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# Identification, molecular structure and expression of two cloned serotonin receptors from the pond snail, *Helisoma trivolvis*

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## SUMMARY

***Helisoma trivolvis*** **has served as a model system to study the functions of serotonin (5-HT) from cellular, developmental, physiological and behavioural perspectives. To further explore the serotonin system at the molecular level, and to provide experimental knockout tools for future studies, in this study we identified serotonin receptor genes from the *H. trivolvis* genome, and characterized the molecular structure and expression profile of the serotonin receptor gene products. Degenerate oligonucleotide primers, based on conserved regions of the *Lymnaea stagnalis* 5-HT$_{1Lym}$ receptor, were used to amplify G protein-coupled biogenic amine receptor sequences from *H. trivolvis* genomic cDNA, resulting in the cloning of two putative serotonin receptors. The deduced gene products both appear to be G protein-coupled serotonin receptors, with well-conserved structure in the functional domains and high variability in the vestibule entrance of the receptor protein. Phylogenetic analysis placed these receptors in the 5-HT$_1$ and 5-HT$_7$ families of serotonin receptors. They are thus named the 5-HT$_{1Hel}$ and 5-HT$_{7Hel}$ receptors, respectively. *In situ* hybridization and immunofluorescence studies revealed that these genes and gene products are expressed most heavily in the ciliated pedal and mantle epithelia of *H. trivolvis* embryos. In adults, widespread expression occurred in all ganglia and connectives of the central nervous system. Expression of both receptor proteins was localized exclusively to neurites when examined *in situ*. In contrast, when isolated neurons were grown in culture, 5-HT$_{1Hel}$ and 5-HT$_{7Hel}$ immunoreactivity were located primarily in the cell body. This is the first study to reveal a 5-HT$_7$ receptor in a molluscan species.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/6/900/DC1

Key words: serotonin receptor, gastropod, mollusc, phylogenetic analysis, receptor expression.

## INTRODUCTION

Among the many neurotransmitter and hormone systems studied to date, serotonin (5-HT) stands out in its relatively large diversity of receptors. In studies on vertebrates, seven families of 5-HT receptors have been identified, including six families of metabotropic G protein-coupled receptors and one family of ionotropic receptors (reviewed in Hoyer et al., 2002). Within these, a total of 14 receptor subtypes have been distinguished in addition to splice variants and RNA-edited isoforms for some of the subtypes. Multiple subunits of the ionotropic 5-HT$_3$ receptors have been characterized, and these may occur in various heteromeric combinations, resulting in further diversification of 5-HT receptors (Hoyer et al., 2002). In the light of the impressive number of receptor subtypes for 5-HT and the well-characterized role played by 5-HT in a multitude of behavioral, physiological and developmental pathways, this neurotransmitter system is well suited to explore the evolution of neurotransmitter receptors from integrated molecular and functional perspectives.

Studies on the molecular evolution of 5-HT receptors support the idea that the various families of 5-HT receptors were established before the radiation of most modern phyla (Peroutka, 1994; Tierney, 2001; Walker et al., 1996). This suggests that the 5-HT receptor families characterized in vertebrate species should have homologs in modern invertebrate species. However, the roughly 500–600 million years of evolution since the separation of chordates from other invertebrate phyla could hamper the identification of homologous receptor subtypes. Alternatively, unique receptor families could have evolved or been lost relatively recently during vertebrate evolution.

Molecular cloning studies on invertebrate 5-HT receptors have in several cases revealed putative homologs to vertebrate 5-HT receptors. To date, four G protein-coupled 5-HT receptors have been cloned from *Drosophila melanogaster* (Colas et al., 1995; Saudou et al., 1992; Witz et al., 1990), and two from *Caenorhabditis elegans* (Hamdan et al., 1999; Olde and McCombie, 1997), all of which are considered to be homologous to known vertebrate receptors (Tierney, 2001). A third nematode 5-HT receptor has been cloned from the parasite *Ascaris suum* (Huang et al., 1999); however, its affiliation with a vertebrate receptor family is more tentative. Somewhat surprisingly, all of these proposed homologies are limited to the 5-HT$_1$, 5-HT$_2$ and 5-HT$_7$ receptor families.

Molluscan model systems have been particularly useful for gaining a comprehensive understanding of 5-HT function through the integration of molecular, cellular and behavioral approaches. Most notably, studies on *Aplysia californica* (Brunelli et al., 1976; Sharma et al., 2003) have elucidated the cellular actions of 5-HT and the underlying mechanisms that are central to the acquisition of short- and long-term forms of learning. Similarly, the critical roles played by 5-HT during feeding behavior have been extensively analyzed in studies on *A. californica* (Hurwitz et al., 2000;

Kabotyanski et al., 2000; Morgan et al., 2000) and *Lymnaea stagnalis* (Straub and Benjamin, 2001; Yeoman et al., 1996). Of the five 5-HT receptors cloned to date from these species, two from *L. stagnalis* (Gerhardt et al., 1996; Sugamori et al., 1993) and one from *Aplysia* (Angers et al., 1998) were proposed to be members of the 5-$HT_1$ and 5-$HT_2$ receptor families (Tierney, 2001). Pharmacological and structural characteristics of the other two *Aplysia* 5-HT receptors precluded their assignment in any of the known 5-HT receptor families (Li et al., 1995; Tierney, 2001).

The pond snail *Helisoma trivolvis* Say 1816 is a gastropod mollusc that has been used to explore in depth the 5-HT neurotransmitter system from a developmental perspective. Experiments demonstrating inhibitory effects of 5-HT on regenerative neurite outgrowth from various identified neurons, most notably buccal ganglion neuron B19, played a pioneering role in establishing neurotransmitters as developmental signals (Haydon et al., 1984; McCobb and Kater, 1988). Neuron B19 was further used to examine the signal transduction pathway of the neurite outgrowth response to 5-HT (Mattson and Kater, 1987; McCobb et al., 1988; Polak et al., 1991; Price and Goldberg, 1993; Zhou and Cohan, 2001), the pharmacological profile of the underlying 5-HT receptors (Price and Goldberg, 1993), and the activity of this response during embryonic development (Goldberg and Kater, 1989).

The experimental tractability of *H. trivolvis* embryos revealed in this earlier work led to their continued use in studies on 5-HT and the realization that this neurotransmitter plays multiple roles during embryonic development. These include the inhibition or facilitation of neurite outgrowth in numerous embryonic neurons (Goldberg et al., 1991; Goldberg et al., 1992), the autoregulation of neurite outgrowth in the serotonergic embryonic neuron C1 (ENC1) (Diefenbach et al., 1995), and excitatory neurotransmission in neural circuits between the ENC1 neurons and their postsynaptic ciliary cells (Kuang and Goldberg, 2001). Furthermore, the signal transduction mechanisms and pharmacological profile of the cilioexcitatory response to 5-HT have been extensively examined (Christopher et al., 1996; Doran et al., 2004; Goldberg et al., 1994).

The molecular cloning of 5-HT receptors from *H. trivolvis* is a logical next step in our studies on the developmental actions of 5-HT. The information gained from this will help build the bioinformatic database required to better understand the evolution of 5-HT receptors (see above). In terms of the *H. trivolvis* model system, it will provide the opportunity to characterize the 5-HT receptors mediating the various known activities of 5-HT in future studies. Furthermore, the cloning of 5-HT receptors will, potentially, reveal further roles for 5-HT through receptor localization studies, as well as provide the opportunity to perform highly specific molecular knockout experiments in exploring these roles.

In this study, we used degenerate oligonucleotide primers based on conserved regions of the 5-$HT_{1Lym}$ receptor to amplify G protein-coupled biogenic amine receptor sequences from *H. trivolvis* genomic cDNA. Sequences were used to generate primers for screening a *H. trivolvis* cDNA library, resulting in the cloning of two putative 5-HT receptors. We present here full nucleotide sequences of the 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ genes, a phylogenetic analysis, and localization of their expression in whole embryos and adult central nervous system (CNS) by *in situ* hybridization and immunochemistry.

