Serotonin receptor cDNA cloned from *Lymnaea stagnalis*

(polymerase chain reaction/guanine nucleotide-binding protein/[³H]lysergic acid diethylamide/invertebrate)

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ABSTRACT Serotonin (5-HT) is a major neurotransmitter that influences various behaviors, neuronal plasticity, learning, and memory in molluscs. Although the physiology of 5-HT transmission in molluscs is well studied, little is known about the pharmacology and diversity of the 5-HT receptor system. Based on the high homology of genes coding for guanine nucleotide-binding protein (G protein)-coupled receptors, we have cloned a gene for the *Lymnaea stagnalis* 5-HT (5HT1ym) receptor. The putative receptor protein, 509 amino acids long, has highest homology with the *Drosophila* 5-HT receptors and mammalian 5HT$_1$ receptors. As revealed by RNA blot-hybridization analysis, two mRNA species of 2.3 and 3.2 kb are detected in the central nervous system of *Lymnaea*. Transient expression of 5HT1ym in COS-7 cells showed saturable [³H]lysergic acid diethylamide binding with an estimated dissociation constant of 0.9 nM. The 5HT1ym receptor exhibited a mixed 5HT-like pharmacology that cannot be precisely categorized with existing mammalian classification nomenclature. However, the 5HT1ym receptor does display some characteristics that have been attributed to the putative mammalian vascular 5HT$_1$-like receptor.

Serotonin (5-HT), a neurotransmitter that mediates a number of effects through several different receptors, is present in both vertebrate and invertebrate species (1, 2). In mammals several 5-HT receptors and receptor subtypes have been identified based on pharmacological and biochemical criteria (3–6). Presently, seven distinct mammalian 5-HT receptors have been cloned. These include the 5HT$_{1A}$ (7, 8), 5HT$_{1B}$ (9), 5HT$_{1C}$ (10, 11), 5HT$_{1D\alpha}$ (12), 5HT$_{1D\beta}$ (13, 14), 5HT$_2$ (11, 15, 16), and the pharmacologically uncharacterized human 5-HT receptor, S31 (17), all of which mediate their effects through coupling to guanine nucleotide-binding regulatory proteins (G proteins). In addition, three 5-HT receptors in *Drosophila melanogaster*—the 5HTdro$_1$ receptor (18), which stimulates adenylate cyclase, and the 5HTdro$_{2A}$ and 5HTdro$_{2B}$ receptors (19), which inhibit adenylate cyclase—have recently been cloned. These receptors in *Drosophila* are most homologous in primary sequence to the human 5HT$_{1A}$ receptor; however, the partial pharmacological characterization of these receptors suggests that they are not identical to the 5HT$_{1A}$ receptor.

The molluscan nervous system contains large amounts of 5-HT, and several 5-HT-containing neurons have been identified therein (20, 21). The effect of 5-HT on various ionic currents and on the cell membrane potential has been studied extensively in molluscs, and on the basis of these experiments the existence of several 5-HT receptors has been postulated (22, 23). 5-HT also has been implicated in many behavioral or physiological processes in molluscs such as

learning, circadian rhythms, feeding, development, and neuronal regeneration (24–32). The 5-HT receptors that mediate the behavioral and/or electrophysiological effects are structurally and pharmacologically not well characterized, and none of these receptors has been cloned.

Using a strategy based on the high degree of sequence homology between genes encoding members of the G-protein-coupled receptor superfamily (33, 34) and the use of nested polymerase chain reactions (PCRs), we cloned the gene coding for a 5-HT receptor from the pond snail *Lymnaea stagnalis* (5HT1ym). The receptor protein displays the highest amino acid homology to the 5HT$_1$ receptor family and to the *Drosophila* 5-HT receptors. This receptor from *L. stagnalis* could not be pharmacologically ranked in the mammalian classification scheme for 5-HT receptors; therefore, we suggest that this receptor be referred to as 5HT1ym.**

