

## Serotonin in *Trichinella pseudospiralis*: An immunocytochemical study

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### Article info

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### Summary

This is the first report on the presence and localization of the neurotransmitter serotonin (5-HT) in the nervous system of the nematode *Trichinella pseudospiralis*, the causative agent of trichinellosis. The orientation of the 5-HT-immunoreactive (5-HT-IR) nerve cells in the adult worm is described. In the anterior region of the worm 5-HT-IR occurs in 7 neurons. Longitudinal nerve cords extend posteriorly from the anterior end. They are connected by transverse commissures. The vulval area is intensively supplied with 5-HT-IR nerve cells and fibres forming a plexus. Two rows of small 5HT-IR structures, hypodermal glands, are visible along the whole nematode body. Because of the conserved structural features among nematodes the 5-HT-IR neurons observed are likely to have counterparts in the model worm, *Caenorhabditis elegans*. Some basic differences are evident and demand further study.

**Keywords:** *Trichinella pseudospiralis*; nematodes; neurotransmitters; serotonin; nervous system

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### Introduction

Trichinellosis is a zoonotic disease caused by parasitic nematodes of the genus *Trichinella* (Gottstein *et al.*, 2009). This disease is a public-health concern throughout the world (Pozio, 2001; Dupouy-Camet, 2000). The main sources of human infection are larvae from meat of pork, game and horse. Although human infections have not been reported for certain genotypes, all *Trichinella* species are considered as human pathogens (Dupouy-Camet *et al.*, 2002). The nervous system of parasitic nematodes plays a key role in the control of all activities of the worm and is thus the main target when new anti-parasitic drugs are developed. The distribution and function of different neurotransmitters have been investigated in numerous helminth groups (Terenina & Gustafsson, 2003, 2014). Nematodes have been extensively studied particularly in *Caenorhabditis elegans* (Chase & Koelle, 2007). A wide range of signaling molecules, including the bioamine serotonin (5-HT), has been identified. A variety of experimental approaches, including

immunocytochemical ones, have revealed 5-HT in 8 classes of neurons in *C. elegans*. Numerous specific functions of 5-HT have been defined. These include, feeding, locomotion, egg laying and copulatory behavior (Horvitz *et al.*, 1982; Desai *et al.*, 1988; McIntire *et al.*, 1992; Loer & Kenyon, 1993; Duerr *et al.*, 1999; Sze *et al.*, 2000). Some studies have been made on neurotransmitters in plant nematodes (Holden-Dye & Walker, 2011). According to Masler (2007, 2008) 5-HT influences the general motility, the egg laying process and the stylet activity of *Heterodera glycines* and *Meloidogyne incognita*. The effect of serotonin on the reproductive behavior of *Heterodera schachtii* has also been recorded (Jonz *et al.*, 2001). The receptors for 5-HT and the genes encoding the enzymes of the 5-HT-synthesis have been identified in *C. elegans* (Loer & Kenyon, 1993; Olde & McCombie, 1997; Hamdan *et al.*, 1999; Sze *et al.*, 2000; Ranganathan *et al.*, 2000; Hobson *et al.*, 2003). Only limited information is available regarding the function of 5-HT in parasitic nematodes. According to Brownlee *et al.* (1995) 5-HT has an effect on the pharyngeal musculature of the parasitic ne-

matode *Ascaris suum*. The presence of 5-HT in the neurons of the pharynx and the tail area of the ventral nerve cord of male *A. suum* has been described by Martin and Donahue (1989), Johnson *et al.* (1996) and Fellowes *et al.* (1999, 2000). According to Rao *et al.* (2011), 5-HT occurs in the nervous system of the highly pathogenic nematode of small ruminants, *Haemonchus contortus*, where it was localized in the amphidal and pharyngeal neurons of adult female and male worms. 5-HT was also found in a ray of sensory neurons and in a few ventral cord motor neurons in adult males. According to Rao *et al.* (2011) 5-HT had an inhibiting effect on the movement of the worms.

