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ACCELERATED COMMUNICATION

Cloning and Expression of a Novel Serotonin Receptor with High Affinity for Tricyclic Psychotropic Drugs

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SUMMARY

We have used the polymerase chain reaction technique to selectively amplify a guanine nucleotide-binding protein-coupled receptor cDNA sequence from rat striatal mRNA that exhibits high homology to previously cloned serotonin receptors. Sequencing of a full length clone isolated from a rat striatal cDNA library revealed an open reading frame of 1311 base pairs, encoding a 437-residue protein with seven hydrophobic regions. Within these hydrophobic regions, this receptor was found to be 41–36% identical to the following serotonin [5-hydroxytryptamine (5-HT)] receptors: 5-HT_2 > 5-HT_{1D} > 5-HT_{1C} > 5-HT_{1B} > 5-HT_{1A} > 5-HT_{1E}. Northern blots revealed a ~4.2-kilobase transcript localized in various brain regions, with the following rank order of abundance: striatum ≫ olfactory tubercle > cerebral cortex > hippocampus. Expression of this clone in COS-7 cells resulted in the appearance of high affinity, saturable binding of (+)-[2-^{125}I] iodolysergic acid diethylamide ([^{125}I]LSD) with a K_d of 1.26 nM. Among endogenous biogenic amines, only 5-HT completely inhibited [^{125}I]LSD binding (K_i = 150 nM). The inhibition of [^{125}I]LSD binding by other serotonergic agonists and antagonists revealed a pharmacological profile that does not correlate with that of any previously described serotonin receptor subtype. In addition, this receptor exhibits high affinity for a number of tricyclic antipsychotic and antidepressant drugs, including clozapine, amoxipine, and amitriptyline. In HEK-293 cells stably transfected with this receptor, serotonin elicits a potent stimulation of adenylyl cyclase activity, which is blocked by antipsychotic and antidepressant drugs. The distinct structural and pharmacological properties of this receptor site indicate that it represents a completely novel subtype of serotonin receptor. Based on its affinity for tricyclic psychotropic drugs and its localization to limbic and cortical regions of the brain, it is likely that this receptor may play a role in several neuropsychiatric disorders that involve serotonergic systems.

The neurotransmitter serotonin (5-HT) has been implicated in a variety of cognitive and behavioral functions. Disruptions in serotonergic systems may be a critical factor in mental disorders such as schizophrenia, depression, and obsessive-compulsive disorder (1). The transduction of serotonergic signals across the neuronal membrane is mediated by a diversity of receptor subtypes that, in mammals, appear to fall into five pharmacologically distinct classes, designated 5-HT_1 to 5-HT_5 (2, 3). The 5-HT_1 subcategory has been further subdivided into five different subtypes, referred to as 5-HT_{1A} to 5-HT_{1E} (4). The primary structures for a number of these receptors have been elucidated by molecular cloning, including the 5-HT_1 (5–19), 5-HT_2 (20, 21), 5-HT_3 (22), and recently the 5-HT_5 (3) subclasses. In addition, the sequences of three different *Drosophila* serotonin receptors, 5-HT_{dro1}, 5-HT_{dro2A}, and 5-HT_{dro2B}, have been reported (23, 24). With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, all of the other members of the serotonin receptor family belong to the large class of receptors that are linked to their effector functions via G proteins.

We now report the cloning from rat brain of a cDNA encoding a novel G protein-linked serotonin receptor that is distinct from any class of previously described 5-HT receptor in both primary structure and pharmacology. This receptor appears to be localized exclusively in the central nervous system, predominately in the corpus striatum but also in various limbic and cortical regions. When expressed in mammalian cells, this receptor exhibits high affinity for serotonin as well as a number of tricyclic antipsychotic and antidepressant drugs, including clozapine, amoxipine, and amitriptyline, suggesting that it may

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; bp, base pair(s); [^{125}I]LSD, (+)-[2-^{125}I]iodolysergic acid diethylamide; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; TM, transmembrane; G protein, guanine nucleotide-binding protein; kb, kilobase(s); SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

mediate some of the therapeutic actions of these important psychotropic agents.

Experimental Procedures

PCR amplification. The PCR amplification was carried out as described previously (25), using cDNA prepared from rat striatal mRNA and 1 μM levels each of the following primers: 5′-GTCGACCCT (GT)T(GT)(CG)GCC(AC)TCA(GT)CA(TC)(GA)G(AG)TCGCTA-3′ and 5′-AAGCTTATGAA(AG)AAGGGCAG(GC)CA(AG)CAGAGG (GT)(CT)(AG)(CA)A-3′. The timing used was 1.5 min at 93°, 2 min at 50°, and 4 min at 72°, followed by a 7-min extension at 72°. The reaction products were purified and size fractionated on a 1% agarose gel. Individual bands were excised, electroeluted, concentrated by ultrafiltration, and ligated into the pCR1000 vector (Invitrogen). Competent INVαF′ cells (Invitrogen) were transformed and mini-preparations of plasmid DNA were prepared for insert sequencing.

DNA sequencing. Nucleotide sequence analysis was performed using the Sanger dideoxy-nucleotide chain-termination method, with Sequenase (United States Biochemical Corp.), on denatured double-stranded plasmid templates. Primers were synthetic oligonucleotides that were either vector specific or derived from previously determined sequence information. cDNA sequence was confirmed through the sequencing of both strands.[1]

cDNA library screening. Recombinants (1×10^6) from a rat striatal cDNA library, constructed in the λ ZAP II vector (Stratagene), were screened with the 538-bp St-B17 PCR fragment, which was ^{32}P-labeled via nick translation. Duplicate nitrocellulose filters were hybridized in 50% formamide, 0.75 M NaCl/0.075 M sodium citrate (5× SSC), 5× Denhardt's solution, 0.02 M Na_2HPO_4, 0.25% SDS, 0.15 mg/ml salmon sperm DNA, with 4×10^6 dpm/ml ^{32}P-labeled probe, for 24 hr at 37°. High stringency washing of the filters was performed with 1× SSC/0.1% SDS at 65° before autoradiography. λ phage found to hybridize to the probe were subsequently plaque purified. *In vivo* excision and rescue of the nested pBluescript plasmids from the λ ZAP II clones were performed using helper phage, according to the Stratagene protocol.

