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REGULAR ARTICLE

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Neuropeptide precursor processing detected by triple immunolabeling

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Abstract Peptides that play critical physiological roles are often encoded in precursors that contain several gene products. Differential processing of a polypeptide precursor by cell-specific proteolytic enzymes can yield multiple messengers with diverse distributions and functions. We have isolated SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide from *Drosophila melanogaster*. The peptides are encoded in the *FMRFamide* gene and have a common C-terminal FMRFamide but different N-terminal extensions. In order to investigate the regulation of expression of FMRFamide peptides, we generated antisera to distinguish between the structurally related neuropeptides. We established a triple-label immunofluorescence protocol using antisera raised in the same host species and mapped the neural distribution of SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide. Each peptide has a unique, nonoverlapping cellular expression pattern, suggesting that the precursor is differentially processed. Thus, our data indicate that *D. melanogaster* contains cell-specific proteolytic enzymes to cleave a polypeptide protein precursor, resulting in unique expression patterns of neuropeptides.

Key words FMRFamide · Neuropeptide · Precursor processing · Proteolytic enzymes · *Drosophila melanogaster* (Insecta)

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Introduction

Peptides that affect critical physiological parameters can often be grouped together based on a common structure and are frequently encoded by genes that contain multiple structurally related products. Differential processing of a precursor to yield peptides with different distributions provides a means by which to regulate gene expression, resulting in diversification of function on a cellular and/or developmental level (Curry et al. 1991; LeBlanc et al. 1991; Brand and Fuller 1993; Mineo et al. 1995). Elucidating the regulatory mechanisms involved in the expression of structurally similar messengers is essential to understanding neuropeptide signaling pathways that are crucial to physiology.

Peptides with a common C-terminal FMRFamide but distinct N-terminal amino acid extensions are present throughout the animal kingdom (Raffa 1988; Greenberg and Price 1992). Since the discovery of FMRFamide (Price and Greenberg 1977), a vast number of structurally related peptides have been identified. Typically, organisms have multiple genes containing numerous FMRFamide peptides (Marks et al. 1995; Price et al. 1996; Edison et al. 1997; Nelson et al. 1998). In *Drosophila melanogaster,* drosulfakinin, *Dsk* (Nichols et al. 1988; Nichols 1992a), dromyosuppressin, *Dms* (Nichols 1992b), and *FMRFamide* (Nambu et al. 1988; Schneider and Taghert 1988) encode many peptides homologous to FMRFamide. We have isolated SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide (Nichols 1992b) encoded in the *FMRFamide* gene.

Although FMRFamide peptides constitute a major class of messengers, relatively little is known about the regulation of gene expression and signaling of this neuropeptide family. Evidence suggests that FMRFamide peptides may act through multiple mechanisms, including peptide-gated ion channels (Lingueglia et al. 1995; Cottrell 1997; Darboux et al. 1998) as well as G protein-coupled receptors (Chin et al. 1994). Determining the cells in which these peptides are present is important in deciphering neurotransmission. Thus, in this paper we

report the neural distribution of *D. melanogaster* SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide.

In order to determine expression, we raised antisera that distinguish among the three structurally related peptides. In order to map peptide distribution within the same tissue preparation, we developed a triple-label immunolocalization protocol that uses antisera raised in different animals of the same host species. SDNFMRFamide-, DPKQDFMRFamide-, and TPAEDFMRFamide-immunoreactive materials are present in nonoverlapping cellular patterns in the central nervous system, indicating that the FMRFamide precursor undergoes differential processing on a cell-specific level. Our data indicate that the *D. melanogaster* FMRFamide precursor is processed at a cellular level to yield peptides that may differ in bioactivity.

Materials and methods

Animals

D. melanogaster Oregon R strain flies were maintained on cornmeal molasses media at 24 C under a 12-h light/dark cycle. Tissue was dissected from third instar larvae.

