Trends in Parasitology

CellPress REVIEWS

Opinion

Reasons to Be Nervous about Flukicide Discovery

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The majority of anthelmintics dysregulate neuromuscular function, a fact most prominent for drugs against nematode parasites. In contrast to the strong knowledge base for nematode neurobiology, resource and tool deficits have prevented similar advances in flatworm parasites since those driven by bioimaging, immunocytochemistry, and neuropeptide biochemistry 20–30 years ago. However, recent developments are encouraging a renaissance in liver fluke neurobiology that can now support flukicide discovery. Emerging data promote neuromuscular signalling components, and especially G protein-coupled receptors (GPCRs), as next-generation targets. Here, we summarise these data and expose some of the new opportunities to accelerate progress towards GPCR-targeted flukicides for *Fasciola hepatica*.

Highlights

Most existing anthelmintics modulate ion channels on helminth excitable membranes, leading to the disruption of neuromuscular function.

GPCRs are targets for more than 30% of the drugs used in human medicine, but only one anthelmintic (emodepside) is known to have a GPCR-directed mode of action.

GPCRs transduce signals from the majority of flatworm neurotransmitters, and have essential functions in the control of motility and reproduction.

Molecular validations of GPCRs have been performed in *Schistosoma mansoni* and *Schmidtea mediterranea* where extensive sequence datasets and functional genomic tools are available.

Fasciola hepatica is now a platform for functional neurobiology, benefitting from a sequenced genome, transcriptome datasets, RNAi methodology, functional assays, and efficient *in vitro* maintenance methods.

More GPCRs have been described in the *F. hepatica* genome than in any other helminth parasite.

Ineffective Flukicides Define the Need for New Control Options

Fasciola spp. liver flukes are digenean trematode parasites with a broad range of mammalian definitive hosts. The *Fasciola* genus defines two species with distinct but overlapping geographical ranges, where *F. hepatica* and *F. gigantica*, respectively, predominate in temperate and tropical locales. Their primary impact is on farmed ruminants, where they were previously (and conservatively) estimated to cause an annual global production loss of more than US$3 billion [1]. Impact estimates from individual countries vary depending on the survey methods used, but taking the UK cattle industry as an example, fasciolosis is estimated to cost farmers £23 million annually [2], while annual impacts of up to US$4.78 billion have been estimated in India [3]. Climate change is predicted to increase these impacts through increased rainfall, by producing more favourable conditions for molluscan intermediate hosts [4]. As a zoonotic agent, *F. hepatica* also poses a threat to human health and causes a neglected tropical disease (NTD) [5]. Of significant concern is the fact that *F. hepatica* strains, resistant to front-line flukicides, have also been reported in humans [6,7].

No commercially viable vaccines are available for liver fluke [8], leaving chemotherapy as the sole control option; in practice, farmers complement chemotherapy with integrated parasite management of their herds [9], taking measures such as restricted grazing, fencing off fluke microhabitats, and using epidemiological forecasting. Anthelmintic resistance is steadily nullifying our arsenal of flukicides. This arsenal currently contains six compounds (albendazole, clorsulon, closantel, nitroxynil, oxyclozanide, and triclabendazole (TCBZ)); *F. hepatica* has evolved resistance in the field to all except oxyclozanide [9]. TCBZ is the flukicide of choice because it is the only available drug that is active against early immature, immature, and adult flukes. Juvenile fluke tunnel through the liver en route from their excystment in the duodenum, to their maturation in the bile ducts. Due to the burrowing of parasites through hepatic tissue and inflammatory damage due to host immune responses against secreted

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immunogenic proteins left by the parasite, this journey causes physical damage to the host liver [10]. This pathology impacts productivity of the host animal and causes financial loss due to liver condemnation at the abattoir. In ovine fasciolosis, this acute stage of the infection can be fatal [2]. In the absence of alternative control options, our continued ability to control *Fasciola* relies on the discovery of new flukicides that are effective against the resistant populations.

Components of flatworm neuromuscular systems have long been considered appealing targets for next-generation anthelmintics, but little progress has been made in the rational discovery of such compounds. In large part this is because the tools and resources needed to facilitate such research have not been available until recently; hence the existing anthelmintics were discovered by empirical phenotypic screening of compound libraries against whole parasites *in vitro* and/or *in vivo* (note that this approach has validated the helminth neuromuscular system as a rich source of anthelmintic targets). The mechanism/target-based screening approach that is now prevalent in the human pharmaceutical industry was not possible in helminths until very recently because we lacked the tools to discover, validate, and screen molecular targets at any significant level. *Schistosoma mansoni* has led the way for flatworm parasites in this regard, where putative anthelmintic targets can now be: (i) identified in a high-quality genome and profiled for expression in several transcriptomes; (ii) functionally validated using RNAi and associated phenotypic assays, and (iii) probed with compound libraries in miniaturised target-based screens. *F. hepatica* is now similarly well resourced, where recent advances in sequence datasets and associated molecular tools and assays designate *Fasciola* as an appealing system for comparative neurobiology, and as a viable flukicide discovery platform.

