

## REVIEW

# Investigating candidate neuromodulatory systems underlying parasitic manipulation: concepts, limitations and prospects

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### Summary

**Studies addressing the functional basis of parasitic manipulation suggest that alteration of the neuromodulatory system is a common feature of manipulated hosts. Screening of the neuromodulatory system has so far been carried out by performing ethopharmacological analysis, biochemical quantification of neurotransmitters and neuromodulators, and/or immunocytochemistry. Here, we review the advantages and limitations of such approaches through the analysis of case studies. We further address whether the analysis of candidate neuromodulatory systems fits the current view of manipulation as being multidimensional. The benefits in combining ethopharmacology with more recent molecular tools to investigate candidate neuromodulatory pathways is also emphasized. We conclude by discussing the value of a multidisciplinary study of parasitic manipulation, combining evolutionary (parasite transmission), behavioural (syndrome of manipulation) and neuroimmunological approaches.**

Key words: behavioural manipulation, ethopharmacology, neuroethology, psychoneuroimmunology, phenotypic engineering, serotonin.

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### Introduction

The ability of an individual to adjust its behaviour to variable environmental stimuli relies on the appropriate neuromodulation of its sensory and motor circuits. Such behavioural flexibility obviously offers an opportunity for a parasite to alter its host's behaviour. This is particularly relevant to manipulative parasites, which alter the habitat choice and defensive behaviour of their hosts in ways that potentially increase their own transmission efficiency (Lafferty and Shaw, 2013). Investigation of the mechanisms underlying parasitic manipulation suggests that alteration of the host's neuromodulatory system is a common feature of manipulated hosts (Thompson and Kavaliers, 1994; Adamo, 2002; Adamo, 2013; Helluy, 2013; Lafferty and Shaw, 2013). Indeed, several studies involving both vertebrate and invertebrate hosts have reported altered levels of neuromodulators or neuropeptides concomitant with a parasite-induced change in behaviour (see references in Table 1), or changes in expression of genes or proteins linked to biogenic amine metabolism in the brain (Hoek et al., 1997; Biron et al., 2005; Ponton et al., 2006; Prandovszky et al., 2011; Biron, 2013).

The exploitation of a host's neural plasticity and signalling by a manipulative parasite raises the question of how specific it is, considering the complex interactions between the neural, endocrine and immune systems in shaping behaviour (Adamo, 2002; Adamo, 2013). Reports on parasitic manipulation have generally focused on one or a few phenotypic traits suspected to increase parasite transmission, but the recent interest in a multidimensional approach is challenging this simplistic view (Cézilly and Perrot-Minnot, 2005; Cézilly and Perrot-Minnot, 2010; Poulin, 2010; Thomas et al., 2010; Cézilly et al., 2013). Parasitic manipulation is better characterized by a suite of traits concomitantly altered whatever the origin of their association (pleiotropic or independent) and its

consequence on parasite transmission (additive or synergistic) (Cézilly and Perrot-Minnot, 2005; Cézilly et al., 2013).

This multidimensional view of parasitic manipulation fits well with the complex interactions between the neuromodulatory, endocrine and immune systems existing throughout the animal kingdom. Their expected complexity also challenges the quest for the proximate mechanisms mediating host behavioural change (Poulin, 1995; Lefèvre et al., 2009; Lafferty and Shaw, 2013). Apart from proteomic studies (Biron, 2013), the study of proximate mechanisms underlying parasitic manipulation has focused on neuromodulatory systems (see Table 1). We critically review here the concepts, limitations and prospects in investigating particular neuromodulatory systems to elucidate the mechanisms underlying host manipulation by parasites. By loose analogy with a candidate gene, a 'candidate neuromodulatory system' is suspected of being involved in the expression of one or several host traits that are altered by a manipulative parasite, and of being the direct or indirect target of the parasite's excretion/secretion (E/S) products. By providing inference concerning the relationship between parasite-induced changes in the neuromodulatory system and changes in behaviour, this research strategy is in line with the association, necessity and sufficiency tests classically used in neuroethology. The investigation of candidate neuromodulatory systems involved in parasitic manipulation more specifically rests on four rationales. (1) Hypothesis testing, based on a putative association between parasite-induced behavioural alterations and a particular neural system suspected to modulate such behaviour. For instance, it has been suspected and subsequently confirmed that the decreased novelty seeking and defensive behaviour of rodents infected with *Toxoplasma gondii* could be mediated by the dopaminergic system (Skallová et al., 2006; Prandovszky et al., 2011). (2) A parsimonious functional argument, based on the intimate connection between the

