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ORIGINAL PAPER

An immunocytochemical, histochemical and ultrastructural study of the nervous system of the tapeworm *Cyathocephalus truncatus* (Cestoda, Spathebothriidea)

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Abstract This study is the first detailed study of the organisation of the neuromuscular system of *Cyathocephalus truncatus* (Cestoda, Spathebothriidea). Five techniques have been used: (1) immunocytochemistry, (2) staining with TRITC-conjugated phalloidin, (3) NADPHdiaphorase histochemistry, (4) confocal scanning laser microscopy and (5) transmission electron microscopy. The patterns of nerves immunoreactive (IR) to antibodies towards serotonin (5-HT) and the invertebrate neuropeptide FMRFamide are described in relation to the musculature. The patterns of NADPHdiaphorase positive nerves and 5-HT-IR nerves are compared. The fine structure of the nervous system (NS) is described. The organisation of NS in the non-segmented, polyzoic *C. truncatus* differs clearly from that in the non-segmented, monozoic *Caryophyllaeus laticeps* and shows distinct similarities with the NS in pseudophyllidean cestodes. This supports the hypothesis that taxon Caryophyllidea and Spatheobothriidea form independent lineages within Eucestoda.

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Introduction

Taxon Spathebothriidea represents a group of polyzoic tapeworms with a polypleroid body type. This means that, unlike other polyzoic eucestodes that have a strobila body type with proglottisation and distinct segmentation, the spathebothriideans have a body with inner proglottisation, but they lack external segmentation. The taxonomic status, phylogeny and evolution of the spathebothriideans are widely discussed in the literature (see Olson and Tkach 2005). The general biology, taxonomy and morphology of this group has been dealt with by Nybelin (1922), Wardle and McLeod (1952), Gibson (1994), Protasova and Roitman (1995), Kearn (1998) and Okaka (2000). The fine structures of the male and female reproductive systems of two spathebotriidean species, the dixenous *C. truncatus* and the monoxenous *Diplocotyle olriki*, have recently been described by Poddubnaya et al. (2005a, b, c, d, 2006, 2007) and Brunanska et al. (2006). Emphasis was placed on those characteristics that might clarify the phylogenetic position of the spathebothriideans among the basal tapeworms and their relationships to the bothriocephalideans, the diphyllobothriideans (Kuchta et al. 2008) and the caryophyllideans (Mackiewicz 2003).

In the discussion about the phylogenetic position of flatworms, the organisation of the nervous system (NS) has been used as one of the discriminating criteria (Reuter et al. 2001; Raikova 2004). The presence of aminergic, cholinergic and peptidergic neuronal signal substances in flatworms has been verified with histochemical and immunocytochemical (ICC) methods (Gustafsson and Maule 2007). The neuronal signal substances have important roles in the regulation of the body musculature and the musculature in association with the attachment organs and the reproductive systems (see Terenina and Gustafsson 2003a; Halton and Maule 2004;

Sebelová et al. 2004). Nitric oxide (NO) represents a new category of neuronal signalling substance: a transmitter gas. Information about the nitrergic NS in flatworms is limited. The NADPHdiaphorase (NADPH-d) reaction, i.e. the evidence for the presence of an active neuronal nitric oxide synthase (nNOS) enzyme, has been described in less than 20 flatworms (see Gustafsson et al. 2003a; Terenina et al. 2006). The pattern of acetycholinesterase in *C. truncatus* has been described by Kotikova and Kuperman (1978). Nothing is known about the aminergic, peptidergic and nitrergic neuronal signal substances in spathebothriidean tapeworms.

This study is the first detailed study of the organisation of the neuromuscular system of *C. truncatus*. Five techniques have been used: (1) immunocytochemistry, (2) staining with TRITC-conjugated phalloidin, (3) NADPH-d histochemistry, (4) confocal scanning laser microscopy and (5) transmission electron microscopy.

Firstly, the patterns of nerves immunoreactive (IR) to antibodies towards serotonin (5-hydroxytryptamine, 5-HT) and the invertebrate neuropeptide FMRFamide are described in relation to the musculature. Secondly, the patterns of NADPH-d-positive nerves and 5-HT-IR nerves are compared. Thirdly, the fine structure of the NS is described.

