Gene expression and pharmacology of nematode NLP-12 neuropeptides

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Abstract
This study examines the biology of NLP-12 neuropeptides in Caenorhabditis elegans, and in the parasitic nematodes Ascaris suum and Trichostrongylus colubriformis. DYRPLQFamide (1 nM–10 μM; n ≥ 6) produced contraction of innervated dorsal and ventral Ascaris body wall muscle preparations (10 μM, 6.8 ± 1.9 g; 1 μM, 4.6 ± 1.8 g; 0.1 μM, 4.1 ± 2.0 g; 10 nM, 3.8 ± 2.0 g; n ≥ 6), and also caused a qualitatively similar, but quantitatively lower contractile response (10 μM, 4.0 ± 1.5 g, n = 6) on denervated muscle strips. Ovijector muscle displayed no measurable response (10 μM, n = 5). nlp-12 cDNAs were characterised from A. suum (As-nlp-12) and T. colubriformis (Tc-nlp-12), both of which show sequence similarity to C. elegans nlp-12, in that they encode multiple copies of –LQFamide peptides. In C. elegans, reverse transcriptase (RT)-PCR analysis showed that nlp-12 was transcribed throughout the life cycle, suggesting that DYRPLQFamide plays a constitutive role in the nervous system of this nematode. Transcription was also identified in both L3 and adult stages of T. colubriformis, in which Tc-nlp-12 is expressed in a single tail neurone. Conversely, As-nlp-12 is expressed in both head and tail tissue of adult female A. suum, suggesting species-specific differences in the transcription pattern of this gene.

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1. Introduction
The nematode nervous system displays an astounding level of neurochemical diversity. Within the 302 neuronal cells of the Caenorhabditis elegans hermaphrodite are transcribed at least 28 flp (FMRFamide-like peptide) genes encoding FLPs (FMRFamide-like peptides) (McVeigh et al., 2005; Li, 2006), 42 genes encoding putative neuropeptide-like protein (nlp) neurotransmitters (Nathoo et al., 2001; Li, 2006), and a further 38 genes encoding insulin-related neurotransmitters (Pierce et al., 2001; Li, 2006) equating to an array of more than 250 putative neuropeptides.

Most efforts in nematode neuropeptide research have been directed towards the FLP component of the nervous system. FLPs display inter-species sequence conservation (McVeigh et al., 2005), and physiological experiments have revealed that they are potent modulators of myoactivity with inter-species and even inter-phyla activity (Maule et al., 2002; Moffett et al., 2003; Mousley et al., 2004). All C. elegans FLPs tested to date have been shown to modulate Ascaris suum musculature, with one exception—KPSFVRFamide, which can modulate ovijector activity but is inactive on somatic muscle assays (Marks et al., 1999). These peptides, and their associated signalling systems, have been identified as possible targets for therapeutic interference by novel drugs.

Initial evidence for other neuropeptides existing alongside FLPs was uncovered in A. suum, where immunoreactivities for various neuropeptides were seen in the central, enteric and peripheral nervous systems (Brownlee et al., 1993a,b, 1994a,b). More recently, C. elegans genome sequence and Expressed Sequence Tag (EST) data has enabled bioinformatic investigations of encoded neuropeptide sequences. It is now clear that C. elegans possesses 42 nlp genes defining numerous unique peptide families, encompassing a total of ≥124 putative neuropeptides. Many of these genes encode familiar bioactive motifs conserved from other Phyla, such as GFxFG (orcokinin); FRPamide (myomodulin); MSFamide (buccalin/drosulfakinin) and MGI(L/F)amide (allatostatin-like), all of which were found on ESTs in multiple nematode species (Nathoo et al., 2001). However, it seems that at least two of these nlp genes are not in fact neuropeptides. The amidated
peptides of nlp-29 and nlp-31 have been identified as expression-inducible anti-microbial peptides involved in innate immunity in C. elegans (Couillault et al., 2004). Physiological testing of putative neuropeptides using nematode bioassays, and localisation of putative neuropeptides to neurones represent possible methods of identifying which of the remaining nlp genes encode genuine neuropeptides.