Expression studies. For transient expression of the St-B17 receptor, COS-7 cells were transfected with the pSRα-B17 construct using the calcium phosphate precipitation method, as described previously (25), or the DEAE-dextran method, as described by Cullen (26). For stable expression of the St-B17 receptor, HEK-293 cells in 150-mm dishes were co-transfected with 30 μg of pSRα-B17 and 3 μg of pMam-neo (Invitrogen) by the calcium phosphate technique and were subjected to selection with 600 μg/ml geneticin (G418) (GIBCO). Resistant colonies were collected and screened for specific hybridization of ^{32}P-labeled St-B17 probe to cellular RNA dot-blots. The expression of St-B17 receptor protein was then assessed by saturation analysis of [^{125}I]LSD binding to each of these cell lines. Specific saturable binding of [^{125}I]LSD with appropriate affinity was observed in each cell line that exhibited positive hybridization with the St-B17 probe, whereas no specific binding was observed in nontransfected 293 cells. One clone was isolated that expressed 800 fmol/mg of protein of St-B17 receptor, and this clone was subsequently used for assessment of intracellular cAMP accumulation.

Radioligand binding and cAMP assays. For [^{125}I]LSD binding assays, cells were harvested 72 hr after calcium phosphate transfection and were either disrupted in a Dounce homogenizer in TME buffer (50 mM Tris·HCl, pH 7.4 at 37°, 10 mM $MgSO_4$, 0.5 mM EDTA) or frozen in 5 mM Tris·HCl, pH 7.4 at 25°, 5 mM $MgCl_2$, 250 mM sucrose, and stored in liquid N_2 before membrane preparation. Crude membranes were prepared from cell homogenates by centrifugation at 43,000 × *g* and resuspension in homogenization buffer at a protein concentration of ~60 μg/ml. For saturation radioligand binding experiments, 50 μl of membrane suspension were incubated in a final volume of 100 μl with increasing concentrations (15–3500 pM) of [^{125}I]LSD (2200 Ci/mmol; DuPont/NEN) in triplicate for 60 min at 37°. 5-HT at 100 μM was used to determine nonspecific binding. Specific binding represented 80–97% of the total binding over the range of concentrations used. For competition binding assays, 50 μl of membrane suspension were incubated with 0.5 nM [^{125}I]LSD and increasing concentrations of competitor for 60 min at 37°. At this concentration of radioligand, total binding represented ≈10,000 dpm, whereas nonspecific binding was ≈300 dpm. Sodium metabisulfate (200 μM) was included as an antioxident in all assays. Binding assays were terminated by rapid filtration through Whatman GF/C filters that had been pretreated with 0.3% polyethyleneimine; filters were then washed with 5 × 3 ml of 50 mM Tris·HCl, pH 7.4, at 4°. Radioactivity levels were determined in a γ counter at an efficiency of ~70%.

For [^{3}H]5-HT binding assays, DEAE-dextran-transfected cells were harvested 48–72 hr after transfection by scraping into ice-cold Dulbecco's phosphate-buffered saline containing 5 mM EDTA. The cells were collected by centrifugation and resuspended by Polytron homogenization in ice-cold 2× TME buffer. The crude membranes were collected by centrifugation and resuspended in ice-cold 2× TME. Of this crude membrane preparation, 0.5 ml containing 50–100 μg of protein was added to chilled tubes containing 0.25 ml of [^{3}H]5-HT (24–27 Ci/mmol; New England Nuclear) (≈3–60 nM final concentration for saturation analyses or ≈5 nM final concentration for competition analyses) and 0.25 ml of competing drugs, all dissolved in 1 mM ascorbic acid to prevent oxidation. Nonspecific binding was determined in the presence of 10 μM 5-HT. After incubation for 20 min at 37°, samples were rapidly filtered through Whatman GF/C filters that had been pretreated with 0.1% polyethyleneimine. The samples were then rapidly washed with an additional 10 ml of ice-cold 50 mM Tris·HCl, pH 7.5, and the radioactivity was quantitated by liquid scintillation counting. In saturation binding experiments, the specific binding represented 60–90% of the total binding, whereas in the competition binding experiments the total binding was represented by ≈5000 dpm and the nonspecific binding was ≈500 dpm.

Intracellular cAMP levels were determined after a 5-min incubation of intact cells with various test compounds at 37° in the presence of the cAMP phosphodiesterase inhibitor Ro-20–1724, using a modification of a previously described cAMP assay (27) wherein the adrenal cAMP-binding protein was replaced with 0.8 mg/ml protein kinase A (Sigma) in water.

mRNA analysis. Poly(A)$^+$ RNA was prepared from rat brain regions using the FastTrack mRNA isolation system (Invitrogen). Poly(A)$^+$ RNA (3 μg) was denatured and subjected to electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde. RNA was transferred to nylon membranes (Genescreen Plus; DuPont) by capillary transfer and was immobilized by UV cross-linking. Northern blots were probed with the entire 2.8-kb cDNA insert of clone St-B17 ^{32}P-labeled by the random priming method. Northern blots were hybridized with 3×10^6 dpm/ml probe in 1 M NaCl, 1% SDS, 10% dextran sulfate, at 60° for 18 hr. Blots were washed in 2× SSC/1% SDS at room temperature, followed by 0.2× SSC/1% SDS at 65°, and were exposed for 15 days at −70° with an intensifying screen.

Results and Discussion

As part of an ongoing effort to identify and clone G protein-linked receptors from rat brain, we have used the PCR technique to selectively amplify cDNA sequences from mRNA purified from rat corpus striatum. Poly(A)$^+$ RNA was used to synthesize cDNA by reverse transcription followed by PCR amplification with a pair of highly degenerate primers, the sequences of which were derived from the third and sixth TM regions of previously cloned G protein-linked receptors. This process resulted in the amplification of several cDNA fragments

[1] The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank under accession number L03202.

ranging from 300 to 500 bp in size, which were preliminarily characterized by DNA sequence analysis (data not shown). Several of these cDNA fragments exhibited sequence homology to previously cloned G protein-coupled receptors; one, clone St-B17, was particularly homologous to several catecholamine receptors and was thus selected for further characterization.

The partial length St-B17 cDNA fragment was used to screen a rat striatal cDNA library to isolate a full length clone. One positive clone containing a cDNA insert of ~2.8 kb was isolated and the nucleotide sequence was determined. The longest open reading frame in this cDNA consisted of 879 bp, which encoded a protein containing sequences homologous to TM regions I-VI of previously cloned biogenic amine receptors (Fig. 1). Further analysis of the cDNA revealed the presence of sequence ~210 bp downstream from the open reading frame that was homologous to the seventh TM region of several receptors. Careful examination of the DNA sequence between these putative TM regions VI and VII revealed the presence of consensus donor and acceptor sequences for intron/exon splice junctions (28) (Fig. 1A), suggesting the possibility that this region contained an unspliced intron harboring several stop codons. This was initially examined by using PCR to amplify this putative intron region from cDNA prepared from rat striatal mRNA. Amplification across this region with primers P3 and P4 (indicated in Fig. 1A) resulted in a primary DNA product of 223 bp, the size predicted for mRNA spliced at the observed donor/acceptor sites, and a minor band of 416 bp, the size expected based on the sequence of the St-B17 cDNA clone (data not shown). We thus initially concluded that the St-B17 cDNA clone was derived from a minor, incompletely spliced, mRNA.

