Serotonin and neuropeptide FMRFamide in the attachment organs of trematodes

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Summary

The serotoninergic and FMRFamidergic nervous system of the attachment organs of trematodes were examined using immunocytochemical techniques and confocal scanning laser microscopy. Adult trematodes from eight families as well as cercariae and metacercariae from ten families were studied. TRITC-conjugated phalloidin was used to stain the muscle fibres. The serotonin- and FMRFamide-immunoreactive (IR) nerve cells and fibres were revealed to be near the muscle fibres of the oral and ventral suckers of the trematodes and their larvae. The results indicate the important role of neurotransmitters, serotonin and neuropeptide FMRFamide in the regulation of muscle activity in the attachment organs of trematodes and can be considered in perspective for the development of new anthelmintic drugs, which can interrupt the function of the attachment organs of the parasites.

Keywords: Trematodes; attachment organs; neurotransmitters; serotonin; FMRFamide; nervous system

Introduction

The parasitic flatworms, trematodes have well-developed adhesive organs represented by oral and ventral suckers. These allow parasites to adhere to the substrate (i.e., to the host organs and tissues) and play an important role in their feeding and, in some cases, their locomotion. The musculature of its adhesive organs consists of longitudinal, circular and radial muscle fibres. Contractions of longitudinal (meridionally oriented) muscles open the sucker while contractions of circularly arranged fibres, in conjunction with the radial muscles that run between the inner and outer surfaces of the sucker close it (Mair et al., 1998; Halton & Maule, 2004; Yastrebov & Yastrebova, 2014; Krupenko & Dobrovolskij, 2015).

It is well known that diseases caused by parasitic flatworms are of medical and agricultural problem as they are harmful to human health as well as they are producing great economic losses in agriculture. That is why studying this animal group is important, not only for solving fundamental medical problems, but also for having great practical significance.

The nervous system of trematodes is well differentiated, consisting of central and peripheral parts, which participate in the regulation of many functions including feeding, locomotion, reproduction and migration. Data on the innervations of trematodes' fixation organs are limited but allowing us to assume on an important role of nervous system in the mechanisms of adhesion of parasites to the host tissues (McKay et al., 1990, 1991; Niewiadomska et al., 1996a,b; McVeigh et al., 2009; Leksanboon et al., 2012).

Several neuronal signal substances, including serotonin and FMRFamide-related peptides (FaRPs) have been identified in central and peripheral nervous systems of flatworm parasites indicating the neurochemical complexity of their nervous system (Gustafsson 1987; Magee et al., 1993; Terenina et al., 2006). Serotonin (5-hydroxytryptamine, 5-HT) appears to be the dominant biogenic
amine in Platyhelminthes. In trematodes 5-HT was identified by
number of biochemical methods in crude extracts of Schistosoma
haematobium, S. japonicum, S. mansoni (Bennett et al., 1969;
Chou et al., 1972), Haplochromis cichlidae, Opisthorchis felineus,
Azygia lucii, Codonoccephalus umigeraus (Terenina & Gustafsson,
2003). Immunocytochemical studies have verified the presence of
serotonin in all trematode species examined so far. 5-HT immuno-
reactivity has been demonstrated in their central and peripheral
nervous systems: in cerebral ganglia, lateral nerve cords, transversal
commissures, in subepithelial and submuscular nerve
plexuses.

The FMRFamide is a member of the neuropeptides FaRPs family
and was firstly isolated from the mollusks Macrocystis nimbosa
(Price & Greenberg, 1977). So far the only four authentic FaRPs
have been isolated from flatworms: YIFRamide (from turbellarian
Bdelloura candida); GYIRFamide (from turbellarian B. candida,
Dugesia tigrina); RYIRFamide (from turbellarian Artioposthia tri-
angulata), QNFRRFaMide (from cestode Moniesia expansa) (see
McVeight et al, 2009 for references). None of FaRPs was isolated
from trematodes, but numerous immunocytochemical investiga-
tions using different antibodies (such as anti-RF, anti-GYIRF and
anti-FMRF) indicate that FaRPs are broadly expressed in their
nervous system (Gustafsson et al., 2002). Endogenous FMRF-like
peptides are remarkably potent in parasitic worms (Day & Maule,
1999) and there are reasons to believe that peptidergic signalling
could be an attractive target for new anthelmintic drugs developing
to treat the infections (Mousley et al., 1999) and there are reasons
to believe that peptidergic signalling could be an attractive target
for new anthelmintic drugs developing to treat the infections (Mousley et al., 2005). In this context, the
attachment organs of parasites may be a convenient model for
drug investigations which will interfere with the function of suckers
and thus entire parasite’s adhesion to the host tissues.

