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Three-Dimensional Reconstruction and Neural Map of the Serotonergic Brain of *Asplanchna brightwellii* (Rotifera, Monogononta)

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ABSTRACT The basic organization of the rotifer brain has been known for nearly a century; yet, fine details on its structure and organization remain limited despite the importance of rotifers in studies of evolution and population biology. To gain insight into the structure of the rotifer brain, and provide a foundation for future neurophysiologic and neurophylogenetic research, the brain of *Asplanchna brightwellii* was studied with immunohistochemistry, confocal laser scanning microscopy, and computer modeling. A three-dimensional map of serotonergic connections reveals a complex network of approximately 28 mostly unipolar, cerebral perikarya and associated neurites. Cells and their projections display symmetry in quantity, size, connections, and pathways between cerebral hemispheres within and among individuals. Most immunopositive cells are distributed close to the brain midline. Three pairs of neurites form decussations at the brain midline and may innervate sensory receptors in the corona. A single neuronal pathway appears to connect both the lateral horns and dorsolateral apical receptors, suggesting that convergence of synaptic connections may be common in the afferent sensory systems of rotifers. Results show that the neural map of *A. brightwellii* is much more intricate than that of other monogonont rotifers; nevertheless, the consistency in neural circuits provides opportunities to identify homologous neurons, distinguish functional regions based on neurotransmitter phenotype, and explore new avenues of neurophylogeny in Rotifera. J. Morphol. 270:430–441, 2009.

KEY WORDS: serotonin; immunohistochemistry; confocal; rotifers; brain

INTRODUCTION

In the quest for a greater understanding of rotifer neurobiology and its utility in studies of behavior and evolution, researchers have employed a variety of methods to reconstruct the rotifer nervous system and map its connections (e.g., Nachtwey, 1925; Clément, 1977; Kotikova, 1995). As noted by Bullock and Horridge (1965): "They are, for animals of their grade of construction, quite well provided with nervous apparatus and often extremely well provided with sensory apparatus." These observations come mostly from studies of a few rotifers in the late 19th and early 20th centuries (Zelinka 1888, 1891; Hirschfelder, 1910; Martini, 1912; Nachtwey, 1925), where researchers employed brightfield optics and histological stains to map out the distribution of sensory devices and the structure of the brain (cerebral ganglion). In many cases, individual connections among cerebral neurons were clearly delimited and revealed, among other things, the apparent constancy of neuron number, position and size within a species.

Additional details of the rotifer nervous system were not revealed until methods in histochemistry were applied on a systematic basis. In particular, histochemical preparations of whole mount specimens allowed for the visualization of neuronal patterns and subsets of neurons defined by neurotransmitter phenotype (Raineri, 1984; Keshmirian and Nogrady, 1987, 1988; Kotikova, 1995, 1997, 1998). These studies yielded significant insights in the organization of the rotifer CNS by establishing the cellular location of likely classes of neurotransmitters, and therefore clues to the probable functions of neurons in different regions of the body. As noted by Clément (1987), a precise mapping of specific neurotransmitters set against the known distribution of sensory devices, muscles, and other organ systems (e.g., the neuroethological diagrams of Clément, 1987) has the ability to add substantially to our knowledge of their roles in various physiological functions and behaviors. To date, however, there are few detailed reports of the locations of specific neurotransmitters in rotifers. Keshmirian and Nogrady (1987) performed the first comparative study of catecholamine distribution; this was followed by Kotikova (1995, 1997, 1998) who performed a comparative analysis of catecholamine distribution among thirteen additional species. However, it was not until Kotikova et al. (2005) applied immunohistochemical methodologies to visualize serotonergic and FMRFamider-

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gic neurons in rotifers that significant differences in neuronal populations were revealed. In particular, Kotikova et al. (1995) exposed differences in the distribution of cells containing serotonin versus FMRFamide-like neurotransmitters, revealing for the first time how different organ systems (e.g., mastax, coronal cilia) may be modulated at the neurochemical level. Later studies by Hochberg (2006, 2007) added further evidence that serotonergic neurons are abundant in rotifers and that understanding their patterns of distribution may have both functional and phylogenetic value.

In some of the earliest investigations of rotifers (Zelinka, 1888, 1891, 1892; Hirschfelder, 1910; Martini, 1912; Nachtwey, 1925; Remane, 1929–1933), researchers documented the structure of the brain as part of a larger monograph on anatomy and taxonomy, but details about specific cerebral connections and the innervation of organ systems came later with the advent of electron microscopy. Specifically, the ultrastructural investigations of Eakin and Westfall (1965), Clément (1975, 1977, 1980, 1987), Clément and Amsellem (1986, 1989), Clément and Wurdak (1984), Clément et al. (1980, 1983), and Wurdak et al. (1983) provided details on the structure and innervation of sensory receptors, muscles and the coronal cilia. However, the only detailed studies on the structure of the cerebral ganglion are those of Clément (1975) on *Trichocerca* and the dissertations of Ware (1971) and Seldon (1972) on *Asplanchna brightwellii*. The latter studies of *A. brightwellii* revealed, among other things, the marked bilateral symmetry of the brain and the relative constancy in number and position of perikarya within the brain (summarized in Ware and Lopresti, 1975). Clément and Wurdak (1991) also provided details on the ultrastructure and innervation of the cerebral eye and photosensitive lateral horns. To date, there is limited information on the distribution of neurotransmitters within the brain of *A. brightwellii*, though some details exist for the closely related congeners, *A. herricki* and *A. priodonta* (Kotikova, 1998).

In this study, I use the well-known rotifer, *A. brightwellii*, as a model for documenting the three-dimensional connections among serotonergic neurons within the rotifer brain. The small size of rotifers creates special challenges for researchers interested in documenting their neurobiology, but species of *Asplanchna* are well suited for investigations of this type for several reasons: (1) the morphology and ultrastructure of many sensory receptors in the apical field (naked head region) are well studied; (2) specimens are large and translucent permitting ease of organ system identification; (3) females are ovoviviparous and often contain live parthenogenic embryos at different developmental stages, allowing a researcher to map the developing nervous system; (4) species undergo cyclical parthenogenesis, thereby sustaining a constant supply of genetic clones for experimentation; and (5) some species are cyclomorphic, providing opportunities to study changes in body size and organization in the absence of genetic variation (e.g., Birky, 1968; Gilbert and Thompson, 1968; Gilbert 1974, 1975). The purpose of the current investigation is to provide the first detailed "wiring diagram" of cerebral connections in female specimens of *A. brightwellii* based on immunohistochemical mapping and 3D reconstruction. Connections within the brain are described, as are some connections to sensory devices outside the brain. A detailed neural map of these connections in females will allow future researchers to make comparisons of nervous system structure between the sexes, between different morphotypes derived from cyclomorphosis (e.g., α- and β-forms of *A. brightwellii*), and among different species for the purpose of homologizing neurons and understanding the evolution of neural circuits in rotifers.

