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ORIGINAL ARTICLE

Virginie Orgogozo · François Schweisguth

Evolution of the larval peripheral nervous system in *Drosophila* species has involved a change in sensory cell lineage

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Abstract A key challenge in evolutionary biology is to identify developmental events responsible for morphological changes. To determine the cellular basis that underlies changes in the larval peripheral nervous system (PNS) of flies, we first described the PNS pattern of the abdominal segments A1–A7 in late embryos of several fly species using antibody staining. In contrast to the many variations reported previously for the adult PNS pattern, we found that the larval PNS pattern has remained very stable during evolution. Indeed, our observation that most of the analysed Drosophilinae species exhibit exactly the same pattern as *Drosophila melanogaster* reveals that the pattern observed in *D. melanogaster* embryos has remained constant for at least 40 million years. Furthermore, we observed that the PNS pattern in more distantly related flies (Calliphoridae and Phoridae) is only slightly different from the one in *D. melanogaster*. A single difference relative to *D. melanogaster* was identified in the PNS pattern of the Drosophilinae fly *D. busckii*, the absence of a specific external sensory organ. Our analysis of sensory organ development in *D. busckii* suggests that this specific loss resulted from a transformation in cell lineage, from a multidendritic-neuron-external-sensory-organ lineage to a multidendritic-neuron-solo lineage.

Keywords Peripheral nervous system · Evolution · *Drosophila* · Cell lineage · Cell migration

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V. Orgogozo · F. Schweisguth
Ecole Normale Supérieure,
UMR 8542, 46 rue d'Ulm,
75005 Paris, France

Present address:
V. Orgogozo (✉)
Department of Ecology and Evolutionary Biology, Princeton University,
Princeton, NJ, 08544, USA
e-mail: virginie.orgogozo@normalesup.org

Introduction

Evolution of the abdominal larval peripheral nervous system (PNS) in *Drosophila melanogaster* and closely related species offers an excellent model system to study the developmental modifications responsible for evolutionary changes. The *D. melanogaster* abdominal larval PNS is composed of a constant number of neurons and associated cells, whose characteristics and positions have been well described and are perfectly reproducible between individuals (Bodmer and Jan 1987; Campos-Ortega and Hartenstein 1997; Dambly-Chaudiére and Ghysen 1986; Ghysen et al. 1986; Hertweck 1931). Three main types of sensory organs can be distinguished: external sensory (es) organs, chordotonal (ch) organs and multidendritic (md) neurons. The es and ch organs are composed of one or several neurons associated with accessory cells. The es organs transduce mechanosensory, chemosensory and possibly stretch information (Green and Hartenstein 1997), while the ch organs are thought to function as stretch or proprio-receptors (Jan and Jan 1993). The md neurons do not have any accessory cells and may function as touch-, proprio-, chemo-, thermo-, or nociceptors (Bodmer et al. 1987; Liu et al. 2003; Tracey et al. 2003), or even as non-sensory secretory neurons (Hewes et al. 2003). The simplicity of the larval abdominal PNS therefore allows the study of evolutionary changes at cellular resolution. Since sensory cells occupy very precise positions and exhibit specific orientations within the body, changes that might have occurred during evolution regarding cell number, position and orientation can be detected. Also, the peripheral location of the larval PNS makes it easily accessible for observation in various species. Finally, the development of sensory organs has been well documented in *D. melanogaster* and many of the genes that regulate the successive steps of sensory development are known (reviewed in Campos-Ortega and Hartenstein 1997; Ghysen and Dambly-Chaudière 1989, 2000; Jan and Jan 2001). Correspondingly, many antibodies have been developed and can be used to follow the different steps of larval sensory organ formation in *D.*

melanogaster (see for example Orgogozo et al. 2001, 2002). Since some antibodies cross-react in other insect species (Meier et al. 1991; Grueber and Truman 1999, and this study), they may be useful to identify variations in sensory organ development between species.

In *D. melanogaster*, the neurons and associated support cells that constitute each larval sensory organ are produced during embryogenesis from a primary precursor (pI) cell. pI cells are singled out from a group of adjacent epithelial cells named the proneural cluster, in a process mediated by the Notch signalling pathway (reviewed in Simpson et al. 1999). Following its specification, each pI cell undergoes a stereotyped sequence of asymmetric cell divisions to generate sensory cells (Bodmer et al. 1989). In the ventral abdominal region, two types of sensory lineages have been observed. The md-es lineage, followed by pI cells located at positions 1–4 and 4a, consists of a series of four-cell divisions and produces a four-cell es organ and an md neuron (Orgogozo et al. 2001; Fig. 1a, c, d). The md-solo lineage, followed by the pI cell located at position 1a, comprises two cell divisions but generates only one cell, an md neuron, because the other progeny cells undergo apoptosis (Orgogozo et al. 2002; Fig. 1b–d). The md-solo lineage has been proposed to be a modified md-es lineage in which two secondary precursor cells undergo apoptosis. In support of this proposal, in embryos in which apoptosis is inhibited, the two cells fated to death divide and together produce an ectopic four-cell es organ indicating that the md-solo lineage has been transformed into an md-es lineage (Orgogozo et al. 2002).

Comparative analysis of first-instar larvae and nymphs from several insect orders indicated that larval abdominal PNS patterns fall into three main types: fixed pattern (in Diptera), fully variable pattern, and variable pattern with some fixed sensory organs (in most other species; Green and Hartenstein 1997 and references therein). In species with a fixed pattern, sensory organs are usually distributed over the entire segment whereas in species with a variable pattern, they typically form one or several rows along the dorsal-ventral axis in the middle of each segment. In both *D. melanogaster* and the grasshopper *Schistocerca gregaria*, sensory neurons are grouped into three regions (ventral, lateral, dorsal) and their axons primarily project onto two nerves (intersegmental and segmental nerves) towards the ventral nerve cord. Although the *Schistocerca* pleural ch organ probably corresponds to the *Drosophila* lch5 ch organ according to its position, it is difficult to compare other sensory organs between *Drosophila* and *Schistocerca*. In the moth *Manduca sexta*, several individual abdominal MD neurons have been proposed to correspond to particular *D. melanogaster* md neurons according to their time of development and their position within the larval body wall (Grueber and Truman 1999).

Morphological modifications of the larval PNS pattern during evolution may result from various developmental changes, such as: (1) appearance/loss of a particular sensory organ associated with emergence/loss of a proneural cluster or pI cell, or with survival/death of sensory cells; (2) re-positioning of sensory cells (through changes in proneural cluster position or migration of sensory cells); (3) change in sensory organ types; etc.

To identify changes in larval PNS pattern in species related to *D. melanogaster*, we examined the PNS of the abdominal segments A1–A7 in several fly species. Then, in order to investigate the developmental changes underlying different patterns, we analysed sensory organ development in these species.

