

A PERITRACHEAL NEUROPEPTIDE SYSTEM IN INSECTS: RELEASE OF MYOMODULIN-LIKE PEPTIDES AT ECDYSIS

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Summary

We identified a set of neuropeptide-expressing cells sited along the respiratory system of *Drosophila melanogaster* using an antibody to the molluscan neuropeptide myomodulin. The number and positions of these 'peritracheal' myomodulin (PM) cells were reminiscent of the epitracheal Inka cells in the moth *Manduca sexta*. These Inka cells release the peptide ecdysis-triggering hormone, which helps elicit ecdysial behavior at the molt, and we show that they are also recognized by the myomodulin (MM) antibody. In both *D. melanogaster* and *M. sexta*, the PM and Inka cells are the only MM-positive cells outside the central nervous system. In both insects, MM immunoreactivity disappears at the end of the molt. In *D. melanogaster*, we have monitored the PM cells throughout development using two enhancer trap lines; the PM cells persist throughout development, but at larval, pupal and adult ecdyses, they display a loss of MM immunoreactivity. This transient loss occurs at a predictable

time, just prior to ecdysis. In contrast, MM-positive neurons in the central nervous system do not show these changes. The PM cells also reveal a concomitant loss of immunostaining for an enzyme contained in secretory granules. The results are consistent with the hypothesis that the PM cells release MM-like peptides just prior to each ecdysis.

In addition, we demonstrate that peritracheal cells of five widely divergent insect orders show a myomodulin phenotype. The peritracheal cell size, morphology, numbers and distribution vary in these different orders. These data suggest that peritracheal cells release MM-like peptides as part of a conserved feature of the endocrine regulation of insect ecdysis.

Key words: molting, ecdysis, peptide, myomodulin, hormone, *Drosophila melanogaster*, *Manduca sexta*, *Schistocerca americana*, Inka, enhancer trap.

Introduction

Insect molting involves the coordination of diverse developmental events with the precise behavioral patterns that produce ecdysis. This coordination is controlled by the neuroendocrine system. Here, we describe a neuropeptide system in *Drosophila melanogaster* that participates in the molting process. The cellular components of this neuropeptide system were detected using an antibody to the molluscan neuropeptide myomodulin (MM). The MM peptides are a large family of neuropeptides that were first identified in the mollusc *Aplysia californica* (Cropper *et al.* 1987), but MM immunoreactivity has also been found in the central nervous system (CNS) of a variety of other organisms including the leech (Keating and Sahley, 1996), crab (Christie *et al.* 1994), locust (Swales and Evans, 1994) and blowfly (Nassel *et al.* 1994). In *D. melanogaster*, the MM antibody recognized both neurosecretory cells in the CNS and a novel set of cells along the tracheae in the periphery (O'Brien and Taghert, 1994). In this report, we describe these *D. melanogaster* 'peritracheal cells' and provide evidence that they are functional homologs of the epitracheal 'Inka' cells described in the lepidopterans

Manduca sexta and *Bombyx mori* (Zitnan *et al.* 1996; Adams and Zitnan, 1997).

Inka cells are endocrine cells, distributed along the tracheae, that produce the ecdysis-triggering hormone (ETH). ETH is a potent stimulator of ecdysial motor outputs by the isolated CNS (Zitnan *et al.* 1996; Adams and Zitnan, 1997) and represents one of three principal neuroendocrine components known to regulate ecdysis behavior. The first hormone identified as a regulator of ecdysis behavior, eclosion hormone (EH), is produced by a small number of brain cells (for a review, see Truman, 1992). These EH neurons release peptide from terminals within the CNS as well as into the circulation (Hewes and Truman, 1991). Ewer *et al.* (1997) have recently shown that ETH activates neurons within the brain, causing a release of EH within the CNS which is, in turn, thought to stimulate the release of the crustacean cardioactive peptide (CCAP) (Stangier *et al.* 1988; Cheung *et al.* 1992; Davis *et al.* 1993), the third endocrine regulator of ecdysis (Ewer *et al.* 1997; Gammie and Truman, 1997a,b). In response to EH, a CCAP-positive network of CNS neurons in Lepidoptera displays a transient elevation of cGMP levels (Ewer

et al. 1994, 1997) and an increase in excitability (Gammie and Truman, 1997a). CCAP is necessary for the transition from pre-ecdysis to ecdysis behavior (Gammie and Truman, 1997b). A transient elevation of [cGMP] is also observed in the Inka cells just prior to ecdysis; EH has been shown to elicit this increase, suggesting a positive feedback system between the EH neurons and Inka cells (Ewer *et al.* 1997).

Several lines of evidence support the idea that many features of the endocrine system controlling ecdysis behavior are conserved among different insects. EH activity and immunoreactivity have been found in diverse insect orders (Truman *et al.* 1981; Truman, 1992), and EH genes have been cloned from two orders (Horodyski *et al.* 1989, 1993; Kamito *et al.* 1992). Ewer and Truman (1996) reported that a CCAP-positive network of neurons that displays a transient increase in [cGMP] at ecdysis is conserved in a variety of insects. To date, ETH peptides have only been identified in the lepidopterans *Manduca sexta* (Zitnan *et al.* 1996) and *Bombyx mori* (Adams and Zitnan, 1997), and it has yet to be established whether the function of ETH peptides is a general feature of insect ecdysis.

In *D. melanogaster*, there is a conserved EH gene (Horodyski *et al.* 1993) whose function has been analyzed by genetically ablating the EH cells (McNabb and Truman, 1997). However, the EH-responsive neurons in *D. melanogaster* and in other higher dipterans may differ from those of most other insects. Only a few CNS neurons displayed an increase in [cGMP] at ecdysis, and they did not correspond to the CCAP-immunoreactive neurons (Ewer and Truman, 1996). An ETH/Inka cell system in *D. melanogaster* has not been identified. In this report, we present evidence that the MM-positive peritracheal cells are homologs of the Inka cell. We use a variety of markers to characterize these cells in *D. melanogaster*, including two enhancer trap lines (Bellen *et al.* 1992; Bier *et al.* 1992) that give reporter expression in peritracheal cells. We consider the functional roles of the peritracheal cells during ecdysis and present evidence that the MM-like peptides are distinct from the ETH-like peptides. Finally, we extend our observations to determine the prevalence of a myomodulin neuropeptide phenotype in cells associated with tracheae in other insect species.

Materials and methods

Fly stocks

The *Drosophila melanogaster* stocks used in this study were an Oregon R wild-type strain, and transformed lines including a *trachealless* enhancer trap line (Wilk *et al.* 1996) and the 36y and c929 enhancer lines. 36y and c929 have a P[gal4] construct (Brand and Perrimon, 1993). To monitor reporter gene expression, the 36y and c929 lines were crossed to either UAS-*lacZ*, in which case the progeny express β -galactosidase (β -gal), or a UAS-GFP line, in which case the progeny express a green fluorescent protein.

Other insect species

We used laboratory stocks of the tobacco hornworm

Manduca sexta (kindly provided by J. Nardi, J. Truman and W. Bollenbacher) and the grasshopper *Schistocerca americana* (kindly provided by M. Bastiani). The representative larval insects from other orders were collected in the environs of St Louis and classified according to Stehr (1987, 1991).

Antibodies

Several antisera and monoclonal antibodies were used in this study, and they are listed in Table 1. We monitored *lacZ* expression using a monoclonal antibody to β -gal (1:2000 dilution) (Promega).

Immunocytochemistry

Whole-mount preparations of CNS, of body wall fillets with tracheae intact or of other tissues as controls were dissected in saline (10 mmol l⁻¹ Tris-HCl, 200 mmol l⁻¹ KCl, 50 mmol l⁻¹ NaCl, 0.2 mmol l⁻¹ CaCl₂) and fixed in either 4% paraformaldehyde or aqueous Bouin's. Tissues were rinsed in phosphate-buffered saline plus 0.1% Triton X (PBST), blocked in 5% normal goat serum, and incubated in primary antibody overnight at 4°C. Tissues were rinsed in PBST and incubated in secondary antibody for 2 h at room temperature (20–22°C), before being rinsed and cleared in 70% glycerol. Preparations were mounted in Vectashield (Vector Labs) for fluorescence microscopy. The secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit, anti-mouse, anti-rat or anti-guinea-pig IgG, Texas-Red-conjugated goat anti-rabbit or anti-mouse IgG and horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG (Vector Labs, Jackson Immunoresearch). All secondary antibodies were used at a dilution of 1:200.

Myomodulin antisera specificity test

For blocking studies using myomodulin peptide, two separate experiments were performed. In the first, 5.8×10^{-4} mol l⁻¹ MM (0.5 μ g peptide ml⁻¹ anti-MM antiserum, diluted 1:5000) was incubated overnight at 4°C before being added to tissue. In the second, MM peptide (M_r 862) was compared with ETH (M_r 2942) and an unrelated *D. melanogaster* neuropeptide DPKQDFMRFamide (M_r 1183); 10 μ g ml⁻¹ of each was preincubated with the anti-MM antisera (1:1500 dilution) overnight at 4°C. ETH and MM were also tested at 1 μ g ml⁻¹. In both experiments, the tissues were then processed as above.

Dot blots

MM, ETH and DPKQDFMRFamide were spotted onto nitrocellulose filters (Schleicher and Schuell) at 0.1, 1.0 and 10 μ g μ l⁻¹, and the filters were dried for 15 min at 80°C and tested for immunoreactivity according to Hawkes *et al.* (1982). Blots were rinsed, blocked in PBS + 1% bovine serum albumin and 10% normal goat serum, and then incubated in MM antisera (1:1000) for 2–4 h at room temperature. After rinsing, the blots were incubated in anti-rabbit HRP-conjugated secondary antibody and developed using the peroxidase substrate 4-chloro-1-naphthol (Sigma).

β -Galactosidase histochemical staining

Wholemount preparations were dissected as above and fixed in 4% paraformaldehyde for 15 min, rinsed in PBS, and then incubated in a solution of 0.2% X-gal (U.S. Biochemicals) in substrate solution at 37°C (Ashburner, 1989). For double-labeling of X-gal and different antibodies, the X-gal-stained tissues were rinsed and refixed for 1 h, and then incubated in primary and secondary antibody as above.

Enhancer trap selection

Approximately 1500 lines from an enhancer trap mutagenesis screen (K. Kaiser, University of Glasgow) were analyzed for reporter gene expression in adult whole-mount CNS preparations. 36y and c929 (Schaefer and Taghert, 1995) were selected for their strong expression in neurosecretory cell terminals on the dorsal surface of nerve cords.