Anthelmintic Discovery and Flatworm Neuromuscular Systems

That the helminth neuromuscular system (NMS) is a promising repository of anthelmintic targets is not an original observation [11,12]. Indeed, helminth neurobiologists have been labouring under this motivation for several decades. Modern flatworm neurobiology originated in the 1980s/1990s, based on biochemical identification of new transmitters, immunolocalisation of those transmitters within the flatworm NMS, or demonstrations of bioactivity in neuromuscular bioassays. Initially, such studies emphasised the presence and activity of known classical neurotransmitters in flatworm nervous systems, but an important change in perspective was the discovery of several examples of novel neuropeptides. These included the **FMRFamide-like peptide (FLP)** (see Glossary) and **neuropeptide F/Y (NPF/Y)** families [13]. These were of interest because of their structural and functional differences from mammalian neuropeptides, raising the prospect of anthelmintics that might selectively target their receptors for therapeutic effect. FLPs in particular were key to this rationale because, in addition to structural distinction from host peptides, they also triggered profound muscle contraction when applied to flatworm tissue, with pharmacological data implicating **G protein-coupled receptors (GPCRs)** as the major receptor type (Figure 1). NPFs, by contrast, were only shown to be myoexcitatory on trimmed *F. hepatica* juveniles [14] and whole *Mesocestoides corti* [15] at high concentrations relative to FLPs. In common with vertebrate NPYs, NPFs employ cAMP-based signalling pathways [16].

These data paint a picture of flatworm motility as mediated by differentially contracting layers of circular, longitudinal, and diagonal muscle [17], under the control of classical transmitter and peptide receptors, with GPCRs playing a major role. Agonist or antagonist anthelmintics acting at such receptors would be expected to disrupt normal motility by triggering muscle paralysis (either **spastic paralysis** or **flaccid paralysis**). These impacts may not be limited to body-wall

Glossary

Classical neurotransmitter: small-molecule chemical transmitter that typically mediates synaptic communication between neurones and other cells. Chemically synthesised by cytosolic enzymes.
Deorphanisation: the process of matching a receptor with its natural ligand. Commonly performed with receptors expressed in a heterologous system and then screened with putative ligands in an activity or binding assay; the most potent ligand is usually hypothesised to be the natural ligand but can only be confirmed through *in vivo* studies.
Flaccid paralysis: the uncontrolled relaxation of muscle, causing impaired function.
FMRFamide-like peptide (FLP): short peptide with a characteristic C-terminal Arg-Phe-NH_2 motif, related to Phe-Met-Arg-Phe-NH_2, a molluscan cardioexcitatory peptide. FLPs exist across invertebrates and most commonly mediate neuronal and/or neuromuscular signal transmission.
G protein-coupled receptor (GPCR): seven-transmembrane cell-surface receptor. Upon binding of an extracellular ligand, the GPCR mediates signal transduction into the cell interior by recruitment of a heterotrimeric guanine nucleotide-binding protein (G protein).
GRAFS nomenclature: a classification system for G protein-coupled receptors (GPCRs) that divides GPCRs in all organisms into one of five classes (glutamate, rhodopsin, adhesion, frizzled/smoothened, secretin).
Ligand-gated ion channel (LGIC): cell-surface ion channel, gated by binding of a specific ligand. Gating opens the channel, permitting ion flow, and de-/hyper-polarisation of the cell membrane.
Neoblast: pluripotent stem cell in flatworms. Responsible for tissue regeneration in planaria, also now implicated in the growth and development of parasitic flatworms.
Neuropeptide: peptide transmitter released by a neurosecretory cell to mediate synaptic or extrasynaptic communication. Synthesised on precursor peptides by protein synthesis, from which mature

muscle, since there is additional **peptidergic** innervation of the reproductive system, attachment structures, and feeding apparatus [18]. Anthelmintics operating at these neuromuscular receptors could therefore have broad, deleterious impacts on various aspects of parasite biology. Despite their considerable appeal, only two planarian peptide GPCRs have been functionally characterised (see below) [19,20], and we have no functional data on any known parasitic flatworm neuropeptide receptors.

Exploring Anthelmintic Targets in the Flatworm Neuromuscular System

Whilst there is a clear rationale for a GPCR-focused approach to anthelmintic discovery (explored further below), there are other targets within parasite NMSs that are worthy of consideration as putative control targets.

Ligand-Gated Ion Channels

Ligand-gated ion channels (LGICs) are validated as targets by the effects of many nematocidal anthelmintics [21]. *In silico* analyses of flatworm genomes show significantly reduced LGIC complements relative to those of human and *Caenorhabditis elegans* [22–24]. The Cys-Loop superfamily in particular, which incorporates the majority of neurotransmitter-gated channels, shows reduced complexity, with most classified as nicotinic acetylcholine receptors (nAChRs). Four *S. mansoni* channels have been functionally characterised, including two ATP-gated P2X channels [25,26], and a glutamate-gated Cl^- channel consisting of homo- or heteromeric combinations of three subunits [27]. An *S. mansoni* ACh-gated Cl^- channel (ACC) subunit (SmACC-1) yields the most persuasive data from a target validation standpoint; this subunit forms a functional, anionic, homomeric channel [28]. Silencing SmACC-1 triggers a hyperactive motility phenotype, suggesting that it mediates inhibitory signalling. Although localisation data are not available, this channel could be responsible for the inhibitory effects of ACh on schistosome muscle cells [29]. ACC channels are of additional interest because they appear unique to invertebrates, and are distinct from the cationic nAChRs of vertebrates [30].

Neurotransmitter Biosynthesis

Neuropeptides and **classical neurotransmitters** are synthesised by distinct cellular pathways that are filled with potential chokepoint targets. Inhibition of these could obliterate downstream signalling capacity [12]. A counter to this argument is the lack of evidence that drugs could differentiate pharmacologically between parasite and host pathway components (since similar protein sequences exist in the nerves of both host and parasite, and these have not yet been functionally compared). RNAi evidence supports the importance of the synthetic enzymes of ACh [31], GABA [32], and octopamine [33] biosynthesis in planarians. While informative, these data have not yet been confirmed in parasitic flatworms [34].