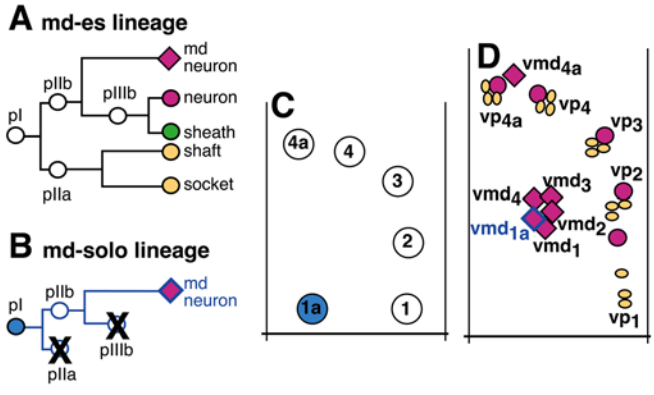

Fig. 1a–d Formation of sensory organs in the ventral region of *D. melanogaster*. **a** Md-es lineage (Orgogozo et al. 2001). **b** Md-solo lineage (Orgogozo et al. 2002). The pIIa and pIIIb precursor cells undergo apoptosis. **c** Diagram of the Cut-positive pI cells. The pI cell at position 1a (*blue cell*) follows an md-solo lineage whereas the ones at positions 1–4 and 4a (*white cells*) follow an md-es lineage. **d** Diagram of the Cut-positive sensory cells in mature PNS. Each es organ is composed of a socket/shaft cell pair (*yellow cell*), a sheath cell (*yellow cell*) and one or several es neurons (*pink circular cell*). md neurons (*diamond-shaped cells*) originate either from an md-es lineage (*encircled in black*) or an md-solo lineage (*encircled in blue*)

Materials and methods

Fly stocks and embryo fixation

The fly stocks used were: *D. melanogaster yw* ; *D. sechellia* 14021-0248.7 and *D. malerkotliana* (gifts of D. Stern); *D. yakuba*, *D. bifasciata*, *D. busckii* 1, and *Zaprionus vittiger* (gifts of P. Santamaria); *D. bifurca* (collected in Mexico, from Bowling Green Stock Center collection), *D. hydei* (collected in Raida, Saudi Arabia), and *D. buzzatii* (collected in Nahal Oren, Israel; gifts of D. Lachaise); *D. ararama* (gift of P. Simpson); *D. virilis* (gift of J.-A. Lepesant); *D. busckii* 2 and *D. duncani* (gift of N. Gompel); and *Megaselia scalaris* (gift of M.-L. Carriou). The *D. busckii* 1 strain was established from a female collected in France while the *D. busckii* 2 strain was from the United States. One-day-laid embryos were collected and fixed according to standard *D. melanogaster* procedures (Rothwell and Sullivan 2000), with the modification that *M. scalaris* and *D. hydei* embryos were dechorionated in bleach for at least 5 min. Fixed embryos from *Calliphora vicina* and *Phormia terraenovae* were kindly provided by P. Simpson.

Immunostaining and microscopy

Embryos were stained as previously described (Orgogozo et al. 2001). Primary antibodies were used at the following dilutions: mouse anti-Cut, 1/1,000 (2B10; DSHB), rat anti-Elav, 1/4 (7E8A10, DSHB), rabbit anti-HRP, 1/500 (Cappel), rabbit anti-Collier, 1/500 (gift of A. Vincent). The RNA-binding protein Elav specifically accumulates in all neurons in *D. melanogaster* (Robinow and White 1988). Anti-Elav antibodies were previously used to stain neurons in the moth *Manduca sexta* (Grueber and Truman 1999) and are, therefore, likely to cross-react between different fly species. The anti-HRP antibodies have been shown to bind to neuronal membranes in all ecdysozoan animals (Haase et al. 2001; Jan and Jan 1982). In *D. melanogaster*, they are known to recognise an epitope of carbohydrate origin, an α1,3-fucosylated, *N*-linked glycan (Fabini et al. 2001; Snow et al. 1987) associated with several cell adhesion molecules and with a Na + K + ATPase (Desai et al. 1994; Sun and Salvaterra 1995; Wang et al. 1994). Staining with anti-HRP antibodies permits us to identify the three types of sensory neurons: es, ch and md neurons. The ch dendrites are surrounded by a scolopale cell which is also HRP-positive (Bodmer et al. 1987), and the es neurons can be reliably distinguished from md neurons because their single dendrite has a small HRP-positive dot at its tip. In *D. melanogaster*, the transcription factor Cut has been observed to accumulate in all the cells that compose an es organ [socket cell, shaft cell, sheath cell, and es neuron(s)] as well as in a subset of md neurons (Blochlinger et al. 1988, 1990; Brewster and Bodmer 1995; Grueber et al. 2003a). Secondary antibodies Cy3-anti-mouse, Cy5-anti-rat and, Alexa488-anti-rabbit were purchased from Jackson Laboratories or Molecular Probes. For *D. hydei*, *C. vicina*, *P. terranovae* and *M. scalaris*, the Cut signal was amplified using biotinylated anti-mouse (1/2000; Jackson Laboratories), avidin DH-biotinylated HRP complexes (Vector Laboratories), TSA-biotin (NEN Life Science), and streptavidin-Alexa568 (Molecular Probes). For each abdominal region (ventral, lateral, dorsal), at least four hemisegments of three embryos were analysed. Developmental stages of *D. busckii* and *C. vicina* embryos were identified according to the extent of germ band retraction, as for *D. melanogaster* embryos. Images were collected on a Leica SP2 confocal microscope and processed using Adobe Photoshop software. Figures show the mean projection of several confocal z-sections. When necessary, the CNS signal detected in the bottom z-sections below the sensory cells was removed to better show the sensory cells in the z-projected images.

Results

To identify changes in the larval PNS pattern among fly species related to *D. melanogaster*, we examined embryos of several species, fixed at the end of peripheral neurogenesis and stained with anti-Cut, anti-Elav and anti-HRP antibodies. The triple staining Cut, Elav, HRP worked in all species analysed herein and allowed the identification of all the es cells as well as all ch and md neurons (see Materials and methods). For a given species, we observed that each abdominal segment A1–A7 displays the same PNS pattern.

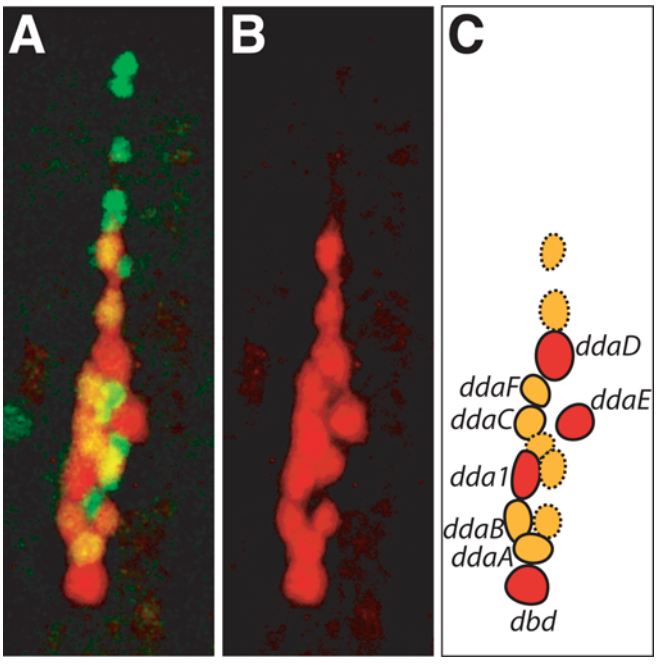

Fig. 2a–c Dorsal sensory cluster in *D. melanogaster* stage 16 embryos. **a** Dorsal abdominal region stained for Cut (*green*) and Elav (*red*). **b** Red channel. **c** Schematic representation of the Elav-positive neurons seen in **a** and **b**. Cut-negative neurons are in *red* and Cut-positive neurons are in *orange*. The five es neurons are *encircled by a dashed line* while the eight md neurons are *encircled with a straight line*. Names have been assigned to each md neuron according to the nomenclature used in Brewster et al. (2001) and Grueber et al. (2002). In all figures, anterior is *left* and dorsal is *up*

The dorsal sensory cluster contains 13 neurons in *D. melanogaster*

Clustering of sensory cells in the dorsal cluster has rendered the number of neurons in this dorsal cluster difficult to determine. First studies indicated that 10 (Ghysen et al. 1986), 11 (Bodmer and Jan 1987), or 12 (Bodmer et al. 1989; Ghysen et al. 1986) neurons are present in the dorsal cluster. Following these studies, the dorsal cluster was usually estimated to contain 12 sensory neurons: 5 es neurons (innervating 3 mono-innervated es organs and 1 doubly innervated es organ), 1 bipolar md neuron and 6 md neurons exhibiting an extensive dendritic arborisation.

Our examination of the *D. melanogaster* PNS pattern revealed that there is an additional md neuron in the dorsal cluster, resulting in a total number of 13 dorsal sensory neurons (Fig. 2a–c). This observation agrees with those of Moore et al. (2002), who also observed 13 sensory neurons in the dorsal cluster (see Fig. 1B in Moore et al.

