Localization of Serotonin in the Nervous System of Biomphalaria glabrata, an Intermediate Host for Schistosomiasis

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ABSTRACT

The digenetic trematode Schistosoma mansoni that causes the form of schistosomiasis found in the Western Hemisphere requires the freshwater snail Biomphalaria glabrata as its primary intermediate host. It has been proposed that the transition from the free-living S. mansoni miracidium to parasitic mother sporocyst depends on uptake of biogenic amines, e.g. serotonin, from the snail host. However, little is known about potential sources of serotonin in B. glabrata tissues. This investigation examined the localization of serotonin-like immunoreactivity (5HTli) in the central nervous system (CNS) and peripheral tissues of B. glabrata. Emphasis was placed on the cephalic and anterior pedal regions that are commonly the sites of S. mansoni miracidium penetration. The anterior foot and body wall were densely innervated by 5HTli fibers but no peripheral immunoreactive neuronal somata were detected. Within the CNS, clusters of 5HTli neurons were observed in the cerebral, pedal, left parietal, and visceral ganglia, suggesting that the peripheral serotonergic fibers originate from the CNS. Double-labeling experiments (biocytin backfill × serotonin immunoreactivity) of the tentacular nerve and the three major pedal nerves (Pd n. 10, Pd n. 11, and Pd n. 12) disclosed central neurons that project to the cephalopedal periphery. Overall, the central distribution of 5HTli neurons suggests that, as in other gastropods, serotonin regulates the locomotion, reproductive, and feeding systems of Biomphalaria. The projections to the foot and body wall indicate that serotonin may also participate in defensive, nociceptive, or inflammation responses. These observations identify potential sources of host-derived serotonin in this parasite–host system. J. Comp. Neurol. 000:000–000, 2012.

INDEXING TERMS: Schistosoma mansoni; pulmonate mollusk; pond snail; miracidium

The parasitic disease schistosomiasis is estimated to presently affect more than 207 million people worldwide (World Health Organization, 2011) and its economic impact on developing countries is second only to malaria (Hotez, 2008; King, 2010). The digenetic trematode blood flukes that cause schistosomiasis require specific aquatic snail species to serve as intermediate hosts, where asexual reproduction generates the cercariae that are capable of infecting their vertebrate definitive hosts. Within the definitive host, schistosomes reproduce sexually to produce eggs that release free-living miracidia into aquatic habitats where they in turn infect the intermediate snail host.

The trematode species Schistosoma mansoni that causes the form of human schistosomiasis found in the Western Hemisphere employs the planorbid snail Biomphalaria glabrata as its major intermediate host (Rollinson and Chappell, 2002; Bayne, 2009; Toledo and Fried, 2010). Early investigations reported the presence of serotonin in B. glabrata, and advanced the hypothesis that biogenic amines could stimulate miracidium swimming and attraction to the foot and tentacle regions of the snail (Chiang et al., 1974; Etges et al., 1975). Moreover, transition from the miracidium to the parasitic primary sporocyst form of S mansoni that occurs within the Biomphalaria integument is proposed to require uptake of serotonin from the snail host (Boyle et al., 2000, 2003; Grant sponsor: National Institutes of Health; Grant numbers: RCMI RR-03051, NIGMS MBRS: GM-08224, GM-061838; Grant sponsor: National Science Foundation; Grant number: DBI-0115825.

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Yoshino et al., 2001; Boyle and Yoshino, 2005). Finally, serotonergic signaling is considered to represent a potential target for parasite manipulation of B. glabrata behavior (Manger et al., 1996; Santhanagopalan and Yoshino, 2000; Boyle and Yoshino, 2002) and snail control strategies (Muschamp and Fong, 2001). To date, however, the sources of host-derived serotonin are not well understood and the neural circuitry that controls Biophphalaria behavior remains largely unexplored.


This study explored the localization of serotonin-like immunoreactive material in the central nervous system and in peripheral tissues of B. glabrata. Emphasis was placed on identification of serotonergic neurons that could participate in the infection process by S. mansoni miracidia and their transformation to parasitic sporocysts; 2) serve as potential targets for parasite manipulation of snail behavior; and 3) provide targets for novel approaches to vector control. Preliminary reports of these observations were presented in abstract form (Delgado et al., 2010, 2011).

**MATERIALS AND METHODS**

**Specimens**

Experiments were conducted on laboratory-reared B. glabrata (6–8 mm shell diameter). These specimens were considered sexually mature, as evidenced by their capacity to lay eggs. Snails were housed in plastic aquaria at room temperature (21–23°C) and fed carrots ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico Medical Sciences Campus (Protocol #3220110).

**Nerve backfills**

Tissues were dissected in snail saline with the following composition (in mmol l⁻¹): NaCl 51.3, KCl 1.7, MgCl₂ 1.5, CaCl₂ 4.1, HEPES 5, pH 7.8. Dissected ganglia were positioned with minutien pins near a small petroleum jelly (Vaseline) enclosure (3–5 mm diameter) on the surface of a Sylgard-lined Petri dish. The nerve of interest was cut and its end was drawn into the Vaseline-lined pool. The saline was withdrawn from the pool and replaced with a saturated solution (1.4 mg / 50 μl dH₂O) of biocytin (Sigma-Aldrich, St. Louis, MO). The enclosure was sealed with Vaseline and the preparation was incubated overnight at room temperature to allow migration of the biocytin. The nerve was then extracted from the pool and the ganglia were re-pinched and washed 3–5 times with saline. Tissues were immersed in 0.5% protease (Type XIV; Sigma-Aldrich) for 30 to 40 minutes and fixed for 1 hour in cold 4% paraformaldehyde prepared in 80 mM phosphate buffer (PB: 24 mM KH₂PO₄, 56 mM Na₂HPO₄, pH 7.4) containing 24% sucrose. Following fixation, tissues were transferred to microcentrifuge tubes, washed 5 times (30 minutes each) with PTA solution (0.1 M PB containing 2% Triton X-100 and 0.1% sodium azide) and incubated in Alexa Avidin 488 (Molecular Probes, Eugene, OR) diluted 1:1,000 to 1:2,000 in PTA (24–48 hours, room temperature). The preparations were assessed daily until the quality of the backfill staining was determined to be sufficient for advancing to immunohistochemical processing.