Material and methods

Specimens of adult *C. truncatus* (Pallas 1781) Kessler, 1868 were recovered from the pyloric caeca of whitefish (*Coregonus lavaretus*) from the White Sea, Russia. The material was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C. For storage, it was transferred to the same buffer with 10% sucrose. The material was embedded in Tissue-Tek, frozen and sectioned at 20 μm using a Bright cryostat. The sections were collected on chrom-alum-gelatin-coated glass slides, dried for about 2 h at room temperature and stained directly.

Immunocytochemistry

Cryostat sections of *C. truncatus* were stained with rabbit-anti-5-HT (Incstar, Stillwater, MN, USA) (1:500) or rabbit-anti-FMRFamide (Peninsula, Belmont, CA, USA) (1:500) according to the method described by Coons et al. (1955). The sections were incubated with the primary antibody for 2 days at 4°C and with the secondary antibody swine anti-rabbit FITC (DAKO) 1:50 for 3 h at 4°C. Controls included omission of the primary antibody and substitution of primary antibody with non-immune rabbit serum.

Phalloidin staining of the musculature

In order to study the relationship between the patterns of 5-HT-IR and FMRF-IR nervous elements and the musculature, staining with TRITC-labelled phalloidin (Sigma, St. Louis, MO, USA) (1:200) was performed for 20 min at 4°C (Wahlberg 1998). The phalloidin staining was performed after the ICC staining. The ICC and the phalloidin staining were performed at the Department of Biology, Åbo Akademi University, Finland.

NADPH-d histochemistry

The NADPH-d staining was performed on cryostat sections. The staining was performed according to Lindholm et al. (1998). In the incubation medium, the final concentration of nitroblue tetrazolium was 1 mg/ml, and that of β-NADPH was 2 mg/ml. For controls, β-NADPH was substituted with β-NAD, β-NADH or β-NADP in concentrations as above (2 mg/ml). The incubation time for sections was 2 h at 37°C. All the above-mentioned chemicals were from Sigma. To exclude coexistence, double staining with NADPH-d and anti-5-HT was performed. The NADPH-d staining was performed before the ICC staining. The staining was performed at the Centre of Parasitology of A. N. Severtsov Institute of Ecology and Evolution, RAS, Moscow. The ICC staining was performed at the Department of Biology, Åbo Akademi University, Finland.

Light microscopy and confocal scanning laser microscopy

The slides stained with NADPH-d were examined with Carl Zeiss microscope Axiostar plus at the Centre of Parasitology of A. N. Severtsov Institute of Ecology and Evolution, RAS, Moscow. The slides stained with anti-5HT, anti-FMRFamide and TRITC-labelled phalloidin were examined with a Leica TCS 4D confocal scanning laser microscope coupled to a Leitz Aristoplan fluorescence microscope at the Department of Biology, Åbo Akademi University, Finland.

Transmission electron microscopy

Adult *C. truncatus* were recovered from the pyloric caeca of whitefish (*C. lavaretus*) and grayling (*Thymallus thymallus*) from Lake Segozero, Karelia, Russia. The worms were processed as described by Poddubnaya et al. (2005a). They were examined in a JEM-1010 C transmission electron microscope at the Institute of Inland Waters, Russian Academy of Sciences, Borok, Russia.

Results

The confocal scanning laser microscopical method made it possible to follow the pattern of muscle fibres stained with TRITC-labelled phalloidin in relation to the pattern of 5-HT-IR and FMRFamide-IR nerve structures respectively.

The musculature

The musculature of *C. truncatus* consists of longitudinal, transverse, dorsoventral and subtegumental muscle fibres. In the funnel-shaped scolex and the neck region, the musculature is well developed (Fig. 1). The layer of longitudinal muscles is the strongest layer, and it divides the body into a medullary and a cortical parenchyma. The walls of the reproductive ducts are surrounded by circular and longitudinal muscle fibres (Figs. 4, 7 and 8).

The 5-HT IR NS

In *C. truncatus*, the central nervous system (CNS) consists of a bilobed brain (= two ganglia connected with a ring commissure) in the scolex and two main cords (MCs), extending in the medullary parenchyma from the brain ganglia to the posterior end of the body. The brain and the MCs consist of a densely interwoven fibrillar neuropile of axons and dendrites. The MCs measure approximately 30 μm in diameter in the neck region and become slightly thinner towards the posterior end of the body. The peripheral nervous system (PNS) consists of numerous thin minor cords, which run in the cortical parenchyma along the outside of the longitudinal muscles. Thin commissures connect the main and the minor cords. The minor cords gradually become thinner, forming nerve plexuses beneath the tegument and on the longitudinal muscles.