Because an additional screen of the cDNA library did not result in the isolation of a full length, completely spliced clone, we decided to use PCR to amplify the predicted coding sequence from rat striatal mRNA using primers P1 and P2 (indicated in Fig. 1A). Two products of ~1.6 and 1.4 kb were obtained, the latter of which was subcloned into pBluescript II SK(+). Sequence analysis confirmed the identity of St-B17 and revealed that the putative intron was absent, with the flanking exons spliced together at nucleotide 873, as predicted. Splicing at this position results in a 1311-bp open reading frame encoding a protein of 437 amino acid residues, with a calculated molecular mass of 46.8 kDa (Fig. 1A). Hydropathy analysis of the deduced amino acid sequence of this protein indicated seven hydrophobic regions (data not shown), predicted to represent putative TM domains. When compared with previously cloned G protein-coupled receptors, the TM regions of St-B17 exhibited high homology to various serotonin (5-HT) receptors, suggesting that it may be a member of this receptor family. Within the TM regions, St-B17 exhibited homologies of 41%, 40%, 39%, 38%, 37%, and 36% to 5-HT_2, 5-HT_{1D}, 5-HT_{1C}, 5-HT_{1B}, 5-HT_{1A}, and 5-HT_{1E} receptors, respectively (Fig. 1B). The third cytoplasmic loop of St-B17, consisting of ~57 residues, would be the shortest of the cloned 5-HT receptors, although similar in length to the 5-HT_{1C} and 5-HT_2 receptors (17, 20, 21). Similarly, whereas the ~117-residue carboxyl-terminal tail of St-B17 would be the longest among the 5-HT receptors, it is similar in length to that found in the 5-HT_{1C} and 5-HT_2 receptors. The combination of a relatively short third cytoplasmic loop and a long carboxyl terminus is common among receptors that couple to the stimulation of either the adenylyl cyclase or phospholipase C signal transduction systems. St-B17 also contains one potential *N*-linked glycosylation site at Asn-9 in the extracellular amino terminus, in addition to several potential sites for phosphorylation by cAMP-dependent protein kinase or protein kinase C in both the third cytoplasmic loop and the intracellular carboxyl-terminal tail.

To definitively establish the identity of the receptor encoded by St-B17, we proceeded with its expression in mammalian cells. Unfortunately, the PCR-amplified, full length, correctly spliced cDNA could not be used directly for expression because of several PCR-generated base substitutions in the 5′ region upstream of the internal *Bam*HI site (indicated in Fig. 1A). We thus constructed a hybrid cDNA in which the entire 3′ sequence downstream of the *Bam*HI site in the intron-containing clone was replaced with corresponding *Bam*HI fragment from the correctly spliced PCR-amplified cDNA. This construct was subcloned into the eukaryotic expression vector pCD-SRα (29), yielding pSRα-B17.

Transient transfection of COS-7 cells with pSRα-B17 resulted in the appearance of high affinity and saturable binding sites for the serotonergic ligand [^{125}I]LSD, which exhibited a K_d of 1.26 ± 0.17 nM (three experiments) and B_{max} values ranging from 2 to 5 pmol/mg of protein (Fig. 2A). Specific binding of [^{125}I]LSD represented ≈95% of total binding at a concentration of 1 nM. No specific binding of [^{125}I]LSD was observed in nontransfected COS-7 cells or in cells transfected with the pCD-SRα vector alone (data not shown). Preliminary characterization of the St-B17 pharmacology indicated that, among several endogenous biogenic amines, including dopamine, melatonin, epinephrine, norepinephrine, and histamine, only 5-HT was capable of completely displacing [^{125}I]LSD binding, exhibiting a K_i of 150 nM (Fig. 2B; Table 1). The Hill coefficient for the 5-HT competition curve was not significantly different from unity and the affinity of 5-HT was not influenced by addition of the guanine nucleotide analogue guanosine 5′-(β,γ-imido)triphosphate (data not shown). It is likely that the apparent lack of effect of guanine nucleotides on 5-HT binding is due to the high level of transient expression obtained in a low percentage of the cultured cells. The resulting excess of receptor relative to G protein thus precludes detection of the high affinity, G protein-coupled state of the receptor. The binding of 5-HT to St-B17 was also investigated directly, using [^{3}H]5-HT as the radioligand. Saturation analysis of [^{3}H]5-HT binding revealed the presence of a single class of high affinity, saturable binding sites in transfected, but not nontransfected, COS-7 cells (Fig. 2C). Analysis of the [^{3}H]5-HT saturation binding data revealed a K_d of 37 ± 5.0 nM (three experiments) and B_{max} values of 1–3 pmol/mg of protein. The binding of [^{3}H]5-HT was similarly not affected by the addition of guanine nucleotides, suggesting recognition of the low affinity, uncoupled state of the receptor. These initial binding data would thus suggest that St-B17 encodes a 5-HT receptor subtype.

Further characterization of the St-B17 pharmacology utilized a variety of drugs that exhibit specificity for various serotonergic receptor subtypes and other binding sites. The average K_i values for compounds competing with affinity higher than ~10 μM are shown in Table 1, with representative competition curves for [^{125}I]LSD binding shown in Fig. 2B. Examination of the rank order of potency for a variety of serotonergic agents revealed that the pharmacology of clone St-B17 does not correspond to that of any previously described serotonin receptor

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                                                                                                   ccaaccccca -432
cgcgcgacacgtggtgatctaacgtactcacacgcccaccccttctcgaagagactgccccggccggaaggcggggagttcggctcctgctcccacatccccagctgtg -324
cccctagccaggaaccccaccccccatcttatggcatccccggtggccctattccatcccagggctctcatccagccccaagctaactttcattgactcgtcacatca -216
gtacccctccccaaacttcttacccgagtactccaggtggccctgcgtaggaggcacccctacaactcctcccgatctcttgaaatcgctgctcgatgacctaagaa -108
     P1
ccccgtcttgccaatactactctaaggtgcagcttcctttctcctcccttgccttcaccctgtacctgcagtcaccatatcccgtcttggtcctcaacccagtcccc -1
ATG GTT CCA GAG CCA GGC CCT GTC AAC AGT AGC ACC CCA GCC TGG GGT CCC GGG CCA CCC CCT GCT CCG GGG GGC AGC GGC 81
Met Val Pro Glu Pro Gly Pro Val Asn Ser Ser Thr Pro Ala Trp Gly Pro Gly Pro Pro Pro Ala Pro Gly Gly Ser Gly