Chemicals

Each of the three antigens was synthesized as a multiple antigenic peptide (MAP), which is composed of a lysyl core onto which the peptide is synthesized at eight branch sites (Postnett and Tam 1989; Nichols et al. 1997). The fluorescently tagged Fab fragments were obtained from Jackson Immunochemicals Research. FITC-labeled whole antibody was purchased from Sigma Chemical, as were all the other chemicals used

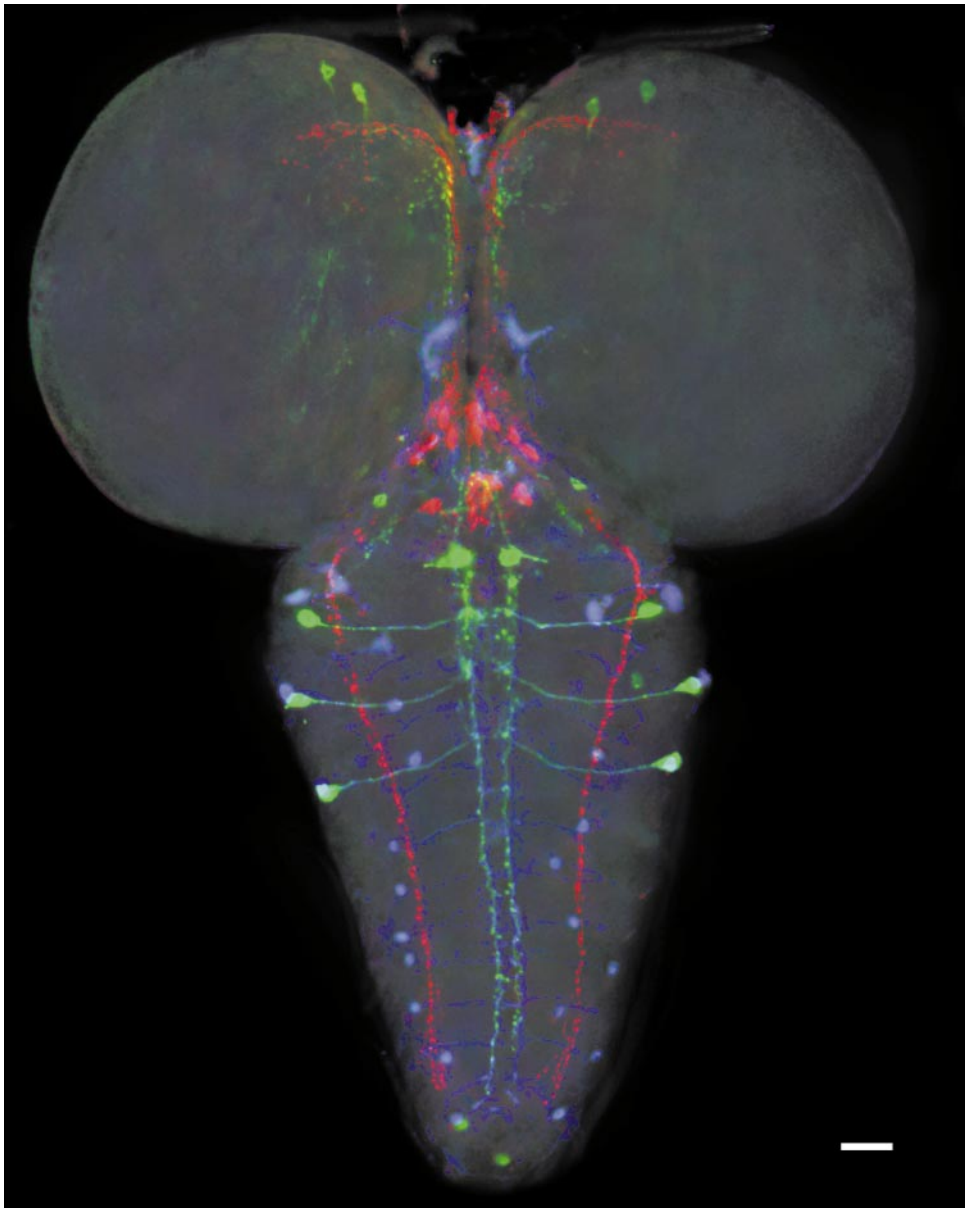


Fig. 1 Triple-label immunofluorescence of the larval *D. melanogaster* central nervous system. SDNFMRFamide, TPAEDFMRFamide, and DPKQDFMRFamide antisera were recognized by Cy3-labeled Fab fragment, Cy5-labeled Fab fragment, and FITC-labeled whole antibody, respectively. Using different combinations of primary antisera and fluorescently labeled second antibody did not change the results. The fluorophores are shown as *red*, *blue*, and *green*, respectively. Although Cy3 and Cy5 are both cyanine derivatives, they are spectrally different. Here, Cy5 is depicted as blue rather than using two shades of red to represent Cy3 and Cy5. *Bar* 50 µm

Antisera production

The three antigens SDNFM-MAP, DPKQD-MAP, and TPAED-MAP were designed to the N-terminal extensions. Antisera were raised in New Zealand white rabbits as previously described (McCormick and Nichols 1993; Nichols et al. 1995a, 1995b). Antisera purification was performed by applying whole sera to affinity columns made by linking the antigen to affi-gel resin according to specifications provided by the manufacturer (BioRad Labs). Specificity was determined by absorbing antisera prior to immunolocalization with the antigen to which it was raised (signal abolished) or preabsorption with either of the other two antigens (signal unchanged). All protocols involving vertebrate animals were approved by The University of Michigan University Committee on Animal Care and Use.

