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Effects of *Microphallus papillorobustus* (Platyhelminthes: Trematoda) on serotonergic immunoreactivity and neuronal architecture in the brain of *Gammarus insensibilis* (Crustacea: Amphipoda)

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The larval flatworm *Microphallus papillorobustus* encysts in the protocerebrum of its intermediate host, *Gammarus insensibilis*, and changes the gammarid's responses to mechanical and photic stimuli. The resulting aberrant escape behaviour renders infected gammarids more susceptible to predation by birds, the definitive hosts of the parasite. We used immunocytochemical methods to explore the mechanisms underlying these subtle behavioural modifications. Whole mounts of gammarid brains were labelled with fluorescent anti-serotonin and anti-synapsin antibodies and viewed using confocal microscopy. Two types of change were observed in infected brains: the intensity of the serotonergic label was altered in specific regions of the brain, and the architecture of some serotonergic tracts and neurons was affected. A morphometric analysis of the distribution of the label showed that serotonergic immunoreactivity was decreased significantly (by 62%) in the optic neuropils, but not in the olfactory lobes, in the presence of the parasite. In addition, the optic tracts and the tritocerebral giant neurons were stunted in parasitized individuals. Published evidence demonstrates changes in serotonin levels in hosts ranging from crustaceans to mammals infected by parasites as diverse as protozoans and helminths. The present study suggests that the degeneration of discrete sets of serotonergic neurons might underlie the serotonergic imbalance and thus contribute to host manipulation.

Keywords: altered behaviour; manipulation; parasites; serotonin; monoamines; neuronal morphology

1. INTRODUCTION

Some larval parasites alter the behaviour of their intermediate host in a way that enhances the predation of the intermediate host by the definitive host of the parasite, thereby enhancing transmission (see reviews in Barnard & Behnke 1990; Combes 1991; Moore 2002). The present study focuses on such a system. The metacercaria of the trematode *Microphallus papillorobustus* encysts in the protocerebrum of *Gammarus insensibilis* (Rebecq 1964) and changes the responses of the gammarid to various environmental stimuli, in particular light and mechanical stimuli, leading to aberrant escape behaviours (Helluy 1982, 1984). In an experimental setting, infected gammarids are, on average, twice as likely as uninfected ones to be preyed upon by birds, the definitive hosts of the trematode. It is important to stress that the parasite does not just induce sluggishness or a general pathological state in the gammarid host. Only very specific behaviours are modified. In addition, the larvae do not induce behavioural alterations from the start of the infection. It is only after several days when the cysts are mature, healthy and infective to the definitive hosts that the behavioural responses are changed (Helluy 1983). Therefore, the trematode is modulating the behaviour of its host with precise timing and in very subtle ways.

Examples of behavioural manipulation by parasites are numerous but the mechanisms underlying ethological changes are by no means well characterized. However, evidence is accumulating that the biogenic amine serotonin (5-hydroxytryptamine, 5-HT) is involved in several host–parasite systems in which infected hosts showing altered serotonin levels are prey of the parasite's next host. Serotonin injected into uninfected gammarids reproduces aspects of the behaviour induced by the acanthocephalan *Polymorphus paradoxus* in *Gammarus lacustris* (Helluy & Holmes 1990); injections of octopamine, dopamine, norepinephrine and GABA fail to reproduce the altered behaviour. Maynard *et al.* (1996) studied the nerve cord of *G. lacustris* and found that the number of serotonergic varicosities is increased in individuals infected by *P. paradoxus*. In sticklebacks parasitized by a tapeworm larva, the concentrations of serotonin and norepinephrine are reduced in the telencephalon of infected fishes as compared with controls (Overli *et al.* 2001). In this same system, the ratios of 5-hydroxy-indoleacetic acid (5-HIAA) to serotonin are significantly elevated in the hypothalami and brainstems of infected sticklebacks, which could indicate enhanced release of the neurotransmitter as a result of increased neural activity. Concentrations of serotonin are also decreased in the brains of mice infected with the