immune, endocrine and neural systems, and the life-long plasticity of the host's neuromodulatory network (Benton et al., 1997; Harzsch et al., 1999; Adamo, 2002; Beltz and Sandeman, 2003; Maier, 2003). According to the psychoneuroimmune hypothesis of parasitic manipulation (Adamo, 2002; Helluy, 2013), these properties of the neural and immune systems have probably been hijacked by parasites with the ultimate consequence of increasing their transmission. (3) A parsimonious evolutionary argument, considering that the phylogenetic conservatism of neuromodulatory, signalling and immune systems may facilitate a molecular cross-talk between the parasite and the host (Salzet et al., 2000; Maier 2003; Caveney et al., 2006). For instance, the increased dopaminergic activity in some area of the brain of *T. gondii*-infected mice is due to the release by the parasite's cyst itself of the rate-limiting enzyme in dopamine synthesis (Prandovszky et al., 2011). (4) An accessible approach to non-model organisms, but with well-understood limitations because of the difficulty of establishing how the parasite hijacks the neurophysiology of its host. Three different approaches have been used so far: an ethopharmacological approach (Kavaliers et al., 2000), and biochemical or immunostaining analyses to quantify different neuromodulators and neurotransmitters in the hemolymph or the brain of infected hosts (Table 1). Ethopharmacology can be defined as 'an evolutionary approach to the study of a drug's effect on neurochemical mechanisms and functions of behaviour' (Parmigiani et al., 1998). For instance, psychoactive drugs have been used to understand the neurochemical basis and the adaptive significance of different forms of intraspecific aggression in mice (Parmigiani et al., 1998). The use of selective opioid peptide receptor agonists and antagonists allowed dissection of the subtle effects of the coccidian parasite *Eimeria vermiformis* on the altered responses of infected male mice to oestrous females, either facilitatory or inhibitory depending on the stage of infection (Kavaliers et al., 1997a). More generally, the ethopharmacological approach has been used to address the proximate and ultimate causation of behaviour from major functional categories (feeding, mating, parental care, stress coping, social interactions); hence, it is highly suitable to address both the proximate mechanisms and evolutionary significance of parasite-induced changes in behaviour (Kavaliers et al., 2000).

We will first review the few cases where the proximate mechanisms of parasitic manipulation have been investigated by focusing on a few 'candidate neuromodulatory pathways', using ethopharmacological analysis and/or biochemical or immunostaining analysis. We will then detail the limitations of such approaches. We will finally propose two directions for the development of a neuroethological approach to understanding the mechanisms underlying parasitic manipulation, emphasizing (1) the interest in framing a one-to-one approach such as one candidate neuromodulatory pathway-one behaviour into a multidimensional view of parasitic manipulation, and (2) from a methodological point of view, the interest in combining ethopharmacology with more recent molecular tools such as functional genomics to investigate candidate neuromodulatory pathways. Throughout, the term 'neuromodulator' or 'neuromodulatory systems' will be used in a broad sense, including neurotransmitter, neuromodulator and neuroendocrine function of biogenic amines, neuropeptides and the gaseous messenger nitric oxide (NO), and their corresponding receptors.

#### Investigating candidate neuromodulatory pathways: concept

The concept associated with the candidate neuromodulatory pathway approach rests on a simple argument: while

neuromodulation provides a powerful means to adjust sensory and motor circuits to variable environmental conditions in vertebrates and invertebrates (Birmingham and Tauck, 2003; Harzsch et al., 1999), it is also a permissive target for a parasite to 'reconfigure' its host's physiology and behaviour to its own benefits (Thompson and Kavaliers, 1994; Adamo, 2002). Neuromodulatory systems, in particular biogenic amines, opioids and the diffusible gaseous signalling molecule NO, are thus considered as potential targets of manipulative parasites (Kavaliers et al., 2000; Adamo, 2002; Helluy and Thomas, 2003; Adamo, 2013; Helluy, 2013). More specifically, the tight connection between the immune system and the nervous system could provide parasites with an indirect and less expensive method of altering host behaviour. From the initial ability to counter the host's immune system, parasites would have secondarily evolved the ability to subvert the bi-directional immune-brain circuitry, eventually modulating sensory-motor processing pathways, in particular those underlying cue-oriented behaviour that facilitate trophic transmission (Adamo, 2002; Adamo, 2013; Helluy and Thomas, 2003; Helluy, 2013; Lafferty and Shaw, 2013).

The first interest in investigating neuromodulatory pathways lies in its feasibility for non-model organisms: insects and crustaceans represent the majority of host species in studies on parasitic manipulation (Moore, 2002) (Table 1) but are not model organisms in functional genomics or proteomics. By contrast, their neurobiology is better known: in arthropods, biogenic amines and neuropeptides mediate a wide range of physiological and behavioural processes, in particular serotonin (5-HT), dopamine, octopamine and NO (Beltz, 1999; Roeder, 1999; Beltz and Kravitz, 2001; Tierney et al., 2003; Libersat and Pflueger, 2004; Colasanti et al., 2010).

