

Cloning and Functional Expression of an *Aplysia* 5-HT Receptor Negatively Coupled to Adenylate Cyclase

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Serotonin (5-HT) is involved in the control of various behaviors in *Aplysia californica*, including reproduction, feeding, locomotion, circadian rhythm, synaptic plasticity, and synaptic growth. The large variety of functions of 5-HT is mediated by different receptor subtypes that are coupled to different second-messenger systems. Here, we report the cloning of a cDNA coding for an *Aplysia* G-protein-coupled 5-HT receptor (5-HT_{ap1}). Its deduced amino acid sequence resembles those of the 5-HT₁ receptor subfamily. When expressed in stable cell lines, 5-HT_{ap1} exhibits high-affinity binding for the serotonergic radioligand [N-methyl-³H]lysergic acid diethylamide. This binding is competed by several 5-HT agonists and antagonists, and the pharmacological profile of inhibition has some similarities with those of 5-HT₁ and 5-HT₇ receptors. Application of 5-HT or its agonists 5-carboxamidotryptamine maleate and (±)-8-

hydroxy-2-(di-*n*-propyl-amino) tetralin hydrobromide on cells transformed with 5-HT_{ap1} produced a dose-dependent inhibition of forskolin-stimulated cAMP accumulation. 5-HT_{ap1} is thus negatively coupled to adenylate cyclase. The production of antiserum against the 5-HT_{ap1} receptor allowed us to examine its expression in animal tissues. The receptor protein is detected in every tissue examined, although it seems only weakly expressed in some samples. The receptor is also found in every ganglia of the nervous system, both in the sheath and in the neurons. 5-HT_{ap1} mRNA is absent from the sheath, indicating that the protein observed there is probably located on the nerve terminals.

Key words: *Aplysia*; 5-HT; 5-HT₁ receptor; G-protein-coupled receptor; adenylate cyclase; inhibition

Serotonin (5-HT) has a large variety of functions both in vertebrates and invertebrates. It plays a role in feeding, circadian rhythm, locomotion, reproduction, and modulation of defensive behavior (Hen, 1993; Weiger, 1997). In mollusks, pharmacological and electrophysiological studies showed that 5-HT is a neuromodulatory neurotransmitter that acts on at least six different receptor subtypes to activate different postsynaptic responses (Gerschenfeld and Paupardin-Trich, 1974; Kadan and Hartig, 1988). In mammals, molecular cloning has shown the existence of at least 14 5-HT receptor subtypes (Hoyer et al., 1994). These receptors, which are members of the G-protein-coupled receptor family, can be classified on the basis of sequence identity and on the nature of the second messenger systems with which they are coupled. The 5-HT_{1,5} and 5-HT_{4,6,7} subtypes inhibit or activate adenylate cyclase, respectively, whereas the 5-HT₂ subtype stimulates phospholipase C. By contrast, the 5-HT₃ subtype is a ligand-gated ion channel (Peroutka, 1995).

In the sensory neurons involved in defensive behavior, 5-HT increases the level of cAMP and activates protein kinase A (Bacscai et al., 1993), protein kinase C (Sugita et al., 1992; Byrne

and Kandel, 1996), and MAP kinase (Martin et al., 1997). These actions of 5-HT are expected to be mediated by different 5-HT receptors (Braha et al., 1990; Mercer et al., 1991; Ghirardi et al., 1992; Emptage and Carew, 1993). In the neuroendocrine bag cell clusters, which are involved in the regulation of the egg-laying behavior, the application of 5-HT inhibits the bag cell afterdischarge that is itself dependent on the stimulation of adenylate cyclase (Jennings et al., 1981). 5-HT has also been reported to inhibit inhibitory interneurons of the tail withdrawal reflex pathway (Xu et al., 1995). Altogether, these results strongly suggest that the major mammalian 5-HT receptor types all have functional homologs in *Aplysia*. Recently, genes encoding two homologous 5-HT receptors have been cloned in *Aplysia*. Both receptors stimulate phospholipase C in response to 5-HT in a dose-dependent manner. However, they could not be readily grouped within any of the mammalian subgroups based on amino acid homologies (Li et al., 1995).

We have cloned and characterized cDNAs coding for an *Aplysia* 5-HT receptor with significant sequence and functional homologies to the members of the vertebrate 5-HT₁ receptor subfamily. Expression of 5-HT_{ap1} in mammalian cells shows that the receptor is able to specifically bind 5-HT at nanomolar concentrations, as well as diverse serotonergic ligands. In addition, 5-HT and 5-HT agonists induce inhibition of adenylate cyclase in cells stably expressing 5-HT_{ap1}. Using RT-PCR and Western blot analysis, we detected expression of this receptor in the CNS and in peripheral tissues.

MATERIALS AND METHODS

Compounds. [2,8-³H]Adenine and [N-methyl-³H]lysergic acid diethylamide ([³H]LSD) were purchased from DuPont NEN (Mississauga, Ontario, Canada). Forskolin, ATP, cAMP, 5-HT, and isobutylmethylxan-

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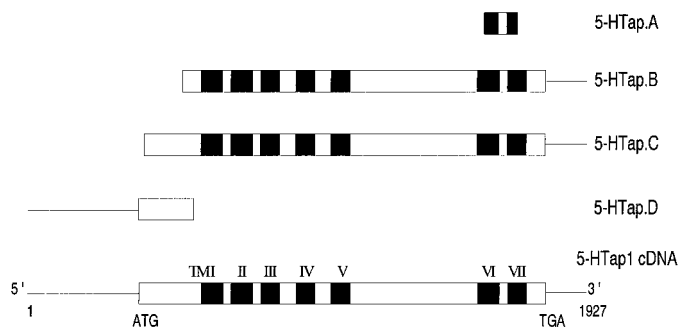


Figure 1. Molecular cloning of the 5-HT_{ap1} receptor cDNA. The 5-HT_{ap.A} fragment was PCR-amplified from DNA isolated from CNS and kidney cDNA libraries, using degenerate primers. The 5-HT_{ap.B} and 5-HT_{ap.C} cDNAs were isolated from a kidney cDNA library using a PCR-screening strategy (Israel, 1993). The 5-HT_{ap.D} was PCR-amplified from DNA isolated from the kidney cDNA library using nested antisense 5-HT_{ap1}-specific primers and a sense (GT10-specific) primer. The reconstituted full-length cDNA is schematized at the bottom. The open reading frame is represented by boxes, and the putative transmembrane domains are indicated by the dark regions. The lines represent the 5' and 3' untranslated regions of the transcript.

