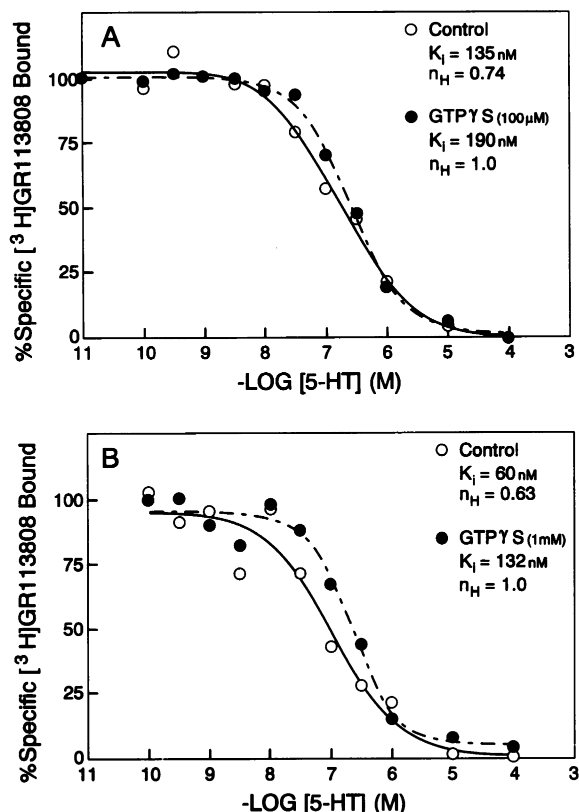

Fig. 5. Inhibition of [^{3}H]GR11380 binding on the cloned rat 5-HT_{4S} (**A**) and 5-HT_{4L} (**B**) receptors by 5-HT, in the absence and presence of GTPγS (100 µM). Membranes harvested from transient transfectants (COS-7 cells) were incubated with [^{3}H]IGR113808 (0.2–0.4 nM) for 30 min at 37°C. Non-specific binding was defined by 50 µM unlabeled 5-HT. Data shown are from a single experiment. Data were analyzed by computer-assisted non-linear regression analysis (Accufit; Lundon Software).

5-HT_{4L}, 2598 ± 154% basal cAMP release (n = 3) $P < 0.05$].

The rank order of affinities obtained from binding assays was different from the rank order of potencies obtained from functional studies (Tables I and II). For example, cisapride had the highest affinity for competing with [^{3}H]GR113808 binding, whereas, in functional studies, 5-MeOT exhibited the highest potency at the cloned 5-HT_4 receptors. The 2-methoxy-4-amino-5-chloro-substituted benzamides (cisapride, BRL-249245 and zacopride) were less potent agonists than 5-HT in stimulating basal cAMP release and displayed different rank orders of potency for 5-HT_{4S} and 5-HT_{4L} clones. As indicated in Table II using the 5-HT_{4S} clone, cisapride, BRL-24924 and zacopride exhibited ~10-, 30- and 100-fold lower potency than 5-HT respectively, whereas with 5-HT_{4L}, these compounds had almost equal potency. All the agonists tested acted as full agonists for both clones, with intrinsic activities ranging between 0.85 and 1.4 (Table II). ICS-205930 (10–100 µM) had a similar effect with the two clones and was found to be a silent antagonist, causing parallel dextral shifts in the concentration–effect curve of 5-HT without altering the maximum response significantly. The estimated K_b value for ICS-205930 was not significantly different between the two clones [5-HT_{4S} = 962 ± 244 nM; 5-HT_{4L} = 607 ± 30 nM (n = 3)] (Figure 6). In the case of 5-HT_{4L}, the K_b

