International Journal for Parasitology 39 (2009) 1243–1252

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Discovery of multiple neuropeptide families in the phylum Platyhelminthes

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ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 20 February 2009
Accepted 11 March 2009

Keywords:
Bioinformatics
Helminth
Neuropeptide precursor
npp

ABSTRACT

Available evidence shows that short amidated neuropeptides are widespread and have important functions within the nervous systems of all flatworms (phylum Platyhelminthes) examined, and could therefore represent a starting point for new lead drug compounds with which to combat parasitic helminth infections. However, only a handful of these peptides have been characterised, the rigorous exploration of the flatworm peptide signalling repertoire having been hindered by the dearth of flatworm genomic data. Through searches of both expressed sequence tags and genomic resources using the basic local alignment search tool (BLAST), we describe 96 neuropeptides on 60 precursors from 10 flatworm species. Most of these (51 predicted peptides on 14 precursors) are novel and are apparently restricted to flatworms; the remainder comprise nine recognised peptide families including FMRFamide-like (FLPs), neuropeptide F (NPF)-like, myomodulin-like, buccalin-like and neuropeptide FF (NPFF)-like peptides; notably, the latter have only previously been reported in vertebrates. Selected peptides were localised immunocytochemically to the *Schistosoma mansoni* nervous system. We also describe several novel flatworm NPFs with structural features characteristic of the vertebrate neuropeptide Y (NPY) superfamily, previously unreported characteristics which support the common ancestry of flatworm NPFs with the NPY-superfamily. Our dataset provides a springboard for investigation of the functional biology and therapeutic potential of neuropeptides in flatworms, simultaneously launching flatworm neurobiology into the post-genomic era.

1. Introduction

The nervous system occupies a position of pivotal importance in flatworm biology. In addition to carrying sensory and neuromuscular signals, it may be responsible for the systemic transmission of developmental and hormonal cues, because as acoelomates these organisms lack the body cavity and circulatory system which would otherwise contribute to such functions. Amidated neuropeptides display constitutive and widespread immunoreactivity in flatworms and are linked to roles in locomotion, reproduction, feeding and larval host-finding (see Marks and Maule, 2008 for review). Physiology studies demonstrate that neuropeptides have contractile and regulatory effects on flatworm muscle and other tissues (Day et al., 1994; Marks et al., 1997; Hrckova et al., 2004; Humphries et al., 2004; Kreshchenko et al., 2008). The breadth of these insights is remarkable given the paucity of structural data on endogenous flatworm neuropeptides – only eight native sequences have been described, defining two structural families: four FMRFamide-like peptides (FLPs; short bioactive peptides with C-terminal -RF.NH_2 motifs) and four neuropeptide Fs (NPFs; 36–39 amino acid peptides with conserved C-terminal $Y_{(-17)}Y_{(-10)}$GRPRF.NH_2 motif) (Curry et al., 1992; Maule et al., 1992, 1993, 1994; Johnston et al., 1995, 1996). FLPs and NPFs are functionally different, reflecting their distinct structures. Available evidence suggests that FLPs function at the flatworm neuromuscular synapse/junction, with a clear myoexcitatory effect on muscle strips and dispersed/individual muscle fibres in vitro (Day et al., 1994; Marks et al., 1997; Moneypenny et al., 2001). These monospecific effects suggest the presence of a single, muscle-based, FLP receptor (Day et al., 1994, 1997). Schistosome NPF displays a potent inhibition of forskolin-stimulated cAMP accumulation in schistosome homogenates (Humphries et al., 2004), indicating conservation of the archetypal neuropeptide Y (NPY) receptor signalling mechanism by NPF receptors. Additionally in regenerating turbellarians, NPF appears to stimulate the mitotic division of neoblasts, the "stem cells" involved in regenerating the head and associated neuromusculature after decapitation (Kreshchenko et al., 2008). This finding suggests that flatworm

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0020-7519/$36.00
doi:10.1016/j.ijpara.2009.03.005

neuropeptides may have core functions in controlling neoblast proliferation during regeneration.

The importance of peptide signalling in flatworm biology implies that recently characterised neuropeptide receptors (G-protein coupled receptors, GPCRs) and associated signalling mechanisms (Omar et al., 2007) represent attractive targets for novel anthelmintics. Improved data on the primary structures of native neuropeptide ligands will provide a source of molecular templates to guide the development of peptidomimetics with potential as next-generation anthelmintics (Greenwood et al., 2005). Despite successful applications of mass spectrometry (MS) to identification of neuropeptides and neurohormones in several invertebrates (Husson et al., 2005; Behrens et al., 2008; Ma et al., 2008; Weaver and Audsley, 2008), no such studies have yet been performed on flatworms. MS-based neuropeptidomic studies are performed on either dissected neural cells/tissues, or gram-scale homogenisations of whole worms (Hummon et al., 2006). Currently, parasitic flatworms preclude both of these methods: the former because flatworms are acoelomate, compromising the selective recovery of neural tissues from surrounding parenchyma and thereby offering source tissues with a very low neural to non-neural tissue ratio; the latter because parasitic flatworms must be cultured in animal hosts, and are therefore extremely difficult to obtain in large quantities. Coupled to these technical difficulties, flatworm neurobiology has suffered from a scarcity of bioinformatic datasets such that knowledge of flatworm neuropeptide diversity has remained static for ~15 years. Only recently have exploitable genomic and transcriptomic datasets provided the opportunity to expand this knowledge-base.

The primary aim of this work was to improve our understanding of native flatworm neuropeptides using in silico analyses of neuropeptide-encoding transcripts. The approach to their discovery utilised web-based basic local alignment search tool (BLAST) searches to identify potential neuropeptide precursors in expressed sequence tag (EST) and genomic databases that are available for six many parasitic species (*Clonorchis sinensis, Echinococcus granulosus, Opisthorchis viverrini, Schistosoma japonicum, Schistosoma mansoni, Taenia solium*), well as a number of free-living forms that serve as model organisms (*Dugesia ryukyuensis*, *Macrostomum lignano* and *Schmidtea mediterranea*). Our initial approach using BLAST searches was followed up by manual curation/inspection and led to the identification of 60 neuropeptide precursors incorporating 96 short peptide transmitters in 10 species of flatworm. Two of the predicted mature peptides were chosen for immunodetection and shown to be expressed in schistosomes.

