The pattern of FMRFamide and serotonin immunoreactive elements in the nervous system of *Aspidogaster conchicola* K. Baer, 1827 (Aspidogastrea, Aspidogastridae)

Oleg Tolstenkov[1], Nadezhda Terenina[1], Natalia Kreshchenko[2], Margaretha Gustafsson[3]

[1]Center for Parasitology of the A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Lenin Avenue 33, 119071 Moscow, Russia

[2]Russian Comparative Neuroendocrinology Research group, Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 1422292, Russia

[3]Department of Biology, Åbo Akademi University, Artillerigatan 6, FIN -20520, Åbo, Finland

Corresponding author: Oleg Tolstenkov; e-mail: otolo@rambler.ru

ABSTRACT. The patterns of the neuropeptide FMRFamide and serotonin (5-HT) immunoreactive (IR) elements in the nervous system of Aspidogaster conchicola (Aspidogastrea, Aspidogastridae) are described using immunocytochemistry and confocal scanning laser microscopy. Both FMRFamid and 5-HT immunoreactivities occur in the bilobed brain, the three pairs of longitudinal nerve cords and many transverse commissures. The adhesive disc is strongly innervated by FMRFamide-IR nerve fibres. Many 5-HT-IR neurones were observed on the reproductive organs.

KEY WORDS: *Aspidogaster conchicola*, nervous system, FMRFamide, serotonin