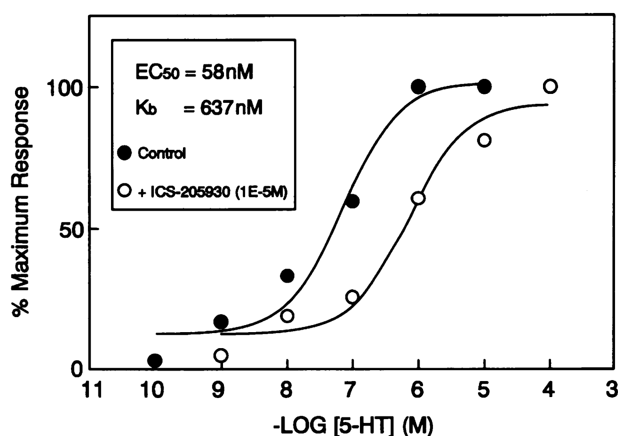

Fig. 6. Stimulation of cAMP production by 5-HT and antagonism by ICS-205930 in transiently transfected COS-7 cells expressing the $5\text{-}HT_{4L}$ receptor. cAMP measurements on intact COS-7 cells were according to Materials and methods. 5-HT concentration–effect curves are represented in the absence (●) and in the presence (○) of ICS-205930. Data were analyzed by computer-assisted non-linear regression analysis (Inplot; Graphpad Inc.). Each curve represents the mean of triplicate determinations from a single experiment representative of at least three separate experiments. Data are presented as percent maximum cAMP released by 5-HT. The dissociation constant of the antagonist (K_b) was calculated according to the formula: K_b = [B]/(A′/A)−1], where [B] is the concentration of antagonist, A′ and A the EC_{50} values of agonist measured respectively in the presence and in the absence of antagonist (Furchgott, 1972).

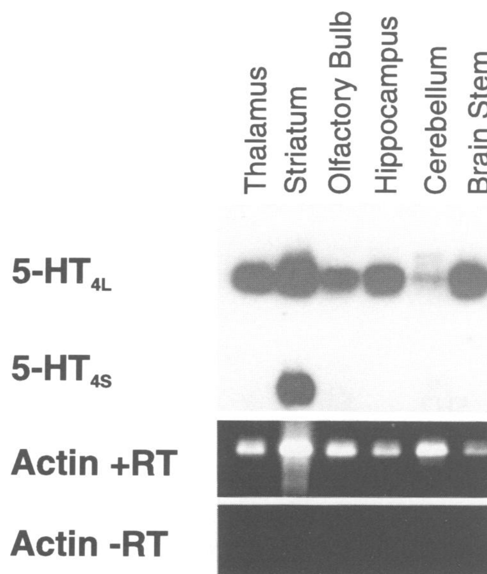

Fig. 7. RT-PCR analysis performed with 60 ng of mRNA from various regions of the rat brain: thalamus, striatum, olfactory bulb, hippocampus, cerebellum and brainstem. The PCR products were analyzed by Southern blotting. The PCR primers and specific probes corresponding to the long and short forms of the $5\text{-}HT_4$ receptor are outlined in Figure 1C and described in Materials and methods. A 2 h exposure of the autoradiogram is shown. A positive control was performed using rat actin primers on mRNA samples treated with (+RT) or without (–RT) reverse transcriptase. The PCR products were analyzed on a 1% agarose gel. A photograph of the ethidium bromide stained gel is shown.

Table II. Pharmacological profile for the cAMP response using the rat $5\text{-}HT_{4L}$ and $5\text{-}HT_{4S}$ genes transiently expressed in COS-7 cells

Drug	EC_{50} or K_b (nM)		Intrinsic activity	
	$5\text{-}HT_{4S}$	$5\text{-}HT_{4L}$	$5\text{-}HT_{4S}$	$5\text{-}HT_{4L}$
5-MeOT	21 ± 6	31 ± 13	1.0	1.0
5-HT	26 ± 3	51 ± 7	1.0	1.0
Cisapride	191 ± 26	413 ± 199	1.2	1.4
α-Me-5-HT	250 ± 91	1038 ± 31	0.9	1.0
BRL-24924	736 ± 129	250 ± 25	1.1	0.9
Zacopride	2740 ± 274	239 ± 33	1.1	1.0
5-CT	5570 ± 808	1411 ± 211	0.85	1.2
ICS-205930	962 ± 244	607 ± 30	0	0

cAMP measurements on intact cells were as described in Materials and methods. EC_{50} values (concentration producing the half-maximal effect) were derived from the analysis of full dose–response curves. The maximum response produced by each drug was normalized to the 5-HT-induced maximum response, which is indicated as having an intrinsic activity of 1.0. Data are means ± SEM of three separate experiments. The apparent dissociation constant of the antagonist (K_b) (ICS-205930) was calculated according to the formula K_b = [B]/(A′/A)−1], where [B] is the concentration of antagonist, A′ and A the EC_{50} values of agonist measured respectively in the presence and in the absence of antagonist (Furchgott, 1972).

value obtained for ICS-205930 from functional studies is comparable with the K_i value derived from binding experiments. However, in the case of $5\text{-}HT_{4S}$, ICS-205930 had ~2- to 4-fold higher binding affinity as compared with the functional studies.