Results*A peritracheal neuropeptide system in Drosophila melanogaster*

We identified a novel site for neuropeptide expression in *D. melanogaster* using an antibody directed against the *Aplysia californica* neuropeptide myomodulin (MM) (Miller *et al.* 1991). MM immunoreactivity has been found in

neurosecretory cells in other molluscs (Santama *et al.* 1994), leech (Keating and Sahley, 1996), locust (Evans, 1994; Swales and Evans, 1994) and blowfly (Nassel *et al.* 1994). In larval *D. melanogaster*, this antibody also labeled several CNS neurosecretory cells but, in addition, it recognized a set of cells associated with the main dorsal trunk of the tracheal system (Fig. 1A–D). There are two labeled cells per segment, one on each side, in at least seven segments. Two previous studies described cells that may be related to these peripheral MM-positive cells of *D. melanogaster*. Pairs of cells found attached to tracheae, at similar positions in each hemisegment in *D. melanogaster* embryos, were named ‘peritracheal cells’ by Hartenstein and Jan (1992) in their study of enhancer trap lines. Also, Zitnan *et al.* (1996) described a segmentally repeated set of epitracheal cells in the lepidopteran *Manduca sexta* and called them ‘Inka’ cells. We therefore refer to the MM-antibody-positive tracheal cells of *D. melanogaster* as peritracheal myomodulin (PM) cells, but since we have not identified PM cells in embryos, we do not know their relationship to the embryonic peritracheal cells of Hartenstein and Jan (1992). In larvae, the PM cell is approximately 10 μ m in diameter and frequently has either a single long process (Fig. 1A,B) or several shorter processes, giving it an astrocytic appearance (Fig. 1C). It has a stereotyped position in each segment, adjacent to the branchpoint from which the transverse

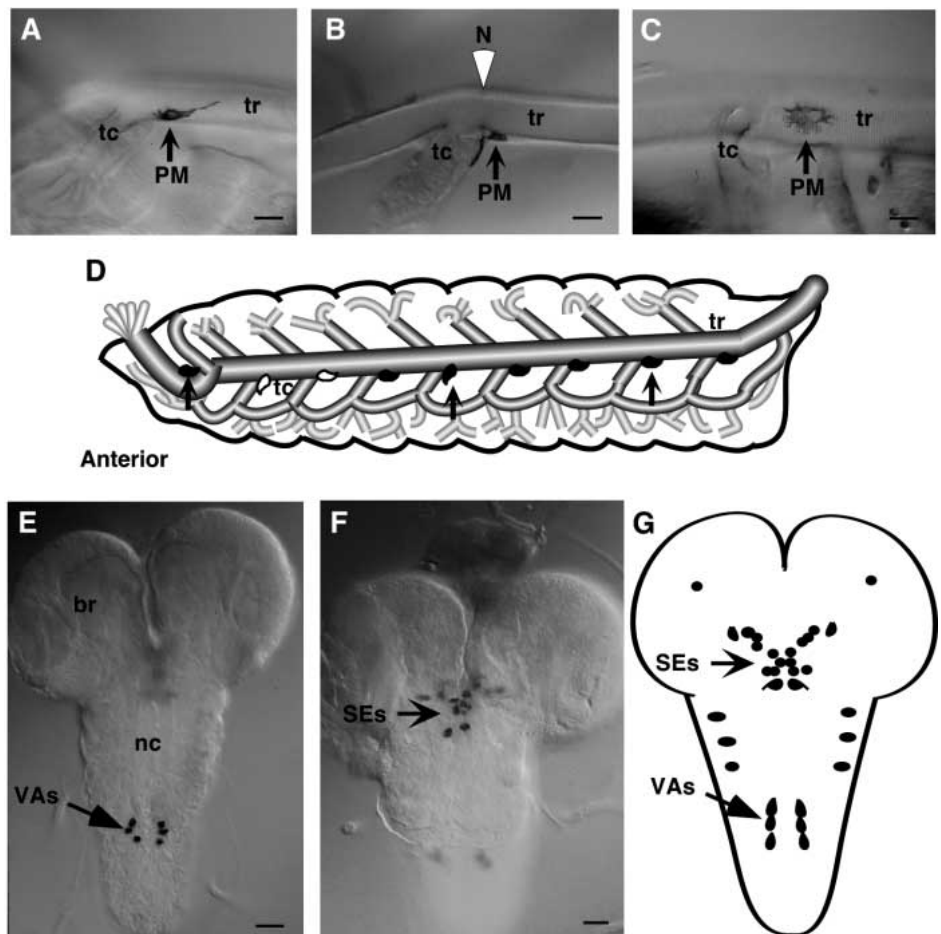


Fig. 1. Immunoreactivity in third-instar *Drosophila melanogaster* larvae visualized using myomodulin (MM) rabbit antisera and horseradish peroxidase (HRP)-conjugated secondary antibodies. Staining occurred in cells along the tracheae (A–D) and in the central nervous system (CNS) (E–G). (A–D) Black arrows point to examples of labeled peritracheal myomodulin cells (PM). tr, main tracheal trunk; tc, transverse connective; N, node. (D) Schematic diagram of the larval tracheal system and the positions of MM-immunoreactive cells. The peritracheal cells indicated in black are always observed, while the peritracheal cells indicated in white (thoracic regions) are infrequently detected. (E) The arrow points to neurosecretory cells on the ventral surface of the nerve cord in abdominal segments (VAs). br, brain; nc, nerve cord. (F) The arrow points to a cluster of subesophageal cells (SEs). (G) Schematic diagram of all the MM-immunoreactive cells in the larval CNS. Scale bars in A–C, 20 μ m, in E and F, 40 μ m.

tracheal connective leaves the main longitudinal trachea (Fig. 1D). It lies close to the tracheal node (Fig. 1B), where the main tracheal trunks break and fuse at each molt (Manning and Krasnow, 1993). The PM cell is occasionally found on the transverse connective. A stained PM cell is routinely visible at the most anterior branchpoint (1) and at branchpoints 4–9, but stained cells at branchpoints 2 and 3 are rare (Fig. 1D).

The pattern of MM immunoreactivity in the CNS of *D. melanogaster* is shown in Fig. 1E–G and is virtually identical to the pattern described for the blowfly (Nassel *et al.* 1994). The most prominently staining cells are in two clusters: the first cluster consisted of three (or occasionally four) pairs of

neurosecretory cells at the ventral midline in abdominal segments of the nerve cord (VAs) (Fig. 1E), and the second cluster included several neurons in the subesophageal region of the brain (SEs) (Fig. 1F). The VA axons exit the CNS on the dorsal surface and project into the transverse nerve, a neurohemal release site for neuropeptides (Nassel *et al.* 1994). There is also weaker staining in the thoracic ventral cells and in a pair of cells in the brain (Fig. 1G) to give a total of approximately 32 MM-positive neurons in the larval CNS. An overnight preincubation of the MM antibody (1:5000) with MM peptide ($0.5 \mu\text{g ml}^{-1}$) completely abolished immunoreactivity in both the neurons and peritracheal cells,

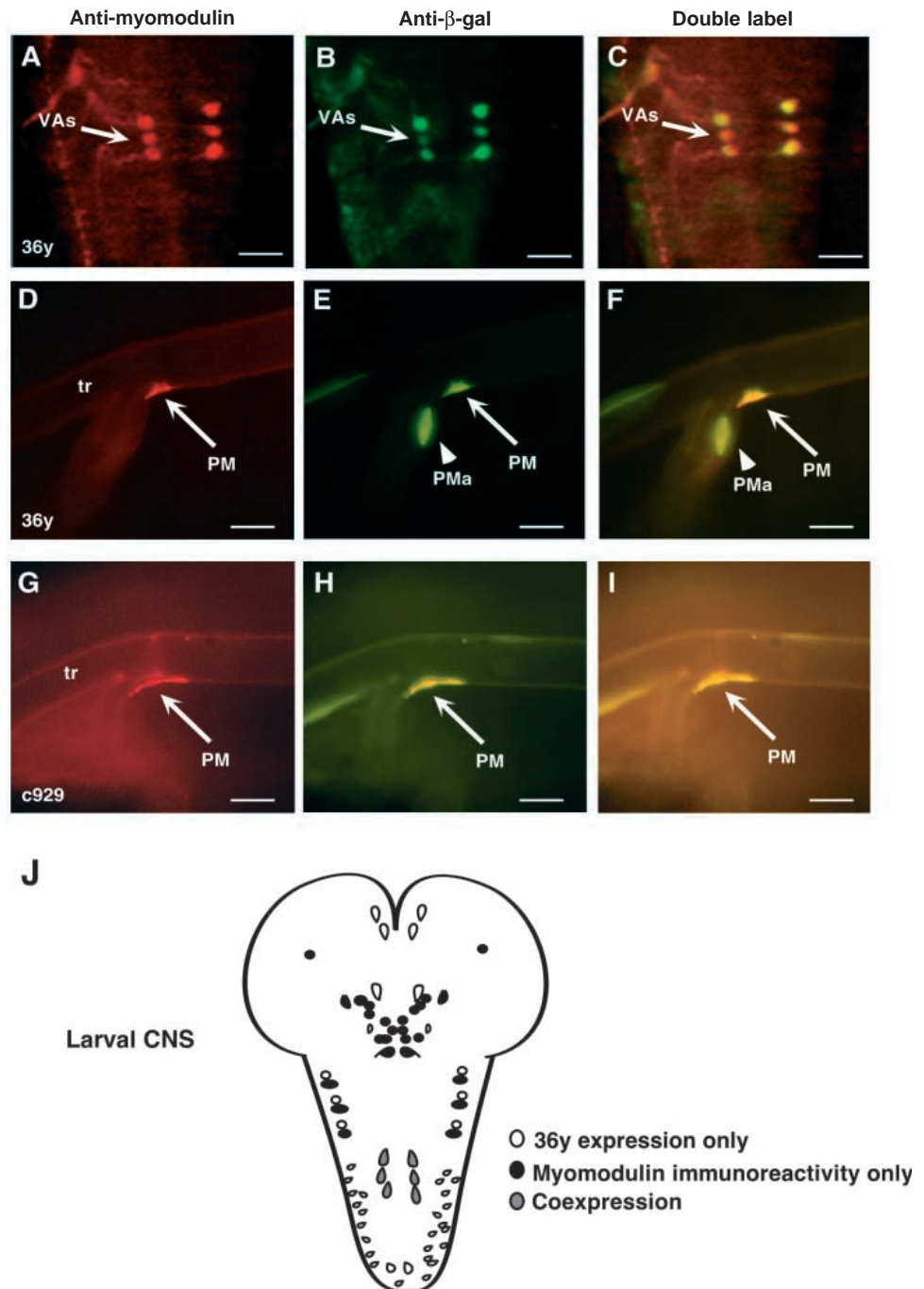


Fig. 2. Double-labeling with MM antisera and β-gal monoclonal antibodies in *D. melanogaster* P(gal4) enhancer trap lines. (A–C) Double-labeling in the CNS of the 36y enhancer line. Arrows point to double-labeled VA cells. (D–F) Double-labeling in the larval peritracheal cell (PM) of the 36y line. Arrows point to the double-labeled PM cell. An arrowhead denotes the second, larger peritracheal cell (PMA) with reporter gene expression. tr, main tracheal trunk. (G–I) Double-labeling in the larval PM cell in the c929 enhancer detector line. Arrows point to the double-labeled PM cell. 36y and c929 flies were crossed to UAS-*LacZ* flies, and β-gal was monitored in the progeny. (J) The differences between MM immunoreactivity and β-gal staining in the CNS of the 36y line. Scale bars in A–C, 50 μm, in D–I, 25 μm.

whereas controls, incubated in antibody alone, gave strong immunostaining (data not shown).