METHODS

Nested PCR. Approximately 200 ng of *L. stagnalis* (Blades Biologicals, Babnbridge, Kent, U.K.) genomic DNA, prepared by using guanidine hydrochloride (35), was submitted to 30 cycles of PCR amplification. The PCR was done under conditions previously described (34) with 10 μg each of degenerate oligonucleotide primers TM3 [5'-ATCYTSAAC-YTSTGYGYSATCAGCRTVGAYMGNT-3'] and TM7 [5'-SAYGGGGTTSAMKGAKSWRTTAGCMCASCCNAAC-CA-3' in which N = A, C, G, or T; V = A, C, or G; Y = C or T; S = G or C; R = A or G; M = C or A; K = G or T, and W = A or T.] (Biotechnology Service Centre, Toronto), corresponding to the highly conserved sequences for the transmembrane regions 3 and 7 of biogenic amine receptors, and with 2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus). Ten microliters of the amplified DNA was resubmitted to an additional 30 PCR cycles in the presence of 2 μg of the TM3 primer and 2 μg of a TM6 degenerate oligonucleotide primer [5'5'-RMHGTKHRNSABGAAGAARGGNRKCCARCA-3' in which N, R, S, K, and M have the same meaning as above; H = C, A, or T; and B = G, T, or C] designed from transmembrane domain 6 of biogenic amine receptors. This DNA was subsequently subjected to electrophoresis in a 1.5% agarose gel and transferred to nylon membrane (Zeta-Probe, Bio-Rad) for Southern blot analysis under high-stringency hybridization conditions by using a ³²P-labeled 1.2 kilobase (kb) *Bam*HI–*Bgl* II fragment that contained the entire coding region for the human D$_2$ dopamine receptor (36). DNA from contiguous gel slices ranging from 350 to 800 bases, corresponding to hybridizing bands, was subcloned

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into the *Sma* I site of the plasmid pSP73 (Promega) and transformed into *Escherichia coli* strain AG-1.

Bacterial colonies were lifted onto nitrocellulose filters (Millipore), prehybridized, hybridized with the D_2 probe, and washed under the same conditions as the Southern blot analysis. Several hybridizing clones were analyzed by sequence analysis by using the Sanger dideoxy chain-termination method with 7-deaza-dGTP and Sequenase v2.0 (United States Biochemical). One clone, Lym 23, revealed the presence of a 800-base-pair (bp) insert that displayed homology to the 5-HT family of receptors from transmembrane domain regions 3 to 6.

Isolation of the cDNA Clone. To obtain the full-length clone, a cDNA library of *Lymnaea* central nervous system (CNS) was constructed into the λ Zap II vector (Stratagene) and screened with the PCR partial clone Lym 23 that was ^{32}P-labeled by nick translation (Amersham). One million independent clones were lifted in duplicate onto colony plaque screen filters (DuPont/NEN). The duplicate filters were hybridized under similar conditions as the Southern blot analysis. Strongly hybridizing λ phage were plaque-purified with subsequent *in vivo* excision and rescue of the pBlue-script plasmids that contained the hybridizing inserts from the λ Zap II clones. A 2.2-kb *Eco*RI–*Xho* I fragment (L17) was sequenced and found to contain an open reading frame of 1527 bases encoding Lym 23.

RNA (Northern) Blot Analysis. Poly(A)$^+$ mRNA was isolated from *Lymnaea* CNS as described elsewhere (37, 38). Two micrograms of the poly(A)$^+$ mRNA was denatured by using glyoxal and dimethyl sulfoxide, electrophoresed in a 1% agarose gel, transferred to nylon membrane (Hybond, Amersham), and probed with a ^{32}P-labeled 2.2-kb *Eco*RI–*Kpn* I fragment encoding L17. The blot was hybridized under stringent conditions as described (36) and was washed once in 1× SSC containing 1% SDS for 10 min at room temperature, once in 1× SSC containing 1% SDS for 15 min at 60°C, and once in 0.5× SSC containing 1% SDS for 15 min at 60°C (1× SSC = 0.15 M NaCl/0.01, M sodium citrate, pH 7). After a 7-day exposure at −70°C to x-ray film (XAR, Kodak) in the presence of one intensifying screen, hybridizing bands were revealed on the autoradiograph.

