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Different Neuropeptides Are Expressed in Different Functional Subsets of Cholinergic Excitatory Motorneurons in the Nematode *Ascaris suum*

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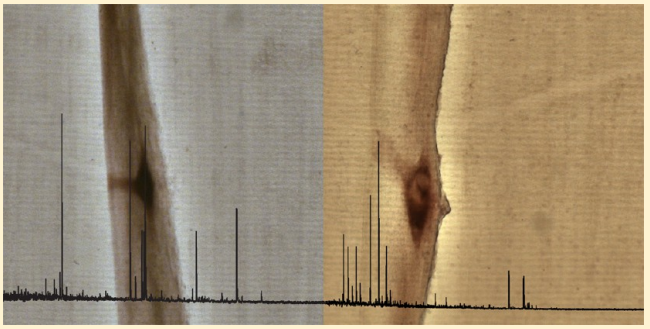
Supporting Information

ABSTRACT: Neuropeptides are known to have dramatic effects on neurons and synapses; however, despite extensive studies of the motorneurons in the parasitic nematode *Ascaris suum*, their peptide content had not yet been described. We determined the peptide content of single excitatory motorneurons by mass spectrometry and tandem mass spectrometry. There are two subsets of ventral cord excitatory motorneurons, each with neuromuscular output either anterior or posterior to their cell body, mediating forward or backward locomotion, respectively. Strikingly, the two sets of neurons contain different neuropeptides, with AF9 and six novel peptides (As-NLP-21.1−6) in anterior projectors, and the six *afp-1* peptides in addition to AF2 in posterior projectors. *In situ* hybridization confirmed the expression of these peptides, validating the integrity of the dissection technique. This work identifies new components of the functional behavioral circuit, as well as potential targets for antiparasitic drug development.



KEYWORDS: *MALDI-TOF MS, de novo sequencing, in situ hybridization, neuropeptides, nematodes, Ascaris suum*

INTRODUCTION

Nematodes are famous for being the first organisms in which the "complete wiring diagram" of the nervous system was determined. This was reported nearly 30 years ago in the free-living nematode *Caenorhabditis elegans*.[1] More recently, such a description has been labeled a "connectome".[2−4] Yet, to understand the neural circuits that control behavior, it is not sufficient merely to describe the anatomical connections of the wiring diagram; even when the basic electrophysiological properties of the neurons and their synapses are added to the morphological description, this "functional wiring diagram" does not adequately describe the circuit that generates behavior. It is a "first-pass" description of the system—necessary, of course, but not sufficient. Importantly, it lacks the incorporation of the functional role of neuromodulators into the description of circuitry. The importance of modulators has perhaps been best described in the stomatogastric nervous system of crustaceans;[5] both biogenic amines and neuropeptides play crucial roles in configuring neural circuits. Simple circuits take on additional layers of complexity, but the full extent of the added complexity is yet to be described.

In the nematode *Ascaris suum*, the inadequacy of the simple connectome approach was demonstrated about 25 years ago.[6,7] At first this was a surprise, because the *A. suum* nervous system, like that of other nematodes, is remarkably simple, both numerically (the entire nervous system comprises only 298 neurons)[8] and morphologically (almost all neurons have at most two branch points). It seemed like the simplest of the so-called simple nervous systems and, therefore, likely to be solved in rapid order. However, the first-pass description is clearly incomplete.[9] It was soon recognized that at least part of what is missing is a description of the activity of neuromodulators. These compounds can affect the activity of the neurons and their synapses, often in intricate ways,[10] so it is essential that a complete description of their structure, cellular localization, and action be added to the basic functional circuit. Neuropeptides are among the most numerous and varied of the known neuromodulators. As in *C. elegans*,[11] it is estimated that there are at least 250 endogenous peptides in *A. suum*;[12,13] we have chemically isolated and sequenced ca. 100 so far, and the majority of those tested to date affect the activity of the neurons and/or muscle in the locomotory system, or locomotory behavior itself.[10,14−17] In retrospect, it is no wonder that a functional circuit description that lacked the role of neuropeptides was a failure.

Initially, our approach to the discovery of endogenous peptides was by chemical fractionation and direct sequence

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determination by Edman degradation.[14,18,19] More recently, we have used mass spectrometry (MS) to detect and then to sequence neuropeptides.[12,13,17,20−22] We have now extended this technique to the single-cell level; mass spectrometric methods are sensitive enough both to detect and to sequence peptides from single dissected neurons.[17,20] This approach is limited only by how successfully we can dissect single neurons. For some neurons, it is relatively easy, but for others, such as the ventral cord motorneurons, the close association between the neurons and hypodermis, in which most of the neurons are embedded, and the many other neural processes in the nerve cord, complicate the dissection.

In this paper, we report our success in dissecting single motorneurons and in cataloguing the neuropeptidome of the different somatic motorneurons. Although the current matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instruments are easily capable of detecting levels of peptides in single cells, careful isolation and preparation of the neurons is necessary to make sure that none of the sample is lost. Glycerol has long been used as a cryoprotectant,[23,24] but more recently, glycerol treatment has been shown to help improve the cells' stability during mechanical manipulation without affecting peptide structure or ion suppression during single-cell analysis by MS.[25−27] We found that the use of an isotonic dissecting solution that contains 30% glycerol was highly successful. Consistent with other work, we find that glycerol treatment confers several benefits: (i) it prevents damage to the cell membrane during dissection, (ii) it loosens adhering cells from each other, probably by transient shrinkage, (iii) it helps to reduce contamination from damaged surrounding tissue, and (iv) it prevents dehydration of the cell once it is exposed to the atmosphere, allowing for better extraction of the analyte(s) when exposed to matrix on the MS target plate.

Analysis of single cells also has important advantages over the analysis of more complex tissue, such as ganglia or the entire nerve cord, because the complexity of the sample is reduced. This reduces the effects of ion suppression, thus increasing the probability of isolating a desired peak for sequencing by tandem MS (MS/MS). The improvements in dissection techniques have now enabled the isolation of ventral cord motorneurons and an enumeration and sequencing of the peptides that each contains.

There are several other important advantages of single-cell MS. First and foremost, it identifies the actual peptide product(s) within each cell, rather than relying on predictions from translated nucleic acid sequences; such predictions are unreliable as the cellular expression patterns of the processing enzymes, especially the proteases, controlling the post-translational modifications of the precursor protein are not yet understood. Since our ultimate goal is to carry out physiological experiments with the peptides, it is crucial to find the fully processed peptides that are actually present in each cell; physiological experiments are very time-consuming, so it is pointless to investigate the bioactivity of a nonendogenous peptide. Second, it identifies the cell in which the peptide is expressed, i.e. one-half of the neuronal signaling dyad mediated by the peptide. Third, it enables comparison of expression in identified cells from individual animals; we have previously noted significant individual-to-individual variation in the expression of peptides, especially in those peptides expressed at low levels.[12,17,20]

In this paper, we describe the peptides present in the excitatory ventral nerve cord motorneurons, and we report an interesting difference in the peptide content of different classes of these motorneurons. They can be divided into two groups, the first of which (DE1, DE3, and VE1) contains a set of shared peptides in neurons where the neuromuscular output region of the neuron is anterior to the cell body; these neurons mediate anteriorly propagating body waveforms[28] and forward locomotion (Stretton, unpublished). In the other group (DE2 and VE2), in which the output is posterior to the cell body (mediating posteriorly propagating waveforms and backward locomotion in *A. suum*), there is a different set of peptides. *C. elegans* has motorneurons, which are anatomically identical to those of *A. suum* (Figure 1b,c). In *C. elegans*, the relationship between the direction of waveform propagation and the morphology of the motorneurons controlling each direction has been established by laser ablation experiments in which either the forward-projecting or the backward-projecting neurons were deleted.[29] In both *A. suum* and *C. elegans*, the anteriorly projecting and posteriorly projecting motorneurons mediate body waveforms that propagate anteriorly or posteriorly, respectively. However, the relationship between the direction of propagation of the body waveform and the direction of locomotion is different in the two worms: in *C. elegans* anteriorly propagating waves drive the worm backward and posteriorly propagating waves drive it forward,[30] whereas in *A. suum* it is anteriorly propagating waves that drive it forward.[31] These differences between *A. suum* and *C. elegans* are due to differences in the interaction between the body movements and the physical environment—moving on a surface versus moving in a tube. However, it is important to reiterate that the motorneurons driving the anteriorly and the posteriorly propagating waves are homologues in the two species.

The cellular expression patterns observed by single-cell MS have been confirmed by *in situ* hybridization (ISH), using riboprobes targeting the transcripts that encode the different peptides. In all cases, the localization by ISH was identical to that seen with single-cell MS. This is an important result, because it validates the dissection technique: the major peptides seen by MS are not due to contamination from other neurons and, in particular, are not due to contamination from input synaptic terminals. Taken together, these experiments identify new components of the functional circuit that describes behavior.

RESULTS AND DISCUSSION

It is important to know the chemical nature of the intercellular signaling molecules that are used in nervous system circuits. Besides the classical neurotransmitters, there are many neuromodulators that affect the properties of neurons and their synapses in important and varied ways, and knowledge of these effects is an essential part of understanding circuit properties. The first step is to identify these molecules, and this is a problem for chemists. The majority of known modulators are peptides, and the recent advances in mass spectrometry, particularly the exquisite sensitivity and accuracy of MS techniques, have opened up new possibilities for the rapid exploration of the neuropeptide complement of nervous systems. We are using the simple nervous system of the nematode *Ascaris suum*, which has a total of only 298 neurons, as a model system. We aim to understand the neural basis of the control of locomotory behavior. It is clear from our previous work that the role of modulators in neural circuitry must be added to our description of the system. Despite extensive studies on the motor nervous system, previously we were not able to define the peptides present in individual motorneurons. Recent improvements in dissection techniques have enabled us to isolate single motorneurons and to both isolate and to sequence their complement of peptides.

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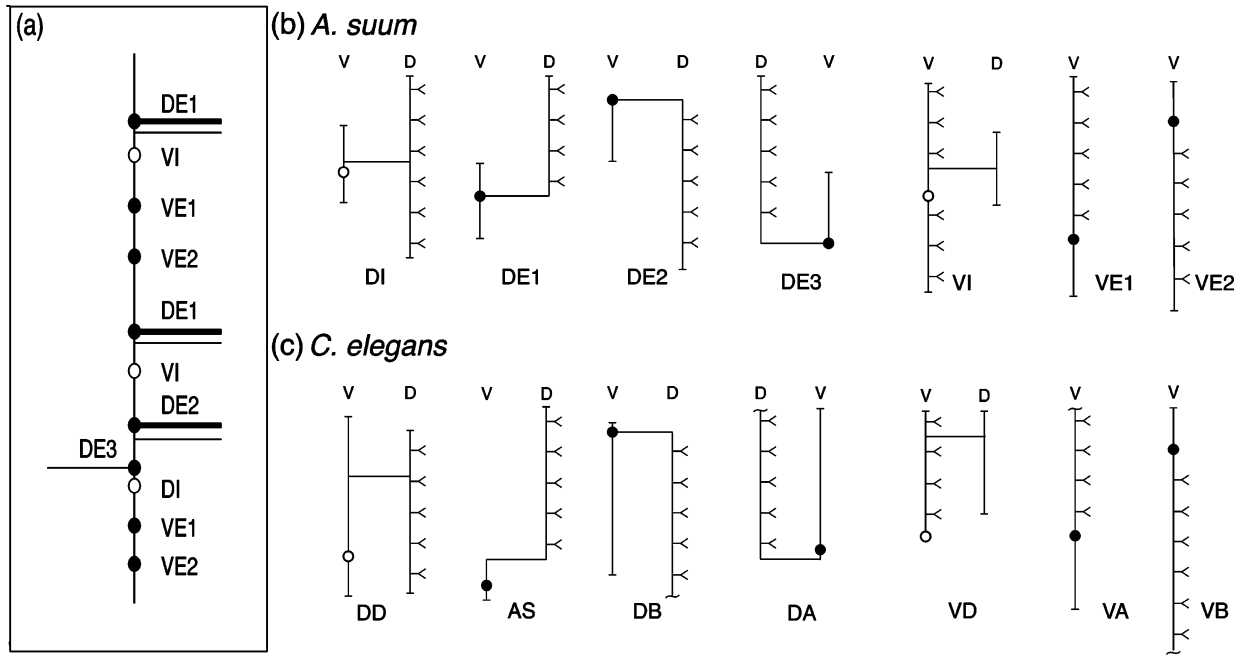

Figure 1. Schematic diagram of the ventral cord motorneurons of *A. suum*. (a) Stereotypical pattern of motorneuron cell bodies found in each segment (segments 2−5) and their associated commissures, adapted from Stretton et al. (1978). Filled circles indicate cell bodies of excitatory motorneurons. Open circles indicate cell bodies of inhibitory motorneurons. DE1 and DE2 commissures have a larger diameter (thicker line) than the inhibitory commissures with which they are paired. (b, c) Schematic diagram of the seven types of (b) *A. suum* and (c) *C. elegans* segmental motorneurons. Circles indicate cell bodies, all found in the ventral nerve cord (V). Filled circles, excitatory neurons; open circles, inhibitory. Vertical lines emanating from the cell body represent the ventral nerve cord processes, and horizontal lines represent commissures that connect dorsal (D) and ventral processes. Forked projections represent axonal synapses onto muscle. Note that the synaptic output from DE1, VE1, and DE3 is anterior to the cell bodies, whereas the output from DE2 and VE2 is posterior to the cell bodies.

