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The mouse 5HT5 receptor reveals a remarkable heterogeneity within the 5HT1D receptor family

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Serotonin (5-HT) is a neuromodulator that mediates a wide range of physiological functions by activating multiple receptors. Using a strategy based on amino acid sequence homology between 5-HT receptors that interact with G proteins, we have isolated a cDNA encoding a new serotonin receptor from a mouse brain library. Amino acid sequence comparisons revealed that this receptor was a distant relative of all previously identified 5-HT receptors; we therefore named it 5HT5. When expressed in Cos-7 cells and NIH-3T3 cells, the 5HT5 receptor displayed a high affinity for the serotonergic radioligand [^{125}I]LSD. Surprisingly, its pharmacological profile resembled that of the 5HT1D receptor, which is a 5-HT receptor subtype which has been shown to inhibit adenylate cyclase and which is predominantly expressed in basal ganglia. However, unlike 5HT1D receptors, the 5HT5 receptor did not inhibit adenylate cyclase and its mRNA was not found in basal ganglia. On the contrary, *in situ* hybridization experiments revealed that the 5HT5 mRNA was expressed predominantly in cerebral cortex, hippocampus, habenula, olfactory bulb and granular layer of the cerebellum. Our results therefore demonstrate that the 5HT1D receptors constitute a heterogeneous family of receptors with distinct intracellular signalling properties and expression patterns.

Key words: cerebral cortex/G protein-coupled receptor/ hippocampus/5HT1D receptor/LSD serotonin

Introduction

Serotonin is a neuromodulator that elicits and modulates a wide range of behaviours such as sleep, appetite, locomotion, sexual activity and vascular contraction (Wilkinson and Dourish, 1991). Both pharmacological studies and molecular cloning of 5-HT receptors have revealed a multiplicity of receptor subtypes. The receptors that have been cloned so far belong to either the ligand-gated ion channel family (5HT3 receptor) or to the large family of receptors that interact with G proteins and share a putative seven transmembrane domains structure (Julius, 1991). Amino acid sequence comparisons have revealed that the G protein-coupled 5-HT receptors can be subdivided into two distinct groups: the 5HT1 group, which contains the mammalian 5HT1A, 1B and 1D subtypes as well as three *Drosophila* 5-HT receptors, and the 5HT2 group, which contains the 5HT2 and 5HT1C subtypes. These receptors differ in their affinity for serotonin, their intracellular signalling properties, their pattern of expression and their subcellular localization (for a review see Hen, 1992).

Pharmacological studies have revealed the existence of additional receptor subtypes such as the 5HT4 receptor as well as a number of '5HT1D-like' receptors (for a review see Peroutka, 1991b). In order to isolate some of these subtypes, we used a strategy based on nucleotide sequence homology between transmembrane domains III and VI of 5-HT receptors. A mouse brain cDNA library was screened and one of the resulting clones was shown to encode a functional 5-HT receptor. Sequence comparisons have revealed that this receptor is a new member of the G protein-coupled receptor family that does not belong to either 5HT1 or 5HT2 groups. We therefore named it 5HT5. The pharmacological profile of the 5HT5 receptor is similar to that of the 5HT1D receptor and suggests that this receptor may correspond to previously described rat 5HT1D binding sites (Herrick-Davis and Titeler, 1988). Furthermore, the 5HT5 receptor is expressed in the cerebral cortex, hippocampus and cerebellum, which are structures that were shown to contain 5HT1D sites in the rat (Herrick-Davis and Titeler, 1988), as well as 5HT1D-like sites in various mammalian species including human (for a review see Peroutka, 1991b).

Our results demonstrate that the 5HT5 receptor is a novel serotonin receptor whose sequence is not closely related to that of any known serotonin receptor, but whose pharmacological profile resembles that of the 5HT1D receptor. These data therefore confirm previous pharmacological studies suggesting that the 5HT1D receptors are a heterogeneous family of receptors.

Results

Isolation of a mouse cDNA clone encoding a new member of the G protein-coupled receptor family: the 5HT5 receptor

Sequence comparisons of serotonin receptors have revealed a striking amino acid sequence conservation particularly in certain putative transmembrane domains such as domains III and VI (Hen, 1992). We therefore decided to use degenerate oligonucleotides corresponding to these two regions to perform a series of PCR experiments on mouse brain RNA. The resulting fragments were subcloned and sequenced. One of these fragments was used to screen a mouse brain cDNA library. We obtained one phage recombinant that contained a 4 kbp long cDNA insert. Sequence analysis revealed one long open reading frame (357 amino acids) and a poly A tail (Figure 1). The hydropathy analysis of this predicted protein revealed seven hydrophobic domains (numbered I–VII in Figure 1), a feature shared