Data on the nervous system and neurotransmitters of *Trichinella* spp. are scarce. The presence of cholinesterase activity (enzyme of cholinergic system) in *Trichinella spiralis* (Ramisz, 1965; De Vos & Dick, 1992) and presence of several catecholaminergic neurons in the anterior end of this nematode (Lee & Ko, 1991) have been described. Despite its medical importance there are no reports dealing with the presence and function of serotonin (5-HT) in *Trichinella*. In the present study the distribution of 5HT in *Trichinella* is investigated. This is thus the first report on the distribution of the neurotransmitter 5-HT in *Trichinella*.

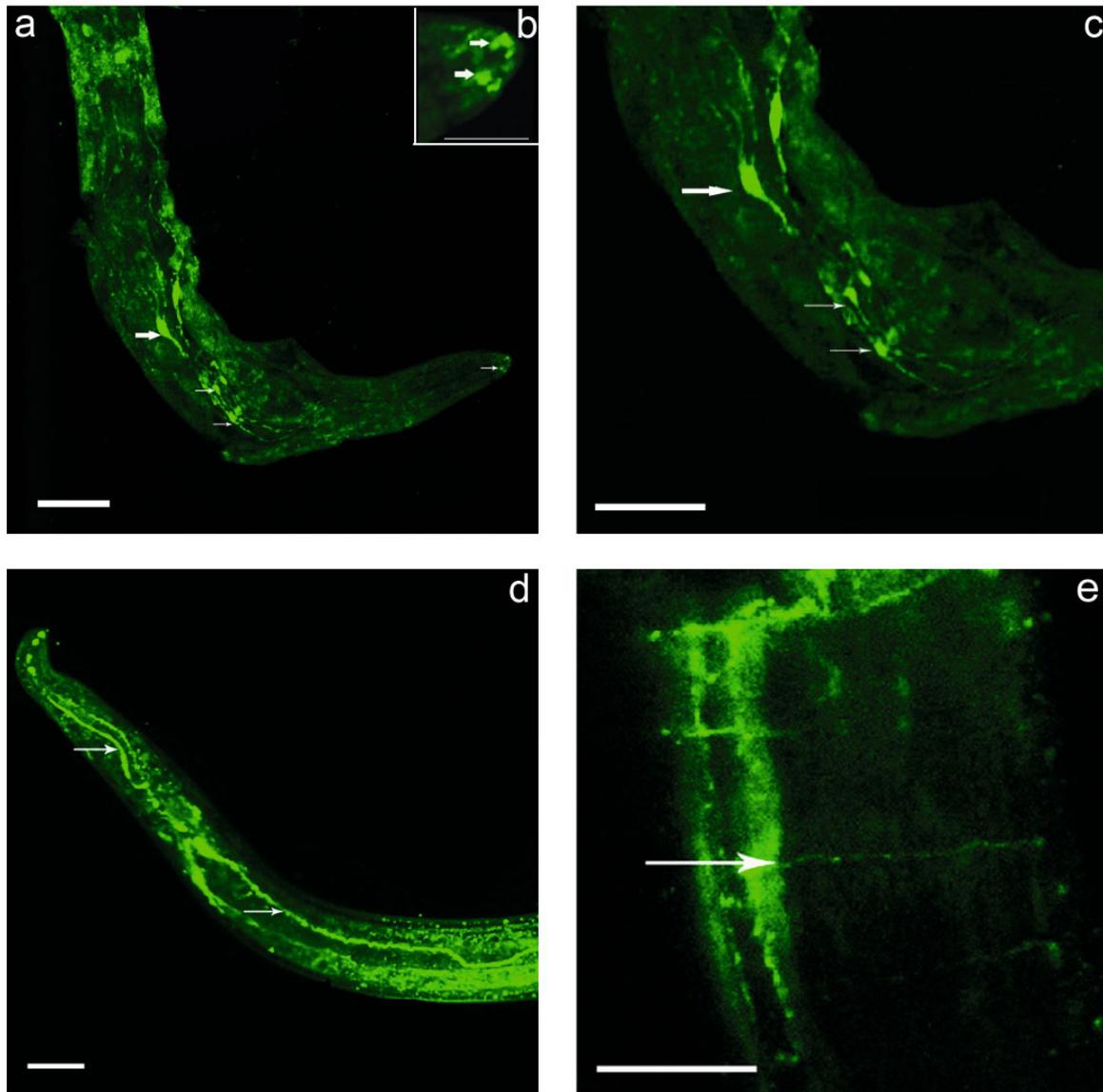


Fig. 1. The pattern of 5-HT-IR neurons and fibres in head region of *T. pseudospiralis*.  
**a** – Three pairs of neurons (large and small arrows) with nerve fibres extending in anterior and posterior direction. Note 5-HT-IR in the tip of the head (small arrow). Bar = 30  $\mu$ m. *Inset*. Magnification of the tip of the head with 5-HT-IR structures (arrows); **b** – Bar = 10  $\mu$ m; **c** – Magnification of the three pairs of neurons (large and small arrows). Bar = 25  $\mu$ m; **d** – The pattern of 5-HT nerve cords (arrows). Bar = 20  $\mu$ m; **e** – Magnification of the longitudinal nerve cord connected by transverse commissures (arrow). Bar = 10  $\mu$ m

## Materials and Methods

### *Nematodes and fixation*

Specimens of adult female and male worms of *Trichinella pseudospiralis* (Garkavi, 1972) were used in this study. The worms were obtained from the small intestines of experimentally infected laboratory rats. The infection was carried out as follows: 1. larvae of *T. pseudospiralis* were derived from domestic pigs from the Kamchatka region in Russia. 2. the pig musculature was digested in artificial gastric juice. 3. the larvae were washed out with warm physiological salt solution and collected. 4. Laboratory rats were infected orally with larvae of *T. pseudospiralis* (10 larvae/1 g of rat weight). 5. The rats were sacrificed at 4, 5 days post infection. 6. the small intestine was removed, slit longitudinally and washed with warm (37 °C) physiological salt solution to remove the contents. 7. adult *T. pseudospiralis* were collected. The material was fixed in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PBS, Sigma) at 4 °C and pH 7.4. For storage, the material was transferred to the PBS buffer with 10 % sucrose.

