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Ap-let neurons—a peptidergic circuit potentially controlling ecdysial behavior in *Drosophila* ☆

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Abstract

Here we describe a novel set of peptidergic neurons conserved throughout all developmental stages in the *Drosophila* central nervous system (CNS). We show that a small complement of 28 *apterous*-expressing cells (Ap-let neurons) in the ventral nerve cord (VNC) of *Drosophila* larvae co-express numerous gene products. The products include the neuroendocrine-specific bHLH regulator called Dimmed (Dimm), four neuropeptide biosynthetic enzymes (PC2, Fur1, PAL2, and PHM), and a specific dopamine receptor subtype (dDA1). For the PC2, Fur1, and PAL2 enzymes, and for the dDA1 receptor, this neuronal pattern represents the vast majority of their total expression in the VNC. In addition, while Dimm and PHM are present in the peritracheal Inka cells in larvae, pupae, and adults, Ap, PC2, Fur1, PAL2, and dDA1 are not. PC2, PAL2, and DA1 receptor expression were all controled by both *dimm* and *ap*. Previous genetic analysis of animals deficient in PC2 revealed an abnormal larval ecdysis phenotype. Together, these data support the hypothesis that the small cohort of Ap-let interneurons regulates larval ecdysis behavior by secretion of an unidentified amidated peptide(s). This hypothesis further predicts that the production of the Ap-let neuropeptide(s) is dependent on each of four specific enzymes, and that a certain aspect(s) of its production and/or release is regulated by dopamine input.
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Keywords: Drosophila; *Apterous*; Ecdysis; Neuropeptides; *Dimmed*; Dopamine receptor; dDA1; PC2; Furin1; PHM; PAL

Introduction

The molting process of insects ends with the highly orchestrated emergence of the animal from the old cuticle. This behavior is called ecdysis for larval and pupal animals, and eclosion for adults. An extensive series of experiments in moths and in *Drosophila* has produced a model of endocrine and neuroendocrine steps that initiate and coordinate ecdysial behaviors (reviewed by Ewer and Reynolds, 2002). Ecdysial behaviors are controlled by at least three distinct peptide hormones, eclosion hormone (EH), ecdysis-triggering hormone (ETH) and crustacean cardioactive peptide (CCAP). The general model features positive interactions between EH and ETH to initiate the ecdysial program, and EH finally causing release of CCAP in a cGMP-dependent manner within the central nervous system (CNS). CCAP then helps trigger the motor activities to complete ecdysial behavior (Gammie and Truman, 1999; Park et al., 2002; Park et al., 2003; Zitnan et al., 1999).

Many observations indicate the model requires additional details and refinement. There are apparent differences in regulation according to stages of development, as indicated by the phenotype of animals lacking EH-producing neurons (McNabb et al., 1997). There are likely species differences as well: *Drosophila* neurons containing CCAP do not display an EH-induced cGMP increase (Ewer and Truman, 1996). Also there is evidence for functional redundancy between control systems: larvae deficient in ETH fail to complete ecdysis at the end of the 1st instar, although many display some ecdysial behaviors (Park et al., 2003). A simple deduction is that other factors, whether normal or compensatory, may be available as part of a more complicated mechanism underlying the control of ecdysial behavior. Therefore, to test the established hypothesis, and to build a more detailed model, it is important to identify other

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signaling components and neural circuits relevant to *Drosophila* ecdysial behaviors.

The genetic analyses of neuropeptide biosynthetic enzymes offer a potential means by which to address ecdysial behaviors. Mammalian prohormone convertase 2 (PC2) is an important endoprotease for neuroendocrine prohormone processing (Seidah et al., 1998; Westphal et al., 1999). The *Drosophila* orthologue is encoded by *CG6438* (also called *amontillado*, *amon*). Recombinant dPC2 displays predictable interactions with the mammalian PC2 regulatory binding partner 7B2 and with the *Drosophila* orthologue of 7B2 (Hwang et al., 2000). *PC2* produces a lethal phenotype in which animals fail to hatch from the egg case, or fail to complete larval ecdysis (Rayburn et al., 2003; Siekhaus and Fuller, 1999). Likewise, animals deficient in production of the amidating enzyme PHM (*PHM*, *CG3832*), fail to produce amidated peptides, and display a lethal phenotype similar to that of *PC2* (Jiang et al., 2000). Synthetic *PHM* hypomorphs fail at later ecdyses, including pupal ecdysis (Jiang et al., 2000). These data are consistent with the possibility that ecdysial behaviors require one or more peptide precursors processed by PC2 and also by PHM. ProETH, which is required for proper larval ecdysis behavior (Park et al., 2000), is possibly one such precursor, as it requires cleavage at dibasic residues and amidation of the ETH peptide (Zitnan et al., 1999). PHM is widely expressed by numerous peptidergic neurons and endocrine cells, such as the ETH-expressing Inka cells, at all stages of development (O'Brien and Taghert, 1998; Jiang et al., 2000). The spatial expression of PC2 has been described by in situ hybridization in embryos, where it is found in numerous neurons, in the Inka cells and in putative gut endocrine cells (Siekhaus and Fuller, 1999).

To address mechanisms of larval ecdysis, we have focused on the expression of such neuropeptide (NP) biosynthetic enzymes in post-embryonic stages. In addition to PC2 and PHM, we studied Furin1 (*fur1*, *CG10772*) and PAL2 (Peptidyl-α-hydroxyglycine α-amidating lyase *PAL2*, *CG5472*). Fur1 is one of two *Drosophila* enzymes related to the mammalian tetra-basic cleaving endoprotease, furin (De Bie et al., 1995; Roebroek et al., 1991, 1992). PAL2 is one of two distinct *Drosophila* PAL-like enzymes (Kolhekar et al., 1997; M. Han, D. Park, P. Vanderzalm, D. Mains, B.A. Eipper, and P.H. Taghert, submitted) that act in series with PHM to catalyze peptide amidation (Eipper et al., 1992). The vast majority of *Drosophila* neuropeptides appear to be amidated (Hewes and Taghert, 2001).

PC2 immunosignals are generally restricted to a small complement of *apterous* (*ap*)-expressing CNS interneurons, but are not found in ETH-producing Inka cells. Furthermore, this complement of *ap* larval neurons is the only neural site in which PC2 and PHM are co-expressed. Apterous is a LIM homeodomain protein that helps regulate neuronal pathway choice and peptidergic cell differentiation (Benveniste et al., 1998; Lundgren et al., 1995). The axons of this *ap*-cell group project to the brain in a fasciculated bundle (Lundgren et al., 1995) and hence may represent a functional unit. We expand and refine the definition of this cellular complement incorporating several, additional cell type-specific markers that are expressed by this neuronal group. We show that this group is the primary site for expression for two other putative neuropeptide biosynthetic enzymes, Fur1 and PAL2, and further that the normal differentiation of these properties is highly dependent on expression of *ap* and one other regulator, *dimmed*. Finally, we show that this group represents one of two specific larval CNS sites that express the D1-like dopamine receptor dDA1 (Kim et al., 2003). Based on this extensive and highly detailed anatomical and molecular signature, we infer a common functional specification to this group of interneurons. We have named them the *Ap-let* cells and hypothesize they secrete a specific neuropeptide(s) acting as a central transmitter to help regulate ecdysial behaviors. We discuss these data in the contexts of ecdysial control mechanisms and peptidergic cell differentiation.

Experimental methods

Fly stocks

The stocks used as controls were either Canton S, or *y w* (Bloomington Stock Center). P[UAS-*GFP*] and P[UAS-*lacZ*] were also obtained from the Bloomington Stock Center. P[$c929^{GAL4}$] (here called *c929*) was derived from a screen conducted in Kim Kaiser's laboratory (Univ. Glasgow; cf., O'Brien and Taghert, 1998). We studied three alleles of *apterous*. *y w* ap^{rk586}/*CyO* (here called *ap*-lacZ) contains a lacZ-bearing enhancer trap inserted within the 5′ end of the *ap* gene; this enhancer trap accurately reports *ap* expression (Benveniste et al., 1998; Cohen et al., 1992). *y w*; ap^{GAL4}/*CyO*, *wg*-lacZ (here called ap^{GAL4}) also contains an enhancer trap insertion within *ap* and appears to be a strong hypomorphic allele (O'Keefe et al., 1998), and *y w*; ap^{p44} contains a deletion of the 5′ end of the *ap* gene and is transcriptional null (Bourgouin et al., 1992). A recombinant stock containing *y w*; ap^{p44}, P[UAS-*tau GFP*], and balanced by *CyO*, *wg*-lacZ, was the kind gift of Stefan Thor and John Thomas. Alleles of *dimm* studied here included the specific P element insertion P{*KG02598*} and the small deficiencies *Rev8*, *Rev4*, and *Rev6* (Hewes et al., 2003).

