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Different Bioactive Neuropeptides are Expressed in Two Sub-Classes of GABAergic RME Nerve Ring Motorneurons in *Ascaris suum*

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Abstract

Neuropeptides can have significant effects on neurons and synapses, but among the ~250 predicted peptides in nematodes, few have been characterized functionally. Here, we report new neuropeptides in the 4 RME nerve ring motorneurons of the nematode *Ascaris suum*. These GABAergic neurons are involved in three-dimensional head movement. Mass spectrometry (MS) of single dissected RMEs detected a total of 12 neuropeptides (encoded by five genes), nine of which are novel. None of these are expressed in the DI/VI inhibitory GABAergic motorneurons that synapse onto body wall muscle. Using peptide sequences obtained by tandem MS, we cloned the peptide-encoding transcripts and synthesized riboprobes for in situ hybridization (ISH). This complementary technique corroborated the results from single-cell MS, showing that the dissections were not contaminated with adhering tissue from other cells. We also synthesized a multiple antigenic peptide to raise a highly specific antibody against one of the endogenous peptides, which labeled the same cells detected by MS and ISH. Our results show that the RMEs can be divided into two subsets: RMED/V (expressing *afp-2*, *afp-15*, *Asu-nlp-58*, and high levels

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Author Contributions

J.J.K. was responsible for transcript identification, ISH, pharmacological and behavioral assays, bioinformatics, and manuscript preparation. C.J.K. was responsible for single-cell dissection, MS, data analysis, transcript identification, ISH, bioinformatics, and manuscript preparation. I.R.V. was responsible for transcript identification, ISH, and ICC. C.B.R. was responsible for behavioral assays. L.A.M. was responsible for transcript identification. M.M.V. was responsible for MS acquisition and data analysis. A.O.W.S. was responsible for data analysis, bioinformatics, and manuscript preparation.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.7b00450. Primer sequences, dot ELISAs, tandem MS spectra of synthetic peptides, MS spectra of AIY/AIM and DVB neurons, homologous neuropeptide transcripts, and multiple alignments from other nematodes (PDF)

Notes

The authors declare no competing financial interest.

of *afp-16*) and RMEL/R (expressing *afp-15* and low levels of *afp-4* and *afp-16*). Almost all of these peptides are bioactive in *A. suum*.

Graphical Abstract

Keywords

Nematode; Neuropeptide; RME motoneuron; Single-cell mass spectrometry; De novo sequencing; In situ hybridization

INTRODUCTION

Nematode nervous systems are well-known as model systems, largely due to their numerical simplicity, with a total of ~300 neurons in both *Caenorhabditis elegans* hermaphrodites and *Ascaris suum* females.^{1,2} However, this simplicity is compounded by the presence of many neuromodulators, the majority of which are neuropeptides. *A. suum* has the additional experimental advantage that its neurons are large enough that single identified neurons can be isolated by manual dissection, and their neuropeptide content can be analyzed by mass spectrometry (MS).^{3–6} The peptides can then be sequenced by tandem MS, all from a single neuron.^{3–6} The peptide-encoding transcripts can also be detected in identified neurons by in situ hybridization in whole-mount preparations,^{4–8} and the sequences of the transcripts give an independent prediction of peptide sequences based on the well-substantiated proteolytic cleavages of precursor proteins at dibasic (or sometimes monobasic) amino acids⁹ followed by the trimming of the C-terminal basic amino acids and subsequent oxidation of C-terminal glycine residues to generate C-terminal amides.¹⁰ In cases where peptide-specific antibodies are available, peptides can be localized by immunocytochemistry.^{4,11,12} Together, these techniques give a robust assessment of the cellular expression of each neuropeptide.

In this paper, we extend our analysis of peptides in GABAergic motoneurons in *A. suum* to the four RME motoneurons of the nerve ring. These neurons innervate the muscles that control the 3-dimensional movement of the head, an important behavior that is involved in feeding and sensory perception.² RME neurons are the only neurons with cell bodies in the nerve ring and are arranged in 4-fold symmetry (Figure 1). They are named RMED, RMEV, RMEL, and RMER according to the dorsal, ventral, left, or right position of the cell body, respectively.^{2,14} They are strongly GABA immunoreactive in both *A. suum*^{14,15} and *C. elegans*.^{16–18} Here, we used single-cell mass spectrometry (MS), in situ hybridization (ISH), and immunocytochemistry (ICC) to search for neuropeptides and their encoding transcripts in the RME neurons. We found a total of 12 peptides encoded by five genes. Two genes (*afp-2* and *Asu-nlp-58*) are expressed in RMEL/R but not RMEV/D. *afp-15* is expressed in all RME neurons; *afp-16* is expressed robustly in RMEL/R neurons and with some variability in RMEV/D neurons, and *afp-4* is more highly expressed in RMEV/D than in

RME/L/R. This variability in expression seen for *afp-4* and *afp-16* is detected by both single-cell MS and by ISH. Four of the transcripts that encode these peptides (*afp-2*, *afp-15*, *afp-16*, and *Asu-nlp-58*) are reported for the first time in *A. suum*, and *Asu-nlp-58* is a member of a novel gene family in nematodes.

Interestingly, none of these peptides are present in the GABAergic VI and DI motoneurons of the ventral cord (which control locomotion); furthermore, peptide As-NLP-22, characteristic of the VI/DI inhibitory neurons,⁶ is not found in any of the RME neurons, providing further evidence that there are multiple subtypes of the GABAergic motoneurons in *A. suum*, as there are in *C. elegans*.¹⁶ We show that most of these peptides are bioactive, affecting locomotory and head searching behavior and/or acetylcholine-induced muscle contraction. To our knowledge, no peptides have been found in the RME neurons of *C. elegans*, suggesting that there may be significant differences between the nervous systems of these two species, which are otherwise strikingly similar in terms of neuronal anatomy and morphology.^{1,7,14,15} This is perhaps not surprising because we previously found several major apparent differences in the cellular expression of peptides encoded by homologous genes in *A. suum* and *C. elegans*.^{3–6} However, the cellular expression of peptides in *C. elegans* depended on the use of reporter genes rather than on the direct detection of gene products (the peptides themselves or their encoding mRNAs) as in *A. suum*.¹⁹ Confirmation by independent techniques is badly needed in *C. elegans*.

The findings reported here are highly relevant to antiparasite drug development efforts and showcase the value of using multiple model systems to study the biology of nematode behavior.

RESULTS AND DISCUSSION

It is clear from our previous work that classical transmitter expression and signaling in the motoneurons is not enough to describe nematode locomotion, and neuromodulators must be incorporated into the functional circuit for behavior.^{20–23} However, identifying the relevant modulators is not a trivial endeavor. In this paper, we utilize single-cell mass spectrometry and in situ hybridization to identify the neuropeptides expressed by the four RME motoneurons of *A. suum*, which control three-dimensional movement of the head. We also synthesized these peptides and examined their effects on *A. suum* muscle contraction and locomotion. Finally, we used a synthetic octameric multiple antigenic peptide to raise a highly specific antibody against one of the endogenous peptides and used it for immunocytochemical localization. Although all of the RMEs are GABAergic, we found that these neurons can be split into two groups (RMEV/D and RMER/L) based on their neuropeptide expression, and these patterns are distinct from the DI and VI somatic inhibitory motoneurons and other GABAergic cells AIY/AIM and DVB. This overall approach combines advances in single-cell peptidomics and synthetic chemistry with biological circuits and identified several novel neuropeptides for future study.

The sequences, discovered by MS and by cloning the peptide-encoding transcripts, are enabling. First, the peptides are synthesized for assays of bioactivity, both physiological and behavioral: almost all of the peptides in the RME neurons are bioactive. Second, the

sequences can be compared with those from many other nematodes from which genomic and/or transcriptomic libraries are available: the peptide sequences are remarkably well conserved, often to the point of identity, compared with nonpeptide-encoding regions. This suggests that the peptides are under positive selection pressure and play an important biological role. It implies the existence of a conserved signaling system consisting of a signaling molecule (the peptide) and its receptor (probably a G-protein coupled receptor). Whether this signaling system is used in a versatile way, with peptide and receptor being expressed in different cells in different nematodes, or whether the cellular expression patterns are conserved as faithfully as the sequences themselves, is a matter for future research. This will require more rigorous analysis of cellular expression patterns of peptides in *C. elegans* and other nematodes and localization studies of peptide receptors.

Anatomical Background.