Current research is focusing on the innervations of the muscula-
ture of the oral and ventral suckers of trematodes and its larvae
(cercariae and metacercariae) with serotoninergic and peptidergic
(FMRMateuratmic) structures. The results obtained in this study
allowed to extend our knowledge about nervous system of attach-
ment organs in selected trematode species, their potential func-
tions and could stimulate further research in this field.

Materials and Methods
Trematodes and fixation procedure
Specimens of adult trematodes (from eight families) and larvae
(cercariae and metacercariae) from ten families, collected in vari-
ous regions in Russia and Belarus were used in the study (see
Table 1).
The material was fixed in 4 % paraformaldehyde (PFA) in 0.1 M
phosphate buffer (PBS, Sigma) at 4 °C and at a pH of 7.4. For stor-
age, it was transferred to the PBS buffer with 10 % sucrose. Part
of the samples (adult trematodes) was embedded in Tissue-Tek,
frozen and sectioned at 20μm on a Bright cryostat. The sections
were collected on chrom-alum-gelatine-coated glass slides, dried

| Table 1. The investigated species of trematodes. |
|-----------------------------------------------|
| **Adults**                                    |
| Opisthorchis felineus Rivolta, 1884 (Opisthorchiidae Loos, 1899) |
| Allocreadium isoporum Loos, 1984 (Allocreadiidae Loos, 1920) |
| Plagiorchis rani Steinb., 1924 (Plagiorchiidae Lühe, 1901) |
| Opisthiophyge ranae Frölich, 1791 (Plagiorchiidae Lühe, 1901) |
| Gorgodera cygnoides Zeder, 1600 (Gorgoderidae Loos, 1899) |
| G. loossi Sinitzin, 1905 (Gorgoderidae Loos, 1899) |
| Paramphistomum cervi Zeder, 1790 |
| (Paramphistomidae Fischeder, 1901) |
| Aspidogaster conchicola K.Baer, 1827 |
| (Aspidogastridae Poche, 1907) |
| **Cercariae** |
| Cercaria parvicaudata Stunkard and Shaw, 1931 |
| (Renicolidae Dolioli, 1939) |
| Plagiorchis elegans Rudolph, 1802 (Plagiorchiidae Lühe, 1901) |
| Cryptocotyle lingua Creplin, 1825 (Heterophyidae Leiper, 1914) |
| Trichobilharzia szidatii Neuhaus, 1952 |
| (Schistosomatidae Stiles and Hassall, 1898) |
| Bilharziella polonica Kowalewski, 1895 |
| (Schistosomatidae Stiles and Hassall, 1898) |
| Sphaerostomum globiporum Rudolphi, 1802 |
| (Opecopidae Ozaki, 1925) |
| Molinella aniceps Molin, 1859 (Echinostomatidae Loos, 1899) |
| Himasthla elongata Mehlis, 1831 (Echinostomatidae Loos, 1899) |
| **Metacercariae** |
| Leucochloridiomorpha lutea von Baer, 1826 |
| (Leucochloridiomorphiid Yamaguti, 1958) |
| Cotylurus sp. (Strigeidae Railliet, 1919) |

for approximately two hours at room temperature, and were either
stained directly or stored at -20 °C. Other samples (cercariae and
metacercariae) were stained as whole mounts.