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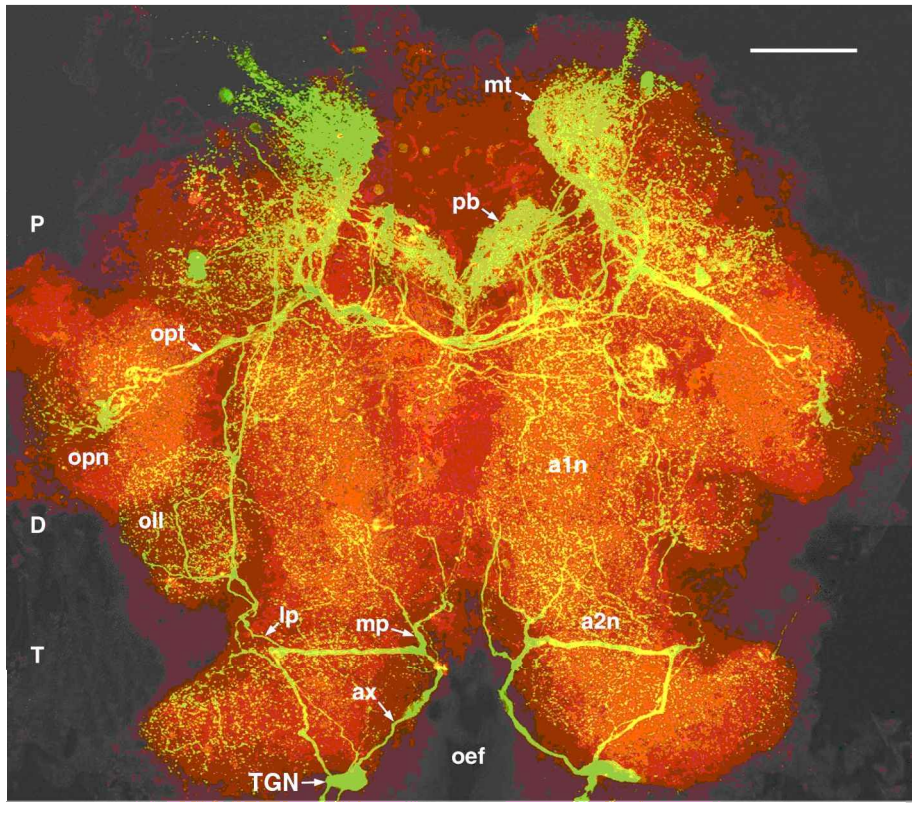

Figure 1. Brain of *G. insensibilis*. Montage of four stacks of 32 confocal scans showing immunoreactivity for serotonin (green label) and synapsin (red outline of neuropils). Anterior is at the top. Abbreviations: ax, axon of TGN; a1n, antenna 1 neuropil; a2n, antenna 2 neuropil; D, deutocerebrum; lp, lateral projections of TGN; mp, medial projections of TGN; mt, medulla terminalis; oef, oesophageal foramen; oll, olfactory lobe; opn, optic neuropil; opt, optic tract; P, protocerebrum; pb, protocerebral bridge; T, tritocerebrum; TGN, tritocerebral giant neuron. Scale bar, 100 μm.

nematodes *Trichinella spiralis* and *Trichinella pseudospiralis* (Abdel Ghafar *et al.* 1996; Terenina *et al.* 1997).

Serotonin can act as a classical neurotransmitter, as a neuromodulator changing the efficacy of synaptic transmission, or as a neurohormone involved in widespread modulation. Ubiquitous in the animal kingdom (Weiger 1997), serotonin is found in few cells with long projections linking multiple regions with a common input (Beltz 1999). These properties also make serotonin a good marker of neuronal architecture. The serotonin label allows the study of identifiable neurons and the comparison of the morphology of their neurites in infected and uninfected brains. The brain or supraoesophageal ganglion of amphipods has been described by Madsen (1960) and by MacPherson & Steele (1980). Groups of cell bodies are found at the periphery, whereas tracts and neuropils (distinct masses of tangled neurites and synaptic connections) are located more internally. The brain of *G. insensibilis* exhibits the typical anatomical pattern of crustacean brains (Sandeman 1982) and consists of three divisions, the protocerebrum, deutocerebrum and tritocerebrum, which correspond to the three major sensory inputs they receive, visual, olfactory and mechanosensory, respectively (see brain structure in figure 1). Because of its size (700–900 μm wide), the brain of *G. insensibilis* lends itself to whole-mount processing and confocal microscopy, and is a convenient model to observe the impact of parasitic cysts on an entire brain. Immunocytochemical methods were applied to brains of infected and uninfected gammarids using two antibodies simultaneously. One revealed serotonin-like immunoreactivity. The other outlined the shape of the neuropils, giving information on the relative location of the serotonergic neurons in the gammarid brain.