Anatomical Background. In the ventral and dorsal nerve cords, there are seven classes of somatic motor neurons that occur in five basic repeating units ("segments"), each comprising 11 motorneurons (four types are present in two copies per repeat, and the other three types in one copy per segment) (Figure 1a).[8] Some innervate dorsal muscle and others innervate ventral muscle. The cell bodies of all seven classes are found in the ventral nerve cord, and the neurons that innervate dorsal muscle send projections to the dorsal nerve cord via semicircumferential commissures. The different classes were initially established on the basis of their distinct cellular morphology (Figure 1b). Subsequent electrophysiological experiments distinguished sets of excitatory and inhibitory motorneurons: in each segment, dorsal muscle is innervated by four excitors (two DE1, one DE2, and one DE3), and one inhibitor (DI), and ventral muscle is innervated by two copies each of the excitors (VE1, VE2) and inhibitor (VI). DE1, DE3, and VE1 neurons have their neuromuscular output regions anterior to the cell body, and DE2 and VE2 neurons have their neuromuscular output posterior to the cell body (Figure 1b). The excitors are cholinergic,[32,33] and the inhibitors are GABAergic.[34]

MS of Excitatory Motor Neurons in the Ventral Nerve Cord. Individual dissected motorneuron cell bodies from the ventral cord were subjected to MALDI-MS analysis. In addition to familiar peaks with mass-to-charge ratios (m/z) matching those of peptides encoded by two previously described *A. suum* genes, *afp-1*[35] and *afp-4*,[36] and the previously identified peptide AF9,[19] there were several novel peptides that had not been identified previously (Figures 2, 3; Table 1). The intensity of the peaks varied; the most intense peaks were consistent for a particular cell type, whereas minor peaks varied from sample to sample of the same cell type.

From the 8 excitatory cells in each segment, two spectral patterns emerged. In spectra from posteriorly projecting VE2 and DE2, the previously identified peptide AF2 (m/z 991.5), commonly thought to be the most abundant peptide expressed in *A. suum*, is often the most intense peak, and was consistently observed. VE2 and DE2 also contained peaks corresponding to the six peptides encoded by *afp-1*: AF3, m/z 857.5; AF20, m/z 875.5; AF14, m/z 905.5; AF4, m/z 958.5; AF13, m/z 1020.5; and AF10, m/z 1541.7 (Figure 2; Table 1).[12] In most cases, these peptides are present at moderate to high intensity. The only exception was AF10, which was absent in 5 out of 10 cells in DE2. Interestingly, spectra from VE2 and DE2 were very similar to spectra from the RID neuron in the dorsal ganglion (DG);[20] AF10 also was absent in about 30% of the spectra from RID neurons.

In the anteriorly projecting DE1, DE3, and VE1 motorneurons, an ion with m/z corresponding to AF9 (GLGPRPLRFa, 1011.7 Da) was observed. These neurons also contained large peaks from a group of novel peptides with m/z of 1018.5, 1271.7, 1322.7, 1327.6, 1516.8, and 1666.8 (Figure 3; Table 1).

Identities of certain functional residues in peptides were initially confirmed by chemical modification (Figures 2, 3). Exposure to Methylene Blue, which oxidizes methionine residues to their sulfoxides, causes a +16 Da mass shift for each oxidized methionine, while on-target acetylation produces a mass shift of +42 Da for acetylation of each N-terminus, as well as an additional +42 Da for each lysine and a fraction of tyrosine residues. In both oxidation and acetylation, modifications are incomplete, which produces spectra that contain both modified

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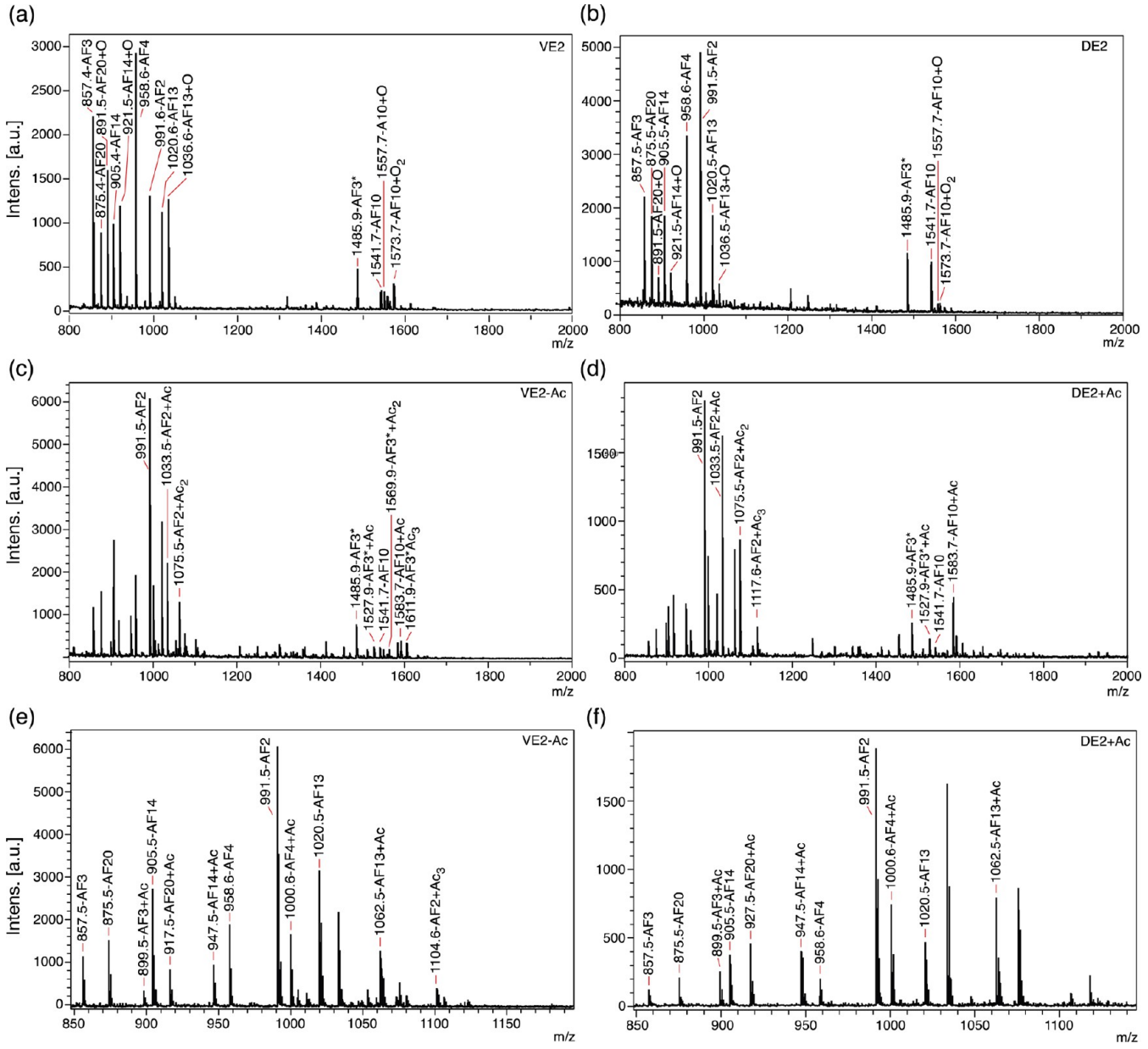

Figure 2. Neuropeptides from single excitatory motorneurons in unmodified and acetylated forms. Mass spectra from individual (a) VE2, (b) DE2 motorneurons with no chemical modifications. Spectra from individual (c) VE2, (d) DE2 motorneurons treated with acetic anhydride adding 42 mass units for each acetylation. (e) and (f) are expansions of (c) and (d), respectively, between 850 and 1200 *m*/*z*. *X*-axis: *m*/*z* is mass-to-charge ratio. *Y*-axis is intensity of MS signal in arbitrary units, a.u. * indicates extended peptide from incomplete cleavage.

and unmodified peaks, allowing for easy comparison between the two. In all cases, peaks expected from the putative sequences were observed in treated cells, confirming the presence of methionine and the number of amino groups in the peptide (Figures 2, 3).

Sequence Determination. *(a). Sequences of Known Peptides.* MS/MS was performed on the peak with *m*/*z* 991.5 (AF2), on a peak presumed to be from the *afp-1* transcript (958.5 Da − AF4), and on a peak with *m*/*z* 1011.7 (AF9). *De novo* sequencing confirmed the predicted amino acid sequence of each peptide, although MS/MS is not able to resolve the (I/L) ambiguities because the two amino acids are isobaric (Figure 4).

(b). Sequences of As-nlp-21 Encoded Peptides. Sequence determination of the six novel peptides from DE1, DE3, and VE1 motorneurons depended on a combination of tandem MS and analysis of sequences from gene transcripts. MS/MS analysis of the peptide with *m*/*z* 1271.7 produced the sequence R[I/L]YDAFSRFP (Figure 5a). To resolve the isoleucine/leucine ambiguity, we performed a tBLASTn search of the *A. suum* genomic survey sequence (GSS) database using both RIYDAFSRFP and RLYDAFSRFP as queries. In the translated sequence (Genbank accession no. E194631.1), we identified an N-terminal dibasic putative cleavage site and a C-terminal stop codon, which would predict the processed peptide RIYDAFSRFP (As-NLP-21.6) with a predicted *m*/*z* of 1271.7. Further analysis of the translated GSS revealed a second putative peptide sequence GGGRSFRLSSLGE (As-NLP-21.4), which has a *m*/*z* of 1322.7, matching that seen in spectra from VE1, DE1, and DE3 neurons. The As-NLP-21.4 sequence corresponds to a peptide first identified by homology from a search of the *A. suum* GSS and EST databases using known *C. elegans* peptides,

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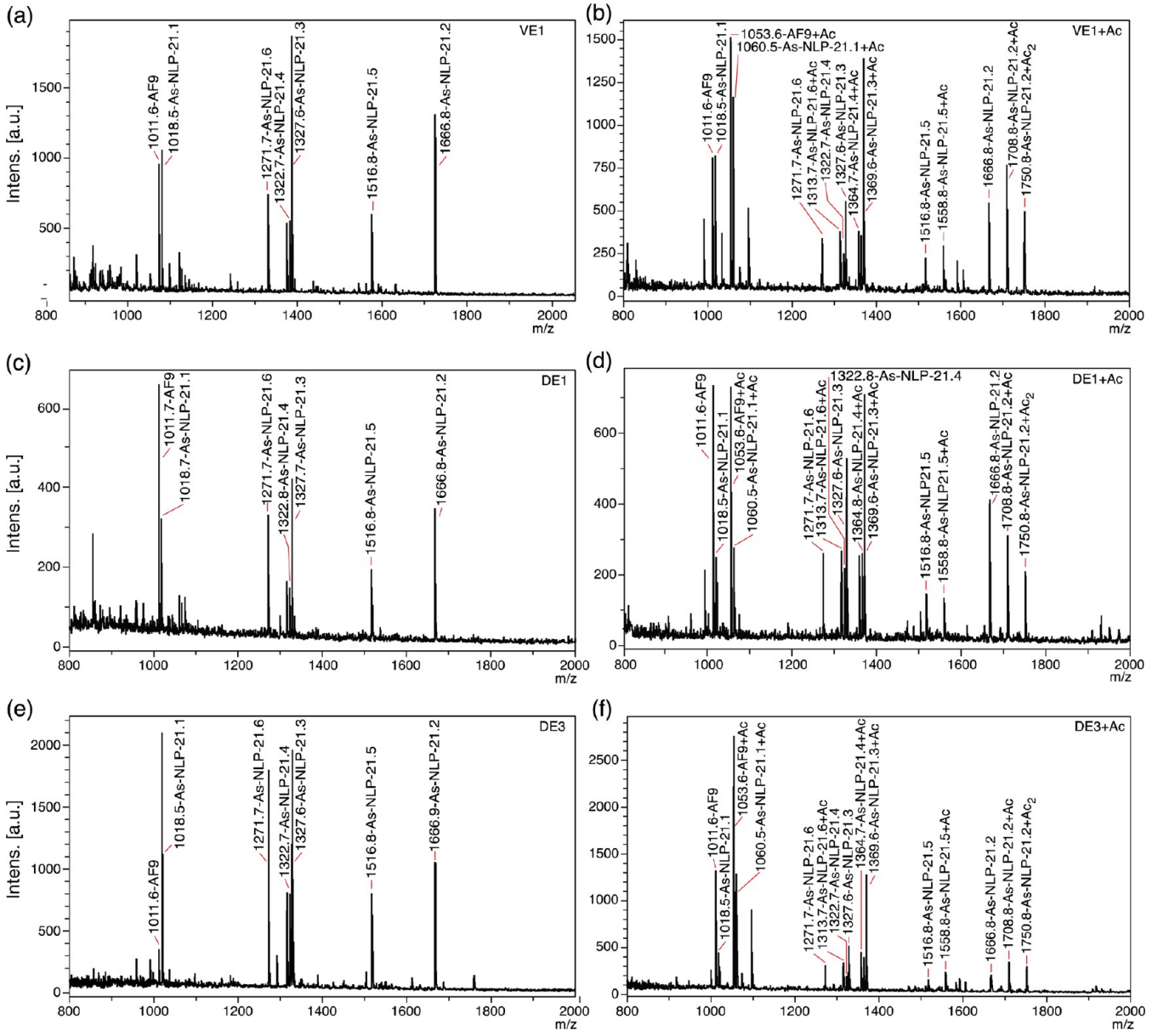

