

Development and organization of glial cells in the peripheral nervous system of *Drosophila melanogaster*

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SUMMARY

We have used enhancer trap lines as markers to recognize glial cells in the wing peripheral nervous system of *Drosophila melanogaster*. Their characterization has enabled us to define certain features of glial differentiation and organization. In order to ask whether glial cells originate within the disc or whether they migrate to the wing nerves from the central nervous system, we used two approaches. In cultured wing discs from glial-specific lines, peripheral glial precursors are already present within the imaginal tissue during the third larval stage. Glial cells differentiate on a wing nerve even in mutants in which that nerve does not connect to the central nervous system. To assess whether peripheral

glial cells originate from ectoderm or from mesoderm, we cultured discs from which the mesodermally derived epithelial cells had been removed. Our findings indicate that peripheral glial cells originate from ectodermally derived cells. As has already been shown for the embryonic central nervous system, gliogenesis in the periphery is an early event during adult development: glial cells, or their precursors, are already present at stages when neurons are still differentiating. Finally, our results also suggest that peripheral glial cells may not display a stereotyped arrangement.

Key words: glial cells, PNS, *Drosophila*, wing

INTRODUCTION

The *Drosophila* peripheral nervous system (PNS) includes the sensory organs, the sensory and motor fibres and the glial cells wrapping the nerves (see Giangrande and Palka, 1990 and references therein). The development of glial cells of the insect central nervous system (CNS) has been extensively investigated. As in vertebrates, CNS glial cells have been divided into classes on the basis of their lineage, position and morphology; the different classes seem to play distinct roles in neurogenesis (Wigglesworth, 1959; Strausfeld, 1976; Meyer et al., 1987; Jacobs and Goodman, 1989; Klämbt and Goodman, 1991; Winberg et al., 1992 and see Discussion). In the case of glial cells in the PNS, the almost complete lack of specific markers has hampered a detailed analysis.

In this study, we describe some enhancer trap lines (O'Kane and Gehring, 1987; Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989) that we have used to start analyzing the development and the overall organization of glial nuclei in the wing PNS. First, we have asked whether glial cells originate at the periphery or migrate there from other tissues and whether they are of mesodermal or ectodermal origin. One hypothesis was that glial cells arise in the central nervous system and use nerves as pathways to migrate to the periphery. In the present study, we show that wing

glial cells are present even in cases where a nerve is not connected to the CNS. This, and results from cultures of imaginal tissues, strongly suggests that glial cells differentiate at the periphery, from ectodermal cells.

Second, we have started a developmental analysis in order to determine when glial cells become detectable. Gliogenesis might have been a late event in PNS development, depending on the presence of well-differentiated sensory organs. We show that some glial cells or their precursors are detectable at early stages of pupal development, when most neuronal precursors are still dividing and axonogenesis has not started yet.

Third, we have used the lines to determine how glial cells are organized. Glial nuclei are distributed throughout the whole length of the nerves. Comparison of staining profiles in wings from the same animal suggests that PNS glial cells do not display a stereotyped organization.

MATERIALS AND METHODS

Fly stocks

Wild-type strains were the *Sevelen* and *Oregon R* stocks. The A289.1F1 line was selected in our laboratory in a screen for lines labeling wing glial cells. It was obtained from the stock centre at Indiana University, Bloomington, and generated in the laboratory

of W. Gehring. 2206 line was originated in A. Spradling's laboratory and selected by M. Schubiger. rA87 and AE2 lines were selected in a screen for glial staining in the embryo in the laboratory of C. Goodman and were kindly provided by V. Auld. A289.1F1 contains an as yet unlocalized insert on the X chromosome. 2206, rA87 and AE2 carry inserts at positions 93B1,2 (Schubiger et al., unpublished data), 30B and 35F, respectively (V. Auld, personal communication).

P8 was identified by M. Schubiger as being a viable *Abruptex* allele. The P8 mutation originated from an EMS mutagenesis in W. Pak's laboratory. P8 homozygous females were crossed with 2206 homozygous males. The progeny was analyzed by staining pupal wings with anti-HRP and anti- β -gal antibodies (see below). The PPS line that carries the *twist* promoter sequences fused with the β -galactosidase gene (Thisse et al., 1991) was provided by F. Perrin-Schmitt.

X-gal staining, antibody labeling and histology

To stain adult wings for β -gal activity (X-gal staining), we followed the procedure described by Blair et al. (1992).

For pupal antibody staining, white prepupae were collected and transferred to a moist chamber at 25°C until they reached the desired stage. Pupae were fixed in 4% formaldehyde (in PBS) and dissected the next day. Wings at 13 hours after pupariation (h AP) were slit open using a tungsten needle and fixed for an additional hour to facilitate antibody penetration, as at this stage the pupal cuticle cannot be peeled off. Fixed wings were washed in PBS, preincubated for 30 minutes at room temperature (RT) in PBS, 0.3% Triton X-100, and 5% normal goat serum, and then incubated overnight (4°C) in the preincubation solution plus the primary antibody (see below). Wings were then washed in PBS, incubated for 2 hours at RT in preincubation solution plus the secondary antibody, washed again in PBS and mounted in 80% glycerol with 4% n-propyl-gallate. For double staining, both primary or both secondary antibodies were added at the same time.

The β -gal protein was detected using a mouse anti- β -gal antibody from Promega (1:1400-1800). Rabbit anti-HRP from US Biochemical (1:600-1600) was used to recognize neurons. The rabbit anti-prospéro antibody (1:5000) and mAb5B12 were kindly provided by H. Vaessin and S. Benzer, respectively. Polyclonal anti-*twist* (1:10000) was a gift from B. Thisse and F. Perrin-Schmitt. The Cy3 goat anti-mouse IgG (1:300) kindly provided by Jackson ImmunoResearch was used to detect the anti- β -gal antibody. The Cy3 fluorophore has a spectrum similar to that of rhodamine, but in our preparations it gave better results in terms of signal-to-noise ratio compared to that obtained with both rhodamine and Texas red. Anti-prospéro and anti-HRP were detected using FITC goat anti-rabbit IgG (1:300). Preparations were ana-

lyzed using epifluorescence illumination and stored at 4°C. Biotinylated horse anti-mouse from Jackson (1:200) was used for the mAb5B12. After rinsing, wings were incubated with the Vector elite A+B complex (1:100 A, 1:100 B) for 1 hour at RT, rinsed in Tris buffer (0.2 M Tris pH 7.6) and stained in 0.8 mg/ml DAB (Sigma) in Tris buffer containing 0.015% H₂O₂. Tissues were then rinsed and mounted in glycerol. For the anti-*twist* we used an HRP goat-anti-rabbit-IgG from Jackson (1:200). After the incubation in the secondary antibody, 2 hours at RT, tissues were stained as described for the mAb5B12.

