A Specific Antibody to Neuropeptide AF1 (KNEFIRFamide) Recognizes a Small Subset of Neurons in *Ascaris suum*: Differences from *Caenorhabditis elegans*

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ABSTRACT

A monoclonal antibody, AF1-003, highly specific to the *Ascaris suum* neuropeptide AF1 (KNEFIRFamide), was generated. This antibody binds strongly to AF1 and extremely weakly to other peptides with C-terminal FIRFamide: AF5 (SGKPTFIRFamide), AF6 (FIRFamide), and AF7 (AGPRFIRFamide). It does not recognize 35 other AF (*A. suum* FMRFamide-like) peptides at the highest concentration tested, nor does it recognize FMRFamide. When crude peptide extracts of *A. suum* are fractionated by two-step HPLC, the only fractions recognized by AF1-003 are those comigrating with synthetic AF1. By immunocytochemistry, antibody AF1-003 recognizes a small subset of the 298 neurons of *A. suum*: these include the paired URX and RIP neurons, two pairs of lateral ganglion neurons in the head, and the unpaired PQR and PDA or -B tail neurons that send processes to

the head along the dorsal and ventral nerve cords, respectively. AF1 immunoreactivity is also seen in three pairs of pharyngeal neurons. Mass spectroscopy (MS) shows the presence of AF1 in the head, pharynx, and dorsal and ventral nerve cords. In *A. suum*, the neurons that contain AF1 show little overlap with neurons that express green fluorescent protein constructs targeting the *flp-8* gene, which encodes AF1 in *Caenorhabditis elegans* (Kim and Li [2004] *J. Comp. Neurol.* 475:540–550); the URX neurons express AF1 in both species, but, in *C. elegans*, *flp-8* expression was not detected in RIP, PQR, and PDA or -B or in the pharynx. Other, less specific monoclonal antibodies recognize AF1, as well as other peptides to differing degrees; these antibodies are useful reagents for determination of neuronal morphology. *J. Comp. Neurol.* 519:1546–1561, 2011.

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INDEXING TERMS: peptide; immunocytochemistry; antibody specificity; monoclonal antibody; neuronal morphology; morphological homology

Since the original isolation of the neuropeptide FMRFamide from the clam *Macrocallista nimbosa* (Price and Greenberg, 1977), many variants of FMRFamide (FMRFamide-like peptides or FLPs) have been identified by immunocytochemistry, by direct isolation, or by cloning the genes encoding the peptides. FLPs are ubiquitous in the animal kingdom and have been found from coelenterates to mammals; most species express multiple FLPs (for review see Li et al., 1999a).

Nematodes in particular are known to contain a great variety of FLPs. In one species, *Caenorhabditis elegans*, the availability of the complete sequence of the genome has allowed an extensive search for genes that encode FLP precursor proteins; 31 such genes (*flp* genes), encoding 71 putative FLPs, have been identified, and new mem-

bers of this family are still being discovered (Li et al., 1999b; McVeigh et al., 2005; Husson et al., 2005, 2007; Li and Kim, 2008). The existence of several of these putative peptides has been confirmed by direct chemical isolation (Marks et al., 1995, 1997, 1999; Davis and Stretton, 1996; Maule et al., 1996; Brownlee and Fairweather,

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1999; Husson et al., 2005, 2007). Other *C. elegans* genes encoding putative peptides with different C-terminal sequences are also being discovered (Li et al., 1999b; Nathoo et al., 2001; Husson et al., 2005, 2007; Li and Kim, 2008; McVeigh et al., 2008; L.A. Messinger, unpublished); it is now clear that there is a rich and complex set of peptides in *C. elegans*.

In the parasitic nematode *Ascaris suum*, there is also evidence for many families of peptides (Sithigorngul et al., 1990; Brownlee et al., 1993; McVeigh et al., 2008). The FLPs are prominent, being present in about 70% of the neurons (Cowden et al., 1993), and there is much variety in their structure. In this laboratory, we have directly sequenced 40 FLPs from *A. suum*: AF1–AF11 and AF13–AF41 (Cowden et al., 1989; Cowden and Stretton, 1993, 1995; Davis and Stretton, 1996, 2001; Yew et al., 2003, 2005, 2007; Jarecki et al., 2010; Jarecki, Andersen, and Stretton, unpublished). In many cases, the *afp* transcripts that encode these peptides have also been characterized, either by cloning or by sequence mining from EST libraries (Nanda, 2004; McVeigh et al., 2005; Yew et al., 2007; Nanda and Stretton, 2010; Jarecki et al., 2010).

AF1 (KNEFIRFamide) occurs in other nematodes. It has been isolated from *C. elegans* (Sithigorngul; reported in Davis and Stretton, 1996). The generation of large EST libraries from 30 species of parasitic nematodes has allowed the discovery of transcripts that encode putative FLPs in parasitic nematodes (McVeigh et al., 2005). Several nematode ESTs include AF1-encoding sequences. In all cases, the predicted precursor proteins include multiple copies of AF1, flanked by classical dibasic amino acid cleavage sites: *Necator americanus* (4× AF1), *Ancylostoma ceylanicum* (4× AF1), *Onchocerca volvulus* (2× AF1; McVeigh et al., 2005), and *A. suum* (5× AF1; McVeigh et al., 2005; revised by Nanda, 2004); the free-living nematodes *C. elegans* and *C. briggsae* each encode four copies of AF1 (Li et al., 1999b). Considering that nematodes are estimated to have diverged about 550 million years ago (Vanfleteren et al., 1994), the sequence conservation of AF1 is remarkable.

A major task is now to determine the role that these peptides play in the overall biology of *A. suum*. Because RFamide immunoreactivity is located in the nervous system of *A. suum*, we conclude that the FLPs are neuropeptides (Cowden et al., 1993). To understand their function in the nervous system, it is necessary to identify both the cells that contain and presumably release the peptide, and the cells that respond physiologically to the peptide. We have previously addressed the sites and mode of action of AF1, showing that it has dramatic effects on the inhibitory motor-neurons in the nerve cords by decreasing their input resistance, thus disrupting their ability to conduct electrical signals (Cowden et al., 1989). This study addresses the cellular distribution of AF1 by generating an antibody with an excep-

tionally high specificity to AF1. This reagent allowed us 1) to trace the processes of the AF1-containing neurons and thereby identify them definitively with homologous neurons in *C. elegans* and 2) to demonstrate that the cellular pattern of expression of AF1 differs in *C. elegans* and *A. suum*. In addition, two other anti-AF1 antibodies with broader cross-reactivity enabled us to trace the processes of additional neurons and to identify them as morphological homologs of *C. elegans* neurons.