## MATERIALS AND METHODS

### Receptor cloning

Degenerate oligonucleotide primers were used to amplify 5-HT receptor sequences from *H. trivolvis* genomic DNA as described previously (Sugamori et al., 1993). In the first round of polymerase chain reaction (PCR), primers transmembrane 3 (TM3) and TM7 (Table 1) were used to amplify from a genomic DNA template (concentration $10^{-4}$ μg $\mu l^{-1}$). $MgCl_2$ concentration ranged from 1 to 5 mmol $l^{-1}$, with a buffer containing 20 mmol $l^{-1}$ Tris-HCl (pH 8.3 at 20°C), 25 mmol $l^{-1}$ KCl, 100 μg $ml^{-1}$ gelatin, 50 μmol $l^{-1}$ each dNTP, Taq DNA Polymerase and Tli DNA Polymerase (Promega, Madison, WI, USA). Touchdown PCR was carried out with an annealing temperature range of 60 to 51°C.

PCR products (10 μl) from the first reaction were used as the template for a second round of PCR using nested primers TM3 and TM6 (Table 1). The re-amplification was performed using the same program as above, over a $MgCl_2$ concentration range of 1 to 9 mmol $l^{-1}$.

PCR products were then cloned into pGEM-T. Individual colonies were picked, boiled, screened by PCR, and the DNA sequenced. PCR primers specific for two distinct receptors were designed based on the initial PCR products, and used to screen sections of a *H. trivolvis* CNS lambda-ZAP cDNA library consisting of 44 fractions (made by Erno Vreugdenhill, 1993, and kindly provided by Garry Hauser and Andy Bulloch, University of Calgary). Primers WJG1157 and WJG1158 were used to identify library sections containing 5-$HT_{1Hel}$, while primers WJG1183 and WJG1184 were

Table 1. Primers used for PCR reactions

| Primer name | Sequence | Use |
|---|---|---|
| TM3 | ATCYTSAACYTSTGYGYSATCAGCRTVGAYMGNT | Deg. PCR |
| TM7 | SAYGGGGTTSAMKGAKSWRTTAGCMCASCCNAACCA | Deg. PCR |
| TM6 | RMHGTKHRNSABGAAGAARGGNRKCCARCA | Deg. PCR |
| WJG1157 | CGGAAAGACAAGTTCCAGTTGACC | 5-$HT_{1Hel}$, screen |
| WJG1158 | ATTATAGCCAGGGTCCTTGCGG | 5-$HT_{1Hel}$, screen |
| WJG1183 | CAGTATGCTATCAAACGCACACCG | 5-$HT_{7Hel}$, screen |
| WJG1184 | CAAGGTGAAGCAGCCCATGATTATC | 5-$HT_{7Hel}$, screen |
| WJG1340 | TAATACGACTCACTATAGGGGTTCCATCAGGGCAGGCACACA | 5-$HT_{7Hel}$, *in situ* |
| WJG1341 | ATTTAGGTGACACTATAGAAAGCCAACACATCTTTTTCTCCTAATCCA | 5-$HT_{7Hel}$, *in situ* |
| WJG1342 | TAATACGACTCACTATAGGGCTTTGGACACGATTAGGGGCTCAC | 5-$HT_{7Hel}$, *in situ* |
| WJG1343 | ATTTAGGTGACACTATAGAAGACCAGAGGATGACCACACAGTAAGTAGAAG | 5-$HT_{7Hel}$, *in situ* |
| WJG1353 | TAATACGACTCACTATAGGGCAATGACTGTGGTGTTTTTCTGCTGTT | 5-$HT_{1Hel}$, *in situ* |
| WJG1354 | ATTTAGGTGACACTATAGAACACCGTCAGCCCCCATAAAGC | 5-$HT_{1Hel}$, *in situ* |
| WJG1355 | TAATACGACTCACTATAGGGGGTTGTCTTTGGCTTTGTTGTGATTGA | 5-$HT_{1Hel}$, *in situ* |
| WJG1356 | ATTTAGGTGACACTATAGAATGAAACATAGCAAAACAAAACAAAAATAAAAC | 5-$HT_{1Hel}$, *in situ* |

Deg. PCR, degenerate PCR.

used to identify those sections containing 5-$HT_{7Hel}$ (Table 1). Out of the cDNA library sections producing PCR bands of the expected size, one was chosen for screening for each putative receptor clone: CNS fraction no. 1 for 5-$HT_{1Hel}$ and CNS fraction no. 4 for 5-$HT_{7Hel}$.

Inserts from plasmids containing the primary cloned PCR products were excised and labeled with $^{32}$P-αdCTP using the Prime-A-Gene labeling system (Promega, Madison, WI, USA). Labeled probes were used for plaque screening of the lambda-ZAP library sections.

Inserts of plaque-purified lambda clones were excised as plasmids (Stratagene, La Jolla, CA, USA) and sequenced on both strands using a primer walking strategy.

### Phylogenetic analysis

A large-scale phylogenetic analysis of G protein-coupled biogenic amine receptors was performed on a set of 768 proteins collected from GenBank (National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). Sequences were aligned using MUSCLE v3.6 (Edgar, 2004). The gapped regions corresponding to poorly conserved N-terminal and C-terminal domains and intracellular and extracellular loops were removed, yielding a final data set of 214 characters. A phylogenetic tree was inferred from this dataset using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). A mixture of 10 different amino acid substitution models was evaluated, and rate variability was modeled as a gamma function with a fraction of sites invariant. Two independent runs consisting of four chains each were carried through a total of 2 000 000 cycles of search, with every 100th cycle being kept. The first 15 001 stored trees from each run were discarded and the remaining 10 000 trees were used to construct the consensus. The two *H. trivolvis* receptors were robustly partitioned into a clade consisting of the type 1, type 5 and type 7 receptors. A representative subset of the vertebrate and invertebrate type 1, type 5 and type 7 receptors and two cnidarian biogenic amine receptors were selected and aligned and the alignment was trimmed to yield a dataset consisting of 252 characters. This dataset was used to infer another phylogenetic tree as described above.

### *In situ* hybridization

Templates based on the 3′ untranslated region (UTR) for 5-$HT_{1Hel}$ and the 5′ UTR for 5-$HT_{7Hel}$ were produced by PCR using specific primers, with nucleotide sequences for either the T7 (sense direction, for control probe production) or SP6 (antisense direction, for experimental probe production) promoter regions on the 5′ end of the appropriate primer (Table 1). PCR products were gel purified and yields quantified, and products were sequenced using dye termination.

RNA probes were transcribed and digoxigenin (DIG) labeled using the DIG-RNA labeling kit (SP6/T7; Roche Diagnostics, Laval, QC, Canada). Labeled probe was quantified by dot blot comparison to standards.

