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Identified *Helix* Neurons: Mutually Exclusive Expression of the Tetrapeptide and Heptapeptide Members of the FMRFamide Family

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Abstract. Extracts were prepared from selected neurons taken from the central ganglia of the pulmonate snail *Helix aspersa,* and parallel radioimmunoassay (RIA) and high pressure liquid chromatography (HPLC) were used to analyze for FMRFamide-related peptides (FaRPs). Some neurons (*e.g.,* the cerebral C3 neuron) contained only the tetrapeptides related to FMRFamide (tetra-FaRPs), whereas two clusters of neurons in the parietal ganglia contain a preponderance of the hepta-FaRPs. Using cloned cDNA probes in molecular hybridization studies, we showed that the C3 neuron (and some other large neurons) contain the mRNA species that encodes the tetra-FaRPs. In contrast, the neurons of the parietal clusters predominantly express the mRNA that encodes the hepta-FaRPs. These results were confirmed by *in situ* hybridization with antisense RNA on whole mount ganglia. Each type of experiment supports the view that FaRP-containing *Helix* neurons express either the tetra-FaRPs *or* the hepta-FaRPs, but not both.

Introduction

The ganglia of molluscs contain several pharmacologically active peptides related to FMRFamide. These peptides have been referred to as the FaRPs and include FLRFamide, FMRFamide itself, and several N-terminally extended forms, most of which are heptapeptides (Price and Greenberg, 1989). In *Helix aspersa,* the hepta-FaRPs are: pQDPFLRFamide, NDPFLRFamide, SDPFLRFamide, NDPYLRFamide, and SEPYLRFamide (Price *et al.,* 1990). Analyses of cDNAs from *H. aspersa* suggest that one species of mRNA encodes both of the tetra-FaRPs (*i.e.,* FMRFamide and FLRFamide), whereas another encodes all of the hepta-FaRPs together with the base sequence for another peptide, pQDPFLRIamide (Lutz *et al.,* 1990; Lutz *et al.,* 1992). Evidence that this latter peptide occurs in *H. aspersa* ganglia had previously been obtained by fast atom bombardment mass spectroscopy (see Bradley, 1982), and it has recently been sequenced (Lesser and Greenberg, pers. comm.).

FaRPs have potent pharmacological actions on peripheral and cardiac muscle cells and neurons. Often considerable differences in potencies are observed between the tetra- and the hepta-FaRPs; in some cases tetra-FaRPs produce opposing actions to the hepta-FaRPs (*e.g.,* Cottrell and Davies, 1987; Lehman and Greenberg, 1987). Such observations have led to the suggestion that there is more than one type of FaRP receptor in molluscan species.

Immunohistochemical studies with different antisera prepared against FMRFamide and related peptides provide evidence for an exclusively neuronal localization of FaRPs (Boer *et al.,* 1980; Schot and Boer, 1982). But we cannot always assume that such antisera stain only FaRPs. A better way to establish the presence of FaRPs in a particular neuron is to analyze extracts of the isolated neuron by RIA. The use of more than one antiserum can be useful in helping to distinguish the different related peptides. Even more information can be gained by subjecting extracts to high pressure liquid chromatography (HPLC), provided that sufficient material is available.

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Immunohistochemistry of *H. aspersa* ganglia has shown that many cells possess FaRP-like immunoreactivity. One such neuron in each cerebral ganglion, the C3 neuron, contains an equivalent of 300 fmoles of FMRFamide (Cottrell *et al.*, 1982). Moreover, a parallel RIA procedure with two antisera revealed that neuron C3 is rich in FMRFamide itself, but contains none of the N-terminally extended forms (Bewick *et al.*, 1990). These observations are in accord with molecular genetics studies suggesting that, in *H. aspersa*, the tetra-FaRPs and hepta-FaRPs are encoded on different species of mRNA (Lutz *et al.*, 1990, 1992).

We wondered whether the tetra-FaRPs and hepta-FaRPs would generally be expressed in different cells, and therefore decided to reinvestigate the distribution of FaRP-containing neurons in *H. aspersa*. Unfortunately, no detailed map of such neurons has been made for this species. Recently, however, Elekes and Nässel (1990) used a commercially supplied FMRFamide antiserum to make a detailed immunohistochemical study of the ganglia of *Helix pomatia*. This species is closely related to *H. aspersa*. For example, Lutz *et al.* (1992) found that the tetra-FaRP precursors are 95% identical at the nucleotide level. Moreover, the ganglia of *H. aspersa* are very similar to those of *H. pomatia*, large neurons and clusters having identical locations in each species. For example, Elekes and Nässel (1990) detected FaRP-immunoreactivity in the *H. pomatia* C3 neuron and in clusters of smaller neurons in the parietal ganglia, which has also been observed in *H. aspersa* (Cottrell *et al.*, 1988). We began this study by assuming that neurons located similarly in the two species contain the same peptides and have therefore used the detailed information provided for *H. pomatia* to investigate *H. aspersa*. We have also undertaken some experiments to investigate the mRNA species that encode the different FaRPs in some of the identified neurons.

Materials and Methods

Specimens of *H. aspersa*, kindly supplied by Dr. R. Koch (Fullerton, California) were used for the peptide analysis experiments. *H. aspersa* collected in Scotland in the locality of St. Andrews were used for the mRNA hybridization experiments.

Radioimmune assays

Individual specified neurons or small cluster of neurons were dissected from desheathed ganglia and transferred to small Eppendorf tubes containing 50 or 100 μl of acetone. The acetone containing the cells was left at −20°C until assayed, but for at least 16 h. The cellular debris was then spun down, the supernatant removed to a clean tube and dried in a Speed-Vac. The residue was resuspended in RIA buffer (100 μl) for immediate assay or in 0.1% TFA for HPLC fractionation. HPLC fractionations were done as previously described (Price *et al.*, 1990) on a Brownlee Aquapore Octyl column (2.1 × 220 mm), and 0.5 min (about 250 μl) fractions were collected. Aliquots (5 μl) were used directly in the RIA; alternatively the fractions were dried in a Speed-Vac, resuspended in RIA buffer, and 25 μl taken for assay. The radioimmunoassays were performed as described previously (Lehman and Price, 1987), using antiserum S253 at a 1/25,000 final dilution, or antiserum Q2 at a 1/600 final dilution. An antiserum developed against EFLRIamide was also used in some experiments at a final concentration of 1/25,000.

