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Cloning and characterization of the rat 5-HT_{5B} receptor

Evidence that the 5-HT_{5B} receptor couples to a G protein in mammalian cell membranes

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A gene encoding a novel G protein-coupled 5-hydroxytryptamine (5-HT) receptor, termed 5-HT_{5B}, was cloned. The ligand binding profile of this receptor is distinct from that of other cloned 5-HT receptors. The 5-HT_{5B} receptor couples to a G protein in COS1 cell membranes; however, activation of the 5-HT_{5B} receptor does not appear to alter either cAMP accumulation or phosphoinositide turnover in a variety of fibroblast cell lines. In the rat brain, 5-HT_{5B} gene expression occurs predominantly in the medial habenulae and hippocampal CA1 cells of the adult. Little expression is seen during embryonic development.

Serotonin; 5-Hydroxytryptamine; 5-Carboxamidotryptamine; G-protein-coupled receptor; Habenula; CA1 pyramidal cell

1. INTRODUCTION

The neurotransmitter 5-hydroxytryptamine (5-HT) modulates a wide array of physiological processes in both the central nervous system and in the periphery. The diverse physiological effects of 5-HT are mediated by a correspondingly diverse set of receptors [1]. Based on primary amino acid sequence, gene structure, pharmacological profile and signal transduction mechanism, these receptors have been classified into four distinct families designated 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 [1]. With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, all known 5-HT receptors are members of the G-protein coupled receptor superfamily.

Despite the unprecedented diversity of 5-HT receptors already known, there is evidence that additional subtypes exist (e.g. [2]). In this report, we present the cloning and characterization of a rat cDNA encoding a novel G protein-coupled 5-HT receptor. During the preparation of this manuscript, identical cDNA sequences from both mouse and rat were reported [3,4]. In keeping with the nomenclature adopted by these authors, we have designated this receptor as the rat 5-HT_{5B} receptor.

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Abbreviations: 5-HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamide; 8-OH-DPAT, 8-hydroxy-2-(di-1-propylamino)tetralin; 5-CT, 5-carboxamidotryptamine; GTI, serotonin-*O*-carboxymethylglycyltyrosinamide; PCR, polymerase chain reaction.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[^{3}H]5-Carboxamidotryptamine ([^{3}H]5-CT) was synthesized by Drs. Ron Mattson and Stephen Hurt. Other drugs were obtained from Sigma (St. Louis, MO), Research Biochemicals (Natick, MA) or were synthesized at Bristol-Myers Squibb.

2.2. *Isolation and characterization of cDNA clones*

Two degenerate oligonucleotides [ATATAAGCTTCTGTG(C/T)GCCAT(C/T)T(T/G)CCCT(G/T)GACCGCTAC] and [TATAGAATTCA(G/T)G(T/A)AGAAGGG(G/C)AGCCAGCA(G/C)A(G/T)C(A/G)(T/C)GAA] corresponding to conserved sequences in the third and sixth transmembrane domains of previously cloned biogenic amine receptors were synthesized. The oligonucleotides were used in the polymerase chain reaction (PCR) with rat forebrain cDNA as template. The PCR was carried out for a total of 30 cycles under the following conditions: 1.5 min denaturation at 94°C, 2.5 min annealing at 55°C and 4 min extension at 72°C. Products in the range of 400–1000 bp were isolated and sequenced. One PCR fragment that had a novel sequence was used to isolate a full-length clone from a λgt11 cDNA library [5]. The complete coding region was then subcloned into a mammalian expression vector (pRc/CMV; InVitrogen San Diego, CA).

2.3. *Transfection and preparation of crude plasma membranes*

COS1 cells (ATCC) were cultured and transfected as described previously [6].

2.4. *Radioligand binding assay*

Binding assays were performed as described [6]. Saturation analyses were performed with 0.15–100 nM [^{3}H]5-CT. Competition experiments were performed with 0.5 nM [^{3}H]5-CT, a concentration which should label almost exclusively (98–99%) the high affinity state of the receptor. In the case of competition experiments, nonspecific binding represented less that 10% of total binding. Data were analyzed by the nonlinear regression program LIGAND [7].

2.5. *In situ hybridization*

^{35}S-labelled oligonucleotides were hybridized to rat brain sections as previously described [8]. After exposure, sections were stained with thionin and printed as negatives alongside their corresponding autoradiographs. Two different 45 base oligonucleotide probes were used. Probe 1 was complementary to nucleotides encoding amino acids 54–68, and probe 2 was complementary to nucleotides encloding amino acids 268–282. Both oligonucleotide probes gave identical patterns of hybridization (see Figs. 3 and 4).