**Wholemount immunohistochemistry**

The dissection, protease, and fixation protocols were performed as described above. Following preincubation with normal goat serum (0.8%, 3–5 hours, room temperature), tissues were immersed (1–7 days, room temperature) in a rabbit polyclonal antiserum (Sigma-Aldrich; #5545; JCN Antibody Data Base v. 7, #4516) diluted 1:2,000 in PTA. Following repeated PTA washes (5 times, at least 30 minutes each, room temperature), ganglia were incubated in secondary antibodies conjugated to fluorescent markers (Alexa 488 goat antirabbit IgG (H+L) conjugate or Alexa 546 goat antirabbit IgG (H+L); Molecular Probes). The second antibody dilutions ranged from 1:1,000 to 1:600 μl and incubation times ranged from 2–10 days. Preparations were examined on a Nikon Eclipse or on a Zeiss Pascal laser scanning confocal microscope (LSCM). Images were captured with the Nikon ACT-1 (v. 2.10) software of the Eclipse or the Zeiss LSM 5 Image Browser (v. 3.1.0.11) program of the LSCM. In most cases, confocal imaging was performed from both the dorsal and ventral surfaces to a depth of 100–150 μm (2–10 μm optical sections), depending on the region of interest. Stacks, z-series, overlays, and calibrations were generated using the ImageJ Program (v. 1.43u, NIH public domain). Images were imported to CorelDraw 10 or Microsoft PowerPoint v. 14.0.0 files for addition of labels, adjustment of brightness/contrast, and organization of panels.

**Controls, nomenclature, and measurements**

The immunogen, serotonin creatinine complex was conjugated with formaldehyde to bovine serum albumin
observed in a minimum of three specimens. All results reported in this study were specified to signify that the serotonergic neurons represent, in most cases, a subset of the entire cluster (see Newcomb et al., 2006). These labels did not further specify the position of the cluster (e.g., dorsal vs. ventral, medial vs. lateral) within the ganglion. Cells were counted on the Nikon fluorescence microscope directly from mounted ganglia. All results reported in this study were observed in a minimum of three specimens.

RESULTS

The general topography of the central ganglia of Biomphalaria is similar to other pulmonates (Slade et al., 1981; Benjamin and Winlow, 1981; Croll and Chiaisson, 1989) and Helisoma trivolvis (Syed et al., 1993). Cluster labels included the ganglion (abbreviated and italicized: cerebral, Ce; pedal, Pe; pleural, Pl; parietal, Pa; visceral, V; buccal, B) and a designation (S) to signify that the serotonergic neurons represent, in most cases, a subset of the entire cluster (see Newcomb et al., 2006). These labels did not further specify the position of the cluster (e.g., dorsal vs. ventral, medial vs. lateral) within the ganglion. Cells were counted on the Nikon fluorescence microscope directly from mounted ganglia. All results reported in this study were observed in a minimum of three specimens.

Cerebral ganglia

Serotonin-like immunoreactivity was observed in four groups of neurons in each cerebral hemiganglion (Fig. 2). Three clusters, CeSB, CeSC, and CeSF, were visible from the dorsal surface (Fig. 2A) and an additional group, CeSD, was observed when preparations were viewed from the ventral surface (Fig. 2B). Frames from a z-stack were examined to estimate the distance of each cluster from the two surfaces of the ganglion. The CeSD cluster was located most superficially on the ventral surface (Fig. 2C). While the CeSB and CeSC clusters were near the dorsal surface, they were commonly observed in sections that were 30–80 μm below the most superficial level, reflecting both their depth and the convex curvature of the surface (Fig. 2D,E). The CeSF cluster was located on the most dorsal surface (Fig. 2E).

No major asymmetries were detected in the distribution of serotonin-like immunoreactive neurons between the two cerebral hemiganglia. The CeSB cluster was composed of 4 to 6 neurons, one of which was significantly larger (40–50 μm) than the others (10–20 μm). The large CeSB neuron, which was found to project to the buccal ganglion (Fig. 3A,B), was designated C1 due to its proposed homology with identified neurons in other gastropods (Sakharov, 1976; Granzow and Rowell, 1981; Kemenes et al., 1989; see Discussion). The CeSC cluster was composed of 4 to 7 small (10–20 μm) cells located slightly anterior to the juncture of the cerebral commissure with each cerebral hemiganglion. The CeSD cluster, the only serotonergic cluster on the ventral surface of the ganglion, consisted of 6 to 10 moderately sized (20–30 μm) neurons. The dorsal CeSF cluster was composed of five brightly stained neurons of varied sizes (20–40 μm in diameter). The axons of most CeSF cluster neurons could be followed as they projected in the anterior direction. These axons then turned sharply, contributing to a prominent fiber bundle projecting toward the two cerebral-pedal connectives (observed in Fig. 2B).