Transfection and Ligand Binding Analysis. The full-length clone was subcloned into the expression vector PCD-PS (39). The cesium chloride-purified construct was introduced into COS-7 cells by electroporation (40). After 72 hr, cells were harvested and membranes were prepared as described (41). Membrane pellets were resuspended in buffer at a concentration of ≈150 μg/ml. For saturation experiments 0.5-ml aliquots of the membrane preparation were incubated in duplicate with increasing concentrations of tritiated lysergic acid diethylamide ([^{3}H]LSD, 65.2 Ci/mmol; 1 Ci = 37 GBq) for 120 min at room temperature in a total volume of 1.5 ml. Competition binding assays were initiated by addition of 0.5 ml of membrane and incubated in duplicate with the indicated concentrations of competing agents (10^{-11} M to 10^{-3} M) and 1 nM [^{3}H]LSD for 120 min at room temperature. All assays were terminated by rapid filtration over Skatron filtermats (Lier, Sterling, VA) and were monitored for tritium by scintillation spectrometry. For all experiments, nonspecific binding was defined in the presence of 10 μM methiothepin. Data were analyzed with the computer program LIGAND as described (41).

RESULTS AND DISCUSSION

A nested PCR approach was used to clone various members of the G protein-coupled receptor family from *L. stagnalis*. *Lymnaea* genomic DNA was subjected to PCR amplification with degenerate oligonucleotide primers (TM3 and TM7) derived from the conserved segments of transmembrane
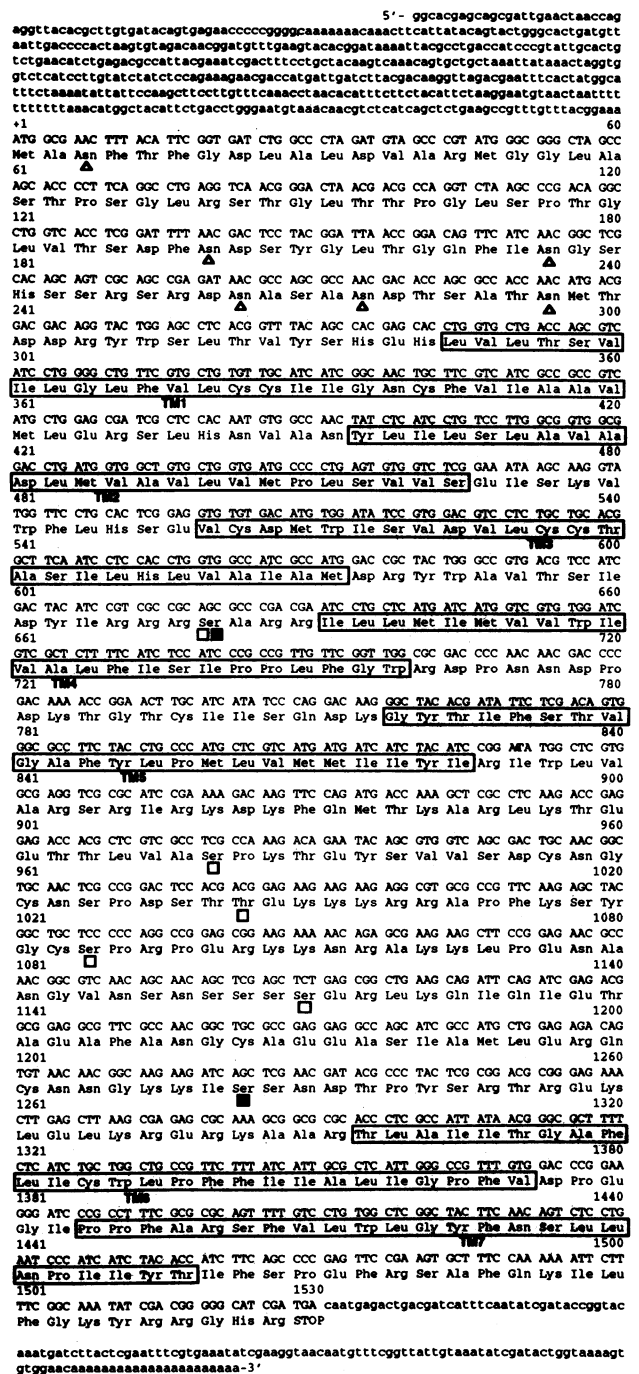

