The NADPH-diaphorase staining reaction in relation to the aminergic and peptidergic nervous system and the musculature of adult *Diphyllobothrium dendriticum*

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SUMMARY

A new category of signalling molecules – transmitter gases – has appeared. Nitric oxide (NO) is generated by nitric oxide synthase (NOS) and diffuses as a short-lived transcellular messenger through the plasma membrane. NADPH-diaphorase (NADPH-d) is a marker enzyme for NO-producing neurons. In this study the pattern of NADPH-d stained neurons in *Diphyllobothrium dendriticum* is described and compared to the pattern of aminergic and peptidergic neuronal elements and to that of the musculature stained with TRITC-labelled phalloidin. NADPH-d staining was observed in neurons in the bilobed brain and along the 2 main nerve cords and in nerve fibres close to the body musculature and the musculature of the reproductive ducts, the walls of the testicular follicles and in sensory endings in the tegument. The NADPH-d staining reaction and the 5-HT or FMRFamide immunoreactivities occur in separate sets of neurons.

Key words: *Diphyllobothrium dendriticum*, nitric oxide, NADPH-diaphorase, serotonin, FMRF-amide, phalloidin.

INTRODUCTION

Nitric oxide (NO) is one of the smallest and lightest molecules known to act as a biological messenger (Snyder, 1991; Vincent, 1995). NO is generated when needed by a family of nitric oxide synthase enzymes (NOS), which produce NO from L-arginine and molecular oxygen. NOS exists in 2 forms, 1 constitutive, occurring mainly in nervous and endothelial tissues, and 1 inducible, occurring mainly in macrophages. Neuronal NOS (nNOS) requires the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor and an increased level of intracellular Ca^{2+} (Moncada, Palmer & Higgs, 1991). Under defined fixation conditions the NADPH diaphorase (NADPH-d) reaction is a histochemical maker for nNOS (for references see Brehmer & Stach, 1997). The NADPH-d activity of nNOS is, in contrast to other NADPH-d enzymes, resistant to fixation with aldehydes (Blottner, Grozdanovic & Gossrau, 1995).

NOS has been detected by histochemical and immunocytochemical methods in the CNS and PNS of vertebrates (for discussion see Vincent, 1995) and invertebrates (for references see Johansson & Carlberg, 1995; Gustafsson *et al*. 1996). To date, only a few reports of the occurrence of NADPH-d positive neuronal elements in flatworms and nematodes have been published. Strong NADPH-d staining was observed in the CNS and PNS of the parasitic worms *Hymenolepis diminuta* (Gustafsson *et al*. 1996), *Azygia lucii, Haplometra cylindracea* (Lindholm, personal observations) and *Ascaris suum* (Bascal *et al*. 1995, 1996). As to the presence of NADPH-d staining in free-living worms, the general picture is that if any staining reaction has been detected so far it has been only a weak one. Elofsson *et al*. (1993) were unable to detect NADPH-d staining in *Dugesia gonocephala* and *Caenorhabditis elegans*. A few NADPH-d stained neurons close to the pharyngeal musculature were observed in *D. tigrina* (Eriksson, 1996). No specific staining was observed in the CNS. The production of citrulline, i.e. a by-product of the synthesis of NO was determined by high pressure liquid chromatography (HPLC) (Eriksson, 1996). In *Planaria torva* very faint staining was observed in the neuropile (Gustafsson, personal communications). To confirm this difference between parasitic and free-living worms, further research has to be done.

In order to complete the picture of the nervous system in *Diphyllobothrium dendriticum*, a study on the occurrence of NADPH-d positive neuronal elements was performed. By combining NADPH-d histochemistry and immunocytochemistry (ICC), the relation between the NADPH-d reaction and aminergic and peptidergic neuronal elements, respectively, was investigated. The parallel patterns of NADPH-d stained nerves and muscle fibres stained with TRITC-labelled phalloidin for actin was also studied.

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MATERIALS AND METHODS

Plerocercoid larvae of *D. dendriticum* Nitzsch, 1824 (Cestoda, Pseudophyllidea) were obtained from whitefish (*Coregonus lavaretus*) from Pyhäjärvi SW Finland. The larvae were excised from the stomach wall. Adult worms were obtained from experimentally infected hamsters. The worms were flushed out from the small intestine with cold saline and fixed overnight in 4% paraformaldehyde (PFA) in 0·1 M phosphate buffer at 4 °C. For storage they were transferred to the same buffer with 10% sucrose. The worms were embedded in Tissue-Tek and sectioned frontally and transversally at a thickness of 20 μm using a Bright cryostat.

