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The glial cell undergoes apoptosis in the microchaete lineage of *Drosophila*

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SUMMARY

Apoptosis plays a major role in vertebrate and invertebrate development. The adult *Drosophila* thoracic microchaete is a mechanosensory organ whose development has been extensively studied as a model of how cell division and cell determination intermingle. This sensory organ arises from a cell lineage that produces a glial cell and four other cells that form the organ. In this study, using an in vivo approach as well as fixed material, we show that the glial cell undergoes nucleus fragmentation shortly after birth. Fragmentation was blocked after overexpression of the caspase inhibitor p35 or removal of the pro-apoptotic genes *reaper*, *hid* and *grim*, showing that the glial cell undergoes apoptosis. Moreover, it seems that fragments are eliminated from the epithelium by mobile macrophages. Forcing survival of the glial cells induces precocious axonal outgrowth but does not affect final axonal patterning and connectivity. However, under these conditions, glial cells do not fragment but leave the epithelium by a mechanism that is reminiscent of cell competition. Finally, we present evidences showing that glial cells are committed to apoptosis independently of *gcm* and *prospero* expression. We suggest that apoptosis is triggered by a cell autonomous mechanism.

Movies available on-line

Key words: Apoptosis, Axogenesis, *gcm*, Microchaete, Phagocytosis, p35, *reaper*, *hid*, *grim*, Asymmetric cell divisions

INTRODUCTION

During ontogeny, the temporal and spatial combination of processes such as cell proliferation, determination, differentiation and death shape multicellular organisms. Of these phenomena, the importance of programmed cell death has been only recently admitted (Kaufmann and Hengartner, 2001). In contrast to pathological cell death, physiological cell death is associated with conservation of the plasma membrane. As a consequence, the intracellular components are contained and do not diffuse into the intercellular space. Several types of physiological cell death have been described, one of them is apoptosis (Schweichel and Merker, 1973). It is characterised by initial DNA condensation followed by nuclear and cytoplasm fragmentation. Further removal of those fragments occurs through phagocytosis. Studies in the worm, the fly and different vertebrates have revealed that the mechanisms underlying apoptosis are conserved (Meier et al., 2000). These mechanisms involve a family of cysteine proteases, known as caspases, which act as executioners. Caspases are the target of negative regulators that inhibit apoptosis, those include endogenous factors such as the Inhibitor of Apoptosis Proteins (IAPs) (Hay, 2000) and external agents such as p35, an antiapoptotic IAP-like factor from baculovirus (Hay et al., 1994). A common trigger for apoptosis is the inhibition and degradation of IAPs, which then releases caspase activity. In *Drosophila*, IAP inhibition and degradation is mediated by the products of the pro-apoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*), *grim* and *sickle* (*skl*) (Goyal et al., 2000; Martin, 2002). In fly, these genes constitute a central regulator of apoptosis as their transcriptional activity is highly regulated by signals that control cell death and survival such as ecdysone and p53, as well as epithelial growth factor (EGF)-, jun-kinase (JKN)- and decapentaplegic (Dpp)-mediated pathways (Rusconi et al., 2000; Moreno et al., 2002).

Mechanosensory organs on the thorax of *Drosophila*, also called microchaetes, have become a model system to study how cell division and cell determination intermingle (Jan and Jan, 1998). Microchaetes are formed by two outer support cells (the socket and the shaft cell) and two inner cells (the neurone and the sheath cell). These cells arise from a primary precursor cell, called pI, after four asymmetric cell divisions that occur during pupal stages of development. The division of pI gives rise to two secondary precursor cells, pIIa and pIIb. During the next cycle of divisions, pIIb divides prior to pIIa to generate a glial cell and a tertiary precursor cell, pIIIb. The division of pIIa produces a socket and a shaft cells. Finally, pIIIb divides, giving rise to a neurone and a sheath cell (Gho et al., 1999).

The asymmetry of these divisions is controlled by the differential segregation of cell determinants during mitosis (Rhyu et al., 1994; Manning and Doe, 1999). For example, during pIIb division, Numb and Prospero are inherited exclusively by the glial cell (Gho et al., 1999). Numb is a negative regulator of the Notch receptor (Rhyu et al., 1994; Guo et al., 1996), therefore among the two pIIb daughter cells, this pathway is only activated in pIIIb, the glia sibling cell.

Prospero, a transcription factor, regulates the expression of several genes. In embryonic cells, Prospero represses neuronal specific genes such as *deadpan* and activates the expression of glial specific genes such as *glial cell missing* (*gcm/glide*) (Vaessin et al., 1991; Freeman and Doe, 2001).

It is generally admitted that glial cells are involved in axonal guidance and neuronal survival (Jones, 2001). In *Drosophila*, glial cells are characterised by their expression of *gcm*, which encodes a DNA-binding protein promoting expression of glial cell determinants such as the homeobox transcription factor Repo (Jones, 2001). In the microchaete lineage, we have previously reported that the glial cell migrates away from the sensory cluster along the axon (Gho et al., 1999). Similar observations were obtained in sensory campaniform in the wing (Van De Bor et al., 2000). In this case, glial cells show precursor properties and divide to generate clonally related glial cells which migrate towards the proximal region of the wing blade.

In this study, we have examined the fate of glial cells in the thoracic microchaete lineage by time-lapse confocal microscopy in living pupae and by immunodetection in dissected nota. This analysis has revealed that glial cells do not actually migrate away but rather undergo apoptosis. Furthermore, glial cells die even after transformation of their fate, suggesting that these cells are committed to programmed cell death independently of the acquired identity. These observations have led us to ask what the role of glial cells is in the final configuration of microchaete organs, particularly during axogenesis.

MATERIALS AND METHODS

Drosophila stocks

The *neuralized*^{p72}-GAL4 (*neu*^{p72}) (Bellaiche et al., 2001), *scabrous*-GAL4 (gift of D. Busson), *elav*-GAL4 (Bloomington) and SOP-GAL4 (stock 109-2-68, gift of Y. Jan) lines were used to express the following constructions: UAS-p35 (Neufeld et al., 1998), UAS-nls-GFP (Shiga, 1996) and UAS-histoneH2B::YFP (UAS-H2B::YFP) (Bellaiche et al., 2001) using the GAL4/UAS expression system (Brand and Perrimon, 1993). The A101 line expresses β-Gal in pI and its progeny (Usui, 1993). The *w*^{1118} line (described in FlyBase) was used as a wild-type stock.

Clonal analysis

Somatic clones were obtained using the FLP/FRT recombination system (Xu and Rubin, 1993). The w; FRT40A *gcm*^{e1} line (gift of V. Rodriguez) were crossed to the flp, FRT40A ubq-nls::GFP (gift of Y. Bellaiche) line to generate *gcm* null somatic clones. The FRT2A H99 (gift of B. Bello) line was crossed to the flp, FRT2A ubq-nls::GFP (gift of J.-R. Huynh) to generate H99 deficient somatic clones. The FRT82B *prospero*^{17} (*pros*^{17}) (Reddy and Rodrigues, 1999) line was crossed to the flp, FRT82B ubq-nls::GFP (gift of J-R. Huynh) to generate *pros*-null somatic clones. To induce mitotic recombination, second instar larvae from these crosses were heat shocked twice at 38°C for 30 minutes at 1 hour interval and kept at 25°C for recovery.