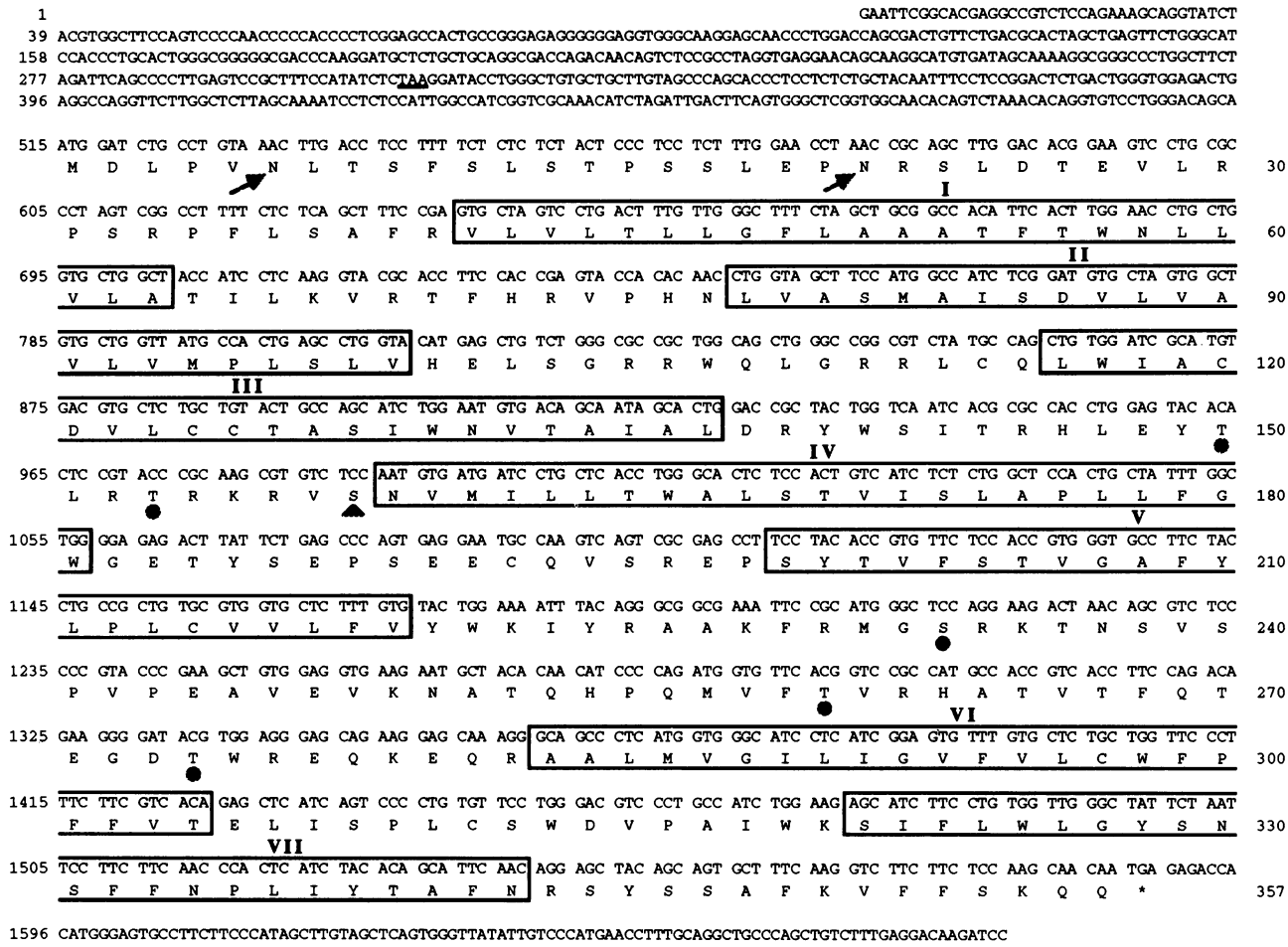

Fig. 1. Nucleotide sequence of the 5HT5 cDNA. The 4 kbp long *Eco*RI−*Xho*I cDNA fragment was sequenced on both strands from the *Eco*RI site to position 1691. The remaining 2300 nucleotides were not sequenced except the 3′ end, which contained a poly A tail. The seven putative transmembrane domains are boxed and numbered (I−VII). Arrows indicate sites of potential N-linked glycosylation. Circles and triangles correspond to consensus sites for phosphorylation by protein kinases C and A, respectively. An in frame stop codon upstream of the ATG is underlined. The asterisk indicates the terminal stop codon.

by all other cloned members of the G protein-coupled receptor family. The amino-terminal end displayed two putative sites for N-linked glycosylation and the presumed cytoplasmic domains contained consensus sites for phosphorylation by protein kinases C and A (Figure 1), features that are also found in most members of that family.

Amino acid sequence comparisons revealed homologies with G protein-coupled receptors in the putative transmembrane domains and short connecting loops, but not in the amino- and carboxy-terminal ends or in the third cytoplasmic loop, which are very variable in sequence and in length within this gene family. Percentages of homology were therefore calculated over the conserved regions (Figure 2A) and used to establish a dendrogram (Figure 2B). The percentages of homology with known receptors are low, the best score being 37% with a *Drosophila* serotonin receptor 5HT-dro2A (Saudou *et al.*, 1992). The dendrogram clearly shows that the 5HT5 receptor does not belong to a subfamily of serotonin receptors such as for example the 5HT1B/1D family or the 5HT2/5HT1C family.

Pharmacological profile of the 5HT5 receptor

To determine whether the 5HT5 cDNA clone encoded a functional receptor, we introduced it into a eukaryotic expression vector and transfected Cos-7 cells with the resulting recombinant. Membranes of transfected cells were then assayed for their ability to bind a number of serotonergic radioligands. While [^{125}I]cyanopindolol, [^{3}H]8-OH-DPAT and [^{3}H]spiperone did not bind to these membranes, [^{125}I]LSD displayed a single saturable binding site: K_d = 340 pM and B_{max} = 1.6 pmol/mg of membrane protein (Figure 3). In a control experiment, [^{125}I]LSD did not bind to mock-transfected Cos-7 cells. To determine the pharmacological profile of the 5HT5 receptor, bound [^{125}I]LSD was displaced with various serotonergic drugs (Table I). These compounds displayed the following rank order of potencies: 2-bromo LSD > ergotamine > 5-CT > methysergide > 5-HT = RU24969 > bufotenine = yohimbine = 8-OH-DPAT (Table I). Ketanserin, (−) pindolol, sumatriptan, dopamine and (−) norepinephrine were inactive.

In some experiments, the competition curves obtained with 5-HT were slightly biphasic and indicated that 5−10% of the [^{125}I]LSD binding sites may have a high affinity for 5-HT. This observation suggested that in the transient Cos-7 expression system, most of the 5HT5 receptor may be in a low affinity state, possibly because of its overexpression in this system. A similar observation had been previously made after expressing the *Drosophila* 5-HT receptors in Cos-7 cells (Saudou *et al.*, 1992). Therefore it was decided

A.