Immunocytochemistry

Immunostaining procedures followed the methods of Benveniste et al. (1998) and of Jiang et al. (2000). In all cases, the fixative was 4% paraformaldehyde in phosphate buffered saline (PBS) containing 7% (v/v) saturated picric acid. Fixation proceeded for 1 h at room temperature. The following dilutions and sources were used for primary antibodies: mouse anti-beta-gal (1:500, Promega Corp., Madison, WI); rabbit anti-PHM (1:750; Jiang et al.,

2000); guinea pig anti-PAL2 (1:500); rabbit anti-PC2 (1:750; Hwang et al., 2000); rabbit anti-Fur1 (1:500; A. Roebroeck, Univ. Leuven); rabbit anti-CCAP (1:500; M. Ekert, University of Jena); mouse anti-dDA1 (1:1000; Kim et al., 2003). Secondary antibodies included Cy3-conjugated anti-mouse, anti-guinea pig, and anti-rabbit (1:500 each; Jackson Labs), and Alexa-488 anti-mouse, anti-guinea pig, and anti-rabbit (1:500 each; Molecular Probes). Images were collected on a Fluoview confocal microscope using 1 μm optical sections at 20 or 40×, or a Nikon E1000 epifluorescent microscope. Adobe Photoshop was used to process and assemble images. Cell counts were performed using a Zeiss Axioplan microscope.

Results

A large subset of the ap-positive VNC cells co-express four peptidergic biosynthetic enzymes

In the embryonic ventral nerve cord (VNC), *ap* expression marks three cell groups (Benveniste et al., 1998; Lundgren et al., 1995), and these are schematized in Fig. 1. In each thoracic and abdominal hemi-neuromere, there is a single *ap* cell that lies mid-laterally on the dorsal aspect; we refer to these collectively as the Dorsal chain. Likewise, each thoracic and abdominal hemi-neuromere contains a pair of *ap* neurons located ventrally. Finally, the third *ap* cell group comprises four neurons that are clustered ventrally and laterally: we refer to this group collectively as the *ap* T cluster (Thoracic). In a minority of prothoracic neuromeres, the T cluster contains five cells (Allan et al., 2003). In this report, we describe examples in the second and third thoracic neuromeres.

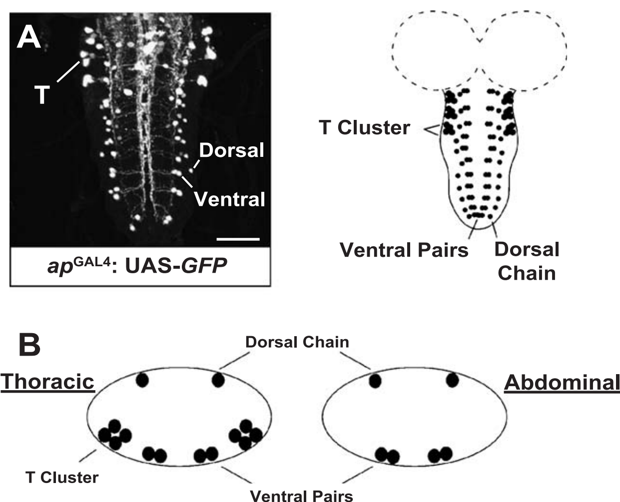

Fig. 1. The pattern of *ap* expression in the embryonic and larval VNC. Top left: a 70-μm confocal stack representing a first instar larval VNC of the genotype *y w*; ap^{GAL4}/*y w*; UAS-*GFP*. Three cell groups are indicated. T—the four cell cluster in the ventro-lateral aspect of each thoracic hemi-neuromere; Dorsal—the chain of single cells in the dorsal aspect of each thoracic and abdominal hemi-neuromere; ventral—the chain of pairs of neurons in the ventral aspect of each thoracic and abdominal hemi-neuromere. Top right: schematic representation of the same image with cell groups marked. Bottom: coronal diagram of the VNC at thoracic (left) and abdominal (right) levels to indicate relative locations of *ap* cell groups in the dorsal/ventral plane. Scale bar = 33 μm.

Fur1 and PC2

Both the anti-Fur1 ($n = 5$, Figs. 2A and 2D) and anti-PC2 antisera ($n = 7$, Figs. 2B and 2E) labeled one or sometimes two of the T cluster neurons. One cell was round and displayed intense *ap*-lacZ which we refer to as Tvb. The other was large and ovoid, and displayed weak *ap*-lacZ expression—the Tv neuron (based on data presented below). Both antisera also labeled most or all of the Dorsal chain *ap*-positive neurons, but none of the ventral chain *ap* neurons. PC2 and Fur1 immunostaining was routinely weak to moderate; Tvb was always more distinctly stained than Tv; Dorsal *ap* cells in anterior neuromeres were more often intensely stained than were homologous neurons in posterior neuromeres (with the exception of the last abdominal neuromere). PC2 immunosignals were also found in a small number of scattered cells of the brain and subesophageal neuromeres, and were typically weak. Likewise, weak Fur1 immunosignals were also found in a small number of additional cells, which were not identical to the additional PC2 cells.

PHM

PHM is widely expressed in the larval CNS (Jiang et al., 2000; Kolhekar et al., 1997) in a heterogeneous pattern that specifically features peptidergic neurons. Most neurons express PHM at very low levels, while several hundred cells in the larval CNS express it strongly; the latter appear to be peptidergic, primarily neuroendocrine neurons (Jiang et al., 2000). Among the four *ap*-positive neurons in the T cell cluster, two neurons were strongly PHM-positive ($n = 6$, Fig. 2C): one of these with a large ovoid cell body was weakly *ap*-positive (presumed Tv), and the other with a smaller, round cell body was strongly *ap*-positive (presumed Tvb). In addition, all Dorsal chain *ap*-positive neurons were strongly PHM-positive ($n = 6$, Fig. 2F), but none of the ventral chain of *ap*-positive neurons was so stained.

PAL2

We have recently found two PAL-like enzymes in *Drosophila* that are likely involved in peptide biosynthesis (M. Han, D. Park, P. Vanderzalm, R. Mains, B. Eipper, and P. Taghert, submitted). While PAL1 is broadly expressed in the larval CNS, a high level of PAL2 expression is prominent in only about 60 neurons (Fig. 3). Additionally, more diffuse PAL2 immunostaining is present in several brain regions, including the developing optic lobes. Because many of the prominent PAL2 neurons resembled *ap* neurons by position, we examined ap^{GAL4}/UAS-*lacZ* larvae stained with anti-PAL2 and anti-β-gal antibodies. In this experiment, 46 of the approximately 60 PAL2-positive neurons in 3rd instar larvae co-expressed *ap* ($n = 10$, Figs. 3A–3D, schematized

