Structure, Function, and Expression of *Drosophila melanogaster* FMRFamide-related Peptides

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ABSTRACT: In 1977, Price and Greenberg[1] identified the tetrapeptide FMRFamide as a cardioexcitatory molecule from mollusc. Subsequent to this discovery, FMRFamide-related peptides (FaRPs) have been identified in both invertebrates and vertebrates.[2,3] Peptides in the FaRP family contain a common RFamide C-terminus and act as modulators and messengers of neural and gastrointestinal functions.[2,3] Like other organisms, *Drosophila melanogaster* contains several genes[4–6] that encode for numerous FaRPs.[7,8] Elucidating the processing and activities of multiple FaRPs encoded in a single precursor is critical to establishing their roles in physiology. In this manuscript, we describe the distribution of FMRFamide immunoreactive materials in the *Drosophila* central nervous system and gut, and correlate it with the expression of specific FaRPs and their activities. The unique distributions[9–13] and biological activities[14,15] of *Drosophila* FaRPs suggest that the precursors are highly processed and the structurally related peptides are not functionally redundant. The complete distribution of FaRPs in the central nervous system and gut as detected by FMRFamide antisera is not accounted for by the sum of the individual expression patterns of the known *Drosophila* peptides. Thus, these data suggest that one or more *Drosophila* FaRPs or structurally related peptides remain to be discovered.

INTRODUCTION

Peptides that play critical roles in physiology can frequently be grouped together based on structure. One peptide family has a common C-terminal RFamide, but distinct N-terminal extensions. The first member of this group to be isolated was FMRFamide, a molluscan cardioexcitatory molecule.[1] Subsequent to this discovery, antisera to FMRFamide have been used to identify FMRFamide-related peptides (FaRPs) from numerous organisms.[2,3] The conservation of structure, function, distribution, and abundance of FaRPs suggests that they play important roles in physiology.

Organisms have more than one gene encoding multiple FMRFamide-related peptides.[4–7] Thus, in order to understand the signal transduction processes associat-

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ed with these important messengers, it is crucial to elucidate their regulation of expression. To this end, we are studying the distribution and processing of FaRP gene products in *Drosophila melanogaster.*[9–13] Three *Drosophila* genes, dromyosuppressin (Dms),[7] drosulfakinin (Dsk),[5] and FMRFamide,[4,6] encode several FaRPs. We have isolated TDVDHVFLRFamide,[6] FDDY(SO3H)GHMRFamide,[7] and DPKQDFMRFamide, SDNFMRFamide, and TPAEDFMRFamide[6] contained in Dms, Dsk, and FMRFamide in *Drosophila*, respectively. We have raised peptide-specific antisera and reported the expression patterns of the individual *Drosophila* FaRPs.[9–13] Here, we use antisera to FMRFamide to identify the family of structurally related peptides, thus providing a complete map of FaRP immunoreactive materials in the *Drosophila* central nervous system and gastrointestinal tissue. Taken together, the expression patterns of all of the known individual FaRPs do not account for the complete pattern of FMRFamide antisera staining. Therefore, these data suggest that at least one other structurally related peptide remains to be identified in *Drosophila.*

MATERIALS AND METHODS

Immunolocalization was performed as previously described.[15–17] Briefly, tissue was dissected in Ca^{++}-free Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.8 mM $MgCl_2$, 0.74 mM Na_2HPO_4, pH 7) and fixed in 4% paraformaldehyde for 4–6 hours at 4°C. After a thorough wash in PTN (0.1 mM $NaPO_4$, 0.3% Triton X-100, 0.1% NaAzide, 0.1% BSA), the tissue was incubated in 1:500 FMRFamide antisera (Peninsula Labs) overnight at 4°C with a slow rotatory motion, rinsed in PTN, and then placed in goat anti-rabbit FITC-conjugated second antibody (Sigma Chemical Co.) or Cy3-conjugated second antibody (Jackson ImmunoResearch Labs, Inc.) for 4–6 hours at 4°C. After extensive washing in PTN, the tissue was rinsed in 4 mM NaCarbonate, pH 9, before being mounted in 80% glycerol containing 5% propyl gallate under a glass coverslip.

Data were collected on a BioRad 600 Kr-Ar laser confocal attached to a Nikon inverted microscope using a 20× or 40× objective. Optical sections or z series were collected using the CoMOS program. Adobe photoshop software, version 3.0, was used to process the data, and images were made with a Kodak XLS8600 printer.

