

# Serotonergic and SCP<sub>b</sub>-like innervation of the atrial complex in *Gyratix hermaphroditus* (Platyhelminthes, Kalyptorhynchia) revealed with CLSM

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**Abstract** The platyhelminth reproductive system is a complex series of canals, glands, and sclerotic components that figure prominently in our understanding of reproductive physiology, taxonomy, and evolution of the Platyhelminthes. Yet, there is limited information on its innervation, especially for free-living species of Rhabdocoela, the most speciose and ecologically diverse assemblage. Here, innervation of the reproductive system in the common marine kalyptorhynch, *Gyratix hermaphroditus*, is studied using confocal laser scanning microscopy (CLSM), fluorescent phalloidin, and antibodies to serotonin (5HT) and small cardioactive peptide b (SCP<sub>b</sub>). Results show that portions of the female atrial system (uterus, female gonopore) are innervated by peptidergic (SCP<sub>b</sub>) neurons that may function to control muscles involved in egg movement. In contrast, portions of the male atrial system (male atrium, male gonopore) are innervated by both peptidergic and serotonergic neurons. These neurons form a complex series of hoops around the musculature of the male atrium that houses prostate stylet type II. It is hypothesized that 5HT is the primary myoexcitatory neurotransmitter, and that it acts either synergistically with SCP<sub>b</sub> to trigger muscle contractions of the male atrium and protract the copulatory stylet, or that SCP<sub>b</sub> plays an inhibitory role during contraction of the male atrium. A comparison of the distribution of peptidergic and serotonergic neurons in *G. hermaphroditus* with other free-living species and parasitic rhabdocoels (Neodermata) reveals similarities in innervation of comparable (though probably not homologous) reproductive organs. These results suggest that the

atrial systems of phylogenetically diverse species may share a common neuronal physiology despite their structural differences and potential independent evolutionary origins within the Platyhelminthes.

**Keywords** Confocal · Serotonin · Neuropeptide · Flatworm

## Introduction

The hermaphroditic reproductive system of rhabdocoel platyhelminths is an often complex series of sacular gonads, ducts, blind canals, and sclerotized hardparts surrounded by a intricate network of muscles. Because there is considerable variability in the size, arrangement, and histological complexity (e.g., epithelial construction, presence of glands, cytological staining, etc.) of both male and female organs, as a whole, the reproductive system remains the basis for most of rhabdocoel (and other platyhelminth) taxonomy. To date, much of our understanding of the structural diversity in rhabdocoel reproductive systems comes from histological observations made for taxonomic purposes. Still, there are several comparative and fine structural studies of rhabdocoel reproductive organs, in particular, the atrial system (e.g., Karling 1956; Schockaert 1974; Reuter 1977; Bunke 1981).

The atrial system, as originally defined by Karling (1956) and extensively detailed by Artois and Schockaert (2003, 2005) for members of Polycystididae (Eukalyptorhynchia), is the series of open and blind ducts (e.g., ejaculatory duct, female bursa, seminal receptacle), glands (e.g., accessory, prostate), and sclerotic hardparts (e.g., cirrus, stylets) of the reproductive system, not including the gonads and vitellaria. The female system is mostly composed of an assortment of

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ducts and glands, while the male system is best represented by ducts, glands, and a variety of sclerotized structures including an armed cirrus or one or more stylets. Many of the open and blind ducts, glands, and sclerotic components are surrounded by an intricate network of muscles (Karling 1956). The homology of atrial organs within the Platyhelminthes has not received widespread attention, calling into question the use of comparable terms to describe similar organs in different lineages (Schockaert 1974). Still, assessments of homology have been conducted for some taxa (e.g., Polycystididae) for the purpose of reconstructing phylogenies and understanding evolutionary trends (Schockaert 1974; Artois and Schockaert 2003, 2005).

To date, functional interpretations of the atrial complex in any platyhelminths are based on structural observations at the light microscopic and ultrastructural levels, and there is limited information on innervation. This is particularly true for free-living rhabdocoels, where most studies of the nervous system have focused on ultrastructure (e.g., Reuter and Lindroos 1979a, b; Bedini and Lanfanchi 1998) or general patterns of cyto- or immunohistochemistry for phylogenetic purposes (Kotikova 1986, 1997, 2001; Kotikova and Joffe 1988a, b; Reuter et al. 1988; Joffe and Kotikova 1991; Reuter and Eriksson 1991). Immunohistochemical observations on innervation of reproductive organs are comparatively rare and generally incidental. Examples include innervation of the genital complex by FMRFamidergic neurons in *Castrella truncata* (Abildgaard, 1789) (Dalyelliidae, Kotikova et al. 2002) and by catecholaminergic and FMRFamidergic neurons in *Gyatrix hermaphroditus* Ehrenberg, 1831 (Kalyptorhynchia, Reuter and Eriksson 1991). Studies on non-rhabdocoels such as species of Seriata also reveal aminergic and peptidergic innervation of genital organs, but again, generally as part of a larger study of the nervous system (e.g., Joffe and Reuter 1993; Johnston et al. 1996; Reuter et al. 1995a, b, c, 1996; Mäntylä et al. 1998). Innervation of reproductive organs in neodermatan rhabdocoels has received more detailed attention (e.g., Maule et al. 1990; Poddubnaya et al. 2005), but again, most are part of a larger study of the nervous system (reviewed in Gustafsson 1992; Halton and Maule 2004).

The purpose of the present investigation is to explore patterns of innervation in the atrial complex of a well known, free-living rhabdocoel, *Gyatrix hermaphroditus*. *G. hermaphroditus* has been the subject of several ultrastructural, cytochemical, and immunohistochemical investigations of the nervous system (Reuter 1975; Reuter and Lindroos 1979a, b; Reuter and Eriksson 1991; Kotikova 1995), so patterns of immunoreactivity in the CNS and PNS are well documented. In fact, the nervous system of *G. hermaphroditus* is much better known than that of most other free-living rhabdocoels, making this species a useful

model for comparative explorations of immunoreactivity in other free-living species. Because numerous sibling species are known from various environments worldwide (the *G. hermaphroditus* species complex sensu Curini-Galletti and Puccinelli 1998), characterization of atrial system innervation in this particular sibling species should provide a basis for comparison with other siblings. In the current study, antibodies to two neurotransmitters are used to characterize innervation of the atrial complex, and thereby gain insights into neuronal control of reproductive activity. Serotonin (5HT) is a well known neurotransmitter that is hypothesized to play a role in sexual (Fairweather et al. 1987) and asexual reproduction of platyhelminths (Gaerber et al. 2007), and stimulates muscle contraction in platyhelminths and other invertebrates (review in Gillette 2006), and is therefore hypothesized to play a central role in stimulating the muscular network surrounding reproductive organs of *G. hermaphroditus*. Small cardioactive peptide b (SCP<sub>b</sub>) is also prevalent in invertebrate nervous systems (Masinoky et al. 1988); however, it is often implicated in modulating muscle activity rather than initiating it (Lloyd et al. 1985; Cropper et al. 1987). Therefore, I hypothesize that SCP<sub>b</sub> will be present in neurons that innervate atrial organs that also receive serotonergic stimulation.