Hybridization analysis of mRNA in different neuronal cell types

Individual neurons or small clusters of neurons were dissected from isolated desheathed ganglia, placed in microcentrifuge tubes containing 0.25 ml of lysis buffer [50 m*M* Tris, pH 8.0, 10 m*M* EDTA, pH 8.0, 0.5% sodium dodecyl sulphate (SDS) and 0.5 mg/ml proteinase K] and incubated at room temperature (about 22°C) for 4 h. The entire tube contents were then loaded onto nitrocellulose filters that had been inserted in a slot-blot apparatus. The filters were air-dried and baked for 2 h at 80°C in a vacuum oven. The filters were hybridized, according to standard techniques (Maniatis *et al.*, 1982), to ^{32}P random-primer labeled DNA probes prepared from either the tetra-FaRP cDNA or hepta-FaRP cDNA isolated from *H. aspersa* (Lutz *et al.*, 1992). After hybridization, the filters were washed stringently (final wash: 0.25 × SSC, 0.1% SDS, 65°C, 2 h [1X SSC contains 0.15 *M* sodium chloride, 0.015 *M* trisodium citrate]), and the bands were visualized by autoradiography; 'Agfa Curix' film was used and exposed for four days at −70°C.

Hybridization to mRNA in situ

Two sets of RNA probes were synthesized: those complimentary to mRNA sequences encoding tetra-FaRPs, and those complementary to mRNA sequences encoding hepta-FaRPs. These antisense probes were transcribed from cDNA cloned in BlueScript plasmids (Lutz *et al.*, 1992) using T7 RNA polymerase as described previously (Kreig and Melton, 1987). The probes were labeled with digoxigenin-11-uridine-5′-triphosphate as a component of the synthesis reaction, as recommended (Boehringer-Mannheim data sheet).

Whole mounts of the cerebral ganglia, and also the suboesophageal ganglia from which the pedal ganglia had been removed, were prepared for *in situ* hybridization according to the method of Tautz and Pfeifle (1989). The preparations were fixed for 20 min, hybridized with the antisense RNA (5 ng/ml in 4 × SSC for 18 h at 37°C), washed for 30 min, and finally stained with FITC-labeled

antibody to digoxigenin (Boehringer-Mannheim) for 2 h. The antibody was diluted 1 in 100 in phosphate buffered saline containing 0.05% Tween 20 (PBS/T) plus 2% bovine serum albumin. The preparations were then washed extensively in PBS/T and mounted in glycerol/PBS (9:1) for viewing on a scanning confocal-laser microscope (Bio-Rad MRC 600). N-propyl gallate (Sigma) was added to the PBS (2.5 g/10 ml) as an antifade agent.

Results

Survey of Single and Clusters of Immunoreactive Neurons detected Histochemically

Refer to Figure 1 for locations of neurons used and Table I for a summary of the results.

Cerebral ganglia

As a test of the dissection and RIA procedures, experiments were first made on the C1 neuron, which contains 5-hydroxytryptamine but does not stain positively with FMRFamide antisera. The level of immunoreactivity in this neuron was lower than the limit of detection, *i.e.*, less than 0.01 pmole/cell (see Table I). This result suggests that general contamination of samples during cell isolation did not pose a problem.

C3 neuron. Previous histochemical and RIA data suggest that this neuron contains one or more FaRPs (Cottrell *et al.*, 1982), and most probably only FMRFamide and FLRFamide (Bewick *et al.*, 1990). Assays of single C3 cells in the present study indicate levels of FMRFamide equivalent to about 0.2 pmole/cell.

Right mesocerebral cluster. The experiments of Elekes and Nässel (1990) showed that a mass of cells in the mesocerebral area of the right cerebral ganglion were stained with the FMRFamide antiserum. Because they are so abundant, we subjected extracts of these cells to HPLC before RIA (see data below).

Pleural ganglia

Neurons equivalent to LPL1 and RPL1. Two large cells, which have the same location as LPL1 and RPL1 detected immunocytochemically in *H. pomatia* by Elekes and Nässel (1990), were isolated from *H. aspersa* pleural ganglia. The level of immunoreactivity assessed by RIA in these *H. aspersa* neurons was low (0.02; 0.08 pmole). When levels in large cells (*i.e.*, those greater than 160 μ in diameter) are less than 0.1 pmole, they are considered low in contrast to the 0.2 pmole detected in the C3 neuron. The C3 neuron has a diameter of about 80 μ, and its volume is therefore only about 1/8th of that of the larger cells. Whether the low levels detected in the large pleural neurons represent contamination from adhering neuropil, or low cellular content of peptide, was not investigated further.