5HT5	mouse	100											
5HT1Bβ	mouse	34	100										
5HT1Dα	human	35	68	100									
5HT1A	human	34	47	46	100								
5HT-dro2A		37	41	39	41	100							
5HT-dro1		35	37	40	43	40	100						
α2	human	33	37	37	38	31	38	100					
D2	rat	30	34	37	33	37	38	36	100				
β1	human	26	31	33	32	32	35	28	32	100			
D1	human	25	34	32	34	33	34	30	31	34	100		
H2	dog	25	33	33	33	32	37	33	33	33	32	100	
5HT1C	rat	27	29	31	27	28	30	32	32	29	26	28	100
5HT2	rat	26	30	29	28	27	27	30	31	28	27	27	67 100

5HT5 mouse
5HT1Bβ mouse
5HT1Dα human
5HT1A human
5HT-dro2A
5HT-dro1
α2 human
D2 rat
β1 human
D1 human
H2 dog
5HT1C rat
5HT2 rat

B.

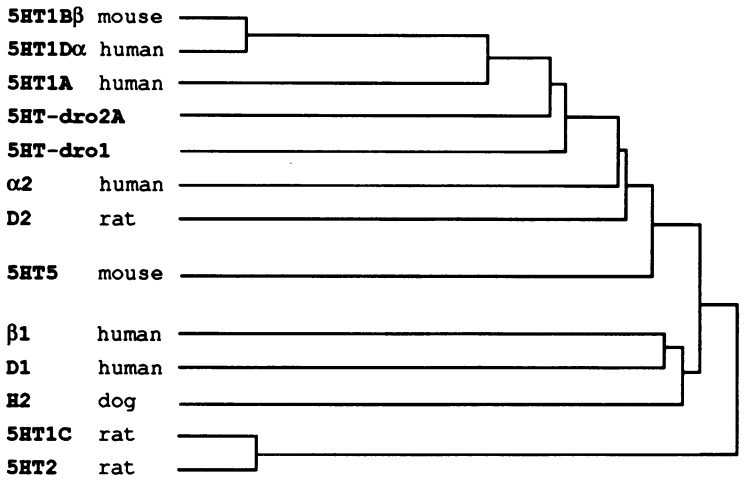

Fig. 2. A. Percentages of amino acid homologies between the 5HT5 receptor and other members of the G protein-coupled receptor family. These percentages of homology were calculated over the sequences that are conserved in this gene family: the transmembrane domains and short connecting loops (Hen, 1992). **B.** Dendrogram. The sequences of the mouse 5HT1Bβ (Maroteaux *et al.*, 1992), human 5HT1Dα (Hamblin and Metcalf, 1991), human 5HT1A (Fargin *et al.*, 1988), *Drosophila* 5HT-dro1 and 5HT-dro2A (Saudou *et al.*, 1992), human α2A adrenergic (Kobilka *et al.*, 1987), rat D2 dopaminergic (Bunzow *et al.*, 1988), human β1 adrenergic (Frielle *et al.*, 1987), human D1 dopaminergic (Dearry *et al.*, 1990), human H2 histaminergic (Gantz *et al.*, 1991), rat 5HT1C (Julius *et al.*, 1988) and rat 5HT2 (Pritchett *et al.*, 1988) receptors were compared and clustered with the program 'clustal' (Higgins and Sharp, 1988). The lengths of the horizontal lines are inversely proportional to the percentages of homology (A).

to introduce the 5HT5 receptor in a more physiological environment by generating stable cell lines expressing variable levels of this receptor. NIH-3T3 cells were chosen because they do not express any endogenous serotonin receptor and because they have been used to characterize various 5-HT receptors. The 5HT5 expression vector was introduced in these cells together with the Neo gene encoding resistance to G418. G418-resistant cell lines were isolated and a Northern analysis was performed with RNA from these cells. We selected two cell lines, NS1 and NS4, expressing high and low levels of 5HT5 mRNA, respectively. Membranes prepared from these cells displayed high affinity [^{125}I]LSD binding sites, indicating that both cell lines expressed the 5HT5 receptor (Figure 4). The Scatchard analysis revealed that [^{125}I]LSD had about the same affinity for both cell lines as for Cos-7 cells: K_d = 240 pM in the case of NS1 cells and K_d = 280 pM in the case of NS4 cells. As expected from the RNA analysis, NS1 cells expressed more 5HT5 receptor as NS4 cells: B_{max} (NS1) = 350 fmol of receptor/mg of membrane protein, while B_{max} (NS4) = 105 fmol/mg. In a control experiment, membranes from wild type 3T3 cells did not bind [^{125}I]LSD. Displacement studies were performed with various compounds (Figure 5). In the case of 5-HT, 5-CT, sumatriptan and 8-OH-DPAT, the resulting competition curves were biphasic. A computer-generated two-site analysis of these data revealed both a high affinity and a low affinity binding site (Table I). In the case of NS1 cells, ~10% of the [^{125}I]LSD sites displayed a high affinity for 5-HT while in the case of NS4 cells, which express lower levels of 5HT5 mRNA, 25% of the sites had a high affinity for 5-HT. These high affinity sites may correspond to receptors coupled with G proteins. Such G proteins may be present in limiting amounts and therefore, when the 5HT5 receptor is expressed at high levels, like in Cos-7, only a small fraction of the receptors may couple with G proteins. To test the hypothesis that the high affinity sites correspond to G protein-coupled receptors, the same competition studies were performed in the presence of GTP, which has been shown to uncouple various receptors (De Lean *et al.*, 1982). The resulting curves were shifted to the right and revealed only a low affinity component, suggesting that the high affinity sites have been converted in low affinity sites due to the uncoupling effect of GTP (Figure 6).

The NS1 and NS4 cells were also used to investigate a possible effect of the 5HT5 receptor on second messenger levels. In a series of preliminary experiments we have not been able to detect any changes in the levels of cAMP or inositol phosphates in response to serotonin, in these cells. In the same conditions, the 5HT1Bβ receptor induced a decrease in cAMP levels (Saudou *et al.*, 1992). Our negative results therefore suggest that the 5HT5 receptor has different intracellular signalling properties than the 5HT1Bβ receptor.