Disc culture

Wandering larvae were rinsed in water, cleaned by immersion in 70% ethanol for 3 minutes and then left to dry. Discs were dissected in *Drosophila* Ringer solution plus 3% neonatal calf serum (DR-NNCS), rinsed several times by transferring to fresh drops of DR-NNCS, and finally transferred to a sterile plastic culture dish containing fresh drops (approx. 150 μ l each) of culture medium that consisted of Shields and Sang M3 medium (Sigma) plus 8% neonatal calf serum. After several washes in culture medium, discs were transferred to a new sterile culture dish with one drop of culture medium supplemented with a cocktail of antibiotics (1.6 \times 10⁻² mg/ml gentamycin + 8 \times 10⁻³ mg/ml tetracyclin) and 0.5 μ g/ml ecdysone. Culture dishes were sealed with Parafilm and incubated at 25°C in a moist chamber to prevent evaporation. After 24 hours, discs were transferred to a new sterile dish containing a fresh drop of antibiotic and ecdysone supplemented culture medium. The transfer was repeated every 24 hours. At the end of the culture period, discs were fixed in 4% paraformaldehyde in PBS for 1 hour and then washed and incubated with antibodies as above.

RESULTS

Identification of glial cells in the wing

The *Drosophila* wing contains two types of external sensory organs: campaniform sensilla on the blade, and, on the costa and the anterior margin (or vein L1), singly or multiply innervated bristles. Eight large campaniform sensilla are located on veins L3, L1 and on the distal radius. For the sake of simplicity, we will limit our analysis to the region containing these sensilla and the anterior margin bristles. Sensory neurons send axons that form nerves L1 and L3 which merge in the proximity of the GSR neuron (Fig. 1). Wing sensory organ development has been exten-

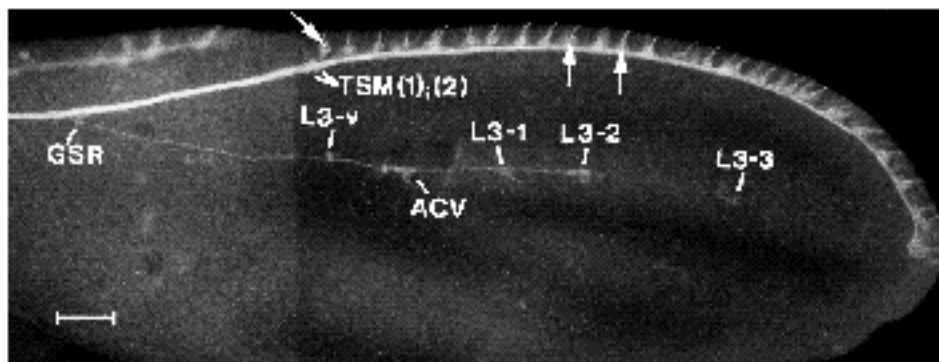


Fig. 1. PNS organization in a developing wing. Wing at 24 h AP stained with anti-HRP. All the sensory neurons have developed. In this and in all the following pictures, distal is to the right, anterior at the top. On the L1 vein (or anterior margin), neurons from the multiply innervated bristles are indicated by arrows (those from singly innervated bristles are not on the same focal plane). Neurons from the large campaniform sensilla are indicated, the giant

sensillum on the radius (GSR), the twin sensilla on the margin (TSM (1) and (2)), and those on L3 nerve: L3-v, neuron of the ventral sensillum on L3 vein; ACV, anterior cross vein neuron; L3-1, L3-2 and L3-3, neurons of the three dorsal large campaniform sensilla on L3. The L1 and L3 nerves form two bundles that meet and merge in proximity of the GSR. Bar, 50 μ m.

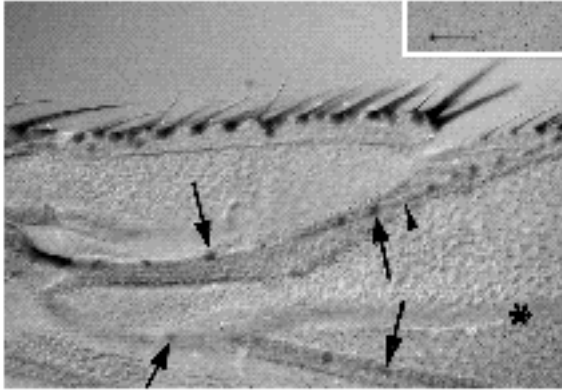


Fig. 2. Staining in an adult wing from the 2206 line. Nomarsky view of an adult wing heterozygous for 2206 stained with X-gal. The picture shows a detail of the proximal region: the L1 nerve (arrowhead) leaves the anterior margin and grows proximally to meet the L3 nerve (not in the same plane of focus). Nuclei of the glial cells that wrap the nerve bundle are visualized as black dots (arrows). No staining was associated with the L2 vein which carries no nerve (asterisk). Bar, 40 μ m.

sively investigated, from the birth of precursor cells to axonogenesis and formation of central projections (Murray et al., 1984; Hartenstein and Posakony, 1989; Huang et al., 1991; Blair et al., 1992; Whitlock and Palka, unpublished data).

The first evidence for glial cells wrapping the wing sensory nerves came from EM sections: at 30 hours after pupariation (h AP), glial processes are present around the L1 and L3 nerves (Murray et al., 1984). As markers we have used enhancer trap lines, some identified in our laboratory, some identified as glial specific in the embryo by Auld and Goodman (1992). To assess whether the staining was specific to glial nuclei, we used several criteria. First, we checked that staining was associated only with innervated veins (Figs 2, 3). Second, by staining with both anti-gal and anti-HRP, a neuronal marker, we ascertained that stained nuclei were not those of the neurons (Fig. 3). Third, we confirmed in wing sections that stained nuclei surrounded the nerve rather than being in the vein epithelium or in the lumen (data not shown). Finally, stained nuclei are flat and elongated (Fig. 3), typical of insect glial cells (see Saint Marie et al., 1982 for a review).

The thecogen is a sensory organ accessory cell that surrounds the dendrite, for this reason also called glial cell. By staining with anti-pros, an antibody specific to that cell (Doe et al., 1991; Vaessin et al., 1991) and with anti-gal, we found that our lines do not mark the thecogen cell (Fig. 4).

Origin of peripheral glial cells: migration or local differentiation?

(a) Glial cells do not use established paths to migrate into the wing blade

Glial cells could either differentiate locally or migrate into the wing blade from other tissues: in vertebrates, peripheral glial cells originate in a central structure, the neural

crest, and use nerves as substrata to migrate to the periphery (Carpenter and Hollyday, 1992; for reviews, see Le Douarin, 1982; Le Douarin et al., 1991). In the insect CNS, glial migration occurs during embryogenesis and during regeneration after wounding (KlÄmbt et al., 1991; Smith et al., 1991).

