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Changes in Cyclic Nucleotides, Locomotory Behavior, and Body Length Produced by Novel Endogenous Neuropeptides in the Parasitic Nematode *Ascaris suum*

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Abstract

Recent technical advances have rapidly advanced the discovery of novel peptides, as well as the transcripts that encode them, in the parasitic nematode *Ascaris suum*. Here we report that many of these novel peptides produce profound and varied effects on locomotory behavior and levels of cyclic nucleotides in *A. suum*. We investigated the effects of 31 endogenous neuropeptides encoded by transcripts *afp-1*, *afp-2*, *afp-4*, *afp-6*, *afp-7*, and *afp-9 - 14*, (*afp*: *Ascaris* FMRFamide-like Precursor protein) on cyclic nucleotide levels, body length and locomotory behavior. Worms were induced to generate anteriorly propagating waveforms, peptides were injected into the pseudocoelomic cavity, and changes in the specific activity (nmol/mg protein) of second messengers cAMP (3'5' cyclic adenosine monophosphate) and cGMP (3'5' cyclic guanosine monophosphate) were determined. Many of these neuropeptides changed the levels of cAMP (both increases and decreases were found), whereas few neuropeptides changed the level of cGMP. A subset of the peptides that lowered cAMP was investigated for effects on the locomotory waveform and on body length. Injection of AF19, or AF34 (*afp-13*), AF9 (*afp-14*), AF26 or AF41 (*afp-11*) caused immediate paralysis and cessation of propagating body waveforms. These neuropeptides also significantly increased body length. In contrast, injection of AF15 (*afp-9*) reduced the body length, and decreased the amplitude of waves in the body waveform. AF30 (*afp-10*) produced worms with tight ventral coils. Although injection of neuropeptides encoded by *afp-1* (AF3, AF4, AF10 or AF13) produced an increased number of exaggerated body waves, there were no effects on either cAMP or cGMP. By injecting peptides into behaving *A. suum*, we have provided an initial screen of the effects of novel peptides on several behavioral and biochemical parameters.

Keywords

Neuropeptides; cAMP; cGMP; *Ascaris suum*; locomotory behavior; parasite

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1. Introduction

Neuropeptides are well established as signaling molecules and are widespread and numerous across animal phyla. They function as neurotransmitters and neuromodulators, affecting many different physiological properties of their target cells [1]. Neuropeptides have been particularly well studied in nematodes, where the discovery of new peptides has been approached by two general methods, chemical analysis of the peptides themselves [2–11], and database mining of genomic or EST libraries [12–15]. Localization studies in nematodes show that the majority of these neuropeptides are expressed in neurons [11, 16–20]. Neurons in nematodes are few in number and are simple geometrically. The adult hermaphrodite *Caenorhabditis elegans* has 302 neurons [21], and adult female *Ascaris suum* has 298 neurons [22], and the morphology of these neurons is, in most cases, unusually simple [21, 23] with at most two branch points. Neuronal morphology is conserved between the two species, with the neurons differing in little else but size. This is remarkable considering that nematodes are estimated to have diverged evolutionarily about 550 million years ago [30].

Both *Caenorhabditis elegans* and *Ascaris suum* contain a large number of peptides; each species has been estimated to encode about 250 different peptides [5, 11]. Many of the peptides are known to have potent activity on subsets of neurons and/or on muscle cells, as well as on locomotory behavior [2, 4–9, 24–29]. The sequences of these neuropeptides are also highly conserved among different species of nematodes, being either identical or very similar, implying strong positive selection pressure on the sequences. The fact that there are so many peptides in nematodes suggests that they may have a correspondingly large number of important functional roles.

In this laboratory, we have explored the role of neuropeptides at the cellular, physiological and behavioral levels. We investigate both the transmitting cell and the receiving cell that constitute the intercellular signaling dyad mediated by the peptide. To identify the cells that produce and presumably secrete these peptides, we use three different approaches: immunocytochemistry with highly specific antibodies against individual peptides [31], *in situ* hybridization with riboprobes derived from cloned genes encoding the peptides [32], or direct analysis of peptide content by mass spectrometry of single dissected neurons [6]. In almost all cases the three techniques give the same answer. The other half of the cellular signaling dyad, namely the cells bearing the receptors for each peptide, has been explored by observing peptide effects on the electrophysiological properties of single neurons [9, 24], on muscle cells [7, 8, 25–27, 29, 33–35], and on levels of cAMP [28, 36].

