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Persistent larval sensory neurones are required for the normal development of the adult sensory afferent projections in *Drosophila*

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SUMMARY

We have tested the hypothesis that larval neurones guide growth of adult sensory axons in *Drosophila*. We show that ablation of larval sensory neurones causes defects in the central projections of adult sensory neurones. Spiralling axons and ectopic projections indicate failure in axon growth guidance. We show that larval sensory neurones are required for peripheral pathfinding, entry into the CNS and growth guidance within the CNS. Ablation of subsets of neurones shows that larval sensory neurones serve specific guidance roles. Dorsal neurones are required for axon guidance across the midline, whereas lateral neurones are required for posterior growth. We conclude that larval sensory neurones pioneer the assembly of sensory arrays in adults.

Key words: Laser ablation, Axon guidance, Metamorphosis, *Drosophila*

INTRODUCTION

During development, insect sensory neurones arise peripherally in stereotyped locations and grow axons to the CNS to synapse with their targets. Within the CNS, the sensory axons form highly organised sensory arrays, such that sensory neurones that serve different sensory modalities terminate in different layers of the CNS (Merritt and Murphey, 1992; Murphey et al., 1989a; Murphey et al., 1989b). Within these different layers, further order is apparent with many systems exhibiting topographic ordering of sensory afferents (Johnson and Murphey, 1985; Newland, 1991). This rigid anatomical organisation of sensory axons is a fundamental feature of sensory systems in many organisms, including mammals, and is a necessary prerequisite for the formation of ordered patterns of synaptic connectivity (Bacon and Murphey, 1984; Burrows and Newland, 1993; Shepherd et al., 1988). Despite the apparent wealth of knowledge concerning the organisation of insect sensory systems, little is known about the mechanisms that control the developmental assembly of these complex arrays. To address these processes, we have been studying the development of the adult sensory system of *Drosophila* and have identified a mechanism of axon guidance that provides a simple means for organising complex sensory arrays.

As part of its life cycle, *Drosophila* undergoes a complete metamorphosis which sees the sensory system assembled during embryogenesis replaced by a new adult sensory system. While this transition sees a turnover of the embryonic sensory system, not all embryonic sensory neurones are eliminated during metamorphosis. A defined subset of embryonic sensory neurones persist into adult stages (Shepherd and Smith, 1996; Tix et al., 1989a; Tix et al., 1989b; Williams and Shepherd, 1999) and retain their central axonal projections within the CNS (Shepherd and Smith, 1996). Furthermore the central projections of these persistent neurones prefigure the central pathways taken by the ingrowing adult sensory axons (Shepherd and Smith, 1996). On the basis of this evidence, it was hypothesised that the axon pathways defined by the persistent neurones might provide a pre-assembled axon scaffold to guide the growth of the adult sensory axons and play an essential role in the development of ordered sensory arrays in the adult CNS.

The idea that persistent neurones might guide adult axon growth has been tested a number of times but with mixed results. The first studies found that persistent sensory neurones serve no role in peripheral axon growth guidance (Kunes et al., 1993; Tix et al., 1989a) while more recent work has shown that embryonic multidendritic neurones in the notum guide adult sensory axon growth towards the CNS (Usui-Ishihara et al., 2000). Importantly, however, all these studies focussed on peripheral pathfinding and did not consider whether persistent neurones are required for axon guidance within the CNS.

The aim of our work was, therefore, to extend these observations and test the role of persistent neurones in guiding adult sensory axon growth within the CNS. To achieve this, we used laser ablation to kill persistent sensory neurones during larval stages and assayed the organisation of the adult sensory afferents within the CNS. The results show that after ablation of persistent sensory neurones, the central afferent projections of the adult sensory neurones on the notum and wing show defects indicative of errors in axon growth. Studies of single sensory axons show that the larval neurones pioneer the central

pathways taken by adult sensory neurones and provide precise guidance cues, which allow neurones to make specific growth decisions within the CNS and ensure that axons innervate the appropriate regions of neuropil.

MATERIALS AND METHODS

Fly strains and crosses

For all ablation experiments, *GAL4-C161*, an insert on the third chromosome, was used (Shepherd and Smith, 1996). In ablation experiments where morphology of the larval projections were visualised, fly stocks with two copies of *UAS-mCD8GFP* were established. In experiments where adult projections were visualised, a single copy of *UAS-mCD8GFP* and a single copy of *UAS-nlsGFP* were used together. This dual reporter combination provided sufficient GFP signal for identification and ablation of the soma while allowing the fine detail of the adult central afferent projections to be revealed.

Laser ablations

The cell bodies of individual neurones were ablated in third instar larvae. These animals were anaesthetised with diethyl ether vapour and placed in phosphate-buffered saline (PBS) under a coverslip. The larvae and neurones of interest were visualised using a Zeiss Axioskop FS fitted with Nomarski, epifluorescence and a low light CCD video camera (JVC). Ablations were observed in real time on the video monitor. Laser light from a pulsed dye laser (VSL 33: Laser Sciences, Newton, MA) was delivered through a Micropoint laser system (Photonics Instruments, Arlington Heights, IL) containing Coumarin 440 dye. The intensity and alignment of the laser was established at the beginning of each experiment. Laser energy was attenuated using neutral density filters until just sufficient to crack a coverslip with the ×63 lens. Thirty pulses were delivered at a frequency of 2 Hz. Survival to eclosed adult was routinely 90%.