C-terminal amidation is a post-translational modification that is essential for the bioactivity of some flatworm neuropeptides [35]. The amidating process differs between parasitic flatworms and their hosts: flatworm parasites employ two separate and sequentially acting enzymes known as peptidylglycine-α-hydroxylating monooxygenase (PHM) and peptidylglycine-α-amidating lyase (PAL); vertebrates employ a single bifunctional enzyme known as peptidylglycine-α-amidating monooxygenase (PAM). Both PHM and PAL have been identified in *S. mansoni*, and are catalytically distinct from mammalian PAM [36,37]. While amidation is essential for neuropeptide bioactivity, and we hypothesise that its inhibition would have deleterious consequences, this hypothesis has not yet been confirmed; although PAL RNAi experiments have been performed in *S. mansoni*, these have not yet yielded any measurably aberrant phenotypes [37].

peptides are excised and post-translationally modified.

Neuropeptide F/Y (NPF/Y): long (36–39 amino acid) invertebrate neuropeptides with C-terminal GRPR-Famide/Yamide signatures. Structurally related to vertebrate NPYs, they signal by inhibiting the accumulation of cyclic AMP (cAMP) second messenger.

Peptidergic: refers to nerves that employ peptide neurotransmitters, that is, neuropeptides.

RNA interference: cellular mechanism for the destruction of target mRNAs, triggered by the presence of complementary double-stranded (ds)RNA. Can be triggered by endogenous dsRNA, as a gene-regulatory mechanism, or by exogenous dsRNA as an experimental method for gene silencing.

Spastic paralysis: the uncontrolled contraction of muscle to the point of tonic spasm, causing impaired function.

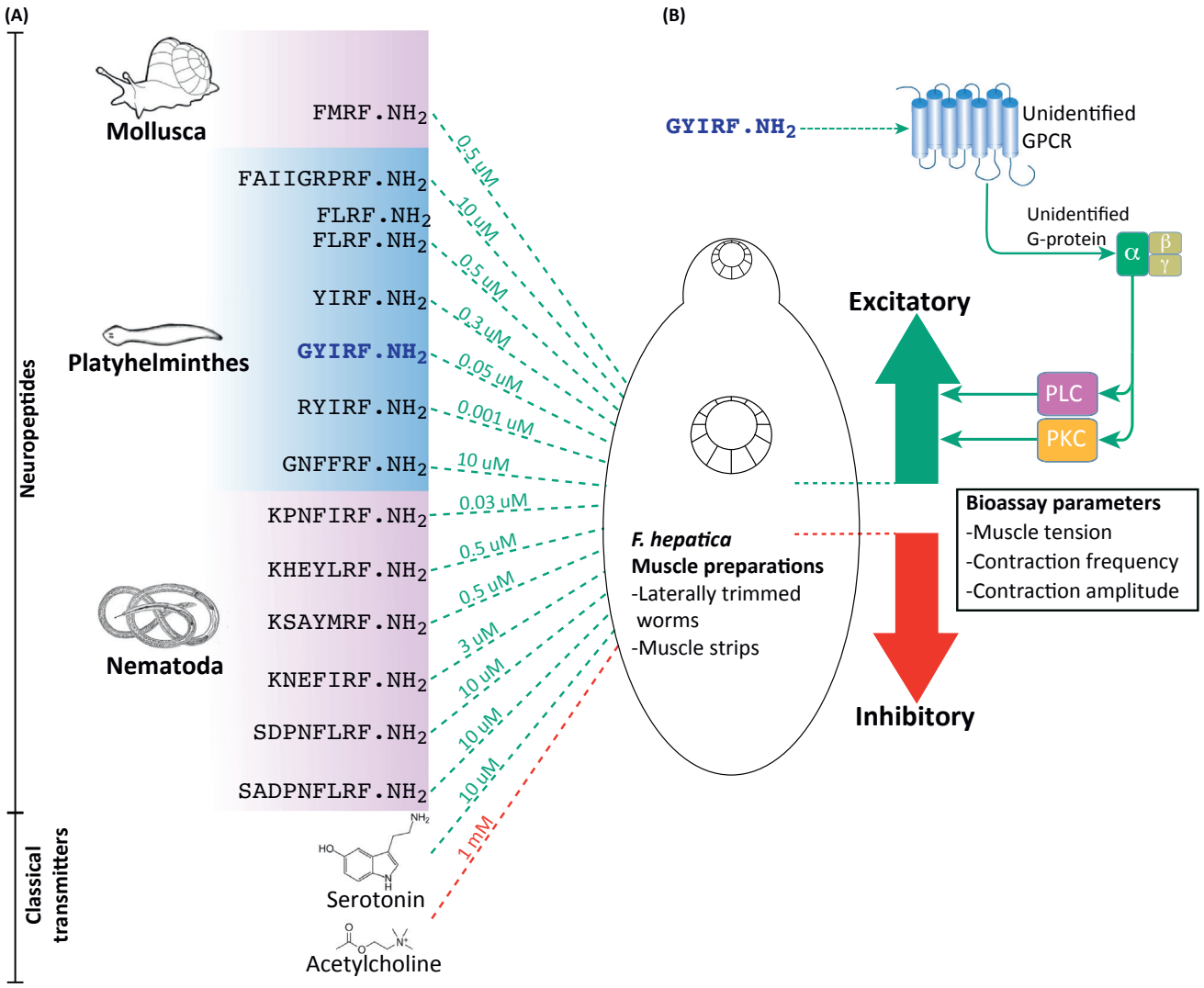

Figure 1. Effects of FMRFamide-like Peptides (FLPs), Neuropeptide F (NPF) and Classical Neurotransmitters on *Fasciola hepatica*. (A) All peptides, and serotonin, have excitatory impacts (green dashed lines); acetylcholine has inhibitory effects (red dashed line) on neuromuscular activity. Peptides originated from the phyla Mollusca, Platyhelminthes and Nematoda as indicated. All peptides are FLPs, except for FAIIGRPRF.NH_2, which represents the C-terminal nine amino acids of the tapeworm *Moniezia expansa* NPF [92]. The minimum reported threshold concentrations are indicated for each transmitter. Data are from neuromuscular bioassays *in vitro,* consisting of either whole trimmed fluke, or fluke muscle strips. (B) Signalling mechanism employed by GYIRFamide: chemical inhibitors implicate signalling through a G protein-coupled receptor (GPCR), with signal transduction through phospholipase C (PLC) and protein kinase C (PKC). See [14,93–97].