2. Materials and methods

2.1. Bioinformatics

An approach based on using BLAST searches (Altschul et al., 1990) was employed to identify putative neuropeptides from phylum Platyhelminthes. In an initial specific approach, known invertebrate neuropeptides flanked by "KR" di-basic cleavage sites were used as search queries. These included adipokinetic and related hormones, allatostatins, bombesins, corazonins, diuretic and antidiuretic hormones, myosuppressins, pigment dispersing factor peptides, proctolins, prothoracicotropic hormones, pyrokinins, sulfakinins, tachykinins and related peptides, and vertebrate opioid peptides. All of these search sequences were derived from multiple source species (Supplementary Fig. S1), but provided no positive matches in flatworms. Latterly, a novel approach using BLAST searches was employed using "degenerate" search strings, designed around the core features of small amidated peptide precursors. Degenerate search strings were constructed according to the template $KRX_{3-11}GKRX_{3-11}GKRX_{3-11}GKRX_{3-11}GKR$, where KR represents prohormone convertase-specific cleavage sites, X_{3-11} represents the variable length (3–11 amino acids) of small propeptides, while G represents the Glycine (Gly) residue which will be processed for C-terminal amidation. In both approaches, search strings were applied to tBLASTn searches of available platyhelminth ESTs in the Genbank database (http://www.ncbi.nlm.nih.gov) using the National Center for Biotechnology Information (NCBI) BLAST server, as well as the *Schistosoma mansoni* (http://www.genedb.org/genedb/smansoni) and *S. mediterranea* (http://smedgd.neuro.utah.edu) genome databases, and two *M. lignano* EST datasets curated in-house at the University of Innsbruck (http://flatworm.uibk.ac.at/mac-est/). Expect values >100,000 were employed to account for the short length of many of the queries. Returns were translated (http://www.expasy.ch/tools/dna.html) and manually examined for neuropeptide motifs and cleavage sites. Cleavage site prediction was supplemented by the ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/) (Duckert et al., 2004). Finally, each prepropeptide sequence was analysed for the presence of an N-terminal secretory signal peptide using the SignalP 3.0 online server (http://www.cbs.dtu.dk/services/SignalP) (Emanuelsson et al., 2007). Sequences were examined for similarity with other genes/proteins using BLASTp and BLASTx tools (http://www.ncbi.nlm.nih.gov/BLAST), as well as InterProScan (http://www.ebi.ac.uk/InterProScan) (Zdobnov and Apweiler, 2001). Finally, transcripts were scored out of five according to how many of the following criteria they fulfilled: (1) Incorporate single or multiple copies of peptide intermediates; (2) Are flanked by mono- or dibasic (K, R, KR, KK or RR) cleavage sites; (3) Possess a C-terminal Gly residue, indicative of post-translational processing to confer amidation in the mature peptide; (4) Possess a hydrophobic N-terminal secretory signal peptide sequence; (5) Display sequence similarity to characterised neuropeptides. Scores can be seen in Supplementary Figs. S2–S26.

2.2. Immunocytochemistry (ICC)

Polyclonal primary antisera were generated by Genosphere, France (anti-GFVRIamide), EZBiolab, USA (anti-AAYMDLPWamide), or in-house (anti-FMRFamide, and anti-NPF, the latter raised against the C-terminal decapeptide, YFAIIGRPRFamide, of *Moniezia expansa* NPF) in New Zealand white rabbits. Adult *S. mansoni*, supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD, USA, were flat-fixed between microscope slides immersed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M PBS (pH7.4), for 4 h at 4 °C, then stored in antibody diluent (AbD, 0.1 M PBS containing 0.1% (v/v) Triton X-100, 0.1% (w/v) BSA and 0.1% (w/v) NaN_3) at 4 °C. Fixed worms were incubated in primary antiserum (diluted 1/200–1/1600 in AbD) for 96 h, followed by a 24 h wash in AbD. Secondary antiserum (goat-anti-rabbit IgG, conjugated to FITC, Sigma–Aldrich), diluted 1/100 in AbD, was applied for 48 h, followed by another 24 h wash in AbD. Finally, worms were incubated in phalloidin-TRITC (Sigma–Aldrich) diluted 1/100 in AbD for 72 h. After a final 24 h wash, worms were mounted in PBS/glycerol (1:9 (v/v)) and viewed on a Leica TCS AOBS SP2 confocal scanning laser microscope. All incubation and wash steps were performed at 4 °C. Controls included omission of primary antisera and preadsorption of primary antisera with relevant antigens. Cross reactivity controls were performed by preadsorbing antisera with similar antigens. Images were labelled and plates assembled using Adobe Photoshop software.

3. Results

3.1. Overview

Using a search strategy based on the BLAST algorithm, we believe this is the first study to describe a global dataset of neuropeptide-encoding transcripts from phylum Platyhelminthes. We

identified 60 prepropeptide transcripts (detailed in Supplementary Figs. S2–S26) from 10 species of parasitic and free-living flatworms, which we have grouped by sequence similarity into 25 sequelogs (Table 1). Sequelogs are genes which encode peptides of similar sequence among different species (Varshavsky, 2004), recognising the sequence similarity of peptides present in multiple species, without implying (or dismissing) any functional or evolutionary relationship between peptides. This system has previously been used to describe nematode FLPs and neuropeptide-like proteins (NLPs) (McVeigh et al., 2005, 2008). From the precursors we predicted a total of 96 distinct novel neuropeptides in the phylum as a whole, representing nine structural families of mature peptide (Tables 1 and 2). In individual species, peptide complements range from single peptides in *Echinococcus granulosus*, *Opisthorchis viverrini* and *Clonorchis sinensis* to 33 peptides identified in *M. lignano*.

We propose the title *neuropeptide precursor* (*npp*) for flatworm neuropeptide genes, and have numbered them consecutively as sequelogs, with a species-specific prefix (e.g. *Sm* for *S. mansoni*, *Smed* for *S. mediterranea*; these are detailed in Supplementary Figs. S2–S26). Our numbering system reflects only the order of discovery, and is open-ended to acknowledge the possibility of future discoveries. Since we consider the phylum as a whole, this system imposes a situation where gene numbers are currently not contiguous in most species, but we anticipate that further gene discovery/genome sequencing will fill many of the gaps in Table 1. All candidate secretory peptide precursors were assessed using the five criteria described in Section 2. The strongest candidates have a score of 5/5, with >70% of transcripts fulfilling at least four of the five criteria. All similarity and identity values that follow are expressed as the mean percentage value across each comparable amino acid position between all predicted peptides. Precursors of all the peptides referred to below are illustrated in Supplementary Figs. S2–S26, together with their database accession numbers.

