Nematology

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Serotonin Regulates the Feeding and Reproductive Behaviors of *Pratylenchus penetrans*

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ABSTRACT

The success of all plant-parasitic nematodes is dependent on the completion of several complex behaviors. The lesion nematode *Pratylenchus penetrans* is an economically important parasite of a diverse range of plant hosts. Unlike the cyst and root-knot nematodes, *P. penetrans* moves both within and outside of the host roots and can feed from both locations. Adult females of *P. penetrans* require insemination by actively moving males for reproduction and can lay eggs both within and outside of the host roots. We do not have a complete understanding of the molecular basis for these behaviors. One candidate modulator of these behaviors is the neurotransmitter serotonin. Previous research demonstrated an effect of exogenously applied serotonin on the feeding and male mating behaviors of cyst and root-knot nematodes. However, there are no data on the role of exogenous serotonin on lesion nematodes. Similarly, there are no data on the presence and function of endogenous serotonin in any plant-parasitic nematode. Here, we establish that exogenous serotonin applied to *P. penetrans* regulates both feeding and sex-specific behaviors. Furthermore, using immunohistochemistry and pharmacological assays, our data suggest that *P. penetrans* utilizes endogenous serotonin to regulate both feeding and sex-specific behaviors.

Plant-parasitic nematodes utilize sophisticated behaviors for both feeding and reproduction. Although efforts to elucidate the biochemical, cellular, and genetic regulation of plant-parasitic nematode behavior are increasing and ongoing (Atkinson et al. 2013, Cotton et al. 2014; Gheysen and Mitchum 2011; Haegeman et al. 2012; Manosalva et al. 2015; Rehman et al. 2016), our knowledge of these mechanisms is still relatively limited. Plant-parasitic nematodes use a protractible stylet for infection and feeding (Decraemer and Hunt 2006). In the Tylenchida plant-parasitic nematodes, the needle-shaped stylet is attached to three stylet protractor muscles that contract to produce a forward thrust of the stylet (Baldwin and Hirschmann 1976; Endo 1983). Reproductive behaviors, including egg laying and male mating, are also likely under neuronal control. Although feeding and reproductive behaviors have been extensively studied in the bacterial-feeding nematode *Caenorhabditis elegans*, there are striking anatomical differences between the structures mediating these behaviors in *C. elegans* and plant-parasitic nematodes (Albertson and Thomson 1976; Endo 1984; Endo et al. 1997).

The neurotransmitter serotonin (5-hydroxytryptamine) is a conserved regulator of various behaviors in animals. In *C. elegans*, serotonin signaling regulates feeding, egg laying, and male mating behaviors (Chase and Koelle 2007; Loer and Kenyon 1993; Sze et al. 2000). Application of exogenous serotonin induces stylet thrusting in several cyst and root-knot species in the absence of a host (Hu et al. 2014; Jonz et al. 2001; Masler 2007). Similarly, exogenous serotonin application can induce spicule eversion behavior in male cyst nematodes (Jonz et al. 2001). However, previous studies examined the effects of exogenously applied serotonin on plant-parasitic nematodes, rather than the role of endogenous serotonin.

Lesion nematodes (*Pratylenchus* spp.) are important migratory endoparasites of numerous crops worldwide (Jones et al. 2013). Lesion nematodes display several behaviors that may be mediated through serotonin signaling. Unlike the sedentary cyst and root-knot nematodes, lesion nematodes move throughout development and feed both endo- and ectoparasitically (Zunke 1990). Upon reaching adulthood, the females lay eggs both within and outside of the roots (Rebois and Huettel 1986). Many species of lesion nematodes are gonochoristic and likely require male-specific mating behaviors.

The primary objective of this study was to elucidate the role of serotonin in *Pratylenchus penetrans* behavior. Therefore, we tested how exogenous serotonin affects feeding and reproductive behaviors of male and female *P. penetrans*. To investigate the role of endogenous serotonin in *P. penetrans,* we used immunohistochemistry to identify putative serotonergic neurons. We tested several pharmacological compounds that affect the endogenous serotonergic signaling pathway. Our results suggest that *P. penetrans* uses endogenous serotonin signaling to regulate specific behaviors. The nervous system is a well-established target for several nematicides and anthelmintics (Holden-Dye and Walker 2011). A better understanding of the neurobiology of plant-parasitic nematodes may lead to novel control strategies.