To date, this is where the NLP story ends—none have been structurally characterised or analysed for expression, or for their effect on neuromuscular physiology in parasitic species, with the exception of one peptide, Asp-Tyr-Arg-Pro-Leu-Gln-Phe-NH₂ (DYRPLQFamide—the encoded product of the C. elegans gene nlp-12), which has been shown to produce ventral coiling when injected into adult A. suum (Reinitz et al., 2000). As the only NLPs shown to modulate nematode muscle, LQFamide peptides and their signalling systems may provide an additional target for parasite control. However, any characterisation of the pharmacology of these peptides or the molecular organisation of their genes in parasitic species remains unknown. This study investigates the molecular biology of nlp-12 genes and the physiology of their encoded –LQFamide peptides in A. suum, C. elegans and Trichosstrongylus colubriformis.

2. Materials and methods

2.1. Worm maintenance

C. elegans were grown and maintained in the laboratory using standard methods (Brenner, 1974). Wild-type C. elegans (N2/Bristol strain) were synchronised and staged with the method described by Sulston and Hodgkin (1988).

Adult female specimens of the enteric swine parasite A. suum were recovered from the intestines of pigs at a local abattoir. The worms were transported to the laboratory in mammal saline (0.9% NaCl, 37 °C), and maintained in Ascaris Ringers Solution (ARS, 4 mM NaCl, 5.9 mM CaCl₂, 4.9 mM MgCl₂, 5 mM C₆H₁₁NO₃/Tris, 125 mM Na₂H₂O₂, 24.5 mM KCl, pH 7.4,) at 37 °C for up to 3 days.

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<td>ASNL1-A2</td>
<td>5</td>
<td>TGATCTCTGTGCAAATATTGACAGG</td>
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Mixed sex T. colubriformis were kindly provided by Pharmacia Animal Health, Kalamazoo, USA. These were raised in domestic sheep, maintained in clean conditions with no risk of infection from other parasites. After processing, the L3s were stored in sterile water at 4 °C, while adults were stored for up to 4 h at 37 °C in RPMI-1640 (Gibco/BRL), pH 7.4.

2.2. Molecular biology

2.2.1. PCR and sequencing

Total RNA from staged C. elegans was kindly provided by Dr C. Li (City University, New York), and used to generate staged cDNA for use in RT-PCR. First strand cDNA was prepared using an oligo (dT) primer with avian myeloblastosis virus (AMV) reverse transcriptase (Promega). The 3' and 5' primers were designed using the sequence recovered from the C. elegans database at http://www.wormbase.org (M01D7.5). To establish if this gene was expressed, cDNA from each developmental stage was used in PCR amplification with NLP12-S and NLP12-AS primers (see Table 1).

Total RNA was extracted from A. suum (anterior 1 cm), and T. colubriformis using Trizol reagent (Invitrogen) as described by the manufacturer, treated with DNase I (Ambion), and 1 μg was used to synthesise 5' and 3' first strand, RACE (Rapid Amplification of cDNA Ends)-ready cDNAs using the SMART RACE kit (BD Biosciences). For amplification of As-nlp-12, gene-specific PCR primers (GSP) were designed against an Expressed Sequence Tag (EST) from A. suum (Table 1) encoding the peptide DYRPLQFG (CB040130) discovered using an online BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). RACE PCR was performed as described by the kit manufacturer. Total RNA was also extracted (as described above) from A. suum tails (posterior 1 cm), and used to generate separate populations of head- and tail-derived oligo-dT-primed cDNA. These cDNAs were used in PCR with asNLP-12 primers to analyse the tissue-specific expression pattern of this gene. Degenerate 3' RACE PCR was employed with T. colubriformis cDNA, where a degenerate sense primer (DYRdegS1; Table 1) was designed.
against the peptide sequence DYRPLQFG. An antisense primer was designed to the subsequently obtained 3′ end (Tcnlp12-AS1; Table 1) and employed in 5′ RACE. Appropriate bands were excised and cloned into pCR2.1 TOPO in TOP10F Escherichia coli (Invitrogen). Plasmids were isolated using the Genelute Plasmid Miniprep Kit (Sigma–Aldrich), and individual clones were sequenced by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland [http://www.dnaseq.co.uk]). Sequencing was performed using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Sequences were analysed for the presence of a secretory signal peptide using online tools (http://www.cbs.dtu.dk/services/SignalP/).