The four GABAergic RME ring motoneurons have been extensively described in the nematodes *A. suum* and *C. elegans*.^{2,14,15} Each RME neuron projects two semicircumferential processes from the cell body into the nerve ring in opposite directions; these two processes meet at a position diametrically opposite the cell body so that each RME neuron covers the entire nerve ring with processes that overlap with those of the other three RME cells.^{2,14} They make neuromuscular synapses onto projections of head muscles into the nerve ring with independent control of dorsal, ventral, left, and right head muscles (in nematodes, neuromuscular synapses are made onto specialized extensions of muscle cells that branch out to neurons rather than the opposite arrangement typical of most animals).

At first sight, the RME neurons look like four members of a class of related neurons, analogous to the seven classes of body motoneurons that control the propagating locomotory waveforms. Each of the seven classes of body motoneurons is present in several copies arranged serially along the body; each class comprises between 5 and 13 cells.^{2,13} In contrast, most other nematode neurons occur as bilateral pairs. The RMEV and RMED neurons also project a prominent process into their respective nerve cords, so already the set of four can be subdivided into two pairs, one that includes 2 bipolar cells (RMEL/R) and the other 2 tripolar cells (RMEV/D).² In *C. elegans*, this subdivision has been strongly reinforced by the analysis and localization of expression patterns of other genes, especially several transcription factors (e.g., *ceh-10* affects *unc-25*/glutamic acid decarboxylase expression in RMED/V, but not RMEL/R;²⁴ *ahr-1* expression promotes RMEL/R differentiation from RMED/V,²⁵ and *tab-1* affects expression of *unc-25* and *unc-47* in RMEL/R¹⁶). It is interesting that the *ahr-1* transcription factor controls the expression of the ventral and dorsal cord projections of RMEV/D. Loss of function of *ahr-1* in RMEL/R causes them to extend a process into the lateral lines; ectopic expression of *ahr-1* in RMEV/D leads to loss of their dorsal or ventral cord processes.²⁵

Mass Spectrometry of Single RME Neurons.

Individual RME neurons were carefully dissected from the nerve ring and transferred to a MALDI-TOF MS target using a protocol developed by this laboratory³⁻⁶ and subjected to MALDI-TOF MS analysis. To characterize the peptides present in each spectrum, we used chemical derivatization, and MS/MS analysis for de novo sequencing. Two patterns emerged

from the resulting spectra, separating the RME neurons into two pairs, RMEV/D and RMER/L, on the basis of their peptide expression.

Peptides in RMEV and RMED.—MS spectra of RMEV and RMED neurons contained a reproducible set of five peaks (Figure 2a, b; Table 1). The mass-to-charge ratio (m/z) of four of the five high-intensity peaks matched the m/z of three previously identified peaks encoded by the *afp-15* transcript (AF7-AGPRFIRFa, 962.6 Da; AF40-AGSARFIRFa, 1023.6 Da; AF39-SPKQKFIRFa, 1149.7 Da³) and AF2-KHEYLRFa, 991.5 Da, encoded by the *afp-4* transcript.⁸ We often use methylene blue to aid identification during dissection. This treatment also aids in preliminary identification of peptides by derivatization because it partially oxidizes methionine residues to the sulfoxide, causing a mass shift of +16 Da. No such shifts were observed for the peptides found among the RMEV/D neurons, suggesting the absence of methionine residues in these peptides. On-target derivatization by acetylation is another quick way to test for the presence of specific amino acids in peptides in a mixture. Acetylation produces a shift of +42 Da for each free amino group, including the α -amino group of the peptide and the ϵ -amino group of any lysine residue present. The amino groups are most reactive; occasionally, there is evidence for additional acetylation of hydroxyls on tyrosine, serine, and/or threonine, although our reaction conditions disfavor these latter modifications. In all cells exposed to acetylation, the expected mass shifts for each predicted amino acid were observed (Figure 2c, d), strengthening the evidence for the putative peptide sequences.

In some of these neurons, lower intensity peaks from the five peptides encoded by *afp-16* were also observed (Supplementary Figure 2; Table 1). For sequences from mass matching of previously identified peptides to be confirmed, MS/MS was performed on m/z 991.5 and 1065.6 (1023.6 + 42, the acetylated form of AF40) (Figure 3). The fifth major peak, with m/z 1222.7, did not match the mass of any previously known or predicted peptide. We were unable to sequence it by MS/MS, and it therefore remains unidentified.

Peptides in RMEL and RMER.—Spectra from RMER and RMEL motoneurons contained several sets of highly reproducible peaks (Figure 4a, b), some the same and others different from those in RMED/V. A peak with the m/z 1051.7 was present in all 22 cells analyzed and was consistently the most intense peak in the spectrum (Figure 4a, b). Two previously identified *A. suum* peptides, AF5 (SGKPTFIRFa) and AF32 (GSDPNFLRFa), share the mass 1051.7 Da.²⁶ Spectra of cells modified by acetylation contained a peak at 1051.7 m/z , another at +42 m/z , and a smaller peak at +84 m/z consistent with double acetylation of the peptide at the N-terminus and at the side-chain of lysine. Together, these peaks match the expected pattern for AF5 and not AF32 (Figure 4c, d). There is also a small peak at 1177.6 m/z suggesting an additional acetylation possibly at the serine and/or the threonine residue in this peptide. Sequencing of the unmodified peak by MS/MS confirmed the sequence to be that of AF5 (Figure 5).

In 11 of 16 RMER and 10 of 22 RMEL neurons, we observed medium-to-small peaks with an m/z corresponding to the three *afp-15* peptides. Spectra of three acetylated RMER and RMEL neurons contained medium-to-large peaks that matched the m/z of the three acetylated *afp-15* peptides seen in RMEV and RMED.

Transcript Identification and de Novo Sequencing of Previously Unidentified Peptides.

The MS data show that the RMEV/D and RMEL/R neurons fall into two sets based on the relative peak amplitude of the endogenous peptides, although there was variability in expression for some peptides. We therefore wanted to use an independent technique, ISH, to examine peptide-encoding mRNA expression in these cells. The transcripts encoding each of the peptides were identified, sequenced, and used to create riboprobes for ISH. In some cases, the sequences also revealed additional predicted peptide products and facilitated de novo sequencing by MS.

afp-2.—The AF5-encoding transcript was first identified by degenerate PCR as part of an effort to identify RFamide-encoding transcripts in the *A. suum* transcriptome. The PCR reaction was performed using a primer for spliced-leader sequence 1 (SL1), a sequence estimated to be present on ~80% of *A. suum* transcripts,²⁷ and a degenerate primer that encoded the amino acids FIRFGKR (AFP2DEG3, 5'-CKYTTICCRAA-ICKDATRAA-3', Supplementary Table 1). The resulting sequence products were then used to design gene-specific primers for amplification of the 3'-end of the transcript. The complete *afp-2* transcript (accession no. JNS47402.1) included SL1, a predicted signal peptide, the AF5 peptide-encoding sequence flanked by basic cleavage sites, and a poly-A tail (Figure 6).

afp-15.—Mass spectra of all four RME neurons contained a peak at m/z 962.6, and subsequent MS/MS analysis confirmed that this peptide was the previously known peptide AF7 (Figure 3). Previous attempts to clone the AF7-encoding transcript using a degenerate primer to the peptide-encoding region paired with a primer for splice-leader sequence SL1 were unsuccessful. The mass spectra also contained a peak at m/z 1023.6, which MS/MS analysis determined to be novel peptide AF40 (AGSARFIRFa, Figure 3). BLAST searches using these sequences yielded a small Genome Survey Sequence (GSS) fragment (GSS 6) containing AF40 and another predicted peptide, SPKQKFIRFa (AF39, 1149.7 Da, also present in spectra from the RMEs), separated by a monobasic cleavage site.²⁸ We used this sequence to design gene-specific primers for 5'- and 3'-RACE and successfully amplified a complete transcript (*afp-15*, accession no. MF737440) containing a signal peptide, AF39, AF40, and, surprisingly, AF7 (Figure 7).

afp-16.—Mass spectra from RMER/L contained large peaks of m/z 1344.8 and 1645.8 (Figure 4; Table 1). MS/MS analysis of these peaks yielded the sequence R(I/L)D(I/L)ND(I/L)A(I/L)RFa and partial sequence DPSN(I/L)N(I/L)RFa (isoleucine and leucine are isobaric and cannot be distinguished by this method, Figure 5). A direct search of the *A. suum* Transcriptome Shotgun Assembly (TSA) database was performed with all I/L permutations of the predicted MS/MS sequences. The search identified the sequence RIDINDLALRFG flanked by two dibasic amino acid residues, thus leading to the predicted amidated peptide RIDINDLALRFamide (AF43, accession no. JI238650.1; calculated mass, 1344.8 Da). In the same reading frame, just C-terminal to AF43 is a predicted peptide with a deduced C-terminal sequence -DPSNLNLRamide matching the partial sequence derived from a series of y-ions in the second spectrum. The full-length sequence deduced from the TSA-1 entry, SSYSFDPSNLNLRFG, is flanked by dibasic cleavage sites, generating the predicted peptide SSYSFDPSNLNLRamide (AF44, accession number MF737441; calculated mass,

1645.8 Da) (Figure 5b; Table 1). In addition, b-ions not used in the initial spectrum were identified, providing further evidence of the predicted peptide sequence. For these peptide sequences to be confirmed, synthetic AF43 and AF44 were subjected to MS/MS and yielded similar fragmentation patterns to the MS/MS patterns of their native forms (Supplementary Figure 3a, b).