2. MATERIAL AND METHODS

(a) *Immunocytochemistry*

Gammarus insensibilis both normal and showing alterations of behaviour were collected in the brackish waters of the south of France (43°25′ N, 3°35′ E). One batch of gammarids was processed in Montpellier, France and the other one was shipped to Boston, USA and kept in the animal facilities at Wellesley College before processing. Brains of *G. insensibilis* were dissected in oxygenated Van Harreveld crustacean saline (in g, l^{-1}: NaCl, 12; KCl, 0.4; $CaCl_2$ $2H_2O$, 1.5; $MgCl_2$ $6H_2O$, 0.25; $NaHCO_3$, 0.2; pH, 7.3–7.4). The brains were pinned onto thin plates made of Sylgard (Dow Corning) using tungsten wire, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight, rinsed copiously in 0.1 M PB with 0.2% Triton X-100 (PBTX) and incubated for 3 h in 2% goat serum (Gibco) diluted in PBTX (methods in Beltz & Burd 1989). After rinsing, the brains were incubated overnight at 4 °C with two primary antibodies diluted in PBTX: a mouse anti-synapsin (SYNORF1) antibody 1 : 40 (gift of E. Buchner, Universität Würzburg, Germany) and a rabbit anti-5HT antibody 1 : 1000 (Diasorin). After rinsing, the brains were incubated overnight at 4 °C with two secondary antibodies (1 : 50) diluted in PBTX: a Texas Red-conjugated goat anti-mouse antibody and an Alexa Fluor 488 goat anti-rabbit antibody (both from Molecular

