The nervous system of *Amphilina foliacea* (Platyhelminthes, Amphilinidea). An immunocytochemical, ultrastructural and spectrofluorometrical study

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SUMMARY

The nervous system of young and adult *Amphilina foliacea* was studied with immunocytochemical, electron microscopical and spectrofluorometrical methods. The general neuroanatomy is described in detail. New data on the structure and development of the brain were obtained. The S-HT and GYIRFamide-immunoreactivities occur in separate sets of neurones. The innervation of the reproductive organs is described. The fine structure of 2 types of neurones in the CNS, a sensory neurone, a ' glial' cell type, the neuropile and the synapses are described. The level of 5-HT varies between 0-074 and 0461 /tg/g wet weight. This is the first detailed study of the nervous system of *A. foliacea.* Earlier data on the structure of the nervous system in *A. foliacea* published in Russian are introduced into the discussion. The study provides data that can be used when considering the phylogenetic position of Amphilinidea.

Key words: *Amphilina foliacea,* nervous system, serotonin, GYIRFamide, ultrastructure, spectrofluorometry.

INTRODUCTION

Taxon Amphilinidea comprises few species. It is geographically widespread, occurring on all continents. The adult worms parasitize fish belonging to Acipenseridae, Osteoglossidae, Haemulidae and Siluridae. The Amphilinideans are monozoic. They are hermaphroditic and have a single set of reproductive organs.

The systematic position and the phylogenetic relationship of the taxon Amphilinidea have long been discussed and controversial opinions prevail. Some authors consider that this group belongs to the taxon Cestodaria (Janicki, 1928, 1930; Fuhrmann, 1930; Bychovsky, 1957; Joyeux & Baer, 1961). Based on the anatomy, morphology and life-cycle of *Amphilina foliacea,* Dubinina (1974, 1982) concluded that these worms form a separate taxon, Amphilinidea, which stands phylogenetically close to the taxon Monogenea. Ehlers (1985) placed Amphilinidea separate from Eucestoda (Fig. 1). In the phylogenetic study of taxon Eucestoda by Hoberg *et al.* (1997), taxon Amphilinidea is regarded as one of the most primitive taxa within Eucestoda, forming a sister group of the tapeworms. Recent data from a molecular test of platyhelminth phylogeny provided by Litvatis & Rohde (1999) show that Amphilinidea and Gyrocotylidea comprise the monophyletic taxon Cestodaria, separate from taxon Eucestoda (Fig. 2).

Amphilina foliacea Rudolphi 1819 is a typical representative of taxon Amphilinidea. Adult *A. foliacea* occurs predominantly in the body cavity of the European species of sturgeon *(Acipenser ruthenus*

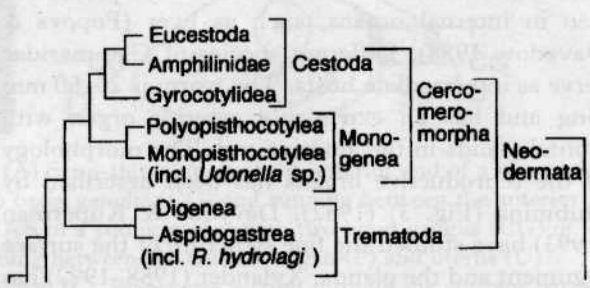

Fig. 1. Morphology-based phylogenetic tree of platyhelminth relationships. Modified after Ehlers (1985). Eucestoda, Amphilinidea and Gyrocotylidea comprise taxon Cestoda.

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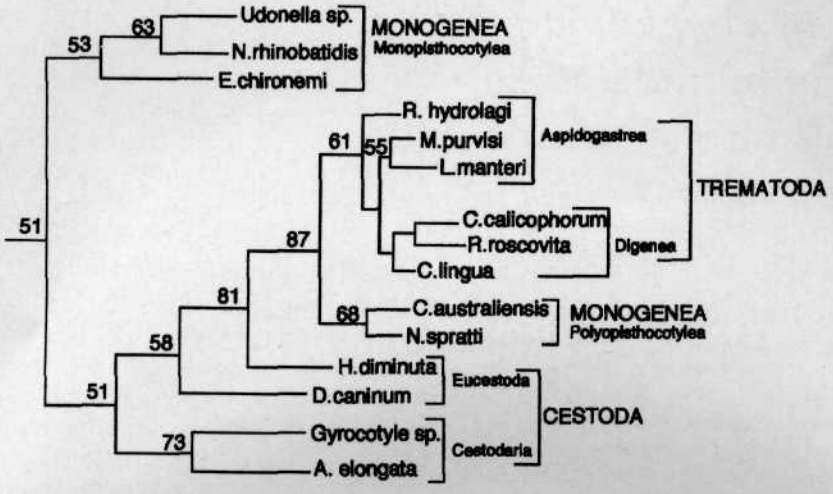

Fig. 2. Parsimony tree of 22 platyhelminth taxa, using the D3 expansion segment of the 28S rDNA gene. Modified after Litvatis and Rohde (1999). *Amphilina elongata* and *Gyrocotyle* sp. comprise taxon Cestodaria.

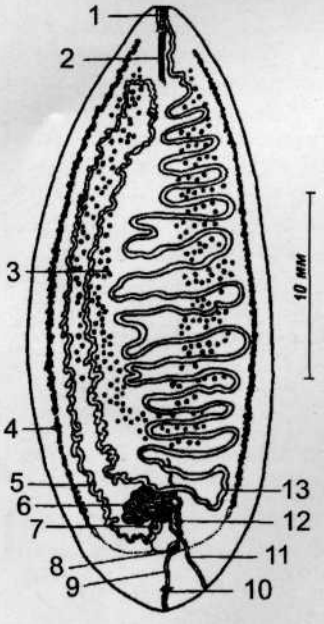

Fig. 3. A schema of the organization of *Amphilina foliacea* (Dubinina, 1974). 1, proboscis; 2, frontal gland; 3, testicular follicles; 4, vitelline gland; 5, uterus; 6, ovaries; 7, ootype; 8, cirrus; 9, ejaculatory duct; 10, embryonic hooks; 11, vagina; 12, spermatic duct; 13, uterus.

L., *A. gueldenstaedti* Brandt, *A. stellatus* Pal.), but also in internal organs, such as liver (Popova & Davydov, 1988). Different species of Gammaridae serve as intermediate hosts. The worm is 20—60 mm long and has an extrudable anterior organ with frontal glands in the anterior end. The morphology of the reproductive organs has been described by Dubinina (Fig. 3) (1982). Davydov & Kuperman (1993) have studied the fine structure of the surface tegument and the glands. Xylander (1988, 1992) has described the fine structure of the reproductive system and the net-like excretory system of Gyrocotylidea and Amphilinidea. As to the nervous system (NS), only scattered data on the general structure (Dubinina, 1982), chemical composition (Shishov, 1991) and fine structure (Biserova & Frolova, 1997a, *b)* are available.