Further investigation of the peptide-encoding TSA-1 entry identified two more predicted peptides with the C-terminal-LXLRamide motif. First was TFVIPTDLALRFG (AF45), which was flanked by dibasic amino acids and preceded an encoded stop codon. The calculated mass of amidated AF45 is 1391.8 Da. The peak corresponding to AF45 was sometimes weak and only observed in 6/16 RMER and 5/22 RMEL neurons.

A partial sequence -NYLASD(I/L)A(I/L)RFa was obtained from MS/MS analysis of the peak with m/z 1726.9 (Figure 5b; Table 1). Cloning of the *afp-16* transcript extended the N-terminus of the TSA-1 sequence to include a predicted signal peptide with initiating methionine and two additional peptides (Figure 8, MF737441). One peptide (AF42, SFPNNYLASD-LALRFa) shares the C-terminal LXLRamide motif and has a calculated mass of 1726.9 Da observed in spectra of 16/16 RMER and 19/22 RMEL neurons. The cloned *afp-16* transcript also included a predicted nonamidated peptide (PepQV, pQEEEEEDGVLPFRFV, m/z 1658.8 Da). A peak with m/z 1658.8 Da was present in 15/16 RMER and 17/22 RMEL. The N-terminus of this peptide is formed as a result of the signal peptide cleavage rather than a conventional basic or dibasic cleavage, resulting in an N-terminal Q that is cyclized to form pyroQ (−17 Da). The C-terminus of PepQV is cleaved at a conventional R residue. MS analysis of cells treated with acetic anhydride did not produce a peak at 1700.8 m/z (1658.8 + 42, Figure 4), consistent with the absence of a free α -amino group due to the N-terminal cyclization of glutamine.

Asu-nlp-58.—Spectra from both RMER and RMEL neurons also included ions with m/z 707.4 and 792.5 (Figure 4; Table 1). MS/MS analysis yielded sequences AV(I/L)SRYa and AA(I/L)(I/L)SRYa (Figure 5f, g). A direct BLAST search of the *A. suum* TSA database yielded a single entry where the calculated masses of two predicted peptides from the deduced sequence (Asu-NLP-58.1: AALLSRYamide, 792.5 Da; Asu-NLP-58.2: AVLSRYamide, 707.4 Da) matched those of the ions seen in the spectra (Figure 5f, g; Table 1). The sequence also contained a predicted N-terminal signal peptide with initiating methionine. We cloned the entire open reading frame using 5′- and 3′-RACE, which included a signal peptide and the expected peptide sequences (accession number MF737439, Figure 9). We also synthesized these peptides and subjected them to MS/MS analysis, which matched the fragmentation patterns observed from *A. suum* neurons (Supplementary Figure 3c, d).

In Situ Hybridization of Peptide-Encoding Transcripts.

As characterized in *C. elegans*,² there are roughly 100 different axons that extend circumferentially in the neuropil of the nerve ring, and a large number of synapses are made between them. In *A. suum*, manual dissection of single neuronal cell bodies from such an environment comes with some risk for contamination by processes from other cells

surrounding the neurons of interest. Although the distinct spectra we observe for the different subsets of RME neurons suggest that the cross contamination between the RME neurons is low, there is still possible contamination from other adjacent cells. The variability of low amplitude peaks in MS spectra also raises the issue of possible cell–cell contamination. Therefore, it is necessary to corroborate cellular expression of peptides by MS with an independent complementary technique like in situ hybridization (ISH) or immunocytochemistry (ICC). ISH uses a gene-specific labeled riboprobe to localize mRNA of peptide-encoding genes. This method not only gives information about the cell of interest but also identifies expression patterns in other cells throughout the preparation being examined. We synthesized digoxigenin (DIG)-labeled antisense probes for *afp-2* (AF5), *afp-4* (AF2), *afp-15* (AF7, 39, 40), *afp-16* (AF42–45 and PepQV), and *Asu-nlp-58* (Asu-NLP-58.1, 58.2) and looked for expression in whole-mount preparations as previously described.^{5,6}

afp-2.—Preparations treated with a labeled *afp-2* probe showed staining in the nerve ring in RMER/L (60% of preparations) and no observable staining in RMEV/D, consistent with single-cell MS data (Figure 10a). Staining of cells was also seen in six pairs of cell bodies in the anterior lateral ganglia, two neurons near the deirid commissures, and two pairs of nerve ring-associated cells located anterior to the nerve ring.

In the VG, there is staining in two bilaterally symmetrical pairs of cells in the VG. One of these pairs is located in the anterior region of the VG. The other pair has the same location and characteristic morphology as the neurons in the two pairs of AIY/AIM interneurons. The AIY/AIM neurons have very similar cell body size and morphology and are nearest neighbors in the VG. They have not yet been individually identified in *A. suum*. One pair of the four AIY/AIM neurons is a subset of the 10 cells in the head that displayed strong GABA-like immunoreactivity.¹⁴ We isolated these cells and analyzed them by single-cell MS. The resulting spectrum from one of the AIY/AIM pair contains a peptide with *m/z* 1198.7, characteristic of peptide As-NLP-22, which is found in GABAergic inhibitory motoneurons as previously reported.⁶ This neuron also contains AF5 (1051.6 Da, Supplementary Figure 4), which is consistent with the ISH results.

The excitatory DVB motoneuron found in the dorsorectal ganglion in the tail also displays strong GABA-like immunoreactivity. Preliminary single-cell MS data show a completely different peptide profile than any of the other GABAergic motoneurons. MS from DVB (Supplementary Figure 14) contains peaks that correspond to peptides found on the previously known *afp-1* transcript and the newly discovered *Asu-nlp-1* transcript (Supplementary Figure 15). A single peptide Asu-NLP-1.3 was sequenced by MS/MS (Supplementary Figure 16).

afp-4.—ISH localization of the *afp-4* transcript in *A. suum* head had previously been described⁸ and did not include positive staining of RMEV or RMED. To further investigate *afp-4* expression in RMEV and RMED, we synthesized a riboprobe to the same 223-nucleotide portion of the *afp-4* transcript used previously⁸ but implemented the optimized ISH protocol used to examine neuropeptide expression in the ventral cord motoneurons.^{5,6} First, we increased the concentration and exposure to proteinase K to digest more of the

thick hypodermis, thereby increasing the permeability of the tissue, and second, we allowed staining to continue overnight. With these modifications, staining of cells in the lateral lines were overall more intense, and preparations now revealed light staining in RMEV, and to a lesser extent RMED (Figure 10b), and consistent staining in ventral cord motoneurons DE2 and VE2.^{5,6} The new protocol produced similar expression levels and intensity of *afp-4*/AF2 in RMEV in ISH (13/29 preparations) and MS (11/20 cells), but RMED was easily lost during the whole mount dissection and washing and was only observed in 2/19 preparations. Given the weak and variable expression of *afp-4* by both techniques and the improved detection after increased exposure to proteinase K, it appears that *afp-4* is expressed at low levels in these cells near the detection limit for both techniques. However, a more quantitative and sensitive method, such as qPCR or single-cell RNA-seq, will be needed to confirm this hypothesis.

afp-15.—ISH of whole mount preparations using the *afp-15* probe showed staining of all four RME neurons at similar prevalences (RMEV, 24/30 preparations; RMED, 15/30; RMER, 21/30; and RMEL, 25/30; Figure 10c). Although RMER/L are usually closely associated with the nerve ring, as in Figure 10a, d, and e, in some cases, after dissection they are found with the lateral lines, as shown in Figure 10c. RMED is the most fragile of the RME neurons. In preparations where the nerve ring appears intact, RMED is always damaged because the cell body is severed from its dorsal cord process. Fairly frequently the cell body is lost from the preparation as in Figure 10c. These results generally agree with the single-cell MS results (RMEV, 19/20 cells; RMED, 21/22; RMER, 11/16; RMEL, 10/22), although MS showed more biased expression in RMEV/D. Because these techniques are only semiquantitative, we cannot yet determine whether these differences are functionally important.

afp-16.—Whole mount preparations exposed to the *afp-16* probe consistently and intensely stained RMEL (20/22 preparations) and RMER (20/22), consistent with MS data (RMEL, 21/22 cells; RMER, 16/16). However, RMEV/D were also *afp-16*-positive in ~70% of the preparations (RMEV, 15/22; RMED, 16/22) (Figure 10d). Detection of peptides encoded by the *afp-16* transcript in RMEV/D single-cell MS spectra was rare with only 3/5 peptides encoded by *afp-16* observed in ~20% of the spectra (RMEV, 5/20; RMED, 4/20). The peaks themselves were usually weak and/or the spectra contained many other peaks corresponding to peptides not known to be expressed by RMED/V, which suggests contamination from surrounding tissue. At present, it is unclear whether the *afp-16* peptides are part of that contamination or are expressed in RMED/V at a level that is at the limit of detection for single-cell MS. However, the *afp-16* transcript is clearly and consistently detected in all four RME neurons by ISH, and staining was often very intense. These conflicting results could be due to a difference in sensitivity between the two techniques or to translational control of this transcript in the cell. Additional experiments will be necessary to determine which case is appropriate here.