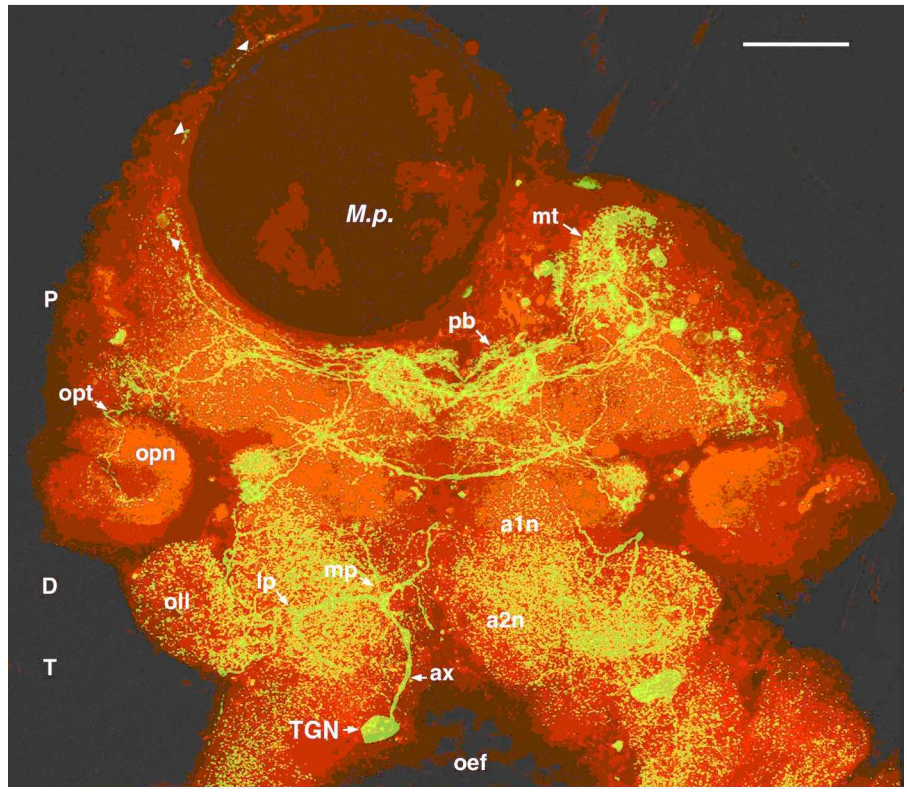

Figure 2. Cyst of *M. papillorobustus* (*M.p.*) in the brain of *G. insensibilis*. Montage of four stacks of 32 confocal scans showing immunoreactivity for serotonin (green label) and synapsin (red outline of neuropils). Note that the projections of the TGN are stunted on the left and ill-defined on the right. Arrowheads show serotonergic varicosities extending anteriorly along the cyst. Anterior is at the top. Abbreviations are the same as those given in figure 1. Scale bar, 100 μm.

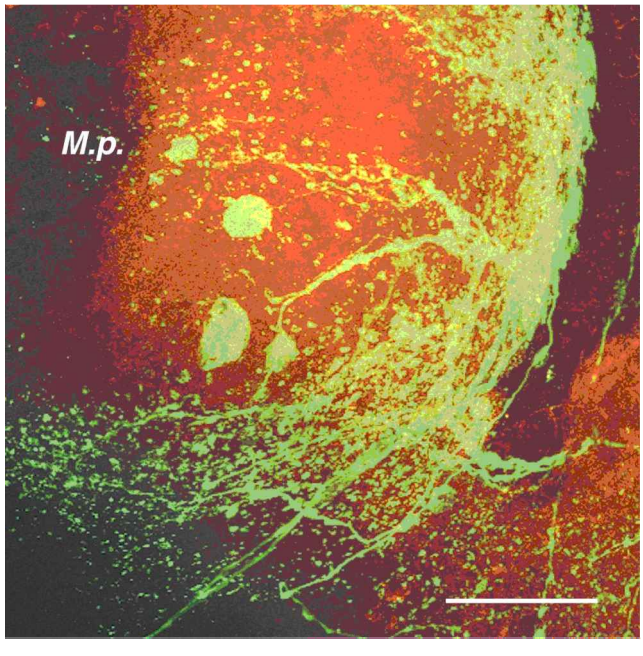

Figure 3. *M. papillorobustus* (*M.p.*) in the medulla terminalis of *G. insensibilis*. Stack of 32 confocal scans showing serotonergic cells and processes (green label) at the surface of a cyst covered with neurites (red label). Anterior is at the top and medial to the right. Scale bar, 50 μm.

Probes). The brains were rinsed in PBTX and then in PB; they were mounted in 80% glycerol and viewed with a Leica TCS-NT scanning confocal microscope equipped with krypton and argon gas lasers to visualize Alexa Fluor 488 and Texas Red, respectively. A total of 25 brains were usable, four uninfected, seven infected with one cyst and 14 infected with more than one cyst (from two to 11 cysts).

(b) *Data analysis*

Hemibrains showing ipsilateral optic neuropils, olfactory lobes and tritocerebral giant neurons (TGNs) were scanned in a single frame with a 20× oil immersion objective (eight hemibrains from four uninfected individuals and 14 hemibrains from seven infected individuals). The confocal images consisted of stacks of scans at 32 levels regularly spaced in whole mount brains (mean ± s.d. interval between scans: 1.82 ± 0.45 μm, $n = 22$) and encompassing the most dorsal and the most ventral signs of serotonin immunoreactivity. Gammarid brains are dorsoventrally flattened and all scans were taken in the horizontal plane of the brains. Individuals infected with a single cyst were considered. A single cyst is sufficient to induce the altered behaviour, is more informative about the preferred microhabitat of the metacercaria and allows blind scoring. Two types of analyses were performed. First, the C-Imaging morphometric system (Compix Inc., Cranberry Township, PA, USA) was used to calculate the percentage of the surface area of the optic and olfactory neuropils labelled with the serotonin antibody. The percentages obtained served as a quantitative assessment of the amount of serotonin in the whole neuropils since the surface areas of the stacked scans were analysed. Second, two observers were presented with the printed confocal images described above. They were asked to rate the optic tracts and the architectures of the TGN, axon, medial projections and lateral projections using a scale from '0, not distinct or deformed' to '5, very clear'. (The morphometric system could not be used for this analysis because the TGNs are too deformed

