Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 928–932, February 1990
Neurobiology

The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors

(phospholipase C/cellular transformation/neurotransmitters)

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Contributed by Richard Axel, October 20, 1989

ABSTRACT **Serotonin exerts its diverse physiological effects by interacting with multiple distinct receptor subtypes. We have isolated a rat brain 5HT2 serotonin receptor cDNA by virtue of its homology with the 5HT1c receptor. The 5HT2 receptor is a member of the family of receptors that are linked to guanine nucleotide-binding proteins and are predicted to span the lipid bilayer seven times. Overall sequence identity between the 5HT2 and 5HT1c receptors is 49%, but identity within the transmembrane domains is 80%. Expression of both the 5HT2 and 5HT1c receptors in transfected mouse fibroblasts activates phospholipase C signaling pathways and promotes cellular transformation. However, RNA blotting shows that these two receptor subtypes are differentially expressed in the central nervous system. In this manner, structurally and functionally homologous receptor subtypes may elicit distinct physiologic actions.**

Serotonin [5-hydroxytryptamine (5-HT)] is a neurotransmitter that mediates a diverse array of physiological responses by interacting with multiple serotonin receptor subtypes. These individual receptor subtypes activate different intracellular signaling systems. The 5HT1c and 5HT2 receptors activate phospholipase C (1, 2), whereas 5HT1a and 5HT1b receptors regulate adenylate cyclase (3–5) or couple to guanine nucleotide-binding proteins (G proteins) that activate ion channels directly (6).

In this study, we have isolated a 5HT2 receptor cDNA and have compared the structure and function of the 5HT2 receptor* with that of other cloned serotonin receptor subtypes (5HT1a and 5HT1c). The 5HT2 receptor belongs to the family of G-protein-coupled receptors, which are thought to traverse the plasma membrane seven times (7), a finding consistent with the studies on the 5HT2 receptor by Pritchett *et al.* (8). The 5HT2 and 5TH1c receptors exhibit striking conservation of amino acid sequence and also modulate similar intracellular signaling pathways. Activation of either receptor in transfected fibroblasts results in a rapid increase in intracellular Ca^{2+}. The activation of either the 5HT2 or the 5HT1c receptor also confers long-term changes in the growth properties of these cells, resulting in cellular transformation and focus formation. These two receptor subtypes, however, exhibit different patterns of expression in the brain. Thus, mammals have evolved two genes, which encode receptors that bind the same ligand and couple to the same intracellular signaling pathway, yet control different physiological functions by virtue of their distinct distributions.

MATERIALS AND METHODS

Library Screening and Sequence Analysis. Three libraries were used in this study: a partial *Eco*RI rat genomic library (9) and a partial *Hae* III rat genomic library (10), both in λ Charon 4A, and a rat whole brain, random-primed cDNA library in λgt11 (11). All probes were ^{32}P-labeled by nick-translation and hybridized to filters under high-stringency conditions (50% formamide/0.9 M NaCl/0.09 M sodium citrate/0.07 M sodium dihydrogen phosphate/7 mM EDTA, pH 7.4, 42°C; washes, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0, 42°C). A full-length coding-region clone was reconstructed from one genomic and two cDNA clones (see Fig. 1). Sequences were determined by the method of Sanger *et al.* (12).

Radioligand Binding Assays. Filter binding assays were performed as described (13, 14). For Scatchard analysis, each sample contained 5 μg of membrane protein in a final volume of 1 ml. The final concentration of ^{125}I-labeled lysergic acid diethylamide (^{125}I-LSD; New England Nuclear) ranged from 0.5 to 8 nM. At 2 nM ^{125}I-LSD, ≈3% of the available radioligand was bound. Nonspecific binding (determined in the presence of 2 μM mesulergine) comprised 2–10% of the total radioligand bound. All other assays contained 1 nM ^{125}I-LSD and 5–10 μg of membrane protein in 1 ml.

