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Some fly sensory organs are gliogenic and require *glide/gcm* in a precursor that divides symmetrically and produces glial cells

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SUMMARY

In flies, the choice between neuronal and glial fates depends on the asymmetric division of multipotent precursors, the neuroglioblast of the central nervous system and the IIb precursor of the sensory organ lineage. In the central nervous system, the choice between the two fates requires asymmetric distribution of the *glial cell deficient/glial cell missing* (*glide/gcm*) RNA in the neuroglioblast. Preferential accumulation of the transcript in one of the daughter cells results in the activation of the glial fate in that cell, which becomes a glial precursor. Here we show that *glide/gcm* is necessary to induce glial differentiation in the peripheral nervous system. We also present evidence that *glide/gcm* RNA is not necessary to induce the fate choice in the peripheral multipotent precursor. Indeed, *glide/gcm* RNA and protein are first detected in one daughter of IIb but not in IIb itself. Thus, *glide/gcm* is required in both central and peripheral glial cells, but its regulation is context dependent. Strikingly, we have found that only subsets of sensory organs are gliogenic and express *glide/gcm*. The ability to produce glial cells depends on fixed, lineage related, cues and not on stochastic decisions. Finally, we show that after *glide/gcm* expression has ceased, the IIb daughter migrates and divides symmetrically to produce several mature glial cells. Thus, the *glide/gcm*-expressing cell, also called the fifth cell of the sensory organ, is indeed a glial precursor. This is the first reported case of symmetric division in the sensory organ lineage. These data indicate that the organization of the fly peripheral nervous system is more complex than previously thought.

Key words: Sensory organ lineage, *glial cell deficient/glial cell missing*, Glia, PNS, Proliferation

INTRODUCTION

The acquisition of cell identity depends on the activity of genes that impose specific fates via the interplay of autonomous and regulatory mechanisms. Multipotent stem cells of the fly embryonic central nervous system (CNS) also called neuroglioblasts (NGBs) give rise to different types of neurons and glial cells (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999 and references therein). The glial cell deficient/glial cell missing (Glide/Gcm) transcription factor is necessary and sufficient to induce gliogenesis (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Bernardoni et al., 1998). *glide/gcm* directs the choice between the neuronal and the glial fate due to asymmetric distribution of its transcript in the neuroglioblast. Upon division, one of the daughter cells, the presumptive glioblast (GB), preferentially accumulates the transcript and takes the glial fate, while the other, the neuroblast (NB), adopts the default neuronal fate (Akiyama-Oda et al., 1999; Bernardoni et al., 1999).

All but one of the larval peripheral glial cells also derive from embryonic NGBs. These glial cells reach the periphery upon migration along axons (Schmidt et al., 1997; Schmid et al., 1999). In the adult, however, the origin of peripheral glial cells depends on the type of tissue. Sub-retinal glial cells originate from outside the eye disc and migrate there subsequently (Choi and Benzer, 1994). In the leg, some glial cells originate within the appendage and some migrate there during pupal development (Giangrande, 1994). Finally, all wing glial cells differentiate within the appendage and then migrate towards the CNS (Giangrande et al., 1993; Giangrande, 1994). In cases in which cells arise at the periphery, it is likely that gliogenesis involves novel genetic controls and cellular mechanisms.

Previous studies have shown that wing glial cells and sensory organs arise from the same regions and that mutations affecting sensory organs also affect glial cells (Giangrande, 1994, 1995). In addition, it has been recently shown that glial cells arise from microchaete precursors (Reddy and Rodrigues, 1999b; Gho et al., 1999). Here we demonstrate that some sensory organ lineages produce glial cells while some others do not, due to different division and gene expression patterns. Glial cells arise from the asymmetric division of a multipotent precursor and require *glide/gcm* to differentiate. Surprisingly, *glide/gcm* is only expressed and required in one daughter cell of that precursor. Thus, the fate choice between neurons and glia taking place in the multipotent precursor is promoted by a yet unknown fate determinant acting upstream of *glide/gcm.* In addition, we demonstrate that gliogenic sensory organs

contain more than five cells, due to proliferation of the *glide/gcm*-expressing cell, which migrates, divides symmetrically and produces mature glial cells. Finally, the rate and the pattern of glial proliferation varies between individuals, suggesting the existence of plasticity in the lineage of gliogenic sensory organs.

MATERIALS AND METHODS

Fly strains

The wild-type strain was *Sevelen*. The sensory organ lineage was visualized using the enhancer trap line *A101* (Huang et al., 1991). The *y w, hs-FLP122* strain used to induce flipase activity was kindly provided by K. Basler. Strains used to generate *glide/gcm* loss-of-function clones were *glide/gcm^{N7-4} P(ry$^+$, hsneo, FRT)40A/CyO, P(y$^+$)* and *y w; P(w$^+$, hspM)21C, P(w$^+$, hspM)36F, P(ry$^+$, hs-neo, FRT)40A; Kg^v A101 (lacZ, ry$^+$), kar^2 ry^{506}/TM6C ry^{CB}*. The *UAS-glide/gcm* transgenic line *M24A* (Bernardoni et al., 1998) and the *heat shock-Gal4* line *w; P(hs-Gal4; w$^+$)*, were used to induce ubiquitous *glide/gcm* expression.