Time-lapse confocal microscopy

In vivo imaging was carried out as described previously (Gho et al., 1999). Confocal images were acquired every 3 minutes on a Leica TCS confocal microscope at 24°C. Time-lapse movies were assembled using NIH image software.

Macrophages labelling

Indian Ink (Pébéo, Gemenos, France) was injected in early third instar larvae. Development was then allowed to proceed at 18°C until pupation. After pupation, samples were transferred at 25°C until dissection and antibody staining.

Immunohistology

Dissected nota from pupae at 22-30 hours after pupal formation (APF) were processed as described previously (Gho et al., 1996). The following primary antibodies were used: rabbit anti-β-galactosidase (Cappel, 1:1000), mouse anti-GFP (Roche, 1:500), rabbit anti-GFP (Santa-Cruz, 1:500), mouse anti-Cut (DSHB, 1:500), rabbit anti-Repo (gift of A. Travers, 1:500), rat anti-Elav (DSHB, 1:10), mouse anti-Futsch 22C10 (DHSB, 1:100) (Hummel et al., 2000), rabbit anti-Croquemort (1:1000) (Franc et al., 1999), guinea-pig anti-Senseless (1:1000) (Nolo et al., 2000). Alexa 488- and 568-conjugated secondary antibodies anti-mouse, anti-rat and anti-rabbit were purchased from Molecular Probes and used at 1:1000. DNA fragmentation was assayed by TdT-mediated dUTP nick end labelling (TUNEL kit, Roche Molecular Biochemicals). Images were obtained on an Olympus BX41 fluorescence microscope (×63 immersion oil objective) equipped with a CoolSnap camera driven by Metaview software (Universal Imaging). Images were processes with NIH Image and Photoshop software.

Only sensory clusters located in row 1 (close to the midline) were considered for statistical analysis.

Physiological assay

Cleaning reflex was tested on decapitated adult as described elsewhere (Corfas and Dudai, 1989).

RESULTS

Glial cells fragment shortly after birth

Time-lapse imaging in living pupae was performed to follow the fate of the glial cell in the thoracic bristle lineage. We used the *neu*^{P72} line to specifically express UAS-H2B::YFP in pI and its progeny cells by way of the GAL4/UAS binary expression system. In vivo imaging of pupae during an 8-hour period beginning at 17 hours after puparium formation (APF) allowed us to follow the entire bristle lineage. Microchaete pI cells underwent a sequence of four cell divisions and generated a cluster of five cells (Fig. 1A). Previous studies have shown that one of these cells acquires a glial identity because it expresses several glial specific markers such as Gcm or Repo. During in vivo imaging, several criteria were used to identify glial cells. First, these cells arose from the division of the pIIb cell, the most anteriorly located secondary precursor cell. Second, they occupied a more basal position than their sister cell, pIIIb. Finally, the glial cells were smaller than the other cells in the cluster, as inferred by the size of the nucleus (Gho et al., 1999).

At about 23 hours APF (on average 1.5 hours after pIIb division), the nucleus of the glial cell condensed (observed as a change in the nucleus shape and in the level of fluorescence) and then rapidly fragmented (Fig. 1A). Fragments dispersed quickly in about 10 minutes, generally towards a posterior direction (arrowheads in Fig. 1B), and disappeared. Before fragmentation, we frequently observed that the glial cell lost contact with the other cells in the cluster (see film clip 1 available at http://ifr-bi.snv.jussieu.fr/FichesPerso/Gho.html). The fragmentation of the glial cell nucleus was observed in every cluster studied. In 90% of the cases (n=18), fragmentation occurred after pIIIb division, when the neurone

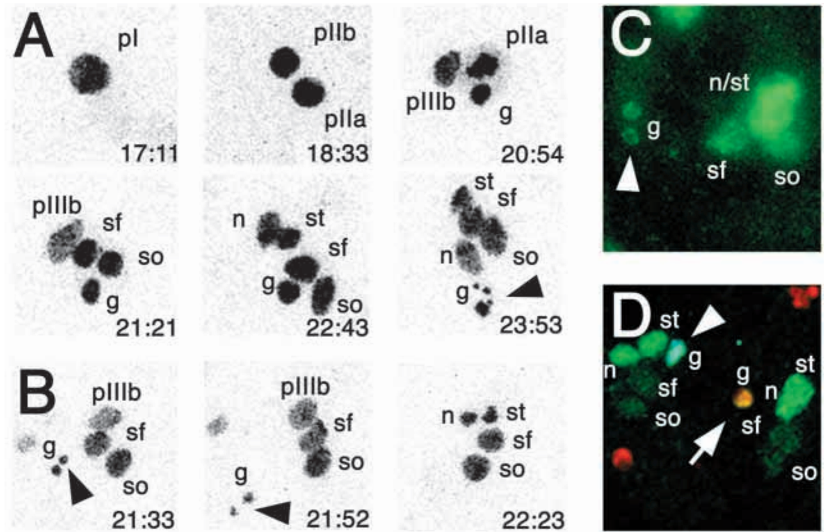

Fig. 1. Glial cells fragment in the microchaete lineage. (A,B) A live *neu*^{P72} UAS-H2B::YFP pupa was observed by time-lapse confocal microscopy. Time (hours:minutes) after puparium formation (APF) is shown at the bottom right of each panel. (A) A pI cell was followed from its initial division (at about 17:15 hours APF) until pIIIb division (at about 22:00 hours APF). Two hours later, the glial cell began to fragment (arrow at 23:53 hours APF). (B) Glial cell fragmentation in another bristle lineage from the same pupae as in A. Note that, in this case, the glial cell began to fragment before the division of pIIIb (at 21:33 hours APF and 22:23 hours APF, respectively). (C) Image taken from an in vivo observation of a *scabrous*-GAL4 UAS-nlsGFP pupae at 23:45 hours APF. The glial cell was identified by the size of its nucleus and lineage criteria. Note glial cell fragments (arrowhead). With this GFP construction, fragmentation was discernible only in rare situations. (D) Glial cells fragment in wild-type pupae. Sensory organ cells were stained with Senseless (green) and Repo (blue) antibodies to identify sensory and glial cells, respectively. Note that the TUNEL-positive cell (red) nearby a sensory cluster is also Senseless-immunoreactive (arrow in D). Also note that Repo-positive glial cells did not co-exist with other TUNEL-positive cells in the same cluster (arrowhead in D). In A and B, each image corresponds to the merging of 12 horizontal confocal optical sections. pI, primary precursor cell; pIIa and pIIb, secondary precursor cells a and b; pIIIb, tertiary precursor cell b; g, glial cell, n, neurone; so, socket cell; sf, shaft cell; st, sheath cell. Anterior is upwards and the view is horizontal.

and the sheath cell were already present. In the other cases, fragmentation was observed before the division of pIIIb (Fig. 1B). Thus, the elimination of the glial cell was independent of the division of its sister cell pIIIb.