5-HT-IR nerve fibres occur in the brain, the MCs, the minor nerve cords and the commissures (Figs. 2 and 3). The 5-HT-IR nerve cell bodies are usually bipolar and measure approximately 20×8 μm. The 5-HT-IR cell bodies occur at the surface of the brain and along the MCs. Many 5-HT-IR cell bodies were also observed in the longitudinal muscle layer. These cell bodies send processes to the main and the minor cords, connecting them (Figs. 2 and 3). The 5-HT-IR nerve fibres end in terminals beneath the basal lamina of the tegument, forming a subtegumental nerve plexus in the whole worm (Figs. 2 and 3). Many 5-HT-IR transverse fibres connect the two MCs. A plexus of thin varicose 5-HT-IR nerve fibres occur in the muscle layers surrounding the reproductive ducts (Fig. 4).

The FMRFamide IR NS

The NS stains strongly with anti-FMRFamide. Staining was observed in the brain, the MCs, the minor nerve cords and in the commissures (Figs. 5 and 6). Thin FMRF-IR nerve fibres extend from the brain between muscle fibres, terminating beneath the basal lamina of the tegument covering the whole surface of the funnel-shaped scolex (Fig. 5). The density of FMRF-IR terminals is as high on the inside as on the outside of the scolex. In addition, FMRF-IR nerve fibres extend from the MCs through the longitudinal muscle layer to the cortical parenchyma, ending in terminals beneath the basal lamina of the tegument, forming a dense subtegumental nerve plexus (Figs. 5 and 6). A FMRFamide-IR nerve plexus surrounds the cirrus sac and the utero-vaginal atrium (Fig. 7). Furthermore, FMRF-IR fibres were observed on the surface of the ovary, the Mehlis glands and the uterine glands (Fig. 8).

The nitrergic NS

Deep blue NADPH-d staining was demonstrated in the CNS of *C. truncatus*. Double staining with NADPH-d and anti-5HT revealed that both occur in the MCs. However, the 5-HT-IR cell bodies that lie close to the MCs do not contain NADPH-d positive material (Figs. 9 and 10). Distinct NADPH-d staining was observed in very thin nerve fibres in close association with all types of muscle, i.e. the main longitudinal, transverse and dorsoventral muscles, as well as subtegumental musculature and the musculature of the reproductive organs.

The fine structure of the NS

The central part of the brain and the MC of *C. truncatus* are composed of densely packed small and large nerve fibres (Fig. 11). No extracellular stroma is present between the nerve fibres. Many of the nerve fibres are devoid of vesicles. However, some nerve fibres contain the following types of vesicles:

1. Small, clear vesicles (sv), measuring about 30 nm in diameter
2. Dense-core vesicles (dcv), measuring 70–120 nm in diameter
3. Large, dense vesicles (ldv), measuring 130–560 nm in diameter
4. Large, lucent vesicles (llv), measuring 90–100 nm in diameter