Immunoreactivity was observed bilaterally symmetric to the midline in the nervous system; thus, reference to one cell indicates the presence of a pair of cells positioned bilaterally symmetric to one another. The positions of the corresponding cells were not always perfectly symmetrical due to the distortion of the tissue that can be caused by placing a coverslip on it.

RESULTS AND DISCUSSION

The presence of FMRFamide immunoreactive material in the *Drosophila* central nervous system is represented by the staining of larval neural tissue (FIGURE 1). Signal is observed in the brain lobes as well as the subesophageal ganglion and ventral nerve cord. In addition to the numerous cells stained by FMRFamide antisera, two immunoreactive fibers were observed to project away from the ventral nerve cord.

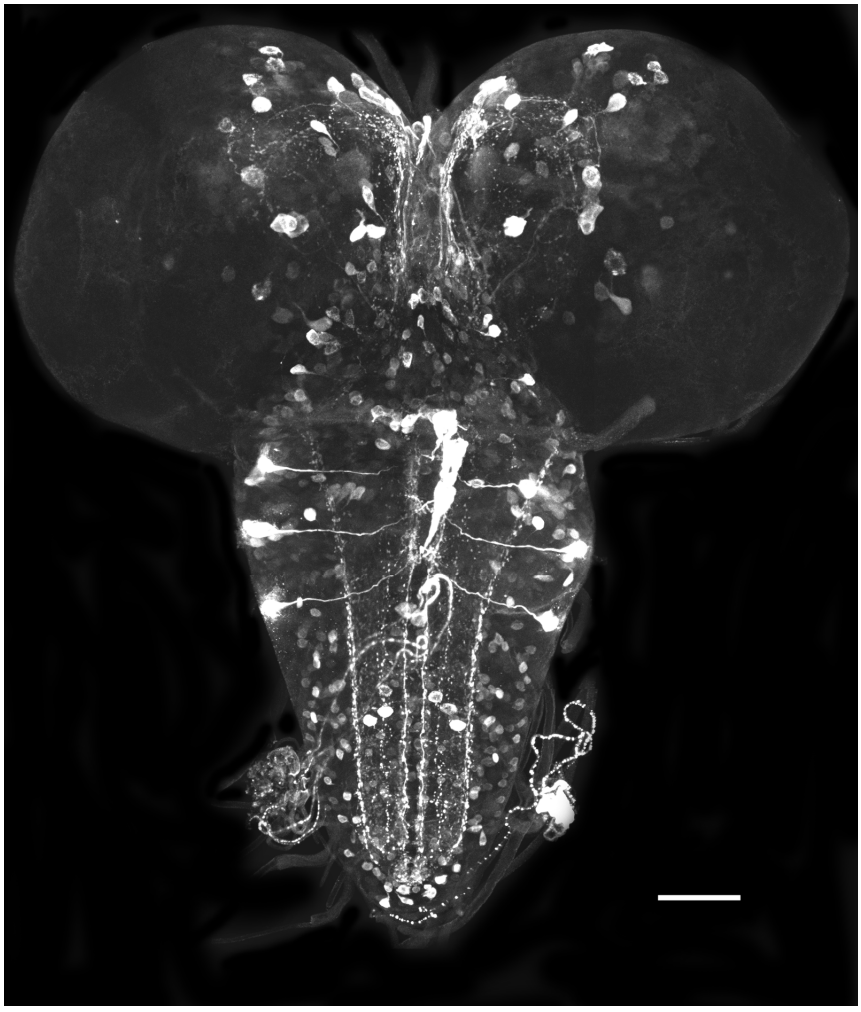

FIGURE 1. FMRFamide antisera staining in the *Drosophila* larval central nervous system. Rabbit FMRFamide antisera (Peninsula) and goat anti-rabbit FITC-labeled second antibody (Sigma) or Cy3-labeled second antibody (Jackson) were used to stain neural tissue prepared as a whole mount. The bar in the lower right-hand corner represents 50 microns.

One immunoreactive fiber extended from the third thoracic ganglion, while the other projected from the posterior abdominal ganglion. The site or sites innervated by these fibers were not identified.