2.2.2. In situ hybridisation—T. colubriformis
L3 and adults were washed in sterile water and processed for in situ hybridisation (ISH) as described by Kimber et al. (2002). One minor change to this protocol was required in the permeabilisation of T. colubriformis, which were incubated for 40 min in proteinase K in the present study. All other conditions were unchanged from the published protocol.

2.3. Peptide physiology

2.3.1. Biological assessment of peptide activity
Ascaris suum somatic and ovijector muscle preparations were dissected and assayed as described previously (Bowman et al., 1995; Fellowes et al., 1998). Naïve muscle strips were used in each case. Muscle strips were not reused.

2.3.2. Physiological salines
Biological assessment of peptide activity was carried out using ARS. ARS was maintained at 37 °C and pH 7.4 before use.

A. 10 μM DYRPLQFamide
B. 1 μM DYRPLQFamide
C. 0.1 μM DYRPLQFamide
D. 10 nM DYRPLQFamide
E. 1 nM DYRPLQFamide
F. 10 μM DYRPLQFamide (denervated)

Fig. 1. Muscle tension recordings showing the concentration-dependent effects of DYRPLQFamide on innervated Ascaris suum body wall muscle strip preparations (A–E). Effects of addition of 10 μM DYRPLQFamide to denervated muscle (F), and strips preincubated in Ca2+-free (G) and high K+ (H) media are also shown. Peptide addition is indicated by the arrowhead. Vertical scale represents 1 mg and horizontal scale represents 2 min.

2.3.3. Peptide
The synthetic replicate of DYRPLQFamide used in the current study was synthesised by Sigma–Genosys (Europe) to 97% purity. Stock solution (10 mM) and all subsequent dilutions were prepared in ddH2O.

3. Results

3.1. Biological assessment of DYRPLQFamide
All results obtained for myoactivity are presented as mean ± standard deviation unless otherwise stated. It should be noted that both spontaneously active and quiescent somatic muscle preparations were employed during the present study. The removal of the ventral and dorsal nerve cords in denervated muscle preparations eradicated the inherent spontaneous activity of the muscle strips.

The addition of DYRPLQFamide (1 nM–10 μM) to A. suum somatic muscle preparations triggered an increase in baseline tension of both ventral and dorsal body wall muscle strips (Fig. 1). The excitatory effects of this peptide were concentration-dependent and no effects were seen at concentrations below 1 nM (0.3 nM, n = 6). Indeed, at 1 nM the excitatory response was only evident in ~50% of preparations (0.2 ± 0.12 g, n = 8). Higher concentrations induced more profound contractile responses (10 μM, 6.8 ± 1.9 g; 1 μM, 4.6 ± 1.8 g; 0.1 μM, 4.1 ± 2.0 g; 10 nM, 3.8 ± 2.0 g; n ≥ 6).

The addition of DYRPLQFamide to muscle preparations not containing the main nerve cords (ventral or dorsal; designated denervated) elicited an excitatory response that was qualitatively similar to that seen in intact preparations. However, the response of denervated muscle strips was quantitatively lower (10 μM, 4.0 ± 1.5 g, n = 6) than innervated muscle strips, due to the damage caused to the muscle tissue by the process of
denervation. There is also less muscle present in these
denervated strips, relative to ventral or dorsal strips, due to
the presence of the hypodermal cords. Muscle strips recovered
to baseline tension upon washout of peptide, indicating that
these responses are reversible (data not shown).