$5\text{-}HT_4$ receptor isoforms are differentially expressed in rat tissues

Since we isolated two different $5\text{-}HT_4$ cDNA clones by screening a library made from whole rat brain, we evaluated the possibility of differential expression in six different parts of the brain. The expression of $5\text{-}HT_4$ transcripts was analyzed by amplification of cDNA derived from RNA isolated from various rat brain tissues (i.e. reverse transcription-PCR or RT-PCR). We were able to examine, selectively, the tissue distribution of each isoform by using pairs of primers specific for each clone, as well as specific internal oligonucleotide probes (Figure 1C). We also demonstrated the presence of cDNA in all tissues corresponding to the constitutively expressed β-actin gene (Figure 7, Actin+RT), as well as the absence of actin PCR product in the minus reverse transcriptase control (Figure 7, Actin–RT). The $5\text{-}HT_{4L}$ splice variant is transcribed everywhere in rat brain except in the cerebellum, where the transcript was barely detectable. Interestingly, the $5\text{-}HT_{4S}$ isoform is transcribed only in the striatum (Figure 7). The hybridization signals are not due to any contaminating genomic DNA, since actin primers failed to yield any PCR products in the minus reverse transcriptase control (Figure 7). Moreover, pairs of primers specific for $5\text{-}HT_{4S}$ and $5\text{-}HT_{4L}$ failed to amplify genomic DNA (Figure 8; see below). In the periphery, duodenum, liver and heart ventricle did not express detectable levels of either isoform. Ileum, colon and bladder expressed both isoforms. The atrium of the heart expressed detectable levels of only the $5\text{-}HT_{4S}$ isoform (Figure 8). These hybridization signals were not due to any contaminating genomic DNA since no bands were observed when RNA was directly amplified (–RT control; data not shown). Moreover, primer sets directed to $5\text{-}HT_{4S}$ and $5\text{-}HT_{4L}$ failed to amplify rat genomic DNA (Figure 8), suggesting that an intron(s), whose size prevents PCR amplification, is contained on the corresponding genomic fragment.

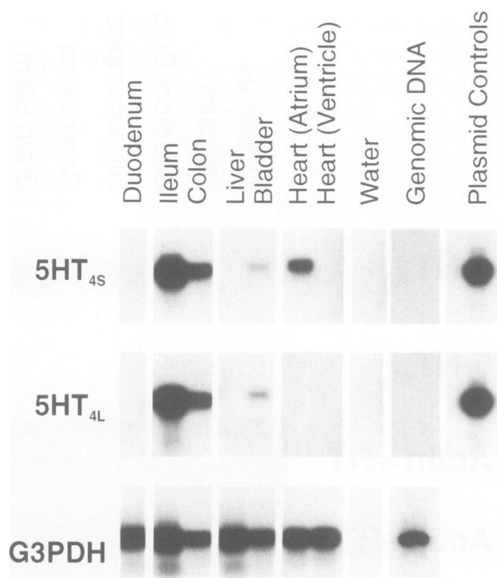

Fig. 8. Peripheral distribution of 5-HT$_{4S}$ and 5-HT$_{4L}$ transcripts. RT-PCR analysis performed with 2.5 ng of mRNA from various organs. The PCR products were analyzed by Southern blotting (see Materials and methods for details). Both isoforms are present in ileum, colon and bladder, but absent in duodenum, liver and heart ventricle. Only the 5-HT$_{4S}$ transcript is present in heart atrium. No 5-HT$_4$-specific PCR product is generated when rat genomic DNA is used as a template.