MATERIALS AND METHODS

Nematodes. *P. penetrans* nematodes were obtained from Dr. Terry Niblack and cultured on monoxenic corn root cultures grown on sterile Gamborg's (1.5% agar) media (Rebois and Huettel 1986). Briefly, sweet corn seed were surface sterilized in 95% ethanol for 3 min followed by 10% bleach for 10 min, and rinsed with sterile water. Sterile seed were placed on Gamborg's media for germination at room temperature. After approximately 7 days, the root was excised from the seed and placed back onto the media. A block of agar containing numerous *P. penetrans* nematodes was then transferred from an old culture onto the fresh media using sterile technique. Cultures were incubated at 20°C for at least 2 weeks before use.

Behavioral assays. To test the effect of serotonin on behavior, a two-factorial (sex and treatment) completed randomized design

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*The *e*-**X**tra logo stands for "electronic extra" and indicates that one supplementary movie is published online.

was used. Each experimental unit contained 20 to 30 nematodes. Nematodes were extracted from cultures by transferring a portion of the agar into sterile 5% M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85.5 mM NaCl, and 1 mM $MgCl_2$) (Brenner 1974). Actively moving adult males and females were picked under a dissecting microscope and incubated in 200 µl of 5 mM serotonin in 5% M9 buffer (treated) or 5% M9 buffer alone (control) for 15 to 20 min at room temperature (20 to 22°C). This concentration of serotonin was previously shown to induce stylet thrusting in cyst and root-knot nematodes (Masler 2007). To ensure that all nematodes were exposed for a similar time, only 5 to 10 mixed male and female *P. penetrans* nematodes were incubated in the serotonin or control as a batch, and multiple batches were tested for the assay. The duration for one batch from the beginning of incubation to the end of the observation did not exceed 40 min. We did not observe any obvious decrease in stimulated behavior in the various compounds that would suggest habituation during the exposure period. The batch of nematodes from the incubation solution was placed immediately into a drop of either 5 mM serotonin in 5% M9 buffer (for treated) or 5% M9 buffer alone (for control) on an 8 to 10% agarose pad made by dissolving agarose in water (Sulston 1976). A coverslip was then gently placed onto the agarose pad with the nematodes. The high concentration of agarose effectively restrained the nematodes for observation while still allowing sufficient movement to ensure that the handling had not killed the nematodes. The number of stylet thrusts in both females and males and vulva muscle contractions in females were counted in each nematode for 1 min at room temperature. We define a stylet thrust as a forward movement of the stylet knobs, cone, and shaft due to contraction of the stylet protractor muscles. Males and females were examined in a random order based on their position on the agarose pad. Males were also examined for the presence of spicule eversion. All behaviors were observed under differential interference contrast microscopy at ×630 or ×1,000 magnification at room temperature. An experimental design similar to the serotonin assay was used to test the behavior of *P. penetrans* following exposure to the following compounds: 2 mM fluoxetine, 10 mM imipramine, 100 mM imipramine, 10 mM octopamine, and 26 mM phentolamine. All chemicals were dissolved in 5% M9 buffer, with the exception of 100 mM imipramine, which was insoluble in 5% M9 and, therefore, dissolved in sterile H_2O. For the imipramine assay, we had only 20 nematodes for each treatment. The concentrations of compounds were based on previous experiments with *C. elegans* and other plant-parasitic nematodes, with the exception of 100 mM imipramine, which was used to test whether a higher concentration would affect vulva contractions (Dempsey et al. 2005; Horvitz et al. 1982; Schroeder and MacGuidwin 2010).

Data from the stylet thrusting assays were compared using a two-way analysis of variance. Because of the lack of a significant main or interaction effect due to sex, data from both sexes were pooled for the figure. Vulva muscle contraction data were analyzed using Student's *t* test. Spicule eversion was never observed in control males and, therefore, was not subjected to statistical analysis.