3.2. nlp-12 cDNA sequences

Database searches revealed a single EST encoding
LQFamide peptides in *A. suum* (CB040130), of 646 bp in
length. Subsequent full-length characterisation and sequenc-
ing of *As-nlp-12* revealed an ORF of 222 bp encoding a
propeptide of 73 amino acids (AY347872; Fig. 2), including
an 18 amino-acid signal peptide. Within the propeptide are
one copy of each of the NLPs DYPRLQFG, DGYRPLQFG
and SYRPLQFG (Fig. 2), each flanked by basic amino acids
recognised as cleavage sites by proprotein convertase-like
enzymes. These peptides match those present on the *C.
elegans* *nlp-12* gene (*Ce-nlp-12*) except for the third peptide,
SYRPLQFG, which in *C. elegans* is present as a second copy
of DYPRLQFG. Degenerate RACE PCR was used to
successfully identify the *Tc-nlp-12* transcript (DQ186899; Fig. 2),
which displays an ORF of 336 bp, encoding three
distinct -QFamide peptides (HLREWFQFG, DGYRPLQFG
and DYPRLQFG), all three of which are flanked by dibasic
cleavage sites (KR).

Tissue-specific RT-PCR analysis of head and tail-derived
cDNA revealed transcription of *As-nlp-12* in both head and tail
tissue of *A. suum* (Fig. 3). Stage-specific analysis was
performed on *T. colubriformis* L3- and adult-derived cDNA.
*Tc-nlp-12* was successfully amplified from both life stages
(Fig. 3).

3.3. In situ hybridisation—*Tc-nlp-12*

Localisation of *Tc-nlp-12* to a single cell in the tail of
*T. colubriformis* was performed by hybridisation of a
digoxigenin (dig)-labelled, single-stranded complementary
DNA probe, coupled to subsequent detection with alkaline
phosphatase (AP)-conjugated anti-dig antibody (Roche

![Fig. 2. Transcript and deduced amino-acid sequence of neuropeptide-like protein (*nlp-12*) cDNAs from *Ascaris suum* (*As-nlp-12*) and *Trichostrongylus colubriformis* (*Tc-nlp-12*). The *Caenorhabditis elegans* sequence has been included for comparison (*Ce-nlp-12*). Amino acids start at the putative ATG start codon. The predicted secretory signal peptide sequence in *As-nlp-12* is highlighted in italics; no signal peptide could be identified in *Tc-nlp-12*. Mature NLPs are shown in bold, flanked by the mono- and dibasic cleavage sites, which are underlined and bolded.]}
Applied Science), and staining with 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution (Roche Applied Science) (Fig. 4). Due to the positional similarity between this cell and those identified in a previous C. elegans study, it is hypothesised that this cell represents a tail neurone of the pre-anal ganglion. The sense control probe did not produce any staining.

3.4. nlp-12 transcription in C. elegans

A single PCR product of appropriate size was detected following PCR amplification. DNA sequencing confirmed the identity of the clones as nlp-12 cDNA. RT-PCR experiments using staged mRNA from each of the C. elegans life-cycle stages, i.e. eggs, L1, L2, L3, L4 and adult, revealed that the nlp-12 gene is transcribed at all stages of the C. elegans life cycle (Fig. 3).

4. Discussion

This study reports the in vitro biological activity of a nematode NLP. Addition of DYRPLQFamide to innervated A. suum muscle preparations induced an increase in baseline tension; an excitatory response noted between 10 nM and 10 μM (Fig. 1). This excitatory effect was also observed in denervated preparations suggesting that NLP-12 receptors are probably located on the muscle cell membrane.