3.2. Some NPPs resemble characterised flatworm, molluscan and/or deuterostome neuropeptides

Two *npp* transcripts encoded peptides remarkably similar to some previously characterised flatworm neuropeptides. A transcript from *M. lignano* (*Ml-npp-23*) encoded 10 copies of YMRFamide (Table 2; Supplementary Fig. S24), a peptide almost identical to both the planarian FLP, YIRFamide, and molluscan FMRFamide (Price and Greenberg, 1977; Johnston et al., 1996). Additionally, a set of peptides bearing a [V/A]FR[F/Y]amide motif, encoded by *Smed-npp-4* in *S. mediterranea* (Table 2; Supplementary Fig. S5), bears notable similarity (57% identity, 86% similarity) to GNFFRFamide, a cestode FLP from *M. expansa* (Maule et al., 1993), although these also resemble molluscan LFRFamides (53% identity, 80% similarity) (Hoek et al., 2005). One of our FLP-encoding transcripts from *S. mansoni* (*Sm-npp-13*) contained two peptides with a C-terminal PQRFamide signature (Table 2; Supplementary Fig. S14) identical to that of vertebrate NPFF and related peptides. Since our methods did not identify similar peptides in any other invertebrate species, this represents the first description of NPFF-like peptides in any invertebrate.

Supplementing the known NPF-encoding cDNAs from *S. mansoni* and *Schistosoma japonicum* (Humphries et al., 2004), and characterised peptides from *M. expansa* (Maule et al., 1992) and *Arthurdendyus triangulatus* (formerly *Arthioposthia triangulata*) (Dougan et al., 2002), we identified novel NPF precursors in the cyclophyllidean cestode *Taenia solium* (*Ts-npp-20*), and the turbellarians *M. lignano* (*Ml-npp-20*) and *S. mediterranea* (*Smed-npp-20*) (Table 1; Fig. 1). In total, NPF-encoding *npp-20* transcripts were identified in seven species. Interestingly, we found multiple NPFs in three species (Fig. 1; Supplementary Fig. S21): these include three distinct NPF-like transcripts in *S. mediterranea* (*Smed-npp-20a*, *-20b* and *-20c*), two distinct NPF-like precursors in *M. lignano* (*Ml-npp-20a* and *-20b*) and a novel second NPF precursor in *S. mansoni* (*Sm-npp-20b*). These represent the first descriptions of multiple NPF genes per se in any flatworm, and the second in invertebrates (Roller et al., 2008 described two NPF-encoding genes in *Bombyx mori*); they are quite distinct from the so-called 'short NPFs' of arthropods which are less than 12 amino acids long and lack the key features of NPY superfamily peptides (Hewes and Taghert, 2001; Huybrechts et al., 2004; Garczynski et al., 2007; Christie, 2008; Ma et al., 2008). All of the NPF peptides that we identified were 36–39 amino acids long and displayed a C-terminal $Y_{(-17)}Y_{(-10)}$RPR[F/Y]amide motif. Additionally, Ml-NPP-20a, Ml-NPP-20b, Sm-NPP-20b and Smed-NPP-20c displayed an N-terminal PP[E/A][R/K]P motif, resembling the polyprolyl PXXPXXP domain of vertebrate NPY superfamily peptides. The six available genomic DNA sequences from *S. mansoni*, *S. mediterranea* and *M. expansa* all displayed conserved phase two introns within the codons of the penultimate arginine residues of the mature peptides, another feature common to vertebrate NPY superfamily genes (Fig. 1).

We identified *npp-6* in five species, encoding peptides with [A/L]XX[L/S]X_oRLamide motifs (where X denotes a variable residue, and X_o denotes a hydrophobic residue) displaying structural similarities with molluscan myomodulin (Table 2; Supplementary Fig. S7). Myomodulin was discovered in molluscs (Cropper et al., 1987), but similar peptides have been recognised in other invertebrates (Christie et al., 1994; Takahashi et al., 1994; Wang et al., 1998; Nathoo et al., 2001). We believe our study provides the first description of myomodulin-like peptides in flatworms. Conservative amino acid differences from molluscan myomodulin (*Lymnaea stagnalis* –MLRLamides) distinguish the NPP-6 peptides from *M. lignano* (AM[R/P]LMRLamide; 46% identity, 80% similarity), *S. mansoni* and *S. japonicum* (AVRLMRLamide; 38% identity, 88% similarity), *E. granulosus* (AIRSLRLamide; 55% identity, 70% similarity) and *O. viverrini* (GLRQLMRLamide; 36% identity, 64% similarity). Buccalin is another peptide first identified in molluscs but recognised in other invertebrates (Cropper et al., 1988; Nathoo et al., 2001). The *M. lignano Ml-npp-9* precursor incorporates 11 copies of a single peptide (GAYSGFLamide) (Table 2; Supplementary Fig. S10), which resembles *Aplysia californica* buccalin (GMDSLAFSGGLamide, 42% identity, 67% similarity). We were unable to identify *npp-9* from any other flatworm species. We believe this finding represents the first description of a putative buccalin-like peptide in phylum Platyhelminthes.

A single transcript from *M. lignano* (*Ml-npp-21*) encoded 15 copies of the tetrapeptide [A/D]PFW (Table 2; Supplementary Fig. S22). These peptides, although not predicted to be amidated since they lack the C-terminal Gly donor, resemble the APGWamides (50% identity, 70% similarity) of the molluscan central nervous system, which are themselves similar to crustacean red pigment concentrating hormone (RPCH) (Kuroki et al., 1990).

3.3. npps *are represented in available genomic datasets*

We used BLAST searches to interrogate all of our EST-derived NPP sequences against *S. mansoni* and *S. mediterranea* genomic data, which represented the two flatworm genome sequences available at the time of our searches (LoVerde et al., 2004; Robb et al., 2008). Using the BLAST tools hosted on the respective genome websites we were able to identify genomic sequences for several of our precursors (Table 1). A total of 11 NPP precursors were identified in *S. mansoni*, including three which were solely genome-derived (*Sm npp-14* through *-16*), with no representative ESTs (although these sequences are represented by *S. japonicum* ESTs). The remaining eight *npp* sequences had dual representation in the *S. mansoni* genome and on ESTs. *S. mediterranea* has 13 *npp* sequelogs, three of which (*Smed-npp-1*, *-21* and *-22*) are only found

Table 1
Neuropeptide precursor (NPP) complement of the phylum Platyhelminthes. Distributions of *npp* genes across flatworm species are detailed, as well as the motifs and proposed similarities of NPP peptides. Numbers in species columns indicate transcript score out of the five criteria described in Section 2.1. Sequences are derived from expressed sequence tags (ESTs) except where indicated.