### *Primary antibody incubation*

For better antibody penetration nematodes were slightly mechanically disrupted prior to the incubation using a blade. Worms were stained with rabbit- anti- 5-HT (Incstar, Stillwater, Belmont, CA, USA) (1:500) primary antibodies in PBS containing 1 % (v/v) Triton X 100 (Sigma) (PBS-T) according to the method described by Coons *et al.* (1955). The samples were incubated in primary antibody for 5 days at 4 °C, then they were washed with PBS-T (3 times during 10 min).

### *Secondary antibody incubation*

Worms were incubated with the secondary antibody goat anti-rabbit Alexa 488 (Molecular Probes, USA) (1:400) in PBS-T for 5 days at 4 °C. Controls included omission of the primary antibody and substitution of primary antibody with non-immune rabbit serum. The stained preparations were examined with a fluorescent microscope Leica DM 1000 (A. N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences, Center of Parasitology) and a Leica TCS SP5 confocal scanning laser microscope (The Pushchino Scientific Center of Russian Academy of Sciences).

## Results

### *Head region and nerve ring*

Seven serotonin-immunoreactive (5-HT-IR) nerve cells were observed in the anterior region of *T. pseudospiralis* (Fig.1a, c). The largest pair of cells (size 9 x 3 µm) stains strongly and is located about 145 µm from the tip of the head. In front of these cells, two pairs of smaller (size 3 x 2 µm) and less intensively stained 5-HT-IR nerve cells are present. In addition, an unpaired 5-HT-IR nerve structures was observed. From the two 5-HT-IR large cells, nerve fibers extend along both sides anteriorly to the head and posteriorly to the vulval area forming two intensively stained longitudinal nerve cords (Fig. 1d). Transversal commissures connect the longitudinal nerve cords (Fig. 1e). In the tip of the head of *T. pseudospiralis*, small 5-HT-IR structures were observed (Fig. 1b). In the head region the pattern of 5-HT-IR nerve cells and nerve cords is similar in both sexes.