Dissections, immunocytochemistry and microscopy

Pupal ventral nerve cords were dissected out in saline (Jan and Jan, 1976) on Sylgard coated dishes under a dissecting microscope. Adult flies were collected as virgins and maintained at 25°C for 24 hours. Flies were anaesthetised with carbon dioxide, the head and abdomen removed and the intact thorax fixed overnight in 4% paraformaldehyde at 4°C on a rotator. Apart from a 30 minute acid permeabilisation step performed with 2N HCl/PBST, immunocytochemistry was as described previously (Smith and Shepherd, 1996). GFP was revealed with an anti-GFP monoclonal antibody mix (Boehringer Roche) at 1:250. An anti-mouse secondary antibody from Vectastain ABC Elite kit was used for visualisation. Stained preparations were dehydrated in ethanol, cleared in xylene and mounted in fluormount. Material was examined on a Zeiss Axiophot. Photographic images were acquired with a Micromax digital camera (Roper Scientific) with Metamorph software (Universal Imaging Corporation™). Figures and montages were assembled using Adobe Photoshop on Macintosh computers. Images were adjusted for brightness and contrast only.

DiI labelling of single neurones and DAB photoconversion

Adult flies were anaesthetised with CO_2 and the thorax isolated by cutting off the head and tip of the abdomen. After overnight fixation in 4% paraformaldehyde (pH 7.4), the preparation was transferred to blob of tacky wax on small circular coverslip and dabbed dry. The macrochaete of interest was removed by gentle side to side movement and neat Fast DiI Oil (Molecular Probes, D3899) applied with broken glass micropipette to the socket. The preparations were transferred to 4% paraformaldehyde and left at room temperature for 7 days. The CNS was dissected out in saline as described above. After viewing under epifluorescence in PBS, the individual CNS was washed for 5 minutes in PBS to remove any paraformaldehyde. Frozen aliquots of 0.2% DAB solution (in 50 mM Tris) were defrosted and centrifuged to bring down any precipitate. The CNS was transferred to a depression slide containing DAB solution and incubated in dark for 5 minutes. A coverslip was placed on the slide and the preparation exposed to epifluorescence (rhodamine filter set) until the DiI signal had completely disappeared. The CNS was washed, cleared and mounted as described above.

RESULTS

Experimental rationale

This work makes use of GAL4 enhancer trap line C161 driving expression of the membrane targeted CD8GFP to visualise identified sensory neurones. Line C161 reveals a subset of sensory neurones in larvae and adult, including neurones that persist through metamorphosis and has been described in detail elsewhere (Shepherd and Smith, 1996; Smith and Shepherd, 1996). Among the neurones revealed by line C161 in larvae is a group of 11 sensory neurones situated in the dorsolateral region of each mesothoracic hemisegment (Fig. 1A). These neurones are individually identifiable and organised into two groups (dorsal and lateral) separated by the dorsal bipolar dendrite neurone (dbd). The lateral group consists of four multidendritic neurones. The dorsal cluster consists of six neurones, including two chordotonal neurones and four multidendritic neurones. The cluster also contains the multidendritic neurones that persist through metamorphosis and become incorporated into the notum (Usui-Ishihara et al., 2000). The neurones in these clusters that are not labelled by line C161 degenerate early in pupation and do not persist (Williams and Shepherd, 1999). The peripheral position, peripheral route and the central projections of the persistent neurones indicate that axons from these clusters exclusively prefigure the pathways of axons from sensory neurones in the wing and notum in the adult. For this reason we used these neurones to test the role of persistent sensory axons in guiding the growth of sensory axons from the wing and notum. The aim was to unilaterally ablate these neurones in larvae, raise these larvae to adulthood and use the GAL4 driven GFP expression to assay the central organisation of axons from sensory neurones on the wing and notum of adults. Although the dorsal and lateral clusters of sensory neurones have axons that prefigure other central axon pathways, for clarity, all of our studies focused exclusively on the effects of the ablations on the axons from wing and notum.

Laser ablation can be used to selectively ablate larval neurones and their axons

With GFP expression each sensory neurone can be identified in living larvae and selectively and individually killed with a laser microbeam, Fig. 1B-D illustrates use of the laser to selectively ablate neurones in the lateral cluster. Fig. 1B shows the lateral cluster and dbd immediately before ablation. Fig. 1C shows the same cluster 12 hours after ablation of the four neurones in the lateral cluster. In this example one neurone (ldaD) has already disappeared and the remaining three neurones exhibit a condensed appearance and signs of cell death. Note that dbd which was not targeted with the laser in this experiment remains unchanged (dbd, Fig. 1B-D). Thirty hours after ablation, only the slightest traces of the ablated neurones are detectable (arrowheads, Fig. 1D) but dbd remains intact. Within 48 hours