Mosaic analysis

glide/gcm clones were generated using the flipase recombinase. *y w, hs-FLP122; Bc* females were crossed with males carrying the flipase target sites and two copies of the *P(w$^+$, hspM)* transposon, which was used as a clone marker (*y w; P(w$^+$, hspM)21C, P(w$^+$, hspM)36F, P(ry$^+$, hs-neo, FRT)40A; Kg^v A101(lacZ, ry$^+$), kar^2 ry^{506}/TM6C ry^{CB}*). The *P(w$^+$, hspM)* transposon displays a MYC epitope-containing protein fused to the heat shock promoter (Evan et al., 1985). *y w, hs-FLP122/Y; P(w$^+$, hspM)21C, P(w$^+$, hspM)36F, P(ry$^+$, hs-neo, FRT)40A/Bc* males obtained in the progeny were crossed with *glide/gcm^{N7-4} P(ry$^+$, hsneo, FRT)40A/CyO, P(y$^+$)* females. Adults were allowed to lay eggs for 1 day. The progeny was heat shocked 21 hours later at 38°C for 30 minutes in order to induce mitotic recombination early during the second larval instar. Female prepupae of the following genotype *y w, FLP122/+; P(w$^+$, hspM)21C, P(w$^+$, hspM)36F, P(ry$^+$, hs-neo, FRT)40A/glide/gcm^{N7-4}, P(ry$^+$, hsneo, FRT)40A* were collected and kept at 25°C until 14 or 33 hours after puparium formation (APF). At these stages, pupae were heat shocked at 38°C for 2 hours and kept for 1 hour at 25°C before dissection. This treatment was necessary to induce the expression of the MYC epitope-containing protein. Clones lacking glide/gcm activity were recognized by the absence of anti-Myc labelling.

Immunohistochemistry and in situ hybridization

Fixation, dissection and antibody incubation were performed as in Giangrande et al. (1993). Wings were mounted in Vectashield (Vector) medium. Third instar larvae were staged according to Maroni and Stamey (1983). The following primary antibodies were used: mouse anti-cMyc 910E.3 (1:100) (Neomarkers), rat anti-Elav (1:2000) (provided by G. Rubin), mouse anti-Elav (1:10) (DSHB), rabbit anti-Repo (1:8000) (provided by A. Travers), rat anti-RK2 (1:1000) (provided by A. Tomlinson), rat anti-Glide/Gcm (1:1000), rat anti-Cut (1:200) (provided by K. Blochlinger), rabbit anti-PH3 (1:50000) (Upstate Biotechnology), rabbit anti-Pros (1:500) (provided by A. Vaessin). Secondary antibodies coupled with Oregon Green (Molecular Probes), Cy3, Cy5, FITC (Jackson), were used at 1:400.

In situ hybridization on pupal wings was performed according to the method of Hughes and Krause (1998) with minor modifications. Digoxigenin-labelled riboprobes were prepared using the *glide/gcm* cDNA. The hybridization signal was detected using a sheep anti-digoxigenin primary antibody and a Cy3-conjugated anti-sheep secondary antibody (1:400) (Jackson). DAPI (50 ng/ml in 0.3% PTX-DEPC) was used to label chromatin. Preparations were analyzed using a confocal microscope (DMRE, Leica).

Ectopic expression analysis

glide/gcm ectopic expression was obtained by crossing the *UAS-glide (M24A)* transgenic line with the *w; P(hs-Gal4; w$^+$)* line. Prepupae coming out of this cross were collected and heat shocked for 1 hour at 38°C at different stages during sensory organ development. *M24A* prepupae were heat shocked and dissected at the same stages as a negative control. Wings were dissected at 16 hours APF and labelled as above.

RESULTS

glide/gcm is required for the differentiation of wing glial cells

To understand how gliogenesis takes place in the peripheral nervous system (PNS), we analyzed the wing, where the array of glial cells and sensory organs has already been described. Mechano- and chemosensory organs differentiate from ventral and dorsal epithelia, most of them being located along the anterior margin (also called L1 vein) and the L3 vein (Murray et al., 1984; Hartenstein and Posakony, 1989; Huang et al., 1991). Axons from the sensory neurons fasciculate into two nerves that navigate along the L1 and L3 veins to reach the thoracic ganglia (Ghysen, 1978, 1980; Palka et al., 1979; Whitlock and Palka, 1995). Like sensory organs, most glial cells differentiate along L1 and L3 (Fig. 1A) and require the activity of proneural genes in order to differentiate (Giangrande et al., 1993; Giangrande, 1995). Subsequently, glial cell nuclei are found along the whole nerves, due to distal to proximal migration along the axonal fibers (Giangrande, 1994).

In an attempt to identify the molecule(s) involved in peripheral glial differentiation, we analyzed the role of *glide/gcm*, which is required for the differentiation of all lateral glial cells in the embryonic CNS (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Since *glide/gcm* is an embryonic lethal mutation, we induced *glide/gcm* mitotic clones using the FLPase recombinase and a strain carrying *myc FRT* that allows the recognition of mutant territories (Xu and Rubin, 1993). The glial phenotypes were then analyzed using the antibody against the reverse polarity (Repo) protein, which is expressed in all but midline glial cells during embryonic and post-embryonic development (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995).

Fig. 1B,C shows an example of a *glide/gcm* null clone straddling the dorsoventral boundary. Almost two thirds of the anterior margin is affected by the mutation. The rest of the margin is wild-type on one or both epithelia. Strikingly, the number of glial cells along L1 is drastically decreased. The few glial cells detected in the mutant territory are not themselves mutant, since they are all Myc positive (Fig. 1C). These wild-type glial cells are likely to have differentiated distally to the clone and subsequently migrated into it by following the axon bundle. Similar results were obtained in clones affecting L3 (Fig. 1D,E). In all cases, no example of *glide/gcm* mutant glial cells were ever observed indicating that gliogenesis strictly depends on *glide/gcm*.

glide/gcm and the sensory organ lineage

In the embryonic CNS, lack of glide/gcm leads to the generation of supernumerary neurons (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). We therefore asked