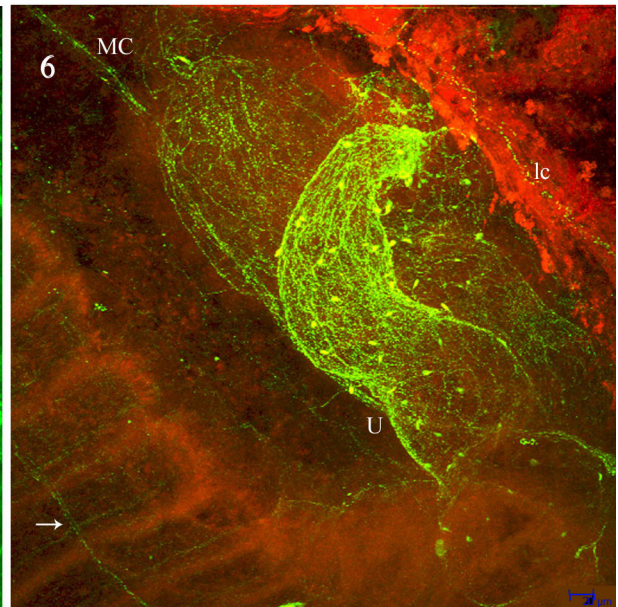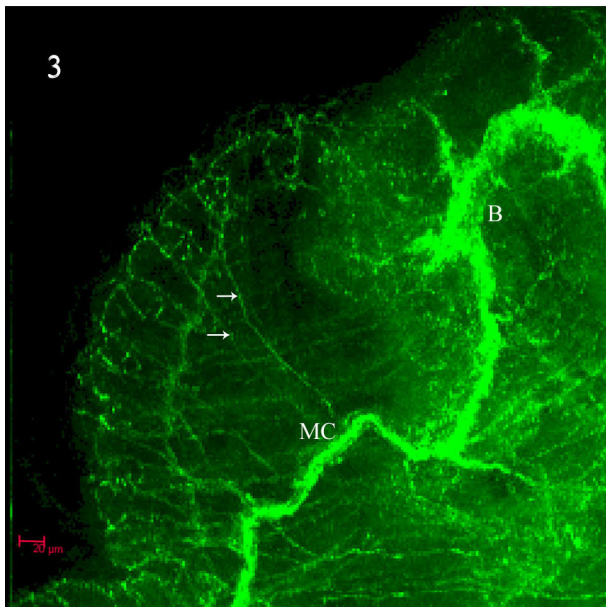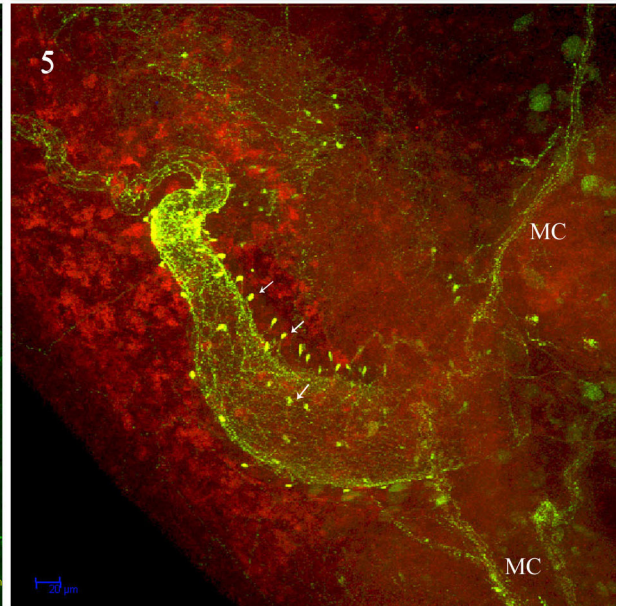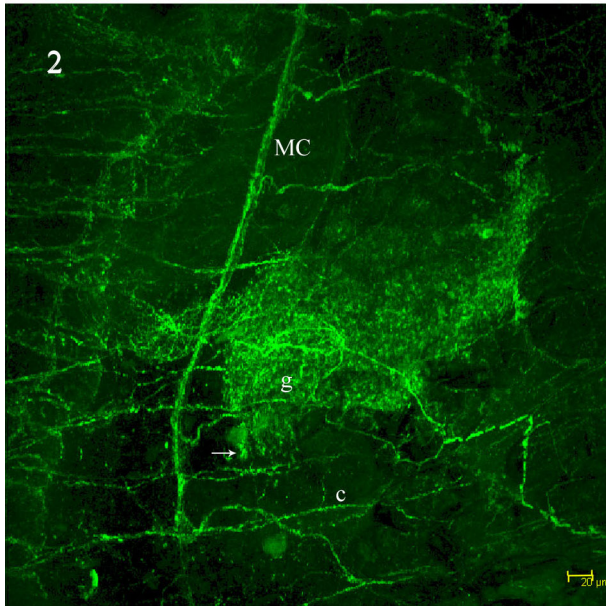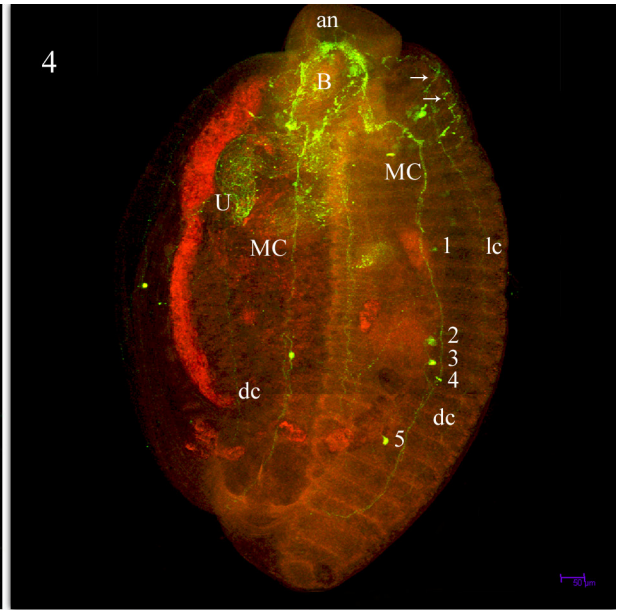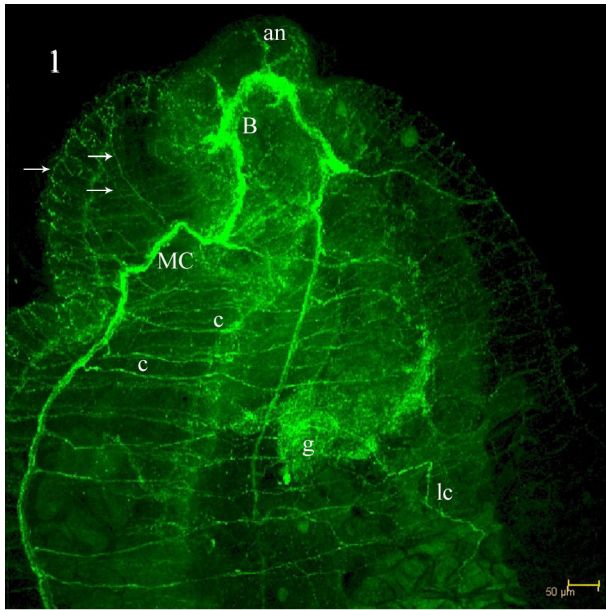
INTRODUCTION

OLSON & TKACH (2005) have recently put together a comprehensive review about the molecular systematics of parasitic Platyhelminthes. According to them Class Trematoda is composed of two subclasses, the Aspidogastrea comprising only a small number of species and the Digenea comprising about 18,000 nominal species. According to SCHLUDERMANN et al. (2005) the aspidogastreans are of great importance as a model in evolutionary studies of parasitic flatworms, i.e., they form a possible link between free-living and parasitic organisms. The aspidogastreans have many ancestral features such as 1) a direct life cycle without larval multiplication, 2) low host and organ specificity, 3) an extremely complicated nervous system, 4) a very great number and variety of sensory receptors (ROHDE, 2001). The aspidogastreans are less modified for a parasitic way of life than other Neodermata groups and they can survive several weeks outside a host animal. They lack the ventral sucker of the digeneans. Instead they have an adhesive disc (Baer's disc) on the ventral surface. The adhesive disc is divided into longitudinal rows of alveoli. The aspidogastreans are found in molluscs, fresh water and marine fish and sea turtles.