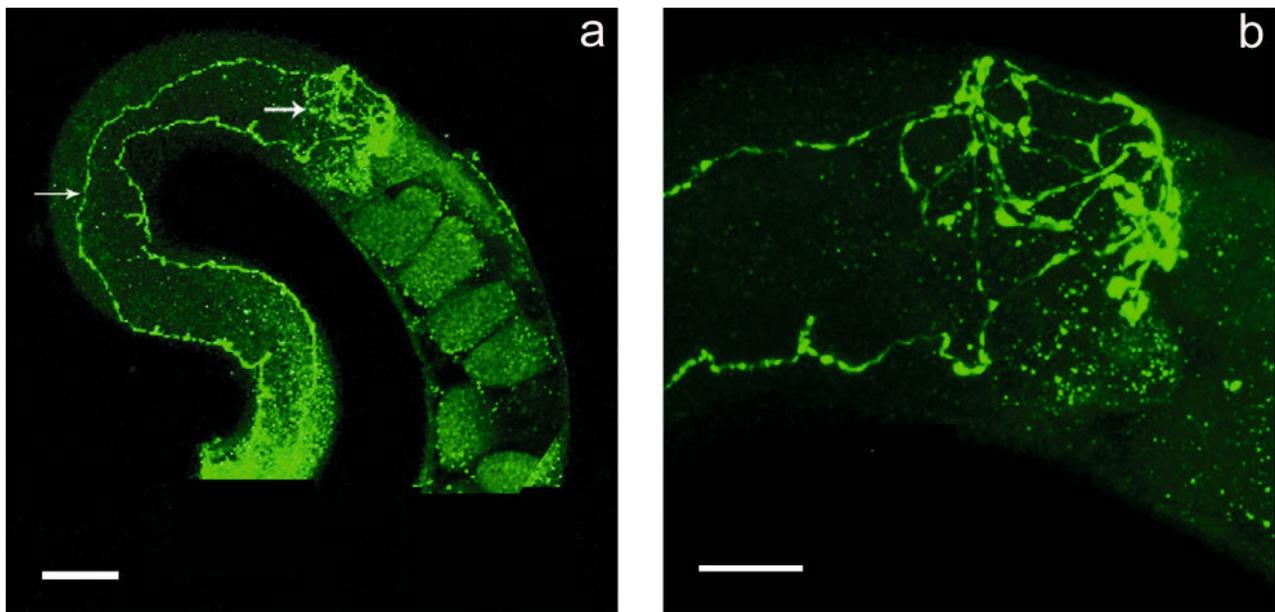


Fig. 2. 5-HT-IR nerve cells and fibres forming a plexus near of vulva in *T. pseudospiralis* (the nerve trunks are noted by arrows also).  
a – Bar =20 µm; b – Large magnification of the 5-HT-IR nerve cells and fibres near of vulva Bar =10 µm

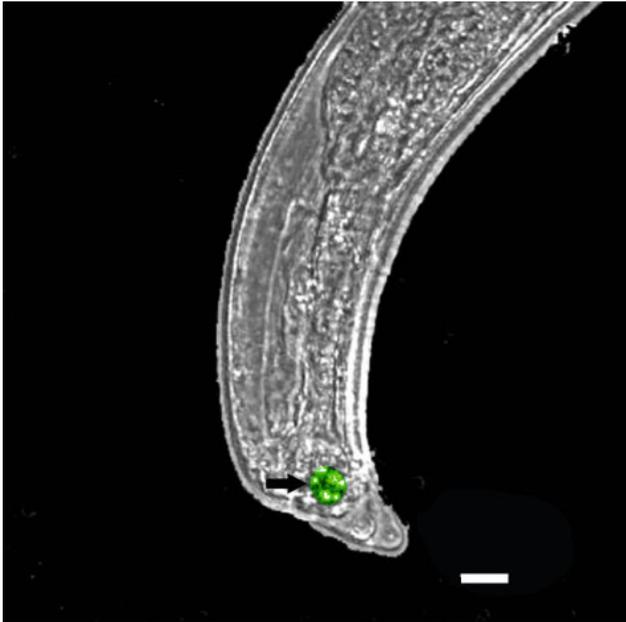


Fig. 3. 5-HT-IR nerve cells in the tail of male of *T. pseudospiralis*. Bar =10  $\mu$ m