NADPH-diaphorase histochemistry

The staining procedure was described in detail by Gustafsson *et al*. (1996). In the incubation medium the final concentration of nitroblue tetrazolium was 1 mg/ml and that of β-NADPH 2 mg/ml. Two staining protocols were followed. (1) Slides taken either directly from the cryostat or after storage in −70 °C to room temperature (RT) for 0·5–1 h. Rinsed in 0·01 M phosphate buffer (PBS) with 0·2% Triton X–100 (PBST) 2 × 10 min. Incubated in incubation medium at 37 °C for 1 h in a covered box. Rinsed in PBST 3 × 15 min. Rinsed in distilled water. Mounted in 50% glycerol/PBS. Stored at 4 °C. (2) In order to minimize the background staining, proceeded as in (1) but, prior to staining, incubated the sections in 0·5% Triton X–100 in PBS at RT for 24 h. The incubation medium contained 0·5% Triton X–100 and the incubation took place at RT for 2 h (Fang *et al*. 1994).

For controls, β-NADPH was substituted with either β-NAD, β-NADH or β-NADP in concentrations as above (2 mg/ml). The incubation was performed for 1 h at 37 °C. All above-mentioned chemicals were from Sigma (St Louis, USA). As a positive control for the β-NADPH-d staining, sections of the small intestine from hamster were used. They were stained according to method (a).

Immunocytochemistry (ICC)

In order to study the relation between NADPH-d positive nervous elements and the pattern of aminergic or peptidergic nervous elements, slides were stained with rabbit-anti-5-HT (1:500) (Incstar, USA) or rabbit-anti-FMRFamide (1:500) (Diagnostika, Sweden) (Price & Greenberg, 1977) according to the method described by Coons, Leduc & Connolly (1955). The sequence of staining was NADPH-d staining according to method 1 first, followed by staining with primary antibody overnight at 4 °C. Rhodamine B isothiocyanate (TRITC) (1:30) was used as secondary antibody. The fluorescent slides were examined with a Leitz Orthoplan microscope combined with filterblock I2. When pairs of images were produced, the fluorescent image was produced first and the NADPH-d stained later. Photographs were produced with an Olympus automatic photomicrographic system, model PM 10ADS. Film T-MAX 400 was used. In order to exclude the possibility of NADPH-d quenching the ICC staining, ICC staining was carried out first and carefully documented on photographic film. The NADPH-d staining was then performed on the same sections and photographed. Controls included omission of the primary antiserum, substitution of primary antiserum with non-immune rabbit serum, and liquid phase pre-adsorption of primary antiserum with 5-HT and FMRFamide in excess.

Phalloidin staining of the musculature

In order to study the relation between the NADPH-d staining and the pattern of muscle fibres, staining with TRITC-labelled phalloidin (Sigma) (1:200) was performed for 20 min at 4 °C (Wahlberg, 1998). The phalloidin staining was performed after the NADPH-d staining. The phalloidin-stained slides were examined with a Leica TCS 4D confocal laser scanning microscope coupled to a Leitz Aristoplan fluorescence microscope.

RESULTS

Staining methods

The best NADPH-d staining result was obtained when the sections were incubated with Triton X–100 (Protocol 2) according to the method of Fang *et al*. (1994). Deep blue NADPH-d staining was observed in the nervous system and very faint background staining was present. In the absence of Triton X–100 the background staining was stronger. When double staining with NADPH-d and ICC was performed, protocol 1 was followed, because long treatment with Triton X–100 destroys the antigenicity. The controls were negative. In the small intestine of the hamster, representing the positive control for staining, deep blue NADPH-d staining was observed in neurons of the Auerbach's plexus.

NADPH-d staining in CNS

Deep blue NADPH-d staining was demonstrated in the CNS of *D. dendriticum*. Fig. 1A shows a transverse section of the scolex with NADPH-d staining in the neuropile of the 2 brain ganglia and in neurons in the brain commissure. From the brain ganglia NADPH-d stained nerves extend through the longitudinal muscle layers to the cortical parenchyma and the nerve plexus surrounding the bothridial muscles. The largest NADPH-d stained neurons (size 10–18 × 18–23 μm) were observed close to the inner border of the brain ganglia in the