Signal Termination

Following activation of synaptic receptors, neurotransmitters must be removed from the synapse to enable the conduction of further impulses, a process known as signal termination. The paralysis of invertebrate crop pests caused by acetylcholinesterase-inhibiting organophosphate pesticides [38] illustrates the impact of inhibiting these processes. Most classical neurotransmitters are removed from synapses by synaptic neurotransmitter reuptake transporters. Several of these have been classified in schistosomes [39]. These are of the sodium-dependent solute carrier (SLC) class that are inhibited by serotonin

(5-hydroxytryptamine; 5-HT)-specific reuptake inhibitor (SSRI) antidepressants, and recreational drugs such as cocaine in the human brain [40]. Silencing of an *S. mansoni* 5-HT transporter (SmSERT) produced a strongly hyperactive phenotype [41], consistent with a role in reuptake of excitatory synaptic 5-HT. ACh and neuropeptide signalling are terminated by enzymatic destruction, respectively by acetylcholinesterases and peptidases. No functional data are available for either of these processes in flatworms [13].

GPCRs Are Druggable, Functionally-Essential Components of the Flatworm NMS

GPCRs are important mediators of signal transduction for a range of flatworm neurotransmitters. They are also druggable targets: 33% of the small-molecule drugs used in human medicine act on GPCRs [42]. The problem remains that we still know little of GPCR diversity or function in *Fasciola.* Can cutting-edge functional genomics data from other parasitic flatworms inform understanding of liver fluke GPCRs?

Eleven flatworm GPCRs have been **deorphanised** to date (Figure 2). These comprise an ACh receptor, two dopamine receptors, a glutamate receptor, two histamine receptors, and a 5-HT receptor from *S. mansoni* [43–49]. The remainder are from planarians, including two 5-HT receptors from *Dugesia japonicum* and *Dugesia tigrina* [50,51], a FLP receptor from *D. tigrina* [20], and an NPF/Y receptor from *Schmidtea mediterranea* [19]. An additional challenge in the path to flukicide discovery is assay miniaturisation to facilitate high-content screening of test compounds. This has been demonstrated for the *S. mansoni* 5-HT receptor [52], a method which has enabled the discovery of new ligands and receptor properties [53,54]. Three flatworm GPCRs bear additional functional information, including two *S. mansoni* GPCRs – a G protein-coupled ACh receptor (SmGAR) [28], and a 5-HT receptor (Sm5HTR) [49]. Both of these trigger reduced motility phenotypes following RNAi. These are the only parasitic flatworm GPCRs proven to be functionally essential, with their importance for parasite motility identifying them as appealing anthelmintic targets. The third functionally annotated flatworm GPCR is a 5-HT receptor from *D. japonicum* (S7.1) [55], for which RNAi triggered impaired motility, and increased the proportion of praziquantel (PZQ)-induced anterior bipolarity (two-headed phenotype) in regenerating worms.

At the time of writing, no parasite peptide-activated GPCRs have been deorphanised. This may reflect the absence of concerted efforts in this regard and/or the unsuitability of the existing heterologous cell (mammalian, insect) expression systems for these receptors. One FLP and one NPF/Y receptor from planarians have been deorphanised [19,20]. Knockdown of the latter (SmedNPYR-1), and its ligand (Smed-NPY-8), removed differentiated germ cells from the gonads, reversing sexual maturity [19,56]. These data encourage the identification and study of parasite NPY receptor (NPYR) orthologues, to determine whether they are similarly important for parasite reproduction. Mirroring the SmedNPYR-1 RNAi phenotype in adult *Fasciola*, for example, would provide powerful support for such a protein's candidacy as a control target.

The Rise of *F. hepatica* as an Experimental System

Despite the focus of this article being the nervous system and flukicide discovery, the dearth of data for *Fasciola* in the work cited above highlights the lack of experimental tools that have served to impede progress in rational approaches to target identification/validation in liver fluke. Until now, *S. mansoni* (with some contribution from planarians) has been the major focus for such experiments. While a useful source of hypotheses for *Fasciola*, such insights are tempered by the many differences between *Fasciola* and schistosomes, not least in the anthelmintics