Immunofluorescence

Triple-label immunofluorescence was performed according to the double-label protocol previously described (Nichols et al. 1995a, 1995b) with the inclusion of a third primary antisera and an additional, spectrally distinct, fluorophore, Cy5. Briefly, central nervous system tissue was dissected in cold Ca^{2+}-free Ringer's (130 mM NaCl, 4 mM KCl, 1.8 mM $MgCl_2$, 0.7 mM Na_2HPO_4, pH 7) and fixed in fresh 4% paraformaldehyde for 4–6 h at 4 °C. After washing in PTN (0.1 mM $NaPO_4$, 0.3% Triton X-100, 0.1% sodium azide, 0.1% BSA) for 1 h at 4 °C, the tissue was incubated in SDNFMRFamide antisera for 8 h and washed for 1 h at 4 °C in PTN. Cy3-labeled goat anti-rabbit Fab fragment (Jackson ImmunoResearch Labs) was then applied for 4 h at 4 °C. The tissue was washed in PTN for 1–2 h at 4 °C, incubated in TPAEDFMRFamide antisera for 8 h, washed for 1 h at 4 °C in PTN, and Cy5-labeled goat anti-rabbit Fab fragment (Jackson ImmunoResearch Labs) was applied for 4 h at 4 °C. The tissue was washed in PTN

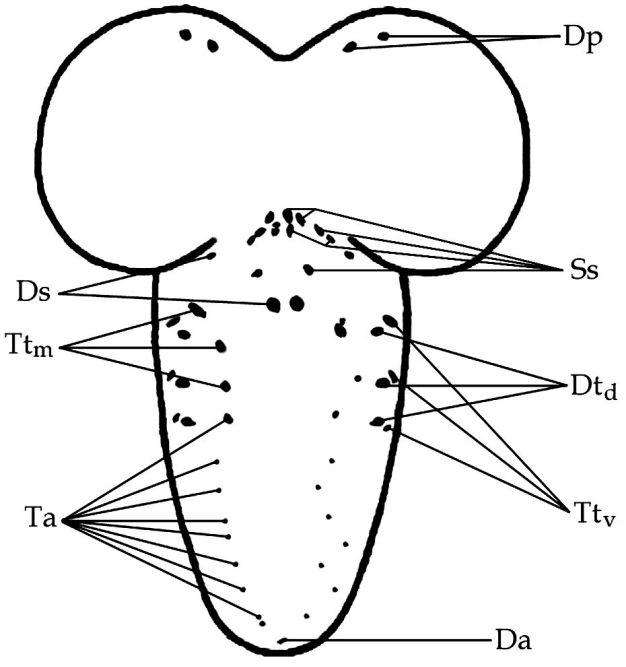


Fig. 2 The triple-label immunofluorescence shown in Fig. 1. *S*, *D*, and *T*, represent SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide, respectively. *a*, *p*, *s*, and *t* represent abdominal, protocerebrum, subesophageal, and thoracic, respectively. The subscripts *d*, *m*, and *v*, refer to dorsal, medial, and ventral, respectively