3. RESULTS AND DISCUSSION

The PCR was used as a first step in the cloning of cDNAs encoding members of the 5-HT receptor family. One PCR fragment was found that, based upon limited sequence identities, appeared to encode a novel 5-HT receptor. This PCR fragment was used to isolate a full length cDNA from a rat forebrain cDNA library. The novel 5-HT receptor encoded by this clone was designated as the 5-HT$_{5B}$ receptor. During the preparation of this manuscript, the sequence of the rat and mouse 5-HT$_{5B}$ receptors was also reported by two other laboratories [3,4]. Our sequence is identical to the rat 5-HT$_{5B}$ receptor sequence reported by Erlander et al. [4].

The deduced amino acid sequence of the 5-HT$_{5B}$ receptor is shown in Fig. 1. Hydropathy analysis revealed the presence of seven strongly hydrophobic regions that presumably constitute transmembrane domains. The 5-HT$_{5B}$ receptor has an N-linked glycosylation consensus sequence within the presumptive N-terminal extracellular domain and numerous consensus phosphorylation sites within the putative third intracellular loop and carboxy terminal domain. These criteria clearly identify the 5-HT$_{5B}$ receptor as a member of the G protein-coupled receptor superfamily.

The 5-HT$_{5B}$ receptor is most homologous (50–52% identity in the transmembrane domains; Fig. 1) to members of the 5-HT$_1$ receptor family. Identity with members of the 5-HT$_2$ receptor family was significantly less (26–28%, Fig. 1). Despite these similarities, the 5-HT$_{5B}$ receptor is sufficiently distinct from other cloned 5-HT receptors to justify its classification in a distinct 5-HT receptor family (i.e. 5-HT$_5$).

The pharmacological characteristics of the 5-HT$_{5B}$ receptor were examined by transiently expressing the