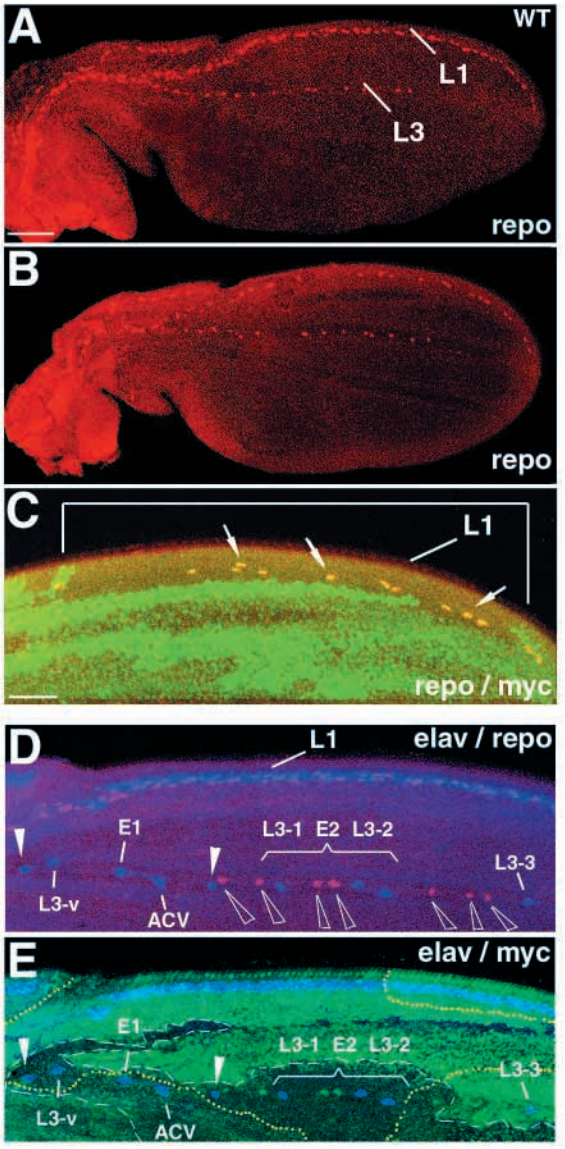

Fig. 1. In absence of Glide/Gcm, peripheral glial cells fail to differentiate and transform into neurons. 36 hours APF wings. In this and all subsequent figures, anterior is to the top, distal to the right. (A) Wild-type (WT) wing labelled with the glial-specific anti-Repo antibody showing uniform glial nuclei along the innervated L1 and L3 veins (L1 and L3). (B-C) Wing displaying *glide/gcm* mutant cells on the anterior margin (see bracket in C) simultaneously labelled with anti-Myc (green) and anti-Repo (red). C shows the double labelling. Note that the few glial nuclei still detectable within the clone do not belong to it, because they are all Myc positive (arrows). (D,E) Wing carrying two *glide/gcm* clones, a dorsal and a ventral one, along the L3 vein, simultaneously labelled with anti-Myc (green), anti-Elav (blue) and anti-Repo (red). L3-v indicates the neuron of the ventral sensillum on L3, ACV, the anterior cross vein neuron, L3-1, L3-2 and L3-3 the neurons of the three campaniform sensilla on L3. E1 and E2 indicate the two 'extra' neurons on L3 (Murray et al., 1984). (D) Double labelling with anti-Elav and anti-Repo. Few wild-type glial nuclei (open arrowheads) are present on the distal part of the L3 vein. (E) Double labelling with anti-Elav and anti-Myc. The dorsal and ventral clones borders are indicated by the white dashed line and the yellow dotted line respectively. Note the presence of two supernumerary neurons along the L3 vein (arrowheads). Bars (A,B) 98 μm; (C-E) 39 μm.

whether lack of wing glial cells is accompanied by an increased number of neurons. As a marker for neuronal nuclei we used the *embryonic lethal abnormal vision* (*elav*) gene (Robinow and White, 1988). We generated mitotic clones as above and simultaneously labelled them with anti-Myc, anti-Repo and anti-Elav, in order to recognize simultaneously the glial and neuronal nuclei as well as the mutant territories. Indeed, more Elav labelling was observed in mutant clones than in wild-type tissues (Fig. 1D,E).

It has been recently shown that sensory organ precursor (SOP) cells of notum microchaetes generate glial cells. These SOP cells divide asymmetrically and produce the PIIa and the PIIb cells, two second order precursor cells that also divide asymmetrically (for reviews, see Horovitz and Herskowitz, 1992; Jan and Jan, 1998). PIIa generates the tormogen and the trichogen. PIIb produces a glial cell and a PIIIb precursor, which in turn divides and generates a neuron and a sheath cell (Reddy and Rodrigues, 1999a, 1999b; Gho et al., 1999). If this type of lineage were common to all sensory organs, the number of supernumerary neurons in *glide/gcm* clones should correspond to the number of sensory organs affected by the mutation. However, clones located along the L3 vein and affecting four sensory organs (in Fig. 1: L3-v, ACV, L3-1, L3-2) only displayed two supernumerary neurons (4 clones were analyzed). Two alternative mechanisms might explain this result: either the fate transformation is not complete in the mutant cells or two types of sensory organs exist, gliogenic and non-gliogenic.

To determine whether all sensory organs produce glial cells, we used two approaches. First, by analyzing *glide/gcm* clones affecting individual sensory organs we detected one supernumerary neuron when L3-1 (6 clones analyzed), L3-3 (n=6) or L3-v (n=1) were mutated, whereas none was detected in clones affecting L3-2 (n=6). No clones affecting only ACV were found.

Second, we analyzed the profile of *glide/gcm* expression in different wing sensory organ lineages. Along the anterior margin, *glide/gcm* is detected in the inner-most cell of chemo- and mechanosensory organs, when the precursors have already divided more than once (data not shown). This indicates that both types of sensory organs can generate glial cells. *glide/gcm* expression was also observed in large campaniform sensilla of the L3 vein. Surprisingly, however, not all of them are *glide/gcm* positive. L3 campaniform sensilla have been divided in two classes, early and late, according to their birth time, central projections and electrophysiological behaviour (Murray et al., 1984; Palka et al., 1986; Dickinson and Palka, 1987; Blair et al., 1992). Early sensilla include ACV and L3-2, late sensilla include L-3v, L3-1 and L3-3. We found that *glide/gcm* is only expressed in late sensilla (Fig. 2 and data not shown). Expression is first detectable in the sub-epithelial cell produced by the PIIb division. In this cell, it persists until the five cell stage, but becomes undetectable later on (Fig. 2D and data not shown). Interestingly, notum macrochaetes behave like the early sensilla in that they do not express *glide/gcm* nor *repo*, although the mutant phenotype has not been assessed (data not shown).

Our results indicate that at least two types of sensory organs exist, a gliogenic one and a non-gliogenic one, and that lack of

Glide/Gcm leads to the complete transformation of glial cells into neurons in the gliogenic ones. Interestingly, supernumerary neurons are not adjacent to the sensory organs, due to migration (Fig. 1D,E).