## Materials and methods

Marine specimens of *G. hermaphroditus* were collected at Capron Shoals off the Atlantic coast of Florida (27° 26.52'N, 80° 13.81'W) in June and July 2004. Dredge samples from 3 to 4 m depth were placed in a bucket and brought back to the Smithsonian Marine Station, Fort Pierce, Florida for extraction. Animals were extracted from the sediments using 7.5% MgCl<sub>2</sub> and identified live prior to immunostaining. Measurements of specimens were obtained with an ocular micrometer. Specimens were fixed for immunostaining within 24 h of extraction and live observation.

Preparation for immunostaining included relaxation of the platyhelminths ( $n = \sim 25$ ) for 15 min in 7.5% MgCl<sub>2</sub> followed by fixation in 5% formalin in 0.1 M phosphate buffer saline (PBS, pH 7.2) for 24 h at 5°C. All subsequent staining steps were performed in 1.5 ml centrifuge tubes at 5°C on an orbital shaker. Specimens were rinsed in 0.1 M PBS for 2 h and then placed in PBT (0.1 M PBS + 0.5% Triton X-100) for 24 h. Animals were transferred to IT-Signal Enhancer (Molecular Probes, Eugene, OR, USA) for 2 h, rinsed in PBT for 15 min, and placed in either rabbit anti-serotonin ( $n = 8$ , diluted 1:200 in PBT, Sigma) or mouse anti-SCP<sub>b</sub> ( $n = 10$ , diluted 1:100 in PBT, courtesy of Dr. Scott Santagata) for 24 h. Five control specimens were omitted from the primary antibody. All specimens were

rinsed in PBT for 24 h and placed in light-protected tubes containing secondary antibody (goat anti-rabbit Alexa-Fluor 546 diluted 1:200 in PBT, goat anti-mouse Alexa-Fluor 546 diluted 1:250 in PBT, Molecular Probes, Eugene, OR) for 48 h. Two control specimens were omitted from the secondary antibody. After immunostaining, specimens were rinsed in PBT for 24 h and stained in Alexa Fluor 488 phalloidin (made following manufacturer's directions, Molecular Probes, Eugene, OR) for 2 h at 5°C. Worms were mounted in Fluormount G (Electron Microscopy Sciences) on glass slides and kept in the dark at 5°C for 24 h prior to examination.

Specimens were examined on a Nikon Eclipse E800 compound microscope equipped with a Biorad Radiance 2000 laser system and Lasershar software (v. 4.1). A series of 0.05 µm optical z-projections were made with Confocal Assistant (v. 4.02). Some confocal data was imported into Volocity 3.1 (Improvision) to render movie files for tracing neurons.

## Results

Descriptions of the nervous system are focused on peripheral innervation of the atrial complex only. Descriptions of the atrial complex follow the terminology of Artois and Schockaert (2003, 2005) for species of Polycystididae. Control specimens showed no fluorescence in the region of the atrial complex; fluorescence signals were only noted for food items in the digestive cavity.

### Small cardioactive peptide b immunoreactivity (SCPb-IR)

SCP<sub>b</sub>-IR was distributed through the nervous system of *G. hermaphroditus*. Strong IR was detected in the cerebral ganglion, a series of anteriorly projecting cerebral neurites, the pharyngeal nervous system, and six posterior nerve cords (not shown). There were several transverse commissures connecting various nerve cords in the posterior half of the body. All nerve cords coalesced into a solid ganglionic mass (gm) at the caudal end (see below).

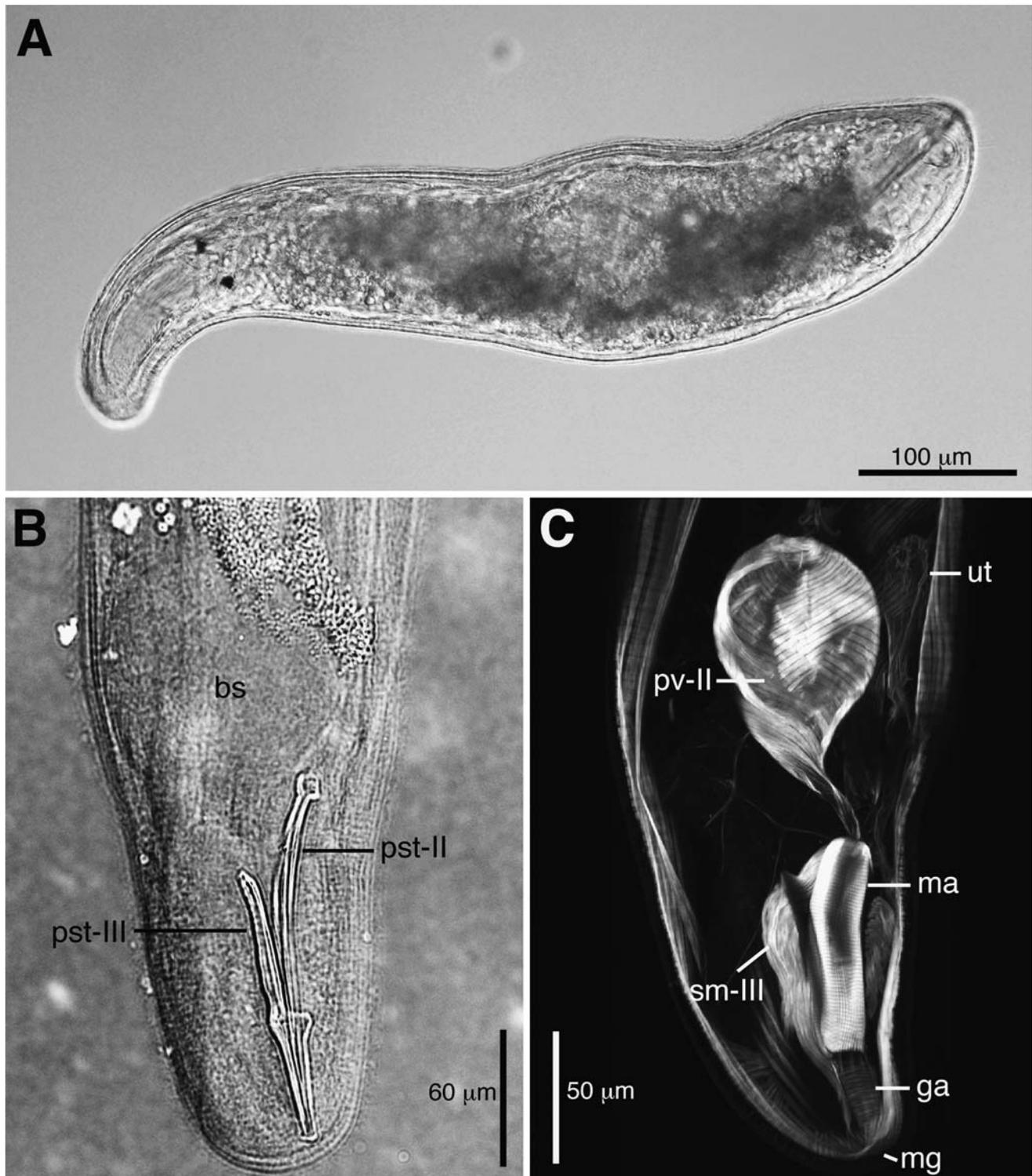
According to Artois and Schockaert (2005), the female atrial system consists of a series of interconnected canals that lead to the gonads, vitellaria, and several ducts and pouch-like organs (bursa, female duct type II, common oviduct, and uterus). Some of these organs (or portions thereof) are wrapped in muscles (Fig. 1). Of these, only neurons that innervate the uterus showed strong SCP<sub>b</sub>-IR. The uterus (ut) is an elongate, bag-like sac lined with longitudinally oriented muscles that form a loose meshwork over its distal end (Fig. 2a). A series of circular muscle fibers forms a sphincter (ms) at approximately one-third length of the uterus from its distal end. The uterine canal (utc) narrows