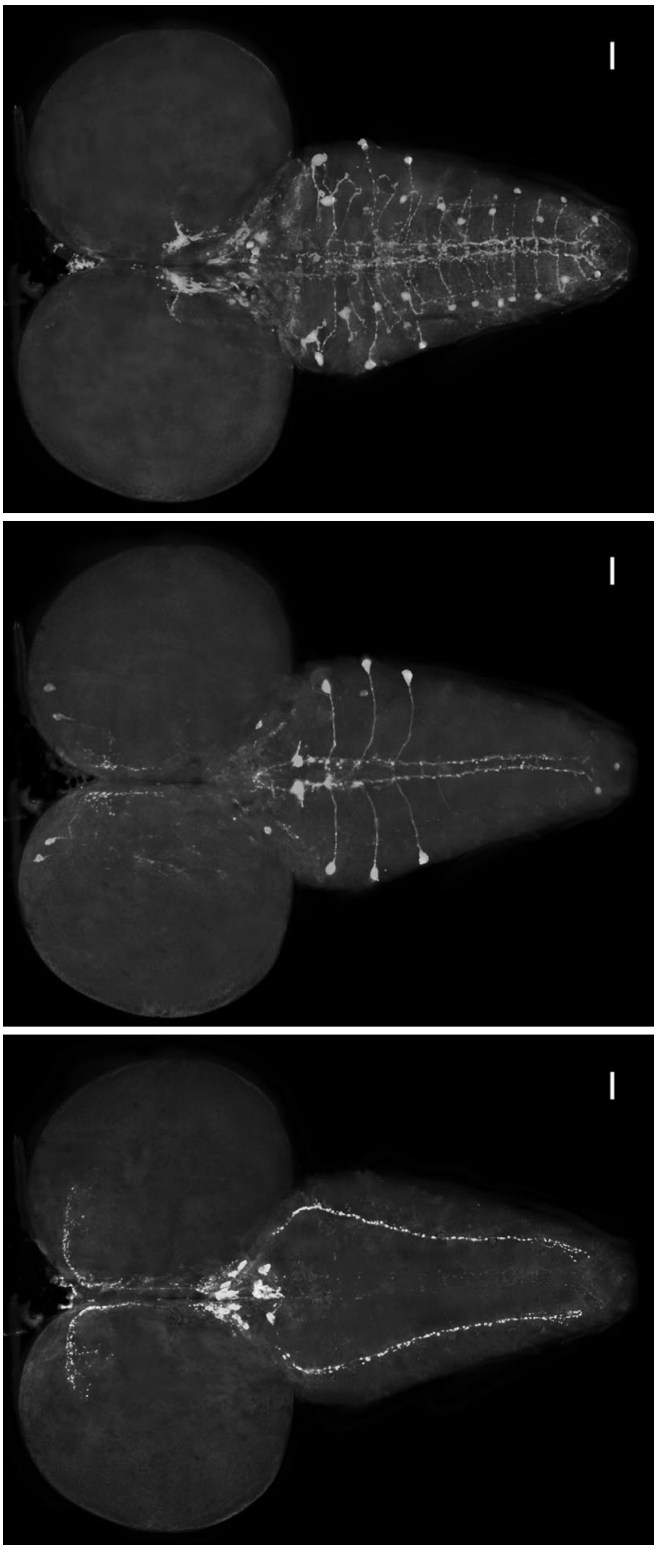


Fig. 3 The triple-label immunolabeling shown in Fig. 1 is presented as individual images. The staining patterns are SDNFMRFamide (*bottom*), DPKQDFMRFamide (*center*), and TPAEDFMRFamide (*top*). *Bars* 50 µm

for 1–2 h at 4 °C, incubated in DPKQDFMRFamide antisera for 8 h at 4 °C, washed in PTN for 1 h at 4 °C, and FITC-labeled goat anti-rabbit whole antibody (Sigma Chemical) was applied for 4 h at 4 °C. The tissue was then washed in PTN for 3 h at 4 °C, rinsed in 4 mM Na_2CO_3, pH 9, and prepared for microscopic analysis by placing it on a glass microscope slide in glycerol containing 0.5% propyl gallate.

Data collection and analysis

Data were collected on a BioRad 600 Kr-Ar laser confocal attached to a Nikon inverted microscope using K1, K2, and W1 filters and 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.

Results

We determined the distribution of SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide in larval neural tissue using triple-label immunostaining (Fig. 1). The reference to one neuron being stained by antisera indicates the presence of a pair of cells, positioned relative to the midline from one another. The preparation of the tissue for microscopy frequently results in the symmetry of the two cells no longer being perfectly bilateral. The drawing in Fig. 2 illustrates the staining pattern observed in Fig. 1 and includes the nomenclature previously assigned to cells stained by FMRFamide antisera (White et al. 1986; Nambu et al. 1988; Schneider and Taghert 1988). In addition, the three different staining patterns comprising the triple immunolabeling are presented as separate images in Fig. 3.

Our triple-label immunofluorescence data indicate that SDNFMRFamide immunoreactivity is contained in several cells present only in the subesophageal ganglion (Figs. 1, 3). In addition, a SDNFMRFamide antisera-stained process projects from a subesophageal cell (Figs. 1, 3). This immunoreactive process, positioned along the midline, projects into the brain, where it turns and forms an arch-like extension into the anterior protocerebrum. Another SDNFMRFamide antisera-stained projection extends from a subesophageal cell to traverse the ventral ganglion (Figs. 1, 3).