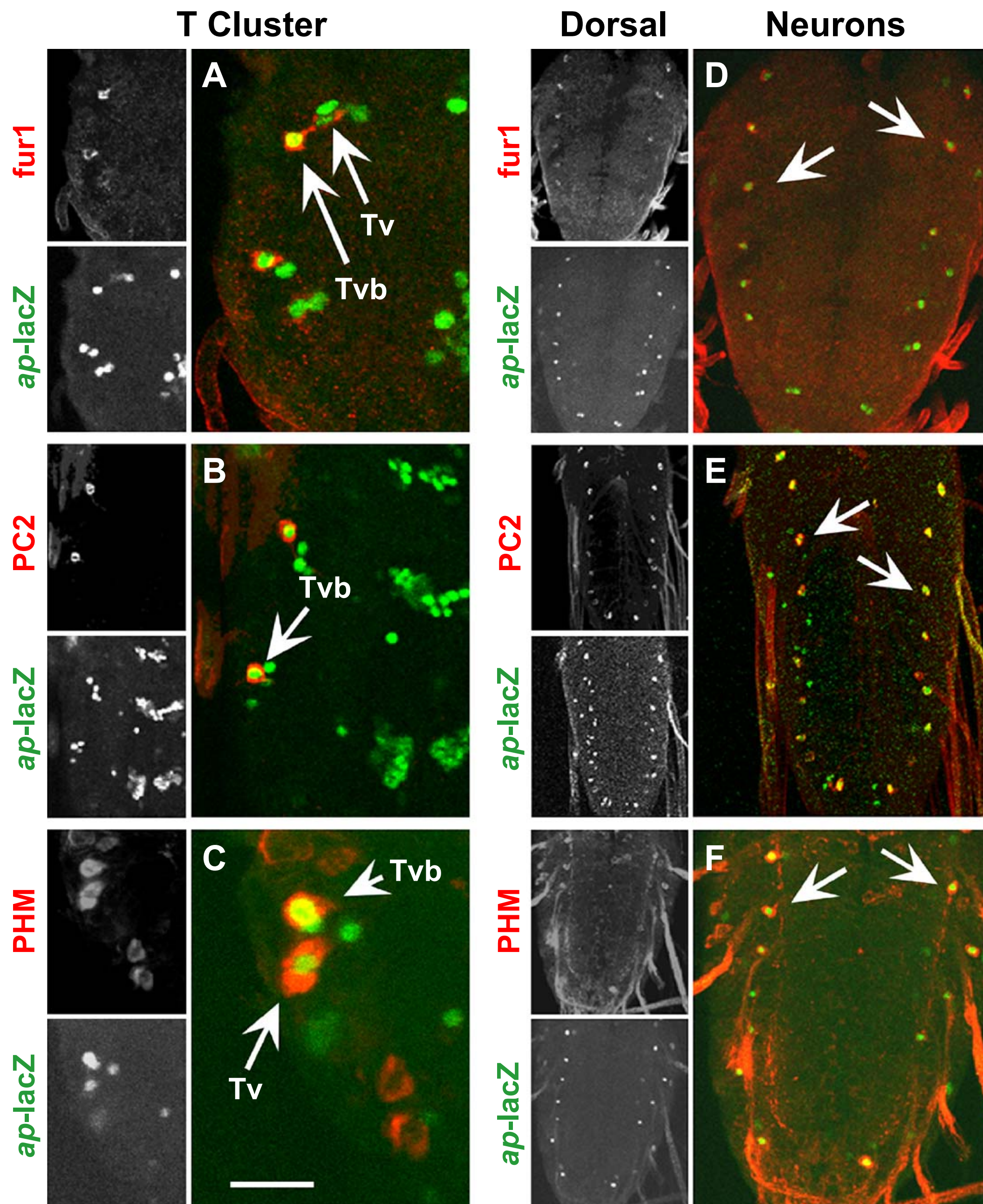

Fig. 2. Co-labeling of diverse *ap* neurons by antibodies to three different neuropeptide biosynthetic enzymes. Left: immunostaining of the T cluster; Right: immunostaining of the Dorsal chain of neurons. These identifications are based on multiple, different antibody stainings; see text for further details. (A) Double immunostaining for fur1 and for *ap*-lacZ in the T cluster reveals that the strong (Tvb) and one of the weak *ap*-lacZ cells (Tv) are Fur1-positive (a 22.5-μm stack). (B) Double immunostaining for PC2 and for *ap*-lacZ in the T cluster reveals that the strong (Tvb) and one of the weak *ap*-lacZ cells (Tv) are PC2-positive (a 23.4-μm stack). (C) Double immunostaining for PHM and *ap*-lacZ in the T cluster reveals that the strong (Tvb) and one of the weak *ap*-lacZ cells (Tv) are PHM-positive (a 16-μm stack). (D) Double immunostaining for Fur1 and for *ap*-lacZ in the region of the Dorsal *ap* chain reveals precise overlap between the two patterns of immunosignals (a 10-μm stack). (E) Double immunostaining for PC2 and for *ap*-lacZ in the region of the Dorsal *ap* chain reveals precise overlap between the two patterns of immunosignals (a 32-μm stack). (F) Double immunostaining for PHM and for *ap*-lacZ in the region of the Dorsal chain reveals precise overlap between the two patterns of immunosignals (a 33-μm stack). An additional example of PHM expression in the Dorsal cells is shown in Figs. 4J–L. Scale bar = 15 μm (A and B); = 7.5 μm (C); = 33 μm (D–F).

in Fig. 3E). In the VNC, two neurons in the T cluster and the Dorsal *ap* neurons expressed PAL2 (Figs. 3A and 3B). Based on cell shapes, sizes, and double staining with *c929* (see below), these T cluster neurons were identified as the Tv and Tvb cells. In addition, PAL2-positive cells were present in the brain (e.g., neuron Br1, Figs. 3B, and 3E–G) and subesophageal neuromeres (Figs. 3C and 3D). In certain genotypes, we observed that most of the *ap*-positive PAL2 staining was greatly reduced or absent, and only *ap*-negative neurons were reliably stained (data not shown). How

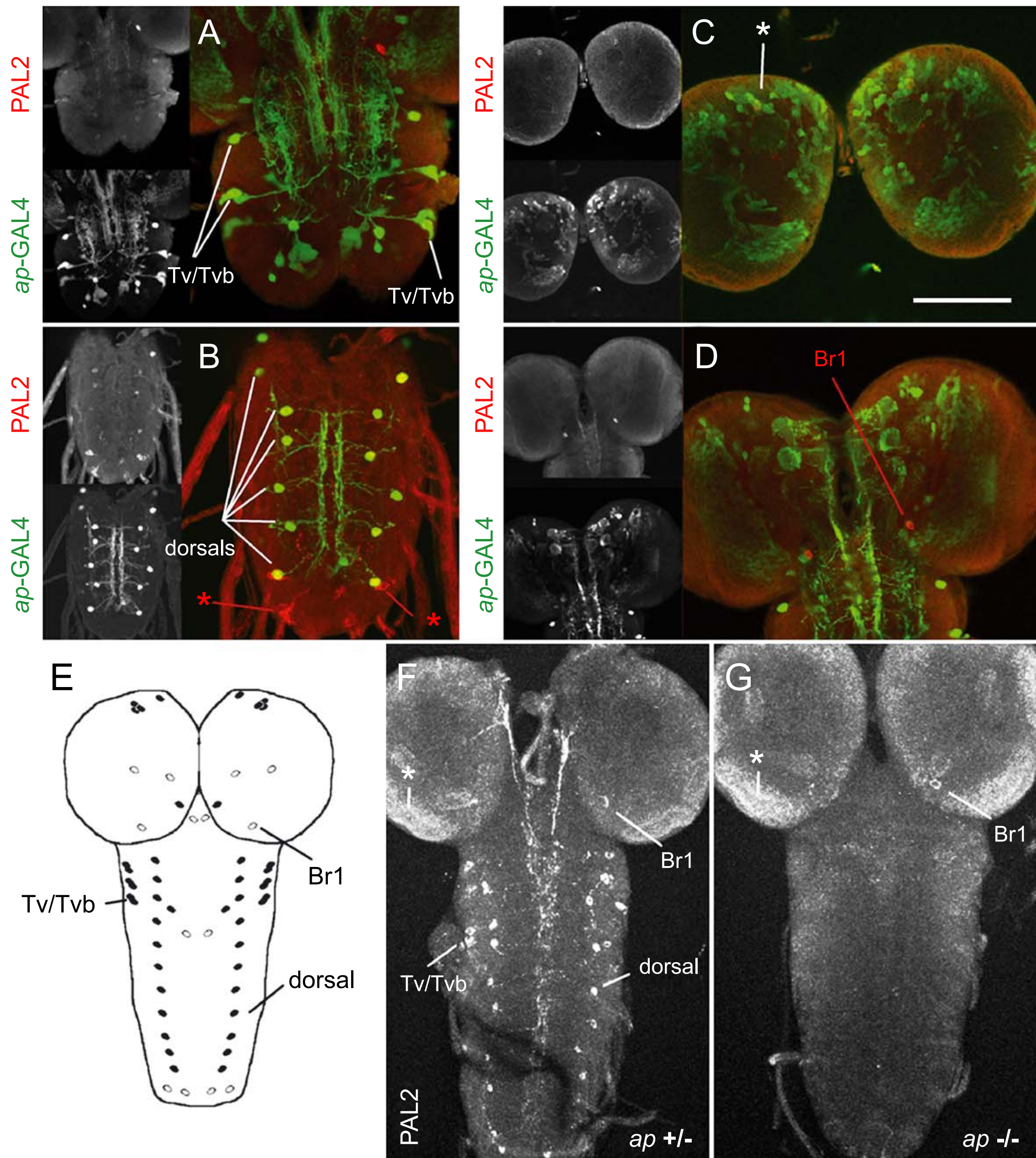

Fig. 3. Most PAL2-positive neurons express *ap*. Ap expression indicated by ap^{GAL4}::UAS-*GFP* expression. (A) Two cells in the T cluster of *ap* neurons are PAL2-positive neurons. (B) The Dorsal *ap* cells are PAL2-positive. Red asterisk indicates non-*ap*, PAL2-positive neurons. (C) PAL2 co-expression in the brain; white asterisk indicates double-labeled neuron in superior protocerebrum. (D) The Br1 PAL-2 in ventral tritocerebrum is *ap*-negative. (E) Diagram of the larval CNS indicating positions and number of PAL2 neurons that co-express ap^{GAL4} (filled circles) and those that do not (open circles). (F) PAL2 staining in an *ap* heterozygote; (G) PAL2 staining in an *ap* trans-heterozygous mutant. *—optic lobe primordia. In the mutant, many *ap*-negative cells (e.g., * and Br1) retain PAL2 expression, while *ap*-positive cells have lost it. Scale bar = 70 µm (A–D); 50 µm (F and G).

precisely this difference correlates with genotype, or with some other unknown variable (e.g., activation state), is presently uncertain.

Double-labeling with anti-enzyme antibodies was performed where possible to confirm tentative cell identifications. The guinea pig anti-PAL2 antibody was used with the rabbit anti-PC2 antibody (n = 5; data not shown), and with the rabbit anti-PHM antibody (M. Han, D. Park, P. Vanderzalm, R. Mains, B. Eipper, and P. Taghert, submitted). In all specimens examined, the double-labeling revealed co-expression of biosynthetic enzymes, as previously indicated by their co-expression with the *ap* reporter and with the reporter for the gene *dimm* (see below).

