Spatial and Temporal Expression Identify Dromyosuppressin as a Brain-Gut Peptide in *Drosophila melanogaster*

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ABSTRACT

The *Drosophila* dromyosuppressin peptide (TDVDHVFLRFamide) is a member of a family of peptides containing the common C-terminal sequence -RFamide. Dromyosuppressin shares a high degree of sequence homology with leucomyosuppressin isolated from cockroach (pEDVDHVFLRFamide) and identity with neomyosuppressin isolated from fleshfly. By means of sequence-specific antisera, the cellular expression pattern of dromyosuppressin immunoreactive material was determined for all stages of *Drosophila* development.

Dromyosuppressin immunoreactivity first appears in two cells of the medial protocerebrum in embryos. The larval stage is characterized by an increase in the number of dromyosuppressin immunoreactive cells in the brain and the first appearance of cellular expression in the ventral ganglion. Immunoreactive fibers extend from the medial protocerebrum cells into the ventral ganglion. Relative to the larval stage, the pupal and adult stages are marked by an increase in the number of immunoreactive cells in the central nervous system and an increase in the arborization of immunoreactive fibers extending from these cells.

Immunoreactivity is present in larvae in two cells near the anus; in the adult gut, expression is observed in two cells in the rectum and immunoreactive fibers in the crop that appear to extend from the central nervous system. In general, the number of cells containing dromyosuppressin immunoreactive material increases throughout *Drosophila* development. However, expression in three cells is restricted to specific developmental periods. These data identify dromyosuppressin as a brain-gut peptide regulated at both a cellular and developmental level.

Key words: FMRFamide, insect physiology, invertebrate peptide, leucomyosuppressin, neuropeptide

The tetrapeptide FMRFamide was first isolated and identified from mollusc as a cardioexcitatory peptide by Price and Greenberg ('77). Since that time, antisera to FMRFamide have been used to identify immunoreactive materials in neural tissue of both vertebrates and invertebrates. Many of the FMRFamide immunoreactive materials isolated from vertebrates and invertebrates contain the C-terminal sequence -MRFamide or -LRFamide, and the entire family of peptides is structurally related by the common C-terminal sequence -RFamide. Studies to date have demonstrated that -RFamide peptides represent an abundant, phylogenetically widespread peptide family. Tissue distribution and activity studies suggest that these peptides act as transmitters, regulators, and modulators in the central nervous system and function in a broad range of important physiological processes.

*Drosophila melanogaster* genes encoding -RFamide peptides include: *drosulfakinin* (*Dsk*) and FMRFamide. The *Dsk* gene encodes two -MFamide-containing peptides designated DSK I and DSK II (Nichols, '87; Nichols et al., '88), and the FMRFamide gene encodes a protein that may be processed to five different -FMRFamide-containing peptides (Nambu et al., '88; Schneider and Taghert, '88). Of the five -RFamide-containing peptides isolated from an extract of adult *Drosophila melanogaster* (Nichols, '92a), four contain -MRFamide and can be predicted from *Dsk* or *FMRFamide*. The fifth peptide, TDVDHVFLRFamide, is not encoded in either *Dsk* or *FMRFamide*.

TDVDHVFLRFamide shares a high degree of sequence homology with peptides isolated from chicken (Dockray et al., '83), cockroach (Holman et al., '86), fleshfly (*F6nagy et al., '92), locust (Robb et al., '89), and snail (Ebberink et al., '87). The fruitfly and fleshfly peptides are identical, while the cockroach, fruitfly, and locust peptides differ only by the

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N-terminal amino acid residue. The cockroach and fleshfly peptides both inhibit gut motility and have been named leucomyosuppressin and neomyosuppressin, respectively. On the basis of sequence homology, the fruitfly peptide has been designated dromyosuppressin.

Although FMRFamide immunoreactivity has been localized to a certain subset of neurons in the Drosophila nervous system (White et al., '86; Chin et al., '90; Schneider et al., '91; O'Brien et al., '91), antisera to FMRFamide recognize DSK peptides, FMRFamide-containing peptides, TDVDHVFLRFamide, and other RFamide-containing peptides. Because the complexity of the RFamide peptide family makes these immunohistochemical data ambiguous, it is difficult to interpret results obtained by using antisera to FMRFamide or any other antigen with the common C-terminal sequence -RFamide. It is important to isolate and determine the structure of the naturally occurring peptides and design sequence-specific antisera to determine the cellular expression patterns of a specific peptide. Immunohistochemical studies will provide information on the neural tracts containing peptides, thus allowing the construction of an anatomical map for the peptides. These data may provide information regarding possible sites of action and suggest biological function(s).