beyond the muscular sphincter and eventually joins the female genital atrium and female gonopore (fg). Closely associated with this sphincter is a region of strong SCP<sub>b</sub>-IR (Fig. 2b). Specifically, IR neurites form a hoop around the uterus at the level of the sphincter. Numerous small IR neurites project anteriorly off the neural hoop and unite in a tiara-like arrangement (tla, Figs. 2b, 5a). A single large IR perikaryon on one ventral nerve cord (vn) sends a neurite to the uterus and connects it to the tiara-like arrangement of neurites (Figs. 2b, 5a). Posteriorly, an IR ring parallel to the longitudinal axis of the body encircles the region of the muscular sphincter around the female gonopore (irr, Figs. 2d, 5a). The ring shows numerous varicosities that might represent small perikarya or multiple neurites.

The male atrial complex is composed of a series of ducts that connect the single testis and seminal vesicle to various glands and organs that function in lubrication and insemination via hypodermic impregnation. The male system has a separate gonopore from the female system and is located at the posterior end of the body (mg, Figs. 1c, 5a). Artois and Schockaert (2003) describe the predominant male atrial components as a seminal vesicle and associated ejaculatory duct, a prostate vesicle type II (pv-II), and prostate stylets type II (pst-II) and type III (pst-III) that are housed in the male atrium (the latter stylet only partially contained) (see Fig. 1b). Muscles line most portions of the male system exclusive of the testis (Fig. 1c). SCP<sub>b</sub>-IR is restricted to that portion of the male atrium that contains prostate stylet type II and is strongly wrapped in an orthogonal muscle grid. In this region, IR neurites form six to eight neural hoops (nh) around the male atrium on the outside of the musculature (Fig. 3a, b). Each hoop appears to branch off of a single longitudinal neurite that lines the muscular male atrium for approximately three-fourth of its length. The most distal neural hoop, closest to the top of prostate stylet type II, may be composed of several neurites. The most proximal neural hoop appears to branch and form a fine meshwork of neurites (Fig. 3b). Prostate stylet type III, which enters the proximal portion of the male atrium, is supplied with its own musculature outside of the male atrium (sm-III, Figs. 1c, 5a). There are no IR neurites associated with this stylet or its musculature. The separation between the base of the male atrium and the beginning of the genital atrium is defined by a change in musculature—only circular muscles line the genital atrium (Fig. 1c). A single neural ring with high IR varicosity (mar1, Figs. 3c, 5a) appears to surround the genital atrium next to a posterior ganglionic mass where the nerve cords unite (gm, Fig. 3c).

### Serotonin immunoreactivity (5HT-IR)

Strong 5HT-IR was present in the cerebral ganglion and at least four nerve cords (not shown). Some perikarya were



**Fig. 1** *Gyratrix hermaphroditus*, habitus and portions of the reproductive system. **a** Habitus, anterior to the left. **b** Posterior end showing portions of the male atrial system including prostate stylet type II (*pst-II*) and prostate stylet type III (*pst-III*) and the seminal bursa (*bs*) of the

present along the length of the nerve cords. Most specimens were heavily flattened from the coverslip making it difficult to distinguish dorsal from ventral nerve cords. All specimens

female atrial system. **c** Phalloidin stained preparation viewed with CLSM, posterior end. Muscular reproductive organs are shown and include the genital atrium of the male system (*ga*), male atrium (*ma*), male gonopore (*mg*), prostate vesicle type II (*pv-II*), and uterus (*ut*)

also had a very fine, highly varicose, subepithelial plexus of neurites distributed throughout the body. Several specimens showed non-specific binding of the secondary antibody to

locomotory cilia and some portions of the epidermis. Control specimens processed without the primary antibody also displayed similar staining; however, there was no staining of nervous structures in these specimens.

Most 5HT-IR was restricted to the male atrial complex, more specifically, the male atrium. 5HT-IR neurites innervate the entire length of the male atrium, from the distal end close to prostate vesicle type II, to the proximal region where it connects with the genital atrium. The entire male atrium is slightly concave as it envelops prostate stylet type II. There are at least four large, multipolar perikarya (pk1–4; Figs. 4a, b, d, 5b) in proximity to the atrium, and several smaller perikarya along its length. Some of these perikarya appear to be associated with the nerve cords, but it was often difficult to distinguish between perikarya of the nerve cords with those directly associated with the male atrium. Still, at least three perikarya on the concave side of the male atrium appeared to be the main source of 5HT-IR neurites that innervated the atrium (Fig. 4a–d). Many of the neurites branched prior to innervation (see di and pi, Fig. 4c), and others made contact with additional smaller perikarya around the periphery of the male atrium. The atrium itself is wrapped in 12–14 neural hoops that appear to have multiple origins, e.g., large perikarya on the concave side of the atrium and smaller perikarya on the periphery of the walls of the atrium (Fig. 4b). Distally, a thin neurite leaves the male atrium and traverses prostate vesicle type II for part of its length (not shown). The neurite could not be followed. Proximally, another thin neurite paralleled the male atrium for a portion of its length beyond the neural hoops and formed a weakly staining, fine meshwork around the genital atrium (Fig. 5b). A highly varicose hoop-like neurite was present around the male gonopore (mar2; Fig. 5b).