Discussion

We have identified cDNA clones encoding the pharmacologically defined 5-HT$_4$ receptor. This receptor exhibits low levels of transmembrane amino acid identity (<50%) and similarity with other serotonin receptors, including those that activate adenylyl cyclase (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a; Monsma *et al.*, 1993; Plassat *et al.*, 1993; Ruat *et al.*, 1993). Surprisingly, two receptor cDNA clones differing in their C-terminus regions have been isolated, a long form with 406 amino acids and a short form with 387 amino acids. Since their amino acid sequence is identical between positions 1 and 359, the mRNAs encoding those two receptors are likely to be generated by alternative splicing, as described for other transmembrane receptors (Vanetti *et al.*, 1992, 1993; Namba *et al.*, 1993; Raychowdhury *et al.*, 1994). Alternative C-terminal tails could lead to differences in their G protein coupling and/or their desensitization characteristics (Namba *et al.*, 1993; Reisine *et al.*, 1993; see below). In addition, the 5-HT$_{4L}$ clone has four protein kinase C phosphorylation sites, whereas the 5-HT$_{4S}$ clone has only three. Based on its low frequency in the cDNA library (see Materials and methods), it is likely that the 5-HT$_4$ receptor mRNA is expressed at low levels in rat brain. The long and short forms of the 5-HT$_4$ receptor are differentially expressed in rat brain and the physiological relevance of the restricted expression pattern of the 5-HT$_{4S}$ receptor remains to be determined. In the cerebellum, the level of expression of each form of the 5-HT$_4$ receptor is barely detectable by PCR and is consistent with low receptor densities reported in binding studies (Grossman *et al.*, 1993; Waeber *et al.*, 1993). In rat peripheral tissues, both isoforms were present in ileum, colon and bladder, whereas both isoforms were absent in duodenum, liver and ventricle of the heart. In the atrium, only the 5-HT$_{4S}$ form was detectable. The physiological relevance of the restricted expression pattern of the 5-HT$_{4S}$ receptor remains to be determined.

The receptor binding data obtained with the rat 5-HT$_{4S}$ and 5-HT$_{4L}$ clones using [^{3}H]GR113808 are very similar to those reported for the pharmacologically defined 5-HT$_4$ receptor by Grossman *et al.* (1993) and Waeber *et al.* (1993) with this ligand, using guinea pig and human brain tissues. Specific [^{3}H]GR113808 binding readily saturated both clones (5-HT$_{4S}$ and 5-HT$_{4L}$). Scatchard analysis of specific binding to both receptors revealed the involvement of a single site. Curve-fitting analysis showed an equilibrium dissociation constant for [^{3}H]GR113808 (0.44 $\pm$ 0.02 nM and 0.58 $\pm$ 0.04 nM for 5-HT$_{4S}$ and 5-HT$_{4L}$, respectively, $n = 3$) which is comparable with the affinity estimates derived from functional receptor assays in rat esophagus (0.3 nM; Grossman *et al.*, 1993) but is ~5-fold lower affinity than that obtained for guinea pig and human brain tissues (0.13–0.2 nM; Grossman *et al.*, 1993; Waeber *et al.*, 1993). The K_d value of [^{3}H]GR113808 for rat brain tissue has not been reported. The rank order of potency of compounds competing for specific [^{3}H]GR113808 binding is very similar for both 5-HT$_{4S}$ and 5-HT$_{4L}$ and is cisapride>5-HT>BRL-24924>5-MeOT>zacopride>α-Me-5-HT>5-CT. This order of potency is similar to that observed in binding assays with guinea pig caudate (Grossman *et al.*, 1993; cisapride>5-HT>BRL-24924>zacopride>5-MeOT>α-Me-5-HT>5-CT), but different from the human caudate (Waeber *et al.*, 1993; cisapride>BRL-24924>5-HT>5-MeOT). These differences may be due to a number of variables, including species differences, proportion of high affinity states of the receptor in the various preparations, type of tissues, methodology or receptor subtypes. Tissue-dependent differences in the desensitization mechanisms of 5-HT$_4$ receptors have also been reported (Ford and Clarke, 1993).

The cloned rat 5-HT$_{4S}$ and 5-HT$_{4L}$ genes transiently expressed in COS-7 cells were coupled to stimulation of adenylyl cyclase activity. The magnitude of this response was large (~20- to 25-fold). Apart from ICS-205930, which acted as a silent antagonist, all the compounds tested, including the benzamides (cisapride, BRL-24924, zacopride) which have been reported to be partial agonists at the 5-HT$_4$ receptor in other systems, displayed full agonist activity. This observation, together with the fact that the potency of the agonists was greater than that expected from the measured K_i values, indicated a significant amount of receptor reserve for agonist-induced stimulation of cAMP release in these transfected cells. For the benzamides, the EC$_{50}$ correlated better with the K_i values, which suggests that a lower receptor reserve exists for these compounds as compared with 5-HT, consistent with their partial agonist activity in native tissues. As anticipated, the affinity of ICS-205930 for antagonism of the 5-HT response of both 5-HT$_4$ isoforms is 1–3 orders of magnitude lower than that of 5-HT$_3$ receptors (Richardson *et al.*, 1985). While the 5-HT$_{4S}$ and 5-HT$_{4L}$ isoforms displayed a similar pharmacological profile, some differences were noted. In general, the potency of agonists for stimulating cAMP release was greater for the 5-HT$_{4S}$ receptor. This could be due to