MATERIALS AND METHODS

Animals

Female *A. suum* were obtained from the small intestines of pigs at local slaughterhouses. They were transported and maintained at 37°C in phosphate-buffered saline (PBS: 8.5 mM sodium phosphate, 150 mM sodium chloride, pH 7.4).

BALB/c mice were obtained from the laboratory of Dr. Robert Auerbach, Department of Zoology, University of Wisconsin–Madison. Mice were housed at 25°C with a 12-hour light/dark cycle and free access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison.

Peptides and reagents

FMRFamide, bovine serum albumin (BSA), ovalbumin (OA), and 3,3'-diaminobenzidine 4HCl (DAB) were purchased from Sigma (St. Louis, MO). Goat anti-mouse IgG H&L horseradish peroxidase conjugate was purchased from Bio-Rad (Hercules, CA).

Peptides AF1, AF2, and AF3 were synthesized by the Biotechnology Center, University of Wisconsin–Madison, via tBOC chemistry. The remaining AF peptides were synthesized on an Applied Biosystems 432 Peptide Synthesizer via Fmoc chemistry. Purity was checked by analytical HPLC and MALDI-TOF mass spectrometry or by paper ionophoresis. Only in the case of AF1 synthesized by tBOC chemistry was more than one product detected. In this case, AF1 was purified from the contaminant (identified by sequence analysis as NEFIRFa, N-terminally truncated AF1) by semipreparative gradient reverse-phase HPLC on a C18 column. Subsequent synthesis of AF1 by Fmoc chemistry gave a single product, AF1.

Peptide conjugation

AF1 and FMRFamide were conjugated to BSA or OA for immunization and ELISA screening. The peptide (0.5 mg AF1 or 0.25 mg FMRFamide) was dissolved in 50 µl 10 mM HCl and added to 20 mg BSA or OA dissolved in 100 µl water, followed by 250 µl 0.2 M phosphate buffer, pH 7.4, and 100 µl 10% freshly prepared formaldehyde from paraformaldehyde. After reaction at room temperature

overnight, the conjugates were dialyzed extensively against distilled water at 4°C.

Production of monoclonal antibodies

Immunization of three BALB/c mice was performed as described by Sithigorngul et al. (1989), except that the primary immunization was with FMRFamide-OA. After immunosuppression, the mice were injected intraperitoneally alternately with AF1-BSA and AF1-OA at 3-week intervals. After the fourth injection of AF1-conjugate, a blood sample was taken to determine the titer of serum antibody against AF1 by ELISA and to detect specific antibodies by immunocytochemistry on whole-mount preparations of *A. suum* heads. Only one of the three mice was taken for fusion, because all showed the presence of anti-AF1 antibodies. A second group of three immunosuppressed mice was then injected alternately with BSA or OA conjugates of FMRFamide. ELISA tests showed that all of the mice had generated anti-FMRFamide antibodies; only one was used for fusion.

Spleen cells from immunized mice were fused with NS1 myeloma cells as previously described (Sithigorngul et al., 1989). The products of each fusion were plated in 23 96-well microculture plates and yielded over 2,000 clones. After 10 days, the wells were screened by ELISA against BSA, FMRFamide-BSA, and AF1-BSA. Those clones that recognized AF1-BSA but not FMRFamide-BSA or BSA alone were then screened by whole-mount immunocytochemistry of *A. suum* heads to determine which clones recognized different subsets of neurons. Selected hybridomas were cloned at least twice by the limiting dilution method with syngeneic (BALB/c) mouse red blood cells as feeder cells. Hybridoma-conditioned medium was used for subsequent analysis.

ELISA

The antigens (1 µg protein or peptide conjugate in 50 µl PBS/well) were incubated in 96-well Immulon 2 microtiter plates (Dynatech) overnight. Blotto (5% or 0.5% Carnation nonfat dry milk in PBS; Johnson et al., 1984) was used as a blocking solution, antibody diluent, and washing solution. Plates were exposed to culture fluid from hybridomas, and binding of mouse antibody to the plates was detected by the indirect immunoperoxidase method using goat anti-mouse IgG H&L horseradish peroxidase conjugate (GAM-HRP) at 1:1,000; bound HRP was detected with 1 mg/ml o-phenylene diamine, 0.05% hydrogen peroxide, in 0.1 M citrate, pH 4.5, and the optical density at 492/340 nm was measured with a Titertex micro-ELISA reader (for details see Sithigorngul et al., 1989).

Immunocytochemistry

Whole-mount preparations were prepared according to the method of Johnson and Stretton (1987). After fixation with 1% paraformaldehyde in PBS overnight at 4°C, wash-

ing with PBS, and blocking with P1+ (10% fetal calf serum and 1% Triton X-100 in P1; P1 is 0.5% NP40 and 0.1% BSA in PBS), the preparations were incubated in 50–100 µl hybridoma-conditioned medium at 4°C for 36–48 hours with gentle rocking. After washing with P1 and incubating for 36 hours in 50–100 µl GAM-HRP diluted 1:1,000 in P1+, the preparations were again washed, and peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% H₂O₂ in PBS. All incubations with antibodies were carried out in a moist chamber at 4°C. The preparations were then dehydrated in a graded ethanol series, cleared in xylene, and mounted in Permount.

Whole-mount preparations of the pharynx, tail, or the entire worm were made using similar procedures. For convenience, the whole worm was usually cut into 3–5-cm pieces after fixation and washing. Serial sections were prepared and analyzed using methods previously described (Guastella et al., 1991). Immunological controls were performed by preabsorbing the antibody with AF1-BSA conjugate at 50 µg/ml.

Characterization of antibodies

Dot-ELISA of synthetic peptides

Cross-reactivity of monoclonal antibodies to the synthetic peptides FMRFamide, AF1-AF11, AF13-AF39, and AF41 was determined by dot-ELISA as described by Sithigorngul et al. (1991).

Dot-ELISA of HPLC fractions of natural peptides

Whole *A. suum* heads (N = 100) were cut off about 1 cm from the tip, and frozen immediately on dry ice. To collect pharynges (N = 100) a longitudinal cut was made along one lateral line to the lips, the worm was spread open in a dish containing cold PBS, the lips were cut transversely and the pharynx pulled free from other tissues and frozen immediately on dry ice. Peptides were extracted in 25 ml acid methanol (methanol:water:acetic acid 90:9:1) for 4 hours at 4°C from freeze-powdered heads or pharynges. The extract was centrifuged at 20,000g for 30 minutes and the supernatant concentrated by rotary evaporation to about 2 ml, then diluted in 10 ml 0.1% trifluoroacetic acid (TFA). This crude extract was applied to an activated C18 cartridge (Waters), washed with 10 ml 0.1% TFA, and eluted with 2 ml 50% acetonitrile (ACN) in 0.1% TFA.