Asu-nlp-58.—In contrast to *afp-16*, *Asu-nlp-58* ISH experiments confirmed the presence of the peptide-encoding mRNA with moderate-to-intense staining of RMER/L (RMER, 14/18 preparations; RMEL, 22/22; Figure 10e). No staining was observed in RMEV/D.

These results agree nicely with the single-cell MS data reported above. Other *Asu-nlp-58*-positive cells included two bilateral pairs of small cells in the lateral lines at the level of the nerve ring and a single moderately stained cell in the RVG (data not shown).

Immunocytochemical Localization of AF5.

When possible, our laboratory endeavors to independently verify the expression of fully processed peptides using highly specific antibodies. These experiments complement the expression of peptide-encoding transcript as determined by in situ hybridization and often show staining of cell processes in addition to the cell body where most mRNA is localized. Generating a specific antibody against AF peptides is a formidable challenge due to their short length and highly conserved C-terminal sequence for which the family is named. Here, we used a multiple antigenic peptide (MAP) as an antigen in the hope of producing a highly specific antibody. The immunogen (SGKPTFIAA-MAP) was specifically designed to exclude the C-terminal -RFamide sequence that is common to all AF peptides. The antibody was immunopurified from serum by absorption onto an Affigel matrix conjugated to authentic AF5 by *N*-hydroxysuccinimide chemistry; furthermore, the ICC localization was carried out on paraformaldehyde-fixed preparations, which produces a different chemical linkage with endogenous protein. We hoped that all these factors together would increase the specificity of the final antibody, increasing the likelihood that the epitope recognized by the antibody would be the N-terminal part of the peptide, which is relatively divergent among AF peptides; our results suggest that we were successful. We tested the antibody against a set of 39 peptides, including those that share C-terminal -FIRFamide (AF1, AF7, AF24, AF39, and AF40) by dot ELISA. No cross-reactivity with any other peptide was seen (Supplementary Figure 1).

The purified anti-AF5MAP antibody robustly stained AF5 in dot ELISAs. When used on whole mount preparations as previously described,⁴ the results were generally more consistent than those observed using the *afp-2* riboprobe. This subset included the RMEL/R neurons, which were stained in approximately half of the 22 preparations (Figure 11).

Bioactivity.

Because the RME neurons are GABAergic motoneurons and presumably inhibitory like the GABAergic DI and VI motoneurons in the nerve cords, we were interested to see how the peptides they express affect locomotion and head searching behavior. Three of these peptides were previously studied,^{21,29–31} and the results are summarized in Table 2. For the newly discovered peptides, we tested their effects on both acetylcholine (ACh)-induced muscle contraction^{4–6} and whole worm locomotory behavior²¹ using established protocols.

afp-15.—In the muscle contractile assay, all of the peptides encoded by *afp-15* significantly inhibited muscle contraction while present in the chamber (10 μ M) followed by a rapid recovery after peptide was washed out (Figure 12a). The strongest responses were observed when preparations were exposed to AF40 and AF7 ($16 \pm 3\%$ and $26 \pm 4\%$ of prepeptide levels, respectively), whereas preparations exposed to AF39 displayed a more modest decrease ($63 \pm 2\%$ of prepeptide levels). In all cases, the contractile response immediately

returned to control levels once peptide was washed out of the test chamber, and no significant changes in baseline muscle tension were observed.

When the 3 *afp-15* peptides were injected simultaneously into intact worms, we saw a cessation of anteriorly propagating locomotory waveforms, stiff, straight body postures, and decreased head searching behavior ($n = 3$). The amplitude of the head searching movements was also decreased and tended to move more laterally than dorsally/ventrally. The effects lasted longer than those observed in the muscle strip assay with recovery beginning around 40 min after injection. No changes in body length were observed.

afp-16.—Behavioral effects of the peptides encoded by *afp-16* had not been previously investigated. We found that only two of these peptides affected muscle contraction in strips of dorsal body wall at 10 μ M. AF42 caused a significant decrease in the contractile response to ACh (~60% of the prepeptide response), and no recovery was observed over 50 min (Figure 12b). In contrast, preparations exposed to AF45 contracted normally while peptide was present but exhibited a delayed small inhibition between 30 and 50 min once peptide was removed from the test chamber. The remaining peptides on the *afp-16* transcript did not have significant effects on ACh-induced contraction. No significant changes in baseline tension of the preparations were observed for any of these peptides.

Intact worms injected simultaneously with all the *afp-16* peptides exhibited large, loose locomotory waveforms as opposed to normal tightly coordinated anteriorly propagating waves ($n = 3$). Head searching behavior was also affected, with the movements being smaller in amplitude and biased toward the dorsal/ventral axis. No changes in body length were observed.

Asu-nlp-58.—Both of the novel peptides encoded by *Asu-nlp-58* had significant effects on muscle contraction; however, their profiles were different (Figure 12c). Asu-NLP-58.1 caused a slight, prolonged inhibition of ACh-induced contraction similar to the response to AF42, although the effect only reached statistical significance from 30 to 50 min. In contrast, preparations exposed to Asu-NLP-58.2 exhibited the initial inhibition of contraction in the presence of peptide ($52 \pm 3\%$ of the prepeptide response) followed by a rapid recovery to control levels once peptide was washed out of the test chamber. This effect resembled the responses recorded in the presence of the *afp-15* peptides. Given the similarity in the amino acid sequences of the two Asu-NLP-58 peptides (Asu-NLP-58.1, AALLSRYa; Asu-NLP-58.2, AVLSRYa), these effects were somewhat surprising but suggest that the sequence differences are important. It will be interesting to determine whether each has a distinct receptor.

Intact worms injected with the *Asu-nlp-58* peptides formed anteriorly propagating locomotory waves, but the wavelength was decreased compared to prepeptide waves, and the worms tended to remain on the bottom of the beaker instead of moving freely through the available space like control worms ($n = 6$). Head searching behavior was initially inhibited upon peptide injection and was biased toward the dorsal/ventral axis, although this may have been affected by the tight locomotory waveforms. Head movements became more frequent

and 3-dimensional around 40 min after peptide injection. No changes in overall body length were observed.

Conservation Among Nematodes.

The morphologies of the neurons in *A. suum* and *C. elegans* are remarkably similar; so similar, in fact, that many neurons in *A. suum* are named for their homologues in *C. elegans*. Considering that nematodes are estimated to have arisen 550 Mya,³² and that *A. suum* and *C. elegans* are in different clades, this is remarkable and suggests either a strong positive selection pressure on the morphology of the neurons or an extremely conservative developmental program that leads to the structure of the nematode nervous system. This similarity further extends to the cellular expression pattern of the classical transmitters acetylcholine,^{33,34} serotonin,³⁵ and dopamine.³⁶ It also applies to GABA, with the exception of two sets of ventral ganglion neurons, which clearly show different GABA expression in *A. suum* and *C. elegans*.^{14,15,33} The conserved subset of GABAergic cells includes 19 ventral cord motoneurons, the DVB neuron in the tail, and the four RME ring motoneurons that are the focus of this study.

Another feature of the nematode nervous system that is highly relevant to the present study is the conserved sequences of neuropeptides, as previously noted.^{3,5,6,37,38} In the present paper, we further extend the sequence comparisons among nematodes to the peptides found in the RME neurons. The sequences of some peptides are almost completely conserved in the majority of nematodes, implying strong selective pressure on function. Others show partial sequence conservation, and it is currently unclear whether this implies functional divergence in different species, tolerance by the receptor(s) for amino acid substitutions at certain positions in the peptide sequence, or concurrent evolution of peptide and receptor.