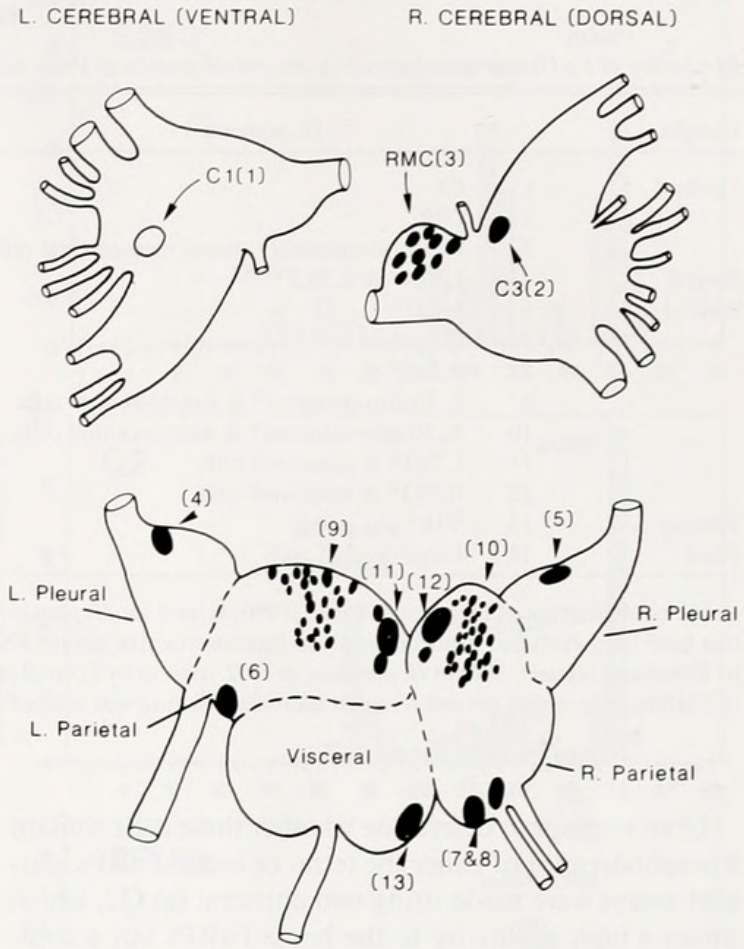

Figure 1. The locations of the neurons taken for RIA from the cerebral, pleural, parietal, and visceral ganglia. The ventral surface of the left cerebral ganglion is shown to indicate the location of the C1 neuron, whereas all of the other ganglia are viewed from the dorsal aspect. The designations that have been given to these different neurons, numbered in brackets, for both *H. pomatia* and *H. aspersa*, are listed in Table I. Immunohistochemical evidence suggests that those neurons marked in black may contain FaRPs (see text).

Parietal ganglia

Neurons equivalent to LPa1, RPa1, and RPa2. The neurons LPa1, RPa1, and RPa2 in *H. pomatia* are all immunoreactive to FaRP-antisera (Elekes and Nässel, 1990), but extracts prepared from neurons in the same locations in *H. aspersa* did not contain appreciable levels of FaRP when tested by RIA (see Table I).

Left and right parietal FaRP-clusters. Histochemical studies in both *H. pomatia* and *H. aspersa* suggest that there are relatively large numbers of small to medium sized FaRP-immunoreactive neurons located rostro-marginally and rostro-centrally in both parietal ganglia. Clusters of cells from these regions were cut out en masse. As shown in Table I, RIA with S253 antiserum provides evidence that such clusters are rich in FMRFamide or related peptides.

Table I

RIA survey of Fa-IR neurons observed in the central ganglia of Helix aspersa *or* Helix pomatia

Ganglia		*H. pomatia*	*H. aspersa*	Content with S253
Cerebral	1	C1	C1	<0.01; <0.01
	2	C3*	C3*	0.20; 0.25
	3	R. Dorso-marginal* rostral mesocerebral cells	R. mesocerebral cells	See Fig. 1
Pleural	4&5	L.PL1* & R.PL1*	Large cells in identical position	0.08; 0.02
Parietal	6	L.Pa1*	D5	0.02; 0.04
	7	R.Pa1*	F1 or F2	<0.01 (2); <0.01
	8	R.Pa2*	F2 or F3	
	9	L. Rostro-marginal* & Rostro-central cells	L. Parietal* Fa-cluster	15.9 (50), 10.8 (50)
	10	R. Rostro-marginal* & Rostro-central cells	R. Parietal* Fa-cluster	9.8 (40), 16.6 (40)
	11	L.Pa3* & associated cells	D1, D2 & D2a	1.22 (2); 0.6 (3)
	12	R.Pa3* & associated cells	F76, F77 & F78	0.26 (2); 0.3 (2)
Visceral	13	V16*	E2 or E4	<0.01 (3)
Pedal	14	Large caudal* cells	Neurons in same location	<0.01; <0.01

The nomenclature of Elekes and Nässel (1990) is used for *H. pomatia* neurons and that of Kerkut *et al.* (1975) for *H. aspersa* neurons. The cells that have been identified histochemically as immunoreactive against FMRFamide antisera (Fa-IR) are marked with an asterisk; they were observed by Elekes and Nässel (1990) in *H. pomatia*, or in *H. aspersa* by Cottrell *et al.* (1982) or Cottrell *et al.* (1988). The values are given in pmole equivalents of FMRFamide, either per cell when an individual neuron was assayed alone, or for a number of cells (number in brackets) assayed together.

In an attempt to determine whether these cells contain a preponderance of either the tetra- or hepta-FaRPs, parallel assays were made using two antisera: (a) Q2, which shows a high sensitivity to the hepta-FaRPs but a comparatively low sensitivity to FMRFamide itself; and (b) S253, with which FMRFamide and the hepta-FaRPs are approximately equipotent (see Bewick *et al.*, 1990, for exact values). With this assay system, extracts rich in FMRFamide should appear to have a higher FaRP-content when tested with S253 than with Q2. On the other hand, extracts rich in the hepta-FaRPs should yield about the same quantitative result with either assay (*i.e.*, the ratio of the two values should be close to 1). The results of two such experiments yielded the following ratios: 0.92 (2.93 pmole/3.11 pmole) and 0.59 (9.28 pmole/15.70 pmole) for the left parietal FaRP-cluster; and 1.03 (16.50 pmole/15.9 pmole) and 0.94 (10.2 pmole/10.81 pmole) for the right parietal FaRP-cluster.

These data suggest that both the left and right parietal clusters are rich in the hepta-FaRPs, an observation substantiated for the right FaRP-parietal cluster by HPLC and for both the left and right clusters by *in situ* hybridization (see below).

Large rostro-medial cells. Elekes and Nässel (1990) provided histochemical evidence that these neurons (LPa3 and RPa3) in *H. pomatia* contain FaRPs. RIAs with antiserum S253 indicated that neurons located in the same position in *H. aspersa* also contain FaRPs (Table I). Several other assays were undertaken on these cells, attempts being made to distinguish between the three large neurons that occur in this location in each ganglion in *H. aspersa* (*i.e.*, D1, D2, and D2a; F76, F77, and F78). In spite of repeated dissections, however, these neurons could not be identified individually by appearance alone. Nevertheless, each member of both groups (left and right) was successfully isolated and assayed (Table II). These experiments showed that each of these cells was immunoreactive

Table II

Analyses of the large rostrally located cells of the left and right parietal ganglia in the position of LPa3 and RPa3 of H. pomatia

	D1, D2, and D2a		F76, F77, and F78	
A	i	0.34	i	0.08
	ii	0.28	ii	0.16
			iii	0.26
B	i	0.24	i	0.2
			ii	0.1
			iii	0.08
C	i	0.32	i	0.12
D	i	0.14	i	0.14
	ii	0.26	ii	0.46
	iii	0.3	iii	0.22
E	i	0.38	i	0.3
	ii	0.26	ii	0.1
	iii	0.42	iii	0.46
Avs		0.29		0.206
(min = 0.14, max = 0.42)			(min = 0.08, max = 0.46)	

Three large cells could usually be observed at each location (D1, D2, and D2a of the left, and F76, F77, and F78 of the right parietal ganglion of *H. aspersa*), but they could not be reliably distinguished by appearance. Individual cells were dissected from five preparations (A to E), but only in preparations D and E were all of the separate cells successfully isolated and assayed. The results suggest that most if not all of these large neurons contain FaRPs. Values are given in pmole equivalents of FMRFamide per cell.

to antiserum S253 and that, of all the *single* cells assayed in the different ganglia, they had the highest level of immunoreactivity.