The distribution of DPKQDFMRFamide immunoreactivity is strikingly different from SDNFMRFamide (Figs. 1, 3). DPKQDFMRFamide antisera stain two cells in the protocerebrum, two cells in the subesophageal ganglion, one cell in each of the three thoracic ganglions, and one cell in an abdominal ganglion (Figs. 1, 3). DPKQDFMRFamide-immunoreactive processes project from the three stained thoracic cells toward the midline, turn, and extend posteriorly along the midline throughout the ventral ganglion as well as anteriorly to the protocerebrum (Figs. 1, 3). In addition, immunoreactive processes project from the medial subesophageal cell into the ventral ganglion and anterior protocerebrum (Figs. 1, 3). Less extensive immunoreactive processes project from the protocerebrum cells (Figs. 1, 3). No immunoreactive projection was observed originating from the lateral subesophageal cell or from the abdominal cell (Figs. 1, 3).

TPAEDFMRFamide-immunoreactive material is present in more cells than either SDNFMRFamide or DPKQDFMRFamide (Figs. 1, 3). TPAEDFMRFamide antisera stains 14 cells in the ventral ganglion: two cells in each of the three thoracic ganglions, and one cell in each of the eight abdominal ganglions (Figs. 1, 3). The lateral thoracic cells containing TPAEDFMRFamide-immunoreactive material are positioned lateral and ventral to those stained by DPKQDFMRFamide antisera (Figs. 1, 4, 5). TPAEDFMRFamide-immunoreactive processes project from each stained thoracic cell toward the midline, turn, and extend posteriorly along the midline into the ventral ganglion (Figs. 1, 3, 4, 5). These processes also extend into the midbrain; however, unlike SDNFMRFamide- and DPKQDFMRFamide-immunoreactive fibers, they do not project into the protocerebrum. Immunoreactive projections were also observed to extend to the midline from the medial thoracic cells stained by

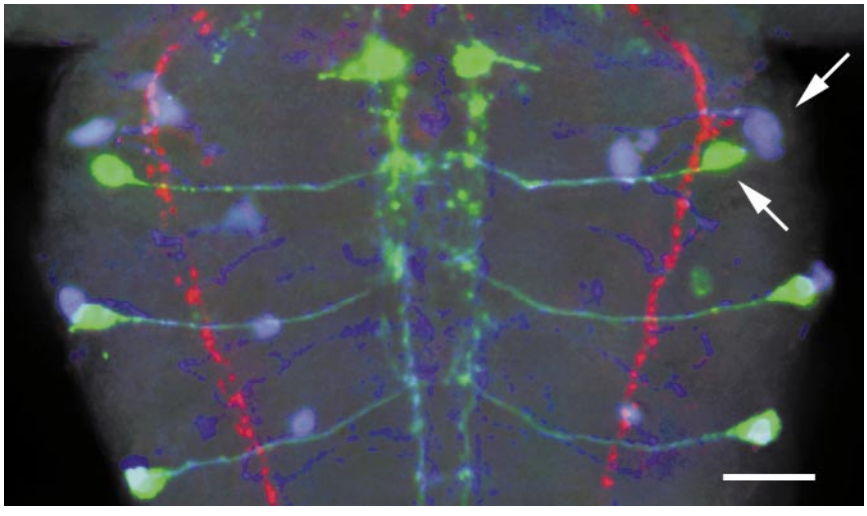
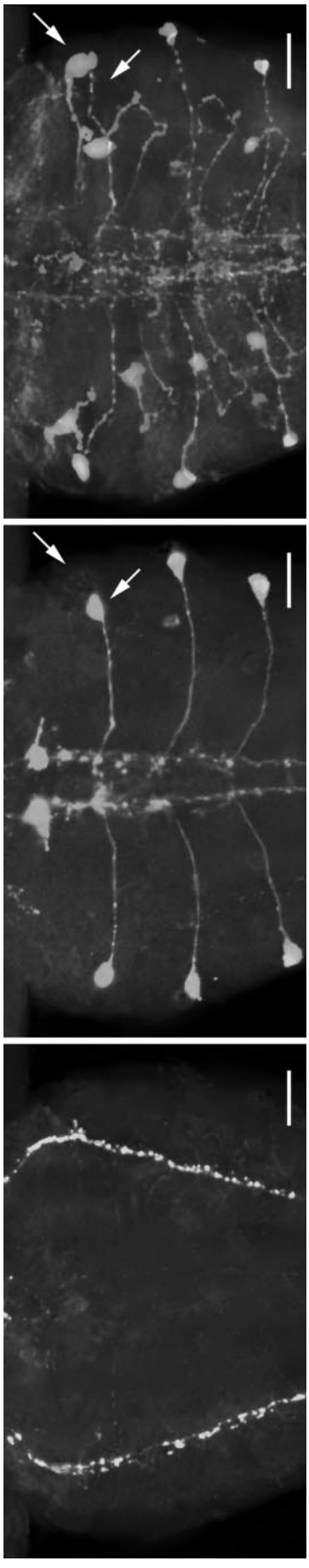