This study is the first detailed investigation of the organization of the nervous system of *A. foliacea.* Firstly, the morphological changes taking place during the development from young to adult worm are followed. Secondly, patterns of nerves immunoreactive (IR) to antibodies towards serotonin (5-hydroxytryptamine, S-HT) and towards the flatworm FMRFamide-related neuropeptide GYIRF amide (Johnston *et al.* 1996) will be revealed. Thirdly, the fine structure of the nervous system is examined and, fourthly, the amount of 5-HT is measured spectrofluorometrically. Results are discussed with respect to the information on the nervous system of *A. foliacea,* a large part of which has been published in Russian.

Recently, in discussing the nervous system of flatworms, Reuter, Mäntylä & Gustafsson (1998) suggested confining the use of the terms (1) central nervous system (CNS) for descriptions of the bilobed brain and the pair of main nerve cords (MCs), and (2) peripheral nervous system (PNS) for all minor cords and the neuronal plexuses associated with the attachment structures, and with the alimentary and reproductive organs. These terms will be used herein.

MATERIALS AND METHODS

Young and adult specimens of *A. foliacea* were obtained from the body cavity of *A. ruthenus* and *A. gueldenstaedti* from the Volga river near Volgograd and Astrakhan (Russia).

Immunocytochemistry

The worms were fixed in Stefanini's fixative at 4 °C, after which they were transferred to 10% sucrose

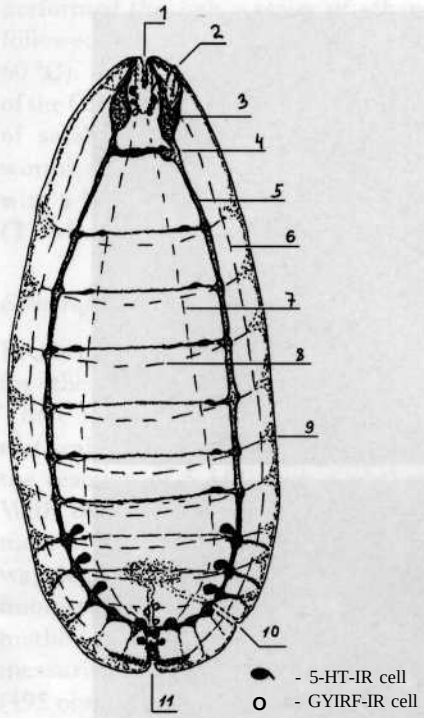

Fig. 4. A schematic drawing of the nervous system of adult *Amphilina foliacea* based on data of the 5-HT and GYIRFamide immunoreactivity. 1, anterior organ; 2, opening of uterus; 3, brain ganglion, 4, transverse commissure; 5, main nerve cord (MC); 6, lateral cord; 7, ventral cord; 8, ring commissure; 9, subtegumental nerve net; 10, vagina; 11, ejaculatory duct. GYIRFamide-IR cell bodies occur close to the anterior organ, in the brain ganglia and close to the vagina and ejaculatory duct.

solution in PBS for 5 days at 4 °C. The worms were embedded in Tissue-Tek and cut at 15 /tm with an ultracryostat. The sections were collected on gelatine-coated slides and double-stained with a mixture of rabbit-anti-5-HT (1:500) (Incstar, USA) and guinea pig-anti-GYIRF-amide (1:500) (Johnston *et al.* 1996) for 36 h, at + 4 °C according to the method described by Coons, Leduc & Connolly (1955). Rhodamine B isothiocyanate (TRITC) (DAKO) conjugated with swine anti-rabbit (1:30) and fluorescein isothiocyanate (FITC) (Cappel) conjugated with goat anti-guinea pig (1:30) were used as secondary antibodies. Incubation time was 1 h, room temperature (RT). The immunostained material was examined using a Leitz Orthoplan and Axioscop OPTON microscopes fitted with filter-blocks 12 and N2; photomicrographs were produced with an Olympus model PM 10 ADS automatic photomicrography system. Controls included omission of the primary antiserum, substitution of the primary antiserum with non-immune rabbit or guinea pig serum, and liquid-phase pre-adsorption of the primary antiserum with 5-HT (Sigma) and GYIRFamide in excess.

Electron microscopy

The worms were fixed in 2 % glutaraldehyde in 0-05 M sodium cacodylate buffer (pH 7·2) at RT for 2 h and then stored in buffer at 4 °C for several days. Post-fixation was performed in 1 % osmium tetroxide in the same buffer for 2'5 h. Dehydration was

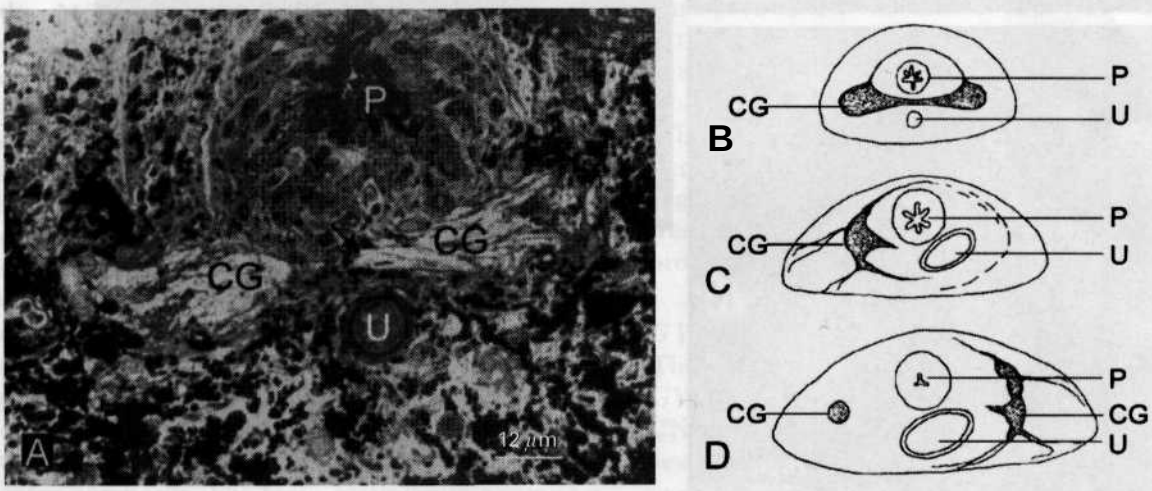

Fig. 5. The development of the brain in *Amphilina foliacea.* (A) Semi-thin section of the anterior end of a young worm with the brain commissure (arrow) connecting the two brain ganglia (CG) and running between the anterior organ (P) and uterus (U). (B) Diagram of the symmetrical CNS in a young worm, with two brain ganglia (CG) of equal size and the connecting median brain commissure running between the anterior organ (P) and uterus (U). (C and D) Diagrams of the asymmetrical CNS in an adult worm. (C) Transverse section through the anterior part of the brain. The left brain ganglion (CG) is larger than the right. The median brain commissure is squeezed between the anterior organ (P) and the uterus (U) and is hardly visible. A thin dorsal and a thick ventral nerve run outside the anterior organ and uterus. (D) Transverse section through the posterior part of the brain. The right brain ganglion (CG) is larger than the left.