After concentration of the eluate, reverse-phase HPLC was performed on a Gilson HPLC system with a 25 × 0.46 cm Microsorb-MV C18 column (Rainin) with two linear gradients, the first from 16–32% ACN in 0.1% TFA over 40 minutes and the second from 32–64% ACN in 0.1% TFA over 40 minutes. The flow rate was 1 ml/minute, and fractions were collected every 1 minute. These fractions were dried (Speed Vac, Savant Instrument) and rehydrated in 10

μ l buffer (100 mM sodium phosphate, 50 mM NaCl, 0.1% sodium azide, and 1 mg/ml RIA-grade BSA, pH 7.4). The final content of each fraction contained material from ~ 10 heads/ μ l. Aliquots of 1 μ l from each fraction were spotted onto nitrocellulose paper for dot-ELISA. Immunoreactivity was estimated by comparison of the staining with 1 μ l spots of serial twofold dilutions of AF1 on the same papers. The AF1-immunoreactive fractions were concentrated and rerun on a C8 column eluted with an acetonitrile/TFA gradient. Synthetic AF1 was also run on the same C18 and C8 columns under identical conditions to establish its elution time for comparison with the natural peptide.

Sample preparation for mass spectrometry of pharynx

The head region was cut longitudinally and pinned flat in a Sylgard-lined Petri dish filled with 170 mM ammonium acetate (Berman et al., 2008). The intestine was removed with forceps, and the lips were cut transversely to allow the pharynx to be pulled free from other tissues. The relaxed pharynx has a triangular lumen; the isolated pharynx was cut longitudinally along the three apices of the lumen, giving three slabs with one of the three pharyngeal nerve cords running along the middle of each slab. With a razor blade, these slabs were then bisected transversely to create a posterior and anterior portion. The six pieces from each pharynx were transferred to a Bruker MTP ground stainless-steel target plate, rinsed on target with 0.5 μ l isopropanol (Schwartz et al., 2003), and allowed to dry. The matrix, α -cyano-4-hydroxycinnamic acid (CHCA; Sigma), was rinsed with 5% acetic acid for 1 minute, then dissolved in 100 μ l 50% acetonitrile, vortexed for 90 seconds, and applied via micropipettes.

Acquisition of mass spectra

A Bruker Ultraflex III MALDI-TOF/TOF MS (Bruker Daltonics, Billerica, MA) equipped with a Smartbeam laser, a reflectron, and a LIFT cell was used to obtain MS spectra. Compass v.1.2 software was used to control the instrument. All spectra were obtained from 50 laser shots per acquisition. Mass spectra were obtained in positive ion reflector mode, with an m/z range of 450–4,000 Da. Polypropylene glycol (PPG) 1000 or polyethylene glycol (PEG) 1500 in methanol was used for external calibration. All spectra were analyzed in Bruker Daltonics flexAnalysis 3.0 software. For all MS spectra, masses were assigned automatically by the software.

Anatomical background

The *A. suum* nervous system can be divided into three connected parts (Fig. 1A). 1) In the head, a series of cephalic ganglia surrounds the nerve ring; these include the

ventral ganglion, two lateral ganglia, the dorsal ganglion, the sensory ganglia anterior to the ring, and the retrovesicular ganglion near the anterior end of the ventral nerve cord; 2) longitudinal nerve cords include the major ventral and dorsal nerve cords and eight minor cords (the four sublateral and the two pairs of lateral cords); all of these cords except for the sublateral cords extend from the head to the tail; 3) in the tail, caudal ganglia surround the rectum; these comprise the two lumbar ganglia converging to the tip of the tail in the lateral lines and two medial ganglia, the preanal ganglion in the ventral cord and the dorsal rectal ganglion.

Most of the neuronal cell bodies are clustered in the ganglia in the head; altogether, there are 162 neuronal cell bodies in these cephalic ganglia (Goldschmidt, 1908; Fig. 1A); a further 30 cell bodies are located in a compact set of ganglia in the tail (Fig. 1A), and the pharynx contains 20 neurons. There are also cell bodies of about 72 motor neurons distributed along the ventral nerve cord (Stretton et al., 1978) and seven pairs of neuronal cell bodies distributed along the lateral lines (see below).

RESULTS

Production of monoclonal antibodies

From one mouse immunized with AF1 conjugates, five hybridoma clones were selected. Three clones produced antibodies (AF1-003, AF1-075, and AF1-259) that were specific to AF1 by ELISA, recognizing AF1, but not BSA or FMRFamide-BSA. In immunocytochemical tests, these three antibodies recognized the same subpopulation of neurons, so, among these three, only AF1-003 will be illustrated and discussed.

Antibody AF1-243 was also specific to AF1 by ELISA, but it recognized a larger subpopulation of neurons than AF1-003. Antibody AF1-62 showed cross-reactivity to all -RFamide peptides tested and recognized a large subpopulation of neurons indistinguishable from those stained with an anti-FMRFamide antiserum specific to C-terminal -RFamide (Marder et al., 1987; Sithigorngul et al., 1990; Cowden et al., 1993).

From a different mouse, immunized with FMRFamide conjugates, the FM-23 hybridoma produced an antibody with broad specificity to all -RFamide peptides tested, and with cross-reactivity different from that of AF1-62. No hybridoma producing an antibody highly specific to FMRFamide itself was found.