Transfection, Focus Formation Assays, and Flow Cytometry. Growth of NIH mouse 3T3 cells, DNA transfections, and focus assays were performed as described (14). For measuring changes in intracellular Ca^{2+}, cells were loaded with indo-1 and analyzed with a flow cytometer (13, 15).

RESULTS

Sequence of the 5HT2 Receptor cDNA. We isolated several cDNAs encoding the 5HT2 serotonin receptor from a rat brain cDNA library by virtue of their homology with the 5HT1c receptor cDNA. Sequence analysis of the cDNA clones revealed a long open reading frame, but none of the clones extended to a putative initiator methionine codon. To obtain the complete coding sequence, we therefore isolated appropriate 5′ genomic clones that overlapped the 5′ cDNA sequence (Fig. 1*a*). The sequence of the cDNA and genomic clones reveals an open reading frame of 1413 nucleotides, encoding a protein of 471 amino acids that exhibits 49% identity with the 5HT1c receptor (Fig. 1*b*). The functional properties of this protein in transfected fibroblasts demonstrate that this clone encodes a 5HT2 serotonin receptor (see below).

The 5HT2 receptor cDNA encodes a protein that shares numerous sequence and structural properties with the family of receptor molecules predicted to span the lipid bilayer seven times (7). The amino-terminal 85 amino acids of the receptor are thought to reside on the extracellular face of the membrane and the seven transmembrane domains would then be linked by three extracellular and three intracellular loops. This 5HT2 receptor shares an overall sequence identity of 49% with the 5HT1c receptor, but the amino acid

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Abbreviations: G protein, guanine nucleotide-binding protein; LSD, lysergic acid diethylamide.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30705).