This study was undertaken to determine the developmental expression pattern of dromyosuppressin in the central nervous system and gut. To identify dromyosuppressin immunoreactive material, sequence-specific antisera were generated and affinity purified, and cross reactivities determined. This manuscript presents data demonstrating that dromyosuppressin expression in the Drosophila central nervous system begins in the late embryo and continues throughout development with an overall increase in the number of cells, arborization of immunoreactive fibers, and intensity of staining. In addition, dromyosuppressin immunoreactive material is observed in both larval and adult gut.

**MATERIALS AND METHODS**

**Staging flies**

*Drosophila melanogaster* Oregon R flies were raised on corneal molasses media and maintained at 25°C. Adults were collected 4 to 6 hours after eclosion, and tissue was dissected from 1–2, 5–7, and 11–13 day old adults. Prepupae were collected and central nervous systems dissected from 23–25, 48, 72, and 94 hour old pupae. Larvae were collected by allowing flies to lay eggs on grape juice medium plates for 21–22 hours, after which the flies were removed and all larvae were discarded. After 1 hour, all larvae were collected; this was repeated over several hours to establish a range of ages. Larvae were dissected at less than 5 hours and at 5 hour intervals for ages 15–95 hours. Embryos, collected on grape juice medium plates, were dissected at 11½–13, 13–16, and 16–20 hours. All developmental stages were verified by morphological changes and no fewer than 12 preparations were examined for each time period.

**Antisera production and purification**

TDVDHV was synthesized by means of an Applied Biosystems model 430A solid phase peptide synthesizer and purified by preparative reversed-phase high performance liquid chromatography (HPLC). The structure of the synthetic peptide was confirmed by mass spectrometry, amino acid analysis, and automated Edman degradation. Antisera were raised in two New Zealand White rabbits to TDVDHV conjugated to thyroglobulin through the cysteinyl residue of the peptide with N-(4-carboxycyclohexylmethyl)-maleimide, a heterobifunctional crosslinker (Yoshitake et al., '79). The initial immunizations were by subcutaneous injections, at multiple sites, of a total of 1 mg of antigen emulsified in Freund's complete adjuvant. Subsequent boosts were given every 2 weeks by subcutaneous injections of a total of 0.5 mg of antigen in Freund's incomplete adjuvant. Antisera titers were analyzed by indirect immunofluorescence of whole mount third instar larval central nervous systems as described (White et al., '86).

A peptide affinity column was made by coupling a TDVDHV-MAP, where MAP represents multiple antigenic peptide (Posnett and Tam, '89), to Affi-gel 10 (Bio-Rad Labs) in dry dimethyl sulfoxide and 1% triethylamine. The amount of peptide coupled to the affinity resin was 2 mg peptide/ml resin. To purify antisera the column was first washed with 10 column volumes of 5 mM sodium phosphate, pH 7.2, prior to antisera application, and then crude antisera diluted 1:1 with 10 mM sodium phosphate, pH 7.2, were applied to the column at a flow rate of 10 ml/hr; the column was then washed with 10 column volumes of 5 mM sodium phosphate, pH 7.2. The binding capacity of the column exceeded the amount of antisera applied.

TDVDHV-specific antisera were eluted from the column with 5 column volumes of 0.1 M sodium citrate, pH 2.5, and the eluant was neutralized by collecting directly into 10 volumes of 1 M Tris, pH 8.0 and subsequently dialyzed.
against 4 liters of 0.01 mM sodium phosphate, pH 7.2, at 4°C for 24 hours. The affinity-purified antisera were then aliquoted, freeze-dried, and stored at –20°C.

Immunochemistry

Tissue was dissected in cold Ringer's solution and prepared for immunohistochemistry according to White et al. ('86). Whole mount preparations fixed in paraformaldehyde and washed in PTN (0.5 M sodium phosphate, pH 7.2 containing 10% Triton X-100 and 1% sodium azide) were incubated for 2 hours or more at 4°C with affinity-purified antisera, washed extensively in PTN, and then incubated for 2 hours or more at 4°C with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibody (Sigma). Controls included no primary antibody, no second antibody, and primary antibody preincubated for 2 hours or longer with FDDYGHMFamide (DSK I), TDVDHVFLRFamide, or FMRFamide at concentrations ranging from 0.0001 M to 0.005 M.