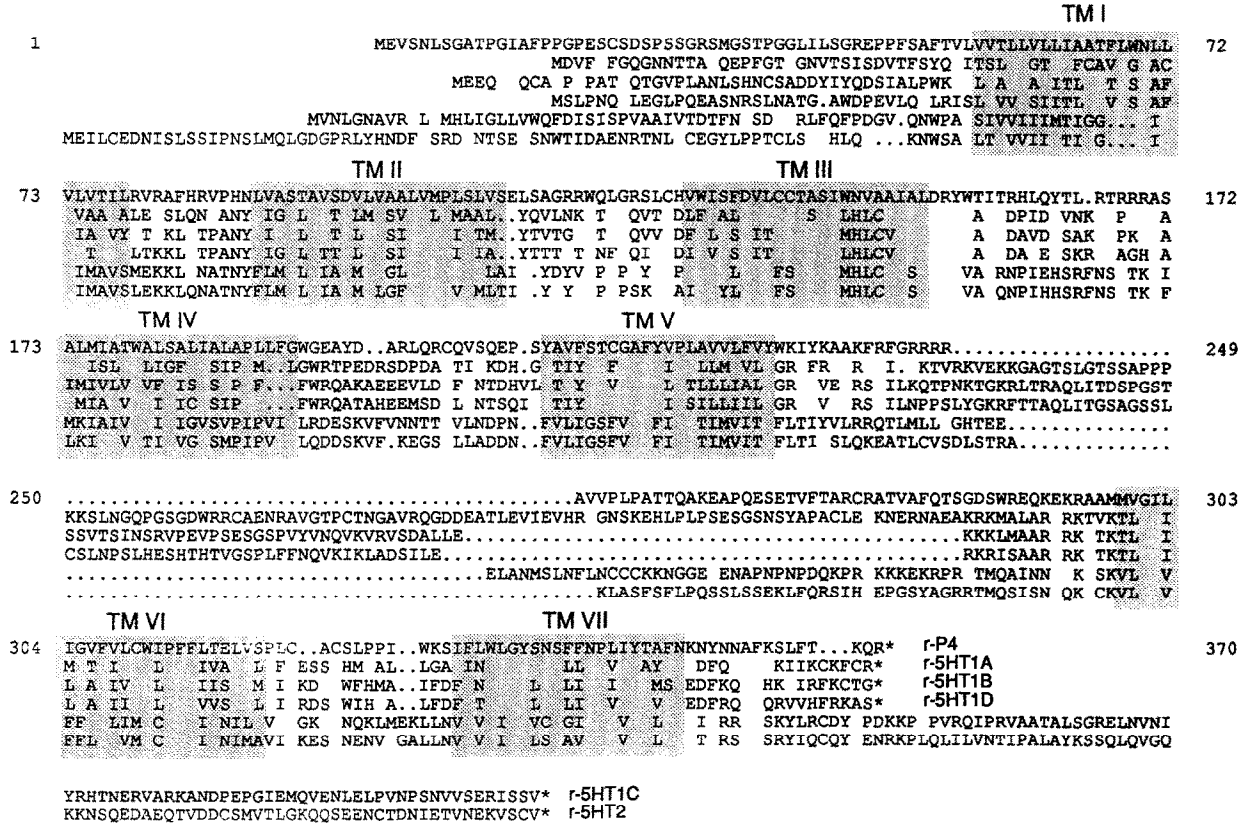

Fig. 1. Amino acid sequence comparison of the rat 5HT$_{5B}$, 5HT$_{1A}$, 5HT$_{1B}$, 5HT$_{1C}$, 5HT$_{1D}$, and 5HT$_2$ receptors. Putative transmembrane domains are indicated.

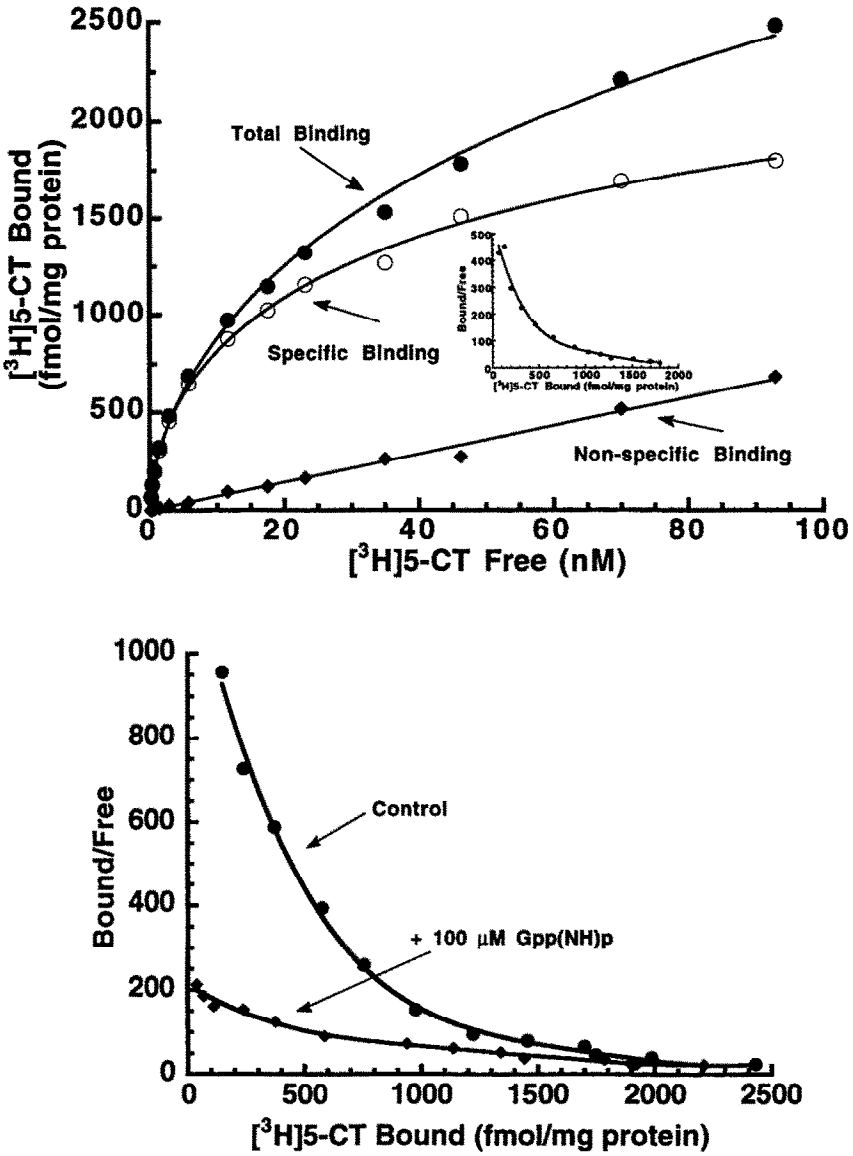

Fig. 2. (Top) Saturation isotherm and the corresponding Rosenthal transformation (inset) of [^{3}H]5-CT binding to the rat 5-HT$_{5B}$ receptor expressed in COS1 cells. Each data point is the mean of duplicate determinations. The experiment was performed two additional times with similar results. (Bottom) Effect of 100 μM Gpp(NH)p on [^{3}H]5-CT binding to the rat 5-HT$_{5B}$ receptor expressed in COS1 cells. See text and the legend to Fig. 2 (top) for details.