In the case of eye and legs, glial cells could migrate from the CNS along existing larval nerves and/or along motor axons (Bolwig, 1946; Zipursky et al., 1984; Jan et al., 1985; Tix et al., 1989). Within the wing blade, however, there is no motor component and no larval nerve (Jan et al., 1985; Tix et al., 1989), therefore glial cells could only enter it at pupal stages using the sensory nerves. To test whether glial cells use such a path, we used an *Abruptex* (*Ax*) allele, *P8*. In some *P8* flies, the L1 nerve forms a neuroma close to the TSM and stops growing (compare Palka et al., 1990), in this case, the nerve does not connect with more proximal fibres (Fig. 5A). The nerve truncation and the neuroma are not due to degeneration of a connected nerve: even at stages when in wild-type flies axons start reaching the base of the wing, no fibres were seen leaving the anterior margin in *P8* flies (data not shown). By crossing a glial line with *P8*, we found stained nuclei on the truncated nerve, on and distal to the neuroma (Fig. 5B), although their number is reduced compared to that found in the wild type.

Finally, glial cells could migrate into the wing by travelling along the tracheal branches that develop during pupal life. However, we have not observed trachea and tracheoles in EM L1 sections taken distal to the L1-L3 junction (data not shown).

(b) Glial precursors are already present in the discs of wandering larva

Glial cells or their precursors could migrate into the wing blade without following an established path. To assess whether glial cells differentiate within the wing, we cultured discs from wandering larvae. In culture, the developmental program is slower and wing size is one-fifth to one-seventh of that found in situ. Nonetheless, most morphogenetic events do take place: wings display the same shape changes that occur during pupal life, neurons differentiate only on the presumptive L1 and L3 veins and send axons that navigate proximally (Fig. 6A).

We cultured discs from the A289.1F1 line for either 72 or 96 hours and found that the tissue was more consistently healthy at 72 hours. For 15 discs cultured for 72 hours, the mean number of stained nuclei on L1 was 16 (Fig. 6B,C), compared to approx. 70 observed in situ (see Table 1). After 96 hours in culture, we did see as many as 30 stained nuclei on L1, but the results were extremely variable (data not shown).

Do glial cells originate from ectoderm?

In the fly embryonic CNS, longitudinal glial cells originate from ectoderm (Jacobs et al., 1989), midline glial cells from mesectoderm (KlÄmbt et al., 1991) and the sheath cell layer of the blood-brain barrier seems to originate from mesoderm (Edwards et al., 1991). While the part of the disc that gives rise to the wing proper contains only ectodermally derived cells, the more proximal part, which gives rise to

