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5-HT₄ Receptors: Gene, Transduction and Effects on Olfactory Memory

Joël Bockaert*, Sylvie Claeyssen, Michèle Sebben, Aline Dumuis

CNRS UPR 9023, Centre CNRS-INSERM de Pharmacologie-Endocrinologie (CCiPE), 141
rue de la Cardonille, 34094 Montpellier cedex 5 France

*Corresponding author: Tel: 33.4.67.14.29.30; Fax: 33.4.67.54.24.32;

E-mail address: bockaert@ccipe.montp

ABSTRACT

In this paper we discuss 1) the primary structures, pharmacology, and brain distribution of cloned 5-HT₄ receptors; 2) the chromosomal localization of the h5-HT₄ receptor; 3) whether benzamides are full or partial agonists because of a species or a coupling difference; 4) the intrinsic activity of 5-HT₄ receptors and inverse agonism of GR125487 in COS-7 cells but not in colliculi neurons; 5) the modulation of 5-HT₄ receptor binding and activity; and 6) the long-term blockade of K⁺ channels by 5-HT₄ agonists and its effect on olfactory memory. We conclude that 1) the cloning of 5-HT₄ receptors in different species using RT-PCR from different tissues reveals the presence of several splice variants for 5-HT₄ receptors differing in the C-terminal part, downstream from the amino acid L₃₅₈; 2) the pharmacological properties of 5-HT₄ receptors are dependent on the cellular context in which they are expressed; and 3) 5-HT₄ agonists can be added to the list of compounds having pro-cognitive properties.

5-HT₄ receptors were first characterized by their ability to stimulate cAMP production in mouse colliculi neurons and by the inability of classical 5-HT₁, 5-HT₂ and 5-HT₃ antagonists to inhibit this function.¹ It was rapidly shown that pharmacologically similar receptors were present in a wide variety of tissues and species including rodent porcine and human brain, guinea-pig ileum and colon, rat esophagus, frog and human adreno-cortical cells, human urinary bladder, human and porcine heart (for a review see ²⁻⁴). Before the characterization of 5-HT₄ receptors, two substituted benzamides, metoclopramide and cisapride were already used to treat dyspepsia, gastroesophageal-reflux disease and to a lesser extent, gastroparesis.² This interesting therapeutical function of 5-HT₄ agonists and the proposed new pathophysiological properties of 5-HT₄ receptors, both in the central nervous system (increase in memory, role in anxiety and possibly in central pain)⁵⁻⁹ and at the periphery (implication in arrhythmia and irritable bowel syndrome),¹⁰⁻¹¹ have stimulated the search and discovery of a very large series of selective and potent agonists and antagonists.^{4,12} Thanks to these pharmacological tools, to the first cloning of rat 5-HT₄ receptors by Gerald *et al.*,¹³ and to the successful labeling of potent 5-HT₄ antagonists and agonists, many questions have now been addressed. Among them, we can quote the tissular and cellular distribution, especially in brain, the possible multiplicity of 5-HT₄ receptors, their coupling characteristics to G proteins, the molecular basis of the difference in efficacy of some of their agonists, especially of benzamides when tested in different preparations, the molecular properties of their desensitization, down-regulation and up-regulation, the difference between their pharmacological behavior when expressed in normal and transfected cells and finally their physiological properties. In this report, we will discuss some of these questions based on our recent work in this field.

PRIMARY STRUCTURES, PHARMACOLOGY AND BRAIN DISTRIBUTION OF CLONED 5-HT₄ RECEPTORS

Comparison between the primary structures of 3 different splice variants of 5-HT₄ receptors in rats, mice and humans

For unclear reasons, but certainly because of the low abundance of the corresponding mRNA as well as the low sequence homologies with other 5-HT receptors, the cloning of the 5-HT₄ cDNA was delayed until 1995. Using a strategy based on nucleotide homologies between transmembrane domains III and V of 5-HT receptors, Gerald *et al.*¹³ isolated two cDNAs coding for G protein-coupled receptors (GPCRs) having 387 and 406 amino-acid residues, respectively.