Antisera specificity

The specificity of the antisera was determined by the choice of antigen, affinity purification, and incubation of the antisera with peptides prior to immunohistochemistry. The antigen used, TDVDHVC, is the N-terminal portion of TDVDHVFLRFamide, which avoids raising antisera to the common C-terminal sequence -RFamide. The peptide also includes a cysteinyl residue as a site for chemically linking a carrier. TDVDHV-specific antisera were purified from the whole sera on a TDVDHV-MAP affinity column. Experiments using antigen-inactivated antisera indicate that fluorescence is from dromyosuppressin-like material since preincubation of the antisera with TDVDHVFLRFamide completely abolished all signal, while preincubation of the antisera with FDDYGHMFamide (DSK I) or FMRFamide did not alter the pattern or intensity of fluorescence observed (data not shown). Larval preparations incubated with either primary or secondary antisera alone did not result in observable fluorescence. Also, the immunoreactivity was restricted to a defined set of neurosecretory cells.

RESULTS

The terminology for comparing the cells expressing dromyosuppressin to those expressing FMRFamide-like materials is from White et al. ('86), Schneider et al. ('91), and O'Brien et al. ('91). The description of the number of immunoreactive cells in the central nervous system takes into account the observation that the cells and fibers were all bilaterally symmetric to the midline, i.e., the report of immunoreactivity in one cell indicates that two immunoreactive cells bilaterally symmetric to each other were pres-
Fig. 3. Expression in the larval central nervous system (dorsal view): A: 55 hours. B: Greater than 65 hours. The immunoreactive fibers that extend from the MP2 cells into the ventral ganglion begin to send out immunoreactive projections (open arrow; A). SP3 cells send immunoreactive fibers to the ring gland (large arrowhead in A and B). An immunoreactive fiber is seen to project from the abdominal ganglia (solid arrow in A and B). T1-3, VC, and A8 cells that appear in the ventral ganglion are illustrated by bars, small arrowheads, and open white arrows, respectively (B). Bars = 50 μm.

Fig. 4. Expression in the larval superior protocerebrum and subesophageal ganglion (dorsal view). Signals are observed in the superior protocerebrum in cells tentatively identified as SP2 cells (horizontal arrows) and in SP3 cells (vertical arrows). The MP2 cells (small white arrows) are out of focus because a whole mount preparation was used. Open arrows point to cells in the subesophageal ganglion. Bar = 50 μm.

Expression of dromyosuppressin immunoreactive material in the embryonic central nervous system

No fluorescence signal was observed in embryos at stage 15 (11.5–13 hours). However, faint signals were observed at stages 16 (13–16 hours) and 17 (> 16 hours) in the brain lobes in two cells in the medial protocerebrum and fibers that project from the cell bodies into the ventral ganglion (data not shown).

Expression of dromyosuppressin immunoreactive material in the larval central nervous system

Fluorescence was observed in larvae at less than 5 hours in the two medial protocerebrum cells (MP2) and the fibers projecting from these cells as well as in two neurosecretory cells (SP3) located in the superior protocerebrum (Fig. 1). The cells are identified as MP2 and SP3 from double-labeling experiments using DSK-specific and TDVDHV-specific antisera (Tibbetts and Nichols '93).

At 15–20 hours, the immunoreactive fibers that project from the MP2 cells into the ventral ganglion began to branch and become more complex (Fig. 2). Although the significance of the pattern formed by these immunoreactive fibers is not known, it is likely that this configuration allows for release of the peptide directly onto as yet unidentified target cell(s). In early larvae, signal from the SP3 cells was faint but during larval development the signal intensified, ent. The signal intensities were strong except where specifically mentioned.
and immunoreactive fibers can be observed to project from the SP3 cells into the ring gland (Fig. 3).

