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A NOVEL RAT SEROTONIN (5-HT$_6$) RECEPTOR : MOLECULAR CLONING, LOCALIZATION AND STIMULATION OF cAMP ACCUMULATION

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Using a strategy based upon nucleotide sequence homology and starting from the sequence of the rat histamine H_2 receptor (Ruat et al., Biochem. Biophys. Res. Commun. 1991, 179, 1470-1478), we have cloned a rat cDNA encoding a functional serotonin receptor (5-HT$_6$). Its coding sequence corresponds to a glycoprotein of 436 amino acids displaying significant homology with other cloned monoaminergic receptors, e.g., various serotonin receptors. Genomic analysis of its gene indicated the presence of at least one intron. The major transcript of the 5-HT$_6$ receptor gene has a size of ~4.1 kb but another minor 3.2 kb transcript was also evidenced. The highest expression, detected by Northern blot analysis as well as by *in situ* hybridization occurs in various serotoninergic areas of rat or guinea pig brain such as striatum, olfactory tubercle, nucleus accumbens and hippocampus, but a faint expression is also detectable in rat stomach.

When transiently expressed in transfected COS-7 cells the 5-HT$_6$ receptor appears to be positively coupled to cyclic AMP production.

Serotonin (5-HT) is a transmitter, namely released from cerebral and gastrointestinal neurons, which affects its target cells via interaction with a large variety of receptors recently defined by cloning of their genes (reviewed in refs. 1-4). There is evidence, however, for the existence of additional 5-HT receptors. For instance, whereas the cloned receptors appear to be coupled either positively to phospholipase C (those of the 5-HT$_2$ subfamily) or negatively to adenylyl cyclase (those of 5-HT$_1$ subfamily), there is evidence for 5-HT receptors stimulating adenylyl cyclase (5-9) or activating [^{3}H]glycogen hydrolysis (10).

Using a nucleotide probe derived from the rat histamine H_2 receptor (11) to screen a cDNA library from rat striatum, we have characterized an additional 5-HT receptor that belongs

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to the superfamily of G protein-coupled receptors. Taking into account the recently proposed nomenclature rules (12) as well as the existence of the not yet cloned 5-HT_4 receptor (9) and of the 5-HT_5 receptor (13) signaling system of which remains to be identified, we propose to name this novel receptor 5-HT_6.

MATERIALS AND METHODS

Library screening. A partial Sau3A cut rat genomic library (11) was screened (10^6 phages) at low stringency with a ^{32}P-labeled DNA fragment corresponding to a coding region of the rat histamine H_2 receptor gene [nucleotides 10 to 644 (11)]. Nitrocellulose filters (BAS 85, Schleicher and Schuell) were hybridized at 42°C for 18 h in 25% formamide, 1 x Denhardt's solution, 4 x SSC (standard saline citrate), Tris-HCl buffer 8 mM pH 7.4, 100 µg/ml of yeast tRNA and 20 µg/ml of denatured salmon sperm DNA with the ^{32}P-probe (2 x 10^6 dpm/ml) and then washed for 2 x 20 min at 42°C in 2 x SSC 0.1% SDS, then for 2 x 20 min at 42°C in 0.2 x SSC 0.1% SDS before autoradiography. One clone (λG7A) was shown to hybridize strongly with the ^{32}P-probe and was further purified and analyzed. Several overlapping reactive DNA fragments were further subcloned in M13mp18 (Boehringer) and both strands were sequenced by the dideoxy chain termination method (14). These fragments encoded an open reading frame of 238 amino acids starting from an initiating methionine. (Met^1 in Fig.1) and including a consensus sequence for an intron in the 3' end.

A ^{32}P-labeled DNA fragment (nucleotides 129 to 640 in Fig.1) was prepared by PCR (15) using λG7A DNA as a template. This probe (2 x 10^6 dpm/ml) was used to screen under high stringency (40% formamide) a rat striatal cDNA library (2 x 10^6 phages) constructed in λZAP II (Stratagene). Eleven clones were plaque purified and plasmids Bluescript KS(+) containing cDNA inserts were recovered using the helper phage R508 according to the manufacturer procedure.

After amplification, both strands of the cDNA inserts were directly sequenced (14) using alkali-denatured plasmid DNA as a template. Two overlapping clones λS7A19 and λS7A20 exhibited an open reading frame encoding a protein of 436 amino acids and containing the open reading frame found in λG7A. A stop codon TGA was found in the 3' end. Genomic nucleotide sequence found in λG7A (nucleotides -319 to 714 in Fig.1) was identical to nucleotide sequences found in λS7A19 and λS7A20.