Cell fragmentation and glial cell elimination were also observed when UAS-H2B::YFP or UAS-nls-GFP constructions were driven by other GAL4 lines such as SOP-GAL4 (which expresses GAL4 in secondary precursor cells and their descendants, not shown) and *scabrous*-GAL4 (which expresses GAL4 in pI and its descendants) (Fig. 1C). This indicates that fragmentation of the glial cell is not an artefact because of the overexpression of the UAS-H2B::YFP construction in the lineage cells nor a positional effect of the transgenic insertions. Similar nuclei fragments were observed in the A101 strain, which express β-Gal under the control of the *neuralized* promoter (not shown). Thus, in all genetic contexts studied, cell fragmentation and glial cell disappearance were observed.

In *w*^{1118} pupae, nuclear staining with DAPI revealed that the glial cell (identified with the sensory cell specific anti-Cut antibodies) had a very condensed nucleus, which was not seen in other cell types suggesting that this cell was in the process of DNA condensation and further fragmentation (not shown). To detect DNA fragmentation in *w*^{1118}, we performed a TUNEL assay. At 23 hours APF, TUNEL staining in wild-type pupae revealed labelled fragments in the proximity of many sensory clusters, suggesting that a cell undergoes apoptosis nearby. To analyse whether the fragments belong to the lineage, we took advantage of Senseless antibodies which preferentially recognised the neurone, the sheath and the glial cells. This double-staining revealed that most Senseless-labelled fragments were also TUNEL-positive (arrow in Fig. 1D). These observations confirm that the TUNEL-positive fragments pertain to the sensory lineage. Furthermore, our experiments showed that sensory clusters are formed either by four cells and a TUNEL-positive fragment or by four cells and a Repo-positive cell (arrowhead in Fig. 1D). Clusters with a cell co-labelled with TUNEL and Repo were rarely observed. This strongly suggests that, in general, the glial cell lost Repo immunoreactivity prior to fragmentation.

All together, our observations reveal that glial cell fragmentation and further elimination is a bona fide phenomenon in the thoracic bristle lineage. Those events are reminiscent of programmed cell death by apoptosis. Therefore, we analysed whether inhibition of apoptosis would result in the loss of glial cell fragmentation.

The glial cell undergoes apoptosis

Genes within the H99 region are involved in the death of the glial cell

To highlight a potential role of the pro-apoptotic genes *rpr*, *hid* and *grim* in the death of the glial cell, we generated somatic clones deficient for the H99 region that covers these three genes.

At 24 hours APF, Repo staining revealed that the glial cell was present in 95% of the clusters within H99 deficient clones (n=83, Fig. 2A), whereas clusters within the twin wild-type clones showed a glial cell in only 8% of the cases (n=59). This striking effect of the H99 deletion on the survival of the glial cell reveals an involvement of the pro-apoptotic genes of the H99 region in the death of the glial cell.

Surprisingly, in the H99 heterozygous parts of the nota, glial cells were visible in 73% of the clusters (n=156, not shown) at 24 hours APF. This increased presence of glial cells in H99 heterozygous clusters compared with wild-type clusters reveals a dose-dependent action of the pro-apoptotic genes of the H99 region.

Finally, at 30 hours APF, fewer glial cells were observed compared with at 24 hours APF. Glial cells were detected in 63% of the clusters within H99-deficient clones (n=89, Fig. 2B), in 45% of the clusters in heterozygous regions (n=144,

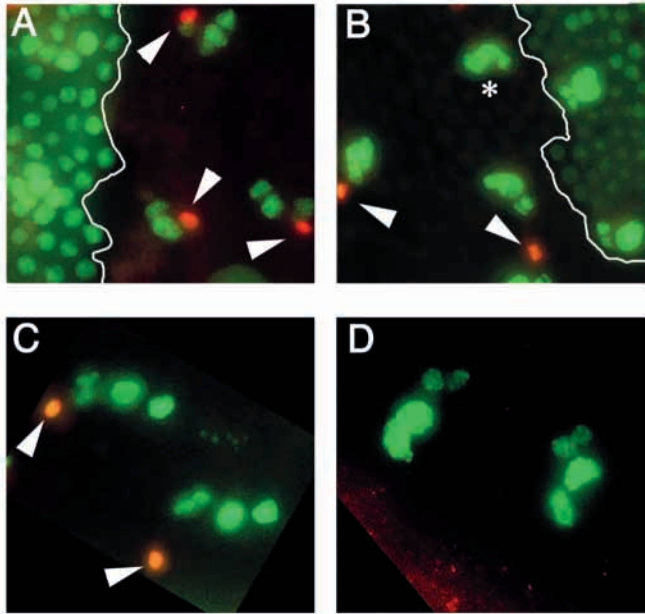

Fig. 2. The glial cell undergoes apoptosis. (A,B) Deletion of the pro-apoptotic genes *rpr*, *grim* and *hid* in H99-deficient clones blocked fragmentation of the glial cell. Clones were detected due to their lack of GFP staining (green); their limits are shown with a white line. Sensory organ cells were identified by anti-Cut immunoreactivity (also in green). Glial cells were immunostained with anti-Repo antibodies (red). Repo-labelled glial cells are observed in clusters inside H99 deficient clones at 24 hours APF (arrowheads in A). Note that, outside the clone, in the homozygous wild-type twin clone (GFP positive cells on the left of the figure), no glial cell was observed. (B) At 30 hours APF, some sensory organs in H99-deficient clones were associated with a glial cell (arrowheads). Note also organs in which the glial cell was not present (asterisk). (C,D) Overexpression of p35 represses glial cell fragmentation. neu^{P72} UAS-H2B::YFP UAS-p35pupae dissected at 24 hours APF (C) and 30 hours APF (D). Sensory cells are in green; Repo immunostaining is in red. Note that glial cells are still present at 24 hours APF (arrowheads in C) and have disappeared at 30 hours APF (D). Note also that no cell fragments are observed. Anterior is upwards and the view is horizontal.

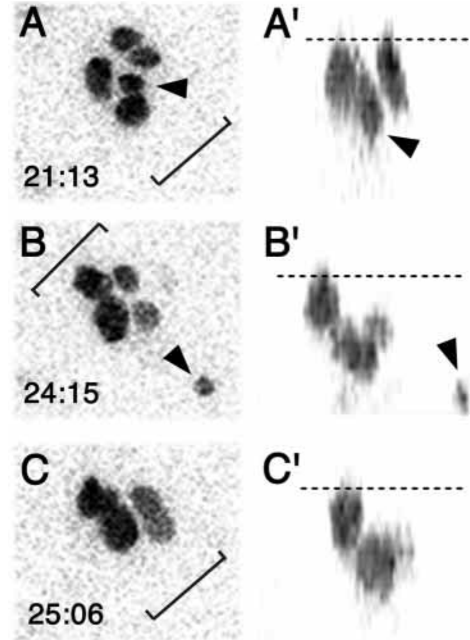

Fig. 3. Inhibition of apoptosis did not prevent the loss of the glial cell. Time-lapse confocal analysis of a neu^{P72} UAS-H2B::YFP UAS-p35 living pupae. Hours:minutes APF is shown at the bottom left of each panel. The glial cell (arrowheads) was maintained alive after overexpression of p35. (A-C) Horizontal views. (A′-C′) Lateral views of the corresponding horizontal views. The glial cell remained near the cluster for about 4 hours after it was produced (A,A′). After this period, the glial cell moved away from the cluster and fell down towards the internal cavity (lateral view at 24:15 hours APF, B′). At about 25 hours APF, the glial cell could not be observed anymore. Note that fragmentation was never observed. Each image corresponds to the merge of 18 horizontal confocal optical sections. Lateral projections were reconstructed from the horizontal sections at the positions showed inside the brackets. Broken line in A′-C′ denotes the dorsal surface. Anterior is upwards in A-C.

not shown) and in none of the clusters within homozygous twin spots (*n*=35, not shown).

p35 overexpression prevents fragmentation of the glial cell

To investigate whether the viral caspase inhibitor p35 could prevent fragmentation of the glial cell, we monitored the presence of Repo-positive cells in neu^{P72} UAS-H2B::YFP UAS-p35 flies.