***P. penetrans* antiserotonin staining.** *P. penetrans* nematodes were extracted from monoxenic cultures and washed with distilled water in a microcentrifuge tube. The antibody staining procedure was modified from previous methods (Hussey 1989). Briefly, nematodes were fixed in 4% formaldehyde at 4°C for 8 to 11 h. After washing with phosphate-buffered saline with Triton X-100 (PBST: 8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, and 0.05% Triton X-100, pH 7.4), nematodes were transferred into a glass well. A razor was used to slice the nematodes into small segments. Nematode sections were incubated in 100 mM Tris (pH 7.5), 1 mM $CaCl_2$, and proteinase K (2 mg/ml) for 30 min at room temperature to facilitate antigen retrieval, then washed three times with PBST. Next, nematode segments were incubated in prechilled methanol at −20°C for 1 min followed by 1 min in prechilled acetone at −20°C, then washed three times in PBST. Nematode

segments were blocked in 1% bovine serum albumin (BSA)/PBST buffer (catalog number A9418; Sigma-Aldrich, St. Louis) overnight at room temperature or 4°C. After blocking, nematode segments were incubated in 1:100 rabbit-derived antiserotonin (catalog number S5545; Sigma-Aldrich) overnight at either room temperature or 4°C, then washed three times with PBST. Finally, nematode segments were incubated in 1:100 goat-derived fluorescein isothiocyanate (FITC)-labeled antirabbit serum (catalog number F9887; Sigma-Aldrich) in 0.1% BSA/PBST for 2 h at 37°C. Samples were washed three times in PBST before observation. Images were acquired using a Zeiss M2 AxioImager and Zen software. Overlay images and scale bars were created using ImageJ.

RESULTS

Similar to studies in sedentary endoparasitic nematodes (Hu et al. 2014; Jonz et al. 2001; Masler 2007; Rolfe and Perry 2001; Schroeder and MacGuidwin 2010), we found that application of serotonin induced stylet thrusting in the migratory parasite *P. penetrans* (Fig. 1; Supplementary Movie S1). We rarely (<10%) observed serotonin-induced pumping of the metacorpus in *P. penetrans.* Although not statistically significant ($P = 0.0966$), we noted a trend of greater sensitivity to serotonin-induced stylet thrusting in female compared with male *P. penetrans* (data not shown).

To investigate the role of serotonin further, we tested the effect of exogenous serotonin on sex-specific structures. We observed continuous spicule eversion in 29 of 31 *P. penetrans* males following exposure to serotonin. Control males never showed spicule eversion ($n = 30$). In females, we found that application of serotonin induced contractions of vulva muscles (Fig. 2).

To determine the presence and localization of endogenous serotonin in *P. penetrans,* we used an antiserotonin antibody. We detected several serotonin-immunoreactive cells in *P. penetrans* (Fig. 3). The brightest and most consistent staining was found in a pair of neurons in the head of the nematodes immediately posterior of the nerve ring (Fig. 3A). Based on their position and serotonin immunoreactivity, we speculate that these cells are homologous to the *C. elegans* ADF amphid neurons (Fig. 4). Anterior to the nerve ring, we detected at least one pair of neurons that have no known positional homolog among the serotonergic neurons in *C. elegans.* Within the esophagus, we only observed occasional and faint staining in a cell located on the ventral side of the metacorpus that is likely homologous to the *C. elegans* NSM neurons. We also detected serotonin-immunoreactive cells in the ventral nerve cord of both sexes. In females, two serotonin-immunoreactive cells were reliably detected in the ventral cord located immediately anterior and posterior of the vulva (Fig. 3B). These cells extended processes that came into close contact with the vulva muscles. These cells appear homologous to the *C. elegans* VC4 and VC5 neurons (Fig. 4). In one *P. penetrans* female, we found a serotonin-immunoreactive neuron anterior to the vulva that we suspect is homologous to the *C. elegans* HSN neuron (data not shown); however, this observation was not consistent. In males, we observed several serotonin-immunoreactive neurons in the ventral cord (Fig. 3C). We suspect that these are homologous to the male serotonergic CP neurons in *C. elegans* (Fig. 4). Because of the requirement to cut the animal to achieve adequate fixation and staining, we could not obtain an accurate count of these male-specific neurons.