From different species of nematodes, we made multiple alignments of the amino acid sequences of the precursor proteins from which the RME peptides are cleaved post-translationally (see Figure 13 and Supplementary Figures 5–13, 17, and 18). In most cases, it is the actual processed peptide that is highly conserved, and intervening sequences diverge to extents that are related to phylogenetic relatedness as seen in these figures where sequences are organized by clade.

The *afp-2* transcript found in RMEL/R is similar to *flp-4* in *C. elegans*. Homologous transcripts have been predicted in 13 other nematode species (Supplementary Figures 5 and 6). *flp-4*-GFP-constructs in *C. elegans* were not expressed in any of the RME neurons.³⁹

AF2, found in the RMEV/D motoneurons in *A. suum*, is encoded by the *flp-14* and *gpf1p-3* transcripts in *C. elegans* and *G. pallida*, respectively.^{39,40} Although the peptide has been isolated from *C. elegans*, GFP reporter constructs for *flp-14* failed to express in any cell.⁴¹ In *G. pallida*, *gpf1p-3* was detected by ISH in two neurons at the dorsal edge of the nerve ring, described by the authors as RMED and RMEL or RMER, although the position of the neurons in the published micrographs makes this identification difficult. If AF2 is not expressed in RMEV in *G. pallida*, this is a significant difference. Multiple alignments of predicted precursor proteins show strong conservation (Supplementary Figures 7 and 8). Especially striking is the presence in all species of multiple copies of AF2 or its close

relative. In all nematodes, the peptide-encoding region is at the C-terminus of the precursor protein. Clade I nematodes include 3 copies, 2 of which are AF2 (KHEYLRFa) and the other KHDYLRFa; clade III nematodes all contain 3 copies of AF2 (except for *Onchocerca volvulus*, which has an I/L exchange in 1 copy), and clade IV nematodes have 2 or 3 copies of the peptide, in most cases all AF2 (in *Meloidogyne* spp. and *Pratylenchus* spp, 1 copy is AF2 and the other KHEFVRFa). In clade V nematodes, there are almost always 4 identical copies of AF2 (in *Teladorsagia circumcincta*, one of the peptides is RHEYLRFa). This family of peptide encoding proteins is also unusual in that there is a fairly well-conserved region N-terminal to the peptide-encoding region itself; MS spectra have not yet detected this fragment, but it is larger than the mass range we usually scan for peptides. Perhaps it is important in the folding of the precursor protein before cleavage or perhaps it is used as a different secreted signaling molecule in its own right.

The *afp-15* transcript expressed in all four RME neurons in *A. suum* is similar to *flp-5* in *C. elegans*. Homologous transcripts have been predicted in 17 other nematode species (Supplementary Figures 9 and 10). *flp-5*-GFP constructs in *C. elegans* were not expressed any of the RME neurons, although unlike *flp-14* it was expressed in other *C. elegans* neurons.³⁹

The five peptides encoded by *afp-16*, found consistently in RMEL/R and sometimes in RMED/V in *A. suum*, most closely resemble the *flp-26* peptides in *C. elegans*. Recent BLAST searches of EST and TSA databases found 12 other nematode species that share the -LXLRFamide C-terminal (Supplementary Figures 11 and 12). The sequences fall into two distinct groups, corresponding to clades III and V. In clade III, there are five distinct predicted processed peptides, whereas there are only two in clade V. BLAST searches return only these sequences, suggesting that there are not other related, undescribed genes in these species. One possibility is that the clade III and V genes are of independent origin but show convergence. Alternatively, they may have diverged from a common ancestor. Examination of homologous transcripts from nematodes in other clades would be interesting.

The *Asu-nlp-58* transcript encodes two peptides that belong to a family of peptides that has not previously been described in nematodes. Searches of nematode EST and TSA databases found 18 other *Asu-nlp-58*-like transcripts, all of which encode two peptides that are highly sequence-related with most of the sequences sharing the -LSRYamide C-terminal motif (Figure 13; Supplementary Figure 13). Among the 19 species identified here, four of five clades are represented (clades I, III, IVb, and V). Species from clade III all share the same AALLSRYG and AVLSRYG sequences. The first peptide in the sequence in clade V is PALLSRYG and the second is A[V/M]LPRYG. Clade IVb is highly similar to clade V, but both peptides have a single residue N-terminal extension. Cellular expression patterns have not yet been reported in these other species.

Comparison of Techniques and Variability in Expression Levels.

Since we discovered that knowledge of the neuronal anatomy, morphology, and classical transmitter expression were not sufficient to predict nematode locomotory behavior,²³ the goal of this laboratory has been to identify the neuromodulators, specifically neuropeptides, that change the activity of the neurons and/or muscles and therefore affect locomotory

behavior in *A. suum*. With the publication of the *A. suum* genome⁴² and transcriptome,^{42,43} many neuropeptide sequences have been predicted. However, because knowledge of the specificity and cellular expression patterns of the processing enzymes that cleave neuropeptides from the precursor protein is not robust, these predictions need to be verified. Our laboratory chose to detect the processed peptides in identified neurons directly through analysis by single-cell MS (and this, of course, automatically gives information on cellular localization) and use ISH (and where possible ICC) to verify cellular expression of their transcripts. Each of these techniques has potential limitations: for example, peptide peaks may be drowned out of MS spectra by ion suppression or inability of the peptide to accept charge, and accurate localization by ISH relies on the probe penetrating through the hypodermis that surrounds neurons in the nerve cords and ganglia to enter the cell and bind to complementary mRNAs (further discussion below). However, by using multiple methods, we gain a clearer picture of what is happening in the cell.

For the vast majority of cases, we see good agreement between MS and ISH, and when a highly specific antibody is available, ICC as well.³⁻⁶ Here, we see clear, consistent expression of the *afp-15* peptides in all four RME neurons and of the *Asu-nlp-58* peptides in RMEL/R by single-cell MS and ISH. However, expression in other cases was more variable than we had seen in previous studies. For example, ISH detected *afp-16* transcript in all four RME neurons, whereas MS was only able to detect peptides in ~20% of RMED/V cells (Table 1). To determine whether variability is representative of true biological differences in expression levels, the possibility of experimental artifact must first be examined. For techniques such as single-cell MS that require dissection of cells from surrounding tissue, the dissection process can result in damage to the cell via mechanical stress or enzymatic overdigestion by collagenase. Further, because neuronal cell bodies in the nerve ring or nerve cords in *A. suum* are often surrounded by processes from other cells, there is also the possibility that a small fragment of extraneous cell processes could accompany the chosen cell body onto the target plate. Out of all 298 neurons in *A. suum*, the four RME cell bodies are arguably the most difficult to dissect without damage or contamination from surrounding tissue. Although they are the only neuronal cell bodies in the nerve ring, they are entangled with processes from many (~100) other neurons. The nerve ring is the central processing center in the worm and many synaptic connections are made there. The dissection process is further complicated by adjacent ganglia; RMED is closely situated to the dorsal ganglion (DG), which contains two other cells, RID and ALA.³ RMEV is buried next to the ventral ganglion, which contains 33 cells, all of which send processes into the nerve ring, joining processes from other neurons in the DG, lateral ganglia, and ventral nerve cord as well as processes of sensory neurons. Use of the vital dye methylene blue (staining neuronal cell bodies) differentiates the four RME cell bodies from the closely associated processes from other neurons, making isolation more manageable. If contamination from processes of other cells was a serious concern, distinct MS spectra of single cells would not emerge. Our results show some clear differences in the spectra of the different subsets of RME neurons with very distinct major peaks. In addition, the use of complementary approaches, in situ hybridization, and immunocytochemistry usually shows that the cells that contain each peptide detected by MS also contain the mRNA that encodes these peptides. Both of these

considerations validate the integrity of the dissection and suggest that peptides from the processes of other cells are not a major source of contamination.

Once cells are isolated for MS analysis, the resulting spectra can also be affected by the peptide content of the cells themselves. For example, some peptides do not readily accept charge and thus are difficult to detect by this method. Other peptides accept charge so well that they sequester charge away from other peptides in the mix, resulting in a spectrum that misrepresents the relative amounts of peptides in the cell and drowning out smaller peaks. Although this could be a concern for de novo identification of peptides, single-cell MS has already identified numerous novel peptides compared to LC-MS/MS studies from tissues and whole ganglia,^{26,44} highlighting the sensitivity of this approach. Our results show that the simplification of the mixture of peptides present in a single neuron compared with that in a ganglion makes a significant reduction in problems due to ion suppression.^{3,5,6}

Our second method of peptide localization is via in situ hybridization. Although this technique, unlike MS, requires prior knowledge of the transcript sequence to design a gene-specific probe, it also allows us to identify all the cells in the preparation that express that particular gene. ISH is only semiquantitative; the intensity of the staining certainly depends on the relative expression level of the gene but could also be impacted by poor penetration of the probe into the tissue/cell or damage to the tissue during dissection and fixation. Our laboratory previously used this technique to localize nine neuropeptide transcripts,^{3-6,8} and in the vast majority of cases, the same subset of cells is stained with medium-high intensity from preparation to preparation. Because the pattern of stained cells is unique to each probe, this suggests that we are seeing gene-specific localization patterns as opposed to artifacts of the technique, such as certain cells taking up probe more easily than others. However, more variability is common among cells that stain more lightly. In these cases, additional evidence is needed to determine whether the transcript is expressed at variable or low levels within the cell or the probe (or staining solution) is not making it into the cell.