higher expression levels (1.5-fold) of this receptor as compared with 5-HT$_{4L}$ receptor. An inverse relationship between receptor number and EC$_{50}$ value for β-adrenergic receptor-mediated stimulation of cAMP levels has been shown previously in several heterologous expression systems (Bouvier *et al.*, 1988; Samama *et al.*, 1993; Whaley *et al.*, 1994). However, BRL24924 and zacopride in particular displayed significantly lower potency for 5-HT$_{4S}$-mediated functional responses than those elicited by the 5-HT$_{4L}$ receptor. In addition, despite the lower expression levels, the 5-HT$_{4L}$ receptor produced significantly greater stimulation of adenylyl cyclase relative to the 5-HT$_{4S}$ receptor. The differential effect of GTPγS on the two receptor isoforms could be accounted for by dissimilarities in the expression levels of the two receptor proteins. If the amount of endogenous G proteins in the host cell becomes rate limiting at high receptor density, then it can be speculated that the relative proportion of agonist high affinity sites, i.e. coupled to G proteins, would be smaller for the higher expressor (5-HT$_{4S}$) than the lower expressor (5-HT$_{4L}$). In such a case, GTPγS would have a smaller effect on 5-HT$_{4S}$, as compared with 5-HT$_{4L}$, agonist high affinity sites, consistent with the present results. Consequently, the coupling efficiency of the 5-HT$_{4L}$ receptor would be expected to be higher than that of the 5-HT$_{4S}$ receptor, which is compatible with the higher maximum response mediated by 5-HT$_{4L}$ receptors. Tissue-specific differences in the coupling of 5-HT$_4$ receptors with G proteins have been described previously (Grossman *et al.*, 1993).

The rank order of efficiencies of compounds obtained from binding studies using the cloned 5-HT$_4$ receptors (cisapride>5-HT>BRL-24924>zacopride) correlated well with that obtained in functional studies using preparations such as guinea pig hippocampus (Bockaert *et al.*, 1990) or mouse embryo colliculi (Dumuis *et al.*, 1989). However, when such a comparison was made using the EC$_{50}$ values obtained from functional responses mediated by the cloned 5-HT$_4$ receptors, the best correlation was obtained with the guinea pig ileum (Craig and Clarke, 1990).

Although 5-HT$_{4S}$ and 5-HT$_{4L}$ are putative splice variants and differ only at their C-terminal tails, they may nevertheless possess different coupling efficiencies to elicit functional responses. This would be consistent with recent evidence from other receptor subtypes, including the somatostatin receptor (SSTR2; Reisine *et al.*, 1993) and prostaglandin EP3 receptor subtypes (Namba *et al.*, 1993), that undergo alternative splicing to generate receptors differing in amino acid sequence only at their C-termini. Although these other receptor splice variants have identical ligand binding properties, they have different characteristics in producing functional responses, as we have observed for the 5-HT$_{4S}$ and the 5-HT$_{4L}$ clones. For the EP3 receptor subtype, it has been demonstrated that the alternatively spliced variants have different specificities for coupling to G proteins which may contribute to the variations in the physiological responses produced by these receptor subtypes (Namba *et al.*, 1993). Whether 5-HT$_{4S}$ and 5-HT$_{4L}$ couple to different isoforms of Gs and/or adenylyl cyclase remains to be investigated. It is noteworthy that the 5-HT$_{4S}$ mRNA is only expressed in the striatum where adenylyl cyclase V is selectively concentrated (Glatt and Snyder, 1993). It will be interesting to see whether the 5-HT$_{4S}$ receptor couples preferentially to adenylyl cyclase V. The 5-HT$_{4S}$ receptor exhibits a weaker adenylyl cyclase response than the 5-HT$_{4L}$ receptor in COS-7 cells. Recent evidence indicates that COS-7 cells contain at least adenylyl cyclase type VI and VII, as detected by PCR-based mRNA detection (Chen and Iyengar, 1993). These cyclases are members of the type V and II cyclase families, respectively.