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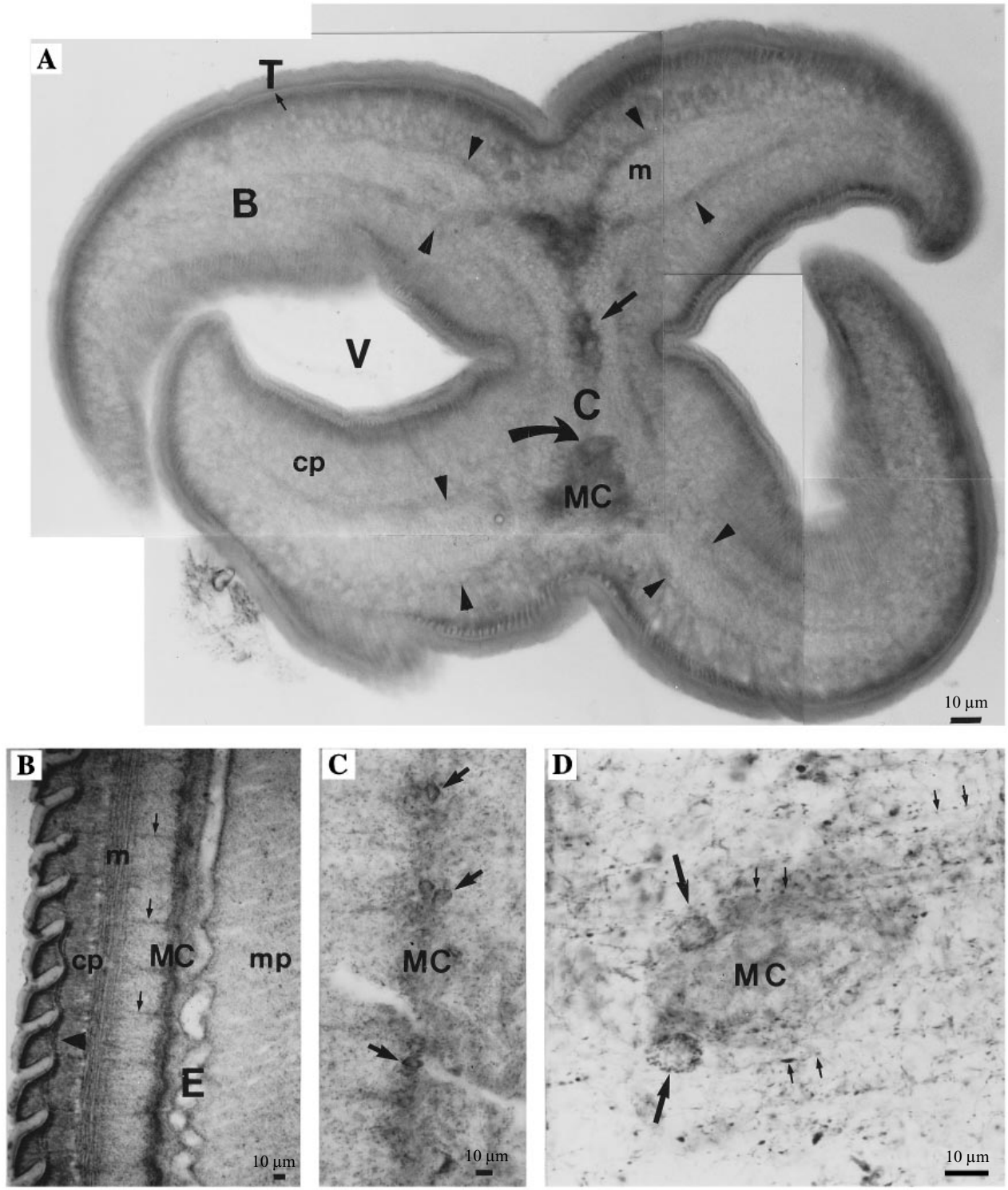

Fig. 1. (A–D) The nervous system of adult *Dipyllobothrium dendriticum* stained with NADPH-diaphorase. (A) Transverse section of scolex showing NADPH-d staining in brain commissure (C) and brain ganglia (MC). Note large neuron (bent arrow) close to brain ganglion, smaller neurons (arrow) in commissure and nerves extending from the ganglia to the cortical parenchyma (cp) forming a nerve plexus (arrow heads) around the bothridial muscles (m). Syncytial tegument (T), basal lamina (small arrow), bothridium (B), place for host villus (V). (B) Frontal section of neck region showing the main nerve cord (MC) and the regular pattern of lateral nerves (small arrows) extending towards the cortical parenchyma (cp). Main excretory duct (E), medullary parenchyma (mp), longitudinal muscle layer (m), bundles of peripheral muscle fibres (arrow head) running longitudinally in cortical parenchyma. (C) Longitudinal section of main nerve cord (MC) showing NADPH-d stained neurons (arrows). (D) Transverse section of main nerve cord (MC) with 2 NADPH-d stained neurons (arrows) at the surface of the neuropile. Note the granular content in the cell bodies and the varicose nerve fibres (arrow pairs) extending from the main nerve cord.