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *Lymnaea* 5HTlym receptor gene. Nucleotides of the cDNA clone are numbered beginning with the first putative initiating methionine (42) with the deduced amino acid sequence presented below the nucleotide sequence. Putative transmembrane (TM) domains are demarcated by boxed regions and indicated below as TM1 to TM7. Putative N-linked glycolsylation sites are indicated by open triangles. Potential phosphorylation sites for protein kinase A (cAMP dependent) and protein kinase C are indicated by closed and open squares, respectively.

domains 3 and 7 of biogenic amine receptors. The DNA amplified by this reaction was subjected to further PCR amplification with the TM3 primer and a degenerate oligonucleotide primer (TM6) constructed from the highly conserved transmembrane 6 domain of biogenic amine receptors. The nested PCR approach was used here to increase the probability that any DNA fragments that were amplified would code for a G protein-coupled receptor. One DNA
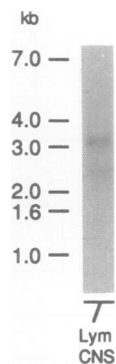
FIG. 2. Northern blot analysis of 5HTlym mRNA in *Lymnaea* CNS. Poly(A)$^+$ mRNA was prepared from *Lymnaea* CNS (37, 38). Two micrograms of the poly(A)$^+$ mRNA was denatured and electrophoresed through agarose, transferred to nylon membrane, and hybridized with a 2.2-kb ^{32}P-labeled nick-translated *Eco*RI–*Kpn* I fragment encoding the entire 5HTlym receptor gene as described in text. The blot was exposed for 7 days at $-70°C$ with an intensifying screen. Sizes of RNA markers are shown to the left.

fragment (Lym 23) of ≈ 800 bp that hybridized to a human dopamine D_2 probe was found by sequence analysis to display considerable deduced amino acid homology to the 5-HT family of receptors. Therefore, Lym 23 was used to screen a *Lymnaea* CNS cDNA Lambda ZAP II library. From this screening, one strongly hybridizing clone (L17) was found to contain a 2.2-kb insert that upon complete sequencing revealed sequence identical to Lym 23.

The complete nucleotide sequence and deduced amino acid sequence are shown in Fig. 1. The longest open reading frame consists of 1527 nucleotides coding for a 509-amino acid protein. Two putative initiating methionines with Kozak sequence are present at amino acid positions 1 and 16 (42). Hydrophobicity analysis of the deduced amino acid sequence (data not shown) revealed the presence of seven stretches of hydrophobic amino acids that represent the seven transmembrane domains characteristic to all G protein-coupled receptors. Comparison of the deduced amino acid sequence of L17 with members of the 5-HT receptor family and other G protein-coupled receptors indicates that the regions of greatest homology occur within these putative transmembrane

domains. The degree of identity between L17 and other G protein coupled receptors within the transmembrane domains is 36% to the $5HT_2$ receptor, 39% to the $5HT_{1C}$ receptor, 40% to the dopamine D_4 receptor, 40% to the β_1 and β_2 adrenergic receptors, 43% to the dopamine D_1 receptor, 45% to the α_{1A} adrenergic receptor, 46% to the dopamine D_2 receptor, 50% to the $5HT_{1D\alpha}$ receptor, 50% to the 5HTdro$_1$ receptor, 53% to the $5HT_{1D\beta}$ receptor, 53% to the $5HT_{1A}$ receptor, 59% to the 5HTdro$_{2A}$ receptor, and 61% to the 5HTdro$_{2B}$ receptor.