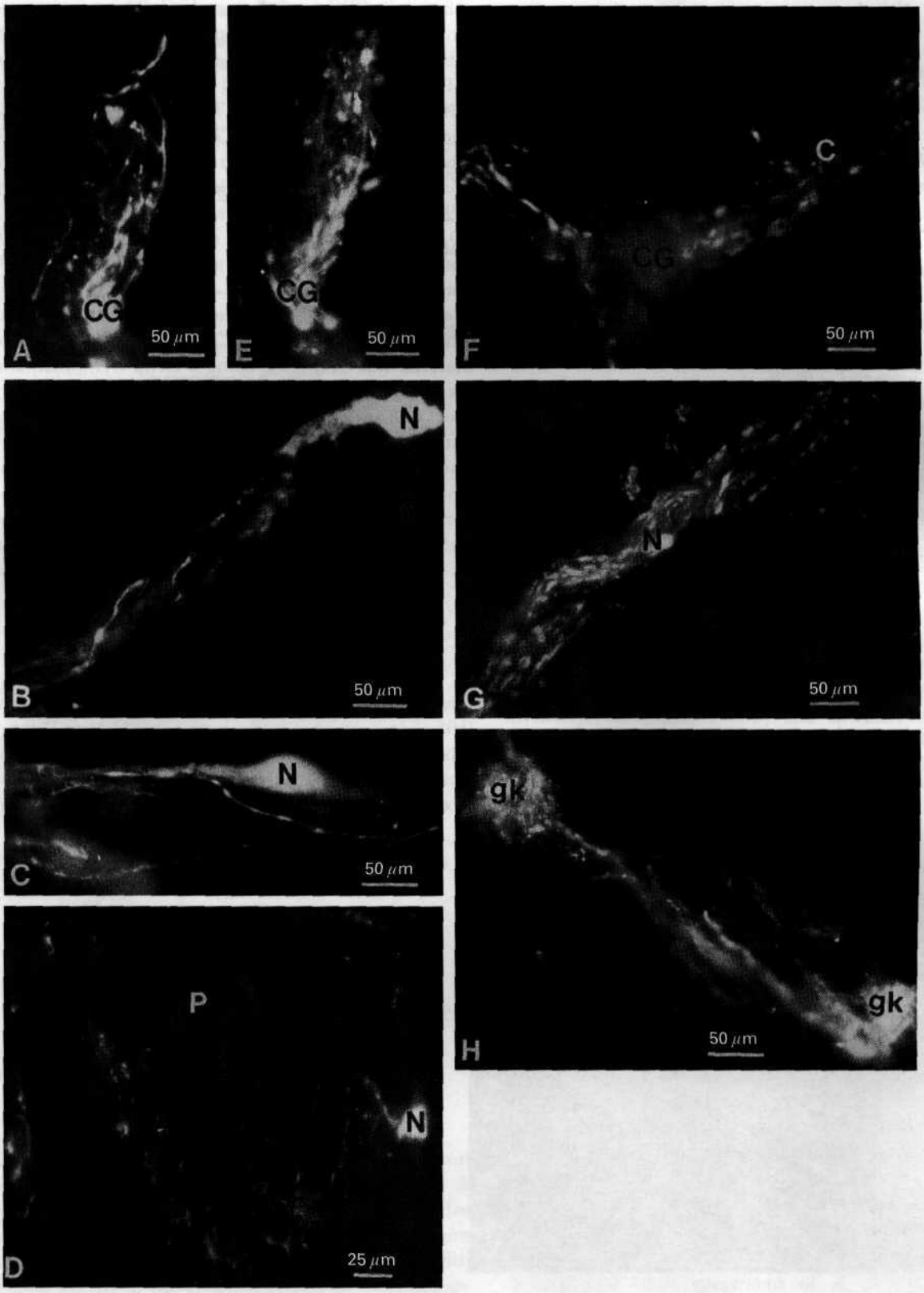

Fig. 6. The pattern of 5-HT- and GYIRFamide-IR nerves in *Amphilina foliacea.* (A) 5-HT-IR nerve fibres in the left brain ganglion (CG). (B) A large unipolar 5-HT-IR neurone (N) in ganglion knot in the posterior end, with processes extending to the tegument. (C) 5-HT-IR bipolar neurone (N) in transverse commissure. (D) 5-HT-IR nerve fibres in the anterior organ (P). 5-HT-IR cell body (N) in right brain ganglion. (E) Image pair of Fig. 4A showing the pattern of GYIRF-IR nerve fibres in the left brain ganglion (CG). (F) GYIRFamide-IR nerve fibres in the right brain ganglion (CG) with part of ring commissure (C) surrounding the uterus. (G) Image pair of (B) showing pattern of GYIRF-IR neurones (N) and processes extending to the tegument. (H) GYIRFamide-IR nerve fibres in two ganglion knots (gk) at MCs and the connecting commissure in posterior end.