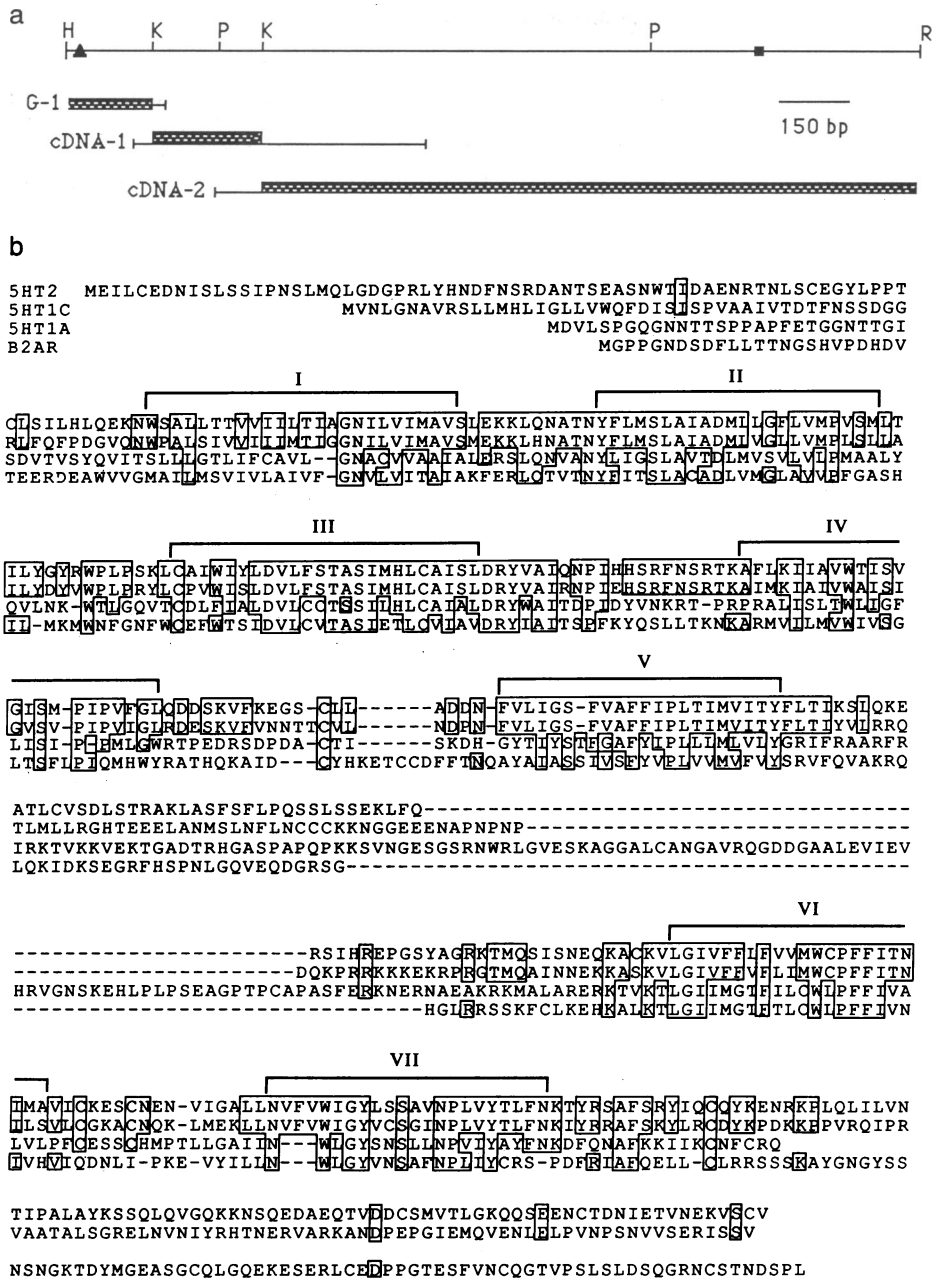

FIG. 1. Homology between the 5HT2 receptor and other G-protein-coupled receptors. (*a*) cDNA and genomic clones used to construct a functional 5HT2 receptor cDNA. The boxed region of each clone denotes the restriction fragment used in the construction (H, *Hpa* I; K, *Kpn* I; P, *Pst* I; R, *Eco*RI). The triangle and the square indicate the positions of the predicted initiation and termination codons, respectively. bp, Base pairs. (*b*) Alignment of the amino acid sequences of the rat 5HT2 receptor (top), rat 5HT1c receptor (second row) (13), human 5HT1a receptor (third row) (16), and hamster β_2-adrenergic receptor (fourth row) (17). The boxed residues are identical in the 5HT2 and 5HT1c receptor sequences. Roman numerals and brackets denote the seven putative transmembrane domains.

identity within the transmembrane domains is 80%. The striking conservation of sequence within the transmembrane domains is consistent with the observation that the two receptors exhibit similar ligand-binding properties and couple to the same intracellular signaling pathways.

In Fig. 1, we present only the amino acid sequence of the 5HT2 receptor, since the nucleotide sequence of a rat 5HT2 receptor has been reported (8). Our sequence differs from that reported at one nucleotide position, altering the reading frame at the amino terminus of the protein. At position 111 in our nucleotide sequence (corresponding to a thymidine), Pritchett *et al.* (8) reported an additional thymidine not present in our sequence. As a consequence, they reported a truncated amino terminus that derives from a different reading frame than that encoding our amino-terminal sequence. The sequence we report has been confirmed by analyzing both strands of two independently isolated genomic clones.

Distribution of Serotonin Receptor mRNA. The distribution of receptor mRNA in the central nervous system was determined by RNA blot analysis (Fig. 2). The 5HT2 receptor RNA was most abundant in the cerebral cortex. Poly(A)$^+$ RNA from the cortex revealed two discrete transcripts between 5 and 6 kilobases in length. Both transcripts were also observed in hypothalamus, hippocampus, spinal cord, and olfactory bulb, at about 10-fold lower abundance (Fig. 2). No hybridization was observed in any peripheral tissues examined. In addition, no hybridization was detected with RNA samples from the choroid plexus, a tissue rich in 5HT1c receptor mRNA (13, 18), thereby demonstrating that the 5HT2 probe is selective for 5HT2 receptor mRNA and does not cross-hybridize with 5HT1c receptor mRNA under our hybridization conditions. The distribution of 5HT2 receptor mRNA is generally consistent with the distribution of 5HT2 receptors in the brain as determined by radioligand binding studies (19). Comparison of 5HT1c and 5HT2 receptor mRNA expression indicates that although the 5HT2 receptor is prevalent in the cerebral cortex, the 5HT1c subtype is more widely distributed in the central nervous system (13, 20) and is far more abundant than the 5HT2 receptor in the brain and spinal cord.