TGG GTG GCT GCC GCG CTG TGC GTG GTC ATC GTG CTG ACA GCA GCC GCC AAT TCG CTG CTG ATC GTG CTC ATT TGC ACG CAG 162
Trp Val Ala Ala Ala Leu Cys Val Val Ile Val Leu Thr Ala Ala Ala Asn Ser Leu Leu Ile Val Leu Ile Cys Thr Gln

CCC GCC GTG CGC AAC ACG TCT AAC TTC TTT CTG GTG TCG CTC TTC ACG TCG GAC TTG ATG GTG GGG TTG GTG GTG ATG CCC 243
Pro Ala Val Arg Asn Thr Ser Asn Phe Phe Leu Val Ser Leu Phe Thr Ser Asp Leu Met Val Gly Leu Val Val Met Pro

CCA GCC ATG CTG AAC GCG CTG TAT GGG CGC TGG GTG TTA GCT CGA GGC CTC TGT CTG CTT TGG ACT GCC TTC GAC GTG ATG 324
Pro Ala Met Leu Asn Ala Leu Tyr Gly Arg Trp Val Leu Ala Arg Gly Leu Cys Leu Leu Trp Thr Ala Phe Asp Val Met

TGC TGC AGC GCC TCC ATC CTC AAC CTC TGC CTC ATC AGC CTG GAC CGC TAC CTG CTC ATC CTC TCG CCG CTG CGC TAC AAG 405
Cys Cys Ser Ala Ser Ile Leu Asn Leu Cys Leu Ile Ser Leu Asp Arg Tyr Leu Leu Ile Leu Ser Pro Leu Arg Tyr Lys

CTG CGC ATG ACA GCC CCG CGA GCC CTG GCG CTC ATC CTG GGT GCC TGG AGC CTC GCG GCG CTT GCC TCC TTC CTA CCC CTC 486
Leu Arg Met Thr Ala Pro Arg Ala Leu Ala Leu Ile Leu Gly Ala Trp Ser Leu Ala Ala Leu Ala Ser Phe Leu Pro Leu

TTG CTG GGC TGG CAC GAA CTG GGC AAA GCT CGA ACA CCT GCC CCT GGC CAC TGC CGC CTA TTG GCC AGC CTG CCT TTT GTC 567
Leu Leu Gly Trp His Glu Leu Gly Lys Ala Arg Thr Pro Ala Pro Gly Gln Cys Arg Leu Leu Ala Ser Leu Pro Phe Val

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CTC GTG GCG TCC GGC GTC ACC TTT TTC CTG CCT TCG GGT GCC ATC TGC TTC ACC TAC TGC AGG ATC CTT CTG GCT GCC CGC 648
Leu Val Ala Ser Gly Val Thr Phe Phe Leu Pro Ser Gly Ala Ile Cys Phe Thr Tyr Cys Arg Ile Leu Leu Ala Ala Arg

AAG CAG GCG GTG CAA GTG GCC TCG CTC ACC ACG GGC ACG GCT GGC CAG GCC TTG GAA ACC TTG CAG GTG CCC AGG ACA CCA 729
Lys Gln Ala Val Gln Val Ala Ser Leu Thr Thr Gly Thr Ala Gly Gln Ala Leu Glu Thr Leu Gln Val Pro Arg Thr Pro
                                                                     P3
CGC CCA GGG ATG GAG TCC GCT GAC AGT AGG CGT CTG GCC ACC AAG CAT AGC AGG AAG GCC TTG AAG GCC AGC CTG ACC CTG 810
Arg Pro Gly Met Glu Ser Ala Asp Ser Arg Arg Leu Ala Thr Lys His Ser Arg Lys Ala Leu Lys Ala Ser Leu Thr Leu
                                                                         873↓
GGC ATC CTG CTG GGA ATG TTC TTT GTC ACC TGG CTG CCC TTC TTT GTG GCC AAC ATA GCT CAG gtaaaatgatgaccgtgaaggtg
Gly Ile Leu Leu Gly Met Phe Phe Val Thr Trp Leu Pro Phe Phe Val Ala Asn Ile Ala Gln

ggatgagcttaggtctgaccggagagacgccatgcttcactgggcaaaggtgggagggaggaggatggctcatctgtggtgcctgtgtctgtgtttctgtcctatccc
                                                                  ↓
tgctgggtgggtagcctgggtccctgcctgggacatggggtgtgatgagtcttatctccacag GCC GTC TGT GAC TGC ATC TCC CCA GGC CTC TTC 906
                                                                 Ala Val Cys Asp Cys Ile Ser Pro Gly Leu Phe
                                                                                    P4
GAT GTC CTC ACA TGG CTG GGG TAC TGT AAT AGC ACC ATG AAC CCT ATC ATC TAC CCG CTC TTT ATG CGG GAC TTC AAG AGG 987
Asp Val Leu Thr Trp Leu Gly Tyr Cys Asn Ser Thr Met Asn Pro Ile Ile Tyr Pro Leu Phe Met Arg Asp Phe Lys Arg

GCC CTG GGC AGG TTC CTG CAT GCG TCC ACT GTC CCC CGG AGC ACC GGC CAG CCC TGC CTC CCC CTC CAT GTG GAC CTC TCA 1068
Ala Leu Gly Arg Phe Leu His Ala Ser Thr Val Pro Arg Ser Thr Gly Gln Pro Cys Leu Pro Leu His Val Asp Leu Ser

CAG CGG TGC CAG ACC AGG CCT CAG CTG CAG CAG GTG CTC GCT CTC CCT CTG CCG CCA AAC TCA GAT TCA GAC TCC GCT TCA 1149
Gln Arg Cys Gln Thr Arg Pro Gln Leu Gln Gln Val Leu Ala Leu Pro Leu Pro Pro Asn Ser Asp Ser Asp Ser Ala Ser

GGG GGC ACC TCG GGC CTG CAG CTC ACA GCC CAG CTT CTG CTG CCT GGA GAG GCC ACA CGG GAC CCC CCG CCA CCC ACC AGG 1230
Gly Gly Thr Ser Gly Leu Gln Leu Thr Ala Gln Leu Leu Leu Pro Gly Glu Ala Thr Arg Asp Pro Pro Pro Pro Thr Arg

GCC ACC ACT GTG GTC AAC TTC TTT GTC ACA GAC TCT GTG CAG CCT GAG ATA CGG CCG CAT CCA CTC AGT TCC CCC GTG AAC 1311
Ala Thr Thr Val Val Asn Phe Phe Val Thr Asp Ser Val Glu Pro Glu Ile Arg Pro His Pro Leu Ser Ser Pro Val Asn
tgaccaggtcaagagctggccattggaggccacattcccggagctctcagcccactctccctgagactaggaggtggtaggtctcctgagagtgtgctgaattgagg
tatctcagctagcccatcttctgctgcagctccttgactgaggggtagtcagacacat....
                                                                                                    P2
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B