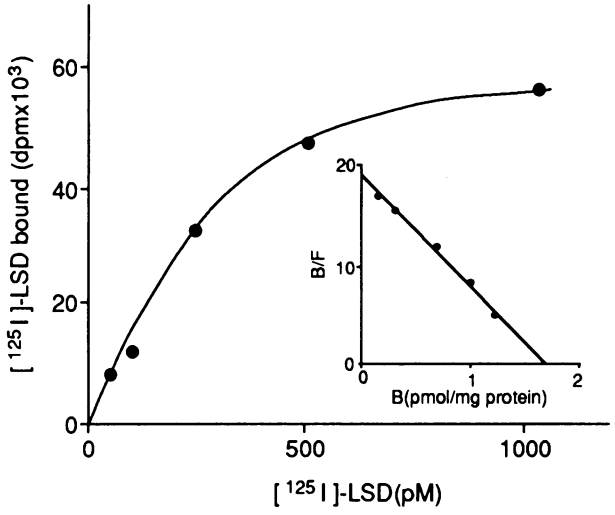

Fig. 3. Saturation isotherm of [^{125}I]LSD binding to membranes of Cos-7 cells expressing the 5HT5 receptor. Membranes were incubated with concentrations of [^{125}I]LSD ranging from 50 pM to 1.25 nM ± 10 μM 5-HT. Specific binding is represented. Inset, Scatchard analysis of [^{125}I]LSD binding: K_d = 340 pM, B_{max} = 1.6 pmol receptor/mg of membrane protein. Data are representative of two independent experiments with each point performed in triplicate.

The 5HT5 receptor is expressed in the central nervous system

Expression of 5HT5 transcripts was analysed by Northern, quantitative PCR and by *in situ* hybridization experiments. The Northern analysis revealed three transcripts in brain and cerebellum (5.8, 5 and 4.5 kb); no transcripts were detected in kidney or liver (Figure 7A). These three transcripts could derive either from a single gene or from several genes that are very homologous to one another.

We used also a more sensitive technique, 'quantitative PCR', to analyse 5HT5 RNA levels in a wider range of tissues. Specific PCR fragments could be amplified from spinal cord and brain RNAs, but not from RNAs prepared from spleen, liver, kidney, lung or heart (Figure 7B). To analyse further the pattern of expression of this receptor, we performed *in situ* hybridization experiments on adult mouse brain sections (Figures 8 and 9). The main sites of expression were the cerebral cortex, the hippocampus, the granular layer of the cerebellum, the habenula and the olfactory bulb. In a control experiment performed in the same conditions with a 'sense' RNA probe, no hybridization was observed. Bright-field observation of the emulsion coated slides revealed that the 5HT5 mRNA was expressed in the granule cells of the cerebellum and of the dentate gyrus and in pyramidal cells of the layers CA1, CA2 and CA3 of the hippocampus. A large number of neurons were stained in all areas of the cerebral cortex. In the olfactory bulb the

Table I. Pharmacological profile of the 5HT5 receptor

	pK_i values				
	5HT5 (Cos-7 cells)	5HT5 (NIH-3T3 cells)		5HT1D (rat cortex)	5HT1D (Calf caudate)
		Low	High		
5-HT	6.6 (7)	6.6 (6)	8.1 (6)	8.7	8.4
5-CT	7.8 (3)	7.9 (3)	9.5 (3)	8.6	8.6
RU 24969	6.5 (2)				7.3
TFMPP	5.6 (2)			6.6	6.2
8-OHDPAT	5.9 (2)	5.8 (2)	7.0 (2)	6.6	5.9
Sumatriptan	4.8 (2)	4.7 (5)	6.8 (5)		7.5
Bufotenine	6.0 (2)				8.1
Methysergide	7.2 (5)	7.1 (2)		7.3	8.4
Ergotamine	8.4 (2)	8.2 (2)			7.8
2-Bromo LSD	8.7 (2)				
Methiothepin	7.0 (2)	6.9 (2)			6.3
Yohimbine	6.0 (2)	6.2 (2)			7.1
(−)Pindolol	4.7 (2)			< 5	5.2
(−)Propranolol	4.9 (2)				5.5
Ketanserin	4.8 (2)			< 5	5.7
Spiperone	5.6 (2)				5.3
Dopamine	4.1 (2)				
(−)Norepin	2.8 (2)				

Data correspond to competition for [^{125}I]LSD binding to membranes of either Cos-7 cells expressing transiently the 5HT5 receptor (first column) or NS4 cells which derive from NIH-3T3 cells and stably express the 5HT5 receptor (second column). IC_{50} values required to displace 50% of [^{125}I]LSD were determined experimentally and converted to pK_i values according to the equation $K_i = IC_{50}/(1 + C/K_d)$ where C is the [^{125}I]LSD concentration (150 pM) and K_d is the equilibrium dissociation constant of [^{125}I]LSD. The data obtained with 5-HT, 5-CT, sumatriptan and 8-OH-DPAT in NS4 cells were best fit by a computer-generated two-site analysis. In these cases both the low affinity and the high affinity pK_i values are indicated. Numbers in parentheses correspond to the number of independent experiments each point being performed in triplicate. Individual pK_i values differed by <20%. The pK_i values for the 5HT1D receptor in rat cortex correspond to the high affinity binding components (Herrick-Davis *et al.*, 1988). In the case of the calf caudate 5HT1D receptor, pK_i values are taken from Waeber *et al.* (1990).

strongest staining was observed in the tufted cells (arrows in Figure 9A and B).

Discussion

Our data indicate that we have isolated a functional serotonin receptor that is expressed predominantly in the central nervous system. The sequence of this receptor reveals that it is a new member of the G protein-coupled receptor family. Although its closest relatives are serotonin receptors, this new receptor does not exhibit a strong homology to any of the already cloned serotonin receptors. Therefore it does not belong to existing subfamilies of serotonin receptors such as the 5HT1B/1D family or the 5HT1C/5HT2 family. Since the numbers 3 and 4 have been already used to designate different subtypes of 5-HT receptors, it was decided to name this receptor 5HT5.