Expression of 5HT2 Receptor in Mammalian Fibroblasts. The functional properties of the cloned protein expressed in transfected mouse fibroblasts provide evidence that it is a

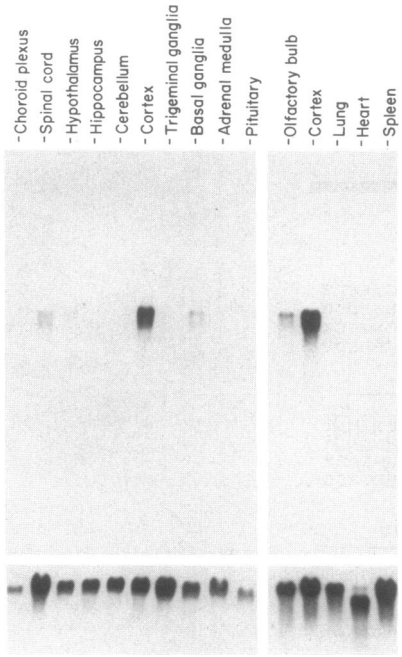

FIG. 2. Tissue distribution of 5HT2 receptor mRNA determined by RNA blot analysis. (*Upper*) Poly(A)$^+$ RNA (2–10 μg) isolated from various brain regions and peripheral tissues was subjected to electrophoresis through a 0.8% agarose/formaldehyde gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled DNA probe prepared from a coding-region fragment of the 5HT2 receptor clone. Tissue regions are indicated above each lane. (*Lower*) Filters were reprobed with a ^{32}P-labeled human α-actin probe to assess relative amounts of RNA in each lane.

5HT2 receptor. A clone encoding the full-length protein was constructed by ligating a 5′ genomic restriction fragment with two overlapping cDNA clones. This full-length 5HT2 receptor cDNA was cloned into the vector pMV7 (21) to produce an expression plasmid, pMV7-SR2. The vector pMV7 contains a murine leukemia virus long terminal repeat that serves as a promoter for expression of the 5HT2 receptor cDNA. This vector also contains an independent expression cassette encoding neomycin phosphotransferase, which renders mammalian cells resistant to G418, a neomycin analogue. G418-resistant colonies expressing 5HT2 receptors were identified and the properties of these cells were compared with those of NIH 3T3 fibroblasts expressing the 5HT1c receptor. Membranes prepared from a single clone expressing 5HT2 receptors, GF6, bound the serotonin receptor ligand ^{125}I-LSD with high affinity, whereas no specific binding was detected with membranes from the parental NIH 3T3 cell line. Scatchard analysis using GF6 membranes revealed a dissociation constant (K_d) for ^{125}I-LSD of 1.6 nM and a site density (B_{max}) of 18 pmol per mg of protein, which corresponds to about 8×10^5 receptors per cell. The K_d we observed is consistent with the K_d for ^{125}I-LSD binding to cortical 5HT2 sites (22). Moreover, the 5HT2-selective antagonists spiperone (10 nM) and ketanserin (10 nM) blocked the binding of ^{125}I-LSD to GF6 membranes (Fig. 3). In contrast, these 5HT2 ligands were much less effective at displacing ^{125}I-LSD from membranes prepared from the cell line P_0 (Fig. 3), which expresses an equivalent density of 5HT1c receptors (14).