receptor in COS1 cells. The agonist radioligand [^{3}H]5-CT demonstrated saturable, high-affinity binding to membranes prepared from transfected COS1 cells (Fig. 2). Saturation analysis revealed binding of [^{3}H]5-CT to two affinity states of the receptor (Fig. 2A), with calculated K_ds of 0.82 nM and 31 nM (n = 3). In the presence of the non-hydrolyzable GTP analogue Gpp(NH)p, the percentage of receptors in the high-affinity state was reduced from 25% to 9% (Fig. 2B), suggesting that this receptor couples with G proteins present in COS1 cell membranes. In contrast, Matthes et al. [3] were unable to convincingly demonstrate guanine nucleotide modulation of agonist binding to the 5-HT$_{5B}$ receptor in COS cells. This discrepancy may be due to the different radioligands used ([^{125}I]LSD vs. [^{3}H]5-CT). In any case, these data are the first demonstration that the 5-HT$_{5B}$ receptor can couple to a G protein in mammalian cell membranes.

The affinities of a variety of serotonergic ligands for the 5-HT$_{5B}$ receptor were determined by competition analysis (Table I). The 5-HT$_{5B}$ receptor displayed high affinity for 5-HT, 5-CT, LSD, dihydroergotamine, methiothepin and methysergide but had low affinity for the biogenic amines norepinephrine, dopamine and melatonin. These data are consistent with the notion that this clone encodes a 5-HT receptor. Interestingly, the 5-HT$_{5B}$ receptor had high affinity for the 5-HT$_{1A}$-selective ligand 8-OH-DPAT and moderately high affinity for the 5-HT$_2$-selective ligand cyproheptadine. However, other ligands with high affinity for 5-HT$_{1A}$ (spiperone, pindolol) or 5HT$_2$ (ketanserin) receptors had very low affinity for the 5-HT$_{5B}$ receptor. In addition, ligands

having high affinity for other 5-HT receptor subtypes (5-HT_{1B}, pindolol; $5HT_{1C}$, mesulergine; 5-HT_{1D}, sumatriptan, GTI, yohimbine; $5HT_3$ and $5HT_4$, zacopride) had very low affinity for the 5-HT_{5B} receptor. Thus, the pharmacological profile of the 5-HT_{5B} receptor is distinct from that of other known 5-HT receptors. The affinities of several ligands were significantly higher than affinities reported by Matthes et al. [3] and Erlander et al. [4]. This is presumably because these authors were measuring binding to the low affinity (uncoupled) state of the receptor whereas we are measuring binding to the high affinity (G protein-coupled) state of the receptor.

Attempts were made to determine the signal transduction pathway(s) activated by the 5-HT_{5B} receptor. 5-HT had no effect on cAMP accumulation or on phosphoinosotide turnover in COS7, 293 or CHO cells transiently expressing the receptor. Furthermore, 5-HT had no effect on these parameters in stable (CHO and 293) cell lines expressing the 5-HT_{5B} receptor. There are a number of possible explanations for this, such as the absence of an appropriate G protein subunit in the cell lines tested or activation of a pathway other than adenylylcyclase or phospholipase C (e.g. ion channel modulation). The exact signal transduction mechanisms of this receptor remain to be elucidated.

The distribution of 5-HT_{5B} receptor mRNA was examined in the adult rat brain by in situ hybridization. Transcripts encoding the 5-HT_{5B} receptor were detected in a very limited number of brain regions (Fig. 3). After long exposure times moderate levels of 5-HT_{5B} receptor mRNA were detectable in hippocampal CA1 cells and in the medial habenula (Fig. 3A,B). Much lower levels of expression were found in the entorhinal cortex (Fig. 3E), subiculum, dorsal raphe nucleus (not shown) and the glomerular layer of the olfactory bulb (not shown for adult, but shown for the neonate in Fig. 5). A faint specific signal was also present in the hippocampal dentate granule cells. No 5-HT_{5B} receptor mRNA could be detected on Northern blots containing 20 μg of poly A^+ RNA from adult rat liver, muscle, lung, heart, kidney and spleen (data not shown). There was also an absence of detectable 5-HT_{5B} receptor transcripts on embryonic days 17 and 19 in non-neuronal tissues (e.g. lung, liver, kidney, pituitary) as assessed by in situ hybridization to sagittal embryo sections (Fig. 4).