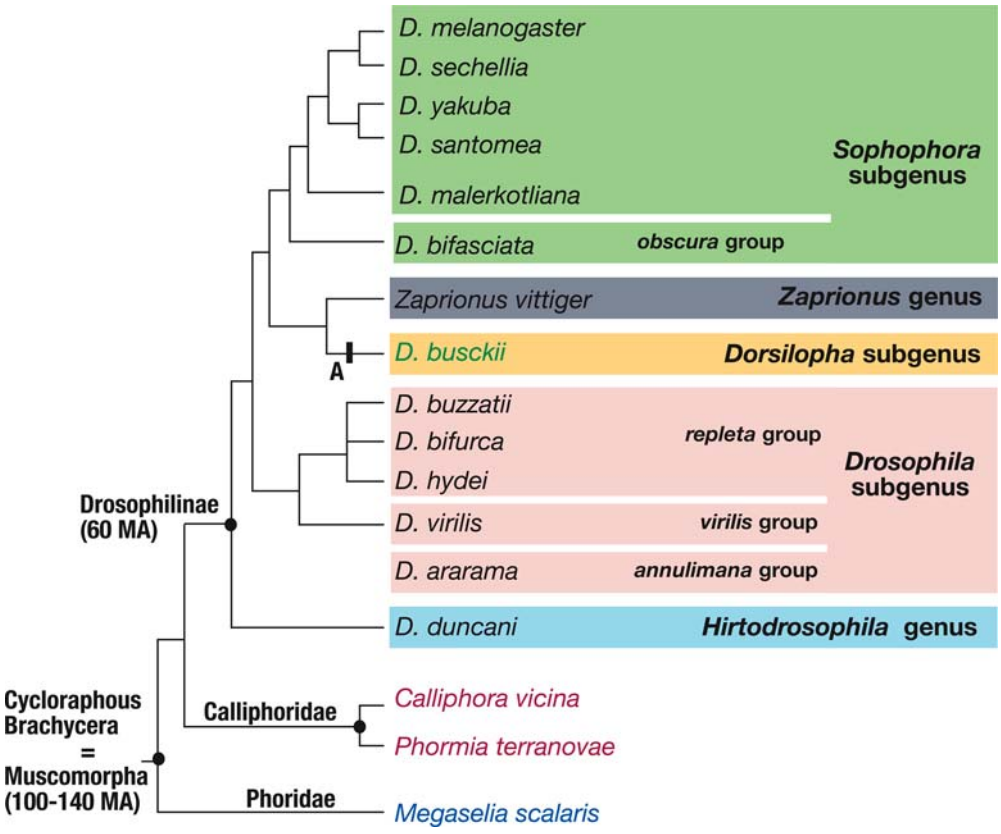

Fig. 3 Phylogenetic tree of the fly species analysed in this study. Adapted from Remsen and O'Grady (2002). Note that branch lengths are not informative. Species with the same abdominal larval PNS pattern are written *in the same colour*. Transformation of the md-es lineage at position 4 into an md-solo lineage may have arisen in *A* during evolution

2002). In former representations of the dorsal sensory cluster, either the Cut-negative dda1 neuron (Grueber et al. 2002, 2003a, 2003b) or one of the Cut-positive md neurons (Brewster et al. 2001; Orgogozo et al. 2002) has been neglected. A complete nomenclature for each dorsal md neuron is proposed based on the nomenclature used in Grueber et al. (2002) and Brewster et al. (2001; Fig. 2c).

Drosophilinae species exhibit the same abdominal PNS pattern as *D. melanogaster*

In all the Drosophilinae species we analysed (*D. sechellia*, *D. malerkotliana*, *D. yakuba*, *D. bifasciata*, *Z. vittiger*, *D. santomea*, *D. bifurca*, *D. hydei*, *D. buzzatii*, *D. ararama*, *D. virilis* and *D. duncani*; Fig. 3) except *D. busckii* (see below), we observed the exact same abdominal PNS pattern as the one in *D. melanogaster*. The number and types of sensory organ cells are identical, and each sensory organ is located at the same relative position and with a similar orientation (Fig. 4a–d and data not shown). According to the phylogenetic tree of Remsen and O'Grady (2002; Fig. 3), this indicates that the PNS pattern seen in *D. melanogaster* was present in the common ancestor of the Drosophilinae flies, and that it has been conserved in all the analysed species, with the noticeable exception of *D. busckii*.

Drosophilinae, Calliphoridae and Phoridae species display similar PNS patterns

We next examined the larval abdominal PNS of three fly species outside the Drosophilinae subfamily, one Phoridae, *Megaselia scalaris*, as well as two Calliphoridae flies, *Calliphora vicina* and *Phormia terranovae*. These three species showed a PNS pattern distinct from the Drosophilinae ones (Figs. 4g, h, 5c, d). Both Calliphoridae exhibit the same PNS pattern, suggesting it was already present in their common ancestor. We noticed many similarities with the *D. melanogaster* larval PNS. As reported for *P. terranovae* (Osborne 1963), we found that in these three species, the number and general position of ch organs is the same as in *D. melanogaster*. Moreover, a Cut-negative md neuron that emits two long dendrites along the antero-posterior axis, named dbp in *D. melanogaster*, is present in the ventral part of the dorsal sensory cluster in each species. A few es organs and md neurons can also be tentatively considered as equivalent between species according to their relative positions (lh1, lp2, lh2, vp5, vp1-3, dda1, ltd, istd, isbd/lbd, lda, vtd1/2, v'dap, vdap, vmd1-4, vmd1a, Fig. 5c, d).

In the ventral region of Calliphoridae flies, two discrete changes were observed relative to Drosophilinae. First, the five ventral es organs are not arranged in a semi-circle similar to the one in Drosophilinae (compare Fig. 4a with g). While es organs appear to be likewise present at the equivalent positions 1–3, the other two es organs (that may correspond to positions 4 and 4a) are not at the same