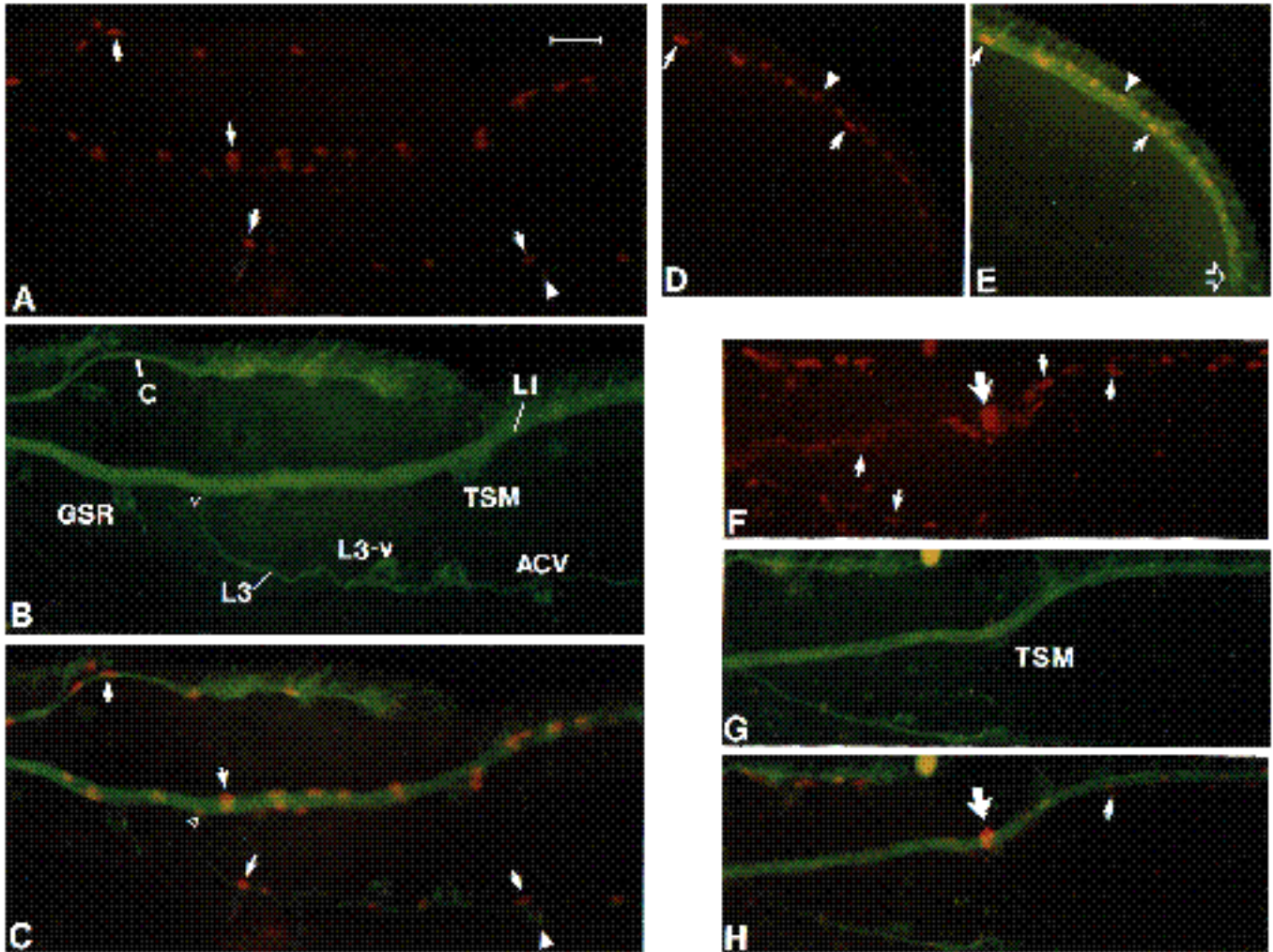


Fig. 3. Details of pupal wings (around 38 h AP) from glial-specific enhancer trap lines stained with anti-HRP and anti-gal. Bar, 25 μ m. (A-C) Proximal region of a 2206 wing stained with (A) anti-gal, (B) anti-HRP, (C) double exposure of the same wing displayed in A and B to show glial and neuronal organization simultaneously. Neurons indicated as in Fig 1. The L1 nerve (L1) merges (open arrowhead) with the L3 nerve (L3) in the proximity of the GSR and grows proximally where it collects axons from the neurons of the small campaniform sensilla in the hinge region. The costal nerve (c) collects axons from the costal neurons. Glial nuclei are located around the L1, L3 and costal nerves (arrows). Often one glial nucleus is present at the L1-L3 junction (open arrowhead). On average 10 stained nuclei are present between the TSM and the L1-L3 junction. On L3, most stained nuclei are located proximal to the ACV. Stain was also observed in neuronal nuclei, for example see filled arrowhead in A and compare with ACV anti-HRP staining in (B,C). Neuronal and glial staining can easily be distinguished by nuclear shape (neuronal nuclei are small and round, glial nuclei are large and elongated) and position (neuronal staining is at the location of neuronal somata, glial staining is adjacent to or far from the neuronal somata) and by the intensity of the staining (weaker in neurons). (D,E) Distal region of a 2206 wing stained with (D) anti-gal, (E) anti-gal and anti-HRP, double exposure of the same wing shown in D. Few nuclei are stained towards the tip of the L1 nerve. Neuronal staining (arrowhead) is distinguishable from glial staining (small arrows), which ends well before the last neurons and axons (open arrow). (F-H) Proximal region of a rA87 wing stained with (F) anti-gal, (G) anti-HRP, (H) double exposure of F and G. The three pictures show the position of the stained cluster relative to nerve and glial cells. (F) Glial staining on L1 and L3 nerves (arrows), the cluster (large arrow) being slightly out of focus, G shows the nerve. (H) The picture was taken at the focal plane of the TSM nuclei cluster. The cluster is located ventrally and/or dorsally to the nerve, but not at the same plane of focus. By the position and by double staining with anti-HRP, it is unlikely that nuclei in the cluster belong to the cells that constitute the TSM. Occasionally only one of the two TSM neurons is present in rA87 wings (G). This polymorphism has also been observed in several wild-type strains. In this line, glial staining proximally to the TSM is always weaker than in the more distal part of the wing blade.

the thorax, contains cells derived from the mesoderm (also called ad epithelial cells), and from the ectoderm (Poodry and Schneiderman, 1970).

To assess whether glial precursors differentiate from ad epithelial cells and then migrate towards the wing proper, we cultured A289.1F1 discs with the proximal part removed

(‘wing blade’ samples, Fig. 6D-G) and compared the results with those obtained with whole discs. To make sure that all ad epithelial cells were contained in the removed part, we ran a parallel experiment. We stained the two parts of operated discs with an antibody against twist, a protein expressed in embryonic mesoderm and ad epithelial cells

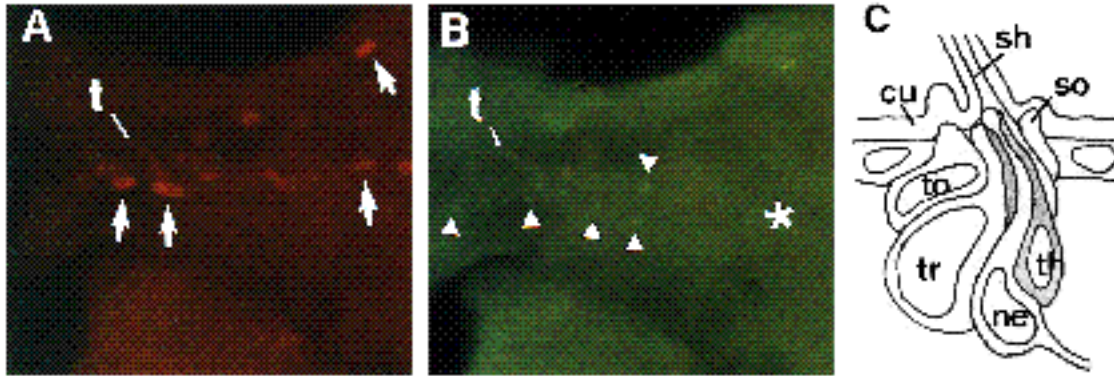


Fig. 4. 2206 wing stained with anti-*-gal* and with a thecamogen-specific antibody. (A) Proximal region of a 38 h AP wing stained with anti-*-gal*. Stained nuclei (arrows) are elongated and line the L1-L3 nerve in the hinge region. (B) Same wing as in A stained with anti-*pros*. Weakly stained small dots (arrowheads) represent the thecamogen nuclei of the small campaniform sensilla of the hinge region. The staining does not colocalize with anti-*-gal*: compare the position of the staining nuclei in A and B relative to the trachea (t). Also note that anti-*pros*, but not anti-*-gal* staining, is absent in the wing region devoid of sensory organs (asterisk). (C) Schematic drawing of a sensory organ (modified from Hartenstein and Posakony, 1989). Cuticle is indicated by cu, the four cells of the sensory organ are indicated as follows: ne, neuron; th, thecamogen; tr, trichogen; to, tormogen. The cuticular structures produced by the tormogen and by the trichogen are the socket (so) and the shaft (sh), respectively.

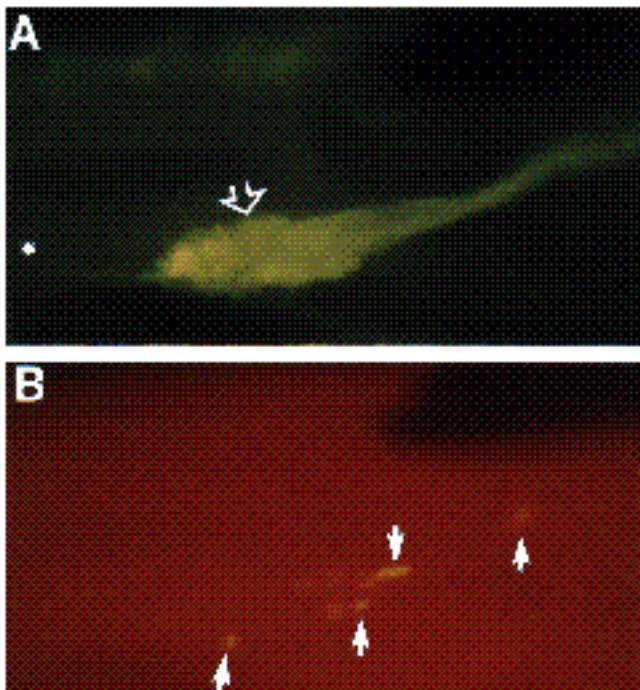


Fig. 5. Glial staining in wings containing a L1 truncated nerve. (A) *P8/X; 2206/+* wing at 36-37 h AP stained with anti-HRP, detail of the proximal region. A bundle of axons leaves the margin but, instead of growing proximally, it stops and forms a neuroma, a thick anti-HRP-positive structure (open arrow). Few fibres exit the neuroma but stop soon after (asterisk), therefore the L1 nerve does not reach the base of the wing and remains disconnected from the CNS. (B) Same wing as in A stained with anti-*-gal*. Stained nuclei are present on the L1 nerve, both on the neuroma and distal to it (arrows).