MATERIALS AND METHODS

Animals

Specimens of *A. brightwellii* Gosse, 1850 were collected from an aquarium at the Smithsonian Marine Station, Fort Pierce, FL. The outdoor aquarium was empty prior to the arrival of Hurricanes Frances and Jeanne during September 2004; rain water accumulated in the aquarium over the course of the two storms. Specimens were collected from the aquarium with a 64-μm sieve and processed for immunohistochemistry in October 2004. Several specimens were photographed alive on a Zeiss compound microscope equipped with Nikon Coolpix 995 digital camera, and whole mounts of trophi were made for taxonomic identification.

Immunohistochemistry

Approximately fifty rotifers were relaxed in 1% $MgCl_2$ for 30 min prior to fixation in 5% paraformaldehyde in 0.1 M PBS for 2 h at 4°C. All immunohistochemical steps were performed in 1-ml centrifuge tubes on an orbital shaker at 4°C. Animals were rinsed in 0.1 M PBS rinse for 30 min and then placed in 0.1 M PBS with 0.5% Triton X-100 (PBT) for 24 h. Specimens were processed in Image-iT FX Signal Enhancer (Molecular Probes, Eugene, OR) for 1 h to prevent nonspecific staining. Specimens were placed in 1° antibody in PBT (n = 30, rabbit anti-serotonin, 1:250 dilution, Sigma Aldrich, MS; n = 10, mouse anti-β tubulin, 1:500 dilution, Sigma Aldrich) for 24 h. Negative control specimens (n = 5 for each antibody) were omitted from 1° antibody and processed identically in the following steps. After a 24 h rinse in PBT, all specimens were transferred to 2° antibody (Alexa Fluor 546 goat anti-rabbit IgG, 1:200 dilution, Molecular Probes; rabbit anti-mouse IgG, 1:200 dilution, Sigma Aldrich, MS) for 24 h, and rinsed in PBT for 24 h. Some specimens were then stained with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR) for 1 h before mounting in Fluoromount G (Electron Microscopy Sciences, PA) on glass slides. Mounts were allowed to harden at 0°C for at least 48 h before examination.

Microscopy and Modeling

Specimens were examined with a Nikon Eclipse E800 compound microscope equipped with Biorad Radiance 2100 laser system and Lasersharp software. Series of 0.05 μm, 0.1 μm, and

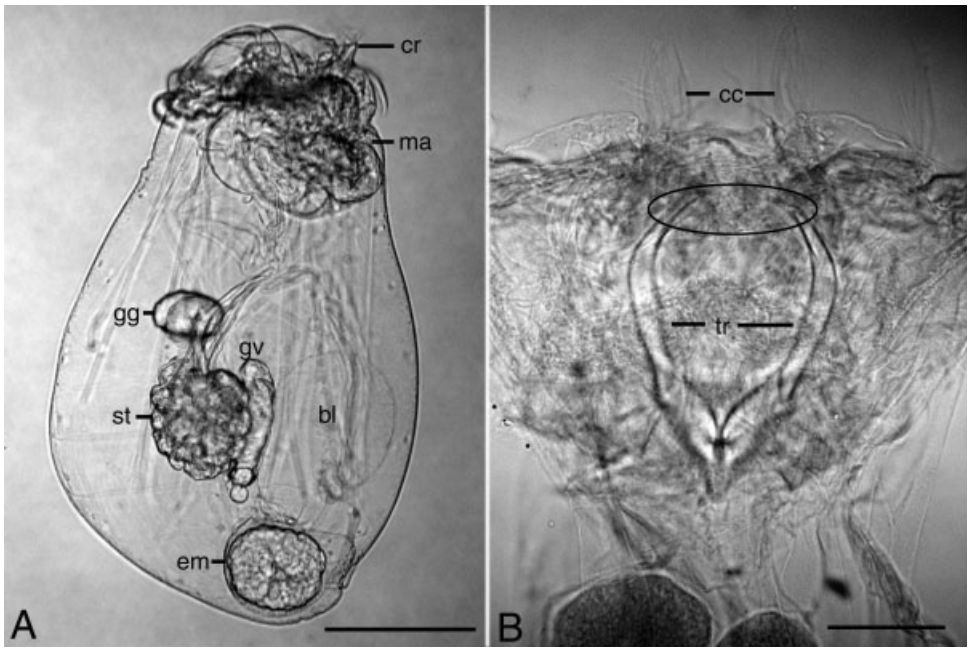

Fig. 1. Live specimens of *Asplanchna brightwellii*. (A) Specimen with corona partially contracted. Scale bar = 250 μm. (B) Squash preparation showing the corona and trophi. Oval outline is the approximate location of the brain. Scale bar = 55 μm. bl, bladder; cc, cilia of the cingulum; cr, corona; em, embryo; gg, gastric gland; gv, germovitellarium; ma, mastax containing the trophi; st, stomach; tr, trophi.

0.2 μm optical z-projections were made with Lasersharp software (v. 4.1) and Confocal Assistant (v. 4.02). To establish the accuracy of specific cerebral connections, specimens in different orientations (e.g., dorsal, ventral, lateral) were observed and high-resolution digital videos made from series of 0.05-μm sections through the entire cerebral ganglion (average of 250 sections at 0.05 μm/section, each optical section with a resolution of 1,024 × 1,024 pixels). This was necessary for four reasons: (1) specimens often contract during fixation, and withdrawal of the corona can create distorted views of the brain; (2) neurites of different cell bodies generally parallel each other closely along their entire length; (3) most neurites show highly tortuous routes, and (4) not all cell bodies stained with equal intensity in all specimens. Confocal image stacks were imported into Volocity (v. 2.6.2 © Improvision) to produce 3-D models. No digital manipulations of images were performed other than adjustments to greyscale, brightness and contrast in Volocity.

Measurements and Cell Coding

Measurements of cell bodies and their projections were performed on confocal images imported into Carnoy 2.1 (© 2001 Peter Schols). Measurements are only approximations: shrinkage and swelling due to chemical processing is unknown as is the degree of compression by the coverslip. Unless otherwise indicated, measurements are of single individuals used as representatives; neurite measurements are from tip of cell body to end of neurite. Measurements of neurite lengths are conservative estimates based on a series of straight lines from cell body to synapse; all neurites are curvilinear in three dimensions.

Perikarya were distributed along the brain midline, off the brain midline but within the brain capsule, and outside of the brain capsule. To follow their general course of connections, perikarya were coded by position within the brain. Those cells directly on or very close to the midline were coded MP# (midline perikaryon # refers to the position of the cell from anterior to posterior along the brain midline). Several cells within the brain capsule and outside its borders made up a linear series of connections that lead to midline perikarya. In these cases, cells were coded in an alphanumeric format. Numbering began at the cerebral midline and proceeded laterally. Secondary and tertiary connections with any cells in the series were coded with a subscript indicating their proximity to the midline perikaryon and their order within the series. For example, if a midline perikaryon designated "MP1" made a linear series of connections (synapses) prior to exiting the brain, the numbering series would proceed as MP1 (midline perikaryon), $MP1_1$ (1st perikaryon connection), and $MP1_2$ (second perikaryon connection). Cells that are not part of a linear series and are positioned off the midline but clearly within the brain capsule were coded PP# (peripheral perikaryon # refers to the position of the cell from anterior to posterior). These coding methods allow for future comparisons of brain cell homology based on positional correspondence and their connections to the midline perikaryon, which in nearly all specimens is the most easily visualized cell. Only those cells with connections that could be reasonably identified were coded. No assumptions were made about the functions of any neurites or perikarya (sensory, motor), thereby alleviating any need for future changes in coding when information on their physiology is at hand.