*H. trivolvis* animals of an inbred lab-raised albino strain were maintained and embryos collected as described before (Goldberg et al., 1994). Embryos were staged as described previously (Diefenbach et al., 1998; Goldberg et al., 1988). Embryos were collected from their egg capsules, rinsed with 0.01 mol $l^{-1}$ phosphate buffered saline (PBS) and immediately fixed in freshly prepared and filtered 4% paraformaldehyde (pH 7.5) in PBS at 4°C for 2 h to overnight. All solutions from this point on were RNase free. Embryos were rinsed twice in PBT (PBS with 0.1% Triton X-100) then dehydrated in an ascending ethanol series in PBT (25%, 50%, 75%) for 10 min each at room temperature, followed by two washes in 100% ethanol. Embryos were incubated at –20°C for at least 2 h. Embryos were rehydrated in a descending (75%, 50%, 25%) ethanol series in PBT at room temperature for 10 min per wash, and then rinsed twice in PBT. Embryos were digested in either 20 μg $ml^{-1}$ proteinase K for 20 min at 37°C or 0.6 mg $ml^{-1}$ trypsin (Sigma-Aldrich, Oakville, ON, Canada) for 5–10 min at 37°C. The digestion was stopped by replacing the enzyme solution with 1 mmol $l^{-1}$ phenylmethylsulfonyl fluoride in PBT for 10 min at room temperature. Embryos were refixed in 0.2% gluteraldehyde/4% paraformaldehyde for 20 min at 4°C. They were washed twice in PBT to remove all fixative, and then incubated in prewarmed (55°C) prehybridization solution [40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× saline sodium citrate (SSC), 10 mmol $l^{-1}$ dithiothreitol (DTT), 1 mg $ml^{-1}$ yeast tRNA, 1 mg $ml^{-1}$ denatured and sheared salmon sperm DNA] for at least 2 h at 55°C with gentle shaking.

Hybridization with 100 ng probe $ml^{-1}$ hybridization solution was carried out at 55°C overnight on a shaking platform. Washes, antibody incubation and antibody washes were performed as described previously (Nieto et al., 1996). Briefly, embryos were washed thrice for 10 min at 55°C in 2× SSC, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and then thrice for 10 min at 55°C in 0.2× SSC, 0.1% CHAPS. Embryos were rinsed in KTBT (50 mmol $l^{-1}$ Tris-HCl pH 7.5, 150 mmol $l^{-1}$ NaCl, 10 mmol $l^{-1}$ KCl, 1% Triton X-100) at room temperature for 10 min and preblocked in 20% fetal calf serum (FCS) in KTBT for at least 2 h at 4°C. Embryos were incubated overnight at 4°C on a rocking platform in 1:2000 diluted anti-DIG alkaline phosphatase-conjugated Fab fragments (Roche Diagnostics, Laval, QC, Canada) in 20% FCS in KTBT. Embryos were washed five times for 1 h in KTBT at room temperature and incubated overnight in KTBT at 4°C.

Chromogenic precipitate formation was carried out in 1 mmol $l^{-1}$ levamisole using either the NBT/BCIP color system (for purple precipitate) or the Fast Red/ HNPP (for red fluorescent precipitate; Roche Diagnostics). Purple-stained embryos were visualized using brightfield microscopy, while fluorescent embryos were cleared with glycerol and visualized using confocal microscopy.

The *in situ* hybridization data presented are representative of the results obtained in three repeat experiments using the purple precipitate and two repeat experiments using the Fast Red precipitate. Sense controls were included in every experiment. In each experiment, at least 10 embryos were included from each embryonic stage tested.

### Immunohistochemistry

Immunofluorescence localization of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors was performed on histological sections from the CNS of mature snails and whole-mounts of cultured identified neurons. The intact CNS or subsets of CNS ganglia were dissected out of mature snails as previously described (Young et al., 1999), fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, and washed three times in PBS for 60 min at room temperature. The tissues were dehydrated through a series of ethanol into toluene, embedded in paraffin wax, cut into 10 μm sections, mounted on slides and incubated for 24 h at 37°C. The sections were washed three times with toluene to remove the paraffin, rehydrated though a series of ethanol and rinsed three times with PBS over 30 min. Primary antibodies to 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ were raised in rabbits against peptides derived from intracellular loop sequences of the respective receptor proteins, as described previously (Doran and Goldberg, 2004). The antibodies were diluted 1:500 in blocking medium (4% horse serum, 0.1%

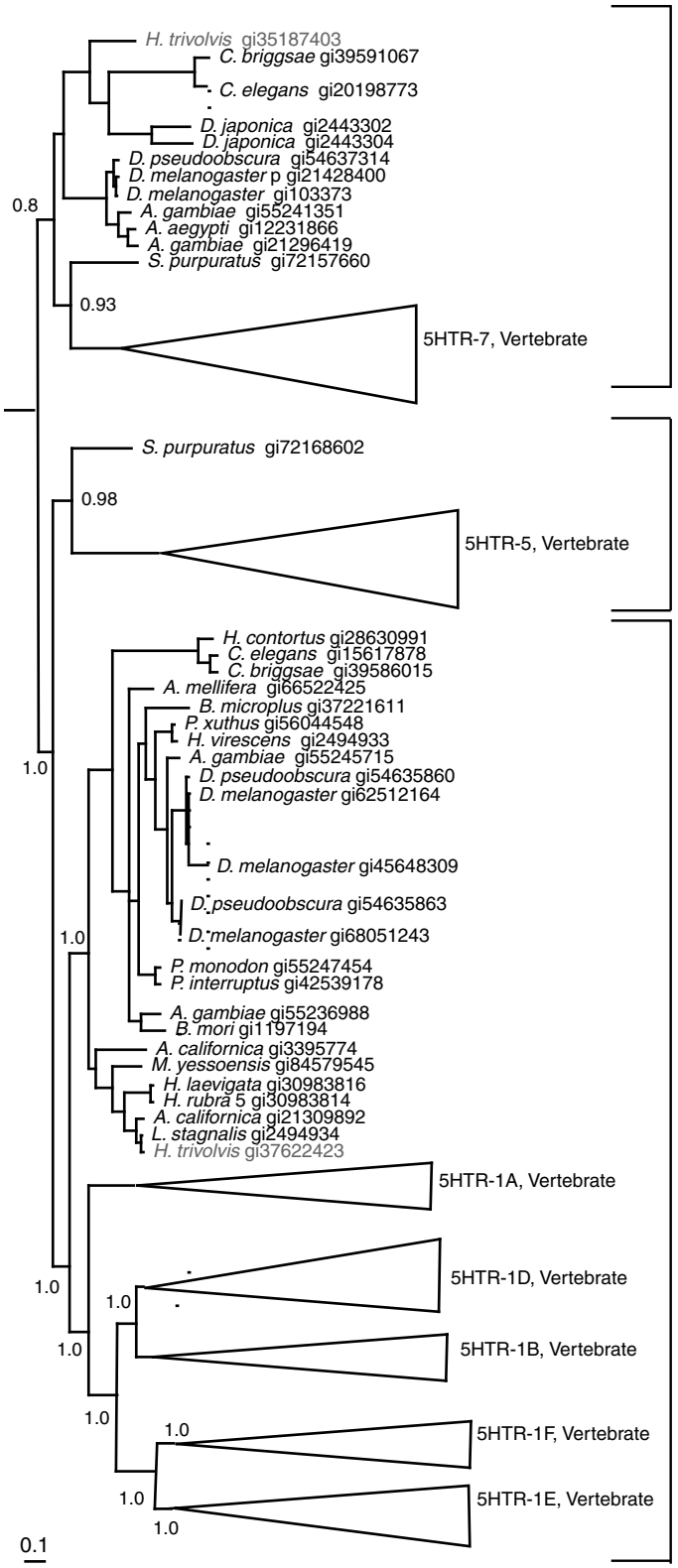


Fig. 1. Phylogenetic reconstruction illustrating the placement of *H. trivolvis* 5-HT receptor proteins as orthologs of 5-$HT_1$ and 5-$HT_7$ receptors in other animals. The tree illustrates the relationship between the two *H. trivolvis* receptors and other receptors of the 5-$HT_1$, 5-$HT_5$ and 5-$HT_7$ families. This figure represents a subtree of a larger phylogenetic tree generated from 768 biogenic amine receptors, aligned and trimmed to yield a matrix of 214 amino acid characters. For simplicity, clades encompassing a large number of vertebrate receptors are represented diagrammatically as triangles. Each sequence is represented by the name of the species from which it was obtained and a GenPept identification number that is uniquely assigned to each sequence. The scale bar represents an amount of evolutionary change corresponding to an expected 0.1 changes per site.