Early and late sensilla lineages

The finding that some sensory organs are not gliogenic prompted us to follow their lineage by triple labelling with anti-Elav, anti-Cut and anti-Prospero (anti-Pros). *cut* is initially expressed in the SOP and in the two daughter cells, PIIa and PIIb (Blochlinger et al., 1990; 1993). Upon divisions of these two cells, it is present in all the progeny, however it is expressed at higher levels in the tormogen and the trichogen cells than in the inner, neuronal and sheath, cells. Anti-Pros identifies PIIb and its progeny (Reddy and Rodrigues, 1999a; Manning and Doe, 1999; Gho et al., 1999).

The lineage of late campaniform sensilla is the same as that of notum microchaete (Reddy and Rodrigues, 1999b; Gho et al., 1999). PIIb divides and produces PIIIb, and a small sub-epithelial cell (Fig. 3B). The small cell accumulates the highest levels of Pros, expresses *glide/gcm* and, later on, *repo* (data not shown). Pros is asymmetrically localized in the cortex of the dividing PIIIb (Fig. 3C). As in microchaete, the daughter that accumulates Pros at high levels is the neuron, however, at later stages, the sheath cell shows higher Pros levels than the neuron, most likely due to de novo synthesis (Fig. 3D).

At early stages, the lineage of non-gliogenic sensilla is similar to that of gliogenic ones. Starting from the division of PIIb, however, there are a number of differences. First, the PIIb cell produces two daughters of equal size: PIIIb and a cell located in the sub-epithelial layer that preferentially inherits Pros (Fig. 3E). This cell, which continuously expresses Pros at high levels and does not express glial-specific genes, corresponds to the sheath cell (Fig. 3F). Second, when PIIIb divides, Pros is present but is not asymmetrically localized (Fig. 3G). The two PIIIb daughters initially accumulate Pros at similar levels but at later stages express it at different levels (Fig. 3H). Interestingly, both cells are labelled by the Elav-neuronal marker: one of them belongs to the early campaniform sensillum, the other, which at late stages accumulates higher levels of Pros and migrates, constitutes one of the 'extra' neurons first described by Murray et al. (1984).

Mode of action of *glide/gcm* in the adult PNS

In the CNS, asymmetric *glide/gcm* RNA distribution dictates the fate choice in the NGB (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). Surprisingly, in the PNS, *glide/gcm* is not detectable in the multipotent precursor, PIIb, suggesting that it does not dictate fate choices (Fig. 2). The analysis of mutant lineages also confirms this hypothesis. If indeed *glide/gcm* were expressed and required to induce a fate choice in PIIb, lack of its product would induce the transformation of the glial cell into a PIIIb. This would entail the presence of two neurons and two sheath cells in a sensory organ consisting of six cells. However, mutant sensory organs never contain two sheath cells and are always composed of five cells, as in the wild-type (Fig. 4A,D and data not shown). All these data strongly suggest that *glide/gcm* is only expressed and required in the PIIb daughter and that its role in the PNS is different from that observed in the CNS.

To better understand the mode of action of *glide/gcm*, we produced gain of function phenotypes by ubiquitously expressing *glide/gcm* at different stages of L3-3 development. Heat shock before SOP birth, at around 0 hour APF, produces no effects (data not shown). Heat shock at 2 hours APF results in the production of a sensory organ consisting of four cells: two cells express the Repo glial-specific marker, while the Elav labelling is completely absent (Fig. 4B). Heat shock at 5 hours APF results in an L3-3 consisting of five cells, three of which express Repo (Fig. 4C). One of the Repo-positive cells also expresses Elav at very low levels. In all cases, the cells that are not Repo-positive express high levels of cut, which identifies them as tormogen and trichogen. These data suggest that the 2-hour APF shock leads to both PIIb daughters adopting the glial fate, while the 5-hour APF shock leads to both PIIIb daughters adopting the glial fate. Surprisingly, in no case did we observe a complete transformation of the sensory organ cells into glial cells (Fig. 4B,C). This result is even more striking when compared to the effects obtained in the embryonic CNS, in which the same *UAS-glide* transgene is able to transform all neural stem cells into glioblasts (Bernardoni et al., 1998). A different competence to acquire the glial fate amongst the cells of the sensory organ lineage is also confirmed by the observation that the fate transformation remains partial even when higher doses of ectopic Glide/Gcm

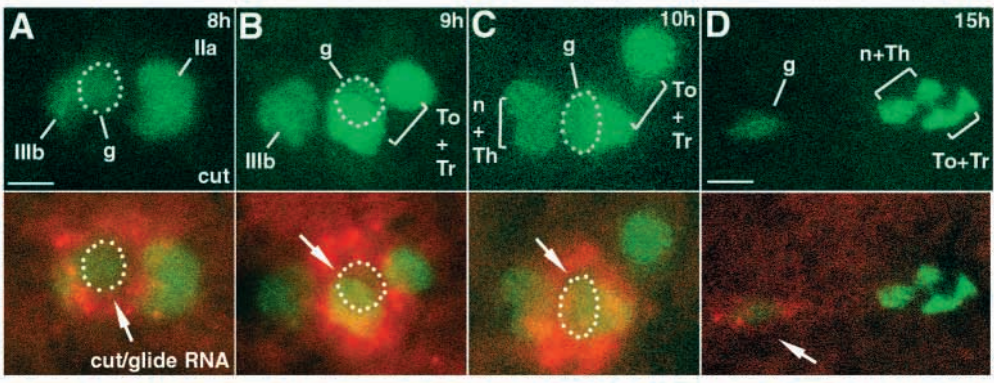

Fig. 2 *glide/gcm* is expressed in campaniform sensilla. (A-D) Confocal projections of the L3-3 sensory organ at different stages (hours APF). Sensory organs were simultaneously labelled with anti-Cut (green), and a *glide/gcm* riboprobe (red). Double exposures are shown in the lower panels. Cut is expressed in the entire lineage of the external sensory organs (Blochlinger et al., 1990; 1993). The position of the glial nucleus is indicated by a white dotted circle (A-C). A-C show respectively the lineage at the three-, four- and five-cell stages, in which *glide/gcm* is expressed at high level. Note the low level of *glide/gcm* expression in D, in which the cell has already started to migrate proximally. The PIIb, the PIIa and the PIIIb precursor cells are indicated; g, the glial cell; To+Tr, the tormogen and trichogen pair; n+Th, the neuron and the thecogen pair. Arrows indicate the *glide/gcm*-expressing cell. Bars, (A-C) 3.1 μm; (D) 6.2 μm.