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STB17                                                  MVHEPGPVNSSTPAWGPGPPPAHGGSGWVAAALCVVIVLT
5HT1A                                          MDVFSFGQGNNTTASQEPFGTGGNVTSISDVTFSYQVITSLLLGTLIF
5HT1B                                MEEQGIQCAPPPPATSQTGVPLANLSHNCSADDYIYQDSIALPWKVLLVALLALITLA
5HT1C                       MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGGRLFQFPDGVQNWPALSIVVIIIM
5HT1D                                          MSLPNQSLEGLPQEASNRSLNATGAWDPEVLQALRISLVVVLSIITLA
5HT1E                                                        MNITNCTTEASMAIRPKTITEKMLICMTLVVITTL
5HT2   MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASNWTIDAENRTNLSCEGYLPPTCLSILHLQEKNWASALLTTVVIILT

STB17  --AAANSLLIVLICTQPAVRNTSNFFLVSLFTSDLMVGLVVMPPAMLNALYG-RWVLARGLCLLWTAFDVMCCSASILNLCLISLDRYL
5HT1A  CAVLGNACVVAAIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLN-KWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYW
5HT1B  -TTLSNAFVIATVYRTRKLHTPANYLIASLAVTDLLVSILVMPISTMYTVTG-RWTLGQVVCDFWLSSDITCCTASIMHLCVIALDRYW
5HT1C  TIGHGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYV
5HT1D  -TVLSNAFVLTTILLTKKLHTPANYLIGSLATTDLLVSILVMPISIAYTTTR-TWNFGQILCDIWVSSDITCCTASILHLCVIALDRYW
5HT1E  -TTLLNLAVIMAIGTTKKLHQPANYLICSLAVTDLLVAVLVMPLSIIYIVMD-RWKLGYFLCEVWLSVDMTCCTCSILHLCVIALDRYW
5HT2   --IAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFSTASIMHLCAISLDRYV

STB17  LILSPLRYKLRMTAPRALALILGAWSLAALASFLPLLLGWHELGKARTPAPGQCRLLASLPFVLVASGVTFFLPSGAICFTYCR-ILLA
5HT1A  AITDPIDYVNKRTPRRAAALISLTWLIGFLISIPPM-LGWRTPEDRSDPDACTISKDHG-YTIYSTFGA-FYIPLLLMLVLYGR-IFRA
5HT1B  AITDAVDYSAKRTPKRAAIMIVLVWVFSISISLPPFF--WRQAKAEEEVLDCFVNTDHVLYTVYSTVGA-FYLPTLLLIALYGR-IYVE
5HT1C  AIRNPIEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTT-CVLNDPN-FVLIGSFVAFFIPLTIMVITYFLTIYVL
5HT1D  AITDALEYSKRRTAGHAAAMIAAVWAISICISIPPLF--WRQATAHEEMSDCLVNTSQISYTIYSTCGA-FYIPSILLIILYGR-IYVA
5HT1E  AITNAIEYARKRTAKRAALMILTVWTISIFISMPPLF--WRSHRRLSPPPSQCTIQHDHVIYTIYSTLGAFYIPLTLILILYYRIYHAA
5HT2   AIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKVF-KEGSCLLADDN--FVLIGSFVAFFIPLTIMVITYFLTIKSL

STB17  ARKQAVQVASLTTGTAGQALETLQVPRTPRPGMESADSRRLATKHSRKALKASLTLGILLGMFFVTWLPFFVANIAQAVCD----CISP
5HT1A  ARFRIRKTVRKVEKKGAGTSLGT-(76)-LERKNERNAEAKRKMALARERKTVKTLGIIMGTFILCWLPFFIVALVLPFCES--SCHMP
5HT1B  ARSRILKQTPNRTGKRLTRAQLI-(33)-KVRVSDALLEKKKLMA-ARERKATKTLGIILGAFIVCWLPFFIISLVMPICKD--ACWFH
5HT1C  RRQTLMLLRGHTEEELANMSLNF-(23)-PRRKKKEKRPRGTMQAINNEKKASKVLGIVFFVFLIMWCPFFITNILSVLCGKACNQKLM
5HT1D  ARSRILNPPSLYGKRFTTAQLIT-(30)-VKIKLADSILERKRISAARERKATKTLGIILGAFIICWLPFFVVSLVLPICRD--SCWIH
5HT1E  KSLYQKRGSSRHLSNRSTDSQNS-(34)-PFDNDLDHPGERQQISSTRERKAARILGLILGAFILSWLPFFIKELIVGLSIY----TVS
5HT2   QKEATLCVSDLSTRAKLASFSFE-(14)-IHREPGSYAGRRTMQSISNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNENVI

STB17  -GLFDVLTWLGYCNSTMNPIIYPLFMRDFKRALGRFLHASTVPRSTGQPCLPLHVDLSQRCQTRPQLQQVLALPLPPNSDSDSASGGTS
5HT1A  ALLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKIIKCKFCRR  422
5HT1B  MAIFDFFNWLGYLNSLINPIIYTMSNEDFKQAFHKLIRFKCTG   386
5HT1C  EKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPDKKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIE
5HT1D  PALFDFFTWLGYLNSLINPVIYTVFNEDFRQAFQRVVHFRKAS   374
5HT1E  SEVADFLTWLGYVNSLINPLLYTSFNEDFKLAFKKLIRCREHT   365
5HT2   GALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQYKENRKPLQLILVNTIPALAYKSS-QLQVGQKKNSQEDAEQTVDDCSMV