Parallel RIAs were made on many single cell extracts prepared from individual neurons in this rostro-medial location. Of 26 cell extracts, 21 had Q2/S253 ratios ranging from 0.05 to 0.35; 3 had values in the range of 0.36 to 0.86; and the remaining two had values of 1.0 and 1.7. These data suggest that most of the large cells in this region contain FMRFamide, but not appreciable levels of the hepta-FaRPs. This conclusion was substantiated by HPLC and also by analysis of the mRNA in these large cells (see below). The significance of the finding that a few cell extracts yielded Q2/S253 ratios close to 1 is not clear. It could have resulted from contamination by some of the surrounding parietal cluster neurons, from inaccurate assays, or possibly from the existence, with the region, of an unidentified heptapeptide-containing neuron. Estimates of the FMRFamide content of all the cells analyzed in this location, based on assays using the S253 antiserum, varied from about 0.02 to 0.2 pmole per cell.

Visceral and pedal ganglia

Large visceral and the large caudal pedal neurons. Histochemical evidence in *H. pomatia* obtained by Elekes and Nässel (1990) suggests that specified neurons in the visceral and pedal ganglia may contain FaRPs. RIAs of individual neurons dissected from the same locations in *H. aspersa* ganglia, however, indicated that these cells do not contain FaRPs (see Table I).

RIA of Extracts Prepared by High Pressure Liquid Chromatography

Right parietal FaRP-cluster

Extracts of these cells yielded large peaks with S253 that correspond to the heptapeptides pQDPFLRFamide, NDPFLRFamide, and SDPFLRFamide (see Fig. 2). There was also a small peak corresponding to FLRFamide and an even smaller one in the position of FMRFamide. The presence of the heptapeptides was confirmed by assays of the fractions with the Q2 antiserum, which also detected a strong peak corresponding to the NDPYLRFa and SEPYLRFamide heptapeptides that were not detected with the S253 antiserum. Analysis of aliquots of the same fractions with yet another antiserum developed against EFLRIamide showed that the right parietal FaRP-cluster also contains pQDPFLRIamide which—together with all the above heptapeptides—is also encoded on the *H. aspersa* mRNA (see Lutz *et al.*, 1992).

These data therefore confirm those from the parallel RIA and support the notion that the neurons of the right FaRP-cluster are rich in the hepta-FaRPs. The ratio of each of the hepta-FaRPs to FMRFamide in the extracts

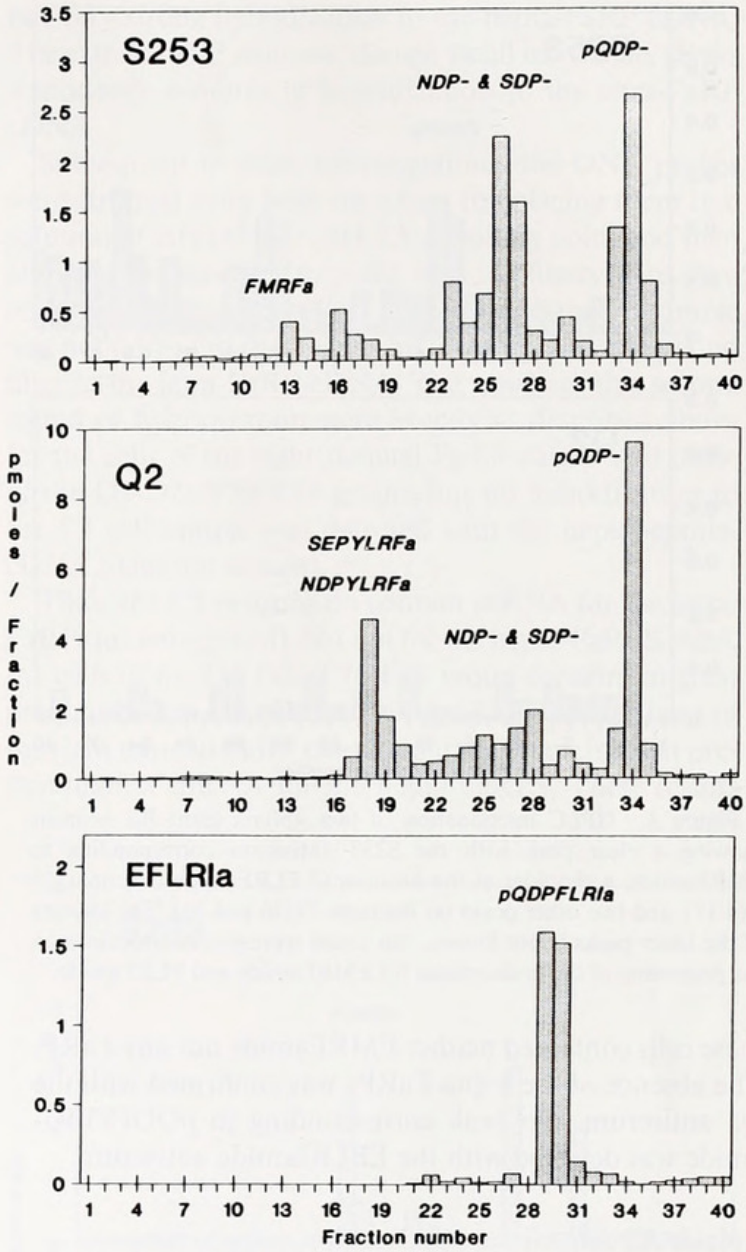

Figure 2. HPLC fractionation of an extract prepared from the parietal cluster of neurons. The fractions were assayed with three antisera: (a) S253, which reacts equally well with both the tetra- and hepta-FaRPs (excluding SEPYLRFamide and NDPYLRFamide); (b) Q2, which is most sensitive to the hepta-FaRPs; and (c) anti-EFLRIa, which selectively reacts with pQDPFLRIamide. For NDP-, SDP-, and pQDP-, the C-terminal is -FLRFa; a = amide. The hepta-FaRPs predominate in the parietal cluster neurons. There is, however, a small peak at the location of FMRFamide; this probably represents some contamination of the sample.

was about 10 to 1. Whether any of the neurons enclosed within the dissected cluster contained any authentic FMRFamide is questionable, because some neuropil, which is rich in FMRFamide, would probably have been included in the sample of cells that were removed en masse.