At 24 hours APF in neu^{P72} UAS-H2B::YFP animals, only 6% of the clusters showed a Repo-positive cells (*n*=65). At the same time in neu^{P72} UAS-H2B::YFP UAS-p35 pupae, glial cells were present in 86% of the clusters (*n*=60, Fig. 2C) and no fragments could ever be seen. At later stages (30 hours APF), the proportion of sensory organs presenting Repo-positive glial cell in p35 overexpressing flies decreased to 15% of the clusters (*n*=40, Fig. 2D).

Taken together, these observations show that glial cells are eliminated from the epithelium by a H99-dependent and p35-sensitive apoptosis. In addition at late stages, we observed that both p35 overexpressing pupae and H99 homozygous clones showed a decrease in glial cell number. Therefore, we monitored the fate of these cells in neu^{P72} UAS-H2B::YFP UAS-p35 living pupae.

Surviving glial cells do not remain in the epithelium

In neu^{P72} UAS-H2B::YFP UAS-p35 living pupae, the glial cell remained associated to the corresponding sensory cluster in an unfragmented form between 21 and 24 hours APF (Fig. 3A), confirming the observations obtained in fixed material. This is about 2 hours longer than in control pupae (see Fig. 1A). After 24 hours APF, the fluorescence of the glial cell gradually weakened as the cell moved away from the cluster (Fig. 3B; see film clip 2 at http://ifr-bi.snv.jussieu.fr/FichesPerso/Gho.html). Moreover, the glial cell progressively fell down into the internal cavity (Fig. 3B′) where it could no longer be observed (Fig. 3C′). It is important to note that fragmentation was never observed. Thus, from these experiments we conclude that, although glial cell fragmentation is inhibited by the viral caspase inhibitor p35, these cells nevertheless leave the epithelium.

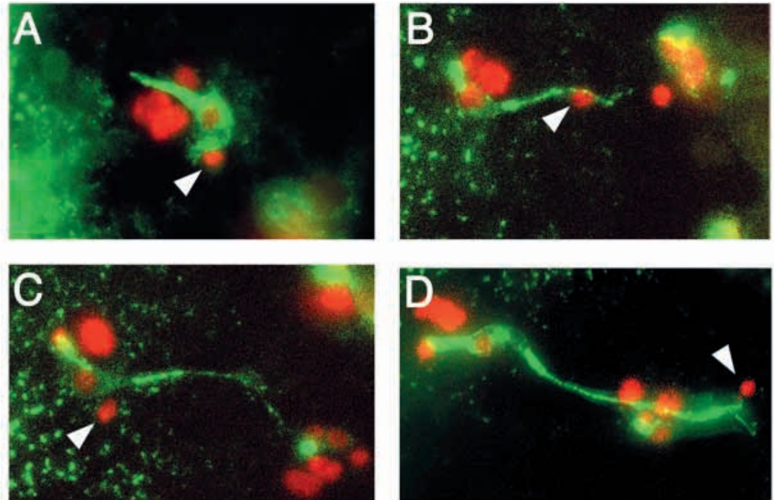

Fig. 4. Ectopic glial cells were frequently associated with growing axons. Growing axons from *neu*^{P72} UAS-H2B::YFP UAS-p35 sensory clusters were immunostained with 22C10 antibodies (shown in green). YFP expressing sensory cells are depicted in red. Glial cells (arrowheads) were maintained alive after overexpression of p35. In A-C, typical positions of the ectopic glial cell are shown relative to the growing axon at 24 hours APF. The glial cell was associated with the growth cone (A), standing along the axon (B) or near the sensory cluster even when the axon was well developed (C). Clusters on the first row of microchaete are shown. Clusters in B and C are from the same notum. Another example of growth cone-associated glial cell is shown on the second row of microchates (D). Anterior is upwards and the view is horizontal.

Ectopically surviving glial cells do not affect axon morphology and connectivity

Given the important role of glial cells in axonal guidance and neuronal survival, it was surprising that in wild-type specimens, most glial cells had already undergone apoptosis at 25 hours APF when axonal growth cones became visible (data not shown). In addition, as shown in Fig. 1B, glial fragmentation sometimes occurred before the division of pIIIb, as such before neurone formation, rendering any interaction between the glial cell and the neurone very unlikely in wild-type flies. Under experimental conditions in which glial cell death was inhibited, we asked whether surviving glial cells would affect the morphology and/or the development of microchaetes.

We first analysed possible ectopic interactions between glial cells and axonal processes. In p35-expressing pupae at 24 hours APF, glial cells were tightly associated with 22C10-positive axonal processes in 72% of the sensory organs studied (n=69). In 55% of these cases, glial cells were located at the growth cone (Fig. 4A, another example of such a location, on the second row of microchaetes, is shown in Fig. 4D), while they were associated to the axon in the remaining 17.5% of the cases (Fig. 4B). In the rest of the sensory organs analysed (27.5%), glial cells remained within the proximity of the cluster (Fig. 4C). The fact that in 72% of the clusters, a close association was observed between the axon and the ectopically surviving glial cell shows that the ectopic cell retains 'glial' characteristics.

Second, we studied the consequence of surviving glial cells on the morphology of the axonal arborisation. At 27 hours APF, the axonal network and its orientation, showed no major difference between wild-type and *neu*^{P72} UAS-H2B::YFP UAS-p35 pupae (Fig. 5A,B, 27 hours). Therefore, it seems unlikely that glial cells provide any guidance clue to the neurones. In addition, we performed physiological studies testing the cleaning reflex in p35-expressing and control adult flies. In both cases, we observed that, after stimulation of the thoracic bristle, the cleaning reflex was elicited normally in p35-expressing adults (not shown). Taken together, these observations suggest that ectopic survival of glial cells has no effect on either the final axonal connectivity or the physiology of the sensory bristle.

The presence of the glial cell promotes axonal outgrowth

Although, glial cell survival does not seem to affect axonal projections, we wondered whether ectopic presence of glial cells could influence the time course of axogenesis. Therefore, we analysed axonal growth at different times of development in *neu*^{P72} UAS-H2B::YFP UAS-p35 pupae. While wild-type specimens exhibited no processes at 24 hours APF (Fig. 5A, 24:00 h), axonal growth cones were already observed 30 minutes before, in p35 overexpressing flies (Fig. 5B, 23:30 h). At 25 hours APF, the first growth cones appeared in *neu*^{P72} UAS-H2B::YFP pupae, whereas axons originating from the first row of pIs were already reaching the second line in *neu*^{P72} UAS-H2B::YFP UAS-p35 pupae (Fig. 5A,B, 25:00 hours).