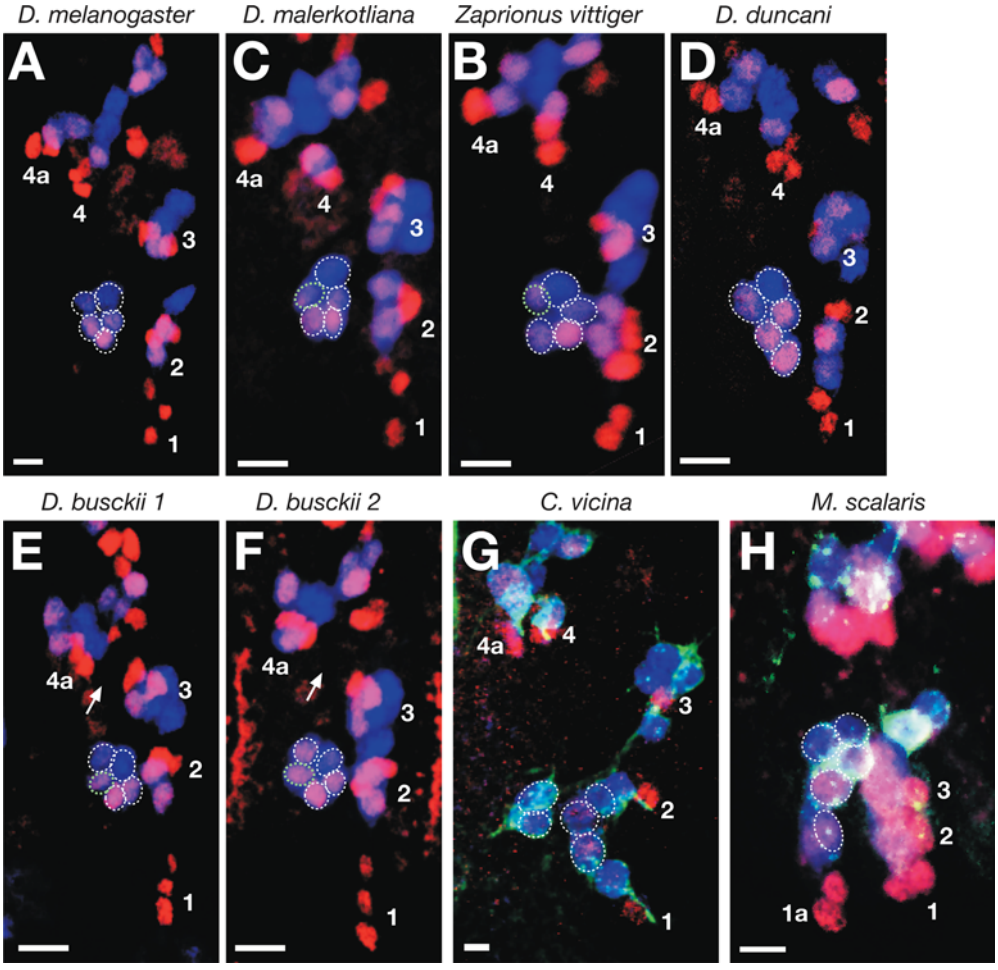

Fig. 4a–h Ventral PNS patterns in various fly species. Ventral region of an abdominal segment A1–A7 in stage 16 embryos stained with anti-Cut (*red*), anti-Elav (*blue*), and anti-HRP (*green*, in **g**, **h** only) antibodies. For sake of clarity, the HRP channel has been omitted in **a**–**f**. In *D. melanogaster* (**a**), *D. malerkotliana* (**b**), *Zaprionus vittiger* (**c**) as well as *D. duncani* (**d**), five mono-innervated external sensory (es) organs are detected in the ventral region at positions 1–4 and 4a. In *D. busckii* embryos (**e**,**f**), only four mono-innervated es organs are observed in the same region at equivalent positions 1–3 and 4a. No Cut-positive cell is detected at equivalent position 4 (*arrow*). In *Calliphora vicina* (**g**), es organs are present at equivalent positions 1–4 and 4a (see text for detail). In *M. scalaris* (**h**), es organs are detected at equivalent positions 1a, 1–3 and two other es organs are present dorsally. In every species, the ventral multidendritic neuron cluster comprises five Elav-positive neurons (*outlined cells*). *Scale bar* is 5 mm.

relative position. The most dorsal is the anterior one (position 4a) in Drosophilinae whereas it is the posterior one in Calliphoridae. In order to elucidate the developmental change underlying this distinct pattern, we examined the positions of the sensory cell clusters during sensory organ development using Cut as a sensory precursor cell marker (Blochlinger et al. 1990). As in *D. melanogaster*, each Calliphoridae ventral es organ is composed of four different cells, a neuron, a sheath cell, a socket cell and a shaft cell. In *D. melanogaster*, the four cells that compose each es organ are produced roughly at their final position from a single pI cell through an md-es lineage (Orgogozo et al. 2001; Fig. 1). In *D. melanogaster* embryos, all sensory cell clusters in the ventral region start to be easily detected from stage 12. At this stage, clusters are arranged at very specific positions named 1–4, 4a, 1a, with positions 1-2-3-4-4a arranged in a semi-circle centered on position 1a (Fig. 6a, b). In stage 12 *C. vicina* embryos, two-or three-cell sensory clusters lie at stereotyped positions that may correspond to positions 1a-1-2-3-4-4a in *D. melanogaster* (n =17; Fig. 6c). Interestingly, a distinct arrangement of positions 4 and 4a is already visible at stage 12 between *D. melanogaster* and *C. vicina*. Whereas both clusters 4 and 4a are located at about the same level along the dorsal-ventral axis in *D. melanogaster* (Fig. 6a, b), cluster 4 is more dorsal than cluster 4a in *C. vicina* (Fig. 6c). This suggests that the difference in es organ positioning in mature PNS of both species results from a difference in sensory precursor cell position.

Second, the five ventral md neurons are not as clustered as in Drosophilinae and they closely abut es neurons (Fig. 4g and data not shown). Since sensory cell clusters appear to be similarly positioned relative to each other in *D. melanogaster* and *C. vicina* (compare Fig. 6a, b with c), this suggests that md neurons originate roughly from similar places in both *D. melanogaster* and *C. vicina*, and that the distinct arrangement of ventral md neurons in both mature PNS is probably achieved through a difference in md neuron migration.

An md-es to md-solo lineage transformation in *D. busckii*

In *D. busckii*, the PNS pattern is identical to the one observed in all other Drosophilinae except that the es organ seen at position 4 in the ventral region of all other Drosophilinae is missing (Figs. 4e, f, 5b). Absence of this es organ was observed in two independent *D. busckii* strains, one collected in France and one in the United States (Fig. 4e, f). Thus, at least two populations of *D. busckii* show this distinctive PNS pattern, suggesting that