and indistinct to be traced in some infected brains.) The anterior part of the brain was hidden and therefore the two observers had no knowledge of the infection status of the individuals they were scoring. To avoid rating neurons damaged during dissection, we used only those TGNs with a clear cell body (six neurons from three uninfected individuals and 11 neurons from six infected individuals). There was a highly significant relationship between the scores given by the two observers (Spearman's rank order correlation coefficient, $r_s = 0.84$, $p < 0.0001$). Thus we used the mean rating value of observers 1 and 2 to compare (Mann–Whitney *U*-test) infected and uninfected individuals. Testing rating values of the two observers independently yielded similar conclusions. The observers were also asked to rate the intensity of the serotonergic label in the olfactory lobes and in the optic neuropils using a scale from '0, absent' to '5, very intense'. Their results show exactly the same trends as those obtained with the C-Imaging system.

3. RESULTS

(a) *Uninfected brains*

The protocerebrum (P) exhibited the highest levels of serotonin immunoreactivity (figure 1) with the medulla terminalis (mt) and the protocerebral bridge (pb) staining most intensely. The optic neuropils (opn) were also labelled. The optic tracts (opt) innervating the optic neuropils were particularly distinct. In the deutocerebrum (D) the olfactory lobes (oll) (primary olfactory centres) and antenna 1 neuropils (a1n) showed moderate levels of immunoreactivity on a par with the staining exhibited by the antenna 2 neuropils (a2n) in the tritocerebrum (T). Approximately 40 (paired) cell bodies showed serotonin immunoreactivity in the brain of *G. insensibilis*, although not all of these cells were labelled in any single individual. Few axons could be traced, with the noticeable exception of the TGN (figure 1). The bilateral TGN has a large ventral cell body (50–60 μm) adjacent to the circumoesophageal foramen (oef). Its medial projections (mp) innervate the neuropils of antenna 1 and antenna 2, and lateral projections (lp) innervate the olfactory lobe and the protocerebrum. After a partial anterior loop, the axon projects posteriorly through the circumoesophageal connective. Serotonin immunoreactivity has not been described in the brain of amphipods but the TGN is clearly homologous to the large tritocerebral serotonergic neuron described by Thompson *et al.* (1994) in isopods.

(b) *Infected brains*

When only one larval cyst was present in the brain ($n = 7$), it was always found in the protocerebrum (figure 2). Within the protocerebrum, it was located in the medulla terminalis ($n = 5$), a zone of higher integration, or more laterally in contact with the optic neuropils ($n = 2$). In the vicinity of the metacercariae, processes were observed extending along the lateral aspect of the cyst (figure 2) or adopting the contour of its ovoid shape (figure 3). Two types of change were noticeable in infected brains: the intensity of the serotonergic label was altered in specific regions of the brain, and the architecture of some tracts and neurons was affected (compare figures 1 and 2). We focused on a comparison of the optic neuropils and olfactory lobes and on the architecture of the optic tracts and of the TGN (figure 4). The surface area of the stacked scans taken through the neuropils was analysed. The percentage of the surface area of the neuropil labelled for serotonin decreased by 62% in the optic neuropils of infected individuals ($n_u = 8$, $n_i = 14$, $z = 2.66$, $p = 0.0078$). In the olfactory lobes this percentage was actually 30% higher in infected than in uninfected brains (figure 4*a*), but this difference was not significant ($n_u = 8$, $n_i = 14$, $z = -1.53$, $p = 0.12$). The TGN was stunted in infected gammarids. The axon (ax), the medial projections, the lateral projections to the olfactory lobes and the protocerebrum were significantly deformed or less distinct (compare figures 1 and 2). The rating system showed that the aberrant architecture of the TGN was found consistently in infected gammarids (figure 4*b*). The axon ($n_u = 6$, $n_i = 11$, $z = 2.46$, $p = 0.013$), medial projections ($n_u = 6$, $n_i = 11$, $z = 3.27$, $p = 0.0011$) and lateral projections ($n_u = 6$, $n_i = 11$, $z = 2.49$, $p = 0.012$) were deemed significantly deformed or less distinct in infected brains. The optic tracts were also dramatically disrupted ($n_u = 8$, $n_i = 14$, $z = 3.2$, $p = 0.0014$). Overall, there was a greater variability in the rating of the serotonergic label in infected than in uninfected gammarids.