(Thisse et al., 1988; Bate et al., 1991): all the staining was contained in the removed part (see Fig. 7A,B). After culturing wing blades for 72 hours, the average number of nuclei stained with anti-*-gal* was 8 (Fig. 6E). While this

is one half the number found in intact cultured discs, we attribute some loss to the additional stress of the surgical manipulation, which may have slowed development. At 96 hours, we saw as many as 25 stained nuclei in operated discs, but again, the results were variable (data not shown).

To test further the hypothesis of the local origin of glial cells, we performed more extensive surgery on the wing disc, removing the portion that forms the anterior and proximal part of the blade (Fig. 7C). When these partial discs evert, they form only the portion of the blade distal to the L1-L3 junction ('distal fragment' samples, Fig. 6H-K). Usually the TSM portion of the L1 nerve is missing, so that both proximal sources of migration and a possible route of travel are missing. Even in these fragments, there was an average of 9 stained nuclei after 72 hours in culture, essentially the same value seen in discs with only the proximal portion removed.

Finally, we analyzed the pupal wings of a transformed line, PPS, that carries the *twist* promoter fused with the *-gal* gene (Thisse et al., 1991). Because of the stability of the *-gal* we could follow the *twist*-positive cells at developmental stages when the *twist* product had been degraded. No staining was observed in 6 and 24 h AP wing blades (data not shown).

Using enhancer trap lines to follow the development of glial cells

In the rA87 line, staining on the anterior margin was detectable at around 9 h AP (data not shown), when most neuronal precursors have not divided yet (Hartenstein and Posakony, 1989); staining was weak and often limited to the distal half of the margin. By 13 h AP (Fig. 8A-C), when some neurons have differentiated and axonogenesis has just started, staining had become stronger and had extended to the position of the TSM: around 80% of the nuclei seen at later stages were already detectable.

At 17 h AP, all neurons on the anterior margin have differentiated and axonogenesis and fasciculation are actively

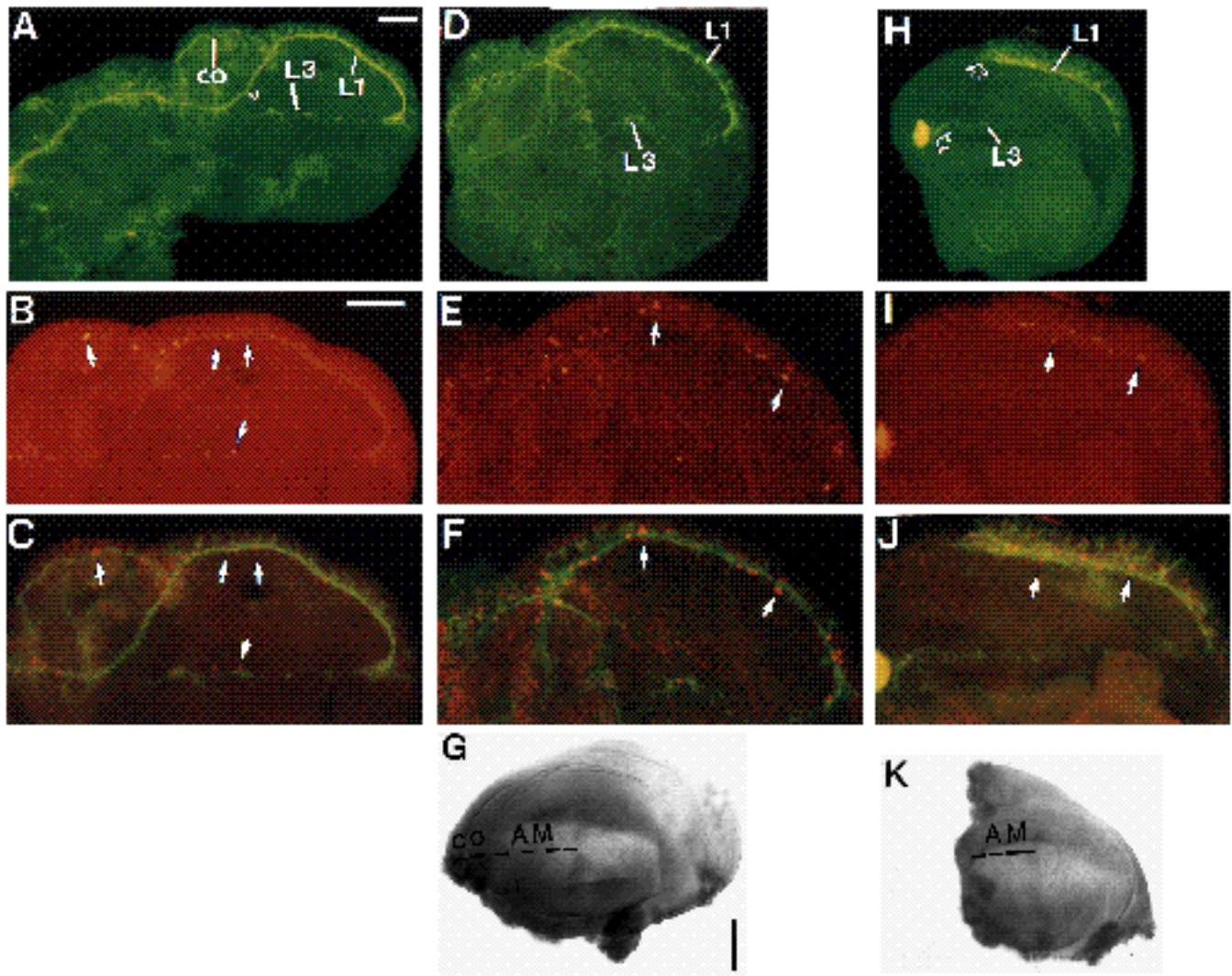


Fig. 6. Glial staining after culturing A289.1F1 wing discs for 72 hours. Bars, 50 μ m. (A-C) In vitro developed whole disc stained with (A) anti-HRP, (B) anti-gal, (C) double exposure of A and B. The L1, L3 and costal nerve are indicated by L1, L3 and co, respectively. Stained nuclei (arrows) are present on all three nerves. The L1-L3 junction is indicated by an open arrowhead. (D-F) In vitro cultures of 'wing blades' stained with (D) anti-HRP, (E) anti-gal, (F) double exposure of D and E. Symbols as in A-C. (G) The portion of the disc put in culture after surgery in the 'wing blade' set of experiments. AM and co indicate the position of the presumptive anterior margin and costa, respectively. (H-J) In vitro cultures of 'distal fragments' stained with (H) anti-HRP, (I) anti-gal, (J) double exposure of H and I. Symbols as in A-C. L1 and L3 nerves stop growing at the point where the disc was cut (open arrows). (K) The portion of the disc put in culture in the 'distal fragment' set of experiments. Symbols as in G.