Immunocytochemistry
Whole mounts and cryostat sections of worms were stained with
rabbit anti-5-HT (Instar, USA) (1:500) or rabbit anti-FMRFamide
(Peninsula, Belmont, CA, USA) (1:500) primary antibodies in PBS
containing 1 % (v/v) Triton X 100 (Sigma) (PBS-T) according to
the method described by Coons et al., (1955). The whole mounts
(cercariae and metacercariae) were incubated with the primary
antibody for five days at 4 °C, and with the secondary goat an-
ti-rabbit Alexa 488 (Molecular Probes, USA) (1:400) antibodies in
PBS-T over five days at 4 °C. The sections were incubated with
the primary antibody for two days and with the secondary antibody
for three hours. Controls included omission of the primary antibody
and the substitution of the primary antibody with non-immune rab-
bit serum.
| Species                          | 5-HT | FMRFamide | References                          |
|--------------------------------|------|-----------|-------------------------------------|
| **Adults**                      |      |           |                                     |
| Bucephaloides gracilesctns      | +    | +         | Stewart et al., 2003a               |
| Haplometra cylindracea          | +    | +         | McKay et al., 1990                  |
| Fasciola hepatica               | +    | -         | Magee et al., 1989                  |
| -                              | -    | +         |                                     |
| Schistosoma mansoni             | +    | +         | Mair et al., 2000                   |
| Opisthorchis felineus           | +    | +         | Tolstenkov et al., 2010             |
| Opisthorchis felineus           | +    | -         |                                     |
| Allocreadium isporum            | +    | -         |                                     |
| Plagiorchis laricola            | -    | +         |                                     |
| Gorgodera cygnoides             | +    | -         |                                     |
| G. loossi                       | +    | -         |                                     |
| Paramphistomum cervi            | +    | -         |                                     |
| Aspidogaster conchicola         | +    | -         |                                     |
| Allocreadium isoporum           | +    | -         |                                     |
| Plagiorchis laricola            | -    | +         |                                     |
| Gorgodera cygnoides             | +    | -         |                                     |
| Cercariae                       |      |           |                                     |
| Echinostoma caproni             | +    | +         | Šebelova et al., 2004                |
| Cercaria eamasculans            | +    | -         | Pan et al., 1994                    |
| Neoastotrema triturii           | +    | +         | Tolstenkov et al., 2012a,b          |
| Cathaemasia hians               | +    | -         |                                     |
| Echinostoma revolutum           | +    | -         |                                     |
| Paramphistomum cervi            | +    | -         |                                     |
| Psilohasmus oxyurus             | -    | +         |                                     |
| Opisthorchis felineus           | +    | +         | Tolstenkov et al., 2010             |
| Molinieula anceps               | +    | -         |                                     |
| Bilharziella polonica           | +    | -         |                                     |
| Trichobilharzia szidati         | +    | -         |                                     |
| Plagiorchis elegans             | -    | +         |                                     |
| Himasthla elongata              | +    | +         |                                     |
| Cryptocotyle lingua             | +    | +         |                                     |
| Cryptocotyle lingua             | +    | +         |                                     |
| Cercaria parvicaudata           | +    | +         |                                     |
| Neoastotrema triturii           | +    | +         |                                     |
| Cathaemasia hians               | +    | -         |                                     |
| Echinostoma revolutum           | +    | -         |                                     |
| Paramphistomum cervi            | +    | -         |                                     |
| Psilohasmus oxyurus             | -    | +         |                                     |
| **Metacercariae**               |      |           |                                     |
| Diplostomum sp.                 | +    | +         | Barton et al., 1993                 |
| Cotylurus erraticus             | +    | +         |                                     |
| Opisthorchis vivenni            | -    | +         | Lecsanboon et al., 2012              |
| Bucephaloides gracilesctns      | +    | +         | Stewart et al., 2003a               |
| Apatemon cobitidis proterohini  | +    | +         | Stewart et al., 2003b               |
| Leucochloridionomorpha lutea     | +    | +         |                                     |
| Cotylurus sp.                   | +    | -         |                                     |

* + substance detected; - substance not detected
Staining of musculature with TRITC-conjugated phalloidin

In order to study the relationship between the patterns of the FMRFamide-IR and 5-HT-IR nervous elements and the musculature, staining with TRITC-conjugated phalloidin (Sigma, USA) (1:200) was performed according to the method described by Wahlberg (1998).

Confocal scanning laser microscopy

The specimens stained with anti-5HT, anti-FMRFamide and TRITC-labelled phalloidin were examined using a fluorescent microscope Leica DM 1000 (A.N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences, Center of Parasitology), a Leica TCS SP5 confocal scanning laser microscope (The Pushchino Scientific Center of Russian Academy of Sciences) and

Fig. 1(a – f). The serotoninergic and FMRFamidergic components of the nervous system in the attachment organs of adult trematodes.

(a, b) *Opisthorchis felineus*: (a) 5-HT-immunoreactive (IR) nerve cells and fibres located near and inside the ventral sucker (arrows), scale bar 50µm; (b) FMRFamide-IR fibres (thin arrows) extending from the main nerve cords towards the ventral sucker (thick arrow) are indicated, scale bar 50µm;

(c) *Allocreadium isoporum*. 5-HT-(IR) nerve cell and fibres located near and inside of the oral sucker (arrows), scale bar 100µm;

(d) *Plagiorchis laricola*. FMRFamide-IR nerve cells (short arrows) and fibres (long arrows) located close to the ventral sucker, scale bar 30µm.