The response of transfected cells to serotonin was analyzed by loading cells with the Ca^{2+}-sensitive dye indo-1 and monitoring changes in the fluorescence emission spectrum of the dye in a flow cytometer (15). Indo-1 undergoes a shift in its fluorescence emission spectrum as a function of Ca^{2+} concentration and serves as a quantitative measure of intracellular Ca^{2+} (23). Cells transformed with either the 5HT1c or the 5HT2 receptor cDNA exhibited a marked increase in intracellular Ca^{2+} when exposed to serotonin, whereas control, untransfected NIH 3T3 cells did not respond to serotonin (Fig. 4).

We used this Ca^{2+} mobilization assay to examine the relative efficacy of drug action at 5HT2 and 5HT1c receptors. The threshold response to serotonin occurs at a concentration of 10 nM and is maximal at concentrations exceeding 1 μM (data not shown). This concentration range is about 100 times greater than that required to activate the 5HT1c receptor in transfected fibroblasts (18). The 5HT2 receptor antagonist spiperone (10 nM) completely abolished the increase in intracellular Ca^{2+} in response to 10 μM serotonin (Fig. 4). In contrast, 10 nM spiperone had little or no effect on serotonin-evoked Ca^{2+} elevation in NIH 3T3 cells expressing the 5HT1c receptor. The selective 5HT2 antagonist ketanserin (10 nM) also blocked Ca^{2+} responses in fibroblasts expressing the 5HT2 receptor but had no effect on NIH 3T3 cells expressing the 5HT1c receptor. The broad-spectrum antagonist mesulergine (10 nM) blocked Ca^{2+} responses in NIH 3T3 cells expressing either of the two receptor subtypes, with a slightly greater potency on cells that express the 5HT1c receptor (Fig. 4). The relative potency of these antagonists in transfected NIH 3T3 cells conforms to the ligand-binding profile of 5HT2 receptors in the rat cortex (19) and is consistent with K_i values obtained for the inhibition of serotonin-induced phosphatidylinositol turnover in rat cortex and choroid plexus preparations (2). This pharmacologic profile confirms that the cDNA we have cloned has the properties of a functional 5HT2 receptor. Moreover, this receptor, like the 5HT1c receptor, couples to intracellular signaling systems that increase intracellular Ca^{2+}.

Activation of the 5HT2 Receptor Leads to Cellular Transformation. We have recently shown that activation of the 5HT1c receptor in transfected NIH 3T3 fibroblasts results in cellular transformation and focus formation (14). We therefore asked whether activation of the 5HT2 receptor also alters the growth properties of transfected fibroblasts. Cells transfected with either 5HT2 receptor cDNA (pMV7-SR2) or 5HT1c receptor cDNA (pMV7-SR1c) were allowed to grow

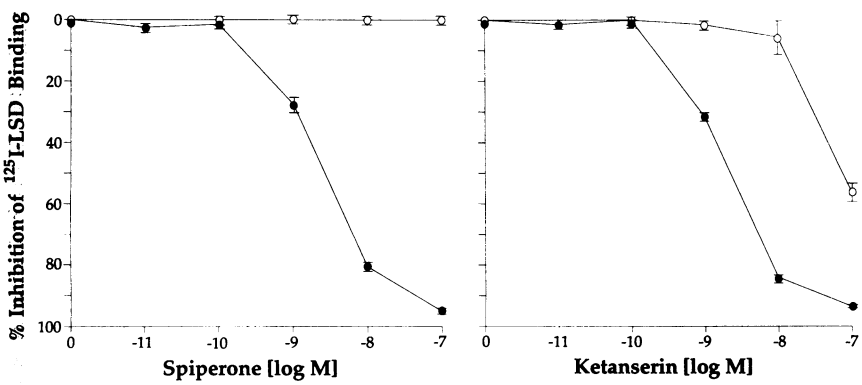

FIG. 3. Pharmacological properties of the 5HT2 receptor expressed in transfected mouse fibroblasts. Membranes were prepared from the transfected NIH 3T3 cell lines GF6 (●) and P_0 (○), which express equivalent numbers of 5HT2 or 5HT1c receptors, respectively. These membranes were incubated with ^{125}I-LSD (1 nM) plus 0–100 nM spiperone or ketanserin. Each sample contained 5 μg of membrane protein and bound $\approx 10^5$ cpm of ^{125}I-LSD in the absence of antagonist. Nonspecific binding was less than 5% of the total counts bound. Values were determined in duplicate.