RESULTS

Female specimens of *Asplanchna brightwellii* were sacciform in shape with body lengths of 550–1,000 μm (Fig. 1A). The horseshoe-shaped germovitellarium (gv) contained approximately 25 to 30 nuclei. The trophi of all fixed, whole mount specimens were examined to confirm taxonomic status: trophi were relatively slender and possessed rami with a small median tooth on the inner margin and fine lamella behind the apex (Fig. 1B); both

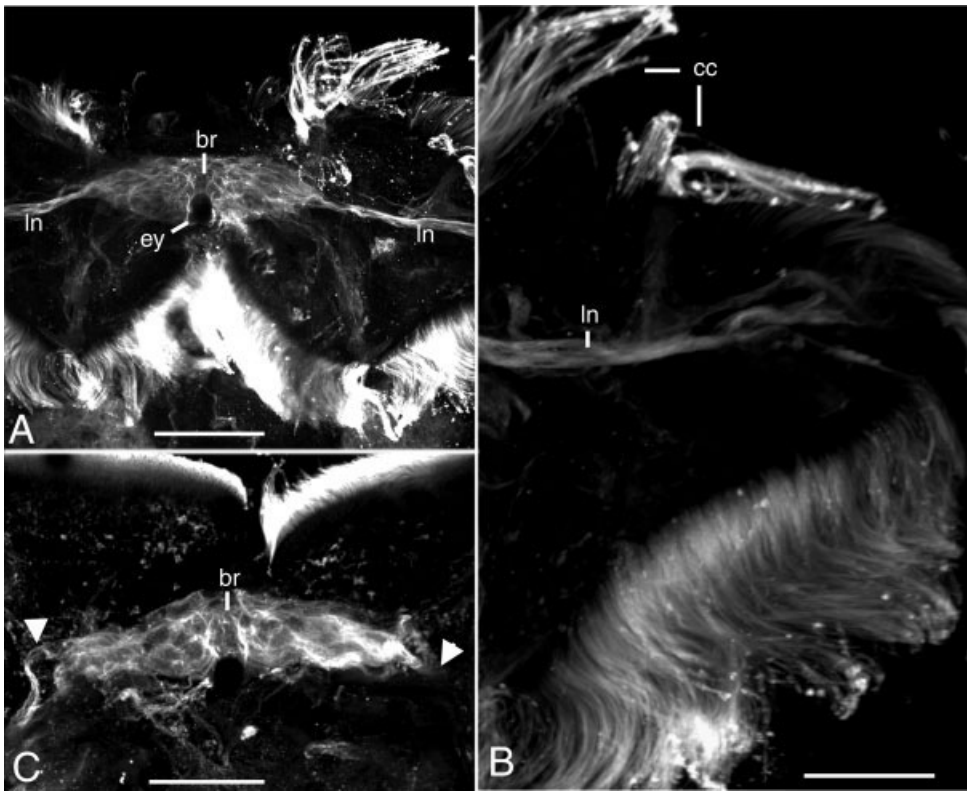

Fig. 2. β-tubulin staining in the coronal region of *Asplanchna brightwellii*. A. Dorsal view of the apical field showing the brain and one pair of laterally projecting neurites. Scale bar = 35 μm. B. Lateral region of the apical field showing possible innervation of the lateral horns. Scale bar = 12 μm. C. Slightly different focal plane of same image in A, revealing a second set of neurites that leave the brain (arrow heads). Scale bar = 35 μm. br, brain; cc, cilia of the cingulum; ey, eyespot; ln, lateral neurite.

apophysis and subapophysis were present on the bulla. Size of trophi fit within the range of variation described by Paggi (2002) and references therein.

Anti-β Tubulin Immunoreactivity (βT-IR)

Anti-β Tubulin staining was performed to visualize the general structure of the brain independent of neurotransmitter phenotype. βT-IR was present in the coronal cilia, brain, and various neurite tracts projecting out from the brain (see Fig. 2). A singular eye (6 μm diameter) was positioned medial and dorsal to the brain (Fig. 2A). The brain showed strong βT-IR, was spindle-shaped, and ca. 82 μm × 21 μm. Two pairs of neurite tracts projected laterally from the brain (Fig. 2A,C). One pair of tracts innervated the lateral sides of the corona including the lateral horns (Fig. 2B). Specific sites of innervation were difficult to determine because of the strong fluorescence signal from the coronal cilia and cilia of the apical receptors. The second pair of neurite tracts was convoluted and projected deep into the body (Fig. 2C); this tract could not be followed.

General Organization of 5HT-IR in the Brain

Anti-serotonin immunoreactivity (5HT-IR) was present in the brain of all adult females. Individual perikarya and neurite tracts were easily visualized in whole mount preparations (see Fig. 3). No staining was evident in the brain of control specimens; non-specific staining was noted for some patches of coronal cilia. Immunoreactivity was mainly restricted to the cerebral ganglion with few neurites in the trunk region. Not all cerebral perikarya or their neurites stained with equal intensity within or between specimens. In most cases where perikarya were weakly immunoreactive, their corresponding neurites were also weakly immunoreactive. Several neurites along the midline of the brain were difficult to trace in all specimens, regardless of the resolution and thickness of the optical sections.

The brain is bilaterally symmetric, located dorsal to the mastax, and surrounded by somatic muscles (Fig. 3A). A faint border (not shown), which may represent a neural lamella (brain capsule), outlined the brain (ca. 97 μm wide, 40 μm long). Many perikarya within the brain border were unipolar with a single fiber directed toward the brain midline (Fig. 3B). The symmetry of the