To further probe the mechanism of endogenous serotonin signaling in *P. penetrans*, we tested the effect of the serotonin reuptake inhibitors fluoxetine and imipramine on serotonin-responsive behaviors in *P. penetrans.* Serotonin signaling is negatively regulated through reuptake (Dempsey et al. 2005). Reuptake inhibitors should increase the level of endogenous serotonin available for signaling. Therefore, application of these reuptake inhibitors should produce results similar to those from the application of exogenous serotonin alone. As expected, both fluoxetine and imipramine caused increased stylet thrusting in *P. penetrans* compared with control nematodes

(Fig. 1). Interestingly, however, these compounds did not affect sex-specific behaviors identically to exogenous serotonin. Although fluoxetine increased the frequency of vulva muscle contractions compared with nontreated nematodes ($P = 0.0342$), the effect was noticeably reduced compared with that seen with exogenous serotonin (Fig. 2). Although not statistically significant ($P = 0.0705$), 10 mM imipramine caused a trend of increased vulva contractions; however, similar to fluoxetine, the effect of imipramine was reduced compared with exogenous serotonin. However, exposure to 100 mM imipramine caused a significant increase in vulva contractions compared with the control that was similar in magnitude to that seen with 5 mM serotonin. Conversely, exogenous fluoxetine application resulted in no apparent spicule eversion in males whereas application of 10 mM imipramine resulted in spicule eversion in 25 of 30 males examined. Interestingly, the spicule eversion behavior of imipramine-exposed males differed from males exposed to serotonin. Although males exposed to serotonin had continuously everted spicules, approximately half of the imipramine-exposed males showed repeated spicule eversion and retraction.

Several studies in *C. elegans* suggest that octopamine acts as a physiological antagonist to serotonin (Chase and Koelle 2007; Horvitz et al. 1982). In our initial serotonin behavioral assays, we observed low levels of spontaneous stylet thrusting in control animals. We hypothesized that, if octopamine antagonizes endogenous serotonin signaling, then exposure to octopamine would reduce spontaneous stylet thrusting in *P. penetrans*. As expected, exposure to 10 mM octopamine significantly reduced spontaneous stylet thrusting (Fig. 1). However, exposure to octopamine did not affect vulva contraction (Fig. 2). To confirm our octopamine results, we tested the effect of phentolamine, an antagonist of octopamine signaling, on stylet thrusting (Evans and O'Shea 1978). Exposure to phentolamine increased stylet thrusting in *P. penetrans* (Fig. 1), suggesting that endogenous octopamine acts to suppress serotonin signaling for stylet thrusting. Interestingly, however, phentolamine had no apparent effect on vulva muscle contraction or spicule eversion (Fig. 2).

DISCUSSION

Our results suggest that endogenous serotonin signaling regulates stylet thrusting behavior in *P. penetrans*. Similar to previous results in sedentary plant-parasitic nematodes (Hu et al. 2014; Jonz et al. 2001; Masler 2007), we found that exogenously applied serotonin induces stylet thrusting in *P. penetrans*. Similar to *C. elegans*, we identified serotonin-immunoreactive cells within and adjacent to the esophagus. However, the specific pattern of staining differed from that found in *C. elegans* and other bacterial feeding nematodes (Horvitz et al. 1982; Loer and Rivard 2007).