Double-labeling experiments were conducted to explore the projection patterns of cerebral serotonergic neurons. Backfills of the cerebral-buccal connective (c-b c.; Fig. 3A) labeled neurons in the ipsi- and contralateral cerebral ganglion, the ipsilateral pleural and parietal ganglia, and the visceral ganglion. When backfilled neurons had a wide range of sizes (10–80 μm) (Fig. 4A). Labeling was blocked following preincubation of the antibody (1:1,000) with serotonin creatinine sulfate coupled to BSA with paraformaldehyde (Immunostar product #20081; 20 μg/ml; overnight; 4°C). It was not reduced following preabsorption with BSA (0.25%; overnight, 4°C).

When possible, the nomenclature for cell clusters and individual neurons were assigned to correspond with designations in more extensively studied pulmonates, such as Lymnaea stagnalis (Slade et al., 1981; Benjamin and Winlow, 1981; Croll and Chiaisson, 1989) and Helisoma trivolvis (Syed et al., 1993). Cluster labels included the ganglion (abbreviated and italicized: cerebral, Ce; pedal, Pe; pleural, Pl; parietal, Pa; visceral, V; buccal, B) and a designation (S) to signify that the serotonergic neurons represent, in most cases, a subset of the entire cluster (see Newcomb et al., 2006). These labels did not further specify the position of the cluster (e.g., dorsal vs. ventral, medial vs. lateral) within the ganglion. Cells were counted on the Nikon fluorescence microscope directly from mounted ganglia. All results reported in this study were observed in a minimum of three specimens.
preparations were processed for serotonin-like immunoreactivity, double-labeling was only detected in C1 (Fig. 3B). This finding is in agreement with observations in several gastropod species, where the sole central serotonergic innervation of the buccal circuitry originates from a single pair of giant cerebral interneurons (Weiss and Kupfermann, 1976; Croll, 1987). A second series of double-labeling experiments examined central projections to the tentacular nerve (Fig. 3C–E). These experiments showed that neurons of the CeSD cluster project to the ipsilateral tentacular nerve. Double-labeling was not detected in any additional neurons in the Ce g. or in other ganglia.

Pedal ganglia

The paired pedal ganglia contained the largest number of serotonergic neurons (Fig. 4). On the dorsal surface, a cluster of 10 to 12 neurons of varying size (10–40 µm) extended across the medial region of each hemiganglion. Based on its position and similarities to a serotonergic

**Figure 1.** *Biomphalaria* central nervous system: topography and experimental manipulations. A: The circumesophageal ring of Bassommartophoran pulmonates consists of paired cerebral ganglia (L Cer g., R Cer g.), pleural ganglia (L Pl g., R Pl g.), and parietal ganglia (L Pa g., R Pa g.). A single visceral ganglion (V g.) is located in the most posterior position of the ring and a pair of buccal ganglia (B g.) is located more anteriorly on the pharynx. The left parietal ganglion is markedly larger than the right, reflecting the sinistral orientation of major reproductive and respiratory structures in *Biomphalaria*. The paired cerebral and pedal ganglia are connected by the cerebral commissure (Cc.) and pedal commissure (Pc.), respectively. The ventral surface of the cerebral ganglia (dark shading) lies in close apposition to the dorsal surface of the pedal ganglia (lighter shading) limiting much of their accessibility and visibility. B: In one of the dissections used in this study (Figs. 4, 7–9, 11), the cerebral commissure was severed prior to fixation and the two cerebral ganglia were reflected laterally to expose the dorsal surface of the pedal ganglia. C: In the other configuration employed in this study (Figs. 2, 3, 12), the pedal commissure was severed and two pedal hemiganglia were reflected laterally. D: Low-power view of serotonin-like immunoreactivity in a nervous system prepared as illustrated in B. When viewing the dorsal surface of the pedal, parietal, and visceral ganglia, the separated cerebral hemiganglia are observed primarily from their dorsomedial aspect. E: When a preparation dissected in the configuration shown in B is observed from the opposite perspective, with the plane of focus on the ventral surfaces of the pedal, parietal, and visceral ganglia (note left—right reversal), the divided cerebral hemiganglia are viewed primarily from their dorsolateral aspect. F: Serotonin-like immunoreactivity in a wholemount CNS oriented as illustrated in C. In this example, the cerebral, parietal, and visceral ganglia are observed from their ventral aspect (note left—right reversal), and the divided pedal ganglia are viewed from a dorsolateral perspective. Scale bar = 200 µm (applies to D–F).
neuron cluster in *Lymnaea* (Croll and Chiasson, 1989), this group was designated the *PdSIa* cluster (Fig. 4A). A prominent band of 5HTII fibers passing through the pedal commissure appeared to link the two *PdSIa* clusters. As in *Lymnaea*, the *PdSIa* cluster was contiguous to an asymmetric *PdSIb* group of 25 to 30 neurons that extended in the lateral direction on the side of the reproductive organs (the left side in *Biomphalaria*). It was usually possible to distinguish the boundary between the two groups of cells, as the *PdSIb* cluster was composed of neurons that were somewhat smaller (10–20 µm) and more uniform in size than the *PdSIa* cluster (Fig. 4A). Additional unpaired large serotonergic neurons were observed in the right hemiganglion (Fig. 4A). One such cell was designated RPdD1 in view of its proposed homology to a giant serotonergic neuron that has been described in other species (Cottrell et al., 1979; Audesirk et al., 1985; Kyriakides et al., 1989; Syed et al., 1993).