$NaN_3$, 0.1% Triton X-100 in 0.1 mol $l^{-1}$ PBS) and applied to sections for 24 h at 4°C under gentle agitation. The sections were washed six times in PBS over 3 h and then exposed to 1:400 diluted goat anti-rabbit IgG conjugated to Alexa 488 (Invitrogen, Carlsbad, CA, USA) for 3 h at 4°C under gentle agitation. The slides were washed six times in PBS over 3 h, mounted in 80% glycerol in PBS, and stored for 3 days at 4°C before viewing. The immunoreactivity data presented are representative of the results obtained from four different experiments on a total of 12 isolated CNS.

Immunolocalization of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ on the identified cerebral ganglion neuron C1 in culture was done according to the protocol used to detect 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ immunoreactivity in cultured embryonic ciliary cells (Doran and Goldberg, 2004). Neuron C1 was isolated and cultured according to the methods of Price and Goldberg (Price and Goldberg, 1993), with one exception. The brain-conditioned medium was prepared using *H. trivolvis* brains that were first washed for 24 h in defined medium, and then incubated in defined medium for 96 h. In control experiments, pre-immune serum from the rabbit used to generate the primary antibody replaced the primary antibody. In addition, control experiments were also performed where the primary antibody was excluded, or the primary antibody was pre-absorbed either with blocking peptides from the sequence used to generate the antibodies (negative control), or with Keyhole limpet hemocyanin, a carrier protein used to generate the antibody (positive control). Sections or whole-mounts were viewed using an Axiovert 135 fluorescence microscope (Zeiss, ON, Canada) equipped with differential interference contrast (DIC) optics. Images were collected with a Retiga Ex digital charged-coupled device camera (Q-Imaging, Burnaby, BC, Canada) linked to a Pentium 4 PC computer running Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada). The immunoreactivity data are representative of the results obtained from three repeat experiments on a total of 14 isolated C1 neurons.

## RESULTS

### Cloning of 5-HT receptors

We used nested degenerate PCR, as described by Sugamori et al. (Sugamori et al., 1993) to amplify fragments of 5-HT receptor genomic DNA. PCR products were cloned, and those with sequences similar to those of known 5-HT receptors were radiolabeled and used as probes to screen *H. trivolvis* CNS cDNA libraries. Two full-length putative 5-HT receptor cDNA clones were isolated in this manner. The sequences of these clones were deposited in GenBank [5-$HT_{1Hel}$ is under accession numbers AY395746.1 (nucleic acid) and AAQ95277.1 (protein), 5-$HT_{7Hel}$ is under accession numbers AY395747.1 (nucleic acid) and AAQ84306.1 (protein)].

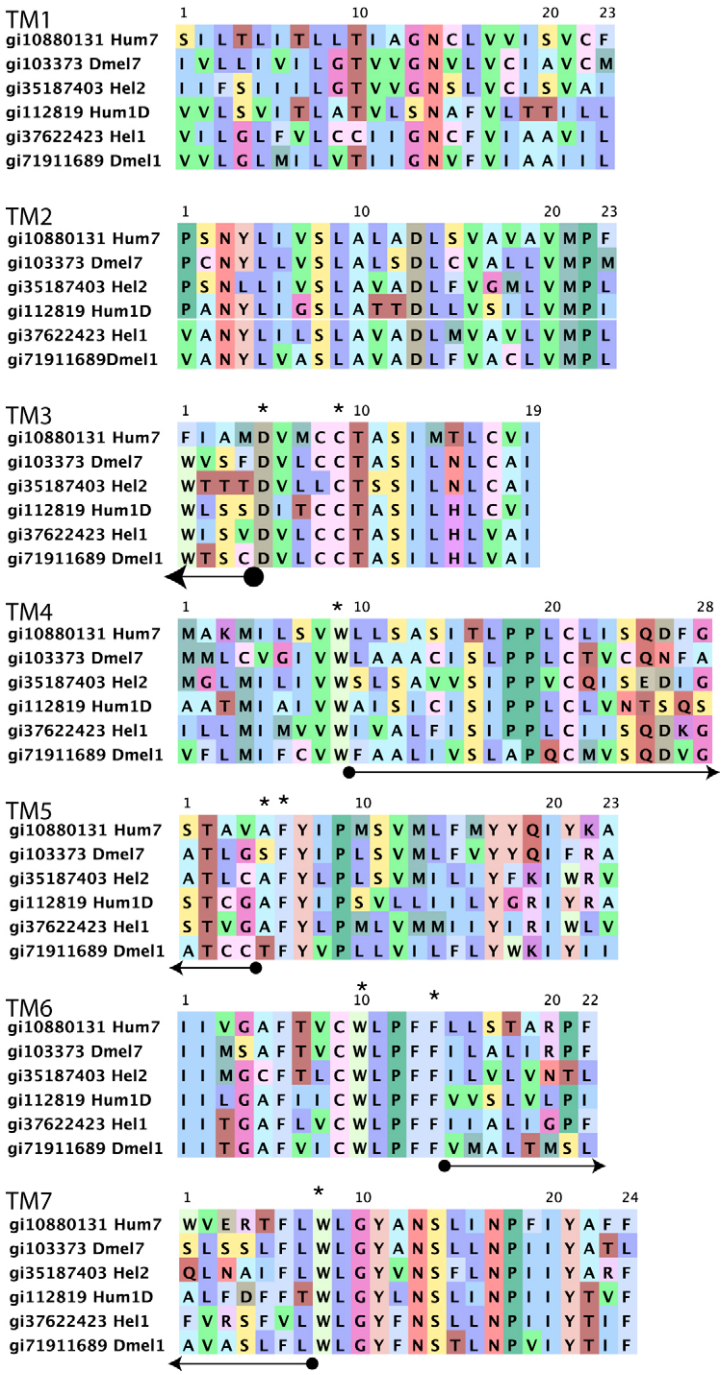


Fig. 2. Alignment of the seven predicted transmembrane helices of the two *H. trivolvis* receptors (Hel1, Hel2) with representative vertebrate (*Homo sapiens*; Hum1D, Hum7) and invertebrate (*Drosophila melanogaster*; Dmel1, Dmel7) 5-HT$_1$ and 5-HT$_7$ receptors. The conserved residues that have been shown to be involved in 5-HT binding to the receptor are marked with asterisks. The arrows along transmembrane (TM) helices 3 to 7 indicate the portion of the sequence that forms the vestibule leading from the extracellular space to the 5-HT binding site.

### Phylogenetic analysis

The amino acid multiple sequence alignment of the two *H. trivolvis* 5-HT receptor proteins with 766 biogenic amine receptors from a variety of organisms yielded a data set of 214 characters after removal of gapped regions (supplementary material Fig. S1). A consensus phylogenetic tree constructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) robustly placed one *H. trivolvis* receptor in a clade of invertebrate type 1 5-HT receptors and the other in a clade of invertebrate type 7 5-HT receptors (Fig. 1 and supplementary material Figs S2 and S3). Thus we have designated one receptor as 5-HT$_{1Hel}$ and the other as 5-HT$_{7Hel}$, based on phylogenetic relationships and the nomenclature suggested by Tierney (Tierney, 2001). The large scale of the phylogenetic analysis supports a robust placement of the 5-HT$_5$ family of receptors as the sister group of the 5-HT$_1$ family, with the 5-HT$_7$ receptor family being the sister group of the 5-HT$_1$/5-HT$_5$ clade. To confirm that the phylogenetic partitioning was not due to an artifact arising from aligning a large number of sequences with widely varying sequence, we performed a similar analysis using selected examples of Type 1, 5 and 7 receptors and two cnidarian receptor sequences as an out group. This alignment of 26 sequences yielded 252 aligned positions, and a phylogenetic tree (not shown) that was completely compatible with the type1,5,7 clade from the larger analysis (Fig. 1).