Two Drosophila melanogaster enhancer trap lines give selective reporter gene expression in both peritracheal cells and CNS neurosecretory cells

We selected two P(gal4) enhancer trap lines on the basis of their reporter expression in identified neurosecretory cells. These two lines (36y and c929) had selective reporter gene expression both in the CNS and in peritracheal cells. In the 36y line, double-labeling experiments using the MM antibody and an antibody against β -galactosidase revealed that both the VA neurosecretory cells (Fig. 2A–C) and the PM cells (Fig. 2D–F) were co-labeled. Both MM immunoreactivity and 36y reporter gene expression were first detectable in the CNS and peritracheal cells in the first larval instar. The 36y line also drives reporter expression in a second peritracheal cell (PMA) that was not MM-immunoreactive in larvae (Fig. 2E,F). The PMA cell was typically found on the transverse tracheal branch, close to the PM cell. It was larger and more rounded than the PM cell, without detectable processes (Fig. 2E,F); the PM cell sometimes had a process extending to the PMA cell (see Fig. 3F). There were other differences between the 36y reporter pattern and MM immunoreactivity: 36y was expressed in additional tissues, including salivary glands, esophagus, hindgut and epidermis, while some MM-positive neurons were not 36y-positive (Fig. 2J). The second reporter line (c929) was also expressed in neurosecretory cells and peritracheal cells; the pattern included approximately 200 CNS neurons (Schaefer and Taghert, 1995) and the PM cells, but not the PMA cells (Fig. 2G–I).

The PM cells are non-neuronal secretory cells

We tested several antibody markers to characterize further the PM and PMA cells (Table 1). First, we determined whether the PM cells are part of the tracheal epithelium. We used an enhancer trap line that has a P-element insertion in the *trachealess* gene and gives *lacZ* reporter gene expression in nuclei of all tracheal epithelial cells. *trachealess-lacZ* is expressed in all tracheal cells from embryonic stage 10 through larval stages (Wilk *et al.* 1996). Double-labeling for X-gal histochemistry and the MM antibody demonstrated that the PM cells are not part of the tracheal epithelium (Fig. 3A). We used an antibody to horseradish peroxidase (anti-HRP), an antibody to the neuron-specific protein ELAV (Robinow and White, 1991) and the monoclonal antibody (Mab) 22C10 (Zipursky *et al.* 1984) as neuronal markers to determine whether the peritracheal cells display a neuronal phenotype. The anti-HRP antibody, which stains all neurons but is not unique to them (Sun and Salvaterra, 1995), gave occasional weak peritracheal cell staining (Fig. 3B). Both the ELAV antibody and the 22C10 Mab were negative for peritracheal cells, but positive for CNS neurons (Table 1). Since ELAV has only been found in post-mitotic neurons to date, and since it is thought to be a general marker of the mature neuronal phenotype (Robinow *et al.* 1988; Robinow and White, 1988), our results indicate that the peritracheal cells are not peripheral neurons.

To test the hypothesis that PM cells are endocrine, we used antibodies against two components of the secretory machinery and an enzyme component of peptidergic secretory granules. Anti-synaptotagmin did not stain the PM cell, while an antibody against human synaptobrevin gave weak but detectable staining (Fig. 3C,D). Because *Aplysia californica* myomodulin is an

Fig. 3. Staining of peritracheal cells in *D. melanogaster* larvae using selected antibodies. (A) Double-labeling of larval tracheae with MM antisera and X-gal in a *trachealess* enhancer line. The arrows point to the immunoreactive PM cells (brown). The nuclei of the cells of the tracheal epithelium are shown in blue. The PM cell does not have a blue nucleus, indicating that it is not part of the tracheal epithelium. tr, main dorsal tracheal trunk; tc, transverse connective. (B) The arrow points to a peritracheal cell stained with an HRP antibody and FITC-conjugated secondary antibodies. (C,D) Double-labeling of the PM cell with MM antisera and a synaptobrevin monoclonal antibody. (C) For MM labeling, the secondary antibody is a FITC-conjugated anti-rabbit IgG. (D) For synaptobrevin labeling, the secondary antibody is a Texas-Red-conjugated anti-guinea-pig IgG. (E) Double-labeling of larval tracheae with X-gal (blue) and an antiserum against the dPHM enzyme (brown) visualized using an HRP-conjugated secondary antibody. The arrow points to the double-labeled PM cell, demonstrating that it is the same as the MM-immunoreactive cell. The PMA cell is not stained with the dPHM antibody (arrowhead). (F) β -gal immunoreactivity in the 36y line. The HRP-conjugated secondary antibody reveals fine processes of the PM cell (arrow). Frequently, a process appears to contact the PMA cell (arrowhead). Scale bars in A, 30 μ m, in B–F, 25 μ m.

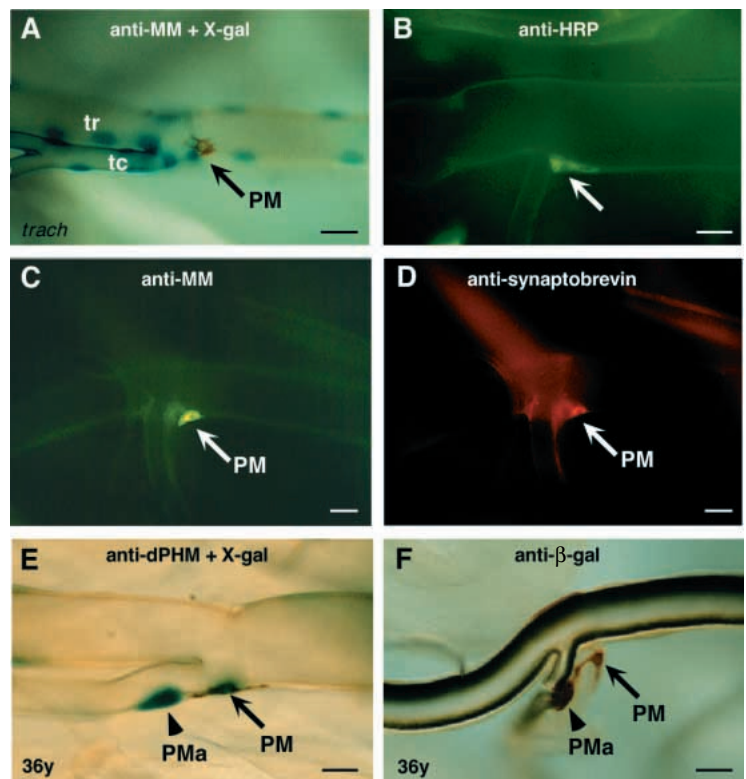


Table 1. Screening for expression in *Drosophila melanogaster* peritracheal cells

	Reference	Immunoreactivity		Source	Dilution
		Peritracheal cells	CNS		
Neuropeptide antibodies					
Anti-myomodulin (<i>Aplysia californica</i>)	1	++	++	Rabbit	1:800–1:5000
Anti-leucokinin (<i>Leucophaea maderae</i>)	2	–	++	Rabbit	1:500
Anti-FMRFamide (<i>Drosophila melanogaster</i>)	3	–	++	Rabbit	1:2500
Anti-FMRFamide precursor (<i>Drosophila melanogaster</i>)	4	–	++	Rabbit	1:2000
Anti-eclosion hormone (<i>Manduca sexta</i>)	5	–	+	Rabbit	1:500
Anti-allatostatin (<i>Manduca sexta</i>)	6	–	++	Rabbit	1:500
Anti-allatostatin (<i>Diploptera punctata</i>)	7	–	++	Mouse	1:2
Anti-SCP (monoclonal) (<i>Aplysia californica</i>)	8	–	+	Mouse	1:50
Anti-SCP (polyclonal) (<i>Aplysia californica</i>)	8	–	–	Rabbit	1:500
Anti-locustamyotropin (<i>Locusta migratoria</i>)	9	–	–	Rabbit	1:2000
Anti-crustacean cardioactive peptide	10	–	–	Rabbit	1:500
Anti-bombyxin (<i>Bombyx mori</i>)	11	–	–	Mouse	1:500
Anti-proctolin (<i>Periplaneta americana</i>)	12	–	–	Rabbit	1:400
Anti-leucomyosuppressin (<i>Leucophaea maderae</i>)	12	–	–	Rabbit	1:500
Other neurotransmitter antibodies					
Anti-Dopa decarboxylase (<i>Drosophila melanogaster</i>)	13	–	++	Rabbit	1:180
Anti-GABA	14	–	++	Rabbit	1:400
Anti-serotonin	14	–	++	Rabbit	1:500
Peptide processing antibodies					
Anti-dFurin 1 (<i>Drosophila melanogaster</i>)	15	–	++	Rabbit	1:1000
Anti-DPHM* (<i>Drosophila melanogaster</i>)	15	++	++	Rabbit	1:100
Secretory protein antibodies					
Anti-synaptotagmin (<i>Drosophila melanogaster</i>)	16	–	+	Rabbit	1:400
Anti-synaptobrevin (human)	17	+	++	Guinea pig	1:500
Neuronal antibodies					
Anti-ELAV (<i>Drosophila melanogaster</i>)	18	–	+	Rat	1:200
Anti-22C10Mab (<i>Drosophila melanogaster</i>)	19	–	+	Mouse	1:5
Anti-horseradish peroxidase	20	+	++	Rabbit	1:500

**Drosophila* peptidylglycine α -hydroxylating mono-oxygenase (amidating enzyme) (Kolhekar *et al.* 1997).

+ weak immunoreactivity; ++ strong immunoreactivity; – no immunoreactivity.