Immunofluorescence appears in ventral ganglion cells that are similar in position to T1–3, A8, and VC cells containing FMRFamide-like immunoreactive material. T1–3 represents three individual cells, T1, T2, and T3, laterally positioned one cell in each of the three thoracic ganglia, A8 is a single cell in the posterior tip of the ganglion, and VC is a chain of approximately seven cells along the midline of the ventral ganglion (Fig. 3). None of the ventral ganglion cells appeared to have immunoreactive fibers, as observed for the cells in the protocerebrum. The intensity of the signal observed was significantly less than that observed for the MP2 and SP3 cells. It is not possible from these experiments to determine whether the difference in intensity is due to the amount of peptide expressed or the affinity of the antisera for the antigen(s) present.

During larval development there was an increase in the number of cells in the central nervous system containing immunoreactive material with the addition of a cell in the subesophageal ganglion and two more cells in the superior protocerebrum (Fig. 4). Overall, the expression of dromyosuppressin immunoreactive material in the larval stage began with just two cell types and progressed to seven. There was a marked increase in the number of immunoreactive cells and the signal intensity with the exception that the signal in the subesophageal cell became less intense during the latter part of the larval stage.

**Expression of dromyosuppressin immunoreactive material in the pupal central nervous system**

Early pupal expression was distinguished by the loss of signal in the superior protocerebrum cells and one of the two MP2 cells, while at the same time two sets of optic lobe cells and two ventral medial subesophageal cells appeared (Fig. 5). As the pupae developed, the number of SP3 cells increased to six and the arborization of the SP3 immunoreactive fibers increased (Fig. 5). The subesophageal cell seen in larvae was not observed in the pupal stage. However, it is important to note that it is difficult to distinguish the different subesophageal cells from one another. The ventral medial subesophageal cells expressing dromyosuppressin are similar in position to the SVM cells containing FMRFamide-immunoreactive materials.

Signal was still observed in cells of the ganglion; however, the immunoreactive fibers were more prominent (Fig. 6). The reduction of cellular signal may, in some part, have resulted from the thickness of the tissue preparations.
Expression of dromyosuppressin immunoreactive material in the adult central nervous system

In general, the cellular pattern of immunoreactivity in the adult was similar to that of the late pupal stage (Fig. 7). However, signal intensity and arborization of immunoreactive fibers increased significantly, and there was a dramatic increase in signal from the varicosities associated with the immunoreactive fibers observed with the adult age (Figs. 7, 8). Cells in a similar position as SC cells were more apparent than in pupae, and an optic lobe cell displayed an increased arborization similar to that observed in the blowfly (Nässel et al. '88).

In addition, dromyosuppressin expression was seen in a cell near the position where SP2 cells are located (Fig. 7). This finding led to the question of whether immunoreactivity in SP2 returns in the adult after disappearing in the pupal stage or if this was a different but similarly positioned cell. Although the exact identity of the cell based on position was difficult to establish in a whole mount preparation, the observation remains an interesting question since expression either returns in only one of the two SP2 cells observed during the larval stage or else begins late in development in a cell not previously seen to express the peptide.

While dromyosuppressin expression in a subesophageal cell thought to be SE was present during the larval stage and absent during the pupal stage, a weak signal was inconsistently observed in the adult. Because the intensity of the signal was low, the question remains as to the exact identity of the cell and the pattern of expression in the SE cell.

The immunoreactivity in the adult ganglion was very similar to that observed in pupae: the signal was mainly from varicosities of the immunoreactive fibers while cellular expression was present but less intense.

Expression of dromyosuppressin immunoreactive material in larval and adult gut tissue

Although no immunoreactivity was seen in the larval proventriculus, midgut, or hindgut, immunoreactivity is observed in two cells near the anus (Fig. 9). Relative to one another, the two cells in the larval gut are at very different focal planes, which may indicate that the larval cells are on opposite sides of the anus.

In the adult gut, immunoreactivity was seen in two cells of the rectum (Fig. 10). Similar to the larva, the cells appeared in very different focal planes, suggesting that they
Fig. 7. Expression in the adult central nervous system (dorsal view): 6 days. Signal is observed in the SP3 and MP2 cells, and cells in the optic lobe and subesophageal ganglion. An open arrow points to a cell in the region of SP2 (refer to text). Bar = 50 μm.

too may be located on opposite sides of the rectum. Immunoreactive fibers extending from the central nervous system were observed in the crop.