In *C. elegans*, the two strongest serotonin immunoreactive neuron classes are NSM and ADF (Horvitz et al. 1982; Loer and Rivard 2007) (Fig. 4). The NSM neurons are located within the metacorpus
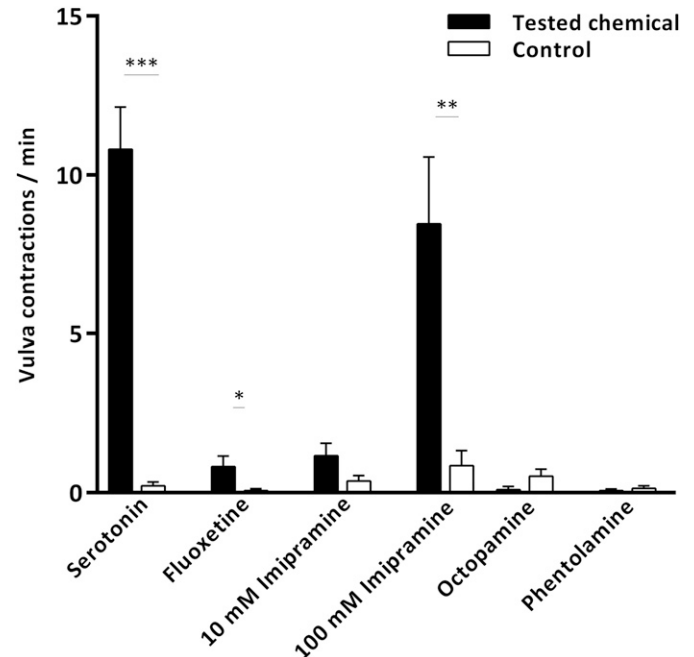
Fig. 2. Serotonin induces vulva contractions in *Pratylenchus penetrans*. 20 to 30 adult female *P. penetrans* nematodes were exposed to either a control solution (white bars, 5% M9, with the exception of the 100 mM imipramine, which was dissolved in H_2O) or one of the following compounds: 5 mM serotonin, 2 mM fluoxetine, 10 mM imipramine, 100 mM imipramine, 10 mM octopamine, or 26 mM phentolamine (black bars). The number of vulva contractions per min were counted for 1 min under a compound microscope. Data from each assay were analyzed using an unpaired *t* test; ***, **, and * indicate $P < 0.001$, 0.01, and 0.05, respectively; error bars = standard error of the mean.
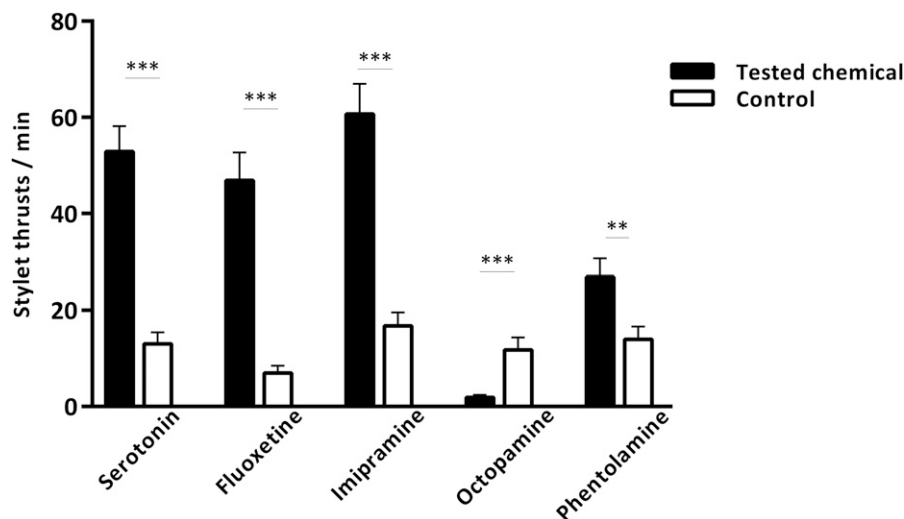
Fig. 1. Serotonin and octopamine signaling affect stylet thrusting behavior in *Pratylenchus penetrans*. Adult male and female *P. penetrans* nematodes were exposed to either a 5% M9 buffer solution (white bars) or one of the following compounds: 5 mM serotonin, 2 mM fluoxetine, 10 mM imipramine, 10 mM octopamine, or 26 mM phentolamine (black bars). The number of stylet thrusts was counted for 1 min under a compound microscope; $n > 30$ per treatment; *** and ** indicate $P < 0.001$ and 0.01, respectively; error bars = standard error of the mean.

of the esophagus whereas the ADF neurons are located outside the esophagus (Albertson and Thomson 1976; Ward et al. 1975). NSM serotonin-immunoreactive homologs are also found in a wide range of bacterial-feeding and animal-parasitic nematode species (Johnson et al. 1996; Loer and Rivard 2007). Interestingly, we found only weak and inconsistent immunoreactivity within the metacorpus of *P. penetrans* where an NSM homolog is expected; however, we cannot completely rule out that our antibody staining was ineffective at penetrating the esophagus. Recent research in *C. elegans* suggests that serotonin produced by the ADF neurons is sufficient to modulate feeding behavior (Cunningham et al. 2012; Song et al. 2013). Therefore, it is possible that serotonin released