Although they were called 5-HT_{4S} and 5-HT_{4L}, we will call them 5-HT_{4(a)} and 5-HT_{4(b)} respectively, according to the NC-IUPHAR recommendations.¹⁴ Performing RT-PCR experiments with poly^{(a)+} RNA from mouse (m) colliculi neurons, rat (r) brain, human (h) atrium, using primers designed to clone forms (a) and (b), we isolated several full length cDNA (each from at least three independent PCR). Sequence analysis (Fig. 1) revealed that we have cloned: 1) the m5-HT_{4(a)} and (b) receptors¹⁵ ; 2) the h5-HT_{4(a)}16 and h5-HT_{4(b)} receptors (see Claeyssen *et al.*, this volume); 3) in addition, we isolated another cDNA (m5-HT_{4(c)}) from mouse (Fig. 1), rat and human brain (see Claeyssen *et al.*, this volume). m and r5-HT_{4(c)} receptors encoding a protein of 372 residues. Although we previously published,¹⁵ as Gerald *et al.*,¹³ that the 5-HT_{4(b)} receptor had 406 amino-acid residues, we realized, after reading the report by Van den Wyngaert *et al.*¹⁷ and reexamining our sequencing gels that, as proposed by Van den Wyngaert *et al.*, the 5-HT_{4(b)} receptor was only 388 amino-acid residues long (Fig. 1). The reason for the sequencing error was the fact that there were indeed 3 and not 2 cytosines after position 1152. This introduced a shift in the reading frame resulting in a stop codon (TAG) at position 1165–1167.

Alignment of the C-termini amino-acid sequences of the three 5-HT₄ forms shows that the divergence in their sequences starts after position L₃₅₈, their last common amino-acid. However, to conclude that all these forms are derived by alternative splicing of a common gene should wait until we know its structure. Therefore, after L₃₅₈, the 5-HT_{4(a)}, (b), (c) receptors have their own specific sequence, the length of which being 29, 30 and 13 residues respectively. The generation of alternative splicing in GPCRs, as a source of functional diversity of coupling, is particularly evident in pituitary adenylyl cyclase activating polypeptide (PACAP) receptors and prostaglandin EP3 receptors.^{18,19} The sensitivity of 5-HT_{4(a)} and (b) to GTPγs is slightly different¹³ and preliminary experiments indicate that they may have a different capacity to be desensitized, the long form 5-HT_{4(b)} being more sensitive to desensitization than the short form 5-HT_{4(a)}.²⁰ In this context, it is interesting to remember that we have described that a rapid and homologous desensitization of 5-HT₄ receptors occurs in colliculi neurons.²¹ This type of desensitization is classically triggered via phosphorylation of the receptors by specific G-protein-coupled receptor kinases called GRKs.²² Although we do not know for sure what are the consensus sequences recognized by GRKs in GPCRs, a recent report proposed that S and T residues downstream of an acidic doublet such as DE, ED or EE in the C-termini of GPCRs, could be the target of GRKs.²³ Examining the C-termini of the three r and m5-HT₄ spliced variants revealed that they have the DE (331–332) doublet in common. It remains to be

demonstrated which of the serines and threonines following this acidic doublet are indeed phosphorylated during desensitization.

The 5-HT₄ receptors have all the canonic residues which characterize the majority of GPCRs (group I) and are not present in the two other groups. Group II GPCRs includes receptors for peptides of the PACAP/VIP/secretin family and group III contains metabotropic glutamate and GABA receptors as well as Ca²⁺ sensing receptors.²⁴ These residues comprise: 1) D₆₆ in transmembrane domain (TMD) II, D₁₀₀ in TMD III engaged in the coupling to G protein and aminergic ligand binding pocket respectively; 2) the cysteines of extracellular loops e1 and e2, likely to be engaged in a disulfide bridge;²⁵ 3) the DRY sequence at the N-terminus of the intracellular loop i2 so important in the structural modification of group I GPCRs associated with activation;^{25,26} 4) the prolines in TMD V, TMD VI and TMD VII; 5) a cysteine (C₃₂₈) which is a potential palmitoylation site.²⁷ r, m and h5-HT₄ receptors have a tyrosine in i2 (Y 129) conserved in many GPCRs of group II which could be, as in β₂-adrenergic receptors, a putative tyrosine kinase phosphorylation site, as well as a tyrosine at the end of TMD VII which is part of a consensus sequence NPXXY₃₁₂ implicated in sequestration.²⁸ 5-HT_{4(b)} and 5-HT_{4(a)} have three putative potential protein kinase C (PKC) phosphorylation sites (Fig. 1). Interestingly, there is no putative PKA phosphorylation site in 5-HT₄ receptors. This may explain why we have found that desensitization of the 5-HT₄ receptor in colliculi neurons and rat esophagus cannot be desensitized by cAMP (See V).^{21,29}