thine (IBMX) were from Sigma-Aldrich (Oakville, Ontario, Canada). 5-Carboxamidotryptamine maleate (5-CT) and methiothepin mesylate were from Research Biochemicals (Natick, MA). Alprenolol hydrochloride, clozapine, dopamine hydrochloride, (\pm)-8-hydroxy-2-(di-*n*-propylamino) tetralin hydrobromide (8-OH-DPAT), ketanserin tartrate, mesulergine hydrochloride, metergoline, methysergide maleate, NAN-190 hydrobromide, *p*-aminophenethyl-*m*-trifluoromethylphenyl piperazine, *R*-(+)-SCH-23390 hydrochloride, spiperone hydrochloride, and yohimbine hydrochloride were generous gifts from BioSignal Inc. (Montréal, Québec, Canada).

PCR amplification and screening of cDNA libraries. Phage DNAs (100 ng) isolated from *Aplysia* kidney and CNS cDNA libraries constructed in the λ GT10 vector were PCR-amplified for 40 cycles (94°C for 1.5 min, 40°C for 2 min, and 72°C for 1 min) in the presence of two primers corresponding to highly conserved 5-HT receptor sequences located in transmembrane domains 6 and 7: 5'-(G,C)IGCITT(T,C)ITHTITG(C,T)TGG(C,T)TGG(C,T)TICCITT(C,T)TT-3' and 5'-TCIGGII(A,T)(G,A)AAIATIG(T,C)(G,A)TA(G,A)ATIA(T,C)IGG(A,C)TT-3'. The PCR products were fractionated on a 4% agarose gel, and the 163 bp fragment (5-HT_{ap.A}), present in both the CNS and kidney samples, was subcloned and sequenced (Fig. 1).

To clone the full-length cDNA, we used the PCR screening method previously described by Israel (1993). Briefly, an overnight culture of *Escherichia coli* strain LE392 was infected with 4×10^6 phages from an *Aplysia* kidney cDNA library. After an incubation at 37°C for 20 min, the culture was diluted to 20 ml and used to fill a 64-well plate with 20,000 phage per well. The plate was incubated at 37°C until the phage titer reached $\sim 1 \times 10^9$ pfu/ml. Phage from eight wells across a row or eight wells down a column were pooled (25 μ l/well) and diluted 1:1 with distilled water. The matrix of 64 wells was therefore reduced to 16 pools, which were used as templates for PCR analysis. PCR reactions were performed in a final volume of 25 μ l using 0.5 μ l of pooled phage culture as template and 200 ng of the degenerate primers described above. The reaction was held 10 min at 94°C to help phage denaturation before PCR amplification. PCR reaction products were electrophoresed through a 3% agarose gel, transferred to a Hybond N+ membrane (Amersham, Oakville, Ontario, Canada), and hybridized with the 5-HT_{ap.A} fragment at high stringency. Phage DNAs in wells located at the junction of positive rows and columns were individually PCR-amplified under the same conditions. Phages in single positive wells were plated and two clones, 5-HT_{ap.B} and 5-HT_{ap.C} (Fig. 1), were isolated using the plaque-lifting method (Sambrook et al., 1989). Their inserts were subcloned and sequenced. To clone the missing 5' end of the transcript, we PCR-amplified the isolated phage DNA from the kidney cDNA library, using a λ GT10-specific primer, 5'-AGCAAGTTCAGCCTGGTTAGTC-3', and two nested 5-HT_{ap1}-specific primers, 5'-GATGAGACTCAGAGGATGAC-3' and 5'-ATACAGCAACAGTTCAGG-3'. The product of the second PCR amplification (5-HT_{ap.D}) was subcloned in pCR 2.1 (Invitrogen, San Diego, CA) and sequenced (Fig. 1).

Cell lines. The coding region of 5-HT_{ap1} was PCR-amplified with the primer pairs 5'-CAGCGAATTCAGAGGATGGGAAGAAACG-3' and 5'-CCGCGAATTCCTACTACGATTCGGTTCAC-3' [nucleotides (nt) 320–1777], digested with *EcoRI*, and subcloned into the pBact-myc vector (Cravchik and Matus, 1993), in frame with the c-myc epitope EOKLISEEDLN (Degols et al., 1991). The pBact-myc/5-HT_{ap1} construct was then partially digested with *HindIII* to remove the full-length c-myc/5-HT_{ap1} fragment, and the resulting fragment was subcloned in pCDNA3/RSV (Jockers et al., 1996). The recombinant plasmid was introduced into HEK 293 cells by calcium phosphate-mediated transfection, and the transfected cells were selected in Geneticin (Life Technologies, Burlington, Ontario, Canada), to establish permanent cell lines. Isolated foci were amplified, and expression of the receptor gene was confirmed by indirect surface immunofluorescence using a monoclonal c-myc-specific antibody (a kind gift of M. Bouvier, Université de Montréal, Montréal, Québec, Canada) and fluorescein-conjugated rabbit anti-mouse Igs (Dako, Mississauga, Ontario, Canada) as the secondary antibody. Cell lines expressing the highest levels of the receptor protein at the cell surface were used in the functional assays.

Ligand binding analysis. HEK cells expressing 5-HT_{ap1} were grown to 90–100% confluence, and membranes were prepared as described by Kohen et al. (1996). Membrane pellets were resuspended in (in mM): 75 Tris, pH 7.4, 5 MgCl₂, and 2 EDTA buffer at a concentration of ~ 250 μ g protein/ml. For saturation experiments, 10 μ g of membrane proteins were incubated in duplicate with increasing concentrations of tritiated lysergic acid diethylamide ([³H]LSD, 71.5 Ci/mmol; 1 Ci = 37 GBq) for 60 min at room temperature in a total volume of 200 μ l. Competition binding assays were done in duplicate with 10 μ g membrane proteins, in the presence of increasing concentrations of the competing agent (10^{-12} – 10^{-4} M) and 1.5 nM [³H]LSD for 60 min at room temperature. Preliminary assays had shown that saturation was reached within 30 min and remained stable for at least 2 hr at room temperature (data not shown). All assays were terminated by rapid filtration over Whatman GF/C glass fiber filters (Xyotech Biosystems, Mt. Royal, Québec, Canada) and rinsed three times with 50 mM Tris, pH 7.4. Nonspecific binding was defined with 10 μ M methiothepin. The amount of bound [³H]LSD was determined by scintillation spectrophotometry (Wallac 1409 liquid scintillation counter).