Six consensus sites for N-linked glycosylation are present in the extracellular amino terminus of L17. Within the second cytoplasmic loop there is a serine residue that may serve as a substrate for either cAMP-dependent protein kinase A or protein kinase C phosphorylation. Several other putative phosphorylation sites for protein kinase A or protein kinase C phosphorylation are located within the large third cytoplasmic loop (Fig. 1). Conserved in L17 is the Asp-175 residue in transmembrane domain 3, which is found in all biogenic amine receptors (43), and the Ser-258 residue in transmembrane domain 5, which is found in all 5-HT receptors except for three *Drosophila* 5-HT receptors. The large third cytoplasmic loop and the short carboxy tail are reminiscent of the motif for G protein-coupled receptors coupled to G_i such as the $5HT_{1A}$ receptor and dopamine D_2 receptor, and as such, L17 receptor may inhibit adenylate cyclase.

Northern blot analysis of *Lymnaea* CNS poly(A)$^+$ mRNA using the 2.2-kb *Eco*RI–*Kpn* I fragment that codes for the entire receptor revealed the presence of 2 mRNA species of 2.3 and 3.2 kb (Fig. 2). The presence of two hybridizing bands may be the result of two different transcription start sites or polyadenylylation sites, or both (44). A more precise distribution of the receptor within the *Lymnaea* CNS and periphery was obtained by PCR with degenerate oligonucleotide primers constructed from transmembrane domain regions 6 and 7 of cDNA derived from heart and CNS total RNA. Sequence analysis revealed that 4 of 6 clones from heart and 12 of 15 clones from CNS were equivalent to 5HTlym. Expression in the CNS was further studied by isolating RNA from individual neurons and using the PCR technique. This demonstrated that the receptor is present specifically in the growth-controlling Light Green Cells of the CNS and in the heart of *Lymnaea*.
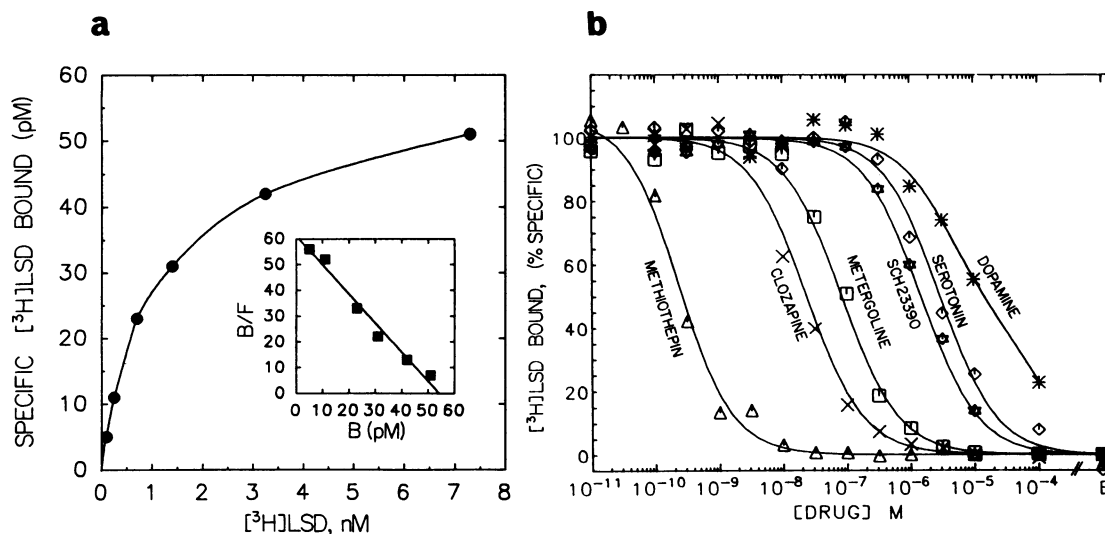