Comparison between the pharmacology of the cloned r, m and h5-HT₄ receptors and the naturally expressed 5-HT₄ receptor in colliculi neurons

Comparing the homologies between r, m and h5-HT_{4(b)} receptors reveals only 11, 25 and 25 amino-acid substitutions between r and m, between r and h and between m and h receptors, respectively. None were, *a priori*, at positions susceptible to modify their pharmacology. Indeed, comparing the binding affinity constants for a series of agonists and antagonists for the m, r, and h5-HT₄ receptors expressed in cell lines, revealed no difference in pharmacology; a significant correlation ($r > 0.95$) was found in each case.^{15,16} Furthermore, comparing the affinities of 11 agonists and 5 antagonists for the cloned m5-HT₄ receptors and the corresponding receptor coupled to AC in mouse colliculi neurons shows an excellent correlation ($r = 0.93$).

Brain Distribution of 5-HT_{4(a)} and 5-HT_{4(b)}

Gerald *et al.*,¹³ reported that the mRNA encoding r5-HT_{4(a)} was only expressed in striatum, whereas the mRNA encoding r5-HT_{4(b)} was expressed in all other brain areas including striatum. Using the same technique, we could not confirm this observation. In contrast, we found that both 5-HT_{4(a)} and 5-HT_{4(b)} mRNA were expressed in all brain areas, in rat and in mouse brain whatever the post-natal development.¹⁵ This finding was confirmed using an *in situ* hybridization approach and specific probes for 5-HT_{4(a)} and 5-HT_{4(b)}.³⁰ The expression pattern of 5-HT_{4(c)} mRNA in different brain areas analyzed was identical to that of 5-HT_{4(a)} mRNA. In conclusion, the general distributions of 5-HT_{4(a)}, (b) and (c) mRNA are not different. This does not mean that they are expressed by the same cells within the various brain areas or that a difference between their somato-dendritic versus axonal distribution does not exist.

CHROMOSOMAL LOCALIZATION OF h5-HT₄ RECEPTOR

Chromosomal localization was accomplished using fluorescence *in situ* hybridization to localize the 5-HT₄ receptor gene in human as described.^{31,32} Analysis of 52 metaphase preparations with the 1.2 kb 5-HT₄ receptor probe using a concentration of 250 ng/ml of cDNA showed a specific labeling on chromosome 5 bands q31-q33 which is syntenic to the region carrying two other receptors positively coupled to adenylyl cyclase, the dopamine D₁ receptor and the β₂-adrenergic receptor.³³

ARE BENZAMIDES FULL OR PARTIAL AGONISTS BECAUSE OF A SPECIES OR A COUPLING DIFFERENCE?

One of the main pharmacological differences between the 5-HT₄ receptors described in different tissues or species, results from the fact that benzamides are either full (mouse colliculi neurons),³⁴ or partial agonists (guinea-pig hippocampal membranes, rat esophagus, distal colon, human heart atria).^{2,35-37} Figure 2 illustrates that renzapride is a full agonist compared to 5-HT in stimulating the 5-HT₄ receptor coupled to AC in colliculi neurons², and is a weak partial agonist in human heart in stimulating L type Ca²⁺ channels (Fig. 2b). It was therefore of interest to examine the reason for which renzapride had such a poor efficacy on human heart 5-HT₄ receptors. Saturation binding with [³H]GR 113808 revealed a population of 8 ± 2 fmol/mg of 5-HT₄ receptors identified in human right atrium with a KD of 0.1 ± 0.03 nM close to that of

the cloned human 5-HT_{4(a)} receptors (not shown). In COS-7 cells expressing a low density of receptors (36 ± 8 fmol/mg), renzapride acted indeed as a partial agonist (Fig. 2c). However, when tested on cells expressing an increasing number of receptors (258 ± 26 fmol/mg), the partial agonism of renzapride was progressively suppressed (Fig. 2d).