Unlike the vertebrate 5-HT$_1$ receptor clade, which contains five paralogs that apparently arose in the common ancestor of vertebrates, the invertebrate 5-HT$_1$ receptor clade generally contains only single homologs from each species that is represented. In the cases where there are more than one paralogous sequence in an organism (e.g. the two 5-HT$_7$ receptors from *Dugesia japonica* and the three 5-HT$_1$ receptors from *Drosophila melanogaster*) the paralogy appears to have arisen late in evolution.

### Molecular characterization

Full-length 5-HT$_{1Hel}$ cDNA is over 4000 base pairs (bp) in length, excluding the poly-adenylated tail. The 5′ UTR is 144 bp long, the 3′ UTR is over 2000 bp and the open reading frame (ORF) is 1509 bp long, predicting a protein of 503 amino acids (aa) in length. The putative 5-HT$_{1Hel}$ receptor has seven hydrophobic helical domains that are strongly predicted by the Transmembrane Hidden Markov Model program (TMHMM) (Sonnhammer et al., 1998), with an extracellular N-terminus that is weakly predicted to contain a non-cleaved signal peptide sequence. The seven transmembrane helices align with the characteristic motifs within transmembrane regions of other seven-pass G protein-coupled receptors (Fig. 2). Interestingly, the species homolog 5-HT$_{1Lym}$ from the related pond snail *Lymnaea stagnalis* (Sugamori et al., 1993) shares a similar protein structure profile, being 508 aa long, having nearly identically sized amino- and carboxy-termini (94 aa and 19 aa, respectively, for 5-HT$_{Lym}$ compared with 95 aa and 19 aa, respectively, for 5-HT$_{1Hel}$) and comparably sized third intracellular loops (150 aa for 5-HT$_{Lym}$, 154 aa for 5-HT$_{1Hel}$).

The full-length 5-HT$_{7Hel}$ cDNA is 4295 bp in length excluding the poly-adenylated tail. The 5′ UTR is 820 bp in length, the 3′ UTR is 2034 bp long and the ORF is 1437 bp in length, predicting a protein of 479 aa. Hydrophobicity analysis based on TMHMM (Sonnhammer et al., 1998) predicts that the putative 5-HT$_{7Hel}$ protein has seven distinct transmembrane regions. Both amino- and carboxy-termini are 70 aa in length, and the third intracellular loop is 97 aa long. There is no predicted cleaved signal peptide at the N-terminus, but the N-terminus is identified as extracellular by TMHMM.

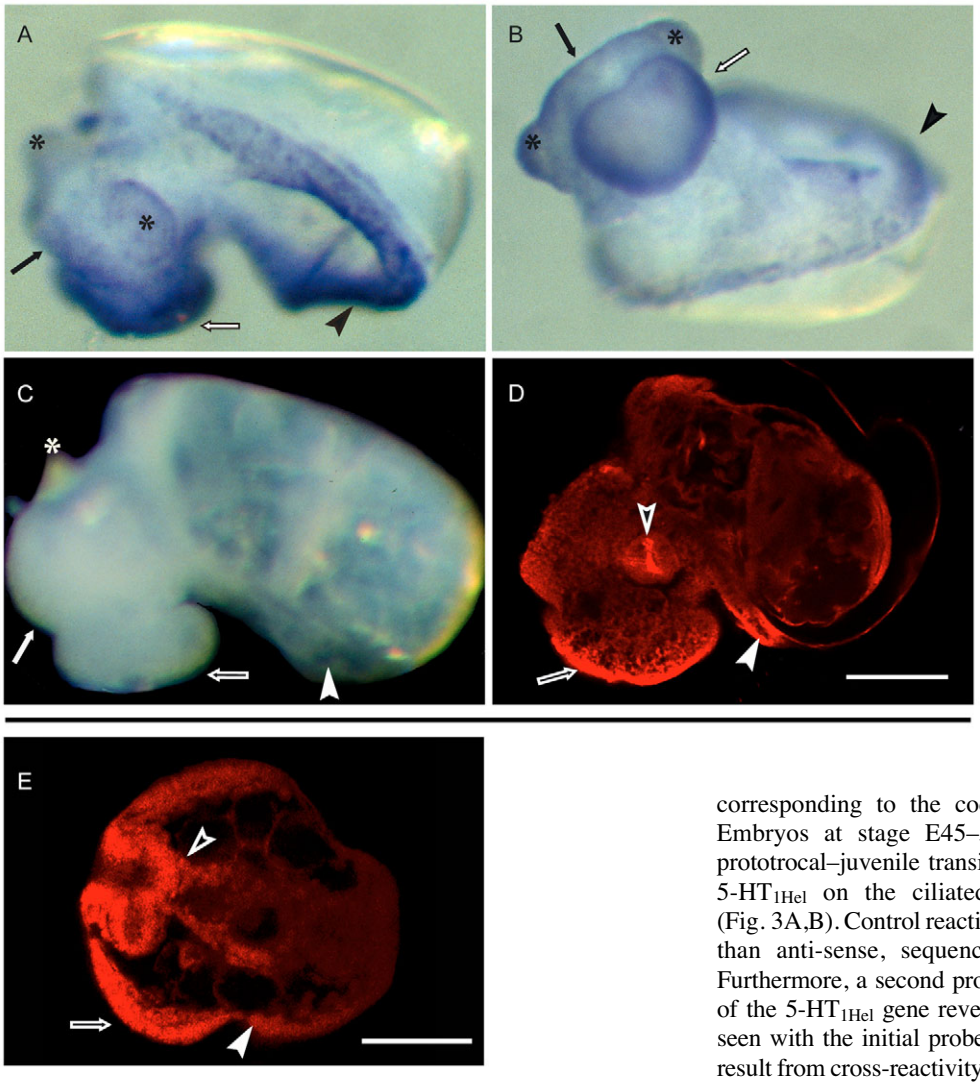


Fig. 3. Localization of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ transcripts in whole-mounts of *H. trivolvis* embryos. *In situ* hybridization of digoxigenin (DIG)-labeled anti-sense RNA probes was immunohistochemically localized with alkaline phosphatase-conjugated anti-DIG antibodies and reactants that form either a purple (A–C; light microscope) or fluorescent red (D and E; confocal microscope) precipitate. Using anti-sense probes in stage E45 embryos (A, B and D), 5-$HT_{1Hel}$ RNA expression was localized mainly to the ciliated epithelium of the foot (open arrows), anterior head (filled arrows), tentacles (*), ciliated mantle (filled arrowheads) and gut (open arrowheads). Using sense probes to 5-$HT_{1Hel}$, no signal was observed (C). In stage E30 embryos (E), 5-$HT_{7Hel}$ RNA expression was localized to the ciliated foot epithelium (open arrows), developing gut (open arrowheads), and ciliated mantle (filled arrowhead). Using sense probes to 5-$HT_{7Hel}$, no signal was observed (not shown). Scale bar, 100 μm.

The regions of both receptors that were identified as transmembrane helices aligned well with each other and with homologous regions from other 5-HT receptors (Fig. 2). The conserved residues that are involved in 5-HT binding are conserved in helices 3–7. The sequences of the helices are more conserved on the cytoplasmic side of the binding cavity than they are in the vestibule of the binding cavity that forms the pathway for 5-HT to reach the ring of binding residues. The DRY (aspartate–arginine–tyrosine) sequence motif that is found at the cytoplasmic boundary of the third transmembrane helix of essentially all biogenic amine receptors (D and R are both present in more than 99% of the sequences and Y is present in more than 95% of the sequences) is present in these two receptor proteins.