TIMOFEEVA (1971) has given a detailed description of the cholinergic nervous system (NS) of *Aspidogaster conchicola*. In general the NS of A. conchicola resembles the NS of digeneans consisting of a distinct orthogon. *A. conchicola* lacks the thick nerve plexus found in monogeneans and cestodes (KOTIKOVA, 1971). SHISHOV (1991) described the patterns of catecholamines and indolamines in *A. conchicola*.

As far as we know nothing is known about the peptidergic NS of *A. conchicola*. In the present study the patterns of neurons and nerve fibres immunoreactive (IR) to FMRFamide in *A. conchicola* are described and compared to those of serotonin (5-HT). To obtain a better picture of the spatial relationships between different parts of the NS and the body musculature, the anti-FMRFamide and anti-5-HT antibodies were used in combination with phalloidin-TRITC staining, which stains F-actin filaments.

MATERIALS AND METHODS

Specimens of adult *Aspidogaster conchicola* K. Baer, 1827 were recovered from the pericard of bivalves (*Anodonta anatina*) from the river Volga, (Tver Region, Russia). The material was fixed in 4% paraformaldehyde in 0.1M phosphate buffer at 4°C. For storage, it was transferred to the same buffer with 10% sucrose. Whole mounts of A. conchicola were stained with rabbit-anti-FMRFamide (Peninsula, Belmont, CA, USA) (1:500) or rabbit anti-5-HT (Incstar, Stillwater, MN, USA) (1:5000) according to the method described by COONS et al. (1955). The whole mounts were incubated with the primary antibody for seven days at 8°C and with the secondary antibody swine anti-rabbit FITC (DAKO) 1:50 for 5 days at 8°C. Controls included omission of the primary antibody and substitution of primary antibody with non-immune rabbit serum. Unfortunately double staining with anti-FMRFamide and anti-5-HT was not performed. In order to study the relationship between the patterns of the FMRF-IR and 5-HT-IR nervous elements and the musculature, staining with TRITC-conjugated phal-

loidin (Sigma, St. Louis, MO, USA) (1:200) was performed for 20min at 8°C (WAHLBERG, 1998). The phalloidin staining was performed after the ICC staining. The ICC and the phalloidin stainings were performed at the Department of Biology, Åbo Akademi University, Finland.

RESULTS

Pattern of FMRFamide immunoreactivity

The plan of the flatworm NS is the so-called orthogon, a rectilinear, ladder-like configuration of longitudinal nerve cords connected at intervals by transverse ring commissures. The NS of *A. conchicola* follows this pattern. The staining with anti-FMRFamide is strong (Fig. 1). The bilobed brain is arch-shaped and measures about 25μm x 300μm. Many FMRFamide-IR nerve fibres extend from the brain in the anterior direction. They form a so-called terminal nerve ring close to the mouth. Three pairs of longitudinal nerve cords extend from the brain to the posterior end of the worm. The two ventral cords are the most prominent and will be called the main cords (MCs). The pairs of lateral and dorsal cords are thinner than the MCs. The longitudinal cords are connected by many transverse ring commissures. The FMRFamide-IR neurones are small and difficult to count. In the region of the genital opening an FMRFamide-IR nerve net including one neurone was observed (Fig. 2). Numerous FMRFamide-IR fibres innervate the adhesive disc (Fig. 3). Many FMRFamide-IR fibres were observed in the subepithelial nerve plexus and surrounding the excretory pore.

Pattern of 5-HT immunoreactivity

The staining with anti-5-HT was weaker than that of anti-FMRFamide (Fig. 4). 5-HT immunoreactivity was observed in the arch-shaped bilobed brain, in fibres extending in the anterior direction forming the so-called terminal ring at the edge of the mouth, the two MCs, the pairs of dorsal and lateral longitudinal cords extending to the posterior end of the worm. Five 5-HT-IR neurones (size 10 x 15 μm) were observed in the brain, two in the anterior nerve fibres and one in the terminal nerve ring. Five 5-HT-IR neurones (size 10x15μm) occur along each MC. The longitudinal cords are connected by a few transverse commissures. Many 5-HT-IR fibres were observed in the subepithelial nerve plexus. Close to the genital opening one 5-HT-IR neurone was observed. Numerous 5-HT-IR neurones (size 4 x 5 μm) and fibres were observed on the surface of the uterus (Figs 5-6). A few 5-HT-IR neurones were observed in connection with the distal parts of the male reproductive system. Four longitudinal and several transverse muscular septa divide the adhesive disc into numerous discrete alveoli. A nerve net composed of thin 5-HT-IR fibres was found inside the adhesive disc (Fig. 6).