We have long been interested in the effects of neuropeptides on the locomotory behavior of *A. suum* [24, 28]. The importance of locomotion in nematode behavior has been summarized by Croll [37, 38]. In parasitic nematodes locomotion is crucial for the migration of the various life form stages during the parasitic life cycle, and, for many adult intestinal nematodes, locomotion is used to maintain position within the intestine. Nematodes make a variety of body movements, including twisting into a corkscrew, and coiling; they also generate waveforms in the body, and the propagation of these waveforms is responsible for locomotion. The number of wave crests within each waveform and the direction of propagation may vary. In *A. suum*, alternating dorsal and ventral muscle contraction and relaxation cycles generate the body bends that constitute the propagating waveform. In the host intestine, where the worm braces its body against the intestinal wall, the direction of waveform propagation is the same as the direction of locomotion, so, unlike in *C. elegans*, forwards propagating waves produce forward locomotion [37]. These movements can be observed in a glass tube with a diameter similar to that of the lumen of the small intestine of the pig.

To establish a baseline of locomotory behavior, all *A. suum* used in this study were ligatured at the head with surgical thread, which induces stereotypical anteriorly-propagating body waves [28, 39]. These waves are initiated in the region of the gonopore in females. For the typical female adult, after placement of the ligature there are generally three crests in the anteriorly propagating waveform (Figure 1). *A. suum* is large enough that neuropeptides can easily be injected into the pseudocoelomic cavity posterior to the ligature. This minimally invasive technique keeps the neuromuscular system intact for monitoring the effects of peptides on behavior and body length, as well as on downstream signal transduction events.

In previous experiments we analyzed the effects of six endogenous *A. suum* peptides and five peptides from *C. elegans*, and showed that most peptides produced significant effects on behavior [28]. Some peptides also produced changes in cAMP levels, and both increases and decreases were seen. In the present paper we extend our previous analyses by assaying the effects of 31 endogenous *A. suum* peptides (Table 1) on both cAMP and cGMP, as well as on locomotory behavior, body length, and body tonus. We report results on peptides encoded by the transcripts *afp-1* [40], *afp-2* [51], and *afp-4* [32], as well as on the more recently discovered peptides encoded by *afp-6* [9], *afp-7* [41], *afp-9*, *afp-10* [42], *afp-11* [42, 51], *afp-12* and *afp-13* [6], and *afp-14* [C. Konop, pers.comm.].

We report for the first time the effects of these peptides on cGMP, a well-known second messenger in other systems: we observed both increases and decreases. We also extend the list of peptides that increase or decrease cAMP levels. There is a loose correlation between the changes in cyclic nucleotides and body length: in general, increases in cAMP are associated with contraction of the body, although there is a marked exception for AF2 which produces the largest change in cAMP, yet no change in body length [28]; similarly decreases in cAMP are generally associated with body relaxation. It is striking that in the case of multiple peptides derived from a single transcript, the activities of the individual peptides are not always identical. Divergent activities of the different products of a single transcript have been observed previously for the *afp-6* transcript in *A. suum* [9], and the *flp-18* peptides of *C. elegans* [43].

One reason for our interest in neuropeptides in nematodes is to address the growing resistance to currently available anthelmintics. Since nematode locomotion is a prime target for anthelmintic action, we aim to identify potential new targets for future anthelmintic development by characterizing the peptide-mediated signaling pathways that are involved in locomotory behavior.

2. Material and methods

A. suum were collected at a local slaughterhouse from the small intestines of freshly killed pigs, and stored at 37 °C in PBS (140 mM sodium chloride, 10 mM sodium phosphate, pH 7.4). In these experiments, large female worms 25–40 cm were used within 30 h of collection.

2.1 Head ligature procedure

In all second messenger assays and all behavioral and body length change determinations, *A. suum* were induced to generate stereotypical anteriorly propagating locomotory waveforms by ligaturing the worm with surgical thread ca. 5 mm posterior to the pharynx.

2.2 cAMP and cGMP second messenger assays

For the second messenger assays, the *A. suum* were injected (27.5 gauge needle) just behind the ligature with 100 µl 10 µM test neuropeptide in *Ascaris* saline (4 mM NaCl, 125 mM sodium acetate, 24.5 mM KCl, 5.9 mM CaCl₂, 4.9 mM MgCl₂, and 5 mM MOPS buffer, pH

6.8), or with control saline, and then placed in a beaker containing PBS at 37 °C. Previous experiments with injection of 100 µl of dye solution showed that there was a ca. 10-fold dilution in the region of the injection, so that target cells were exposed to ca. 1 µM peptide [39]. After observing general behavioral effects, namely body tonus immediately after injection (stiff or relaxed), the shape of the body (straight or curved) and whether or not propagating waveforms were affected, the worms were frozen in liquid nitrogen 5 – 7 minutes after injection. A ca. 2 cm piece of worm from the injected region was excised and stored at –80 °C.