from outside of the *P. penetrans* esophagus leads to stylet thrusting behavior. In addition to the putative ADF homologs, we consistently found serotonin-immunoreactive cells outside of the esophagus in a position just anterior of the nerve ring in *P. penetrans*. There are no known serotonergic cells at this position in *C. elegans*. One caveat to our antibody staining is that very little is known about the neuroanatomy of *P. penetrans* (Endo et al. 1997; Han et al. 2016; Trett and Perry 1985). Although our data demonstrate the presence of serotonin in *P. penetrans,* our assignment of individual cells as homologs to *C. elegans* is based solely on serotonin immunoreactivity, the position of the cell body, and, when available, the morphology of associated neuronal processes.
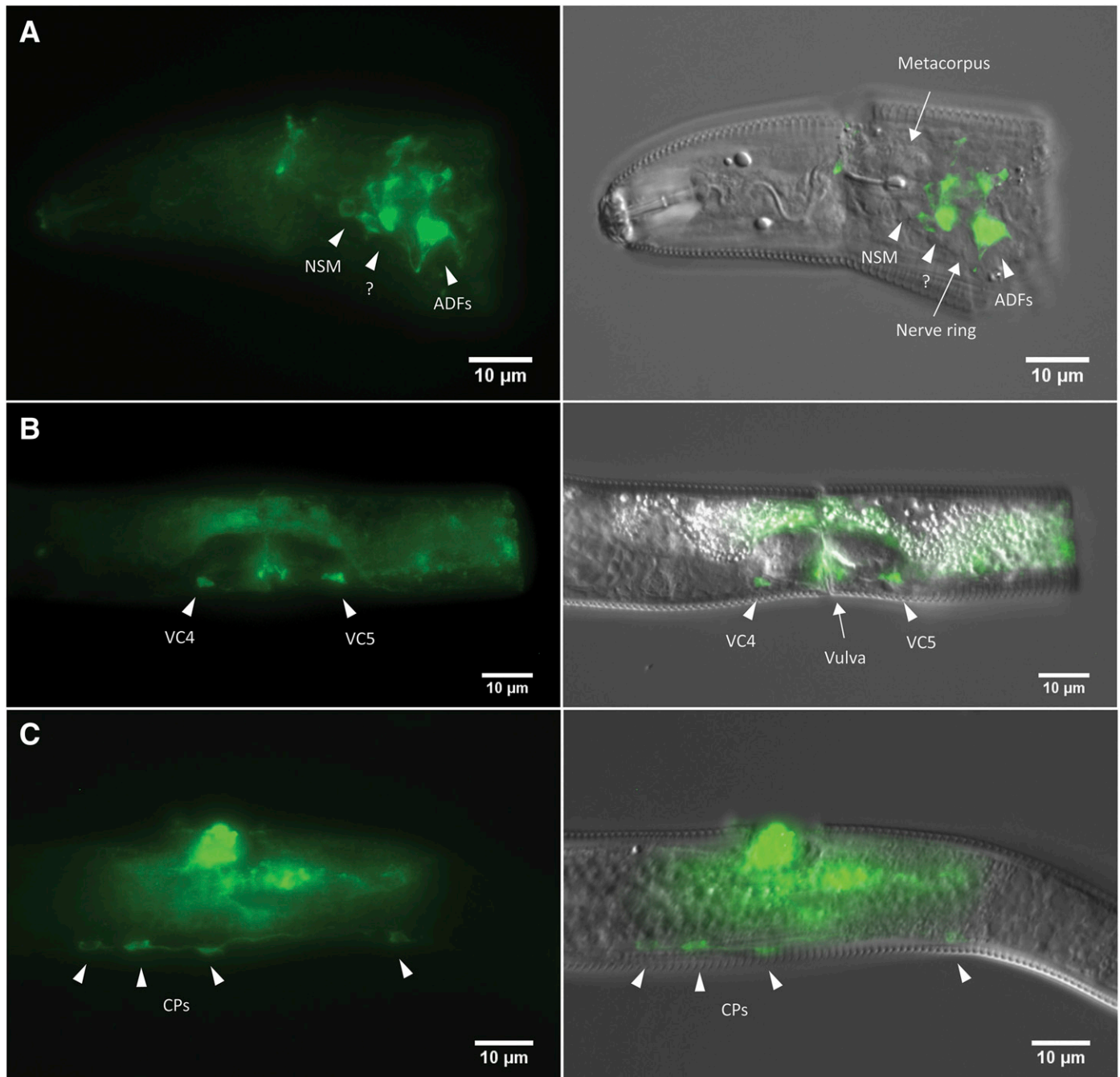
Fig. 3. Antibody staining reveals endogenous serotonin in *Pratylenchus penetrans.* Fluorescence (left) and differential interference contrast overlay (right) images of antiserotonin staining. All images are arranged anterior to the left and dorsal aspect to the top. Arrowheads indicate serotonin immunoreactive cells. **A,** Anteriors of both sexes have two pairs of serotonin immunoreactive cells. Putative ADF homologs and one unidentified pair (question mark) were consistently and strongly immunoreactive. Unidentified neurons are anterior to the nerve ring whereas ADF are posterior to the nerve ring. The putative NSM homologs in the metacorpus are weakly and inconsistently immunoreactive. **B,** In the vulva region of adult females, the putative VC4 and VC5 neuron homologs are strongly immunoreactive. **C,** In the ventral nerve cord of the male, several putative CP neuron homologs are strongly immunoreactive.
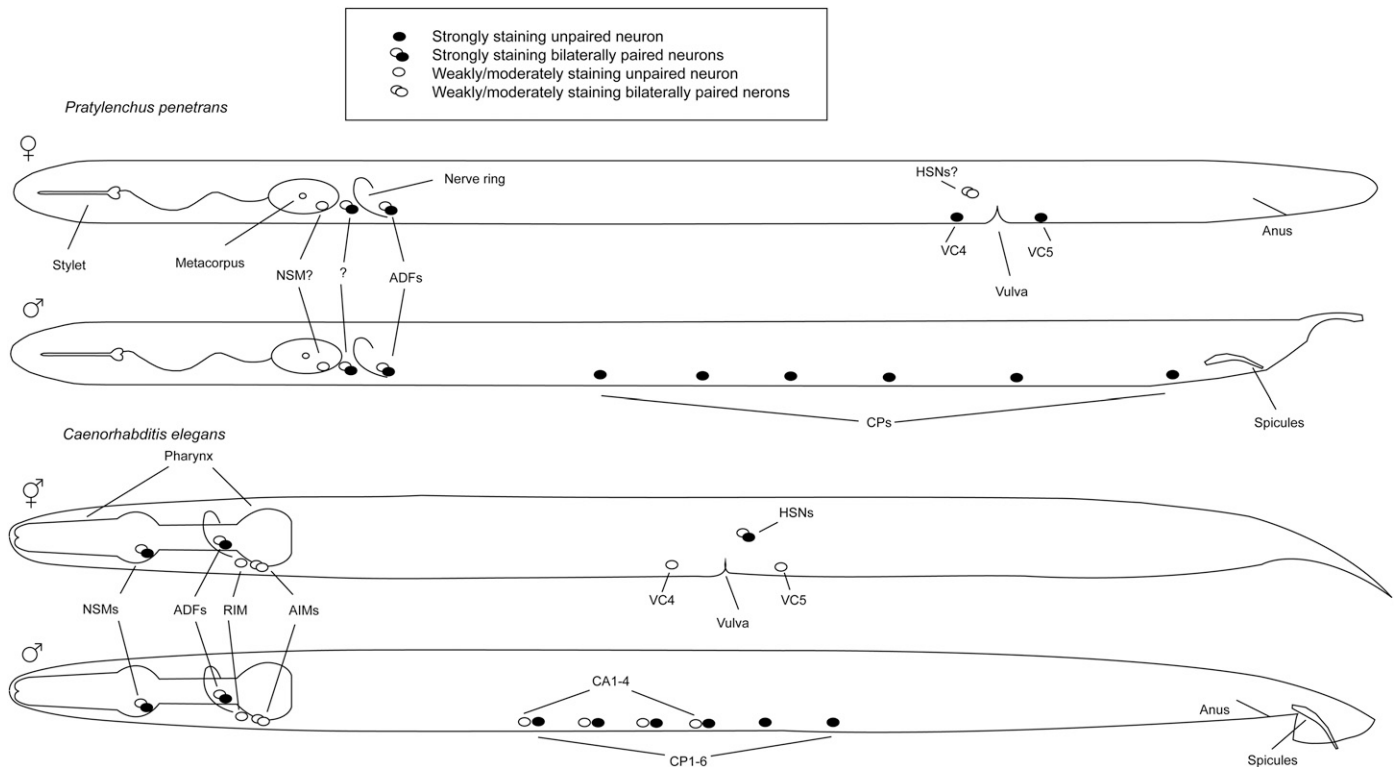
Fig. 4. Schematic of antiserotonin immunoreactivity in *Pratylenchus penetrans* and *Caenorhabditis elegans* as determined by this study and previous research, respectively (Duerr et al. 1999; Loer and Kenyon 1993; Loer and Rivard 2007).