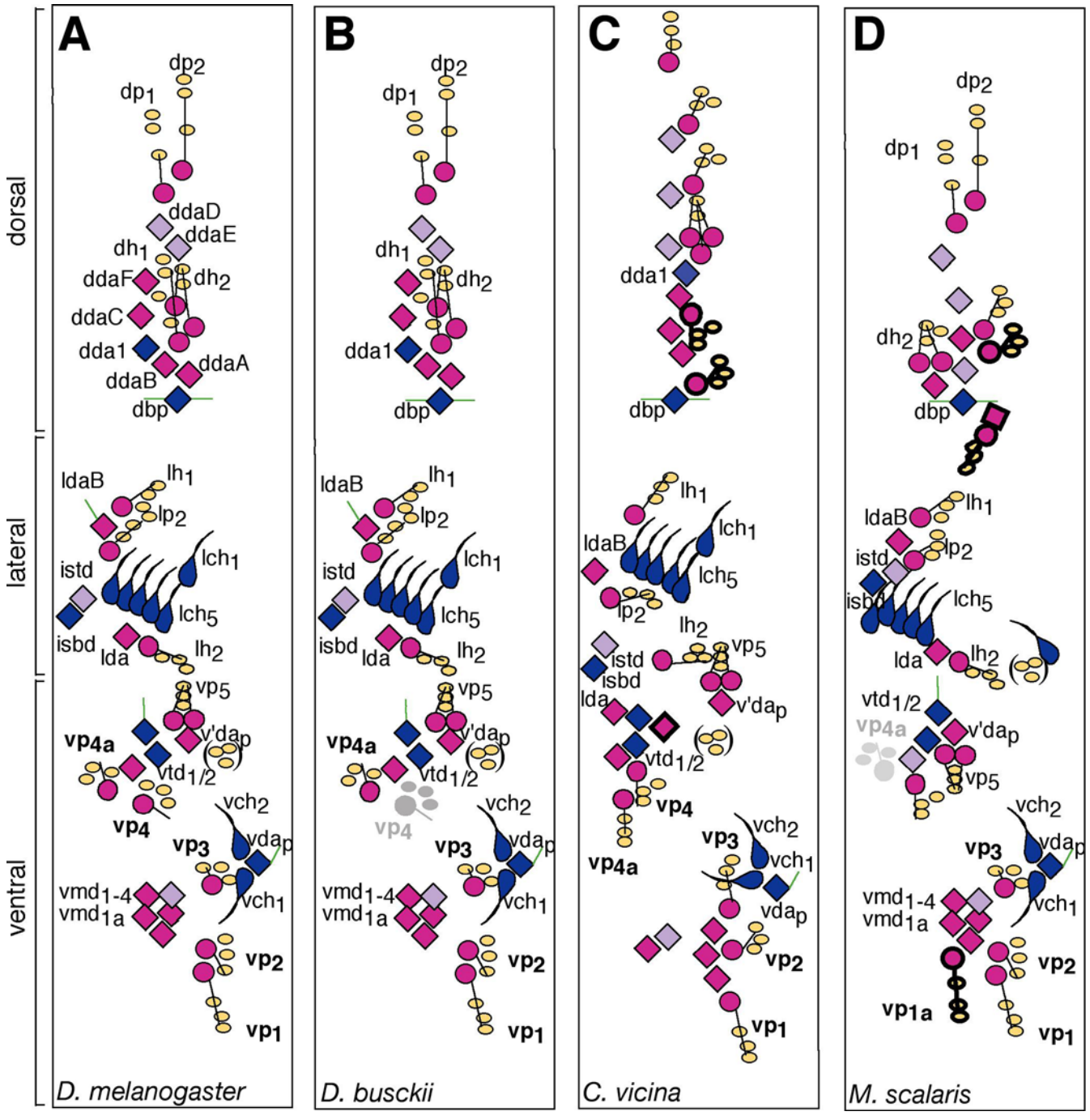

Fig. 5a–d Diagram of the embryonic abdominal PNS in segments A1–A7 of *D. melanogaster* (**a**), *D. busckii* (**b**), *Calliphora vicina* (**c**) and *Megaselia scalaris* (**d**). Schematic representation as in Fig. 1. Each es neuron emits a single dendrite (*black line*) along a particular orientation. The md neurons accumulate either a high level of Cut (*dark pink*), a low level of Cut (*light purple*), or no Cut (*blue*). Some md neurons have long and thick HRP-positive dendrites (*green line*). The ch neurons (*pear-shaped blue cells*) are either isolated or clustered in array. A group of three Cut-positive cells without any associated neuron (*yellow cells in brackets*) was also detected in the ventro-lateral region of all fly species. These three cells may form an epidermal gland (V. Orgogozo and F. Schweisguth, unpublished results). **b** In *D. busckii*, the four-cell es organ at position 4 is missing (*grey cells*). **c** In *C. vicina*, as well as in *Phormia terraenovae* (data not shown), we detected one ectopic Cut-positive md neuron in the ventro-lateral region and two ectopic mono-innervated es organs in the dorsal region compared to the *D. melanogaster* PNS pattern (cells *outlined with a thick black line*). **d** In *M. scalaris*, we detected three ectopic mono-innervated es organs and possibly one ectopic Cut-positive md neuron compared to the *D. melanogaster* PNS pattern (cells *outlined with a thick black line*). Furthermore, the four-cell es organ at position 4a appears to be missing compared to the *D. melanogaster* pattern (*grey cells*). Note however that the number of md neurons in the dorsal region has not been determined with certainty in *M. scalaris*

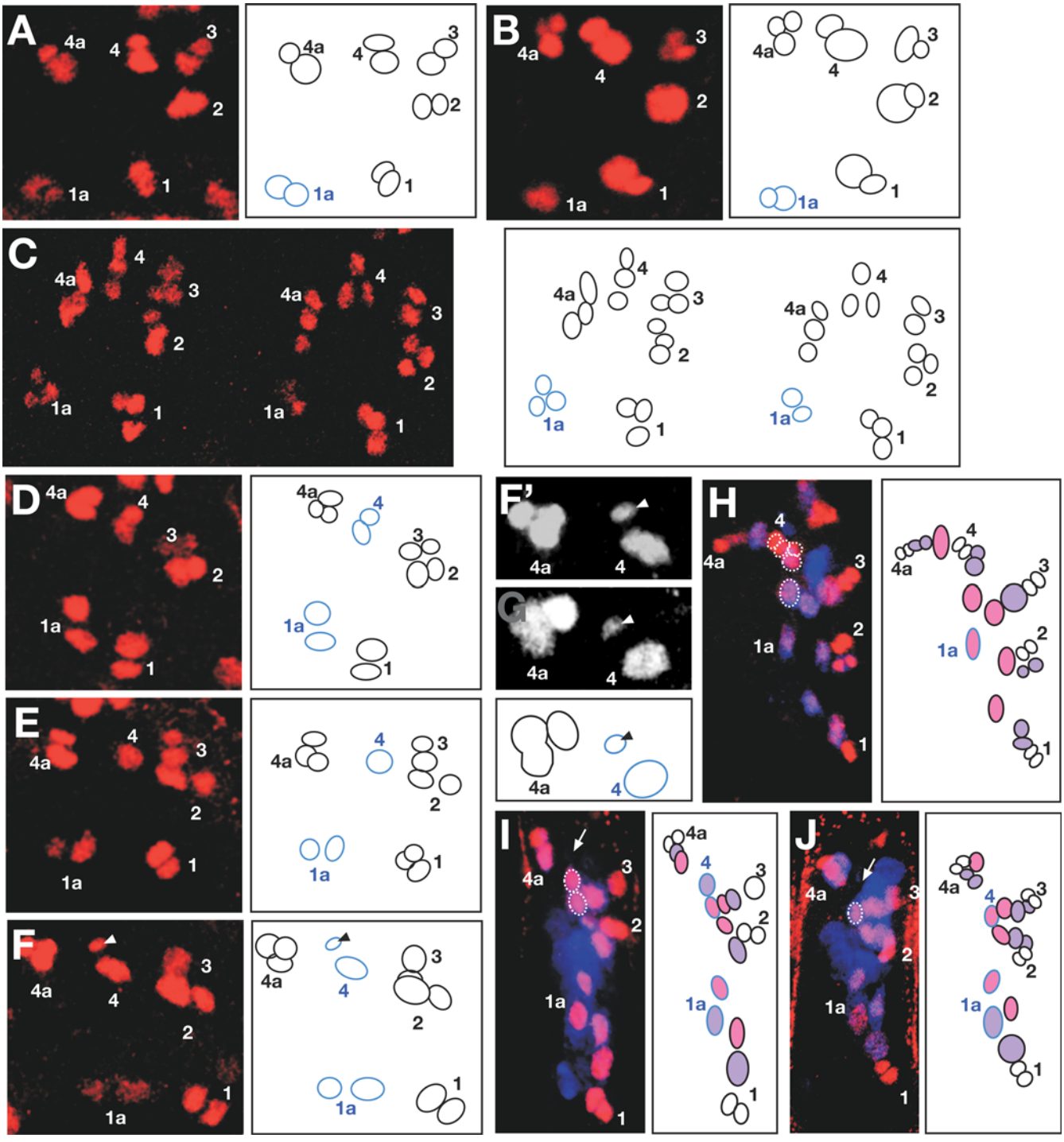

Fig. 6a–j Analysis of sensory organ formation in *Calliphora vicina* and *D. busckii*. Confocal images show the ventral region stained for Cut (*red* in **a**–**f**, *white* in **f′**, **g**) and Elav (*blue* in **h**–**j**). Corresponding schematic representations of the Cut-positive cells are presented for each confocal image. In the schematic representations, sensory precursor cells that may originate from an md-solo lineage are *outlined in blue*. Cells accumulating a high level of Elav are in *pink* while cells accumulating a low level of Elav are in *purple*. **a**–**e** At stage 12, two- or three-cell clusters are detected at positions 1–4, 4a and 1a in *D. melanogaster* embryos (**a**, **b**) as well as in *C. vicina* embryos (**c**). In stage 12 *D. busckii* embryos (**d**, **f**), similar two- or three-cell clusters are detected at the equivalent positions 1, 2, 3, 4a and 1a. However, only one (**e**, **f**) or two (**d**) cell(s) are detected at the equivalent position 4. **f′**, **g** Positions 4 and 4a at stage 12 *D. busckii* (**f′** is a high magnification of **f**). A Cut-positive dot is observed at position 4 near the Cut-positive cell (*arrowhead* in **f**, **f′**, **g**). In stage 13 *D. melanogaster* embryos (**h**), a five-cell cluster is detected at positions 4 (*outlined cells*). In stage 13 *D. busckii* embryos (**i**, **j**), only one (**j**) or two (**i**) Cut-positive cells are detected at this equivalent position (*outlined cells and arrows*)

this pattern may be characteristic of the *D. busckii* species. According to the phylogenetic tree established by Remsen and O'Grady (2002; Fig. 3), the most parsimonious evolutionary scenario is that the es organ at position 4 has been lost during evolution in the branch leading to *D. busckii* (see Discussion).