Figure 3. Neuropeptides from single excitatory motorneurons in unmodified and acetylated forms. Mass spectra from individual (a) VE1, (c) DE1, (e) DE3 motorneurons with no chemical modifications. Spectrum from an individual (b) VE1, (d) DE1, (f) DE3 treated with acetic anhydride, which acetylates free amines (N-terminus, K) and to a lesser extent Y, adding 42 mass units for each acetylation. X axis: m/z is mass-to-charge ratio. Y axis is intensity of MS signal in arbitrary units, a.u.

and then confirmed by LCMS and MS/MS from a peptide extract of *A. suum* heads.[13]

MS/MS of synthetic As-NLP-21.6 showed a highly similar fragmentation pattern to that of the endogenous peptide, serving as further confirmation of the peptide sequence (Supplementary Figure 2a). Despite several attempts, As-NLP-21.4 could not be sequenced by MS/MS.

The *As-nlp-21* gene was cloned by 5′ and 3′ rapid amplification of cDNA ends (RACE) using the target sequence (5′-CCCAAGCTGCTCAAACGAAACGATCGTCC-3′) from the GSS to design primers (Supplementary Table 2). The product yielded two overlapping sequences that were fused to produce the full *As-nlp-21* transcript (GenBank accession no. KP311680; Figure 5b), encoding a predicted protein with an initiating methionine and predicted N-terminal signal peptide, six putative peptide products (As-NLP-21.1 through As-NLP-21.6), named for the order in which they appear in the transcript, and a C-terminal stop codon. None of these peptides is C-terminally amidated. MS/MS of the peak with m/z 1516.8 confirmed the identity of peptide As-NLP-21.5 (Figure 6a). Besides As-NLP-21.4, As-NLP-21.5, and As-NLP-21.6, the full-length transcript encodes three additional predicted nonamidated peptides (As-NLP-21.1, GGGRPFTLVD; As-NLP-21.2, AGGRFFKI-EDVDALE; and As-NLP-21.3, GGGRPFYTQTDE). All have predicted m/z that correspond to intense peaks consistently seen in VE1, DE1, and DE3 motorneurons by MS (Figure 3). MS/MS spectra partially confirmed the peptide sequences (Figure 6). For peptides As-NLP-21.1 and -21.3, a characteristic peak at m/z 328.1 (b_4 ion) was observed in tandem MS spectra (Figure 6; Supplementary Figure 2). In MALDI, as well as electrospray ionization (ESI), fragmentation of peptides with an internal proline often preferentially cleaves N-terminal to that residue.[37]

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Table 1. Comparison of Peptides Localized in the Excitatory Ventral Cord Motor Neurons by MALDI-MS and ISH

neuron	gene	peptide name	sequence	obsd [MH+]	ISH
VE2, DE2 (posteriorly projecting)	*afp*−1	AF3	AVPGVLRFa	857.5	
		AF20	GMPGVLRFa	875.5	
		AF14	SMPGVLRFa	905.5	
		AF4	GDVPGVLRFa	958.5	+
		AF13	SDMPGVLRFa	1020.5	
		AF3*	ENEKKAVPGVLRFa	1485.9	
		AF10	GFGDEMSMPGVLRFa	1541.7	
	afp−4	AF2	KHEYLRFa	991.5	+
	afp−14	AF9	GLGPRPLRFa	1011.6	+
VE1, DE1, DE3 (anteriorly projecting)	*As*−*nlp*−21	As−NLP−21.1	GGGRPFTLVD	1018.5	
		As−NLP−21.6	RIYDAFSRFP	1271.7	
		As−NLP−21.4	GGGRSFRLSSLGE	1322.7	
		As−NLP−21.3	GGGRPFYTQTDE	1327.6	+
		As−NLP−21.5	AGARSFPVLEDLLE	1516.8	
		As−NLP−21.2	AGGRFFKIEDVDALE	1666.8	

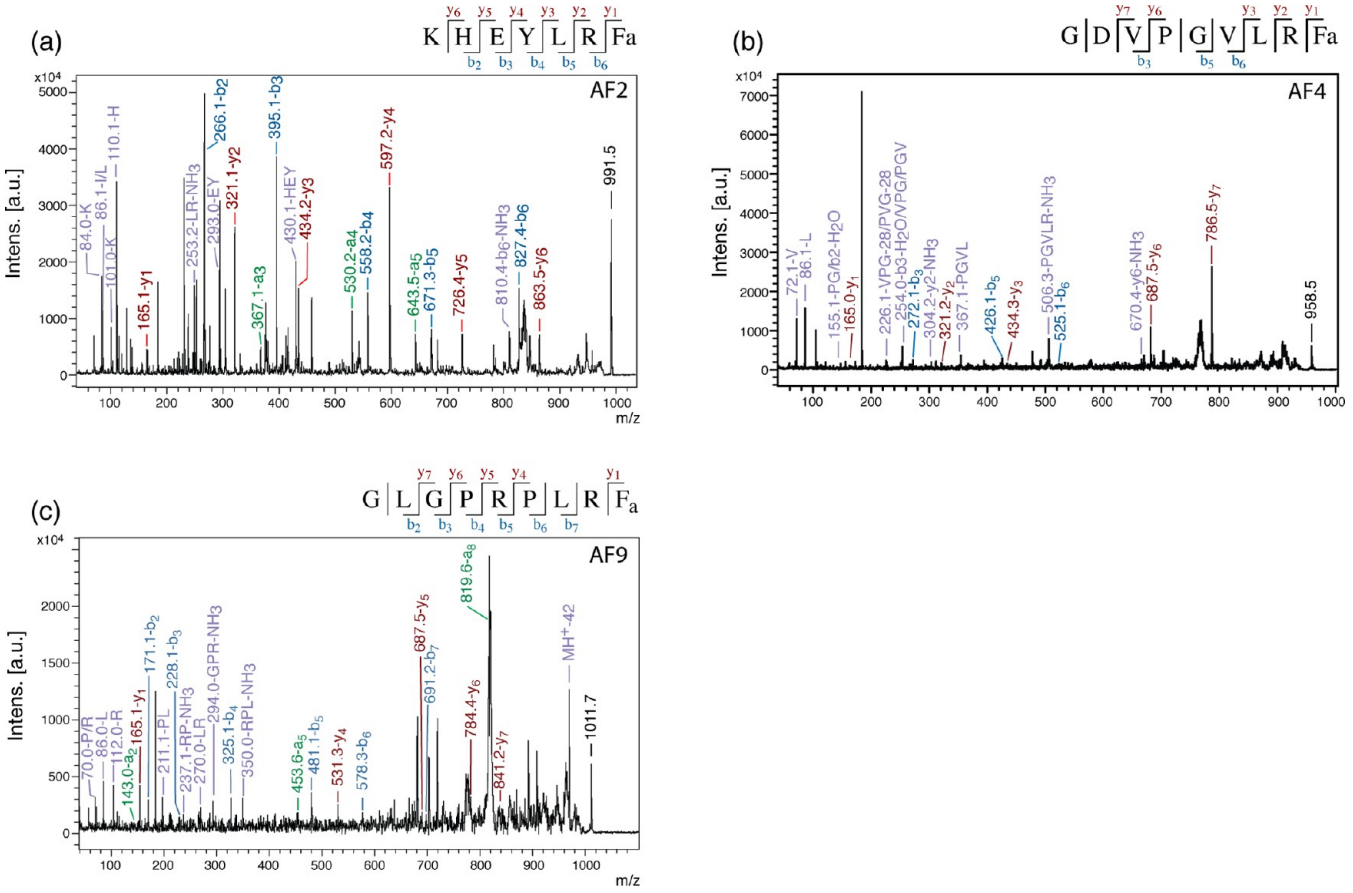

Figure 4. MS/MS spectra of known peptides from the excitatory ventral cord motorneurons. Peaks representing a (green), b (blue), y (red), and high intensity internal fragment and immonium (purple) ions are labeled, and b and y ions are summarized in the sequence at the top of each spectrum. (a) MS/MS spectrum from DE2 of AF2 (*afp-4*), *m*/*z* 991.5. (b) MS/MS spectrum from DE2 of AF4 (*afp-1*), *m*/*z* 958.5 (c) MS/MS spectrum from DE1 of AF9 (*afp-14*), *m*/*z* 1011.7.

We also observe b_4-NH_3 (−17 *m*/*z*), which we have found to be a typical neutral loss for a fragment ion with an arginine (R) residue.

Localization of the *As-nlp-21* Transcript by *In Situ* Hybridization. To confirm the localization of the As-NLP-21 peptides identified by MS, we synthesized an RNA probe targeting a 431 bp section of the peptide-encoding transcript (Figure 5b). This probe labeled the DE1, DE3, and VE1 neurons in the ventral nerve cord with moderate to high intensity (Figure 7a−c; 10/10 preparations), supporting the results obtained by single-cell MS. No staining was observed in DE2 or VE2 (Figure 7d,e), or negative control preparations using a sense probe

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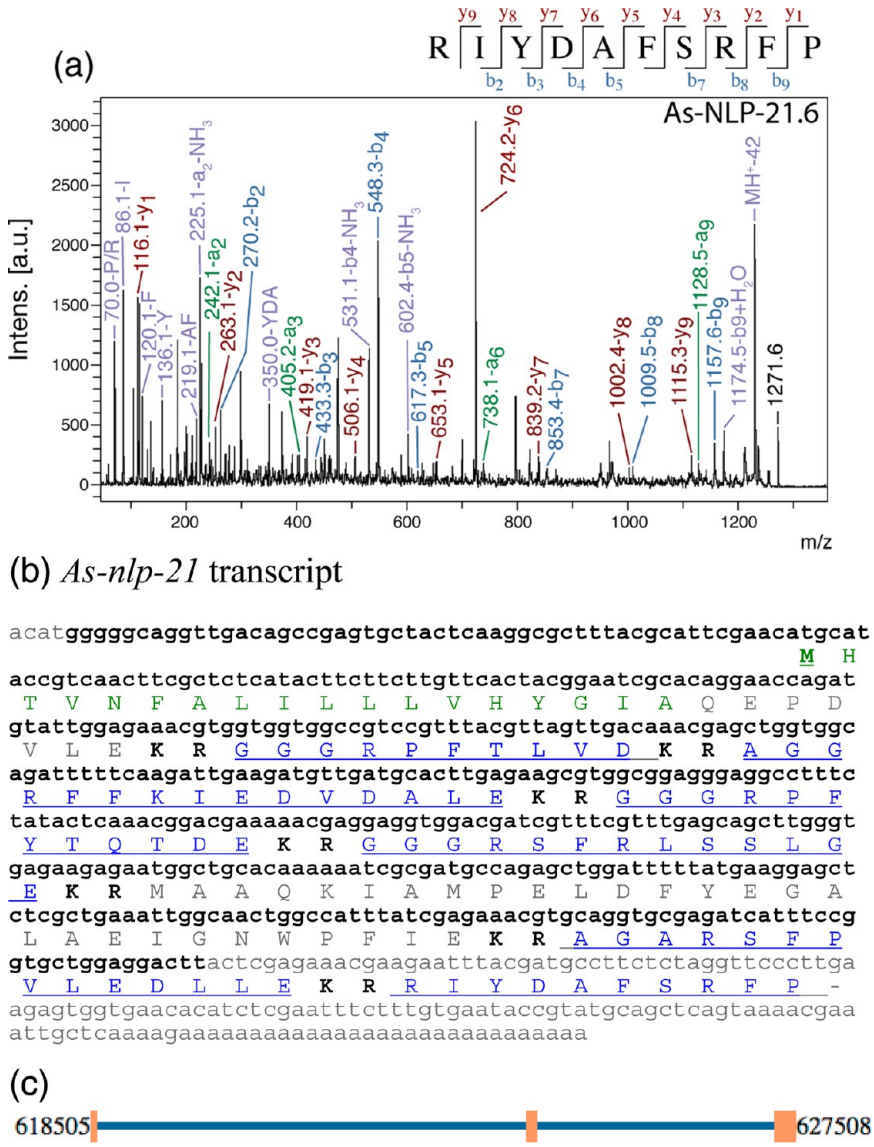

Figure 5. Sequencing As-NLP-21 peptides. (a) MS/MS sequencing of As-NLP-21.6. Peaks representing a (green), b (blue), y (red), and high-intensity internal fragment (purple) ions are labeled, and b and y ions are summarized in the sequence at the top of the spectrum. (b) *As-nlp-21* transcript; bold nucleotides indicate portion of the transcript targeted by the *As-nlp-21* antisense riboprobe. Blue and underlined indicates encoded peptide while bold amino acids indicate basic cleavage sites. Green amino acids indicate signal peptide. (c) *As-nlp-21* gene model. The gene consists of three exons of 93, 149, and 298 bases separated by 5459 and 3007 base introns.