1, Miller *et al.* (1991); 2, Nassel and Lundquist (1991); 3, Taghert and Schneider (1990); 4, Chin *et al.* (1990); 5, Truman and Copenhaver (1989); 6, Zitnan *et al.* (1993); 7, Stay *et al.* (1992); 8, Masinovsky *et al.* (1988); 9, Schoofs *et al.* (1991); 10, Dirksen *et al.* (1992); 11, Mizoguchi *et al.* (1987); 12, Meola *et al.* (1991); 13, Beall and Hirsh (1987); 14, White and Valles (1985); 15, Roebroek *et al.* (1993); Kolhekar *et al.* (1997); 16, Littleton *et al.* (1993); 17, Shone *et al.* (1993); 18, Robinow and White (1991); 19, Zipursky *et al.* (1984); 20, Sun and Salvaterra (1995).

amidated neuropeptide (Cropper *et al.* 1987), we tested whether the PM cell contains enzymes involved in amidation. Specifically, we used an antibody made against the *D. melanogaster* enzyme peptidylglycine α -hydroxylating mono-oxygenase (dPHM) (Kolhekar *et al.* 1997), which helps catalyze amidation of secretory peptide intermediates (Eipper *et al.* 1992). We found that the PM cell was strongly positive for the dPHM antibody. Because the dPHM and myomodulin antibodies were both made in the rabbit, we used the 36y line to show that both antibodies independently label the same peritracheal cell (Fig. 3E). Together, these results suggest that PM is a secretory cell which produces an MM-like precursor that is processed to create amidated peptides.

Finally, as listed in Table 1, we tested several other neurotransmitter and neuropeptide antibody markers. Several antibodies stained the CNS quite strongly, but all were negative for the PM and PMa cells (Table 1).

Disappearance of MM immunoreactivity in the PM cells at ecdysis

Larval ecdysis

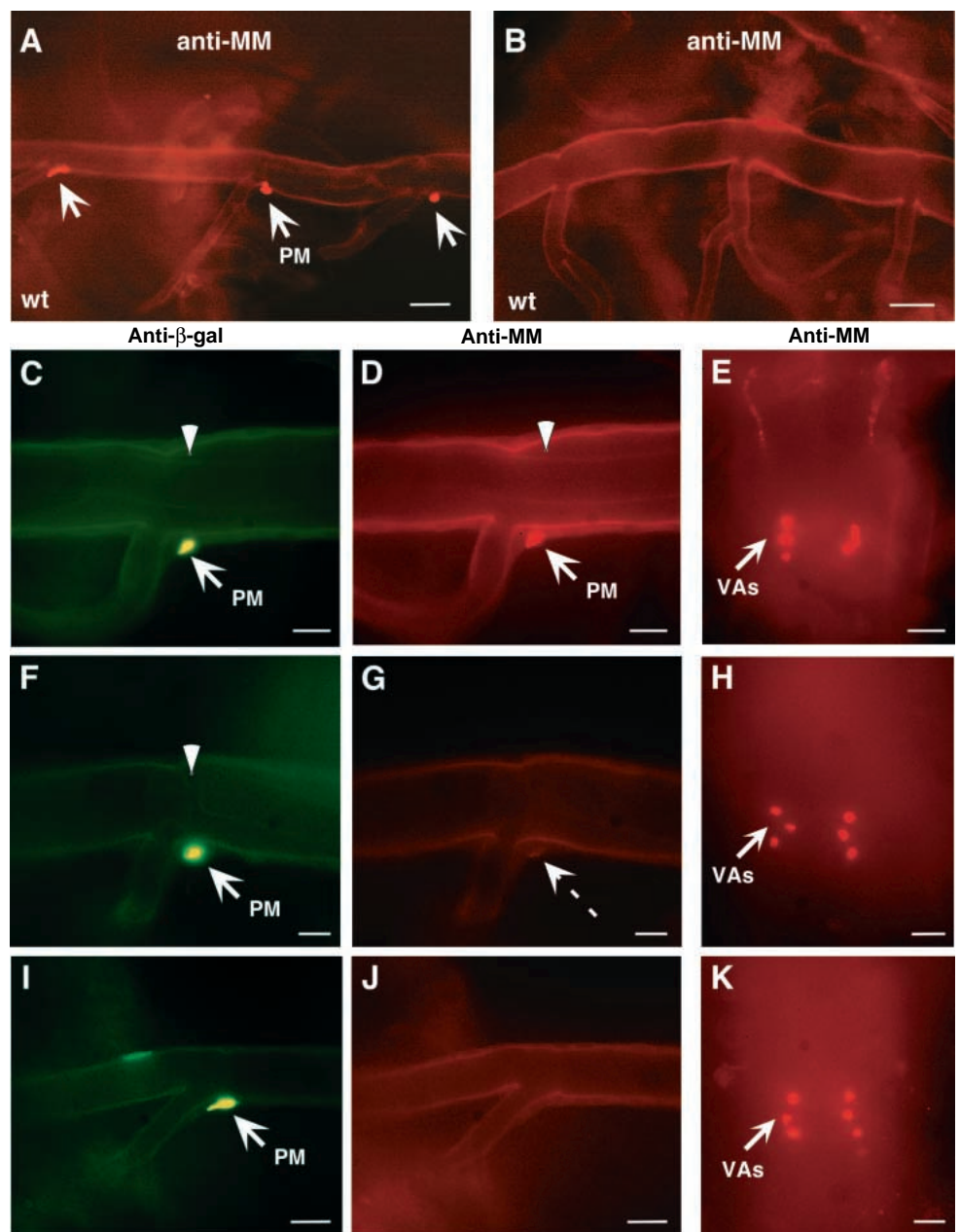
Our characterization of the peritracheal cells suggested that they were endocrine cells, so we monitored both reporter gene expression and MM immunoreactivity throughout development. The previous finding that epitracheal cells in *M. sexta* release peptides at the end of the molt (Zitnan *et al.* 1996) directed our attention to ecdysial stages in *D. melanogaster*. The VA neurons of the CNS showed constant MM immunoreactivity and reporter expression throughout development; in contrast, the PM cells lost MM immunoreactivity at ecdysis (Fig. 4). Second-instar larvae, prior to molting, gave strong immunoreactivity in the PM cells (Fig. 4A), while ecdysing larvae showed no MM immunoreactivity in PM cells (Fig. 4B). We used c929 larvae to monitor the time of this disappearance. Second-instar larvae, close to ecdysis, were selected by the differentiation of new

mouthparts (Ewer and Truman, 1996) and dissected at different stages. The first stage we monitored was prior to differentiation of complete mouthparts, when two sets of mouth hooks were visible. At this double mouth hook stage, both layers of tracheal cuticle were visible and there was strong MM staining in the PM cells (62/63 cells stained, five animals) (Fig. 4C,D) as well as in the VA neurons (Fig. 4E). Subsequently, at the double mouth parts stage (when the larvae were minutes from ecdysis and the old tracheal cuticle was breaking at the nodes), there was a sudden loss of MM immunoreactivity in the PM cells (11/47 cells stained, four animals) (Fig. 4F,G), while strong staining persisted in the VA neurons (Fig. 4H). During and just after ecdysis, there was no MM immunoreactivity in the PM cells (0/50 cells stained, four animals) (Fig. 4I–K). MM immunoreactivity reappeared approximately 1 h after ecdysis (data not shown).

Adult eclosion

The rapid disappearance of PM cell MM immunoreactivity at the end of the larval molt suggested that the peptides had been released. To determine whether MM-like peptides may also be released at subsequent ecdyses, we first established that peritracheal cells are still present in the adult. We used the 36y and c929 lines and showed that adult peritracheal cells are both MM-immunoreactive and reporter-gene-positive (Fig. 5A,B). As expected, dPHM was also expressed in adult PM cells (Fig. 5C). The number of stained adult PM cells was more variable than for larval PM cells, in part because the adult tracheae are more fragile and difficult to dissect than the larval tracheae, and also because the pattern was less precise in its segmental arrangement. The PM cells in some segments were not routinely found near tracheal branchpoints or at nodes, but

Fig. 4. Changes in MM immunoreactivity in *D. melanogaster* during larval ecdysis. (A) Peritracheal cell immunoreactivity in wild-type (wt) larvae before ecdysis from the second to the third instar. Arrows point to the stained PM cells. (B) Immunoreactivity during ecdysis from the second to the third instar. No labeled PM cells are detected along the tracheae. (C–K) c929 larvae were double-labeled with the MM and β -gal antibodies. Texas-Red- and FITC-conjugated secondary antibodies were used, respectively. (C,D) Double-labeling of the PM cell prior to ecdysis when double mouth hooks are visible. Both antibodies bind to the PM cell. The old tracheal cuticle is still visible (arrowhead). (E) MM immunoreactivity in the VA cells in the CNS in the same larva. (F,G) Double-labeling of the PM cell within minutes of ecdysis from the second to the third instar when the old tracheal cuticle is breaking at the node (arrowhead in F). (F) The PM cell is labeled with the β -gal antibody (arrow). (G) The PM cell is barely visible with the MM antibody (dashed arrow). (H) MM immunoreactivity in the VA cells in the CNS at the same stage (arrow). (I,J) Double-labeling of the PM cell within 15 min of ecdysis from the second to the third instar. (I) The PM cell is labeled with the β -gal antibody (arrow). (J) The PM cell is not detected with the MM antibody. (K) MM immunoreactivity remains strong in the VA cells in the CNS in the same larva. Scale bars in A and B, 50 μ m, in C–K, 25 μ m.