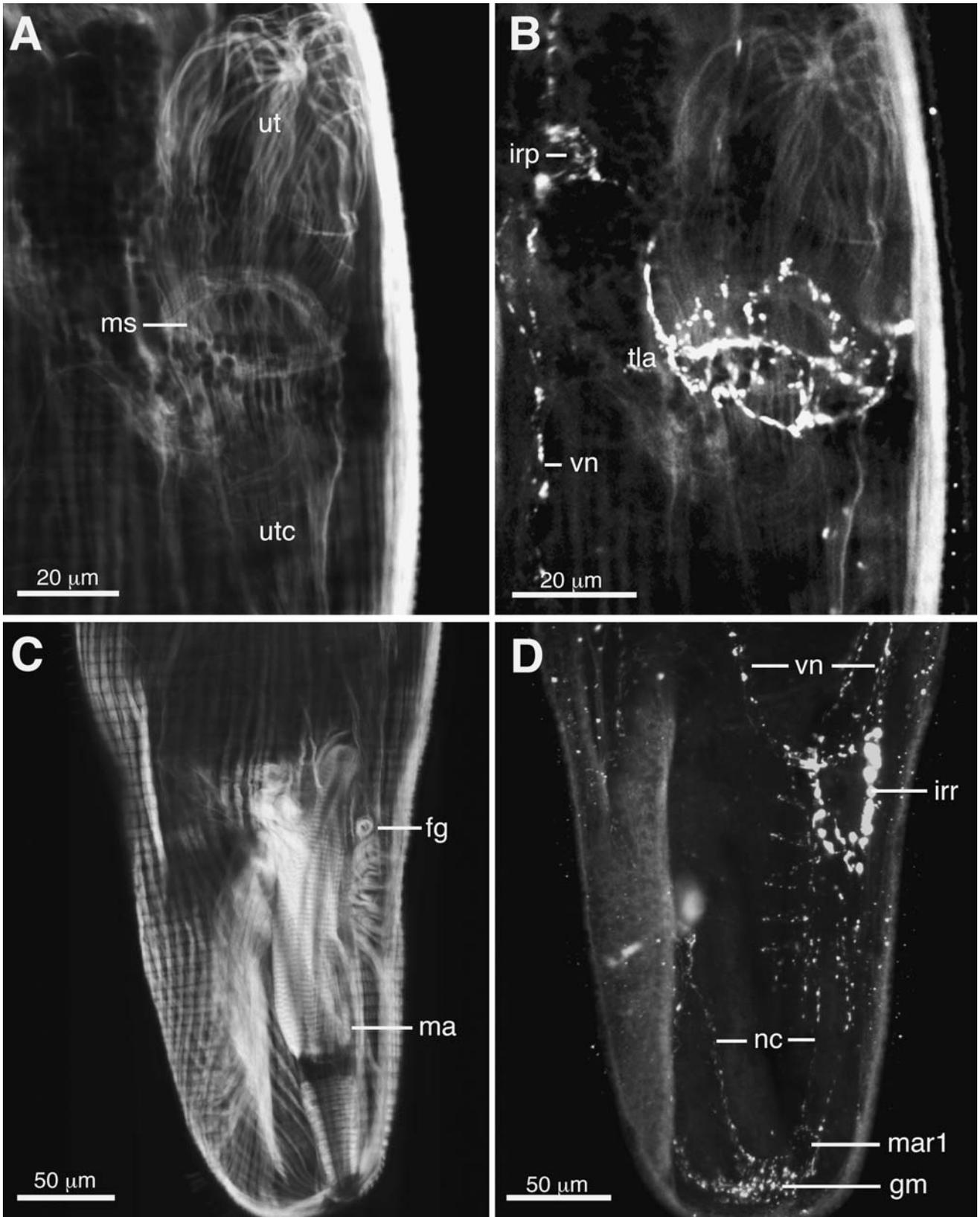
## Discussion

The platyhelminth nervous system has been the subject of numerous investigations since the late nineteenth century, yet no studies have focused on innervation of the reproductive system in free-living species despite its structural complexity and importance in taxonomy and phylogenetics. In fact, much of what is known about innervation of the genital complex, especially from an immunohistochemical perspective, is derived from studies of only two groups, the Tricladida and Neodermata (e.g., Maule et al. 1990; Fairweather and Halton 1992; Marks et al. 1995; Reuter et al. 1995a, b, c, 1996; Armstrong et al. 1997; Mair et al. 1997; Mäntylä et al. 1998; review in Halton and Maule 2004), while only incidental observations have been made of free-living rhabdocoels (Reuter and Eriksson 1991; Reuter et al. 1995a, b, c; Kotikova et al. 2002).

The purpose of the present investigation was to perform a detailed examination of the reproductive system of a free-living rhabdocoel and document specific patterns of innervation using CLSM and immunohistochemistry. *Gyratix hermaphroditus*, a well known meiofaunal kalyptorhynch the world over (e.g., Karling 1955; Reuter 1961; Karling and Schockaert 1977; references in Curini-Galletti and Puccinelli 1998), is also a well studied species from histochemical, karyological and ultrastructural perspectives (e.g., Reuter 1977; Reuter and Lindroos 1979a, b; Reuter et al. 1988; Reuter and Eriksson 1991; Kotikova 1995; Sopott-Ehlers and Ehlers 1998). To date, a variety of neuroactive substances have been documented in the CNS and PNS of *G. hermaphroditus* including catecholamines (Reuter and Eriksson 1991; Kotikova 1995), serotonin (Reuter et al. 1998) and several neuropeptides such as bovine pancreatic polypeptide, FMRFamide, Leu-enkephalin, substance P, and vasopressin (Reuter et al. 1998). Among these substances, only FMRFamide has been demonstrated in the atrial complex, though specific patterns of innervation were not described. Most other neuroactive substances were restricted to the CNS and peripheral innervation of the pharynx. There is no information of the physiological actions of any neurotransmitters in *G. hermaphroditus*.