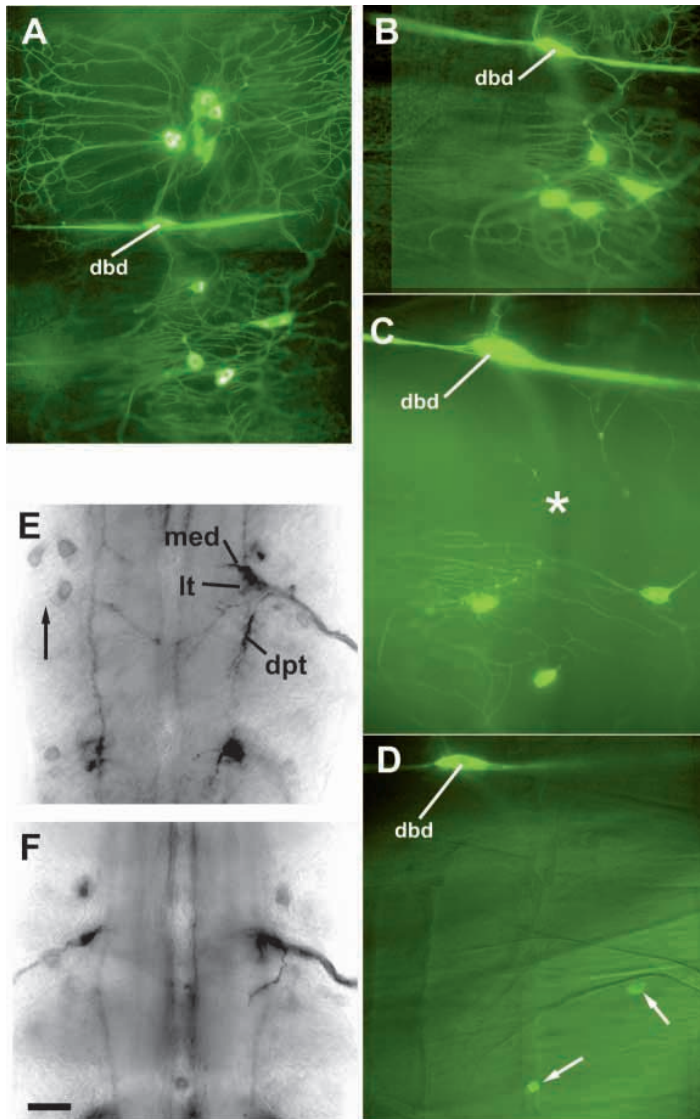

Fig. 1. Selective ablation of sensory neurones. (A-D) Dorsal is upwards and anterior towards the right. (A) Detail of the sensory neurones revealed by GAL4 line C161 driving expression of UAS-CD8GFP in a living third instar larva. Eleven sensory neurones of the dorsal and lateral clusters in the mesothorax. The two clusters are separated by the dorsal bipolar dendrite neurone (dbd). The lateral group, ventral to dbd, consists of four multidendritic neurones (ldaA, ldaB, ldaC and ldaD). The dorsal cluster, dorsal to dbd, consists of six neurones, including two chordotonal neurones (dch1 and dch2), and four multidendritic neurones (ddaA, ddaB, ddaC, ddaD). (B) Detail of the lateral cluster and dbd in a live specimen prior to laser ablation. (C) The same neurones as shown in B 12 hours after laser ablation of the four neurones ventral to dbd. One of the neurones is missing (*) and the other three neurones exhibit a condensed cell body, loss of dendrites and blebbing. (D) The same neurones 30 hours after ablation, only 2 neurones are visible and exhibit shrunken morphology and no dendritic structures. (E) Central projections of the neurones in line C161 revealed immunocytochemically with anti-GFP. The panel shows a dorsal view of the mesothoracic neuromere of the ventral nerve cord of a pupa 48 hours after unilateral laser ablation of the neurones in the dorsal and lateral cluster neurones. On the intact (right-hand) side the normal pattern of central projections is seen in which it is possible to identify three major axon pathways (med, lt and dpt). On the ablated (left-hand) side there are no central projections evident (arrow). (F) Dorsal view of the mesothoracic neuromere 48 hours after ablation of only the lateral cluster neurones. On the intact (right-hand) side the normal pattern of axons is present. On the ablated (left-hand) side only the med projection is evident. Scale bar: 50 μm.

of ablation, all traces of the targeted neurones in the periphery are gone.

To ensure that ablation of neurones removed the central axon projections of the targeted neurones, we used GFP expression to visualise the central projections of ablated neurones in early pupal stages before the arrival of adult sensory axons. Fig. 1E shows the typical outcome of this illustrating the CNS of a pupa approximately 12 hours after puparium formation and 48 hours after the unilateral ablation of the dorsal and lateral groups of neurones in the mesothorax. There is complete loss of axons entering the CNS via the dorsal mesothoracic nerve on the ablated left-hand side (arrow, Fig. 1E). On the untreated side, the normal pattern of axons is evident (Fig. 1E) with the axons forming three pathways within the CNS (Fig. 1E). One projects to the midline (med, Fig. 1E) and the other two project posteriorly and prefigure known pathways of the adult CNS (Whitlock and Palka, 1995), the dorsal posterior tract (dpt, Fig. 1E) and the lateral tract (lt, Fig. 1E). It is not possible to determine how many axons there are in each fascicle at light microscope level but both dpt and lt appear to consist of a single axon and the medial fascicle appears to contain more than one axon. Ablation of the different groups of neurones deletes different axon bundles in the CNS. Ablation of both the dorsal and lateral neurone clusters, for example, results in the loss of all three pathways (left side of Fig. 1E), while ablation of the lateral cluster neurones alone eliminates both posterior fascicles (dpt and lt) and leaves the medial projection intact (Fig. 1F). Ablation of only the dorsal cluster produces the reciprocal result by eliminating the medial projection and leaving dpt and lt intact (data not shown). This suggests that the different subsets of neurones may provide different guidance cues within the CNS with the dorsal cluster neurones providing guidance for medially directed neurones and the lateral cluster guiding posteriorly directed axons.