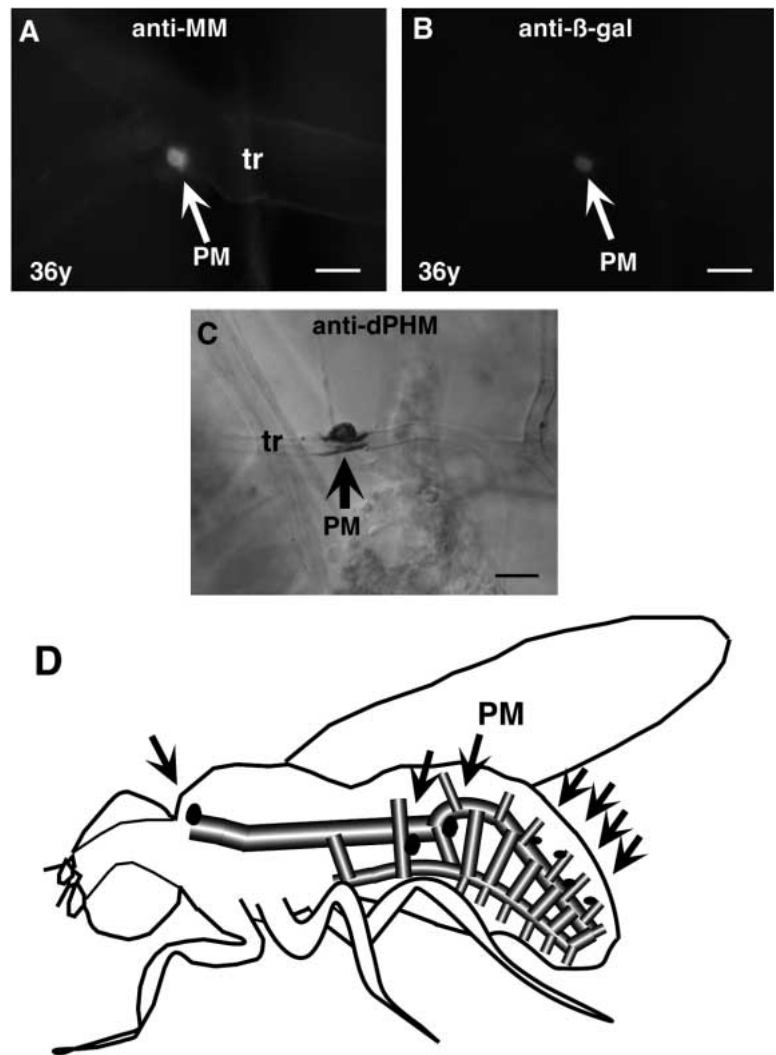


Fig. 5. Double-labeling of *D. melanogaster* adult peritracheal cells in the 36y line with MM, visualized using a FITC-conjugated secondary antibody, and with β -gal, visualized using Texas-Red-conjugated secondary antibodies. (A,B) Arrows point to the double-labeled PM cell. tr, main tracheal trunk. (C) Labeling of adult peritracheal cell with the dPHM antibody and an HRP-conjugated secondary antibody. Double-labeling experiments (not shown here) demonstrated that, as in the larva, it is the MM-positive peritracheal cells in the adult (PM) that express the dPHM enzyme (arrow). (D) Diagram of the adult tracheal system. Black cells denote MM-immunoreactive peritracheal cells in the adult. Thoracic cells were not observed, so the thoracic tracheal system was not included. There are characteristic cells at the two most anterior branchpoints in the abdomen; more posteriorly, the cells are often not at branchpoints and appear more randomly distributed than in the corresponding segments in the larva. Scale bars in A–C, 25 μ m.

instead showed more variable positioning along tracheal branches (Fig. 5D). We did not observe a PMa cell in the adult 36y line; the cell either stops expressing the 36y reporter in the adult or it dies during development.

To assess PM cell activity at adult eclosion, pharate adults were staged according to Kimura and Truman (1990). We again used the enhancer lines to monitor the PM cells and found both reporter gene expression and MM immunoreactivity in pharate adults (Fig. 6A,B), but at adult eclosion there was virtually no detectable MM immunoreactivity (Fig. 6C,D). Occasionally, a remaining spot of immunoreactivity within the PM cell could still be detected (Fig. 6D). Pharate adults were then staged to determine precisely when MM immunoreactivity disappears (Fig. 7). After the meconium moves posteriorly, pharate adults are within 10 h of eclosion (Kimura and Truman, 1990). At this stage (called smooth, prior to fluid reabsorption and 10–7 h before eclosion), all PM cells had strong MM immunoreactivity. Likewise, at the smooth/grainy and grainy stages (during fluid reabsorption and 7–1 h prior to eclosion), most of the PM cells were still MM-immunoreactive. After tracheal air-filling, at the white stage (1–0.7 h prior to eclosion),

most of the PM cells had lost MM immunoreactivity. At the ptilinum extended stage (0.7–0 h before eclosion), virtually all the PM cells had lost MM immunoreactivity (Fig. 7). These data demonstrate that there is a sudden loss of immunoreactivity shortly before adult eclosion. After adult eclosion, weak MM immunoreactivity returned within 1 h in the PM cells (Fig. 7).

Head eversion–pupal molt

We then monitored both reporter gene expression and MM immunoreactivity during metamorphosis. Pupation occurs at stage P4ii, when the head everts in the puparium (Bainbridge and Bownes, 1981). At all prepupal stages prior to this, we found reporter gene expression and MM immunoreactivity in the PM cells. During pupal stages P5–P6, the PM cells continued to display strong reporter expression, but they were negative for MM immunoreactivity (Fig. 6G,H). This rapid disappearance of MM immunoreactivity closely correlated with head eversion at pupation. To define this time more precisely, we selected 36y and c929 puparia that were within 4 h of pupation (buoyant puparia, Rodriguez *et al.* 1990) and aged them for selected times before immunostaining with MM and β -gal antibodies. We

found that there was strong MM immunoreactivity within 1–0.5 h of head eversion (84/84 cells stained, seven animals) (Fig. 6E,F), but at head eversion the staining had disappeared (0/92 cells stained, eight animals) (Fig. 6G,H). MM immunoreactivity in the CNS persisted throughout pupation, as it had during larval ecdysis and adult eclosion. MM immunoreactivity in the peritracheal cells returned by the pharate adult stage (P7) (Bainbridge and Bownes, 1981).

If the MM-like peptides are being released at the ecdyses, we would also expect to see a simultaneous decrease in dPHM immunoreactivity. Since many of the biosynthetic enzymes that produce the final forms of secretory peptides are packaged with their substrates within secretory granules (Eipper *et al.* 1992), we monitored dPHM immunoreactivity around the time of head eversion and adult eclosion (Fig. 6I–L). Just prior to adult eclosion (Fig. 6I) and head eversion (Fig. 6K), dPHM

immunoreactivity was strong, but at adult eclosion (Fig. 6J) and head eversion (Fig. 6L) there was no dPHM immunoreactivity in PM cells. Although we did not monitor MM-like peptides in the hemolymph at ecdysis, the rapid and complete loss of MM immunoreactivity at precise developmental times, and the concomitant loss of dPHM immunoreactivity, strongly suggests that the PM cell releases MM-like neuropeptides at ecdysis.

Metamorphosis of the peritracheal cell system

Because the positions of larval and adult PM cells differed (see above), we wished to know whether the two sets of cells were identical or whether the larval set was replaced at metamorphosis. Using the 36y and c929 lines and focusing on metamorphosis from the time prior to head eversion until adult eclosion, extensive observations made before, during and after metamorphosis suggested that there is continuous reporter

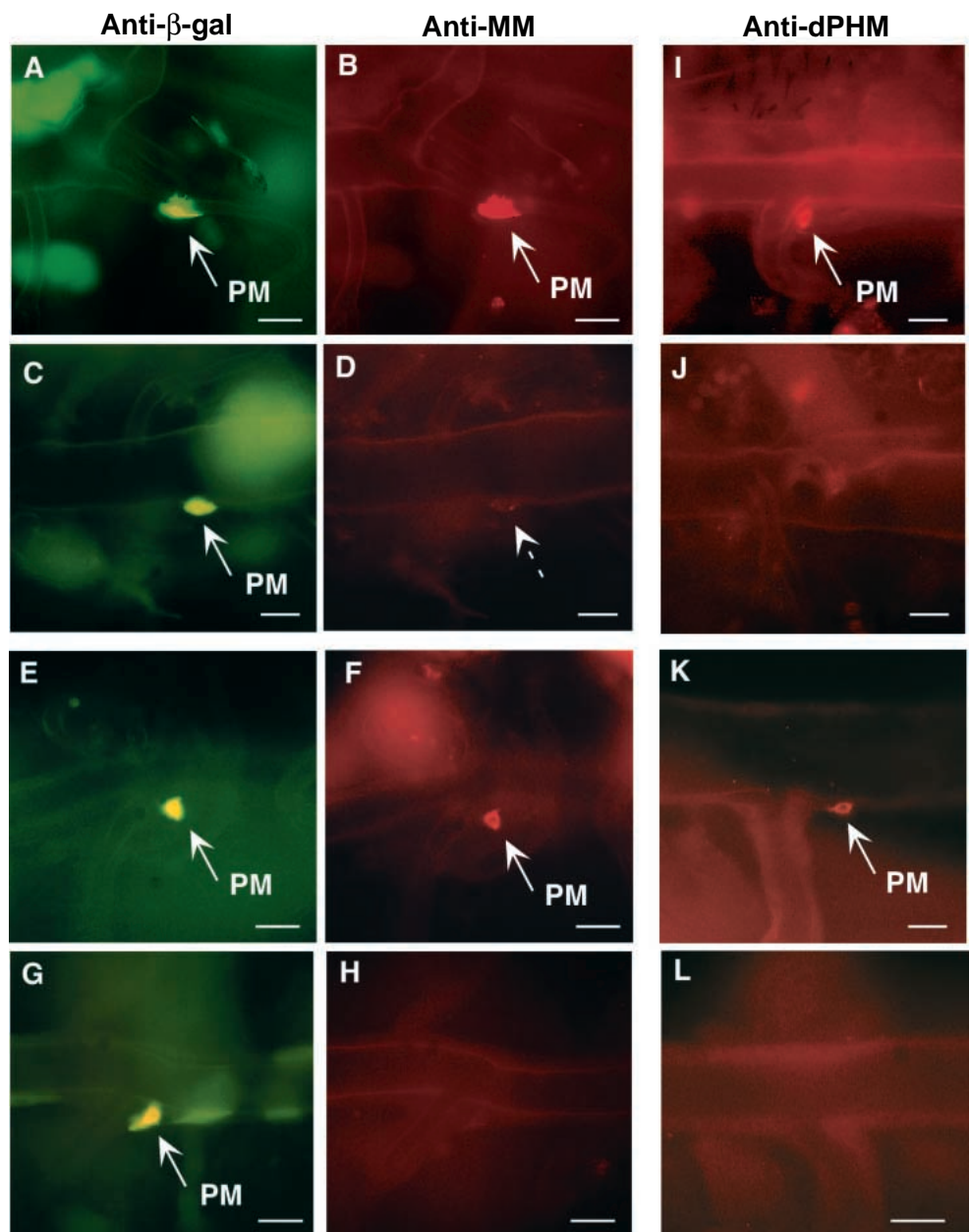


Fig. 6. Changes in MM and dPHM immunoreactivity in *D. melanogaster* PM cells at adult eclosion and pupal ecdysis. (A,B) Double-labeling with β -gal visualized with a FITC-conjugated secondary antibody and MM visualized with Texas-Red-conjugated secondary antibody in the c929 line prior to adult eclosion (P15, after the meconium has moved posteriorly). Arrows point to the double-labeled PM cell. (C,D) Double-labeling in the 36y line at adult eclosion. The PM cell is stained with the β -gal antibody (arrow) but is barely detectable with the MM antibody (dashed arrow). (E,F) Double-labeling with β -gal and MM antibodies in the c929 line prior to head eversion (within 60 min of head eversion). Arrows point to the double-labeled PM cell. (G,H) Double-labeling in the c929 line during head eversion. The PM cell is stained with the β -gal antibody (arrow), but no MM immunoreactivity is detectable. (I) dPHM immunoreactivity in PM cells in a wild-type pupa before adult eclosion (P14) (arrow). (J) No dPHM immunoreactivity is detected at adult eclosion. (K) The arrow denotes dPHM immunoreactivity prior to head eversion. (L) No dPHM immunoreactivity was detected at head eversion. Scale bars, 25 μ m.