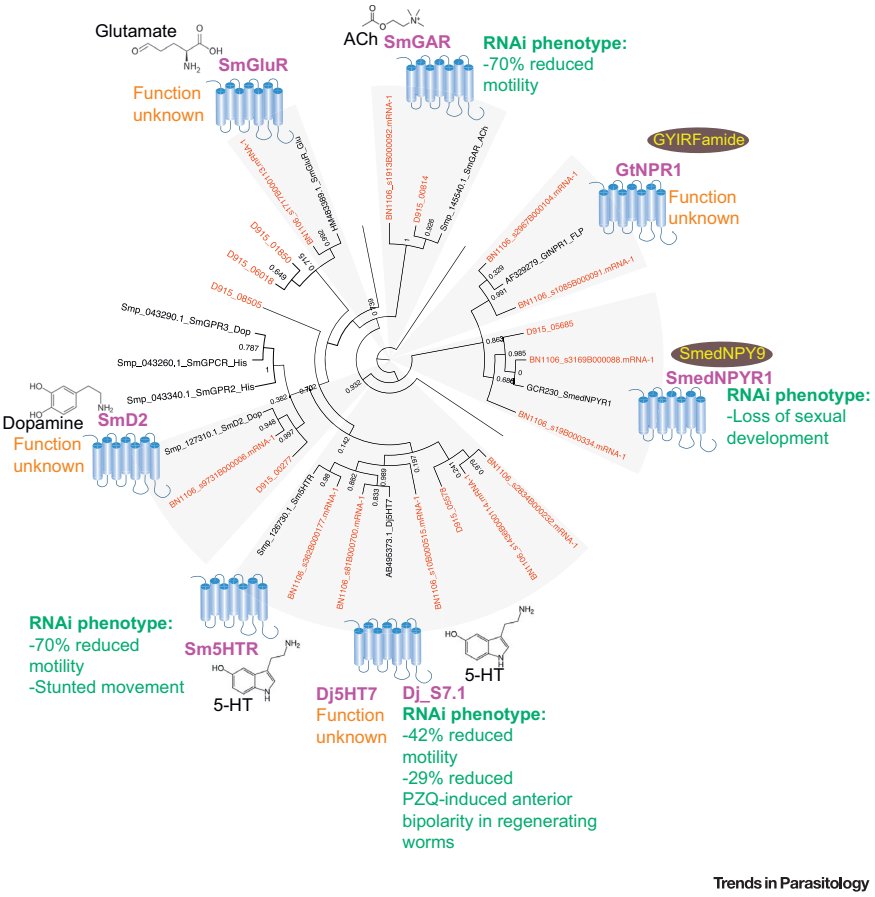

Figure 2. *Fasciola hepatica* Orthologues of Deorphanised Flatworm G protein-coupled receptors (GPCRs). Maximum likelihood phylogeny of ten flatworm GPCRs deorphanised in heterologous systems (black), alongside their closest orthologues from *F. hepatica* (red). *F. hepatica* displays seven orthologues of deorphanised GPCRs, illustrated by a GPCR graphic alongside the receptor name, ligand, and function (RNAi phenotype) where known. Species prefixes: Dj, *Dugesia japonicum*; Gt, *Girardia* (= *Dugesia*) *tigrina;* Sm, *Schistosoma mansoni*; Smed, *Schmidtea mediterranea*. Phylogenetic tree adapted from McVeigh *et al.* [75]. Abbreviations: D2, dopamine receptor; GAR, G-protein-coupled acetylcholine receptor; GluR, glutamate receptor; GYIRFamide, abbreviated primary sequence of flatworm neuropeptide; NPR1, neuropeptide receptor #1; NPY9, neuropeptide Y #9; NPYR1, neuropeptide Y receptor #1; 5HTR/5HT7, 5-hydroxytryptamine receptor. See [19,20,43–46,49,50,55].

used to control them. Schistosomiasis (*S. mansoni*) is controlled by praziquantel (PZQ) or oxamniquine (OX), with PZQ considered the drug of choice due to its broad activity against all schistosome species. PZQ is also effective against the majority of human cestode parasites, and even against other liver flukes (*Clonorchis sinensis*, *Opisthorchis viverrini*, *Opisthorchis felineus*), but it is ineffective against *Fasciola* [57]. OX is effective only against *S. mansoni* [58], and is not considered an effective flukicide. The effectiveness of fasciolicides against human schistosomiasis is similarly unconvincing: albendazole's efficacy in urinary schistosomiasis [59] is supported by limited evidence; clorsulon has schistosomicidal activity in murine infections [60], while triclabendazole has only weak and inconsistent schistosomicidal activities [61]. Although closantel inhibits schistosome motility *in vitro* [62], it is not an effective schistosomicide *in vivo*. There is no evidence for the impacts of either nitroxynil or oxyclozanide on schistosomes. *Fasciola* and *Schistosoma* also differ in basic biology – they have very different life histories, adopt different reproductive strategies (schistosomes are dioecious, *Fasciola* are

hermaphroditic), and differ at the molecular level (the *F. hepatica* genome is approximately four times larger than that of *S. mansoni* [22,63]). In terms of neurobiology, neither species' nervous system has been mapped in fine detail; although some neurotransmitters have been immunolocalised in both species, there are no data on cell–cell synaptic connectivities in any flatworm that would enable comparative analysis of expression patterns between *Schistosoma* and *Fasciola*. All these differences make it difficult to rationalise comparative datasets between these flukes. So, while direct orthologues of receptors and signalling molecules might exist in both species, they could have species-specific functions and target utility. Experiments would preferably be performed directly in *Fasciola*, using a tailored suite of tools and resources. This would have the additional benefit of providing comparative data from at least two systems that would enhance understanding of flatworm NMS function more broadly. Only within the past few years has such research in *Fasciola* become possible thanks to development of an appropriate molecular toolbox.

What's inside the *Fasciola* Molecular Toolbox?

Figure 3 describes the tools and resources currently available for *F. hepatica*. The transcriptomes of intrahepatic *Fasciola* [64,65] were the first high-quality sequence datasets for liver fluke, and represented a significant advance from the small expressed sequence tag (EST) dataset that had previously been available [66]. These resources are now complemented by two independent *F. hepatica* genomes from geographically distinct fluke, and a series of developmentally staged transcriptome datasets [63,67]. These resources permit, for the first time, genome-wide identification of the sequence diversity and developmental expression of putative control targets.