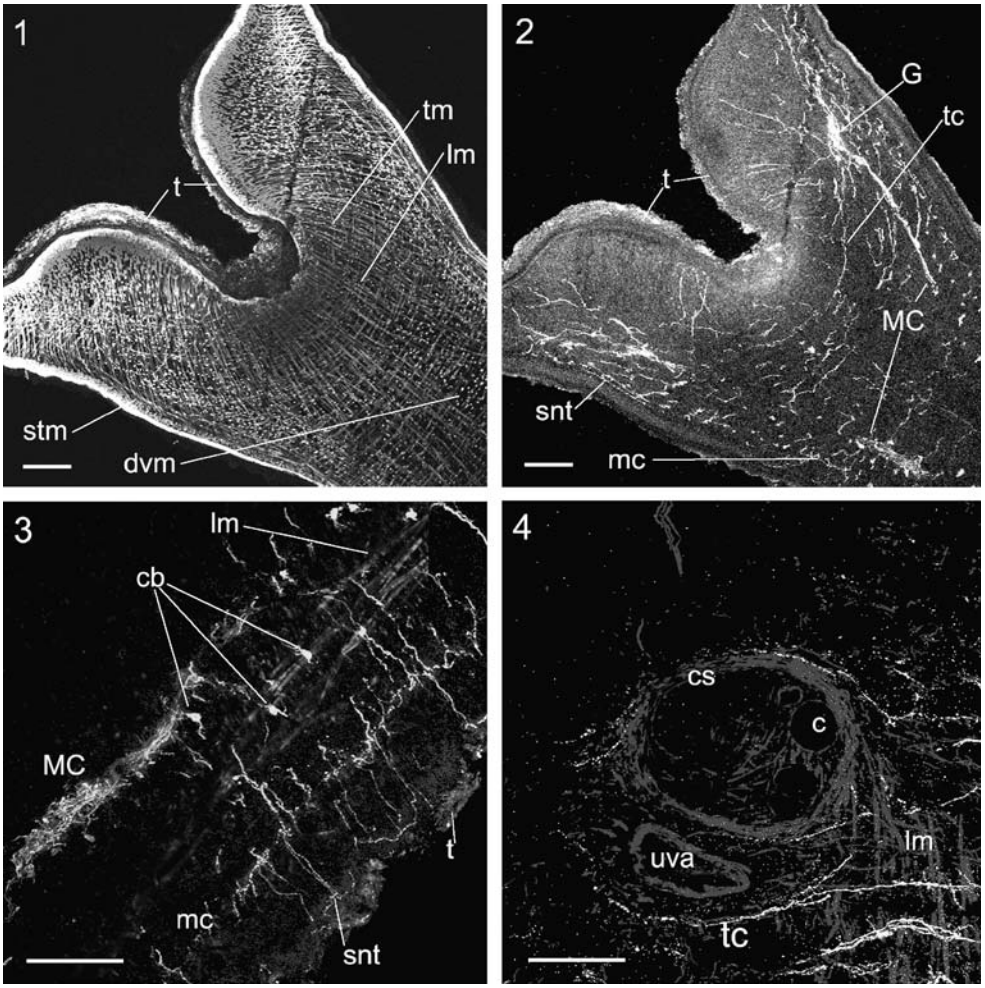

Fig. 1 Frontal section of scolex and neck region showing the longitudinal muscles (*lm*), transverse muscles (*tm*), dorsoventral muscles (*dvm*), subtegumental muscles (*stm*) and tegument (*t*). *Bar*=100 μm

Fig. 2 Image pair of Fig. 1 showing the pattern of 5-HT-IR nerves. Brain ganglion (*G*), main nerve cords with cell bodies (*MC*), minor nerve cord (*mc*), transverse commissure (*tc*), subtegumental nerve terminals (*snt*) and tegument (*t*)

Fig. 3 Section of neck region, showing the pattern of 5-HT-IR nerves. Main nerve cord (*MC*), longitudinal muscles (*lm*), 5-HT-IR nerve cell bodies (*cb*), minor nerve cord (*mc*), subtegumental nerve terminals (*snt*) and tegument (*t*). *Bar*=100 μm

Fig. 4 Pattern of 5-HT nerves around the reproductive ducts. Cirrus sac (*cs*), cirrus (*c*), uterovaginal duct (*uva*), longitudinal muscles (*lm*) and transverse nerve commissure (*tc*). *Bar*=100 μm

The vesicles occur in different combinations in the nerve fibres. Nerve fibres containing sv and dcv were observed in the CNS and the PNS. The most common type of synapse contains sv and dcv. The sv outnumber by far the dcv (Fig. 12). Both types of vesicle are tightly accumulated on the presynaptic side. Generally, the dcv are located farther from the synaptic site than the sv. The synaptic cleft is 20 nm wide and filled with material of moderate density. Both pre- and postsynaptic densities occur. The synapses measure 200–400 nm in length. Shared and single synapses were observed (Fig. 13).

Nerve fibres containing ldv occur in the CNS and the PNS. These nerve fibres run very close to the muscle fibres, and many terminate beneath the basal lamina of the tegument, forming a subsurface nerve plexus (Figs. 14 and 15). Generally, these nerve fibres are packed with ldv. A few omega figures indicating release from the ldv were observed (Figs. 14 and 16). Only seldom were a few llv observed in the same nerve fibres. The muscle fibres are composed of tightly packed thin and thick myofilaments (Figs. 14, 15, 16, 17). Extracellular filaments were observed between the muscle fibres (Fig. 16). Figure 17 shows a nerve cell body containing ldv. The mitochondria are round and contain few cristae. Many free ribosomes but very few RER membranes occur in the cell body.

Figure 18 shows nerve fibres close to the Mehlis gland. Some of the nerve fibres do not contain vesicles. Some of them contain sv and dcv. Neurotubules were observed in the nerve fibres.

