Fish and Shellfish Immunology 87 (2019) 286–296

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

In vitro effects of the neuroactive substances serotonin and γ-aminobutyric acid on leucocytes from sticklebacks (*Gasterosteus aculeatus*)

Ivan A. Kutyrev[a,b,*], Frederik Franke[b], Joachim Kurtz[b], Jörn P. Scharsack[b]

[a] Institute of General and Experimental Biology, Siberian Branch of the Russian Academy of Sciences, Sakhyanovoi st. 6, 670047, Ulan-Ude, Russian Federation
[b] Institute for Evolution and Biodiversity, Animal Evolutionary Ecology, University of Münster, Hüfferstr. 1, 48149, Münster, Germany

ARTICLE INFO

Keywords:
Parasite immunomodulation
Serotonin
γ-aminobutyric acid
Cestodes
In vitro leucocyte responses
Gasterosteus aculeatus
Schistocephalus solidus

ABSTRACT

The majority of parasites have evolved strategies to evade the immune responses of their hosts. Neuroactive substances produced by cestodes are possible candidate molecules for regulating host immune responses. The neurons of helminths can synthesize a wide range of molecules that are identical to the ones functioning in their host organisms, and host lymphocytes have receptors for these neuroactive substances. We hypothesized that in teleost fish, antihelminthic immune responses are regulated via 5-hydroxytryptamine (5-HT, or serotonin) and γ-aminobutyric acid (GABA). In the present study, we investigated the *in vitro* influence of serotonin, GABA and *Schistocephalus solidus* (helminth) antigens on basic characteristics of the three-spined stickleback *Schistocephalus solidus* cellular immune response. Head kidney leucocytes (HKLs) were analysed by flow cytometry for cell viability and the frequency of leucocyte subsets (the granulocyte-to-lymphocyte ratio) and by a chemiluminescence assay for the production of reactive oxygen species (ROS). In short-term (2-h) HKL cultures, 5-HT did not change the total numbers of live HKLs, but the production of ROS decreased significantly with all 5-HT concentrations. In long-term (96-h) cultures, high 5-HT concentrations induced a decrease in leucocyte viability. This coincided with elevated ROS production in cultures with all 5-HT concentrations. In short-term (2-h) HKL cultures, GABA did not change the total numbers of live HKLs, but the production of ROS decreased significantly with high (100 nmol L^{-1}) GABA concentrations. In long-term (96-h) cultures, high and medium concentrations of GABA (100 nmol L^{-1} and 10 nmol L^{-1}) elevated the numbers of live HKLs compared to controls. The granulocyte-to-lymphocyte ratios generally increased upon exposure to GABA at all concentrations. All concentrations of GABA alone elevated the ROS production of HKLs compared to controls. In the present work, we showed that the neuroactive substances serotonin and GABA regulate the teleost immune system. Our study supports the hypothesis that these substances might be immunomodulators in tapeworm–fish parasite-host interactions.

1. Introduction

Coevolution between hosts and their parasites is predicted to be dynamic and rapid, because fitness costs caused by parasites are high, parasites are ubiquitous, and they often evolve rapidly. The majority of parasites have evolved strategies to evade the immune responses of their hosts [1]. The capacity of helminth parasites to modulate the immune system underpins their longevity in vertebrate hosts [2]. There is, consequently, intense interest in understanding the molecular basis of helminth immunomodulation [3,4]. Most of the published work on immune evasion mechanisms of helminths deals with mammalian hosts, whereas interactions of parasites with fish hosts have been, to date, insufficiently investigated [5]. It has been shown that cestodes can regulate antihelminthic immune responses of fish via excretory/secretory products [6,7]. However, the potential function of immunomodulating molecules produced by fish parasites is not yet well understood.

Possible candidates for substances that are produced by cestodes to regulate host immune responses are certain neuroactive substances. The neurons of helminths can synthesize a wide range of molecules that are identical to those functioning in the host organism, and thus, helminths can manipulate vital functions of the host—in particular, its immune

Abbreviations: 5-HT, 5-hydroxytryptamine; Ctrl CM, culture medium alone; Ctrl HCl, control concentration of HCl solvent; GABA, γ-aminobutyric acid; G/L, granulocyte-to-lymphocyte ratio; HKL, head kidney leucocytes; LPS, *Salmonella typhosa* lipopolysaccharides; PBS, phosphate-buffered saline; RLU, relative luminescence units; ROS, reactive oxygen species; SS, *Schistocephalus solidus* antigens

* Corresponding author. Institute of General and Experimental Biology, Siberian Branch of the Russian Academy of Sciences, Sakhyanovoi st. 6, 670047, Ulan-Ude, Russian Federation.
E-mail address: kutyrevi@yahoo.com (I.A. Kutyrev).

https://doi.org/10.1016/j.fsi.2019.01.022
Received 7 September 2018; Received in revised form 10 January 2019; Accepted 13 January 2019
Available online 18 January 2019
1050-4648/

responses and behaviour [8]. It has been suggested that the behavioural changes of sticklebacks towards higher predation susceptibility, due to infection by the cestode *Schistocephalus solidus*, is facilitated by crosstalk between the neuroendocrine and immune systems [9–11].

In the absence of a circulatory system, the neurons of cestodes secrete hormone-like substances, neuropeptides, and other bioactive molecules with a broad range of actions [12]. For example, in the model organism *D. dendriticum*, which parasitizes fish, immunocytochemical evidence suggests the presence of 13 neuropeptides, 2 growth factors, and 2 biogenic amines [13,14]. It was shown that cestodes in fish have ciliated and unciliated free nerve endings that contained light-coloured vesicles in the bulb. This fact supports the idea that the sensory nerve endings serve a secretory function [15,16].

It is known that a common pool of molecules is shared by the immune and neuroendocrine systems, both in invertebrates and vertebrates [17]. Interestingly, the immune cells themselves do express components of the neuronal neurotransmitter system. Lymphocytes, the central cells of the mammalian immune system, have many characteristics of neuroendocrine cells, including receptors for neuroendocrine peptides and hypothalamic-releasing-factor receptors. Accordingly, the involvement of the neurotransmitters in neuroimmunomodulation has been shown [18,19].

In humans, serotonin is a neurotransmitter and hormone that contributes to the regulation of various physiological functions. 5-HT is also a potent immunomodulator and affects various immune cells through its receptors and via the recently identified process of serotonylation [20]. Serotonin can influence the mobility and proliferation of lymphocytes, phagocytosis, cytolytic properties, and the synthesis of chemokines and cytokines. Changes in 5-HT levels have been reported in patients with inflammatory bowel disease, allergic airway inflammation, and rheumatoid arthritis [20]. The expression of 5-HT receptors has been identified in rodent and human innate immune cells, including neutrophils, eosinophils, monocytes, macrophages, dendritic cells (DCs), mast cells, and natural killer (NK) cells [21,22]. Serotonin performs numerous functions in helminths, from the regulation of carbohydrate metabolism to the regulation of motor activity, behaviour, and interactions between the parasite and the host [23,24].

The physiological role of γ-aminobutyric acid (GABA) is best studied in the brain, where GABA is the main inhibitory neurotransmitter. In recent years, it has become evident that cells of the human immune system may also produce GABA and express GABA-A ion channels, GABA transporters, and the GABA-B receptor. GABA has a number of effects on immune cells, such as the activation or suppression of cytokine secretion and the modification of cell proliferation and cell migration [25]. GABA appears to have a role in autoimmune diseases such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis and may modulate the immune response to infections [25]. It has been shown that GABA-ergic systems can reduce the phosphorylation of signalling kinases of macrophages and reduce the autoimmune inflammatory process in the nervous system [26]. GABA-ergic signalling plays an important role in the immunological pathogenesis of different parasite infections [27,28].

As in other vertebrates, the innate immune system of fish provides the first line of immune defence against parasites. Both innate and adaptive immune responses are mounted by fish to control parasite infections; however, innate immunity seems to play a more important role in antihelminthic host immune response [29].

The potential function of immunomodulating molecules produced by fish parasites is, to date, not well understood. Only information about the influence of a potential cestode-secreted immune regulating molecule—prostaglandin (PG) E_2—on fish immunity is available. It is known that the cestode *Diphyllobothrium dendriticum* produces PGE_2 and PGD_2 and secretes them in response to their fish host's (*Coregonus migratorius)* blood serum [16]. It was shown that the influence of PGE_2 on fish head kidney leucocytes (HKLs) is time and concentration dependent. In short-term (2-h) HKL cultures, PGE_2 did not change the total numbers of live HKLs. In long-term (96-h) cultures, high ($0.1\ \mu mol\ L^{-1}$) PGE_2 concentrations induced a sharp decrease in leucocyte viability, while low ($0.1\ pmol\ L^{-1}$) and intermediate ($0.1\ nmol\ L^{-1}$) concentrations of PGE_2 caused elevated leucocyte viability. This coincided with the reduced production of reactive oxygen species (ROS) in cultures with high PGE_2 and elevated ROS production in cultures with low PGE_2 [30]. Kutyrev et al. [16] showed that the body of *D. dendriticum* contains PGE_2 in concentrations close to the high PGE_2 concentrations used for *in vitro* experiments [30]. Thus, cestodes can produce PGE_2 in host tissues, which potentially induce a strong immunosuppressive effect on leucocyte viability and ROS production [16,30].