Structural family	*npp* gene	Encoded peptide motif[a]	Most similarity	*Schistosoma mansoni*	*Schistosoma japonicum*	*Macrostomum lignano*	*Schmidtea mediterranea*	*Dugesia ryukyuensis*	*Dugesia japonica*	*Echinococcus granulosus*	*Opisthorchis viverrini*	*Taenia solium*	*Clonorchis sinensis*
FMRFamide-like peptides (FLPs)	3[b]	AI[L/V]LTR[F/Y]G					4						
	4[b]	-[V/A]FR[F/Y]G	Cestode GNFFRFamide				5						
	11	-N[T/M]RW[P/H/T]SR[L/F/W][G/L]				3							
	13[c]	-F[M/V]PQRFG	Vertebrate neuropeptide FF	5									
	19[b,c]	FX_oF[N/D]L[R/K]DTRWG NXXIYXXESGXRXNXGXRFG		3	4		3						3
	23	YMRFG	Turbellarian YIRFamide; Molluscan FMRFamide			5							
L/M/Iamides	1[c]	-[S/G/A][F/Y]VR[L/M/I]G		4	4	4	4						
	2[b,c]	RGX_oIG		4	4		3	4					
	6[b,c]	[A/L]XX[L/S]X_oRLG	Molluscan myomodulin	5	5	4	4			5	5		
	7	EWQ[R/L]GSRLG				4							
	9	GAYSGFLG	Molluscan buccalin			5							
	14[c]	GLRNMRMG		3	4								
	15[c]	VQFLRLG SAYPYVG		4	4								
	16[c]	-YLWD[V/T]RLG		3	4								
	22	A[K/S]Y[F/I]R[L/M]G				4	4						
	24	-[G/R]LLG				4							
	25	A[Y/W]YXSPRLG				4							
Unamidated peptides	21	APFW	Molluscan APGWamide			4							
	10	-TPS[Y/D]SW						2					
Neuropeptide F (NPF)	20[b,c,d]	$Y_{(-17)}Y_{(-10)}$GRPR[F/Y]G	NPF/Neuropeptide Y superfamily	5	5	5	5					5	
PWamides	5[b,c]	-[A/N][Y/W]X[D/V][M/L/V]PWG		4	4	4	4	4					
SGFamides	8[b]	-Q[R/A]W[S/Y]SG[F/Y]G				3	3						
D_5amides	17[b,c]	DX_3[G/F]G		3	3								
Amidated tripeptides	12[b]	NY[Y/F]G					3		3				
VVamides	18[b]	GAEFFIRRVVG					3	4					

[a] Single letter annotation for amino acids: X, variable amino acid; X_o, hydrophobic amino acid; residues in parentheses are alternatives for that position; subscript numbers indicate the number of intervening amino acids; a hyphen at N-terminus indicates variable (sequence/length) extensions.
[b] Sequelog represented in *S. mediterranea* genome database.
[c] Sequelog represented in *S. mansoni* genome database.
[d] NPF-like peptides are encoded on multiple genes in *S. mansoni*, *M. lignano* and *S. mediterranea*, see Supplementary Figs. S2–S26 for details.

Table 2
Mature peptides predicted from flatworm neuropeptide precursor (*npp*) genes. Peptides were predicted from the prepropeptides detailed in Supplementary Figs. S2–S26. Where there was ambiguity in cleavage site identification, these were selected according to criteria described by McVeigh et al. (2005). Note that NPP-20 peptides are omitted; these are detailed in Fig. 1.

Schistosoma mansoni	***Sj-npp-15***	***Ml-npp-21***	***Smed-npp-12***
Sm-npp-1	VQFLRLamide	13x APFW	3x NYFamide
AFVRLamide	SAYPYVamide	2x DPFW	***Smed-npp-18***
2x GFVRIamide	***Sj-npp-16***	***Ml-npp-22***	2x GAEFFIRRVVamide
Sm-npp-2	NYLWDTRLamide	8x ASYIRMamide	***Smed-npp-19***
GMIamide	SYLWDVRLamide	***Ml-npp-23***	FYFDLRDTRWamide
Sm-npp-5	***Sj-npp-17***	10x YMRFamide	***Smed-npp-22***
AAYMDLPWamide	DDFRGamide	***Ml-npp-24***	3x AKYFRLamide
AAYIDLPWamide	DHRPFamide	GAYYGLLamide	***Dugesia ryukyuensis***
Sm-npp-6	***Sj-npp-19***	GPYHRLLamide	***Dr-npp-2***
AVRLMRLamide	FLFNLRDTRWamide	6x AGYRGLLamide	5x GLIamide
Sm-npp-13	NADIYESESGPRHNIGNRFamide	4x AGFRGLLamide	2x GMIamide
HFMPQRFamide	***Macrostomum lignano***	VYQRLLamide	***Dr-npp-5***
YTRFVPQRFamide	***Ml-npp-1***	***Ml-npp-25***	PNWKDMPWamide
Sm-npp-14	PSFVRMamide	AYYASPRLamide	3x SAWRDMPWamide
GLRNMRMamide	ASYVRMamide	7x AWYVSPRLamide	GAWRDMPWamide
Sm-npp-15	***Ml-npp-5***	AYYKSPRLamide	NAWRDMPWamide
VQFLRLamide	7x AYGVVPWamide	***Schmidtea mediterranea***	***Dr-npp-10***
SAYPYVamide	AYGAVPWamide	***Smed-npp-1***	5x TMTPSYSW
Sm-npp-16	***Ml-npp-6***	SFVRLamide	3x TPSYSW
NYLWDTRLamide	3x AMRLMRLamide	3x ASFVRLamide	4x NTMTPSYSW
Sm-npp-17	AMPLMRLamide	***Smed-npp-2***	NTMTPSYSWI
DDFRGamide	***Ml-npp-7***	7x GLIamide	4x ITMTPSDSW
DHRAFamide	NAWERGSRLamide	***Smed-npp-3***	***Dr-npp-18***
Sm-npp-19	4x EWQRGSRLamide	AILLTRYamide	GAEFFIRRVVamide
FLFNLKDTRWamide	EWQRGSRLS	AIVLTRFamide	***Dugesia japonica***
NHGSRFamide	EWQLGSRLS	***Smed-npp-4***	***Dj-npp-12***
Schistosoma japonicum	***Ml-npp-8***	SSVFRFamide	NYYamide
Sj-npp-1	2x PDQRWSSGFamide	SVAFRFamide	NYFamide
AFVRLamide	3x AGQRWSSGFamide	RGVAFRFamide	***Echinococcus granulosus***
GFVRLamide	AGQRWSSGFV	GSVFRYamide	***Eg-npp-6***
GFVRIamide	***Ml-npp-9***	QSVFRYamide	AIRSLRLamide
Sj-npp-2	11x GAYSGFLamide	***Smed-npp-5***	***Opisthorchis viverrini***
GMIamide	***Ml-npp-11***	PNWKDMPWamide	***Ov-npp-6***
GFMamide	WNTRWTSRLamide	5x SAWRDMPWamide	GLRQLMRLamide
Sj-npp-5	3x WNTRWPSRLamide	***Smed-npp-6***	***Clonorchis sinensis***
AAYMDLPWamide	WNTRWPSRSL	AYRLMRMamide	***Cs-npp-19***
AAYIDLPWamide	WNMRWHSRFamide	AVRLMRMamide	FFFDLRDTRWamide
Sj-npp-6	3x NTRWPSRFamide	AVRLMRLamide	
AVRLMRLamide	2x NMRWPSRLamide	***Smed-npp-8***	
Sj-npp-14	WNTRWPSRFamide	DPRFSDQVWHSGYamide	
GLRNMRMamide	WNTRWPSRWL	NYYNRFDGAWYSGYamide	
	NTRWPSRLamide	NDAFDGAWYSGYamide	
		NYYNRFDGQAWYSGYamide	
		NDAFDGQAWYSGYamide	