In summary, most of the cells that display intense staining for PAL2, PC2, and Fur1 immunosignals were identical, and they co-expressed *ap*. We refer to them hereafter as the *Ap-let* cohort of neurons. Specifically, they include the Tvb and the Dorsal chain of *ap* cells. The

exclusion of Tv from the Ap-let group is based on (i) gene expression differences (very weak and inconsistent staining by the antibodies to the PC2, Fur1, and PAL2 enzymes, and weak *ap* reporter expression, compared to the strong expression for these of Tvb and Dorsal chain cells) and on (ii) the difference in axon projection patterns between Tv versus that of Tvb and the Dorsal chain cells.

ap regulates NP biosynthetic enzyme expression in Ap-let neurons

We tested the hypothesis that *ap* regulates expression of these NP biosynthetic enzymes by staining for PC2 and PAL2 in *ap* mutant (ap^{GAL4}/ap^{p44}) and control larvae. Previous studies have demonstrated that the *ap* neurons survive in this mutant (Benveniste et al., 1998): this heteroallelic combination produced a *dFMRFa* expression phenotype that was quantitatively similar to that of homozygous (hypomorphic) ap^{GAL4} or homozygous (null) ap^{p44} mutants. In the T cluster, and in Dorsal cells, both PC2 and PAL2 expression was lost in *ap* trans-heterozygous mutant larvae (Table 1 and Figs. 3F and 3G). The immunosignals that remained in mutant tissues were found only in *ap*-negative neurons (e.g., Br-1, Fig. 3G).

dimm is expressed in many VNC ap neurons and also controls their differentiation

The *c929* line (O'Brien and Taghert, 1998) was useful in further characterizing the Ap-let neurons. *c929* contains a P element inserted in the *ATF-4* (*crc*) gene at 39CD (*CG8669*, Hewes et al., 2000). The spatial expression pattern of GAL4 in the P element primarily reflects a distant bHLH gene called *dimmed* (*dimm*). *dimm* normally regulates the differentiation of neuroendocrine lineages (Hewes et al., 2003). In the larval CNS, the expression patterns and levels of *c929* and PHM are highly correlated, thus making the *c929* pattern an accurate indicator of high-level amidated, peptidergic activity levels. We double immunostained *c929*/UAS-*lacZ* larvae for β-gal and for Fur1, PC2, PHM (n = 8, Fig. 4) or for PAL2 (n = 6; data not shown). Those experiments indicated that the Fur1/PC2/PHM/PAL2-positive pair of neurons in the T cluster cells was *c929*-positive, and thus corresponded to the Tv neuron and the Tvb neurons (Figs. 4A–4C). The Tvb neuron, as indicated by its cell size and shape, was typically stained more brightly for *c929* than the Tv neuron. In addition, the Dorsal chain of *ap* neurons was *c929*-positive (Figs. 4D–4F; cf., Hewes et al., 2003). Recently, we showed that *dimm* regulates Fur1 expression in the *ap*-positive Dorsal cells (Hewes et al., 2003). Similarly, PAL2 expression was dependent on *dimm*: In a severe hypomorphic *dimm* allelic combination, PAL2 expression was strongly reduced, though not eliminated in 1st instar CNS (Table 1). Thus, *dimm* is expressed by Ap-let neurons and controls their expression of NP biosynthetic enzymes.

The dDA1 dopamine receptor is also expressed specifically by Ap-let neurons

One of the *Drosophila* D1-like dopamine receptors, dDA1 (*CG9562*), is expressed in a subset of the larval and adult CNS neurons (Kim et al., 2003). In the VNC, dDA1 immunoreactivity is evident in a single Dorsal neuron in each thoracic and abdominal hemi-neuromere, and a single lateral neuron in each thoracic hemi-neuromere. This expression pattern is similar to that of the Ap-let group described above. To test whether dDA1 is expressed in Ap-let neurons, the larval CNSs of ap^{GAL4}/*UAS-GFP* or of *c929/UAS-GFP* were stained with the dDA1 antibody (Kim et al., 2003; Figs. 5A and 5B, n = 5 each). All of the dDA1 cells were positive with ap^{GAL4} (Figs. 5A and 5B), and with *c929* (data not shown), and they included the Dorsal chain and one of the two Tv neurons. This indicates the dDA1-positive T neuron is either Tv or Tvb. When the CNS was double-stained with anti-FMRFa antibody that stains the larval Tv neuron, the dDA1 and dFMRFa immunosignals were in distinct cells (n = 13, Fig. 5C insert). Together, these data indicate that the dDA1-positive cell in the T cluster was the Tvb neuron. Similar to PHM, PC2, Fur1, and PAL2, none of the *ap*-positive ventral neuron pairs expressed dDA1 immunosignals.

ap and dimm both control expression of the dDA1 dopamine receptor in Ap-let neurons

We next explored potential regulation of dDA1 expression levels by *ap* and *dimm*. We stained the larval CNS

Table 1
Incidence of anti-PC2 and anti-PAL2 immunostaining in larval CNS of different *ap* and *dimm* genotypes

Genotype	PC2[a] (n)	Tvb PC2	Dorsals PC2	PAL2[b] (n)	Br1 PAL2	Tv/Tvb PAL2	Dorsals PAL2
$ap^{GAL4}/+$	4	22/24 (92%)	55/88 (63%)	16[c]	20/32 (63%)	118/192 (62%)	256/352 (73%)
$ap^{p44}/+$	4	23/24 (96%)	72/88 (82%)				
ap^{p44}/ap^{GAL4}	8	1/48 (2%)	0/176 (0%)	17	21/34 (62%)	2/204 (<1%)	0/374 (0%)
$dimm^{P1}/+$ or *Df(2R)Rev 4*/+				9	N.D.[d]	102/198 (52%)	26/108 (24%)
$dimm^{P1}$/*Df(2R)Rev 4*				6	N.D.	2/72 (3%)	19/132 (14%)

[a] 1st instar larvae; Tv staining by anti-PC2 was typically weak, and was not scored; n = number of larvae studied.
[b] n = number of larvae studied; *ap*—early 3rd instar larvae; *dimm*-1st instar larvae.
[c] These two genotypes were analyzed together.
[d] Not determined—Br1 staining was rarely seen during the 1st instar.