## Innervation of the atrial complex

The current study demonstrates that portions of the atrial complex in *G. hermaphroditus* are strongly innervated by peptidergic (SCP<sub>b</sub>) and serotonergic (5HT) neurons, in addition to FMRFamidergic neurons previously confirmed by Reuter et al. (1988). The SCP<sub>b</sub> peptidergic component however, has a wider distribution throughout the atrial complex when compared to FMRFamide or serotonin. In particular, SCP<sub>b</sub>-IR neurons innervate portions of both the female atrial complex (uterus and female gonopore) and male atrial complex (male atrium and gonopore), in addition to the CNS and other portions of the PNS (Fig. 5a). Within the female complex, the uterus receives significant innervation in the form of an intricate, hoop-like neural lattice around the uterine sphincter. Similarly, SCP<sub>b</sub>-IR neurons also form a ring around the female gonopore, which bears a muscular sphincter. The presence of SCP<sub>b</sub> in neurons that innervate muscular organs suggests a potential excitatory role in muscle contraction. In gastropods, where most information on SCP<sub>b</sub> physiology is documented, the neuropeptide appears to play a role in muscular contractions of the heart, gut and radula (Lloyd et al. 1984, 1985; Richmond et al. 1986; Prior and Welsford 1989); evidence suggests it may also function in modulating ciliary activity (Candellario-Martinez et al. 1993). Its distribution in species of Platyhelminthes has received comparatively less attention, with the exception of one endoparasite (*Diphyllbothrium*



◀ **Fig. 2** Phalloidin staining and anti-SCP<sub>b</sub> immunoreactivity (IR) of the female atrial system. **a** Loose arrangement of muscles at the distal end of the uterus (*ut*). A muscular spincter (*ms*) separates the uterus proper from the narrow uterine canal (*utc*). **b** SCP<sub>b</sub>-IR around the uterus. IR-perikaryon (*irp*) of the ventral nerve cord (*vn*) innervates the tiara-like arrangement (*tla*) of IR neurons around the uterine sphincter. **c** Posterior end of specimen showing muscles associated with the female gonopore (*fg*) and male atrium. **d** SCP<sub>b</sub>-IR in the caudal end showing ventral nerve cords innervating a ring of neurites (*irr*) around the female gonopore. All main nerve cords (*nc*) unite in a ganglionic mass (*gm*) at the caudal end. An additional ring of neurites surrounds the base of the male genital atrium at the posterior end of the body (*mar1*)

*dendriticum* Nitzsch, 1824, Gustafsson and Wikgren 1989) and one free-living macrostomid (*Microstomum lineare* (Müller, 1773), Reuter and Palmberg 1989). In the tapeworm *D. dendriticum*, SCP<sub>b</sub>-IR is mostly restricted to peripheral innervation of the bothridial (attachment organ) musculature. In *M. lineare*, SCP<sub>b</sub>-IR neurons are limited to the CNS and pharyngeal plexus. In neither species is there any evidence that SCP<sub>b</sub>-IR neurons innervate reproductive organs.

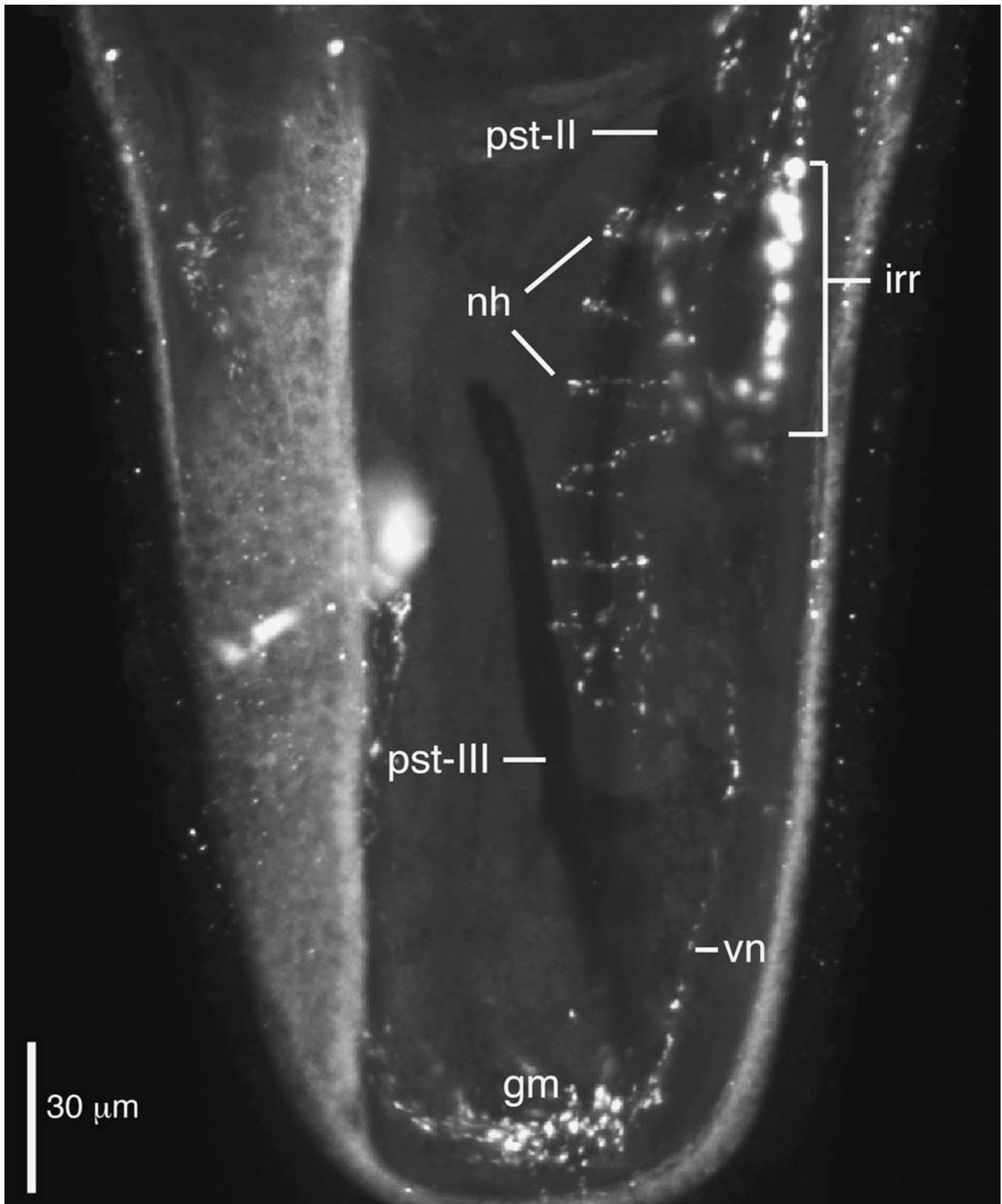
The function of SCP<sub>b</sub> as a stimulator/modulator of muscle contraction in molluscs fits with its known IR distribution in platyhelminths, where researchers have hypothesized that SCP<sub>b</sub> functions to increase the frequency and/or magnitude of muscle contractions for host attachment (*D. dendriticum*, Gustafsson and Wikgren 1989) or pharyngeal movement during feeding (Reuter and Palmberg 1989). In *G. hermaphroditus*, SCP<sub>b</sub>-containing neurons innervate muscular portions of the female atrial complex, which suggests their potential role in stimulating muscles that control egg position within the uterus and oviposition through the female gonopore. Similarly, SCP<sub>b</sub>-IR neurons also innervate the muscular male atrium (*aka* stylet protractor apparatus of Reuter 1977), forming a series of neural hoops around that portion of the atrium that houses the needle-like prostate stylet type II (pst-II). The distal opening of pst-II receives sperm from the seminal vesicle (along the ejaculatory duct) and secretions from prostate vesicle type II (pvt-II). The proximal end of pst-II is positioned in a groove of the second stylet, pst-III that functions as a guide to orient pst-II for insemination. Pst-III has its own muscle supply outside of the male atrium on the distal end, while the proximal end is located inside the male atrium close to the male gonopore. Through peristaltic contractions of circular muscles that line the male atrium, which are presumably stimulated or modulated by SCP<sub>b</sub>, pst-II is pushed down the male atrium and directed by pst-III through the male gonopore.