Antibody specificity

Cross-reactivity with synthetic peptides

AF1-003

When the specificity of the antibodies was tested by dot-ELISA (Table 1, Fig. 2), antibody AF1-003 showed high specificity to AF1 (~ 10 fmole/spot) with cross-reactivity only to very high levels (10–40 pmole/spot) of AF5, AF6, and AF7,

Figure 1. A: Diagrams of head and tail ganglia. At left is shown an intact 30-cm female worm in a locomotory posture; the extent of the pharynx is shown by the shaded area near the tip of the head; insets show the neuronal cell bodies of the head (top; modified from Goldschmidt, 1908) and tail ganglia (bottom) after the worm has been cut longitudinally and flattened after muscle, pharynx, intestine, and gonad have been removed; the lips have been cut away. The dotted line in the tail shows the rectum. B: Diagrams of cell bodies and neurites of paired URX neurons. C: Paired RIP neurons. The diagrams are radial projections and show the major neurites of the featured neurons (varicosities and fine branches are not indicated). The lighter background image is of the head structures as in A. NR, nerve ring; VC, ventral nerve cord; DC, dorsal nerve cord; VG, ventral ganglion; DG, dorsal ganglion; RVG, retrovesicular ganglion; LL, lateral line; LLL, left lateral line; RLL, right lateral line; AC, amphidial commissures; DeC, deirid commissures. Adjacent to the NR, the ganglia between the DC and the lateral lines are the subdorsal ganglia (both anterior and posterior to the NR), and those between the VC and the lateral lines are the subventral ganglia (anterior to the NR).

which share the C-terminal sequence -FIRFamide; there was no detectable cross-reactivity observed at the highest amount (40 pmole/spot) of FMRFamide, nor of peptides AF2-AF4, AF8-AF11, AF13-AF39, or AF41. Four non-AF peptides (TF, TL, TT, and NY), which are also endogenous peptides from *A. suum*, were also tested and did not cross-react. Peptides TT and TL are products of the *afp-13* transcript (Jarecki et al., 2010); peptide NY has been isolated,

and peptide TF is predicted (I. Viola, unpublished) from the *afp-11* transcript. AF1-003 immunoreactivity on dot-ELISA was blocked when antibody AF1-003 was preabsorbed with an AF1-BSA conjugate.

AF1-243, AF1-62, and FM-23

Antibody AF1-243 also showed high specificity to AF1 (Table 1), but it showed greater cross-reactivity to other

TABLE 1.

Cross-Reactivity of Monoclonal Antibodies to Synthetic-RFamide Peptides¹

Peptide	Monoclonal antibodies			
	AF1-003	AF1-243	AF1-62	FM-23
FMRFamide	—	—	640	40
AF1 (KNEFIRFamide)	10	<1	<1	40
AF2 (KHEYLRamide)	—	—	80	40
AF3 (AVPGVLRamide)	—	—	640	20
AF5 (SGKPTFIRamide)	40,000	640	10	40
AF6 (FIRamide)	10,000	640	10	80
AF7 (AGPRFIRamide)	40,000	640	10	40
AF8 (KSAYMRamide)	—	—	640	40
AF9 (GLGPRPLRamide)	—	—	160	40
AF11 (SDIGISEPNFLRamide)	—	—	20	40

¹The numbers represent the least amount of peptide (fmole/spot) that gives a visible signal on nitrocellulose membrane with dot-ELISA. —, No detectable signal at the level of 40 pmole/spot.

peptides with C-terminal -FIRamide (AF5, AF6, and AF7). Antibody AF1-62 also recognized AF1 more specifically than the other AF peptides tested but had an even higher degree of cross-reactivity to AF5, AF6, and AF7; it also bound to other -RFamide peptides to varying degrees. Antibody FM-23 was a broad-specificity antibody that bound to a similar extent to all FMRFamide-like peptides tested. Because these three antibodies were less specific to AF1 than antibody AF1-003, we did not test their cross-reactivities as extensively.

Cross-reactivity with *A. suum* extracts separated by HPLC

Head extract

The peptide-enriched extracts were fractionated by HPLC on a C18 column, and the fractions were assayed by dot-ELISA (Fig. 3A). Antibody AF1-003 detected strong immuno-

reactivity in only two adjacent fractions with elution times corresponding to authentic AF1. When these two fractions were concentrated and rerun on a C8 column with an acetonitrile/TFA gradient, again the immunoreactivity comigrated with synthetic AF1 (not shown).

Antibody AF1-243 detected strong immunoreactivity in the same fractions as AF1-003 but also detected weak immunoreactivity in nearby fractions with elution times similar to those of AF5 and AF7 (Fig. 3A). It also detected very weak immunoreactivity in additional earlier fractions. Antibody AF1-62 detected immunoreactivity in a larger number of fractions than the previous antibodies, with varying intensity (Fig. 3A). Antibody FM-23 showed a strong immunoreactivity with a large number of HPLC fractions distributed across the entire gradient (not shown), which was very similar to the results obtained with an anti-RFamide serum (Cowden et al., 1993).

Pharynx extract

In HPLC fractions of an extract made from pharynxes (Fig. 3B), antibody AF1-003 detected immunoreactivity with the same elution time as synthetic AF1. Antibody AF1-243 gave similar results but also detected low immunoreactivity in later nearby fractions with elution times similar to AF5 and AF7, but not in any fraction with elution times earlier than AF1. The earlier fractions would have included peptide AF6, which was not detectable in pharyngeal extracts. Antibody AF1-62 detected immunoreactivity in the fractions recognized by the more specific antibodies and in several additional fractions; there appeared to be enrichment of some of the more hydrophobic peptides (eluting later) from the pharynx (Fig. 3B). Antibody FM-23 showed a larger number of immunopositive fractions, although fewer than those for the head extract (not shown).

Figure 2. Dot-ELISA of antibody AF1-003 against synthetic endogenous *A. suum* peptides. Aliquots of 5 and 1 pmole of each peptide were spotted with BSA on nitrocellulose paper and cross-linked with glutaraldehyde. After exposure to antibody AF1-003, bound antibody was detected by indirect immunoperoxidase staining with a second antibody. Only AF1 shows detectable immunoreactivity.

Figure 3. Chromatogram of head extract (A; N = 100) and pharynx extract (B; N = 100) separated by gradient reversed-phase HPLC on a C18 column and assayed for immunoreactivity by dot-ELISA. The top trace records the OD at 214 nm. The graphs below represent the immunoreactivity of each fraction after dot-ELISA with monoclonal antibodies AF1-003, AF1-243, and AF1-62. Dot intensity is estimated by comparison with dot-ELISA of a standard twofold dilution series of AF1 and is recorded on a linear scale. The elution times of synthetic AF1, AF5, AF6, and AF7, determined in separate experiments, are shown.

Immunocytochemistry

The *A. suum* nervous system is very similar to that of the small, free-living nematode *C. elegans*. From electron micrographs of serial sections of the whole nervous system, 118 different types of neuron have been recognized among the 302 neurons in *C. elegans* (White et al., 1986). The morphology of the motor neurons in the ventral and dorsal nerve cords is very similar in the two species (Stretton et al., 1978; Johnson and Stretton, 1987), and many other neurons in the head and tail ganglia also have similar morphology in these two nematodes (Sithigorngul et al., 1990, 1996; Guastella et al., 1991; Cowden et al., 1993; Yew et al., 2007). As part of the results reported here, additional neurons have been identified in *A. suum* with morphologies similar to those reported for *C. elegans*; in these cases, we have assigned them the names of their *C. elegans* counterparts (White et al., 1986).