Central projections of wing sensory neurones are disrupted by ablation of persistent neurones

We first examined the effects of ablating all 11 labelled neurones in the dorsal and lateral groups to remove all persistent neurones in the region. The neurones were ablated in early third instar larvae and the adult projections visualised using the C161 enhancer-trap line. In virtually all treated specimens (24 out of 25), ablation of the neurones had significant effects on the organisation of all dorsally originating sensory afferents on the ablated side. These defects are visible even at relatively low magnifications, where it is possible to see that axons entering the CNS on the ablated side are clearly abnormal (left side in Fig. 2A). At this level, the most obvious indication of a defect is that there is asymmetry in the organisation of sensory axons at or around the entry point to the CNS. Axon fascicles are poorly formed, for example, the dorsal anterior tract (dat, Fig. 2A) on the ablated side is not as tightly bundled as its contralateral

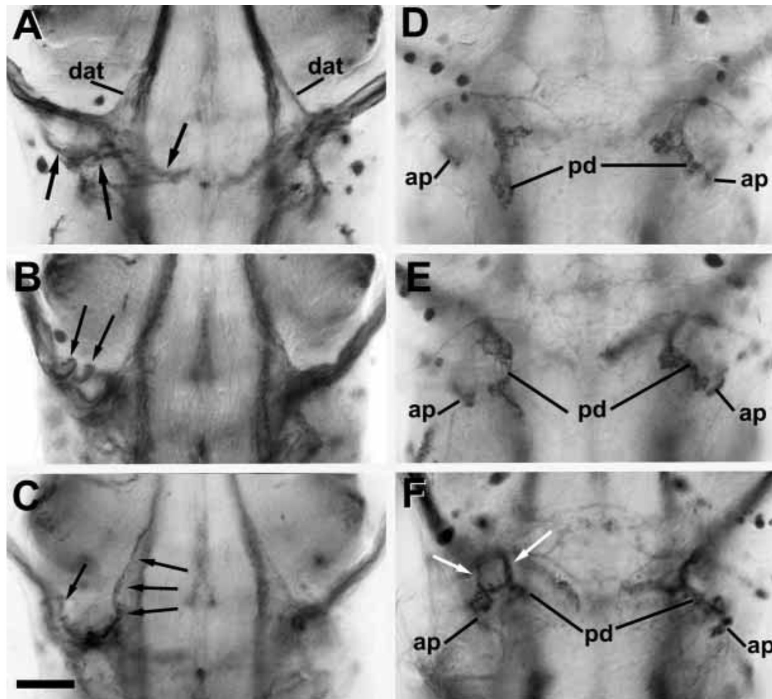

Fig. 2. The effects of laser ablation on the central projections of adult sensory neurones. Six different specimens showing the mesothoracic neuromere of the adult ventral nerve cord with sensory projections revealed immunocytochemically using GAL4 line C161 driving expression of CD8-GFP. In all panels, the ablated side is the left-hand side and anterior is upwards. (A-C) Illustrate typical defects in the central projections from the wing and notum. Typical defects include poor axon fasciculation, pathway errors and axon spiralling. (D,E) Defects in the formation of a specific terminal arborisation the posterior-dorsal projection (pd). Refer to text for detailed descriptions. Scale bar: 30 μm.

homologue. Other fascicles take abnormal routes (arrows, Fig. 2A), often making abrupt changes in the direction. Such asymmetry and defects were never seen in untreated individuals (*n*>250). On closer examination, three basic types of defect were observed with the projections on the ablated side.

Axon spiralling

The most readily identified defect is that the axons in the peripheral nerve exhibit distinct spiralling. In untreated specimens, the axons in the peripheral nerve normally form orderly and evenly arrayed axons, which can be described as being laminar and show no abrupt changes in direction relative to each other. This can be seen in the untreated right-hand side of the specimens in Fig. 2A,B. In the peripheral nerves of most ablated specimens (24 out of 25) axon fascicles twist to produce a helical structure (arrows, Fig. 2A,B), producing contorted axon fascicles. The spiralling is most pronounced at the point at which the axons enter the ganglion.

Pathway defects

In untreated specimens, the dorsally originating axons segregate shortly after entering the CNS to form a stereotyped array of axon fascicles, which project to specific domains of neuropil (Fig. 2A). This pattern of projections is invariant in untreated specimens.

In 24 out of 25 ablated specimens this pattern of projections shows deviations from normal. Typical pathway defects can be seen in Fig. 2A-C. Axon fascicles are poorly formed and split inappropriately as they enter the CNS, often leaving the nerve before the normal branch point. The abrupt change in trajectory is a common feature seen in many preparations. Fig. 2C illustrates a typical example as axons of the dorsal anterior tract (see arrows) enter the CNS and project incorrectly along a posterior pathway, although they eventually undertake an acute turn to correct this error and turn anteriorly.

Disrupted terminal arborisations

Although pathway defects are evident in ablated specimens, the overall pattern of projections shows similarity with the untreated side and many axons still reach their correct target area albeit via abnormal routes. Having innervated the correct neuropilar domain, however, there are still errors of detail in the terminal arborisations. These errors are most clearly seen in a specific and discrete terminal projection called the posterior dorsal tract (pd). This projection is formed by proprioceptive sensory neurones in the wing hinge and is readily identifiable in all specimens (Fig. 2D-E).