The absence of immunoreactivity in the remaining regions of both the adult and larval gut was further examined. In addition, larvae and adults were analyzed for immunoreactivity outside of the central nervous system and gut. Two approaches were used to monitor the quality of experiments and presence of signal: 1) central nervous systems were incubated along with the gut tissue; and 2) DSK I antisera that localize to the midgut (Nichols, '92b) were used. These studies indicated that the lack of signal in the midgut was not due to technical difficulties (data not shown). In addition to the control studies, the high signal to low background staining in the gut preparations suggest that no immunoreactivity went unobserved. No cell expressing dromyosuppressin was observed in tissue other than the central nervous system and gut.

DISCUSSION

The insect brain contains neurosecretory cells that differ from other neurons by the presence of membrane-bound secretory granules and are thought to make use of biologically active peptides as messenger substances (Scharrer, '58, '59, '82). To learn about the role(s) of peptidergic neurons in Drosophila brain, we have undertaken the task of isolating and identifying the structure of naturally occurring peptides containing -RFamide and determining cellular expression by using sequence-specific antisera. The expression pattern of specific neuropeptides used in conjunction with genetic and molecular studies may provide the opportunity to determine peptide function(s).

Sequence-specific antisera were used to determine the developmental expression pattern of dromyosuppressin, an abundant peptide isolated from Drosophila. Dromyosuppressin expression began early in development in the embryonic central nervous system and had a net increase in both the number of cells and signal intensity through development. Immunoreactivity was first observed in the brain and then in the ventral ganglion. At each of the numerous time points studied, signals were observed suggesting that the neuropeptide gene is expressed without disruption throughout Drosophila development. While the vast majority of cells continue to express dromyosuppressin, the expression in three cells—a MP2 cell, and cells in similar positions to a SE cell and the SP2 cells—was restricted to specific developmental periods.

The patterns of gut expression in larva and adult are similar, suggesting that the peptide may serve a similar function at both stages. In addition, immunoreactive fibers are observed in the crop, an adult structure that utilizes
extensive muscle activity. The presence of dromyosuppressin immunoreactivity in the gut as well as the central nervous system indicates that control of gene expression is tissue-specific as well as developmental and that dromyosuppressin is a brain-gut peptide.

The question of whether DMS and other peptides containing -RFamide are expressed in the same cells is interesting from functional and regulatory aspects. Dromyosuppressin appears to be expressed in some of the same cells that have been identified as containing FMRFamide immunoreactive material. However, since antisera to FMRFamide recognize DMS and DSK peptides as well as the five -FMRFamide-containing peptides encoded in the FMRFamide gene, the immunohistochemical data obtained using antisera to FMRFamide are ambiguous, and the cellular expression patterns of the FMRFamide gene products are not known.

Meola and co-workers used antisera raised to the cockroach leucomyosuppressin peptide to study leucomyosuppressin-like immunoreactivity in the adult stable fly (Meola et al., '91). The expression pattern differs between fruitfly and stable fly, with the majority of leucomyosuppressin-like immunoreactivity in stable fly observed in the thoracic ganglion. The basis of the difference in expression between the fruitfly and the stable fly is not clear. However, possibilities may include a difference in species-specific expression and/or differences in the avidity of the antisera. No information was reported as to whether immunoreactivity was observed in stable fly earlier in development or in the gut.

Fig. 8. Expression in the adult central nervous system (dorsal view): 11 days. Increased signal intensity is observed in the older adult central nervous system. (Signal originates from one MP2 cell.) Bar = 50 μm.

Fig. 9. Expression in the larval gut (dorsal view). A: Two immunoreactive cells (white arrows) near the posterior of the larval gut (an open arrow points to the posterior spiracles). B, C: Enlargements of the same cells. Bars = 50 μm.
peptides exert in vivo. *Drosophila melanogaster* provides the opportunity to utilize an organism amenable to genetic manipulation as well as biochemistry, molecular biology, and physiology to study the role(s) of this peptide with the possibility of elucidating function.

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### LITERATURE CITED


![Fig. 10. Expression in the adult: A: Central nervous system, proventriculus, and crop. B: Rectal cells. Immunoreactive fibers observed in the crop (A) extend from the central nervous system. At two different planes of focus, illustrated by B and C, two immunoreactive cells and fibers projecting from the cells can be observed in the rectum. Bars = 50 μm.](image-url)