According to the classical pharmacological³⁸ and operational models,³⁹ if one supposes that the agonist (H) interacts with the receptors (R) following a simple mass action kinetic:

$$RH = RT \frac{H}{Kd + H} \quad (\text{Equation 1})$$

and that a chain of hyperbolic coupling steps (including the interaction of RH with G proteins),

occurs between the formation of RH and the final response. Then, the final response E (proportional to RHG) is expected to be a hyperbolic function of the ligand concentration, as generally observed. In addition, the final response (E) (cAMP production) is also a hyperbolic function of RH:

$$E = ET \frac{RH}{C_t + RH} \quad (\text{Equation 3}).$$

It is possible to determine the coupling constant (C_t) for 5-HT and renzapride as well as maximal cAMP which can be produced (ET) when RH is very high compared to C_t . Plotting $E_{\max}$ (maximal activity obtained for saturating H concentrations and for a given receptor concentration (R)) as a function of RT (the concentration of RH at saturating H concentration) which was experimentally modified (Fig. 2e) gives hyperbolic functions. Plotting $E_{\max}$ as a function of E/RT gives a straight line. ET is equal to $E_{\max}$ when $E_{\max}/RT = 0$. In COS-7 cells, the coupling constant for 5-HT was 2 times higher than the coupling constant for renzapride (135 ± 9 and 70 ± 3 fmol/mg protein, respectively). As indicated in Figure 2f, ET values were identical for 5-HT and renzapride. This implies that at high receptor density, renzapride is a full agonist. Interestingly in LLC-PK1, a similar analysis also gave a 2-fold difference between C_t values for 5-HT and renzapride (46 ± 4 and 26 ± 2 fmol/mg protein, respectively) and again similar values of ET for the two drugs.

The conclusion from these experiments is that the difference of efficacies of benzamides compared to 5-HT, when tested in different preparations, is due to the fact that when occupied by benzamides, the affinity of RH for G protein (C_t) is lower than when occupied by 5-HT (Figs. 2c and d). Therefore, when the receptor density is low, like in human heart, the maximal

response which can be obtained at saturating ligand concentrations ($E_{\max}$) is lower (lower efficacy) when H is benzamide than when L is 5-HT.

INTRINSIC ACTIVITY OF 5-HT₄ RECEPTORS AND INVERSE AGONISM OF GR125487 IN COS-7 CELLS BUT NOT IN COLLICULI NEURONS

When one compares the basal cAMP production in mock transfected COS-7 cells and in cells transfected with 123 fmol/mg protein of 5-HT₄ receptors, it is clear that it was 2-fold higher in the latter than in the former (Fig. 3). This is the result of an intrinsic activity of the receptors because it cannot be inhibited by RS 100235 a potent antagonist of the receptor.⁴⁰ It is now accepted that receptors exist in equilibrium between two conformations, an inactive conformation R and an active conformation R*. Spontaneous isomerization occurs between these two forms, but only R* interacts productively with the G protein.^{41,42} In the absence of agonist, there are two main possibilities for increasing the absolute copies of R*. One is to introduce a mutation in the receptor,⁴³⁻⁴⁵ the other is to increase the number of receptors keeping the same isomerization ratio between R and R*.⁴⁶⁻⁴⁸ This result in an absolute increase in R*. Generally, it is necessary to highly increase (above 1 pmol/mg protein) the receptor concentration to obtain a significant increase in basal activity. What is surprising with 5-HT₄ receptor is that a doubling of basal activity is obtained for physiological receptor concentrations (around 100 fmol/mg protein). It has been shown with β and α adrenergic receptors, that the C-terminal part of i3 is very important for obtaining a constitutively activated receptor. Comparing the C-terminal part of i3 in 5-HT₄ receptors and β_2 -adrenergic receptors, two receptors coupled to Gs, indicates that among the last 8 residues, 5 are identical (Fig. 3b). In addition, the three different residues (T₂₅₃, T₂₅₅, A₂₅₈ in 5-HT₄ receptors and K₂₆₇, H₂₆₉ L₂₇₂ in β -adrenergic receptors) are those which confer, when mutated, a high constitutive activity (constitutively active mutant = CAM) to β_2 -adrenergic receptors⁴³ (Fig. 3b). In particular, the A₂₅₈ in native 5-HT₄ receptor is homologous to L₂₇₂ in β_2 native adrenergic receptors which was mutated to A₂₇₂ in CAM. It would be interesting to exchange the 3 different residues between 5-HT₄ and β_2 -adrenergic receptors to see if the intrinsic activity of the 5-HT₄ receptor were to be decreased, whereas that of β_2 adrenergic receptor were to be increased.