Transient expression in COS-7 cells. A 1.8 kb HindIII-SacII DNA fragment comprising the full length coding sequence of λS7A20 was excised from the Bluescript plasmid and purified by Gene-clean II (Bio 101). It was ligated at the HindIII-BglII site of an expression vector derived from the $pSVD_2$ (16), using a SacII-BglII adaptor. Cos-7 cells (96 mm petri dishes) were transfected with 30 µg of pSV5-HT_6, the expression vector described above, using the calcium phosphate method according to the Stratagene protocol. Fourty-eight hours later, cells were harvested, put into 96-well plates and incubated for 24 h at 37°C. Cells were washed twice (10 min at 37°C) and incubated for 15 min with the appropriate drugs in Dulbecco's modified Eagle's medium containing 0.1 mM isobutylmethylxanthine and 0.05% ascorbate. Intracellular cAMP levels were determined by radioimmunoassay (DuPont) as described (17).

Northern blot analysis. Poly(A)$^+$ mRNAs from Wistar rats or Hartley guinea pigs were subjected to Northern blot analysis as described (17) . Hybridization was carried out overnight at 42°C with a probe (15 x 10^6 dpm/ml) corresponding to nucleotides 129-640 in Fig.1 and ^{32}P-labeled by nick translation. Blots were washed three times in 2 x SSC/0.1 % SDS at 42°C for 30 min and twice in 0.2 x SSC/0.1 % SDS at 42°C for 30 min. They were then exposed to X-ray films at -80°C for 4 days with two intensifying screens.

In situ hybridization. Brain sections were prepared and incubated essentially as described (18). For hybridization, each brain section was covered with a buffer (65 % formamide, 10 % dextran