Adenylate cyclase activity. The cAMP content of cells stably expressing 5-HT_{ap1} was measured by the prelabeling technique as described by Ansanay et al. (1992). Cells were cultured in 12-well plates. When apparent confluence was reached, cells were incubated with 2 μ Ci/ml [³H]adenine. After 2–3 hr, the cultures were washed and incubated with 2.5 mM IBMX, 2.5 μ M forskolin, and the indicated drugs in a final volume of 1 ml PBS for 20 min at 37°C. The reaction was stopped by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid. Cells were scraped with a rubber policeman, and 100 μ l of a solution of 5 mM ATP and 5 mM cAMP were added to the mixture. Cellular proteins were centrifuged at 5000 \times g. [³H]ATP and [³H]cAMP were separated by sequential chromatography on Dowex and alumina columns (Bio-Rad, Mississauga, Ontario, Canada) (Salomon et al., 1974). cAMP formation corresponded to the conversion: [³H]cAMP/(³H]ATP + [³H]cAMP) \times 1000. Results are expressed as a percentage of the maximal cAMP accumulation.

Antibody production and immunoblotting. The 5-HT_{ap1} sequence corresponding to the third cytoplasmic loop was PCR-amplified with the primer pair 5'-ATCAGAGCTCAGATATATCGCGACGTCGG-3' and 5'-AACAAAGCTTGCCAGACTTTCCTTTCTCGC-3' (nt 1068–1523), digested with *SacI* and *HindIII*, and subcloned into the pQE30 prokaryote expression vector (Qiagen, Mississauga, Ontario, Canada), thus fusing a 6XHis tag to its N-terminal extremity. This domain of the protein was chosen because of the very low conservation of the primary structure of this region among different G-protein-coupled receptors. The fusion protein was purified on a nickel-nitrilo-tri-acetic acid resin column and used to inoculate rabbits for antibody production as described previously (Aloyz and DesGroseillers, 1995). For Western blotting, plasma membranes from freshly dissected tissues were prepared on a sucrose cushion as described in Bawab et al. (1992), electrophoresed on a 10% SDS polyacrylamide gel, and electroblotted onto nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat dry milk for 1 hr at room temperature and then incubated with the primary antibody for 1 hr. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Dako) and immunoreactive bands were detected by a chemiluminescent substrate reaction (Pierce, Rockford, IL).

RT-PCR. Reverse transcription experiments were performed on total RNA extracted from the desheathed ganglia of the nervous system and from the sheath itself. RNA was purified using Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA was reverse transcribed using an oligo-dT₁₆ primer and Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Foster City, CA) as described previously (Angers and DesGroseillers, 1998). A DNA fragment encompassing nt 1068-1523 was amplified using the same primers and conditions as described above. The presence of contaminating genomic DNA was monitored by amplification of the same samples without reverse transcription. The PCR products were fractionated on a 2% agarose gel and transferred to a Hybond N+ membrane (Amersham) for hybridization with a specific oligonucleotide probe. As a positive control, we used actin primers as described by DesGroseillers et al. (1994).

RESULTS

Isolation and structure of the gene encoding 5-HT_{ap1}

The striking sequence conservation of transmembrane domains six and seven of G-protein-coupled receptors was used in a PCR approach to isolate a DNA fragment encoding the corresponding region of a putative 5-HT receptor in *Aplysia*. Using degenerate oligonucleotide primers, we amplified a 163 bp fragment that shared 56% amino acid sequence identity with mammalian 5-HT_{1D} receptors. These primers and the PCR-amplified fragment were then used to isolate two clones from a kidney cDNA library, using a PCR-based screening strategy (Fig. 1). The missing 5' end of the transcript was PCR-amplified with DNA isolated from the cDNA library, using nested oligonucleotides derived from the cDNA and a paired primer derived from the λGT10 vector sequence.

The reconstituted cDNA is 1927 nt long. Its longest open reading frame of 1476 nt codes for a putative protein of 492 amino acids (Fig. 2). Hydrophobicity analysis of the deduced amino acid sequence revealed the presence of seven stretches of hydrophobic residues that represent the seven transmembrane domains characteristic to all G-protein-coupled receptors (data not shown). Amino acid sequence identity between 5-HT_{ap1} and other 5-HT receptors within the transmembrane domains and adjacent regions is 62.4% to *Lymnaea* 5-HT_{lym1}; 54.7% to *Drosophila* 5-HT_{dro2A-B}; 51.8% to human 5-HT_{1A}; 49.6% to human 5-HT_{1D}; 49.3% to human 5-HT_{1F}; 47.8% to human 5-HT_{1E}; 47.1% to mouse 5-HT_{1B}; 42.7% to mouse 5-HT_{5A-B}; 41.7% to mouse 5-HT₇; 41.2% to 5-HT_{dro1}; 35.6% to rat 5-HT_{2C}; 35.5% to *Lymnaea* 5-HT_{lym2}; 35.2% to rat 5-HT₄; 30.6% to *Aplysia* 5-HT_{apB1-2}; and 29.6% to rat 5-HT₆. A dendrogram analysis of amino acid sequence comparisons within the transmembrane domains indicates that 5-HT_{ap1}, along with other invertebrate 5-HT receptors, is associated with the mammalian 5-HT₁ receptor family (Fig. 3).

Six consensus sites for N-linked glycosylation are present in the extracellular N terminus of 5-HT_{ap1} (Fig. 2). Within the third cytoplasmic loop, seven serine or threonine residues may be used as a substrate for phosphorylation by protein kinase C. Another potential phosphorylation site for protein kinase C is found within the C-terminal intracellular domain. A cysteine residue is also found in this domain, suggesting that the receptor may be palmitoylated. The structure of the receptor, with its large third cytoplasmic loop and its short C-terminal tail, is similar to that of the vertebrate 5-HT₁ receptor family, which groups receptors coupled to G_i (Boess and Martin, 1994).