Since we found that intrinsic activity of 5-HT₄ receptors can be obtained for physiological concentrations of receptors which are expressed in COS-7 cells, it was interesting to check if an intrinsic activity is also present in neurons expressing similar concentrations of

receptors (60 ± 23 fmol/mg protein). To perform this experiment, we needed to find an inverse agonist able to reverse the intrinsic activity of 5-HT₄R*. We found that GR125487 is an inverse agonist in COS-7 cells transfected with 98 ± 16 fmol/mg protein of 5-HT₄ receptor; it inhibited the basal cAMP formation, whereas it was not able to inhibit basal cAMP formation in neurons expressing similar amounts of receptors. Therefore, it seems that there is less R* present in neurons than in COS-7 cells for the same total R + R* concentration. Several explanations can be proposed. Either the isomerization constant is different, due to the cellular context or the concentration of G is different leading to less R*G in neurons than in COS-7 cells. Whatever the explanation, these results provide another illustration of the difference in pharmacology which could be found when the same molecules of receptors are expressed in different cellular contexts.

MODULATION OF 5-HT₄ RECEPTOR BINDING AND ACTIVITY

One of the very important physiological characteristics of receptors is their ability to modulate the signal they deliver to the cell depending upon the intensity and duration of the stimulation they receive. An over-stimulation will desensitize the receptor, a stimulation lower than normal will hypersensitize the receptor. There are several steps in the desensitization of GPCRs. The first step is an uncoupling between receptors and G proteins and is believed to be a consequence of their phosphorylation by GRKs, this is the desensitization step *per se*. When the desensitization is triggered by receptor occupation and GRKs without the participation of second messenger-activated kinases, it is called “homologous desensitization”. The desensitization triggered by second messenger-activated kinases are called “heterologous desensitization”. A second step consists of “sequestration” of receptors in a weakly defined compartment and a third one consists of a “down-regulation” of the receptor concentration.

Homologous desensitization of 5-HT₄ receptors in colliculi neurons

5-HT desensitized very rapidly in various preparations. In colliculi neurons and rat esophagus, we have analyzed the molecular mechanisms of this desensitization.^{21,29} More than 50% desensitization occurred following a 5 min exposure to 5-HT. This rapid desensitization was not mediated nor produced by cAMP or other agonists that increase cAMP in these cells. This suggested that the desensitization was homologous. In the β -adrenergic system, homologous desensitization is dependent on phosphorylation of the agonist-occupied receptor

by GRK. A good correlation is observed between the affinities (pEC_{50}) of a series of agonists and the percentage of desensitization they induce. No correlation was obtained between the efficacies (E_{max}) of 5-HT₄ agonists and their abilities to desensitize 5-HT₄ receptors. This indicates that homologous desensitization is a function of mean time of receptor occupancy by the agonist. This is consistent with the fact that receptor kinases (such as β ARK) phosphorylate only occupied receptors. One consequence of this observation is that following activation with agonists having a low potency (like metoclopramide), receptor desensitization has a much slower time-course than in the presence of potent agonists (like cisapride). During rapid desensitization (a 5-HT stimulatory period shorter than 30 min), the homologous desensitization is not associated with any loss in 5-HT₄ binding sites labeled with [³H]GR 113808.⁴⁹ However, if the 5-HT₄ receptor stimulation is prolonged over 30 min, a loss of binding sites is apparent. This indicates that *in vivo*, the level of expression of 5-HT₄ receptors may be related to the tonic activation of the receptor. In preliminary experiments, we found that desensitization of 5-HT₄ receptors (m5-HT_{4(a)} and m5-HT_{4(b)}), transiently transfected in COS-7 cells, is very weak compared to that found in colliculi neurons.