STB17  GLQLTAQLLLPGEATRDPPPPTRATTVVNFFVTDSVEPEIRPHPLSSPVN 437
5HT1C  MQVENLELPVNPSNVVSERISSV   460
5HT2   TLGKQQSEENCTDNIETVNEKVSCV 449
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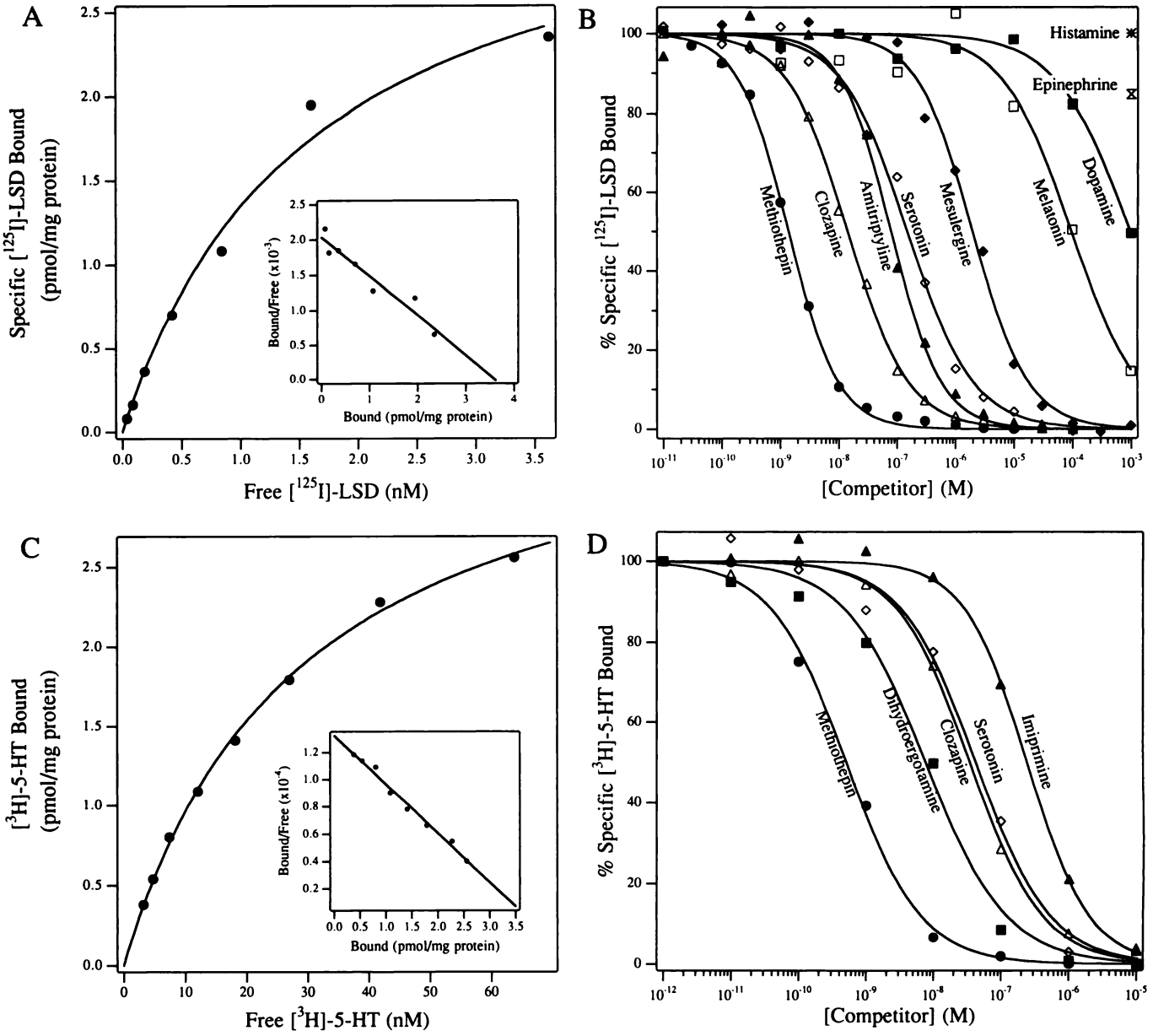


Fig. 2. Radioligand binding analysis of St-B17 receptor transiently expressed in COS-7 cells. Transient expression and radioligand binding assays were performed as described in Experimental Procedures. A, Saturation analysis of specific [^{125}I]LSD binding to membranes prepared from COS-7 cells transiently transfected with construct pSRα-B17. The experiment shown is representative of three independent experiments, each conducted in triplicate. *Inset*, Scatchard analysis of saturation binding data. In this experiment, the K_d and B_{max} values were 1.5 nM and 3.4 pmol/mg of protein, respectively. B, Pharmacological analysis of [^{125}I]LSD binding to COS-7 cell membranes expressing clone St-B17. Competition curves shown are representative of three independent experiments conducted in triplicate. Average K_i and standard error values are given in Table 1. C, Saturation analysis of [^{3}H]5-HT binding to membranes prepared from COS-7 cells transiently transfected with construct pSRα-B17. The experiment shown is representative of three experiments conducted in triplicate. *Inset*, Scatchard analysis of saturation binding data. In this experiment the K_d and B_{max} values were 27.8 nM and 3.7 pmol/mg of protein, respectively. D, Pharmacological analysis of [^{3}H]5-HT binding to COS-7 cell membranes expressing clone St-B17. Competition curves shown are representative of three independent experiments conducted in triplicate. Average K_i and standard error values are given in Table 1.

Fig. 1. Nucleotide and deduced amino acid sequences of cDNA clone St-B17. A, The nucleotide coding sequence is shown in *upper case letters*, noncoding sequence is shown in *lower case letters*, and deduced amino acid sequence is shown *below the nucleotide sequence*. The numbering of the nucleotide sequence begins with the first methionine of the open reading frame. Sequences of PCR primers described in the text are enclosed by *boxes*. *Solid inverted triangle*, position of the *Bam*HI restriction site used for construction of an intronless version of this clone. *Arrows*, putative intron/exon splice junctions. An asparagine residue representing a putative glycosylation site is *underlined*. In the interest of brevity, not all of the 3′ untranslated sequence is shown. B, Alignment of the deduced amino acid sequence (single-letter code) of clone St-B17 with the rat 5-HT$_{1A}$ (6), 5-HT$_{1B}$ (7), 5-HT$_{1C}$ (17), 5-HT$_{1D}$ (10), and 5-HT$_2$ (20) and human 5-HT$_{1E}$ (19) receptors. Conserved amino acid residues are *boxed*. Putative TM domains are indicated *above the sequences*.

TABLE 1

Pharmacology of St-B17 expressed in COS-7 cells

Increasing concentrations of the indicated compounds were used to inhibit the binding of either 0.5 nM [^{125}I]LSD or 5 nM [^{3}H]5-HT to membranes of COS-7 cells transiently transfected with clone St-B17. [^{125}I]LSD competition assays were performed as described in Fig. 2; those for [^{3}H]5-HT were performed as described previously (8). K_i values were obtained from graphically determined IC_{50} values by the method of Cheng and Prusoff (31) and are presented as the geometric mean ± standard error (three experiments), calculated according to the method of De Lean *et al.* (32). The following compounds were tested and found to exhibit K_i values of > 10 μM: zimelidine, zacopride, NAN-190, citalopram, BIMU-1, metoclopromide, fenfluramine, MDL-722222, 8-OH-DPAT, BRL-24924, BRL-43694A, GR-38032F, ICS-205,930, ketanserin, melatonin, spiroperidol, tyramine, DAU-6215, DAU-6285, dopamine, epinephrine, histamine, idazoxan, LY-278,584, mazindol, norepinephrine, octopamine, paroxetine, and pindolol.