Serotonin-immunoreactive neurons were present in four bilateral clusters on the ventral surface of the pedal ganglia (Fig. 4B). The largest ventral cells (30–50 µm) comprised the *PdSA* cluster lining the posteromedial border of each hemiganglion. An expansive cluster of 25 to 30 neurons extended across the entire anterior margin of each *Pd* g., from the commissure to the base of the middle pedal nerve (*Pd* n. 11). As it was not possible to partition this assembly into the three divisions (A, B, and C) described by Slade et al. (1981) in *Lymnaea*, it was...
collectively denoted PdSB. Finally, two clusters were distinguished in the lateral region of each hemiganglion, corresponding to the L region in Lymnaea (Slade et al., 1981). The more medial group, denoted PdSLa, consisted of 7 to 11 neurons and the lateral PdSLb clusters were composed of 6 to 10 cells (Fig. 4B). The PdSLb cluster was located on the lateral margin of the Pd g., where previous authors described extensions of the dorsal Ib clusters wrapping around to the ventral surface in Lymnaea (Slade et al., 1981; Croll and Chiasson, 1989). However, unlike PdSLb cells, several neurons in this group had distinctive elongated perikarya and appeared to project in the medial direction. Double-labeling experiments described below support their association with the ventral surface of the Pd g. (Fig. 4B).

**Buccal, pleural, parietal, and visceral ganglia**

No serotonin-immunoreactive cell bodies were detected in the buccal ganglion (Fig. 5A), the left and right pleural ganglia (Fig. 5B,C), or in the right parietal ganglion (Fig. 5D). Several large-caliber fibers coursed through these ganglia, giving rise to finer collaterals that produced a rich innervation of substantial portions of the neuropil core. In the left parietal ganglion, immunoreactive cell bodies covered the posterior part of the dorsal surface (Fig. 5E). A cluster of smaller cells (10–20 µm) was located near the parietal-visceral connective (LPaSD) and a group of slightly larger neurons (20–30 µm) was located more laterally (LPaSE), in the central part of the ganglion.

On the dorsal surface of the visceral ganglion, a group of smaller (10–20 µm) serotoninergic neurons (VSC) was located near the left parieto-visceral connective (Fig. 5E). Slightly more laterally, larger cells (20–40 µm) extended toward the right parieto-visceral connective (VSH, see Fig. 3. Projections from serotonergic neurons in the cerebral ganglion. A,B: Serotonergic projection to the buccal neuromuscular system originates from C1. A: Neurobiotin backfill of the cerebral-buccal connective (C-b c.) labels several cell bodies in the anterior region of the cerebral ganglion (ventral surface of the left Ce g. shown). B: After processing for serotonin-like immunoreactivity (same preparation as A), double-labeling was only detected in C1 of the CeSB cluster. The intensity of 5HTI observed in the C1 was typically weaker than the other neurons in the CeSB cluster, possibly reflecting its comparatively large nucleus or lower somatic serotonin levels. C-E: Serotonergic projection to the tentacular nerve originates in the CeSD cluster. C: Neurobiotin backfill of the tentacular nerve labeled neurons throughout the cerebral ganglia, including a cluster of tightly apposed cells near the ventral surface of the ipsilateral hemiganglion (enclosed by white dashed ellipse; right hemiganglion shown). D: After processing for serotonin-like immunoreactivity, neurons of the CeSD cluster (white dashed ellipse) appeared to correspond to the cells labeled by the tentacular nerve tracing. E: Overlay of C,D images confirmed double-labeling (cells appear yellow) of all neurons in the ipsilateral CeSD cluster. Scale bars = 50 µm in B (applies to A); 100 µm in E (applies to C,D).
Benjamin and Winlow, 1981; Syed et al., 1993). A separate group (VSA) of large neurons (30–50 µm) was located in the anterior region of the Vg. The VSA group could also be observed from the ventral perspective of the ganglion (Fig. 5F).

Innervation of the cephalopedal integument

As infection of B. glabrata by S. mansoni miracidia is commonly achieved by boring through the cephalopedal or tentacular epithelium (see Maldonado, 1967), it was of interest to examine the serotonergic innervation of these tissues. Wholemount processing for serotonin-like immunoreactivity was performed on the anterior region of the foot following a lengthwise mid-dorsal incision and removal of the buccal mass and CNS (Fig. 6). When viewed from the exterior surface, the epithelium exhibited patches of punctate 5HTli stippling (Fig. 6A). In contrast, the internal surface of the pedal epithelium was uniformly lined with a plexus of varicose serotonergic fibers (Fig. 6B). The entire muscular lining of the anterior foot, as well as the lips and more dorsal portions of the head were also innervated by a more diffuse serotonergic fiber system (Fig. 6C). No peripheral serotonergic cell bodies were detected. The remnants of the pedal nerves that remained following removal of the nervous system contained several serotonergic fibers, however, suggesting that the peripheral innervation originates from the CNS.

Figure 4. Serotonin-like immunoreactivity in the pedal ganglia. A: Dorsal surface of the paired pedal ganglia. Serotonergic neurons of varied sizes (10–40 µm) are located in the bilateral PdSlA clusters that form a band across the dorsal surface of each hemiganglion. The left PdSlA cluster is contiguous with the unilateral PdSlb cluster of smaller (10–20 µm) neurons which extend to the lateral edge of the ganglion. A bundle of immunoreactive fibers is present in the pedal commissure (Pd c.). The giant RPdD1 neuron is visible on the posterior edge of the R Pd g. B: Serotonin-like immunoreactivity on the ventral surface of the pedal ganglia (same preparation as A). Large (20–60 µm) neurons are present in the central PdSA clusters on each side of the Pd c. The bilateral PdSB clusters form prominent bands across the anterior edge of each hemiganglion and the PdSlA and PdSlb clusters of smaller (10–20 µm) neurons are located more laterally. Additional large immunoreactive cells that are not part of these clusters are observed on both surfaces of the Pe g. Scale bar = 100 µm.
This hypothesis was supported by the observation of numerous 5HTli fibers in each of the three major pedal nerves at their origin in the pedal ganglion (Fig. 6D; superior pedal n., Pd n 10; middle pedal n., Pd n 11; and inferior pedal n., Pd n 12).