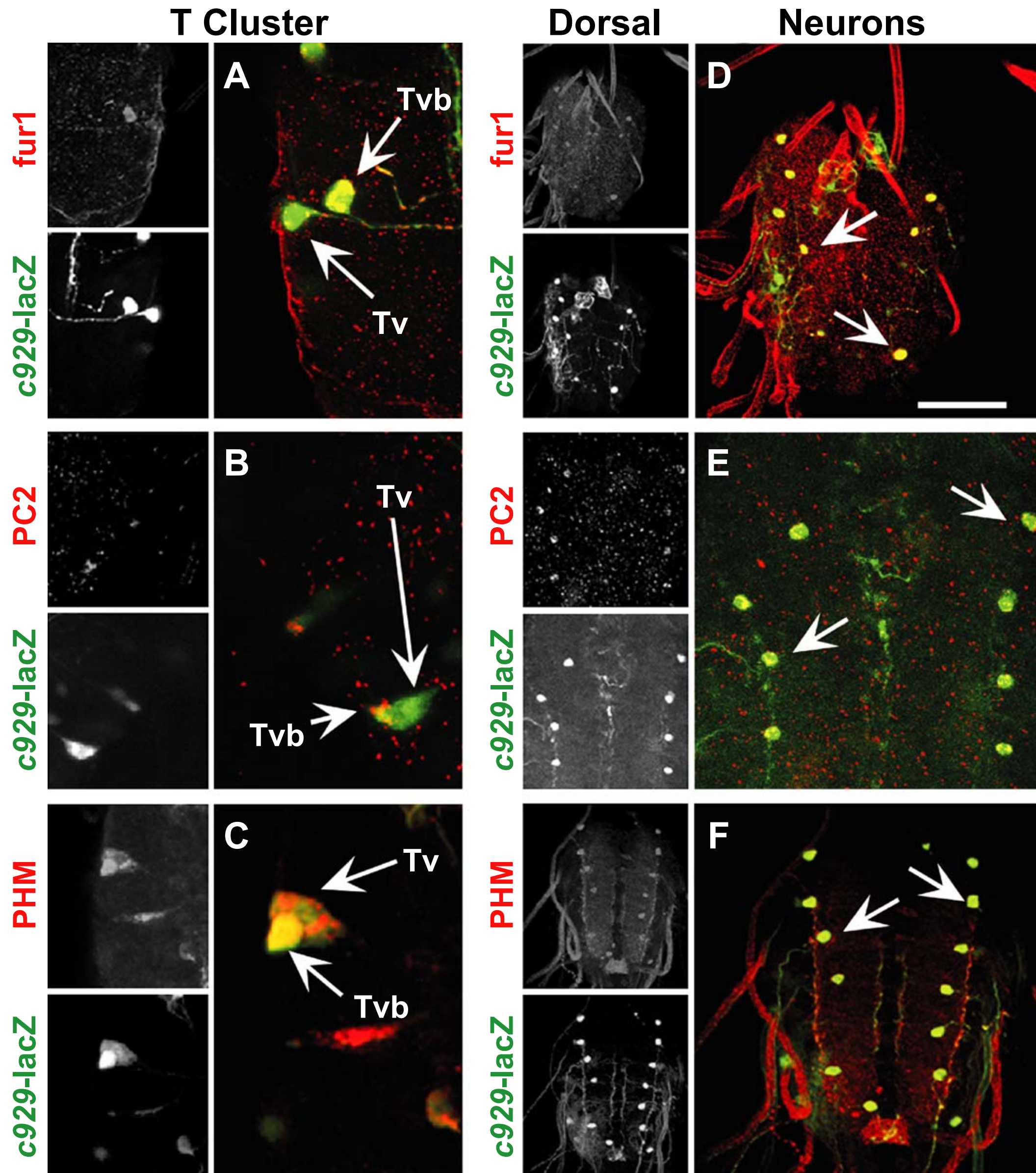

Fig. 4. Co-labeling of Fur1/PC2/PHM *ap* neurons by *c929*. Left: immunostaining of neurons in the T cluster. Right: immunostaining of the Dorsal chain of *ap* neurons. (A) Double immunostaining for Fur1 and for *c929*-lacZ reveals that the Fur1-positive cells Tvb and Tv are both *c929*-positive (a single focal image). The strong *c929*-lacZ cell (Tvb) displays moderate Fur1 immunosignals; the weaker *c929*-lacZ cell (Tv) displays weak Fur1 immunosignals. Similar results were observed by double immunostaining for PC2 and *c929*-lacZ (B—a single focal image) and for PHM and *c929*-lacZ (C—a 25-μm stack). (D) The precise overlap of Fur1 and *c929*-lacZ reveals the Dorsal cells are also *c929*-positive (a 10-μm stack). Similar results were obtained by double staining with PC2 and *c929*-lacZ (E—a 32-um stack) and with PHM and *c929*-lacZ (F—a 33-um stack). Scale bar = 10 μm (A and B); 7.5 μm (C); 33 μm (D and F); 20 μm (E).

of homozygous ap^{GAL4} and trans-heterozygous ap^{GAL4}/ap^{p44} with anti-dDA1 antibody. As depicted in Fig. 5E for the case of the trans-heterozygote, dDA1 immunosignals were not detectable in the VNC of *ap* mutants. In the brain lobes, dDA1 and *ap* were also co-expressed in the mushroom bodies (MB; Fig. 5D); however, this dDA1 expression was not abrogated in *ap* mutants (Fig. 5E). The larval dDA1 expression pattern is conserved in adult VNC (Kim et al., 2003) and was absent in *ap* trans-heterozygous mutants as well (data not shown; n = 6). Therefore, the expression of dDA1 in both developing and adult Ap-let neurons required normal *ap* expression. dDA1 expression also depended on *dimm* activity. We tested larvae trans-heterozygous for the small *dimm* deficiencies *Rev4* and *Rev8*: together, these represent a strongly hypomorphic allelic combination (Hewes et al., 2003). All dDA1 immunostaining in Ap-let cells was also lost in the mutant, but it was retained in the MB (Figs. 5F and 5G). The data for these experiments are summarized in Table 2.

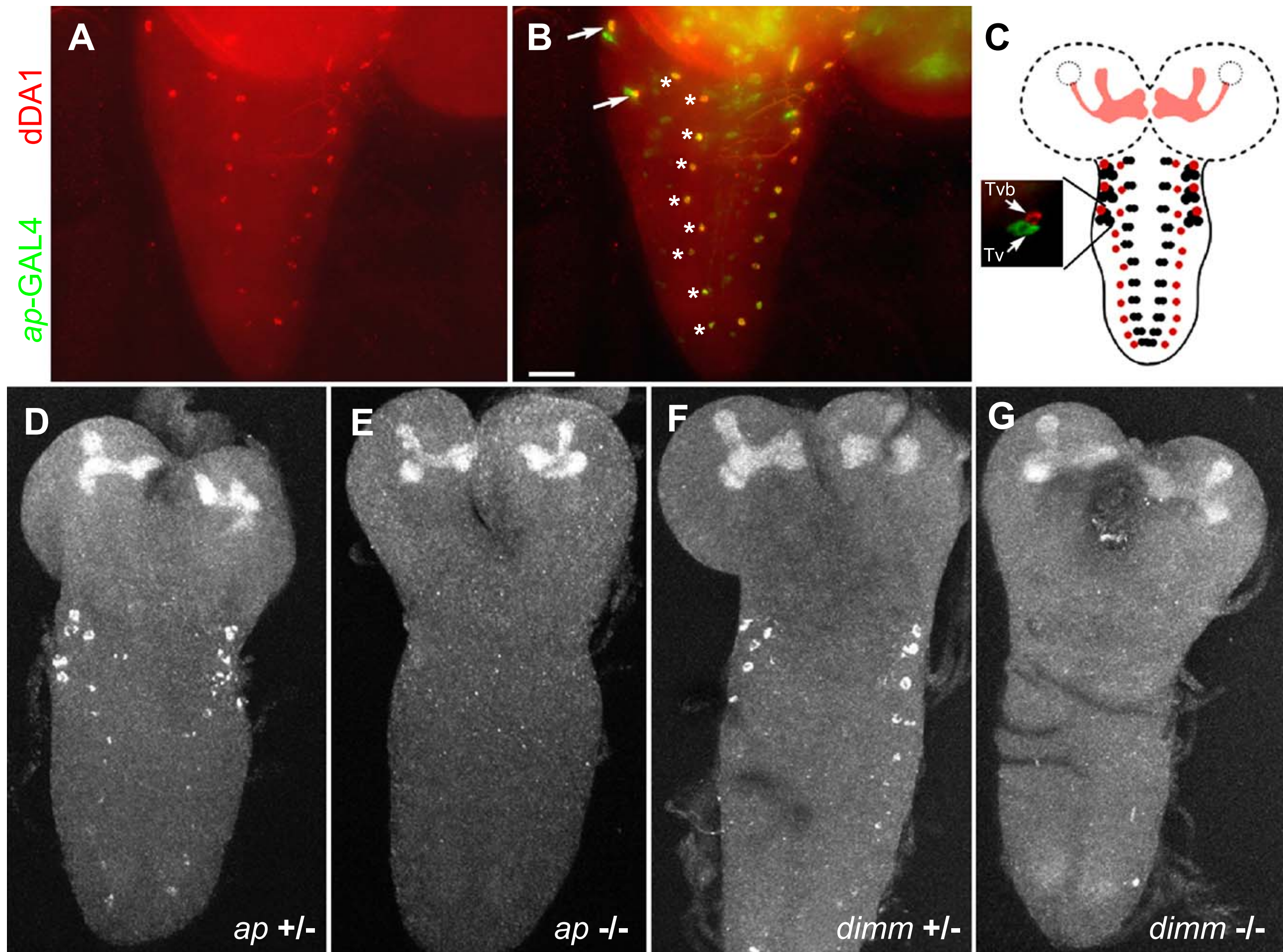

Fig. 5. dDA1 immunoreactivity in *ap*- and *c929*-positive cells in the larval VNC. (A) dDA1 immunoreactivity in ap^{GAL4}/UAS-*GFP*. (B) A composite image of (A) with GFP expression in *ap* neurons. The co-localization of dDA1 and GFP is marked with an asterisk in Dorsal neurons in each of eight abdominal hemi-segments and in the 2nd and 3rd thoracic hemi-segments. In addition, the co-localized lateral neurons in the 2nd and 3rd thoracic hemi-segments are marked with arrows. The Dorsal neurons in the 2nd and 3rd thoracic segments, and one lateral neuron in the 1st thoracic segment are hidden under the brain lobe, thus are not shown. (C) A schematic presentation of dDA1-positive neurons (red circles) among *ap* neurons (black circles). Inset shows that distinct T cluster cells are labeled by anti-dDA1 (red) and anti CT-FMRFa (green) antibodies. (D and E) dDA1 immunostaining in the CNS of *ap* heterozygous (D) and trans-heterozygous animals (E, ap^{GAL4}/ap^{P44}). The dDA1 immunoreactivity is absent in the VNC, but not in the MB, of *ap* mutants (E). (F) dDA1 immunostaining in the CNS of *dimm* heterozygotes (*Rev8*/+ *or Rev4*/+). (G) dDA1 immunostaining in the CNS of *dimm* trans-heterozygotes (*Rev8/Rev4*). In *dimm* mutants, dDA1 immunoreactivity is absent in the VNC, but not in the MB. Images in (A) and (B) were generated by stacking multiple images taken at different focal planes using an epifluorescent microscope. Scale bars = 50 μm (A, B); 20 μm (D–G).