Genomic and transcriptomic datasets are essential starting points for interrogation with functional-genomic tools. We, and others, have made successful efforts to develop and optimise a functional genomics platform in *F. hepatica* based on **RNA interference (RNAi)** technology [68–71]. RNAi is a powerful tool because it permits transcriptional silencing in nonmodel organisms where genetic manipulations are difficult. As such, it is ideally suited to functional studies of liver fluke (RNAi also appears functional in *F. gigantica* [70]). In support of these methods we have also addressed a key impediment of *in vitro* study, by developing a simple maintenance system in which juvenile flukes grow and survive for more than 1 year [72]. This method is a major advance because it supports *in vitro* analysis of gut and reproductive development using simple tools, and enables the long-term quantification of RNAi or chemotherapy phenotypes based around development, survival, and **neoblast** proliferation. We have developed additional assays for quantifying key neuromuscular phenotypes of motility and migration, and have used these to show that silencing calmodulins impacts the growth and motility of juvenile *F. hepatica* [73]. Tools for transcript localisation also exist; as a complement to immunocytochemical methods [73] we have adapted *in situ* hybridisation (ISH) methods from *Schmidtea* [74] (Figure 3). These tools enable spatial localisation of mRNA transcripts as well as potentially spatial deorphanisation of putative receptors and ligands based on colocalisation profiles.

Exploring GPCR Biology in *F. hepatica*

To recap, GPCRs are prime flukicide targets because they: (i) are involved in neuromuscular control of key parasite functions; (ii) represent the majority of NMS receptors, since they transduce signals from all known neuropeptides and many classical transmitters; and (iii) have well-established druggability – GPCRs are molecular targets for approximately 33% of the small-molecule drugs used in human medicine [42]. How can we exploit *Fasciola*'s molecular toolbox to understand liver fluke GPCRs in the context of flukicide discovery?

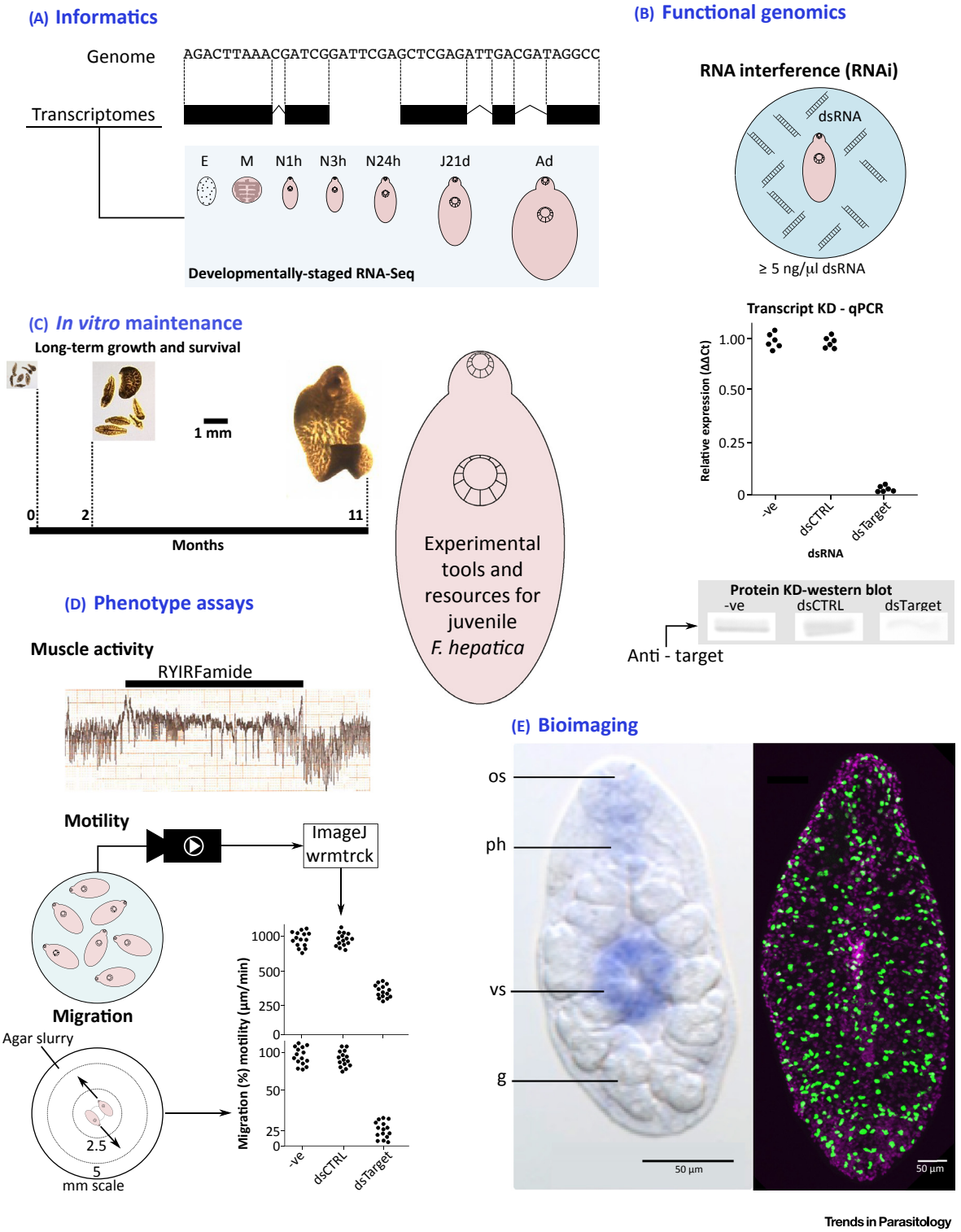

Figure 3. The *Fasciola hepatica* Molecular Toolbox. Neurobiology and flukicide discovery research benefits from *Fasciola*'s growing molecular toolbox. (A) Two genomes are available from geographically distinct *F. hepatica*, as well as transcriptomes from developmentally staged intramammalian fluke (Ad, adult; E, Egg; J21d, *(See figure legend on the bottom of the next page.)*