on ESTs, and five of which are solely represented in the genome (*Smed-npp-5*, *-6*, *-12*, *-17* and *-18*). The remaining five *Schmidtea* sequelogs display both genomic and EST representatives.

3.4. GFVRIamide and AAYMDLPWamide localise to the schistosome nervous system

To verify the neuropeptide status of our predicted peptides, we raised polyclonal antisera to two novel schistosome peptides – Sm-NPP-1 (GFVRIamide) and Sm-NPP-5 (AAYMDLPWamide) – and used those in standard ICC approaches. Both sera demonstrated strong and specific immunoreactivities (IR) in elements of the *S. mansoni* nervous system (Fig. 2). GFVRIamide (Fig. 2A) displayed restricted expression, localising to a pair of CNS neurones within the brain ganglia which projected posteriorly along the main nerve cords, but was not apparent in any of the peripheral innervation to the somatic muscle or organs. In contrast, AAYMDLPWamide-IR (Fig. 2B) was almost exclusively peripheral in location, with IR in nerves visibly branching from the ventral nerve cords to innervate the somatic muscle on the worm flanks, as well as the oral and ventral suckers, the oesophagus and the female reproductive system.

4. Discussion

We believe that this study reports the first description of new flatworm neuropeptides in ~15 years and represents a major expansion in our knowledge of flatworm neuropeptide biology. We have described 25 *npp* sequelogs in phylum Platyhelminthes which encode putative neuropeptide precursor proteins grouped into nine distinct families of neuropeptide (Table 1). While most of the predicted peptides are completely novel, showing little similarity with peptides from other genera, several NPPs appear similar to characterised neuropeptides from molluscs and even vertebrates. Although none of the previously characterised flatworm FLPs were identified, some similar peptides were predicted. As well as *Smed-npp-4* peptides (-[V/A]FR[F/Y]amides), which display some C-terminal similarity to the *M. expansa* peptide