The tapeworm *S. solidus* and its host, the three-spined stickleback (*Gasterosteus aculeatus*), are a well-suited model for research on the influence of cestodes on fish immunity. Techniques for culturing the parasite *in vitro* and generating infective stages that can be used to experimentally infect sticklebacks have been developed, and the system is increasingly used as a laboratory model to investigate aspects of host-parasite interactions [31–34]. The teleost head kidney is a site of antigen presentation, leucocyte activation, proliferation, and maturation, and consequently, it interacts closely with immunological activity in the periphery. Accordingly, information derived from studies with HKLs can be regarded as representative of immune activity in the periphery, even if specific interactions at the site of infection might remain concealed [29,34].

In teleosts, neuroactive substances might be important molecular agents for modulating host immunity during parasitosis. As deduced from studies in mammals, serotonin and GABA might regulate inflammation at the site of contact between cestode and fish tissues [23,24,27,28]. However, information on the role of neuroactive substances in cestode-fish interactions is limited.

Here, we hypothesize that the fish antihelminthic immune response is regulated via serotonin and GABA. Hence, the first object of our *in vitro* study was to explore whether the neuroactive substances serotonin and GABA regulate the teleost immune system. Another question was whether the substances had stimulating or inhibiting effects on leucocyte activity. We also aimed to illustrate whether the influence of serotonin and GABA is time-dependent. The last objective was to estimate the combined action of these neuroactive substances and helminth antigens on leucocyte activity. In the present study, we investigated the *in vitro* influence of serotonin, GABA, and *S. solidus* antigens on basic characteristics of the three-spined stickleback cellular immune response. HKLs were analysed by flow cytometry for cell viability and the frequency of leucocyte subsets (the granulocyte-to-lymphocyte [G/L] ratio) and by a chemiluminescence assay for ROS.

2. Material and methods

2.1. Propagation of Gasterosteus aculeatus

The sticklebacks were laboratory-raised offspring of individuals originating from the brook Ibbenbürener Aa (52°17′31.76″N, 7°36′46.49″E) in the area of Münster, Germany. They were raised and kept in groups of 10–20 fish in 16-L tanks connected to a water filtration/recirculation system (VeWaTech, Germany) at 18 °C and a day:night cycle of 16:8 h. During the first weeks after hatching, the fish were fed daily *ad libitum* with *Artemia* sp. nauplii and later with frozen mosquito larvae and occasionally frozen cladocerans. The sticklebacks were maintained and treated in accordance with the EU Directive 2010/63/EU for animal experiments and the local animal welfare authorities under the project number 87–51.04.2010.A297.

2.2. Preparation of Schistocephalus solidus antigens

Five *S. solidus* plerocercoids, with an individual weight of 113–207 mg, were collected from three naturally infected sticklebacks

from the Ibbenbürener Aa. The plerocercoids were washed with phosphate-buffered saline (PBS). Subsequently, the worms were individually homogenised in PBS (5 μL mg^{-1} wet weight) using a ball mill (120 s, 29 Hz; MM 301; Retsch, Germany). In the second homogenisation step, the samples were pooled, PBS was added (15 μL mg^{-1} original wet weight), and the material was sonicated (60 s, duty cycle 10%, power 60%; Sonoplus HD 2070, Bandelin, Germany) on ice. Afterwards, solid particles were removed by centrifugation (600 × *g*, 4 °C, 10 min). Subsequently, the protein content was determined colorimetrically with a Bradford assay, and aliquots were stored at −80 °C.

2.3. Culture media

Media for cell separation and cultivation and phosphate-buffered saline (PBS) were diluted with 10% (v/v) distilled water to adjust their osmotic pressure according to stickleback serum osmolarity. For the isolation of HKLs, diluted Leibovitz 15 (L-15) medium with 2×10^4 IU L^{-1} sodium heparin was used (heparinised medium). Washing procedures were conducted with diluted L-15 without heparin (wash medium). For cultivation experiments, diluted L-15 was supplemented with 10^5 IU L^{-1} penicillin, 100 mg L^{-1} streptomycin, 4 mmol L^{-1} L-glutamine, 5% (v/v) foetal bovine serum (FBS; all chemicals: SIGMA, USA), and 2% (v/v) carp serum (culture medium). Carp serum was used as a source of piscine serum because of the limitations inherent in the preparation of sufficient amounts of stickleback serum. It was heat-inactivated for 30 min at 56 °C, 0.2-μm filtered, and stored at 20 °C until use. A stock solution of 40 μM serotonin creatinine sulfate monohydrate (5-HT; SIGMA, USA) was prepared in 0.1 M HCl and further diluted with culture media to obtain the desired concentrations. A solution of 40 μM γ-aminobutyric acid (GABA) (SIGMA, USA) was prepared in water and further diluted with culture media to obtain the desired concentrations. Stock solutions of 5-HT and GABA were aliquoted and stored at −80 °C until use.

2.4. Leucocyte isolation

Media and cells were kept on ice, and washing procedures were performed at 4 °C. Sticklebacks were anaesthetised by a blow to the head and killed by incision of the brain. Cell suspensions from head kidneys were prepared by forcing the tissues through a 40-μm nylon screen (BD-Falcon, USA) with heparinised medium. Isolated HKLs were centrifuged (600 × *g*, 5 min), washed once with wash medium, centrifuged again, and resuspended in culture medium. The numbers of viable cells (determined by the exclusion of propidium iodide-positive cells) were quantified by means of flow cytometry.

2.5. Leucocyte cultivation

From each stickleback, several leucocyte cultures were prepared, which enabled us to test the impact of different substances/concentrations on the same individual. For short-term (2-h) and long-term (4-day) cell-culture experiments, HKLs were incubated in half-area flat-bottomed 96-well microtitre plates (10^5 cells $well^{-1}$ in a final volume of 100 μl). All setups were made in duplicates. 5-HT was added in final concentrations of 10 pmol L^{-1}, 1 pmol L^{-1}, and 0.1 pmol L^{-1} [35,36]. GABA was added in final concentrations of 100 nmol L^{-1}, 10 nmol L^{-1}, and 1 nmol L^{-1} [37,38]. Mitogen stimulation of cultured leucocytes was induced with *Salmonella typhosa* lipopolysaccharides (LPS; SIGMA, USA) in a concentration of 20 mg L^{-1} [30], but only in the long-term cultures, since short-term cultures did not reveal significant LPS stimulation in previous experiments [7]. Parasite antigens were added to a final concentration of 10 mg L^{-1} (protein fraction) [30]. Control cultures were incubated with medium alone and with 4 nM HCl (corresponding to the amount of solvent present in 10 pmol L^{-1} 5-HT) as a solvent control. The cultures were incubated for 2 h and 4 d at 20 °C in a water-vapour-saturated atmosphere with 3% CO_2.

After incubation, culture plates were placed on ice (30 min) to detach adherent cells. HKLs were resuspended by repeated pipetting, and from each well, 65 μL of cell suspension was used for the respiratory burst assay (see 2.7), and 25 μL was used for the flow cytometric analysis (see 2.6).

2.6. Flow cytometric analysis

Suspensions of freshly isolated and cultured HKLs were analysed by flow cytometry with a FACS Canto II (BD, USA). Tubes were kept on ice until measurement. Samples were supplemented with propidium iodide (2 mg L^{-1}; SIGMA, USA) to detect dead cells. For the adjustment of cell numbers for *in vitro* assays and the vital cell counts after *in vitro* cultures, total cell counts were determined with the standard cell dilution assay [39] in a modified form: 15×10^3 green fluorescent standard beads (4.5 μm, Polyscience, USA) were added to each sample. Standard beads (green-fluorescence positive) could easily be discriminated from viable cells (propidium-iodide negative, green-fluorescence negative). Forward- and sideward-scattered light profiles (FSC/SSC characteristics) of at least 2×10^4 events were acquired in linear mode; fluorescence intensities at wavelengths of 530 nm and 585 nm were acquired at log scale. Cellular debris with low FSC/SSC characteristics and dead cells (propidium-iodide positive) were excluded from further evaluation. Granulocytes and lymphocytes were identified according to their characteristic FSC/SSC profiles [40]. All flow cytometry data were analysed with FACS Diva (v. 6.1.2, BD) software. Absolute numbers of cells in individual samples were calculated according to N (vital cells) = events (vital cells) × number (standard beads)/events (standard beads).

2.7. Production of reactive oxygen species by head kidney leucocytes

After *in vitro* stimulation, the respiratory burst activity of HKLs was quantified in a lucigenin-enhanced chemiluminescence assay modified after Scott & Klesius (1981), as described by Kurtz et al. (2004) [40]. From each culture well, 65 μL of cell suspension were transferred to individual wells of a white 96-well plate (Nunc Maxi Sorp surface; Thermo Fisher, USA), prefilled with 20 μL $well^{-1}$ of 2.5 g L^{-1} lucigenin solution (N,N′-dimethyl-9,9′-biacridiniumdinitrate; SIGMA, USA) in PBS and 95 μL $well^{-1}$ RPMI-1640 medium (SIGMA, USA) diluted with 10% (v/v) distilled water. Plates were incubated for 30 min at 20 °C in a water-vapour-saturated atmosphere with 3% CO_2 to enable lucigenin uptake by the cells. For each duplicate culture, one received 20 μL zymosan suspension from *Saccharomyces cerevisiae* (7.5 μg L^{-1}; SIGMA, USA) in PBS to initiate the production of ROS, and the other received diluted RPMI-1640 medium as a control. Relative luminescence units (RLU) per well were measured every 5 min for 3.5 h at 20 °C in a Tecan Infinite 200 multimode reader (Tecan, Switzerland). For data analyses, the area under the kinetic curve (RLU area; integral from t_0 to $t_{3.5\,h}$ of kinetic RLU curve) was determined with Magellan software v. 6.5 (Tecan, Switzerland).