The overall pattern of FMRFamide immunoreactive material found by staining with antisera to FMRFamide contains more cells than the combined expression patterns of individual FaRPs established using peptide-specific antisera.[9–13] This is ascertained by comparing the distribution of FMRFamide antisera staining with the

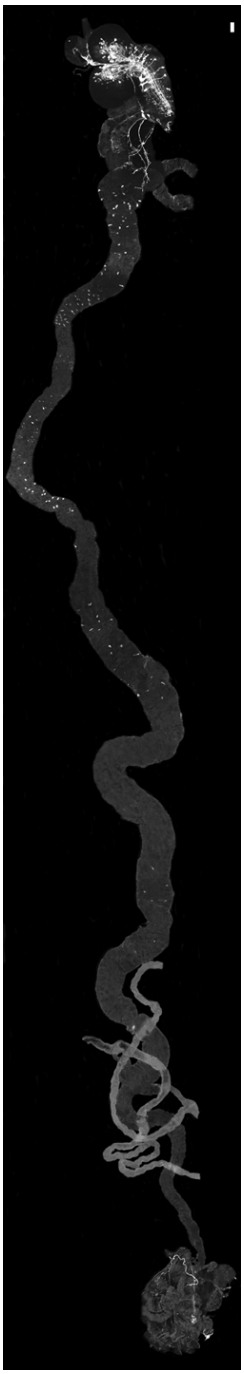

FIGURE 2. FMRFamide antisera staining in the *Drosophila* larval gastrointestinal tract. Rabbit FMRFamide antisera (Peninsula) and goat anti-rabbit FITC-labeled second antibody (Sigma) or Cy3-labeled second antibody (Jackson) were used to stain gastrointestinal tissue prepared as a whole mount. The bar in the lower right-hand corner represents 50 microns.

expression patterns of the known *Drosophila* FaRPs, TDVDHVFLRFamide (DMS), FDDYGHMRFamide (DSK I), and DPKQDFMRFamide, SDNFMRFamide, and TPAEDFMRFamide.[9–13] Several cells in the brain, subesophageal ganglion, and ventral nerve cord contain FMRFamide immunoreactive material, but are not stained by any of the peptide-specific FaRP antisera. Thus, these data suggest that *Drosophila* neural tissue contains one or more novel FMRFamide-related peptides.

Based on *Drosophila* FaRP genomic sequences and on our immunolocalization data, we conclude that the unidentified peptides are not encoded by known genes. For instance, the Dms gene encodes only DMS; thus, it does not include the unidentified peptides. In addition to DSK I, the Dsk gene encodes DSK II and DSK 0; however, antisera to DSK I would recognize DSK II (GGDDQFDDYGHMRFamide), and DSK 0 (NQKTMSFamide) is recognized by FMRFamide antisera. SPKQDFMRFamide and PDNFMRFamide, although not isolated in our purification protocol, are encoded by the FMRFamide gene; however, our polyclonal antisera to DPKQDFMRFamide and antisera to SDNFMRFamide would recognize SPKQDFMRFamide and PDNFMRFamide, respectively.

While FMRFamide antisera stain *Drosophila* neural tissue consistently, there were two distinct levels of signal, one more intense than the other. The difference in intensities may be explained by the amount of material present, or the cross-reactivity of the antigen and antisera. Our study did not address the basis for the differences in staining intensity observed.

The presence of FMRFamide immunoreactive material in the *Drosophila* gastrointestinal tract is represented by the staining of larval gut (FIGURE 2). Peptide-specific antisera have been used to determine the expression of individual *Drosophila* FaRPs in gut. TDVDHVFLRFamide is present in fibers that innervate the crop and in cell bodies of the rectum,[9] and TPAEDFMRFamide immunoreactive material is present in several cells in the midgut.[13] However, the identity of the material representing the FMRFamide-like immunoreactivity in the proventriculus and foregut is not known, and TPAEDFMRFamide immunoreactivity does not represent the entire staining pattern in the midgut. Thus, the total number of cells containing FMRFamide immunoreactive material is not accounted for by the expression of the known *Drosophila* FaRPs. Therefore, these data suggest that a novel FMRFamide-like material(s) is present in *Drosophila* gut.

FMRFamide-like immunoreactive materials are present in adult as well as early development. The complexity of FMRFamide antisera staining is retained during the life cycle of the animal as shown by the immunoreactivity in the adult central nervous system (FIGURE 3) and gastrointestinal system (FIGURE 4). Again, the cellular expression of the individual *Drosophila* FaRPs does not account for the complete staining pattern generated from FMRFamide antisera.[9–13] Thus, these data indicate that one or more uncharacterized *Drosophila* FaRPs may exist.