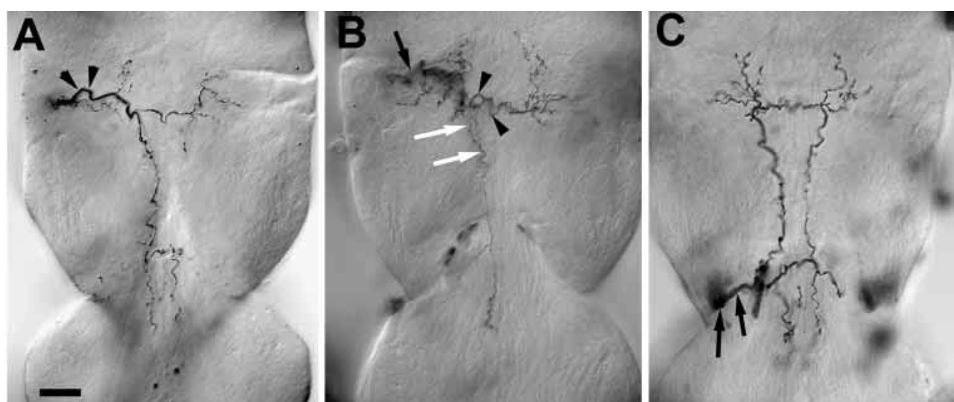

Fig. 3. DiI staining of the psc neurone. (A) Central projections of the psc neurone in an untreated specimen. The neurone is formed by a single robust axon (arrowheads) entering the CNS via the dorsal wing nerve and exhibits the stereotypical features previously described for this neurone. (B) The psc neurone following unilateral ablation of the dorsal and lateral groups of sensory neurones. The neurone shows disruption to its structure at the point of entry (black arrow), as well as repeatedly crossing the midline (arrowheads) to form the posterior process (white arrows). (C) An unusual example of the psc neurone central projection following unilateral ablation of dorsal and lateral clusters. In this example, the psc has entered the CNS ectopically via the metathoracic nerve (arrows) and has formed an elaborate central projection in which elements of the normal psc projection can be recognised. Scale bar: 30 μm.

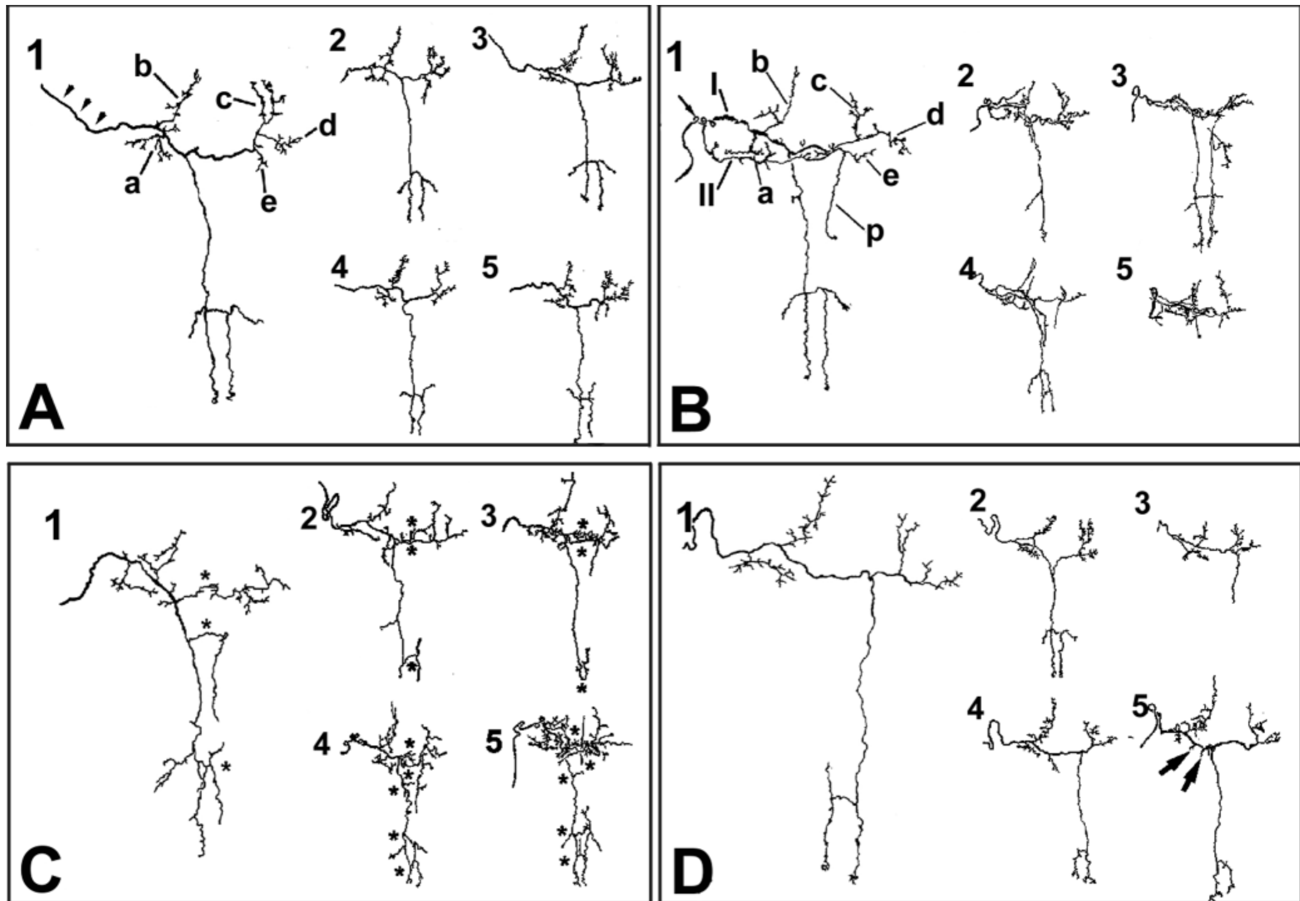

Fig. 4. Detailed structure of the psc neurone in untreated and ablated specimens. (A) Five examples of the psc neurone in untreated specimens showing the consistent structure of the central projections. (B) Five examples of the psc neurone following unilateral ablation of the dorsal and lateral groups of neurones. These examples show axon splitting at the entry points as well as duplication of key structures. (C) Five examples of the psc neurone following unilateral ablation of only the dorsal groups of neurones. These examples do not show axon splitting at the entry point but all show defects associated with crossing the midline (*). (D) Five examples of the psc neurone following unilateral ablation of only the lateral groups of neurones. These examples show defects with the formation of the posterior projection. Refer to text for detailed descriptions. Lowercase lettering refers to the identity of the different sub-branches in the psc neurone.