Tissue preparation for the determinations of cAMP and cGMP were made as previously described [28]. Briefly, the frozen tissue was homogenized in 750 µl ice-cold 6% TCA, and then centrifuged, and the supernatant extracted with ether to remove TCA. cAMP and cGMP were assayed by ELISA (BioVision cAMP or cGMP assay kit) or RIA (New England Nuclear). Both samples and standards were acetylated to improve the sensitivity of the assays [44]. The 1% Triton X-100 soluble protein in each TCA pellet was determined (Bradford reagent [45]) in order to calculate the specific activity of each cyclic nucleotide (nmol/mg protein). A Student's t-test was used to test for significance of the neuropeptide-induced changes compared to controls.

Most determinations of cyclic nucleotides were made by ELISA. In our previously reported experiments [28], cAMP was assayed by RIA, so to ensure that the results with ELISA were comparable some determinations were made by both RIA and ELISA. Additional validation of the ELISA assays was accomplished by spiking *A. suum* samples with a series of cAMP or cGMP standards. In each case, the arithmetic sum of the standard plus the amount contributed by the worm was found, showing that there was no interference from *A. suum* material in the assays.

2.3 Photo-documentation of locomotory posture and body length determinations

For the body length determinations, worms were ligatured as described above, and placed in a glass tube (18 mm internal diameter × 41 cm in length fitted with stoppers) containing PBS maintained at 37 °C. The worm was photographed with a Nikon D50 digital camera before and after injection against a background transparent centimeter grid. Images for body length determinations were made 3–6 minutes before and after injection. The images were encoded with a random file name and stored in batches of 50 to 100 files, then body lengths were measured in ImageJ (NIH) using images magnified up to 4X on the screen in order to reduce ambiguity about the positions of the lips and the tail. At least ten determinations of each worm's body length were averaged to give the body length for each worm. Worms that showed coiling were not measured. After the analysis was complete, the images were decoded. The average percent change in body length and the standard error for each treatment was computed and compared to control worms. A Student's t-test was used to test for significance.

2.4 Source of neuropeptides

Neuropeptides were synthesized by the UW-Madison Biotechnology Center, and were over 99% pure as determined by HPLC. The molecular mass of each peptide was determined by MALDI-TOF MS as a further control of the quality of the synthesis.

3. Results and Discussion

3.1 Head – Restricted Behavior

The utility of a head ligature for inducing the stereotypical Head-Restricted Behavior [39] was first discovered when we were attempting to refine the markedly varied behavior

observed when *A. suum* is placed in a large beaker of PBS. In this environment, the locomotory behavior is largely unpredictable, consisting of alternating bouts, of variable duration, of forwards- and backwards-moving waveforms, superimposed upon apparently random body bends that can sometimes form knots. By prolonged observation, it is possible to observe the effects of injected peptides on locomotory behavior [9, 24, 28], but we (J.E. Donmoyer and A.O.W. Stretton, unpublished) sought a way to induce standardized behavior by applying to the head (where the sensory receptors are concentrated) compounds that might act as attractants (like the presence of the opposite sex) or repellents (like garlic), while leaving the rest of the body free for observation of locomotory behavior. We devised an apparatus consisting of a glass trough with two compartments separated by a sheet of dental rubber dam. The head was inserted through a hole in the dam, and we varied the diameter of the hole to minimize leakage. When the head was squeezed tightly enough to prevent leakage, the worm generated forwards-moving waves without the necessity of chemical stimulation. We found that a head ligature produced the same effect, generating the standardized behavior we were seeking. A similar effect is produced by decapitation, but the useful lifetime of the preparation is reduced as the turgor pressure goes down due to leakage of the pseudocoelomic fluid. The basis for the behavioral bias produced by the ligature is not clear. The ligature is placed posterior to the pharynx, so the nerve ring and its associated sensory neurons are well separated from the rest of the body. The appropriate tightness of the ligature was determined by injections of Fast Green solution (10 mg/ml) into the posterior or anterior side of the ligature: when the ligature was tight enough to produce the standardized behavior, there was no diffusion between the injected and non-injected side. We presume that the ligature prevents conduction of nerve signals past the ligature, but have no proof for this assumption. Many of the peptides we investigate in this paper are encoded by the *afp-11* transcript that is expressed in the paired AVK neurons in the ventral ganglion [51]. The cell bodies of these neurons are separated from the body by the ligature, but the ventral cord processes of AVK extend into the region of the worm posterior to the ligature [51], which is where injected peptides act, so we surmise that the behavioral effects of injected peptides encoded by *afp-11* are relevant to the natural effects of peptides released from the AVK processes.