Up regulation of 5-HT₄ receptors in vivo

Lesions of 5-HT neurons with 5,7-dihydroxytryptamine induced an up-regulation of 5-HT₄ receptor density in the rostral but not the caudal part of the caudate putamen, in the nucleus accumbens, and in the hippocampus.⁵⁰ The reason for the absence of up-regulation in the caudal part of the caudate putamen is not clear because these areas received the highest 5-HT innervation. Even more unexpected was the fact that dopaminergic denervation also induces an up-regulation of 5-HT₄ receptors in the caudal but not the rostral part of the caudate-putamen, in the globus pallidus but not in the substantia nigra. In line with this result, it has recently been published that striatal 5-HT_{1B} and 5-HT₂ receptors can be up-regulated after dopaminergic denervation. These data reinforce the view that complex reciprocal 5-HT/dopamine interactions are involved in the basal ganglia function.⁵¹ The fact that up-regulation of 5-HT₄ receptors was restricted to the rostral part after 5-HT denervation and to the caudal part after dopaminergic innervation may reflect differential influences exerted by the two monoaminergic systems in striatal regions.⁵⁰

LONG-TERM BLOCKADE OF K⁺ CHANNELS BY 5-AGONISTS AND EFFECT ON OLFACTORY MEMORY

Long-term inhibition of K⁺ channels

In colliculi neurons, voltage-clamp experiments show that 5-HT₄ receptors inhibited a delayed rectifier voltage-dependent K⁺ current. This effect was mimicked by cAMP and other agents able to increase cAMP (forskolin, isoproterenol, PACAP) and blocked by PKA inhibitors. In current-clamp experiments, 5-HT₄ receptors and cAMP have been shown to slower the falling phase of the action potential, inhibit the after-hyperpolarization (AHP) and spike accommodation. A remarkable observation was that after a short application (a few seconds) of 5-HT or 8-Br-cAMP all the effects described above slowly developed and remained stable for more than 30 min after washout of the drugs.⁵² Indeed, a complete recovery from the blockade was observed only after 2 hours. The blockade could be prolonged up to 4 hours in the presence of okadaic acid, an inhibitor of phosphatases 1 and 2A. We have shown that these long-term effects were not due to a prolonged activation of PKA, but to a prolonged inhibition of phosphatase activity. A tentative hypothesis is that the prolonged inhibition of phosphatases may result in a prolonged phosphorylation of some K⁺ channels. A delayed rectified K⁺ channel, having similar characteristics as the one recorded here, has been cloned (KV3.2) and is inhibited after PKA-induced phosphorylation. The implication of the inhibitor of protein phosphatase 1 (PP1), which is activated by PKA, is likely since preliminary results indicate that blockade of the K⁺ channel is reduced in PP1 mice deficient (PP1 knock-out mice provided by P. Greengard). Such a long-lasting broadening of the action potential and reduction of the AHP may result in prolonged depolarization and activation of Ca⁺⁺ channels. This may explain the stimulation, by 5-HT₄ receptors, of neurotransmitter release including acetylcholine,^{53,54} dopamine,^{55,56} and serotonin.⁵⁷

Effect of BIMU -1, an agonist of 5-HT₄ receptors on social and associative olfactory memories

The observation that 5-HT₄ receptor agonists are able to stimulate acetylcholine release in both the peripheral and central nervous systems,⁵³ associated with its ability to produce a long-term blockade of K⁺ channels,⁵² is reminiscent of a similar role of 5-HT receptors in

Aplysia.^{58,59} In this animal, 5-HT receptors positively coupled to AC are involved in learning-induced short and long-term facilitations of the synaptic transmission between sensory and motor neurons.

We therefore examined the effect of BIMU1, a 5-HT₄ agonist which is also a 5-HT₃ antagonist, on social olfactory recognition in rats, a behavioral test which has been previously shown to access short-term memory and be sensitive to cholinergic drugs. This test is based on the investigation of an unfamiliar juvenile by an adult rat during two distinct 5-min periods of presentations (P1 and P2). At a 30 min delay between each presentation, adults recognize the juvenile as indicated by a reduction of the exploration period (P2 compared to P1) of the juvenile by the adult ($P2/P1 < 1$) (Fig. 4a). In contrast, after a 2 hour delay, all the adults have lost their memory for the juvenile ($P2/P1 = 1$) (Fig. 4a). The recognition of the juvenile is specific: a different juvenile is recognized as a new congener after 30 minutes $P2/P1 = 1$. BIMU1 administered after the first presentation enhanced short-term memory (*i.e.*, recognition of the juvenile after a 2 hour delay). A 5-HT₃ antagonist (ondansetron) had no effect, whereas a very specific 5-HT₄ antagonist (GR125487 at 10 mg/kg) was highly effective in blocking the BIMU1 effect. This indicated that the positive effect of BIMU1 on short-term memory was due to its 5-HT₄ agonistic properties rather than to its 5-HT₃ antagonistic properties.⁵