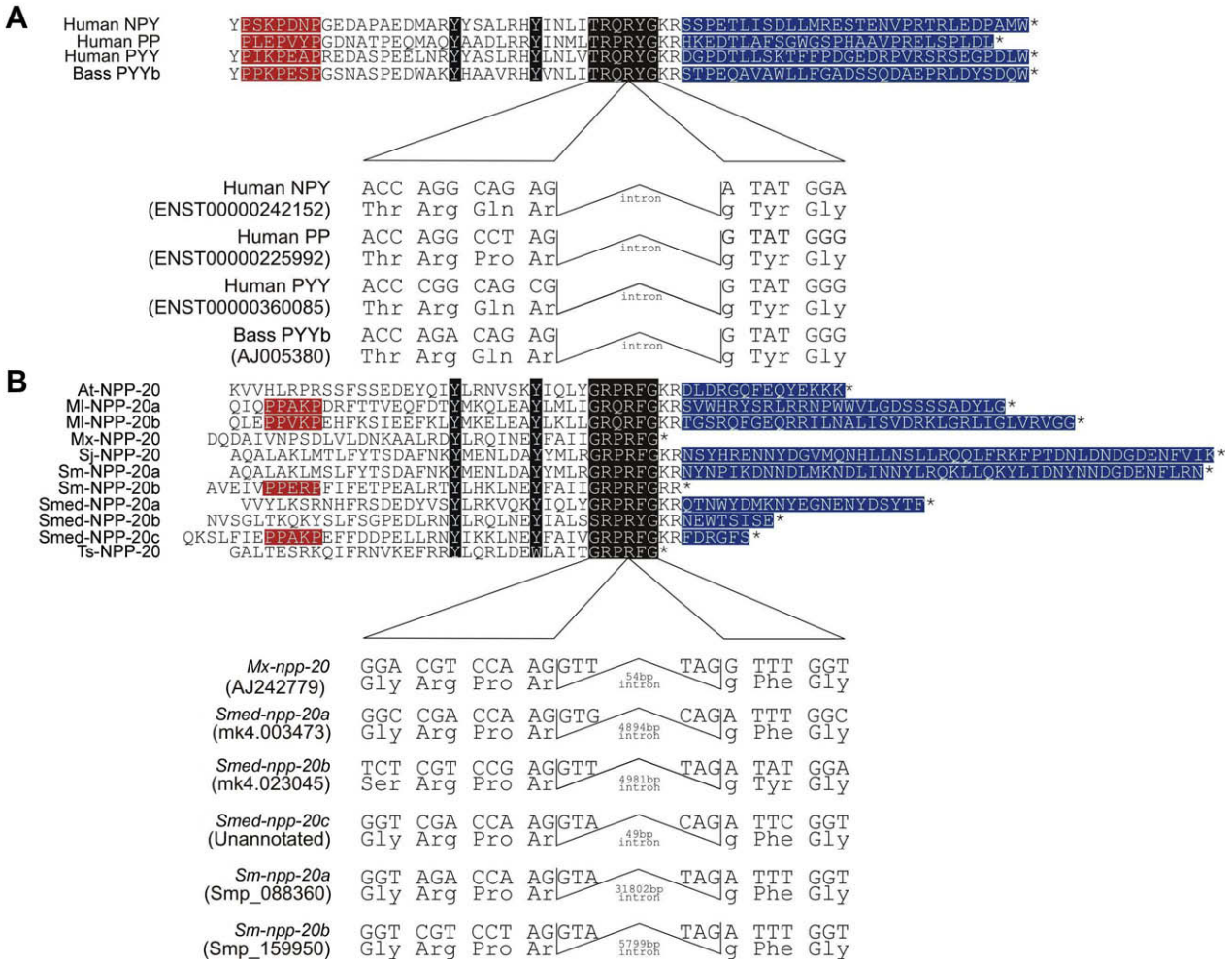

Fig. 1. Similarities of flatworm neuropeptide Fs (NPFs) with the vertebrate neuropeptide Y (NPY) superfamily. (A) Alignment of NPY superfamily members from selected species; PP, pancreatic polypeptide; PYY, peptide tyrosine-tyrosine; PY, peptide tyrosine. Position of conserved intron is indicated. Human sequences obtained from Ensembl (www.ensembl.org). (B) Alignment of all 11 known flatworm NPFs (Neuropeptide Precursor-20, NPP-20) identified by this study and previous work (Mair et al., 2000; Dougan et al., 2002). In (A) and (B), predicted mature peptide sequences are shown, as are C-terminal cleavage recognition sites and/or translation stop signals (*), as well as the C-flanking peptide of NPY (CPON, denoted by blue or dark grey highlighting) where present. Conserved residues characteristic of NPY superfamily members are highlighted in black; polyprolyl domains are highlighted in red (or underlined). Intronic architecture of available genomic *npp-20* sequences is displayed. Genomic accession numbers are provided; relevant databases are detailed in Section 2. *Moniezia expansa* sequence reported in Mair et al. (2000). Intron sizes and positions are indicated. At, *Arthurdendyus triangulatus* (formerly *Artioposthia triangulata*); Ml, *Macrostomum lignano*; Mx, *Moniezia expansa*; Smed, *Schmidtea mediterranea*; Sj, *Schistosoma japonicum*; Sm, *Schistosoma mansoni*; Ts, *Taenia solium*.

GNFFRFamide (Maule et al., 1993), we discovered YMRFamide, an *M. lignano* peptide encoded by *Ml-npp-24*. Previous structure-activity studies on YIRFamide (one of the most potently myoactive peptides tested in flatworm neuromuscular bioassays) showed that substitution of Isoleucine for Methionine at position two did not significantly alter the peptide's potency on *S. mansoni* dispersed muscle fibres (Day et al., 1997), suggesting that YMRFamide should retain a myoexcitatory function. While YIRFamide/YMRFamide have not yet been identified outside of the Turbellaria, YIRFamide's potent contractile effect on schistosome muscle fibres cannot be ignored, and suggests that a similar peptide could reside within the *S. mansoni* genome. In a rational approach to drug discovery, this peptide's muscle-based receptor represents an attractive drug target. The absence of a YIRFamide-like peptide from the *S. mansoni* genome, as well as discrepancies in *Schmidtea*, (where *Smed-npp-1*, *-22* and *-23* are represented by ESTs but do not appear in the current genome database), raise an important point regarding the current levels of genome coverage in these projects. Despite the reported 7x coverage of the *S. mansoni* genome (LoVerde et al., 2004; Haas et al., 2007), and 10x coverage of the *S. mediterranea* genome (Cantarel et al., 2008), our data suggest that some stretches of these genomes remain unsequenced. It is possible that further *npp* genes will be uncovered as genome curation continues to shed new light on these areas.

The overwhelming presence of C-terminal glycine residues on the peptides in our dataset (Table 2) suggests that the vast majority will be C-terminally amidated. We deliberately focused our search protocol on amidated peptides because previous experiments illustrate that C-terminal amidation appears to be one of the key structural components required for maintenance of bioactivity of flatworm neuropeptides (Day et al., 1997). However, in other organisms (particularly *Caenorhabditis elegans*), many non-amidated peptides are present and appear to play important roles in worm biology. While the identifications of *Ml-npp-22* and *Dr-npp-10*, which appear to lack an amidation site, illustrate that our search protocol was capable of uncovering at least some non-amidated peptides, one should note the possibility that further non-amidated peptides await discovery in flatworms.

While several NPPs resemble neuropeptides originally described in flatworms, molluscs and even vertebrates, the majority