occurring (Fig. 8D-F). A thick nerve bundle could clearly be distinguished along the margin but most axons stopped in the proximity of the TSM. We did not find staining proximal to the TSM, however, since, as at 13 h AP, wings were often damaged proximally because of the difficulty of dissection, we cannot exclude the existence of a few stained nuclei in that region. At this stage, a stained nucleus was always found on the growing axon from the distal-most campaniform sensillum on the L3 vein (Fig. 8G).

The 5B12 antibody recognizes embryonic CNS and PNS glial cells at stages when the sensory and motor nerves establish their paths, and continues to mark glial cells at later embryonic stages (Fredieu and Mahowald, 1989). We could not detect any staining in 25 h AP wings incubated

with 5B12, even though by this stage L1 and L3 axons have formed thick nerves (data not shown).

Enhancer trap lines mark subsets of the glial cell population

By counting the stained nuclei in the different lines we found that most, if not all, of them only mark subsets of the whole glial cell population (Table 1). The highest numbers of stained nuclei were, on average, 74 for the L1 nerve and 12 for the L3 nerve (Table 1). By crossing animals from the AE2 and 2206 lines and counting the stained nuclei in their progeny, we found that the number is higher in 2206/+; AE2/+ than in the individual lines (Fig. 9 and Table 1). This suggests that the 2206 and AE2 markers are

Table 1. Average number of stained nuclei in flies from different glial-specific enhancer trap lines

Line		L1	TSM	L3
2206/+	(#4)	29-34 (32)	8-13 (10)	3-8 (6)
AE2/+	(#3)	40-46 (43)	6-10 (8)	6-8 (7)
AE2/AE2	(#12)	44-59 (49)	6-12 (10)	4-12 (6)
AE2/+; 2206/+	(#4)	59-65 (62)	14-17 (15)	10-13 (12)
A289.1F1/+	(#31)	56-84 (67)	13-24 (16)	NA
A289.1F1/A289.1F1	(#9)	64-86 (74)	13-20 (17)	NA
rA87/+	(#7)	52-60 (55)	9-12 (11)	NA
rA87/rA87	(#9)	62-73 (70)	8-18 (14)	6-15 (12)

indicates the number of wings analyzed. Numbers indicate the maximum and minimum values, average values are shown in parentheses. 'L1', 'TSM', 'L3' indicate the regions where nuclei were scored: in all cases, the most proximal point was the L1-L3 junction. In the case of 'L1' and 'L3', the distal point was the distal tip of the nerve; in the case of 'TSM', the position of the twin sensillum on the margin. NA means not assessed. Wings were scored at 36-40 h AP depending on the line. Since in the A289.1F1 line, other cells (trachea, sensory organ) were stained in addition to glia, we limited our analysis to L1 nerve, where glia could be unambiguously identified by their position and morphology. In one line, rA87, the number of stained nuclei increased in homozygous conditions. In the other cases, although we did not attempt to do a statistical analysis, the value range (minimum and maximum values) did not seem to change significantly when one or two doses of -gal were expressed.

not identical, although we cannot say whether they are recognizing distinct or overlapping groups of glial nuclei.

An interesting feature of the rA87 line was the presence, in addition to the nuclei on L1 and L3, of 1-4 clusters of 2-5 stained nuclei at the position of the TSM (Fig. 3F-H). These nuclei were not elongated but round and more intensely stained than the other nuclei; in the adult wing they were the only stained nuclei (not shown). If they are indeed glial nuclei, they could represent a different class of glial cells. It is notable that they are located at the point where the anterior margin axons make a deviation of 45° and start following the path pioneered by the early TSM axon.

Although we have not undertaken a detailed analysis outside the wing, we detected staining in other discs and in embryos at positions where the PNS develops and in the adult and embryonic CNS, with all the lines we have studied (data not shown; Auld and Goodman, 1992).

The number and position of glial nuclei are not fixed

We noted that the number and position of stained nuclei varied among wings of the same line. This did not depend on differences in sex and stage since we still found variability in left and right wings from the same animal (Table 2 and data not shown). If a glial-specific gene were only transiently expressed in a given cell, the consequence of slight differences in the developmental program between the left and right side would be an asymmetrical distribution of the mRNA and product of that gene. However, as it is known that the -gal is rather stable and remains detectable for several hours after induction (Bellen et al., 1989), our assay detects all the cells in which the reporter has been activated. Finally, staining variability is common to all the markers that we have so far analyzed: five independent lines plus two lines carrying an insert on a CyO chromosome (data not shown).

DISCUSSION

Although the presence of glial cells in the insect PNS was detected several decades ago (Wigglesworth, 1959), most studies have concentrated on the development of CNS glial cells. In the present study we have used the enhancer trap detector system to start analyzing the development and the organization of the wing glial cells.

Origin of peripheral glial cells

We have asked whether *Drosophila* glial cells differentiate at the periphery or outside the disc. We have shown that glia can develop at their normal sites in the absence of a nerve connected with the CNS. Results from the disc cultures indicate that glial precursors are already present within wing discs of wandering larvae: either they were born in the disc or they had migrated there before discs were dissected. Since in culture the wing itself is reduced in length, the number of neurons on the anterior margin is reduced to between one half and one third (Milner, 1977; Murray, unpublished observations) and the rate of cell division is lower (Bullmore, 1977), it is likely that the reduction of