Right mesocerebral cell cluster

Analysis of HPLC fractions with the more selective S253 antiserum indicated, against our expectations, that

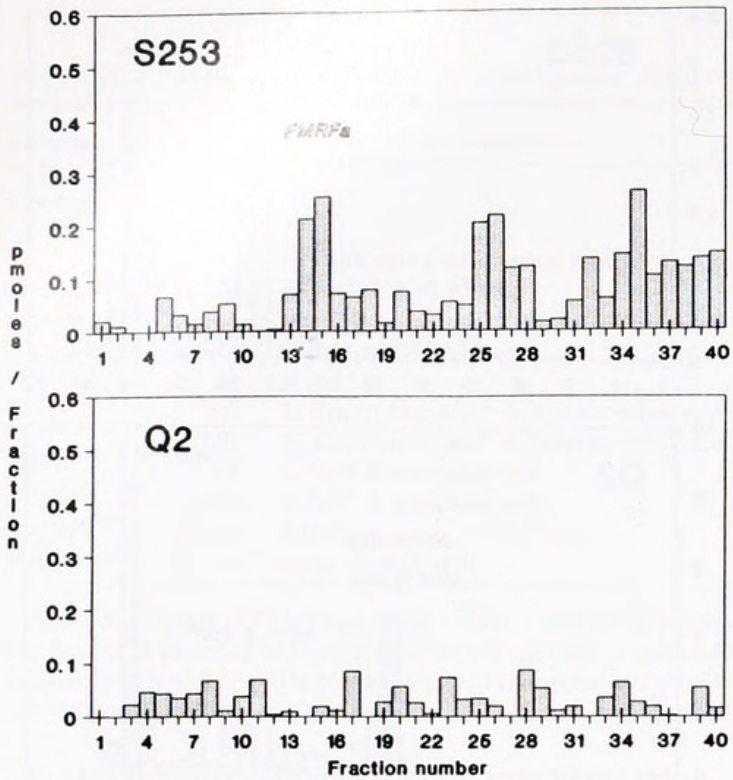

Figure 3. HPLC fractionation of two *Aplysia* giant R2 neurons showing a clear peak with the S253 antiserum corresponding to FMRFamide, a shoulder at the location of FLRFamide (fractions 16 and 17), and two other peaks (at fractions 25/26 and 34). The identity of the latter peaks is not known, but could represent intermediates in the processing of the prohormone for FMRFamide and FLRFamide.

these cells contained neither FMRFamide nor any FaRP. The absence of the hepta-FaRPs was confirmed with the Q2 antiserum. No peak corresponding to pQDPFLRIamide was detected with the EFLRIamide antiserum.

Aplysia *R2 cell*

An extract of two of the large R2 *Aplysia* neurons known to contain FMRFamide (Schaeffer *et al.*, 1985) was fractionated and assayed with the S253 and Q2 antisera (Fig. 3). Available evidence indicates that *Aplysia* ganglia do not contain hepta-FaRPs. With S253, the major peak corresponded to FMRFamide with a shoulder corresponding to FLRFamide in fractions 16 and 17 predicted from what is known of the structure of the precursor peptide. Unexpectedly, some other peaks corresponding to higher molecular weight peptides, as yet unidentified, were also observed with S253. These peptides were not detected when the Q2 antiserum was used. Further, there was no peak corresponding to the position of pQDPFLRIamide.

Cerebral C3 neurons

The C3 neurons are much smaller than the *Aplysia* R2 neurons and more were required for HPLC. Eight cells were isolated over a period of 19 days. Fractionation of the extract of these cells and RIA with S253 showed several peaks. The first corresponded to oxidized FMRFamide (Fig. 4). The presence of the oxidized form of the peptide was probably related to the relatively long period taken to amass sufficient cells. Oxidized FMRFamide is only