We next investigated the developmental change underlying the novel pattern seen in *D. busckii*. In *D. melanogaster*, the es organ at position 4 is associated by lineage with the md neuron named vmd4. Thus, we wondered whether the absence of an es organ at position 4 in *D. busckii* is associated with the absence of the vmd4 neuron. In *D. melanogaster*, the vmd4 neuron migrates to the center of the semicircle of the vp1-4a es organs, where it clusters with four other md neurons, the three vmd1–3 neurons originating from the pI cells at positions 1–3 (Orgogozo et al. 2001; Fig. 1d) and the vmd1a neuron. The vmd1a neuron can be identified using an antibody for the transcription factor Collier (Col; Crozatier et al. 1996). Col accumulates only in three neurons in the abdominal PNS, vmd1a, vmd4a, and the dorsal md neuron ddaC (M. Crozatier and A. Vincent, personal communication). Noticeably, in *D. busckii*, the ventral md cluster contains five md neurons including a single Col-positive neuron, as in *D. melanogaster* (Fig. 7a, b). This indicates that *D. busckii* also possesses a vmd4 neuron even though no es organ is associated with vmd4.

A simple developmental model to account for the presence of the vmd4 neuron and the absence of an associated es organ in *D. busckii* is that a pI cell forms at position 4 in *D. busckii* as in *D. melanogaster* but that this pI cell undergoes an md-solo lineage in *D. busckii* instead of an md-es lineage (Fig. 7c, d). The md-solo lineage comprises two successive asymmetric cell divisions and generates one md neuron whereas its two other progeny cells, named pIIa and pIIb, undergo apoptosis (Fig. 1b). To test this hypothesis, we examined sensory precursor cells in the ventral region during *D. busckii* neurogenesis. If this hypothesis is correct, then sensory precursor cells must be detected at position 4.

In stage 12 *D. busckii* embryos, clusters of sensory precursor cells are arranged in a pattern similar to *D. melanogaster*, at the equivalent positions 1–4, 4a and 1a (n =164 hemisegments; Fig. 6d, f), indicating that a pI cell is indeed generated at position 4 in *D. busckii*. As in *D. melanogaster*, each *D. busckii* cluster contains two or three sensory precursor cells at positions 1–3, 4a, and 1a.

However, at position 4, clusters never comprise three Cut-positive cells, but only two (69%, n =16; Fig. 6d) or one (31%; Fig. 6e, f). A single Cut-positive cell at position 4 is never detected in *D. melanogaster* at this stage (n =15). The unique Cut-positive cell located at position 4 in *D. busckii* is probably not a pI cell since pI cells are known to accumulate a low level of Cut. We hypothesise that this cell is a pIIb cell and that its sibling pIIa cell has undergone apoptosis. Consistent with this interpretation, we sometimes observed at this stage at position 4 in *D. busckii* some small Cut-positive dots, which we interpreted as nuclear remnants of a dying cell (n=2 hemisegments; arrowheads in Fig. 6f, f', g). According to this view, the two-cell clusters observed at position 4 in *D. busckii* at stage 12 are either pIIa-pIIb cell pairs (before cell death of pIIa) or both pIIb daughter cells (after cell death of pIIa).

At late stage 13, four or five Cut-positive cells are present at position 4 in *D. melanogaster* (Fig. 6h and data not shown). These cells are the two strongly stained Cut-positive socket and shaft cells, the Elav-positive md

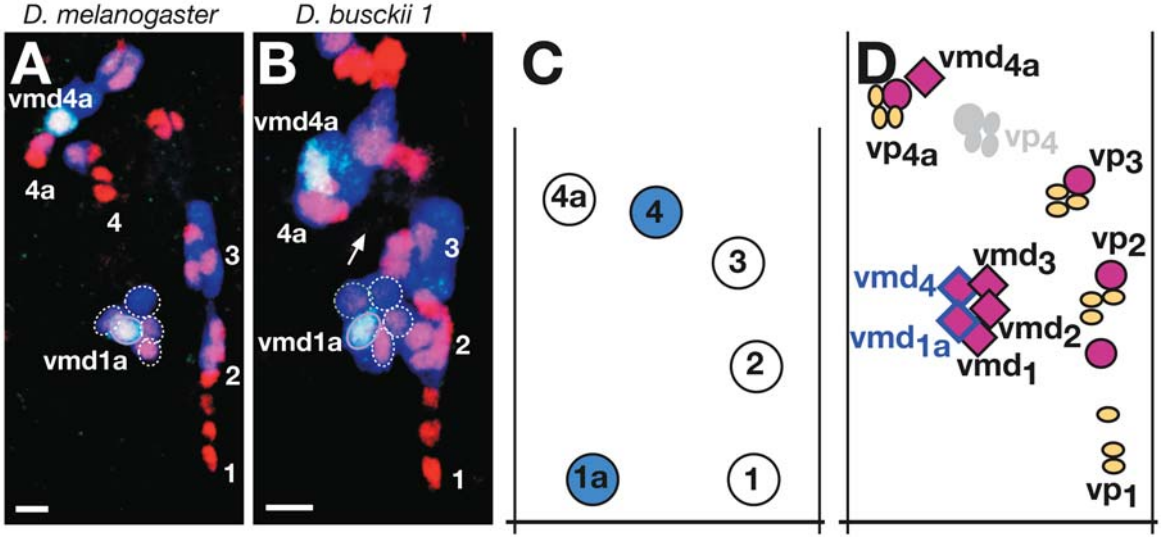

Fig. 7a–d Sensory organs in the ventral region of *D. busckii*. **a**, **b** Ventral region of an abdominal segment A1–A7 at stage 16 in *D. melanogaster* embryos (**a**) and *D. busckii* embryos (**b**) stained for Cut (*red*), Elav (*blue*) and Col (*green*). In both species, five md neurons (*outlined cells*) are clustered in the ventral region, including one Col-positive neuron (named vmd1a). **c** Diagram of the presumed Cut-positive pI cells in the ventral region of *D. busckii*. pI cells following an md-solo lineage are in *blue*. **d** Diagram of the Cut-positive sensory cells in mature *D. busckii* PNS. Schematic representation as in Fig. 1. Md neurons originating from an md-solo lineage are *encircled in blue*

neuron, as well as the Elav-positive pIIIb cell or its two Elav-positive daughter cells. In *D. busckii*, only one (50%) or two (50%) Cut-positive cells are detected at this position (n =124; Fig. 6i, j). These cells accumulate Elav and one of them seems to migrate to the final position of the vmd4 neuron. These observations indicate that the cell pair is very likely a vmd4a-pIIIb cell pair and that the unique cell must be the remaining vmd4 neuron after its sibling pIIIb cell has undergone apoptosis. All these observations suggest that the pI cell appearing at position 4 in *D. busckii* follows an md-solo lineage and not an md-es lineage as in *D. melanogaster*.

Discussion

Stability of the larval PNS pattern throughout evolution

Our study reveals that, with the exception of *D. busckii*, the larval abdominal PNS pattern did not change during evolution of all the Drosophilinae species analysed here. However, other aspects of the larval PNS not studied here may have changed during evolution, including morphology of sensory structures, axonal projections, type of sensory receptors expressed by sensory neurons, and sensory organs located in the head, thorax and posterior region.

Strikingly, the stability of the Drosophilinae larval PNS during evolution contrasts sharply with the higher variability of the adult PNS pattern. Indeed, many variations in the adult PNS pattern on the thorax, wings, legs, abdomen and head have been reported within Drosophilinae species (Ashburner 1989; Garcia-Belido 1983; Grimaldi 1990 and references therein). Within the *melanogaster* species subgroup for example, species that diverged 2–5 million years ago (Powell 1997; Lachaise et al. 1988) exhibit distinct PNS patterns on the male genitalia, the female ovipositor and the maxillary palp (Ashburner 1989). PNS on the thorax, wings, legs, and head are also variable within the Drosophilinae species analysed here (Grimaldi 1990; Ashburner 1989). Similarly, the two Calliphoridae species analysed here have identical larval but distinct adult PNS patterns on the thorax (Skaer et al. 2002) and other adult parts (McAlpine 1989). These comparisons of larval vs adult PNS suggest that the adult PNS evolved more rapidly than the larval PNS.