Here, we show that FMRFamide immunoreactivity is present throughout development in the *Drosophila* central nervous system and gastrointestinal tract. The production of structurally related peptides in unique cellular distributions provides the animal with a powerful means to regulate the generation of numerous, novel messengers to modulate physiological activities. The widespread distribution and the large number of cells containing FaRPs during the life cycle of the animal support the conclusion that these peptides play several important roles in physiology. Our

previous studies show that, for the most part, the expression patterns of individual FaRPs are not overlapping.[9–13] For instance, DPKQDFMRFamide, SDNFMRFamide, and TPAEDFMRFamide encoded in the *Drosophila* FMRFamide gene are distributed in distinct cellular expression patterns and do not coexist.[10,11,13,15] The

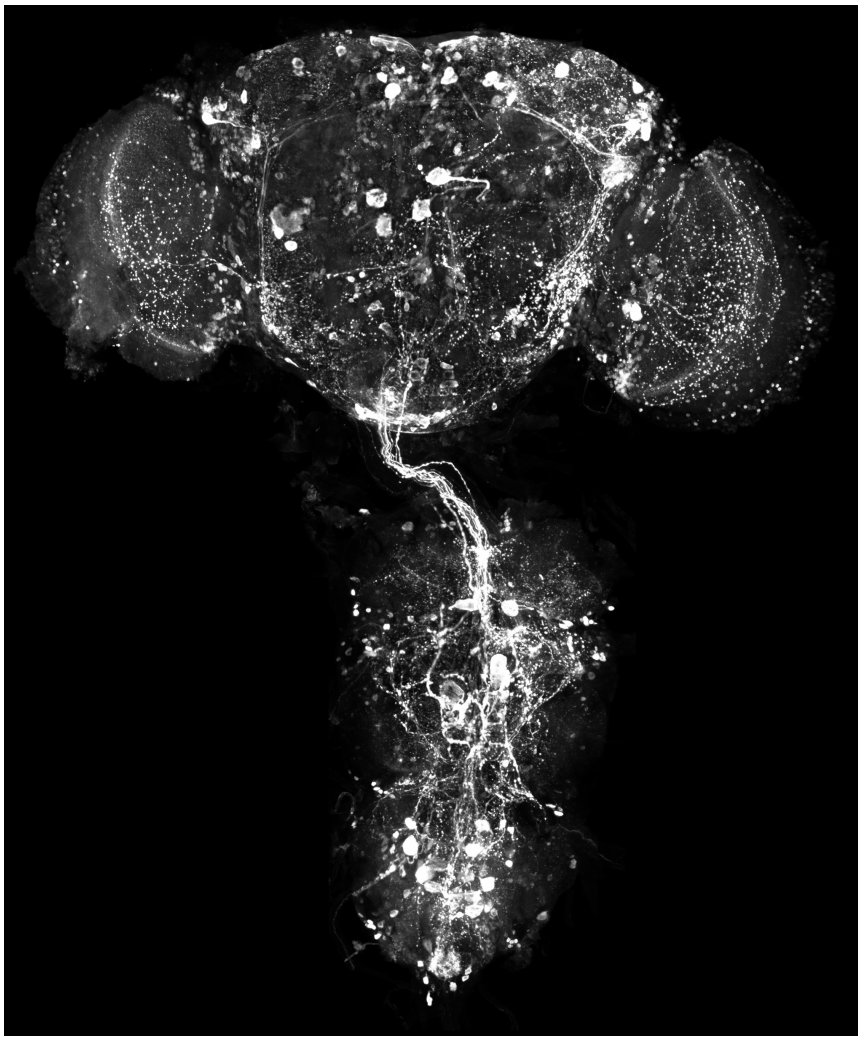

FIGURE 3. FMRFamide antisera staining in the *Drosophila* adult central nervous system. Rabbit FMRFamide antisera (Peninsula) and goat anti-rabbit FITC-labeled second antibody (Sigma) or Cy3-labeled second antibody (Jackson) were used to stain neural tissue prepared as a whole mount.