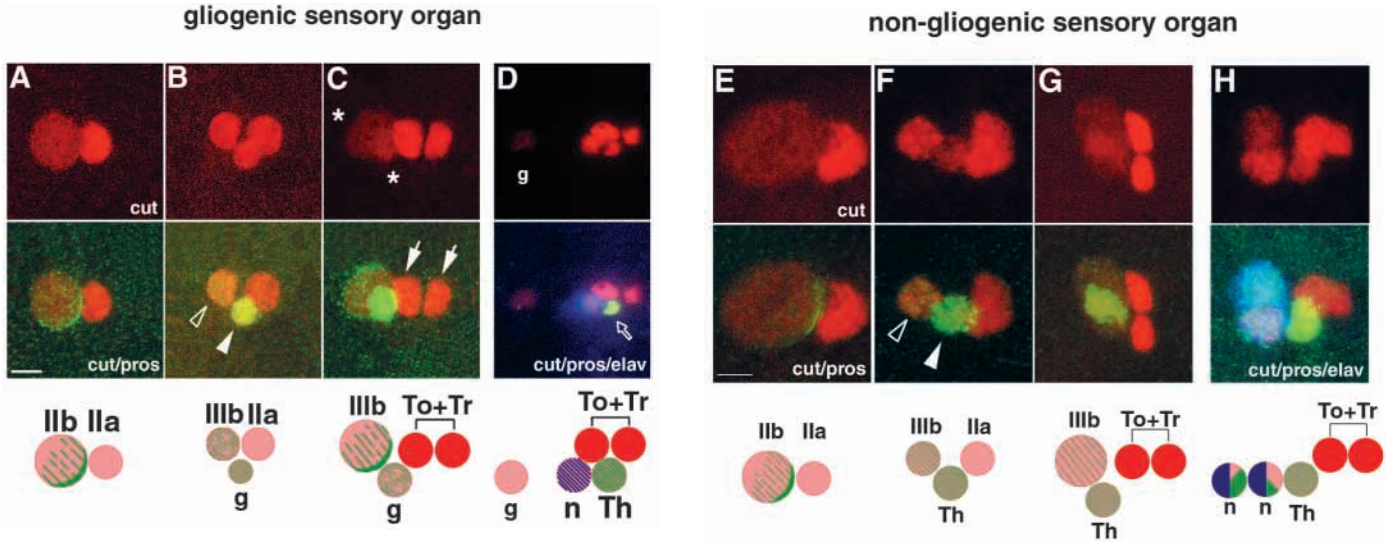

Fig. 3. Developmental analysis of gliogenic and non-gliogenic mechanosensory organs. Confocal projections of sections taken along the Z axis; distal is to the right. Triple labelling with anti-Cut (red), anti-Elav (blue) and anti-Pros (green) at different stages. (A-D) Gliogenic sensory organ (L3-3). Bottom panels show double or triple labelling. (A) At around 6 hours APF, the SOP has divided once. The proximal cell, which corresponds to the PIIb, is dividing and expresses Pros asymmetrically. (B) PIIb divides at 8 hours APF and produces a small sub-epithelial cell, and a large daughter cell that stays in the epithelium layer. The large cell inherits low levels of Pros (open arrowhead), whereas the small one shows high Pros levels (white arrowhead). The small and the large cells correspond to the presumptive glial cell and to the PIIIb precursor, respectively. (C) By around 10 hours APF, the PIIa cell has divided and generated the trichogen and the tormogen cells, which are located in the epithelial layer. These two cells express Cut at high levels (arrows) whereas PIIIb and the glial cell express it at low levels (asterisks). PIIIb is dividing to produce the neuron and the thecogen cell. During the division Pros is localised basolaterally. (D) An L3-3 at about 14 hours APF. The glial cell has already started to migrate. The thecogen cell expresses Pros at high levels (open arrow). (E-H) Non-gliogenic sensory organ (L3-2). Bottom panels show the double or the triple labelling. (E) 4 hours BPF wing. The SOP has divided and produced PIIa and PIIb, which is more proximally located and expresses Pros asymmetrically at division. (F) PIIb has produced two cells of equal size. One of them expresses Pros at high level (the thecogen cell, white arrowhead) and is located below the epithelium. The other daughter cell, which expresses Pros at low levels, corresponds to PIIIb (open arrowhead). (G) Pros is expressed in the dividing PIIIb (around WP stage), but is symmetrically distributed. PIIa has divided and produced the tormogen and the trichogen, which express high levels of Cut. (H) By around 4 hours APF, PIIIb daughters express the neuronal marker Elav. One of them expresses Pros at higher levels and Cut at lower levels than the other. Schematic representation of the two lineages are shown under each stage to show cell organization and product localization along the Z axis: apical is to the top. Large cells indicate cells at mitosis. Symbols are as in the previous figure. Color coding reflects the triple labelling results. Color intensity indicates the levels of expression of each antigen. Bar, (A-C) 6.2 μm, (D) 8.2 μm, (E-H) 3.1 μm.