Larval Inka cells express c929 and PHM, but not ap-lacZ, PC2, PAL2, Fur1, or dDA1

We examined larval Inka cells for their ability to stain with the panel of antibodies that define Ap-let neurons. We double-stained larval progeny (two to four specimens each) of the cross *c929* × *UAS-lacZ* with antibodies to β-gal, and either to PC2, PAL2, Fur1, PHM, or dDA1. In addition, we examined four larvae of the line *ap-lacZ* with antibodies to β-gal. As previously observed (O'Brien and Taghert, 1998), *Drosophila* Inka cells are positive for *c929* (*n* = 52 hemi-segments), and also for PHM (*n* = 18/18, data not shown). However, Inka cells were not stained by antibodies for PC2 (*n* = 0/9), Fur1 (*n* = 0/10), PAL2 (*n* = 0/15), dDA1 (*n* = 0/22) or *ap*-lacZ (*n* = 0/28, data not shown).

Table 2
Incidence of anti-dDA1 immunostaining in 1st instar larval CNS in different *ap* and *dimm* genotypes

Genotype	*n*	Tvb dDA1	Dorsals dDA1
ap^{P44}, + or ap^{GAL4}/+	6[a]	32/36 (89%)	94/132 (71%)
ap^{P44}/ap^{GAL4}	14	2/84 (2%)	0/308 (0%)
Rev8/+ *Or Rev4*/+	9[a]	50/54 (93%)	59/198 (30%)
Rev8/*Rev4*	10	3/60 (5%)	0/122 (0%)

[a] These two genotypes were analyzed together.

Ap-let neurons in later developmental stages

Ap-let neurons (the Dorsal neurons and the Tvb, Fig. 6) maintained their cellular properties (*ap-lacZ*, *c929*,

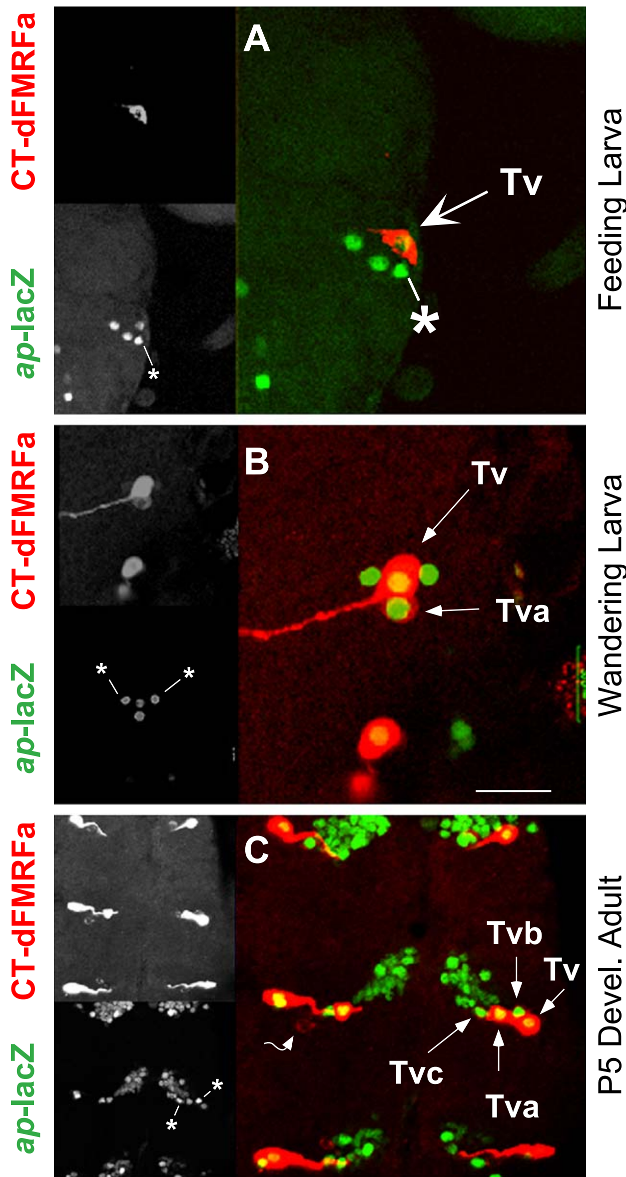

Fig. 6. CT-dFMRFa peptidergic neurons in the T cell cluster of a first instar *ap*-lacZ larva. (A) In the third thoracic neuromere, single large, tear-dropped cell body Tv, is stained by the CT-dFMRFa antibody (18 μm stack). Immunostaining for β-gal reveals four adjacent nuclei, among which Tv is weakly stained. Tvb, which is strongly stained for *ap*-lacZ, is marked by an asterisk. (B) In the 3rd instar wandering stage, the second weakly staining *ap* neuron of the T cluster, Tva, displays CT-dFMRFa expression. The two neurons strongly staining for the *ap* reporter (Tvb and Tvc) are marked by asterisks. (C) At the P5 stage of developing adults, all four T cluster neurons display some CT-dFMRFa immunosignals. Those of Tv and Tva are strong; those of Tvb and Tvc are extremely weak and transient. The two neurons strongly staining for the *ap* reporter (Tvb and Tvc) are marked by asterisks. Note that adjacent, unidentified, *ap*-negative cells also express CT-dFMRFa immunosignals (curved arrow). Scale bar = 5 μm (A and B); 20 μm (C).

PHM, PC2, Fur1, PAL2, and dDA1 expression) throughout pupal and adult stages. At all stages, these neurons represented the principal sites of PC2 and Fur1 expression Supplemental Figure 1 and dDA1 expression (Kim et al., 2003) in the VNC. Regarding the other *ap* VNC neurons, each cell within the four cell T cluster could be distinguished by a particular set of properties, including reproducible patterns of *ap-lacZ* expression. Larval clusters included a single nucleus with very bright *ap-lacZ* staining (Tvb, Fig. 7), and a second nucleus (Tvc) with a moderately bright level of staining ($n > 10$, Fig. 6A). The remaining two nuclei (Tv and Tva) typically had low-to-moderate *ap*-lacZ levels. This pattern persisted in developing adult (P5, $n = 8$, Fig. 6B) and adult stages ($n = 5$, supp. Fig. 1), although the differences were sometimes less pronounced.

In larvae, the Tv neuron is a neuroendocrine cell that projects its axon to a neurohemal organ at the dorsal midline and it is the only cell in the *ap* T cluster expressing the neuropeptide gene *dFMRFa* (Allan et al., 2003; Benveniste et al., 1998; Schneider et al., 1993). It is one of the two weak *ap-lacZ* cells, and has a large, ovoid cell body throughout all larval stages ($n = 10$, Figs. 6A–C). In wandering larvae, a second *dFMRFa* neuron appears near the T cluster in the mesothoracic neuromere (Silber, 1997) and later differentiates as the neuroendocrine Tva neuron of the adult (O'Brien et al., 1991). As shown in Fig. 6B, when Tva first expresses dFMRFa at the wandering larval stage, it expresses *ap*-lacZ weakly, and hence also represents an *ap* T cluster cell. Tva expresses *ap* weakly and Tvc expresses *ap* moderately ($n > 10$ for both embryonic and larval stages). Tv and Tva maintained strong dFMRFa and weak to moderate *ap*-lacZ expression through adult development. Surprisingly, there was a brief stage (approximately P5), when all four T cluster neurons in neuromere T2 expressed dFMRFa immunosignals: two did so strongly (Tv and presumed Tva) and two weakly (Tvb and Tvc; Fig. 6C, $n = 5$). This transient and coordinate expression of dFMRFa by all *ap* T cluster neurons was also noted in the other thoracic neuromeres, but not studied in detail. In contrast to other *ap* T cells, Tvc was not positive for any of the enzymes examined in this study nor for dDA1.