FIG. 3. Pharmacological profile of the 5HTlym receptor: (*a*) Saturation isotherm of [^{3}H]LSD binding to membranes prepared from COS-7 cells transfected with PCD-L17. (*Inset*) Scatchard plot of the same data. (*b*) Pharmacological specificity of [^{3}H]LSD binding to COS-7 cells. Representative curves are shown for the concentration-dependent inhibition of [^{3}H]LSD binding (1 nM) with various serotonergic agonists and antagonists. Data were analyzed by using the computer program LIGAND (41). Assays were conducted as described in text; the results shown are representative of two independent experiments conducted in duplicate.

To classify L17, the pharmacological profile was determined. The full-length clone was subcloned into the expression vector PCD-PS and introduced into COS-7 cells by electroporation. Membranes prepared from COS-7 cells transiently expressing the gene product bound [^{3}H]LSD in a saturable and dose-dependent manner with an estimated K_d of 0.9 nM and a receptor density of 0.8–1.2 pmol/mg of protein (Fig. 3a). The addition of salt (120 mM NaCl) and nonhydrolyzable guanine nucleotides [120 mM NaCl, 200 μM Gpp(NH)p] to the buffer resulted in a small shift in the observed K_d (1.5 nM) of [^{3}H]LSD with a large decrease in B_{max} (0.6–0.8 pmol/mg of protein), suggesting that the expressed receptor is capable of G protein interactions.

The rank order of potency for inhibition of [^{3}H]LSD binding to COS-7 membranes by various serotonergic agonists and antagonists is listed in Table 1 and illustrated in Fig. 3b. 5-HT was the most potent displacer of [^{3}H]LSD binding compared with other naturally occurring endogenous neurotransmitters such as noradrenaline, octopamine, tyramine, tryptamine, and dopamine. The indolealkylamines 5-HT and 5-carboxamidotryptamine, which normally bind to mammalian 5HT$_1$ receptors (5HT$_{1A}$, 5HT$_{1B}$, or 5HT$_{1D}$) with high affinity (1–10 nM), displayed an affinity lower by a factor of 10–100 for the expressed receptor. The ergot alkaloids (ergotamine, lisuride, methysergide, and metergoline) displayed affinities similar to mammalian 5HT$_{1A}$ or 5HT$_{1D}$ sites. Methiothepin, a nonselective 5-HT antagonist, displayed an affinity ≈1 order of a magnitude higher than binding to any cloned mammalian 5-HT receptor. Other antagonists, in particular quipazine and propranolol, had affinities similar to those for binding to 5HT$_{1D}$ receptors. Although clozapine bound to the receptor with a dissociation constant of ≈12 nM, ketanserin, a 5HT$_2$ receptor antagonist, and SCH23390, a 5HT$_{1C}$ receptor antagonist, bound with poor affinity, suggesting that the receptor is not a 5HT$_2$-like receptor.

The finding that L17 displays a pharmacological profile that appears to be composed of a multiple 5HT$_1$ receptor type (5HT$_{1A}$, 5HT$_{1B}$, and 5HT$_{1D}$) rather than a 5HT$_2$ type suggests that L17 is a 5HT$_1$-like receptor homologue. Whether this receptor represents an additional subclass of 5HT$_1$-like receptors not yet discovered in mammals remains to be established; however, the presence of a vascular 5HT$_1$-like receptor that is neither 5HT$_{1A}$ or 5HT$_{1D}$ and for which methiothepin is a potent antagonist has been reported to exist (45–47). Furthermore, the finding that L17 is present in the *Lymnaea* heart suggests that this 5-HT receptor may be