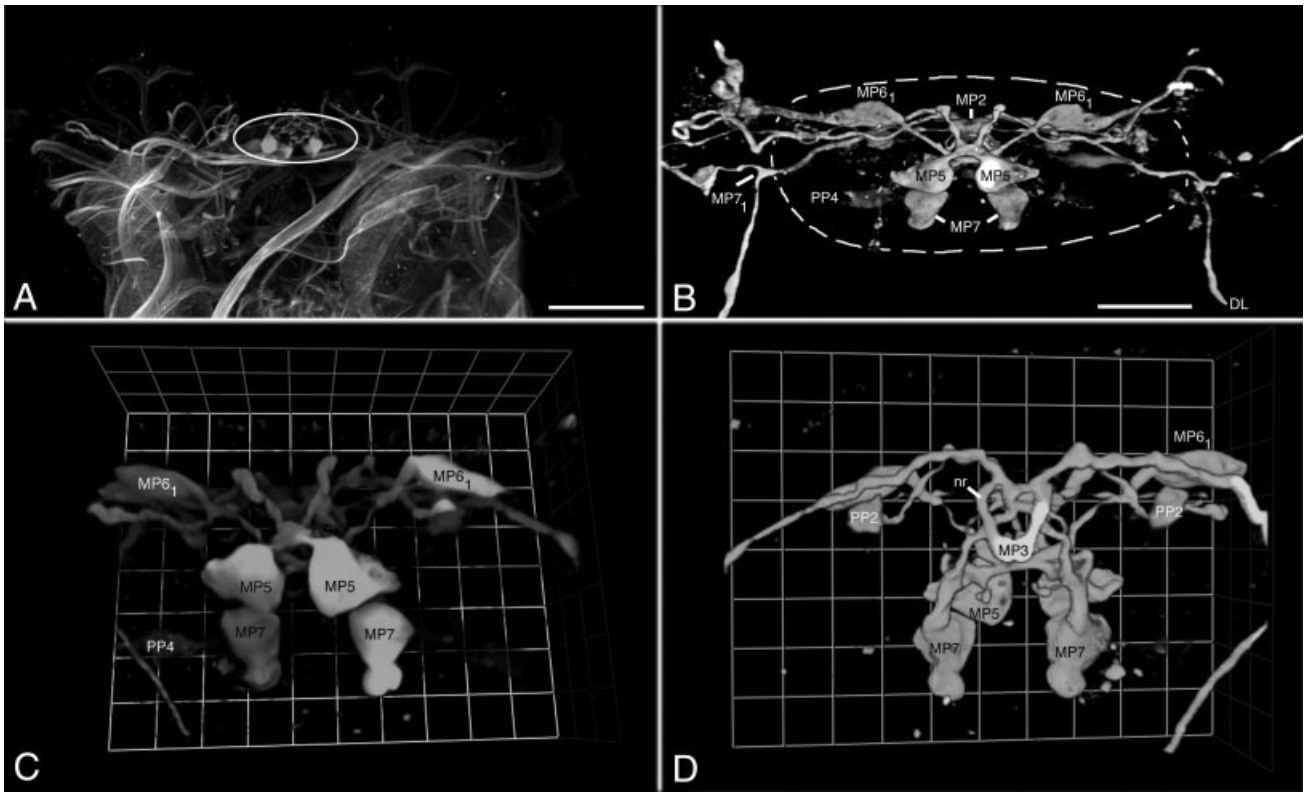

Fig. 3. Serotonergic immunoreactivity (5HT-IR) in the brain of *Asplanchna brightwellii*. (A) Position of the brain at the anterior end of a dual stained specimen (phalloidin for muscles, 5HT-IR for nerves). Circle indicates 5HT-IR in the brain. Scale bar = 80 μm. (B) Computer-rendered ventral view of the brain. Circle outlines the approximate area visualized in A. Scale bar = 25 μm. (C) Computer-rendered 3D model of the brain in ventral view. Box scale = 5.4 μm. (D) Computer-rendered 3D model of the brain in dorsal view. Box scale = 5.4 μm. Cell numbers correspond to position along the anterior-posterior axis of the brain (explanation in text); MP, midline perikaryon; PP, peripheral perikaryon.

brain was displayed in the position, size and IR intensity of each perikaryon and in the general circuitous route of each neurite within the brain (Fig. 3B–D). However, in some specimens, a perikaryon on one side of the brain stained more intensely than its contralateral counterpart. Many perikarya stained very weakly and appeared to lack neurites altogether. These cells were most noticeable in the posterior region of the brain and generally of similar size (ca. 5 μm × 3–4 μm) and spindle shaped. A few neurites within the brain did not have an identifiable cell body (see later).

Connections Among 5HT-IR Cerebral Perikarya

A total of eight perikarya (four pairs) were present directly along the midline of the brain (MP1-4); three additional pairs of perikarya were present very close to the midline (MP5-7) and had neurites that crossed the midline as decussations (Figs. 3B–D, 4, 6). MP1 is the most anterior pair of perikarya (ca. 2 μm diameter, Fig. 4B), though without any obvious connections to other cells or neurites. MP2 is a pair of small perikarya (ca. 2–3 μm diameter) directly posterior to and abutting MP1; a single neurite extends laterally from each cell and exits the brain on the ipsilateral side (Figs. 4B, 6). MP3 is a pair of small cell bodies (ca. 2–3 μm diameter) positioned dorsal to most other perikarya in the brain (Figs. 3D, 4A, 6); these cells are connected to a thick pair of neurites that loop up from below, i.e., from the ventrally positioned MP5. The voluminous MP5 perikarya (ca. 8 μm × 10 μm) send neurites to the midline that decussate and create an intricate series of neurite branches (described below) prior to exiting the brain along its antero-ventral side as potential nerve cords (see Fig. 6). The nerve cords are somewhat convoluted in the head region, straighten out in the region of the mastax, and appear to terminate at approximately 50% body length (Fig. 5A). Back at the brain midline, and following the MP5 decussation, each neurite produces a dorsal branch that then bifurcates (see Fig. 6 for details): one branch projects posteriorly where it synapses with MP4, a pair of small perikarya (ca. 2–3 μm diameter) at the brain midline. The second branch forms a thick, dorsally projecting ring-shaped neurite (nr) that synapses on MP3 (Figs. 3D, 4A, 6).