Fig. 4 An enlargement of the thoracic ganglia of the triple labeling shown in Fig. 1. SDNFMRFamide-immunoreactive material is seen as *red*, while DPKQDFMRFamide and TPAEDFMRFamide are *green* and *blue*, respectively. *Arrows* are used to point to two neurons in one ganglion. One cell is stained by DPKQDFMRFamide antisera (*lower arrow*), and the other stained by TPAEDFMRFamide antisera (*upper arrow*). *Bar* 50 µm



TPAEDFMRFamide antisera (Figs. 1, 3, 4, 5). Immunoreactive fibers also extend toward the midline from the TPAEDFMRFamide antisera-stained abdominal cells (Figs. 1, 3).

Discussion

Regulation of peptide signaling is critical to physiology, hence several mechanisms exist for modulating neurotransmission. One mode of regulation is at the post-translational level with cell-specific proteolytic processing enzymes cleaving a polypeptide protein precursor to produce neuropeptides in unique expression patterns (Curry et al. 1991; LeBlanc et al. 1991; Brand and Fuller 1993; Mineo et al 1995). Through differential processing, structurally related peptides present in the same precursor can have different cellular or developmental distribution patterns and diverse biological activities. *D. melanogaster* contains enzymes important for post-translational modifications (Baeuerle et al. 1988; Neckameyer and Quinn 1992; Kolhekar et al. 1997). Our interest in neuropeptide gene regulation and processing led us to determine the distribution of SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide to suggest whether the FMRFamide precursor undergoes differential processing.

In order to establish cellular location, we raised three antisera that distinguish between these structurally related peptides. The use of a Fab fragment to detect a primary antisera prevents a subsequently applied primary antisera from binding to unbound sites on a previously applied secondary antisera (Negoescu et al. 1994; Nichols et al. 1995a, 1995b; Nichols et al. 1997). Thus, antisera used in multiple immunolabeling can be raised in the same host species, which provides several advantages related to animal care and housing, technical skills required for antisera collection, and availability of second antibodies. While previous protocols report detection of two antigens in the same preparation (Würden and Homberg 1993; Negoescu et al. 1994; Nichols et al. 1995a, 1995b; Nichols et al. 1997), the ability to identify three antigens expands the type of experiment that can be performed.

Our immunostaining data indicate that no two of the three FMRFamide peptides are expressed in the same cell, but that each one is uniquely distributed in the larval central nervous system. Based on position, the peptides are expressed in cells detected by antisera generated to the FMRFamide precursor and antisera raised to the FMRFamide peptide (Nambu et al. 1988; Schneider and Taghert 1988; Schneider et al. 1993; Nichols et al.

Fig. 5 The triple-label immunolabeling shown in Fig. 4 is presented as individual images. The staining patterns are SDNFMRFamide (*bottom*), DPKQDFMRFamide (*center*), and TPAEDFMRFamide (*top*). The *two arrows* in the same locations as in Fig. 4 remain in the center and bottom panels to emphasize the positions of the two cells relative to one another and their presence or absence. *Bars* 50 μm

1995a, 1995b). The extensive trajectories of the immunoreactive processes into the anterior protocerebrum and the thoracic and abdominal ganglia suggest that SDNFMRFamide and DPKQDFMRFamide are delivered to several targets away from their site of production and that the peptides may have multiple actions. It is also likely that TPAEDFMRFamide acts at several sites.

The ability to simultaneously detect the distribution of multiple neuropeptides in the same tissue is important in determining gene regulation, processing, and signaling. Utilizing triple-label immunostaining, we have determined that SDNFMRFamide, DPKQDFMRFamide, and TPAEDFMRFamide are expressed in distinct, nonoverlapping cells and processes. Thus, our study supports the conclusion that *D. melanogaster* contains cell-specific proteolytic enzymes to differentially process a polypeptide protein precursor.

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