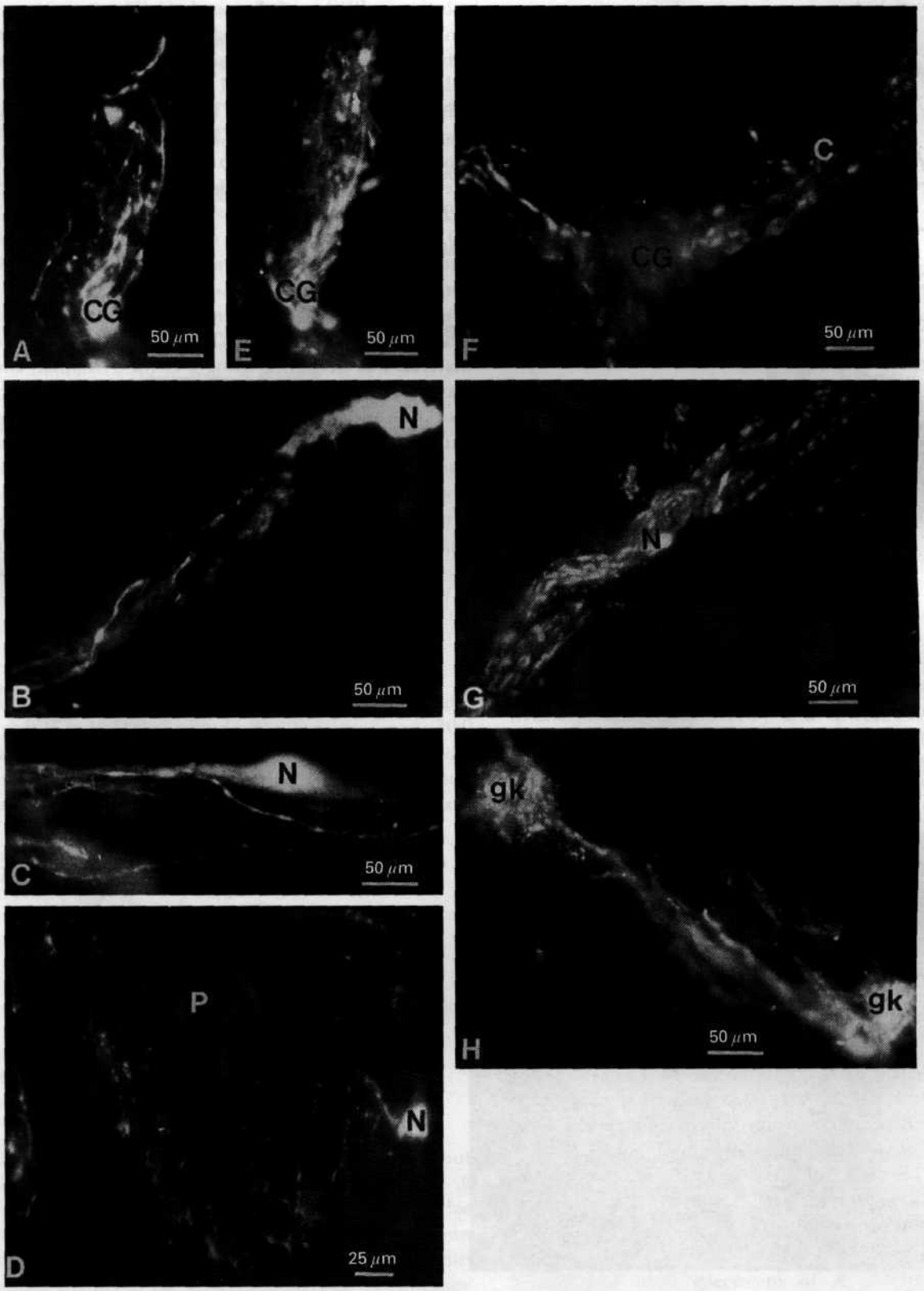

Fig. 6. The pattern of 5-HT- and GYIRFamide-IR nerves in *Amphilina foliacea.* (A) 5-HT-IR nerve fibres in the left brain ganglion (CG). (B) A large unipolar 5-HT-IR neurone (N) in ganglion knot in the posterior end, with processes extending to the tegument. (C) 5-HT-IR bipolar neurone (N) in transverse commissure. (D) 5-HT-IR nerve fibres in the anterior organ (P). 5-HT-IR cell body (N) in right brain ganglion. (E) Image pair of Fig. 4A showing the pattern of GYIRF-IR nerve fibres in the left brain ganglion (CG). (F) GYIRFamide-IR nerve fibres in the right brain ganglion (CG) with part of ring commissure (C) surrounding the uterus. (G) Image pair of (B) showing pattern of GYIRF-IR neurones (N) and processes extending to the tegument. (H) GYIRFamide-IR nerve fibres in two ganglion knots (gk) at MCs and the connecting commissure in posterior end.

performed through a series of ethanol and acetone, followed by embedding in Araldite (37 °C and 60 °C). In order to be able to study the development of the CNS in *A. foliacea* a series of semi-thin sections of small (4 mm long) and larger (7—8 mm long) worms were cut. Ultrathin sections were examined with a JEM 100C transmission electron microscope (TEM).

Spectrofluorometry

Fresh and frozen specimens *of A. foliacea* were used for the spectrofluorometrical analysis of 5-HT. Three methods were used. (1) The spectrofluorometrical method of Maikel *et al.* (1968) is based on the reaction of 5-HT with o-phthaldialdehyde. (2) With the method of Udenfriend (1962), measurement of excitation (295 nm) and emission (450 nm) wavelengths was done after induction of 5-HT fluorescence with strong acid (3 M HC1). (3) With the method of Snyder, Axelrod & Zweig (1965), measuring excitation (385 nm) and emission (495 nm) wavelengths was done after treatment with ninhydrin in a hot near-neutral solution. The fluorescence was measured in a Hitashi MPF-4 spectrofluorimeter using 1-cm quartz cuvettes. Serotonin creatinine sulphate (5-hydrotryptamine, Reanal) was used as the standard.

RESULTS

Gross morphology of the nervous system

Fig. 4 shows the gross morphology of the nervous system of adult *A. foliacea.* From the brain, the MCs run posteriorly. Transverse commissures connect the MCs. At the crossing points between the transverse commissures and the MCs, ganglion knots occur. During development from young to adult, the CNS undergoes changes. Fig. 5 A shows an overview of the anterior end of a young worm and the symmetrical CNS with 2 brain ganglia of equal size and the connecting brain commissure. The brain commissure is located between the anterior organ and uterus. In the brain ganglia nerve cells, nerve fibres and processes from muscle cells occur. Thin nerves extend to the periphery. Fig. 5 B is a diagram of the symmetrical CNS of a young worm. Fig. 5 C shows the asymmetrical CNS in an adult worm. A dislocation of the brain ganglia has taken place, due to the growth and development of the uterus, which opens anteriorly. The brain commissure occupies a restricted site between the proboscis and uterus. The left brain ganglion is larger that the right. In addition to the brain commissure a thin dorsal and a thick ventral commissure run outside the anterior organ and the uterus, connecting the brain ganglia. Fig. 5 D shows a transverse section of the CNS of an adult worm somewhat more distally. Here the right brain ganglion is larger than the left.

Immunocytochemistry

5-HT immunoreactivity in CNS. The nervous system stains strongly with anti-5-HT (Fig. 6A-C). In the brain ganglia, numerous 5-HT-immunoreactive (5-HT-IR) neurones (size 3-5 *μm*) and 5-HT-IR varicosities were observed (Fig. 6A). The MCs extending from the brain to the posterior end contain many 5-HT-IR nerve fibres. In the posterior end, close to the 3 most posterior ganglion knots, 1 large, uni- or pseudo-unipolar 5-HT-IR neurone (size 10 *μm* x 18 μm) per ganglion knot was observed (Figs 4 and 6B). Processes from these cells extend radially towards the tegument. Caudally, the MCs run close to the vagina and the ejaculatory duct (Fig. 4). Close to the openings of the vagina and the ejaculatory duct numerous (about 5) ganglion thickenings of MCs were observed. In the transverse commissures, bipolar 5-HT-IR neurones were observed (Fig. 6C).

5-HT-immunoreactivity in the PNS. In the proboscis a 5-HT-IR ring commissure, 2 thin 5-HT-IR nerves, a 5-HT-IR nerve net, and 2 large symmetrical unipolar and several smaller 5-HT-IR neurones were observed (Fig. 6D). The uterus opening is situated close to the anterior organ. It is surrounded by a 5-HT-IR ring commissure. From the anterior organ, nerves extend to the uterus opening. 5-HT immunoreactivity was observed in the minor nerve cords: 2 lateral, 2 dorsal and 2 ventral cords (Fig. 4). The ring commissures and the subtegumental nerve net with thin processes to the tegument show 5-HT immunoreactivity (Fig. 4). In the posterior end, the ring commissures are more numerous than in the anterior end. 5-HT-IR nerves surround the ejaculatory duct and the vagina. In the apical part of the concavity in the posterior end, large amounts of 5-HT-IR neuronal elements were found (Fig. 4). In the basal part of the concavity, 3 pairs of large 5-HT-IR cell bodies were observed. In the vaginal wall only 2 thin varicose 5-HT-IR processes were found. 5-HT-IR nerve fibres send varicose processes to muscle fibres (Fig. 7B).