(Supplementary Figure 3a−c). The antisense probe also labeled many cell bodies in the head of the worm, including two pairs of cells associated with the nerve ring, seven pairs of cells and one unpaired cell in the lateral ganglia, seven pairs of cells in the ventral ganglion, and two pairs of cells in the retrovesicular ganglion (Supplementary Figure 3d). An additional four pairs of cells were sometimes stained in the lateral lines, but staining was weak and highly variable.

Identification of *afp-1*, *afp-4*, and *afp-14* Peptide-Encoding Gene Transcripts and Localization by ISH. *(a). afp-14.* Several previous attempts to clone the transcript that encodes AF9 (*afp-14*) using primers designed to SL1 (a 5′ splice leader sequence found in ca. 80% of *A. suum* transcripts)[38] and a gene-specific primer were unsuccessful. However, by using primers for 5′ and 3′ RACE, designed from AF9 GSS information (GenBank accession no. ED426005.1), we successfully amplified overlapping 3′ and 5′ sequences of the *afp-14* transcript (GenBank accession no. KP311679). The deduced sequence contained an N-terminal signal peptide with an initiating methionine, the AF9 peptide-encoding region with C-terminal glycine (typically the precursor for C-terminal amidation), immediately followed by a stop codon, and an N-terminal dibasic cleavage site (Figure 8). The full-length transcript was confirmed by PCR. The genomic structure, showing introns and exons, is shown in Figure 8b. To synthesize an antisense riboprobe complementary to a 266-nucleotide portion of the *afp-14* transcript for ISH, we used specific primers AF9FWDPROBE1 (5′-GCGTACCGTTTGTACGACCT-3′) and AF9REVPROBE2 (5′-CATTAGCGAGAAGCCGAGAG-3′). We observed medium to intense staining in DE1 and DE3 motorneurons (Figure 9) but no staining in DE2, VE2, or the inhibitory motorneurons DI and VI (data not shown). VE1 neurons were also stained, but this staining is highly variable and often weak (Figure 9c). This could be due to lower expression levels in this cell type or insufficient penetration of the probe. VE1 is a relatively small cell and buried deep in the cord, which

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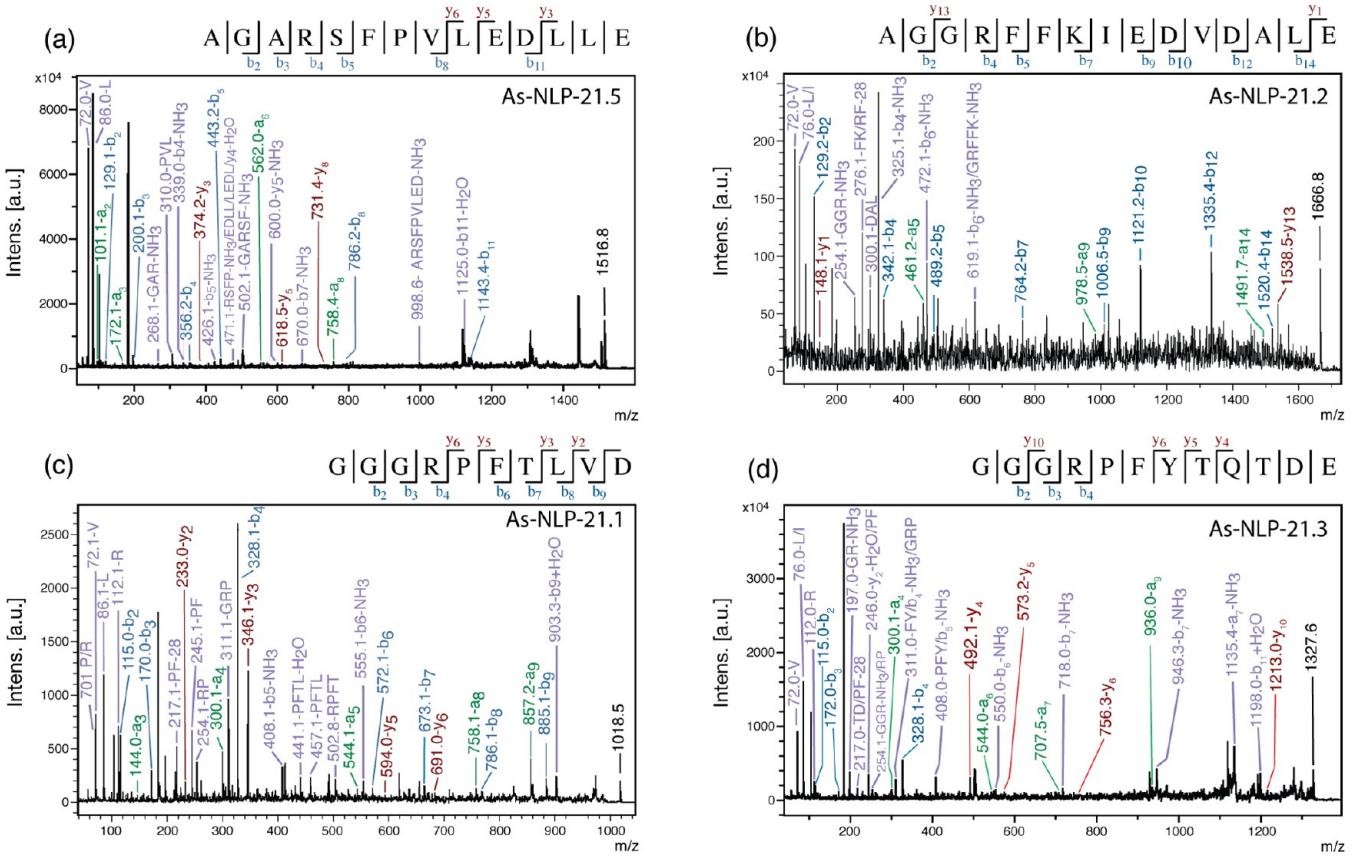

Figure 6. MS/MS of peptides encoded by *As-nlp-21*. Peaks representing a (green), b (blue), y (red), and high-intensity internal fragment and immonium (purple) ions are labeled, and b and y ions are summarized in the sequence at the top of each spectrum. The names of the peptides are below the sequences. (a) MS/MS spectrum from DE1 of As-NLP-21.5, *m/z* 1516.8. (b) MS/MS spectrum from DE1 of As-NLP-21.2, *m/z* 1666.8. (c) MS/MS spectrum from DE1 of As-NLP-21.1, *m/z* 1018.5. (d) MS/MS spectrum from DE1 of As-NLP-21.3, *m/z* 1327.6.

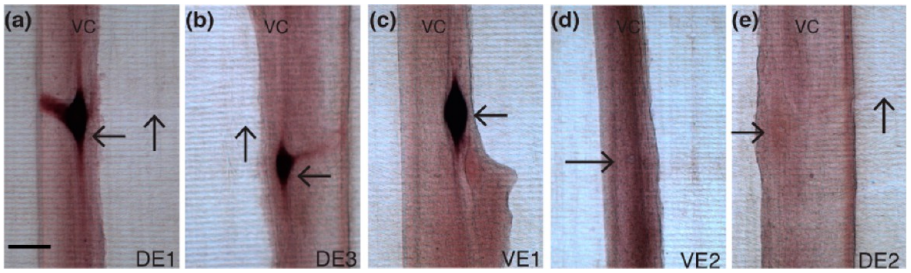

Figure 7. Confirmation of *As-nlp-21* expression in DE1, VE1, and DE3 motorneurons in *A. suum* by *in situ* hybridization. Positive *As-nlp-21 in situ* hybridization staining in DE1 (a), DE3 (b), and VE1 (c). No staining was observed in VE2 (d) or DE2 (e) VC = Ventral nerve cord. Vertical arrows indicate commissures and horizontal arrows indicate cell bodies. Scale bar = 100 μm.

might make access of the probe more difficult. This variability is consistent with the MS of VE1 cells. In 3 out of 11 cells, we saw moderate to intense peaks for AF9 (*m/z* 1011.7), in 4/11 cells, the AF9 peak was of low intensity, and in 4/11 cells the AF9 peak was not detected. No staining was observed in DE2 or VE2 (Figure 9d,e) or negative control preparations using a sense probe (Supplementary Figure 4a–c). ISH of *afp-14* using the antisense probe also detected two other cells in the head. These are a bilateral pair believed to be either the ADA, FLP, or RMG neurons because of their position in the lateral lines; all of these send projections into the deirid commissure bundle which is observable in these preparations (Supplementary Figure 4d).

(b). afp-1, afp-4. Previous experiments in this laboratory investigated the localization of *afp-1* and *afp-4* peptide-encoding transcripts in the head of *A. suum* by ISH;[36] however, these preparations did not include the segment 2 motorneurons analyzed by MS. To corroborate our single-cell MS data by ISH, we made *afp-1* and *afp-4* riboprobes that targeted the same region of the transcripts used in those experiments,[36] and we confirmed the expression of both peptide-encoding mRNAs in VE2 and DE2 motorneurons (Figure 10). We saw no expression in any other ventral cord motorneurons. Negative controls with the *afp-1* and *afp-4* sense probes showed no staining (Supplementary Figure 5).

In Other Nematodes. *Sequence and Cellular Expression Patterns.* Nematodes are the most prevalent phylum on the planet and inhabit a wide variety of ecological niches, yet their nervous systems are highly conserved. The chemical messenger systems used to control behaviors like locomotion are also highly conserved; this includes the structure of neuropeptides expressed

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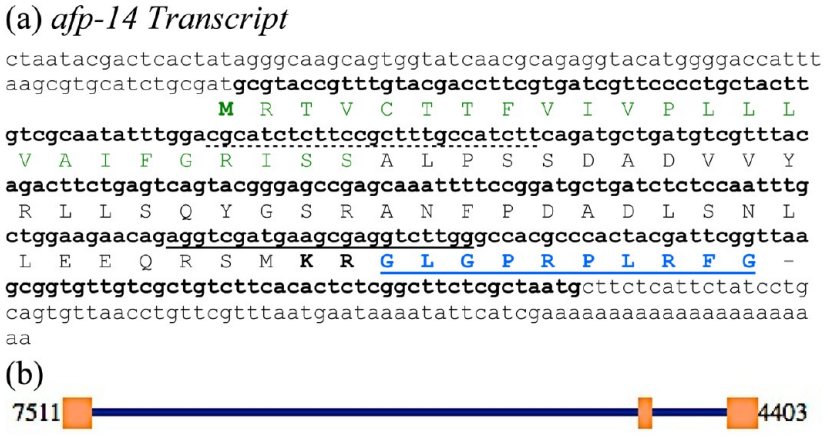

Figure 8. Nucleotide sequence and deduced amino acid sequence of *afp-14*. (a) The *afp-14* transcript was found by using unique gene specific primers for 5′RACE, underlined with a solid black line, and 3′RACE, dotted underlined. Bolded nucleotides indicate the portion of the transcript targeted by the *afp-14* riboprobe. Blue, bold, and underlined indicates the encoded peptide with an N-terminal dibasic cleavage site in bold. Green indicates the signal peptide with the putative start site bolded. (b) The structure of the *afp-14* gene consists of 3 exons 134, 67, and 144 bp in length. Exons are separated by introns of 2429 and 337 bp.