Similar results were obtained when glial cells were forced to survive in H99-deficient clones. Fig. 5C shows a H99 clone in a pupa at 25 hours APF, sensory neurones inside the clones extend their axons towards the second row of microchaetes (Fig. 5C, arrowheads), whereas axons from sensory organs in the contralateral H99 heterozygous heminotum are still absent or just appearing. The fact that, in H99 heterozygous tissue, axogenesis occurs at the same time as in control tissue was a surprise because, under these conditions, glial cells survive longer than in wild type (see above). This suggests that H99 heterozygous glial cells, even if they survive longer, are unable to affect neuronal development (see Discussion).

Early axogenesis was not due to an advanced rate of neuronal development because, first of all, the time of the pIIIb division was not altered and, second, the determination of neurones, as measured by Elav immunoreactivity, was similar in wild-type and in p35-overexpressing pupae (data not shown). Furthermore, the time course of axonal appearance was not affected after overexpression of p35 using the neuronal-specific driver *elav*-GAL4 (not shown).

Therefore, we conclude from these observations that the presence of the glial cell promotes axonal differentiation and outgrowth.

Glial cell death is independent of glial identity

In order to test the role of cell identity acquisition in triggering apoptosis of glial cells, we analysed the pattern of apoptosis in *gcm* mutant somatic clones. No Repo-immunostained cell was

revealed in clusters inside *gcm* clones compared with, at most, one cell for clusters outside the clone, confirming that pIIIb sibling cell did not acquire glial cell identity in *gcm* mutant clones (data not shown). Thus, at 24 hours APF, two types of sensory organs were observed in *gcm* clones (Fig. 6A), those composed of four cells (82%, *n*=66) and those composed of five cells (18%, *n*=66). Interestingly, a similar situation was observed outside *gcm* clones. There, clusters were composed of four (84%, *n*=62) or five cells (16%, *n*=62). In addition, two Elav-positive cells were detected in five-cell clusters inside *gcm* clones (arrowhead in Fig. 6A), this situation was never observed in clusters outside *gcm* clones. In four-cell clusters within *gcm* clones, only one cell was Elav positive, verifying that the entire lineage had been produced at this pupal age. The absence of Repo-positive cells in *gcm* clones and the extra neurone observed in five-cells clusters confirmed that in absence of *gcm*, glial cells adopted a neuronal fate. Moreover, the number of cells by cluster (four or five) and the number of neurones in each cluster (one or two, respectively) observed in *gcm* clones suggests that one extra neurone was eliminated from the five-cell clusters. This was confirmed by the observation of Elav-positive fragments in *gcm* clones (arrowhead Fig. 6B), suggesting that the extra Elav-positive cell underwent programmed cell death. These results indicate that the glial cell undergoes apoptosis independently of *gcm* expression. As this cell dies when it acquires a glial or a neuronal identity, this could indicate that apoptosis is triggered in the future glial cell before this cell is determined.

Glial cell death is independent of *prospero* expression

Previous studies have shown that the glial cell strongly expresses the transcription factor Prospero (Gho et al., 1999). Therefore, we investigated a potential involvement of this gene in glial cell apoptosis using mosaic flies for the null allele $pros^{17}$. No Prospero-immunostained cell was observed in clusters inside $pros^{17}$ mutant clones, confirming the amorph quality of this allele. Surprisingly, one Elav-positive neurone and one Su(H)-immunoreactive socket cell were observed in every sensory clusters inside the clones (data not shown). This strongly suggests that the bristle lineage occurred normally in absence of Prospero, allowing the study of the glial cell fate under these conditions.

Sensory organs in $pros^{17}$ clones at 24 h APF were composed by five (29%, *n*=69) or four (71%, *n*=69) Cut-positive cells. In addition, one Repo-immunoreactive cell was observed in every five-cell cluster within the clone, showing that Repo-positive glial cells are formed in the absence of Pros. Furthermore, the lack of Repo-positive cell in four-cells clusters suggests that the Repo-positive glial cell was eliminated from the cluster as in control tissue (see above). This was confirmed by the observation of Repo-positive (arrows in Fig. 6C) as well TUNEL-