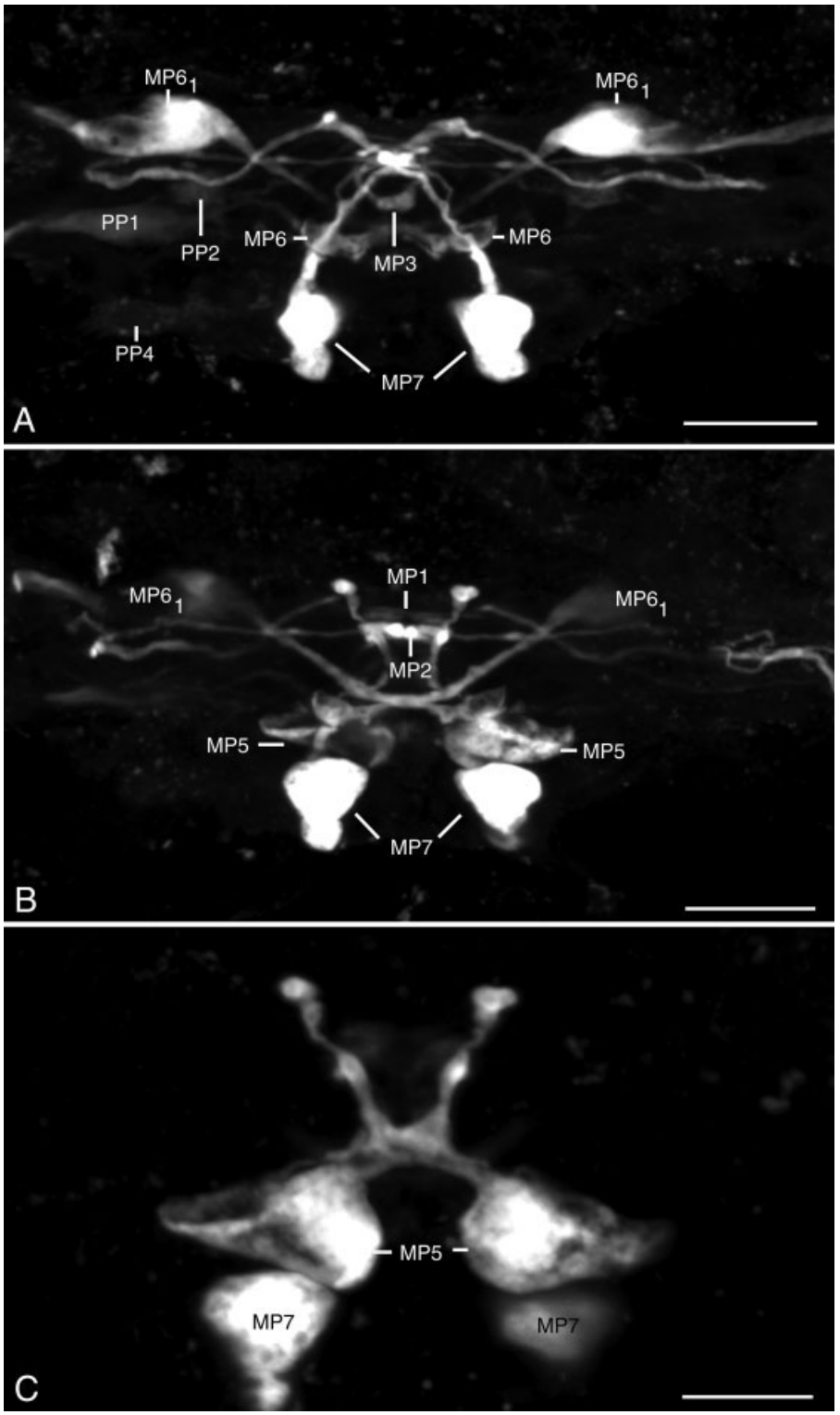

Fig. 4. Confocal images of serotonergic immunoreactivity in the brain of *Asplanchna brightwellii*. Images represent successive focal planes from dorsal (A) to ventral (C), revealing the three main decussations in a single specimen. Scale bars = 11 μm. Cell numbers correspond to position along the anterior-posterior axis of the brain (explanation in text); MP, midline perikaryon; PP, peripheral perikaryon.

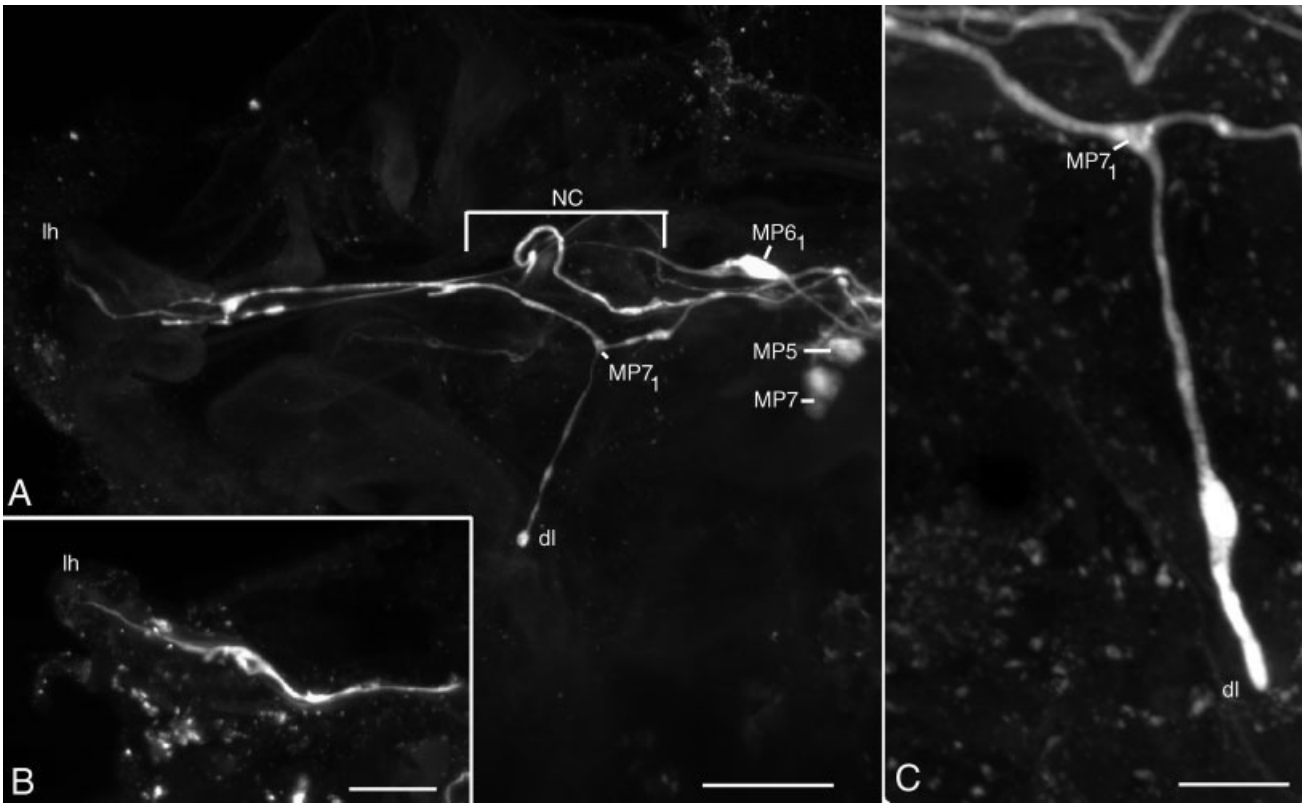

Fig. 5. Possible innervation of sensory receptors by serotonergic neurites that extend from the brain of *Asplanchna brightwellii*. (A) Confocal image of the anterior end, left side, revealing innervation of the lateral horn. Scale bar = 20 μm. (B) Closeup of lateral horn from A. Scale bar = 6 μm. (C) Confocal image of a neurite that appears to innervate the dorsolateral receptor. Scale bar = 5 μm. Cell numbers correspond to position along the anterior-posterior axis of the brain (explanation in text). Dl, dorsolateral receptor of the apical field; lh, lateral horn; MP, midline perikaryon; NC, region where the nerve cord follows a circuitous route prior to extending toward the trunk; PP, peripheral perikaryon.