A Southern blot analysis of genomic DNA (Fig. 4) reveals the presence of a single band when the restriction enzyme used to digest the DNA has no restriction site in the region covered by the

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5' - AAAAAAGAAAATCTGCCTAAATCATCTGTCCAGATCTGTACCTTTATCCTCGAT 55
TTACAAGAACAAATACGACTCCTACAGTGGTGGACGAAATGTCGACTCGAGGCCTCGC 115
TGGAGGCACACAGGTTCCGGCCCGAGCTTAGTGTCCAGGATCTGCTCAGTTTGACAACA 175
ACGCACACGAGAGTAACTCGTCAACCCCTGTCAACAGAGTCAATAGGTCCGTGGTCAACAAA 235
GATTGACCCACACGACTAGATTGAGATAGCCCCCGCTTTTGTTCACAGCAGCGGGG 295
1
M S W H N I S V Q R M G R N A S L L E A
ATGTCGTGGCATAACAATTCGGTACAGAGGATGGGAAGAAACGCTTCGCTATTGGAAGCC 355
21
N M Y A M F P G Y N I T T G W P P R D N
AATATGTACGCCATGTTTCCCGGTACAACATCACGACGGTTCGCCCTCCAGAGATAAC 415
41
N S N N S G Y F D Q V T V N G S D R P E
AATTCACAAATTCGGGTACTTCGATCAAGTAAACCCTCAATGGCAGTACCGCCCTGAA 475
61
L L R Q Y N Y H E P K Y E E P V M I V I
CTGTGGCAGCAGTATAACTACCATGAACCTAAATGAAGAGCCTGTGATGATTGTTCATA 535
81
C I F L G C M I L A I I L G N I F V I T
TGATCTTTCTAGGATGCATGATCTTAGCCATATTTCTCGGTAATATCTTTGTAATAACT 595
101
A I L V E K S L Q G V S N Y L I L S L A
GCCATCTGGTGGAGAAATCTCTCCAGGTTGTGCCAACCTACCTCATCTGTCTCTGGCA 655
121
V T D L L V A V L V M P L S L I Y E I S
GTGACTGATCCTCTGTGGCTGTCTGTCTGTCATGCCCTCTGAGTCAATCTACAGAGATCAGC 715
141
I H W F L G N A V C D M W V S M D V L C
ATCCATTTGGTTTCTAGGCAACGCCCTCTCGATATGTGGTGTCTATGGATGTTCTCTGCG 775
161
C T A S I L H L V A I A F D R Y W A V S
TGTACAGCTTCCATATACATCTCGTGGCTATAGCGTTTGTATCGATACTGGCAGTCTCA 835
181
N I D Y V R S R N A R Q I L L M V A I V
AACATCGACTACGTTCCGAGTCGGAATGCAAGGCAGATCTCTCTGATGGTGGCCATCGTG 895
201
W T V S V F I S I S P L F G W R H D S D
TGGACCGTGTCCAGTGTATATATCGATATCGCCTTTGTTCGGTTCGGAGCATGTCGAGC 955
221
D P E L T G Q C L I S Q D H G Y T V F S
GACCCGAGCTGACAGGACAAATGTCTGATCTCCGAGGATCACGGGTACCCGTTCTCCG 1015
241
T V G R F Y C P L L L M L V I N F K I Y
ACGGTAGCCCGTCTACTGCCCACGTCTGTGATGCTGTGATCAACTTAAAGATATAT 1075
261
R A A R Y R I R K K R F G G R G G K H Q
CGCCGAGCTCGTACAGAATACGAAAGAAACGCTTCGGTGGACGGGAGGAGAAACATCAA 1135
281
A L H V P L P A V T V D T S H R Q I L A
GCGTTACACGTTCCGTTACCGGCTGTAAACGGTCGACACGTCACATCGACAAATACTCGCA 1195
301
T L E G D V S Q D G I S M Y M P S C T N
ACTCTCGAAGGTGAGCTAAGCCAGGACGCGCATCAGCATGTATATGCCCTCTCGACAGAAC 1255
321
A M T S T R V E H G E T A R C P G E W S
GCTATGACGTCACACCGGGTGGAAACATGGAGAGACCCGCTAGATGTCAGGAGAAATGGTCA 1315
341
T R R P A G S K P D S H V V Q H T D R P
ACGGTCTCGTCCAGCAGGATCCAAACCTGACTCGCATGTTGTCCAACACTGACCGTCCC 1375
361
S M H L L A S P Y P V Q E P R S R F Q Q
AGCATGACCTGTAGCGTCCCCCTACCCCGTTCAGGAACTCGTTCGCCCTTCCAACA 1435
381
Q R H Q K T E T H R Q R V V F R R E K M
CAACGTCACAAAAGACTGAGACGACAGACAAAGAGTCTGCTTCCGGCAGAGAAAATG 1495
401
E M A R E R K V W L R V L G I I T G A F
GAGATGGCCGAGAAAGAAAGTCTGGCTCAGAGTGTAGGAATATTATCCGGAGCGTTT 1555
421
V V C W L P F F V V A V V K P M C G T P
GTGTGTGTGGCTGCCCTTCTTCGTGGTGGCAGTCTGTGAAACCGATGTGCGGCACACCCG 1615
441
C D M P S Y V Y S L F L W L G Y V N S L
TGTGACATGCCTAGCTACGTGTACAGTCTCTTCTGTGGCTCGGCTACGTTAATAGCCTC 1675
461
I N P I I Y T I F N P S F R C A F N K I
ATCAATCCCATCATTTACACCATCTCAACCCAGGCTTCGATGTGGTTCATTAAGATT 1735
481
F L R R I K S V N R I T stop
TTCCTACGTCGGATCAAGTCAGTGAACCGAATACCTAGTACATGTCATGTCATGTTGTGCG 1795
TGTATTCGGCATGCAAAATATTATTCATGTCATCTTCAATCTAGTATTGCTACCATGT 1855
ACTTCCCATGACCAACAGGCATTTGTAGTCAACAAATCGAGCATATTTGTGTTACAAA 1915
AAAAAAAAAAAA -3' 1927

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Figure 2. Nucleotide and deduced amino acid sequence of the 5-HT_{ap1} cDNA. Putative transmembrane regions are boxed and numbered Tm I-VII. Arrows indicate the position of consensus sites for N-linked glycosylation. Serines and threonines that are within a consensus sequence for phosphorylation by protein kinase C are indicated by circles (●). A potential palmitoylation site is indicated by a triangle (▲). The nucleotide sequence is available in the GenBank database under accession number AF041039.