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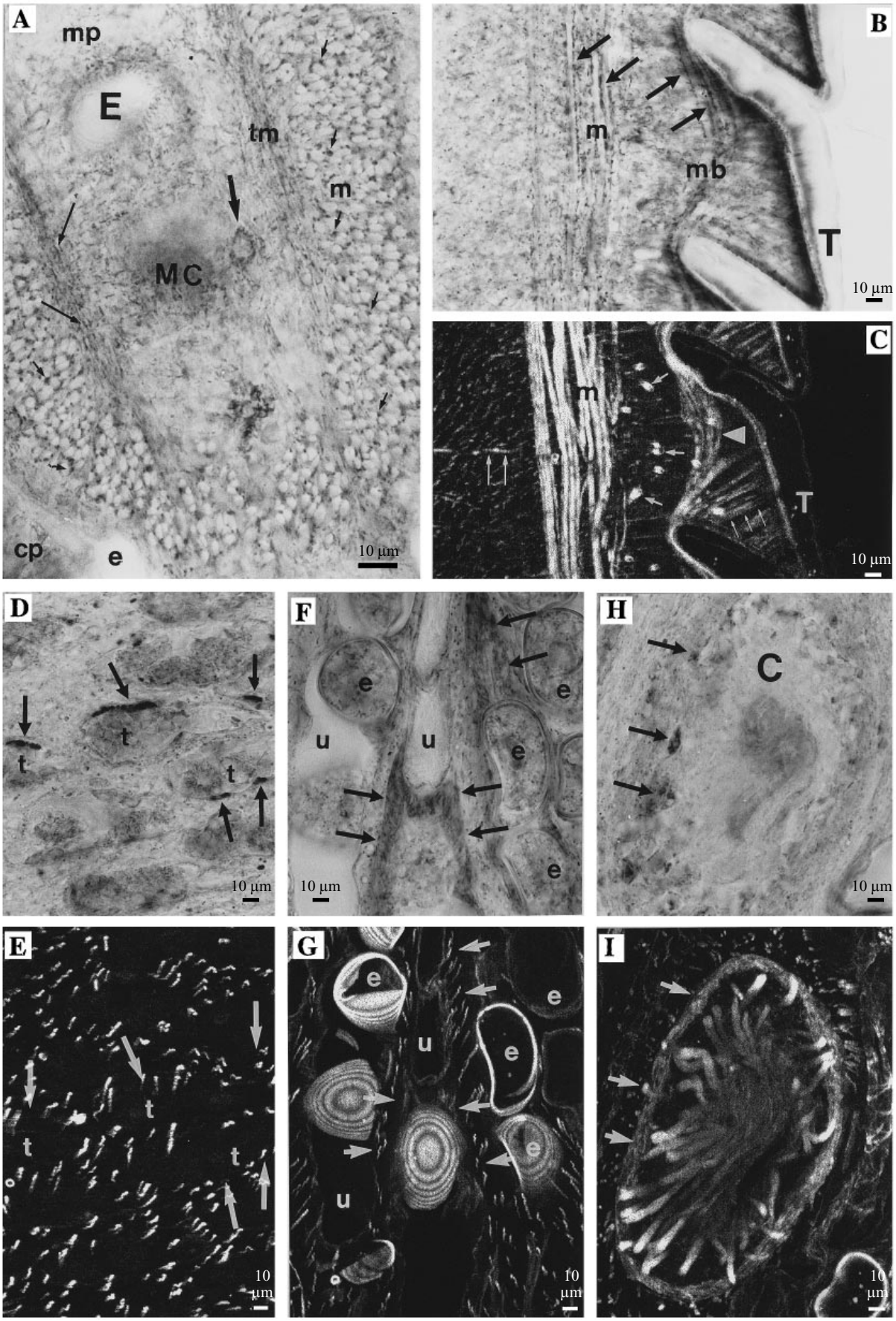

Fig. 2 (A–I) The pattern of NADPH-d and TRITC-phalloidin stained muscles in *Diphyllobothrium dendriticum*. (A) Transverse section of neck region. Note large NADPH-d stained neuron (large arrow) close to the main nerve cord (MC). The muscle fibres (m) in the main longitudinal muscle layer are cut transversally, the transverse muscle fibres

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commissure. They occur in 2 pairs; 10–12 smaller NADPH-d stained neurons (size $6 \times 10\ \mu$m) form the mid-part of the commissure. Fig. 1B shows a frontal section of the neck region with distinct NADPH-d staining along the main nerve cord (MC). A regular pattern of NADPH-d stained lateral nerves extending from the MC towards the cortical parenchyma and ending at the border between the proglottides was observed. Fig. 1C and D shows NADPH-d stained neurons (size $7 \times 14\ \mu$m) along the MC. A few smaller neurons (size $6 \times 7\ \mu$m) were also observed close to the MCs. The NADPH-d stained neurons are localized at the surface of the neuropile of the MC, not inside (Fig. 1D). Varicose nerve processes encircle the neuropile of the MC and extend outwards.