DISCUSSION

As expected the NS of *A. conchicola* contains peptidergic (FMRFamide-IR) and 5-HT-IR nervous elements. The presence of cholinergic, catecholaminergic and indolaminergic substances in *A. conchicola* had previously been demonstrated by TIMOFEEVA (1971) and SHISHOV (1991). This means that *A. conchicola*, with reference to the main categories of neuronal mediators, resembles all other flatworm species so far examined. The presence of acetylcholine (ACh), FMRFamide- and 5-HT-IR innervation of the mouth, adhesive disc and reproductive system indicates that the above-mentioned neuronal mediators take part in the regulation of the muscle activity in these vital parts. A physiological role for ACh as an inhibitory neurotransmitter in most trematodes and cestodes is indicated by the fact that their muscular activity is reduced by cholinomimetics and by cholinesterase inhibitors, ultimately producing a flaccid paralysis. 5-HT appears to be the dominant biogenic amine in all flatworm species examined, and there is good experimental evidence that it serves a variety of functions, most notably that of excitatory neurotransmission (GUSTAFSSON & HALTON, 2001). The function of neuropeptides such as FMRFamide-like peptides (FLPs) in flatworms is as yet unclear but their ubiquitous occurrence throughout the flatworm NS implies a fundamental role in the nerve-muscle physiology. All FLPs so far isolated and sequenced from flatworms have been shown to be myoexcitatory in a concentration-dependent manner when applied exogenously to living muscle cells or muscle strips from free-living and parasitic flatworms (MCVEIGH et al., 2005).

In 1998 REUTER et al. introduced new concepts into the flatworm neurobiology. The terminology of the longitudinal nerve cords had long been very confusing. In order to obviate these difficulties, two new terms were coined, the 'main

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Fig. 1-3. – The pattern of FMRFamide-IR nerve fibres in *Aspidogaster conchicola.*
Fig. 1. – Anterior part of the animal. Arrows: innervation of the adhesive disc
Fig. 2. – The innervation of the reproductive organs. Arrow: neurone.
Fig. 3. – The innervation of the adhesive disc. Arrows: nerves from the MC to the adhesive disc.

Figs 4-6 – The pattern of 5-HT-IR nerve fibres in *Aspidogaster conchicola*.
Fig. 4. – neurones along the MC indicated with 1, 2, 3, 4, 5; arrows: nerves to adhesive disc.
Fig 5. – The innervation of the reproductive organs. Note many small 5-HT-IR neurones (arrows) at the surface of the uterus.
Fig. 6. – The innervation of the adhesive disc. 5-HT-IR nerves form a regular network in the adhesive disc. Many small 5-HT-IR neurones on the wall of uterus. Arrow: innervation of the adhesive disc.

Abbreviations: B: bilobed brain; an: anterior nerves; c: transverse commissures; dc: dorsal nerve cord; g: genital pore; lc: lateral nerve cord; MC: main nerve cord; U = uterus.

nerve cords' (MCs) and the 'minor nerve cords'. The MCs are defined as the two most prominent nerve cords in the worm. Irrespective of their disposition as ventral, dorsal or lateral, the MCs originate as multifibre outgrowths or rootlets from each of the brain ganglia and are associated with more neurones that any other nerve cord. The minor cords comprise all other longitudinal cords. The concept of MCs provides for the possibility of dividing the flatworm NS into a central nervous system (CNS) and a peripheral nervous system (PNS). The CNS comprises the bilobed brain and the MCs; the PNS comprises all of the minor cords and the nerve plexuses. The ventral nerve cords of *A. conchicola* fill the criteria for MCs and make it possible to divide the NS into a CNS and a PNS.

In order to establish their phylogenetic position, the aspidogastreans have been investigated from many different angles, from light, confocal scanning laser, transmission and scanning electron microscopy to molecular biology (Rhode, 1994, 2001, 2002; Levron et al., 2009; Gao et al., 2003; Olson & Tkach, 2005). Raikova (2004) investigated the neuroanatomy of basal bilaterians (Xenoturbellida, Nemertodermatida, Acoela) and its phylogenetic implications. She pointed out that "the NS characters seem very useful to unravel the acoel taxonomy at a low level (family, genus and species), within a closely related monophyletic group. The use of NS characters at a higher level may be misleading, because of a high degree of homoplasy and the fact that the NS evolution has taken place independently and in parallel in many monophyletic lines and not within Acoela as a whole." This means that the ICC results obtained in this study have no implications for the discussion of the phylogenetic position of the aspidogastreans but they still form a piece in the general jigsaw puzzle of the flatworm NS.

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