Discussion

Gross anatomy

The plan for the flatworm NS is the so-called orthogon, a rectilinear, ladder-like configuration of longitudinal cords connected at intervals by transverse commissures (see Halton and Gustafsson 1996). Thirty years ago, Kotikova and Kuperman (1978) described the pattern of acetycholinesterase in *C. truncatus*. They found four ganglia connected by a nerve ring in the scolex, two main lateral

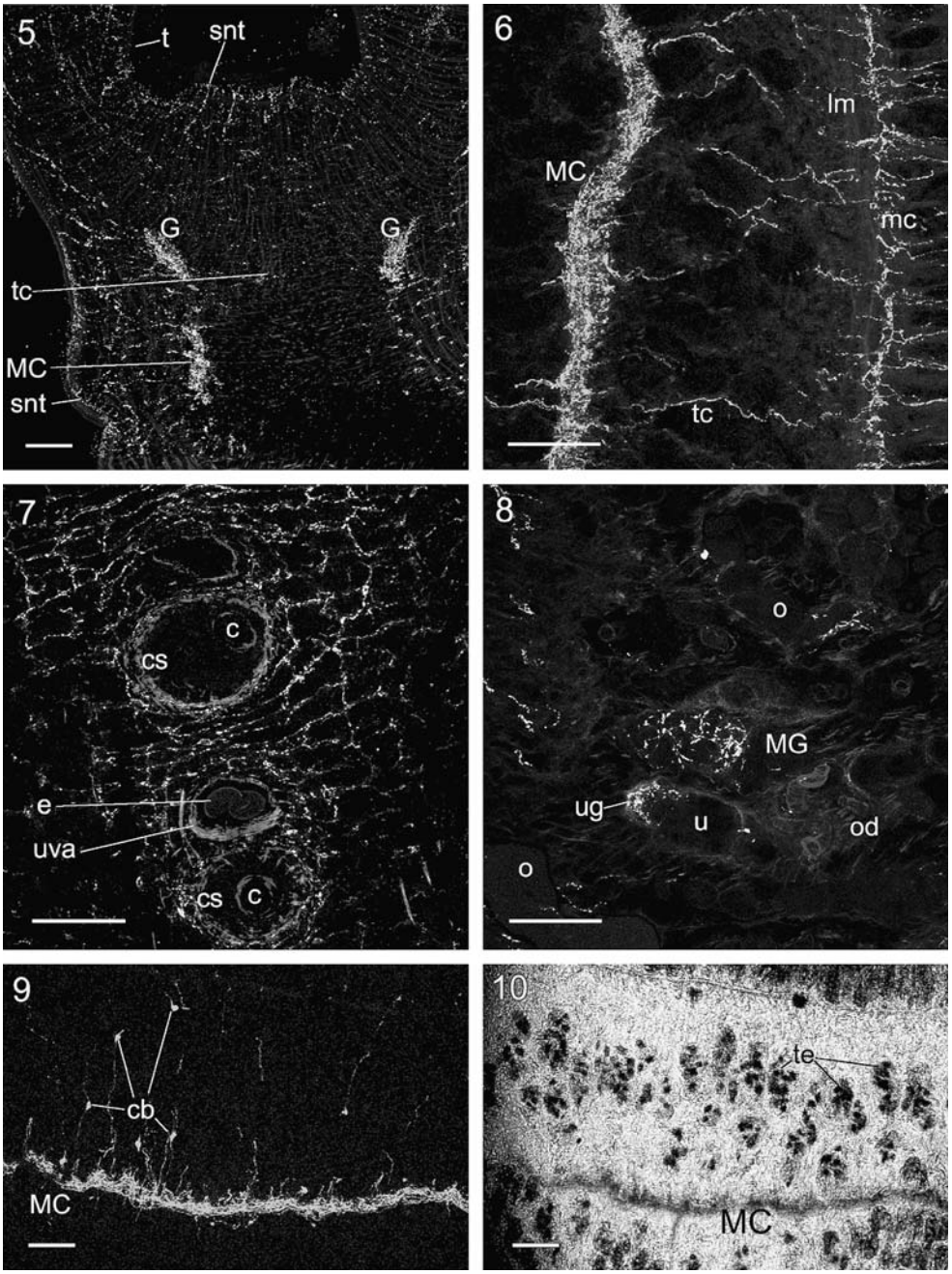

Fig. 5 Frontal section of scolex and neck region showing the pattern of FMRF-IR nerves. Brain ganglion (*G*), main nerve cord (*MC*), transverse nerve commissure (*tc*), subtegumental nerve terminals (*snt*) and tegument (*t*). *Bar*=100 μm