Table I

Affinity values (K_i) of various drugs for the cloned rat 5-$HT5_B$ receptor

Compound	K_i (nM)
5-CT	1.3 ± 0.3
LSD	3.2 ± 0.6
5-HT	7.8 ± 3.6
Dihydroergotamine	31.0 ± 2.0
Methysergide	40.7 ± 5.3
Methiothepin	44.2 ± 12.3
8-OH-DPAT	46.0 ± 6.9
Cyproheptadine	117 ± 13
GTI	582 ± 48
Sumatriptan	807 ± 163
Spiperone	> 1000
Dopamine	> 1000
Norepinephrine	> 1000
Melatonin	> 1000
Ketanserin	> 1000
Pindolol	> 1000
Mesulergine	> 1000
Yohimbine	> 1000
Zacopride	> 1000

K_i values were obtained as described in Materials and Methods. Values are the means ± S.E.M. from at least three separate experiments, each performed in duplicate.

The discrete expression of the 5-HT_{5B} receptor mRNA in the brain permits reasonable hypotheses to be formulated concerning its role in normal CNS function. Expression in hippocampal CA1 cells and, to a lesser extent, in dentate granule cells, subiculum and entorhinal cortex is interesting because these regions can be viewed as an excitatory circuit loop [9]. The 5-HT_{5B} receptor may, therefore, be involved in tonically modulating the general tone of this circuit. If so, the 5-HT_{5B} receptor could affect processes such as learning and memory consolidation. Expression of the 5-HT_{5B} receptor mRNA in the habenula is less informative because the exact function of the habenula complex is uncertain (e.g. see [10,11]). However, animals with habenular lesions have pronounced difficulties learning tasks in stressful situations. Therefore, the 5-HT_{5B} receptor in the habenula may participate in circuits involved in the acquisition of adaptive behaviour under demanding conditions [11].

To determine if the 5-HT_{5B} receptor gene was developmentally regulated, its expression was studied during late embryonic (E17 and E19) and early postnatal (P0–P15) stages of the rat (Figs. 4 and 5). No general tissue expression of the $5HT_{5B}$ receptor gene could be detected in either the E17 or E19 embryo (Fig. 4). In these embryonic stages, however, a hybridization signal was detectable overlying one single brain nucleus located in the ventral brainstem area, possibly corresponding to the nucleus raphe pallidus. On the day of birth (P0), no signal was present in hippocampus or in any other part of the forebrain examined with the exception of a faint signal over the entorhinal crotex (Fig. 5, P0). By P6, the essential adult pattern of expression was established, with the levels in the dentate granule cells declining by P15. Thus, the 5-HT_{5B} receptor is only associated with a mature adult phenotype. This is in contrast to some other 5-HT receptors, where significant embryonic expression occurs (e.g. the 5-HT_{1B} receptor).

The cloning of the 5-HT_{5B} receptor by us and others [3,4] has resulted in the identification of a previously unknown 5-HT receptor subtype. The 5-HT_{5B} receptor is particularly interesting because its expression appears to be limited to neurons in a few specific limbic regions.