Inset: the pattern of TRITC-phalloidin labeled F-actin in the ventral sucker;

(e) *Gorgodera cygnoides*. Max projection shows the pattern of 5-HT-IR nerve fibres (arrows) among the muscle fibres of the ventral sucker staining with TRITC-phalloidin, scale bar 50µm;

(f) *Paramphistomum cervi*. 5-HT-IR nerve fibres located among the muscles of the ventral sucker (arrows), scale bar 100µm.
Fig. 2(a – d). The serotoninergic and FMRFamidergic nerve elements in the attachment organs of adult trematodes.

(a, b) *Opisthioglypbe ranae.* 5-HT-IR nerve cells and fibres located near the oral (a) (large arrow) and ventral (b) suckers, scale bar 30μm.

Inset: the pattern of TRITC-phalloidin labeled F-actin in the suckers.

(c) *Gorgoda loossi.* 5-HT-IR nerve cell located near the ventral sucker.

Note the 5-HT-IR nerve fibres extending to the musculature of the ventral sucker (arrows), scale bar 50μm;

(d) *Aspidogaster conchicola.* 5-HT-IR nerve fibres observed in the adhesive disc situated on the ventral body surface (arrows), scale bar 20μm.

![Image](attachment-organ.png)

Fig. 1c) and in the ventral sucker of *Plagiorchis laricola* (Fig. 1d), *Gorgoda cygnoides* (Fig. 1e) and *Paramphistomum cervi* (Fig. 1f). 5-HT-IR nerve cells and fibres can be seen near the muscles of the oral (Fig. 2a) and ventral (Fig. 2b) suckers of *Opisthioglypbe ranae.* Thin, 5-HT-IR fibres innervate the ventral sucker of *Gorgoda loossi* (Fig. 2c). The adhesive disc located on the ventral surface of *Aspidogaster conchicola,* a representative of the ancient trematode group, is strongly innervated by 5-HT-IR nerve fibres (Fig. 2d).

**Cercariae**

The 5HT- and FMRFamidergic nerve structures have been found in attachment organs of trematode larvae – cercariae and metacercariae. Figs. 3 and 4 show the FMRFamidergic fibres running to the ventral and oral suckers of *Cercaria parvicaudata* (Fig. 3a, b) and *Plagiorchis elegans* (Fig. 3c, d), the ventral sucker of *Cryptocotyle lingua* (Fig. 3e) and the oral sucker of *Molinella anceps* (Fig. 4e) and *Himasthla elongata* (Fig. 4h).

Positive 5-HT-immunoreactivity has been revealed in the nerve fibres running to the ventral sucker of cercariae of *Cryptocotyle lingua* (Fig. 3f), the oral and ventral suckers of *Trichobilharzia szi-
Fig. 3(a – f). The serotoninergic and FMRFamidergic components of the nervous system in the attachment organs of cercariae.

(a, b) Cercaria parvicaudata. FMRFamide-IR nerve fibres extended to the ventral (a) and oral (b) suckers (arrows). The brain commissure is marked with a thick arrow (b). Scale bar 20 μm;

(c, d) Plagiorchis elegans. FMRFamide-IR nerve fibres located near the ventral (c) and oral (d) suckers (long arrows). The main nerve cords are marked with a thick arrow (с), scale bar 20 μm;

(e, f) Cryptocotyle lingua. FMRFamide-IR(e) and 5-HT-IR(f) fibres near the ventral sucker (long arrows). The main nerve cord is marked with a thick arrow (f), scale bar on (e) - 15 μm; on (f) - 20 μm.

dati (Fig. 4a, b), the ventral sucker of Bilharziella polonica (Fig. 4c), Sphaerostomum globiporum (Fig. 4d) and H. elongata (Fig. 4g), and the oral sucker of M. aniceps (Fig. 4f).

Metacercariae

The ventral sucker of Leucochloridiorhapha lutea metacercariae is strongly innervated with 5-HT-IR and FMRFamide-IR nervous fibres extending from the main longitudinal nerve cords (Fig. 5a, b). The innervations of the ventral sucker with 5-HTergic fibres have been observed in the metacercariae of Cotylurus sp. (Fig. 5c). The summary data relating to the identification of 5-HT- and FMRFamide-IR components of the nervous systems in the oral and ventral suckers of trematodes are presented in Table 2.