Exogenous serotonin stimulated sex-specific behaviors in *P. penetrans*. Similarly, we found immunoreactive cells that may serve as a source of endogenous serotonin in proximity to these sex-specific structures. In *C. elegans* hermaphrodites, the serotonergic HSN neurons innervate the vulva muscles and regulate egg laying behavior (Fig. 4). The VC4 and VC5 motor neurons of *C. elegans* hermaphrodites are serotonin immunoreactive but do not express the gene required for serotonin biosynthesis (Sze et al. 2000). Therefore, the VC4 and VC5 neurons of *C. elegans* are thought to take up serotonin produced by HSN (Anderson et al. 2013). Interestingly, in *P. penetrans*, we found serotonin immunoreactivity in the putative VC4 and VC5 neurons even in the absence of apparent serotonin immunoreactivity in an HSN homolog (Fig. 3). We also observed serotonin immunoreactivity in the ventral cord of male *P. penetrans*. We speculate that these serotonin-immunoreactive cells are homologous to the serotonergic CP motor neurons found in *C. elegans* and several other free-living nematodes (Loer and Rivard 2007; Sze et al. 2000). Ablation of CP neurons in *C. elegans* results in males defective for turning during mating (Loer and Kenyon 1993). Similar to results in *Heterodera schachtii* and *Panagrellus redivivus,* we found that serotonin induced spicule eversion in *Pratylenchus penetrans* (Croll 1975; Jonz et al. 2001). Unlike in *H. schachtii* males (Jonz et al. 2001), we did not observe any ejaculation from *P. penetrans* following exposure to serotonin. Given the proximity of the *P. penetrans* CP neurons to the spicule, it is reasonable to assume that serotonin released from these neurons modulates spicule insertion during mating.