The second reason for investigating a candidate neuromodulatory pathway is to allow phenotypic engineering, i.e. the production of a novel phenotype in an individual. Initially, phenotypic engineering was performed to address life-history trade-offs, and achieved by hormonal manipulations (Ketterson et al., 1992). In the study of parasitic manipulation, phenotypic engineering consists in mimicking parasite-induced changes in one or several phenotypic traits in uninfected individuals, or in cancelling them in infected hosts, in order to assess the contribution of such alterations to parasite transmission. Although the value of phenotypic engineering for the demonstration of a causal link between increased transmission and phenotypic alteration has been emphasized (Cézilly et al., 2010; Thomas et al., 2010), its use remains very limited in the framework of studies on parasitic manipulation. Recently, we have used pharmacological manipulation of the reaction to light to assess the consequence of the decrease in photophobia induced by the fish acanthocephalan *Pomphorhynchus tereticollis* in its intermediate amphipod host *Gammarus pulex* on vulnerability to predation (Perrot-Minnot et al., 2012). Uninfected amphipods were made photophilic by injecting a mixture of serotonin and its selective reuptake inhibitor, fluoxetine; they were mixed with uninfected conspecifics injected with vehicle solution, and then exposed to predation by fish. Photophilic amphipods were not more vulnerable to fish predation than photophobic (control) ones, thus questioning the actual contribution of altered photophobia to parasite transmission in this system.

Third, investigating candidate neuromodulatory pathway allows the estimation of multiple effects on several traits. Such an approach is particularly relevant if consistent differences in behavioural traits among individuals (i.e. behavioural types or personalities) (Dall et al., 2004) and intra-population or intra-

Table 1. Review of studies suggesting the involvement of biogenic amines or neuropeptides in the parasite-induced alteration of invertebrate host behaviour

Method	Host species	Parasite species	Results	Reference
<b>Ethopharmacology</b>				
Injection: serotonin, dopamine, octopamine, noradrenaline	Amphipod <i>Gammarus lacustris</i>	<i>Polymorphus paradoxus</i> Acanthocephala	Only 5-HT injection mimics clinging behaviour in uninfected gammarids	Helluy and Holmes, 1990
Injection of serotonin	Amphipod <i>Gammarus pulex</i> , <i>Gammarus roeseli</i>	<i>Pomphorhynchus laevis</i> , <i>Pomphorhynchus tereticollis</i> Acanthocephala	Injection of 5-HT reversed the natural photophobia of uninfected gammarids	Tain et al., 2006; Tain et al., 2007
Injection of octopamine or blood from post-emergence parasitized larvae	Moth <i>Manduca sexta</i>	<i>Cotesia congregata</i> Hymenoptera	Injection of octopamine mimics the decreased peristaltic activity in the foregut (related to decreased feeding)	Miles and Booker, 2000
Injection of dopamine, reserpine and dopamine receptor antagonist	Cockroach <i>Periplaneta americana</i>	<i>Ampulex compressa</i> Hymenoptera	Injection of dopamine mimics venom-induced grooming. Depletion of monoamines mimics venom-induced non-paralytic hypokinesia and reduced escape response	Weisel-Eichler et al., 1999; Weisel-Eichler and Libersat, 2002
Injection of opioid receptor agonist and antagonist	Cockroach <i>Periplaneta americana</i>	<i>Ampulex compressa</i> Hymenoptera	Pre-stinging injection of opioid receptor antagonists decreases the venom-induced hypokinesia (by increasing the threshold of escape behaviour): evidence for opioid receptor ligands in venom	Gavra and Libersat, 2011
Injection of a selective dopamine uptake inhibitor (GBR 12909)	Mouse	<i>Toxoplasma gondii</i> Apicomplexa phylum	Injection increases activity and decreases exploration in infected animals (opposite pattern to uninfected ones): changes in dopaminergic system as a proximate cause of manipulation	Skalova et al., 2006
<b>Biochemical analysis</b>				
HPLC-ED on brain extracts	Crab <i>Macrophallus hirtipes</i>	<i>Maritrema</i> Trematode <i>Profilicollis</i> sp. Acanthocephala	Increase in 5-HT content in the brain of crabs co-infected with both parasites	Poulin et al., 2003
HPLC-ED on haemolymph extracts	Crab <i>Hemigrapsus crenulatus</i>	<i>Profilicollis antarcticus</i> Acanthocephala	Increase in hemolymph dopamine content, but not 5-HT, in infected crabs	Rojas and Ojeda, 2005
HPLC-ED on haemolymph extracts	Cricket <i>Nemobius sylvestris</i>	<i>Paragordius tricuspidatus</i> Nematomorpha	Increased concentration of taurine and decreased concentration of tyrosine and valine in the brain of infected manipulated crickets	Thomas et al., 2003
GC-MS and HPLC-ED on venom	<i>Periplaneta americana</i> and other cockroaches	<i>Ampulex compressa</i> Hymenoptera	Dopamine present in the venom	Moore et al., 2006
HPLC-ED on haemolymph extracts	Moth <i>Manduca sexta</i>	<i>Cotesia congregata</i> Hymenoptera	Increased octopamine content in the brain, and in thoracic and abdominal ganglia	Adamo and Shoemaker, 2000
Brain monoaminergic concentrations by HPLC-ED	Three-spined stickleback <i>Gasterosteus aculeatus</i>	<i>Schistocephalus solidus</i> Cestode	Elevated 5-HIAA:5-HT ratio, or lower 5-HT and noradrenaline concentration, depending on brain area	Overli et al., 2001
Brain concentrations of monoamines and their metabolites by HPLC-ED	Killifish <i>Fundulus parvipinnis</i>	<i>Euhaplorchis californiensis</i> Trematoda	Altered 5-HT and dopamine metabolism in specific brain regions of infected fish: altered locomotion and arousal.	Shaw et al., 2009
<b>Immunohistochemistry and immunocytochemistry</b>				
Immunocytochemistry (anti-5-HT) on nerve cord	Amphipod <i>Gammarus lacustris</i>	<i>Polymorphus paradoxus</i> Acanthocephala	Increased 5-HT immunoreactivity in the third thoracic ganglion of infected hosts	Maynard et al., 1996