Fig. 6 The pattern of FMRF-IR nerves in mid region. Main nerve cord (*MC*), minor nerve cord (*mc*), transverse commissure (*tc*) and longitudinal muscles (*lm*). *Bar*=100 μm

Fig. 7 A network of FMRF-IR nerves around the reproductive ducts. Cirrus sac (*cs*), cirrus (*c*), uterovaginal duct (*uva*) and egg (*e*). *Bar*=100 μm

Fig. 8 The pattern of FMRF-IR nerves close to Mehlis gland (*MG*), ovary (*o*), uterine gland (*ug*), uterus (*u*) and ovary duct (*od*). *Bar*=100 μm

Fig. 9 The patter of 5-HT-IR nerves in main cord (*MC*) and nerve cell bodies (*cb*) in nerves extending from the main cord towards the tegument. *Bar*=100 μm

Fig. 10 Image pair of Fig. 9 stained with NADPHd. Positive NADPHd reaction occurs in the main cord (*MC*) and close to muscle fibres. Testes (*te*)

nerve cords extending longitudinally in the medullary parenchyma, numerous small nerve cords running along the outside of the longitudinal muscles and nerve plexuses beneath the tegument. According to Protasova and Roitman (1995), the NS in *C. truncatus* consists of two brain ganglia and two MCs. In the scolex, four minor cords branch out from the ganglia and form nerve plexuses.

The serotoninergic, peptidergic and nitrergic NS of *C. truncatus* has never been described before. The ICC analysis of the patterns of 5-HT-IR and FMRFamide-IR nerves also gives, in addition to information about the occurrence of the above-mentioned neuronal signal substances, a picture of the general neuroanatomy of the worm. The ultrastructural part deepens the knowledge of the NS. The NS of *C. truncatus* follows the general plan for NS in flatworms with a bilobed brain, two MCs, many minor cords, connecting commissures and nerve plexuses.

The 5-HT IR NS

5-HT-IR nerves have been described from the NS in all flatworm taxons investigated so far (see Terenina and Gustafsson 2003a; Biserova 2004; Halton and Maule 2004; Raikova 2004). The pattern of 5-HT-IR nerves in