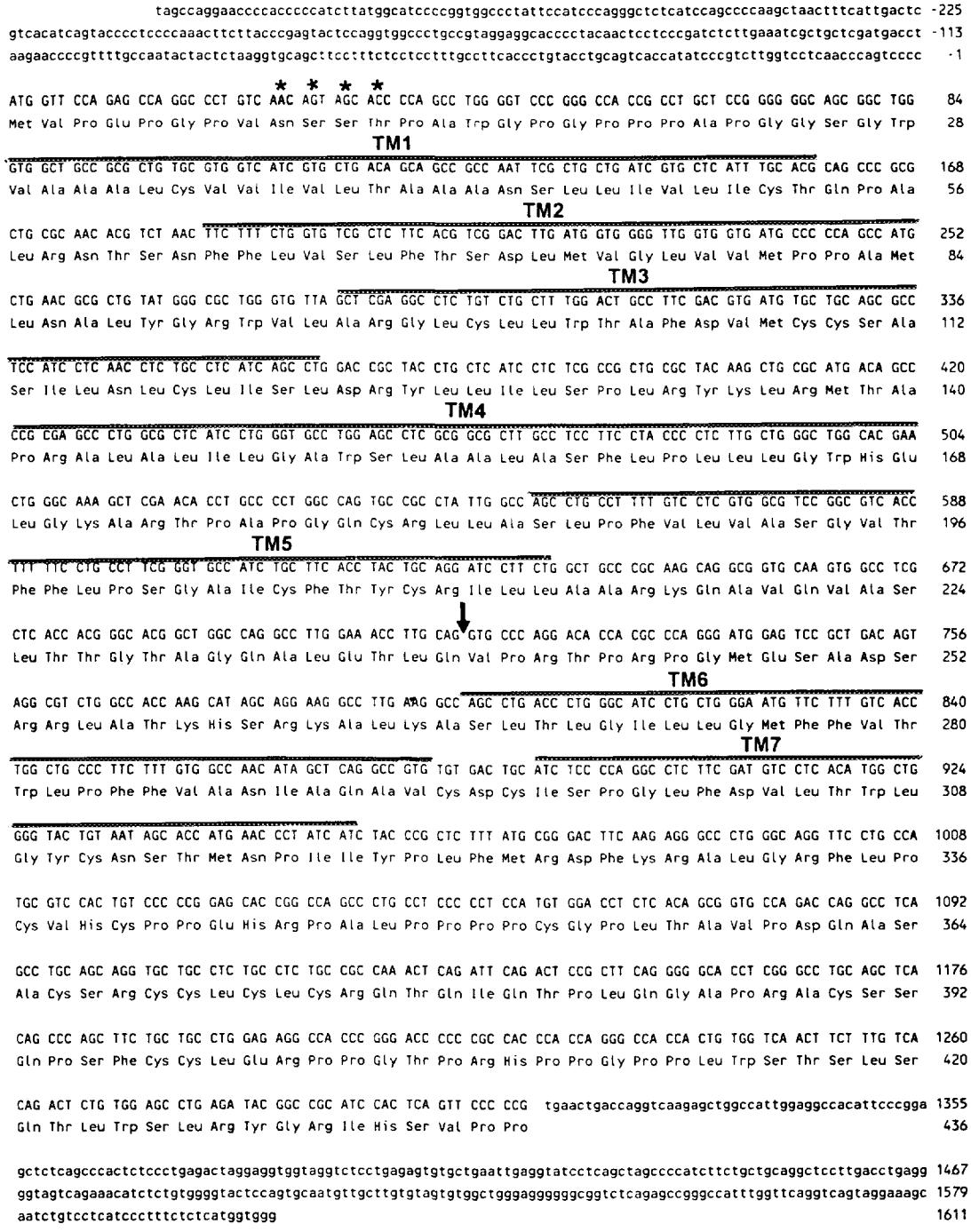

Figure 1. Nucleotide and deduced amino acid sequences of a rat cDNA encoding the 5-HT$_6$ receptor. Both sequences are numbered from the first ATG (Met^1) of the open reading frame. Putative transmembrane regions (TM) are overlined and numbered 1 through 7. (*) represents a consensus glycosylation site. The arrow indicates the position of the intron.

sulfate, 1 x Denhardt's solution, 4 x SSC, 0.1 % sodium pyrophosphate, 100 µg/ml tRNA, 100 µg/ml denaturated salmon sperm DNA) containing 4 $x10^6$ dpm of a labeled antisense- or sense-probe. The latters were synthetized by PCR and corresponded to nucleotides 359-597 in Fig.1. After subcloning in pGEM-4Z (Promega), ^{32}P-labeled antisense- or sense-strand RNA probes were prepared by *in vitro* transcription using a Riboprobe kit (Promega).

RESULTS

Screening of a rat genomic library with a probe encoding putative transmembrane domains (TM1-TM5) of the rat histamine H_2-receptor DNA led to the identification of DNA fragments encoding an open reading frame of 238 amino acids beginning with a consensus ATG initiator codon (GTCCCCATGGTT) (19), a nonsense codon (TAA) at position -240 and an intron sequence (TTGCAGGTACCC) (20) starting after amino acid 238.

The full length nucleotide sequence was obtained by screening a rat striatal cDNA library with a partial length DNA fragment of λG7A. A protein of 436 amino acids with an estimated molecular weight of 46,922 daltons and exhibiting seven clusters of 20-25 hydrophobic residues was deduced (21). A comparison of this sequence with that of various members of the superfamily of G protein-coupled receptors revealed significant homology, particularly with the putative transmembrane domains of some serotonin receptors i.e. 40-45 % with the 5-HT_{dro1} (22), 5-HT_{1C} (23) and 5-HT_2 (24) receptors.

Transient expression of the encoded protein in COS-7 cells transfected with the pSV5-HT_6 expression vector led to cAMP accumulation upon stimulation by serotonin (table 1), whereas no significant accumulation occurred upon addition of 10 µM serotonin to non transfected COS-7 cells (data not shown). In addition, 5-methoxytryptamine (0.01 µM) and 5-carboxamidotryptamine (1 µM) were found to stimulate by 5 fold over basal level cAMP accumulation in transfected COS-7 cells (data not shown).

These data indicate that the cloned protein encodes a novel serotonin receptor (5-HT_6) positively coupled to adenylate cyclase.

Northern blot analysis of various rat tissues revealed two transcripts of 4.1 kb and ~3.2 kb (Fig. 2A). In cerebral tissues, the highest expression was observed in striatum but a signal was also easily detected in the cerebral cortex, hippocampus and hypothalamus. In peripheral tissues, a low expression was detected in the stomach. Northern blot analysis of guinea pig tissues performed with the same probe revealed a single transcript of ~3.8 kb. The strongest signal was found in striatum and olfactory tubercles (Fig. 2B). A moderate expression was also

TABLE 1

STIMULATION OF cAMP ACCUMULATION IN COS-7 CELLS TRANSIENTLY TRANSFECTED WITH pSV5-HT_6

Drugs	cAMP accumulation	
	fmol/well	% of basal level
None	81 ± 3	100
Forskolin, 10 μM	880 ± 60**	1,086
Serotonin, 0.01 μM	187 ± 36*	231
" 3 μM	643 ± 71**	794

*P < 0.01 ; **P < 0.001 as compared to basal level (Student's t test).