GYIRFamide immunoreactivity in CNS and PNS. The nervous system stains strongly with anti-GYIRFamide. Staining was observed both in the CNS (Fig. 6E-F) and the PNS, and was especially associated with the reproductive organs such as the uterus opening, the ovaries, the ootype, the vagina and the opening of the ejaculatory duct (Fig. 4). In the wall of the anterior organ, the GYIRFamide-IR neurones are more numerous and have larger nuclei than the 5-HT-IR neurones. In the brain ganglia the GYIRFamide-IR processes are thinner than the 5-HT-IR processes but more densely packed. Double staining with anti-5-HT and anti-GYIRFamide showed that the 2 neuronal signal substances occur

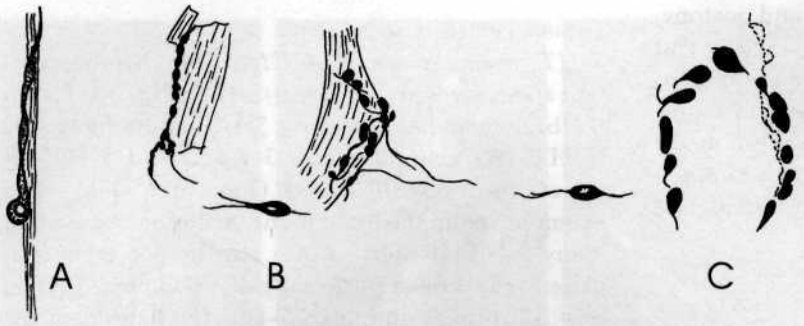

Fig. 7. (A) Schematic drawing of close contact between a GYIRFamide-IR neurone and a muscle firbre. (B) 5-HT-IR neurone innervating muscle fibres. Note the many varicosities. (C) The distribution GYIRF-IR and S-HT-IR elements in posterior part of the MC; black - GYIRF-IR, dotted line - 5-HT-IR.

in separate sets of neurones and fibres (Figs 6B, G and 7C). GYIRFamide-IR neurones (size 4—5μm) occur in the MCs. Large unipolar GYIRFamide-IR neurones (size 12x15μm; nucleus 10 μm in diameter) were observed close to the longitudinal muscle fibres. From these cells large processes (size 9-5μm in diameter), showing weak GYIRFamide immunoreactivity, and extending to the longitudinal muscles fibres in the subtegument were found (Fig. 7A). The same types of neurones were observed in the wall of the concavity in the posterior end and in the wall of the ejaculatory duct. The vaginal wall has a richer supply of GYIRFamide-IR elements than 5-HT-IR. The ganglion knots in the posterior end of MCs showed considerably more GYIRFamide immunoreactivity than 5-HT immunoreactivity (Fig. 6H). From each ganglion knot GYIRF-IR radial nerves extend (Fig. 6G).

Ultrastructure of the CNS

Cell types. In the brain ganglia, small (size 6.5 μm—9.0 μm in diameter) and large (size 9 μm to 16—24 μm in diameter) neurones were observed (Fig. 8A and B). They are usually round and lie in pairs or as groups of 3 cells close to the neuropile. All neurones contain small mitochondria with dark matrix and few cristae, free ribosomes, small amounts of rough endoplasmic reticulum (RER), Golgi complex and vesicles of varying electron density and dimension (Fig. 8 A—C). Rosettes of beta-glycogen occur in the perikaryon and in processes (Fig. 8B). The large neurones are characterized by a single deep invagination of the plasma membrane (Fig. 8B). The nucleus (size 5.4 x 7·8μm) is oval or round. The main part of the neuroplasma is shifted to one pole of the cell, where most part of the free ribosomes, the electron-dense vesicles and the neurotubules are located (Fig. 6B). Sometimes electron-lucent vacuoles were found. In this type of neurone the RER is best developed. Fig. 8C shows a sensory neurone in the subtegument of proboscis. Dense vesicles (dv) (size 50—80 nm) occur in the cell body.

Along the MCs only a few neuronal cell bodies, with electron-dense cytoplasm and dv, occur. The nucleus/cytoplasm ratio is 1:1.

Types of vesicles. In the neurones the following types of vesicles have been found (1) small electron translucent vesicles, (size 35-40 nm) (sv); (2) dense vesicles (size 55-100 nm) (dv). Occasionally a dense core was observed in these vesicles; (3) large electron-dense vesicles (size 85—140 nm) (ldv).

The neurones contain either dv or ldv, but not both types simultaneously (Fig. 6 A—C). The sv were mainly observed in processes together with the dv. Large neurones contain usually ldv.

"Glial" cells. 'Glial' cells were observed in the periphery of the MCs. They often occur in pairs. The nucleus is round and contains a dense round nucleolus and blocks of chromatin. The cytoplasm is electron translucent and contains some glycogen particles, a few lipid droplets and microtubules, some ribosomes and smooth ER (Fig. 8D). Thin processes from the 'glial' cells surround the MCs, forming layers of 8-10 membranes (Fig. 8E). 'Glial' processes penetrate the MCs, surrounding groups of nerve fibres with 4—6 layers and pairs of neurones with 2-3 layers. Between the processes of the 'glial' cells fine extracellular material was observed. Synaptic vesicles are lacking. Tight junctions were observed between 'glial' processes and nerve fibres.

Neuropile and synapses in brain ganglia and the main nerve cord {MC)

The central part of the brain ganglia and MC is composed of small and large nerve processes (Fig. 9). Large translucent processes form groups of 4 each. In these processes, microtubules, mitochondria, beta-glycogen and occasionally light vesicles, were observed. Chemical synapses are rare between the light processes. Small processes filled with synaptic vesicles formed 3—4 'dark' zones in the neuropile. Most of the synapses were found in the dark zones.