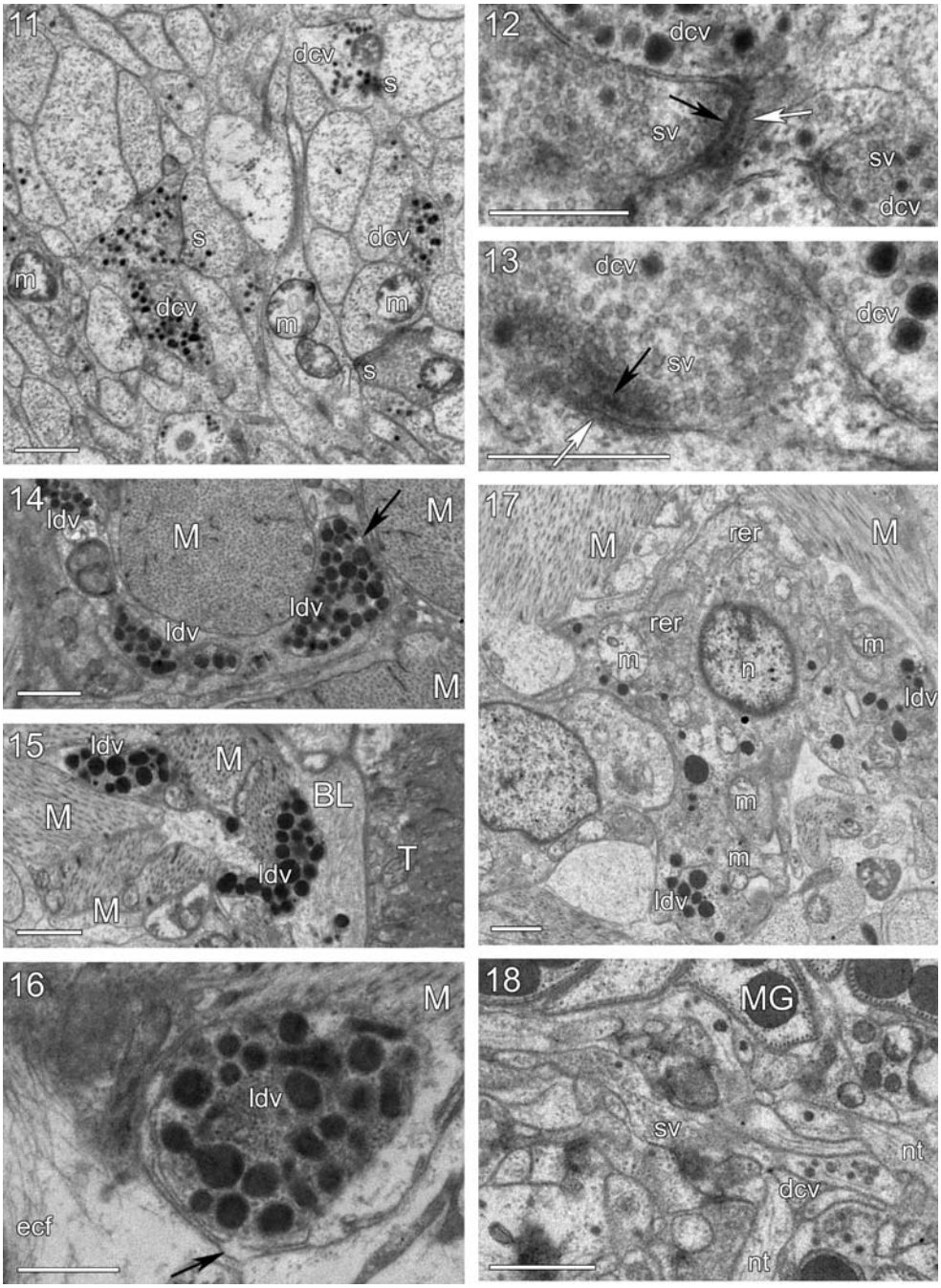

Fig. 11 Small magnification of neuropile in main nerve cord showing the tightly packed nerve fibres. Dense core vesicles (*dcv*) occur in some nerve fibres. Mitochondrion (*m*), synapse (*s*). *Bar*=1 μm

Fig. 12 Synapses containing small clear vesicles (*sv*) and dense core vesicles (*dcv*). Presynaptic density (*black arrow*), postsynaptic density (*white arrow*). *Bar*=0.5 μm

Fig. 13 Synapses containing small clear vesicles (*sv*) and dense core vesicles (*dcv*). Presynaptic density (*black arrow*), postsynaptic density (*white arrow*). *Bar*=0.5 μm

Fig. 14 Nerve fibres containing large dense vesicles (*ldv*) running between muscle fibres (*M*). The *arrow* points to an omega figure, which indicates neuronal release. *Bar*=1 μm

Fig. 15 Nerve fibre containing large dense vesicles (*ldv*) close to basal lamina (*BL*) of tegument (*T*). Muscle fibres (*M*). *Bar*=1 μm

Fig. 16 Large magnification of nerve fibre containing large dense vesicles (*ldv*). The *arrow* points to an omega figure, which indicates neuronal release. Extra cellular filaments (*ecf*). Muscle fibre (*M*). *Bar*=0.5 μm

Fig. 17 Nerve cell body containing large dense vesicles (*ldv*). Nucleus (*n*), mitochondria with short cristae (*m*), rough endoplasmatic reticulum (*rer*) and muscle fibres (*M*). *Bar*=1 μm

Fig. 18 Nerve fibres close to Mehlis gland (*MG*). Dense core vesicles (*dcv*), small clear vesicles (*sv*) and neurotubules (*nt*). *Bar*=1 μm

C. truncatus conforms to that of other tapeworms, and the 5-HT-IR cell bodies are of the same size as in other parasitic flatworms (Gustafsson et al. 1985; Terenina et al. 2006). 5-HT is generally regarded as the main excitatory neurotransmitter of motor activity in flatworms (see Halton and Maule 2004).

The FMRFamide IR NS

This is the first demonstration of a neuropeptide in a spathebothriidean flatworm. FMRFamide has been localised with ICC methods in flatworms from all taxons studied so far (see Day and Maule 1999; Gustafsson et al. 2002; Halton and Maule 2004; Gustafsson and Maule 2007). The pattern of FMRFamide-IR nerves in *C. truncatus* conforms to that of other tapeworms. Generally, FMRFamide and 5-HT occupy separate sets of neurones and fibres in flatworms (see Gustafsson et al. 2002; Halton and Maule 2004). Unfortunately, double staining with anti-5-HT and anti-FMRF was not performed in this study. FMRFamide belongs to the FaRP neuropeptide family and has been shown to be myoexcitatory in a concentration-dependent

manner when applied exogenously to isolated muscle cells or muscle strips from free-living and parasitic flatworms (Day and Maule 1999). The authors point out that the potent, specific, and immediate action of FaRPs on flatworm muscles suggest that they are acting as fast transmitters rather than as modulators.