In untreated specimens, the pd is formed from a single axon bundle that projects dorsally to create a discrete comma shape arborisation (pd, see right-hand side of Fig. 2D) which terminates adjacent to a second projection called the accessory projection (ap). In all experimental preparations analysed for the structure of pd (n=15), the pd is recognisable and, of these, 12 showed disruption. Typical defects are illustrated in Fig. 2D-F and are best seen by comparing the ablated side with the intact side of each preparation. On the experimental side, the pd projections are different. The nature of the defects showed variation and the three most frequently observed examples are shown. The most common defect (nine out of 12) illustrated in Fig. 2D is that the pd is present but positioned ectopically, its main projections run posteriorly instead of laterally and it fails to contact the ap. In other examples, the pd projection was not only ectopic but also poorly formed, as shown in Fig. 2E where the pd not only fails to contact the ap but has a fragmented appearance. In the third example (two out of 12) (Fig. 2F), the pd is formed by two separate axon bundles (arrows, Fig. 2F) instead of the usual one, and it fails to contact the ap.

Labelling of single identified neurones

Line C161 provided a simple means of visualising and ablating the persistent neurones, but the numbers of axons labelled in this line made it difficult to resolve the effects of ablations on the growth of single neurones. We therefore further analysed the consequences of ablations by dye staining single sensory neurones. We chose to examine the central projections of the sensory neurone associated with one of the macrochaetes on the notum, the posterior scutellar bristle (psc). The psc was chosen for three reasons: first, its axon is likely to be guided by the ablated neurones; second, its morphology has been extensively characterised (highly stereotyped and invariant in wild-type flies); and third, it is suited to retrograde staining with DiI.

DiI staining of psc in untreated specimens (n=25) showed that its structure is exactly as described by Ghysen (Ghysen, 1980). Thus, this neurone has a single entry point into the CNS and the entire central projection is formed by a single axonal process that projects medially (arrowheads, Fig. 3A, Fig. 4A1-5). The neurone forms two small sub-branches in the ipsilateral mesothoracic neuromere (branches a and b, Fig. 4A). The primary axonal branch bifurcates to form two secondary axon

processes. One of these processes extends across the midline and forms three contralateral sub-branches, one projecting laterally, the second posteriorly and the third anteriorly (branches c-e, Fig. 4A). The second process projects posteriorly to the metathoracic neuromere and produces a characteristic arborisation. Staining of the same neurone in multiple specimens shows that the basic scaffold of this neurone is invariant but there is limited variability in the size of local sub-branches (Fig. 4A1-5).

The psc is disrupted following ablation of persistent sensory neurones

The analysis of singly stained psc neurones revealed remarkable detail on the effects of the ablations and showed that the absence of persistent neurones resulted in major defects of adult sensory axon growth. In ablated specimens, staining of the psc neurone on the treated side revealed a number of characteristic defects in the structure of the psc axon. The most obvious feature of the psc neurone in ablated specimens was axon spiralling. In 14 of the 15 specimens examined, the psc axon shows spiralling and abrupt changes of directions at or around the point of entry to the ganglion (arrow, Fig. 3B). This is evident in the examples shown in Fig. 4B where the psc axon forms a corkscrew (arrow) with three complete turns shortly after entering the CNS. Similar abrupt turning and spiralling is seen in other examples (Fig. 4B1-5).

In virtually all ablated specimens (13 out 15), the psc axon splits shortly after entering the CNS (arrow, Fig. 4B) to produce two or three independent axonal projections. This results in duplication of structures within the normal psc axon morphology. The most frequently duplicated structures are the contralateral process and the posterior process. In the main specimen shown in Fig. 4B, for example, instead of the psc neurone being formed by a single primary axon, the axon splits to produce two ectopic secondary axons (I and II, Fig. 4B). Axon I projects medially producing the ipsilateral sub-branches (a and b) and bifurcates a second time to produce an axon that crosses the midline and another that projects posteriorly to the metathorax. The contralateral part of axon I produces only the anterior and lateral contralateral sub-branches (c and d). Axon II also projects medially, via an incorrect tract, and crosses the midline to create a second commissural process that produces the posterior contralateral sub-branch (e) and an additional posteriorly projecting axon (p). Note that both secondary axons produce a posteriorly directed axon. Other examples of the psc in ablated individuals exhibited similar defects in the structure of the psc axon (Fig. 4B1-5) and all show duplications of either the commissural process or posterior axon or both. The example shown in Fig. 4B5 has a particularly devastated structure; the axon has split to produce four secondary axons, fails to make a posterior axon and shows ectopic growth and errors. These errors were not seen in untreated specimens.

Interestingly, the only psc in the experimental preparations that did not show axon spiralling at the point of entry was one remarkable preparation in which the mesothoracic psc neurone enters the CNS via the haltere nerve in the metathorax. In other words, the neurone became lost in the periphery and entered the CNS via the homologous route in the wrong segment (Fig. 3C).

Do different persistent neurones ‘pioneer’ different pathways?

We have already shown that the axons from the persistent neurones in the two clusters of sensory neurones form distinct pathways in the CNS (Fig. 1E,F) suggesting the possibility that different subsets of neurones may provide different, specific guidance cues within the CNS. To test this idea, we therefore decided to analyse the effects of ablating subsets of neurones and examining the resultant effects on the growth of the psc neurones.