2.8. Statistics

The normal distribution of the data were tested by Shapiro-Wilk tests and by visual inspection of histograms. With normally distributed data, repeated-measure ANOVAs were performed with fish as a repeat factor, treatment as an independent factor, and immune traits as dependent variables. If significant effects were detected with the ANOVAs, significant differences between groups were identified by *post-hoc* Tukey tests. For short-term cultures, *post-hoc* tests were performed on the overall ANOVAs. Due to high number of treatments, data from the long-term cultures (with a total of 12 treatments) were split for presentation as graphs according to *in vitro* treatments (Figs. 3 and 5). For long-term cultures, the *post-hoc* tests were performed after data splitting into the resulting groups, each with four members (control culture and

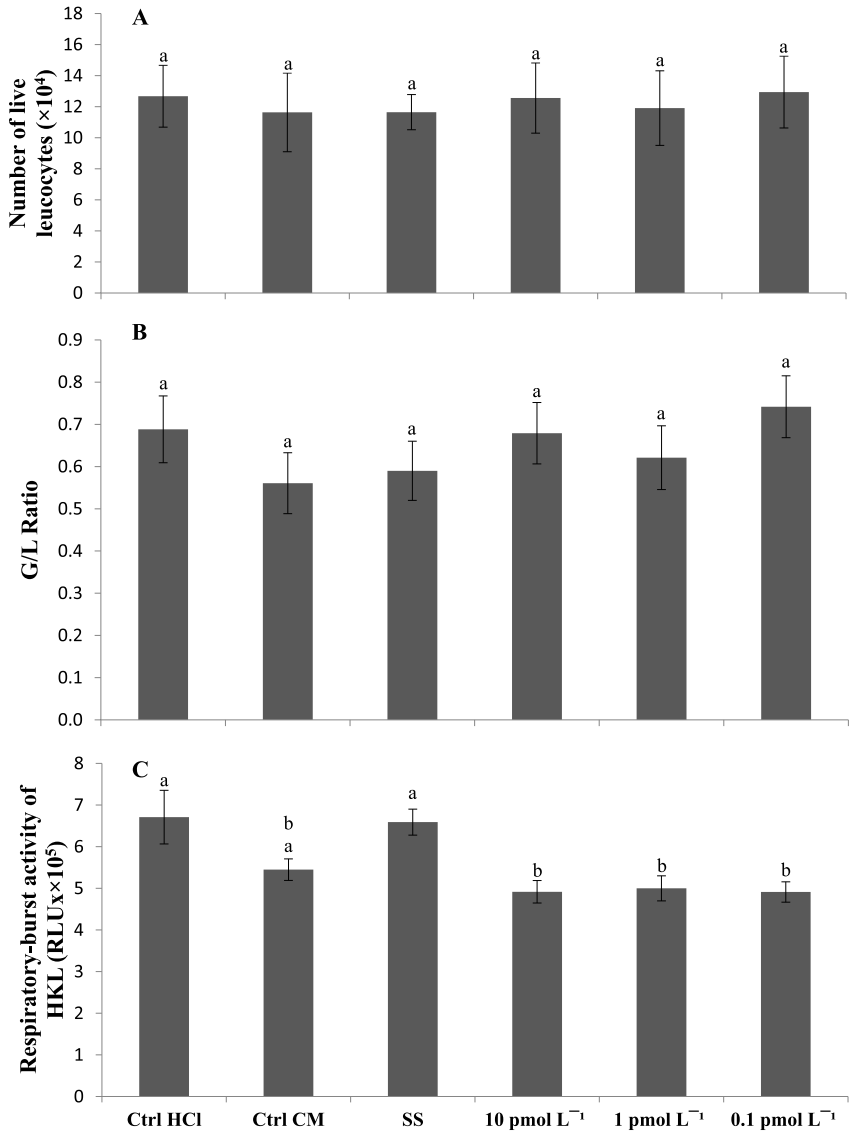

Fig. 1. Short-term *in vitro* leucocyte responses. Head kidney leucocytes (HKLs) from sticklebacks (n = 8) were incubated for 2 h without (Ctrl HCl = solvent control; Ctrl CM = culture medium control) and with serotonin at concentrations of 10 pmol L^{-1}, 1 pmol L^{-1}, and 0.1 pmol L^{-1} and with *S. solidus* antigens (10 μg mL^{-1}). (A) Total numbers of live HKLs per culture. (B) Granulocyte-to-lymphocyte (G/L) ratio. (C) Respiratory burst activity of HKLs. Mean values ± SE are given. Different letters indicate significant differences ($p < 0.05$).

cultures with three concentrations of substances), if the overall model ANOVAs were significant. Differences between treatments were considered significant at $p < 0.05$. All statistical tests were performed using Statistica software v. 6.0.

3. Results

3.1. Serotonin (5-HT)

3.1.1. Responses of head kidney leucocytes to 5-HT in short-term cultures

To analyse the influence of 5-HT, stickleback HKLs were incubated in medium alone (Ctrl CM), with a control concentration of HCl solvent (Ctrl HCl), with different concentrations of 5-HT, and with *S. solidus* antigens.

The absolute number of live head kidney leucocytes was not influenced by the solvent (HCl) and the tested concentrations of 5-HT after 2 h of cultivation (Fig. 1A). The granulocytes to lymphocytes ratio did not change during 2 h of 5-HT exposure (Fig. 1B).

Exposure of HKL to all concentrations of 5-HT significantly reduced the ROS production compared to solvent controls. However, respiratory burst activity of cultures with serotonin addition is not different to the medium control (Fig. 1C). Incubation of HKL with *S. solidus* antigens did not change ROS production.

3.1.2. Responses of head kidney leucocytes to 5-HT in long-term culture

For further characterization of the immune modulatory activity of 5-HT, HKL were incubated in medium with the control concentration of HCl (Ctrl HCl), different concentrations of 5-HT, *S. solidus* antigens (SS) and LPS. In previous experiments, differences in the viability and respiratory burst activity of HKL incubated with medium alone and the control concentration of HCl for 4 days were not detected (Fig. 2). Therefore, only solvent was used as the control in the present experiment.

The addition of high concentrations of 5-HT (10 pmol L^{-1}) reduced the numbers of live HKLs during 4 days of incubation compared to corresponding solvent controls (Fig. 3A). The addition of *S. solidus* antigens (SS) alone decreased the numbers of live HKL. In combination with *S. solidus* antigens (SS), all concentrations of 5-HT increased the number of live HKLs (Fig. 3B). The addition of LPS alone did not change the numbers of live HKLs. 5-HT in combination with LPS had no

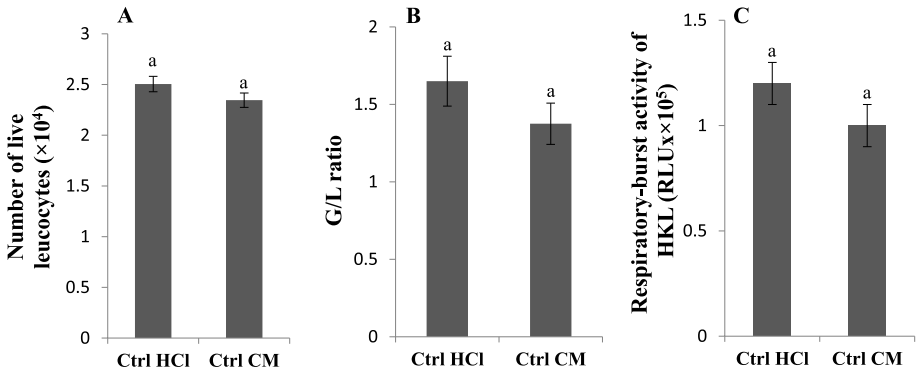

Fig. 2. Long-term *in vitro* leucocyte responses. (A) Total numbers of live HKLs per culture. (B) Granulocyte-to-lymphocyte (G/L) ratio. (C) Respiratory burst activity of cultured HKLs. Head kidney leucocytes (HKLs) from sticklebacks (n = 5) were cultured for 4 days (Ctrl HCl = solvent control; Ctrl CM = culture medium control. Mean values ± SE are given.