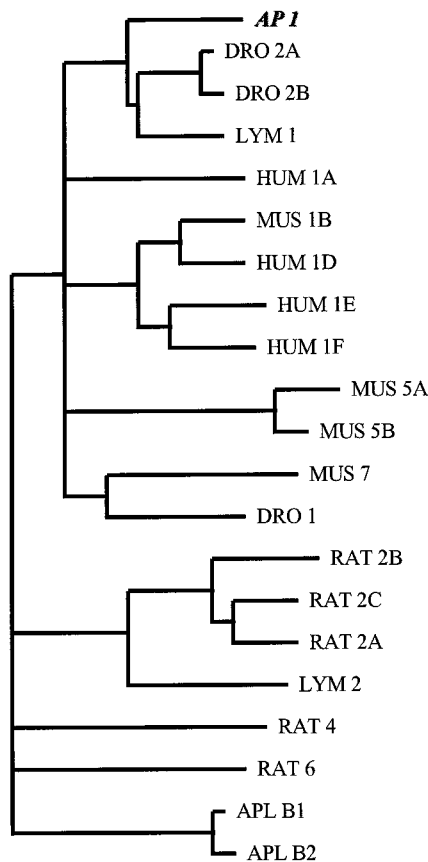


Figure 3. Phylogenetic analysis of 5-HT receptors. Amino acid sequences of 5-HT receptors were retrieved from the GenBank database. Their amino acid sequence, excluding their N termini and their third cytoplasmic loops, were aligned with the corresponding amino acid sequence of 5-HT_{ap1}, using the ClustalW package (Thompson et al., 1994). The alignment was then used for phylogenetic comparisons using the PHYLIP package (J. Felsenstein, 1993, PHYLIP 3.5.1c; University of Washington, Seattle, WA). Analysis was performed with a bootstrap procedure that computes the probability of occurrence of the branches for 100 possible trees. Branching order was determined using the Fitch-Margoliash algorithm included in the PHYLIP package. Only branches occurring in >80 trees are represented. *HUM*, human; *MUS*, mouse; *RAT*, rat.

probe and two bands when the restriction site is present in the cDNA probe sequence. This indicates that the gene is probably intronless. This conclusion was confirmed by partial sequencing of a genomic clone and PCR amplification of genomic DNA (data not shown). The Southern blot analysis also indicates that 5-HT_{ap1} is unique in the genome and does not have a close homolog. This is in contrast to the *Aplysia* 5-HT_{apB1} and 5-HT_{apB2} receptors, which share 86% identity at the nucleotide level (Li et al., 1995).

Pharmacology

The coding region of 5-HT_{ap1} was cloned in an expression vector in fusion with a c-myc epitope at its N terminus. The vector was transiently expressed in COS-7 and HEK 293 cells, and the expression of the receptor at the plasma membrane was monitored with the anti-myc antibodies (data not shown). The receptor with its myc tag was then used to transfect mammalian HEK 293 cells to establish permanent cell lines. Membranes isolated from one of these cell lines bound [³H]LSD in a saturable and dose-dependent manner with an estimated K_d of 0.56 (0.03 nM)

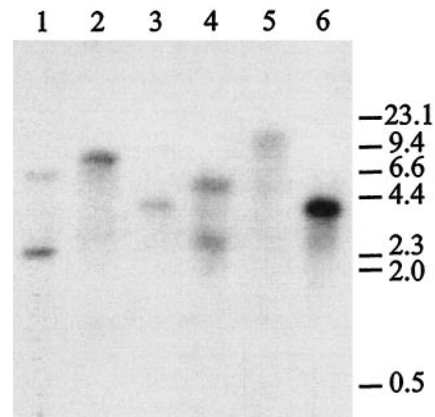


Figure 4. Southern blot analysis of the 5-HT_{ap1} gene. Genomic DNA was isolated from ovotestis, purified, and digested with *Bam*HI (lane 1), *Bgl*II (lane 2), *Eco*RI (lane 3), *Hind*III (lane 4), *Sac*I (lane 5), or *Xba*I (lane 6), as described previously (Wickham and DesGroseillers, 1991). Digested DNA was run on a 1% agarose gel, transferred to a Hybond N+ membrane, and hybridized with a ³²P-labeled 5-HT_{ap1} cDNA fragment (nt 457–1927). *Bgl*II, *Eco*RI, *Sac*I, and *Xba*I sites are not present in the probe sequence, whereas *Bam*HI and *Hind*III appear once. The molecular weight marker is λ phage DNA digested with *Hind*III. The molecular weights are indicated in kilobase pairs.

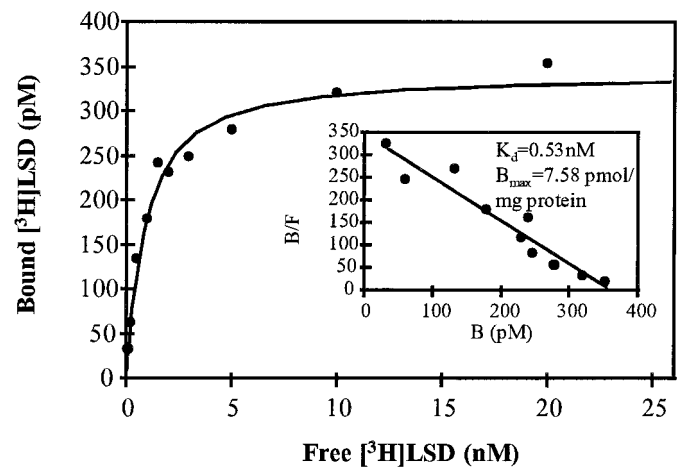


Figure 5. Saturation analysis of [³H]LSD binding on the 5-HT_{ap1} receptor. Membranes harvested from stable cell lines expressing 5-HT_{ap1} were incubated with increasing concentrations of [³H]LSD (0.1–20 nM) for 1 hr at room temperature. Nonspecific binding was defined in the presence of 10 μ M unlabeled methiothepin. Results are those of a single experiment, but similar results were obtained in six different binding experiments. The K_d and B_{max} values were determined with the AllFit for Windows 2.1 computer program (C. De Léan and A. De Léan, 1993, Université de Montréal, Montréal, Québec, Canada). *Inset*, Scatchard plot of the same data.

and a receptor density of 6.89 (0.61 pmol/mg of protein; $n = 3$) (Fig. 5). Similar results were obtained from two independent cell clones and from transiently transfected COS-7 cells, for a mean K_d of 0.51 (0.13 nM; $n = 6$). Of course, receptor density varied in each transfection experiment and in each clone tested.