A third method for determining neuropeptide expression is immunocytochemistry. Generally, ICC will stain both the cell body and its major processes as opposed to only the cell body as is typical of ISH, providing better cell identification. However, generating a suitably specific antibody is a formidable task. Many of the neuropeptides identified in the *A. suum* nervous system belong to the FMRFamide-like peptide class, and the conserved C-terminus provides ample opportunity for cross-reactivity. In the present study, we increased the specificity of the AF5 antibody by using a MAP antigen and by affinity purification of the antiserum, which selects for relatively high affinity antibodies. When specific antibodies are available, peptide-specific subsets of cells are consistently stained.

In other cases, there was much more variability from preparation-to-preparation (or cell-to-cell) across techniques, namely, in the cases of *afp-2* and *afp-4*. For *afp-2*, MS detected AF5 in 73% of RMER cells, 60% of preparations by ISH, and 50% of preparations by ICC (Table 1). Similarly, *afp-4* was detected in roughly half of RMED/V/R cells by MS and in RMEV by ISH, whereas staining in RMED and RMER was only seen in 3–7% of preparations. We attempted to increase permeability of the cells (and thereby penetration of the riboprobe) by increasing the proteinase K incubation step in the ISH protocol, and this helped reveal more

cells but still did not reach the consistency seen for other transcripts. In these cases, seeing variability at both the transcript and peptide level, we suspect that the observed variability is real and due to low-level expression of these transcripts, which can be near the limit of what we can detect. For resolving these issues, more sensitive and especially more quantitative approaches, such as qPCR or single-cell RNA-seq, will be needed.

Encouragingly, most of the peptides found in the RME neurons had significant effects on muscle contraction or locomotory behavior. The majority of effects were inhibitory in nature, although AF5 and AF7 caused hyperactivity and tremors in the head, and AF2 had primarily excitatory activity in muscle, DE2, and intact worms.^{21,29} These results, combined with the peptide localization in the RME motoneurons, confirm that these are relevant components of the locomotory circuit.

Overall Conclusions.

This study reports the neuropeptide expression profiles of the four RME inhibitory motoneurons of the head using single-cell MS, ISH, and in one case, ICC. Although these techniques showed nearly identical results in the ventral nerve cord^{5,6} and dorsal³ and ventral⁴ ganglia, we saw greater variability in the RME neurons, and in some cases, the variability was noted among multiple techniques. This suggests that the variability cannot be explained by experimental artifact of any one technique alone and is likely indicative of either low-level transcript/peptide expression near the limit of these techniques or true variability from cell-to-cell. An alternative method that is more quantitative and sensitive will be needed to investigate these possibilities, and we are currently using single-cell RNA-seq to attempt to solve this issue.

We also report the identification of nine novel peptides from three different transcripts and found that many of these had significant inhibitory activity on muscle contraction and whole worm behavior. Homologues of these peptides were identified in other nematode species, in some cases with sequences identical to the peptides in *A. suum*, implying strong selection pressure on the amino acid sequences. Further study will be needed to identify the receptors and mechanism of action for these peptides and determine how they fit into the locomotory circuit.

METHODS

Animals.

Live *Ascaris suum* were collected from pig intestines at a slaughterhouse and washed with phosphate-buffered saline (PBS, 140 mM sodium chloride, 10 mM sodium phosphate, pH 6.8–7.5) at 37 °C to remove chyme. They were transported to the laboratory and maintained in PBS, which was changed daily. Worms were used within 3 days of collection.

Sample Preparation for Mass Spectrometry.

Adult female *A. suum* were injected with 0.1–0.3 mL of 2 mg/mL collagenase (Sigma Blend H) in *Ascaris* saline (4 mM sodium chloride, 125 mM sodium acetate, 24.5 mM potassium chloride, 5.9 mM calcium chloride, 4.9 mM magnesium chloride, 5 mM 3-(N-

morpholino)propanesulfonic acid (MOPS) buffer, pH 6.8) and incubated for 1.5–2 h at 37 °C to dissociate the muscle tissue. A 6–7 cm portion of the worm anterior to the gonopore was removed and transferred to a Sylgard-lined dish, and single identified neurons were dissected out as previously described^{5,6} in isotonic glycerol dissecting solution (170 mM ammonium acetate in 30% glycerol).^{45–47} For better visualization, some preparations were bathed in 0.8 mM methylene blue in 170 mM ammonium acetate for 20–30 s then cleared with 170 mM ammonium acetate and dissected as described above. Mass acquisition, on-target chemical modification, and interpretation of spectra were performed as previously described.^{5,6}

Database Searches.

Database searches for transcripts encoding novel peptides that had been sequenced by MS/MS were conducted using methods described in recent publications.^{5,6} For these searches, program settings were modified for searching short sequences using a word size of 2, an *E* value of 20,000, and a PAM30 matrix. Search results were examined for peptide cleavage sites and C-terminal glycine for amidated peptides. Signal peptides were predicted using Signal P (<http://www.cbs.dtu.dk/services/SignalP/>).

For multiple alignment of homologous transcripts encoding similar peptides in other nematodes, both the *A. suum* predicted precursor protein and its homologue in *C. elegans* were entered as queries in BLAST searches of the Expressed Sequence Tag (EST) and the Transcribed Sequence Assembly (TSA) databases in NCBI and Nematode.Net. The word size was 6, and the BLOSUM62 matrix was used. Selected sequences were imported into MEGA7⁴⁸ for alignment by MUSCLE.⁴⁹ The alignments were imported into Jalview⁵⁰ for display.

Peptide Synthesis.

Peptides were synthesized by the University of Wisconsin-Madison Biotechnology Center. The quality of the synthesis was monitored by LC/MS and HPLC, and the purities are reported in Supplementary Table 2.

Transcript Identification and in Situ Hybridization.

RNA isolation, reverse transcription, and PCR/RACE reactions were performed as previously described^{5,6} using the primer pairs listed in Supplementary Table 1. The target sequences of each riboprobe are shown in Figures 6–9. The in situ hybridization protocol was performed as previously described.^{5,6}

Antibody Characterization.

The N-terminal portion of the AF5 sequence with two spacer alanines (SGKPTFIAA) was synthesized onto a backbone of seven branched lysines with eight amino groups. This generated an octameric multiple antigenic peptide (MAP) molecule (adapted from ref 51), which was used for immunization of rabbits without conjugation to carrier protein (Panigen Inc., Blanchardville, WI). Polyclonal antibodies were purified from whole serum via affinity chromatography on AF5-conjugated silica bead columns (0.5 mg/mL of AF5 in MOPS

buffer, pH 7.5–8.0 incubated overnight at 4 °C with 1–2 mL of Affigel Beads (Bio-Rad, Hercules, CA) suspended in MOPS as previously described.⁵²

Antibody specificity was tested via dot-ELISA⁵³ on nitrocellulose paper spotted with 39 known AF peptides^{3,4,26,44,52,54–56} in a 1 mg/mL bovine serum albumin (BSA) solution in PBS and 1 mg/mL of BSA as a negative control (Supplementary Figure 1). Blots were spotted with 1 μ L of the peptides at concentrations of 1 and 5 μ g/mL, and the antibody was tested at 1:250 and 1:1,000 dilutions. At both concentrations, the anti-AF5 antibody showed robust staining for AF5 and no cross-reactivity with BSA or the other peptides (Supplementary Figure 1). Antibody specificity was further corroborated by comparing the cellular expression patterns observed by immunocytochemistry with the results of in situ hybridization with probes designed from the peptide-encoding transcript and with the results of direct chemical identification of peptides by single-cell MS.

Whole Mount Immunocytochemistry (ICC).

Female *Ascaris suum* were injected with collagenase, dissected, fixed with paraformaldehyde, and prepared as previously described.⁴ The anti-AF5 antibody was diluted to the working concentration in P1+ (0.1% BSA, 0.5% NP-40, 0.5% TritonX-100, 10% normal calf serum in PBS, pH 7.3–7.4). In some preparations, anti-AF5 antibody was used at 1:50 or 1:20. Negative controls were treated without primary antibody.