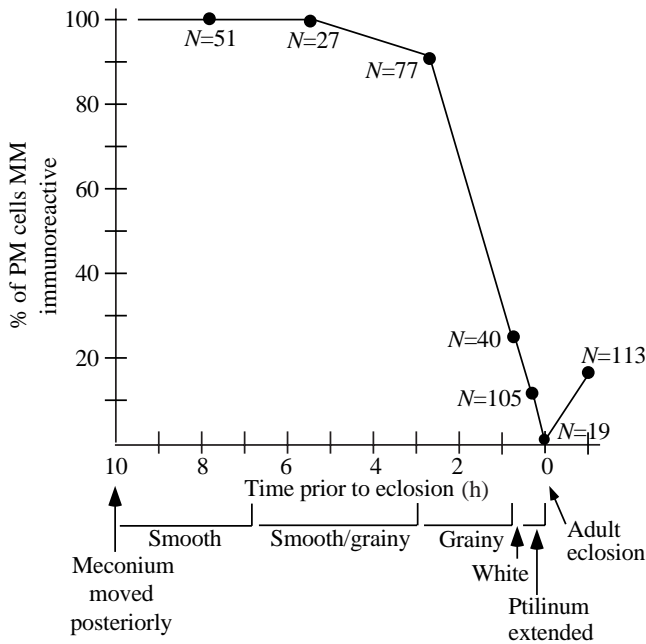


Fig. 7. Temporal assessment of the disappearance of MM immunoreactivity around adult eclosion. Double-labeling with the β -gal and MM antibodies of *D. melanogaster* pupae, prior to adult eclosion, staged according to Kimura and Truman (1990). 36y and c929 pupae were selected if the meconium had moved posteriorly (within 10 h of eclosion); the operculum was removed and pupae were monitored and staged. At the smooth and smooth/grainy stages, 100 % of β -gal immunoreactive cells were MM-immunoreactive (five and three animals represented, respectively). At the grainy stage, 90 % of PM cells were double-labeled (nine animals represented). At the white stage, 23 % were double-labeled (five animals represented) and at the ptilinum extended stage, 11 % of the PM cells were double-labeled (13 animals represented). At adult eclosion, 0 % of the β -gal stained cells were MM immunoreactive (three animals represented). Within an hour of adult eclosion, 19 % of the PM cells were double-labeled (14 animals represented).

gene expression in PM cells throughout all developmental stages. The number of adult PM cells was similar to the number of larval PM cells, and the positions of at least some of the cells were retained (see Figs 1, 5). Although there are significant morphological changes in the tracheae as the larval airways degenerate and the adult airways form (Manning and Krasnow, 1993), the positions of PM cells at branchpoints 1, 4 and 5 were relatively invariant. PM cells in more posterior segments were more likely to have altered positions. Their positions began to change at head eversion, when the larval tracheae are degenerating. Although each PM cell could not be followed throughout metamorphosis, the consistent numbers and retained positions of at least some of them indicate that they persist into the adult. PM cells also exhibited significant, though transient, shape changes around the time of head eversion (P4–P6) (Bainbridge and Bownes, 1981), becoming enlarged and more irregular (Fig. 8A–C). The PM cell at branchpoint 1, in particular, frequently exhibited dramatic shape changes and enlarged to 20–25 μ m in diameter

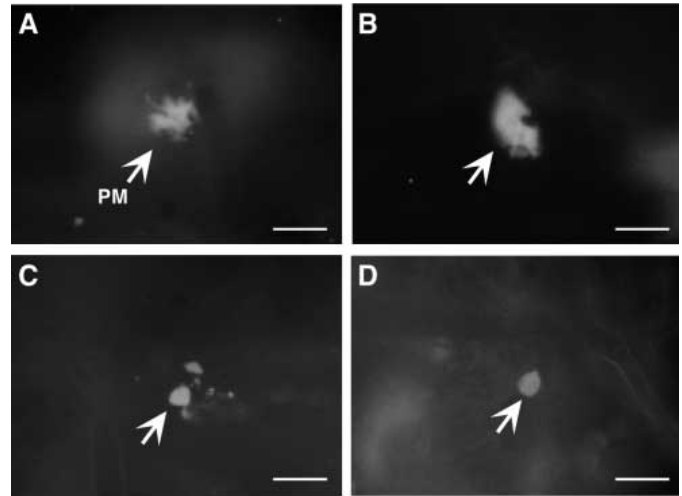


Fig. 8. Changes in peritracheal cell morphology during metamorphosis. (A–C) Examples of PM cell changes during pupal stages P5–P6. Note the increase in size and the irregular shape compared with cells at earlier pupal stages. Tracheae are not in focus, but the arrows denote PM cells. (D) By the pharate adult stage (P7), the PM cell returns to a smaller, more regular shape (arrow). Scale bars, 25 μ m.

(Fig. 8A). By pharate adult stages, the PM cells returned to a more compact shape that persisted in the adult (Fig. 8D). We also wanted to determine when the PMA cells lost reporter gene

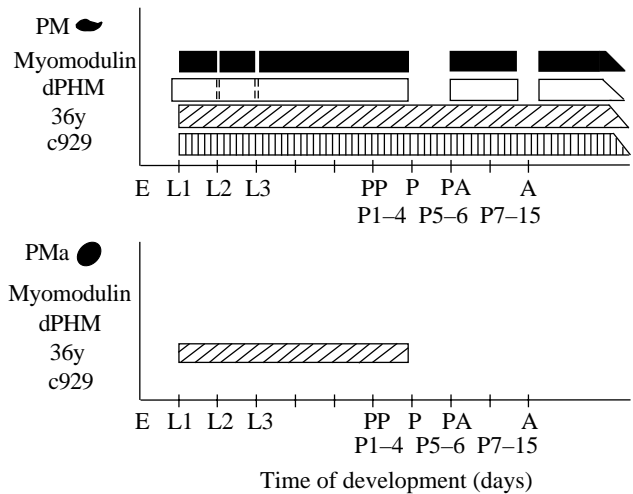


Fig. 9. Summary of staining in the *D. melanogaster* peritracheal cells during development. MM immunoreactivity is shown with the black bar, dPHM immunoreactivity is shown with the white bar, 36y reporter gene expression is shown with the diagonally striped bar, and c929 expression is shown with the vertically striped bar. The dashed lines in the white bar indicate that dPHM immunoreactivity was not assessed at larval ecdyses, but we predicted a decrease on the basis of the results for pupal and adult ecdyses. Developmental stages are embryo (E), first-, second- and third-instar larvae (L1, L2, L3, respectively), prepupa (PP), pupa (P), pharate adult (PA) and adult (A). The corresponding stages, P1–P15, are according to Bainbridge and Bownes (1981). The slashes on the x-axis indicate days of development, which are approximate for 25 °C.

gene expression in the 36y line. The PMa cell lost 36y reporter expression at head eversion (Fig. 9). Having no other markers with which to identify this cell subsequently, we do not know whether it persists. A developmental summary of staining in the PM and PMa cells with the various markers is illustrated in Fig. 9.

MM immunoreactivity in the Manduca sexta Inka cell: disappearance at ecdysis

Our finding that the *D. melanogaster* peritracheal cells are likely to release MM-related peptides at the completion of the larval, pupal and adult molts implicated these cells in ecdysial events. In *M. sexta*, the large endocrine Inka cells, adjacent to

the spiracles along the tracheae, release ETH at the molt to help regulate ecdysis behavior (Zitnan *et al.* 1996; Ewer *et al.* 1997). *D. melanogaster* PM cells show similarities with the lepidopteran epitacheal system, suggesting that these PM cells could be Inka cell homologs. The number of cells and their segmental arrangement is similar, and both sets of cells appear to release peptide at the end of the molt. We tested the MM antibody on second- and third-instar *M. sexta* larvae and detected a single cell per hemisegment along the trachea, as in *D. melanogaster* larvae. The MM-positive cell was larger than in *D. melanogaster* (approximately 50 μm in diameter compared with approximately 10 μm) and more rounded, like the Inka cell (Fig. 10A). We determined that the MM-positive