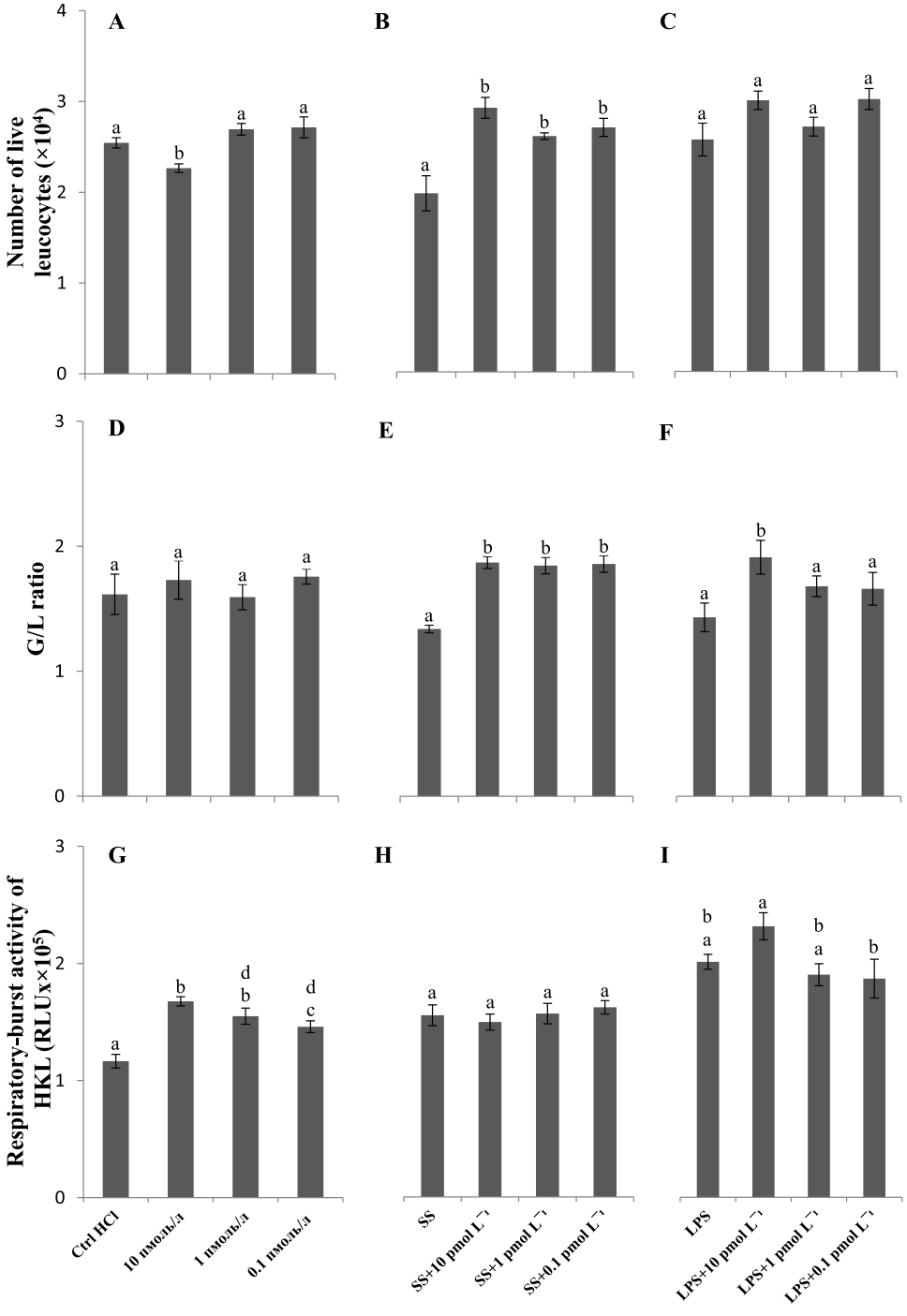

Fig. 3. Long-term *in vitro* leucocyte responses. (A–C) Total numbers of live head kidney leucocytes (HKLs) per culture. (D–F) Granulocyte-to-lymphocyte (G/L) ratio. (G–I) Respiratory burst activity of cultured HKLs. HKLs from sticklebacks (n = 6) were cultured for 4 days as a control (Ctrl HCl = solvent control) with serotonin at concentrations of 10 pmol L^{-1}, 1 pmol L^{-1}, and 0.1 pmol L^{-1}, *S. solidus* antigens (SS; 10 μg mL^{-1}), and lipopolysaccharides (LPS; 20 mg mL^{-1}). In addition, serotonin was combined with SS and LPS. Mean values ± SE are given. Different letters indicate significant differences within each of the four-membered (control culture and cultures with three concentrations of substances) model ANOVAs ($p < 0.05$).

significant effect on the number of live HKLs (Fig. 3C).

5-HT alone did not change the G/L ratio of HKLs (Fig. 3D). The addition of *S. solidus* antigens (SS) alone did not change the G/L ratio of HKLs, but the ratio increased upon exposure to 5-HT at all concentrations in combination with *S. solidus* antigens (Fig. 3E). This coincided with a prominent increase in HKL viability at all concentrations of 5-HT in combination with SS. LPS alone did not change the G/L ratio. Only the highest concentration of 5-HT in combination with LPS elevated the G/L ratio (Fig. 3F).

All concentrations of 5-HT alone elevated the ROS production of HKLs compared to the solvent control (Fig. 3G). Only for high concentrations of 5-HT did this coincide with decreased HKL viability (Fig. 3). *S. solidus* antigens and LPS alone each increased the ROS production. 5-HT in combination with *S. solidus* antigens and LPS did not change the ROS production (Fig. 3H and I).

3.2. γ-aminobutyric acid (GABA)

3.2.1. Responses of head kidney leucocytes to GABA in short-term cultures

To analyse the influence of GABA, stickleback HKLs were incubated in medium alone, with different concentrations of GABA and with *S. solidus* antigens. The absolute number of live HKLs was not influenced by the tested concentrations of GABA after 2 h of cultivation (Fig. 4A). The G/L ratio did not change during 2 h of GABA exposure (Fig. 4B).

The exposure of HKLs to high concentrations of GABA (100 nmol L^{-1}) significantly reduced the ROS production compared to control media (Fig. 4C). Low GABA concentrations (10 nmol L^{-1} and 1 nmol L^{-1}) had no significant effect on the ROS production of HKLs compared to controls.

3.2.2. Responses of head kidney leucocytes to GABA in long-term culture

For further characterization of the immune modulatory activity of GABA, HKLs from sticklebacks were incubated in media with different concentrations of GABA, S. solidus antigens, and LPS. High and medium concentrations of GABA (100 nmol L^{-1} and 10 nmol L^{-1}) increased the survival of live HKLs during 4 days of incubation compared to the corresponding controls (Fig. 5A). *S. solidus* antigens alone decreased the survival of live HKLs. In combination with *S. solidus* antigens, high concentrations of GABA (100 nmol L^{-1}), the number of live HKLs was elevated (Fig. 5B). LPS alone did not change the survival of live HKLs. GABA in combination with LPS also had no significant effect on the number of live HKLs (compared with the influence of LPS alone; Fig. 5C).

The G/L ratio of HKLs was generally increased upon exposure to GABA alone at all concentrations (Fig. 5D). This coincided with elevated HKL viability induced by high and medium concentrations of GABA (100 nmol L^{-1} and 10 nmol L^{-1}). The addition of *S. solidus* antigens alone did not change the G/L ratio. High and medium concentrations of GABA (100 nmol L^{-1} and 10 nmol L^{-1}) in combination with *S. solidus* antigens elevated the G/L ratio (Fig. 5E). This coincided with increased HKL viability induced only by high concentrations of GABA (100 nmol L^{-1}). The addition of LPS alone did not change the G/L ratio. GABA in combination with LPS had no significant effect on the G/L ratio (compared with the influence of LPS alone; Fig. 5F).

All concentrations of GABA alone elevated the ROS production of HKLs compared to controls (Fig. 5G). This coincided with elevated HKL viability induced by high and medium concentrations of GABA (100 nmol L^{-1} and 10 nmol L^{-1}). *S. solidus* antigens and LPS alone each increased the ROS production. At the same time, GABA in combination with *S. solidus* antigens (SS) and LPS had no significant effect on G/L ratio (compared with the influence of SS and LPS alone respectively) (Fig. 5H and I).

4. Discussion

In recent years, there has been increasing interest in understanding the molecular basis of helminth immunomodulation [41]. It has been shown that cestodes can regulate an antihelminthic immune response of fish via excretory/secretory products [7,16]. The neurons of helminths can synthesize a wide range of molecules that are identical to the ones functioning in the host organism and thus, helminths can manipulate vital functions of the host—in particular, its immune responses and behaviour [12,34].

With the present research, we showed that the neuroactive substances serotonin and GABA regulate the teleost immune system. We examined whether the substances had stimulating or inhibiting effects on leukocyte activity. Another question was the influence of time and concentration on the action of the neuroactive substances. Our study showed that serotonin and GABA may have different influences on leucocyte function in sticklebacks, which depend on exposure duration. In short-term cell cultures, both neuroactive substances had (immune-) suppressive action on the leucocyte production of ROS. After only 2 h of incubation, all concentrations of 5-HT and high concentrations of GABA supressed the ROS production of HKLs, which, after this relatively short time, did not coincide with losses in cell viability (Figs. 1 and 4).

In contrast, in long-term cultures, serotonin and GABA activated ROS production. Leucocyte viability did not change in short-term cultures under the influence of serotonin and GABA. In long-term cultures, a high concentration of serotonin supressed the numbers of live leucocytes. However, a supplementation of cultures with *S. solidus* antigens and serotonin increased the survival of leucocytes (Fig. 3B), particularly granulocytes (Fig. 3E), but did not modulate ROS activity (Fig. 3H). GABA increased the survival of leucocytes, particularly granulocytes, both alone (Fig. 5 A, D) and in combination with *S. solidus* antigens (Fig. 5 B, E). However, a supplementation of cultures with *S. solidus* antigens and GABA did not change the ROS production (Fig. 5H).

In sum, serotonin had more immunosuppressive potential compared with GABA. Our study also showed that in long-term cultures, serotonin and GABA in combination with *S. solidus* antigens increased leucocyte viability and G/L ratios without any influence on ROS production. This suggests that in long-term cultures, serotonin and GABA compensated for the negative influences of SS. However, serotonin and GABA may lose their physiological activity in the course of cultivation. In natural systems, the constant secretion of neuroactive substances at the contact site of parasite and host tissues may play a leading immunomodulatory role.

We can propose several reasons for changes in serotonin and GABA immunosuppressive potential depending on exposure duration. For example, it may be the consequence of change in the expression of leucocyte receptors for serotonin and GABA. The diversity of the immunomodulating effects of serotonin is determined by the heterogeneity of serotoninergic receptors [42]. It is known that about 13 different serotonin receptors are expressed in rats. Of those, six receptors are expressed in leucocytes of the spleen, thymus and peripheral blood [43]. At least two types of GABA receptors are known in mice: $GABA_A$ and $GABA_B$. T-lymphocytes of mice express $GABA_A$ receptors [44]. Information about serotonin and GABA receptors on competent immune cells in fish is absent. In the present study, the physiological activity of serotonin and GABA probably decreased over time, and a compensatory response of leucocytes was observed. It is known that GABA in solution has five different conformations, and different GABA conformations bind with different receptors [45].