To promote the study of GPCR biology in *Fasciola*, we have profiled GPCRs from the *F. hepatica* genome [75]. Using homology-driven searches of the *F. hepatica* genome [63,67] and a stringent annotation pipeline, we identified 147 GPCRs. Under **GRAFS nomenclature** [76], this comprises three glutamate, 135 rhodopsin, three adhesion, five frizzled, and one smoothened GPCR. This designates *F. hepatica* with the largest GPCR complement of any parasitic helminth (117, 64, and 83 GPCRs have been reported in the genomes of *S. mansoni*, *Schistosoma haematobium* and *Echinococcus multilocularis*, respectively) [23,77,78]. Whether this larger GPCR complement reflects higher functional diversity, or is a consequence of gene duplication within *F. hepatica*'s much larger genome (1.1–1.3 Gb vs 0.1 Gb and 0.3 Gb for *F. hepatica, E. multilocularis* and *Schistosoma* spp., respectively [22,23,63,67]) is unclear at present. Clearly, functional analyses are essential to our understanding of liver fluke GPCRs. Below, we suggest some areas of *Fasciola* GPCR biology that have significant potential for liver fluke control, and can be studied with the tools and resources now available in *Fasciola*.

Are Flatworm-Specific Rhodopsins Divergent Peptide Receptors?

Rhodopsin-class GPCRs are important because they represent the largest class of GPCRs, and include receptors for a wide range of neurotransmitters, as well as photoactivated opsins, such as are found in invertebrate photoreceptor cells. Amongst the 135 rhodopsins in *F. hepatica* [75] are 18 sequences that bear diagnostic rhodopsin motifs, but lack significant sequence similarity with any non-flatworm GPCRs in the ncbi nr database (searchable at https://blast.ncbi.nlm.nih.gov/Blast.cgi). These 'flatworm rhodopsins' (fwRhods) are probably rhodopsins that diverged from a common ancestor within the flatworm lineage. Fifty-eight sequences with the same definition also exist in other flatworm genomes, with the vast majority in trematodes. Phylogenetic analysis places fwRhods as additional members of existing classifications of flatworm-specific rhodopsins, including the platyhelminth rhodopsin orphan family (PROF1) clade in *E. multilocularis, S. mansoni*, and *S. mediterranea* [23,77], and the srfa, srfb, srfc, RhoL and RhoR clusters from *S. mediterranea* [19].

These data are interesting from an evolutionary standpoint, suggesting that these rhodopsins have diverged in the phylum Platyhelminthes until they show very little overall similarity with non-flatworm rhodopsins. Key questions are whether they have coevolved with similarly unique ligands, or whether their divergence indicates new functionality not seen in other rhodopsins. These questions lack answers at present since no data exist on their expression patterns, ligand interactions, or functions. If the protein sequence divergence of fwRhods reflects difference in function or pharmacology that can be harnessed, they would have obvious appeal as anthelmintic/flukicide targets with high selectivity for parasite over host receptors. To clarify these uncertainties we must address three key questions. First, what are their functions? Transcriptomic data show that several fwRhods are expressed most abundantly in invasive juveniles [75], the most pathogenic stage of the *Fasciola* life cycle. *In vitro* RNAi phenotypes associating with motility, sensory function, growth or protease secretion (all of

ex vivo liver stage juveniles, 21 days post rat infection; M, metacercariae; N1h, newly-excysted juvenile (NEJ) 1h post-excystment; N3h, NEJ 3h; N24h, NEJ 24h) [63,67,75]. (B) RNA interference (RNAi) can be triggered in *F. hepatica* by incubation of juvenile flukes in double-stranded (ds)RNA trigger, with impacts on target transcript monitored by quantitative (q)PCR, and suppression of target protein monitored using Western blot [68–71,73]. (C) Juvenile worms can be maintained for at least 11 months *in vitro*, using standard cell culture conditions [72]. Images show *in vitro* fluke growth over time, all three images shown at the same 1 mm scale. (D) Phenotype assays, which can be used in concert with RNAi or drug screens, include force-transducer measurement of muscle activity in whole worms (trace shows excitatory impact of RYIRFamide neuropeptide on whole fluke), digital quantification of whole worm motility using video analysis and ImageJ software, and migration of juveniles through a soft agar substrate [14,49,73,93,95]. (E) Bioimaging methods include *in situ* hybridisation (left panel; image shows localisation of paramyosin transcript in muscle of ventral sucker (vs), oral sucker (os) and pharynx (ph)) and confocal scanning laser microscopy showing mitotic activity of neoblast-like cells (right panel shows a juvenile fluke following incubation in 5-ethynyl-2-deoxyuridine, EdU, such that proliferating cells appear green) [72,74]. Drawings are not to scale; data in B and D graphs are representative.

which are quantifiable using existing assays), would provide compelling support for fwRhods as appealing receptors that, if targeted, could diminish the pathogenicity of juvenile liver fluke. Second, in which tissues/cells are they expressed? Localisation data would complement RNAi-derived functional insights, and could be achieved using commonly used immunolocalisation methods (following generation of appropriately sensitive and specific antisera), or through ISH. Colocalisation of receptors with putative ligands would also help to answer the third pertinent question on fwRhod biology – what are their ligands? Phylogenetic analysis suggests that fwRhods are more similar to peptide than amine receptors [75], so a testable hypothesis is that they represent divergent peptide receptors. If they can be successfully expressed in a heterologous screening system, a rational starting point for deorphanisation efforts would be to probe them with fluke neuropeptides. We have identified 36 neuropeptide precursor (*npp*) genes/transcripts in *F. hepatica,* encoding 43 predicted mature peptides. Most of these encode peptide motifs not seen in mammalian systems, reflecting the diversity of fwRhod receptors. Identifying a receptor's native ligand can provide a starting point for the development of mimetic ligands as receptor-targeting therapeutics [79,80].