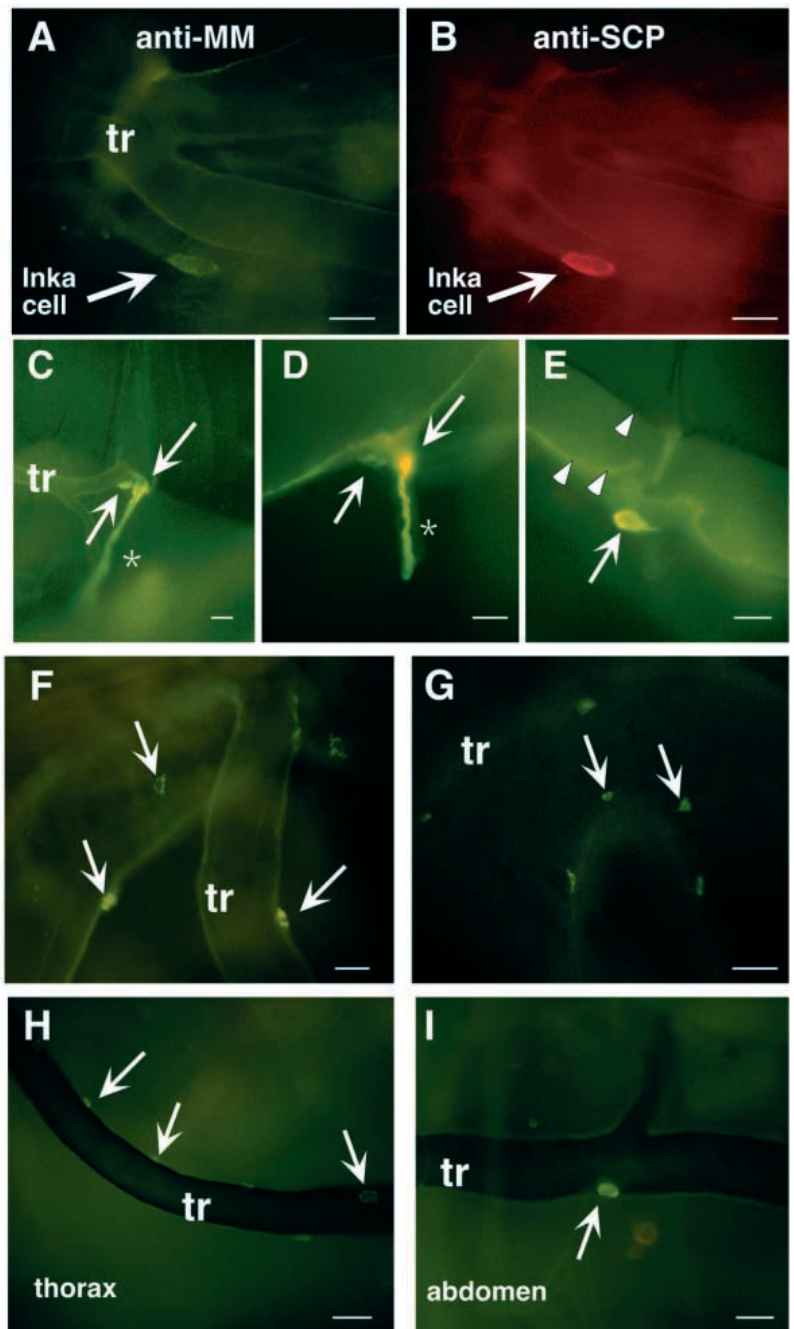


Fig. 10. Interspecific comparison of MM immunoreactivity along tracheae in larval insects. (A,B) Double-labeling of *Manduca sexta* (Lepidoptera) trachea with MM antisera and an SCP monoclonal antibody. The arrow points to the Inka cell identified with the SCP antibody. The Inka cell is adjacent to the spiracle in each segment (not visible). (A) For the MM antibody, a FITC-conjugated secondary antibody was used. tr, main tracheal trunk. (B) For SCP, a Texas-Red-conjugated anti-mouse secondary antibody was used. (C–E) Examples of MM staining in the mosquito larva (Diptera, species not determined). (C) Arrows point to two strongly immunoreactive cells that were seen in each segment at the same tracheal branchpoint. The asterisk denotes a stained process that was attached to the epidermis. (D) Arrows point to the same two cells in another preparation at higher magnification. In this case, one of the cells is more weakly stained. (E) The arrow points to a single immunoreactive cell that was seen in each segment. In this preparation, the second cell was not observed in any segment. The single arrowhead points to old tracheal cuticle, the double arrowhead points to new tracheal cuticle. (F) MM immunoreactivity in peritracheal cells of the grasshopper *Schistocerca americana* (Orthoptera). Arrows point to cells distributed all along the tracheae. (G) MM immunoreactivity in a damselfly larva (Odonata, species not determined). Arrows point to labeled cells distributed all along the tracheae. (H,I) MM immunoreactivity in peritracheal cells of a beetle larva (Coleoptera, species not determined). (H) In thoracic segments, stained cells were distributed all along the tracheae. (I) In abdominal segments, there was a single cell per segment at the main tracheal branchpoint. Scale bars, 50 μm .

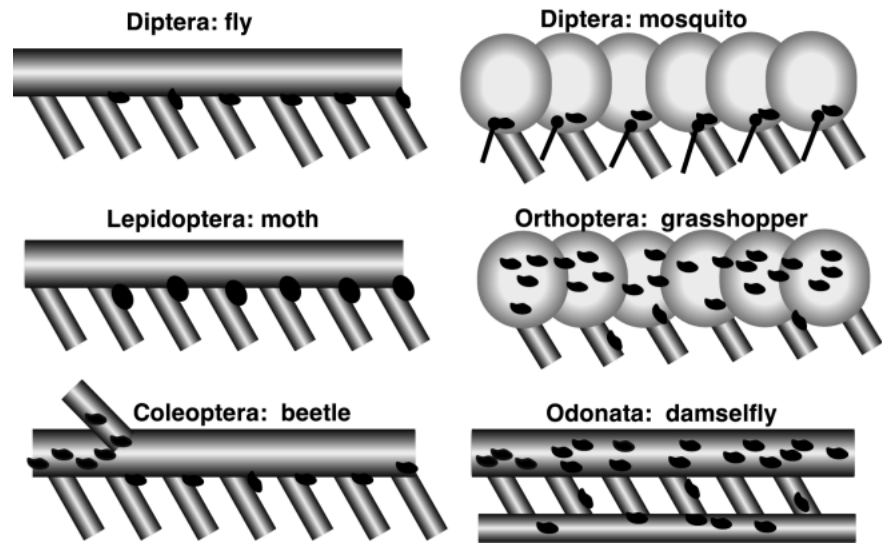


Fig. 11. Schematic summary of the patterns of MM immunoreactivity in peritracheal cells (black) from representative insects in different orders. The tracheae are either more tube-like or more sac-like (mosquito and grasshopper). In Lepidoptera, the cells are larger and positioned adjacent to the spiracle. In the other insects, the cells are smaller and not necessarily positioned near the spiracles. For all drawings, anterior is to the left and dorsal to the top.

cells in *M. sexta* were the Inka cells by double-labeling with the SCP and MM antibodies (Fig. 10A,B). The SCP monoclonal antibody specifically stains the Inka cell and is thought to recognize ETH because the three C-terminal amino acids of SCP and ETH are identical (Zitnan *et al.* 1996). As reported in Zitnan *et al.* (1996), we also found that SCP immunostaining disappeared from the Inka cell at larval ecdysis. When the SCP immunoreactivity disappeared, the MM immunoreactivity also disappeared (data not shown). We tested the SCP antibody on *D. melanogaster* larvae, but found no peritracheal cell immunoreactivity (Table 1).

These immunocytochemical results suggested either that the *M. sexta* Inka cells co-release MM-like peptides and ETH or that the MM antibody is cross-reacting with the ETH peptide. To test the latter hypothesis, we assayed the interaction of the MM antibody with *M. sexta* ETH peptide in both immunocytochemical blocking experiments and dot blot analyses. We preabsorbed the MM antibody (1:1000) with MM (*A. californica*), ETH (*M. sexta*) or DPKQDFMRFamide (*D. melanogaster*) peptides at $10 \mu\text{g ml}^{-1}$ and $1 \mu\text{g ml}^{-1}$, and then tested the antibody for immunoreactivity on *D. melanogaster* larval preparations. MM peptide completely blocked the antibody, while ETH and DPKQDFMRFamide had no effect. Similarly, the ETH and DPKQDFMRFamide peptides produced no detectable signal when evaluated by dot blot, while the MM peptide produced a prominent signal (data not shown). The results did not support the hypothesis that the MM antibody cross-reacts with *M. sexta* ETH in the *M. sexta* Inka cell, but instead suggest that distinct ETH- and MM-like peptides are released from the Inka cell.

Peritracheal MM-like neuropeptide expression is evolutionarily conserved

To determine whether peritracheal MM neuropeptide expression is a widespread phenomenon in insects, we screened for MM immunoreactivity in insects of several additional orders including Orthoptera, Coleoptera, Odonata

and another dipteran, a mosquito. In general, strong staining occurred in all the insects tested, both in the CNS and among peritracheal cells. However, the MM-positive peritracheal cells varied considerably in their morphology and distribution along the tracheae (Figs 10, 11). In the mosquito, there was immunoreactivity in two cells per segment along the trachea (Fig. 10C). One cell was quite similar to the *D. melanogaster* PM cell in size and morphology, while the other cell was unusual in having a process connected to the epidermis. We observed three different patterns of staining: (1) strong staining in both cells (Fig. 10C); (2) strong staining in the cell with the connection to the epidermis and weaker staining in the other cell (Fig. 10D); and (3) staining only in one cell (Fig. 10E). In Fig. 10E, where only one cell is stained, the trachea contained two distinct cuticular layers, suggesting that these immunocytochemical changes were correlated with the molt cycle.

In an orthopteran, *S. americana* (Fig. 10F), and an odonatan (damselfly larva) (Fig. 10G), the MM-positive cells were small (approximately $15 \mu\text{m}$ in diameter) and similar in appearance to those found in *D. melanogaster*, but their number was not restricted to one cell per segment. Tracheae in these animals had numerous MM-positive cells scattered on both the major and minor branches (Fig. 10F,G). In a coleopteran (beetle larva), there was a combination of phenotypes that resembled patterns found in both orthopterans and dipterans. In thoracic segments, numerous antibody-positive cells were scattered along the trachea (Fig. 10H); in abdominal segments, there was one cell per segment at a branchpoint (Fig. 10I), as in *D. melanogaster*. These results indicate that the peritracheal cell system is widely conserved in insects, although its specific pattern is quite variable (Fig. 11).

Discussion

A conserved peritracheal neuropeptide system in insects

In this report, we have identified a peripheral site of

myomodulin-like expression in the dipteran *D. melanogaster*. We found that this peritracheal myomodulin neuropeptide system was conserved in representative larvae of diverse insect orders including Lepidoptera, Orthoptera, Odonata and Coleoptera. Such conservation suggests an important function for this peritracheal system. The interspecific comparison also revealed differences in peritracheal cell size, morphology and distribution along the tracheae. In Orthoptera, Odonata and Coleoptera, the peritracheal cells were similar in size and morphology to those in *D. melanogaster*, but they were distributed much more abundantly along the tracheae. This species variation in number and distribution may reflect the larger size of these larvae. As in *D. melanogaster* larvae, the lepidopteran *M. sexta* had a single immunoreactive peritracheal cell per hemisegment, but this cell was significantly larger than its *D. melanogaster* counterpart. The larger cell size in *M. sexta* may reflect the larger size of the larva. The identification of the *M. sexta* peritracheal cells as Inka cells implied that all MM-immunoreactive peritracheal cells in various insects are functional homologs of the Inka cells. The variations observed between species may reflect specializations to enable a conserved function to be carried out most efficiently in different insects.