A summary of cellular phenotypic profiles for Ap-let neurons

Fig. 7 presents a summary of information that describes cell type-specific features of the *ap* neurons in the T cluster and in the Dorsal chain. Briefly, the T cluster contains two pairs of cells distinguishable by their level of *ap*-lacZ expression, two are weak, and two are moderate-to-strong. The Tv (weak *ap*-lacZ) and Tvb (strong *ap*-lacZ) neurons appear to be differentiated peptidergic neurons from the late embryo through the larval stages. Tv expresses dFMRFa, while the presumed Tvb neuropeptide(s) is unknown. The Tvb axon projects to the brain along with that of Tvc (Lundgren et al., 1995; S. Thor, personal communication). The Dorsal chain *ap* cells are most similar in profile to the Tvb cells in that they share the same set of molecular markers and their axons fasciculate

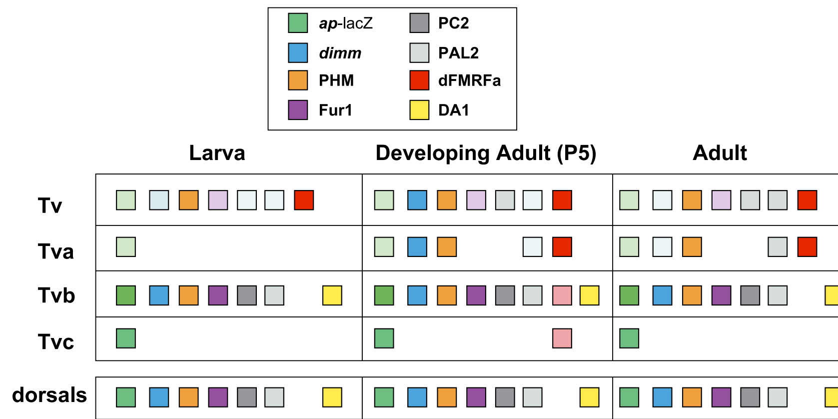

Fig. 7. Block diagram to represent the interpretation of *ap* VNC cell identities and properties throughout all developmental stages. Two *ap* VNC groups are described—the T cluster and the Dorsal chain—during both embryonic and post-embryonic developmental stages. The intensity of the color for a given marker denotes the relative strength of staining observed on a consistent basis (i.e., Tvb was always the strongly stained for *ap*-lacZ and had a small round cell body with the staining properties indicated).

with those of Tvb neurons and project to the brain (Lundgren et al., 1995). Likewise, their presumed neuropeptide product(s) is unknown. The pattern of the Ap-let neurons (Tvb and Dorsal cells) resembles the pattern of CCAP immunoreactive cells in the VNC (Ewer and Truman, 1996). However, Ap-let neurons do not express CCAP because these two sets of cells are not overlapping (Supplemental Figure 2). The Tva cell is weakly *ap*-positive in embryos; its larval fate is unknown. It acquires a peptidergic phenotype at metamorphosis, as indicated by its prominent co-expression of *c929*, PHM, and dFMRFa. Likewise, its adult axon projection mirrors that of Tv, and both are therefore neuroendocrine neurons. The Tvc cell remains largely undefined, except for moderate *ap* expression beginning in the embryo, and weak, transient dFMRFa expression during metamorphosis.

Discussion

The Ap-let cohort of neurons

We identified a small cohort of *ap* neurons that represent the majority of CNS neurons that are immunostained for three putative NP biosynthetic enzymes, PC2, Fur1, and PAL2. We further associated that pattern with expression of a distinct neuroendocrine-specific transcription factor, the bHLH protein Dimm. Finally, the cohort of neurons also features specific expression of aminergic receptor, the dDA1 dopamine receptor. To distinguish this remarkably complex pattern of cellular differentiation, we have dubbed this group of neurons the Ap-let cohort (Fig. 8A). We have chosen to name the group not simply because it displays a distinctive set of molecular markers. Rather, we believe it warrants a special name to call attention to its putative functions in regulating ecdysial behaviors, according to the following hypothesis.

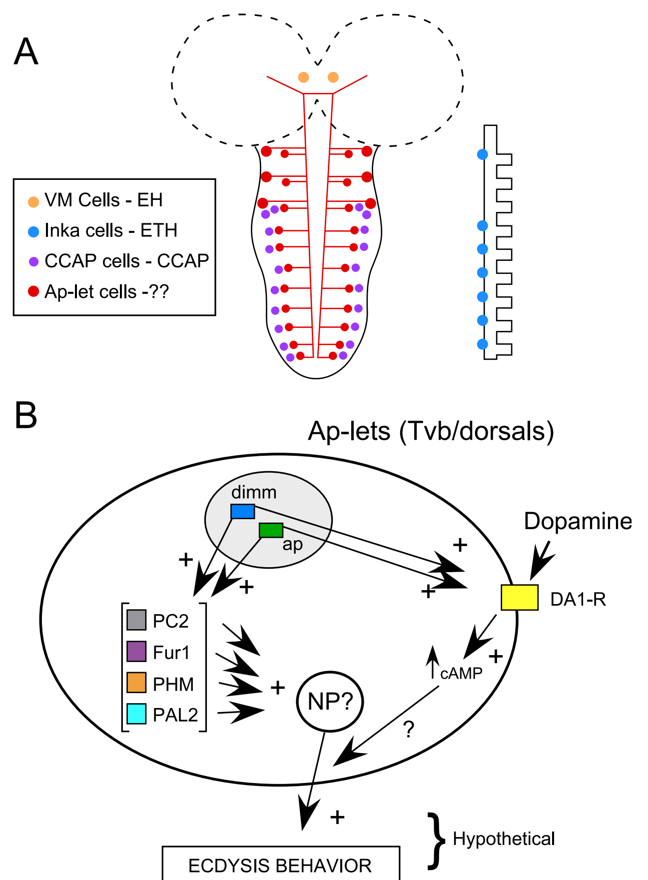

Fig. 8. Summary of Ap-let neuron properties. (A) Neural and endocrine cells involved in control of ecdysial behaviors. The hypothetical roles of VM cells secreting EH and the Inka cells secreting ETH are supported by genetic evidence. The hypothetical roles of the CCAP neurons secreting CCAP and the Ap-let neurons secreting an unknown NP are suspected, but not proven. (B) Flow diagram indicating regulatory relationships between gene products identified within Ap-let neurons. For simplicity, only a single putative effect of elevated cAMP levels (on the secretion of a hypothetical NP) is shown. This diagram features a hypothetical effect of the secreted NP on ecdysis: see text for discussion of this hypothesis.

Hypothesis—the Ap-let cohort contributes to the performance of ecdysial behaviors

We hypothesize that the Ap-let set of neurons contributes to ecdysial behaviors in larvae, and perhaps also in later stages as well. The principle reason to propose this hypothesis relates the phenotype of PC2-deficient animals: a failure to complete embryonic hatching and later larval ecdyses (Rayburn et al., 2003; Siekhaus and Fuller, 1999). PC2 transcripts are detected in Inka cells in stage 17 embryos, but not in post-embryonic animals (Siekhaus and Fuller, 1999). We have found that the main site of PC2 accumulation throughout post-embryonic stages is the Ap-let neurons, while none is detectable in Inka cells (Fig. 8A). Given that PC2-deficient animals display a severe disruption in the ability to produce normal larval ecdysial behavior, we surmise the involvement of the PC2-expressing neurons (the Ap-let cohort) in supporting normal ecdysial behavior. The hypothesis is supported by the highly related phenotype of PHM-deficient animals (Jiang et al., 2000). Severe PHM hypomorphs typically die at larval molts, while synthetic hypomorphs survive through larval stages, but subsequently display defects in head eversion behaviors that are associated with pupal ecdysis. While PHM is widely expressed, its expression within the Ap-let cohort is consistent with its necessity to produce an amidated peptide(s) within Ap-let neurons. Furthermore, the lack of detectable PC2 expression by post-embryonic Inka cells excludes the simple hypothesis that PC2 supports ecdysial behaviors by processing of pro-ETH peptides in Inka cells. Rather, the focus turns to the CNS, and specifically to the Ap-let cohort.

Despite its complexity, the pattern of the Ap-let cohort remained largely invariant throughout all post-embryonic stages examined. The association of *ap* with three NP biosynthetic enzymes, and with a fourth, more broadly expressed enzyme, PHM, suggests this distinct neuronal group produces a similar, amidated bioactive peptide(s) (Fig. 8B). Until very recently, there was no indication of which NP gene may be co-expressed by this group (see recent reviews of the *Drosophila* neuropeptides by Nässel, 2002 and by Taghert and Veenstra, 2003). A very recent report by Verleyen et al. (2004) describes a cell group that greatly resembles the Ap-lets: these neurons express the newly discovered *Drosophila* neuropeptide gene called *NPLP1* (CG3441). Therefore, follow-up studies may pursue this possible identity, and ask about the phenotype of animals deficient in production of *NPLP1*. Likewise, there is now good reason to evaluate the potential role of dopamine in regulating ecdysial behaviors via its effects on Ap-let neurons. Studies in numerous animals including *Drosophila* have indicated roles for dopamine signaling in the control of motor behavior, including locomotion (Rothenfluh and Heberlein, 2002) and aggression (Baier et al., 2002). The specific association of dDA1 expression with the Ap-let cohort suggests a special role of dopamine in controlling some aspect of Ap-let cell physiology. There is at present no evidence with which to support any particular function for dopamine inputs in regulating Ap-let physiology—it could affect NP release and/or its synthesis, or affect transcriptional regulation of the *ap* and *dimm* regulators (Fig. 8B). Analysis of its possible functions awaits the creation of specific *dDA1* alleles or loss-of-function reagents. Regardless of its specific targets, its actions are likely excitatory, since the dDA1 receptor causes elevation of cAMP. We note a special association between *ap* and dDA1, not only in the Ap-let cohort but also in the MBs. It will be interesting to see if there is a similar association of *ap* and D1-like receptors within specific interneuronal sets in other animals. The phylogenetic conservation of each genetic component described herein suggests this behaviorally relevant circuit may display evolutionary parallels in other animals.