A pair of eye-shaped perikarya, MP6 (ca. 5 μm long), is dorsal to and abuts the MP5 perikarya (Fig. 3A). A single neurite projects off each cell toward the midline, decussates, and synapses with a second, larger eye-shaped perikaryon, $MP6_1$ (ca. 11 μm × 4 μm), close to the anterior border of the brain (Figs. 3, 4A,B). Each perikaryon is associated with a single neurite that projects laterally outside of the brain capsule. MP7 are large, posteriorly positioned perikarya (ca. 13 μm × 8 μm) close to the brain midline (Figs. 3, 4, 6). Both cells send a single long neurite (ca. 13–15 μm) towards the midline where they decussate through the neural ring (nr), extend along the contralateral, antero-dorsal margin of the brain, and synapse on $MP7_1$ close to the brain margin (Figs. 3, 4A,B, 6). Two neurites connect with $MP7_1$: one neurite projects dorsally outside the brain capsule and may innervate a sensory organ (below); the second neurite projects laterally to a perikaryon just outside the brain capsule, $MP7_2$ (an unusual case of weak IR in the cell body (not shown) but strong IR in the neurite).

There are at least five pairs of peripheral perikarya within the brain capsule (PP1-PP6). All perikarya were highly variable in shape. The spindle-shaped PP1 and oval PP2 send neurites medially toward the brain midline where they synapse with MP2 or cells in proximity to MP2 (Figs. 4A, 6). PP3 are oval, weakly immunoreactive cells that receive a connection from outside the brain capsule and send a single long neurite toward the brain midline (see Fig. 6); the point of connection could not be determined. PP4 are spindle-shaped perikarya that send single neurites medially toward MP6 where they may synapse (Figs. 4A, 6). PP5 are spindle-shaped perikarya that send single neurites postero-dorsally outside the brain capsule. These neurites synapse with a second perikaryon, $PP5_1$, which in turn sends a single projection posteriorly toward the trunk (see Fig. 6); its site of innervation could not be determined.

5HT-IR Innervation of Apical Sensory Devices

There are six pairs of sensory organs in the naked apical field of the corona (terminology of Wurdak et al., 1983): one pair of dorsolateral receptors, one pair of lateral horns, two pairs of apical recep-

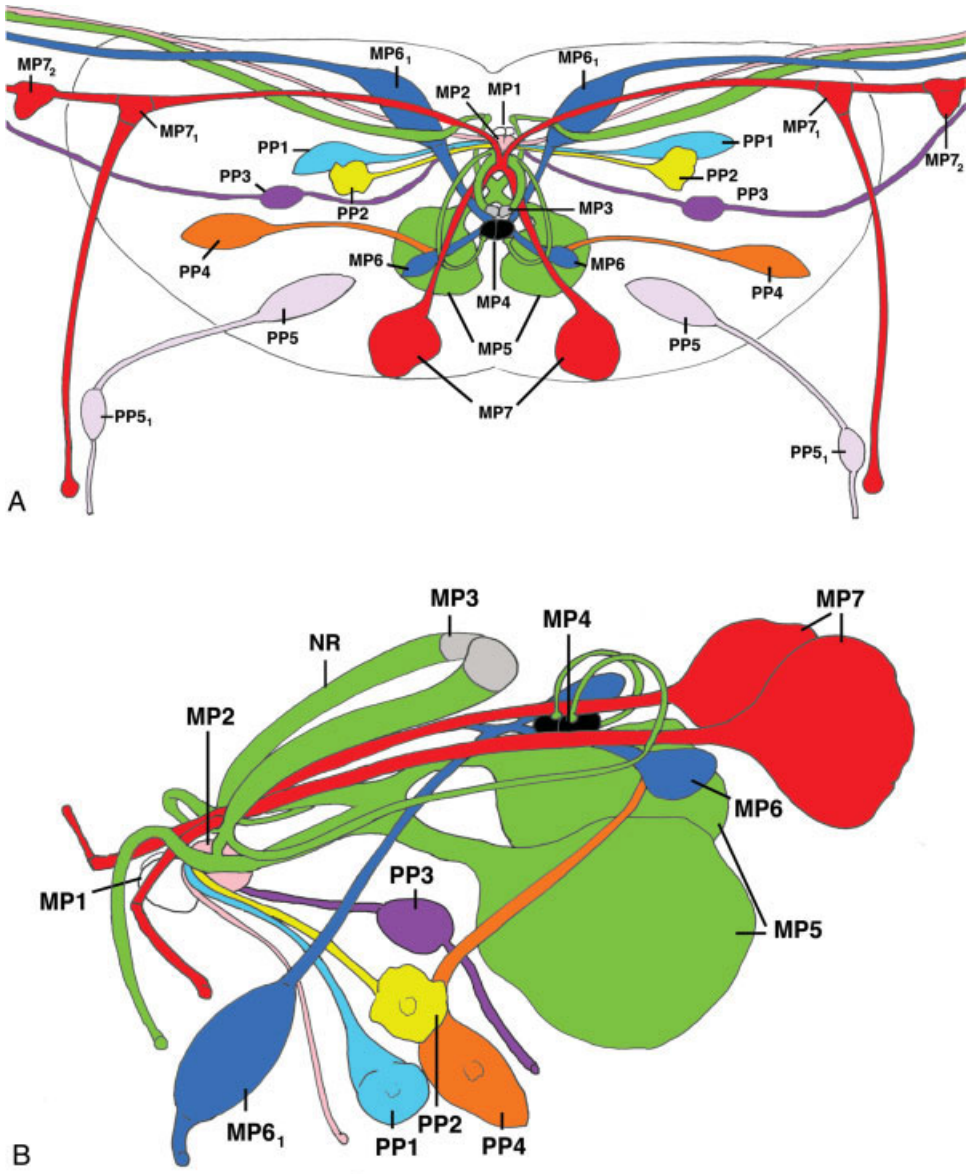

Fig. 6. Neural map of cerebral connections in *Asplanchna brightwellii* based on 5HT-IR, confocal laser scanning microscopy and 3D modeling. Colors of perikarya and neurites correspond between A and B. (A) Dorsal view of serotonergic connections. (B) Lateral view of serotonergic connections. Cell numbers correspond to position along the anterior-posterior axis of the brain (explanation in text). MP, midline perikaryon; PP, peripheral perikaryon.

tors (inner and outer), one pair of ventrolateral receptors, and one pair of oral receptors. At least five pairs of neurites supply the apical field and may innervate sensory organs distributed across the corona (Figs. 5, 6). Three pairs of perikarya, $MP6_1$, $MP7_2$, and PP3, send neurites toward the lateral horns, although their precise connections could not be determined with accuracy (Figs. 5, 6). $MP7_1$ appears to send a single neurite dorsally for ca. 30 μm prior to innervating the region around the dorsolateral receptors (dl, Figs. 5A,C, 6A). The weakly staining PP5 also send a pair of neurites in the dorsal direction, but it is difficult to determine if these neurites connect with sensory receptors or bend deeper into the body. There are additional neurites of undetermined origin around the apical field that might innervate the remaining sensory receptors; unfortunately, all specimens prepared as whole mounts had flattened apical fields, making it impossible to accurately trace the route of individual neurites.