Drosophila melanogaster PM cells are homologous to Inka cells

Cellular arrangement

We demonstrated that the MM-immunoreactive cells along the tracheae in *M. sexta* were the Inka cells, which have recently been shown to produce a peptide that is a potent trigger of ecdysis behavior (Zitnan *et al.* 1996). We monitored the ETH peptide in Inka cells with an SCP antibody and were able to co-label with the MM antibody. Although the converse experiment would have further supported this homology between the two cell types, the SCP antibody did not recognize any cells along the tracheae in *D. melanogaster*. However, the peritracheal cells in *D. melanogaster* and the Inka cells in *M. sexta* were the only cells outside the CNS that were MM-immunoreactive. Furthermore, the cellular arrangement is virtually identical. In both species, there is typically an immunoreactive cell in an anterior segment and in abdominal segments, but not in thoracic segments (Ewer *et al.* 1997).

Further support for the homology of the two cell types came from examining neuropeptide biosynthetic enzymes. ETH is an amidated peptide (Zitnan *et al.* 1996) and requires the C-terminal amide for its activity (Adams and Zitnan, 1997); therefore, the antibody against dPHM (Kolhekar *et al.* 1997), an enzyme necessary for peptide amidation, could identify potential Inka cell homologs. The PM cells were the only cells along the tracheae that had intense dPHM immunoreactivity, and thus are the only good candidates to be Inka cell homologs in *D. melanogaster*.

Release of peptides at ecdysis

The activity of the PM cells during development also resembles that of the Inka cells. The sudden decrease in MM

immunoreactivity in *D. melanogaster* PM cells associated with larval ecdysis, head eversion (larval–pupal ecdysis) and adult eclosion supports the hypothesis that MM-like peptides are released at the culmination of the molt. In each instance, the loss occurred predictably, less than an hour prior to ecdysis. The concomitant loss of dPHM immunoreactivity provides further evidence that the PM cells undergo regulated exocytosis at these times, although conclusive proof of peptide release would require monitoring for MM-like peptides in the hemolymph.

We found that the MM immunoreactivity of Inka cells also disappeared during ecdysis in *M. sexta*. When ETH was present prior to the larval molt (monitored using the SCP antibody), we found MM immunoreactivity. At ecdysis, when the Inka cells were not visible using the SCP antibody, we could detect no MM immunoreactivity outside the CNS. Our results in the mosquito also demonstrated a correlation between progression through the molting cycle and MM antibody staining. A loss of staining in one of two peritracheal cells was correlated with the presence of both old and new tracheal cuticle. Taken together, our results provide strong evidence that *D. melanogaster* peritracheal cells are the Inka cell homologs; we therefore propose that the MM antibody labels the Inka cell homologs in several insects.

In *M. sexta*, an elevation in [cGMP] in response to EH occurs in the CNS (Morton and Truman, 1985; Morton and Guinta, 1992) in the transverse nerve (at pupation only) (Morton, 1996, 1997) and in the Inka cells (Ewer *et al.* 1997). This increase in [cGMP] in the Inka cells can cause release of ETH (Ewer *et al.* 1997). The cGMP response in the CNS at ecdysis appears to be limited in higher dipterans. In most insects, the network of neurons that reveal an increase in [cGMP] are also CCAP-immunoreactive. In contrast, very few neurons display a cGMP response at eclosion in *D. melanogaster*, and they do not coincide with the CCAP-immunoreactive cells (Ewer and Truman, 1996). Whether the *D. melanogaster* PM cells will display fluctuations in [cGMP] during its release events remains to be determined.

Identity and function of MM-like peptides in peritracheal cells

The MM antibody may cross-react with the ETH peptide in *M. sexta*, although our results do not support this hypothesis. The MM antibody was prepared against *Aplysia californica* myomodulin C (GWSMLRL-NH₂), which does not have an obvious sequence similarity to the ETH peptide (SNEAISPFDQGMGYVIKTNKNIPRM-NH₂). Furthermore, the ETH peptide did not block any MM immunoreactivity in *D. melanogaster*, nor did the MM antibody recognize ETH peptide on a dot blot assay. Our results suggest that MM-like peptides are co-released with ETH from the Inka cells in *M. sexta*. No MM peptides have been purified from insects, although other peptides with sequence similarities at the C termini have been reported, including pheromone-biosynthesis-activating neuropeptide (PBAN), diapause hormone (Sato *et al.* 1993; Xu *et al.* 1995)

and the leucopyrokinins (Schoofs *et al.* 1991). This family of peptides has in common the C-terminal pentapeptide sequence FX_PRL-NH₂, where X is Gly, Ser, Thr or Val (Nachman *et al.* 1991, 1993). Peptides in this family have been shown to accelerate pupariation in another dipteran, the fleshfly *Sarcophaga bullata* (Nachman *et al.* 1997; Zdarek *et al.* 1997). The RL-NH₂ C terminus, in common with the MM C terminus, may be recognized by the MM antibody. In *M. sexta*, RPLC analysis indicated that there were additional peptides in the epitracheal glands, some of which had ecdysis-triggering activity (Zitnan *et al.* 1996). An MM-like peptide could be one of these.

This study implies a role for MM-like peptides at ecdysis, but their precise function and targets remain to be determined. We do not detect a decrease in MM immunoreactivity in the CNS at ecdysis, so release of MM-like peptides at this time appears to be specific to the PM cells. Whether the MM antibody is recognizing distinct MM-like peptides in the CNS and tracheae, or whether the antibody is recognizing the same peptides but their functions have diverged in the two tissues, will be determined when the insect MM peptides and genes have been identified. The molluscan myomodulin gene (Lopez *et al.* 1993; Miller *et al.* 1993; Kellett *et al.* 1996) encodes a family of related peptides that are known to have modulatory effects in circuits involved in feeding behavior (Cropper *et al.* 1987, 1991; Lloyd, 1988; Miller *et al.* 1991; Hooper *et al.* 1994; Santama *et al.* 1994), reproduction (Li *et al.* 1994; van Golen *et al.* 1996) and cardiovascular function (Miller *et al.* 1991). Related peptides appear to be present in the CNS of a broad spectrum of organisms (Miller *et al.* 1993; Christie *et al.* 1994; Nassel *et al.* 1994; Swales and Evans, 1994; Brezina *et al.* 1995; Kellett *et al.* 1996; Keating and Sahley, 1996). Roles in locomotion and cardiovascular function have been suggested in these other organisms (Evans, 1994; Keating and Sahley, 1996).

Do peritracheal cells have multiple functions?

Whether the identified peritracheal cells in a variety of insects serve the ecdysis-triggering function demonstrated by the Inka cells in lepidopterans remains an important question. The lack of cross-reactivity between ETH peptides and the MM antibody suggested that, at least in *M. sexta*, distinct peptides are released. By extension, we hypothesize that in other insects peritracheal cells release both ETH- and MM-like peptides. However, it is not known whether there are ETH-like peptides in insect orders other than the Lepidoptera. An ETH antibody has recently been reported, but it has not been used to examine non-lepidopteran insects (Adams and Zitnan, 1997). As mentioned above, the SCP antibody, which is thought to recognize ETH because of the similarity of the three C-terminal amino acids (Zitnan *et al.* 1996), did not stain any cells along the tracheae in *D. melanogaster*. Thus, if there is an ETH activity in *D. melanogaster*, the peptide does not contain the SCP epitope that appears to be present in *M. sexta*.

In addition to a possible hormonal role in affecting ecdysis behavior, peritracheal cells may have a local, paracrine

function within the tracheae during the molt. There are numerous changes that the tracheae must undergo at each molt; some occur at each ecdysis and some are specific for certain developmental changes. For example, the tracheal cuticle must break at the node of each segment so that old cuticle can be shed through the spiracle, and then new tracheal cuticle must fuse to form the new tracheal trunks. There are repeated phases of liquid/gas exchange in the tracheal lumen associated with the molts. At metamorphosis, the larval tracheae degenerate, and imaginal tissues form new tracheae (Manning and Krasnow, 1993). In *D. melanogaster*, the loss of MM immunoreactivity was temporally associated with various tracheal changes. At larval ecdysis, the putative release of peptide was closely associated with the breaking at the nodes of the old tracheal cuticle. At larval-pupal ecdysis (head eversion), the loss of MM immunoreactivity correlated with the rapid degeneration of the larval tracheae. At adult eclosion, the putative release of MM-like peptides was closely associated with tracheal inflation (Kimura and Truman, 1990).

Peritracheal cells may have additional functions that are unrelated to molting, as suggested by the persistence of PM cells into the adult in *D. melanogaster*. In the vertebrate respiratory system, there is good evidence that, at least in some species, neuroendocrine cells act as chemoreceptors sensitive to oxygen levels (Youngson *et al.* 1993). It is possible that insect neuroendocrine cells positioned along the tracheal respiratory system may have evolved similar functions. Neuroendocrine cells in the vertebrate lung are also thought to regulate some aspects of lung development. It has been hypothesized that bombesin/gastrin-like peptides produced in lung neuroendocrine cells are involved in regulating branching (King *et al.* 1995). In summary, there may be multiple functions for peritracheal cells; there may be distinct ETH-like activities and MM-like activities at ecdysis, there may be endocrine and paracrine functions, and different peritracheal cells may serve distinct functions.

A genetic link between the CNS and peritracheal neuropeptide-expressing cells in Drosophila melanogaster

The co-expression of reporter gene and myomodulin-like peptides in both CNS and peritracheal cells in the 36y line indicates that the genetic programs for these cells have a common element. We would expect that the secretory aspects of both neurosecretory cells and peritracheal cells would require expression of several common genes; 36y and c929 may be reporting on two genes of this category. It does not seem likely that the 36y enhancer is reporting on a myomodulin gene because there is incomplete overlap between the MM immunoreactivity and the 36y expression pattern. There is 36y reporter expression in tissues other than the CNS and peritracheal cells. Conversely, not all of the MM-immunoreactive neurons are positive for 36y reporter gene expression. A molecular analysis of the 85C region surrounding the P element has identified several cDNAs, none of which encodes myomodulin sequences (M. A. O'Brien and P. H. Taghert, unpublished results).

In conclusion, we have identified a CNS–peritracheal myomodulin neuropeptide system that is conserved in insects from at least five orders. Several markers indicated that the peritracheal cells in *D. melanogaster* are non-neuronal endocrine cells. The results from Lepidoptera and Diptera suggest that the peritracheal myomodulin system plays a role in regulating ecdysis. In the present study, the *D. melanogaster* P[gal4] enhancer trap lines 36y and c929 were critical for monitoring the presence of the peritracheal cells during metamorphosis. In future studies, these enhancer trap lines will allow for targeted misexpression of genes in peritracheal cells. A molecular characterization of their sites of insertion may lead to the identification of additional gene products important for peritracheal cell function.

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