4. DISCUSSION

The intensity of the serotonergic immunocytochemical label was altered in specific regions of the brain in gammarids infected with the cerebral metacercaria of *M. papillorobustus*. In addition, the optic tracts were deformed, and various projections of the TGN showed signs of degeneration in the presence of the larva. The absence of a serotonergic label in part of an axon could indicate that serotonin is not detectable in an otherwise normal fibre. However, many TGNs were fully labelled in infected brains but appeared deformed or stunted indicating that the morphology of the neurons and, presumably, their synaptic fields were modified.

The fact that serotonin levels are selectively altered in the optic neuropils of *G. insensibilis* is parallel to the findings of Terenina *et al.* (1997) and of Overli *et al.* (2001) who showed that serotonin concentrations are decreased significantly only in specific parts of the brain in infected hosts. In the case of *M. papillorobustus–G. insensibilis*, the decreased serotonergic levels in the optic neuropils are likely to be related to the aberrant photic behaviour manifested by infected gammarids. However, one would have to assume that serotonin levels must be within an optimal range to ensure appropriate photic behaviour. This could explain why injections of serotonin in uninfected *G. lacustris* (increased concentrations of serotonin) reproduce the photic behaviour of *G. lacustris* infected with *P. paradoxus* (Helluy & Holmes 1990). Alternatively, reduced levels of serotonin could indicate increased release and degradation of this amine.

The present results show that not only are the levels of serotonin altered in specific regions of the brain but also the integrity of serotonergic neurons is widely affected. The TGNs are deformed even though these neurons are located in the most posterior region of the brain (tritocerebrum) and the parasites in the most anterior part of the brain (protocerebrum). For this reason, a direct purely mechanical influence of the parasite on surrounding tissues is not the most likely hypothesis. Alternatively,