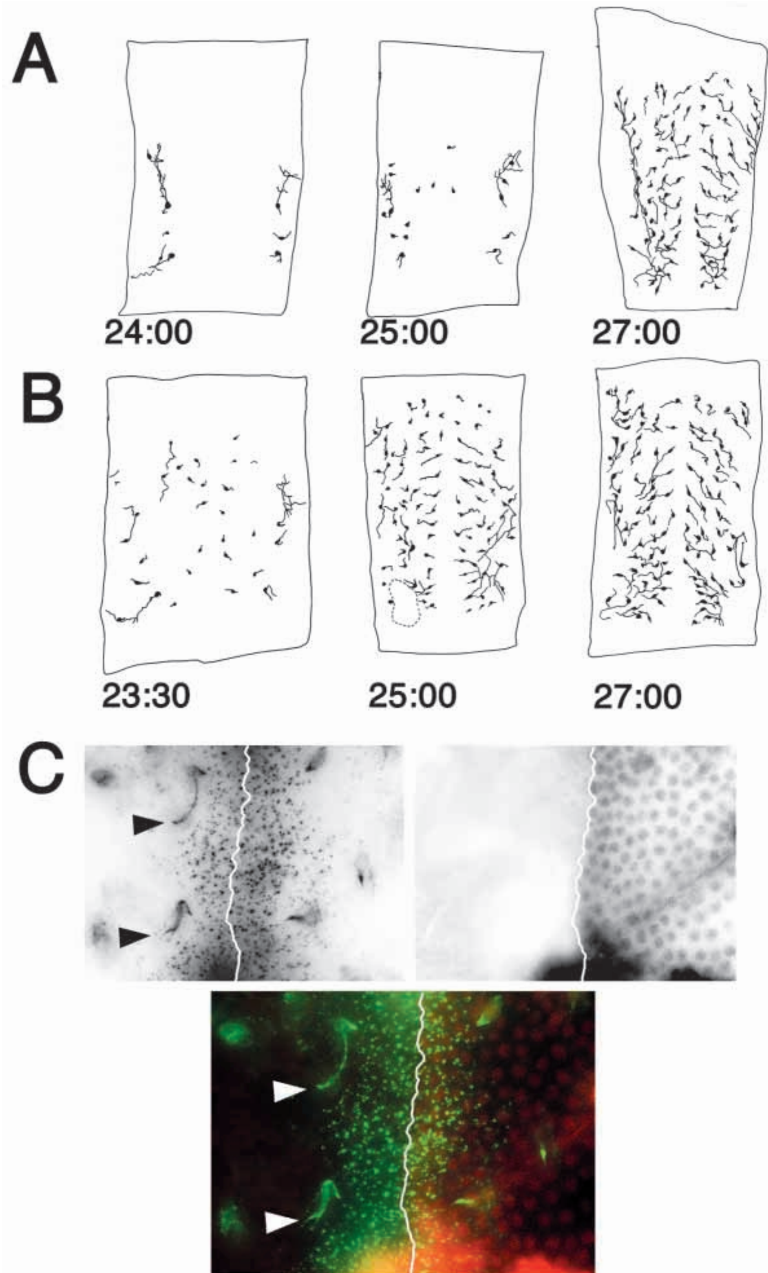

Fig. 5. Ectopic glial cells promote precocious axogenesis. (A,B) Camera lucida drawing of nota stained with 22C10 antibodies at different times of development. In each case, the entire region between the two dorsocentral macrochaetes is depicted. (A) Wild-type nota. At 24 hours APF, only axons from macrochaetes were present. Axogenesis of microchaetes starts at around 25 hours APF. (B) Nota from *neu*^{P72} UAS-H2B::YFP UAS-p35. Microchaete axogenesis initiates at about 23:30 hours APF. Note that in nota older than 27 hours APF, no major difference regarding the orientation of axonal processes could be discerned. (C) H99-deficient clone stained with 22C10 antibody (shown in green on the bottom overlay image). The clone was detected because of its lack of GFP staining (shown in red on the bottom overlay image), its medial limit is shown with a white line. Each channel is shown individually (22C10 top left and GFP top right). The midline is associated with a dotted 22C10 immunoreactivity of unknown nature. Note that axon projections are more developed in sensory organs inside the clone (arrowheads) than the counterpart axons in the contra-lateral region (H99 heterozygous). Limits of clones are indicated with a white line. Anterior is upwards and the view is horizontal.

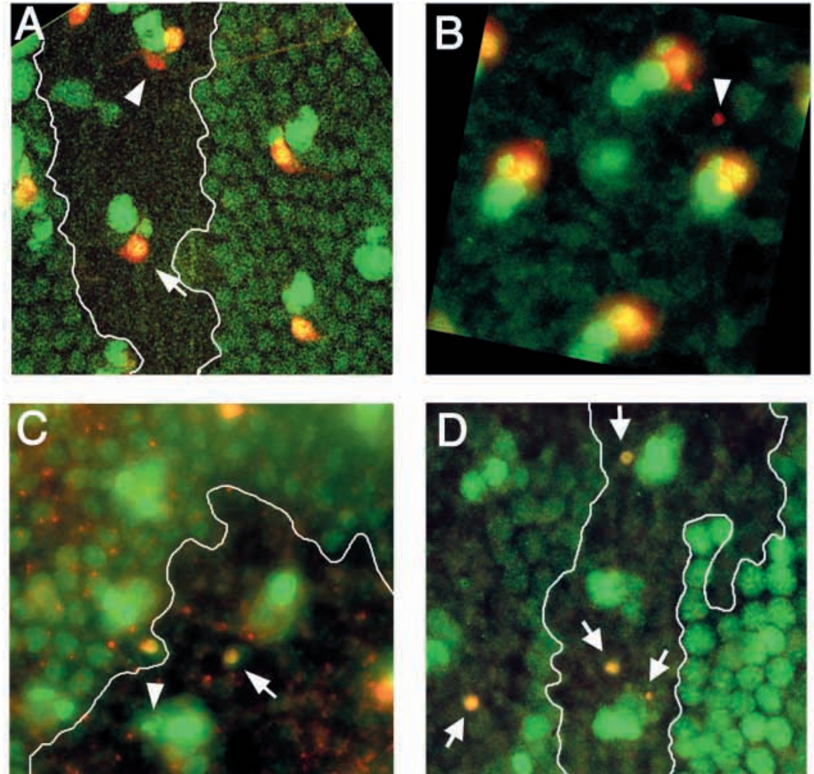

Fig. 6. The apoptosis of the glial cell is independent of glial identity and of *pros* expression. (A,B) gcm^{e1}/gcm^{e1} somatic clones at 24 hours APF are shown. Clones were detected because of their lack of GFP staining (green), their limits are shown with a white line (in A). Sensory organs cells were detected with anti-Cut antibodies (also in green). Neurones were identified by Elav-immunoreactivity (red). (A) At 24 hours APF, two types of sensory clusters were observed inside the gcm^{e1}/gcm^{e1} clone: four-cell clusters (arrow) and five-cell clusters (arrowhead). In these cases, an extra Elav-positive cell could be detected (arrowhead). Note that clusters with two Elav-positive cells were never observed outside the clone. (B) Detailed view from another gcm^{e1}/gcm^{e1} clone. The arrowhead shows Elav-positive cell fragments next to a four-cell cluster. (C,D) $pros^{17}/ pros^{17}$ somatic clones at 24 hours APF are shown. Clones were detected because of their lack of GFP staining (green), their limits are shown with a white line. Sensory organs cells were detected with anti-Senseless antibodies (in green, C,D) and with anti-Cut antibodies (also in green, D). (C) At 24 hours APF, some cell fragments co-labelled with Senseless and Repo (red) were detected within $pros^{17}$ clones (arrow), other fragments did not show any Repo staining (arrowhead). (D) Glial cell fragmentation could be detected using TUNEL staining (red) inside $pros^{17}$ clones as well as outside the clones (arrows). Anterior is upwards and the view is horizontal.

positive cell fragments (arrows in Fig. 6D) co-labelled with senseless nearby sensory clusters within $pros^{17}$ clones. These results show that the glial cell undergoes apoptosis independently of *pros* expression.

Fragments are phagocytosed

Macrophages are known to be involved in the removal of apoptotic cells. Therefore, we analysed the behaviour of macrophages during the period of glial cell fragmentation. In embryos, macrophages can be visualised by immunostaining against the membrane protein Croquemort (Crq) (Franc et al., 1999). However, we failed to detect Crq-immunoreactive cells in the notum. As an alternative procedure, we labelled macrophages by injecting Indian Ink into larvae. Macrophages are known to accumulate ink following its injection into the general cavity (see Lanot et al., 2001).

Indian Ink was injected in neu^{P72}UAS-H2B::YFP early third instar larvae. Fifty percent of the injected larvae (n=250) survived until pupation and only 20% of the pupae were still alive when dissected at 24 hours APF. Dissected nota from these pupae frequently exhibited black-labelled multicellular structures located at posterior lateral positions. In addition, spread throughout the nota, we observed subepithelial groups of ‘black-dots’, which we interpreted as macrophages containing ink conglomerates. We next examined the glial cell fragments, identified as highly YFP-positive particles. This analysis showed YFP-positive nuclear fragments surrounded by ‘black-dots’ suggesting that fragments were engulfed by ink marked macrophages (Fig. 7). This evidence together with the observation that fragments move away (see arrowhead in Fig. 1B) from the clusters indicates that they are phagocytosed by mobile macrophages.

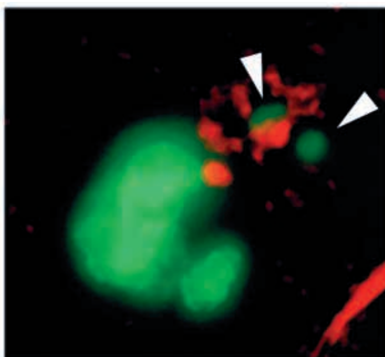

Fig. 7. Cell fragments are engulfed by macrophages. neu^{P72} UAS-H2B::YFP third instar larvae were injected with Indian Ink and dissected at pupal stage (23 hours APF). Macrophages which have incorporated Indian Ink depicted black inclusions of different sizes (red). Sensory organ cells expressing GFP are in green. To avoid reduction in green staining after superposition with a transmitted image, the Indian Ink image was depicted in negative. Note that GFP-positive fragments (arrowheads) are surrounded by an Indian Ink-labelled cell. Anterior is upwards and the view is horizontal.