#### Mid body, vulval and tail regions

In the vulval area, a complex structure of intensively stained 5-HT-IR nerve cells and nerve fibers occurs (Fig. 2a, b). The nerve cell bodies are bipolar. Two brightly stained longitudinal nerve fibres extend from the head end of the body to the group of 5-HT-IR cells located close to the vulva. Posterior to the vulval region no longitudinal nerve cords were observed.

In the tail of the male of *T. pseudospiralis*, several 5-HT-IR structures were observed (Fig. 3), probably relating to male specific receptors.

#### Row of sublateral body structures

Along the whole length of the body, two rows of small (size about 1  $\mu$ m) 5-HT-IR structures were observed (Fig. 4a, b). In the anterior end, they formed single rows. Further back they occurred in pairs representing sublateral rows of hypodermal glands.

#### Discussion

The nervous systems of parasitic nematodes, and in particular that of *Trichinella pseudospiralis* - the causative agent of human trichinellosis - are poorly described. In this study, we describe the pattern of 5-HT-IR neurons and nerve processes in adult of *T. pseudospiralis* and in the tail end of the male worms. In the head region, one pair of large neurons, two pairs of small neurons and an additional single neuron occur, some of which equipped with processes extending in anterior and posterior direction. Based on the present knowledge it is difficult to compare the 5-HT-IR nervous system of *T. pseudospiralis* with that of *C. elegans*. However, it is tempting to look for homologies between the two worms. In the anterior region of *C. elegans*, two pairs of 5-HT-IR neurons have been described in detail by Jafari *et al.* (2011). In *C. elegans*, the first pair is formed by the right and left pharyngeal neurosecretory motor neurons (NSMR and NSML). The pair of large 5-HT-IR neurons in *T. pseudospiralis* could be homologous to the above mentioned neurons in *C. elegans*. In *C. elegans* the second pair is formed by the right and left amphid chemosensory neurons (ADFR and ADFL), which are defined by having two amphid sensory dendrites each. The small 5-HT-IR structures observed in the tip of the head of *T. pseudospiralis* could be homologous to the double dendrites of ADFR and ADFL in *C. elegans*.

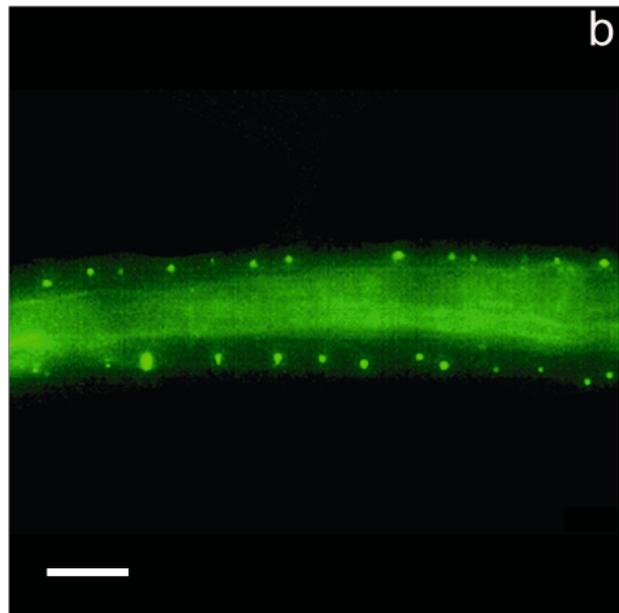
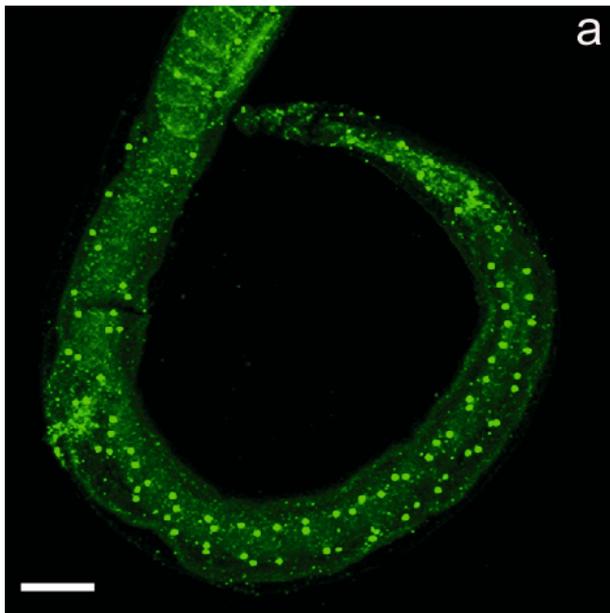