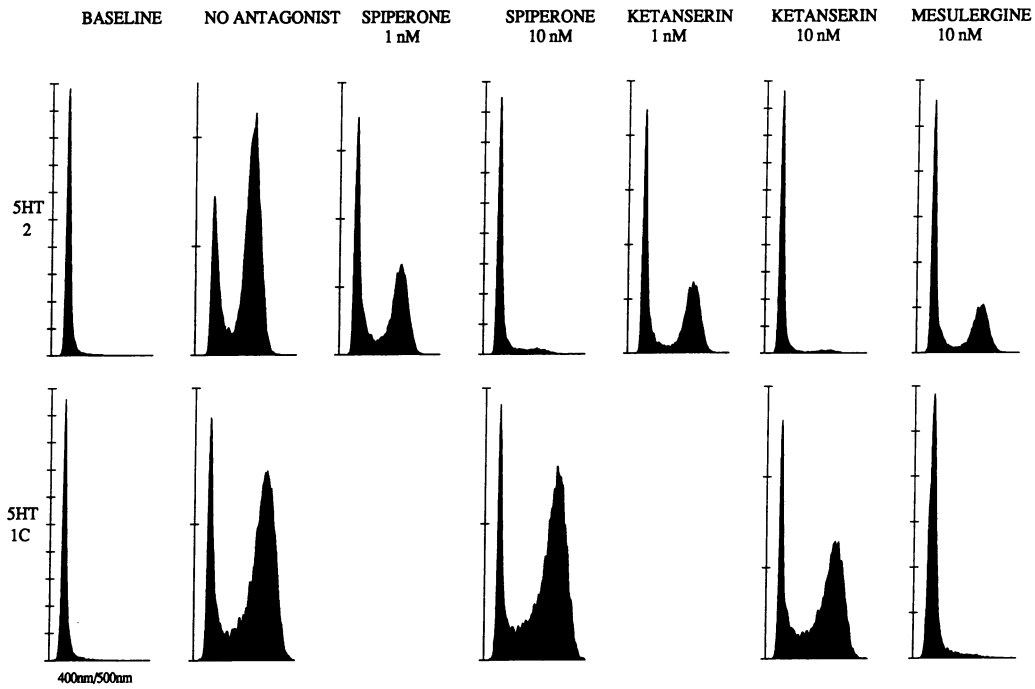

FIG. 4. Activation of 5HT2 receptors in transfected mouse fibroblasts. NIH 3T3 cells transfected with either the 5HT1c or 5HT2 receptor cDNA were loaded with the Ca^{2+}-sensitive dye indo-1. Changes in the level of intracellular free Ca^{2+} following exposure to serotonin were monitored with a flow cytometer. The ratio of fluorescence emission was monitored at 400 and 500 nm. Increases in intracellular Ca^{2+} are denoted by a shift to the right in the histograms. Cell number is plotted on the ordinate. In the absence of serotonin (baseline) cells remain at low, resting Ca^{2+} levels. In the presence of serotonin (10 μM) cells show an increase in intracellular Ca^{2+} (see shift in histograms). The ability of a given antagonist to block this response was determined by adding the drug to cells, at the concentration indicated, prior to serotonin exposure. Each histogram represents data collected from 15,000 cells. Cells expressing 5HT1c receptors were clonally derived. Cells expressing 5HT2 receptors were derived from approximately 30% of primary transfected fibroblasts pre-sorted on the basis of serotonin sensitivity.

to confluence and the appearance of foci was scored after 2–3 weeks. Both pMV7-SR2 and pMV7-SR1c generated transformed foci in 5 μM serotonin with roughly equivalent frequencies (Table 1). The frequency of focus formation with both clones was about one-fourth the frequency of G418-resistant colonies. The appearance of foci was dependent upon transfection with vectors containing the serotonin receptor cDNA. Transformed foci were never observed with the expression vector (pMV7) alone (data not shown; ref. 14).