Table 1. Continued

Method	Host species	Parasite species	Results	Reference
Immunohistochemistry and immunocytochemistry (cont.)				
Immunocytochemistry (anti-5-HT) on the brain	Amphipod <i>Gammarus insensibilis</i>	<i>Microphallus papillorobustus</i> Trematoda	Serotonergic activity depressed in optic neuropils but not in optic lobes, and altered architecture of some serotonergic tracts and neurons, in infected hosts	Helluy and Thomas, 2003
Immunocytochemistry (anti-NOS, anti-glutamine synthase) on the brain	Amphipod <i>Gammarus insensibilis</i>	<i>Microphallus papillorobustus</i> Trematoda	Increased NOS immunoreactivity and astrocyte-like glial cells at the host–encysted parasite interface in the brain giving rise to neuroinflammation	Helluy and Thomas, 2010
Immunocytochemistry (anti-5-HT) on the brain	Amphipod <i>Gammarus pulex</i>	<i>Pomphorhynchus laevis</i> , <i>Pomphorhynchus tereticollis</i> Acanthocephala	Increase in brain 5-HT immunoreactivity in infected manipulated <i>Gammarus pulex</i> (but not in infected unmanipulated <i>Gammarus pulex</i> and <i>Gammarus roeseli</i> ), with respect to reaction to light	Tain et al., 2006; Tain et al., 2007
Immunohistology on the brain (proliferative activity of neuroblasts using DNA staining)	Cricket <i>Nemobius sylvestris</i>	<i>Paragordius tricuspidatus</i> Nematomorpha	Twofold increase of mitotic index: enhanced neurogenesis in the mushroom bodies of infected crickets	Thomas et al., 2003
Immunostaining of brain section, antibody raised against dopamine and parasite-specific tyrosine hydroxylase	Mouse	<i>Toxoplasma gondii</i> Apicomplexa phylum	Increased dopamine metabolism in infected neural cells; intracellular tissue cysts encode a tyrosine hydroxylase specific to <i>T. gondii</i>	Prandovszky et al., 2011

In the above cases, invertebrate hosts were used as intermediate hosts by trophically transmitted parasites or as a food store and shelter by parasitoid larvae.

It should be emphasized that in none of these studies has an active manipulation of host neuromodulatory systems by the parasite been demonstrated, but rather a coincidence between the patterns observed in the host's brain and infection by a manipulative parasite.

5-HT, serotonin; NO, nitric oxide; NOS, nitric oxide synthase.

species correlations among behavioural traits (i.e. behavioural syndromes) (Sih et al., 2004) are established in hosts of manipulative parasites. For instance, does the serotonergic system modulate photophobia, refuge use, glycogen storage and immune defences in *G. pulex*, a set of traits concomitantly altered by the fish acanthocephalan parasite *Pomphorhynchus laevis* (see references in Cézilly et al., 2013)?

Finally, a neglected contribution of ethopharmacology is in the consideration of other behavioural traits than the ones that are generally studied. In stress and addiction studies, biogenic amines and neuropeptides have been commonly investigated for their role in learning, motivation, decision-making, fear and anxiety-like behaviour, and social and agonistic interactions. Interestingly, most of these traits are used in the categorization of coping styles (or personality) as proactive and reactive, and their associated level of behavioural flexibility (Coppens et al., 2010). By contrast, studies on parasitic manipulation have essentially focused on behaviours that could increase trophic or vector-borne transmission in heteroxenous parasites – more specifically, cue-oriented behaviours (such as phototaxis, chemotaxis, rheotaxis or wind-evoked behaviour, geotaxis, etc.) – or on defensive behaviours. The possibility that these parasite-induced alterations in host behaviour result from changes in decision-making rules or motivational state has not been considered so far, despite the interest, from a

neuroethological point of view, of addressing the cognitive processes underlying behavioural manipulation. There are a few exceptions, however. Neurophysiological investigations on how parasitoid wasps make their host ‘zombies’ have revealed the astonishing potency of the venom cocktail in affecting decision-making processes and motivation level, rather than sensory–motor systems (Libersat et al., 2009; Libersat and Gal, 2013). In the classical model of parasitic manipulation, *T. gondii*-infected rodents, the innate aversion of rodents to the urine of cats – the definitive host – is specifically turned to attraction in *T. gondii*-infected rodents, without a more general alteration in cognitive ability such as learned fear, neophobia and anxiety-like behaviour (Vyas et al., 2007).