NADPH-d in PNS

Distinct NADPH-d staining was observed in nerve fibres in close association with all types of muscles i.e. the main longitudinal, transverse and dorsoventral body muscles as well as the smaller bundles of muscles running longitudinally in the cortical parenchyma, the subtegumental musculature and the musculature of the ducts of the reproductive organs, such as the cirrus sac and uterus (Figs 1B, 2A–I, 3A). In order to illustrate this, image pairs of NADPH-d stained nerve fibres and muscles stained with TRITC-phalloidin are presented. The NADPH-d positive fibres follow the sarcolemma, innervating the muscles. Fig. 2B and C shows the parallel patterns of NADPH-d stained nerve fibres and phalloidin stained muscles. In Fig. 2D–I comparisons between NADPH-d stained nerves and phalloidin stained muscles around the testicular follicles, the uterus ducts and in the cirrus sac are shown. NADPH-d stained terminals were also observed between the muscle fibres in the wall of the main excretory ducts (Fig. 3A).

NADPH-d staining in sensory endings

Varicose NADPH-d stained nerve fibres extend towards the subtegumental region, where some end in terminals beneath the basal lamina of the tegument and some penetrate the lamina, forming sensory endings (Fig. 3B). The sensory endings are shaped like thin ovals (size $4–5 \times 2\ \mu$m) reaching one/third of the syncytial tegument, i.e. they do not penetrate the tegument. No NADPH-d stained sensory endings were observed in the scolex region. In a frontal section of $20\ \mu$m thickness of the neck region 1 sensory ending/3·3 proglottides was found, while in the mature region 1 sensory ending/1 proglottid was found. In transverse sections of $20\ \mu$m thickness of the mature region, about 7 NADPH-d stained sensory endings were observed/section. No specific localization for those endings was observed. They occurred all along the surface of the body.

NADPH-d histochemistry and immunocytochemistry

Double staining with NADPH-d and anti-5-HT or anti-FMRF revealed that coexistence does not occur. No quenching was observed. Fig. 3C shows the pattern of NADPH-d stained neurons in a longitudinal section of the MC. Fig. 3D shows the pattern of 5-HT-immunoreactive (IR) neurons and nerve fibres in the same section. The patterns do not overlap. Both cell types lie in close proximity to each other. Fig. 3E shows the pattern of NADPH-d stained neurons and fibres in a transverse section of the MC. Fig. 3F shows the pattern of 5-HT-IR neurons and fibres in the same section. Processes from the 5-HT-IR neurons extend towards the NADPH-d stained neurons and encircle them. The cell bodies are of the same size. The NADPH-d stained neurons have short processes while the 5-HT-IR neurons have long varicose processes. Both cell types lie in the periphery of the neuropile of the MC.

Double staining with NADPH-d and anti-FMRF revealed that coexistence does not exist. Fig. 3G shows the pattern of NADPH-d stained neurons in a longitudinal section of the MC. Fig. 3H shows the pattern of FMRF-IR fibres in the same section. The NADPH-d stained neurons are localized on the lateral border of the MC. No FMRF-IR cell body

(tm) are cut longitudinally. NADPH-d staining is evident as a network of fibres around both types of muscle fibres (small arrows and long thin arrows). Main excretory duct (E) in medullary parenchyma (mp), minor excretory duct (e) in cortical parenchyma (cp). (B) Longitudinal section of neck region showing NADPH-d staining in nerve fibres (arrows) along main longitudinal muscles (m) and peripheral muscle bundles (mb). Tegument (T). (C) CSLM image pair of (B) showing pattern of TRITC-phalloidin. Staining is seen in the main longitudinal muscles (m), the peripheral muscle bundles, the individual dorsoventral muscle fibres (arrow pair), muscle fibres (triple arrow) spreading out from the peripheral muscle bundles towards the tegument (T) and in the flame cells (small arrows) in cortical parenchyma. (D) NADPH-d staining (arrows) in the walls of testicular follicles (t). (E) CSLM image pair of (D) showing the pattern of TRITC-phalloidin around testicular follicles (t). Single muscle fibres surround the follicles. (F) NADPH-d staining (arrows) in the walls of uterus ducts (u). Egg (e). (G) CSLM image pair of (F) showing the pattern of TRITC-phalloidin around uterus ducts. Single muscle fibres (arrows) occur in the walls. Egg (e). (H) NADPH-d staining (arrows) in the cirrus sac (C). Note that the NADPH-d staining occurs in the peripheral parts, not centrally. (I) CSLM image pair of (G) showing the pattern of TRITC-phalloidin in the cirrus sac.