Cells were incubated for 15 min with the drugs at the indicated concentration. Data are representative of two to three independent experiments, conducted in quadruplicate. (means ± S.E.M. ; n = 8-16.)

easily detected in other central regions. Among the peripheral tissues tested, a faint signal was only observed in adrenals.

In situ hybridization with the antisense probe revealed a highly heterogeneous distribution of transcripts in the rat brain, whereas the corresponding sense probe led to a weak

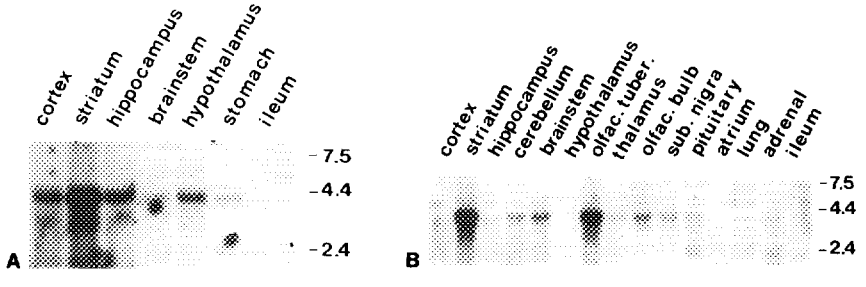

Figure 2. Northern blot analysis of the 5-HT_6 receptor gene transcripts from various rat (A) or guinea pig (B) tissues. Poly(A)$^+$ mRNAs (8 μg/lane) were used. Blots were exposed for 4 days at -80°C with intensifying screens. Molecular sizes (kb) are indicated.