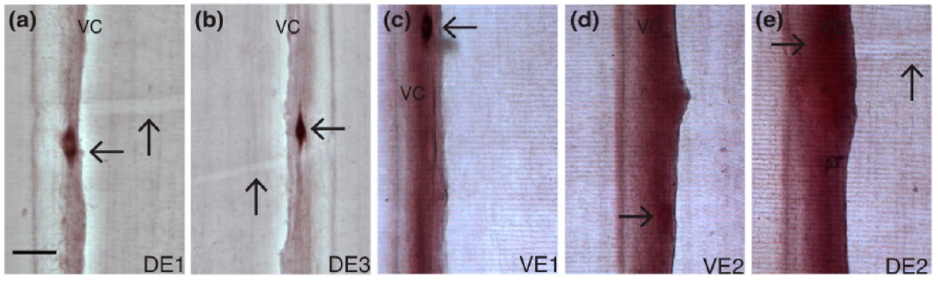

Figure 9. Confirmation of *afp-14* (AF9) expression in DE1, VE1, and DE3 motorneurons in *A. suum* by *in situ* hybridization. Positive *afp-14 in situ* hybridization staining in DE1 (a), DE3 (b), and VE1 (c). No staining was observed in VE2 (d) or DE2 (e) VC = Ventral nerve cord. Vertical arrows indicate commissures and horizontal arrows indicate cell bodies. Scale bar = 100 μm.

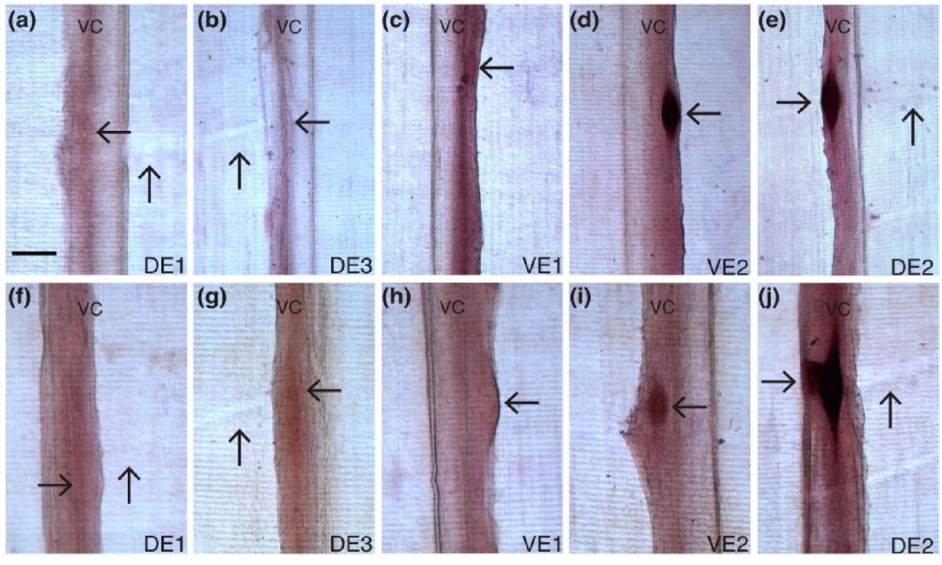

Figure 10. Confirmation of *afp-1* (AF3, 4, 10, 13, 14, 20) and *afp-4* (AF2) expression in VE2 and DE2 motorneurons in *A. suum* by *in situ* hybridization. Positive *afp-1* ISH staining in (d) VE2 and (e) DE2. Positive *afp-4* ISH staining in (i) VE2 and (j) DE2. No staining was observed in DE1, DE3, or VE1 for neither *afp-1* (a-c) nor *afp-4* (f-h) VC = Ventral nerve cord. Vertical arrows indicate commissures. Horizontal arrows indicate cell bodies. Scale bar = 100 μm.

by individual nematode species. Previous work suggests that cellular expression of orthologous peptides is in most cases quite different between *A. suum* and *C. elegans*.[17,20,36,39,40] Our current investigation finds some instances of overlap in cellular expression of peptide-encoding genes in the ventral cord motorneurons. We suspect that, in some cases, there are true

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Table 2. Comparison of Orthologous Peptides Localized within the Excitatory Ventral Cord Motor Neurons in *A. suum* and *C. elegans*

A. suum				*C. elegans*[a]			
gene	peptide name	sequence	VCMN Expression	gene	peptide name	sequence	VCMN Expression
afp-14	AF9	GLGPRPLRFa	VE1,DE1,DE3	*flp-21*	FLP-21	GLGPRPLRFa	NONE
As-nlp-21	As-NLP-21.1	GGGRPFTLVD	VE1,DE1,DE3	*Ce-nlp-21*	Ce-NLP-21-1	pQYTSELEEDE	VA, VB, DA, DB, AS
	As-NLP-21.2	AGGRFFKIEDVDALE			Ce-NLP-21-2	GGARAMLH	
	As-NLP-21.3	GGGRPFYTQTDE			Ce-NLP-21-3	GGARAFSADVGDDY	
	As-NLP-21.4	GGGRSFRLSSLGE			Ce-NLP-21-4	GGARAFYDE	
	As-NLP-21.5	AGARSFPVLEDLLE			Ce-NLP-21-5	GGARAFLTEM	
	As-NLP-21.6	RIYDAFSRFP			Ce-NLP-21-6	GGARVFQGFEDE	
					Ce-NLP-21-7	GGARAFMMD	
					Ce-NLP-21-8	GGGRAFGDMM	
					Ce-NLP-21-9	GGARAFVENS	
					Ce-NLP-21-10	GGGRSFPVKPGRLDD	
afp-1	AF3	AVPGVLRFa	VE2, DE2	*flp-18*	FLP-18-1	(DFD)GAMPGVLRFa	VA, VB, DA, DB, AS
	AF4	GDVPGVLRFa			FLP-18-2	EMPGVLRFa	
	AF10	GFGDEMSMPGVLRFa			FLP-18-3	(SYFDEKK)SVPGVLRFa	
	AF13	SDMPGVLRFa			FLP-18-4	EIPGVLRFa	
	AF14	SMPGVLRFa			FLP-18-5	SEVPGVLRFa	
	AF20	GMPGVLRFa			FLP-18-6	DVPGVLRFa	
afp-4	AF2	KHEYLRFa	VE2,DE2	*flp-14*	FLP-14	KHEYLRFa	N/A

In *A. suum*, all of the peptides were localized by MALDI-TOF MS and *in situ* hybridization. [a]In *C. elegans*, peptide encoding genes were localized using GFP constructs[11,53,54,70] *C. elegans* neurons are homologs of *A. suum* neurons as follows: VA (VE1), VB (VE2), DA (DE3), DB (DE2), AS (DE1)

differences in expression of homologous genes between species, but at this point it is too early to be completely certain about the comparisons made between the two species because the techniques used to identify expression of peptide-encoding genes in the two species are different. In *A. suum*, the techniques we used, namely, single-cell MS, ISH and immunocytochemistry, directly detect the peptides or the transcripts that encode them, whereas in *C. elegans*, GFP constructs are used to detect peptide-encoding genes.[11] The interpretation of the results from using these constructs relies on adequate knowledge of the *cis*-regulatory domains that interact with the numerous transcription factors and regulators to control expression of different genes. The use of SAGE (Serial Analysis of Gene Expression, which detects mRNA) on distinct populations of *C. elegans* neurons identified significant differences from expression data obtained from GFP reporter strains,[41] calling into question, in each particular case, the completeness of the control sequences used with GFP constructs. With a better understanding of the regulatory mechanisms that control gene expression, reporter constructs are becoming more reliable in *C. elegans*.[41−43]

We show that the posteriorly projecting VE2 and DE2 motor neurons express *afp-1* (AF3, 4, 10, 13, 14, 20) and AF2. *Afp-1* is orthologous to the *C. elegans* gene *flp-18*, while AF2 (encoded by *afp-4*) is orthologous to the *C. elegans* gene *flp-14*. Both genes are among the most broadly conserved genes in nematodes,[44−46] with the sequence of AF2 being completely conserved, and peptides encoded by *afp-1* orthologs maintaining a conserved PNFLRFamide C-terminus. GFP reporter constructs for *Ce-flp-14* did not identify any cells in *C. elegans*, while ISH experiments in *G. pallida* using a *Gp-flp-14* specific probe did not identify any of the ventral cord motorneurons but instead the RME motorneurons that innervate muscles of the head.[47]

ISH for *flp-18* failed in *C. elegans*, but *flp-18*:GFP-constructs in *C. elegans* do show expression in excitatory ventral cord motorneurons (Table 2).[48] *Flp-18* has been shown to work in a homeostatic manner to regulate excitation/inhibition balances in locomotion via two G-protein coupled receptors, NPR-1 and NPR-5.[49] Both AF9 and the *flp-18* peptides were identified as ligands for NPR-1.[50−52]

AF9 is also highly conserved and found broadly in nematodes; we show that it is expressed in cholinergic motorneurons VE1, DE1, and DE3 in *A. suum*; however, the *C. elegans* ortholog *Ce-flp-21* is reported to be expressed in ADL, ASE, and ASH sensory neurons, the URA motorneurons, the MC, M2, and M4 pharyngeal neurons[11] but not in any of the motorneurons in the ventral nerve cord.

The neuropeptide-like protein (NLP) gene *As-nlp-21* is expressed in VE1, DE1, and DE3 motorneurons. Searches of nematode ESTs identified orthologous *nlp-21* peptides in 11 species from 3 different clades (Supplementary Figure 6).[53,54] The alignment of peptide sequences predicted from nematode *nlp-21* precursor proteins suggests that they share a common motif: [G/A][G/A][A/G]RXF[X_{0-19}] (Figure 11, Table 2). Predicted nematode *nlp-21* peptides have a conserved N-terminal sequence and a C-terminus that is highly variable, both in sequence and length. Currently, NLP localization data are restricted to *A. suum* and *C. elegans*. Expression of *nlp-21* is observed in both nematodes in VA, AS, and DA (VE1, DE1, and DE3 in *A. suum*), but in *C. elegans*, *nlp-21*:GFP is also reported in VB and DB[54] (VE2 and DE2); however, in *A. suum*, there is no expression of the orthologous transcript in these neurons. Both nematodes also show a number of unidentified cells in the head, and *Ce-nlp-21*:GFP was also expressed in the pharynx, intestine, and tail.

Bioactivity. To date, over 100 peptides have been isolated and sequenced from *A. suum*.[10,12,13,17−20,22] Each new peptide needs to be studied for its biological function. The functions of AF2, AF9 and the six peptides encoded on the *afp-1* transcript (AF3, 4, 10, 13, 14, 20) have been studied extensively by electrophysiological recording from individual motorneurons.[10] These peptides have been shown to cause a variety of effects in both the motorneurons and/or the muscle they innervate.