Many larval sensory axons survive at metamorphosis in insects and act as guidance cues for organising the ordered array of adult sensory neurons (Bate 1976; Jan et al. 1985; Shepherd and Smith 1996; Tix et al. 1989; Usui-Ishihara et al. 2000; Williams and Shepherd 1999, 2002). In *D. melanogaster*, sensory neurons persisting through metamorphosis into adult stages have been identified in the ventral and lateral abdominal regions (Williams and Shepherd 1999), and some of them have been shown to be required for the normal development of adult sensory projections (Usui-Ishihara et al. 2000; Williams and Shepherd 2002). Since hypothetical changes in the pattern of these larval neurons are likely to modify the organisation of the adult nervous system, it is possible that the persistent neurons that function as guidance cues may be subjected to more constraints than other sensory neurons and should therefore be more stable during evolution. Accordingly, the neurons that persist through metamorphosis in *D. melanogaster* (vch2, vp2, dbd, ltd, isbd/lbd, lda, one vtd neuron, v'dap and vmd3/vbd) were detected in all Drosophilinae, Calliphoridae as well as Phoridae studied here. This argues that the guidance role of the larval PNS during metamorphosis may indeed constrain the larval PNS pattern.

An md-es to md-solo sensory lineage switch

In *D. melanogaster*, the pI cell at position 4 produces an es organ as well as the vmd4 neuron (Orgogozo et al. 2001). Our analysis of the vmd4 lineage suggests that the pI cell at position 4 in *D. busckii* does not follow an md-es lineage as in *D. melanogaster* but an md-solo lineage, accounting thereby for the presence of a vmd4 neuron and the absence of vp4 organ in *D. busckii*. Thus, the difference between *D. busckii* and *D. melanogaster* larval PNS patterns appears to relate to the cell death pattern within the sensory lineage at position 4. We propose that the pIIa and pIIIb cells at position 4 survive and produce a set of four es cells in *D. melanogaster*, whereas they undergo apoptosis in *D. busckii*. In the md-solo lineage in *D. melanogaster*, the two cells fated to die specifically express the pro-apoptotic genes *reaper* and *grim* (Orgogozo et al. 2002). It would therefore be interesting to examine the expression of the *reaper* and *grim* homologs in *D. busckii*. Unfortunately, we have been unable to clone portions of the *D. busckii reaper* and *grim* genes that are large enough to generate specific in situ probes (data not shown).

Analysis of the PNS pattern in species closely related to *D. busckii* would be helpful to establish when this change appeared during evolution. *D. busckii* belongs to the *Dorsilopha* subgenus. Two other members of this subgroup have been described (Toda 1986) but to our knowledge, none of them is maintained in laboratory. As the phylogenetic position of the *Dorsilopha* subgroup within the Drosophilinae tree remains uncertain (Grimaldi 1990; Kwiatowski and Ayala 1999; Remsen and O'Grady 2002; Thomas and Hunt 1993), no species closely related to *D. busckii* have been clearly established, thus preventing their study.

Given the undefined position of *D. busckii* within the Drosophilinae tree, polarity of the evolutionary change at position 4 is difficult to determine within Drosophilinae. Our study of the outgroup species *C. vicina* suggests that the es organ at position 4, although displaced, is present in *C. vicina*. Therefore, the most parsimonious evolutionary scenario would be that the es organ at position 4 has disappeared in the branch leading to *D. busckii* during evolution. A change from md-es towards md-solo lineage

may thus have led to the loss of the es organ at position 4 in *D. busckii*.

We further note that other gains (or losses) of mono-innervated es organs were observed between Drosophilinae and Calliphoridae/Phoridae species without any modification of the number of neighbouring md neurons (see Fig. 5, for example vp4a seems to be absent in *M. scalaris*). This thus raises the possibility that these PNS differences have arisen similarly through md-es/md-solo lineage switches.

Distinct arrangement of sensory cells in *C. vicina* and variability at position 4

The arrangement of es organs corresponding to positions 4 and 4a differs between *C. vicina* and *D. melanogaster*. Our analysis suggests that this difference in es organ arrangement results from a difference in the positioning of the sensory precursor cells. This change is observed at position 4 in the ventral region. Interestingly, the es organ at position 4 is also the one that has evolved in the *D. busckii* pattern. This suggests that the es organ at position 4 may be more evolutionary labile than es organs at other positions. Interestingly, sensory cells at position 4 appear to have undergone distinct developmental changes during evolution—changes in relative position as well as in cell lineage. This suggests that the observed evolutionary variants at position 4 probably resulted from mutations in different genes.

Noticeably, in the abdominal segment A8 of *D. melanogaster*, the pI cell arising at position 4 follows an md-solo lineage and thus produces no es organ (Orgogozo et al. 2002). Moreover, when apoptosis is blocked, the es organ generated by this pI cell is located more dorsally than the es organ at position 4a, in a pattern similar to the one observed in *C. vicina* (see Fig. 3D in Orgogozo et al. 2002). Therefore, the intersegmental variation in the es organ at position 4 within *D. melanogaster* mirrors the observed interspecific variation, namely the difference in cell lineage for *D. busckii* and the difference in relative cell position for *C. vicina*. This suggests that morphological evolution may arise from modification of pre-existing genetic switches that regulate intersegmental variation.

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References

Ashburner M (1989) *Drosophila*. A laboratory handbook. Cold Spring Harbor Laboratory Press, New York

Bate M (1976) Pioneer neurones in an insect embryo. Nature 260:54–56

Blochlinger K, Bodmer R, Jack J, Jan LY, Jan YN (1988) Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila*. Nature 333:629–635

Blochlinger K, Bodmer R, Jan LY, Jan YN (1990) Patterns of expression of Cut, a protein required for external sensory organ development, in wild-type and *cut* mutant *Drosophila* embryos. Genes Dev 4:1322–1331

Bodmer R, Jan YN (1987) Morphological differentiation of the embryonic peripheral neurons in *Drosophila*. Roux's Arch Dev Biol 196:69–77

Bodmer R, Barbel S, Sheperd S, Jack JW, Jan LY, Jan YN (1987) Transformation of sensory organs by mutations of the cut locus of *D. melanogaster*. Cell 51:293–307

Bodmer R, Carretto R, Jan YN (1989) Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. Neuron 3:21–32

Brewster R, Bodmer R (1995) Origin and specification of type II sensory neurons in *Drosophila*. Development 121:2923–2936

Brewster R, Hardiman K, Deo M, Khan S, Bodmer R (2001) The selector gene *cut* represses a neural cell fate that is specified independently of the *Achaete-Scute*-Complex and *atonal*. Mech Dev 105:57–68

Campos-Ortega JA, Hartenstein V (1997) The embryonic development of *Drosophila melanogaster*. Springer, Berlin Heidelberg New York

Crozatier M, Valle D, Dubois L, Ibnsouda S, Vincent A (1996) Collier, a novel regulator of *Drosophila* head development, is expressed in a single mitotic domain. Curr Biol 6:707–718

Dambly-Chaudiére C, Ghysen A (1986) The sense organs in the *Drosophila* larva and their relation to embryonic pattern of sensory neurons. Roux's Arch Dev Biol 195:222–228

Desai CJ, Popova E, Zinn K (1994) A *Drosophila* receptor tyrosine phosphatase expressed in the embryonic CNS and larval optic lobes is a member of the set of proteins bearing the "HRP" carbohydrate epitope. J Neurosci 14:7272–7283

Fabini G, Freilinger A, Altmann F, Wilson IB (2001) Identification of core alpha 1,3-fucosylated glycans and cloning of the requisite fucosyltransferase cDNA from *Drosophila melanogaster*. Potential basis of the neural anti-horseadish peroxidase epitope. J Biol Chem 276:28058–28067