In addition to peptidergic innervation, the male atrium also receives innervation from serotonergic neurons that form a similar pattern of neural hoops around the atrial musculature (Fig. 5b). Serotonergic innervation is more extensive than peptidergic innervation with SCP<sub>b</sub>, and there are a larger number of IR perikarya on and around the male

atrium. Like SCP<sub>b</sub>, serotonin is known to function as an excitatory neurotransmitter, and serotonergic neurons are often considered the primary myoexcitatory cells in molluscs (Lloyd et al. 1984, 1985). In *G. hermaphroditus*, where neurons with two different neurotransmitter phenotypes (5HT, SCP<sub>b</sub>) innervate the same organ (male atrium), there are at least two hypotheses to explain this dual innervation. First, the neurotransmitters may function simultaneously or in synchrony to vary the force of atrial muscle contraction. For example, the firing of serotonergic neurons may generate the initial contractions of the atrial musculature, thereby moving pst-II toward the male gonopore. Simultaneous (or sequential) firing of SCP<sub>b</sub>-containing peptidergic neurons would then increase the overall frequency of excitatory stimulation (e.g., Zoran et al. 1989), allowing for the forces of successive atrial muscle twitches to summate and thereby force pst-II (up to 160 µm in local specimens) through the male gonopore and into a receptive partner. A second and alternative hypothesis is that the peptide and amine neurotransmitters have different functional roles. For example, one neurotransmitter may function in initiation of muscle contraction (e.g., 5HT), while the second neurotransmitter plays an inhibitory role. Evidence of SCP-related peptides as potential inhibitors is found in a study of the clam *Mercenaria mercenaria* (Linnaeus, 1758), where the neuropeptides appear to initiate muscle relaxation (Candelario-Martinez et al. 1993).

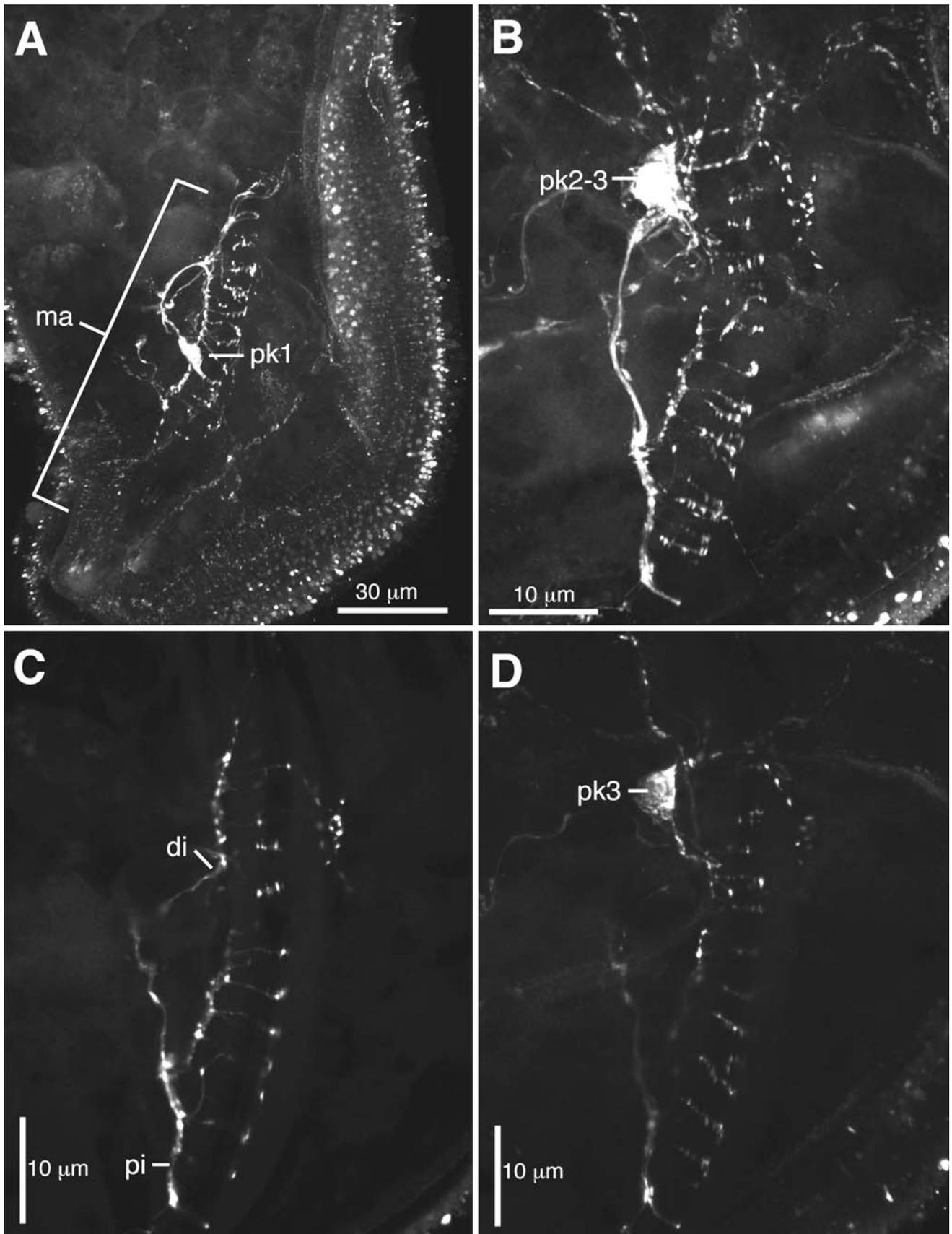
Curiously, there was no serotonergic or SCP<sub>b</sub>-like peptidergic innervation of the somatic muscles (sm-III) that supply pst-III. The presumed function of these muscles is to push pst-III through the gonopore and guide pst-II into a receptive partner for insemination. The identity of the neurotransmitters that initiate contraction of these muscles is unknown, and it seems unusual that these muscles would be controlled by a different neurotransmitter than those that control the atrial muscles of pst-II. Coordinated contraction of both muscle sets would appear necessary for simultaneous protraction of both stylets.