### Localization in embryos and the CNS of adult *H. trivolvis*

Localization of the 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ gene transcripts was initially examined using *in situ* hybridization in *Helisoma* embryos, where 5-HT has been shown to be involved in the regulation of ciliary beating (Diefenbach et al., 1991; Kuang and Goldberg, 2001), neurite outgrowth (Diefenbach et al., 1995; Goldberg and Kater, 1989; Goldberg et al., 1991; Goldberg et al., 1992) and neuronal intracellular calcium concentration (Goldberg et al., 1992). The RNA DIG labeled probe for 5-$HT_{1Hel}$ was based on the full-length insert corresponding to the coding region between TM3 and TM6. Embryos at stage E45–50, which represents the end of the prototrocal–juvenile transition, displayed consistent expression of 5-$HT_{1Hel}$ on the ciliated foot, tentacles and ciliated mantle (Fig. 3A,B). Control reactions using the corresponding sense, rather than anti-sense, sequences revealed no expression (Fig. 3C). Furthermore, a second probe that was generated from the 3′ UTR of the 5-$HT_{1Hel}$ gene revealed a similar expression pattern to that seen with the initial probe, suggesting that the expression did not result from cross-reactivity with other receptor gene sequences. This latter probe was revealed using Fast Red labeling of anti-DIG immunoreactivity and confocal microscopy, confirming that the whole-mount techniques were sufficient to reveal staining in the interior of the embryos (Fig. 3D). This approach revealed additional expression where the neurites of serotonergic neurons innervate foot ciliary cells (Koss et al., 2003), as well as within the gastrointestinal tract.

*In situ* hybridization experiments on the 5-$HT_{7Hel}$ receptor gene revealed a similar expression pattern to that described for 5-$HT_{1Hel}$ (Fig. 3E). Confocal optical sectioning of Fast Red–labeled embryos showed expression of 5-$HT_{7Hel}$ in the region where the pedal cilia are innervated by the ENC1 neurons, as well as in the gastrointestinal tract and at the ventral mantle (Fig. 3E). Expression was also observed in the primordial tentacles, whereas no expression was observed when a sense probe was used (data not shown).

The expression of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors was also characterized in histological sections of the CNS from mature snails. Using antibodies raised against peptides from within distinct intracellular loop regions of the receptor proteins (Doran and Goldberg, 2004), immunohistochemistry revealed widespread expression of both receptors throughout the CNS (Figs 4 and 5). Whereas neuritic staining of both receptor proteins occurred in the neuropile, connectives and peripheral nerves in every ganglion of the CNS, neuronal somata appeared unstained in all cases. The absence of cell body staining precluded an analysis of the expression

profile in known identified neurons. Interestingly, when cerebral ganglion neuron C1 was isolated and grown in cell culture, 5-$HT_{1Hel}$ (Fig. 5C–E) and 5-$HT_{7Hel}$ (data not shown) immunoreactivity was observed primarily in the cell body. The widespread expression of the 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors in both embryos and mature snails suggests that these receptors may mediate some of the well-established developmental and physiological actions of 5-HT.

## DISCUSSION

Studies on the *H. trivolvis* model system have made a major contribution to the understanding of diverse functions of the 5-HT system from molecular, cellular, developmental and physiological perspectives. Both to further explore this broader question at the molecular level and to provide experimental knockout tools to test functional hypotheses, our objective in the present study was to identify 5-HT receptor genes from the *H. trivolvis* genome, and to provide an initial characterization of the molecular structure and expression profile of the 5-HT receptor gene products. Through a molecular cloning strategy that relied upon the structure of the first 5-HT receptor gene cloned from the pond snail *Lymnaea stagnalis* (Sugamori et al., 1993), we cloned two 5-HT receptor genes from *Helisoma trivolvis*. The deduced gene products both appear to be G protein-coupled 5-HT receptors, with typically well-conserved structure in the functional domains and high variability in the vestibule entrance of the receptor protein. Our phylogenetic analysis placed these receptors in the 5-$HT_1$ and 5-$HT_7$ families of 5-HT receptors, respectively, and they are thus named the 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors according to the nomenclature system described by Tierney (Tierney, 2001). *In situ* hybridization and immunofluorescence studies revealed that these genes and gene products are expressed in a variety of embryonic and mature tissues, including widespread expression in a subset of neurites, but not cell bodies, throughout the CNS.

### Phylogenetic relationships of the 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors

Phylogenetic analysis clearly identifies the two new receptors as homologs of type 1 and type 7 5-HT receptors from other

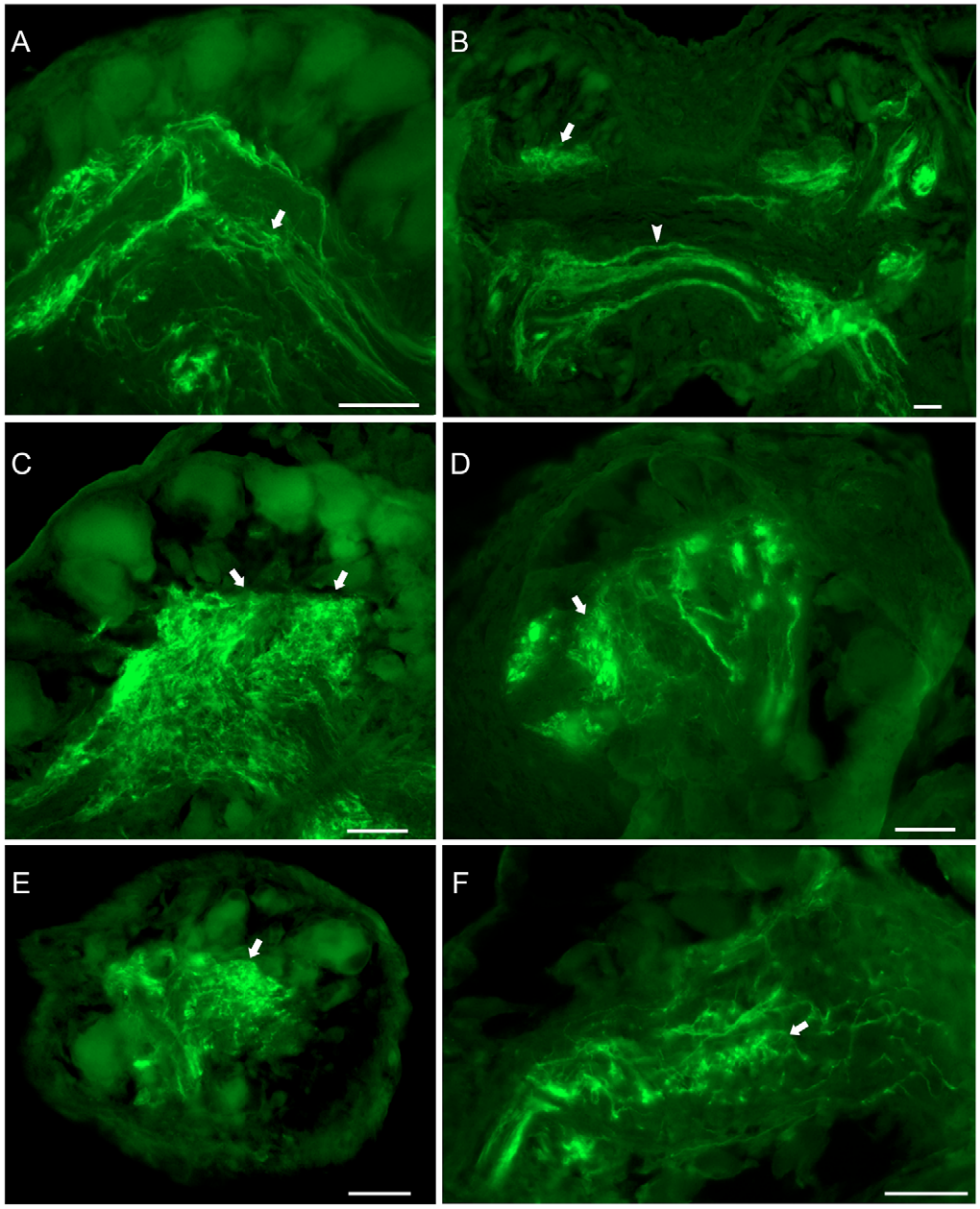


Fig. 4. Immunofluorescence expression of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ in the CNS of mature *H. trivolvis*. 5-$HT_{1Hel}$-like immunoreactivity (A) and 5-$HT_{7Hel}$-like immunoreactivity (B–F) was observed in select neurites within neuropile (arrows) and neurite tracts (arrowhead) in all ganglia of the CNS. The expression observed in the cerebral ganglia (A and B), pleural ganglia (C and D) and buccal ganglia (E and F) was representative of the pattern seen in all ganglia. Immunoreactivity was not observed in neuronal somata. Scale bar, 30 μm.