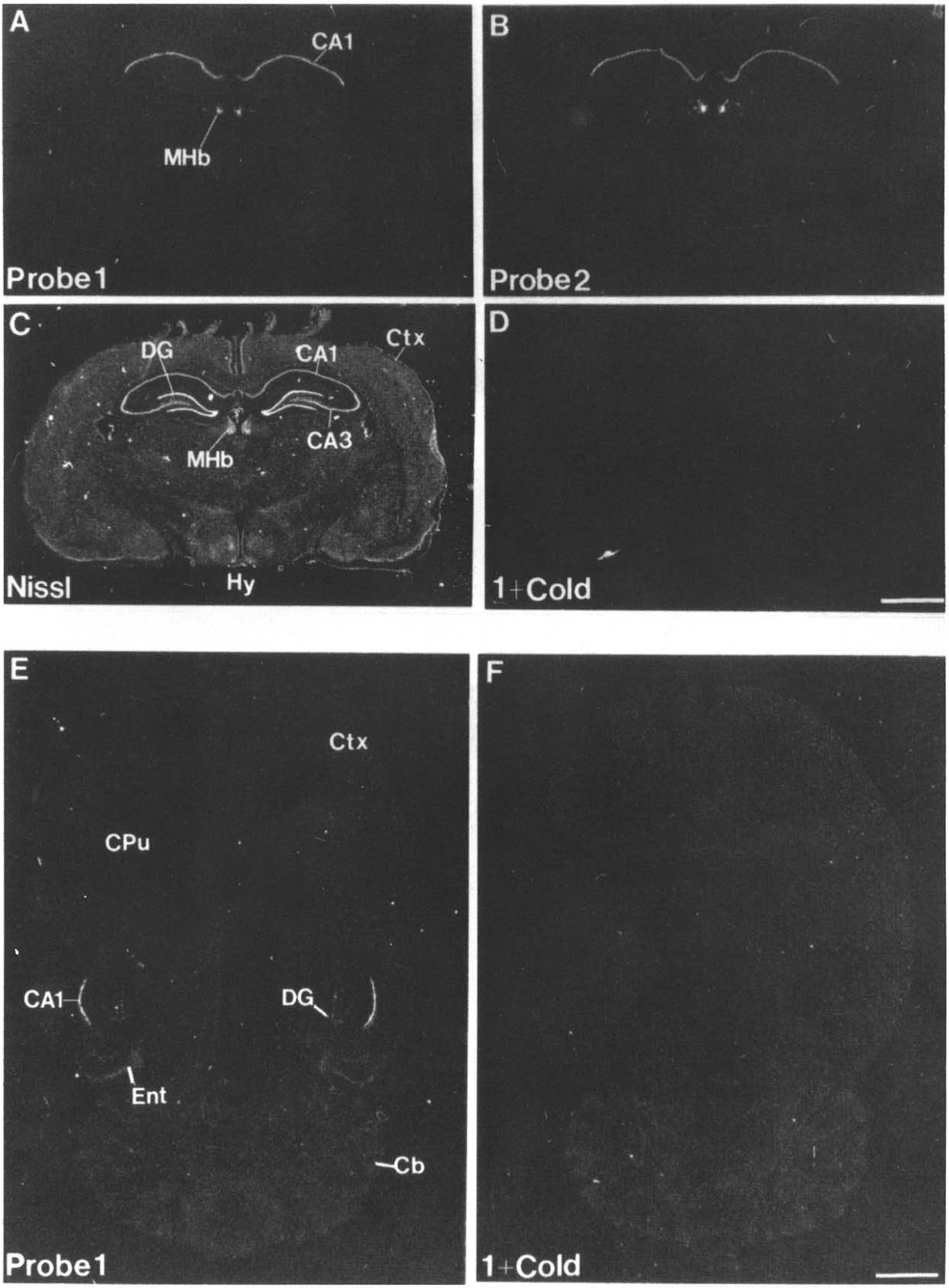

Fig. 3. Distribution of 5-HT_{5B} receptor mRNA in adult rat brain coronal (A–D) and horizontal (E–F) sections. Probes 1 and 2 are oligonucleotides targeting different regions of the 5-HT_{5B} receptor mRNA (see section 2). Cb, cerebellum; CPu, caudate-putamen; CTx, cortex; DG, dentate granule cells; Ent, entorhinal cortex; Hy, hypothalamus; MHb, medial habenula. C, negative Nissl stain of the section that produced the autoradiograph in A. Exposure time, 8 weeks. Scale bar in D, 2.4 mm. Scale bar in F, 2.5 mm.