The NADPH-d positive NS

To date, the pattern of NADPH-d has been studied in less than 20 flatworms (see Gustafsson et al. 2003a; Terenina et al. 2006). Cellular signalling mediated by NO involves the highly regulated synthesis of NO by nNOS, the diffusion of NO into adjacent target cells and the synthesis of the second messenger cGMP (Garthwaite and Boulton 1995). An nNOS-like enzyme has been identified by radiometric analysis in *Hymenolepis diminuta* and *Fasciola hepatica* (Terenina et al. 2000, 2003). The patterns of cGMP-IR nerves have been described in adult *H. diminuta* and *F. hepatica*, plerocercoids of *Diphyllobothrium dendriticum* and cercaria of *Diplostomum chromatophorum* (Gustafsson et al. 2003a, b; Terenina and Gustafsson 2003b). The effect of a NO donor on the synthesis of cGMP in *H. diminuta* has been followed by radiometric analysis (Onufriev et al. 2005). When studying the pattern of the NADPH-d reaction in flatworms, a close association to the muscle fibres has consistently been observed. A myoinhibitory role of NO in flatworms has been suggested (Gustafsson et al. 2001). For the first time, the presence of NADPH-d staining in nerve fibres in a spathebothriidean flatworm has been demonstrated. The pattern of NADPH-d in *C. truncatus* conforms to that in other flatworms (Gustafsson et al. 1996, 2001; Lindholm et al. 1998). Further studies are needed.

The fine structure of the NS

In flatworms, which lack a coelom and a circulatory system, integration takes place through versatile and highly secretory neurones engaged in the synthesis and export of material by axonal transport in vesicles (Halton and Gustafsson 1996). They release the neuronal mediators to the intercellular space close to target cells or organs, in a synaptical or non-synaptical paracrine way (Gustafsson 1992). A diversity in both size and structure of vesicles has been recognised in the NS of flatworms, and they have been used as markers for the different neuronal cell types. The small clear vesicles (sv) of the synaptic type are regarded as cholinergic or as vesicles for recapturing membranes or for retrieval of Ca^{2+}. The dense-cored vesicles (dcv) are regarded as aminergic and the large dense vesicles (ldv) as peptidergic. However, the results of immunogold-labelling experiments at the electron microscopical level have shown these broad categories to be unreliable, with immunoreactivities for neuropeptides most often observed in dcv. In all probability, vesicle ultrastructure likely depends on the developmental stage observed, the processing state of the neuroactive substances involved and the co-existence of neuroactive substances (Halton and Gustafsson 1996). This is the first study of the fine structure of the NS in *C. truncatus*. Four kinds of vesicles were observed in the nerve fibres. The vesicles and the synapses are of the same type as those found in the NS of *D. dendriticum*, *Triaenophorus nodulosus* and *Amphilina foliacea* (Gustafsson 1984; Biserova et al. 1996, 2000). The ldv-filled nerve terminals beneath the basal lamina of the tegument in *C. truncatus* correspond to the nerve terminals identified by the ICC analysis. The same pattern has been observed in many tapeworms. The tegument is the nutrient-absorbing surface of tapeworms, and the need for innervation of this surface is obvious (Gustafsson 1992).

Phylogenetic aspects

Taxon Spathebothriidea is regarded to be related to both the monozoic taxon Caryophyllidea and the polyzoic taxon Pseudophyllidea (see Olson and Tkach 2005). The NS of the non-segmented, monozoic *Caryophyllaeus laticeps* (Caryophyllidea) has recently been described by Biserova (2004). The level of centralisation in *C. laticeps* is very low. No brain ganglia, no neuropile and no brain commissures were observed. The organisation of NS in the non-segmented, polyzoic *C. truncatus* thus differs clearly from the organisation of the NS in the non-segmented, monozoic *C. laticeps* and shows distinct similarities with the NS in pseudophyllidean cestodes (see Halton and Maule 2004). According to Poddubnaya et al. (2006) and Brunanska et al. (2006), taxons Caryophyllidea and Spatheobothriidea form independent lineages within Eucestoda.

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