Immunocytochemical staining with different monoclonal antibodies

AF1-003 immunoreactivity

Antibody AF1-003 recognized a very small subset of cells in the head. The strongest immunoreactivity was in

two pairs of cells, one subdorsal pair close to the nerve ring and one pair in the lateral ganglia (Fig. 1B,C; 1 and 2 in Fig. 4A) and in one process in the dorsal nerve cord (Fig. 4B) and another in the ventral nerve cord (5 in Fig. 4A). There is weaker, but consistent, staining of two more pairs in the lateral ganglia (3 and 4 in Fig. 4A). In rare preparations, one or more other neurons were weakly stained. The following description is based on the analysis of immunostained whole mounts of 185 heads, 24 female tails, and 92 pharynges and of three sets of immunostained serial sections.

URX neurons

The two strongly stained subdorsal neurons (Fig. 1B; 1 in Fig. 4A) typically have fusiform cell bodies. Each has a short, stalk-like process joining the cell body to the nerve ring. Examination of immunostained whole mounts in which the ring is kept intact shows that the process bifurcates on entering the ring, sending one branch to the dorsal midline and the other ventrally toward the ventral midline, where it branches into fine terminals with many varicosities (not shown); neither the dorsal nor the ventral process crosses the midline. These features can also be seen in serial sections, which further show that these cell bodies lie in the

Figure 4. Whole-mount preparations of *A. suum* treated with monoclonal antibody AF1-003. **A:** Head. **B:** Dorsal nerve cord. **C:** Tail. **D:** Pharynx. Anterior is to the top. DC, dorsal nerve cord; LC, lateral nerve cord in the lateral line; NR, nerve ring; VC, ventral nerve cord; VG, ventral ganglion; R, rectum. In A–C, numbers indicate individual neurons: 1 = URX; 2 = RIP; 3, 4 = lateral ganglion neurons; 5 = PDA or -B process; 6 = PQR process. In the preparation shown in A, the PDA or -B process in the ventral nerve cord ends in a prominent anterior varicosity. In D, three pairs of cells (labeled 2, 3, and 5) are stained. Scale bar = 100 μ m.

pseudocoelomic cavity, posterior to the nerve ring. In *C. elegans* there are two pairs of subdorsal neurons, URX and CEPD, with cell bodies in the pseudocoelomic cavity posterior to the nerve ring (White et al., 1986). The morphology of the *A. suum* subdorsal neurons corresponds to that of the *C. elegans* URX neurons, except that in *A. suum* we do not see an elongated anterior subdorsal sensory process projecting to the lips. In most preparations, the end of the cell body is extended into a short process, and occasionally there is a tangled fine process that looks as though it had snapped back after breaking under tension; possibly these are the missing sensory processes or fragments of them. If

the *A. suum* URX neurons do have long anterior processes, it is possible that they were broken while being prepared for whole-mount immunocytochemistry, because the processes are not embedded in hypodermis like other processes that project into the lips. In stained serial sections, however, we could trace only short anterior processes emerging from these cells, so this is most likely a morphological difference between the URX neurons of *A. suum* and *C. elegans*.

RIP neurons

In the lateral ganglia, strong immunoreactivity was found in one pair of cells with cell bodies just anterior to

the nerve ring (Fig. 1C; 2 in Fig. 4A); each cell body gives rise to a short, stalk-like process that then enters the nerve ring and crosses in the dorsal ring to the contralateral side to the position of its paired cell body (the two processes from the paired neurons are in close apposition in the ring). It then turns and runs anteriorly along the lateral line toward the lips, at first close to the bundle of processes projecting to the amphid sensillum but later usually projecting more medially and ending in a flattened process, often with short, spiky projections, about halfway between the nerve ring and the tip of the head (Fig. 4A). Darkly stained granules can often be seen in the anterior processes of these cells. In sections, these processes are seen running close to the pharynx, and, toward their anterior ends, they are seen touching its outside wall. Taken together, these morphological features resemble those of the RIP neurons of *C. elegans*, which innervate the pharynx (White et al., 1986).

PQR and PDA or -B neurons

Strong immunoreactivity was found in a single varicose process in the ventral nerve cord (5 in Fig. 4A) and in the dorsal nerve cord (6 in Fig. 4B); each of these two processes projects anteriorly from a cell body in the tail ganglia and usually terminates shortly before reaching the ventral or dorsal ganglion. In sections, these processes extend short extensions to the interface between the neurons in the nerve cords and the ends of muscle arms, where neuromuscular junctions and synapses to other neurons occur (Cowden et al., 1993). The process in the ventral cord was intensely stained throughout its length and could be traced to a cell body situated in the left lumbar ganglion in the tail (5 in Fig. 4C). This cell is equivalent to the PQR neuron in *C. elegans*.

The process in the dorsal cord was intensely stained in the area close to its anterior end (Fig. 4B), but the staining faded as the process ran posteriorly, becoming intermittent and finally fading completely about halfway along the worm. The cell body of this process was in the preanal ganglion (6 in Fig. 4C). The cell body was stained lightly with antibody AF1-003 but was stained more darkly with antibody AF1-243 (6 in Fig. 5C), and the process could be traced through the whole worm. This neuron sends a process via the right lumbar commissure to the dorsal cord and runs toward the head; it is equivalent to PDA or PDB in *C. elegans*.

Amphidial neurons

Lighter but consistent staining occurred in two pairs of neurons in the region of the cluster of neuronal cell bodies that innervate the amphid. One pair (3 in Fig. 4A) is a typical member of the group of amphidial neurons. It has one process that runs anteriorly toward the lips in the

anterior lateral line. Another process joins the bundle that constitutes the amphidial commissure and enters the nerve ring at the ventral ganglion and then runs ipsilaterally to the dorsal ganglion. These neurons are not sufficiently distinct morphologically to assign a *C. elegans* equivalent; there are 12 neurons that share these features (White et al., 1986). In the other pair of more lightly stained neurons (4 in Fig. 4A), each neuron has a process that runs into the nerve ring directly.

Rare preparations showed weak and inconsistent staining of other neurons with antibody AF1-003. These neurons include the asymmetrical neurons RIS and RIR in the ventral ganglion, the pair of RIG neurons in the retrovesicular ganglion, and the RMEV and RMED neurons of the nerve ring. Usually, only one of these inconsistently stained neurons was seen in each exceptional preparation.