Discussion

Due to well-developed muscle elements of the oral and ventral suckers, trematodes are able to attach themselves securely to the host organs and tissues. The innervations of the attachment organs are not always mentioned in the description of a general morphology of the trematodes nervous system. Only a few studies pointed out the presence of nerve structures in trematodes' oral and ventral suckers. Serotoninergic and peptidergic nerve fibres have been found in the oral suckers of the adults of Bucephaloides gracilescens and in the oral and ventral suckers of Hapalometa cylindracea, Schistosoma mansoni, Fasciola hepatica and Opisthorchis felineus (Stewart et al., 2003a; McKay et al., 1990,
Fig. 4(a – h). The serotonergic and FMRFamidergic components in the attachment organs of cercariae of trematodes.

(a, b) *Trichobilharzia szidati*. 5-HT-IR fibres in the oral (a) and ventral (b) suckers (arrows); The main nerve cord is marked with a thick arrow (b), scale bar 10μm;

(c) *Bilharziella polonica*. 5-HT-IR fibres extending towards the ventral sucker (thin arrows), scale bar 10μm. Note the nerve cells near the ventral sucker (thick arrows).

Inset: the pattern of TRITC-phalloidin labeled F-actin in the ventral sucker;

(d) *Sphaerostomum globiporum*. 5-HT-IR cells near the ventral sucker (arrow), scale bar 10μm;

(e, f) *Moliniella aniceps*. FMRFamide-IR (e) and 5-HT-IR (f) fibres in the oral sucker (thin arrows), scale bar 20μm. The nerve cells are marked by thick arrows (f);

(g, h) *Himasthla elongata*. (g) 5-HT-IR fibres extended to the ventral sucker (arrows). Inset: the pattern of TRITC phalloidin labeled F-actin in the ventral sucker. (h) FMRFamide-immunoreactivity in the oral sucker (arrow), scale bar 20μm.
Based on own data and exo-genic groups, including the most ancient subclass, Aspidogastrea. The results not only revealed for the first time the innervations of the attachment organs in trematode species not studied before, but also confirmed and expanded the data already available on this issue for several species (M. anceps, B. polonica, T. szidati, C. parvicaudata, O. felineus). It can be concluded that the presence of studied neuromediators in the innervations of the attachment organs is characteristic for phylum Trematoda. We found that FMRF-immunoreactivity in the fixation organs of some trematodes studied herein was more intensive than 5-HT-immunoreactivity. This is true for Opisthochis felineus (adults), metacercariae of Leucochloridiodoroma lutea and cercariae of Cryptocotyle lingua. A question on possible differences in localization pattern of each neurotransmitter between the adult trematodes and their larvae is an interesting one. Based on own data and existing literature it is difficult to perform such comparative analysis as it requires simultaneous staining and investigation of the different life stages in one trematode species, which were not available at the same time. In some cases the immunoreactivity to serotonin or FMRFamide was not enough pronounced (or even absent) in attachment organs, what could be due to a limited quality of samples. However, the presence of nerve structures could not be ruled out.

In general, in flatworms serotonin acts as an excitatory neurotransmitter. The exogenous 5-HT can induce or enhance the motility of muscle strips or contractions of individual muscle fibres prepared from monogeneans, trematodes, and cestodes (reviewed by Halton & Maule 2004). FaRPs have also been shown to be myoexcitatory in a concentration-dependent manner when were applied exogenously to isolated muscle fibres or muscle-strips from free-living Bdelloura candida and Procerodes littoralis (Johnston et al., 1996; Moneypenny et al., 2001) and parasitic flatworms Schistosoma mansoni, Fasciola hepatica, Mesostoides corti (Day et al., 1994; Marks et al., 1997; Hrčkova et al., 2002). It was showed that FaRPs are acting through different types of receptors involving different second messenger pathways than was shown for serotonin (Zamanian et al. 2011; Patocka et al. 2014). The innervations of the copulatory organ and genital tracts by FMRF-IP nerve fibres have also suggested a role of FaRPs in the reproductive system of platyhelminths (Gustafsson et al. 2002). In summary, present study revealed the pattern of innervations of the attachment organs with serotoninergic and peptidergic nerve structures in adults and larvae of trematodes from various taxonomic groups, which have different life cycles, hosts and localization within them. The innervations of the oral and ventral suckers in trematodes imply an important role of the nervous system, namely its serotoninergic and peptidergic components in the regulation of the function of trematodes' adhesive organs. Based on our observations and similar studies on various trematode species, we can conclude that the innervations of trematode fixative organs with serotoninergic and peptidergic (FaRPs) neurotransmitters are widely represented (if not universal) characteristic of this class of parasitic flatworms.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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