The rank order of potency of various serotonergic agonists and antagonists for the inhibition of [³H]LSD binding to HEK membranes is listed in (Table 1) and illustrated in Figure 6. Briefly, 5-HT_{ap1} showed high-affinity binding to 5-HT, which is comparable to the affinity of 5-HT for the mammalian 5-HT_{1A,B,D-F} and 5-HT₇ receptors (Hoyer et al., 1994). Compounds that show high

Table 1. Affinities of various compounds that compete with the binding of 1.5 nM [³H]LSD to the membranes of HEK 293 cells stably transfected with the 5-HT_{ap1} gene

Drug	K _i (nM)
5-CT	1.04
Methiothepin	2.62
PAPP	9.50
5-HT	13.23
Methysergide	15.01
Clozapine	56.63
8-OH-DPAT	73.96
Metergoline	90.66
Yohimbine	222.37
Mesulergine	245.52
Ketanserin	288.18
SCH 23390	616.14
NAN 190	>1000
Dopamine	>1000
Spiperone	>1000
Alprenolol	>1000

Affinity estimates are given as K_i values in nanomolar concentrations and were determined by a computer-assisted nonlinear curve analysis (De Léan and De Léan, 1993). K_i values are expressed as the mean of at least three different determinations.

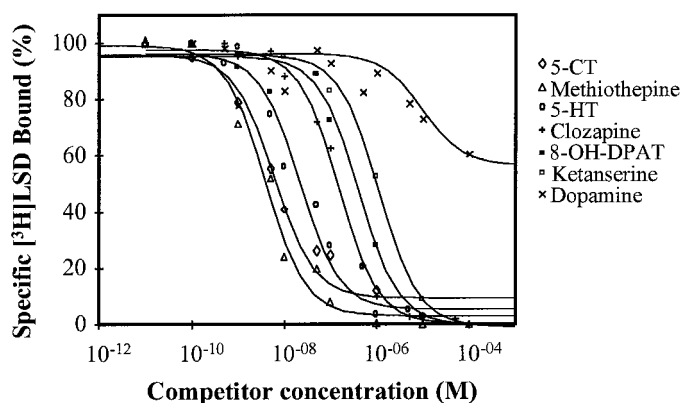


Figure 6. Inhibition of specific [³H]LSD binding to the *Aplysia* 5-HT_{ap1} receptor. Membranes from stable cell lines expressing 5-HT_{ap1} were incubated with 1.5 nM [³H]LSD in the presence or absence of increasing concentrations of unlabeled competitors. Nonspecific binding was defined in the presence of 10 μM unlabeled methiothepin. Results at each concentration are presented as a percentage of the specific binding in the absence of the competitor. Results are from a single experiment but are representative of three such experiments. Data were analyzed by a computer-assisted nonlinear analysis (C. De Léan and A. De Léan, 1993, AllFit for Windows 2.1; Université de Montréal).

binding affinity to the mammalian 5-HT₁ receptors, such as 5-CT, methiothepin, PAPP, and methysergide, also bound 5-HT_{ap1} in the nanomolar range. By contrast, metergoline, clozapine, mesulergine, and ketanserin, compounds that bind to 5-HT₂ receptors with high affinity, showed a lower affinity for 5-HT_{ap1}. NAN-190, a very specific 5-HT_{1A} antagonist, showed very poor affinity for 5-HT_{ap1}. Interestingly, 8-OH-DPAT, a 5-HT_{1A}-specific agonist, bound 5-HT_{ap1} with a better affinity than most other 5-HT receptors, except for 5-HT_{1A} itself. Very high concentrations of dopamine, spiperone, or alprenolol were necessary to displace [³H]LSD binding, confirming the serotonergic nature of 5-HT_{ap1}. Overall, the pharmacological profile of 5-HT_{ap1} seems related to

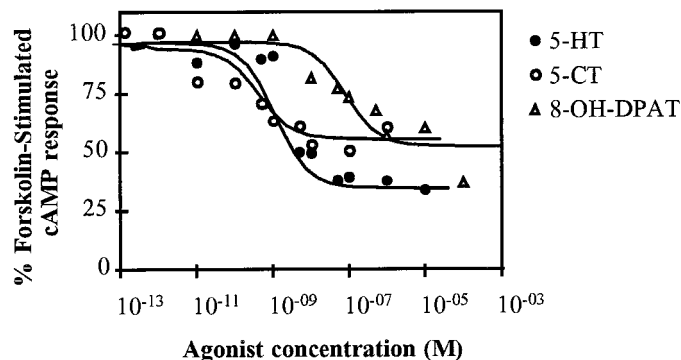


Figure 7. 5-HT- and agonist-induced decrease in cAMP levels in cell lines expressing the 5-HT_{ap1} receptor. cAMP levels are expressed as a percentage of the value obtained with 2.5 μM forskolin (100%) in the absence of 5-HT or agonists. The values are the mean of an experiment done in triplicate and are representative of three such experiments. Data were analyzed by a computer-assisted nonlinear analysis (C. De Léan and A. De Léan, 1993, AllFit for Windows 2.1; Université de Montréal).

the 5-HT₁ and 5-HT₇ receptor profiles, although not clearly associated with either one.

Functional coupling

The molecular structure and pharmacological profile of 5-HT_{ap1} suggested that this receptor is coupled to G_i and therefore could inhibit adenylate cyclase. To verify this hypothesis, we measured the accumulation of [³H]cAMP in the 5-HT_{ap1}-expressing HEK cells after application of 5-HT or agonists. As expected, forskolin-induced cAMP accumulation was efficiently inhibited by 5-HT and the other serotonergic agonists, 5-CT and 8-OH-DPAT, in a dose-dependent manner (Fig. 7). The concentrations of agonists at which inhibition of adenylate cyclase was effective corresponded to those that were shown to be necessary for competing [³H]LSD binding. 5-HT produced no inhibition of cAMP accumulation in the presence of 100 nM methiothepin. 5-HT concentrations ranging from 10 nM to 10 μM had no effect on cAMP levels in untransfected HEK cells treated with forskolin (data not shown). Altogether, these experiments demonstrate that 5-HT_{ap1} is functionally coupled to the mammalian G_i subunit, and that it inhibits adenylate cyclase and cAMP accumulation in the heterologous expression system.