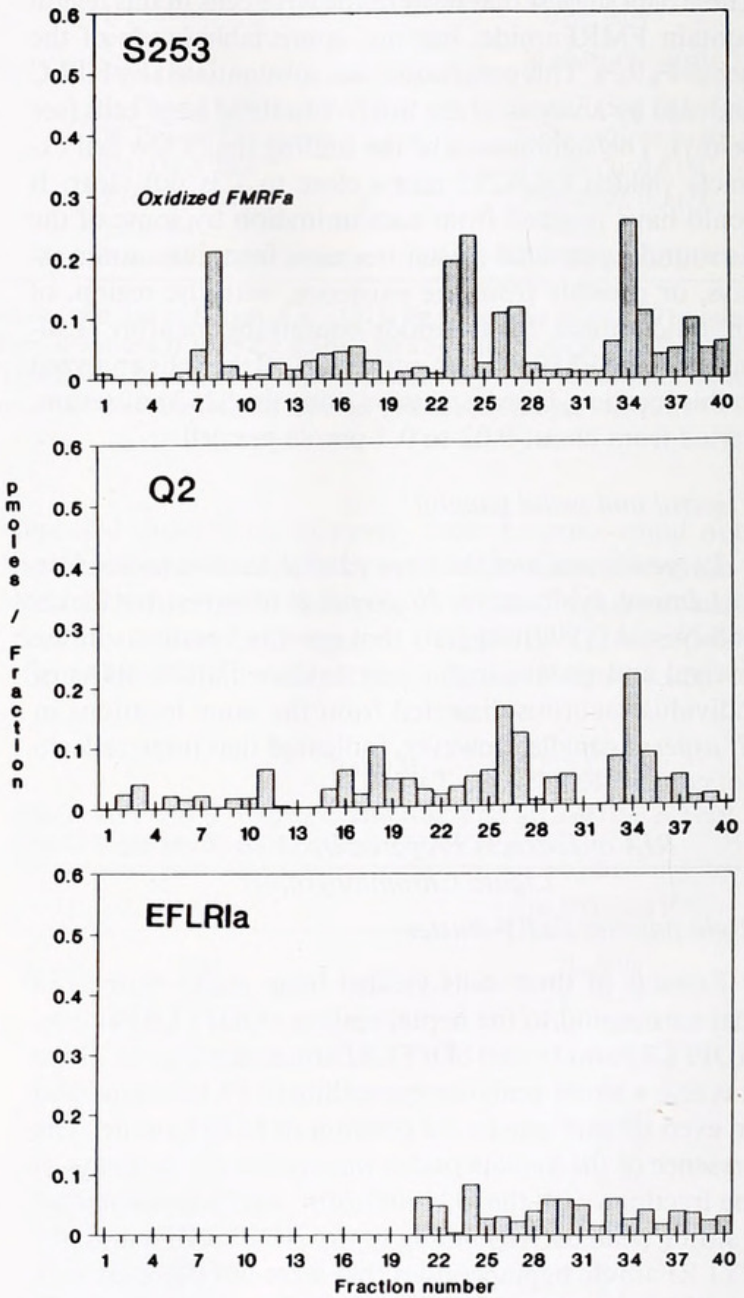

Figure 4. HPLC fractionation of an extract of eight C3 neurons. The C3 neuron is only about 100 μ in diameter and occurs singly in each cerebral ganglion. Several are required to provide sufficient material to detect the peptides by HPLC. The peak at fraction 8, with S253 antiserum, corresponds to oxidized FMRFamide. As S253 reacts about four times less intensely with oxidized FMRFamide that with FMRFamide itself, FMRFamide must be the most abundant FaRP in the extract. A small peak at fractions 16 and 17 corresponds to FLRFamide. Other peaks corresponding to higher molecular weight peptides are also seen; as with the *Aplysia* R2 neuron (Fig. 3). These unknown peptides could also be processing intermediates.

about one quarter as reactive with S253 as the unoxidized form. Thus FMRFamide is the predominant peptide in the C3 neuron, as previous experiments have shown. A small peak corresponding to FLRFamide was also observed in fractions 16 and 17; and three other peaks indicative of higher molecular weight peptides appeared, two of them eluting in positions similar to those of the unidentified peptides seen in the extract of the *Aplysia* R2 neuron. The identity of none of these peaks is known, although at least one of them (at fraction 34) does appear to react with the Q2 antiserum. Neither SEPYLRFamide nor NDPYLRFamide were observed with the Q2 antiserum, nor was pQDPFLRIamide with the EFLRIamide antiserum.

Large cells in the location of LPa3 and RPa3

Extracts were prepared from three preparations, each containing eight large neurons that are located in the vicinity of LPa3 and RPa3 of *H. pomatia* (Elekes and Nässel, 1990): *i.e.*, D1, D2, D2a, F76, F77, and F78 of *H. aspersa* (Kerkut *et al.*, 1975). RIA of HPLC fractions provided clear evidence that at least some of the neurons in this group contain FMRFamide (see Fig. 5). At present, we cannot distinguish between these large cells based on their appearance alone.

Analysis of mRNA from Single Neurons and Small Clusters of Neurons

The mRNA contents of the following cell types were examined by hybridization, as described above: C3 neurons; cells from the right parietal FaRP cluster; and the group comprising the D1, D2, D2a and the F76, F77, F78 neurons, which corresponds to the large neurons in the region of LPa3 and RPa3 of *H. pomatia* (see Elekes and Nässel, 1990).

Two filters were prepared. Onto each were loaded both the contents of about 35 cells from the right parietal FaRP cluster and the contents of about 15 cells of the D1-D2a and F76-78 groups. Additionally, onto only one of the filters (because of the difficulty in isolating large numbers of intact C3 neurons) we loaded the contents of five C3 neurons (see lane C in Fig. 6a). The filter with the C3 neuron sample was hybridized to the tetra-FaRP cDNA of *H. aspersa*, and the other filter to the hepta-FaRP cDNA. The results of this experiment are shown in Figure 6. A strongly hybridizing band appears in the D1-D2a/F76-F78 sample when it is probed with the tetra-FaRP cDNA, while only a very faint band is produced when it is probed with the hepta-FaRP cDNA. Conversely, the sample from the cells of the right parietal FaRP cluster shows hardly any hybridization to the tetra-FaRP cDNA, but very strong hybridization to the hepta-FaRP cDNA. The extract of C3 neurons, though small in volume, shows a moderate amount of hybridization to the tetra-FaRP cDNA.

Subsequent to these hybridizations, the DNA probes were stripped from both the filters (by placing them in a solution of 20 m*M* Tris, pH 7.5 at boiling point and then allowing the solution to cool), and the filters were then reciprocally re-probed—*i.e.*, the filter with the C3 sample was hybridized to the hepta-FaRP cDNA and the second filter to the tetra-FaRP cDNA. The results of this second round of hybridization were exactly as described above for the cells of the right parietal FaRP cluster and those of the D1-D2a/F76-F78 group; but no hybridization to the C3 cell sample was detected with the heptapeptide-cDNA (data not shown).

Thus, the C3 neurons do contain mRNA for the tetra-FaRPs (as anticipated), but not for the hepta-FaRPS. Also, the cells of the D1-D2a/F76-F78 group contain, in great predominance, mRNA for the tetra-FaRPS; and those of the right parietal FaRP cluster contain, again in great predominance, mRNA for the hepta-FaRPS. These results