Pharyngeal neurons

In the pharynx, antibody AF1-003 showed strong immunoreactivity in three pairs of neurons in the pharyngeal subventral nerve cords, two pairs situated anterior to (2, 3 in Fig. 4D) and one pair situated posterior to the pharyngeal nerve ring (5 in Fig. 4D). The neurons in the subventral cords project almost to the anterior end of the pharynx, where they terminate in a blunt ending. The posterior neurons project to the dorsal nerve cord of the pharynx, and, as they project anteriorly, they give off varicose branches; there are also several varicose processes that originate from the cell bodies of these neurons (Fig. 4D). To confirm that immunoreactivity in the pharynx was due to AF1, we analyzed two dissected pharynges by MS, dividing each pharynx into six pieces as described above. After mounting on the MS target and application of matrix, each piece was analyzed in six separate locations. Although not all spectra contained a peak at m/z 952.5, the peak was seen in at least one spectrum from all pieces analyzed (Fig. 6). The calculated m/z for protonated AF1 is 952.537.

Immunological controls

When the AF1-003 antibody was preabsorbed with an AF1-BSA conjugate, all staining was blocked.

Antibody AF1-243 immunoreactivity

Although the immunoreactivity of the less specific antibodies is peripheral to our original aim of determining the cell-specific expression of AF1, they were useful neuroanatomical tools, in that they allowed for the further identification of *A. suum* neurons morphologically equivalent to their *C. elegans* homologs. Antibody AF1-243 staining was examined in 117 heads, 20 tails, and 91 pharynges.

Figure 5. Whole-mount preparation of *A. suum* treated with monoclonal antibody AF1-243. **A:** Head. **B:** Higher magnification of right lateral ganglion in a different preparation. **C:** Tail. **D:** Pharynx. Abbreviations and cell identities as in Figure 4. 8 = ADL; 12 = RMEV; 13 = RMED; 15 = AVK; 16 = RIS; 17 = RIR; 19 = PHA. In A, the other numbers are given to neurons for which the identification is not definitive. In C, the commissure from PQR (6), which supplies the dorsal nerve cord process extending to the head, is shown. In the pharynx, four pairs of neurons (labeled 2–5) are stained. Scale bar = 100 μ m for A,C,D; 50 μ m for B.

There was strong staining in all neurons recognized by antibody AF1-003 (1–6 in Figs. 5A–C), with additional staining of five pairs of neurons in the lateral ganglia, four pairs and two asymmetric neurons in the ventral ganglion, and one pair of sensory neurons in the subventral and subdorsal ganglia (Fig. 5A,B). We describe only these additional cells.

In the lateral ganglia, there was strong staining of a pair of unidentified monopolar neurons (7 in Fig. 5A,B) which have a large process that runs in the amphidial commissure to the ventral ganglion, then enters the nerve ring and runs ipsilaterally toward the dorsal cord. This

neuronal cell body lacks a process projecting to the amphid. In *C. elegans* there are six pairs of neurons, AIB, AIZ, AVB, RIB, RIC, and RIM, with processes in the amphidial commissure but lacking processes projecting anteriorly to the amphid sensillum.

There was a second pair of strongly stained lateral ganglion neurons (8 in Fig. 5A,B), each with an anterior process that ran to the amphid sensillum in the ventrolateral lip, and another process that entered the nerve ring. In *C. elegans*, the ADL neurons share these morphological characteristics. They are amphidial neurons that are unique in that they do not send a process into the amphidial commissure.

Figure 6. MALDI-TOF mass spectrum of dissected pharynx. A peak with m/z (mass to charge ratio) of 952.5, the calculated m/z of protonated AF1, is present.

On the ventral side of the lateral ganglion posterior to the nerve ring, a pair of unidentified monopolar neurons was stained (9 in Fig. 5A,B), and two more pairs of unidentified, small sensory neurons stained lightly (10, 11 in Fig. 5B). In the nerve ring, the RMEV neurons (12 in Fig. 5A) and the RMED neurons (13 in Fig. 5B) were stained lightly and inconsistently; these neurons were previously identified as GABA immunoreactive (Guastella et al., 1991). In the subventral and subdorsal ganglia, light staining occurred in four sensory neurons (14 in Fig. 5A) along with their supporting cells.

In the ventral ganglion, strong immunoreactivity was seen in the AVK neurons, a pair of large neurons that makes up the most posterior neurons in the ganglion (15 in Fig. 5A). The RIS neuron, the largest and most posterior of the four asymmetric neurons (16 in Fig. 5A), is also strongly stained. This neuron is just anterior to the right AVK neuron, and its stained process can be traced to the nerve ring, which it enters to the right (Yew et al., 2007). Another asymmetric neuron, the RIR neuron (17 in Fig. 5A), is also strongly stained; this neuron sends a fine process to the ventral midline in the nerve ring, where it bifurcates. Light staining was found in three pairs of small, unidentified, monopolar neurons close to the ring (18 in Fig. 5A).

In the lateral lines, this antibody showed light staining in PVD and PVM neurons (Fig. 7). In the tail (Fig. 5C), in addition to the strong staining of the two cells PQR and PDA or -B (5 and 6 in Fig. 5C) recognized by antibody AF1-003, there was strong staining of one pair of neurons (19 in Fig. 5C), the PHA phasmid neurons in the lumbar ganglia. In the pharynx, antibody AF1-243 showed additional staining in a pair of neurons (4 in Fig. 5D) in the sublateral cords close to the pharyngeal nerve ring.

AF1-62 and FM-23 immunoreactivities

Antibody AF1-62 and FM-23 recognized all neurons recognized by the previously described anti-AF1 antibodies to-

gether with many additional neurons in all ganglia in the head, tail, and pharynx (Fig. 8). Extra immunoreactive neurons in the pharynx included several asymmetric cells (7–10

Figure 7. Diagram of the position of neurons (solid circles) in left and right lateral lines. The two vertical lines represent the ventral nerve cord, and the horizontal lines represent the position of dorsoventral commissures as they exit the ventral cord. The positions of some of the morphologically equivalent neurons in the left and the right lateral lines are different. Roman numerals indicate the five repeating units found in the motor nervous system. As indicated by the brackets, there is preparation-to-preparation variation in the location of some of these cell bodies, especially SDQ and the right AVM neuron. The circles at the anterior and posterior ends represent the head and tail ganglia. Vu, vulva.