Double-labeling experiments (biocytin backfill / serotonin immunohistochemistry) were performed on the three major pedal nerves to localize central serotonergic neurons that project into the foot (Figs. F7–F9). The superior pedal nerve (Pedal nerve 10), which innervates the most anterior portion of the foot (see Fig. 5C), originates from the dorsolateral region of the Pd g. (Fig. 6D). Backfills of Pd n. 10 labeled neurons throughout the CNS (Fig. 7A). After processing for serotonin-like immunoreactivity, double-labeling was only detected in the ipsi- and contralateral PdSl a clusters (Fig. 7B–H) on the dorsal surface of the pedal ganglia. Interestingly, the number of double-labeled cells in the contralateral Pd Sl a cluster (Fig. 7F–H) exceeded the number in the ipsilateral Pd g (Fig. 7C–E).

The middle pedal nerve (Pd n. 11), which innervates more central portions of the foot, originates from the ventrolateral margin of the Pd g. (see Fig. 6D). Backfills of Pd
n. 11 labeled neurons throughout the CNS (Fig. 8A). After processing for serotonin-like immunoreactivity (Fig. 8B), double-labeling was observed in the ipsilateral PdSLb cluster (Fig. 8C–E) and in one additional cell on the ventral surface of the ipsilateral Pd g. (Fig. 8F–H). No contralateral double-labeled cells were detected. Within the PdSLb cluster, 3 to 5 of the larger neurons were consistently double-labeled. Their perikarya had an elongated form, with initial processes that were oriented toward the origin of Pd n. 11 (see Fig. 8E). The single double-labeled cell, which was located in the posteromedial quadrant of the ventral surface, had a more rounded form (Fig. 8H).

The inferior pedal nerve (Pd n. 12), which innervates more posterior regions of the foot, descends from the ventral surface of the Pd g. to reach the integument directly below the CNS (Fig. 6D). In our experiments Pd n. 12 was often reflected in the anterior direction to perform the backfill protocol and to facilitate subsequent visualization of the Pd g. (Fig. 9A). Backfills of Pd n. 12 produced labeling of neurons in all central ganglia (Fig. 9A). Subsequent immunostaining for serotonin (Fig. 9B) disclosed double-labeling in the majority of ipsilateral PdSLa neurons (Fig. 9A2,B). Two additional double-labeled cells were located in the medial quadrant of the ventral surface of the ipsilateral Pd g. (Fig. 9C–E). The position, size, and shape of the larger of these neurons resembled the medial double-labeled cell observed in backfills of Pd n. 11 (Fig. 8F–H), suggesting that this cell could project to both nerves. No contralateral double-labeled cells were observed.

Serotonergic innervation of the reproductive system

A survey of the Biomphalaria hermaphroditic reproductive system suggested the involvement of serotonin in...
F10 both female and male reproductive functions (Fig. 10). The most distal structures of the female reproductive tract, including the gonopore, spermathecum, and uterus, were innervated by a dense plexus of 5HTli fibers (Fig. 10A). In the male reproductive tract, serotonin-immunoreactive fibers were observed on the spermiduct (Fig. 10B), as well as on distal structures such as the penis retractor muscle and penis sheath that are critical determinants for the expression of male reproductive behavior (Fig. 10C). No peripheral 5HTli cell bodies were associated with the reproductive organs that were examined.

The neural control of female reproductive function in gastropods is mediated predominantly via the intestinal nerve of the visceral ganglion (see de Vlieger, 1968; Ferguson et al., 1993). Biocytin backfills of the visceral nerve labeled neurons in each of the circumesophageal ganglia. C–E: Double-labeling in the left PdSla cluster. C: Enlarged image of area enclosed by the dashed rectangle in A. Several neurons were labeled in the region of the serotonergic PdSla cluster. Only two cells (arrows) were subsequently shown to contain 5HTli. D: Enlarged image of area enclosed by dashed rectangle in B. Arrows indicate two neurons in the PdSla cluster that correspond to backfilled cells in C. E: In an overlay of C,D, only the two neurons denoted by arrows in those panels appear white. F–H: Double-labeling in the right PdSla cluster. F: Enlarged image of area enclosed by the dotted rectangle in A. Several neurons were labeled in the region of the serotonergic PdSla cluster. Seven neurons (marked by asterisks) were subsequently shown to contain 5HTli. G: Enlarged image of area enclosed by dotted rectangle in B. Asterisks mark neurons in the PdSla cluster that correspond to the backfilled cells in F. H: In an overlay of F,G, the neurons marked by asterisks in those panels appear white. Scale bars = 200 μm in B (applies to A); 50 μm in E,H (applies to C,D,F,G).
When backfilled preparations were processed for 5HTii (Fig. 11B), double-labeling was observed in two neurons near the posterior pole of the left parietal ganglion and in a cluster of superficial dorsal V g. cells belonging to the VSH cluster (Fig. 11C–E). The remaining ganglia were devoid of double-labeled neurons, with the exception of the R Pd g., where a single large serotonergic cell was located on the posterior margin of the ganglion (Fig. 11F–H). This cell corresponded to the RPdD1 neuron described above. RPdD1 was previously shown to project to the intestinal nerve in the sinistral pond snail *Helisoma trivolvis* (see Syed et al., 1993).