After insemination, the stylet complex must be withdrawn back into the male atrium and into resting position. The muscles that might perform this function are unknown. Regrettably, there is no data on potential inhibitory neurotransmitters that might innervate the atrial muscles, thereby suppressing atrial contractions and allowing the retraction of pst-II and pst-III. Some studies suggest that FMRFamide-like neurons may function in such an inhibitory capacity (Lloyd et al. 1987), but acetylcholine is generally considered the primary inhibitor of muscle contraction in most platyhelminths (Day et al. 1996; Pax et al. 1996). My previous hypothesis (above) that SCP<sub>b</sub> may play an inhibitory role in stylet movement is also a possibility. Future research on the neurons that innervate the male atrial system, in particular, those that innervate the somatic muscles



**Fig. 3** SCP<sub>b</sub>-IR in the male atrial complex. **a** Posterior end showing the regions of the male atrium that are innervated by IR neural hoops (*nh*) that may branch off the ventral nerve cords (*vn*) prior to their fusion in the ganglionic mass (*gm*) at the caudal end. The muscular

male atrium does not fluoresce, nor do prostate stylets type II (*pst-II*) or III (*pst-III*). The ring of neurites (*irr*) that surround the female gonopore is in proximity to a ventral nerve cord and the male atrium. See Fig. 2d for neurites around the base of the male genital atrium

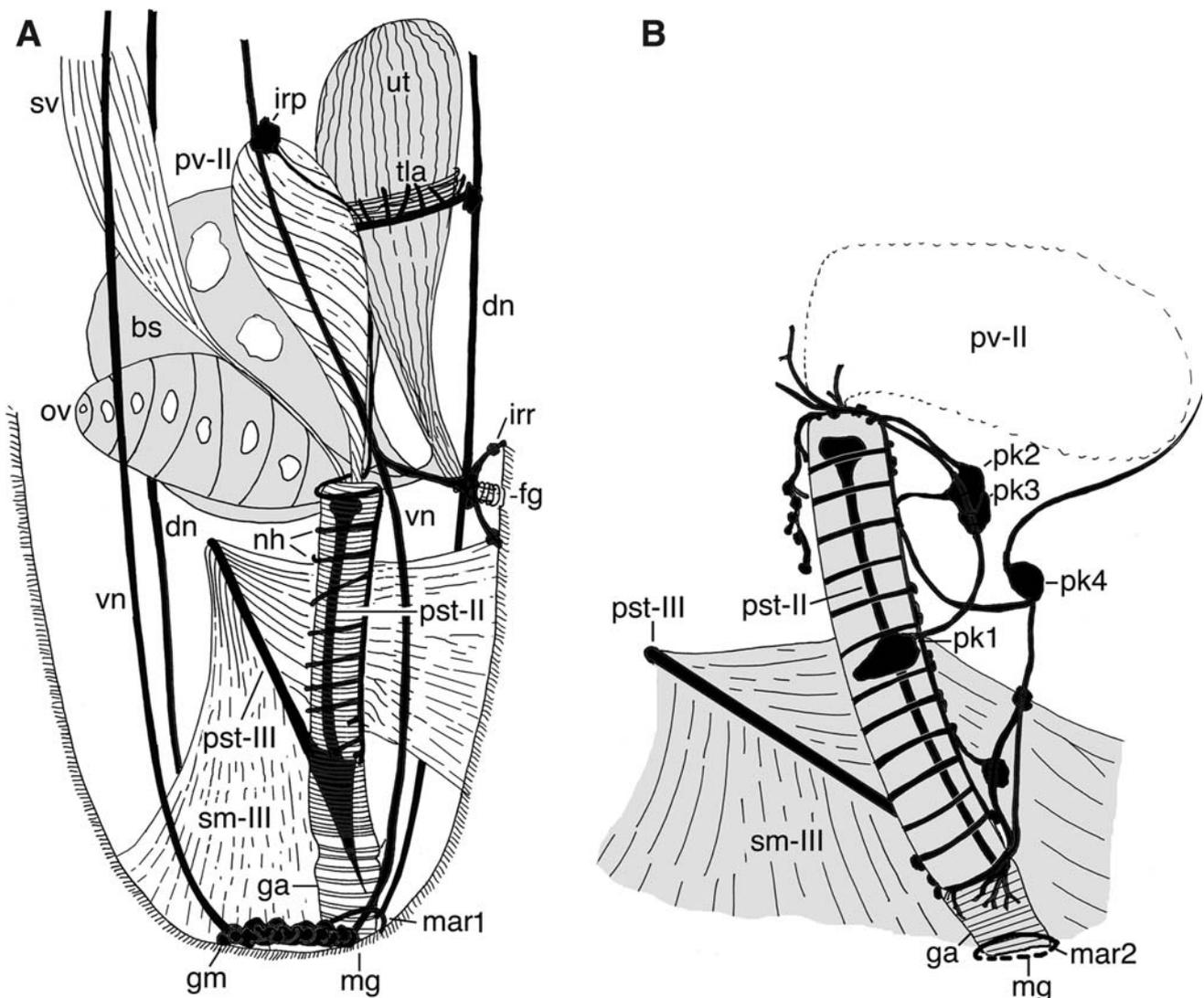


◀ **Fig. 4** Anti-5HT (serotonin) immunoreactivity in the male atrial complex. **a** Caudal end of specimen showing the region of the male atrium (*ma*) and its innervation by a large IR perikaryon (*pk1*). **b** Closeup of distal end of male atrium (different specimen) revealing two overlapping IR perikarya (*pk2–3*) that appear to innervate the male atrium. **c** Confocal slice through the male atrium revealing a neurite that branches and innervates the proximal (*pi*) and distal (*di*) portion of the male atrium. **d** Confocal slice through the same male atrium revealing an IR perikaryon (*pk3*) that is partially hidden in B

of *pst-III*, or patterns of cholinergic and FMRFamideergic neurons in the atrial system in general, would greatly enhance our knowledge of this process.