DISCUSSION

Species of *Asplanchna* have been the subject of research for over a century. To date, asplanchnids are some of the most common rotifers in studies of neurobiology, where histology and electron micros-

copy have elucidated several aspects of nervous system structure and function (e.g., Eakin and Westfall, 1965; Ware, 1971; Seldon, 1972; Clément, 1975, 1977, 1980, 1987; Ware and Lopresti, 1975; Clément and Wurdak, 1991; Clément et al., 1980, 1983; Wurdak et al., 1983). More recently, histochemical methodologies have been applied to study the general distribution of enzymes in neurotransmitter metabolism (Nogrady and Alai, 1983) and the organization of catecholamines in whole mount specimens (Keshmirian and Nogrady, 1987, 1988; Kotikova, 1998). These studies revealed the basic bilateral symmetry of the cerebral ganglion and the innervation of various organ systems. They also revealed an apparent species-specific pattern of catecholaminergic neurons that might have phylogenetic value (see Kotikova, 1998).

The current study adds to this general knowledge of rotifer neurobiology by illuminating patterns of connections among cerebral neurons using anti-serotonin immunohistochemistry (5HT-IR). The general pattern of immunoreactivity in the serotonergic brain revealed a bilaterality similar to that observed with light microscopy (Nachtwey, 1925), transmission electron microscopy (Ware, 1971; Seldon, 1972; Ware and Lopresti, 1975) and histofluorescence (Kotikova, 1998). The latter study utilized the GAIF method for labeling catecholamines in *Asplanchna herricki* and *A. priodonta*; this method may also label indolamines such as serotonin (Lindvall and Bjorklund, 1974). Kotikova (1998) states that catecholamines are present in approximately 6 to 11 cerebral cells of the rotifers studied, or 3–7% of the total population of brain cells. Dual labeling of serotonin and specific catecholamine neurotransmitters (e.g., dopamine, norepinephrine, epinephrine) or their biosynthetic enzymes (e.g., tyrosine hydroxylase) has yet to be performed, so the extent of colocalization within specific neurons is undetermined. For these reasons, it is premature to homologize cerebral cells with different neurotransmitter phenotypes among species of *Asplanchna*.

Brain Structure and Innervation of the Apical Field

The brain of *A. brightwellii* is bilaterally symmetric, surrounded by a neural lamella (brain capsule), and displays constancy in number and position of perikarya, confirming earlier developmental and ultrastructural observations (Ware, 1971; Seldon, 1972; Ware and Lopresti, 1975). Previous researchers have questioned the presence of a neural lamella around the rotifer brain, but Ware (1971) demonstrated a "loosely fitting structureless membrane" that apparently demarcates the neuron cell bodies. Within the 196-200 neurons that make up the brain (Ware, 1971; Ware and Lopresti, 1975), approximately 28 5HT-IR perikarya were visualized with immunohistochemistry and CLSM in this study —only 10 perikarya (5 bilateral pairs: MP3, MP5, MP6, $MP6_1$, MP7) display strong immunoreactivity, and even this immunoreactivity varies within and among specimens. The reasons for this variation are unknown. Some of the largest perikarya, and consequently those that display the greatest immunoreactivity, are the cells that presumably innervate the sensory organs of the apical field (see Figs. 5, 6). Neurons in the MP6 and MP7 series of connections innervate the lateral horns and possibly the dorsolateral receptors of the apical field. The most voluminous perikarya, MP5, may form connections with the ventral nerve cords.

Consistent with the small size and relative simplicity of rotifer anatomy, the number of serotonergic connections within the brain of *A. brightwellii* is low, but still comparatively higher than most other rotifers examined with similar methods (see Kotikova et al., 2005; Hochberg, 2006, 2007). Moreover, there is no distinct cerebral neuropil in *A. brightwellii* as there is in other microscopic invertebrates. For example, the cerebral ganglion of gastrotrichs and nematodes has at its center a nerve ring neuropil (defined ultrastructurally in gastrotrichs, Ruppert, 1991; and nematodes, Wright, 1991). In species of Acoela, similar commissures are well defined with 5-HT immunohistochemistry (Raikova et al., 1998). A commissure-like neuropil was not observed in *A. brightwellii* by Ware (1971) using TEM: "...there are no apparent organizations of large number of fibers within the cerebral neuropil, and certainly no regions which one might call glomeruli." Ultrastructural observations of rotifers such as *Philodina* and *Trichocerca* (Clément, 1977; Clément and Wurdak, 1991) confirm this description, and show that a well-defined commissural mass *sensu* Acoela, Gastrotricha or Nematoda is not present in rotifers.

As stated, the specificity of neuronal connections within the brain, and in regions around the corona, is a consistent feature among the specimens examined. Unfortunately, not all connections or perikarya were easily visualized even at the highest magnifications and resolution, hindering a complete understanding of connections within the brain (e.g., connections of PP1 and PP2; the connections of MP5 to the presumed nerve cords) and outside it (e.g., connections to many of the apical receptors). Moreover, a nuclear dye such as propidium iodide was not used as a counterstain, so there remains some uncertainty about the precise number and position of perikarya. Still, several intracerebral pathways and some pathways to sensory devices in the apical field could be determined with some accuracy, and may lead to a better understanding of their functions. For example, serotonergic neurons innervate the dorsolateral receptors of the apical field, lateral horns and

perhaps even the presumed chemosensitive pits at the base of the horns (Fig. 5; see Wurdak et al. 1983 for descriptions of the sensory devices). The lateral horns are characteristic structures in the apical field of both female and male individuals of *Asplanchna* (Wurdak et al., 1983). Clément and Wurdak (1991) note that the lateral horns in females of *A. brightwellii* are innervated by at least three neurons. Two of these neurons make contact with the horn apex and may be photosensitive; a third neuron appears to innervate the small pit at the base of the horn and may be chemosensitive. This description fits well with the results of the current investigation, where three serotonergic neurons also supply the region around the lateral horns (see Fig. 5): $MP6_1$, $MP7_2$, and PP3. Interestingly, the voluminous perikarya of MP7 are connected through a series of neurites ($Mp7_1$, $MP7_2$) to both the lateral horns (or chemosensitive pits at the base of the horns) and the dorsolateral apical receptors. Presumably, then, MP7 receives afferent sensory information from both sets of organs. It should be noted that this latter description is somewhat tentative; neurons in this region were circuitous and their sites of innervation were often difficult to trace. Further studies using CLSM and immunogold TEM may clarify which of the three serotonergic neurons innervates the lateral horns and ciliated pits, and therefore which cerebral perikaryon ($MP6_1$, MP7, PP3) receives information pertaining to perceived light levels and/or water chemistry.

Unfortunately, serotonergic pathways to other sensory structures identified by Wurdak et al. (1983), such as the inner and outer apical receptors, oral receptors, and ventrolateral receptors, could not be traced with accuracy in *A. brightwellii*. According to Clément and Wurdak (1991), most small, ciliated structures present within the apical field possess a similar ultrastructure and are probably tactile in function. Their presumed roles may be in sensing water-borne vibrations or contact with conspecifics and/or prey. This was confirmed in a study by Joanidopoulos and Marwan (1998), where experimental manipulation of the inner and outer apical receptors of male *A. sieboldi* produced a behavioral response indicative of mechanoreception. Further immunohistochemical analysis with anti-serotonin or antibodies to different neurotransmitters may provide a clearer picture of innervation and sensory physiology for these receptors.