The central control of male reproductive structures is mediated via the penial nerve, which originates from the dorsal surface of the left cerebral hemiganglion. The majority of neurons labeled with backfills of the penial nerve (p n.) were located in clusters on the left side of the CNS (Fig. 12A). These included the prominent ventral lobe of the cerebral ganglion and a cluster of 15 to 20 cells in the pedal ganglion near the cerebral-pedal connective. When backfilled preparations were processed for 5HTii, double-labeling was only observed in the pedal ganglion (Fig. 12B–E). These cells belong to the PdSlb cluster described above (see Fig. 4A), the major asymmetric serotonergic...
cluster in the Pd g. Notably, not all of the PdS1b neurons were backfilled and not all of the neurons backfilled in this region were serotonergic (Fig. 12E).

**DISCUSSION**

This study surveyed serotonin-like immunoreactivity in the central nervous system and peripheral tissues of *B. glabrata* (Fig. 13). Previous observations suggest participation of serotonin in the *B. glabrata–S. mansoni* host-parasite system (Etges et al., 1975; Manger et al., 1996; Boyle et al., 2000, 2003; Boyle and Yoshino, 2005). Hypotheses concerning the functional properties of the *Biomphalaria* serotonergic system are also suggested by studies conducted in related gastropods (Jing and Gillette, 2000, 2003; Katz et al., 2001; Weragoda and Walters, 2007).

**Serotonergic innervation of the cephalopedal integument**

Early investigators used microfluorimetry and histochemical fluorescence microscopy to demonstrate the presence of serotonin in the cephalopedal tissues of *B. glabrata* (Chiang et al., 1974). Etges et al. (1975) reported that *S. mansoni* miracidia were activated by serotonin.
and proposed that serotonin could form part of a complex attractant "miraxone" released by *B. glabrata* (see Cherinin, 1970). The patches of serotonin-immunoreactive punctae observed on the external surface of the foot (Fig. 6A) may reflect an anatomical substrate for serotonin release to the surrounding medium.

Several studies have demonstrated the involvement of serotonin in transformation from the free-living *S. mansoni* miracidium to the parasitic mother sporocyst stage that occurs following penetration of the skin. Infection is followed by an increased dependence on uptake of exogenous serotonin by primary sporocysts where it produces muscular contractions that promote the emergence of daughter sporocysts (Boyle et al., 2000, 2003; Boyle and Yoshino, 2005). Moreover, increased motility of the mother sporocyst is promoted via surface-exposed serotonin receptors (Boyle et al., 2000). The subcutaneous serotonergic fiber plexus and the axons innervating the muscles of the cephalopedal regions (Fig. 6B,C) could serve as sources of host-derived serotonin required for transformation of the *S. mansoni* miracidium to its parasitic sporocyst form and for its subsequent phase of multiplication.

In several opisthobranch and pulmonate species, a plexus of serotonin-immunoreactive fibers innervates the pedal sole ciliary cells that produce locomotion (Syed et al., 1988; Moroz et al., 1997; McKenzie et al., 1998). The network of subcutaneous 5HTIi fibers observed after removal of the peripheral muscles (Fig. 6B) is likely to correspond to this ciliary motor plexus. As peripheral serotonergic cell bodies are rarely detected, it is thought that this innervation originates from central neurons (Moroz et al., 1997). Longley (2008) described seven serotonergic neurons in the pedal ganglion of *Lymnaea stagnalis appressa* that give rise to the pedal sole plexus. The serotonergic neurons labeled in this study with backfills of the pedal nerves are likely to include cells that contribute to the subcutaneous pedal plexus of *Biomphalaria*.

Serotonin-immunoreactive fibers have also been observed innervating the body wall musculature of several gastropods (McPherson and Blankenship, 1991, 1994; Satterlie et al., 1995; Moroz et al., 1997; McKenzie et al., 1998). In the swimming opisthobranch *Aplysia brasiliana* and in the pteropod *Clione limacina*, serotonergic neurons project from the pedal ganglion to specialized parapodial structures that produce undulating movements (McPherson and Blankenship, 1991; Satterlie et al., 1995). In nonswimming *Aplysia* species, e.g., *Aplysia californica*, pedal serotonergic neurons potentiate motor neuron-evoked contractions in the foot and body wall (McPherson and Blankenship, 1992). In all cases, the serotonergic neurons exert modulatory actions, i.e., they enhance the contractions produced by motor neurons but do not themselves cause contractions. It was proposed that these serotonergic modulatory neurons act to enhance escape crawling following noxious stimuli (McPherson and Blankenship, 1992).