In mammals, neuroactive substances are actively involved in the regulation of immunological functions. Recent findings have implicated the GABAergic system in immune cell functions, inflammatory conditions, and diseases in peripheral tissues. Interestingly, the specific effects may vary among immune cell types with the stage of activation and may be altered by infectious agents [25,46,47]. On the one hand, GABAergic systems participate in stimulating influences on immune reactivity [18,48,49]. On the other hand, GABA reduces the phosphorylation of signalling kinases of macrophages, reduces the autoimmune inflammatory process in the nervous system [26,50], and

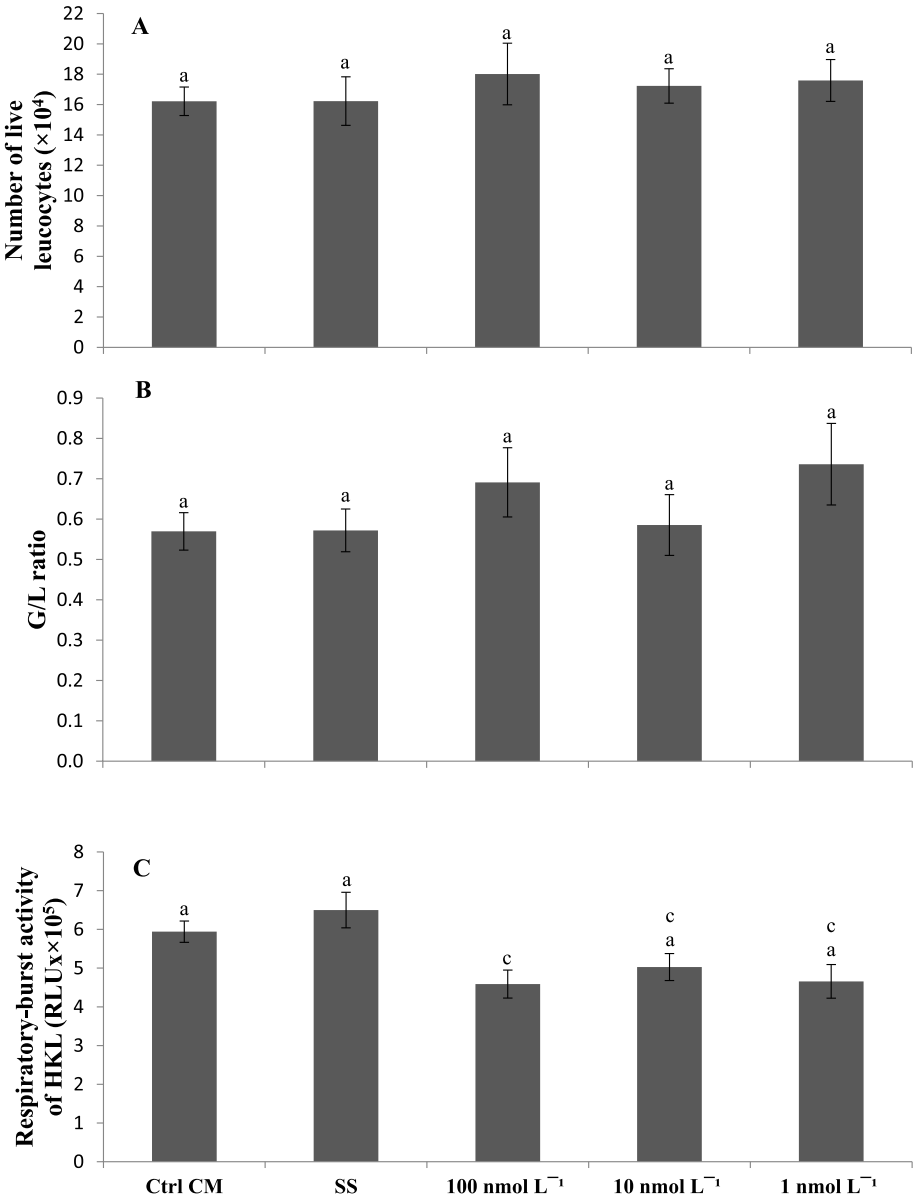

Fig. 4. Short-term *in vitro* leucocyte responses. Head kidney leucocytes (HKLs) from sticklebacks (n = 8) were incubated for 2 h without (Ctrl CM = culture medium control) and with GABA at concentrations of 100 nmol L^{-1}, 10 nmol L^{-1}, and 1 nmol L^{-1} and with *S. solidus* antigens (SS, 10 μg mL^{-1}). (A) Total numbers of live HKLs per culture. (B) Granulocyte-to-lymphocyte (G/L) ratio. (C) Respiratory burst activity of HKLs. Mean values ± SE are given. Different letters indicate significant differences ($p < 0.05$).

inhibits antigen-specific proliferation of T-lymphocytes [44]. In some cases, serotoninergic systems are also involved in inhibitory mechanisms of neuroimmunomodulation [18], promote anti-inflammatory action [51,52], and suppress the innate immune response [53]. In other cases, serotonin is needed for the optimal functioning of macrophages and monocytes, as the initiation of delayed-type hypersensitivity and stimulation of the production of chemotactic factors by serotonin has been described [54–59]. However, the influence of serotonin on the expression of histocompatibility complexes MHCI and MHCII by macrophages [60] and B-lymphocyte functioning [61–63] is ambiguous.

In fish, the immune system is also regulated by neuroendocrine mediators and neurotransmitters [64–70]. However, studies on this topic mainly addressed catecholamines, corticosteroid hormones, adrenocorticotropic hormone, adrenaline, and acetylcholine, but investigations of the influence of serotonin and GABA on fish immunity have not been carried out.

Information about parasites using neuroactive substances as immunoregulators is sparse. Recently, it was shown that the obligate intracellular parasite *Toxoplasma gondii* exploits cells of the immune system to disseminate. Dendritic cells are considered the gatekeepers of the immune system but can, paradoxically, also function as “Trojan horses” to mediate the dissemination of *T. gondii*. Previous work has shown that *Toxoplasma* hijacks the migratory machinery of dendritic cells by inducing secretion of the neurotransmitter GABA and by activating GABAergic signalling pathways, thereby making infected dendritic cells hypermigratory *in vitro* and *in vivo* [28]. Results from Huby et al. [71] suggest a modulatory role for acetylcholinesterase secreted by the nematode parasites *Trichostrongylus colubriformis* and *Nematodirus battus* on the proliferation of the epithelial cells of their rodent host. A mitogenic effect of acetylcholinesterase was shown at low concentrations. In contrast, an inhibitory effect was noted at high concentrations.

In the present study, we did not analyse parasite extracts for the presence of serotonin and GABA. Accordingly, we cannot directly prove