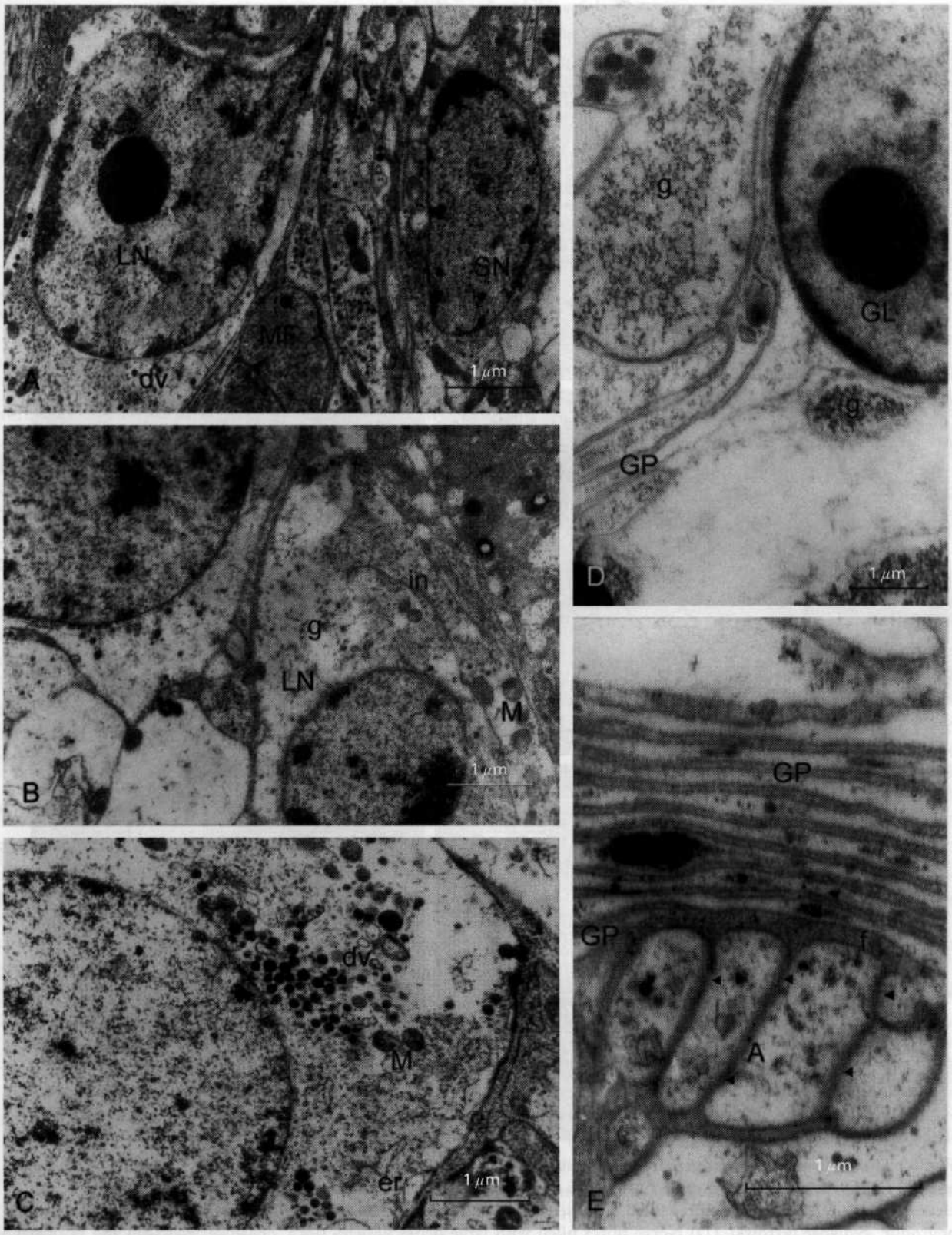

Fig. 8. The fine structure of neurones and nerve fibres in *Amphilina foliacea.* (A) A large neurone (LN) with dense vesicles (dv) and a small neurone (SN) in brain ganglion. The neuropile is composed of nerve fibres filled with vesicles. Note muscle fibres (MF) in neuropile. (B) Large neurone (LN) in brain ganglion. Note invagination of plasma membrane (in), glycogen (g), mitochondrion (M). (C) Sensory neurone in subtegument of the anterior organ with dense vesicles (dv), mitochondrion (M), and endoplasmic reticulum (er). (D) Nucleus of a 'glial' cell (GL) in main nerve cord. 'Glial' processes (GP), glycogen (g). (E) Eight layers of processes (GP) from 'glial' cells surround the main nerve cord. Note group of 5 nerve fibres (A) forming a synaptic glomerulus. Thin processes (arrows) filled with fibrous extracellular material (f) surround the glomerulus.