Table 1. Dissociation constants for [^{3}H]LSD binding to cloned 5HTlym receptor

Drug	K_i, nM	Drug	K_i, nM
Methiothepin	0.1	NAN-190	732
Lisuride	0.7	SCH 23390	770
LSD	0.9	8-OH-DPAT	1100
Clozapine	12	5-HT	1562
Ergotamine	22	Quipazine	1909
Methysergide	40	Ketanserin	2554
Metergoline	55	(−)-Propranolol	3077
5-CT	282	Noradrenaline	3777
PAPP	410	Dopamine	9130

Dissociation constants (K_i, in nM) of various serotonergic agonists and antagonists are listed in order of their potency for the 5HTlym receptor. The dissociation constants were obtained by using the computer program LIGAND (41). Values represent the means of two independent experiments conducted in duplicate with results that varied by less than 15%. 5-CT, 5-carboxamidotryptamine; PAPP, p-aminophenylethyl-m-trifluoromethyphenyl piperazine; NAN-190, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide; SCH 23390, (3R)-(+)-8-chloro-2,3,4,5-tetrahygro-3-methyl-5-phenyl-1H-3-benzazepine; 8-OH-DPAT, (±)-8-hydroxy-N,N-dipropyl-2-aminotetralin.
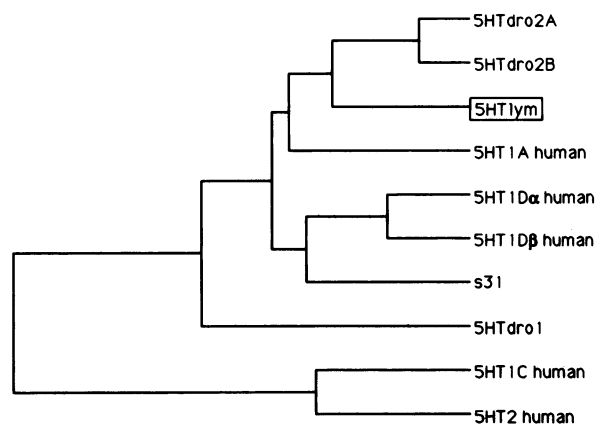

FIG. 4. Phylogenetic tree showing the calculated evolutionary relationships of 5HTlym to other cloned serotonin receptors. The amino acid sequences excluding the amino termini and third cytoplasmic loops were compared for 5HTlym and several serotonin receptors using the computer program GENEWORKS which first aligns the sequences and then calculates the evolutionary relationships of the aligned sequences by the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method (48). The length of the horizontal lines connecting one amino acid sequence to another is proportional to the estimated genetic distance between the sequences.

analogous to such a vascular 5-HT receptor. On the other hand, the phylogenetic differences may be so great between molluscs and mammals that the pharmacological profile of a receptor from an invertebrate species may not be comparable with a mammalian species.

From an evolutionary point of view, it appears that 5HT$_1$-like receptors arose early in evolution before the divergence of invertebrates and vertebrates. When the evolutionary relationships were determined for the aligned amino acid sequences excluding the amino termini and third cytoplasmic loops for 5HTlym and several other cloned 5-HT receptors using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method (48), 5HTlym and the *Drosophila* 5HT receptors were categorized with mammalian 5HT$_1$ receptors (Fig. 4). 5HTlym in particular appeared to relate most closely to the *Drosophila* 5HTdro$_{2A}$ and 5HTdro$_{2B}$ receptors and to the human 5HT$_{1A}$ receptor over the defined regions.

Because it is not possible to classify this receptor according to the classification scheme used to define previously cloned mammalian 5-HT receptors, we propose that this receptor be referred to as 5HTlym. The localization of this receptor to defined neurons within the CNS of *Lymnaea* and further characterization may help to determine, for example, if it is involved in any behaviors such as learning and memory as described for *Aplysia*. The finding that 5HTlym is present in the *Lymnaea* heart and that vascular 5HT$_1$-like 5-HT receptors (45–47) have been postulated to exist in mammals that appear not to be of a previously defined 5HT$_1$ receptor type suggests that 5HTlym may belong to an additional subclass of 5HT$_1$-like receptors common to mammals.

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