The amino acid sequence, pharmacological binding profile and the functional coupling obtained from cells expressing 5-HT$_{4S}$ and 5-HT$_{4L}$ clones indicate that these cDNAs encode a member of the 5-HT receptor family, the pharmacologically defined 5-HT$_4$ receptor. All the unique pharmacological characteristics described above define 5-HT$_{4S}$ and 5-HT$_{4L}$ clones as the adenylyl cyclase stimulatory '5-HT$_4$' receptor that has been examined by functional responses in the ileum (Craig and Clarke, 1989, 1990), colon (Wardle *et al.*, 1994), hippocampus (Shenker *et al.*, 1987), esophagus (Baxter *et al.*, 1991), embryonic collicular neurons (Dumuis *et al.*, 1989), atrium (Kaumann *et al.*, 1990), adrenal (Lefevre *et al.*, 1992) and bladder (Corsi *et al.*, 1991), and distinguish these clones from all other subtypes of 5-HT receptor. Differences in the distribution, signaling pathways and, possibly, desensitization mechanisms of the 5-HT$_{4S}$ and 5-HT$_{4L}$ clones indicate that alternative splicing may be a mechanism by which functional diversity is established for 5-HT$_4$ receptors.

Materials and methods

PCR amplification

The third (III) and fifth (V) transmembrane (TM) domains of the following receptors were aligned and used to synthesize a pair of degenerate primers: 5-HT$_{1A}$, 5-HT$_{1C}$, 5-HT$_2$ and the 5-HT$_{1D\alpha/\beta}$ receptors. Primers 3.17 and 5.5 [5′-TGGAATTCTG(C/T)G(C/T)IAT(A/C/T)(G/T)CICTGGA(C/T)(A/C)G(C/G)TA-3′], [5′-CATIA(G/C/A)I(G/A)IIA-(G/A)IGG(T/G/A/)AT(G/A)(T/A)A(G/A)AAIGC-3′) were used to amplify 5 μg of poly(A)$^+$ RNA from rat brain that was reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase. PCR was performed on single-stranded cDNA under the following conditions: 94°C for 1 min, 50°C for 2 min and 72°C for 3 min for 40 cycles. Following PCR, 90 μl of the reaction were phenol:chloroform extracted and precipitated; 10 μl were visualized on a gel using ethidium bromide staining. After precipitation, the sample was treated with T4 DNA polymerase and digested with *Eco*RI prior to separation on a 1% agarose gel. The DNA fragments (200–400 bp) were isolated from the gel, phosphorylated and cloned into pBluescript (*Eco*RI–*Sma*I cut). Recombinant clones were analyzed by sequencing. One fragment 270 bp in length, named S10, was identified. This sequence contained a 'TM IV' like domain and represented a potentially new serotonin receptor. The corresponding full length cDNA was isolated from a rat brain cDNA library.

cDNA library construction, screening and sequencing

Rat brains were dissected from adult male CD rats (Charles River Laboratories) and total RNA was prepared by the guanidine thiocyanate method (Kingston, 1987). Poly(A)$^+$ RNA was purified with a FastTrack kit (Invitrogen Corp., San Diego, CA). Double-stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA according to Gubler and Hoffman (1983). The resulting double-stranded cDNA was ligated to *Bst*XI–*Eco*RI adaptors (Invitrogen Corp.), the excess adaptors were removed by chromatography on Sepharose CL 4B (Pharmacia LKB) and the DNA was then size selected on a Gen-Pak Fax HPLC column (Waters, Millipore Corp., Milford, MA) (Zhao *et al.*, 1992). High molecular weight fractions were ligated in pCDM8 cut by *Bst*XI (Invitrogen Corp.). The ligated DNA was electroporated in *Escherichia coli* MC 1061/P3 (Gene Pulser, Bio-Rad). Before amplification, the library was divided into pools of 2.5–5$\times$10^4 independent clones. After 18 h amplification, the pools were stored at –85°C in 20% glycerol. One hundred pools of the cDNA library, representing 3.2$\times$10^6 primary clones

(1.9 kb average size inserts), were screened using exact PCR primers derived from the S10 PCR clone sequence.One μl (4×10^6 bacteria) of each amplified pool was subjected directly to 40 cycles of PCR and the resulting products analyzed by agarose gel electrophoresis and Southern blotting. Two out of four positive pools were analyzed further and, by sib selection and plating out, two individual full length cDNA clones, 5-HT_{4S} and 5-HT_{4L}, were isolated. Double-stranded DNA was sequenced with a sequenase kit (US Biochemical, Cleveland, OH) according to the manufacturer. Nucleotide and peptide sequence analyses were performed with GCG programs (Genetics Computer Group, Madison, WI).