The Ap-let cohort contains two morphologically distinct cell types—the Tvb and the Dorsal cells. To what extent is it fair to assume they share functions, simply because they share phenotypic features? There are many examples of neurons that share transmitter properties but which likely participate in different functional circuits. For example, pigment dispersing factor (PDF) is expressed in circadian pacemaker neurons of the brain, and in large non-pacemaker neuroendocrine neurons of the VNC (Helfrich-Förster, 1995). Of these cell groups, only PDF neurons in the brain display clock properties, and PDF expression in brain neurons specifically is affected by clock gene mutations (Park et al., 2000). Likewise, the 17 different *dFMRFa* cell types of the larval VNC are controlled by several distinct transcriptional regulatory mechanisms (Benveniste and Taghert, 1999; Schneider et al., 1993). That feature suggests such neurons, despite sharing a common peptide output, are dedicated to different functions. In contrast, both the Tvb and Dorsal Ap-let neuron cell types project axons to the brain along a common, fasciculated pathway. In addition, the two cell types display *common* mechanisms of regulation (by *ap* and by *dimm*), and both display common receptor (dDA1) and NP biosynthetic enzyme (PAL2, Fur1, PC2) expression profiles. While these two cells types are clearly different by position, at present, every other property they exhibit suggests a similarity of function.

The four cells in the T cluster display characteristic levels of *ap* reporter expression that are correlated with their distinct cell fates. We have interpreted these levels to represent durable features of the individual cells. This assumption is difficult to prove without resort to real-time observations. It is supported, however, by the strong correlations between levels of *ap* reporter and expression of peptidergic cell markers (e.g., dFMRFa is limited to the cells with moderate *ap*-lacZ, dDA1 and strong *c929* was limited to the cell with the highest *ap*-lacZ levels, etc.). Thus, the T cluster represents a spatially segregated set of neurons that displays many common features, as well as several unique, cell-specific features. Such details describe lineally related cells in other parts of the *Drosophila* CNS,

of which the best known are the progeny of the 7–3 neuroblast (Lundell and Hirsh, 1998; Novotny et al., 2002). The NB 7–3 progeny include several serotonin-expressing interneurons, as well as other non-aminergic cell types. Whether T cluster neurons share lineal ancestries is not yet known.

ap and dimm regulate coordinated neuroendocrine cell differentiation in Ap-let neurons

We focused on the approximately 90 *ap* neurons in the 11 thoracic and abdominal neuromeres of the VNC. Of these 90 *ap* neurons, 42 (including the Ap-lets) display clear evidence of moderate-to-strong NP production by virtue of cell type-specific expression of NPs, NP biosynthetic enzymes and/or of reporter genes whose spatial patterns are themselves highly correlated with peptidergic cell fates. Previous studies have suggested that *ap* mutant animals have specific neuroendocrine deficits (Altaratz et al., 1991; Shtorch et al., 1995), and two studies specifically implicated *ap* in the regulation of peptidergic differentiation in the dFMRFa-expressing Tv neuron (Benveniste et al., 1998), and in a leukokinin-expressing neuron of the brain (Herrero et al., 2003). The present results extend this suggestion, and further indicate a notable (though not exclusive) association between *ap* regulation, and the assumption of a peptidergic cell fate. The effect of *ap* mutations on NP dFMRFa in the Tv neuron was partially penetrant, while the same genotypes had a much stronger effect on NP biosynthetic enzyme expression in the same identified cell. There are several potential reasons to explain this difference, but regardless of the strength of the effect, it is clear that *ap* can affect expression of both a NP gene and of NP biosynthetic enzymes in a single identified neuron. Such observations suggest that, within individual cells, *ap* has coordinate control over each of several genes that contribute to the differentiation of a peptidergic phenotype. By an alternative model, *ap* may control levels of specific NPs only, and those levels may in turn feedback to control levels of auxiliary factors such as biosynthetic enzymes. While we favor the first hypothesis for simplicity, we cannot rule out more complex alternatives at the present time. Certain *ap* neurons (e.g., the ventral chain of the VNC) do not appear to differentiate a peptidergic/neuroendocrine cell fate. Likewise, many peptidergic/neuroendocrine neurons in the *Drosophila* CNS do not express *ap*. While most of the other peptidergic neurons express PHM, they likely utilize endoproteolytic biosynthetic enzymes distinct from PC2 and Fur1.

We also showed that *dimm* regulates expression of several NP biosynthetic enzymes in Ap-let neurons in a manner consistent with a previous hypothesis of *dimm* functions (Hewes et al., 2003). Namely, *dimm* was proposed to support the regulated secretory pathway by controlling levels of granule components in dedicated secretory cells. Potential *dimm* targets include all constituents of secretory granules including peptide hormones, biosynthetic enzymes, and granule membrane components. The regulation of dDA1 receptor levels by *dimm* appears to represent an exception to that hypothesis, in that receptors are presumably placed in the cell membrane by constitutive insertion. Recent reports suggest, that following receptor activation, μ opioid receptors in sensory neurons are inserted into the plasma membrane from stores within secretory granules (Bao et al., 2003). Given its control by *dimm*, it will be interesting to establish the subcellular localization of dDA1 in Ap-let neurons. The possible relationship between *ap* and *dimm* is presently unclear. While these factors regulate common events in certain peptidergic neurons, it is uncertain whether they do so via common or independent mechanisms, or whether they influence each other's expression. In most cases of co-expression, *ap* and *dimm* expression is temporally coincident. In the Tva neuron, however, the two genes are turned on at different times. *ap* expression commences in the embryo, right after the birth of the cell; *dimm* expression is delayed for many days until adult development, when the cell first displays peptidergic differentiation. This rank order of appearance may also suggest an epistatic relation between Ap and Dimm in the functional hierarchy within peptidergic neurons, but that hypothesis requires further experimental evaluation.

The delayed differentiation of the Tva cell has precedent: Certain *Drosophila* motor neurons innervate adult-specific muscles, but lack synaptic targets during larval stages. For example, the MN5 neuron makes a short axonal segment, reaching only into the start of the peripheral nerve; the full differentiation of this cell type is not displayed until during adult development (Consoulas et al., 2002). Likewise, *Drosophila* photoreceptors have distinct genetic requirements for early determination and for later cell type-specific differentiation (Mollereau et al., 2001). Interestingly in the moth *Manduca*, the homologous neuron (also called Tva) is fully differentiated during embryogenesis as an FMRFa-positive neuroendocrine neuron along with the Tv neuron in second thoracic segment (Wall and Taghert, 1991).

The present experiments have led to the construction of a provisional map that relates gene expression to individual neuronal phenotypes within the T cluster and Dorsal cell *ap* groups (Fig. 8). This resolution at the level of uniquely identifiable cells is akin to that applied to *Drosophila* neuroblasts (e.g., Doe and Skeath, 1996) and photoreceptors (e.g., Krämer and Cagan, 1994). It provides a basis for further molecular genetic analysis of the regulatory factors that generate such precise cellular diversity. Recent results of Allan et al. (2003) implicate another factor, the zinc finger protein Squeeze, in regulation of dFMRFa expression by Tv neurons. Furthermore, their studies also demonstrated retrograde influences on Tv dFMRFa expression, mediated by BMP signaling. It will be of interest to integrate the action of those regulatory factors with the cellular properties we have described here.

Defining patterns of NP biosynthetic enzyme expression

Siekhaus and Fuller (1999) previously described a more complex pattern of PC2 RNA during embryonic development than we have described for post-embryonic PC2 immunosignals. In addition, *Fur1* RNA patterns in embryonic stages display greater complexity than we have described with antibody stains in post-embryonic stages (Roebroek et al., 1993). There are several possible factors that may explain these differences. First and most obvious is that both previous methods measured the distribution of the relevant RNAs, while we examined distributions of presumed protein immunosignals. Regulation at the level of translation or protein stability could reduce the scope of protein distribution. Second, the developmental stages examined were different—the broad embryonic patterns of *PC2* and *Fur1* expression may resolve to more restricted post-embryonic patterns. In fact, *PC2* RNA signals decrease in complexity even during embryonic times (Siekhaus and Fuller, 1999), such that by the 1st larval instar, the pattern greatly resembles that which we have described with an anti-PC2 antiserum. In addition, we have used only single antibodies for individual gene products and that may not have reacted equally to all putative PC2 or Fur1 isoforms (none yet described). These various considerations indicate that NP biosynthetic genes may undergo complex developmental regulation and these features bear further investigation. Regardless of the complexity of embryonic expression, we note finally that the post-embryonic expression patterns we have defined appear stable through all larval, pupal, and adult stages. They call attention to a circumscribed group of neurons, here called the Ap-lets, for their probable role in regulating larval and pupal ecdysial behaviors.

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