Garcia-Belido A (1983) Comparative anatomy of cuticular patterns in the genus *Drosophila*. In: Goodwin BC, Holder N, Wylie CC (eds) Secondary comparative anatomy of cuticular patterns in the genus *Drosophila*. Cambridge University Press, London, pp 227–255

Ghysen A, Dambly-Chaudière C (1989) Genesis of the *Drosophila* peripheral nervous system. Trends Genet 5:251–255

Ghysen A, Dambly-Chaudière C (2000) A genetic programme for neuronal connectivity. Trends Genet 16:221–226

Ghysen A, Dambly-Chaudiere C, Aceves E, Jan LY, Jan YN (1986) Sensory neurons and peripheral pathways in *Drosophila* embryos. Roux's Arch Dev Biol 195:281–289

Green P, Hartenstein V (1997) Structure and spatial pattern of the sensilla of the body segments of insect larvae. Microsc Res Tech 39:470–478

Grimaldi D (1990) A phylogenetic, revised classification of genera in the Drosophilidae (Diptera). Bull Am Mus Nat Hist 197:1–139

Grueber WB, Truman JW (1999) Development and organization of a nitric-oxide-sensitive peripheral neural plexus in larvae of the moth, *Manduca sexta*. J Comp Neurol 404:127–141

Grueber WB, Jan LY, Jan YN (2002) Tiling of the *Drosophila* epidermis by multidendritic sensory neurons. Development 129:2867–2878

Grueber WB, Jan LY, Jan YN (2003a) Different levels of the homeodomain protein cut regulate distinct dendrite branching patterns of *Drosophila* multidendritic neurons. Cell 112:805–818

Grueber WB, Ye B, Moore AW, Jan LY, Jan YN (2003b) Dendrites of distinct classes of *Drosophila* sensory neurons show different capacities for homotypic repulsion. Curr Biol 13:618–626

Haase A, Stern M, Wachtler K, Bicker G (2001) A tissue-specific marker of Ecdysozoa. Dev Genes Evol 211:428–433

Hertweck H (1931) Anatomie und Variabilität des nervensystems und der Sinnesorgane von *Drosophila melanogaster* (Meigen). Z Wiss Zool 139:559–663

Hewes RS, Park D, Gauthier SA, Schaefer AM, Taghert PH (2003) The bHLH protein Dimmed controls neuroendocrine cell differentiation in *Drosophila*. Development 130:1771–1781

Jan LY, Jan YN (1982) Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. Proc Natl Acad Sci USA 79:2700–2704

Jan YN, Jan LY (1993) The peripheral nervous system. In: Bate M, Martinez-Arias A (eds) The development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, New York, pp 1207–1244

Jan YN, Jan LY (2001) Asymmetric cell division in the *Drosophila* nervous system. Nat Rev Neurosci 2:772–779

Jan YN, Ghysen A, Cristoph I, Barbel S, Jan LY (1985) Formation of neuronal pathways in the imaginal discs of *Drosophila melanogaster*. J Neurosci 5:2453–2464

Kwiatowski J, Ayala FJ (1999) Phylogeny of *Drosophila* and related genera: conflict between molecular and anatomical analyses. Mol Phylogenet Evol 13:319–328

Lachaise D, Cariou ML, David JR, Lemeunier F, Tsacas L, Ashburner M (1988) Historical biogeography of the *Drosophila melanogaster* species subgroup. In: Hetch MK, Wallace B, MacIntyre RJ (eds) Evolutionary biology. Plenum Press, New York, pp 127–173

Liu L, Yermolaieva O, Johnson WA, Abboud FM, Welsh MJ (2003) Identification and function of thermosensory neurons in *Drosophila* larvae. Nat Neurosci 6: 267–273

McAlpine JF (1989) Phylogeny and classification of the Muscomorpha. In: McAlpine JF, Wood DM (eds) Secondary phylogeny and classification of the Muscomorpha, vol 32. Research Branch Agriculture Canada, pp 1397–1502

Meier T, Chabaud F, Reichert H (1991) Homologous patterns in the embryonic development of the peripheral nervous system in the grasshopper *Schistocerca gregaria* and the fly *Drosophila melanogaster*. Development 112:241–253

Moore AW, Jan LY, Jan YN (2002) *hamlet*, a binary genetic switch between single- and multipledendrite neuron morphology. Science 297:1355–1358

Orgogozo V, Schweisguth F, Bellaiche Y (2001) Lineage, cell polarity and *inscuteable* function in the peripheral nervous system of the *Drosophila* embryo. Development 128:631–643

Orgogozo V, Schweisguth F, Bellaiche Y (2002) Binary cell death decision regulated by unequal partitioning of Numb at mitosis. Development 129:4677–4684

Osborne MP (1963) The sensory neurones and sensilla in the abdomen and thorax of the blowfly larva. Q J Microsc Sci 104:227–241

Powell JR (1997) Progress and prospects in evolutionary biology: the *Drosophila* model. Oxford University Press, London

Remsen J, O'Grady P (2002) Phylogeny of Drosophilinae (Diptera: Drosophilidae), with comments on combined analysis and character support. Mol Phylogenet Evol 24:249–264

Robinow S, White K (1988) The locus *elav* of *Drosophila melanogaster* is expressed in neurons at all developmental stages. Dev Biol 126:294–303

Rothwell WF, Sullivan W (2000) Fluorescent analysis of *Drosophila* embryos. In: Ashburner M, Hawley S, Sullivan B (eds) Secondary fluorescent analysis of *Drosophila* embryos. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp 141–157

Shepherd D, Smith SA (1996) Central projections of persistent larval sensory neurons prefigure adult sensory pathways in the CNS of *Drosophila*. Development 122:2375–2384

Simpson P, Woehl R, Usui K (1999) The development and evolution of bristle patterns in Diptera. Development 126:1349–1364

Skaer N, Pistillo D, Simpson P (2002) Transcriptional heterochrony of *scute* and changes in bristle pattern between two closely related species of blowfly. Dev Biol 252:31–45

Snow PM, Patel NH, Harrelson AL, Goodman CS (1987) Neural-specific carbohydrate moiety shared by many surface glycoproteins in *Drosophila* and grasshopper embryos. J Neurosci 7:4137–4144

Sun B, Salvaterra PM (1995) Characterization of nervana, a *Drosophila melanogaster* neuronspecific glycoprotein antigen recognized by anti-horseradish peroxidase antibodies. J Neurochem 65:434–443

Thomas RH, Hunt JA (1993) Phylogenetic relationships in *Drosophila*: a conflict between molecular and morphological data. Mol Biol Evol 10:362–374

Tix S, Bate M, Technau G (1989) Pre-existing neuronal pathways in the leg imaginal discs of *Drosophila*. Development 107:855–862

Toda MJ (1986) Drosophilidae (Diptera) in Burma. I. The subgenus *Dorsilopha* Surtevant of the genus *Drosophila*, with descriptions of two new species. Kontyu 54:282–290

Tracey WD, Jr., Wilson RI, Laurent G, Benzer S (2003) *painless*, a *Drosophila* gene essential for nociception. Cell 113:261–273

Usui-Ishihara A, Simpson P, Usui K (2000) Larval multidendrite neurons survive metamorphosis and participate in the formation of imaginal sensory axonal pathways in the notum of *Drosophila*. Dev Biol 225:357–369

Wang X, Sun B, Yasuyama K, Salvaterra PM (1994) Biochemical analysis of proteins recognized by anti-HRP antibodies in *Drosophila melanogaster*: identification and characterization of neuron specific and male specific glycoproteins. Insect Biochem Mol Biol 24:233–242

Williams DW, Shepherd D (1999) Persistent larval sensory neurons in adult *Drosophila melanogaster*. J Neurobiol 39:275–286

Williams DW, Shepherd D (2002) Persistent larval sensory neurones are required for the normal development of the adult sensory afferent projections in *Drosophila*. Development 129:617–624