3.2 Effects of neuropeptides on cAMP and cGMP responses, body length and locomotory behavior

Almost all of the neuropeptides tested here (Table 1) are AF peptides, with C-terminal RFamide. Peptides NY, TT and TL are intervening sequences between AF peptides encoded by *afp-11* or *afp-13*, and are known to be expressed since they were detected by single-cell MS [6]. Peptide TF was predicted, but its expression has not yet been confirmed.

The effects of neuropeptides on levels of cAMP and cGMP are shown in Figures 2 – 5 as fold changes from control levels after injection of saline. Numerical values of specific activity are presented in Supplementary Table 1. Control injections produced no significant changes in cAMP or cGMP, and no change in posture; the worms still have at least three propagating wave crests in the anterior region (Figure 1). Control injected worms did not show a significant change in body length (Table 2). Basal levels of cAMP and cGMP were in good agreement with the values reported in *A. suum* by Thompson et al. [36]

3.2.1 Effects of peptides encoded by *afp-1* (AF3, 4, 10, 13)—In *A. suum* treated with neuropeptides AF3 (AVPGVLRFa), AF4 (GDVPGVLRFa), AF10 (GFGDEMSMPGVLRFa), and AF13 (SDMPGVLRFa), all encoded by *afp-1* [40], there are no significant changes in either cAMP or cGMP levels (Figure 2). However, these peptides do have strong effects on locomotory behavior. In head-ligated worms generating anteriorly propagating locomotory waves, injection of any of the neuropeptides encoded by

the *afp-1* transcript increased the number of crests in the locomotory waveform. This was also observed after peptide injection into worms with or without ligatures and free in a beaker, rather than in a tube [24], and we have confirmed these results. A significant decrease in body length (contraction) was produced after injection with AF10 [28]. Previous studies have shown that these peptides have very little effect on the electrophysiological properties of motor neurons [24]. The *afp-1* neuropeptides were not investigated further in this study.

3.2.2 Effects of peptides encoded by *afp-2* (AF5), *afp-4* (AF2), and *afp-7* (AF1)

—As shown in Figure 2, AF5 (SGKPTFIRFa) produced a significant decrease in cAMP (0.029 nmol/mg $\pm$ 0.004, $p < 0.05$). In worms without ligatures, Davis and Stretton [24] described the behavioral effects of AF5 as being similar to those of AF1, with marked decreases in locomotory movements and reduced amplitude of body waves. The increase in body tonus immediately after injection of AF5 was also similar to those observed for AF1 [24]. AF5 and AF1 are similar in that they share a FIRFamide C-terminal sequence, and each has a lysine residue seven amino acids from the C terminus.

Figure 2 also shows the changes in cAMP and cGMP produced by AF2 (KHEYLRFa, *afp-4*) or AF1 (KNEFIRFa, *afp-7*). We previously demonstrated that both AF1 and AF2 dramatically elevated cAMP [28], but the effects on cGMP levels were not determined. In the present experiments, AF1 increased cAMP 6-fold (comparable to previous results obtained with a different assay [28]) and increased average cGMP 6-fold, although the range was large (17 – 0.7 times control), and increases were seen in only 6/16 cases; the reasons for this high variability are unknown. In contrast, AF2 dramatically increased cAMP, by 175-fold, but had no effect on cGMP levels. Thompson et al. [36] have confirmed our results on the effects of AF1 and AF2 on cAMP levels, but did not observe any effect of AF1 on cGMP levels.

In another experiment, we followed the time course of the responses of cGMP to a higher dose (100 μ M) of AF1. We confirmed that cAMP levels continue to rise for up to 45 minutes after injection, as in previous experiments [28], but there was no significant change in cGMP during the same time period. This contrasts with the effects on cGMP of the lower dose of AF1, suggesting that the peptide may have multiple sites and/or mechanisms of action on cGMP.

In a few experiments we injected control saline or 10 μ M AF1 into the head region anterior to the ligature, and assayed for cAMP and cGMP in heads. In control worms, the specific activity for cGMP was below the detection limit of our assay. In AF1-treated worms, cAMP was elevated relative to control but control specific activity of cAMP from the head was much lower than from the more posterior region of the worm (data not shown). Because of the low levels of cyclic nucleotides that could be detected from single heads, these experiments were not pursued further.

We previously found that AF1 injections produce worms that are paralyzed, have stiff tonus and shallow waveforms [28]. AF1 also increases muscle tension [4] and depolarizes the excitatory motor neuron DE2 [24]. The response to AF2 is different. The posture produced by injection of AF2 is similar to that produced by AF1 (contracted shallow crests, not shown) [28], but AF2-injected worms make intense thrashing jerky flexions, perhaps associated with backward propagating waveforms. AF2 also produces a biphasic change in muscle tension, an initial relaxation followed by a sustained contraction [2].