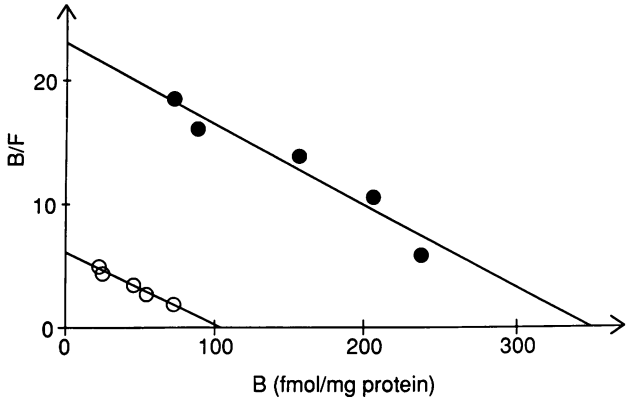

Fig. 4. [^{125}I]LSD binding to membranes of NS1 and NS4 cells. NS1 and NS4 cells are NIH-3T3 derivatives that stably express the 5HT5 receptor (Materials and methods). Saturation isotherms were performed as described in legend to Figure 3. The Scatchard analysis is represented. Open circles correspond to NS4 cells: K_d = 280 pM, B_{max} = 105 fmol/mg of membrane protein; closed circles correspond to NS1 cells: K_d = 240 pM, B_{max} = 350 fmol/mg. Data are representative of two independent experiments with each determination performed in triplicate.

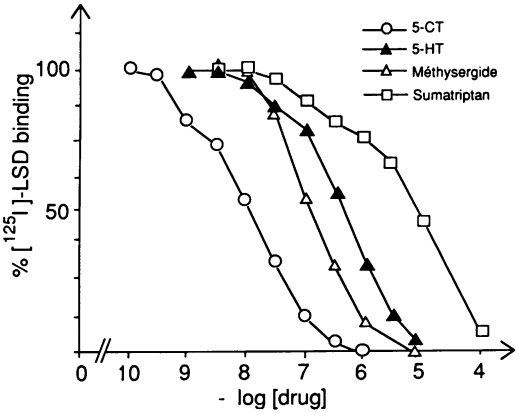

Fig. 5. Competition displacement of [^{125}I]LSD from membranes of NS4 cells. Membranes of NS4 cells were incubated with 150 pM of [^{125}I]LSD and various concentration of the following drugs: 5-CT (open circles), 5-HT (closed triangles), methysergide (open triangles) and sumatriptan (open squares). The displacement curves obtained with 5-CT, 5-HT and sumatriptan were best fit by a computer-generated two-site analysis. 20–25% of the binding sites had a high affinity for these compounds; the high and low affinity pKi values are indicated in Table I. Data are representative of five independent experiments with each determination performed in triplicate.

The pharmacological profile of the 5HT5 receptor, transiently expressed in Cos-7 cells, was unusual and did not correspond to the profile of any of the previously characterized serotonin receptors. However, these studies were performed in cells where the 5HT5 receptor was overexpressed. Therefore the low affinity pK_i values obtained with agonists such as 5-HT may correspond to receptors that were not coupled to G proteins. In support of this hypothesis is the fact that when the 5HT5 receptor was expressed at lower levels, such as in the stable cell line NS4, it displayed two affinities for 5-HT, a high affinity and a low affinity, which was similar to the low affinity observed in Cos-7 cells (Table I). Furthermore, the high affinity sites were converted into low affinity sites by GTP suggesting that they correspond to receptors coupled to G proteins. If one considers the high affinity pK_i values of the 5HT5 receptor obtained in NS4 cells, then the pharmacological

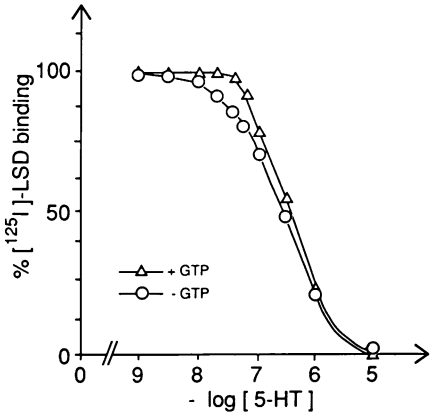

Fig. 6. Effect of GTP on [^{125}I]LSD binding to NS4 cells. Membranes of NS4 cells were incubated with 150 pM of [^{125}I]LSD and various concentrations of 5-HT in the presence or in the absence of 100 μM GTP. The displacement curve obtained in the absence of GTP was best fit by a computer-generated two-site analysis (pKi values are shown in Table I). In the presence of GTP the displacement was monophasic: pK_i = 6.6. Data are representative of three independent experiments with each determination performed in triplicate.

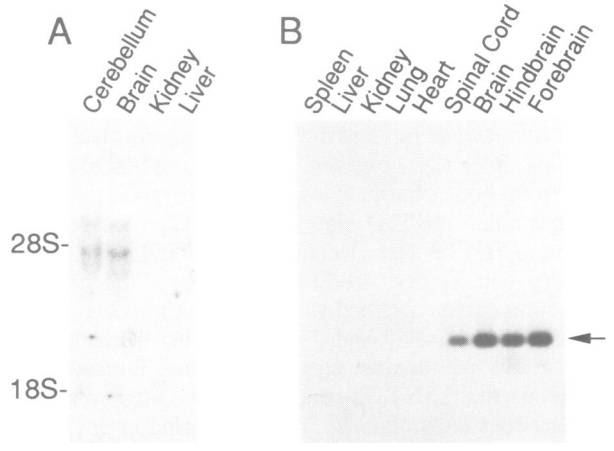

Fig. 7. Distribution of 5HT5 transcripts. **A.** Northern blot analysis of poly(A)$^+$ RNA (5 μg) from various organs. Three transcripts were detected in cerebellum and brain, but not in liver or kidney. The probe used is the ^{32}P-labelled *Eco*RI–*Xho*I cDNA fragment. **B.** Quantitative PCR analysis performed with 1 μg of total RNA from various organs. A 404 bp specific PCR product (arrow) is detected in spinal cord, hindbrain and forebrain, but not in all other organs tested.