### Comparative aspects of innervation

The diversity of neuroactive substances in peripheral neurons that innervate reproductive organs has received significant attention in some platyhelminth clades, particularly for species of Seriata (Joffe and Reuter 1993; Reuter et al. 1995a, b, c, 1996; Johnston et al. 1996; Mäntylä et al. 1998) and Neodermata (Maule et al. 1990; reviewed in Gustafsson 1992; Armstrong et al. 1997; Mair et al. 1997; Halton and Maule 2004). In the course of these studies, researchers have noted both peptidergic and serotonergic innervation of male and female systems. Among proseri-



**Fig. 5** Schematics of innervation in the hermaphroditic reproductive system of *G. hermaphroditus* based on anti-SCPb and anti-5HT immunohistochemistry. Vitellaria and testis not shown. **a** SCP<sub>b</sub> peptidergic innervation. **b** Serotonergic innervation (only the male atrium is innervated). Abbreviations: *bs* seminal bursa, *dn* dorsal nerve cord, *fg* female gonopore, *ga* genital atrium, *gm* ganglionic mass, *irp* immunoreactive perikaryon, *irr* immunoreactive neural ring, *mar1* SCP<sub>b</sub>-IR

neural ring around the male genital atrium, *mar2* 5HT-IR neural ring around the male genital atrium, *mg* male gonopore, *nh* neural hoops around the male atrium, *ov* ovary, *pk1–4* 5HT-IR immunoreactive perikarya, *pst-II* prostate stylet type II, *pst-III* prostate stylet type III, *pv-II* prostatic vesicle type II, *sm-III* somatic muscles that supply *pst-III*, *sv* seminal vesicle, *tla* tiara-like arrangement of SCP<sub>b</sub>-IR neurons around the uterine sphincter, *ut* uterus, *vn* ventral nerve cord

ates, both RFamide and neuroactive peptide F (NPF) are known from the male copulatory organs of *Bothriomolus balticus* Meixner, 1938 (Joffe and Reuter 1993) and *Archilopsis unipunctata* (Fabricius, 1826) (Reuter et al. 1995a). Among species of Tricladida, GYIRFamide is present in neurons innervating the penis bulb of *Bdelloura candida* (Girard, 185) (Johnston et al. 1996); RFamide is present in neurons innervating a variety of male and/or female organs in *Dendrocoelum lacteum* Oersted, 1824, *Polycelis tenuis* Ijima, 1884 (Tricladida, Reuter et al. 1996) and *Planaria torva* (Mäntylä et al. 1998); and neuroactive peptide F (NPF) is present in neurons innervating both male and female organs in *Procerodes littoralis* Strom, 1768 (Reuter et al. 1995b) and *Planaria torva* Müller, 1773 (Mäntylä et al. 1998). These neuropeptides, among others, are also noted for neurons in the genital system of neodermatans where they innervate a suite of male and female organs (see review by Maule et al. 1990).

While homology among the various reproductive organs of proseriates, triclads, neodermatans, and free-living rhabdocoels such as *G. hermaphroditus* remains unclear, it is interesting to note the diversity of peptide transmitters that innervate these same organs. What is the function of this diversity? In vertebrates, there is redundancy at both the level of the neuropeptide and the receptor, i.e., different peptides can act on the same receptor, and one peptide can act on multiple receptors (Holmgren and Jensen 2001). However, this redundancy does not necessarily translate into equal physiological effects since different peptides can evoke different responses from the same receptor, and related receptors may have different second messenger systems (Holmgren and Jensen 2001). Is there a similar form of redundancy in the peptidergic system of platyhelminth reproductive organs? This question remains difficult to answer without detailed pharmacological assays of platyhelminth reproduction. For example, there is little evidence for multiple FMRFamide-related peptide (FaRP) receptors in platyhelminths (Geary et al. 1999; but see the variety of receptors for the biogenic amines, Ribeiro et al. 2005), which would suggest that peptide diversity in organ system innervation displays a form of vertebrate-like redundancy. In fact, different FaRPs often evoke similar myoexcitatory effects in monogeneans (Moneypenny et al. 1997), while in nematodes, different FaRPs can evoke qualitatively and quantitatively different effects (Fellowes et al. 1998).

Compared to the neuroactive peptides, the biogenic amine serotonin (5HT) is less pronounced in peripheral neurons that innervate reproductive structures of most seriates and neodermatans. For example, there is apparently no serotonergic innervation of female organs in any examined seriate, and innervation of male organs is either limited to the male copulatory organ (Joffe and Reuter 1993; Reuter et al. 1995a, b, c) or nearly absent from the reproductive

system (Reuter et al. 1996; Mäntylä et al. 1998). Similarly, among neodermatans, female reproductive organs receive only limited serotonergic innervation (e.g., the uterus of *Discocotyle sagittata* (Leuckart, 1842), Cable et al. 1996; and *D. botthrium*, Lindholm et al. 1998) or none at all (e.g., Maule et al. 1990). However, in *Fasciola hepatica* Linnaeus, 1758, there is extensive innervation of the ootype and Mehlis gland complex (Fairweather et al. 1987), highlighting the variability in reproductive organ physiology among parasitic species, perhaps related to their host environment.

While much attention has been directed at species of Neodermata to understand neural control of egg production and its potential as a chemotherapeutic target (e.g., Armstrong et al. 1997), similar efforts would also benefit our understanding of free-living species. Patterns of immunoreactivity, especially among neuroactive substances with known or presumed reproductive roles (e.g., 5HT, SCP<sub>b</sub>, FMRFamide, ACh), would provide knowledge of neuromuscular function and reproductive physiology that are currently lacking from this large and phylogenetically diverse assemblage. A comparative examination would also provide data for testing hypotheses of atrial system function, the homology of reproductive organs (e.g., Polycystididae, Artois and Schockaert 2003, 2005), and neural evolution (see Fig. 4 in Reuter and Gustafsson 1989).

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