organisms. Not unexpectedly, 5-HT$_{1Hel}$ grouped entirely within a molluscan clade of 5-HT$_1$ receptor genes, with the 5-HT$_{1Lym}$ receptor being the closest ortholog. The non-molluscan 5-HT$_1$ receptor sequences in our analysis were from arthropod and nematode species. In every case, sequences from the same phylum were more similar to each other than to those from different phyla. Furthermore, arthropod and nematode sequences were more closely related to each other than they were to the molluscan sequences, as expected from the ecdysozoan relationship between arthopods and nemotodes. The position of the 5-HT$_{1Hel}$ gene in the phylogentic tree produced in our analysis supports the hypothesis that a common ancestor of the ecdysozoans and lophotrochozoans contained 5-HT$_1$ receptors.

Interestingly, the phylogenetic relationships for 5-HT$_7$ genes were quite different, with 5-HT$_7$ genes from various insect species grouping together as expected, but the two nematode genes were more related to 5-HT$_{7Hel}$ and planarian genes than to the insect genes. 5-HT$_7$ sequences from additional arthropods, nematodes, planarians and molluscs are required to confirm this surprising separation of insect and nematode genes.

### Expression and function of 5-HT$_{1Hel}$ and 5-HT$_{7Hel}$ receptors in *H. trivolvis*

Invertebrates have long been known to express a wide variety of 5-HT receptors, based on the variety of cellular responses, signal transduction elements and pharmacological profiles associated with the actions of 5-HT (Peroutka, 1994; Tierney, 2001). However, only after the primary structure of many different 5-HT receptor proteins was revealed through molecular cloning did it become evident that most, if not all, invertebrate 5-HT receptors belong to one of the seven major families of mammalian 5-HT receptors. It is not yet clear whether all invertebrate 5-HT receptors in a particular family couple to the same signal transduction elements (see below). Furthermore, the pharmacological profile of specific invertebrate receptors is less likely to match that of their mammalian homologs, as the changes in molecular structure during 600 million years of evolution beyond the divergence of invertebrates and vertebrates would probably result in altered receptor binding characteristics (Tierney, 2001).

Several invertebrate 5-HT$_1$ receptor genes have been cloned to date, including at least seven from molluscan species. Of particular

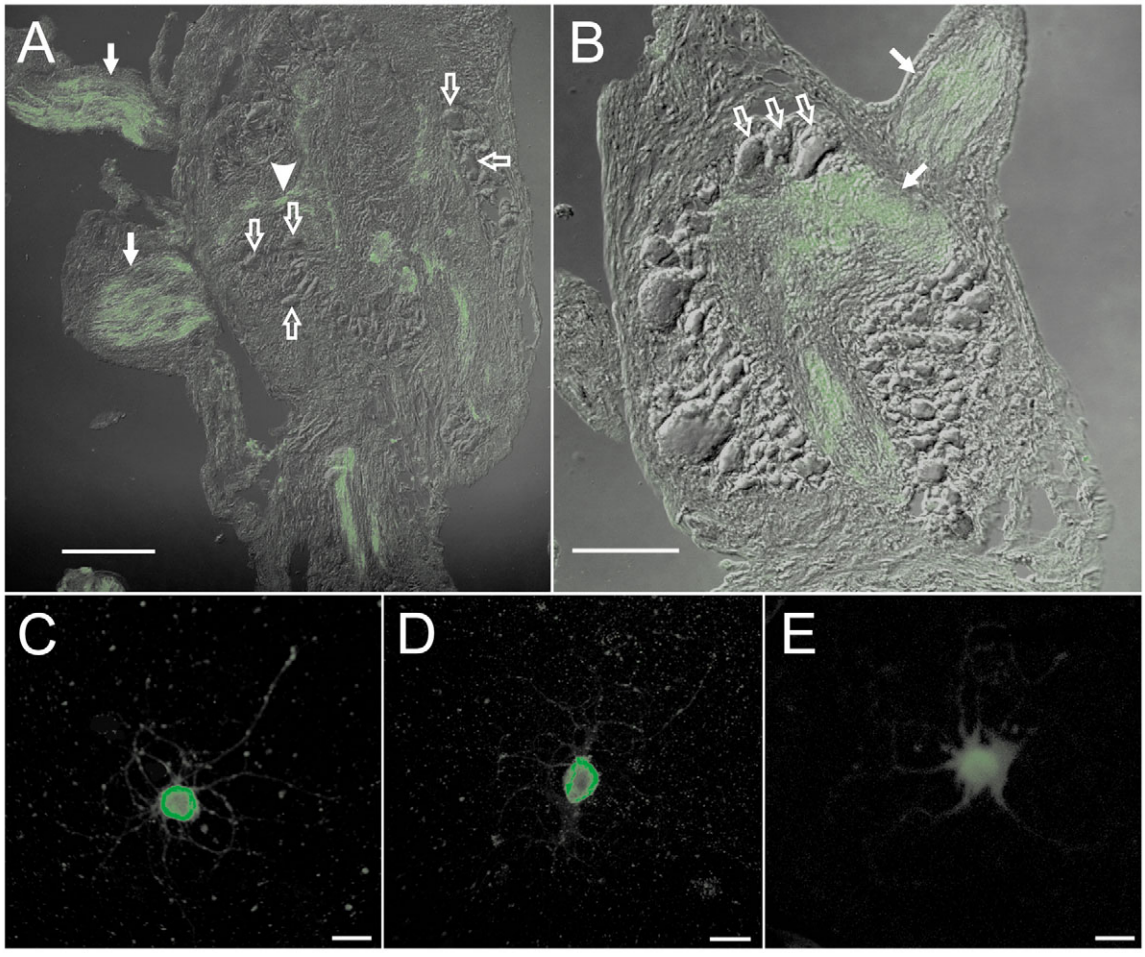


Fig. 5. *In situ* and cultured neurons display different cellular localization of 5-HT$_{1Hel}$ and 5-HT$_{7Hel}$ receptors. *In situ*, 5-HT$_{1Hel}$-like immunoreactivity (A) and 5-HT$_{7Hel}$-like immunoreactivity (B) was restricted to neurites in neuropile (filled arrows) and neurite tracts within connectives and nerves (arrowhead; also see Fig. 4). Cell bodies (open arrows) were always unstained as revealed by overlaying the differential interference contrast pattern on the fluorescence signal. In contrast, after isolation and culture of cerebral neuron C1, 5-HT$_{1Hel}$-like immunoreactivity occurred primarily in the cell body (C and D). Preabsorption of antibody with Keyhole limpet hemocyanin (D) and replacement of antiserum with pre-immune serum (E) provided positive and negative controls for antibody specificity, respectively. Scale bar, 50 μm.

interest is the 5-$HT_{1Lym}$ receptor cloned from *Lymnaea stagnalis* (Sugamori et al., 1993), which provided the template for the original primers used in the present study. While a phylogenetic analysis suggested that this receptor also fell into the 5-$HT_1$ family of receptors, binding analysis indicated a mixed pharmacology relative to the 5-$HT_1$ profile of vertebrate receptors, typical for invertebrate 5-HT receptors (Goldberg et al., 1994). In contrast to 5-$HT_1$ receptors, the only invertebrate 5-$HT_7$ receptor genes cloned to date are from arthropod, roundworm and possibly flatworm species (Hobson et al., 2006; Pietrantonio et al., 2001; Witz et al., 1990). Therefore, the present study is the first to describe the molecular structure of a 5-$HT_7$ receptor from a molluscan species.