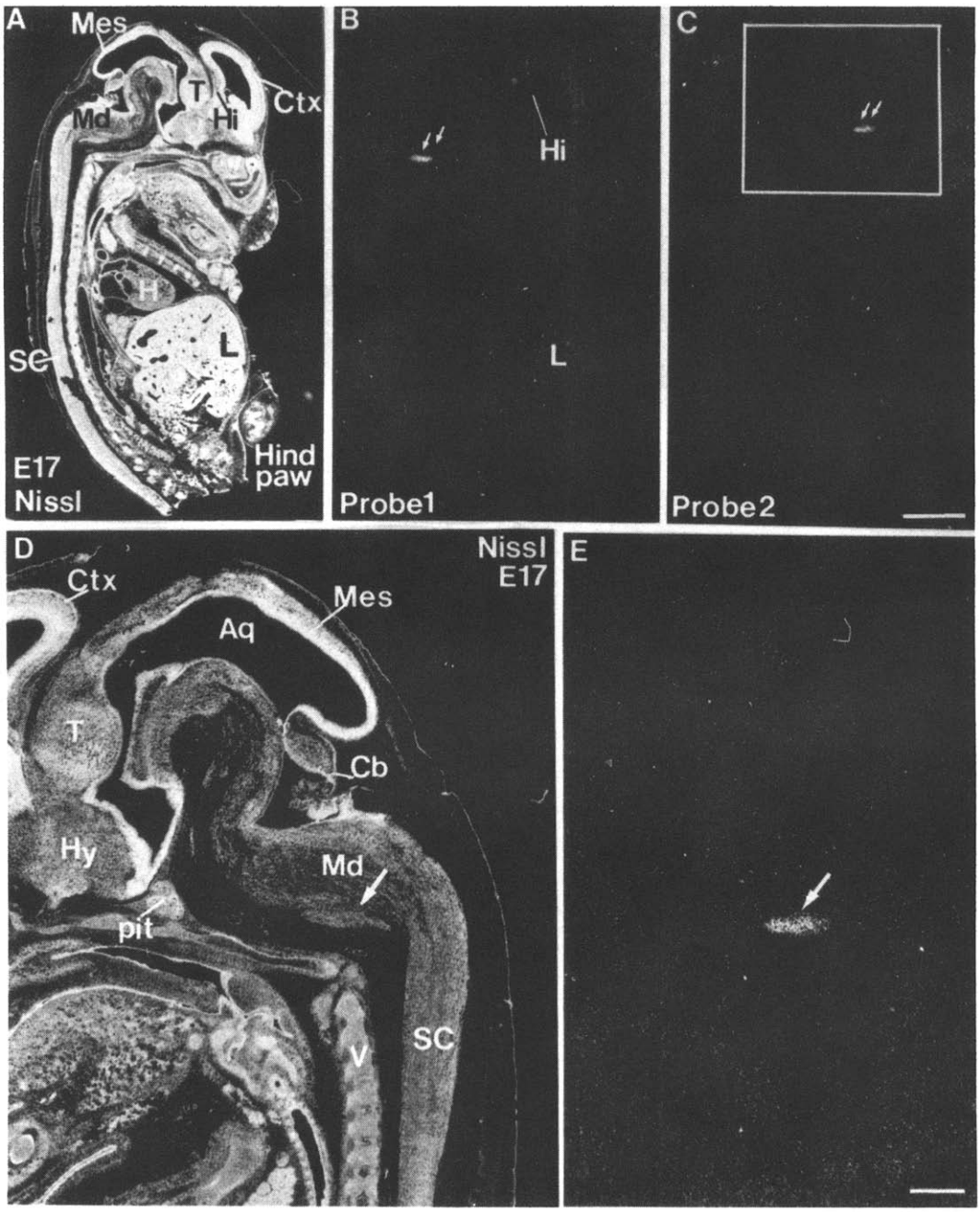

Fig. 4. Expression of the 5-HT_{5B} receptor mRNA in rat E17 embryos. A, Nissl stain of whole embryo; B,C, corresponding X-ray film autoradiographs produced with probes 1 and 2, respectively. The specifically hybridizing region on the ventral surface of the medulla (possibly the nucleus raphe pallidus) is marked by the two arrows. No signal is present in the rest of the brain, or in heart, liver or bone. D (Nissl stain) and E (X-ray film autoradiograph) are higher magnification prints of the regions boxed in C. The positively hybridizing region is marked by an arrow. Scale bar in C, 2.5 mm and scale bar in E, 0.7,mm; Aq, aqueduct; Cb, cerebellum; Ctx, neocortex; H, heart; Hi, hippocampus; Hy, hypothalamus; L, liver; Md, medulla; Mes, mesencephalon; Pit pituitary; SC, spinal cord; T, thalamus; V, vertebral column.

This receptor is also interesting because it does not utilize signal transduction pathways activated by the other known 5-HT receptor subtypes. As shown for the first time in the present study, however, the receptor clearly has the capability to couple to G proteins. The availability of cell lines expressing this receptor will play an important role in the design of subtype selective drugs, which will be especially crucial in future studies investigating the functional roles *in vivo* of the rat and human 5-HT_5 receptor subtypes.