Neuromuscular GPCRs Are Expressed in Invasive Juveniles

Interrogation of *F. hepatica* RNA-Seq datasets supports the expression of 101 GPCRs across egg, metacercariae, newly excysted juvenile (NEJ), liver-stage juvenile and adult datasets [63,75]. Particularly intriguing is that 64 GPCRs show their highest relative expression in NEJ or liver-stage juveniles. These life stages are the most motile phases of the intramammalian fluke life cycle. This motility is apparent in the neuromuscular coordination exhibited during escape from the metacercarial cyst and transit through the gut mucosa into the peritoneum (NEJ), followed by burrowing into the liver capsule and tunnelling through the liver *en route* to the bile ducts (liver-stage juvenile). These behaviours define the acute phase of infection. Damage caused by liver transit can lead to excessive bleeding and shock, and can be fatal for sheep. Even in cattle, this pathology has economic consequences for farmers through condemnation of damaged livers at slaughter. Since many of the GPCRs expressed in these invasive stages are predicted to have neurotransmitter ligands (e.g., ACh, FLPs, 5-HT), it seems likely that their relatively high expression in these motile parasites reflects their importance to neuromuscular coordination. This hypothesis must be tested in functional genomics experiments coupled with appropriate assays. Chemotherapeutic disruption of these receptors could impede neuromuscular coordination of invasive juveniles, and might hinder juvenile migration and the onset of liver pathology. TCBZ is the only existing flukicide with activity against early immature, immature and adult fluke, but its role in sustainable parasite control is compromised by widespread anthelmintic resistance [9]. GPCRs with core neuromuscular functions in invasive juveniles and adults could facilitate identification of the next generation of flukicides needed to fill the void that would be left by the failure of TCBZ.

Accelerating the Pipeline Towards Deorphanisation

We have established that compelling flukicide targets exist amongst the *F. hepatica* GPCR cohort. In concert with functional classification, a directed drug-discovery approach requires knowledge of the receptor's native ligand [79,80]. These data are most often gathered by functional expression of a receptor in a heterologous expression system, followed by the screening of putative compounds in an *in vitro* receptor-activation assay. Without pre-existing knowledge of the identity of putative ligands, screening is performed blind – this is a difficult task given the sheer number of potential GPCR ligands. Fortunately, in the context of NMS function, the majority of receptors of interest are within the rhodopsin family. We can use homology tools to delineate rhodopsins as peptidergic or aminergic. Within these categories, we have placed 17 GPCRs on a high-confidence list of priority candidates for deorphanisation. These

candidates were classified by positional conservation of ligand-interacting residues relative to those in mutationally verified GPCRs from model species. These classifications increase the confidence of predictions based on phylogenetic or homology analyses [75]. These priority GPCRs include seven octopamine receptors, five 5-HT receptors, two acetylcholine receptors, and three NPF/Y receptors, classified with respect to non-flatworm orthologues [81–89]. These include direct orthologues of the deorphanised and functionally characterised *S. mansoni* Sm5HTR and SmGAR receptors [43,49], attractive because of their importance for schistosome motility. The NPF/Y GPCRs similarly include orthologues of SmedNPYR-1 [19]. The latter orthologue is an appealing candidate for study because it could enable deorphanisation and functional characterisation of the first parasitic flatworm neuropeptide receptor. Although a neuromuscular function has not yet been demonstrated for octopamine in flatworms, octopamine receptors have similar rationale since they would provide the first direct evidence for octopamine signalling in parasitic flatworms. Providing an *in silico* method for the prioritisation of candidate receptors could help to 'accelerate the pipeline' towards deorphanisation of these *Fasciola* GPCRs.

Concluding Remarks

Despite major importance for food security, human zoonotic medicine, and the widespread onset of resistance to existing flukicides, there is little evidence for progress in flukicide discovery or target validation. Relatively higher attention has been paid to vaccine development, but with little progress to date in ruminant hosts. From an industrial perspective this asymmetry is difficult to sanction, given the global market for liver fluke control. The global market opportunity for a liver fluke vaccine is estimated at US$182M [9]; since drugs generate relatively higher revenue than vaccines [90,91], there clearly exists a large and sustainable (due to the inevitable development of resistance and consequent need for new drugs, and the lack of a vaccine for liver fluke) market for new flukicides that match or exceed the spectrum of TCBZ.

The recent growth of informatics resources for *Fasciola* and the development of an associated molecular toolkit promote studies on liver fluke biology, which will prioritise targets for validation and screening pipelines. To this end, we believe these new opportunities will reinvigorate research on parasite neuromuscular signalling systems, driving more targeted functional studies and advancing drug-discovery efforts (see Outstanding Questions). In highlighting the research tools now available for *Fasciola*, and by proposing several avenues for investigation, we hope that this article will encourage helminth neurobiologists to consider adopting the *Fasciola* experimental system to help translate their basic research and inform new target-validation studies.

Outstanding Questions

Is neuromuscular function a valid target for the control of juvenile stage liver fluke, and are there nerve/muscle receptors that are common to both juvenile and adult flukes that are critical to their survival?

Do GPCR complements and functions vary between species or between flatworm classes? Can we identify GPCRs with conserved function across cestodes, trematodes, and monogeneans, as broad-spectrum (pan-phylum) anthelmintic targets?

Heterologous expression of helminth GPCRs is challenging. Will we be able to use existing paradigms to heterologously express the receptors with most promise as flukicide targets? Will these paradigms achieve stable expression compatible with industrial compound screening of candidate flukicides? Could flatworm neoblasts be used as vehicles for ectopic GPCR expression and screening?

Does redundancy exist amongst flatworm GPCRs and/or their peptide ligands? If so, is this redundancy reflected *in vivo*, and what implications does this have for therapeutic intervention against these targets?

Could CRISPR/Cas9 genome editing methods be added to the *Fasciola* molecular toolbox? Might the unembryonated egg of *Fasciola* represent a valid target stage for genetic transformation?

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