We next demonstrated that focus formation is dependent upon the activation of the 5HT2 receptor. In initial experiments we asked whether the generation of foci by the 5HT2 receptor cDNA can be blocked by the 5HT2-selective antagonist spiperone. NIH 3T3 cells were transfected with either pMV7-SR2 or pMV7-SR1c and the appearance of foci was scored in the presence of spiperone. Spiperone (0.1 μM) completely blocked the generation of foci after transfection with the 5HT2 receptor cDNA (Table 1). In contrast, spiperone had no effect on the ability of the 5HT1c receptor cDNA to elicit foci. Focus formation with either pMV7-SR2 or pMV7-SR1c was effectively inhibited by the broad-spectrum antagonist mesulergine.

Furthermore, we have shown that focus formation with 5HT2 receptor cDNA is dependent upon the addition of exogenous serotonin. Although serum contains significant levels of serotonin, the frequency of focus formation with pMV7-SR2 increases with increasing concentrations of added serotonin (Table 1). The effective concentration of serotonin determined in this assay is consistent with the EC_{50} for 5HT2 receptor-mediated activation of Ca^{2+} mobilization in transfected cells. The frequency of focus formation with 5HT1c receptor cDNA remains constant over this concentration range, a finding consistent with the high affinity (EC_{50} = 10–50 nM; refs. 2 and 18) exhibited by this receptor for serotonin. Taken together, these results demonstrate that activation of either the 5HT1c or the 5HT2 receptor on the surface of transfected fibroblasts results in transformed foci.

DISCUSSION

The diverse functions of serotonin in the mammalian brain are mediated by multiple, distinct serotonin receptor subtypes. We previously isolated a functional cDNA encoding the 5HT1c receptor. In this study, we cloned the gene encoding the 5HT2 receptor and compared the function of the 5HT2 and 5HT1c receptors in transfected fibroblasts. Our

Table 1. Transfected fibroblasts exhibit a transformed phenotype as a consequence of serotonin receptor activation

		Foci, no./μg of plasmid DNA			
Antagonist	DNA	5 μM	1 μM	0.5 μM	0.1 μM
None	pMV7-SR1c	25	25	28	20
	pMV7-SR2	18	8	3	0
Spiperone	pMV7-SR1c	28	13	20	18
(0.1 μM)	pMV7-SR2	0	0	0	0
Mesulergine	pMV7-SR1c	3	0	0	0
(1 μM)	pMV7-SR2	0	0	0	0

NIH 3T3 fibroblasts were transfected with the neomycin (G418)-resistance expression vector pMV7 containing a functional 5HT1c (pMV7-SR1c) or 5HT2 (pMV7-SR2) receptor cDNA. Forty-eight hours after the addition of DNA, the cells were split into several Petri dishes to determine the efficiency of focus formation in a variety of culture conditions. Focus formation was scored in medium containing 3% calf serum plus exogenously added serotonin (0.1, 0.5, 1, or 5 μM, final concentration) in the presence or absence of the antagonist spiperone or mesulergine. The efficiency of transfection was determined by scoring G418-resistant colonies in medium containing 10% calf serum and 0.5 mg of G418 per ml. Total G418-resistant colonies obtained per microgram of plasmid DNA were 115 (pMV7-SR1c) and 105 (pMV7-SR2).

findings indicate that the 5HT2 and 5HT1c receptors modulate similar cellular functions and also exhibit striking conservation in amino acid sequence.