5-HT_{ap1} protein distribution

Plasma membrane extracts from various *Aplysia* tissues were prepared and used in an immunoblot analysis to detect the presence of the receptor. Different bands ranging in size from 45 to 71 kDa were detected in the gill, heart, hermaphroditic duct, kidney, and ovotestis extracts (Fig. 8A). To determine whether the differences in the size of the receptor can be explained by different patterns of glycosylation, kidney membrane extracts were treated with deglycosidase F (New England Biolabs, Mississauga, Ontario, Canada) and separated by SDS-PAGE. After treatment, the observed molecular weight of the receptor is decreased to 45 kDa, which is the size expected from the cDNA sequence (Fig. 8B). In the CNS, the protein is detected in all ganglia, as well as in the sheath, with the strongest signal observed in the abdominal and pedal ganglia (Fig. 9A). These results were confirmed by RT-PCR experiments. The mRNA could be amplified from all desheathed ganglia but not from the sheath itself (Fig. 9B). There is a poor correlation between the mRNA and

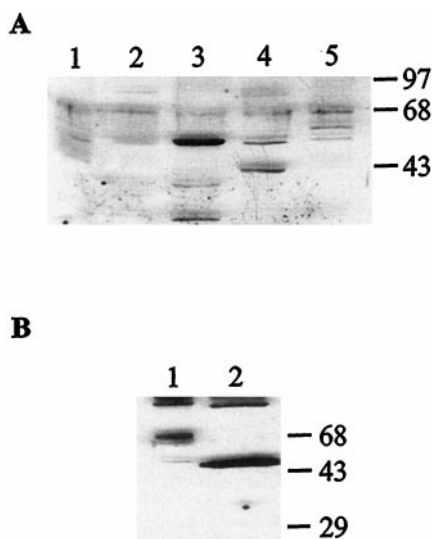


Figure 8. Western blot analysis of the expression of the 5-HT_{ap1} receptor in different *Aplysia* tissues. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting with antiserum raised against the third cytoplasmic loop of the 5-HT_{ap1} receptor. *A*, Thirty micrograms of cytoplasmic membrane protein extracts prepared from gill (lane 1), heart (lane 2), hermaphroditic duct (lane 3), kidney (lane 4), and ovotestis (lane 5). *B*, Fifty micrograms of kidney plasma membrane proteins before (lane 1) or after (lane 2) treatment with deglycosidase F to remove N-linked sugars. Molecular weight marker positions are indicated in kilodaltons.

protein levels, suggesting that the protein is expressed at the level of the nerve terminals.

DISCUSSION

We have isolated and characterized an *Aplysia* 5-HT receptor that belongs to the G-protein-coupled receptor family. Its structure and biochemical properties are closely related to those of the invertebrate 5-HT_{lym1} and 5-HT_{dro2A-2B} and to those of the vertebrate 5-HT₁ receptor subtypes. The absence of introns in the gene, the presence of a large third cytoplasmic loop and a short C-terminal domain in the protein, and a functional coupling to the inhibition of adenylate cyclase in stably transfected cells are all features of the 5-HT₁ receptor subfamily. 5-HT_{ap1} is therefore clearly associated with this vertebrate receptor subfamily. Two other 5-HT receptors have recently been cloned in *Aplysia* (Li et al., 1995). These receptors are closely related to each other and are linked to the phospholipase C pathway. They show very little sequence identity with 5-HT_{ap1} and therefore are clearly members of another subfamily of 5-HT receptors. In *Lymnaea*, a 5-HT receptor with some similarities to vertebrate 5-HT₁ receptors has been cloned (Sugamori et al., 1993). However, its functional coupling to a second messenger system has not been determined, and its pharmacological profile is different from that of 5-HT_{ap1}, suggesting that they may not be homologous.

Like other invertebrate G-protein-coupled receptors (Saudou et al., 1992; Li et al., 1995; Gerhardt et al., 1997), 5-HT_{ap1} is efficiently coupled to mammalian G-proteins when expressed in mammalian cells. This underlines the high conservation among species of members of the G-protein-coupled receptor subfamily and the functional conservation of mechanisms that link these receptors to G-proteins. In this respect, it is not surprising to observe that the biochemical characterization of G_{sα}, G_{oα}, and G_{iα}, as well as G_β, in the *Aplysia* nervous system has demon-

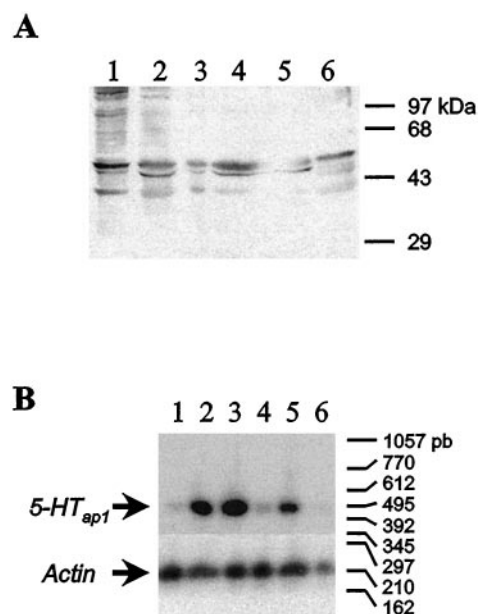


Figure 9. Western blot and RT-PCR analysis of the expression of the 5-HT_{ap1} receptor in the CNS. *A*, Fifty micrograms of total protein extracted from desheathed ganglia and sheath alone were resolved by SDS-PAGE and analyzed by immunoblotting with antiserum raised against the third cytoplasmic loop of the 5-HT_{ap1} receptor. Abdominal (lane 1), buccal (lane 2), cerebral (lane 3), pedal (lane 4), and pleural (lane 5) ganglia from five animals were pooled. The sheath extract (lane 6) was prepared from a single animal. *B*, RT-PCR analysis was conducted on total RNA isolated from the same samples as in *A*. The PCR products were analyzed by Southern blotting probed with a 5-HT_{ap1}-specific oligonucleotide. The same procedure was followed for amplification of the control (actin). The sizes of the bands were estimated by comparison with the DNA size markers on the original ethidium bromide-stained gel.

strated that these proteins are very similar to their mammalian counterparts (Critz et al., 1986; Vogel et al., 1989).