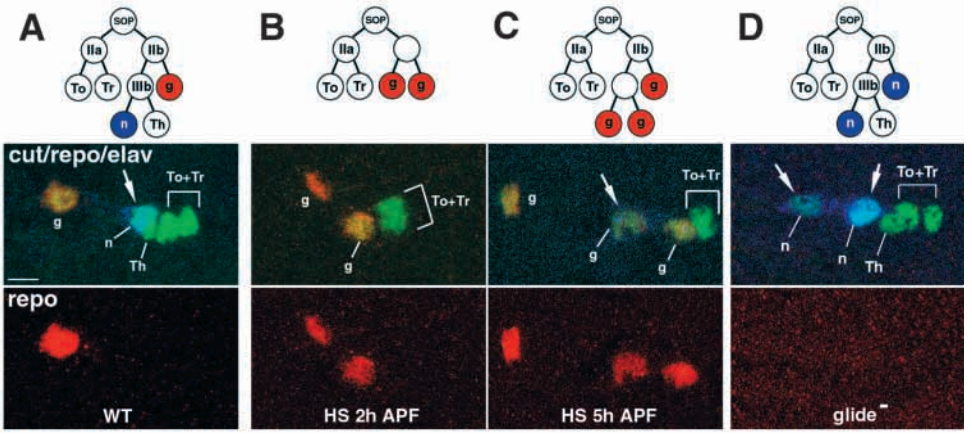

Fig. 4. Effects of gain and loss of function *glide/gcm* mutations. (A) Wild-type and (B-D) mutant L3-3 at 15 hours APF. (Top) Schematic representations of the lineage. (Middle) Triple labelling with anti-Cut (green), anti-Repo (red), and anti-Elav (blue). (Bottom) Repo labelling. At this stage L3-3 is composed of five cells located at stereotyped positions. Symbols as in the previous figures. (A) Tormogen and trichogen cells are located distally and express Cut at higher levels than the other cells of the lineage. The neuron is more proximally located and expresses Elav (arrow). The glial cell is Repo-positive and has started to migrate proximally. (B) *hs-Gal4; UAS-glide* wing heat shocked at 2 hours APF. Note that the sensory organ is composed of four cells. The two distal cells are strongly Cut-positive, which identifies them as tormogen and trichogen cells. The two proximal cells are Repo-positive. No Elav labelling can be detected in the cluster. (C) *hs-Gal4; UAS-glide* wing heat shocked at 5 hours APF. The sensory organ is composed of five cells. The two distal cells express Cut at high levels, as in the wild type. The three proximal cells are Repo-positive. Note that one of them is located at the position of the presumptive neuron and expresses Elav at very low levels (arrow). (D) *glide/gcm* mitotic clone: L3-3 is composed of five cells. The two distal cells express Cut at high levels while the two proximal cells express Elav (arrows). The proximal-most neuron has started to migrate. No Repo labelling is detectable. Bar, 12 μm.

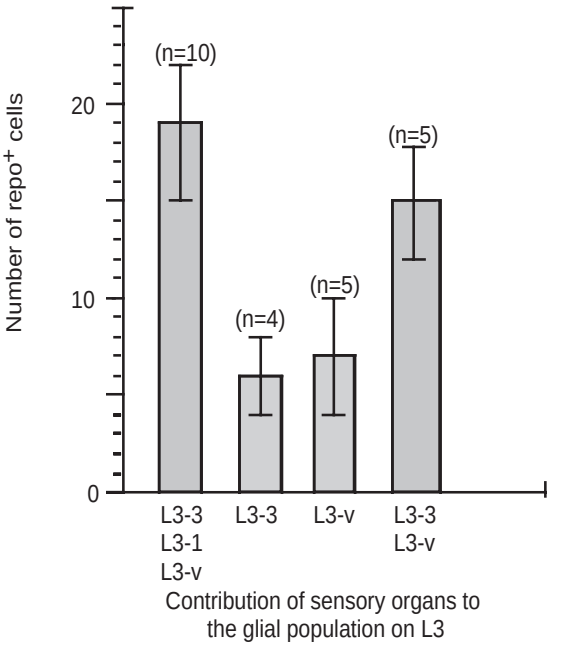

Fig. 5. Relative contribution of gliogenic lineages to the glial population on the L3 vein of wild-type wing. Numbers of Repo-positive cells at 36 hours. *n* indicates the number of samples analyzed. Bars indicate the highest and the lowest values. The number of glial cells were deduced by analyzing *glide/gcm* clones affecting sensory organs individually or in combination. For example, the number of L3-3 associated glial cells (second column) was obtained by counting the Repo-positive cells present in wings carrying a clone that affects the two other gliogenic sensory organs, L3-v and L3-1.

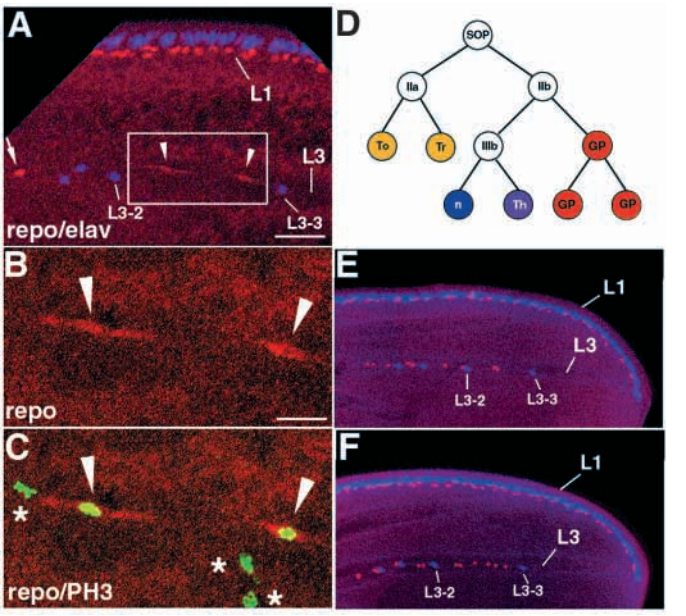

Fig. 6. Glial proliferation. (A-C) 22 hours APF wing simultaneously labelled with anti-Repo (red), anti-Elav (blue) and anti-PH3 (green). (A) Double anti-Repo and anti-Elav labelling. (L3-3) and (L3-2) indicate respectively the neurons of the L3-3 and the L3-2 campaniform sensilla neurons and the L1 and L3 veins are indicated. At this stage, two Repo-positive cells (arrowheads) are present between the L3-3 and the L3-2 neurons. Compared to the labelling in more proximal cells (arrow), Repo seems to be localized in the cytoplasm of the dividing cells. (B,C) Higher magnification of the region highlighted in A showing (B) Repo and (C) double Repo/PH3 labelling. The two Repo-positive cells (arrowheads) are PH3-positive. Asterisks indicate PH3-positive nuclei that do not belong to glial lineages. (D) Schematic drawing showing the division of the glial precursor cell (GP). Symbols as in previous figures. (E,F) 36 hours APF wings simultaneously labelled with anti-Repo (red) and anti-Elav (blue). Note that the number of glial nuclei varies between wings of identical stage (compare E with F). Bars: (A,B) 14 μm; (C) 39 μm; (E,F) 75 μm.

remains partial even when higher doses of ectopic Glide/Gcm are provided (data not shown).