Tissue damage or inflammation caused by penetration of the cephalopedal skin of *Biomphalaria* by miracidia could stimulate release of serotonin from the subcutaneous or neuromuscular axons observed in this study. In addition to its proposed motor functions considered above, Walters and coworkers advanced the hypothesis that serotonin can induce hyperexcitability of peripheral sensory axons following noxious stimuli in *Aplysia* (Billy and Walters, 1989; Weregoda and Walters, 2007). In

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**Figure 10.** Serotonergic innervation of reproductive structures. A: Serotonergic fibers covered the distal structures of the female reproductive tract, including the spermathecum (Sp) and the uterus (Ut). The fiber network diminished abruptly near the juncture of the Ut with the nidamental gland (Ng). B: Serotonin-like immunoreactivity was observed in more proximal segments of the reproductive tract, including the spermiduct (Sd) as it coursed in contiguity with the larger oviduct (Ov), which was not innervated. C: Serotonergic fibers were also found on distal regions of the male reproductive tract, including the penis retractor muscle (Prm) and the preputium (Pp). The most intense staining was observed lining the region of insertion of the Pp by the Prm. Scale bar = 200 μm (applies to all panels).
support of this proposal, serotonergic pedal ganglion neurons with projections to the periphery were shown to respond to aversive stimuli with increased firing rates (Marinesco et al., 2004a). Moreover, levels of serotonin in the hemolymph of *Aplysia* are increased following sensitizing stimuli (Levenson et al., 1999).

**Figure 11.** Localization of serotonergic neurons that project to the intestinal nerve. A: Biocytin backfill of the intestinal nerve (Int n) labeled neurons in all of the central ganglia (dorsal surface shown). B: Serotonin-like immunoreactivity on the dorsal surface of the same preparation as A. Double-labeling was detected in two large neurons near the posterior pole of the L Pa g. and in several cells associated with the VSH cluster in the visceral ganglion (dashed rectangles in A,B). Double-labeling was also observed in the giant RPeD1 cell in the right pedal ganglion (dotted rectangles in A and B). B: Double-labeling in cells on the dorsal surface of the left parietal ganglion and the visceral ganglion. C: Enlarged image of area enclosed by the dashed rectangle in A. Backfills of the intestinal nerve labeled several neurons in the L Pa g. and the V g. D: Serotonin-like immunoreactivity, same field as C, area enclosed by dashed rectangle in A. Clusters of neurons were labeled in the L Pa g. and the V g. E: Overlay of C,D images disclosing double-labeling (white) in two large neurons at the posterior pole of the L Pa g. and in the VSH cluster of the visceral ganglion. F-H: Double-labeling in the RPeD1 neuron. F: Enlarged image of area enclosed by the dotted rectangle in A. Backfills of the intestinal nerve labeled a single giant neuron in the posterior region of the right pedal ganglion. G: Serotonin-like immunoreactivity in the same field as C (enclosed by dotted rectangle in B) marked the giant RPeD1 neuron. H: Overlay of F,G images demonstrates double-labeling in RPeD1. Scale bars = 200 μm in B (applies to A); 100 μm in E (applies to C,D); 50 μm in H (applies to F,G).
Higher-order serotonergic neurons

A large distinctive neuron in the anterior region of the cerebral ganglion has been observed in a wide range of gastropods (Cottrell and Macon, 1974; Gerschenfeld and Paupardin-Tritsch, 1974; Berry and Pentreath, 1976; Sudlow et al., 1998). While different names have been assigned to these neurons in various species (Helix, Limax: metacerebral giant cells [MCG; Kandel and Tauc, 1966; Wieland et al., 1987]; Helisoma: serotonergic cerebral cell [SCC; Granzow and Fraser Rowell, 1981]; Aplysia: metacerebral cell [MCC; see Weiss and Kupfermann, 1976], Lymnaea stagnalis: C1 or cerebral giant cell [CGC; see Alania et al., 2008]; Tritonia: C1 [Fickbohm et al., 2001]) strong arguments have been advanced proposing their homology (Sakharov, 1976; Weiss and Kupfermann, 1976; Croll, 1987). As a metacerebrum was not readily observed in the Biomphalaria cerebral ganglion, and several additional neurons of comparable dimensions were detected, we selected the nondescriptive nomenclature of C1 for the apparent homolog in B. glabrata.

The buccal ganglia lack 5HT-immunoreactive somata in all gastropods that have been examined to date (Ono and...
McCaman, 1984; Longley and Longley, 1986; Croll and Chiasson, 1989; Kemenes et al., 1989; Satterlie et al., 1995; Sudlow et al., 1998; Fickbohm et al., 2001). In all species, the C1 homologs project to the buccal ganglion where they produce modulatory actions on the feeding system (Gillette and Davis, 1977; Kupfermann and Weiss, 1981; Straub and Benjamin, 2001). As the C1 homologs are not sensu stricto participants in the feeding central pattern generator (CPG) circuit, this modulatory architecture is designated "extrinsic" (Katz, 1995, 1998). The ability of single serotonergic neurons to exert such broad and coherent actions on the circuits controlling ingestive behavior could provide precise targets for parasite-induced modification of snail feeding and growth (Williams and Gilbertson, 1983; see also de Jong-Brink et al., 2001).

The large neuron in the posterior region of the right pedal ganglion was designated RPdD1 due to its proposed homology with the RPdD1 neuron of the sinistral pulmonate *Helisoma* (Syed et al., 1993) and the dextral *Lymnaea* (Cottrell et al., 1979; Audesirk et al., 1985; Kyriakides et al., 1989). As observed in those species, the *Biomphalaria* RPdD1 axon projects through the ipsilateral pleural and parietal ganglia to the visceral ganglion, giving rise to fibers in the parietal and intestinal nerves (Fig. 11H). While the RPdD1 neuron of *Helisoma* was found to possess some synaptic interactions with interneurons involved in the control of respiratory behavior, its function remains incompletely understood (Syed et al., 1993).