DNA transfection

The full coding regions of the 5-HT_{4S} and 5-HT_{4L} genes were subcloned in the correct orientations in the mammalian expression vector pEXJ.BS (a vector derived from pcEXV-3; Okayama and Berg, 1983; Miller and Germain, 1986). For transient expression, COS-7 cells were transfected by the DEAE–dextran method, using 1 μg of DNA/10^6 cells (Warden and Thorne, 1968; Cullen, 1987).

Tissue localization studies

CNS. Rat brain areas were dissected from adult male CD rats (Charles River Laboratories) and total RNA extracted using the guanidine isothiocyanate/CsCl cushion method (Kingston, 1987). Polyadenylated RNA was purified on oligo(dT)–cellulose (Pharmacia, Piscataway, NJ) and cDNA was prepared from 1 μg of mRNA with random hexanucleotide primers (0.2 ng/μg of mRNA) using AMV reverse transcriptase and the conditions recommended by the manufacturer (Seikagaku, Rockville, MD). An aliquot of the first strand cDNA, corresponding to 60 ng of RNA, was diluted (1:25) in a 50 μl PCR reaction mixture (200 μM dNTPs final concentration) containing 1.25 U of *Taq* polymerase in the buffer supplied by the manufacturer (Perkin Elmer Cetus, Norwalk, CT), and 1 μM of primers S10-4/S10-13 for 5-HT_{4S} (coordinates 787–804 for S10-4 and 1200–1217 for S10-13; Genbank accession number U20906) and S10-4/S10-15 for 5-HT_{4L} (coordinates 787–804 for S10-4 and 1189–1206 for S10-15; Genbank accession number U20907). PCR was performed under the following conditions: 94°C for 1 min, 50°C for 2 min and 72°C for 3 min for 40 cycles, with pre- and post-incubation of 95°C for 5 min and 72°C for 10 min, respectively. The PCR products were run on a 1.5% agarose gel and subjected to Southern blot analysis with ^{32}P-5′-end-labeled probes internal to the PCR primers (probe S for 5-HT_{4S}, coordinates 1213–1257; probe L for 5-HT_{4L}, coordinates 1253–1297; see Figure 1C).

Periphery. Rat tissues were dissected and poly(A)$^+$ RNA extracted using the Micro-Fast Track kit (Invitrogen, San Diego, CA). Poly(A)$^+$ RNAs were treated with DNase (BRL, Gaithersburg, MD) to remove any contaminating genomic DNA, according to the manufacturer's protocol. cDNA was prepared from mRNA with random hexanucleotide primers using reverse transcriptase (Superscript II; BRL). An aliquot of the first strand cDNA [2.5 ng of poly(A)$^+$ RNA] was amplified in a 25 μl PCR reaction mixture (200 μM dNTPs final concentration) containing 1.2 U of *Taq* polymerase in the buffer supplied by the manufacturer (Perkin-Elmer Corp.), and 1 μM of primers, using a program consisting of 30 cycles of 94°C for 2 min, 68°C for 2 min and 72°C for 3 min, with a pre- and post-incubation of 95°C for 5 min and 72°C for 10 min, respectively. A forward PCR primer common to both human 5-HT_{4S} and 5-HT_{4L} was designed against the third intracellular loop, 5′-GACCAGCACAGCACACATCGCAT-3′, and reverse PCR primers, specific to 5-HT_{4S} and 5-HT_{4L}, were designed to their corresponding C-terminal regions: 5′-GGACTCTGGGTCATTGTGTATGGG-3′ and 5′-TACAGCTGTCTTCTAGGTCATTGTC-3′, respectively. The PCR products were run on a 1.5% agarose gel and transferred to charged nylon membranes (Zetaprobe GT, BioRad). Filters were hybridized with ^{32}P-5′-end-labeled probes and washed under high stringency. The hybridization primers were designed to the 5-HT_{4S}- and 5-HT_{4L}-specific C-terminal regions and flanked by the PCR primers discussed above: 5′-TTTGCATAGTGGTCAACACCAGGAACTGGAGAAGTTGCCCATACA-3′ and 5′-ACTTCTCCTTTGGTGGCTGCTCAGCCAGTGATACGTAGGCCCCAG-3′, respectively. Under the above conditions, PCR products identified on Southern blots using the isoform-specific probes and isoform-specific PCR primers showed no cross-reactivity to the non-targeted template (data not shown). To assess relative quantities of cDNAs from different tissue sources, similar PCR and Southern blot analyses were conducted with primers and probe directed to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA).