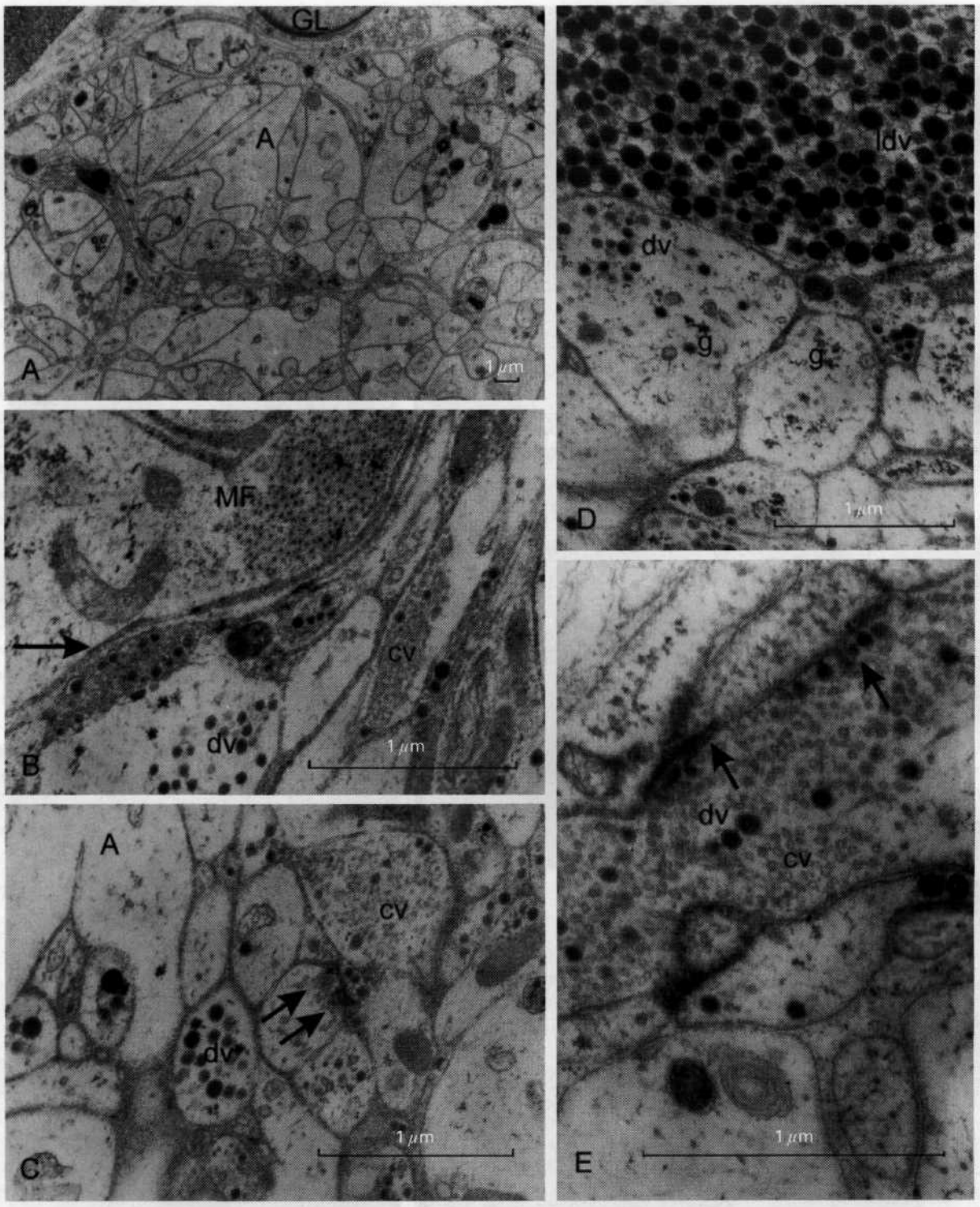

Fig. 9. The neuropile of brain ganglion and main nerve cord (MC) of *Amphilina foliacea.* (A) Overview of neuropile of MC. (B) Contact between muscle fibre (MF) and nerve fibre (arrow) in periphery of MC. Dense vesicles (dv), clear vesicles (cv). (C) Nerve fibres filled with dense vesicles (dv) and clear vesicles (cv) form a 'dark' zone in the neuropile. Synapse (arrows). (D) Nerve fibres filled with large dense vesicles (ldv), dense vesicles (dv) and glycogen particles (g) in MC. (E) En-passant synapses (arrows) from nerve fibres filled with dense vesicles (dv) and clear vesicles (cv) in MC.

The above-mentioned 3 types of vesicles occur in the processes (Fig. 9B-E). One of them can dominate or they can exist in various combinations. Axons with both dv and sv form most of the synaptic contacts. Both single and shared synapses were observed (Fig. 9B-C, E). The synaptic cleft is about 20 nm. A thickening of the post-synaptic membrane is common. Some axons formed 'en passant' synapses (Fig. 9E).

Thin processes from muscle cells pass close to the

Table 1. Concentration of 5-HT in *Amphilina foliacea*

5-HT content (*μg/g* wet wt) determined with		
o-Phthaldialdehyde (Maickel *et al.* 1968)	Ninhydrin (Snyder *et al.* 1965)	HC1 (Undenfriend, 1962)
0-078-0-211 (11)	0.090-0.461 (7)	0.074-0.420 (18)

brain ganglia, some of them penetrating into the central part of the neuropile. The processes branch strongly and they contain lipid drops and ribosomes. Nerve terminals containing sv and dv often form synapses on the surface of these muscle fibres (Fig. 9B).

In the MCs 'dark' zones with numerous synapses were observed both centrally and peripherally. Occasionally so-called synaptic glomerules were found in the MCs. They are composed of small aggregations of thin nerve fibres surrounded by a common sheet of intercellular substance (Fig. 8E). Up to 10 processes of equal size can form a synaptic glomerulus. Occasionally, they form rosette-like structures with a small central part filled with fibrillar intercellular matrix and surrounded by a single layer of intercellular material. The synaptic glomerules are often surrounded by 2–4 layers of processes from the 'glial' cells. Chemical synapses between the processes in a glomerulus are rare. However, the parallel membranes of the processes suggest that electrical transmission of signals is possible.

Spectrofluorometry

5-HT was identified in tissue extracts *oi A. foliacea* by the 3 spectrofluorometrical methods used (Table 1). The concentration of 5-HT in *A. foliacea* varied from 0-074 to 0-461 *fig/g* wet weight. The level of 5-HT in small worms, weighing from 0-03 to 0-2 g, was 0-236 ± 0-035 /tg/g wet weight (29 ± 4 ng per worm). The level of 5-HT in large worms, weighing from 0-35 to 3-0 g, was 0-197 ± 0-03 *fig/g* wet weight (180 ± 61 ng per worm) and not significantly different from that of the small worms. No difference in the concentration of 5-HT was observed between anterior and the posterior regions of the worm.

DISCUSSION

The nervous system of *A. foliacea* has never been described in detailed before. The immunocytochemical analysis of the pattern of 5-HT-IR and GYIRFamide-IR gives, in addition to information about the occurrence of the above-mentioned neuronal signal substances, also a clear picture of the general neuroanatomy of the worm. The ultrastructural part of the study deepens the knowledge of the neurocytology and the spectrofluorometrical part informs about the level of 5-HT of the nervous system. The immunocytochemical method has proven to be an excellent one for neuroanatomical studies (see Gustafsson, 1991, 1992; Gustafsson, Nässel, & Kuusisto, 1993; Reuter & Gustafsson, 1995, 1996). Both the gross morphology and fine details in the nervous system have been revealed.

Gross anatomy

The nervous system *oi A. foliacea* follows the general plan for NS of flatworms (Reuter & Gustafsson, 1995; Reuter *et al.* 1998). The brain of adult *A. foliacea* is clearly non-symmetrical. Janicki (1928) reported the presence of a ring commissure, connecting the brain ganglia and being broader on the ventral side in *A. linguloidea. A* similar observation was made by Kotikova (1971) when studying *A. foliacea.* This is the first time a median commissure passing between the anterior organ and uterus has been observed. This commissure is easy to distinguish in young worms but in adult worms it becomes squeezed between the developing uterine ducts. In the posterior end of *A. foliacea* the nervous system is very well developed. Due to the well-developed posterior NS, the posterior end of *A. foliacea* was earlier believed to be the head region (Cohn, 1904). The posterior end is richly supplied with sensory receptors (Dudicheva & Biserova, 2000).

Already Dubinina (1982) described ganglion knots in *A. foliacea* at the crossing points between the MCs and the transverse commissures. In our study it has been shown that, in addition to the transverse commissures, also the ring commissures connect at the ganglion knots. In the free-living flatworms *Procerodes littoralis* (Reuter *et al.* 1995 a), *Dugesia tigrina* (Reuter *et al.* 1995 6), *Dendrocoelum lacteum* (Reuter *et al.* 1996), and *Planaria torva* (Mantyla *et al.* 1998), ganglion knots at the crossing points of the MCs and the transverse commissures have been observed.

5-HT immunoreactivity

5-HT-IR nerves have been described from the nervous system in all flatworm taxa investigated so far (see Reuter & Gustafsson, 1995). The pattern of 5-HT-IR neurones and fibres in *A. foliacea* is distinct, as is the case in most flatworms (Biserova *et al.* 1996). 5-HT was first demonstrated in *A. foliacea*

by Terenina (1988) in a spectrofluorometrical study. After application of the glyoxylic acid method, Shishov (1991) described 5-HT in large cells (size 25-50 *µm)* and fibres in the longitudinal cords, and in smaller cells (size 4-10µm) and fibres along the transversal commissures *oi A. foliacea.* The immunocytochemical technique enabled us to demonstrate, in addition to the large cells (size 10—18 µm) in the posterior ganglion knots and the bipolar cells in the transverse commissures (size 3—5 *µm),* also small cells (size 3-5 *µm)* in the brain ganglia. The muscular ducts of the reproductive system are well innervated with 5-HT-IR nerves. 5-HT is generally regarded as the main excitatory neurotransmitter of motor activity in flatworms (Terenina, Gustafsson & Reuter, 1995).