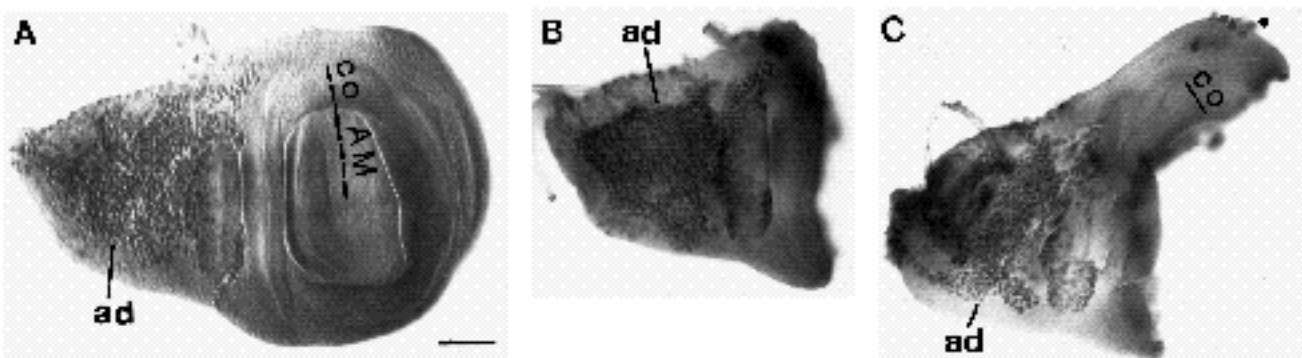
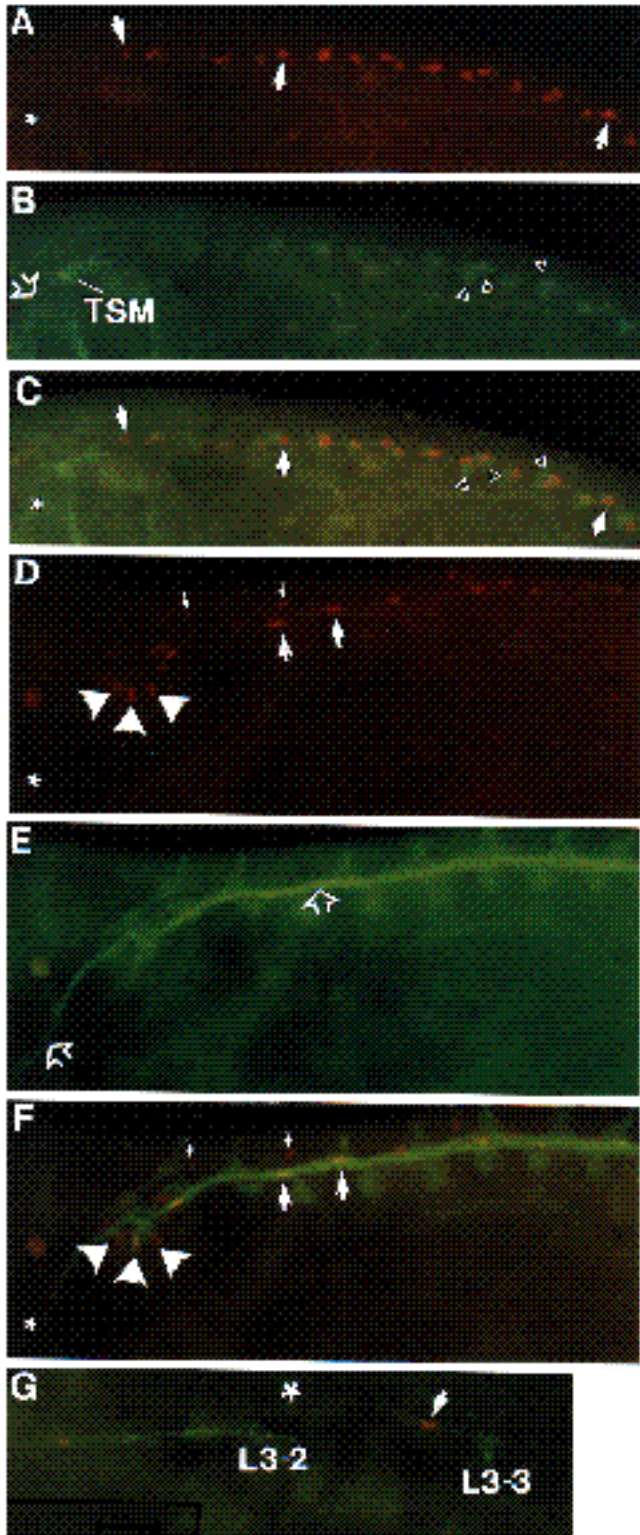


Fig. 7. Anti-twist staining in third instar wing discs. Bar, 50 μ m. (A) Wing disc at wandering larva stage. Symbols as in Fig. 6; ad indicates the adepithelial cells. (B,C) The portions of the disc that were cut off in the 'wing blade' and in the 'distal fragment' experiments, respectively. All the anti-twist staining is contained in these portions. Few unidentified cells were occasionally found associated with the disc. These cells were not on the same focal plane of the disc epithelium and their position was not constant. It is possible that cells not intrinsic to the disc got stuck to the epithelium during the fixation and dissection of the larval tissues.



stained glial nuclei is due to the culture system. By culturing discs we eliminated the possibility that glial cells differentiate from migrating blood cells, by culturing operated discs we removed ad epithelial cells. The fact that glial precursors can differentiate from a wing disc portion devoid of *twist*-expressing cells supports the hypothesis that peripheral glial cells originate from ectoderm.

Fig. 8. Staining in rA87 wings at early pupal stages. Bar, 25 μ m. (A-C) Proximal part of the wing at around 13 h AP stained with (A) anti-gal, (B) anti-HRP, (C) double exposure of A and B. Axonogenesis has started but there is no continuous nerve bundle yet. Anti-gal-positive nuclei were mainly observed adjacent to the anti-HRP-positive cells. Glial nuclei (arrows) and neurons (open arrowheads) are present along the anterior margin, from the tip to the TSM region. No glial staining is observed proximal to the TSM (asterisk), although a pioneer fibre containing the TSM(1) has grown proximally (open arrow) and has already established connection with the L3 nerve (not shown). In a few preparations, one stained nucleus has been detected along the pioneer fibres, close to the TSM. (D-F) Proximal part of the wing at around 17 h AP stained with (D) anti-gal, (E) anti-HRP, (F) double exposure of the wing shown in D and E. Axons on the anterior margin have formed a thick bundle distal to the region of the TSM, but only a few fibres are seen proximal it (open arrows). The stained nuclei on the anterior margin are of two types: elongated nuclei lining the nerve (large arrows) and periodically spaced nuclei laying toward the anterior margin (small arrows). As at 13 h AP, no staining has been found proximal to the TSM (asterisk). At the position of the TSM, a rosette of round nuclei surrounds the network of axons (large arrowheads). These nuclei could correspond to the clusters detected at later stages. (G) Same wing as in (D-F), detail of the L3 nerve, double exposure. The axon of the distal-most sensillum, L3-3, is growing proximally but has not yet reached (asterisk) the L3-2 neuron soma. A stained elongated nucleus (arrow) is already lining the axon.