The 5HT2 receptor shares sequence and structural properties with the family of receptor molecules predicted to span the lipid bilayer seven times. The 5HT2 receptor shares an overall sequence identity of 49% with the 5HT1c receptor, but the amino acid identity within the putative transmembrane domains is over 80%. The stringent sequence conservation within the transmembrane domains is likely to reflect the fact that these regions of the molecule are required for the maintenance of two primary functions shared by the 5HT1c and 5HT2 receptors, ligand binding and G-protein-mediated activation of phospholipase C. These two serotonin receptors therefore define a new family of G-protein-coupled receptors.

Families of G-protein-coupled receptors have also been identified for cholinergic and adrenergic transmitters. The muscarinic acetylcholine receptors define a family of receptors that are highly homologous to one another (24, 25), even though many of these receptors couple to different intracellular signaling pathways (26). Similarly, individual members of the adrenergic receptor family also couple either to phospholipase C or to adenylate cyclase (7). These observations suggest the existence of a superfamily of G-protein-coupled receptors, comprised of multiple families, each binding a distinct ligand. Duplication and mutation within a given family could generate novel receptors that maintain ligand specificity but couple with different G proteins, thus providing a mechanism whereby a single neurotransmitter activates different intracellular signaling pathways.

However, the 5HT1a receptor gene is clearly a member of the adrenergic receptor gene family (16, 27). In this instance, duplication and mutation has apparently altered the ligand-binding properties of a member of the adrenergic receptor gene family such that it now binds serotonergic rather than adrenergic ligands. This finding, together with our studies on the 5HT2 and 5HT1c receptors, indicates that the serotonin receptors are encoded by at least two gene families. The 5HT1c and 5HT2 receptors define one family, whereas the 5HT1a receptor has evolved from a distinct adrenergic receptor gene family. Mutation within a given family can therefore generate variant receptors that either bind different ligands or couple to different G proteins.

Our studies not only demonstrate structural conservation between the 5HT2 and 5HT1c receptors but suggest that the two receptors may activate common intracellular signaling systems. Activation of either receptor subtype elicits an increase in intracellular Ca^{2+} in *Xenopus* oocytes (ref. 8; D.J., unpublished data) or transfected fibroblasts. Moreover, activation of either the 5HT1c or the 5HT2 receptor subtype in transfected fibroblasts results in the appearance of transformed foci. These results demonstrate that the expression and activation of either the 5HT2 or the 5HT1c receptor in a given cell type has similar biochemical and functional consequences. However, the particular functional consequences of serotonin receptor activation are critically dependent on the cellular environment. Activation of the 5HT2 and 5HT1c receptors on neurons modulates membrane excitability, whereas in fibroblasts activation of the same receptors modulates cell growth.

The 5HT2 and 5HT1c receptors provide a particularly clear example of the evolution of receptor subtypes that bind the same ligand and couple to identical signaling systems. This apparent redundancy may enhance the potential for diversity in receptor signaling by altering the affinity of receptor subtypes for serotonin. The affinity of the 5HT1c receptor is approximately 2 orders of magnitude greater than that of the 5HT2 receptor. Thus, limiting concentrations of neurotransmitter may selectively activate only the high-affinity receptor subtype, 5HT1c. It is also possible that the 5HT1c and 5HT2 serotonin receptors may activate distinct intracellular signaling pathways not apparent in our assay systems. Alternatively, the generation of two structurally distinct but functionally homologous genes may permit independent genetic regulation of the expression of these two receptors in different subsets of neurons.

We thank I. Schieren for cell sorting and analysis; B. Han for cell culture assistance; S. Peroutka and T. Branchek for the gifts of mesulergine, spiperone, and ketanserin; and G. Lemke, D. Colman, R. Dunn, and J. Boulter for providing genomic and cDNA libraries. This work was supported by the Howard Hughes Medical Institute (D.J., K.N.H., T.M.J., and R.A.) and by grants from the National Institutes of Health (R.A. and T.M.J.).

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