Figure 8. Whole-mount preparations of *A. suum* treated with AF1-62 antibody. **A:** Head. **B:** Pharynx (focused on subventral nerves). **C:** Tail. **D:** Pharynx (focused on pharyngeal dorsal nerve). Abbreviations as in Figure 4. SDG, subdorsal ganglion; SVG, subventral ganglion. In the pharynx, there are five pairs of stained neurons (2–6 in C) and four unpaired neurons (7 and 8 in C, 9 and 10 in D) Scale bar = 100 μ m.

in Fig. 8B,D). The neurons stained are similar to those stained by rabbit anti-FMRFamide antisera (Cowden et al., 1993). Antibodies more specific to AF1 (AF1-003 and AF1-243) recognize overlapping subsets of neurons that are fully included in those recognized by the broadly specific antibodies AF1-62 and FM-23. These less specific antibodies allowed recognition of the complete set of neurons in the lateral lines, so they are presented here, even though they do not contain detectable AF1 immunoreactivity. The lateral line neurons comprise seven pairs (Fig. 7), and they correspond morphologically to paired neurons BDU, CAN, ALM, SDQ, PVD, and PDE, and the AVM/PVM pair of *C. elegans*. Figure 7 shows the placement of these neurons relative to

the five repeating units (labeled I–V) of the *A. suum* motor nervous system (Stretton et al., 1978).

DISCUSSION

For this study we have generated several monoclonal antibodies that recognize AF1 (KNEFIRFamide) with varying degrees of specificity. One antibody in particular, AF1-003, is highly specific to AF1. When tested by dot-ELISA against synthetic AF peptides, the cross-reactivity to AF1 is at least 1,000 times stronger than to peptides AF5, AF6, and AF7, which share a C-terminal -FIRFamide sequence with AF1. There is no detectable cross-

reactivity with any of the other 35 AF peptides sequenced to date. In whole mounts and sections of *A. suum*, AF1-003 recognizes a very small subset of neurons, identified by their morphological similarity to neurons in *C. elegans*.

Two other, less specific antibodies, AF1-243 and AF1-62, recognize other peptides besides AF1, but to different extents. These antibodies proved to be useful reagents for morphological studies.

Antibody specificity

There are two separate concerns regarding the cross-reactivity of these antibodies; one is with other identified AF peptides, which we can test with dot-ELISA, as described above; the other is with as yet unidentified peptides, which might or might not be sequence related to AF peptides. A large variety of neuropeptides remains to be purified in *A. suum*; some of them are immunologically related to CCK or other known peptides from other organisms (Sithigorngul et al., 1990; Brownlee et al., 1993). In addition, other peptides have been found or predicted in *A. suum* (Smart et al., 1992; McVeigh et al., 2005, 2008), although it is not known whether they are neuropeptides.

To address the issue of cross-reactivity with unknown peptides, crude peptide-enriched extracts of *A. suum* were fractionated by HPLC, and each fraction was assayed by dot-ELISA with each of the antibodies. Antibody AF1-003 detected a single, sharp peak with chromatographic properties identical to those of authentic AF1; the same result was obtained when the immunoreactive peak material was rechromatographed on a different HPLC column. The strict interpretation of the results of this test is that, other than AF1, there are no detectable immunoreactive peptides with different chromatographic properties. This test is more stringent than is usually applied to the determination of cross-reactivity of antibodies.

The second antibody studied, antibody AF1-243, clearly recognizes more than one peptide in HPLC fractions. The highest immunoreactivity coincides with AF1, but there is also immunoreactivity in the flanking fractions where AF5 and AF7 elute. In addition, there is a small peak of more hydrophilic immunoreactivity of unknown nature. The neurons that are recognized by this antibody include all of those recognized by antibody AF1-003. However, a new subset of neurons is also recognized, and we suspect that these cells may contain one or more AF peptides, but not AF1.

Antibody AF1-62 recognizes even more peptides than AF1-243 in the HPLC fractions and, not surprisingly, stains a larger subset of neurons in whole mounts. Both AF1-62 and AF1-243 also recognize the neurons stained by AF1-003. The neurons recognized by the least specific antibody, FM-23 (which was not selected for its specific-

ity for AF1), were indistinguishable from those stained by the well-characterized antibody raised against FMRFamide (Marder et al., 1987; Cowden et al., 1993).

Differential localization

Differential localization of individual AF peptides by immunocytochemistry is not easy, because they are structurally related; they share, in almost all cases, C-terminal -RFamide. Most available antisera made against FMRFamide or other FLPs show a high degree of cross-reactivity to all other FLPs (Marder et al., 1987; Kivipelto et al., 1989). Very recently, in situ hybridization (Nanda and Stretton, 2010) and mass spectrometry (MALDI-TOF MS) of single dissected neuronal cell bodies (Jarecki et al., 2010) have been shown to be valid techniques for peptide localization in identified neurons, although so far they have been applied to a small fraction of the neurons or peptide-encoding transcripts in *A. suum*. However, each of these techniques has limitations that make corroboration by other techniques highly desirable. In MALDI-TOF MS, the problem of ion suppression in mixtures means that the relative heights of peaks might not represent their true relative abundance, even to the point of there being complete absence of peaks for some peptides that are present in the sample. For in situ hybridization, the strength of the staining might not be quantitatively related to abundance of transcript, so it is unclear whether low-level expression is reliably detectable: in addition, the detection of possible alternatively spliced transcripts may require different riboprobes. Furthermore, the relationship between the level of transcript and the level of processed peptide product is not clear.

Given that all three techniques have drawbacks, our aim was to generate better antibodies, each highly specific to a single peptide, and to use them as improved immunocytochemical probes. Although the AF peptides indeed have sequence similarities, they also have differences, and we have been able to exploit these differences to obtain antibodies specific to single peptides.

Compared with the population of neurons stained by broad-specificity anti-RFamide antibodies, the subset of neurons recognized by antibody AF1-003 is small. Because this antibody is so specific, we conclude that these neurons are the major sites where peptide AF1 is expressed. This conclusion is supported by the results of mass spectrometry on dissected ganglia: AF1 has a mass to charge ratio (m/z) of 952.537 in the singly protonated state, and ions with this m/z were detected in the dissected nerve ring and ventral and dorsal nerve cords (Yew et al., 2005), where AF1-003 immunoreactivity was found. Furthermore, mass shifts induced by acetylation of these tissues showed in each case that, as predicted for AF1, the peptide contained two amino groups, an alpha

amino group and an amino group contributed by a lysine residue (Yew et al., 2005).