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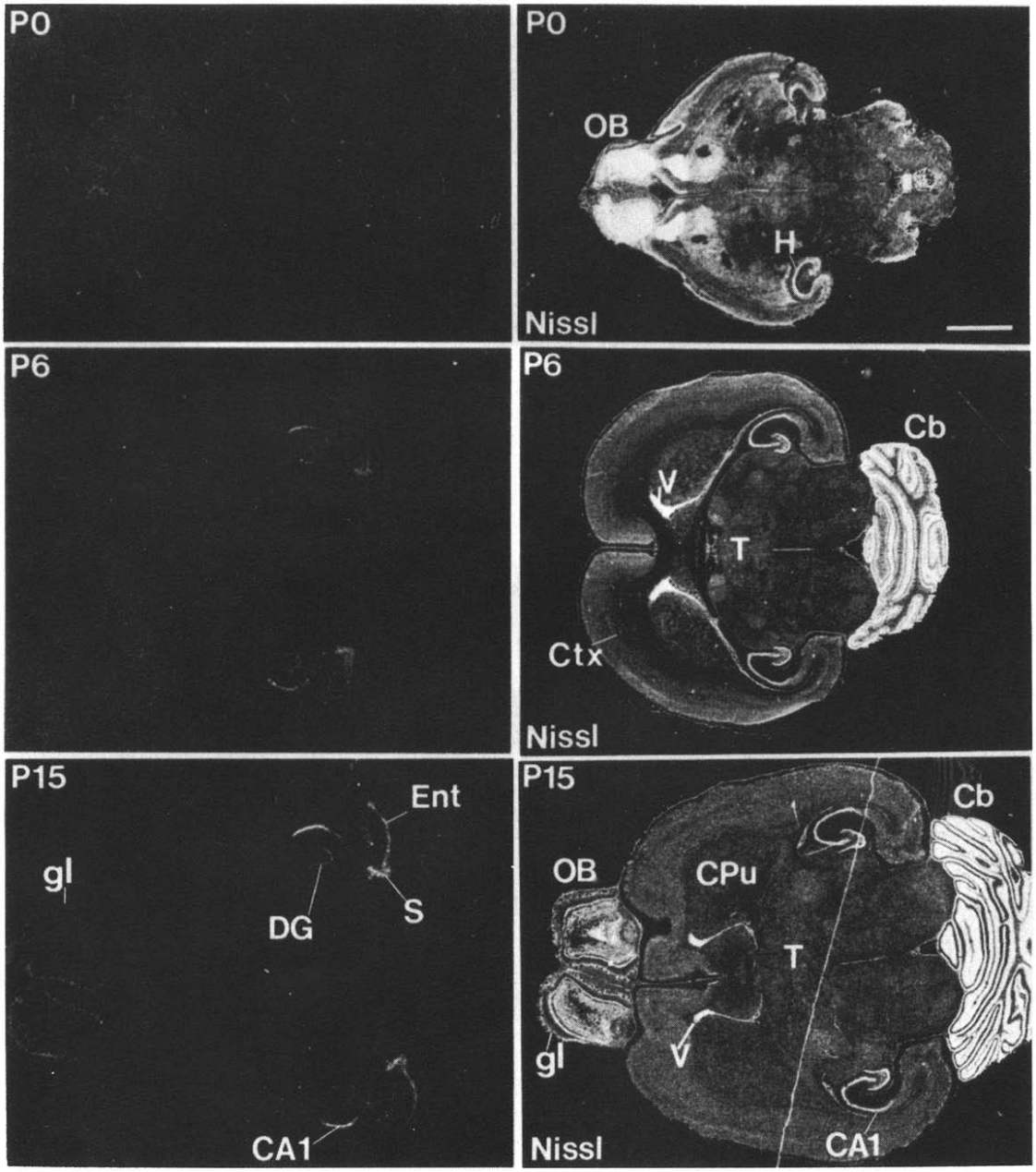

Fig. 5. Postnatal (P0 to P15) developmental expression of the 5-HT_{5B} receptor mRNA in the rat. The left-hand column shows the X-ray film autoradiographs, and the right hand column shows the corresponding Nissl stains of horizontally cut sections. All images are printed to the same scale, thus representing the increase in brain size with age. Scale bar, 2.7 mm. P0 is the day of birth; Cb, cerebellum; CPu, caudate-putamen; Ctx, cortex; Ent, entorhinal cortex; gl, glomerular layer; H, hippocampus; OB, olfactory bulb; S, subiculum; T., thalamus; V, ventricular proliferative zone.

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