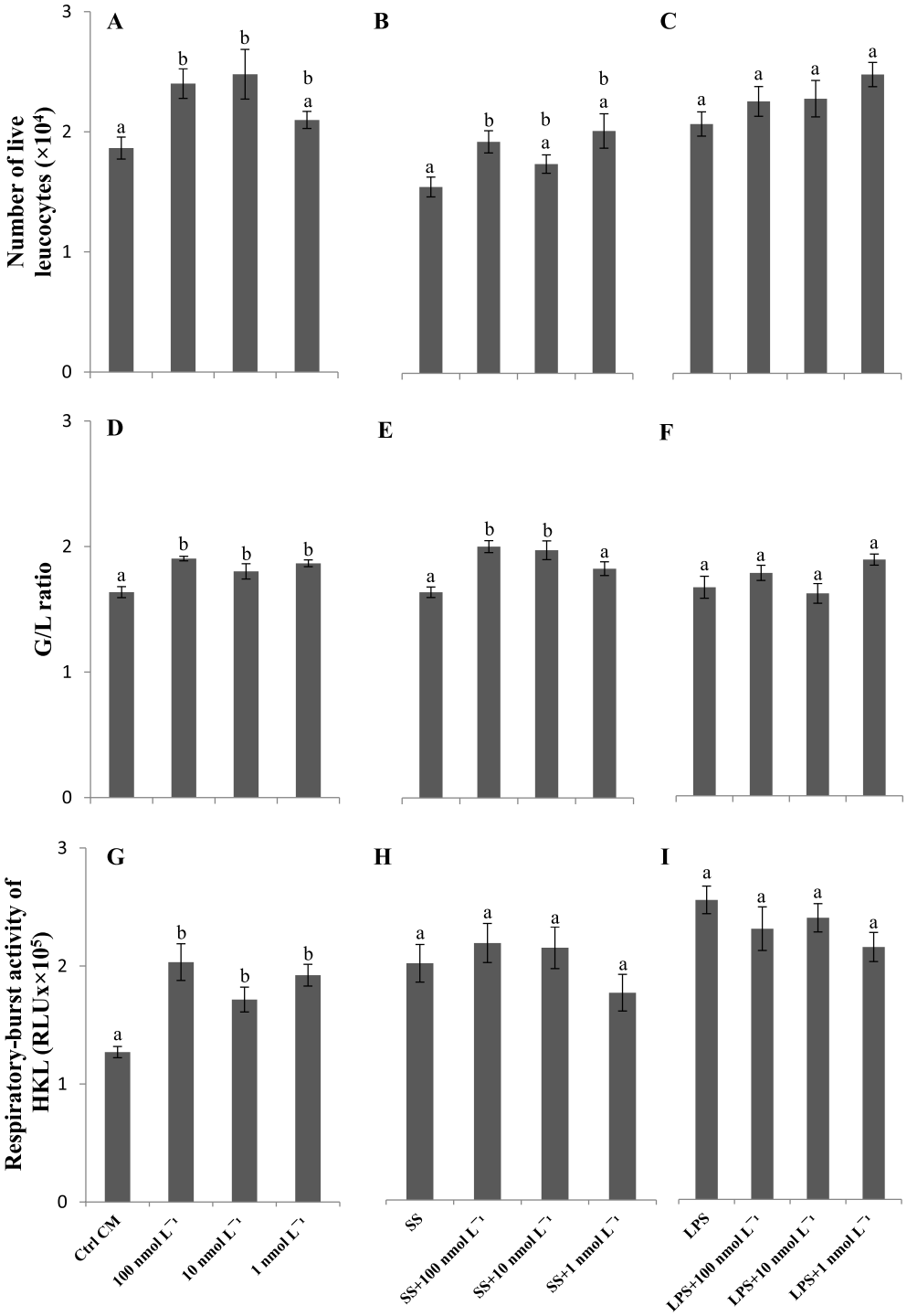

Fig. 5. Long-term *in vitro* leucocyte responses. (A–C) Total numbers of live head kidney leucocytes (HKLs) per culture. (D–F) Granulocyte-to-lymphocyte (G/L) ratio. (G–I) Respiratory burst activity of cultured HKLs. HKLs from sticklebacks (n = 6) were cultured for 4 days as culture medium controls (Ctrl CM), with GABA at concentrations of 100 nmol L^{-1}, 10 nmol L^{-1}, and 1 nmol L^{-1}, *S. solidus* antigens (SS; 10 μg mL^{-1}) and lipopolysaccharides (LPS; 20 mg mL^{-1}). In addition, GABA was combined with SS and LPS. Mean values ± SE are given. Different letters indicate significant differences within each of the four-membered (control culture and cultures with three concentrations of substances) model ANOVAs ($p < 0.05$).

that *S. solidus* is producing and using these substances in parasite–host relations. However, we showed that stickleback leucocytes are responsive to these substances, and therefore, *S. solidus* might use those, or they at least are likely playing a role in the infection. However, it is a well-known fact that the nervous system of cestodes produces serotonin and GABA [12,14,15]. Serotonin has been shown to be the predominant bioamine present in cestodes [72,73]. In particular, Wikgren et al. [74] revealed the presence of serotonin in the nervous system of *S. solidus*. Serotonin and GABA were described not only in the central part of the cestode's nervous system, but also in the periphery, in the subtegumental region [14,74,75]. Since cestodes have sensory endings that contain vesicles, which excrete into small cavities in their tegument [15,16], we can propose that cestodes might secrete neuroactive substances (including serotonin and GABA) into the site of contact between parasite and host tissues.

It was shown recently that parasites including cestodes produce extracellular vesicles that contain substances with immunomodulatory capacity [76,77]. There is some information about the role of serotonin and other monoamine neurotransmitters in the infection of *G. aculeatus* with *S. solidus*. The concentrations of monoamine neurotransmitters were measured in the brain of parasitized and nonparasitized sticklebacks. The ratios of 5-hydroxyindoleacetic acid: serotonin were significantly elevated in both the hypothalami and brainstems of infected sticklebacks. The concentrations of serotonin and norepinephrine were

significantly reduced in the telencephalons of infected fish as compared with controls [78]. Hence, *S. solidus*, during the infection of *G. aculeatus*, might use the neuroactive substances serotonin and GABA in immunological parasite-host interactions.

The levels of serotonin and GABA in helminths and their hosts have been studied previously, and serotonin is the most examined in this respect. Serotonin has been described in cestodes, trematodes, nematodes, monogeneans, and acanthocephalans [79]. In cestodes, serotonin is the dominant biogenic amine. Only some cestodes species have dopamine. For example, dopamine is the dominant biogenic amine in trematodes. In cestodes, the concentration of serotonin ranges from 0.05 to 6.0 μg/g wet weight [79–84]. There are no data on serotonin concentrations in *S. solidus*. However, adult *S. pungitii* contain 0.5–1.7 μg/g, and larvae contain 0.19–0.9 μg/g [79]. There are some data on the correlation of serotonin levels in helminths and their hosts. For example, *Hymenolepis diminuta* from the rat intestine contains 2.9–6.0 μg/g of serotonin, while the serotonin concentration in the rat intestine is 4–32 μg/g and depends on feeding [82]. Under the influence of reserpine, the serotonin concentration decreased from 0.492 to 0.131 μg/g in *H. diminuta* and from 1.820 to 0.889 μg/g in the rat intestine [73]. Cestode–fish parasite-host systems are less studied in this respect. It is also known that the serotonin concentration in *Triaenophorus nodulosus* parasitizing the intestine of pike was 0.297–1.411 μg/g and in the pike intestine was 0.560–2.854 μg/g [84]. The influence of *S. solidus* on the serotonin concentration in the brain of *G. aculeatus* was noted above.

Information about GABA concentrations in helminth-host systems is scarce. For the nematode *Nippostrongylus brasiliensis*, the GABA concentration was 0.095 pg per worm [85], and for the trematode *Fasciola hepatica*, it was1.7 pg/mg [86]. For free-living platyhelminths—the planarians *Polycelis nigra*, *Dugesia gonocephala*, and *Schmidtea mediterranea*—GABA concentrations were 10.3–24.7, 4.8, and 7.3 pg/mg, respectively [86–88]. The cestode *Moniezia expansa* contained 12.9 pg/mg of GABA [86]. Information about the influence of parasite invasion on the GABA level in hosts is available only for *Echinococcus granulosus*. It was shown that *E. granulosus* contains 1.5–3.0 pg/mL of GABA. Interestingly, in the plasma of horses and mice infected with *E. granulosus*, GABA levels were 0.5 and 1.6 pg/mL, respectively, whereas in non-infected hosts GABA was not detected [89].

In our *in vitro* experiments we used serotonin in the concentrations 1.76, 0.176, and 0.0176 pg/mL. GABA was used in concentrations of 10, 1, and 0.1 pg/mL. In our *in vitro* experiments, we observed physiological but not toxic actions of serotonin and GABA on fish leucocytes. It is quite likely that *in vivo* cestodes, including *S. solidus,* secrete serotonin and GABA in similar concentrations at the place of contact between parasite and host tissues to regulate host immunity.

In conclusion, we have shown that the neuroactive substances serotonin and GABA regulate the teleost immune system. In cestode–fish systems, serotonin and GABA may be used by parasites to supress host immune responses. Theoretically, *in vivo* cestodes can constantly secrete serotonin and GABA in the host tissues, which will have mainly immunosuppressive effects. Moreover, cestodes might change the levels of neuroactive substances in host organisms using alternative mechanisms. Further research is required to clarify whether cestodes indeed secrete neuroactive substances, including serotonin and GABA, at the place of contact with host tissues. Also, the detection of serotonin and GABA levels in the hosts of cestodes and its correlation with host immune status will be of importance.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

We thank Janine Büscher, Hannah Janssen, and Alexander M. Schmidt for their assistance during the experiments, Barbara Hasert and Joe Lange for technical support, and Nadezhda Terenina for providing full-text articles. We are grateful to two anonymous reviewers for their helpful suggestions. The project was funded by grants of the German Research Foundation (DFG) to Jörn P. Scharsack (SCHA-1257/2-1) within the Priority Programme SPP 1399 on Host-Parasite Coevolution, the German Academic Exchange Service to Ivan A. Kutyrev (A/11/04242), and the Russian Foundation for Basic Researches to Ivan A. Kutyrev (grants № 16-04-01213, 18-34-20015). This study was performed under the theme of Russian government task (government registration № AAAA-A17-117011810039-4).