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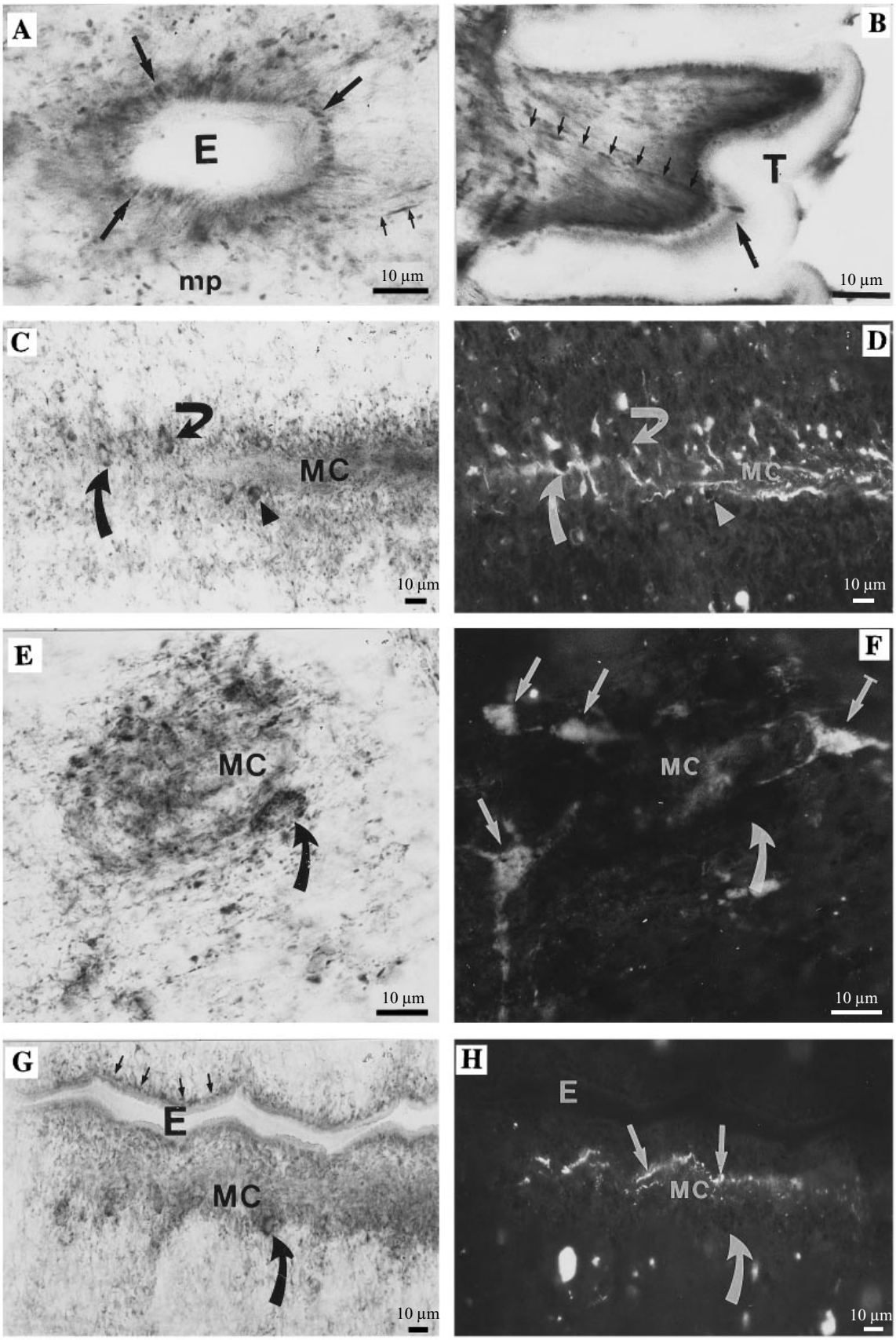

Fig. 3 (A–H). Pattern of NADPH-d staining in *Diphyllobothrium dendriticum*. (A) Large magnification of main excretory duct (E) with NADPH-d stained terminals (arrows) beneath the basal lamina of the duct. Varicose nerve fibres (arrow pair) in medullary parenchyma (mp). (B) Large magnification of NADPH-d positive sensory ending (arrow) in syncytial tegument (T). A varicose nerve fibre extends from the sensory ending inwards (small arrows). (C) Longitudinal section of the main nerve cord (MC) with 3 cell bodies showing NADPH-d reaction (bent arrows

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occurs along the MC, only a few fibres, which run mainly along the medullary border.

DISCUSSION

The results of this study show strong NADPH-d staining in the CNS and PNS of *D. dendriticum*. Gustafsson *et al*. (1996) reported strong NADPH-d staining in the nervous system of *H. diminuta*. Preliminary results indicate strong NADPH-d staining in the nervous system of *Azygia lucii* and *Haplometra cylindracea* (Lindholm, personal observation).