In Table 1 we review the studies illustrating the first two of these reasons for investigating candidate neuromodulatory systems, while the third and fourth are the main prospects for the candidate neuromodulatory approach (see ‘Investigating candidate neuromodulatory pathways: prospects’, below). The importance of four neuromodulatory systems to the study of parasitic manipulation – the serotonergic, dopaminergic, nitric and opioid systems – should be particularly emphasized. These messenger substances have pervasive effects on sensory, motivational and motor networks, on learning and memory, on energy balance and homeostasis, and on immunity. They operate both in the central



nervous system and in peripheral organs, and can interact with each other. Pleiotropic effects of biogenic amines have been reported (Mitayake et al., 2008; Riemensperger et al., 2011). Neuromodulatory systems may also combine to coordinate behavioural responses to a particular situation (*sensu* Sih et al., 2004). For instance, opioid, serotonergic and GABAergic systems may all be involved in the elaboration of two components of defensive behaviour in rodents: fear-induced analgesia and behavioural avoidance in facing a predator (Kavaliers et al., 1997b). Such an adaptive response to predation risk is reduced in parasitized mice compared with healthy ones. This pattern is found in parasites with different transmission routes, i.e. in the nematode *Heligmosomoides polygyrus* with direct transmission between conspecifics (Kavaliers et al., 1997b) or in the trophically transmitted cestode *Taenia crassiceps* (Gourbal et al., 2001), thus questioning the adaptive significance of such parasite-induced alterations (Kavaliers et al., 2000; Gourbal et al., 2001). Single or interacting neuromodulatory systems are therefore likely candidates for the pleiotropic effects and multidimensionality seen at the phenotypic level. The role of some of these systems in parasitic manipulation is presented elsewhere in this issue (Helluy, 2013; Lafferty and Shaw, 2013), and will not be reviewed in detail here.

The gaseous messenger NO acts as a major signal molecule in the olfactory system and in the immune defence of major invertebrate phyla (Benton et al., 2007; Colasanti et al., 2010). The increased level of NO synthase immunoreactivity and the numerous astrocyte-like glial cells around cysts of *Microphallus papillorobustus* in the brain of infected *Gammarus insensibilis* provided evidence for a chronic neuroinflammatory response triggered by mature cysts of the trematode in its intermediate host (Helluy and Thomas, 2010). According to the neuroimmune hypothesis of parasitic manipulation, such a neural immune defence

potentially affects neuromodulation and, as a consequence, could be implicated in parasite-induced behavioural alterations.

In crustaceans and insects, dopamine controls the release of hormones regulating several functions such as gonad maturation, carbohydrate metabolism, pigmentation and osmotic balance. This biogenic amine is also involved in learning processes, sometimes in interaction with serotonin (Tierney et al., 2003). Elevated dopamine levels have been reported in the crab *Hemigrapsus crenulatus* infected by the acanthocephalan *Profilicollis antarcticus*, and linked to increased oxygen consumption and altered body postures of infected individuals (Rojas and Ojeda, 2005). More recently, Prandovszky and colleagues demonstrated that *Toxoplasma gondii* induces a significant increase in dopamine metabolism in neural cells, probably by releasing tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis (Prandovszky et al., 2011).

Serotonin is widely present in the animal kingdom, and modulates important behaviours from simple taxis to memory, learning, response to stress, sexual and agonistic behaviour, physiological process (including control of energy balance), circadian rhythms and neurogenesis (Weiger, 1997; Harzsch et al., 1999; Il-Han et al., 2010; Zhang et al., 2011). The role of this biogenic amine in parasite-induced changes in behaviour has been reported in some host–parasite models (Table 1) but without elucidation of how the parasite interferes with the host's serotonergic system. However, its implication as a modulator of several traits altered by the parasite can still be investigated (Fig. 1) (Cézilly et al., 2013; Helluy, 2013). Whether the serotonergic system is the direct target of the acanthocephalan parasite *P. laevis* or not, it is likely that the increased 5-HT immunoreactivity in the brain of infected *G. pulex* results from altered 5-HT metabolism, with potentially multiple cascading effects on several phenotypic traits (Fig. 1). For instance, the