Both AF1 and AF2 can elevate cAMP and they have been shown to have distinct receptors in *A. suum* [29]. Brief application of AF2 produces a lasting potentiation of ACh responses

in muscle strips. It is thought that multiple interacting second messenger systems elements are involved in the AF2 regulation of muscle, but our results suggest that cGMP is not one of them.

3.2.3 Effects of peptides encoded by *afp-6* (AF21, 22, 23)—Figure 3 shows changes observed in levels of cAMP and cGMP following treatment with AF21 (AMRNALVRFa), AF22 (NGAPQPFVRFa), and AF23 (SGMRNALVRFa), which are encoded by the *afp-6* transcript [9]. All three neuropeptides produced significant decreases in cAMP and one of them, AF23, significantly decreased cGMP. Each of the three *afp-6* peptides produced a significant lengthening of the body (AF23>AF22=AF21) and abolition, or near abolition, of the body waveform (Figure 1, Table 2). *A. suum* treated with AF22 showed loss of waveform propagation and a reduction in the amplitude of most waveforms; the waveform in the region near the gonopore where the waveforms normally originate remained static. AF21 and AF23 produced similar locomotory posteriors that were noticeably more flattened than the posture from AF22 injections.

It is interesting to compare these responses to the electrophysiological effects of these peptides that were described previously [9]. The responses of muscle to 10 μ M macrophoretic application of peptide showed that AF21, 22, and 23 all produced muscle hyperpolarization and relaxation, with relative potency AF22>AF21=AF23. However, the effects of AF22 on motor neurons AF22 differed dramatically from effects of AF21 and AF23. Whereas AF22 hyperpolarized excitatory motoneurons DE1 and DE2 and inhibitory motoneuron DI, with little effect on input resistance, AF21 and AF23 produced strong depolarization in DE1, DE2, and DI, accompanied by a large reduction in input resistance [9].

While in the electrophysiological experiments AF21 and AF23 had similar effects, in the second messenger assay AF21 and AF23 have different actions. The effects of these three peptides on motor neurons clearly involve signal transduction pathways in addition to cyclic nucleotides, and merit further study.

Among nematodes, all three peptides are highly conserved, which implies a strong positive selective pressure on each of these sequences. AF22 is structurally quite distinct from AF21 and AF23. Although AF21 and AF23 are sequence related, they have separately conserved sequences; the AF21-related consensus is AMRNALVRFa, while the most common sequence for the AF23-related peptides is (A/S)GMRNALVRFa, sometimes with two additional N-terminal amino acid extensions [9]. In several other nematodes there are two separate gene families, one of which encodes all three AF21, 22 and 23 sequelogs, while the other encodes only one of these peptides, AF21 or its close relative AMRNSLVRFa [5]. At least in *C. elegans*, it is known that both genes, *flp-11* and *flp-32*, are expressed [5].

3.2.4 Effects of peptides encoded by *afp-9* (AF15) and *afp-10* (AF30)—*Afp-9* encodes three copies of AF15 (AQTFVRFa). Although AF15 has no effect on either cAMP or cGMP (Figure 3, Supplementary Table 1), it is a potent inhibitor of locomotion. After injection of AF15, the body of the worm is stiff and contracted (Table 2); waveform propagation is inhibited, although there are some regions of the body that adopt low amplitude body bends (Figure 1). These effects are not correlated with changes in cyclic nucleotide levels.

Peptide AF30 (APNKILMRFa), which is encoded by *afp-10* [42], produces a decrease in cAMP, but has no significant effect on cGMP (Figure 3). AF30 produced ventral coils in 4 out of 6 *A. suum*. We have no information on effects on body length since ventral coiling interferes with the measurements. It is interesting that peptide AF8 (encoded by *afp-3* [32]),

which was tested previously [28], also produces ventral coils, but since AF8, unlike AF30, does not change cAMP levels, we conclude that ventral coiling does not necessarily involve changes in cAMP. AF8 (KSAYMRFa) has been shown to relax dorsal muscle and contract ventral muscle [46], consistent with the generation of ventral coils. It may be relevant that both AF8 and AF30 share a C-terminal –MRFa sequence.

3.2.5 Effects of peptides encoded by *afp-11* (AF 11, 25, 26, 27, 28, 29, 31, 32, 33, 41, and TF and NY)—The effects on cAMP and cGMP produced by the twelve different neuropeptides encoded by *afp-11* are shown in Figure 4. These peptides include ten AF peptides, AF11 [3], AF25 [10], and AF26 [10], and the recently discovered peptides AF27, AF28, AF29, AF31, AF32, AF33, AF41, as well as two non-RFamide peptides, TF and NY which are intervening sequences between AF peptides in the AFP-11 precursor protein [42, 51]. These peptides produced multiple classes of responses when injected into *A. suum* (see also Supplementary Table 1). Peptides AF25, AF26, AF29, and AF41 significantly lowered cAMP. Neuropeptides AF31 and NY slightly elevated cAMP (but not cGMP) and produced low amplitude body waveforms, but these effects on locomotion were less dramatic than those of other neuropeptides that lowered cyclic nucleotides. For example, neuropeptides AF26 (KPNFLRFa) and AF41 (KPNFIRFa) significantly lowered both cyclic nucleotides and caused body wall relaxation.