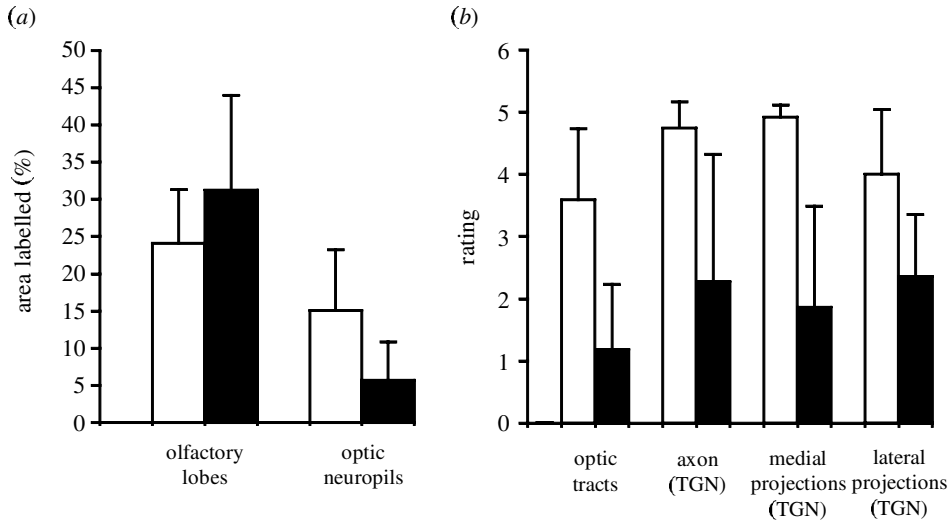

Figure 4. Assessment of the serotonergic label in various structures of the brain in uninfected (open bars) and *M. papillorobustus*-infected (filled bars) gammarids. All confocal images analysed are stacks of 32 scans regularly spaced in whole mount brains. (*a*) Mean percentages of the surface areas of the olfactory lobes (n.s.) and optic neuropils ($p = 0.008$) labelled for serotonin (surface area of the stacked scans through the neuropils) (uninfected, $n_u = 8$; infected, $n_i = 14$). (*b*) Mean rating of the morphology of the optic tracts ($n_u = 8$; $n_i = 14$) and of the TGN ($n_u = 6$; $n_i = 11$), from '0, not distinct or deformed' to '5, very clear'. The mean rating values of two observers are shown. The Mann–Whitney *U*-test is used to compare structures from infected and uninfected individuals. Error bars represent one standard deviation; *p*-values for optic tracts (0.001), axon (TGN) (0.01), medial projections (TGN) (0.001) and lateral projections (TGN) (0.01).

the parasite could produce substances such as cell-adhesion molecules (e.g. N-cam, cadherins and integrins) or growth factors that attract or redirect serotonergic terminals (review in Gilbert 2000). Indeed, fibroblast growth factors (FGF-2, FGF-4 and FGF-8) have been found at the surface of the metacercaria of *Euhaplorchis californiensis* located in the meningeal space of a killifish (La Clair and Lafferty (unpublished data) cited in Lafferty *et al.* 2000). These results are of particular interest because fibroblast growth factors have a broad range of effects on the growth and differentiation of serotonergic neurons in both vertebrates (e.g. Lindholm *et al.* 1994) and invertebrates (e.g. Condron 1999). The above hypotheses have little explanatory potential in the case of haemocoelian parasites manipulating their host behaviour. However, the morphology of serotonergic neurons could be altered in the vicinity of neural cysts in a variety of hosts, even in humans. Berdoy *et al.* (2000) emphasized that serotonin could be involved in the personality shifts observed in patients infected with cysts of *Toxoplasma gondii* (Flegr *et al.* 1996), a highly prevalent protozoan in humans, who represent a dead-end host for that parasite. Similarly, Abdel Ghafar *et al.* (1996) mention that the decrease in the levels of serotonin and norepinephrine found in *T. spiralis*-infected brains could contribute to the mental and motor abnormalities exhibited by infected mice and possibly also by infected humans.

Another compelling theory could explain the degeneration of serotonergic neurons. Briefly, the parasite would use a host defence reaction—the synthesis of nitric oxide (NO)—to modify the development and pattern of discrete neural networks in the brains of infected hosts. This is plausible considering the following facts. NO is known to be a major effector molecule of macrophage toxicity against many parasites including helminths (Oswald *et al.* 1994). Encapsulation of *M. papillorobustus* has been observed in gammarids (Helluy 1982; Thomas *et al.* 2000). NO is present in crustaceans where it may play a general part in the development of discrete neural networks (Scholz *et al.* 1998). Therefore, NO released by immunocompetent cells at the interface between host and parasite tissues could alter the distribution of serotonergic terminals, disrupting neural connectivity. Overli *et al.* (2001) have already pointed out the link between parasites, immune response and monoaminergic systems. The exploitation of a phylogenetically conserved host defence mechanism could explain how manipulation has evolved repeatedly in systems involving different taxa of hosts and parasites. Furthermore, the exploitation of a host immune response would be advantageous for the parasite because it would not require the synthesis of energetically expensive substances and therefore would cut down on the 'cost of manipulation'.

The present immunocytochemical study suggests that the cerebral larva of a trematode affects neuromodulation in an invertebrate brain by disrupting the morphology and connectivity of serotonergic neurons, thus contributing to the altered responses to environmental stimuli exhibited by infected hosts. Published biochemical evidence has demonstrated that changes in serotonin concentrations are involved in several host–parasite systems in which infected hosts are prey of the parasite's next host. The degeneration of indolaminergic neurons and the ensuing neuroendocrine imbalance might be a widespread mechanism underlying host manipulation involving predation.

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