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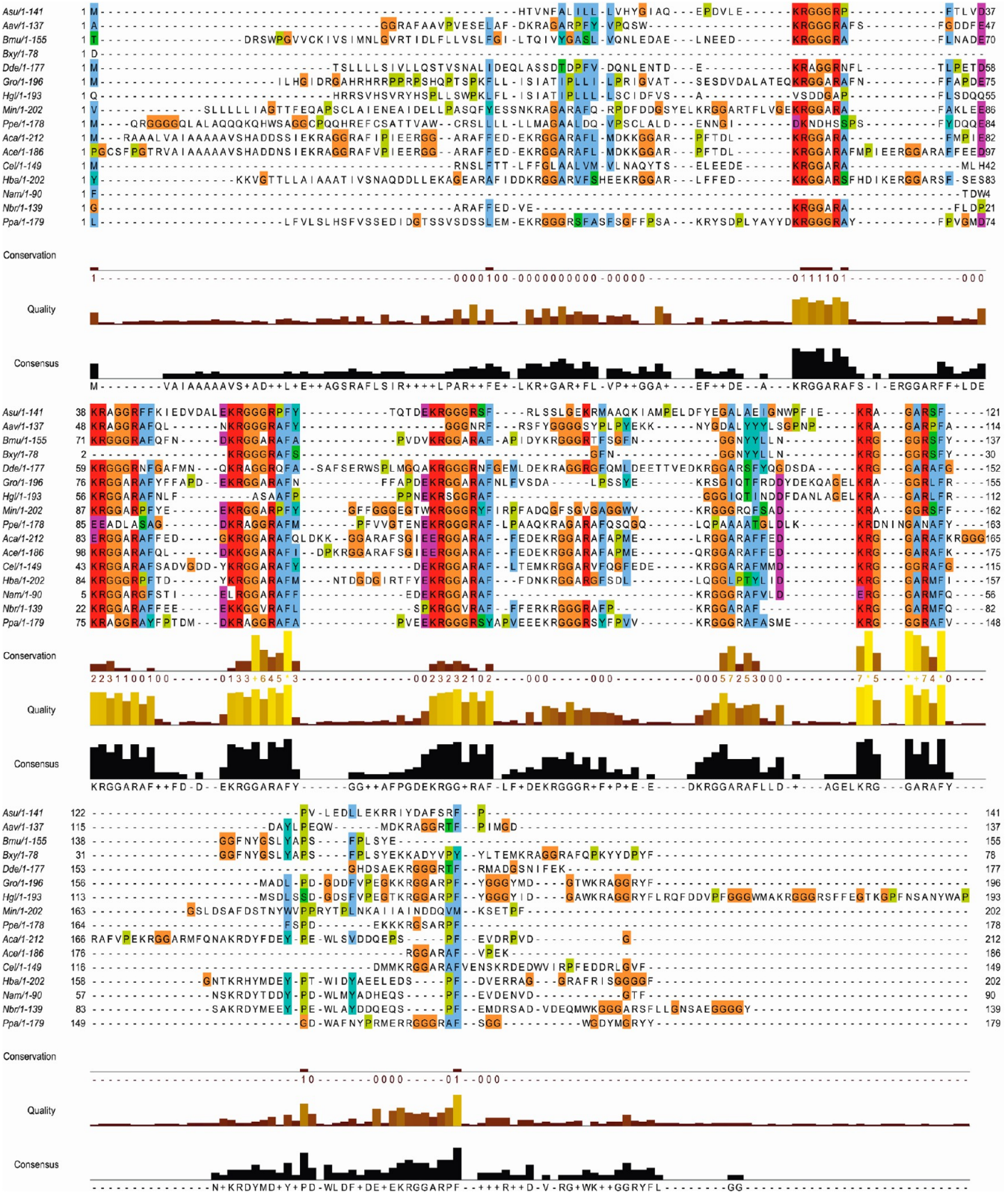

Figure 11. *As-nlp-21* multiple alignment by MEGA5.1[65] of *nlp-21*-like transcripts from different nematodes. Results were presented in Jalview[67] and show strong conservation of the predicted encoded peptides. Asu = *Ascaris suum* (Clade III), Aav = *Aphelenchus avenae* (IVb), Bmu = *Bursaphelenchus mucronatus* (IVb), Bxy = *Bursaphelenchus xylophilus* (IVb), Dde = *Ditylenchus destructor* (IVb), Gro = *Globodera rostochiensis* (IVb), Hgl = *Heterodera glycine* (IVb), Min = *Meloidogyne incognita* (IVb), Ppe = *Pratylenchus penetrans* (IVb), Aca = *Ancylostoma caninum* (V), Ace = *Ancylostoma ceylonicum* (V), Cel = *Caenorhabditis elegans* (V), Hba = *Heterorhabditis bacteriophora* (V), Nam = *Necator americanus* (V), Nbr = *Nippostrongylus brasiliensis* (V), Ppa = *Pristionchus pacificus* (V).

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We show that AF9 is expressed in DE1, VE1, and DE3. From our ISH experiments, there is no evidence to suggest expression of AF9 in any other ventral cord neurons (i.e., interneurons). An interesting observation is that AF9 causes a strong depolarization of DE2 and a weak but significant hyperpolarization of DI. This may explain the dorsal coiling posture shown by Davis and Stretton (2001). A few synapses have been shown between DE1 and DE2. Possibly these mediate the effect of AF9 on DE2, although it is also possible that the interaction is nonsynaptic, involving diffusion of the peptide from its presumptive release site from DE1, DE3, and/or VE1 to receptors on DE2.

AF2 elicits a strong depolarization of DE2 motorneurons and a biphasic change in input resistance.[10] In muscle tension experiments, AF2 produces a biphasic response, with an initial relaxation followed by contraction and rhythmic activity.[18] The explanation for these effects is not yet clear. Perhaps there is an autoreceptor on DE2 axons, although it is not certain that the electrophysiological effects of AF2 on DE2 neurons are direct. When AF2 was injected into whole worms, it caused a cessation of body waveform propagation; this was accompanied by a more than 100-fold increase in cAMP in muscle.[15] AF2 has also been shown to potentiate the response of nicotinic ACh receptors in *A. suum*.[55,56]

The *afp-1* peptides produced small depolarizations in DE2 motorneurons and had little effect on DI motorneurons, yet when injected into whole worms, these peptides caused an increase in the total number of body waveforms, and a dramatic effect on their amplitude.[10] We are still seeking a better understanding of these results.

Preliminary experiments to characterize the bioactivity of the six novel peptides encoded by the *As-nlp-21* gene were carried out using the dorsal muscle strip assay. No significant effect by any of the peptides was observed on acetylcholine (ACh)-induced muscle contraction when compared to controls (Supplementary Figure 7). The *C. elegans* ortholog, Ce-NLP-21, has been shown to be trafficked in dense-core vesicles in the cholinergic ventral cord motorneurons, and secreted into the extracellular fluid, but no postsynaptic target or primary function has been identified.[57]

CONCLUSIONS AND FUTURE DIRECTIONS

By single-cell MS, we have shown that there are 2 functional classes of *A. suum* excitatory somatic motorneurons, which contain distinct subsets of neuropeptides. The anteriorly projecting excitatory motorneurons (DE1, DE3, and VE1) express six novel peptides encoded by the *As-nlp-21* transcript, and AF9, encoded by the *afp-14* transcript. The posteriorly projecting excitatory motorneurons (DE2 and VE2) express AF2 (encoded by *afp-4*) and the six related peptides encoded by the *afp-1* transcript. Cellular localization was confirmed by *in situ* hybridization. The biological activity of most of these previously identified peptides has been reported.[10,18] Here we report preliminary experiments on the biological activity of the novel peptides we discovered. All of the biological effects described in this paper involve responses to single peptides. Now that we know that the excitatory motorneurons contain multiple peptides, it will be important in the future to examine possible interactions between them, using a combination of behavioral, muscle contractile, and electrophysiological assays. Initially, the interest will be in pairwise interactions, but we realize that there may be differences in time course of the effects of different peptides. It may be that some peptides set a background context against which other peptides (or neurotransmitters) act. There is overlap between the output regions of different motorneurons (i.e., the muscle cells at a particular position in the worm receive input from all the motor axons in that region). Furthermore, the muscle cells in the dorsal or ventral fields are interconnected with electrical synapses. Thus, any context setting might be produced not only by interaction between the peptides in each cell but also by interactions between the two distinct sets of excitatory neurons (the anterior-projectors and the posterior-projectors) that we have identified by their function in this paper.

METHODS

Animals. Live *A. suum* were collected from pig intestines at a slaughterhouse, and maintained in phosphate buffered saline (PBS, 140 mM sodium chloride, 10 mM sodium phosphate, pH 6.8−7.5) at 37 °C. The PBS was changed daily and 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. After cutting longitudinally between the dorsal nerve cord and the right lateral line, the preparation was pinned open, the lips were cut off, and the pharynx was removed. The preparations were rinsed three times with 170 mM ammonium acetate before the isotonic glycerol dissecting solution was added (170 mM ammonium acetate in 30% glycerol).[20,27,58−60] Dissecting needles made from electrolytically sharpened tungsten wire were used to isolate a desired neuron from the many neuronal processes and hypodermis in which it is embedded. 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.

Isolated cells were transferred to a Bruker MTP stainless steel MALDI target. Cells were cleared with 0.5 μL isopropanol[61] to remove the glycerol and any remaining extracellular salts. Individual neurons were spotted with 60−100 nL of saturated α-cyano-4-hydroxycinnamic acid (CHCA; Sigma, St Louis, MO) in 70% acetonitrile (ACN; Fisher Scientific, Waltham, MA), 1% trifluoroacetic acid (TFA; Sigma) or saturated 2,5-dihydroxybenzoic acid (DHB; Sigma) in 1:1 HPLC grade methanol and water (Fisher Scientific) using a Nanoliter Cool Wave Syringe II.[62]

Mass Acquisition. A Bruker Ultraflex III MALDI-TOF/TOF MS (Bruker Daltonics, Billerica, MA) equipped with a Smartbeam laser and LIFT-TM cell was used to obtain MS and MS/MS spectra with Bruker Compass v. 1.2 software. Because the cells contain relatively small quantities of peptide, all spectra were obtained from 50 laser shots per acquisition. MS spectra were obtained in positive ion reflectron mode, with an m/z range of 500−4000. Instrument settings were optimized for maximum detection sensitivity using the Bruker Flex Control software: ion source 1 voltage, 25.0 kV; ion source 2 voltage, 20.5 kV; reflector 1 voltage, 26.5 kV; reflector 2 voltage, 14.5 kV; lens voltage, 9.5 kV. Synthetic peptide standards were not suitable for calibration of the instrument because the laser intensity needed for analysis of single cells was often far too high when analyzing pure standard, making it impossible to identify the monoisotopic peak of the ion needed for accurate calibration. Therefore, a reference

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spectrum from a freshly dissected *A. suum* AVK neuron, with known peptide peaks previously described by Jarecki et al. (2012), was used for external calibration of the instrument.

Acetylation of Peptides. Following deposition of the cell onto the target surface, 0.5 μL of isopropanol was applied to each cell to wash away the glycerol solution, followed by 0.5 μL of methanol/acetic anhydride (3:1). The cells were allowed to dry before each wash. Cells were then covered with matrix as described above.

Oxidation of Peptides. In cells exposed to Methylene Blue, methionine residues were partially oxidized to the sulfoxide, with a mass shift of +16 Da, and tryptophan residues gave rise to +16 and +32 adducts.[20]

Assignment of Peaks and Interpretation of Mass Spectra. Spectra were analyzed using Bruker Daltonics flex-Analysis 3.0 software. The software automatically assigned masses to peaks in each MS spectrum. Each MS/MS spectrum underwent background subtraction and smoothing before the automatic assignment of masses. In some cases, peak m/z values were added manually. Peaks were considered significant if they were twice the intensity of the baseline noise.

Molecular masses and ion fragmentation patterns were calculated by Protein Prospector MS-Product (http://prospector.ucsf.edu). Spectra containing peaks with m/z values corresponding to masses ±0.2 Da of *A. suum* peptides were temporarily assigned. Confirmation of the assignments was carried out by MS/MS and chemical modifications to the peptides. *De novo* sequencing of unidentified peaks was carried out by hand. Verification of peptide sequence was carried out by comparing the experimental MS/MS spectrum to that of synthetic peptide. Further verification was carried out by comparing sequences to tBLASTn searches and cloning of *A. suum* gene sequences. Raw spectral image files were acquired with Bruker Daltonics flex- Analysis 3.0 software and transferred to Adobe Illustrator (San Jose, CA) for annotation.

Database Searches. Database searches were conducted using methods described in recent publications.[13,17,20] Briefly, all predicted peptide sequence assignments were searched using tBLASTn (National Center for Biotechnology Information [NCBI], Bethesda, MD; http://www.ncbi.nlm.nih.gov/BLAST/) against *A. suum* ESTs and a library of 447 546 genomic survey sequences (GSS). For all 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 translated using the ExPASy Translate tool (http://au.expasy.org/tools/dna.html) and examined for putative peptide cleavage sites, and C-terminal glycine, a precursor for C-terminal amidation of peptides. Genomic sequences were analyzed for mRNA splice sites directly by PCR cloning of the mRNA sequences and comparison with genomic sequences.[63,64] Signal peptides were predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/).

Multiple Alignment. BLAST searches of the nematode EST library were performed using both As-NLP-21 and Ce-NLP-21 as queries. Selected sequences were imported into MEGA5.1[65] for alignment by MUSCLE.[66] The alignments were imported into Jalview[67] for display.

Peptide Synthesis. Peptides were synthesized by the University of Wisconsin Biotechnology Center (UWBC). The integrity of the synthesis was monitored by MALDI-TOF MS and high-pressure liquid chromatography (HPLC). Two of the *As-nlp-21* peptides were >95% pure (As-NLP-21.4, As-NLP-21.5). The remaining peptides were <95% pure by HPLC (Supplementary Table 1).