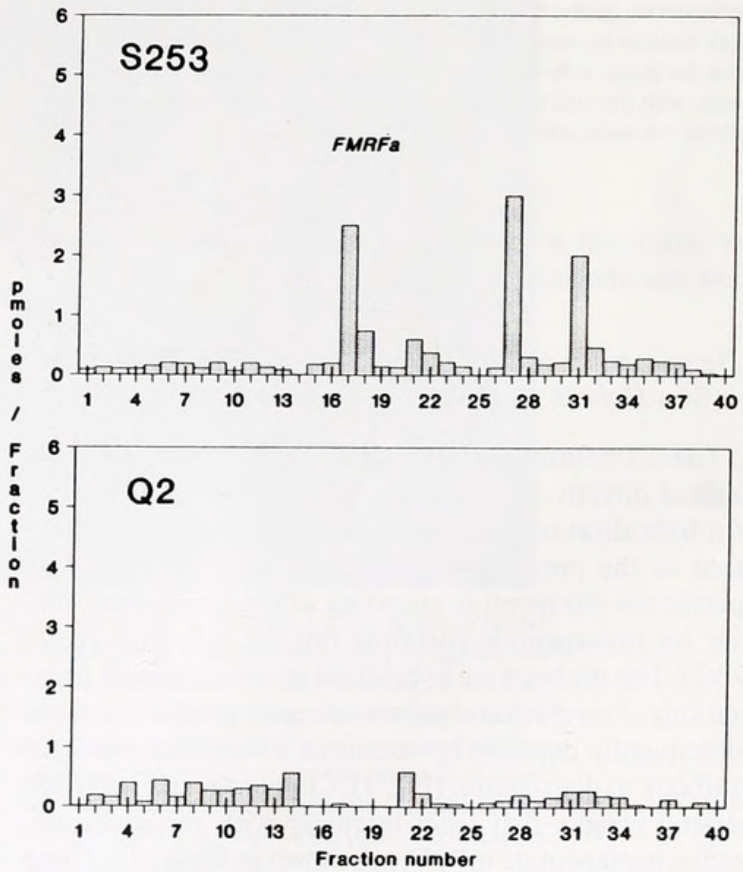

Figure 5. HPLC fractionation of the large parietal neurons, taken from three ganglion preparations, in the location of D1, D2, D2a, F76, F77, F78. This extract was run on a new column that retained FMRFamide slightly more effectively than the column used for the other extracts. A major peak corresponding to FMRFamide is seen, as are two other peaks reflective of higher molecular weight peptides of unknown identity.

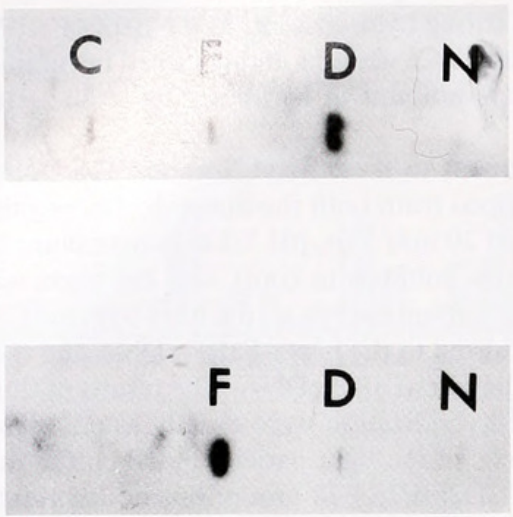

Figure 6. Autoradiographs of filters with various cell extracts hybridized with either (top) tetra-, or (bottom) hepta-FaRP cDNA probes of *H. aspersa* (Lutz *et al.*, 1992). The cells analyzed were: C, C3 neurons; F, the right parietal cell cluster; and D, neurons from the groups D1-D2a, F76-F78. The control, N, was a sample of physiological solution taken from the organ bath during dissection of the cells. Neither of the probes hybridized with the control sample, while the tetra-FaRP cDNA hybridized strongly to the sample from the cells of the D1-D2a/F76-F78 groups (D), and moderately to the C3 cell sample (C). The cells of the parietal cluster (F) reacted much more strongly with the hepta-FaRP cDNA probe than with the tetrapeptide probe. No reaction with the heptapeptide probe was seen with the C3 neuron sample, and only a weak reaction was seen with sample D. Subsequent stripping of the probes from the filters, followed by reciprocal re-probing gave exactly the same result, with the additional observation that no hybridization to the C3 sample was seen with the hepta-FaRP cDNA.

are consistent with the immunohistological and HPLC data described above.

Detection of FaRP-Encoding mRNA in Neurons by In Situ *Hybridization with Whole-Mounted Ganglia*

The C3 neuron and the parietal cell clusters were examined directly for tetra- and hepta-FaRP mRNA by *in situ* hybridization. Antisense RNA probes were synthesized in the presence of digoxigenin-labeled UTP, one specific for tetrapeptide encoding mRNA, the other specific for heptapeptide encoding mRNA, (*cf.*, Lutz *et al.*, 1992). The probes were hybridized to whole-mount preparations of cerebral and suboesophageal ganglia and were subsequently detected by means of a fluorescein-labeled antibody to digoxigenin (Fig. 7). Cells in the left and right parietal ganglia that react intensely with the probe detecting heptapeptide mRNA are shown in Figure 7a. These cell clusters correspond to ones that are rich in hepta-FaRPs and that do not react with the probe detecting the tetrapeptide mRNA (data not shown). In contrast, the C3 neuron reacted with the probe detecting the tetrapeptide mRNA (Fig. 7b), but not with the probe for the heptapeptide mRNA (data not shown).

Discussion

Identification of FaRP-containing neurons in H. aspersa

Some of the neurons in *H. aspersa,* in the same locations as cells in *H. pomatia* that react immunocytochemically with FMRFamide antiserum, contain detectable levels of FaRPs, but others do not. The absence of FaRPs in some of these cells may be due to the species difference, or because some of the neurons detected immunocytochemically do not contain FaRPs. Certainly, FMRFamide antisera have been used to identify non-FMRFamide peptides in neurons of, for instance, coelenterates (see Grimmelikhuijzen and Graff, 1986) and echinoderms (Elphick *et al.*, 1991).

Differential localization of the tetra- and the hepta-FaRPs

Helix neurons that possess FaRPs appear to be of two sorts. Either they contain the tetrapeptide FMRFamide (and also presumably FLRFamide, although this is difficult to detect because of its lower concentration), as in the C3 neuron, and some of the large parietal neurons (D1, D2, D2a, F76, F77 and F78), *or* they contain the hepta-FaRPs, as in the right and left parietal clusters. This conclusion, suggested by earlier studies with the C3 neurons (Bewick *et al.*, 1990), is supported by the HPLC data and also by the parallel RIAs of the parietal clusters and the large parietal neurons (D1, D2, D2a, F76, F77, F78) reported here.