References

[1] P. Schmidt-Hempel, Evolutionary Parasitology: the Integrated Study of Infections, Immunology, Ecology, and Genetics, Oxford University Press, Oxford; New York, 2011.
[2] R.M. Maizels, M. Yazdanbakhsh, Regulation of the immune response by helminth parasites: cellular and molecular mechanisms, Nat. Rev. Immunol. 3 (2003) 733–743.
[3] S. Imai, K. Fujita, Molecules of parasites as immunomodulatory drugs, Curr. Top. Med. Chem. 4 (2004) 539–552.
[4] M.J.G. Johnston, J.A. Macdonald, D.M. McKay, Parasitic helminths: a pharmacopoeia of anti-inflammatory molecules, Parasitology 136 (2009) 125–147.
[5] A. Sitja`-Bobadilla, Living off a fish: a trade-off between parasites and the immune system, Fish Shellfish Immunol. 25 (2008) 358–372.
[6] M.J. Taylor, D. Hoole, *Ligula intestinalis* (L.) (Cestoda: pseudophyllidea): polarization of cyprinid leucocytes as an indicator of host- and parasite-derived chemoattractants, Parasitology 107 (1993) 433–440.
[7] J.P. Scharsack, A. Gossens, F. Franke, J. Kurtz, Excretory products of the cestode, *Schistocephalus solidus*, modulate in vitro responses of leukocytes from its specific host, the three-spined stickleback (*Gasterosteus aculeatus*), Fish Shellfish Immunol. 35 (2013) 1779–1787.
[8] I. Barber, J.P. Scharsack, The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish, Parasitology 137 (2010) 411–424.
[9] Ø. Øverli, M. Pall, B. Borg, M. Jobling, S. Winberg, Effects of Schistocephalus solidus infection on brain monoaminergic activity in female three-spined sticklebacks Gasterosteus aculeatus, Proc. Roy. Soc. Lond. B. 268 (2001) 1411–1415.
[10] J.P. Scharsack, K. Koch, K. Hammerschmidt, Who is in control of the stickleback immune system: interactions between *Schistocephalus solidus* and its specific vertebrate host, Proc. Roy. Soc. Lond. B 274 (2007) 3151–3158.
[11] B.S. Dezfuli, J.A. DePasquale, G. Castaldelli, L. Giari, G. Bosi, A fish model for the study of the relationship between neuroendocrine and immune cells in the intestinal epithelium: *Silurus glanis* infected with a tapeworm, Fish Shellfish Immunol. 64 (2017) 243–250.
[12] D.W. Halton, M.K.S. Gustafsson, Functional morphology of the platyhelminth nervous system, Parasitology 113 (1996) S47–S72 Supplement S1.
[13] M.K.S. Gustafsson, D.W. Halton, A.G. Maule, M. Reuter, C. Shaw, The gull-tapeworm, *Diphyllobothrium dendriticum* and neuropeptide F: an immunocytochemical study, Parasitology 109 (1994) 599–609.
[14] N.M. Biserova, I.A. Kutyrev, Localization of prostaglandin E_2, γ-aminobutyric acid, and other potential immunomodulators in the plerocercoid *Diphyllobothrium dendriticum* (Cestoda), Biol. Bull. 41 (2014) 242–250.
[15] M.K.S. Gustafsson, The cells of a cestode: *Diphyllobothrium dendriticum* as a model in cell biology, in: M.K.S. Gustafsson, M. Reuter (Eds.), The Early Brain. Proceedings of a Symposium on Invertebrate Neurobiology. Acta Acad Series B. 50, Abo Academy Press, Vaasa, Finland, 1990, pp. 13–44.
[16] I.A. Kutyrev, N.M. Biserova, D.N. Olennikov, Z.V. Korneva, O.E. Mazur, Prostaglandins E_2 and D_2 – regulators of host immunity in the model parasite *Diphyllobothrium dendriticum*: an immunocytochemical and biochemical study, Mol. Biochem. Parasitol. 212 (2017) 33–45.
[17] E. Ottaviani, D. Malagoli, C. Franceschi, Common evolutionary origin of the immune and neuroendocrine systems: from morphological and functional evidence to in silico approaches, Trends Immunol. 28 (2007) 497–502.
[18] L. Devoino, G. Idova, E. Alperina, M. Cheido, Brain neuromediator systems in the immune response control: pharmacological analysis of pre- and postsynaptic mechanisms, Brain Res. 633 (1994) 267–274.
[19] T. Kawli, F. He, M.W. Tan, It takes nerves to fight infections: insights on neuroimmune interactions from *C. elegans*, Dis. Mod. Mech. 3 (2010) 721–731.
[20] M.S. Shajib, W.I. Khan, The role of serotonin and its receptors in activation of immune responses and inflammation, Acta Physiol. 213 (2015) 561–574.
[21] G.P. Ahern, 5-HT and the immune system, Curr. Opin. Pharmacol. 11 (2011) 29–33.
[22] N.L. Baganz, R.D. Blakely, A dialogue between the immune system and brain, spoken in the language of serotonin, ACS Chem. Neurosci. 4 (2012) 48–63.
[23] T.E. Mansour, Serotonin receptors in parasitic worms, Adv. Parasitol. 23 (1984) 1–36.
[24] D.W. Halton, A.G. Maule, Flatworm nerve-muscle: structural and functional analysis, Can. J. Zool. 2 (2004) 316–333.
[25] Z. Jin, S.K. Mendu, B. Birnir, GABA is an effective immunomodulatory molecule, Amino Acids 45 (2013) 87–94.