Dorsal-only ablations

In specimens in which only the dorsal group neurones were ablated, the psc neurone consistently showed defects (Fig. 4C). As with the combined dorsal and lateral ablations, all of the specimens (n=10) exhibited axon spiralling and changes of direction at the entry point to the CNS (Fig. 4C1-5). In dorsal-only ablations, however, the psc showed significant differences from the dorsal and lateral ablations. One such difference is that only one dorsal-only specimen exhibited axon splitting, all other specimens resembled the untreated psc neurone with the major bifurcation of the axon occurring adjacent to the midline to form the contralateral and posterior axons. Most of the dorsal-only ablation group (9/10) also showed defects associated with crossing the midline with the axon crossing the midline as many five times in extreme examples (Fig. 4C1-5). The main specimen in Fig. 4C, for example, crosses the midline at three different points (*) compared with the normal two. Of these, the anteriormost and posteriormost are in the normal locations, but the third is ectopic. In the remaining examples (Fig. 4C2-5), it is evident that axons cross the midline ectopically and repeatedly (*). The examples in Fig. 4C4,5 are quite extreme, with multiple crossing points, particularly around the normal commissure in the mesothorax, where it is evident that there are many processes projecting either partially or completely across the midline. Most specimens were normal with respect to the posterior process; only a small number (2/10) showed any indication of defects with the posterior process and these were manifest as a thinning of the axon (Fig. 3B).

Lateral-only ablations

Lateral-only ablations also caused significant defects in the psc neurone (Fig. 4D1-5), which were different and complementary to those produced by dorsal-only ablations. All lateral ablation specimens (10/10) showed axon spiralling at the point of entry to the CNS. Splitting of the axon was seen in only two specimens. The majority of specimens showed no defects associated with crossing the midline, although one showed errors in the position of commissural process (Fig. 4D2) and none showed signs of supernumerary commissural processes. The most striking defect in the lateral-only specimens was with the posterior process. The majority of specimens (9/10) showed defects with some aspect of the posterior process. Of these, three formed the posterior process contralateral to the entry point as opposed to the normal ipsilateral location (Fig. 4D3,4). Four had a posterior process that failed to extend into the metathorax. The specimen shown in Fig. 4D3 has a posterior process that was both contralateral and incomplete. The remaining specimens had a complete posterior process but showed deviations from normal. The types of deviation observed included being in an incorrect longitudinal tract. The example in Fig. 4D2 has a normal looking posterior process but it is too lateral, as evidenced by the abnormal trajectory taken by the process that crosses the midline. Another variation seen in two specimens was a normally located process associated with multiple

attempts to generate the posterior process (arrows, Fig. 4D5). Furthermore, in most preparations, the posterior process is thinner than normal.

DISCUSSION

In this paper, we have re-addressed the role of persistent larval neurones as pioneers for growing adult sensory axons during metamorphosis in *Drosophila*. We have shown that these neurones play an essential role in guiding the assembly of the central afferent projections of adult sensory neurones. The work shows that selective ablation, during larval life, of the dorsal and lateral groups of sensory neurones in the mesothorax caused defects in the central projections of sensory neurones on the adult wing and notum. The defects caused by ablations, disorganised axon spiralling in the peripheral nerve, meandering axon growth, ectopic projections are all indicative of failure in axon growth guidance mechanisms. These data show that persistent neurones provide essential guidance cues for axon growth guidance and are required not only for peripheral pathfinding but are also essential for directed axon growth within the CNS.

Peripheral pathfinding

The first stage at which persistent axons are required is in peripheral pathfinding. In all ablated specimens, there is disruption of axons within the peripheral nerve, suggesting that the ingrowing adult axons lack the cues needed for their normal organised pathway to the CNS. This is consistent with recent work that has shown that the dorsal multidendritic neurones in the mesothorax, including the neurones ablated in this study, are required for normal sensory axon growth in the notum (Usui-Ishihara et al., 2000). Furthermore, our data show that guidance provided by the persistent neurones, while important for axon growth guidance, is not essential for axons to bridge the gap between the periphery and CNS. It therefore seems that there is redundancy in peripheral guidance and that, in the absence of the preferred guidance cues, sensory axons can locate alternative substrates for guiding axon growth to the CNS. Possible alternate guidance cues might include the motoneurones that innervate the flight musculature (Fernandes and Keshishian, 1996), other sensory neurones, not labelled by line C161, or glia. An interesting corollary to this is the single DiI-labelled psc neurone, which after ablation of mesothoracic neurones enters the CNS via the metathoracic nerve. Evidently this neurone entered the CNS via the wrong nerve root, but showed none of the axon spiralling seen in ablated specimens. One interpretation of this is that this axon became lost in the periphery and found an alternative substrate, the metathoracic homologue of its intended guide that led it to the CNS via the metathoracic route. A similar defect was seen by Usui-Ishira and co-workers (Usui-Ishihara et al., 2000) in the periphery. Clearly our evidence shows that persistent neurones are needed for normal peripheral axon growth but are not essential for axons to bridge the gap between periphery and CNS.