Membrane preparation

Membranes were prepared from transiently transfected COS-7 cells which were grown to 100% confluence. The cells were washed twice with phosphate-buffered saline (PBS), scraped from the culture dishes into 5 ml of ice-cold PBS and centrifuged at 200 *g* for 5 min at 4°C. The pellet was resuspended in 2.5 ml of ice-cold Tris buffer (20 mM Tris–HCl, pH 7.4 at 23°C, 5 mM EDTA), and homogenized by a Wheaton tissue grinder. The lysate was subsequently centrifuged at 200 *g* for 5 min at 4°C to pellet large fragments which were discarded. The supernatant was collected and centrifuged at 40 000 *g* for 20 min at 4°C. The pellet resulting from this centrifugation was washed once in ice-cold Tris wash buffer and finally resuspended in final buffer containing 50 mM Tris–HCl and 0.5 mM EDTA, pH 7.4 at 23°C. Membrane preparations were kept on ice and utilized within 2 h for the radioligand binding assays. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Radioligand binding assays

Binding was performed as follows. Radioligand binding studies were achieved at 37°C in a total volume of 250 μl of buffer (50 mM Tris, 10 mM $MgCl_2$, 0.2 mM EDTA, 10 μM pargyline, 0.1% ascorbate, pH 7.4 at 37°C) in 96-well microtiter plates. Initial screening of clones was performed using [^{3}H]5-HT (5–10 nM; Dupont-NEN, Boston, MA) as a radioligand. However, the radiolabeled antagonist [^{3}H]GR113808 was found to produce a very robust binding signal and thus was used for all subsequent studies. Saturation studies were conducted using [^{3}H]GR113808 (Amersham, Arlington Heights, IL) at 8–10 different concentrations ranging from 0.005 to 2.5 nM. Displacement studies were performed using 0.2–0.4 nM [^{3}H]GR113808. The binding profile of drugs in competition experiments was established using 10–12 concentrations of compound. Incubation times were 30 min for both saturation and displacement studies. Non-specific binding for [^{3}H]GR113808 binding was defined in the presence of 50 μM 5-HT. Binding was initiated by the addition of 50 μl membrane homogenates (10–20 μg protein). The reaction was terminated by rapid filtration through pre-soaked (0.5% polyethyleneimine) filters using the 48R Cell Brandel Harvester (Gaithersburg, MD). Subsequently, filters were washed for 5 s with ice-cold buffer (50 mM Tris–HCl, pH 7.4 at 4°C), dried and placed into vials containing 2.5 ml of Ready-Safe scintillation cocktail (Beckman, Fullerton, CA) and radioactivity was measured using a Beckman LS 6500C liquid scintillation counter. The efficiency of counting of [^{3}H]GR113808 averaged between 45 and 50%. Binding data were analyzed by computer-assisted non-linear regression analysis (Accufit and Accucomp, Lundon Software, Chagrin Falls, OH). IC_{50} values were converted to K_i values using the Cheng–Prusoff equation (1973).

Measurement of cAMP formation

For measurement of intracellular cAMP accumulation, transiently transfected COS-7 cells were incubated in Dulbecco's modified Eagle's medium containing 5 mM theophylline, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 10 μM pargyline and/or appropriate concentrations of antagonists for 20 min at 37°C, 5% CO_2. Serotonin or other agonists were then added at appropriate concentrations in the absence or presence of FSK (10 μM) and incubated for an additional 10 min at 37°C, 5% CO_2. The medium was aspirated and the reaction stopped by the addition of 100 mM HCl. The plates were stored at 4°C for 15 min, centrifuged for 5 min at 500 *g* to pellet cellular debris and the supernatant aliquotted and stored at –20°C prior to assessment of cAMP formation by radioimmunoassay (cAMP Radioimmunoassay kit, Advanced Magnetics, Cambridge, MA). Radioactivity was quantitated using a Packard COBRA Auto Gamma Counter equipped with data reduction software. Functional data were fitted to a four-parameter logistic equation to obtain response parameters (EC_{50}, E_{max}, nH; Inplot, GraphPad, San Diego, CA).

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