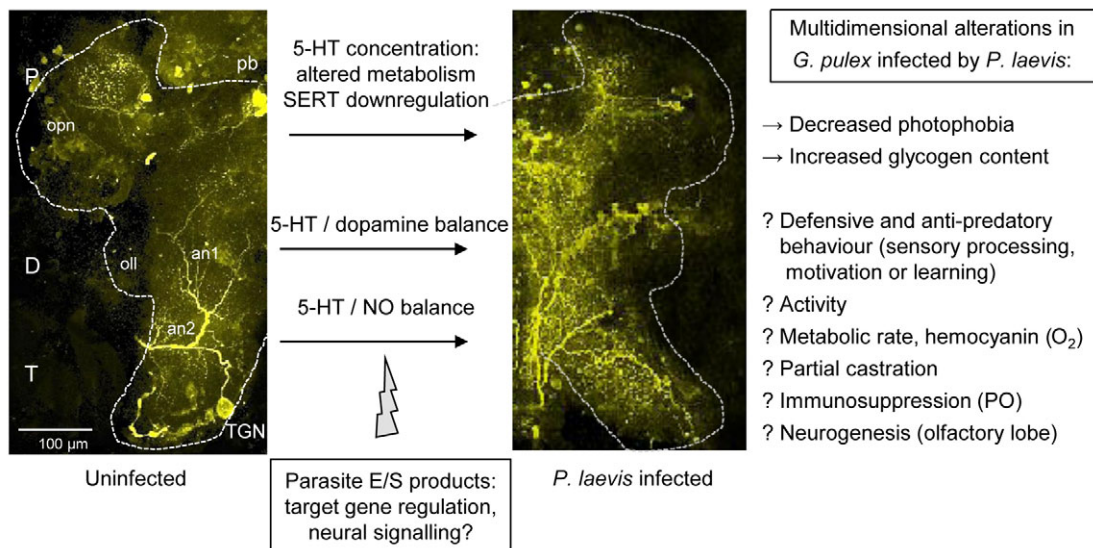


Fig. 1. Possible implication of the serotonergic system in parasitic manipulation of the amphipod *Gammarus pulex* by the acanthocephalan *Pomphorhynchus laevis*. Images show 5-HT (serotonin) immunoreactivity (green) within half of the brain of (A) a representative uninfected and (B) a representative *P. laevis*-infected amphipod. The parasite may affect several phenotypic traits in its host *via* alteration of 5-HT brain concentration/sequestration (metabolism or re-uptake), and/or changing 5-HT/dopamine or 5-HT/nitric oxide (NO) balance. A correlation between increased brain 5-HT immunoreactivity and decreased photophobia has been shown in this host–parasite system, and the regulation of glycogen metabolism by 5-HT is highly suspected. The role of 5-HT in other traits altered by *P. laevis* is not known, but has been reported for 5-HT, dopamine and NO in crustaceans. Under the scenario of a change in 5-HT/dopamine or NO balance, such pleiotropic effects are thus plausible. Abbreviations: a1n, antenna 1 neuropil; a2n, antenna 2 neuropil; D, deutocerebrum; E/S, excretion/secretion; oll, olfactory lobe; opn, optic neuropil; P, protocerebrum; pb, protocerebral bridge; SERT, serotonin transporter; T, tritocerebrum; TGN, tritocerebral giant neuron. Scale bar, 100  $\mu$ m. Images modified from Tain et al. (Tain et al., 2006).

cystacanth of *P. laevis* and *P. tereticollis* (the last larval stage, infective to definitive hosts) modulates the reaction to light of *G. pulex* through a change in its host's serotonergic system, possibly mediated by 5-HT<sub>2</sub> receptors (Tain et al., 2006) (M.-J.P.-M., unpublished results). It remains to be investigated whether *P. laevis*- and *P. tereticollis*-induced changes in the serotonergic system of *G. pulex* are implicated in other behavioural and physiological traits known to be altered by the parasite, and to be potentially modulated by 5-HT (Fig. 1).

#### Investigating candidate neuromodulatory pathways: limitations

Although the exploration of these neurophysiological changes can provide evidence that one or several neuromodulators play a key role in one or a few behavioural dimensions of parasitic manipulation, it also has several limitations.

First, the neuromodulatory and signalling network is complex: several neuropeptides or amines may act together to modulate a given behaviour, while a single neuromodulator may regulate several behaviours (Harris-Warrick and Marder, 1991; Adamo, 2013). Although this may fit well with the multidimensionality of parasitic manipulation, it challenges a full understanding of the underlying neurophysiological process.

Second, it does not tell us how the parasite is manipulating its host, nor does it identify the direct target of the parasite's E/S products in the host. There is a causality problem akin to 'the chicken or the egg' causality dilemma: whenever one identifies a functional change associated with the manipulative process or the manipulated phenotype (be it a neurophysiological change, a gene or a protein differentially expressed or produced), is it the cause or a side effect of parasitic manipulation? Unravelling the role of a particular neuromodulator in an altered behaviour is no proof of the direct impact of a parasite on its regulation. However, such a direct modulatory effect of a parasite's secretion on a host's biogenic amine metabolism has been evidenced in the venom of a cockroach parasitoid (for reviews, see Libersat et al., 2009; Libersat and Gal, 2013) (Table 1) and is strongly suspected from the expression of a specific *T. gondii* tyrosine hydroxylase gene modulating dopamine level in rat brain (Prandovszky et al., 2011).