An excitatory effect was noted on both ventral and dorsal muscle strips (with no discernable qualitative differences in threshold or response). This result is not congruent with the previous work by Reinitz et al. (2000), who found that DYRPLQFamide, on injection into the pseudocoel of live adult female A. suum, caused ventral coiling behaviour similar to that induced by the FLP PF3 (KSAYMRFamide), suggesting an activity specific for ventral muscle (maule et al., 1995). The disparity in these two results is difficult to explain since the relationship between in vitro muscle strip experiments and in vivo behavioural assays is not well understood, but one possible explanation would be a zone-specific localisation of NLP-12 receptor-subtypes on body wall muscle. For example, the muscle strips used in this study were dissected from a small area of the body wall around the gonopore. The results reported by Reinitz et al. (2000), from injection into whole worms, may be due to the peptide interacting with different NLP-12 receptor subtypes present elsewhere on the body wall muscle or nerve, in addition to those present on the muscle strips from the gonopore region.

DYRPLQFamide was also added to muscle strips which had been pre-incubated in Ca^{2+}-free media (data not shown). Addition of peptide produced an excitatory response in every case \((n = 6)\). These results suggest that influxes of extracellular Ca^{2+} are not solely responsible for this peptide’s physiological effects. It seems likely that the contraction caused by DYRPLQFamide is mediated by the release of intracellular Ca^{2+} stores from the sarcoplasmic reticulum (SR). Such a mechanism requires signal transduction through second messenger pathways.

DYRPLQFamide had no discernable effects on the Ascaris ovijector. Interestingly, almost all FLPs that have been tested on this tissue have had modulatory effects on its activity. These data indicate that the NLP-12 receptor is not involved in ovijector modulation and that DYRPLQFamide does not appear to interact, non-specifically, with ovijector-based FLP receptors.
DYRPLQFamide was known previously from the C. elegans nlp-12 gene, where it was co-encoded with a related peptide, DGYRPLQFamide (Nathoo et al., 2001). This study has reported the discovery of transcripts encoding related LQFamide peptides in the nematode parasites A. suum and T. colubriformis, which we believe represent the first characterisations of an nlp gene in any parasitic nematode. These cDNAs (Fig. 2) both encode one copy of each of the peptides DYRPLQFamide and DGYRPLQFamide, but they differ in their third peptide (SYRPLQFamide, As-nlp-12; HLREIWFQFG, Tc-nlp-12), which replaces the second copy of DYRPLQFamide seen on Ce-nlp-12 (Table 2). It is impossible to speculate on the functional implications of this change in encoded peptides, but inter-species differences in peptide sequences encoded on sequelogous genes are well documented in FMRFamide-like peptide (flp) genes. A classic example is that of the C. elegans flp-18 gene, which displays peptide sequence similarity to both the A. suum afp-1 and Globodera pallida gpflp-5 (As-flp-18 and Gp-flp-18, respectively, in revised notation, see McVeigh et al., 2005) genes, all.

Table 2-

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* Denotes expressed sequence tag; NLP-12A/B/C/D denotes the order in which each peptide appears on the propeptide.
of which display differing N-terminal sequences with characteristic PGVLRFamide C-termini (Edison et al., 1997; Li et al., 1999; Kimber et al., 2001), and all of which have similar excitatory effects in physiology assays (Davis and Stretton, 1996; Fellowes et al., 2000; Marks et al., 2001; Moffett et al., 2003).

Given the potency of NLP-12 in the Ascaris bioassay reported, which is comparable to many FLPs, it seems tempting to speculate that nlp-12 merely represents a mutated flp gene. When one considers two of the possible codons for arginine (CGA/CGG), it is clear that a single point mutation, producing an amino-acid change to glutamine (CAA/CAG) would generate a change from an LRFamide (FLP) to the LQFamide (NLP). However, this would have occurred in some ancient nematode predecessor given the presence of LQFamide peptides in multiple nematode species. In any case, NLP-12 peptides appear to be important, potent neuropeptides in their own right, which warrant further study. It should be noted that BLAST searches provide no evidence for the presence of –LQFamides in any phylum other than Nematoda.