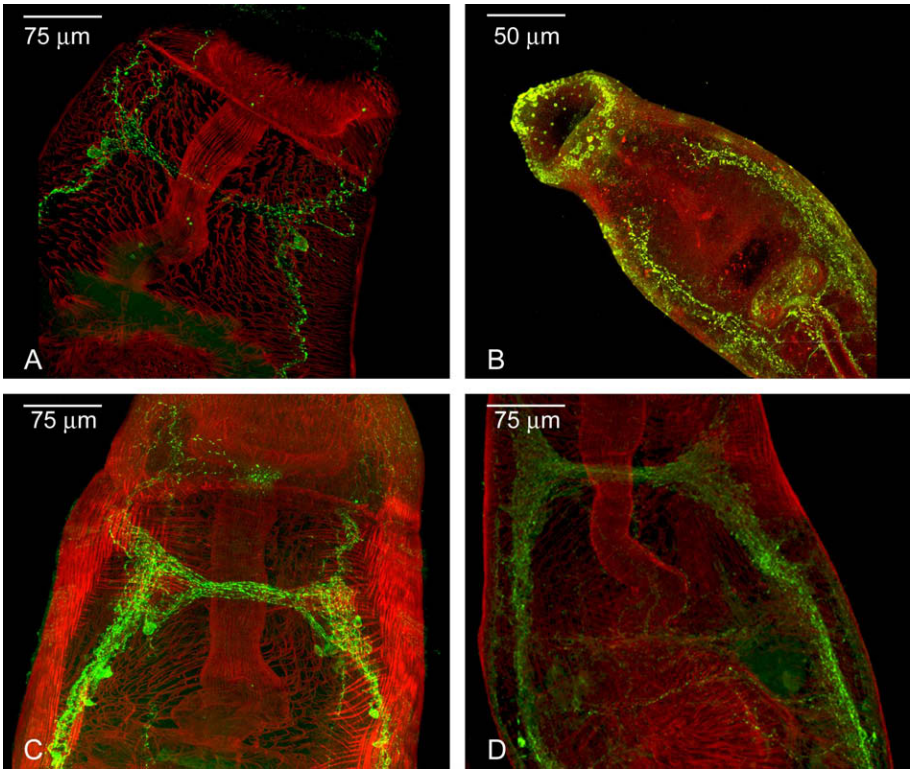

Fig. 2. Immunocytochemical localization of neuropeptides in anterior of adult *Schistosoma mansoni*. (A) GFVRIamide-immunoreactivity (IR) is restricted to two brain neurones which project across the cerebral commissure, anteriorly towards the oral sucker, and posteriorly along the main nerve cords; (B) AAYMDLPWamide-IR appears in peripheral nerves innervating the somatic musculature, oesophagus and oral and ventral suckers; both neuropeptide F-IR (C) and FMRFamide-IR (D) are apparent in the brain, central and peripheral nerves. C and D are shown for comparison of A and B with known neuropeptide expression patterns. In all images, anterior is towards the top of the page; green staining (FITC) represents peptide immunoreactivity, while red staining represents filamentous actin in muscle detected by TRITC-conjugated phalloidin.

of predicted peptides (52 putative peptides encoded by 15 sequelogs) are novel and show little or no obvious sequence similarity with neuropeptides from other genera (Tables 1 and 2). Such pathogen-specific neuropeptides, in their absence from host species, represent particularly promising avenues for drug development. Provided that their endogenous receptors are ligand selective, drugs targeting these receptors should be pathogen-specific and less likely to exhibit host-toxicity through non-specific interactions with host receptors. All of these transcripts score at least 3/5 on our criteria-based system (completely novel peptides can only score a maximum of 4/5, see Section 2), indicating that they are strong candidates for consideration as secretory peptide precursors. The nematode *C. elegans* represents an analogous system which illustrates the validity of our method of bioinformatics-based gene identification/peptide prediction. Most of the currently known *C. elegans* neuropeptide genes were initially identified by similarity-based sequence searches similar to the methods used in the current study (Nelson et al., 1998; Li et al., 1999a, 1999b). To date, the majority of these have been validated by neuronal localisation, knockout analysis and/or peptide physiology – none of those original discoveries have been proven to represent "false positives". Most of our genome-derived peptides are also represented by ESTs, strengthening the validity of their prediction. Even *S. mansoni* genes *Sm-npp-14* through *-18*, while not represented by *S. mansoni* ESTs, are indirectly supported by *S. japonicum* ESTs, suggesting that these genes are probably transcribed.

The main issue encountered in our analyses was the absence of identifiable signal peptides from some precursors (*npp-8*, *-10*, *-11*, *-12* and *-17*). In all of these cases, the associated ESTs represent 3′-directed (or otherwise partial) sequences which do not support full-length open reading frames (ORFs) with an N-terminal signal peptide. Mono- and di-basic cleavage sites were used to predict mature peptide sequences, using both manual annotation and the ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/) (Duckert et al., 2004). It should be noted that until MS methods are developed for, and applied to, flatworms to confirm the presence of ions corresponding to our predicted peptides, those predictions remain equivocal. In the absence of an established neuropeptide comparator with which to validate their status at the level of primary structure, other methods must be used to confirm their identity as neuropeptides. To this end, ICC or in situ hybridisation experiments are required to localise expression of *npp* peptides or transcripts to secretory tissues. As a first step, we raised Sm-NPP-1 (GFVRIamide) and Sm-NPP-5 (AAYMDLPWamide) antisera; both revealed strong and specific IRs in the schistosome nervous system, with distinct staining patterns, confirming these motifs as neuropeptides and strengthening the validity of our approach to neuropeptide discovery and annotation.

Schistosome *Sm-npp-13* encoded two peptides with 100% C-terminal identity (PQRFamide) to NPFF-like peptides. In vertebrates, NPFF (and the closely-related peptides NPAF and NPSF) are thought to be involved in nociception and opiate-induced analgesia (Yang et al., 2008). Peptides with similar motifs exist throughout vertebrates, including mammalian RFamide-related peptides (RFRPs), gonadotropin inhibitory hormone (GnIH) and frog growth hormone releasing peptide (fGHRP), all of which possess PQRFamide or similar motifs (LPXRFamide). Prior to this study, PQRFamides had only been identified in vertebrates, including species belonging to ancient vertebrate lineages (e.g. the agnathan *Petromyzon marinus* (Osugi et al., 2006)). Our identification of PQRFamides in phylum Platyhelminthes represents the first description of these peptides in invertebrates. In light of current opinion on the

molecular phylogeny of animal genera (Ruiz-Trillo et al., 1999; Philippe et al., 2005), the NPFF progenitor may have arisen in a shared ancestor of Lophotrochozoa and Deuterostomia, which would entail the loss of NPFF from Ecdysozoans. Precedent exists for such a relationship; genes have been described which are shared between *S. mansoni* and Deuterostomia but absent from Ecdysozoa (Venancio et al., 2007), these are thought to have been "co-opted" by schistosomes, perhaps by horizontal gene transfer, to perform a role in host-parasite interactions. It is interesting to consider such a role for NPP-13 in schistosomes since NPFF has been shown to stimulate or inhibit T-cell proliferation at low or high concentrations, respectively (Lecron et al., 1992; Minault et al., 1995). The ability to modulate the proliferation of T-cells by the release of Sm-NPP-13 would have obvious survival benefits for an intravascular parasite. However, until Sm-NPP-13 expression is demonstrated in a locale consistent with excretory/secretory release from schistosomes, such a hypothesis remains speculative.