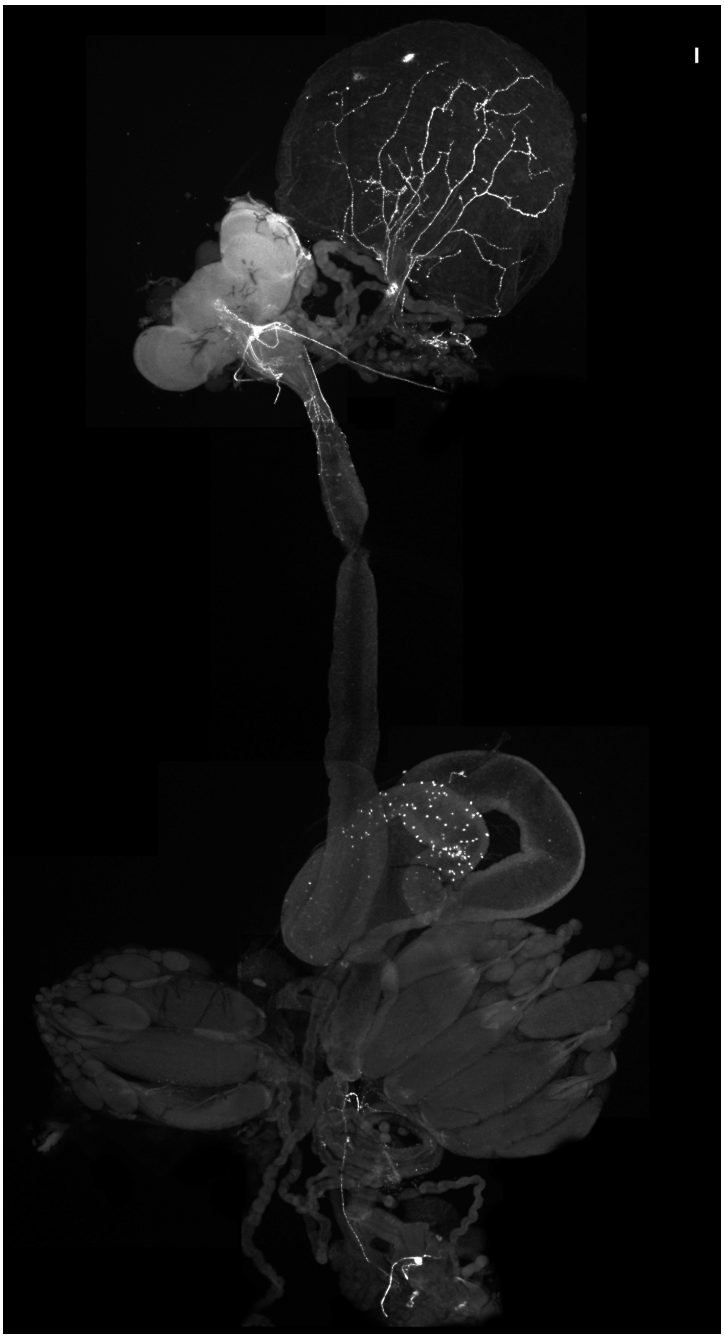

FIGURE 4. FMRFamide antisera staining in the *Drosophila* adult gastrointestinal tract. Rabbit FMRFamide antisera (Peninsula) and goat anti-rabbit FITC-labeled second antibody (Sigma) or Cy3-labeled second antibody (Jackson) were used to stain gastrointestinal tissue prepared as a whole mount. The bar in the lower right-hand corner represents 50 microns.

FaRPs, TDVDHVFLRFamide and FDDY(SO3H)GHMRFamide, encoded in the *Drosophila* Dms and Dsk genes, respectively, are not expressed in the same cells.[16]

Several conclusions can be made by comparing the expression of individual FaRPs and the distribution of FMRFamide immunoreactivity. Since the peptides are not expressed in the same cells, one conclusion is that there is not simple redundancy of function among the structurally related peptides. The peptides can affect similar tissues, but may receive unique regulatory cues due to the differences in cellular distribution. Indeed, the activities of *Drosophila* FaRPs are different. DMS acts as a neurotransmitter to regulate heart rate, while SDNFMRFamide modulates heart rate.[14] In contrast, DPKQDFMRFamide and TPAEDFMRFamide do not affect heart rate. Another finding is that the FaRP polyprotein precursors undergo differential processing on a cell-specific level.[9–13] In addition, our data support the hypothesis that one or more unidentified substances recognized by FMRFamide antisera are present in *Drosophila* neural and gut tissues.

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