Curiously, there did not appear to be any evidence for serotonergic innervation of locomotory cilia in the corona of *A. brightwellii*. In free-swimming rotifers, the corona is the main source of locomotion, where two concentric rings of cilia, the trochus and cingulum, create metachronal patterns of ciliary movement. Species of *Asplanchna* lack a trochus, and the cingulum forms a discontinuous ring of cilia around the apical field (Wurdak et al., 1983). In many invertebrates, serotonin is the dominant neurotransmitter for regulating ciliary activity, and is present in neurons that innervate the ciliary bands of larval invertebrates (Hay-Schmidt, 2000) and nerve cords that parallel ciliary bands in microscopic invertebrates (e.g., Hochberg and Litvaitis, 2003). In some rotifers, serotonin is present in neurons that parallel the coronal cilia (e.g., species of *Conochilus*, Hochberg, 2006), but immunoreactivity is absent in other species (e.g., *Notommata copeus*, Hochberg, 2007). The absence of direct serotonergic innervation of the cingulum in *A. brightwellii* may point to a form of indirect control through muscles that insert on the ciliary rootlets (see Clément, 1987). Keshmirian and Nogrady (1987) demonstrated catecholamines in the coronal region that may correspond to these sites of innervation. Alternatively, other neurotransmitters might function to control ciliary activity in these species.

Comparisons Among Rotifera

Immunohistochemical studies of the rotifer nervous system are relatively rare, leaving many questions unanswered regarding structural diversity and the variety of neurotransmitter phenotypes in the brain of this important group of microinvertebrates. To date, only three studies have examined the structure of the rotifer nervous system using serotonin immunohistochemistry: the study of Kotikova et al. (2005) on *Euchlanis dilatata*, *Plationus patulus* (formerly *Platyias patulus*, see Segers, 2007), and *Asplanchna herricki*, and the studies of Hochberg (2006, 2007) on species of *Conochilus* and *Notommata*, respectively. These studies revealed substantial diversity in the numbers of 5HT-IR perikarya and their patterns of distribution. In particular, Kotikova et al. (2005) revealed the relatively low numbers of serotonergic perikarya compared to peptidergic perikarya for all three species. Similarly, low numbers of 5HT-IR perikarya were also noted by Hochberg (2006, 2007) for species of *Conochilus* and *Notommata*.

Of interest to the current study are two observations made by Kotikova (1998) and Kotikova et al. (2005): (1) neurons in some rotifers appear to make X-shaped patterns in the brain; and (2) *A. herricki* has few 5HT-IR cerebral perikarya and a relatively simple neuron map. With regards to the first observation, the brachionid rotifers *Plationus patulus* and *Beauchampiella eudactylota* (formerly *Manfredium eudactylotum*, see Segers, 2007), and a species of *Philodina*, all have a characteristic X-shaped pattern of neurons in their brains (Kotikova, 1998; Kotikova et al., 2005). To this list, we may add *A. brightwellii*, which bears several pairs of neurites that decussate at the brain midline and give the appearance of a similar X-shaped

pattern. Whether the patterns observed in brachionid and philodinid rotifers are structurally similar to that of *A. brightwellii* will require additional observations at greater spatial resolution. Still, the fact that these patterns are similar in presumably distantly related species, as are other patterns (e.g., ring-shape) among additional groups of rotifers (see Kotikova, 1998; Kotikova et al., 2005), suggests that convergence in brain structure among distantly related lineages may have a comparable functional basis. Additional neurobiological studies of congeners should be performed and may open up an entirely new avenue or rotifer research: rotifer neurophylogeny (e.g., see Harzsch, 2006).

As mentioned previously, the asplanchnid brain consists of approximately 200 cells (Ware, 1971), and only a small proportion of these cells are immunoreactive to serotonin antibodies (this study). In *A. brightwellii*, there are ca. 28 serotonergic cells in the brain, but only four in the brain of *A. herricki*. The reason for this disparity is unknown and difficult to interpret in either a functional or phylogenetic context. Anatomically, both species are very similar, though *A. herricki* possesses some vestiges of a foot that might indicate its more basal status within the family (see also Walsh et al., 2005 for phylogeny of Asplanchnidae). One potentially significant difference between the species is found in the ability of females of *A. brightwellii* to produce polymorphic daughters under certain environmental conditions (cyclomorphosis, see Birky, 1968; Gilbert and Thompson, 1968; Gilbert 1974). Cyclomorphosis is linked to extraordinary morphotypes with larger bodies (but apparently no change in cell number, Birky and Field, 1966), and is known from *A. brightwellii*, *A. intermedia*, *A. sieboldi*, and *A. silvestrii*; *A. intermedia* and *A. sieboldi* are closely related to *A. brightwellii* (Walsh et al., 2005). How this might relate to differences in cerebral architecture and neurophysiology is unknown.

Future Directions

In an inspired review of rotifer biology, Wallace (2002) presented numerous challenges to the future of research on rotifer anatomy, ecology, neurobiology, and evolution. Among those challenges in neurobiology, Wallace posed two queries: (1) Will new technologies and procedures be employed to better characterize rotifer neurobiology? (2) Are neuroanatomical characters useful for studies of phylogeny? The use of CLSM and immunohistochemistry in this and other studies is not new, but their application to describe the circuitry of the rotifer brain is novel, and may herald numerous opportunities to explore sensory physiology in rotifers and the evolution and development of their neural circuits. Species of *Asplanchna* would be particularly amenable to such studies because of their large size, ovoviviparous development, and relative ease of culture. These factors also make species of *Asplanchna* excellent model systems for exploring neuroanatomical parallels with other more anatomically complex animals. For example, the serotonergic brain of *A. brightwellii* has at least three decussation pathways similar to those of other model organisms such as the fruit fly (Kidd et al., 1998) and mouse (Shu and Richards, 2001). In many bilaterally symmetric animals, sensory information is transmitted from one side of the body to the other through axons that cross at the body midline. These decussations are the result of local guidance cues from neurons and midline glia that direct patterns of axon outgrowth from one side of the brain to the other (Jacobs and Goodman, 1989; Kidd et al., 1998). The relative simplicity of the afferent sensory circuits in *A. brightwellii* may demonstrate the simplest decussation pathways known in the animal kingdom: glia are absent (Clément and Wurdak, 1991), pathways from sensory receptors to the cerebral ganglion are made up of few cells, and the two distinct submodalities of putative chemoreception and photoreception are carried by two parallel paths that converge on one pair of large cerebral cells (MP7). Without glia to serve as guidance posts for axon pathfinding, and other sensory receptors (e.g., inner and outer apical receptors) apparently utilizing different chemical messengers, species such as *A. brightwellii* may provide important clues on the development, function and evolution of decussation within the metazoan CNS.

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