The vertebrate NPY superfamily encompasses four similar, highly conserved peptides that share a common evolutionary origin – NPY, peptide YY (PYY) and pancreatic polypeptide (PP) in tetrapods, and pancreatic peptide Y (PY; now considered to be a duplicate of PYY and renamed PYYb) in fish (Sundstrom et al., 2008). Many invertebrates possess similar peptides which most commonly terminate in phenylalaninamide and are therefore known as NPFs. All NPFs exhibit structural correlates with NPY superfamily peptides – most notably, their C-terminal R[P/Q]R[F/Y]amide resembles the terminal RXR[Y/F]amide motif of the NPY family, while both NPF and NPY-like peptides display conserved tyrosyls at positions 10 and 17 from the C-terminus. At the N-terminus, not all identified flatworm NPFs share the characteristic and functionally important triprolyl (PXXPXXP) domain found in almost all NPY superfamily peptides (Day and Maule, 1999), clouding the relationship of NPF with NPY so that their common ancestry has been considered uncertain. Here, we identified NPF-like genes and peptides in four species of flatworm, four features of which support a common ancestry for NPF and NPY family peptides (Fig. 1): (i) Flatworms possess multiple NPF genes. The NPY family is thought to have arisen by sequential gene duplication events, which in a first instance generated NPY and PYY genes from a single ancestral coding sequence in teleost fishes followed by a local expansion of PYY to PY and PP (Sundstrom et al., 2008). We found three genes encoding NPF-like peptides in *S. mediterranea* (Smed-NPP-20a, -20b and -20c), while *S. mansoni* and *M. lignano* both possessed two (Sm-NPP-20a, -20b and Ml-NPP-20a, -20b, respectively). All seven of the associated transcripts were amplified by reverse transcriptase (RT)-PCR, supporting their expression in adult worms (data not shown). While some evidence exists for a multiplicity of short peptides that resemble NPF in some invertebrates (four peptide YFs [PYFs] in the tiger prawn (Sithigorngul et al., 2002), and multiple variants of "short NPFs" in insects (Hewes and Taghert, 2001; Huybrechts et al., 2004; Garczynski et al., 2007; Christie, 2008; Ma et al., 2008), only from the silkworm, *B. mori*, have true multiple NPF genes been previously described (Roller et al., 2008); we believe our data represent the first description of multiple NPF-encoding genes or NPF-like peptides in any flatworm. (ii) Some flatworm NPFs display N-terminal polyprolyl domains. *S. mansoni* Sm-NPP-20b, *Schmidtea* Smed-NPP-20c and *Macrostomum* Ml-NPP-20a and -20b, all display an N-terminal PPXXP motif, which resembles the PXXPXXP domain of NPY family peptides. This domain is important in allowing NPY family peptides to assume a functionally-important tertiary structure known as the PP fold (Glover et al., 1984). (iii) Flatworm NPF genes share gene structure with the NPY family. All known NPY family peptides have a phase two intron within the codon for their penultimate arginine residue, which was also identified in the *M. expansa* NPF-encoding gene, but failed to amplify in a PCR-based approach in *A. triangulatus* and *S. mansoni* (Mair et al., 2000; Dougan et al., 2002; Humphries et al., 2004). Here, we have identified a further five genomic NPF sequences from the *S. mansoni* and *S. mediterranea* genomes, all of which display introns of variable size in the canonical position at phase 2 in the codon for the penultimate arginine (Fig. 1). (iv) Another typical feature of NPY family genes is the presence of a C-terminal extension of the NPF precursor protein (CPON, C-flanking peptide of NPY), immediately following the NPF peptide. Of the nine flatworm NPFs reported in this study, seven display a CPON domain on their precursor. These four factors, allied with the knowledge that schistosome NPF displays functional conservation of NPY's archetypal cAMP-based signalling mechanism (Humphries et al., 2004), lend strong support to the hypothesis that vertebrate NPY family peptides are evolutionarily related to NPF.

Due to their etiological importance as disease-causing pathogens it is certainly the parasitic members of phylum Platyhelminthes that are of greatest concern to most humans. Therefore, an important application for these data is as a resource for those interested in anthelmintic discovery, since this is, to our knowledge, the first study to describe multiple neuropeptide ligands from flatworms which can be used to "deorphanise" peptide receptors (such as the recently-reported *Girardia tigrina* GPCR (Omar et al., 2007)) following their heterologous expression. Given the importance of neuropeptides in the core neuromuscular functions of parasitic flatworms, we anticipate that some neuropeptide receptors could represent attractive, rational targets against which new, non-peptide, anthelmintic drugs could be directed (Mousley et al., 2004); indeed, a rich seam of potential receptors await exploitation, since preliminary screening suggests that both *S. mansoni* and *S. mediterranea* genomes contain at least 20 neuropeptide-like GPCR genes (M. Zamanian, unpublished data). Attention must be paid to investigating the bioactivity of new neuropeptides if we are to validate and prioritise their receptors in terms of drug target potential. Various methods are available for functional characterisation of bioactive peptides, including established dispersed muscle-fibre bioassays (Day et al., 1994; Moneypenny et al., 2001), as well as newer techniques being investigated by our laboratories such as computer-based behavioural analyses of the effects of peptides on whole worms. Silencing of *npp* expression using RNA interference (RNAi) is another potential avenue of investigation since RNAi is now an established technique in several of the species represented in our dataset (Sanchez Alvarado and Newmark, 1999; Krautz-Peterson et al., 2007; Pfister et al., 2008), however, our attempts at silencing neuronal genes in numerous flatworm species have shown, at best, limited success (Atkinson L, McVeigh P, Pierson L, unpublished data). Nevertheless, RNAi remains the most powerful method available for investigating gene function in flatworms, and large-scale RNAi-based screening of neuropeptide and receptor genes must be attempted if we are to begin to dissect the intricacies of the flatworm nervous system. In describing 51 flatworm-specific putative neuropeptides, our study has significantly widened the scope for such functional investigations. We hope that the data presented in this study will catalyse much needed further research into the roles of individual neuropeptides and receptors in these important pathogens.

Acknowledgements

We acknowledge the laboratories responsible for generating the publicly available genomic and transcriptomic resources for flatworms, without which this study would not have been possible. This work was funded by The National Institutes of Health grant ROI-AI49162. A research studentship (for LA) from the Department of Education and Learning for Northern Ireland and a Wellcome Trust equipment grant (069411) is also acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2009.03.005.

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