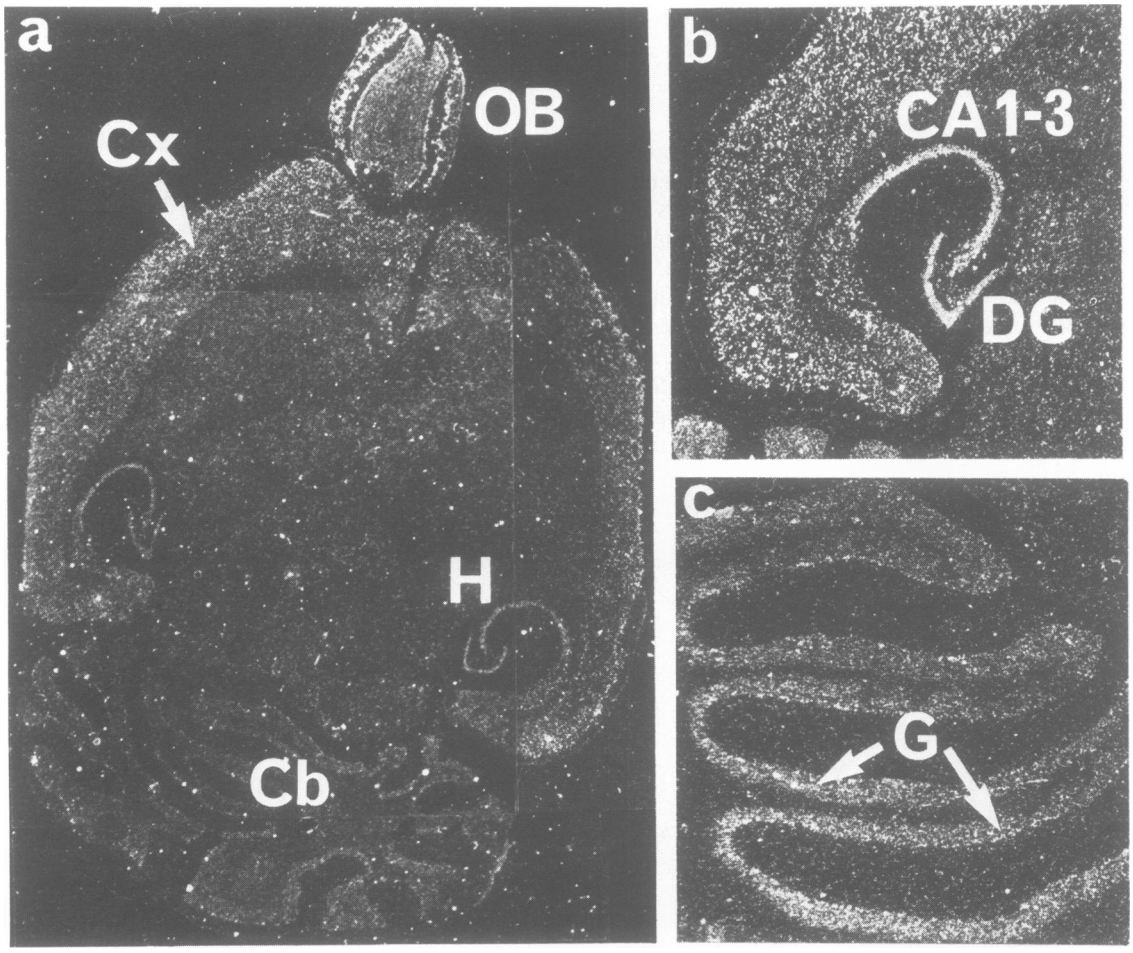

Fig. 8. *In situ* hybridization. The RNA probe was prepared as described in Materials and methods. In the experiment presented (panels a–c), the probe corresponds to the full-length cDNA plasmid (Figure 1). The same results were obtained with a shorter probe corresponding to the coding region (see Materials and methods). **a**. Dark field microscopy of the emulsion autoradiograph of an horizontal section through an adult mouse brain (8 mm wide). **b** and **c**. 2-fold magnifications of the hippocampal and cerebellar regions seen in panel a. Abbreviations: Cx, cerebral cortex; OB, olfactory bulb; H, Hippocampus; Cb, Cerebellum; CA 1–3, CA1, CA2 and CA3 hippocampal areas; DG, dentate gyrus; G, granular layer of the cerebellum.

profile of this receptor becomes similar to that of the 5HT1D receptor (Table I).