In addition, ethopharmacological approaches have so far attempted to mimic, in uninfected hosts, the alteration of behaviour induced by parasites in infected hosts, but none has reported on the pharmacological reversal of manipulation in the infected-manipulated host. This is necessary to further confirm that parasite modulation of the neuromodulatory system studied does indeed induce the observed change in phenotypic trait.

#### Investigating candidate neuromodulatory pathways: prospects

The multidimensionality of host manipulation by the parasite should be taken into account in the prospective study of candidate neuromodulatory pathways, as it is now acknowledged (Cézilly and Perrot-Minnot, 2005; Thomas et al., 2010; Cézilly and Perrot-Minnot, 2010; Cézilly et al., 2013). Multidimensionality refers to the behavioural, metabolic, immune and neuropathological alterations found in many host-parasite systems where parasitic manipulation has been most thoroughly characterized (Cézilly and Perrot-Minnot, 2005; Cézilly and Perrot-Minnot, 2010) [but see Thomas et al. for a restriction of this definition to phenotypic changes that are involved in transmission processes (Thomas et al., 2010)]. Intra-individual or intra-population correlations between these phenotypic components would be indicative of a 'manipulative syndrome'

(Cézilly and Perrot-Minnot, 2010; Poulin, 2013). Phenotypic correlations between parasite-induced phenotypic alterations and their proximate mechanisms remain to be established. Some of the limitations of the candidate neuromodulatory approach introduced above come from the fact that our understanding of the pattern of parasitic manipulation is still fragmentary, thus limiting our ability to generate hypotheses on the mechanisms underlying a manipulative syndrome. We propose here that a detailed (and un-biased) characterization of a manipulated phenotype by its multidimensional components is necessary to investigate the role of particular neuromodulatory systems (Cézilly et al., 2013). It should be done in two complementary ways: by investigating the existence of a manipulative syndrome at the intra-specific level, and by documenting non-random associations of altered traits across host-parasite associations sharing similar transmission constraints (Cézilly and Perrot-Minnot, 2005; Cézilly and Perrot-Minnot, 2010; Poulin, 2010; Thomas et al., 2010; Cézilly et al., 2013). More specifically, three main questions must be answered. First, are there pleiotropic relationships between the multiple traits altered by a manipulative parasite? Second, does multidimensionality in parasitic manipulation involve altered components of the immune system? Third, are behavioural alterations specific, or do they reflect a more general impairment of cognitive processes?

To address pleiotropy in multidimensionality, pharmacological modulation of the level of a neuromodulator can be achieved using precursors or a rate-limiting enzyme responsible for its synthesis, selective reuptake inhibitors, or a specific neurotoxin (for instance, 5-hydroxytryptophan, fluoxetine and the serotonergic neurotoxin 5,7-dihydroxytryptamine have been classically used to modulate serotonin levels). There is no experimental evidence for pleiotropy between behavioural components of multidimensional parasitic manipulation yet, but the orchestration of such pleiotropic effects by neuromodulatory systems is known, in particular for monoaminergic systems. For instance, mutant *Drosophila* flies that selectively lack the tyrosine hydroxylase enzyme responsible for dopamine biosynthesis in their brain show reduced activity and arousal, are less photophilic, have a lower startle-induced negative geotaxis and impaired climbing ability, and show impaired aversive olfactory learning and decreased motivation to feed (Riemensperger et al., 2011). The similarity of these multiple effects of neural dopamine deficiency in *Drosophila* with parasite-induced behavioural alterations in some well-known host-parasite systems (Fig. 1, Table 1) is striking.

According to the psychoneuroimmune hypothesis of parasitic manipulation and by analogy with a 'sickness syndrome', behavioural adjustments are expected in response to signals from immune cells (Maier and Watkins, 1999; Adamo, 2002; Adamo, 2013; Helluy, 2013). So far, the empirical evidence for the neuroimmune hypothesis of parasitic manipulation is still limited to a few host-parasite systems (Adamo, 2002; Helluy and Thomas, 2010). Cornet and colleagues failed to find any relationship between the intensity of photophobia reversal and immune parameters in gammarids either naturally or experimentally infected by *P. laevis* (Cornet et al., 2009). By contrast, cerebral larva of the trematode *M. papillorobustus* triggers a neuroinflammatory response at the host-parasite interface in the brain of the amphipod *G. insensibilis* (Helluy and Thomas, 2010). Communication between the brain's nervous and immune systems is still best evidenced in the 'sickness behaviour' of infected animals, or in the immune origin of neurodevelopmental disorders and consecutive selective cognitive impairment (Maier, 2003; Bilbo and Schwarz, 2009), independently of parasite transmission

enhancement. Indeed, immune activation and immune products such as the pro-inflammatory cytokines can have a pervasive effect on the brain-mediated host behavioural defence against pathogens and parasites (Maier, 2003). The key role of cytokines in the brain-immune network strongly suggest an important role in behavioural regulation (Maier and Watkins, 1999; Maier, 2003; Adamo, 2013; Helluy, 2013). Given the broad range of behaviours potentially affected by a manipulative parasite, from simple taxis to sensory processing, mood and cognition, the neuroimmune hypothesis of parasitic manipulation deserves deeper investigation.