The pharynx contains three pairs of neurons recognized by antibody AF1-003. As would be expected if this reactivity were due to AF1, there is a single peak of immunoreactivity, with the chromatographic mobility of AF1, in extracts of dissected pharynges. MS of pharynges also chemically confirms the presence of AF1 (Fig. 5). Comparison of HPLC fractions of extracts of whole heads and pharynges by dot-ELISA with antibodies AF1-243 and AF1-62 shows that pharynges contain fewer FLPs than extracts of heads, confirming the results of Cowden et al. (1993).

The single processes that contain AF1 immunoreactivity in the dorsal and ventral nerve cords are the processes that were previously described from immunocytochemical experiments with a broad specificity anti-RFamide antiserum (Cowden et al., 1993). The small number of immunoreactive neurons staining with antibody AF1-003 allowed the cell bodies of these neurons to be identified. The dorsal varicose process, previously identified as D-var (Cowden et al., 1993), originates in a cell in the preanal ganglion and most closely resembles the PDA or PDB neurons of *C. elegans*. In the dorsal nerve cord, the varicosities of D-var usually extend projections to the ends of the muscle arms of dorsal muscle cells and in the dorsal cord make both neuromuscular synapses and synapses to other neurons, including the ventral inhibitory (VI) motoneurons (Cowden et al., 1993). Physiological experiments show that the VI motoneurons have receptors for AF1: intracellular recordings have shown that AF1 opens channels in inhibitory motoneurons, producing a large decrease in input resistance and effectively short-circuiting their electrical activity (Cowden et al., 1989). Furthermore, when injected into the pseudocoelomic cavity, AF1 blocks the propagation of locomotory waves, suggesting that the role of AF1 in the locomotory system of *A. suum* is an important one.

The ventral varicose process that anatomically appears to be the ventral equivalent of D-var arises from the cell body of the PQR neuron in the left lumbar ganglion. Like D-var, it sends projections to the ends of muscle arms at the neuron-muscle interface in the nerve cord. It is interesting that the AQR cell, thought to be in the same class as PQR in *C. elegans* (White et al., 1986), does not contain AF1 immunoreactivity, so in *A. suum* the AQR and PQR neurons probably belong to different functional classes of neurons.

Comparison with *C. elegans*

The fact that AF1 is expressed in so few neurons in *A. suum* allowed us to distinguish their morphology and especially the extent and position of their processes in

TABLE 2.
Comparison of Expression of AF1 in *A. suum* and *C. elegans*

Neuron	<i>A. suum</i>	<i>C. elegans</i> ¹
RIP	+	–
URX	+	+
Amphidial neurons	One pair	ASE
PVM	–	+
PQR	+	–
PDA/B	+	–
Pharynx	Three pairs	–

¹Li et al. (1999b).

the nerve ring. Thus we were able to identify the neurons with their anatomical counterparts in *C. elegans*.

C. elegans expresses AF1 (also named *ce-FLP8*; Sithigorngul, reported in Davis and Stretton, 1996; Li et al., 1999a,b; Li and Kim, 2008), but, as shown in Table 2, we find little overlap between the AF1-immunoreactive neurons in *A. suum* and the neurons that express reporter constructs in which the promoter of the *flp-8* gene, which encodes AF1, was placed upstream of green fluorescent protein (GFP; Li et al., 1999b). Unlike the RIP neurons of *A. suum*, the RIP neurons in *C. elegans* do not express AF1 (Li et al., 1999b). The *A. suum* RIP neurons end in close proximity to the pharynx and contain dark immunoreactive granules. We presume that these endings are sites of release of AF1 and that neurally released AF1 has important effects on pharyngeal activity: exogenously applied AF1 causes a potent inhibition of the *A. suum* pharynx (Brownlee and Walker, 1999; R.E. Davis, personal communication).

In addition to the differences in the RIP neurons, there are other striking differences in the neurons expressing AF1 in these two nematodes (Table 2). The ventral and dorsal nerve cord processes of the PQR and PDA or -B neurons, in which there is strong AF1 immunoreactivity in *A. suum*, show no such expression in *C. elegans*. Similarly, there is no expression of AF1 in the pharynx of *C. elegans*, whereas *A. suum* has three pairs of AF1-immunoreactive neurons.

The URX neurons of both nematodes express AF1, and one of the *A. suum* amphidial neurons that expresses AF1 might be equivalent to ASE in *C. elegans*, where expression of the *flp-8* gene is seen (at present we cannot distinguish the 12 amphidial neurons of *A. suum* individually). Thus, although there is some overlap in the cellular expression of AF1, the differences are more striking than the similarities. Although it is not always certain that the GFP constructs used for determining cellular expression in *C. elegans* include all the sequences that are normally used to control gene expression (Kim and Li, 2004), Li and Kim (2008) report that antibody AF1-003 stains the same neurons that express the *flp-8*-GFP construct, confirming the cellular expression of the *flp-8* gene in *C. elegans*.

In addition, there may be species differences in expression during development. Indeed, there appears to be no detectable *flp-8* transcript beyond the L1 larval stage in *C. elegans* (Li et al., 1999b), although AF1 immunoreactivity is present in adults (Li et al., 1999a).

We have previously shown comparable differences in the cellular expression patterns of GABA, AF2, and AF8, which are chemically identical in *A. suum* and *C. elegans*, as well as for several other families of peptides that are chemically closely related in the two species (Guastella et al., 1991; Nanda and Stretton, 2010; Jarecki et al., 2010). The morphological and chemical homology between these two species is not matched by homology in the cellular expression.

Diversity of AF peptides

Why are there so many AF peptides? So far, 40 AF peptides have been isolated and sequenced, and there are several additional peaks of RFamide immunoreactivity that have not yet been resolved into pure peptides. Analysis of the *C. elegans* genome has shown the existence of 31 genes that encode precursor proteins with predicted cleavage sites giving rise to 71 putative peptides with C-terminal RFamide (Li et al., 1999b; Li and Kim, 2008).

It has been suggested previously (Stretton et al., 1991) that one reason for the existence of so many neuropeptides in nematodes may be to allow differences in behavior to be produced by different species. Structurally, the nervous systems of nematodes appear to be very conservative: in *A. suum* and *C. elegans*, the same individual cells can be recognized readily from their morphology. To produce different behaviors, appropriate to different ecological niches, in species in which the basic structure of the nervous system is essentially the same, nematodes may have diversified the intercellular signaling mechanisms by varying the cellular expression patterns of neuropeptides and/or their receptors. The differences that we observe in cellular expression of AF1 support this idea.

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