Until recently there was little evidence of heterogeneity within the 5HT1B/1D receptor family. Most authors believed that the 5HT1B and 5HT1D receptors were species variants of a same receptor subtype, the 5HT1B receptor being present in mice and rats and the 5HT1D receptor in all other mammals. Recently, however, indications of heterogeneity came from both pharmacological studies and molecular cloning studies. 5HT1D sites were found in rat brain in addition to 5HT1B sites (Herrick-Davis and Titeler, 1988; Weisberg and Teitler, 1992). Furthermore, the biphasic competition curves obtained with compounds such as 5-CT, sumatriptan, RU24969 and TFMPP on brain membranes from various mammalian species including human, have suggested that 5HT1D and 5HT1B sites may be heterogeneous (Asarch *et al.*, 1985; Heuring *et al.*, 1986; Leonhardt *et al.*, 1989; Sumner and Humphrey, 1989; Mahle *et al.*, 1991a; Peroutka, 1991a; Beer *et al.*, 1992). In addition, molecular cloning studies identified two 5HT1B/1D receptor subtypes, α and β, in mice (Maroteaux *et al.*, 1992), rats (Voigt *et al.*, 1991) and human (Weinshank *et al.*, 1992). The RNA corresponding to the α subtype is expressed in very low amounts in the mouse brain (our unpublished observation); this receptor, which has a 5HT1D-like pharmacological profile (Voigt *et al.*, 1991), may therefore be very rare and has probably not been detected by classical pharmacological techniques. In contrast, the 5HT1Bβ subtype is abundant, it is expressed predominantly in striatal neurons projecting to the substantia nigra (Hen, 1992; Maroteaux *et al.*, 1992) and it has a 5HT1B pharmacological profile. The 5HT1Bβ receptor therefore most probably corresponds to the 5HT1B sites that have been characterized in the substantia nigra of mice and rats (Schoeffter and Hoyer, 1989; Bouhelal *et al.*, 1988). The human counterpart of this receptor is the 5HT1Dβ receptor (Adham *et al.*, 1992).

The mouse and rat 5HT1Bβ receptors, which have a 5HT1B pharmacological profile, cannot account for the 5HT1D sites that have been reported in rats, because these sites were detected in conditions where the 5HT1B sites were blocked (Herrick-Davis and Titeler, 1988). Because of its very low abundance, the rat 5HT1Dα is also an unlikely candidate for these 5HT1D sites that are relatively abundant in most brain areas (Herrick-Davis and Titeler, 1988). We therefore believe that the 5HT5 receptor may correspond to the 5HT1D sites observed in the rat brain, or to a fraction of these sites. In keeping with this hypothesis, is the fact

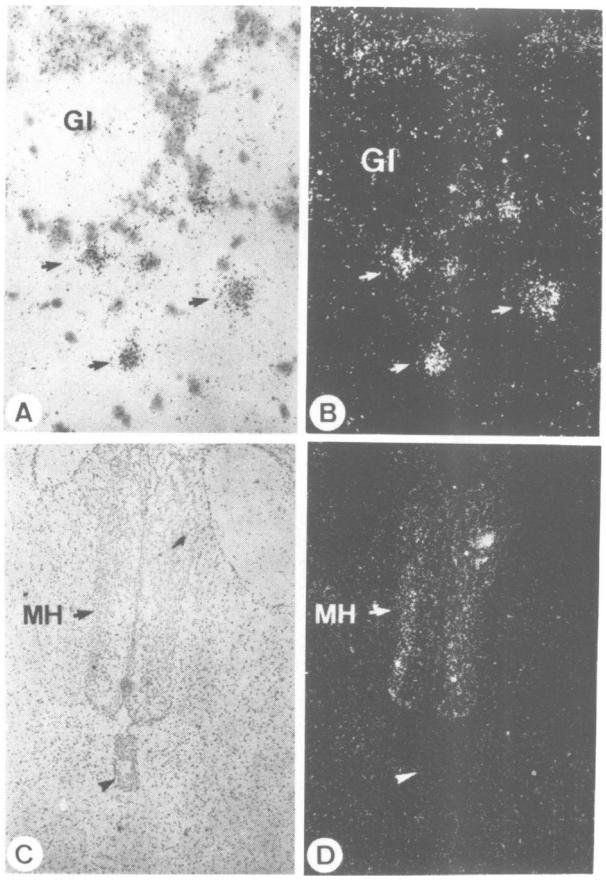

Fig. 9. *In situ* hybridization. The same probe as in Figure 8 was hybridized to adult mouse brain horizontal sections. Panels A–B correspond to the glomerular layer of the olfactory bulb (100 μm wide). Panels C–D represent a region containing the habenula and the dorsal third ventricule (1 mm wide). **A** and **C**. Bright-field microscopy of emulsion autoradiographs counterstained with toluidine blue to reveal cell bodies. Autoradiographic grains are most abundant over tufted cells (arrows in panel A). **B** and **D**. Dark-field photomicrographs of the same emulsion-coated slides as in panels A and C, respectively. The lowest arrow in panels C and D indicates a group of cell bodies that may correspond to the subcommissural organ and that does not express the 5HT5 RNA. Abbreviations: Gl, olfactory glomerulus; MH, median habenula.

that the 5HT5 receptor is expressed in cortex, hippocampus and cerebellum, brain regions where the rat 5HT1D sites were also found.

The 5HT5 receptor may also correspond to some of the 5HT1D-like sites that have been reported in the brain of various mammalian species and that could have slightly different pharmacological profiles due to species differences. However, the high affinity of 5-CT and ergotamine for the 5HT5 receptor, indicates that this receptor does not probably correspond to the 5HT1E sites that have a low affinity for 5-CT and ergotamine and have been found in human brain (Leonhardt *et al.*, 1989) and possibly in rat brain (Weisberg and Titeler, 1992).

It is also interesting to notice that unlike the 5HT1B and 5HT1D receptors, the 5HT5 receptor does not inhibit adenylate cyclase in NIH-3T3 cells. No effect on phospholipase C could be detected either. *In vivo*, this receptor may therefore interact with a different signalling system such as ion channels. Consistent with this hypothesis is the fact that, while 5HT1D receptors have been shown to inhibit adenylate cyclase in substantia nigra, no such coupling could be detected in other brain regions where 5HT1D receptors are also found, such as cortex and striatum (Waeber *et al.*, 1990). These authors actually postulated on that basis, a possible heterogeneity of 5HT1D receptors. Because of its expression in cerebral cortex, the 5HT5 receptor may correspond to some of the previously reported cortical 5HT1D sites.