DISCUSSION

Microchaete glial cells are eliminated by apoptosis

In this study we show that, during microchaete development, the glial cell undergoes apoptosis soon after birth because: (1) DNA fragmentation was observed in fixed material using

TUNEL assay and in vivo using the UAS-H2B::YFP construction; (2) anticaspase p35 was able to inhibit cell fragmentation; and finally (3) no cell fragmentation was observed in H99 deficient clones. The apoptosis of the glial cell occurred in every sensory organ studied on the notum. As such, it is surprising that it went previously unobserved (Gho et al., 1999). The failure to detect the glial cell death in our previous study can be explained by the UAS-nls-GFP construction used at this time for the in vivo recordings. The fluorescence of this transgene is weaker and more diffuse than the UAS-H2B::YFP construction employed in the present study. The use of UAS-H2B::YFP allows us to distinguish clearly the glial nucleus fragmentation, a situation exceedingly difficult to visualise with UAS-nls-GFP (although not impossible when one knows what to look for, Fig. 1C). Furthermore, in our previous study, we described the migration of the glial cell out of the sensory cluster. We believe that the weak and diffuse fluorescence of nls-GFP lead us to observe only those situations in which glial fragmentation left large pieces of nuclei. Thus, we probably followed fragments of glial cell moving away from the clusters, possibly, as we show in the present study, during phagocytosis by mobile macrophages.

Inhibition of apoptosis does not prevent loss of glial cells from the epithelium

Overexpression of the caspase inhibitor (p35) or removal of pro-apoptotic genes (H99 deletion) blocks apoptosis of the glial cell, as evidenced by the absence of nuclear fragmentation. Nevertheless, in both cases, the glial cell disappears later on from the epithelium. We propose three possibilities to explain this disappearance: loss of specific markers, cell migration or cell death.

Time-lapse study of *neu*^{P72} UAS-H2B::YFP UAS-p35 specimen showed that YFP was always present in this cell until it went too deep in the specimen to be observed. We believe that this movement towards the inside of the pupa is the reason for the loss of Repo-positive cells observed between 24 and 30 hours APF. Therefore the hypothesis that the glial cell disappearance is due a loss of markers seems unlikely.

The possibility that surviving glial cells migrate away could be in agreement with the observation that those cells tend to be associated to the subepithelial axonal processes. Nevertheless, the drop of these cells, perpendiculary to the plane of the neuronal processes, suggests that they lost their close associations with the axons. This drop appears reminiscent of a process know as 'cell competition' in which cells lose their epithelial position and fall down into the internal cavity (Simpson, 1979).

We favour the hypothesis that surviving glial cells in H99-deficient clones as well as in p35 overexpressing pupae still present characteristics that either trigger a redundant programmed cell death or are recognised by executioner cellular agents. Accordingly, phagocytosis of exceeding cells after apoptosis inhibition was recently observed in embryos (Mergliano et al., 2002).

Glial cell survival promotes axonal outgrowth

The ability of glial cells to induce ectopic expression of neuronal specific markers such as Futsch protein has already been observed (Klambt et al., 2001). Consistently, our data show that ectopically surviving glial cells induce a premature outgrowth of 22C10-positive axonal processes. Nevertheless, this observation is difficult to reconcile with the fact that axonal outgrowth was similar between wild-type and H99 heterozygous pupae where glial cells showed prolonged survival. However, as we observed that these cells undergo nucleus fragmentation (data not shown), we think that the H99 heterozygous glial cells present apoptotic characteristics that prevent them from promoting axonal outgrowth.

We believe that the premature axogenesis is indeed due to the action of the ectopic glial cells for the following reasons. First, similar observations were obtained when apoptosis was blocked by two different procedures, overexpression of p35 and H99 deletion. This ruled out the possibility that premature axogenesis could be a consequence of the genetic background on the sensory cells. Second, overexpression of p35 in the neurone using *elav*-GAL4 had no effect on the timing of axogenesis. This excludes the possibility of cell-autonomous effects of p35 on neurones. Finally, the time of neuronal determination was unaffected after apoptosis blockade. These observations strongly suggest that premature axogenesis is promoted by the presence of the glial cell that has been forced to survive.

The close association observed between p35 overexpressing glial cells and axons could reveal a common clue recognised by both cells and used for pathfinding. Alternatively, the glial cell could migrate towards the axon or vice versa. From our study, we cannot distinguish between these hypotheses. Nevertheless, the similarity of the axonal arborisation in control and p35-expressing flies strongly suggests that ectopic glial cells do not have major influence on axonal pathfinding. If some subtle effect occurs, the normal functionality of bristles observed in p35 expressing flies would suggest that the precise path of the axon does not matter for making a functional connection.

Our results also revealed that, under normal conditions, axonal outgrowth occurred in the absence of glial cells. Thus, microchaete glial cells are dispensable for axonal guidance, connectivity and sensory function. Instead, the principal clue to guide microchaete axons of the thorax seems to be a scaffold of persistent larval multidendrite neurones (Usui-Ishihara et al., 2000). Thus, we suggest that the acquisition of glial cell identity by one of the cell in the microchaete lineage is an evolutionary relic.

Mechanisms responsible for the apoptosis of the glial cell

Diverse studies of developing vertebrate and invertebrate embryos have demonstrated the essential role of extrinsic factors in promoting or preventing cell survival (see Rusconi et al., 2000; Durand and Raff, 2000). Two types of extrinsic signals can be distinguished: the endocrinal signal, which has a global action on the organism; and the paracrine signals, which act locally. During the bristle lineage, it is unlikely that hormonal signals or other endocrine factors are involved in glial cell death, as apoptosis occurred in the absence of any synchrony. A synchrony would be expected for a large-scale signal unless cells exhibit different responsiveness at different times. Our study revealed that ecdysone, an essential hormone controlling metamorphosis, did not trigger glial cell death. More precisely, glial cell death occurred normally in *ultraspiracle* mutant clones that affect nuclear ecdysone

receptor complex (data not shown) (Thummel, 1995). Among the paracrine signals analysed, we focused our attention on the EGF and JNK pathways, both of which are involved in triggering apoptosis in *Drosophila* (Rusconi et al., 2000; Adachi-Yamada et al., 1999). However, in our system, neither upregulation of the EGF pathway (by overexpression of the activators Secreted Spitz, Rhomboid and an activated form of the EGF receptor) (Klambt, 2000) nor blockade of the JNK pathways (in *basket* mutant clones) (Noselli, 1998) had an effect on glial cell apoptosis (data not shown).