The secondary antibody was a goat-antirabbit polyclonal antibody conjugated to horseradish peroxidase (GAR-HRP, Bio-Rad Laboratories) used at 1:500 in P1+. The preparations were exposed to staining solution (0.006% H₂O₂, 0.03% 3–3'-diaminobenzidine-4-HCl in PBS), dehydrated through an ethanol series, and mounted on slides with Permount (Fisher Scientific) as previously described.⁴

Pharmacological and Behavioral Experiments.

Peptide effects on acetylcholine-induced muscle contraction were assayed as previously described.^{5,6} Hydrophobic peptides (AF42–45, Asu-NLP-58.1–2, denoted with “*h*” in Figure 12) were first dissolved in 10% DMSO and then diluted to the working concentration with water (final concentration of 0.001% DMSO in the test chamber). Separate muscle strips were used for each tested peptide and compared to saline-only or DMSO controls as appropriate. Responses were reported as a percentage of the maximum ACh contraction observed for each muscle strip and plotted as an average $\pm$ SEM for each experimental condition. Significance was determined separately for each time point using unpaired *t*-tests with a threshold of *P* < 0.05. Peptide effects on behavior in intact worms were assayed as previously described.²¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AC	amphidial commissure
ACh	acetylcholine
AF	<i>Ascaris</i> FMRFamide-like
afp	<i>Ascaris</i> FMRFamide-like precursor protein transcript
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHCA	α -cyano-4-hydroxycinnamic acid
DC	dorsal nerve cord
DeC	deirid commissure
DG	dorsal ganglion
DHB	2,5-dihydroxybenzoic acid
ELISA	enzyme-linked immunosorbent assay
EPSP	excitatory postsynaptic potential
EST	expressed sequence tag
flp	FMRFamide-like peptide gene
GABA	γ -aminobutyric acid
GFP	green fluorescent protein
GSS	genomic survey sequence
ICC	immunocytochemistry
ISH	in situ hybridization
LC	liquid chromatography
LLL	left lateral line
MALDI-TOF	matrix-assisted laser desorption/ionization–time-of-flight
MS	mass spectrometry

MS/MS	tandem mass spectrometry
<i>m/z</i>	mass-to-charge ratio
NLP	neuropeptide-like protein
<i>nlp</i>	neuropeptide-like protein gene
NR	nerve ring
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RLL	right lateral line
RVG	retrovesicular ganglion
SL1	spliced-leader 1
TSA	transcriptome shotgun assembly
VC	ventral nerve cord
VG	ventral ganglion

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Figure 1.

Diagram of RME anatomy, modified from Guastella et al., 1991.¹⁴ (a) Diagram of the head ganglia of *A. suum*. The worm has been split to the right of the dorsal axis and opened flat. Neuronal cell bodies and commissural processes are shown. The nerve ring (NR), ventral ganglion (VG), dorsal ganglion (DG), lateral ganglia (LG), and retrovesicular ganglion (RVG) are indicated. LLL, left lateral line; RLL, right lateral line; VC, ventral nerve cord; DC, dorsal nerve cord; DeC, deirid commissures; AC, amphidial commissures. (b) Drawing of the cephalic region illustrating GABA-immunoreactive neurons. The RME cells (labeled RMEV, red; RMED, orange; RMEL, green; RMER, blue) are found in the nerve ring; each sends two lateral processes into the nerve ring (the processes truncated in the diagram). RMEV and RMED send processes down the ventral and dorsal cords, respectively. Anterior is to the top of the diagrams.

Figure 2. Mass spectra from single identified RME neurons. Spectra collected from (a) RMEV and (b) RMED with no chemical modification. Spectra collected from (c) RMEV and (d) RMED treated with acetic anhydride to acetylate free amines (N-terminus, lysine) and, to a lesser extent, tyrosine residues, causing +42 Da mass shift (+Ac). Expansions of 950–1250 m/z range from (e) RMEV-ac and (f) RMED-ac. X -axis, m/z , is mass-to-charge ratio. Y -axis is intensity of MS signal in arbitrary units (a.u.).

Figure 3.

MS/MS of peptide peaks from RMEV/D. MS/MS spectrum of (a) m/z 991.5, previously identified as AF2, (b) m/z 962.6, previously identified as AF7, and (c) m/z 1065.6, novel peptide AF40. Peaks representing N-terminal b-ions (blue), C-terminal y-ions (red), a-ions (green), and high intensity internal fragment and immonium ions (purple) are labeled, and b and y ions are summarized in the sequence at the top of each spectrum. X-axis, m/z , is mass-to-charge ratio. Y-axis is intensity of MS signal in arbitrary units (a.u.).

Figure 4.

Mass spectra from single identified RMER and RMEL neurons. Spectra from unmodified (a) RMER and (b) RMEL neurons. Spectra collected from (c) RMER and (d) RMEL neurons treated with acetic anhydride. X -axis, m/z , is mass-to-charge ratio. Y -axis is intensity of MS signal in arbitrary units (a.u.).

Figure 5.

MS/MS of peptide peaks from RMER/L: (a) m/z 1051.6, AF5; (b) m/z 1726.9, AF42; (c) m/z 1344.8, AF43; (d) m/z 1645.8, AF44; (e) m/z 1658.8, PepQV; (f) m/z 792.5, Asu-NLP-58.1; (g) m/z 707.4, Asu-NLP-58.2. Peaks representing b-ions (blue), y-ions (red), a-ions (green), and high intensity internal fragment and immonium ions (purple) are labeled, and b and y ions are summarized in the sequence at the top of each spectrum. X-axis, m/z , is mass-to-charge ratio. Y-axis is intensity of MS signal in arbitrary units (a.u.).

ggtttaattacccaagtttgaggcttgaaaacaatggctgccttcaagaccactgttttt
M A A F K T T V F
tcatgttttggttacattgatgctcactttatgttcgtggagagcatcaacgctgagctg
S C L V T L M L T L F V V E S I N A E L
gataacggagatatcacaatggcaaaggacgatatgcttcgggcactctacaattacata
D N G D I T M A K D D M L R A L Y N Y I
ctttatgagcgcaaaggtgcacacgattttgatggatttgacgtgcacaacgatcaggc
L Y E R K G A H D F D G F E R A Q R S G
aaaccaacgttcattcgttttggaagaggagtgcgaatattgtaacgaatccagaaaac
K P T F I R F G K R S A N I V T N P E N
atgtgaagatattgcttcaataattcaacaatgcaatttgacgtgccctaaaataagatc
I -
acctgcataaaattttttttgtgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 6.
afp-2 transcript (JN547402.1). Signal peptide, blue and italic; putative neuropeptide, red and underlined; putative basic cleavage sites, bold. The *afp-2* riboprobe sequence is denoted by bolded, italicized, underlined nucleotides. Primer to the SL1 spliced-leader sequence is indicated in orange.

acatggggaattcaaaat**gctctccagattacaactggttgtgctcgccttagctctgtgt**
 M L S R L Q L V V L A L A L C
cttcttcaagtgaccacctcacagctatcagaaacattcgatcgtgatgaacggtcacccg
 L L Q V T T S Q L S E T F D R D E R S P
aaacagaaattcatccgttttgaagggctggtagcgcgagattcatcaggttcggtagg
 K Q K F I R F G R A G S A R F I R F G R
agttctcccgatctatgggatgaatcgaaagggctcttcagaaatcgacgaaatgaaacgt
 S S P D L W D E S K G S S E I D E M K R
gctgggccgagatttattcgattcggatgagtattttaatgcaaaaaatcgccattcaaa
 A G P R F I R F G -
 aatcacataaaattcgttcattataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 7.

afp-15 transcript (MF737440). Signal peptide, blue and italic; putative neuropeptides, red and underlined; putative basic cleavage sites, bold. The *afp-15* riboprobe sequence is denoted by bolded, italicized, underlined nucleotides.

ggtttaattacccaagtttgagatccaccttcgcatgatgatca**cgtccagttttgtcgca**
M M I T S S F V A
gtgttggtgcttataattgctgttcatgtgtgcgcacaggaagaggaaggatggcgtg
V L V L I I A V H V C A Q E E E E D G V
ctgcctgaaagggttcgtacgctcatttccgaacaactatttggcctcagatctggctctt
L P E R F V R S F P N N Y L A S D L A L
cgatttggaaaagcgatcttgggaaatgaatggcagctcaaagcgaagaattggtatcaac
R F G K R S W E M N G S S K R R I G I N
gaaaagcgaagaattgacatcaacgatcttgccttgagattcggcaaacgtagctcctac
E K R R I D I N D L A L R F G K R S S Y
tcattcgacccttccaacttaaacttgaggtttggttaagcgatcgtttgagggtatgctg
S F D P S N L N L R F G K R S F G G M L
gaaggattcgatgagaagaggactttcgtgattcccactgatctggcgcttcggttcgga
E G F D E K R T F V I P T D L A L R F G
aagaagtgatcaatattgactactactagcacttcaaatcattgatcaatatgactgttg
K K -
cgtcgttcgaaatcacatgaacttatcgatgtgttcggttcatagcggcactgagtgcgca
tcacagagtgcttcaagtagagtatagaagactcttcaataaagaaaaaaaaaaaaaaaa
aaaaaaa