Concerning a possible function of the 5HT5 receptor, due to the similarity between this receptor and the 5HT1D receptor, it is conceivable that some of the effects attributed to the 5HT1D receptors such as their involvement in motor control, feeding, anxiety and depression (Wilkinson and Dourish, 1991), may actually be mediated by the 5HT5 receptor. The 5HT5 receptor may also be involved in some of the physiological effects evoked by 5HT1D-like receptors. In the cerebellum for example, a 5HT1D-like receptor has been shown to inhibit the release of glutamate from granule cell nerve endings (Raiteri *et al.*, 1986). In the hippocampus, a number of effects mediated by 5HT1D-like receptors have also been reported, such as a stimulation of phosphoinositide turnover (Conn and Sanders-Bush, 1985) and a modulation of K-conductances (Colino and Halliwell, 1987; Yakel *et al.*, 1988).

Our results confirm previous pharmacological studies indicating that the 'non-5HT1A, −1B, −1C and −2' receptors constitute a heterogeneous family of receptors. Some of these 5HT1D receptors are close relatives such as the 5HT1Dα and 5HT1Dβ receptors. Others, like the 5HT5 receptor, are very different not only in their amino acid sequence, but also in their expression pattern and in their intracellular signalling properties. The availability of the genes encoding the various 5HT1D receptors should allow us, via gene targeting techniques, to dissect the individual roles of each of these receptor subtypes in the mouse.

Materials and methods

Isolation and sequence of the 5HT5 cDNA

A nested PCR experiment was performed with the following oligonucleotides: (i) AGAACTAGTGGATCCAA(A/G)AA(A/G/C/T)GG(A/G/C/T)A(A/G)-CCA(A/G)CA; (ii) CTTGATATCGAATTCGA(T/C)(A/G)T(A/G/C/T)CT-(A/G/C/T)TG(C/T)TG(C/T)AC; (iii) GGTATCGATAAGCTTAT(C/T/A)-GC(C/T)CT(A/G/C/T)GA(C/T)(C/A)G(A/G/C/T)TA. 5 μg of adult mouse brain RNA were reverse transcribed in the presence of 500 ng of oligo (i) and 200 units of MMLV reverse transcriptase (BRL). Half of that reaction was then amplified for 30 cycles in the presence of 5 units of Taq polymerase (Cetus) and 1 μg of oligonucleotides (i) and (ii). One-twentieth of that reaction was amplified for 30 more cycles with oligonucleotides (i) and (iii). The resulting products were digested with *Bam*HI and *Hin*dIII, inserted in the Bluescript plasmid and sequenced. One of resulting fragments that exhibited homology with 5-HT receptors, was labelled by random priming and used to screen a mouse brain cDNA library constructed in the Uni-Zap phage (Stratagene). Positive phages were isolated, the cDNA inserts were recovered in the Bluescript plasmid and sequenced on both strands by the dideoxynucleotide technique using successive synthetic oligonucleotides.

Expression of the 5HT5 receptor in cultured cells

The *Eco*RI–*Xho*I cDNA fragment (Figure 1) was inserted between the *Eco*RI and *Xho*I sites of expression vector p513, which is a derivative of pSG5 (Green *et al.*, 1988) containing a multiple cloning site. The resulting recombinant was introduced into mouse NIH-3T3 cells by calcium phosphate-mediated transfection together with the recombinant pRSVneo, which encodes resistance to G418 (20 μg of 5HT5 recombinant and 1 μg of pRSVneo per 10 cm dish). Transformed clones were selected in the presence of 0.5 mg of G418/ml. Isolated foci were amplified and total RNA was prepared and analysed for expression of 5HT5 RNA. Two clonal cell lines were selected (NS1 and NS4) that expressed variable levels of 5HT5 RNA as determined by Northern blot analysis.

For transient expression of the 5HT5 receptor, Cos-7 cells were transfected

by the calcium phosphate technique with the 5HT5 recombinant alone (20 μg per 10 cm dish) and harvested 48 h after transfection.

Radioligand binding assay

Membranes were prepared as described in Amlaiky and Caron (1985). [^{125}I]LSD saturation and competition binding experiments were performed with 10–20 μg protein per sample in a final volume of 250 μl in 50 mM Tris–HCl (pH 7.4) at 37°C for 10 min. Reactions were terminated by filtration under vacuum over Whatman GF/C glass fibre filters and rinsed four times with 4 ml of 50 mM Tris–HCl (pH 7.4). Non-specific binding was defined with 10 μM 5-HT. Radioactivity was determined in a gamma counter.

Northern and quantitative PCR analysis

Poly(A)$^+$ RNA was prepared, fractionated on a 1% agarose–formaldehyde gel and transferred to a nitrocellulose filter. The probe was the *Eco*RI–*Xho*I cDNA fragment that was ^{32}P-labelled by random priming and hybridized to the filter at high stringency (42°C, 50% formamide, 5×SSC, 1×Denhardt's, 20 mM sodium phosphate buffer pH 6.5, 0.1% SDS and 100 μg/ml tRNA). Washes were performed at high stringency (60°C, 0.1×SSC and 0.1% SDS).

For the quantitative PCR analysis we used the following oligonucleotides: (i) CTTCTGCTCCCTCCACGTATC and (ii) CGCCACCTGGAGTACACACTC corresponding to positions 1351 and 947, respectively (Figure 1). 1 μg of total RNA was reverse transcribed with 200 units of MMLV reverse transcriptase and 300 ng of oligonucleotide (i) for 1 h at 37°C. Half of that reaction was amplified in the presence of 5 units of Taq polymerase (Cetus) and 500 ng of oligonucleotides (i) and (ii) for 20 cycles. These PCR products were transferred to filters and hybridized as described above.

In situ hybridization

In situ hybridizations were performed on cryostat sections of adult mouse brains (~8 weeks old) as described by Hafen *et al.* (1983). The probe used was a single stranded RNA probe produced in the presence of [^{35}S]CTP and as a template, either the full-length cDNA plasmid (Figure 1) or a plasmid containing a PCR fragment corresponding to most of the 5HT5 coding region (from positions 375–1351 in Figure 1). The antisense probe was produced in the presence of T7 polymerase and a template linearized by *Eco*RI, while for the sense probe, we used T3 polymerase and a template linearized by *Xho*I.

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