Glial cell proliferation in the peripheral nervous system

glide/gcm is expressed in a cell of the sensory organ and ceases to be expressed after that cell starts migrating, whereas *repo* is expressed in the glial lineage until terminal differentiation (Fig. 2). By comparing the profile of expression of the two genes, we found that while the number of *glide/gcm*-positive cells corresponds to that of gliogenic sensory organs, the number of Repo-positive cells is much higher. For example, on L3 it goes from 15 to 22 (vs. three gliogenic sensory organs). A possible explanation for this result is that the sensory organ lineage contains more than five cells, due to the division of the *glide/gcm*-expressing cell. We therefore determined the precise number of glial cells arising from each sensory organ as well as the profile of glial proliferation in an individual sensory organ.

Analysis of *glide/gcm* clones affecting L3 gliogenic sensory organs individually or in combination allowed us to establish the relative contribution of each sensory organ to the total number of glial cells. Fig. 1D shows an example of clones affecting all sensory organs but L3-3, which specifically allows the identification of all L3-3 glial associated cells. For each sensory organ, the analysis was performed on several clones. The histogram in Fig. 5 shows that the three L3 gliogenic sensory organs produce on average six cells. This analysis confirms that the *glide/gcm*-expressing cell is a glial precursor cell and shows that the number of divisions producing glial cells is comparable amongst the sensory organs.

The profile of proliferating glial cells during development was obtained by double labelling with anti-Repo and anti-PH3, an antibody that recognizes the phosphorylated form of histone H3 present in the chromatin of dividing cells (Table 1 and Fig. 6). Since glial cells migrate from distal to proximal during development (Giangrande, 1994), we focussed on the gliogenic lineage most-distally located and counted nuclei between L3-3 and L3-1. While at 9 hours APF only one Repo-positive cell is present in that region (data not shown), five to eight Repo-positive cells can be detected at 36 hours APF (Table 1). Between 17 hours and 30 hours APF several mitotic figures were observed, concomitant with the progressive increase in Repo-positive cells. Thus, the fifth cell of the sensory organ proliferates and produces several glial cells. While this cell expresses both *glide/gcm* and *repo*, its daughters only express *repo*. We will refer to this cell as to GP for glial precursor.

Given the final number of glial cells (Figs 1D, 5; Table 1), it is clear that more than one division occurs. At least two

Table 1. Glial proliferation in the L3-3 sensory organ

	L3-3 to L3-1 region	
Hours APF	$Repo^+$ cells	$Repo^+$ and $PH3^+$ cells
17	**1-2** (1.0)	**0-1** (0.1)
20	**1-2** (1.6)	**0-1** (0.1)
22	**2-6** (2.1)	**0-2** (0.4)
23	**2-6** (3.4)	**0-1** (0.4)
24	**4-7** (4.8)	**0-1** (0.2)
30	**5-8** (5.8)	**0-1** (0.1)
36	**4-8** (5.2)	**0** (0)

Twenty pupal wings were scored at different developmental stages (hours APF). In the second and third columns the highest and the lowest number of cells detected at each stage is shown in bold. The average number of glial cells at each stage is in parentheses.

modes of proliferation can account for the results observed: (i) the GP divides asymmetrically and produce another glial precursor and a differentiated glial cell; (ii) the GP divides symmetrically and produces two proliferating daughters. Two Repo-positive dividing cells were indeed observed in the L3-3 to L3-1 region (Fig. 6), strongly supporting the model in which both GP daughters have proliferative potentials.

It is important to notice that the time of division is not fixed, since at any given stage the number of proliferating glial cells varies from wing to wing (Table 1 and data not shown). Similarly, the number of cell divisions is not tightly fixed within a given lineage, since the total number of glial cells is not constant amongst the different wings analyzed (Fig. 6E,F). The average number of glial cells associated with each sensory organ as well as the average number of glial cells on L3 suggest that more than two rounds of cell division take place in average for each GP (Fig. 5).

It is worth noticing that clones lacking *glide/gcm* display sensory organs composed of five cells carrying only one additional neuron indicating that this neuron does not proliferate. This explains why the number of additional neurons in mutant territories was always lower than the number of Repo-positive cells found in corresponding regions in the wild-type background (see Fig. 1).

DISCUSSION

glide/gcm is necessary for the differentiation of all peripheral glial cells

The present data demonstrate that peripheral glial cells strictly depend on *glide/gcm* in order to differentiate. Therefore, one single gene promotes all the types of adult and embryonic, central and peripheral glial cells, the only exception being midline glia, which arise from mesectoderm and are under a different genetic control (Granderath and Klambt, 1999). PNS and CNS glial cells originate from multipotent precursors that divide asymmetrically and produce neuronal and glial precursors. Interestingly, while the Glide/Gcm protein has the role of inducing gliogenesis in central and peripheral glial precursors, the transcript displays a different regulation depending on the tissue. In the CNS, *glide/gcm* RNA expression and asymmetrical distribution in the NGB dictates the fate choice between glia and neurons (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). In the PNS, the gene is not expressed in the multipotent precursor, PIIb, but in its daughter, the GP, which divides symmetrically and only produces glial cells. This indicates that yet unknown factors, and not *glide/gcm* transcripts, promote the choice between the glial and the PIIIb fate in PIIb. Such factors are then necessary to activate *glide/gcm* in GP. Thus, the behavior of *glide/gcm* is context dependent.