Differential localization of mRNAs for the tetra- and the hepta-FaRPs

Two mRNA species encode the FaRPs in *Helix* (Lutz *et al.*, 1990, 1992). One species encodes FMRFamide and FLRFamide, and the other encodes all the hepta-FaRPs and pQDPFLRIamide. The C3 neurons and the some of the large parietal neurons (D1, D2, D2a, F76, F77, F78) possess mRNA encoding the tetra-FaRPs, whereas neurons of the parietal clusters are abundant in mRNA encoding the hepta-FaRPs. Bearing in mind the technical problem of isolating the different neurons uncontaminated with other cell types and neuropil, especially with the relatively large amount of tissue taken for the parietal cluster samples, the differential distribution of the mRNA, and also of the peptides, is striking.

The data suggest that either one of the mRNAs or the other is expressed, at least in the neurons studied here. This view is supported by hybridization of antisense RNA probes to mRNA *in situ.* In whole-mount ganglia, we saw that the C3 neuron expresses the mRNA encoding the tetra-FaRPs, whereas the parietal clusters express the mRNA encoding the hepta-FaRPs. The results of recent *in situ* hybridization experiments on *Lymnaea stagnalis*

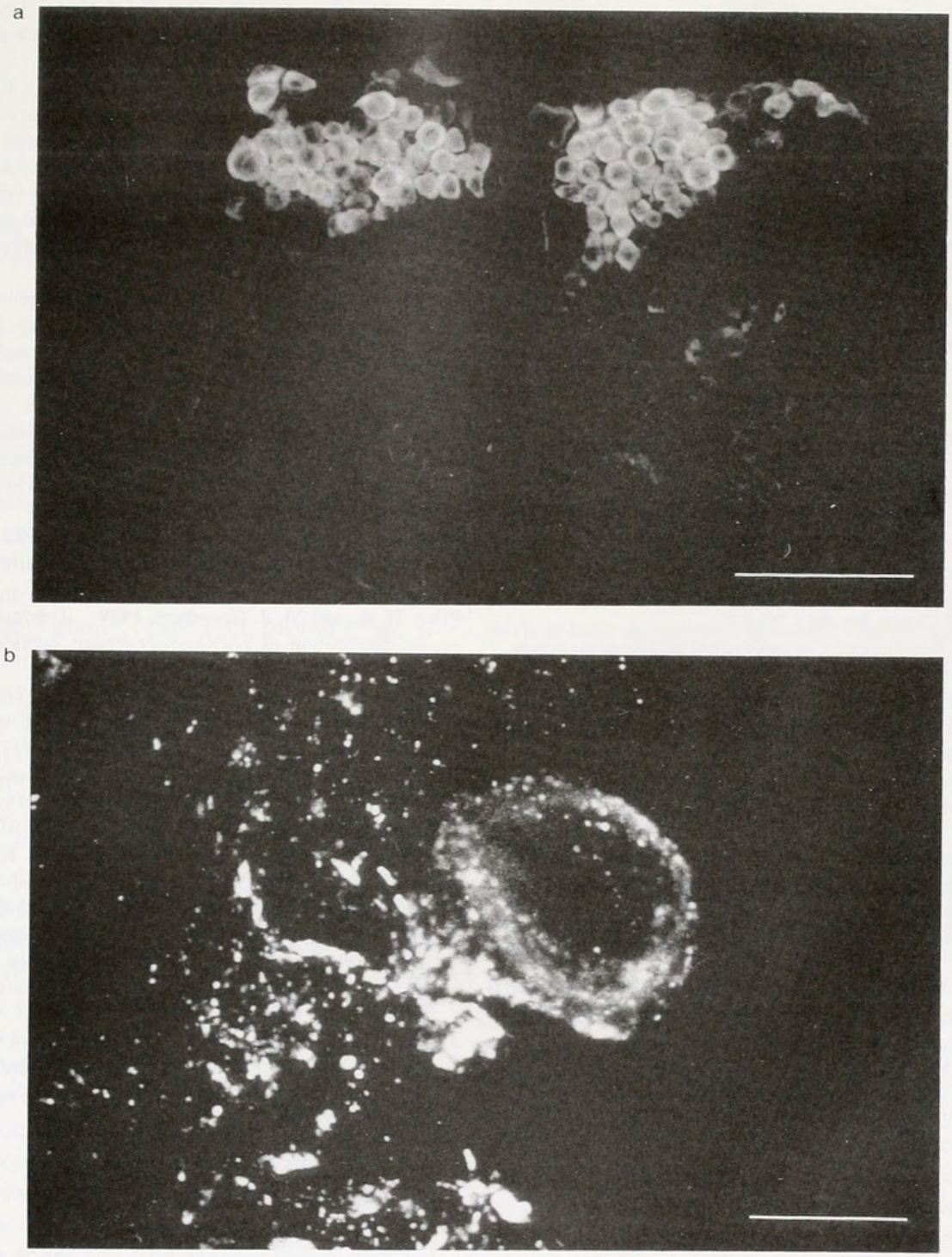

Figure 7. Hybridization of specific antisense probes *in situ* to mRNA encoding either tetra- or hepta-FaRPs. Hybridized mRNA is detected using fluorescein-labeled antibodies directed against the probes. (a) Whole mount of the visceral and left and right parietal ganglia hybridized with the probe specific for heptapeptide-encoding mRNA. The left and right parietal cell clusters are seen to fluoresce strongly; cf. with Figure 1. (b) Whole mount of a cerebral ganglion hybridized with the probe specific for tetrapeptide-encoding mRNA. Only the C3 neuron is seen to fluoresce. Note the higher magnification and the presence of some punctate fluorescence, some of which was non-specific, close to the axon hillock of the C3 neuron. Hepta-FaRP mRNA is not detected in the C3 neuron, nor is tetra-FaRP mRNA detected in the parietal cell clusters (not shown here). The scale bar corresponds to 250 μm in (a) and 50 μm in (b).

provide good evidence that, in this species too, the tetra-FaRPs are expressed in some neurons and the hepta-FaRPs in others (Saunders *et al.*, 1992).

The use of the antisense RNA probe for the heptapeptide mRNA defines most clearly the number of neurons comprising the heptapeptide-containing clusters, their packing together, and their topography in the parietal ganglia of *H. aspersa*. Because none of the cells within these clusters stained with the probe for the tetra-mRNA, they would appear to provide a homogeneous source of

neurons for further studies on the synthesis of the hepta-FaRPs carried out with a range of biochemical and molecular biological techniques.

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