We have also tested the effect of BIMU1 on an olfactory associative task.⁷ In this task, rats deprived of water for 48 h were trained to perform two odor-reward associations. Each odor had to be associated with a specific reward, one arbitrarily designed as positive (S^+) and the other as negative (S^-). Rats had to approach the odor and water ports to interrupt the light beam only when the positive odor had been delivered. Response to odor designated as negative, resulted in presentation of non-adversive light, water was not delivered. Individual trials were presented in a random fashion. A daily session was made up of 60 trials and five sessions were performed at a rate of one session per day (Fig. 4b, c). Four groups of rats (7 animals each) were used, a group injected with saline, two groups injected with BIMU1 (10 mg/kg) (in the presence or absence of the antagonist GR125487 at 10mg/kg) and a group injected with GR125487. Drug injections were done before the third session. Whatever the group, the performances were improved across sessions reaching a score higher than 80% on session 5. However, the BIMU1 group reached this performance on session 4. The latency to give a response was different between S^+ and S^- only on session 4, for the control group, whereas this difference appeared just after the injection on session 3 for the BIMU1 group. The performance

of the GR125487 and BIMU1 plus GR125487 groups were not significantly different from the performance of the control group.⁷

A reversal test was performed one month later. Thus, the odor that had served previously as an S⁺ stimulus in the training session was now an S⁻ stimulus and vice versa. The BIMU1 group had difficulty in rapidly reversing their behavioral responses to previously learned associations. This indicated that the BIMU1 effect was not transient, but correlated to a long-term memory process. Several other studies are in favor of a role for 5-HT₄ in memory. RS 67333, a potent and selective 5-HT₄ agonist reverses the rat performance deficits induced by atropine in the Morris water maze learning test,⁶ an effect reversed by a selective antagonist. RS 17017, another 5-HT₄ agonist has been found to improve the computer-assisted delayed matching to sample task in macaques.⁶⁰ BIMU1 and BIMU8 improve learning performance after hypoxia-induced amnesia in mice.⁸

CONCLUSION

The three main conclusions of these studies are the following:

The cloning of 5-HT₄ receptors in different species using RT-PCR from different tissues, reveals the presence of several splice variants for 5-HT₄ receptors differing in the C-terminal part, downstream from the amino acid L₃₅₈. Although not formal, this may indicate that there is only one gene coding 5-HT₄ receptors. Heterogeneity has been suggested on the basis of difference in potencies and intrinsic activities of 5-HT₄ agonists on different tissues. As shown here, receptor density can explain the fact that benzamides have lower or equal efficacy as 5-HT depending on tissues. However, there are still some observations yet to be explained, for example RS57639 is a competitive antagonist and partial agonist in the colon and esophagus, respectively, whereas another drug RS23597 is a partial agonist and competitive antagonist in the colon and esophagus, respectively.⁶¹ So far, the three splice variants found, differing mainly in their C-termini, do not seem able to modify the pharmacology of the receptor.

The pharmacological properties of 5-HT₄ receptors are dependent on the cellular context in which they are expressed. Indeed, for similar receptor concentrations, a constitutive activation was found in COS-7 cells but not in neurons. Reciprocally, homologous desensitization was much stronger in colliculi neurons than in COS-7 cells.

5-HT₄ agonists can be added to the list of compounds having pro-cognitive properties.

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FIGURE LEGENDS

Figure 1

Putative structural model of m5-HT₄ receptors. Comparison of the amino acid sequences of the three cloned m5-HT₄ receptors, m5-HT_{4(a)}, m5-HT_{4(b)}, m5-HT_{4(c)}. Y*, potential sites of tyrosine kinase; Y**, potential sites for sequestration; C, site of potential palmitoylation; ▲, consensus sites for phosphorylation by protein kinase C; □ consensus sites of phosphorylation by calmoduline kinase; ED and DE, acidic doublet putatively recognized by G protein-coupled receptor kinase (GRK).