The NADPH-d stained neurons of *D. dendriticum* are localized to the CNS i.e. the bilobed brain and the 2 MCs, as defined by Reuter *et al*. (1996). In the brain commissure, 4 large and 10–12 smaller NADPH-d stained neurons were observed. In earlier investigations 4 5-HT-, 4 FMRF-, 1 RFamide-, 1 small cardiac peptide B-, 3 substance P-, 1 basic fibroblast growth factor (bFGF)-, and 1 neuropeptide F-IR neurons have been reported from the brain commissure (Gustafsson, 1990, 1991; Gustafsson & Eriksson, 1992; Gustafsson, Nässel & Kuusisto, 1993; Gustafsson *et al*. 1994). The 4 large NADPH-d stained neurons in the commissure are of the same size as those detected by ICC. The group of smaller cells forms a new size category of neurons in the brain commissure. The result of this study implies that about 50% of the cell population in the brain commissure of *D. dendriticum* comprise NADPH-d positive cells. In the brain of *H. diminuta* 6 serotoninergic (size 5–7 × 3 μm) and 8 peptidergic neurons (size 4–6 × 2–3 μm) have been described by McKay *et al*. (1991). In this worm about 30 NADPH-d positive neurons (size 5–6 μm) were reported by Gustafsson *et al*. (1996), indicating that the proportion of NADPH-d positive neurons is slightly larger in the brain of *H. diminuta*. In adult *Triaenophorus nodulosus* the total number of cells in the bilobed brain has been determined to approximately 80, with 11 cells in the commissure and about 35 cells in each ganglion (Biserova *et al*. 1996). The estimation was made using electron microscopy and thus includes all types of cells.

In *D. dendriticum* the NADPH-d stained neurons along the MCs fall into 2 size categories. The larger cells (7 × 14 μm) correspond in size to aminergic and peptidergic neurons detected by ICC. The shape of the NADPH-d stained neurons differs from that of aminergic and peptidergic neurons. They have short blunt processes while aminergic and peptidergic neurons have long varicose processes (Gustafsson, 1991; Gustafsson *et al*. 1994). In *H. diminuta* the NADPH-d stained neurons are bipolar with thin processes running along the medullary border of the MC (Gustafsson *et al*. 1996).

In tapeworms the MCs grow constantly. In *D. dendriticum* undifferentiated cells migrate from the surrounding parenchyma into the MCs, renewing the population at a rate of 16% per day and differentiating to neurons (Gustafsson, 1976). In *D. dendriticum* cells IR to bFGF occur in the MCs (Gustafsson & Eriksson, 1992). According to Peunova & Enikolopov (1995) nerve growth factor induces the production of NO in cultures of nerve cells. NO, in turn, causes nerve cells to differentiate. The presence of both a growth factor (bFGF-IR nerve cells) and NO producing cells in the MCs of *D. dendriticum* makes the milieu favourable for neuronal cell differentiation.

According to Feelish & Martin (1995), NO might have been one of the first biological signalling molecules. NO diffuses through cell membranes and binds to and influences numerous target proteins such as soluble guanylate cyclase, which results in an increase of cyclic GMP (cGMP) levels (Bredt, 1995). cGMP is an important intracellular signal molecule involved in regulation of various cellular events, such as smooth muscle relaxation (for references see Koesling, Humbert & Schultz, 1995). Expression of nNOS in the sarcolemma region of visceral and somatic striated muscle fibres was demonstrated by Grozdanovic *et al*. (1995). In *D. dendriticum* NADPH-d staining was detected in nerve fibres close to all types of musculature. The same pattern was seen in *H. diminuta* (Gustafsson *et al*. 1996) and in *D. tigrina* (Eriksson, 1996). Little is known about the function of neuronal signal substances in flatworms. However, 5-HT is regarded as a stimulatory neurotransmitter affecting muscle contraction (Halton & Gustafsson, 1996). A cooperation between the stimulating 5-HT and the relaxing NO may be suggested. Bascal *et al*. (1995) demonstrated intense

and arrow head). (D) Image pair of (C) showing the pattern of 5-HT immunoreactivity in the main nerve cord (MC). Note that the NADPH-d positive cell bodies (bent arrows and arrow head) are surrounded by 5-HT-IR fibres. (E) Transverse section of main nerve cord (MC) stained with NADPH-d. Note large NADPH-d positive neuron (bent arrow) at the surface of the neuropile. (F) Image pair of (E) showing the pattern of 5-HT-IR cell bodies around the main nerve cord (MC). In the neuropile the two substances do not overlap. Co-existence does not occur. The 5-HT-IR neuron (marked arrow) to the right sends a process to the NADPH-d stained neuron (bent arrow). (G) Longitudinal section of main nerve cord (MC) stained with NADPH-d. Note neuron (bent arrow) at the surface of the neuropile. Main excretory duct (E) with NADPH-d stained terminals (arrow pairs). (H) Image pair of (E) showing the pattern of FMRF-IR nerve fibres running in the neuropile of the main nerve cord (MC). Co-existence does not occur. The bent arrow indicates the position of the NADPH-d stained neuron. Main excretory duct (E).