Fig. 4. a, b – 5-HT-IR structures representing sublateral rows of hypodermal glands along the body of *T. pseudospiralis*. Bar =20  $\mu$ m

Furthermore, Jafari *et al.* (2011) describe three adjacent ring inter neurons, RIH (unpaired), AIMR and AIML (right and left) in *C. elegans*.

We suggest that one of the smaller pairs of neurons in *T. pseudospiralis* is of the same type as the amphidial (ADFs) in *C. elegans*. The other pair of neurons and the unpaired neuron in *T. pseudospiralis* are perhaps homologous to the ring inter neurons AIMs and the single RIH (unpaired interneuron) in *C. elegans*.

Of the 5-HT-IR pairs of neurons in the anterior end of the model worm, *C. elegans*, two pairs have been shown to be serotonin producing. Members of one pair have been defined as NSMR and NSML (right and left pharyngeal neurosecretory motoneurons). The members of the other pair are noted as, ADFR and ADFL (right and left amphid chemosensory neurons) and are defined as having two amphid sensory dendrites each. The serotonin produced by these two cell pairs has been shown to diffuse into extracellular spaces (Jafari *et al.*, 2011). On the other hand the authors reported three adjacent ring inter neurons, RIH (unpaired), AIMR and AIML (right and left), instead, to transport serotonin (noted as serotonin reuptake transporters, SERT) controlling in this way a behavioral response to food deprivation. In the present study it was found difficult to actually pinpoint the identity of the neurons observed although, because of the surprising similarity among nematode structure, the serotonin reacting neurons found in our study are likely to be homologues of the neurons defined in *C. elegans*.

In *Haemonchus contortus*, which infects small ruminants, four 5-HT-IR neurons situated within the pharynx have been found (Rao *et al.*, 2011). The authors suggest that the pair of brightly stained neurons, located posteriorly to the nerve ring, is homologous to the neurosecretory motor neurons (NSM) in *C. elegans* (Albertson & Thompson, 1976) and *Ascaris suum* (Johnson *et al.*, 1996). However, in *H. contortus* these cell bodies are located posterior to the nerve ring, whereas in *C. elegans* they are situated anterior to the nerve ring. The difference in the location of these cell bodies in the two species has been discussed by Rao *et al.* (2011). In *H. contortus*, the NSM cell bodies are located in the anterior mid region of the pharynx (Rao *et al.* 2011). In *A. suum*, the same cells have been shown to be located at the very posterior end of the pharynx (Johnson *et al.* 1996). We suggest that the pair of large 5-HT-IR neurons in the head region of *T. pseudospiralis* is homologous to the NSM in *A. suum* and *H. contortus* (Johnson *et al.*, 1996; Rao *et al.*, 2011). In *H. contortus*, the two neurons, located anterior to the NSM, are supposed to be amphidial neurons (Rao *et al.*, 2011).