The CeSF cluster of five serotonergic neurons (Fig. 2A) is noteworthy due to its possible role in responding to noxious stimuli, such as penetration of miracidia. Katz et al. (2001) described common features of a conserved cluster of five serotonergic neurons on the dorsal surface of the cerebral ganglion in several opisthobranch groups. In the nudibranch *Tritonia diomedea*, the notaspid *Pleurobranchaea californica*, and the gymnosome *Clione limacina* three of these neurons act as interneuronal neuron of the dextral *Lymnaea* (Cottrell et al., 1979; Audesirk et al., 1985; Kyriakides et al., 1989). As observed in those species, the *Biomphalaria* RPdD1 axon projects through the ipsilateral pleural and parietal ganglia to the visceral ganglion, giving rise to fibers in the parietal and intestinal nerves (Fig. 11H). While the RPdD1 neuron of *Helisoma* was found to possess some synaptic interactions with interneurons involved in the control of respiratory behavior, its function remains incompletely understood (Syed et al., 1993).

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modulators of swimming (Getting, 1981; Arshavsky et al., 1992; Katz et al., 1994; Satterlie and Norekian, 1995, 1996; Jing and Gillette, 1999). In each of these species, as well as in non-swimming opisthobranchs, the neurons in this cluster respond to noxious stimuli and appear to contribute to a general arousal state (see also Jing and Gillette, 2000; Xin et al., 2001; Marinesco et al., 2004b; Jing et al., 2009). It was proposed that the rhythmic opisthobranch swim interneurons evolved from a nonrhythmic system of serotonergic cells that was responsive to noxious stimuli and that activated several motor systems (Katz et al., 2001). While functional properties of possible homologs in pulmonates have not been examined (see Croll and Chiasson, 1989), we hypothesize that neurons in the CeSF cluster could similarly contribute to the general arousal state (increased heart rate, locomotion, and feeding) observed in Biomphalaria following infection by S. mansoni miracidia (Lee and Cheng, 1971; Williams and Gilbertson, 1983; Boissier et al. 2003).

Serotonergic neurons and the control of reproduction

The array of neuron clusters labeled with backfills of the Biomphalaria penial nerve resembled a mirror image of the pattern observed in dextral pulmonates (Li and Chase, 1995; de Boer et al., 1996; Koene et al., 2000). While the prominent cluster of neurons at the origin of the penial nerve in the cerebral ganglion corresponds to the cerebral extension of the sinistral snail Helisoma trivolvis (Young et al., 1999), we adopted the more widely employed nomenclature of ventral lobe (see Koene, 2010). Previous investigators showed that serotonergic pharmacological antagonists caused penis eversions in Biomphalaria and postulated that serotonin signaling promotes penis retraction (Muschamp and Fong, 2001; Fong et al., 2005). This hypothesis is supported by two findings from the present study: 1) the presence of serotonergic fibers and varicosities on the penis sheath and the penis retractor muscle (PRM; Fig. 10C), and 2) the demonstration of a serotonergic projection to the penial nerve from the PeSlb cluster (Fig. 12). In Helix pomatia, serotonin was shown to produce relaxation of the PRM (Wabnitz and von Wachtendonk, 1976). Moreover, stimulation of individual neurons in the lateral region of the right pedal ganglion produced either PRM contraction or relaxation (Eberhardt and Wabnitz, 1979). Evidence from several species supports the serotonergic phenotype of the pedal Ib cluster (Croll and Chiasson, 1989; Hernádi et al., 1989; Kemenes et al., 1989).

Infection of B. glabrata by S. mansoni has complex effects on reproduction, including a reduction of egg laying referred to as “parasitic castration” (Minchella and Loverde, 1981; Crews and Yoshino, 1989; Boyle and Yoshino, 2000). Manger et al. (1996) measured significant decreases in the concentration of serotonin in extracts of the CNS and plasma during the prepatency period of infection. As injection of infected snails with exogenous serotonin was found to restore control levels of egg laying, it was proposed that parasitic castration is attributable, at least in part, to the diminished levels of serotonin caused by larval S. mansoni infection (Manger et al., 1996). While ovulation in pulmonates is initiated by neuroendocrine factors (Geraerts et al., 1988), several behavioral components of the egg-laying process are triggered by feedback to the CNS, primarily via the intestinal nerve (Ferguson et al., 1993). The double-labeling of neurons in the left parietal, visceral, and right pedal ganglion observed in our experiments with backfills of the intestinal nerve can be expected to reflect the broadest spectrum of potential serotonergic cells involved in motor and sensory function of the genital tract. As this approach will also result in labeling of neurons projecting to additional peripheral structures innervated by the intestinal nerve (see de Vlieger, 1968), more refined anatomical and electrophysiological approaches will be required to identify individual neurons involved in the control of specific stages of oogenesis, and the feedback control of egg-laying behavior.

CONCLUSION

The serotonergic neural system described in this study could act at the interface between S. mansoni and B. glabrata at all stages of the infection process, ranging from the initial response to miracidium penetration to later phases when competition for energy and resources can occur (see de Jong Brink et al., 2001; Bayne, 2009). Numerous structural and functional commonalities have been recognized between the serotonergic systems of mollusks and mammals, the two hosts of S. mansoni (Jacobs and Gelperin, 1981). In both groups, small assemblies of serotonergic neurons or even individual cells have the capacity to broadly impact neural circuits and behavior (Weiger, 1997; Gillette, 2006). This neural architecture led to the proposal that pivotal aminergic systems could provide effective channels for host responses to infection and for parasite manipulation of host behavior (see Helluy and Holmes, 1990; Maynard et al., 1996; Adamo, 2002; Lefèvre et al., 2009). Our observations should stimulate further investigation of these hypotheses at the level of individual identified neurons.

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