Drug	K_i	
	Versus [^{125}I]LSD	Versus [^{3}H]5-HT
	nM	
Methiothepin	1.84 ± 0.2	0.39 ± 0.06
Lisuride	8.19 ± 0.5	5.3 ± 1
Clozapine	12.9 ± 2	20 ± 3
Dihydroergotamine	13.1 ± 0.9	5.4 ± 5
2-Br-LSD	17.1 ± 1	
Pergolide	29.9 ± 7	
Metergoline	30 ± 0.4	61 ± 23
Amoxipine	30.4 ± 2	
Lergotrile	36.5 ± 1	
5-Methoxytryptamine	38.9 ± 4	18 ± 2
Ritanserin	44.1 ± 4	16 ± 2
Mianserin	45.7 ± 9	38 ± 7
Clomiprimine	53.8 ± 3	
5-Hydroxy-*N*-ω-methyltryptamine	58 ± 8	
Loxapine	64.8 ± 0.6	56 ± 2
Amitriptyline	69.6 ± 7	82 ± 6
N,*N*-Dimethyl-5-methoxytryptamine	79.8 ± 4	
1-(1-Napthyl)piperazine	104 ± 14	
5-Benzyloxytryptamine	110 ± 18	
Cyproheptidine	134 ± 6	
Doxepin	136 ± 7	
Nortryptyline	148 ± 3	
5-HT	151 ± 13	56 ± 9
Dihydroergocryptine	161 ± 13	
Imipramine	209 ± 23	190 ± 3
N,ω-Methyltryptamine	342 ± 32	
Methysergide	372 ± 73	
Tryptamine	438 ± 15	
TFMPP[a]	482 ± 37	
CGS 12066B	738 ± 67	
5-Carboxamidotryptamine	774 ± 84	253 ± 20
PAPP	806 ± 75	
Mesulergine	1720 ± 250	
Fluoxetine	1770 ± 290	
mCPP	2300 ± 340	
2-MPP	3060 ± 320	

[a] TFMPP, 1-(3-(trifluoromethyl)phenyl)piperazine; PAPP, *p*-aminophenylethyl-*m*-trifluoromethylphenylpiperazine; mCPP, 1-(3-chlorophenyl)piperazine; 2-MPP, 1-(2-methoxyphenyl)piperazine.

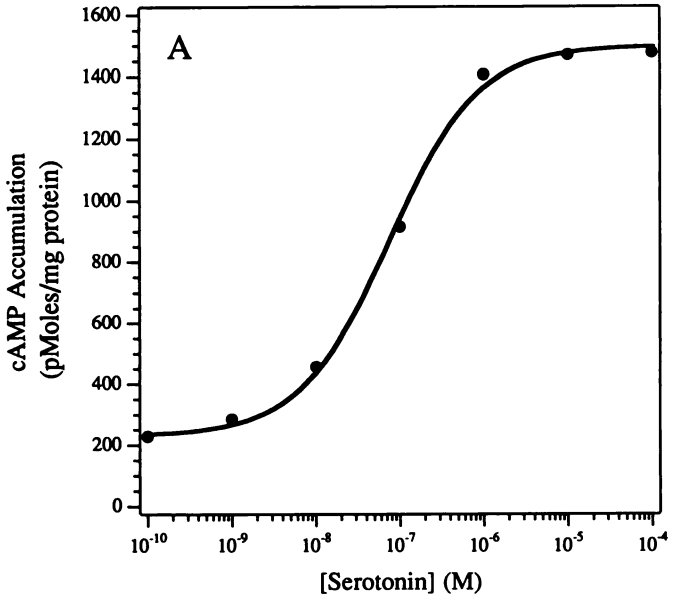
subtype. A number of drugs selective for 5-HT_3 and 5-HT_4 receptors (i.e, MDL-72222, ICS-205,930, and DAU-6285) exhibited virtually no affinity for St-B17, whereas agents selective for other 5-HT receptor subtypes, such as 8-OH-DPAT (5-HT_{1A}), CGS 12066B (5-HT_{1B}), mesulergine (5-HT_{1C}), and ketanserin (5-HT_2), bound with relatively low affinity. Ergot alkaloids, especially ergoline derivatives (i.e., LSD, lisuride, or pergolide), displayed relatively high affinity for St-B17, as did the nonselective serotonergic antagonist methiothepin. Interestingly, the atypical and typical antipsychotics clozapine and loxapine, respectively, also exhibited high affinity for St-B17, as did several tricyclic antidepressant drugs (i.e., amoxipine, clomiprimine, and amitriptyline), which all exhibited K_i values of <100 nM. In general, the drugs that exhibited the greatest affinity for St-B17 (i.e., K_i of <100 nM) are tricyclic, ergoline, or tryptamine derivatives.

Competition for [^{3}H]5-HT binding by a number of drugs revealed, with a few exceptions, the same rank order of potency as for inhibition of [^{125}I]LSD binding (Fig. 2D; Table 1). However, for some drugs the K_i values determined by competition with [^{3}H]5-HT were up to 5-fold lower than those determined by competition with [^{125}I]LSD, with the exception of clozapine, metergoline, and amitriptyline, which exhibited somewhat greater potency in competition with [^{125}I]LSD.

Although the pharmacological profile of St-B17 does not correspond to that of previously defined 5-HT receptor subtypes, it does resemble the profile described by Conner and Mansour (30) for 5-HT stimulation of adenylyl cyclase activity in the NCB-20 neuroblastoma cell line. In these cells, 5-HT activates adenylyl cyclase with an EC_{50} of ~300 nM and a variety of tryptamine derivatives exhibit EC_{50} values similar to their K_i values for inhibition of [^{125}I]LSD binding to St-B17. Similarly, for antagonism of 5-HT-stimulated adenylyl cyclase activity in NCB-20 cells a variety of compounds, including methiothepin, mianserin, amitriptyline, and cyproheptidine, also exhibit apparent K_i values and a rank order of potency similar to those observed for inhibition of [^{125}I]LSD binding to St-B17. In light of this observation, we examined the coupling of St-B17 to either stimulation or inhibition of adenylyl cyclase in transiently transfected COS-7 cells. Despite several attempts, however, we were unable to demonstrate any effects of 5-HT on adenylyl cyclase activity in the transiently transfected cells (data not shown).