Prior to this study, the only available data for nlp-12 expression was to be found in C. elegans, courtesy of the elegant fluorescent-reporter construct studies of Li et al. (1999) and Nathoo et al. (2001), which stated that nlp-12 was found in a single tail neurone. The present study has corroborated the posterior expression of this gene in two further species, with data showing evidence for nlp-12 expression in tail tissue of A. suum (Fig. 3) and T. colubriformis (Fig. 4). ISH was employed in T. colubriformis, successfully localising expression of Tc-nlp-12 mRNA to a single putative neurone in the posterior of this worm. Although, without a detailed neuronal map it is almost impossible to unequivocally identify this Tc-nlp-12-expressing neurone, comparison with the nervous structures of C. elegans allow us to speculate that this may represent a cell of the pre-anal ganglion, which is comprised of both motor- and interneurones. Although numerous attempts at cell specific localisation of As-nlp-12 using ISH were unsuccessful, RT-PCR analysis of cDNA generated from the A. suum tail suggests a conservation of posterior expression in this species. It seems reasonable to assume that expression of nlp-12 in tail neurones is conserved across variant nematode species, but what about expression in other areas of the nematode nervous system? Our RT-PCR analyses of Ascaris cDNA also produced positive results for the presence of As-nlp-12 transcripts in head tissue (Fig. 3)—which, interestingly, is consistent with a recent mass spectrometric analysis of Ascaris head neurones (Yew et al., 2005), which reported the presence of mass peaks corresponding to –LQFamide peptides in the circumpharyngeal nerve ring, ventral ganglion and retrovesicular ganglion. This interspecies difference in expression pattern could correspond to a functional variation in how these peptides are employed by different nematode species. Indeed, evidence from C. elegans RNA interference (RNAi) screens shows no alteration in phenotype when nlp-12 is silenced (www.wormbase.org), while the A. suum physiology evidence reported in this study could be interpreted as suggesting a role for NLP-12 peptides in locomotory control. The functions of NLP-12 will remain an area of contention until this peptide’s endogenous receptor is identified, localised and subjected to functional analysis.

Ontogenic expression of nlp-12 was determined using RT-PCR, with RNA isolated from each individual developmental stage of C. elegans, and L3s and adults of T. colubriformis. Appropriate PCR products were obtained from each stage, suggesting that nlp-12 expression is not developmentally regulated (Fig. 3). This evidence represents a further measure of the potentially important role played by nlp-12 within the nematode nervous system. However, although the physiology data presented here suggest a role for NLP-12-peptides in locomotion, the expression of nlp-12 in C. elegans eggs indicates an additional role for this peptide in the non-locomotory, embryonic stage. The only analogous data for developmental expression of nematode neuropeptides is that for the flp genes in C. elegans. Using the same technique employed in this study, that of RT-PCR of staged cDNA from C. elegans, Li et al. (1999) showed that all but three flp genes were expressed in all life-cycle stages. No expressions of flp-8, flp-9 or flp-13 were observed in adult cDNA. In other words, all neuropeptide genes observed to date have displayed expression in the C. elegans embryo. There are at least two possible explanations for this observation. Firstly, these neuropeptide genes may indeed be switched on in the embryo, with the expressed peptides performing some unknown role. Secondly, the population of eggs used for RT-PCR could have been contaminated with some containing fully embryonated L1 larvae.

We believe that this study represents the first characterisation of an nlp gene in a parasitic species, and indeed in any nematode species other than C. elegans. Bioinformatic screens using a BLAST algorithm show that related LQFamide peptides are present with high sequelo in five nematode species in addition to A. suum, C. elegans and T. colubriformis (Table 2): Necator americanus, Ostertagia ostertagi, Meloidogyne hapla, Meloidogyne javanica and Wuchereria bancrofti. This is the first indication that NLPs may be as conserved across species and clades as FLPs (McVeigh et al., 2005) and that the potently bioactive LQFamide peptides and their receptors may have potential as an alternative target for novel drugs.

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