G YIRFamide immunoreactivity

This is the first demonstration of a neuropeptide in an Amphilinidean flatworm. GYIRFamide and 5-HT in *A. foliacea* occupy separate sets of neurones and fibres, as is the case in most flatworms (see Halton & Gustafsson, 1996). GYIRFamide belongs to the FMRFamide-related peptides (FaRPs), which have been localized with immunocytochemical methods in flatworms from all taxa studied so far (Halton *et al.* 1994). GYIRFamide was isolated from the free-living flatworm *Bdelloura candida* by Johnston *et al.* (1996). GYIRFamide-IR neurones and fibres have been reported from flatworms belonging to different taxa e.g. a monogenean, *Polystoma nearcticum* (Armstrong *et al.* 1997), a planarian, *Girardia tigrina* (Kreshchenko *et al.* 1999), a digenean, *Echinostoma caproni* (Humphries *et al.* 2000) and a cestode, *Moniezia expansa* (Maule, personal communication). An especially high density of GYIRFamide-IR nerves was observed close to the reproductive organs in the posterior part of *A. foliacea.* A close association between GYIRFamide-IR nerves and the somatic musculature was noted, with giant GYIRFamide-IR nerves innervating the muscle fibres. In regenerating *G. tigrina,* GYIRFamide-IR nerves are closely associated to the developing muscle fibres (Kreshchenko *et al.* 1999). According to Johnston *et al.* (1996) synthetic GYIRFamide causes dose-dependent contractions of muscle fibres isolated from *B. Candida.* Day, Bennet & Pax (1994) have shown that flatworm neuropeptides closely related to GYIRFamide cause concentration-dependent contractions of muscle fibres in the human blood fluke, *Schistosoma mansoni.*

Ultrastructure

Two types of ganglion neurones, one type of sensory neurone and a ' glial' cell type could be differentiated in *A. foliacea.* As in all flatworms studied so far the ganglion neurones of *A. foliacea are* of a highly secretory nature, with vesicles of different density and size (for references, see Xylander 1987; Gustafsson, 1992; Reuter & Gustafsson, 1995). Correlates of the 2 types of ganglion neurones in *A. foliacea* occur in *Triaenophorus nodulosus,* in which 5 types of neurones have been described (Biserova *et al.* 1996), in *Diphyllobothrium dendriticum* (Gustafsson & Wikgren, 1981) and in *Grillotia erninaceus* (Biserova, 1991a). In the free-living flatworm *Geocentrophora waginii* 'light' and large multipolar neurons have been described by Bœckerman, Reuter & Timoshkin (1994).

Neurones characterized by several deep invaginations of the neurilemma and an amoeba-like shape have been described from actively moving flatworms (Golubev & Kaschapova, 1975; Pluzhnikov & Pospekhov, 1990; Bœckerman *et al.* 1994; Biserova *et al.* 1996; Biserova, 19976; Mäntylä *et al.* 1998). The large ganglion neurones of *A. foliacea* have only 1 deep invagination and the small neurones lack invaginations. *A. foliacea* lives a rather sedative life in the body cavity of fishes, squeezed between the inner organs. Even when freed in saline, *A. foliacea* moves only very slowly.

'Glial' cells

' Glial' cells are rare in flatworms (for discussion see Reuter & Gustafsson, 1995). Data from both free-living and parasitic flatworms exist (Rohde, 1970, 1971; Golubev, 1988; Bœckerman *et al.* 1994; Bedini & Lanfranchi, 1998; Mäntylä *et al.* 1998). Typical 'glial' cells lack vesicles and have a thin cytoplasm with a few organelles. They often form branches or lamellae and are believed to perform supporting, isolating and metabolic functions. The morphology of the 'glial' cells of *A. foliacea* conforms to that of 'glial' cells from other flatworms. Pluzhnikov & Pospekhov (1990) have discussed the role of the intercellular matrix as glia in cyclophyllid tapeworms. According to Biserova (1997 a) the cells of the excretory system of *T. nodulosus* perform 'glial' tasks. The processes surround the brain ganglion and penetrate deep into the neuropile.

The synapses and non-synaptic release sites

The morphology of the synapses of *A. foliacea* falls within the range of that reported from flatworms in general (see Reuter & Gustafsson, 1995). In the study of the fine structure of the CNS of adult *A. foliacea* Biserova & Frolova (19976) described paracrine contacts between nerve fibres containing ldv (size 110—170 nm) and large muscle fibres close to the brain ganglia.

Spectrofluorometry

Biogenic amines are often of irregular occurrence among members of the phylum Platyhelminthes (Terenina, 1984, 1991; Eriksson, Gustafsson & Akerlind, 1993 a; Eriksson, Reuter & Timoshkin, 19936; Eriksson, 1995; Reuter & Gustafsson, 1995; Terenina, Gustafsson & Reuter, 1995 ; Biserova *et al.* 1996). In cestodes, however, 5-HT seems to be ubiquitous. 5-HT has been detected in all taxa that have been tested through radioenzymatic, histochemical, and immunocytochemical methods (for references see Terenina *et al.* 1995). The level of 5-HT in *A. foliacea* (0.074-0.461 µg/g wet wt) is of the same magnitude as that in plercercoids of *D. dendriticum* (6.0 + 0.7 nmol/g fresh wt; Eriksson *et al.* 1993 *a),* in tetrathyridia of *Mesocestoides vogae* (0.333-1.146 *µg/g* wet wt; Terenina *et al.* 1995), and in adult *T. nodulosus* (0.297—1.411µg/g wet wt; Biserova *et al.* 1996). The excitation and emission spectra of the extracts of *A. foliacea* obtained with the methods used in this study are identical with those of known samples of 5-HT extracted by the same methods.

Cestodes are able to actively absorb 5-HT from their environment (Harriri, 1975; Gyr, Gruner & Mettrick 1983). Their 5-HT could thus be of host origin. The levels of 5-HT, in *Hymenolepis diminuta* and in the intestine of its rat host are positively correlated (Cho & Mettrick, 1982; Terenina, Shalaeva & Pomogaev, 1986).

The phylogenetic position of Amphilinidea

The phylogenetic position of Amphilinidea has long been debated (see Introduction section). Recent data from a molecular test of platyhelminth phylogeny provided by Litvatis & Rohde (1999) show that Amphilinidea and Gyrocotylidea comprise the monophyletic taxon Cestodaria. Morphological justification for such a taxon can be found in the presence of 5 pairs of hooks and a body that is not divided into proglottids. Litvatis and Rohde found no support for a monophyletic Cestoda (i.e. Cestodaria and Eucestoda) meaning that tapeworms and Amphilinidea are not closely related. According to Dubinina (1974, 1982) taxon Amphilinidea and Monogenea share certain morphological characteristics. The results of this study support the opinions of Dubinina (1974, 1982) and Litvatis & Rhode (1999) placing Amphilinidea separately from tapeworms.

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