RNA Isolation and cDNA Preparation. *A. suum* heads (N = 20−30) were flash-frozen in liquid nitrogen and ground to a fine powder. Total RNA was isolated using a Nucleospin Nucleic Acid Purification Kit (Clontech, Mountain View, CA). First strand cDNA was generated for PCR using a Superscript First Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY). Rapid Amplification of cDNA Ends (RACE)-ready cDNA for 5′- and 3′-RACE was created from total RNA using a SMARTer RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions.

Transcript Identification by PCR and RACE. To obtain information about the transcripts that encoded each peptide, primers were designed for PCR and RACE from sequences in the *A. suum* EST library. RACE reactions were performed using an Advantage 2 PCR Kit (Clontech) according to the manufacturer's instructions with the appropriate primers in a 50 μL reaction as follows: 2 μL of *A. suum* cDNA or 2 μL of cDNA reaction solution with no reverse transcriptase as a control, 5 μL of 10× PCR Gold buffer (Applied Biosystems, Foster City, CA), 2−8 μL of 25 mM $MgCl_2$, 1 μL each of 10 mM dNTPs, 1 μL of each primer (20 μM), and 0.25 μL of AmpliTaq Gold DNA polymerase (Applied Biosystems; 5 U/μL). The PCR conditions were programmed into the Eppendorf Mastercycler Gradient as follows: 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 66 or 68 °C for 30 s, 72 °C for 1 min.

PCR and RACE products were run on a 1% agarose gel, and novel bands were excised and purified using a Qiaquick Gel Purification Kit (Qiagen, Chatsworth, CA). The purified products were cloned into *E. coli* using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and the plasmid DNA was isolated with a Qiagen Miniprep Kit. Automated sequencing was carried out by the DNA Sequencing Facility at the UWBC. Sequence electropherograms were viewed on Chromas Lite software (Technelysium Pty Ltd.). Sequences were analyzed using the ExPASy Translate Tool and T-Coffee Multiple Sequence Alignment Tool (Swiss Institute of Bioinformatics), and signal peptide sequences were identified using the SignalP 4.0 Server (Technical University of Denmark).

Riboprobe Synthesis. The primer pairs listed in Supplementary Table 2 were used to amplify a selected region by PCR. Products were cloned and sequenced to confirm the fidelity of the sequence, and to determine the orientation of the insert in the vector. The target sequences of each riboprobe are shown in Figures 5, 8. The constructs were linearized using restriction enzymes NotI and SpeI (New England Biolabs, Beverly, MA), and purified (Gel Extraction kit, Qiagen). Linearized plasmids were used as a template to synthesize an antisense (experimental) and a sense (negative control) digoxigenin-labeled riboprobe (Maxiscript SP6/T7 kit, Ambion, Austin, TX; digoxigenin-11-dUTP, Roche Applied Science, Indianapolis, IN), as described in Nanda and Stretton (2010). To remove unincorporated nucleotides, the reactions were run through NucAway Spin Columns (Ambion). Probe integrity and concentration were determined by gel electrophoresis with Sybr Gold staining (Molecular Probes, Eugene, OR) and nylon membrane dot blots (Roche Applied Science protocol).

Localization of *afp-1, -4, -14*, and *As-nlp-21* by *In Situ* Hybridization. *A. suum* whole mount preparations were prepared by injecting large female worms with 0.1−0.3 mL of 2 mg/mL collagenase in autoclaved *Ascaris* saline and incubating in a beaker of PBS at 37 °C for 1.5−2 h. The anterior 6 cm of the

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worm was taken and rinsed in autoclaved 100 mM phosphate buffer (pH 7.4) and the heads were transferred to a Sylgard-coated dissecting dish. The heads were cut longitudinally between the dorsal nerve cord and the right lateral line, the lips were removed, and the heads were pinned flat. Any remaining muscle cells were gently removed with forceps. The preparations were rinsed several times in 100 mM phosphate buffer, and fixed in 1% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) on a rocking table at 4 °C overnight.

After fixation, the head preparations were processed as previously described,[36] with the following modifications: incubation in proteinase K solution was increased to 30 min at 37 °C, and staining was allowed to occur for up to 16 h in the dark, or until staining of the cells was evident, and background staining began to occur. The staining reaction was stopped with Milli-Q water, and the heads were rinsed with Milli-Q water (2 × 10 min). The preparations were mounted in Clear-Mount (Electron Microscopy Sciences, Hatfield, PA) and allowed to dry. Photomicrographs were taken with a Zeiss AxioCam MRc camera on a Zeiss Universal microscope.

Characterization of As-NLP-21 Bioactivity. Muscle tension assays were performed using standard protocols.[68,69] Strips of dorsal muscle were obtained by cutting large female *A. suum* longitudinally along the lateral lines and isolating a 2 cm strip approximately 1 cm posterior to the head. Each preparation contained dorsal muscle cells (all longitudinal) and the motor axons of the dorsal nerve cord. The ends of the preparation were tied with silk thread. One end was attached to a fixed hook in a 7 mL chamber containing normal *Ascaris* saline at 37 °C and stirred by bubbling nitrogen gas, and the other end was tied to a FORT25 force transducer (World Precision Instruments, Sarasota, FL). The output of the transducer was routed through a TBM4M transbridge (WPI) and recorded on a computer using Data-Trax or LabScribe2 software (WPI), and the data were analyzed for tension/time relationships.

Baseline contractions were measured by adding 5 μM ACh to the chamber by micropipette and waiting until the contraction reached its maximum tension, followed by rinses of *Ascaris* saline. The preparation was then exposed to 10 μM peptide solution in *Ascaris* saline for 10 min (in the case of As-NLP-21.1 and As-NLP-21.2, the peptide was first dissolved in 10% DMSO, then diluted into *Ascaris* saline, resulting in a final concentration of 0.001% DMSO in the chamber). The chamber was rinsed, fresh peptide was added, and ACh-induced contraction was measured by adding 5 μM ACh immediately after exposure to peptide solution, followed by rinses with peptide-free *Ascaris* saline. Muscle strip contraction in response to ACh was measured at 10, 20, 30, 40, and 50 min after exposure to peptide, with rinses of peptide-free *Ascaris* saline between doses of ACh. For comparison, control muscle strips were subjected to ACh-induced contraction in peptide-free *Ascaris* saline (or 0.001% DMSO in *Ascaris* saline) at the same time points. Responses of the muscle strips were reported as a percentage of the maximum ACh contraction observed for each muscle strip.

Responses for each peptide group were reported as the mean ± S.E., and displayed using Prism 6 software (GraphPad, San Diego, CA). Significance between peptide and control groups was determined using unpaired *t*-tests, with significance levels set as $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

Tandem MS spectra of synthetic peptides, additional *in situ* hybridization data, effects of *As-nlp-21* peptides on muscle tension, primer sequence data, peptide purities, and nematode EST and GSS sequences referenced in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

C.J.K. was responsible for single-cell dissection, MS, bioinformatics, data analysis, transcript identification, ISH, and manuscript preparation. J.J.K. was responsible for transcript identification, ISH, muscle tension assays, bioinformatics, and manuscript preparation. M.S.S. was responsible for single-cell dissections. M.M.V. was responsible for MS and data analysis, A.O.W.S. was responsible for data analysis, bioinformatics, and manuscript preparation.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACh, acetylcholine; AF, *Ascaris* FMRFamide-like; *afp*, *Ascaris* FMRFamide-like precursor protein transcript; CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; EST, expressed sequence tag; *flp*, FMRFamide-like peptide gene; GSS, genomic survey sequence; ISH, *in situ* hybridization; MALDI-TOF, matrix-assisted laser desorption/ionization−time-of-flight; MS, mass spectrometry; *m*/*z*, mass-to-charge ratio; NLP, neuropeptide-like protein; *nlp*, neuropeptide-like protein gene; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; VC, ventral nerve cord

■ REFERENCES

(1) White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc., B 314*, 1−340.

(2) Towlson, E. K., Vértes, P. E., Ahnert, S. E., Schafer, W. R., and Bullmore, E. T. (2013) The Rich Club of the *C. elegans* Neuronal Connectome. *J. Neurosci. 33*, 6380−6387.

(3) Kleinfeld, D., Bharioke, A., Blinder, P., Bock, D. D., Briggman, K. L., Chklovskii, D. B., Denk, W., Helmstaedter, M., Kaufhold, J. P., Lee, W. C., Meyer, H. S., Micheva, K. D., Oberlaender, M., Prohaska, S., Reid, R. C., Smith, S. J., Takemura, S., Tsai, P. S., and Sakmann, B. (2011) Large-