Small 5-HT IR structures are visible in the tip of the head of *T. pseudospiralis*. We assume that they are related to the cephalic sense organs described by transmission and scanning electron microscopy in *T. spiralis*, *T. pseudospiralis* and other *Trichinella* species (Kim & Ledbetter, 1980; Hulinská & Shaikenov, 1984; Lichtenfels *et al.*, 1983). Our immunocytochemical data suggest that the small 5-HT-IR structures in the tip of the head apparently are localized to ADFL and ADFR dendrites in the lateral amphids and

that the serotonergic nervous system takes part in the function of head sense structures in *T. pseudospiralis*.

In the vulval region of *T. pseudospiralis*, abundant innervation by serotonergic neurons was observed. Intensely stained 5-HT-IR nerve elements formed a complex network comprising neurons and nerve fibers. We suggest that the serotonergic nervous system participates in the egg laying of *T. pseudospiralis*.

When comparing the 5-HT innervation of the vulval region of different nematodes differences appear. In *T. pseudospiralis*, the innervation of the vulval region is more extensive than in free living nematodes, where only single 5-HT-IR neurons have been observed (Loer & Rivard, 2007).

An interesting observation in our study was the presence of 5-HT-IR structures located along the whole body of *T. pseudospiralis*. Nematodes of the order Trichinellida (Ley & Blaxter, 2002) are characterized by having bacillary bands (rows of hypodermal gland cells) present also in Dioctophymatida (e.g. *Eustrongylides*). The ultrastructure of the of hypodermal gland cells of trichinellid nematodes studied earlier indicated that the region contains glandular and non-glandular cells (Wright, 1963, 1968; Sheffield, 1963; Jenkins, 1969; Bruce, 1970; McDarby *et al.*, 1987) and, occasionally, neurons and sensory receptors (Wright, 1968; Jenkins, 1969; Wright & Chan 1973). According to Zdjarska and Nebesarov (2000) the sense receptors in *Capillaria pterophylli* (trichinellid nematode of Capillariidae) occur in association with the hypodermal gland cells in a limited part of the nematode body, i.e. in the proximal region between the level of the nerve ring and the end of stichosome.

Studies of the surface morphology of *Trichinella* spp. (*T. spiralis*, *T. nativa*, *T. pseudospiralis*) by scanning electron microscopy have shown the presence of small pores in the hypodermal gland cells running along the nematode body (Kim & Ledbetter, 1980; Lichtenfels *et al.*, 1983). Lichtenfels *et al.* (1983) found rows of hypodermal gland cells to initiate 50 µm from the anterior end in *Trichinella* spp. and to reach 25 µm from the posterior end. While mainly single, pores of the hypodermal gland cells were found to be organized in double subventral rows in the more central regions of the worms. In newborn larvae, muscle larvae, intestinal larvae, and adults of *Trichinella spiralis*, the presence of hypodermal gland cells with pores was demonstrated using light, scanning and transmission electron microscopy by Kozek (2005). The proposed functions of the hypodermal gland cells are osmoregulatory and absorptive (Wright, 1963; Wright & Chan, 1973), and secretory (Reichels 1955; Bruce 1974). A sensory function has been suggested by the presence of nerve endings (Wright & Chan, 1973; Kozek, unpublished observations) (See Kozek, 2005).

It is evident that 5-HT-IR in the structures located along the body of *T. pseudospiralis* is connected with the hypodermal gland cells of the worm. The function of serotonin in these structures is unknown. Further studies are needed.

In conclusion, our results present the first report on the identification and localization of the neurotransmitter serotonin in *T. pseu-*

*dospiralis*. The localization of the 5-HT-IR structures in the nematode are concentrated to the nervous system of the anterior end and the vulval regions as well as to stained structures running along the trichinella body, which probably correspond to structures of the hypodermal glands previously described in *Trichinella* species. Further studies are needed to clarify the function of serotonin in *T. pseudospiralis*.

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