Figure 8.

afp-16 transcript (MF737441). Signal peptide, blue and italic; putative neuropeptides, red and underlined; putative basic cleavage sites, bold. The *afp-16* riboprobe sequence is denoted by bolded, italicized, underlined nucleotides. Primer to the SL1 spliced-leader sequence is noted in orange.

atcaattggtcattcatgcacaaattttagcgaatgtc ***gataagcacacagttgttcgctctca***
M S I S T Q L F V S
tttgtcatactcactaataatggttaactgctgaattcgcaacgaacgacatatgttgagcca
L C I L T L M V T A E F A T N D I L E P
actaaacggcgagcacttttgcagatacggtagggcgctactttcca agatatgggaaa
T K R ***A A L L S R Y G R A V L S R Y G K***
cgatcgctaccacaatcaggatattcatcgtttggtgagcaaacgatgggtgattatt
R S L P Q S G Y S S F G E Q T D G G L F
ttatgcccagacaatatggtgaagttatgcaatgcataccatatcacttaacggttaacgttaa
L C R Q I Y G E V M Q C I P Y S R -
acgattatgcaataaattgcaattaaaattgcaaacgattacaatcgagacgtaacaatt
gcaaattttgttgaaattcaattagaaattttgcacttcatttgattgaataaatgcattta
ttcaacgttgatttaattatgtgttaaaataaaatcgtcatttttcaacaatatatgcg
atatgttggaattcatttagtaataaaattttcacagatgctaaaaa
aaaaaaaaaaaaaaaa

Figure 9.

Asu-nlp-58 transcript (MF737439). Signal peptide, blue and italic; putative neuropeptides, red and underlined; putative basic cleavage sites, bold. The *Asu-nlp-58* riboprobe sequence is denoted by bolded, italicized, underlined nucleotides.

Figure 10.

In situ hybridization of RME neurons. Whole mount preparations stained with the (a) *afp-2*, (b) *afp-4*, (c) *afp-15*, (d) *afp-16*, and (e) *Asu-nlp-58* riboprobes. (f) Negative control preparation treated with *afp-16* sense probe. Stained RME neurons are indicated by arrows. NR, nerve ring; RLL, right lateral line; LLL, left lateral line; VC, ventral nerve cord. Scale bar, 100 μ m. Anterior is to the top of each panel.

Figure 11. Anti-AF5 immunocytochemistry. Whole mount preparations exposed to anti-AF5MAP antibody showed staining in RMEL/R. Stained RME neurons are indicated by arrows. NR, nerve ring; LLL, left lateral line; RLL, right lateral line; VC, ventral nerve cord. Scale bar, 100 μ m. Anterior is to the top.

Figure 12.

Pharmacological effects of RME peptides on muscle contraction. ACh-induced contraction was measured with exposure to (a) *afp-15*, (b) *afp-16*, and (c) *Asu-nlp-58* peptides. Contractile response to 5 μ M ACh was plotted as the average response for each experimental group reported as a percentage of the maximum contraction obtained before exposure to peptide for each preparation. Peptide (10 μ M) was introduced at 0 min and washed out at 10 min (denoted by shaded box), followed by 40 min of recovery. Error bars show SEM. * P < 0.05. ^aPeptides dissolved in DMSO (see Methods).

Figure 13.

Alignment of nematode sequences related to Asu-nlp-58. Nematode sequences are organized alphabetically by clade (denoted by Roman Numerals). Tpe, *Trichinella pseudospiralis*; Tsp, *Trichinella spiralis*; Alu, *Ascaris lumbricoides*; Asu, *Ascaris suum*; Tca, *Toxocara canis*; Aav, *Aphelenchus avenae*; Bxy, *Bursaphelenchus xylophilus*; Mch, *Meloidogyne chitwoodi*; Min, *Meloidogyne incognita*; Mja, *Meloidogyne javanica*; Rsi, *Radopholus similis*; Aca, *Ancylostoma caninum*; Cbg, *Caenorhabditis briggsae*; Cel, *Caenorhabditis elegans*; Cja, *Caenorhabditis japonica*; Cre, *Caenorhabditis remanei*; Nbr, *Nippostrongylus brasiliensis*; Oos, *Ostertagia ostertagi*; Tci, *Teladorsagia circumcincta*. Full sequences and accession numbers are reported in Supplementary Figure 13.

Table 1.

Summary of RME Peptide Expression by MS, ISH, and ICC^a

Transcript	Peptide	Peptide Sequence	Mass(M+H)	Technique	RMEV	RMED	RMER	RMEL
<i>atp-2</i>	AF5	SGKPTFIRFa	1051.8	MS	3 [*] /22 (14%)	4 [*] /22 (18%)	16/22 (73%)	22/22 (100%)
				ISH	0/43 (0%)	0/43 (0%)	15/43 (60%)	15/43 (60%)
				ICC	0/22 (0%)	0/22 (0%)	11/22 (50%)	11/22 (50%)
				Consensus				
<i>atp-4</i>	AF2	KHEYLRFa	991.5	MS	11/20 (55%)	14/22 (64%)	8/16 (50%)	0/22 (0%)
				ISH	13/29 (45%)	2/29 (7%)	1/30 (3%)	0/30 (0%)
<i>atp-15</i>	AF7 AF40 AF39	AGPRFIRFa AGSARFIRFa SPKQKFIRFa	962.7 1023.8 1149.9	MS	16/20 (80%)	21/22 (95%)	10/16 (63%)	10/22 (45%)
				MS	19/20 (95%)	20/22 (91%)	11/16 (69%)	10/22 (45%)
				MS	19/20 (95%)	19/22 (86%)	11/16 (69%)	10/22 (45%)
				ISH	24/31 (77%)	15/30 (50%)	21/30 (70%)	25/30 (83%)
				Consensus				
<i>atp-16</i>	AF43 AF44 PepQV AF42 AF45	RIDINDLALRFa SSYSFDPSNINLRFa pQEEEEEDGVLPERFV SFPNNYLASDLALRFa TFVIPTDLALRFa	1344.8 1645.8 1658.8 1726.9 1391.8	MS	1 [*] /20 (5%)	2 [*] /22 (9%)	16/16 (100%)	20/22 (91%)
				MS	5/20 (25%)	4 [*] /22 (18%)	16/16 (100%)	21/22 (95%)
				MS	2/20 (10%)		16/16 (100%)	17/22 (77%)
				MS	2/20 (10%)	1/22 (5%)	16/16 (100%)	19/22 (86%)
				MS	0/20 (0%)	0/22 (0%)	7/16 (44%)	8/22 (36%)
				ISH	15/22 (73%)	15/22 (68%)	20/22 (91%)	20/22 (91%)
<i>Asu-nlp-58</i>	Asu-NLP-58.1 Asu-NLP-58.2	AALLSRYa AVLSRYa	792.6 707.5	MS	0/20 (0%)	0/22 (0%)	15/16 (94%)	20/22 (91%)
				MS	0/20 (0%)	0/22 (0%)	13/16 (81%)	13/22 (59%)
				ISH	0/21 (0%)	0/21 (0%)	14/18 (78%)	22/22 (100%)
				Consensus				

^a Proportion of cells/preparations where peptide/transcript was detected is noted by cell type. Consensus is shaded gray for >50% detection by one method and <50% detection by another and shaded black for detection in >50% of cells/preparations by at least two methods.

* Includes MS samples that contained desired cell body with additional process(es) that could not be removed prior to analysis.

Table 2.

Summary of physiological and behavioral data for previously studied peptides

peptide	DE2 electrophysiology ^a		DI electrophysiology ^a		locomotory behavior ^a	cAMP ^b
	V _m	EPSP	R _{in}	V _m R _{in}		
AF2	strong depolarization	no change	weak decrease	no change decrease	decreased movement; more linear body posture; head tremor	small decrease
AF5	biphasic: depolarization then hyperpolarization	no change	decrease	no change decrease	increase movement; head tremor	large increase
AF7	weak hyperpolarization then weak depolarization	5× increase	no effect	depolarization decrease	decreased movement; more linear body posture; head tremor	N/A

^aDavis and Stretton, 2001.²¹

^bReinitz et al., 2011.³¹ V_m, membrane potential; R_{in}, input resistance; EPSP, excitatory postsynaptic potential frequency.