Our experiments with serotonin reuptake inhibitors also provided evidence that sex-specific behaviors in *P. penetrans* are regulated by endogenous serotonin. However, unlike the stylet-thrusting assays, application of imipramine and fluoxetine did not produce a size effect similar to that of exogenous serotonin. One possible explanation is that the dose of fluoxetine and imipramine required to induce stylet thrusting is lower than that required for vulva muscle contraction or continuous spicule eversion. Although both fluoxetine and imipramine inhibit the reuptake of serotonin, they can also act outside of the serotonin reuptake pathway to regulate *C. elegans* egg laying

(Dempsey et al. 2005; Weinshenker et al. 1995). Therefore, the inconsistent effects of fluoxetine and imipramine in *P. penetrans* sex-specific behaviors compared with *C. elegans* might indicate that *P. penetrans* uses a partially distinct neuronal signaling pathway.

A better understanding of the mechanisms regulating behaviors in plant-parasitic nematodes could lead to novel control strategies. The anthelminthic ivermectin is safe for mammals due, in part, to its affinity for invertebrate-specific glutamate neurotransmitter receptors (Geary 2005). The application of synthetic peptides designed to inhibit acetylcholinesterase in the head sensory neurons disrupted the cyst nematode host-sensing behavior (Wang et al. 2011). Despite the conservation of the serotonin biosynthesis pathway among animals, serotonin receptors can vary among different animal species (Komuniecki et al. 2004). Our results demonstrate the presence of endogenous serotonin in *P. penetrans*. The identification of specific serotonin receptors in plant-parasitic nematodes may provide new targets to selectively interfere with serotonin signaling.

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