Taken together, the results from the disc cultures, from the glial staining pattern in *Ax* and from the staining profile in the *twist*-gal line, strongly suggest that glial cells differentiate from ectodermally derived cells within the disc, in the region that will give rise to the adult wing.

Development of peripheral glial cells

We have found that glial staining is detectable when most or all neurons have not yet differentiated. This suggests that at least the first steps of differentiation may not require the presence of neurons. Because the number of stained nuclei is lower than at later stages it is likely that glial precursors are still dividing. By using these lines as glial markers, it will be possible to birthdate the glial cells in bromodeoxyuridine (BrdU) incorporation experiments. The comparison of sensory organ and glial precursor birthdates will also shed some light about the relationship between sensory organ lineage and glial development.

The observation that staining is detectable when neurogenesis is still taking place opens the possibility that peripheral glial cells play a role in axonal guidance and fasciculation. Laser ablation experiments in the grasshopper embryo have shown the importance of the segment boundary cell, a primitive CNS glial cell, in the establishment of the intersegmental nerve (Bastiani and Goodman, 1986). In *Drosophila*, Klämbt et al. (1991) have shown that some CNS glial cells are necessary for the formation and the maintenance of the commissures.

Organization of peripheral glial cells

Glial-specific enhancer trap lines showed variability in number and position of stained nuclei. We cannot formally rule out the possibility that the observed variability is due to a problem of threshold expression of -gal. If that were

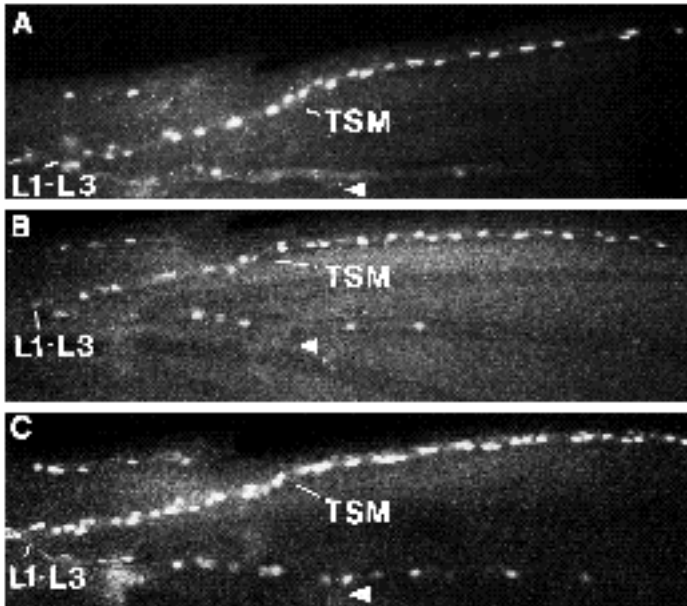


Fig. 9. Subsets of the glial population recognized with two enhancer trap lines. 36-38 h AP wings stained with anti- β -gal: (A) 2206/+; (B) AE2/AE2 or AE2/+, genotype not assessed; (C) AE2/+; 2206/+. Compare nuclear staining along L1 and L3 in the three genotypes. See for example staining on L1 nerve between the TSM region and L1-L3 junction or staining on L3 in the region distal to the anterior cross vein (arrowhead).

Table 2. Variations in the number of stained nuclei in left and right AE2 wings from the same animal

Animal	L1		TSM		L3	
	L	R	L	R	L	R
1	48	53	11	10	8	4
2	44	43	6	11	8	5
3	54	59	12	11	6	6
4	50	49	8	9	8	6
5	48	46	11	9	12	10
6	50	46	9	7	8	11

Six AE2/AE2 animals were dissected at 38 h AP. Each animal was processed separately to compare staining between left (L) and right (R) wings. Each row indicates the results obtained for a single animal. L1, TSM, L3 indicate the regions where nuclei were scored.

the case, glial cells organization would be constant, but some glial cells would express levels of β -gal too low to be detected, which might lead to a variable staining pattern. Although it is possible that, in a given line, β -gal expression is submitted to position effects on the P element insert, variability within a line is not a frequent feature of enhancer trap lines, see, for example in the PNS, the stereotyped pattern observed in sensory organ-specific lines such as A37 and A101 (Ghysen and O'Kane, 1989; Huang et al., 1991; Blair et al., 1992). Since all the lines (at least five independent insertions) displayed the same behaviour, we think that staining pattern variability reflects a real feature of glial organization, although it is possible that only sub-populations of peripheral glial cells are variable.

Different classes of glial and sheath cells have been described in the insect CNS. Although most, if not all, of

the analyzed lines mark subsets of the wing glial cells, more detailed analyses will be required to assess whether the staining patterns reflect the existence of distinct types of glial cells in the PNS.

Neurogenesis and gliogenesis

The availability of the glial markers make it now possible to determine which genes are required for glial differentiation. An obvious question is: do genes required for the early steps of neuronal development also affect gliogenesis? Proneural genes like those of the *achaete-scute complex* (Garcia-Bellido and Santamaria, 1978) and neurogenic genes like *Notch* (*N*) (Lehmann et al., 1983) are the first candidates for such an analysis (for reviews see Ghysen and Dambly-Chaudière, 1989; Giangrande and Palka, 1990; Campuzano and Modolell, 1992; Simpson et al., 1992).

Ax mutations are alterations of the *N* protein that induce lack of some sensory organs in the wing and thorax (Palka et al., 1990; De Celis et al., 1991; Heitzler and Simpson, 1993). The fact that the *P8* mutation, which is an *Ax* allele, induces loss of some neurons (Schubiger and Giangrande, unpublished results) and reduction of glial staining in the wing, indicates that its effects on glial cells parallel those observed on neurons. This also correlates well with the finding that *N* embryos, where there is over-production of CNS neurons, also display over-production of longitudinal glial cells (Jacobs et al., 1989). Preliminary data obtained with other mutations of the two classes suggest the existence of a genetic pathway common to neurogenesis and gliogenesis (Giangrande, unpublished data).

The mAb5B12 and the polyclonal antibodies anti-twist and anti-prospero were a gift of S. Benzer, F. Perrin-Schmitt and H. Vaessin, respectively. The *P8* stock and the *PPS twist*- β -gal line were kindly provided by W. Pak and F. Perrin-Schmitt, respectively. We thank V. Auld for communicating unpublished results and for the AE2 and the rA87 lines. We are grateful to M. Schubiger, B. Taylor and people in the laboratory for helpful suggestions and to G. Richards, M. Schubiger and P. Simpson for comments on the manuscript. We thank K. Wilson for technical assistance and B. Boulay, S. Metz and C. Werlé for help with the figures. This work was supported by an NSF grant to J. P. and by CNRS and INSERM.

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