The molecularly identified invertebrate 5-HT receptors generally couple to their respective effector systems in a similar way to their vertebrate homologs (reviewed by Tierney, 2001). Similar to the vertebrate 5-$HT_1$ receptors, activation of invertebrate 5-$HT_1$ receptors decreases cyclic AMP levels through $G_{\alpha i}$ protein-mediated inhibition of adenylate cyclase. Likewise, both vertebrate and invertebrate 5-$HT_7$ receptors couple to $G_{\alpha s}$ proteins, causing activation of adenylate cyclase and cyclic AMP production. There are, however, some invertebrate 5-HT receptors that do not belong in any of the seven 5-HT receptor families, even though they couple normally to an effector pathway. For example, the 5-$HT_{AAp}$ and 5-$HT_{BAp}$ receptors cloned from *Aplysia* do not group in any of the known families, even though they activate the enzyme phospholipase C (PLC), a coupling characteristic of 5-$HT_2$ receptors (Li et al., 1995). Likewise, the MOD-1 5-HT receptor of *C. elegans* is a ligand-gated chloride channel that does not appear to be a member of the 5-HT3 family of ligand-gated 5-HT receptors (Ranganathan et al., 2000).

Although confirmation of the coupling characteristics of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors awaits functional analyses performed both in an expression system and *in situ*, it is reasonable to hypothesize that these receptors act in the same manner as their vertebrate and invertebrate homologs. Evaluation of various 5-HT-mediated responses in *H. trivolvis* therefore allows for a tentative association of the receptors cloned in this study with particular response pathways. One such pathway is the 5-HT-induced inhibition of neurite outgrowth in buccal ganglion neuron B19 (Haydon et al., 1984; Price and Goldberg, 1993). When regenerating in cell culture, the growth cone motility and neurite elongation of neuron B19 are reversibly inhibited by 5-HT. This response is thought to be transduced by a cascade involving the activation of adenylate cyclase and elevation of cyclic AMP levels, activation of cyclic nucleotide-gated sodium channels, depolarization and action potential activity, influx of extracellular calcium, activation of calcium/calmodulin-dependent protein kinase (Polak et al., 1991), and cytoskeletal rearrangement (Welnhofer et al., 1999). This scheme leads to the prediction that 5-$HT_{7Hel}$ receptors may mediate this response through their adenylate cyclase-stimulating activity. Unfortunately, both the anti-5-$HT_{1Hel}$ and anti-5-$HT_{7Hel}$ antibodies only stained neurites in the intact CNS, such that the absence of soma staining precluded a determination of whether the identifiable neuron B19 expressed these receptors. Staining cultured B19 neurons, or staining *in situ* preparations after neuron B19 has been injected with a fluorescent marker, could possibly reveal whether the 5-$HT_{7Hel}$ receptors are expressed in these neurons. Interestingly, neither 5-$HT_7$ receptors nor members of the 5-$HT_4$ or 5-$HT_6$ receptor families, all of which induce elevations in cyclic AMP levels, have been cloned in *Aplysia californica*, in which serotonergic pathways involving elevations in cyclic AMP have been well characterized (Barbas et al., 2003).

Another well-studied 5-HT pathway in *Helisoma* underlies a behavioural response to hypoxia during embryonic development. *Helisoma* embryos contain a pair of unique sensorimotor neurons that detect hypoxia and stimulate ciliary beating in postsynaptic ciliary cells through the release of 5-HT (Kuang et al., 2002; Kuang and Goldberg, 2001). Cell culture studies have revealed that the cilioexcitatory response to 5-HT occurs through a highly complex signal transduction pathway that may include multiple 5-HT receptor subtypes (Doran and Goldberg, 2004), PLC and protein kinase C (PKC) activation (Christopher et al., 1999; Doran and Goldberg, 2006), constitutive nitric oxide activity (Doran et al., 2003) and release of calcium from intracellular stores (Christopher et al., 1996; Doran and Goldberg, 2004). Since inhibitors of PLC or PKC only partially block the cilioexcitatory response (Christopher et al., 1999; Doran and Goldberg, 2006), there may be more than one type of 5-HT receptor expressed on ciliary cells, with each one mediating a component of the entire response through different pathways. The current *in situ* hybridization experiments and previous immunofluorescence experiments (Doran et al., 2004) together suggest that both 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors are expressed in embryonic ciliary cells, thus supporting the multi-receptor model. However, the expected coupling mechanisms of these types of 5-HT receptors do not easily fit into what is currently known about the signal transduction of the cilioexcitatory response. Previous experiments indicated that the response is not mediated by an elevation of cyclic AMP levels, which would be expected from a 5-$HT_7$ receptor (Christopher et al., 1996). Furthermore, the possibility that a 5-$HT_1$-mediated decrease in cyclic AMP levels is involved in the response has not yet been explored. One possibility is that while an elevation in cyclic AMP may not be involved in producing the primary response, it is involved in producing the long duration plateau in the response that persists beyond the removal of 5-HT (Gallin et al., 2006), as well as the response facilitation that occurs upon repeated exposure of the animal to anoxia (Kuang et al., 2002). Determining whether the 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors couple typically to the cyclic AMP system or operate through atypical signal transduction pathways, and their specific roles in the cilioexcitatory responses to 5-HT, awaits a functional examination of expressed receptors and molecular knockout experiments on ciliary cells.

The *in situ* hybridization experiments in this study and previous immunolocalization experiments on embryos (Doran et al., 2004) revealed widespread expression of both receptor subtypes in all ciliated regions. Whether this expression indicates roles for these receptors in the regulation of ciliary activity, as proposed above, or more fundamental roles in regulating the development of these tissues remains to be determined. Expression was also observed in regions of the embryo associated with neural tissue, such as the regions containing ENC1 somata at early stages of embryonic development (data not shown), and the regions where ENC1 neurons innervate pedal ciliary cells at later stages (Fig. 3). In the adult CNS, both receptor proteins were expressed widely and selectively in neurons throughout all ganglia of the CNS, corroborating the data from embryos that both of these are neural receptors. To our surprise, CNS expression was entirely limited to neurites within neuropiles and connectives, with none seen in neuronal somata. Although our preliminary Western blot experiments did not confirm that each antibody recognizes only a single antigen, the striking restriction of CNS immunoreactivity to neurites argues that the antibodies were highly selective for receptor proteins. In an autoradiographic study of lysergic acid diethylamide binding in the CNS of *Aplysia californica*, Kadan and Hartig (Kadan

and Hartig, 1988) reported that 5-HT receptors were most prominently localized to the neuropile, with relatively few neurons displaying somatal expression. In the present study on tissue sections, the intense immunoreactivity seen in numerous neurites suggests that the absence of cell body staining correctly reflected an absence of actual expression. Perhaps the small number of labeled cell bodies seen in *Aplysia* represents the expression of a 5-HT receptor subtype distinct from 5-$HT_1$ or 5-$HT_7$ receptors. On the other hand, the expression of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ immunoreactivity in the cell body of cerebral ganglion neuron C1 only when it was regenerating in culture suggests that expression in the cell body may be more likely to occur under conditions of neuronal growth, such as during development or regeneration. This will be tested in future immunolocalization experiments on *Helisoma* embryos to confirm the expression in embryonic neuronal somata. In any event, the widespread neuritic expression of 5-$HT_{1Hel}$ and 5-$HT_{7Hel}$ receptors throughout the CNS supports the hypothesis that these molecules are critical to numerous physiological processes in the *Helisoma* CNS.

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