Guidance into the CNS

There is also evidence that the persistent neurones guide axon growth as they enter the CNS. In all ablated preparations, there is aberrant axon growth indicated by axon spiralling, misdirected growth and abrupt changes of direction at or around the entry point to the CNS. The nerve entry point is a complex mesh of axon pathways where axons are faced with a diverse choice of growth directions but must make specific choices as they defasciculate to innervate their prospective target domains. The entry point is therefore likely to be a region where a growing axon may need a pre-established pathway for it to recognise and follow, as proposed in the labelled pathway hypothesis (Goodman, 1996). In the labelled pathway hypothesis, growing axons are provided with a pre-established scaffold of axon pathways that represent all of the available directions of growth. The selection of correct pathway is made by the axon selecting the correct axon(s) to follow by means of a pathway specific molecular marker. Thus, after ablation of the persistent neurones, the growing adult axons no longer have the necessary guides through this complex environment and they make errors as they seek their intended pathway to the target domain. Interestingly, despite gross errors and changes of direction, these neurones innervate their normal target domains with reasonable fidelity.

The effects of the ablation on the axons at the nerve entry point also suggest that neurones within the two clusters of neurones studied here play different or additive roles. When both the dorsal and lateral groups of larval neurones are ablated, the axon of the psc shows extreme defects. In such cases, the psc axon not only shows spiralling and meandering growth, but also the axon splits into multiple secondary axons. The neurone is no longer formed by a single robust axon but by a series of smaller secondary axons. Our interpretation of this is that ablation of both neurone groups has removed all the persistent axons needed by the adult sensory axons and that in their absence they become lost. In response to the lack of normal cues, the growth cone splits to form separate growth cones that grow independently to explore the environment. This response of the growth cone to the absence of an anticipated cue is comparable with that seen with the growth of pioneer sensory axon in the embryonic grasshopper limb after ablation of the guide post cells (Bentley and Caudy, 1983), where the formation of multiple axons is seen.

By contrast, when only one cluster of neurones is ablated, the defects in psc are not as severe. After ablation of only one cluster, the psc still shows abnormal axon growth, but the axon does not split at the entry point. This suggests that at this particular choice point, the persistent axons in dorsal and lateral clusters can substitute for each other. Thus, in the absence of both clusters, there are no pioneer axons and growing axons struggle through the complex array of axon pathways at the entry point. With only one cluster, ablated axons from the other cluster provide guidance cues and enable the psc to navigate through this region without fragmenting. Once the axon has passed through this point, the psc shows consistent defects to indicate that neurones in the different clusters are required for specific growth choices within the CNS.

Guidance within the CNS

The ablation of subsets of the persistent neurones reveals that the axons of different persistent neurones serve specific guidance roles within the CNS. In dorsal-only ablations the defects in psc were mostly associated with crossing the midline whereas in lateral-only ablations the psc had defects with the posterior projection. Thus, neurones in the dorsal cluster are required for guidance of adult neurones to and across the midline, whereas neurones in the lateral cluster are required to guide the posteriorly directed growth.

These observations are consistent with pathways taken by

axons in the larval clusters. Elimination of the lateral cluster eliminates the posterior projections (dpt and lt), whereas ablation of the dorsal group eliminates the medial bundle and leaves the posterior projections intact. This correlates with the defects caused by ablations. Thus, when the dorsal cluster is ablated, the medial projection is missing and adult axons lack a guide to the midline and make errors crossing the midline. At the same time, the lt projection remains intact, the adult axons encounter the posterior guide provided by these axons and the posterior projection is normal. In lateral-only ablations, the situation is reversed, with posterior cues missing and medial cues intact. Consequently, adult axons cross the midline normally but lack the posterior guides provided by lateral neurones and seek alternative routes. Interestingly, the posterior projection is often formed contralaterally, where the neurone presumably contacts the homologues of its guidance cues and follows them. Obviously after ablation of both clusters, both sets of guides are missing and axons face a compound problem and make a greater number of errors.

It is conceivable that defects with the posterior projections could be due to incomplete staining of psc, but we have reasons not to suspect this. First, this is the only group where this problem was manifest and there is no obvious why this problem should be restricted to this group of treatments. Furthermore, the ectopic axon on the contralateral side is seen only with this treatment and cannot be explained by staining problem or artefact. Therefore we conclude it is a defect and not an artefact.

One axon or many?

The differential role of the dorsal and lateral neurone clusters raises an important question. Are the guidance roles the property of groups of neurones or can they be attributed to single neurones. Thus, it is possible that the resolution of the guidance cues may be even more refined. Owing to experimental limitations, we restricted this study to ablating small groups of neurones rather than single neurones. Our work shows that the clusters serve specific roles in guiding adult sensory neurone growth. It now remains to be determined whether specific neurones within the clusters are responsible. Clearly the examination of the CNS in early pupal stages suggests that there is not a huge number of persistent axons emerging from the ablated neurone clusters, suggesting that guidance roles may be provided by a small number of neurones. Further study is required to ascertain this.

What does this mean for the formation of sensory arrays?

One of the key features of sensory systems is that sensory axon terminals in the CNS show clear modality specific and somatopic organisation. While the functional significance of this order is known, the developmental mechanisms that establish the order are relatively unknown. The results presented here provide evidence for a simple mechanism by which sensory arrays can be formed. This mechanisms suggests that development of sensory axon arrays can be controlled by a relatively small set of sensory neurones born early in development to define the basic framework of the adult sensory order. Thus development of the adult system is achieved by adult sensory axons growing along the appropriate axon pathways with the adult order emerging as an expansion of the prepattern laid down early in development. Thus, in the case of the psc neurone, the pioneer axons are responsible for: guiding its entry into the CNS; guiding it through the maze of axon pathways at the entry point; providing alternative cues to guide growth across the midline; and establishing its posterior axon. By extrapolation, it is possible to see that a relatively small number of pioneer axons could provide a comprehensive axonal framework that might underlie the structure of the entire sensory system. Thus, adult sensory arrays can be regarded as an expansion of a relatively precise but simple array of pioneer axons.

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