Two models can account for the acquisition of the glial fate in the adult PNS. The SOPs of gliogenic and non-gliogenic sensory organs already have different identities, due to the expression of specific cell fate determinants, which in turn results in the differential activation of *glide/gcm* in one of the PIIb daughter cells. Alternatively, the difference between the two types of sensory organs is only established in the PIIb cell. Interestingly, two molecules are expressed and asymmetrically distributed in PIIb: *numb* and *pros* (Uemura et al., 1989; Chu-Lagraff et al., 1991; Vaessin et al., 1991; Rhyu et al., 1994; Knoblich et al., 1995; Wang et al., 1997; Gho et al., 1999; this paper). Interestingly, most of the Pros and Numb proteins are inherited by the daughter cell that takes the glial fate (Gho et al., 1999; Reddy and Rodrigues, 1999b; this paper). All this evidence suggests that *numb* and/or *pros* participate to the fate choice taking place in the PIIb cell and act upstream of *glide/gcm*. It will be interesting to investigate the precise role and mode of action of such molecules in peripheral gliogenesis and in *glide/gcm* induction. Moreover, it will be important to determine whether the acquisition of the glial fate is completely autonomously regulated or whether it is also dependent on signalling between cells, like all the other fates of the sensory organ lineage (see for example: Hartenstein and Posakony, 1990; Guo et al., 1996).

glide/gcm and the sensory organ lineage

Sensory neurons and glial cells are lineage related. Unexpectedly, however, only some sensory organs express *glide/gcm* and are gliogenic. The fact that *glide/gcm* expression was never detected in early sensilla indicates that the differentiation of the glial precursor responds to fixed cues. Interestingly, while both mechano- and chemosensory organs express *glide/gcm* and are gliogenic, sensory organs of the same type such as sensilla campaniformia (late versus early) and bristles (micro- versus macrochaetes) may behave differently with respect to their gliogenic potentials and their lineage tree (microchaetes: Gho et al., 1999; Reddy and Rodrigues, 1999b; macrochaetes: this paper). In the embryo, none of the sensory organs is gliogenic except for the bipolar dendrite precursor, which divides once and produces one neuron and one peripheral glial cell; in addition, lineage analyses have shown that some embryonic sensory organs are composed of four instead of five cells (Bodmer et al., 1989; Brewster and Bodmer, 1995).

The developmental pathways leading to peripheral glial differentiation are at least as variable as those described for the sensory organs: some types of glial cells are lineage related to the cells of the sensory organ (wing and some leg glial cells, microchaete-derived glial cells, bipolar dendrite associated glial cell in the embryo), some are not (sub-retinal glial cells, some leg glial cells, embryonic PNS glial cells).

Therefore, different mechanisms of glial and sensory organ differentiation exist, depending on the tissue and on the stage of development. The identification of mutations affecting

specific pathways will be instrumental in understanding the molecular bases of these differences.

Cell proliferation in gliogenic sensory organs

Mechanosensory organs have been previously shown to contain at the most five cells (Vervoort et al., 1997; Reddy and Rodrigues, 1999b; Gho et al., 1999). Our data indicate that gliogenic sensory organs undergo additional rounds of proliferation and that the cell expressing *glide/gcm* is indeed a glial precursor. The fact that proliferation occurs when the fifth cell has migrated out of the sensory organ cluster is most likely the reason for this division being overlooked in the past, since sensory organ cells have always been considered as being physically associated. It is important to notice that *glide/gcm* expression ceases as proliferation and migration start, suggesting a role of this gene in preventing the two events. The observation that the *glide/gcm*-expressing cell divides during development raises several important questions concerning the mode and the pattern of proliferation within the sensory organ. The present data enable us to answer a number of such questions, even though further analyses will be necessary to fully understand the cellular and molecular mechanisms of glial proliferation.

First, we have shown that while all the other divisions in the sensory organ lineage take place within the epithelium, the divisions of the glial precursor and its progeny take place within the vein space, with no direct contact with epithelial cells, and do not show evident signs of asymmetry. Moreover, the plane of these divisions is always parallel to that of the epithelium, while that of the other PIIb daughter is orthogonal to it. This is the first reported case of symmetric division within the sensory organ lineage. It will be interesting to determine whether other sensory precursors producing two daughters with similar fates also divide symmetrically. In particular, precursors producing two neurons such as the IIIb of non gliogenic sensory organs (this paper) and the secondary precursors of chemosensory organs (see Ghysen and Dambly-Chaudière, 1993 for a review) have been described, but their mode of division has not been established yet.

Second, we have found that both GP daughters are able to proliferate, indicating that GP does not divide in a stem cell mode. Third, the number of cell divisions is rather similar amongst different sensory organs, suggesting that the proliferative potentials and the cues regulating proliferation are conserved amongst the gliogenic lineages. Fourth, the number of glial cells as well as the time of division within a given sensory organ vary between individuals, suggesting the existence of cell-cell interactions in the regulation of proliferation. In the future, it will be important to determine the molecular nature of the proliferative signal(s) as well as the relative role of extrinsic cues (underlying axons, extracellular matrix, vein epithelial cells) in glial proliferation. Although a number of molecules have been shown to promote glial proliferation in vertebrates (see for example Orentas and Miller, 1998), none has so far been identified in flies.

Finally, we have shown that glial cells migrate while they are still proliferating, as has been shown in vertebrates (see for example Le Douarin, 1982; Le Douarin et al, 1991; Bronner-Fraser, 1993; Zhou et al., 1990; Gansmuller et al., 1991). In the case of oligodendrocytes, it has indeed been proposed that some cells start to migrate before the entire population of precursors has stopped dividing (Gansmuller et al., 1991). Thus, it will be possible to use *Drosophila* as a model system to study the processes of glial migration and proliferation.

The unexpected diversity of progeny and mode of division in the fly PNS indicate that the sensory organ lineage is much more complex than previously described. Given our results on the gliogenic lineages, we speculate that, while early aspects of sensory organ differentiation do respond to fixed cues, late events such as the generation of glial cells may at least in part depend on regulatory mechanisms. We are currently developing techniques that will enable us to follow these events in the living organism.

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