Reasoning that the negative adenylyl cyclase data might be due to the low percentage of COS-7 cells actually transfected in these cultures, we stably transfected HEK-293 cells with the St-B17 receptor. HEK-293 cells were used because they do not endogenously express serotonin receptors. We selected one transfected HEK-293 cell line, expressing 800 fmol/mg of protein of St-B17 receptor, for cAMP analysis. As shown in Fig. 3A, serotonin caused a potent, dose-dependent increase in cAMP levels in transfected HEK-293 cells, with an average EC_{50} value of 145 ± 40 nM (three experiments), whereas there was no detectable response in nontransfected cells (data not shown). Pharmacological analysis of the cAMP response (Fig. 3B) indicated that the serotoninergic agonists 5-methoxytryptamine and 5-carboxamidotryptamine were also able to elicit an increase in cAMP levels. The ergot alkaloids lisuride and dihydroergocryptine also stimulated cAMP accumulation, although these drugs appeared to function as partial agonists at the St-B17 receptor. Amoxipine, methiothepin, and clozapine all appeared to act as antagonists of this receptor, because they had no significant effect on cAMP levels on their own but were able to substantially inhibit the response elicited by 5-HT. These data thus indicate that the St-B17 receptor is functionally linked to activation of the adenylyl cyclase signal transduction system.

The distribution of mRNA encoding St-B17 was evaluated by Northern blot analysis of poly(A)$^+$ RNA prepared from a variety of rat brain regions, as well as other peripheral tissues. A single transcript of ~4.2 kb was observed in various brain regions, with highest expression appearing to occur in the corpus striatum (Fig. 4). St-B17 mRNA was also observed in the amygdala, cerebral cortex, and olfactory tubercle, whereas



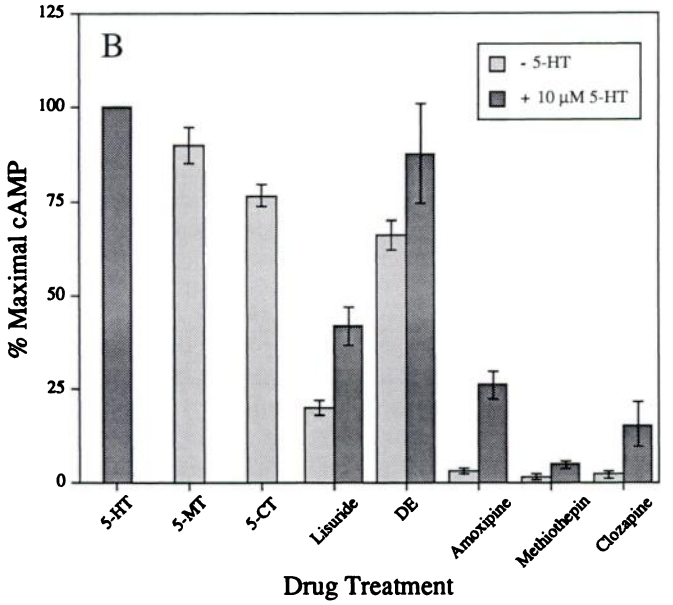


Fig. 3. Accumulation of intracellular cAMP in HEK-293 cells stably expressing the St-B17 receptor. Intact cells were incubated with various test compounds and intracellular cAMP levels were assayed as described in Experimental Procedures. A, Dose-response curve for stimulation of cAMP accumulation by serotonin in HEK-293 cells. Data are representative of three independent experiments, each conducted in triplicate. In the experiment shown, the basal cAMP level was 230 pmol/mg of protein. B, Pharmacological analysis of serotonin-stimulated cAMP accumulation in HEK-293 cells. Cells were incubated with the drugs with or without 5-HT. All drugs were assayed at 10 μM final concentration. Data are representative of three independent experiments, each conducted in triplicate. *5-MT*, 5-methoxytryptamine; *5-CT*, 5-carboxamidotryptamine; *DE*, dihydroergocryptine.

it was absent, or present in undetectable levels, in the cerebellum, hippocampus, hypothalamus, medulla, olfactory bulb, pituitary, retina, and thalamus. Similarly, no transcript was observed, even after prolonged exposures, in Northern blots of mRNA from rat heart, lung, kidney, liver, spleen, pancreas, smooth muscle, skeletal muscle, stomach, ovary, prostate, or testes (data not shown).

Taken together, our results have led us to conclude that we

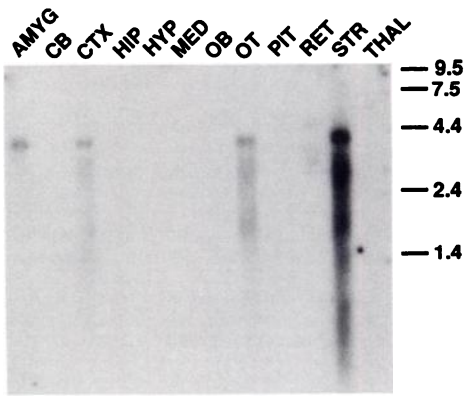


Fig. 4. Northern blot analysis of St-B17 RNA transcripts in rat brain. Northern blots were prepared and analyzed as described in Experimental Procedures. Each lane contained 3 μg of poly(A)$^+$ RNA. *AMYG*, amygdala; *CB*, cerebellum; *CTX*, cerebral cortex; *HIP*, hippocampus; *HYP*, hypothalamus; *MED*, medulla; *OB*, olfactory bulb; *OT*, olfactory tubercle; *PIT*, pituitary; *RET*, retina; *STR*, striatum; *THAL*, thalamus. Locations of RNA size markers (kb) are indicated.

have cloned a novel serotonin receptor that exhibits a distinct pharmacological profile not previously described. This unique pharmacology, together with the relatively low level of homology (<50%) of St-B17 to previously cloned 5-HT receptor subtypes, indicates that this receptor does not belong to any of the previously defined 5-HT$_1$ to 5-HT$_4$ subcategories of 5-HT receptors. In addition, St-B17 represents the first mammalian serotonin receptor to be cloned that is coupled to the activation of adenylyl cyclase. So-called 5-HT$_4$ receptors have been characterized in mammalian tissues as being linked to the stimulation of adenylyl cyclase, yet our pharmacological data clearly indicate that St-B17 is not a 5-HT$_4$ receptor. Thus, in view of its distinct structural and pharmacological characteristics and in light of the very recent cloning and description of a "5-HT$_5$" serotonin receptor (3), we suggest that St-B17 be designated a 5-HT$_6$ receptor.

The localization of 5-HT$_6$ receptor mRNA to limbic and cortical brain regions and the relatively potent interactions of several therapeutically important drugs, including the atypical antipsychotic clozapine, the typical antipsychotic loxapine, and the tricyclic antidepressants amoxipine, clomiprimine, amitriptyline, and nortriptyline, with this receptor suggest that it may play an important, although hitherto unappreciated, role in several neuropsychiatric disorders that involve serotonergic systems. The molecular cloning of this novel 5-HT$_6$ receptor will likely result in the development of drugs with specificity for this site, allowing further investigations of its role *in vivo* and possibly improvement of current therapies for several neuropsychiatric disorders.

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