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NADPH-d staining in the motorneurone commissures of *A. suum*, indicating a potential role for NO as a neurotransmitter at the neuromuscular junctions.

The role of the main excretory ducts for the circulation in *D. dendriticum* has been discussed by Lindroos (1991). They are lined with a syncytial epithelium, a fibrous lamina and small muscle fibres (Lindroos, 1983; Wahlberg, 1998). They are surrounded by cells IR to histamine (Wikgren *et al.* 1990) and terminals IR to PHI (Gustafsson, Lehtonen & Sundler, 1986). The NADPH-d stained terminals between the muscle fibres along the main excretory ducts may be involved in the pumping function performed by the ducts.

Data about the innervation of the reproductive systems of flatworms are sparse. In this study NADPH-d staining was observed in the walls of the testicular follicles. In the same location, IR towards porcine peptide histidine isoleucine (PHI) was described by Gustafsson *et al.* (1986). NADPH-d staining was also noted in the muscular wall of the cirrus sac and uterus ducts. Nerves IR to 5-HT, FMRF, and NPF have been described from the same locations (Gustafsson *et al.* 1994). Thus, the picture of the innervation of the reproductive system in the gull-tapeworm is slowly emerging.

NADPH-d stained sensory endings were observed totally embedded in the distal tegument of *D. dendriticum*. They increased in number from the neck region towards mature regions, indicating a function in connection with reproduction. Three types of sensory receptors have been described in *D. dendriticum* (Gustafsson, 1990). The most common type is onion-shaped, totally embedded in the distal tegument and similar to the NADPH-d stained sensory ending. According to Lindroos & Still (1990), the lamina surrounding this type of sensory ending is IR towards laminin but not towards fibronectin or collagen IV. The NADPH-d stained sensory endings detected in the tegument of *H. diminuta* are smaller than those of *D. dendriticum* (Gustafsson *et al.* 1996). Bascal *et al.* (1995) demonstrated selective NADPH-d staining in the PNS innervating the sensory organs of *A. suum*. NADPH-d positive fibres have been described in peripheral olfactory sensory cells and in the neuropile of the olfactory lobes of the crayfish *Pasifastacus leniuscules* (Johansson & Carlberg, 1994; Johansson & Hallberg, 1995) and in the chemosensory neuropiles of *Apis mellifera* and *Drosophila melanogaster* (Muller & Biker, 1994). NO has been shown to be involved in the feeding response of *Hydra* – the most primitive olfactory-like behavioural phenomenon present in multicellular organisms (Colasanti, Lauro & Venturini, 1995).

Double staining with NADPH-d and anti-5HT or anti-FMRF shows that co-existence does not occur. The NADPH-d positive substance is localized to cells that are IR neither to 5-HT nor to FMRF. The 5-HT-IR fibres encircle the NADPH-d positive neurons. In *H. diminuta* a similar pattern with separate sets of nitrergic and serotoninergic neurons, in close contact with each other was observed (Gustafsson *et al.* 1996). Generally, in flatworms, neuroamines and neuropeptides occur in separate sets of neurons (Gustafsson, 1991; Reuter & Gustafsson, 1995). This seems to hold true also for the NADPH-d reaction. According to Bascal *et al.* (1996) NADPH-d staining and the synthetic neuropeptide SALMFamide co-localizes in a distinct subset of neurones in the ventral, dorsal and lateral ganglia of *A. suum*.

The nervous system of the gull-tapeworm *D. dendriticum* is one of the best known within Class Cestoda (Gustafsson, 1992). The cholinergic nervous system was described by Öhman-James (1968). Among the bioamines, serotonin (5-HT) was detected by ICC and HPLC methods (Gustafsson *et al.* 1985; Eriksson, Gustafsson & Åkerlind, 1993). Neurons with histamine-like immunoreactivity were described by Wikgren *et al.* (1990). DOPA, dopamine, noradrenaline and adrenalin have been detected by HPLC (Eriksson, Gustafsson & Åkerlind, 1993). So far 16 neuropeptides have been detected by ICC (for references see Gustafsson, 1992; Gustafsson *et al.* 1994). The results of this study show indirect evidence of a new category of neuronal messenger molecules, the gaseous transmitter NO, in the nervous system of the gull-tapeworm.

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