DOI: 10.1021/cn5003623
ACS Chem. Neurosci. 2015, 6, 855−870

scale automated histology in the pursuit of connectomes. *J. Neurosci. 31*, 16125−16138.
(4) Bargmann, C. I. (2012) Beyond the connectome: how neuromodulators shape neural circuits. *BioEssays 34*, 458−465.
(5) Marder, E., Calabrese, R. L., Nusbaum, M. P., and Trimmer, B. (1987) Distribution and partial characterization of FMRFamide-like peptides in the stomatogastric nervous systems of the rock crab, *Cancer borealis*, and the spiny lobster, Panulirus interruptus. *J. Comp. Neurol. 259*, 150−163.
(6) Stretton, A., Donmoyer, J., Davis, R., Meade, J., Cowden, C., and Sithigorngul, P. (1992) Motor behavior and motor nervous system function in the nematode Ascaris suum. *J. Parasitol. 78*, 206−214.
(7) Meade, J. A., and Stretton, A. O. W. (1989) Activity of Motorneurons during *Ascaris* Locomotion. *Soc. Neurosci. Abs. 15*, 1299.
(8) Stretton, A. O., Fishpool, R. M., Southgate, E., Donmoyer, J. E., Walrond, J. P., Moses, J. E., and Kass, I. S. (1978) Structure and physiological activity of the motoneurons of the nematode *Ascaris*. *Proc. Natl. Acad. Sci. U.S.A. 75*, 3493−3497.
(9) Davis, R. E., and Stretton, A. O. (1996) The motornervous system of *Ascaris*: electrophysiology and anatomy of the neurons and their control by neuromodulators. *Parasitology 113* (Suppl), S97−117.
(10) Davis, R. E., and Stretton, A. O. (2001) Structure−activity relationships of 18 endogenous neuropeptides on the motor nervous system of the nematode *Ascaris suum*. *Peptides 22*, 7−23.
(11) Kim, K., and Li, C. (2004) Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *J. Comp. Neurol. 475*, 540−550.
(12) Yew, J. Y., Kutz, K. K., Dikler, S., Messinger, L., Li, L., and Stretton, A. O. (2005) Mass spectrometric map of neuropeptide expression in *Ascaris suum*. *J. Comp. Neurol. 488*, 396−413.
(13) Jarecki, J. L., Frey, B. L., Smith, L. M., and Stretton, A. O. (2011) Discovery of neuropeptides in the nematode *Ascaris suum* by database mining and tandem mass spectrometry. *J. Proteome Res. 10*, 3098−3106.
(14) Cowden, C., Stretton, A. O., and Davis, R. E. (1989) AF1, a sequenced bioactive neuropeptide isolated from the nematode *Ascaris suum*. *Neuron 2*, 1465−1473.
(15) Reinitz, C. A., Herfel, H. G., Messinger, L. A., and Stretton, A. O. (2000) Changes in locomotory behavior and cAMP produced in Ascaris suum by neuropeptides from Ascaris suum or *Caenorhabditis elegans*. *Mol. Biochem. Parasitol. 111*, 185−197.
(16) Reinitz, C. A., Pleva, A. E., and Stretton, A. O. (2011) Changes in cyclic nucleotides, locomotory behavior, and body length produced by novel endogenous neuropeptides in the parasitic nematode *Ascaris suum*. *Mol. Biochem. Parasitol. 180*, 27−34.
(17) Jarecki, J. L., Viola, I. R., Andersen, K. M., Miller, A. H., Ramaker, M. A., Vestling, M. M., and Stretton, A. O. (2012) Three independent techniques localize expression of transcript afp-11 and its bioactive peptide products to the paired AVK neurons in Ascaris suum: *in situ* hybridization, immunocytochemistry, and single cell mass spectrometry. *ACS Chem. Neurosci. 4*, 418−434.
(18) Cowden, C., and Stretton, A. O. (1993) AF2, an *Ascaris* neuropeptide: isolation, sequence, and bioactivity. *Peptides 14*, 423−430.
(19) Cowden, C., and Stretton, A. O. (1995) Eight novel FMRFamide-like neuropeptides isolated from the nematode *Ascaris suum*. *Peptides 16*, 491−500.
(20) Jarecki, J. L., Andersen, K., Konop, C. J., Knickelbine, J. J., Vestling, M. M., and Stretton, A. O. (2010) Mapping neuropeptide expression by mass spectrometry in single dissected identified neurons from the dorsal ganglion of the nematode *Ascaris suum*. *ACS Chem. Neurosci. 1*, 505−519.
(21) Jarecki, J. L. Identification and Localization of Neuropeptides in *Ascaris suum* By Mass Spectrometry, *Thesis*, University of Wisconsin-Madison, December 2010.
(22) Yew, J. Y., Davis, R., Dikler, S., Nanda, J., Reinders, B., and Stretton, A. O. (2007) Peptide products of the afp-6 gene of the nematode *Ascaris suum* have different biological actions. *J. Comp Neurol 502*, 872−882.
(23) Miyamoto, M. D. (1975) Binomial Analysis of Quantal Transmitter Release at Glycerol Treated Frog Neuromuscular-Junctions. *J. Physiol-London 250*, 121−142.
(24) Pegg, D. E. (1976) Long-term preservation of cells and tissues: a review. *J. Clin. Pathol. 29*, 271−285.
(25) Rubakhin, S. S., and Sweedler, J. V. (2008) Quantitative measurements of cell-cell signaling peptides with single-cell MALDI MS. *Anal. Chem. 80*, 7128−7136.
(26) Nemes, P., Rubakhin, S. S., Aerts, J. T., and Sweedler, J. V. (2013) Qualitative and quantitative metabolomic investigation of single neurons by capillary electrophoresis electrospray ionization mass spectrometry. *Nat. Protoc. 8*, 783−799.
(27) Rubakhin, S. S., Greenough, W. T., and Sweedler, J. V. (2003) Spatial profiling with MALDI MS: Distribution of neuropeptides within single neurons. *Anal. Chem. 75*, 5374−5380.
(28) Walrond, J. P., and Stretton, A. O. (1985) Excitatory and inhibitory activity in the dorsal musculature of the nematode *Ascaris* evoked by single dorsal excitatory motonerons. *J. Neurosci. 5*, 16−22.
(29) Chalfie, M., Sulston, J. E., White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1985) The neural circuit for touch sensitivity in *Caenorhabditis elegans*. *J. Neurosci. 5*, 956−964.
(30) Wen, Q., Po, M. D., Hulme, E., Chen, S., Liu, X., Kwok, S. W., Gershow, M., Leifer, A. M., Butler, V., Fang-Yen, C., Kawano, T., Schafer, W. R., Whitesides, G., Wyart, M., Chklovskii, D. B., Zhen, M., and Samuel, A. D. (2012) Proprioceptive coupling within motor neurons drives *C. elegans* forward locomotion. *Neuron 76*, 750−761.
(31) Johnson, A. O., Stretton, A. O. (1980) Neural Control of Locomotion in *Ascaris*; Anatomy, Electrophysiology, and Biochemistry, In *Nematodes as Biological Models* (Zuckerman, B., Ed.), pp 159−195, Acedemic Press, New York.
(32) Johnson, C. D., and Stretton, A. O. W. (1985) Localization of Choline-Acetyltransferase within Identified Motoneurons of the Nematode *Ascaris*. *J. Neurosci. 5*, 1984−1992.
(33) Angstadt, J. D., Donmoyer, J. E., and Stretton, A. O. (1989) Retrovesicular ganglion of the nematode *Ascaris*. *J. Comp. Neurol. 284*, 374−388.
(34) Johnson, C. D., and Stretton, A. O. (1987) GABA-immunoreactivity in inhibitory motor neurons of the nematode *Ascaris*. *J. Neurosci. 7*, 223−235.
(35) Edison, A. S., Messinger, L. A., and Stretton, A. O. (1997) afp-1: a gene encoding multiple transcripts of a new class of FMRFamide-like neuropeptides in the nematode *Ascaris suum*. *Peptides 18*, 929−935.
(36) Nanda, J. C., and Stretton, A. O. (2010) In situ hybridization of neuropeptide-encoding transcripts afp-1, afp-3, and afp-4 in neurons of the nematode Ascaris suum. *J. Comp. Neurol. 518*, 896−910.
(37) Khatun, J., Ramkissoon, K., and Giddings, M. C. (2007) Fragmentation characteristics of collision-induced dissociation in MALDI TOF/TOF mass spectrometry. *Anal. Chem. 79*, 3032−3040.
(38) Maroney, P. A., Denker, J. A., Darzynkiewicz, E., Laneve, R., and Nilsen, T. W. (1995) Most mRNAs in the nematode *Ascaris lumbricoides* are trans-spliced: a role for spliced leader addition in translational efficiency. *RNA 1*, 714−723.
(39) Sithigorngul, P., Nanda, J. C., and Stretton, A. O. W. (2003) A strategy for isolating rare peptides: isolation and sequencing of a large peptide present in a single neuron of the nematode *Ascaris suum*. *Peptides 24*, 1025−1033.
(40) Sithigorngul, P., Jarecki, J. L., and Stretton, A. O. (2011) A specific antibody to neuropeptide AF1 (KNEFIRFamide) recognizes a small subset of neurons in *Ascaris suum*: differences from *Caenorhabditis elegans*. *J. Comp Neurol 519*, 1546−1561.
(41) Etchberger, J. F., Lorch, A., Sleumer, M. C., Zapf, R., Jones, S. J., Marra, M. A., Holt, R. A., Moerman, D. G., and Hobert, O. (2007) The molecular signature and *cis*-regulatory architecture of a *C. elegans* gustatory neuron. *Genes Dev. 21*, 1653−1674.
(42) Zhang, F., O'Meara, M. M., and Hobert, O. (2011) A left/right asymmetric neuronal differentiation program is controlled by the *Caenorhabditis elegans* lsy-27 *zinc*-finger transcription factor. *Genetics 188*, 753−759.

DOI: 10.1021/cn5003623
ACS Chem. Neurosci. 2015, 6, 855−870

(43) Kratsios, P., Stolfi, A., Levine, M., and Hobert, O. (2012) Coordinated regulation of cholinergic motor neuron traits through a conserved terminal selector gene. *Nat. Neurosc. 15*, 205−214.
(44) McVeigh, P., Geary, T. G., Marks, N. J., and Maule, A. G. (2006) The FLP-side of nematodes. *Trends Parasitol. 22*, 385−396.
(45) McVeigh, P., Leech, S., Mair, G. R., Marks, N. J., Geary, T. G., and Maule, A. G. (2005) Analysis of FMRFamide-like peptide (FLP) diversity in phylum Nematoda. *Int. J. Parasitol. 35*, 1043−1060.
(46) McCoy, C. J., Atkinson, L. E., Zamanian, M., McVeigh, P., Day, T. A., Kimber, M. J., Marks, N. J., Maule, A. G., and Mousley, A. (2014) New insights into the FLPergic complements of parasitic nematodes: Informing deorphanisation approaches. *EuPA Open Proteomics 3*, 262−272.
(47) Kimber, M. J., Fleming, C. C., Prior, A., Jones, J. T., Halton, D. W., and Maule, A. G. (2002) Localisation of *Globodera pallida* FMRFamide-related peptide encoding genes using *in situ* hybridisation. *Int. J. Parasitol. 32*, 1095−1105.
(48) Li, C., Kim, K., and Nelson, L. S. (1999) FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *Brain Res. 848*, 26−34.
(49) Stawicki, T. M., Takayanagi-Kiya, S., Zhou, K., and Jin, Y. (2013) Neuropeptides function in a homeostatic manner to modulate excitation-inhibition imbalance in *C. elegans*. *PLoS Genet. 9*, e1003472.
(50) Kubiak, T. M., Larsen, M. J., Nulf, S. C., Zantello, M. R., Burton, K. J., Bowman, J. W., Modric, T., and Lowery, D. E. (2003) Differential activation of ″social″ and ″solitary″ variants of the *Caenorhabditis elegans* G protein-coupled receptor NPR-1 by its cognate ligand AF9. *J. Biol. Chem. 278*, 33724−33729.
(51) Rogers, C., Reale, V., Kim, K., Chatwin, H., Li, C., Evans, P., and de Bono, M. (2003) Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nat. Neursci. 6*, 1178−1185.
(52) Dossey, A. T., Reale, V., Chatwin, H., Zachariah, C., deBono, M., Evans, P. D., and Edison, A. S. (2006) NMR analysis of *Caenorhabditis elegans* FLP-18 neuropeptides: implications for NPR-1 activation. *Biochemistry 45*, 7586−7597.
(53) McVeigh, P., Alexander-Bowman, S., Veal, E., Mousley, A., Marks, N. J., and Maule, A. G. (2008) Neuropeptide-like protein diversity in phylum Nematoda. *Int. J. Parasitol. 38*, 1493−1503.
(54) Nathoo, A. N., Moeller, R. A., Westlund, B. A., and Hart, A. C. (2001) Identification of neuropeptide-like protein gene families in *Caenorhabditis elegans* and other species. *Proc. Natl. Acad. Sci. U.S.A.*, 14000−14005.
(55) Puttachary, S., Robertson, A. P., Clark, C. L., and Martin, R. J. (2010) Levamisole and ryanodine receptors. II: An electrophysiological study in *Ascaris suum*. *Mol. Biochem. Parasitol. 171*, 8−16.
(56) Robertson, A. P., Clark, C. L., and Martin, R. J. (2010) Levamisole and ryanodine receptors. I: A contraction study in *Ascaris suum*. *Mol. Biochem. Parasitol. 171*, 1−7.
(57) Goodwin, P. R., and Juo, P. (2013) The scaffolding protein SYD-2/Liprin-alpha regulates the mobility and polarized distribution of dense-core vesicles in *C. elegans* motor neurons. *PLoS One 8*, e54763.
(58) Rubakhin, S. S., and Sweedler, J. V. (2007) Characterizing peptides in individual mammalian cells using mass spectrometry. *Nat. Protoc. 2*, 1987−1997.
(59) Rubakhin, S. S., Churchill, J. D., Greenough, W. T., and Sweedler, J. V. (2006) Profiling signaling peptides in single mammalian cells using mass spectrometry. *Anal. Chem. 78*, 7267−7272.
(60) Nemes, P., Knolhoff, A. M., Rubakhin, S. S., and Sweedler, J. V. (2012) Single-cell metabolomics: changes in the metabolome of freshly isolated and cultured neurons. *ACS Chem. Neurosci. 3*, 782−792.
(61) Schwartz, S. A., Reyzer, M. L., and Caprioli, R. M. (2003) Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation. *J. Mass. Spectrom. 38*, 699−708.
(62) Hilker, B., Clifford, K. J., Sauter, A. D., Jr., Sauter, A. D., 3rd, and Harmon, J. P. (2009) The measurement of charge for induction-based fluidic MALDI dispense event and nanoliter volume verification in real time. *J. Am. Soc. Mass. Spectrom 20*, 1064−1067.
(63) Jex, A. R., Liu, S., Li, B., Young, N. D., Hall, R. S., Li, Y., Yang, L., Zeng, N., Xu, X., Xiong, Z., Chen, F., Wu, X., Zhang, G., Fang, X., Kang, Y., Anderson, G. A., Harris, T. W., Campbell, B. E., Vlaminck, J., Wang, T., Cantacessi, C., Schwarz, E. M., Ranganathan, S., Geldhof, P., Nejsum, P., Sternberg, P. W., Yang, H., Wang, J., Wang, J., and Gasser, R. B. (2011) *Ascaris suum* draft genome. *Nature 479*, 529−533.
(64) Wang, J., Czech, B., Crunk, A., Wallace, A., Mitreva, M., Hannon, G. J., and Davis, R. E. (2011) Deep small RNA sequencing from the nematode *Ascaris* reveals conservation, functional diversification, and novel developmental profiles. *Genome Res. 21*, 1462−1477.
(65) Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol. 28*, 2731−2739.
(66) Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res. 32*, 1792−1797.
(67) Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., and Barton, G. J. (2009) Jalview Version 2–a multiple sequence alignment editor and analysis workbench. *Bioinformatics 25*, 1189−1191.
(68) Ash, A. S., and Tucker, J. F. (1967) The bioassay of gamma-aminobutyric acid using a muscle preparation from *Ascaris lumbricoides*. *J. Pharm. Pharmacol. 19*, 240−245.
(69) Kass, I. S., Larsen, D. A., Wang, C. C., and Stretton, A. O. (1982) *Ascaris suum*: differential effects of avermectin B1a on the intact animal and neuromuscular strip preparations. *Exp. Parasitol. 54*, 166−174.
(70) Husson, S. J., Clynen, E., Baggerman, G., De Loof, A., and Schoofs, L. (2005) Discovering neuropeptides in Caenorhabditis elegans by two dimensional liquid chromatography and mass spectrometry. *Biochem. Biophys. Res. Commun. 335*, 76−86.

DOI: 10.1021/cn5003623
ACS Chem. Neurosci. 2015, 6, 855−870