Peptides AF26 and AF41 were investigated further for effects on body length using different concentrations of the peptides. AF26 at higher concentration (100 μ M) produces a similarly shaped worm to that of AF41 injected at a lower concentration (5 μ M). Immediately after injection of AF41, all of the anteriorly propagating waveforms are abolished and the posture is severely flattened. AF41 injections after AF26 produces the more flattened posture and the effects are long lasting (> 15 minutes) (N= 10 worms; data not shown). Tested at the same concentration, AF26 has a much less dramatic effect in the behavioral assay, as propagating waveforms are still present, although the number of wave crests present is reduced and the wavelength is elongated. These results are notable, in that the sequences of these two neuropeptides only differ in that the isoleucine in AF41 is replaced by leucine in AF26; both peptides are endogenous in *A. suum*.

Three independent detection techniques, MALDI-TOF MS of single neurons [42], ICC, and ISH [42, 51, 52] indicated localization of neuropeptide AF11 to AVK neurons. The AVK neurons are paired neurons with cell bodies in the ventral ganglion. Their processes traverse the nerve ring and then project posteriorly in the ventral nerve cord; they are ventral cord interneurons.

Previous experiments with related peptides PF1 (SDPNFLRFa) and PF2 (SADPNFLRFa), isolated from *Panagrellus redivivus*, showed peptide-induced relaxation of *A. suum* muscle [47, 48]. This relaxation involved hyperpolarization of muscle cells by a mechanism independent of GABA activity.

3.2.6 Effects of peptides encoded by *afp-12* (AF36), *afp-13* (AF 19, 34, 35, and TT and TL), and *afp-14* (AF9)—*Afp-12* encodes AF36 (VPSAADMMIRFa), the only predicted peptide product of this precursor protein [6]. AF36 is co-expressed with AF8 and the six peptides encoded by *afp-13* in the ALA neuron of the dorsal ganglion. AF36 did not change levels of either cAMP or cGMP nucleotide level (Figure 5) yet produced a flaccid response when it was injected (data not shown).

Afp-13 encodes 6 peptides, all of which have been detected by single-cell MS in the ALA neuron of the dorsal ganglion [6]. Three of these are AF peptides, and the others are either intervening sequences or flanking sequences relative to the AF peptides. Two AF peptides,

AF19 (AEGLSSPLIRFa) and AF34 (DSKLMDPLIRFa), which share a C-terminal PLIRFa sequence, produce a marked reduction in cAMP (Figure 5). The third AF peptide, AF35 (DPQQRIVTDETVLRFa), had no effect on cAMP levels. Two of the remaining peptides, peptides TT (TPPEEDLLGRFT) and TL (TNIMGENRLNRNL), produced small but significant increases in cAMP. Small but significant reductions in cGMP levels were induced by AF19 and peptide TL. AF19 and AF34 increased the body length by more than 7% (Table 2) and dramatically inhibited locomotion and locomotory posture (Figure 1).

The *afp-14* transcript encodes one copy of AF9 (GLGPRPLRFa), and no other predicted peptide (C. Konop, personal communication). AF9 lowered cAMP, but had no effect on cGMP (Figure 5). Mostly, the body pieces taken for cyclic nucleotide determinations were straight, although sometimes tightly curved at the anterior end near where the injection was made and then dramatically flattened posteriorly. AF9 increased the body length by more than 7% (Table 2) and dramatically inhibited locomotion and obliterated the locomotory posture (Figure 1).

3.2.7 Locus of cAMP and cGMP changes—Previously we concluded that the large AF2-induced elevation of cAMP most probably occurred in muscle [28]. In the experiments reported here, we made no attempt to locate the cellular source of the changes in cAMP or cGMP. The preparations include body wall (cuticle, hypodermis, muscle, excretory canal, and neurons in the nerve cords) and intestine. Cells in any of these tissues might produce the changes in cyclic nucleotides. In *C. elegans*, the majority of the receptor guanylate cyclases are expressed in sensory neurons in the head [49]. If the receptor guanylate cyclases of *A. suum* are similarly expressed, these receptors are not present in the region where we sampled.