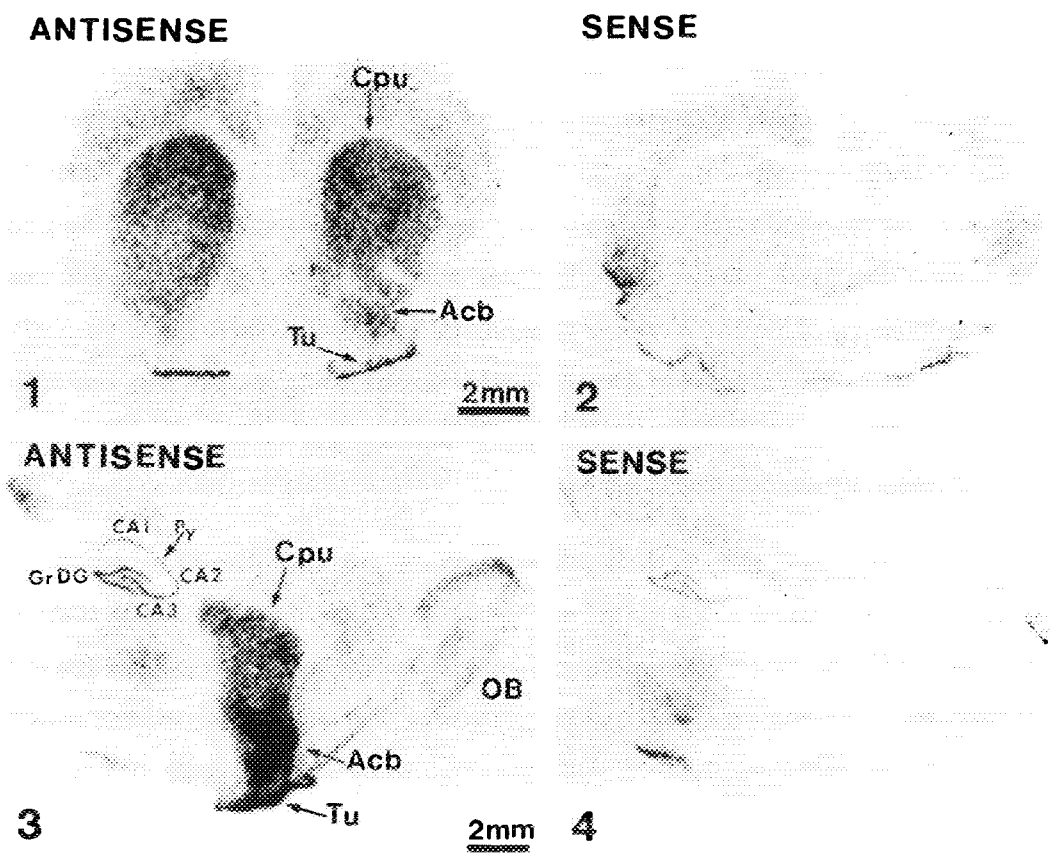

Figure 3 . Localization of the 5-HT_6 receptor gene transcripts in frontal and sagittal sections of rat brain by *in situ* hybridization. ^{32}P-labeled antisense- (1 and 3) or sense-strand (2 and 4) RNA probes were used. Abbreviations : Acb, nucleus accumbens ; CA1-3, fields CA1-3 of Ammon's horn (Py, pyramidal cell layer) ; Cpu, caudate putamen ; DG, dentate gyrus (Gr, granulate layer) ; OB, olfactory bulb ; Tu, olfactory tubercle.

and rather uniform signal (Fig. 3). The highest levels were observed in the olfactory tubercles, striatum and nucleus accumbens. In striatum, the labeling was heterogeneous, possibly indicating a preferential localization in striosomes or matrix. Hybridization signals of moderate intensity also occurred in the hippocampus (CA_1, CA_2 and CA_3 fields and dentate gyrus), olfactory bulb and cerebral cortex.

DISCUSSION

The 5-HT_6 receptor represents the first mammalian serotonin receptor to be cloned that is coupled to the activation of adenylyl cyclase. That this receptor was previously detected in functional studies remains unclear. A positive coupling of the 5-HT_{1A} receptor to adenylyl cyclase in rat brain has been suggested (5, 6). It is clear, however, that the amino acid sequence of the 5-HT_6 receptor differs from that of the cloned 5-HT_{1A} receptor (25). The regional distribution of the 5-HT_6 receptor does not appear to correspond to that of the 5-HT_4 receptor, also positively coupled to adenylyl cyclase (9). The 5-HT_6 receptor may also differ from the 5-HT receptor responsible for a high-affinity activation of adenylyl cyclase in rat cerebral cortex, striatum and hippocampus (7).

The novel 5-HT_6 receptor is characterized by the presence of a consensus glycosylation site located in the amino terminal tail (Asn^9), of highly conserved cysteine residues (Cys^{99} and Cys^{180}) presumably involved in a disulfide bond connecting the first and second extracytoplasmic loops and of various consensus sites for phosphorylation by protein kinases C in the third intracytoplasmic loop and in the C terminus. Interestingly, a consensus "leucine zipper" motif (26) is present in the end of the putative third transmembrane domain (Leu^{115} to Leu^{136}) but its structural or functional role remains unclear. An aspartate residue (Asp^{106}) in TM3, conserved in all aminergic receptors and shown by site-directed mutagenesis (27) to be responsible for salt-linking the ammonium group of amines, presumably plays a similar role in serotonin binding. The structure of this novel serotonin receptor is characterized by a short third cytoplasmic loop (~50 amino acids) and a rather long C-terminal tail (~120 amino acids), two features widely found in receptors positively coupled to adenylyl cyclase (28).

The novel 5-HT_6 receptor displays significant but rather limited homology with other 5-HT receptors, the highest being found with a *Drosophila* serotonin receptor positively coupled to adenylyl cyclase (22). Significant homology also occurs with the rat histamine H_2 receptor, which accounts for its cloning using a probe derived from the sequence of the latter. The 5-HT_6 receptor gene, like those of the 5-HT_2 subfamily (29), i.e. the 5-HT_{1c} and 5-HT_2 itself, has its coding sequence interrupted by at least one intron. The latter occurs at the level of the third intracytoplasmic loop, where an intron is also present in various dopamine receptor genes (30).

The 5-HT_6 receptor gene seems to be expressed in many serotoninergic brain areas and in the gastrointestinal tract, as shown by Northern blot analysis. This approach, as well as *in situ* hybridization, indicates that, in the rat, the highest expression level occurs in the basal forebrain (striatal complex), hippocampus, hypothalamus and cerebral cortex. A slightly different distribution seems to occur in guinea pig brain. Species differences in the localization and pharmacology of the same receptor is not uncommon among members of the large 5-HT receptor family (3). Stable expression of the 5-HT_6 receptor in a transfected cell line is required to establish precisely its pharmacology in several species and is in progress in our laboratory.

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