Figure 2

Effect of receptor densities on dose-response curves for 5-HT and renzapride on endogenous 5-HT₄ receptors in mouse colliculi neurons, in human heart and on human 5-HT_{4(a)} receptors transiently expressed in COS-7 cells. (a) Effect of 5-HT and renzapride on stimulation of cAMP production in colliculi neurons. (b) Effect of 5-HT and renzapride on increasing Ca²⁺ current (ICa) in human cardiomyocytes, data from.³⁷ (c) Human 5-HT_{4(a)} receptors transiently expressed at low density in COS-7 cells (36 ± 8 fmol/mg protein). Effect of 5-HT and renzapride on stimulation of cAMP production expressed as a percentage over basal cAMP produced in mock-transfected cells (control). The percentage conversion of [³H]-ATP to [³H]-cAMP in control was 0.158 ± 0.04. (d) Same experiment as in (c) except that h5-HT_{4(a)} receptors are expressed at higher concentrations (258 ± 26 fmol/mg protein). (e) cAMP production (E_{max}) for maximal concentrations of 5-HT and reenzapride (above 1 mM) was measured at different concentrations of h5-HT_{4(a)} receptor (RH) expressed in COS-7 cells. E were plotted as a function ofd RH. (f) E_{max} plotting as a function of E_{max}/RH gives a straight line, the slope of which is C_t E_t is equal to E_{max} when E_{max}/RH = 0. In COS-7 cells, the coupling constant for 5-HT was 2-fold higher than the coupling constant for renzapride (135 ± 70 ±± 3 fmol/mg, respectively). E_t was identical for 5-HT and renzapride. This means that a high receptor density renzapride is a full agonist.

Figure 3

(a) (b) Effect of classical 5-HT₄ antagonists on agonist-independent cAMP production in intact or COS-7 cells and in mouse colliculi neurons. The effects of RS 100235 and GR 125487 on cAMP production were evaluated on human 5-HT_{4(a)} receptors, transiently transfected in COS-

7 cells expressing 98 ± 16 fmol/mg protein or in colliculi neurons expressing endogenous 5-HT₄ receptors (60 ± 23 fmol/mg protein). The percentage conversions of [³H]-ATP to [³H]-cAMP in mock-transfected COS-7 cells and in colliculi neurons were 0.162 ± 0.015 and 0.055 ± 0.018 (basal), respectively. cAMP productions were measured during 30 min and expressed as the percent over control. The presented typical experiment is representative of three separate experiments. Results are mean $\pm$ standard error of triplicate determinations. (c) Comparison of amino acidic sequences of the C-termini of the intracellular loop i3 in m5-HT₄, r5-HT₄, h β 2AR and CAM β 2AR (CAM=constitutive activated mutant).⁴³

Figure 4

(a) Role of 5-HT₄ receptors on social memory; effect of a 5-HT₄ agonist, BIMU1. Social recognition of juvenile conspecifics by adult rat at a delay of 30 (P₂₃₀) and 120 min (P₂₁₂₀) after P1 ((P1) = first exploration period and (P2) = second exploration period). Investigation times were followed after i.p. administration of BIMU1 (10 mg/kg) at a delay of 120 min after the first presentation. *y axis*: RID values (RID = P2/P1, ratio of investigation duration), comparison between the same and different juveniles at P₂₃₀ and P₂₁₂₀ by adult rats after i.p. administration of PBS (control) and at P₂₁₂₀ by adult rats after i.p. administration of BIMU1 (10 mg/kg). (b)(c) Involvement of 5-HT₄ receptors in an olfactory associative learning task. Mean performance ($\pm$ SE) obtained across the five 60-trial sessions by rats injected with BIMU1 (10 mg/kg) (n=7) or saline (control) rats (n = 7). The intertrial interval was 15 sec. (b) mean correct response rate to make two odor-reward associations. (c) Mean latencies (in seconds, S⁺ are the latencies recorded for the positive odor and S⁻ are the latencies for the negative odor).

(a)

m5-HT₄ receptors

(b)

m5-HT ₄ (a)	358	387
	LRYTVLHSGHHQELEKLPINHNDPESLESCF	
m5-HT ₄ (b)		388
	LRDAVECGGQWESRCHLTATSPLVAAQPSDT	
m5-HT ₄ (c)		371
	LSFPLLFRNRVPVPV	

Figure 1

Figure 2

Figure 3

Figure 4