4 Summary

Instead of using isolated *A. suum* dissected preparations, the behavior and other bioassays were performed on whole *A. suum*, modified by ligaturing the head; this induces stereotypical anteriorly propagating body waveforms, and leaves intact the neuromuscular system that generates locomotory behavior. A standard dose of the neuropeptide of interest is injected into the worm. While it is known from other studies that neuropeptides can work in tandem with classical neurotransmitters at the same synapse [50], in the behaving worm any essential co-ligands would have to be supplied by the endogenous system, and it is not clear whether the concentrations are appropriate.

Presumably through a variety of different G protein coupled receptors, cyclic nucleotide levels are regulated by *A. suum* neuropeptides, and both increases and decreases were seen. Decreases in cAMP correlated with reduced amplitude and/or abolition of waveforms, loss of waveform propagation and increases in body length. Large increases in cAMP are correlated with body contraction and intense jerky thrashing movements (AF2). Neuropeptides AF23, AF26, AF41 and AF19 lower both cAMP and cGMP, and these treatments also produced relaxed linear shaped worms. cGMP is also lowered by TL (*afp-13*) but the consequence of this on behavior is not obvious.

The changes in cyclic nucleotides we have observed are correlated with the administration of particular peptides, but we do not know whether these effects are direct. Injected peptides might be activating the release of other signaling molecules from cells, and these molecules might be the agonists that affect the cyclic nucleotide levels. Identification and cellular localization of peptide receptors, and the demonstration at the single cell level that cyclic nucleotide levels are changed in that cell by injected peptide, are important goals for future work.

The complete description of neuropeptides present in the *A. suum* nervous system is essential if we are to understand the role of the nervous system in the organism's behaviors, and this description is still far from complete. However, the demonstration that the endogenous neuropeptides we have investigated here can disrupt locomotory behavior makes them interesting potential targets for the future development of anthelmintics. Their receptors are in a separate class from those targeted by drugs that affect the cholinergic or GABAergic systems. Peptidergic pathways are thus important in their potential for overcoming genetic drug resistance to the first generation of anthelmintics, many of which affect cholinergic or GABAergic neurotransmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AF	Ascaris FMRFamide-like peptide
afp	Ascaris FMRFamide-like Precursor protein

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Figure 1.

Postures of worms after injection of neuropeptides. Heads are to the right. All injected peptides disrupted locomotory behavior, either completely abolishing all movements and producing a straight worm, or producing a severe reduction in the generation and propagation of locomotory waveforms. Control: Digital image of *A. suum* generating a typical locomotory waveform. Ligaturing the head induces anterior propagation of waveforms. Generally there are three waveforms present in the anterior region and they propagate in the anterior direction. After injection of control saline there is no change in behavior.

Figure 2.

cAMP and cGMP fold changes relative to control after injection of peptides encoded by *afp-1*, *afp-2*, *afp-4*, and *afp-7*. Peptides were injected in 100 μ l volumes at a concentration of 10 μ M unless indicated otherwise. Black bars, cAMP; gray bars, cGMP (this convention is used in subsequent figures). Since AF2 and AF1 caused elevation of cAMP far beyond the scale of the other treatments shown, their calculated cAMP fold changes were divided as indicated. Standard error bars are shown. Significance, (Student's t-test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Figure 3. cAMP and cGMP responses to injection of 10 μ M neuropeptides encoded by the transcripts *afp-6*, *afp-9*, and *afp-10*. Neuropeptides AF21, AF22, and AF23 (*afp-6*) and AF30 (*afp-10*) significantly lowered cAMP. A significant decrease in cGMP was observed following AF23 injections. Standard error bars are shown. Significance (t-test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4. cAMP and cGMP responses to injections of the twelve neuropeptides encoded by *afp-11*. Key observations are that AF25 and AF29 both decrease cAMP; AF26 (KPNFLRFa) and AF41 (KPNFIRFa) decrease both cAMP and cGMP. Neuropeptides AF31 and NY showed slight elevations of cAMP. Standard error bars are shown. Significance (t-test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5. cAMP and cGMP responses to injections of the neuropeptides encoded by *afp-12*, *afp-13*, and *afp-14*. AF19 and AF34 dramatically decreased cAMP levels whereas peptide TT and TL (not RFamide-like) elevated cAMP. Standard error bars are shown. Significance (t-test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1