[26] R. Bhat, R. Axtell, A. Mitra, Inhibitory role for GABA in autoimmune inflammation, Proc. Natl. Acad. Sci. U.S.A. 6 (2010) 2580–2585.
[27] S.Z. Hasnain, D.J. Thornton, R.K. Grencis, Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection, Parasite Immunol. 33 (2011) 45–55.
[28] S. Kanatani, J.M. Fuks, E.B. Olafsson, L. Westermark, B. Chambers, M. Varas-Godoy, et al., Voltage-dependent calcium channel signaling mediates $GABA_A$ receptor-induced migratory activation of dendritic cells infected by *Toxoplasma gondii*, PLoS Pathog. 13 (2017) e1006739.
[29] P. Alvarez-Pellitero, Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects, Vet. Immunol. Immunopathol. 126 (2008) 171–198.
[30] I.A. Kutyrev, F. Franke, J. Büscher, J. Kurtz, J.P. Scharsack, *In vitro* effects of prostaglandin E_2 on leucocytes from sticklebacks (*Gasterosteus aculeatus*) infected and not infected with the cestode *Schistocephalus solidus*, Fish Shellfish Immunol. 41 (2014) 473–481.
[31] J.D. Smyth, Studies on tapeworm physiology. 1. The cultivation of *Schistocephalus solidus* in vitro, J. Exp. Biol. 23 (1946) 47–70.
[32] C. Arme, R.W. Owen, Infections of the three-spined stickleback, *Gasterosteus aculeatus* L., with the plerocercoid larvae of *Schistocephalus solidus* (Müller, 1776), with special reference to pathological effects, Parasitology 57 (1967) 301–314.
[33] K. Hammerschmidt, J. Kurtz, Ecological immunology of a tapeworms' interaction with its two consecutive hosts, Adv. Parasitol. 68 (2009) 111–137.
[34] I. Barber, J.P. Scharsack, The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish, Parasitology 137 (2010) 411–424.
[35] L. Šmejkal-Jagar, M. Boranić, Serotonin, serotoninergic agents and their antagonists suppress humoral immune reaction *in vitro*, Res. Exp. Med. 194 (1994) 297–304.
[36] G. Ménard, V. Turmel, E.Y. Bissonnette, Serotonin modulates the cytokine network in the lung: involvement of prostaglandin E_2, Clin. Exp. Immunol. 150 (2007) 340–348.
[37] C. Suñol, D.A. García, GABAA receptor binding and ion channel function in primary neuronal cultures for neuropharmacology/neurotoxicity testing, Neuromethods 56 (2011) 481–493.
[38] A.K. Bhandage, Z. Jin, S.V. Korol, Q. Shen, Y. Pei, Q. Deng, GABA regulates release of inflammatory cytokines from peripheral blood mononuclear cells and CD4+ T cells and is immunosuppressive in Type 1 diabetes, EBioMedicine 30 (2018) 283–294.
[39] K. Pechhold, T. Pohl, D. Kabelitz, Rapid quantification of lymphocyte subsets in heterogeneous cell populations by flow cytometry, Cytometry 16 (1994) 152–159.
[40] J.P. Scharsack, M. Kalbe, R. Derner, J. Kurtz, M. Milinski, Modulation of granulocyte responses in three-spined sticklebacks (*Gasterosteus aculeatus* L.) infected with the tapeworm (*Schistocephalus solidus*, Muller 1776), Dis. Aquat. Org. 59 (2004) 141–150.
[41] M.J.G. Johnston, J.A. Macdonald, D.M. McKay, Parasitic helminths: a pharmacopoeia of anti-inflammatory molecules, Parasitology 136 (2009) 125–147.
[42] R.I. Sepiashvili, I.P. Balmasova, L.N. Staurina, Serotonin and its immune and physiological effects, Ross. Fiziol. Zh. Im. I.M. Sechenova 99 (2013) 17–32.
[43] J. Stefulj, B. Jernej, L. Cicin-Sain, I. Rinner, K. Schauenstein, mRNA expression of serotonin receptors in cells of the immune tissues of the rat, Brain Behav. Immun. 14 (2000) 219–224.
[44] J. Tian, C. Chau, T.G. Hales, D.L. Kaufman, $GABA_A$ receptors mediate inhibition of T cell responses, J. Neuroimmunol. 96 (1999) 21–28.
[45] F. Cavagnini, G. Benetti, C. Invitti, G. Ramella, M. Pinto, M. Lazza, et al., Effect of gamma-aminobutyric acid on growth hormone and prolactin secretion in man: influence of pimozide and domperidone, J. Clin. Endocrinol. Metab. 51 (1980) 789–792.
[46] M. Auteri, M.G. Zizzo, R. Serio, GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation, Pharmacol. Res. 93 (2015) 11–21.
[47] A. Barragan, J.M. Weidner, Z. Jin, E.R. Korpi, B. Birnir, GABAergic signalling in the immune system, Acta Physiol. 213 (2015) 819–827.
[48] S.H. Oh, C.H. Oh, Brown rice extracts with enhanced levels of GABA stimulate immune cells, Food Sci. Biotech. 12 (2003) 248–252.
[49] A.M. Abdou, S. Higashiguchi, K. Horie, M. Kim, H. Hatta, H. Yokogoshi, Relaxation and immunity enhancement effects of γ-Aminobutyric acid (GABA) administration in humans, Biofactors 26 (2006) 201–208.
[50] T. Crowley, J.F. Cryan, E.J. Downer, O.F. O'Leary, Inhibiting neuroinflammation: the role and therapeutic potential of GABA in neuro-immune interactions, Brain Behav. Immun. 54 (2016) 260–277.
[51] M. de las Casas-Engel, A. Dominguez-Soto, E. Sierra-Filardi, R. Bragado, C. Nieto, A. Puig-Kroger, et al., Serotonin skews human macrophage polarization through HTR2B and HTR7, J. Immunol. 190 (2013) 2301–2310.
[52] J.J. Kim, H. Wang, J.D. Terc, B. Zambrowicz, Q.M. Yang, W.I. Khan, Blocking peripheral serotonin synthesis by telotristat etiprate (LX1032/LX1606) reduces severity of both chemical- and infection-induced intestinal inflammation, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (2015) G455–G465.
[53] A. Anderson, H. Laurenson-Schafer, F.A. Partridge, J. Hodgkin, R. McMullan, Serotonergic chemosensory neurons modify the *C. elegans* immune response by regulating G-protein signaling in epithelial cells, PLoS Pathog. 9 (2013) e1003787.
[54] K. Hellstrand, S. Hermodsson, Serotonergic 5-HT1A receptors regulate a cell contact-mediated interaction between natural killer cells and monocytes, Scand. J. Immunol. 37 (1993) 7–18.
[55] M.R.I. Young, J.P. Matthews, Serotonin regulation of T-cell subpopulations and of macrophage accessory function, Immunology 84 (1995) 148–152.
[56] H. Matsuda, H. Ushio, G.P. Geba, P.W. Askenase, Human platelets can initiate T cell dependent contact sensitivity through local serotonin release mediated by IgE antibodies, J. Immunol. 158 (1997) 2891–2897.
[57] M. Freire-Garabal, M. Nunez, J. Balboa, P. Lopez-Delgado, R. Gallego, T. Garcıa-Caballero, Serotonin upregulates the activity of phagocytosis through 5-HT1A receptors, Br. J. Pharmacol. 139 (2003) 457–463.
[58] J.E. Ghia, N. Li, H. Wang, M. Collins, Y. Deng, R.T. El-Sharkawy, et al., Serotonin has a key role in pathogenesis of experimental colitis, Gastroenterology 137 (2009) 1649–1660.
[59] Z. Mikulski, Z. Zasłona, L. Cakarova, P. Hartmann, J. Wilhelm, L.H. Tecott, et al., Serotonin activates murine alveolar macrophages through 5-HT2C receptors, Am. J. Physiol. Lung Cell Mol. Physiol. 299 (2010) 272–280.
[60] U. Nannmark, L. Sennerby, L.M. Bjursten, G. Skolnik, U. Bagge, Inhibition of leukocyte phagocytosis by serotonin and its possible role in tumor cell destruction, Cancer Lett. 62 (1992) 83–86.
[61] J.C. Jackson, R.J. Cross, R.F. Walker, Influence of serotonin on the immune response, Immunology 54 (1985) 505–512.
[62] R.S. Gray, F.M. McCorkle, K.M. Denno, Modulation of chicken plaque-forming cells by serotonin and dopamine, Poultry Sci. 70 (1991) 1521–1526.
[63] R. Mössner, K. Lesch, Role of serotonin in the immune system and in neuroimmune interactions, Brain Behav. Immun. 12 (1998) 249–271.
[64] C.M. Flory, Phylogeny of neuroimmunoregulation: effects of adrenergic and cholinergic agents on the *in vitro* antibody response of splenic leukocytes from the rainbow trout, *Oncorhynchus mykiss*, Dev. Comp. Immunol. 14 (1990) 283–294.
[65] C.M. Flory, C.J. Bayne, The influence of adrenergic and cholinergic agents on the chemiluminescence and mitogenic responses of leukocytes from the rainbow trout, *Oncorhynchus mykiss*, Dev. Comp. Immunol. 15 (1991) 135–142.
[66] C.J. Bayne, S. Levy, Modulation of the oxidative burst in trout myeloid cells by ACTH and catecholamines: mechanism of action, J. Leukoc. Biol. 50 (1991) 554–556.
[67] C.J. Bayne, S. Levy, The respiratory burst of rainbow trout, *Oncorhynchus mykiss* (Walbaum), phagocytes is modulated by sympathetic neurotransmitters and the `neuro-peptide ACTH, J. Fish. Biol. 38 (1991) 805–811.
[68] Y.K. Narnaware, B.L. Baker, M.G. Tomlinson, The effect of various stresses, corticosteroids and adrenergic agents on phagocytosis in the rainbow trout, *Oncorhynchus mykiss*, Fish Physiol. Biochem. 13 (1994) 31–40.
[69] K. Yuwaraj, N. Norman, Y.S. Woo, Neuroendocrine regulation of immune function in fish, in: P.D.P. Rao, R.E. Peter (Eds.), Neural Regulation in the Vertebrate Endocrine System, Springer, Boston, 1999, pp. 219–231.
[70] G. Nardocci, C. Navarro, P.P. Cortes, M. Imarai, M. Montoya, B. Valenzuela, et al., Neuroendocrine mechanisms for immune system regulation during stress in fish, Fish Shellfish Immunol. 40 (2014) 531–538.
[71] F. Huby, S. Mallet, H. Hoste, Role of acetylcholinesterase (AChE) secreted by parasitic nematodes on the growth of the cell line from epithelial origin HT29-D4, Parasitology 118 (1999) 489–498.
[72] M.K.S. Gustafsson, M.C. Wikgren, Peptidergic and aminergic neurons in adult *Diphyllobothrium dendriticum* Nitzsch, 1824 (Cestoda, pseudophyllidea), Z. Parasitenkd. 64 (1981) 121–134.
[73] N.B. Terenina, N.M. Shalaeva, E.B. Overchuk, Serotonin in *Hymenolepis diminuta* (Cestoda) (concentration in helminths of various ages, correlation with the level of serotonin in the intestines of the host, and influence of reserpine), Trudy-Akademiia nauk SSSR, Gel'mintologicheskoi laboratorii, 34 1986, pp. 115–119.
[74] M. Wikgren, M. Reuter, M. Gustafsson, Neuropeptides in free-living and parasitic flatworms (Platyhelminthes) an immunocytochemical study, Hydrobiologia 132 (1986) 93–99.
[75] N.M. Biserova, I.A. Kutyrev, K. Jensen, GABA in the nervous system of the Cestodes *Diphyllobothrium dendriticum* (Diphyllobothriidea) and *Caryophyllaeus laticeps* (Caryophyllidea), with comparative analysis of muscle innervation, J. Parasitol. 100 (2014) 411–421.
[76] G. Coakley, A.H. Buck, R.M. Maizels, Host parasite communications - messages from helminths for the immune system: parasite communication and cell-cell interactions, Mol. Biochem. Parasitol. 208 (2016) 33–40.
[77] M.E. Ancarola, A. Marcilla, M. Herz, N. Macchiaroli, M. Pérez, S. Asurmendi, et al., Cestode parasites release extracellular vesicles with microRNAs and immunodiagnostic protein cargo, Int. J. Parasitol. 47 (2017) 675–686.
[78] Ø. Øverli, M. Páll, B. Borg, M. Jobling, S. Winberg, Effects of *Schistocephalus solidus* infection on brain monoaminergic activity in female three-spined sticklebacks *Gasterosteus aculeatus*, Proc. Roy. Soc. Lond. B 268 (2001) 1411–1415.
[79] N.B. Terenina, The serotonin and dopamine content of helminths in different classes, Zh. Evol. Biokhim. Fiziol. 25 (1989) 566–571.
[80] T.T. Chou, J. Bennett, E. Bueding, Occurrence and concentrations of biogenic amines in trematodes, J. Parasitol. 58 (1972) 1098–1102.
[81] M. Hariri, Occurrence and concentration of biogenic amines in *Mesocestoides corti* (Cestoda), J. Parasitol. 60 (1974) 737–743.
[82] D.F. Mettric, C.H. Cho, Changes in tissue and intestinal serotonin (5-HT) levels in the laboratory rat following feeding and the effect of 5-HT inhibitors on the migratory response of *Hymenolepis diminuta* (Cestoda), Can. J. Zool. 60 (1982) 790–797.
[83] K. Eriksson, M. Gustafsson, G. Akerlind, High-performance liquid chromatographic analysis of monoamines in the cestode *Diphyllobothrium dendriticum*, Parasitol. Res. 79 (1993) 699–702.
[84] N.M. Biserova, M.K.S. Gustafsson, M. Reuter, N.B. Terenina, The nervous system of the pike-tapeworm *Triaenophorus nodulosus* (Cestoda: pseudophyllidea): ultrastructure and immunocytochemical mapping of aminergic and peptidergic elements, Invertebr. Biol. 115 (1996) 273–285.
[85] D.M.S. Watts, A.M. Atkins, Mechanism-based inhibitors of GABA-transferase increase the GABA concentration in a nematode parasite, Biochem. Soc. Trans. 14 (1986) 1035–1036.
[86] K.S. Eriksson, A.G. Maule, D.W. Halton, P.A. Panula, C. Shaw, GABA in the nervous

system of parasitic flatworms, Parasitology 110 (1995) 339–346.
[87] S.M. Rawls, G.W. Stagliano, T. Gomez, R.B. Raffa, Measurement of GABA and glycine in planarians, Pharmacologyonline 1 (2007) 1–7.
[88] K. Rangiah, D. Palakodeti, Comprehensive analysis of neurotransmitters from regenerating planarian extract using an ultrahigh-performance liquid chromatography/mass spectrometry/selected reaction monitoring method, Rapid Commun. Mass Spectrom. 27 (2013) 2439–2452.
[89] H. Hurd, *Echinococcus granulosus*: a comparison of free amino acid concentration in hydatid fluid from primary and secondary cysts and host plasma, Parasitology 98 (1989) 135–143.