The pharmacological profile of 5-HT_{ap1} is also reminiscent of those of 5-HT₁-like receptors. The K_d and K_i values demonstrated the high affinity of the 5-HT_{ap1} receptor for LSD, 5-CT, methiothepin, and 5-HT and are comparable to the values obtained with vertebrate 5-HT₁ receptors (Boess and Martin, 1994). Interestingly, these ligands bind to all vertebrate 5-HT₁ receptor subtypes with high affinity. By contrast, ligands that show a high specificity to the 5-HT_{1A} receptors, such as 8-OH-DPAT and NAN-190, do not bind 5-HT_{ap1} efficiently. Their binding efficiency is more closely related to that of the 5-HT₇ receptor than to those of other vertebrate 5-HT₁ receptors (Ruat et al., 1993). These results suggest that the structure and function of the 5-HT_{ap1} receptor are probably closely related to those of an ancestor gene, which existed before the divergence of the 5-HT receptor subtypes in vertebrates, and that it kept characteristics of more than one receptor subtype. Therefore, 5-HT_{ap1} may be closer to the prototype of the early 5-HT_{1,7} receptors.

This view is in agreement with previous phylogenetic studies that suggested that the major subfamilies of 5-HT receptors diverged early in evolution to form three major subclasses: the 5-HT₁ (which includes 5-HT₅ and 5-HT₇), 5-HT₂, and 5-HT₆ receptor subclasses (Peroutka and Howell, 1994; also see Fig. 3). This divergence occurred before the evolution of vertebrates from invertebrates, and one can expect to find members of these major subclasses in invertebrate species (Peroutka, 1994). Further division within the 5-HT₁ and 5-HT₂ subfamilies seems to have

occurred after the separation of vertebrates and invertebrates, and homologs of these subtypes may not be found in mollusks. More than one 5-HT₁ receptor may nevertheless be found in *Aplysia* and in other invertebrates, but they will have emerged independently from vertebrate receptors.

The pharmacological profile of a receptor depends on the conservation of amino acids in and around the binding site of the receptor (Hibert et al., 1991; Ho et al., 1992; Chanda et al., 1993; van Rhee and Jacobson, 1996). Therefore, it is not surprising to find that the pharmacological profiles of mollusks and mammals are difficult to compare, considering the large phylogenetic distances. Nevertheless, whatever its pharmacological profile, the characterization of the second messenger pathway with which the receptor is linked clearly associates 5-HT_{ap1} to the 5-HT₁ subfamily and not to 5-HT₇.

Very few 5-HT-dependent pathways have been characterized on a pharmacological basis in *Aplysia*. None of these generated a 5-HT_{ap1}-like pharmacological profile. In earlier works, binding experiments using crude membrane preparations of different tissues revealed a [³H]LSD binding site with a dissociation constant of 0.63 nM (Drummond et al., 1980). Similar results were obtained on tissue sections using [¹²⁵I]LSD (Kadan and Hartig, 1988). Although this value is consistent with our data on the 5-HT_{ap1} receptor expressed in mammalian cells, competition of the LSD binding sites in the crude tissue membranes with 5-HT gave K_i values at least two orders of magnitude higher than the one observed for 5-HT_{ap1} (Drummond et al., 1980; Kadan and Hartig, 1988). In addition, Drummond et al. (1980) demonstrated that there was a good correlation between the amount of [³H]LSD binding sites and the amount of 5-HT-induced adenylate cyclase activity in most tissues. Altogether, these results suggest that although 5-HT_{ap1} is expressed in all these tissues, it was not recognized in these binding assays. It also suggests that more than one 5-HT receptor is present in most *Aplysia* tissues, and therefore, the binding assay with *Aplysia* tissue membranes probably recognized a heterogeneous population of receptors, with similar affinities for LSD.

More recently, Ram et al. (1994) reported the existence of an *Aplysia* 5-HT_{1A}-like receptor in the *Aplysia* buccal muscle. In this system, 5-HT is known to potentiate the acetylcholine-elicited contractions. This effect was shown to be efficiently competed by NAN-190 in the nanomolar range and mimicked by 8-OH-DPAT in the micromolar range, thus defining a 5-HT_{1A}-like site. However, this receptor is likely to be different from 5-HT_{ap1}, because we showed that 5-HT_{ap1} does not bind NAN-190 at these concentrations. These last observations strongly suggest that at least one other 5-HT₁-like receptor exists in *Aplysia*.

The application of 5-HT has generally been associated with an increase in cAMP levels in *Aplysia*. Nevertheless, application of 5-HT often triggers inhibitory responses, although the downstream pathway is not yet determined (Jennings et al., 1981; Xu et al., 1995). In addition, functional coupling of a receptor may vary in different cell types, and stimulation of a single receptor subtype may mediate different responses under different physiological conditions. For example, the mammalian 5-HT_{1A} receptor has been shown to inhibit adenylate cyclase via its interaction to G_i protein and also to nonenzymatically mediate the opening of K⁺ channels via an interaction with a different, unidentified G-protein subunit (Andrade et al., 1986). Moreover, it is known that the stimulation of different G_i-coupled receptors in different cell types leads to the activation of MAP kinase through the G_{βγ} subunits (Koch et al., 1994). The presence of 5-HT_{ap1} in many

Aplysia tissues suggests that it may play a variety of roles linked to the inhibition of adenylate cyclase, activation of MAP kinase, and/or modulation of other pathways. It will be particularly interesting to determine whether 5-HT_{ap1} is expressed in some sensory neuron clusters of the CNS (Storozhuk et al., 1998) and whether it is involved in the 5-HT-induced activation of MAP kinase in these cells. This activation is implicated in the establishment of long-term facilitation in cultured neurons (Martin et al., 1997). Further investigation of the roles of the 5-HT_{ap1} receptor in this animal is susceptible to give interesting new data on this aspect of cell signaling.

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