A. suum neuropeptides and the transcripts that encode them.

Neuropeptide	Transcript	Sequence	<i>C. elegans</i> homolog
AF1	<i>afp-7</i>	KNEFIRFa	<i>flp-8</i>
AF2	<i>afp-4</i>	KHEYLRFa	<i>flp-14</i>
AF3	<i>afp-1</i>	AVPGVLRFa	<i>flp-18</i>
AF4	<i>afp-1</i>	GDVPGVLRFa	<i>flp-18</i>
AF10	<i>afp-1</i>	GFGDEMSMPGVLRFa	<i>flp-18</i>
AF13	<i>afp-1</i>	SDMPGVLRFa	<i>flp-18</i>
AF5	<i>afp-2</i>	SGKPTFIRFa	<i>flp-4</i>
AF21	<i>afp-6</i>	AMRNALVRFa	<i>flp-11, flp-32</i>
AF22	<i>afp-6</i>	NGAPQPFVRFa	<i>flp-11</i>
AF23	<i>afp-6</i>	SGMRNALVRFa	<i>flp-11</i>
AF15	<i>afp-9</i>	AQTFVRFa	<i>flp-16</i>
AF30	<i>afp-10</i>	APNKILMRFa	<i>flp-28</i>
AF11	<i>afp-11</i>	SDIGISEPNFLRFa	<i>flp-1</i>
AF25	<i>afp-11</i>	NNFLRFa	<i>flp-1</i>
AF26	<i>afp-11</i>	KPNFLRFa	<i>flp-1</i>
AF27	<i>afp-11</i>	PADPNFLRFa	<i>flp-1</i>
AF28	<i>afp-11</i>	SAEPNFLRFa	<i>flp-1</i>
AF29	<i>afp-11</i>	NAEPNFLRFa	<i>flp-1</i>
AF31	<i>afp-11</i>	TPSNNFLRFa	<i>flp-1</i>
AF32	<i>afp-11</i>	GSDPNFLRFa	<i>flp-1</i>
AF33	<i>afp-11</i>	SNQAQNFLRFa	<i>flp-1</i>
AF41	<i>afp-11</i>	KPNFIRFa	<i>flp-1</i>
TF	<i>afp-11</i>	TAPPLTFa	
NY	<i>afp-11</i>	NDQFDREY	
AF36	<i>afp-12</i>	VPSAADMMIRFa	<i>flp-24</i>
AF19	<i>afp-13</i>	AEGLSPLIRFa	<i>flp-13</i>
AF34	<i>afp-13</i>	DSKLMDPLIRFa	<i>flp-13</i>
AF35	<i>afp-13</i>	DPQQRIVTDETVLRFa	
TT	<i>afp-13</i>	TPPEEDLLGRFT	
TL	<i>afp-13</i>	TNIMGENRLNRNL	
AF9	<i>afp-14</i>	GLGPRPLRFa	<i>flp-21</i>

Accession numbers. *afp-1*: U15279; *afp-4*: AY386834; *afp-6*: AY705391; *afp-9*: BM518433; *afp-12*: HM125966; *afp-13*: HM125967; *flp-1*: F23B2.5; *flp-4*: C18D1.3; *flp-8*: F31F6.4; *flp-11*: K02G10.4; *flp-13*: F33D4.3; *flp-14*: Y37D8A.15; *flp-16*: F15D4.8; *flp-18*: Y48D7A.2; *flp-21*: C26F1.10; *flp-24*: C24A1.1; *flp-28*: W07E11.4; *flp-32*: R03A10.2.

Table 2Effects on body length produced from 10 μ M injection of neuropeptides.

Treatment	Mean % Change ^a	Posture
Control	0.4 $\pm$ 0.2 (20)	2–3 propagating waveforms
AF1	–5.3 $\pm$ 0.6 (13) ***	shallow crest, no propagation
AF30	n.d. (6)	ventral coil
AF21	3.8 $\pm$ 0.7 (5) ***	linear, no propagation
AF22	4.7 $\pm$ 0.7 (8) ***	broad static waveform
AF23	10.6 $\pm$ 1.5 (7) ***	linear, no propagation
AF15	–2.3 $\pm$ 2.2 (6) *	shallow crest, no propagation
AF25	4.5 $\pm$ 1.4 (5) ***	linear, no propagation
AF26 ^b	6.2 $\pm$ 0.6 (5) ***	linear, no propagation
AF29	7.1 $\pm$ 2 (5) ***	linear, no propagation
AF31	7.4 $\pm$ 2 (6) ***	linear, no propagation
AF33	0.5 $\pm$ 1 (5) ***	broad static waveform
AF41 ^c	4.5 $\pm$ 0.7 (5) ***	linear, no propagation
AF19	8.7 $\pm$ 2.0 (6) ***	linear, no propagation
AF34	7.7 $\pm$ 2.2 (8) ***	linear, no propagation
AF9	7.8 $\pm$ 1.5 (8) ***	linear, no propagation

^a *t*-test was applied to determine significance.* $P < 0.05$;** $P < 0.01$;*** $P < 0.001$.^b 100 μ M injected.^c 5 μ M injected.