How specific are behavioural alterations and are more general cognitive processes such as learning, motivation, fear and anxiety-like behaviour worth investigating? Such behaviours have commonly been studied in the neuroethology of vertebrates and invertebrates; however, their modulation by parasites in relation to transmission enhancement has so far been limited to *T. gondii*-infected rodents (Vyas et al., 2007). More specifically, one should clarify whether the altered defensive behaviour of infected-manipulated hosts is the expression of unconditioned responses (reflex or motivational processing) versus conditioned effects (learning processing), by using classical learning paradigms. Two lines of evidence offer a promising avenue for progress in the study of parasitic manipulation in an explicit neuroethological framework. First, several studies have correlated altered serotonergic pathways to parasitic manipulation of a phenotypic trait (Table 1). Interestingly, the serotonergic and dopaminergic systems are implicated in memory and aversive learning in invertebrates (Giurfa, 2006; Il-Han et al., 2010; Riemensperger et al., 2011). In host-parasite systems with trophic transmission, enhancement of memory formation by predator detection could be compromised by infection through disruption of 5-HT-mediated long-term memory formation (Il-Han et al., 2010). Second, plasticity towards long-term environmental changes, learning and memory relies on persistent neurogenesis (Harzsch et al., 1999). Many behaviours, in particular anti-predatory ones, are based on previous experience and hence connected to neuronal plasticity. Life-long neurogenesis occurs in the main integrative centres in insects (mushroom body) and in the olfactory pathway of crustaceans and vertebrates (see Harzsch et al., 1999; Beltz and Sandeman, 2003; Benton et al., 2007). Interestingly, enhanced neurogenesis in the cricket *Nematobius sylvestris* infected with the nematomorph *Paragordius tricuspidatus* has been reported (Thomas et al., 2003; Biron et al., 2005). Given the implication of the serotonergic, dopaminergic and possibly nitregeric systems in behavioural manipulation of several amphipods (Table 1), the reported bidirectional interaction between the nitregeric and serotonergic systems in the modulation of neurogenesis in lobster (Benton et al., 2007) is worth considering in other crustacean and insect models.

Finally, from a methodological point of view, we emphasize the interest in combining ethopharmacology with transcriptomics or proteomics, and with functional genomics such as quantitative RT-PCR. The common strategies employed so far for the identification of neuromodulatory systems (biogenic amines, peptides, NO synthases) have involved differential quantification by chromatography or immunostaining of a single or a few monoamines, amino acids, peptides or enzymes. Complementary strategies could be used to unravel the mechanisms underlying parasitic manipulation, and implemented as follows. The differential screening of the host proteome or transcriptome between infected-manipulated individuals and non-manipulated ones (uninfected and infected) could reveal candidate proteins or

mRNA in the host associated with a manipulated phenotype (as the cause or the consequence of altered behaviour and physiology). Phenotypic engineering *via* pharmacology or gene silencing (RNAi) could then be used to validate these as targets of the manipulative parasite. In parallel, the analysis of a parasite's secretome could be used to screen for stage-dependent molecules released by a manipulative parasite that could trigger the observed phenotypic changes. Following the identification of proteins potentially modulating host behaviour, injection of isolated fractions could be used to validate the role of such molecules in the manipulation process. Finally, the growing availability of expressed sequence tag (EST) databases should also offer the possibility of screening for genes of interest in the parasite's genome, followed by gene expression studies to validate their effects in the manipulation process.

### Conclusions

Candidate neuromodulatory systems are worth investigating to understand the interplay between a manipulative parasite and its host, as illustrated by several case studies. Such an approach can be used either to generate a hypothesis as to which pathway is likely to be targeted by a manipulative parasite, either directly or indirectly, or to indicate which behaviour could be altered, based on a reported change in a particular neuromodulatory system. In addition, phenotypic engineering of host behaviour by pharmacological manipulation would allow estimation of the contribution of a given altered trait to parasite transmission. The future challenge will be (1) to reconcile specificity in host manipulation (if any) to the broad and complex effects of neuromodulatory systems, (2) to better characterize parasitic manipulation in terms of its multidimensional phenotypic components and to understand how these changes are orchestrated by neuroimmune and endocrine systems, and (3) to combine such approaches with omics techniques and functional genomics. We advocate here a detailed characterization of a manipulated phenotype in terms of its multidimensional components, using an integrative approach combining evolutionary ecology (parasite transmission), behaviour (syndrome of manipulation) and neuroethology.

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