As a consequence, we favour the idea that an intrinsic mechanism is involved in the apoptosis of the glial cell. We have first ruled out a potential involvement of *gcm* in this process. This result was unexpected, knowing the key role of *gcm* in determining glial cell identity, as revealed by the absence of Repo- and gain of Elav-expression in sensory organs within *gcm* clones (our data) (Van De Bor and Giangrande, 2002). Other intrinsic factors potentially involved in glial apoptosis are Numb and Prospero, which segregate during pIIb division into the glial cell (Gho et al., 1999). According to this possibility, those intrinsic factors, when inherited by one pIIb daughter cell, would trigger apoptosis in the glial cell or would prevent it in its sibling pIIIb cell. Recent studies have shown that Numb prevents apoptosis mediated by the Notch pathway in an embryonic sensory lineage (Orgogozo et al., 2002). This observation is difficult to reconcile in our system, as glial cells receive Numb during the asymmetric cell division of its progenitor, pIIb. In the bristle lineage, Numb plays an essential role in cell determination and any modification of the segregation of this cell determinant has consequences for the cell identity (Rhyu et al., 1994; Wang et al., 1997; Reddy and Rodrigues, 1999). This precludes any well-defined experiment designed to test a possible role of this factor on the glial apoptosis.

Previous studies have shown that Prospero promotes pIIb cell fate. Thus, *pros*-overexpression transforms the pIIa cell into its sibling pIIb (Reddy and Rodrigues, 1999; Manning and Doe, 1999). In *pros* null clones, we observed at least two of the pIIb cell progeny, the neurone and the glial cell. This shows that removal of *pros* gene does not strongly affect the pIIb identity. In addition, we also observed glial cell apoptosis. Two conclusions can be drawn from these results. Firstly, Prospero seems sufficient but not necessary to induce pIIb identity and secondly, this gene is not involved in triggering glial cell apoptosis.

A common pattern of cell divisions at the basis of sensory organ lineages

In the *Oncopeltus* larvae, nuclear breakdown in one of the five cells that makes up the mechanosensory organs has been described (Lawrence, 1966). Moreover, it has been shown that, for a period, these organs comprise five cells but only four survive throughout the life of this bristle, confirming that one cell of the sensory cluster is eliminated. Although the origin of the cell that underwent nuclear breakdown was not conclusively determined in these early observations, we believe that this cell is the neurilemma cell, which is homologous to the *Drosophila* glial cell of microchaete organs. Thus, these observations strongly suggest that the elimination of the glial cell in mechanosensory organs has been conserved throughout evolution.

Several cell lineages that give rise to various *Drosophila* sensory organs share the same common pattern of cell divisions (Fig. 8) (Orgogozo et al., 2001; Orgogozo et al., 2002; Gho et al., 1999; Brewster and Bodmer, 1995; Van De Bor et al., 2000). This core sensory lineage is composed by a primary precursor cell which divides giving rise to two secondary precursor cells. One of these cells produces three inner cells after two rounds of divisions and the other gives rise two outer cells after one division. We propose that diversity amongst the different lineages arises from adding different processes to this core (Fig. 8). Two types of processes may be distinguished: first, processes in which cell identity is changed without altering the configuration of the common core pattern of cell divisions; and, second, processes in which new complexity is added to this core without profound modifications in cell identity (red and blue arrows in Fig. 8, respectively). Chordotonal organs and es-md sensory organs in the embryo are two examples of the former type of process (Brewster and Bodmer, 1995; Orgogozo et al., 2001). Both of them originate from precursor cells after a cell lineage identical to the core

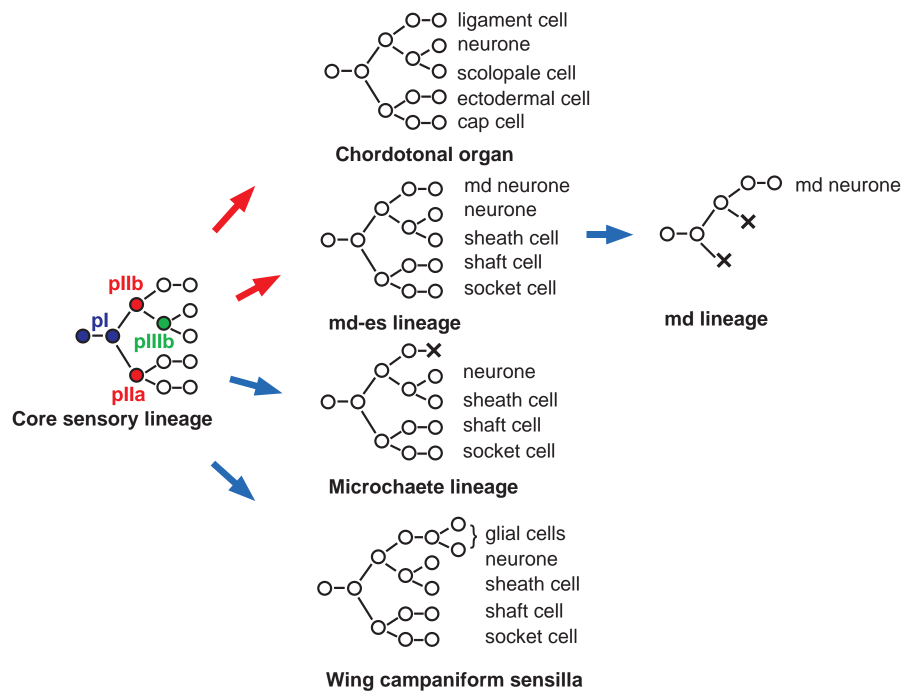

Fig. 8. Proposed core cell lineage of sensory organs in *Drosophila* and creation of diversity. Sensory diversity is generated when different processes are added to this basic pattern of cell division. Two processes may be distinguished: first, processes in which cell identity is changed without altering the configuration of the core pattern of cell divisions (red arrows); and, second, processes in which new complexity is added to this core without profound modifications in cell identity (blue arrows).

sensory lineage discussed before. However, the identities of the cells are different. Chordotonal cell lineage gives rise to an internal sensory organ while es-md lineage produces a mono-innervated organ and a multidendritic neurone (Fig. 8). Situations that involve processes of the second type, which add complexity without extensive changes in cell identity, are found in microchaete (this work), in embryonic md-lineage (Orgogozo et al., 2002) and in wing campaniform sensilla (Van De Bor et al., 2000). Specifically, the cell lineages at the origin of microchaete and embryonic md-neurones are similar to the core of sensory lineages. However, programmed cell death occurs in one inner cell (the glial cell) in microchaetes and in the outer secondary precursor cell and the tertiary precursor cell in the md-lineage (Orgogozo et al., 2002). Complexity can also increase by adding cell proliferation. Thus, in campaniform sensilla of the wing, the same cell, which dies in the thoracic microchaete lineage, shows proliferative characteristics and after symmetric division produces several mature glial cells (Van De Bor et al., 2000). In the same way, it seems very likely that such extra proliferative features are involved in the formation of chemosensorial bristles (Nottebohm et al., 1994).

In conclusion, *Drosophila* sensory organs appear to be homologous structures that originate from an ancestral cell lineage and possess a common core of cell divisions. In agreement with Bellaiche and Schweisguth (Bellaiche and Schweisguth, 2001), we propose that sensory organ diversity arises by adding new features to this core, which is mainly achieved through cell proliferation, cell determination and programmed cell death. It is interesting to note that these three processes are also identified as a source of morphological and functional variation during the formation of complex systems such as the central nervous system of vertebrates.

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