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Lineage, Migration, and Morphogenesis of Longitudinal Glia in the Drosophila CNS as Revealed by a Molecular Lineage Marker

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Summary

Previous studies described three different classes of glial cells in the developing CNS of the early Drosophila embryo that prefigure and ensheath the major CNS axon tracts. Among these are 6 longitudinal glial cells on each side of each segment that overlie the longitudinal axon tracts. Here we use transformant lines carrying a P element containing a 130 bp sequence from the *fushi tarazu* gene in front of the *lacZ* reporter gene to direct β-galactosidase expression in the longitudinal glia. Using this molecular lineage marker, we show that 1 of the "neuroblasts" in each hemisegment is actually a glioblast, which divides once symmetrically, in contrast to the typical asymmetric neuroblast divisions, producing 2 glial cells, which migrate medially and divide to generate the 6 longitudinal glial cells. As with neuroblasts, mutations in *Notch* and other neurogenic genes lead to supernumerary glioblasts. The results indicate that the glioblast is similar to other neuroblasts; however, the positionally specified fate of this blast cell is to generate a specific lineage of glia rather than a specific family of neurons.

Introduction

Glia have been shown to play important roles during the development of the nervous system, particularly as permissive, and sometimes instructive, substrates for migrating neurons and extending growth cones (e.g., Rakic, 1971; Silver et al., 1982; Bastiani and Goodman, 1986). Previous studies on the Drosophila embryo (Jacobs and Goodman, 1989) revealed three different classes of early glial cells in the developing CNS that appear to play important roles in the formation of the major CNS axon tracts: 6 longitudinal glial cells on each side prefigure the longitudinal tracts, 6 midline glial cells help establish the anterior and posterior commissures, and the segment boundary cell establishes the location of the intersegmental nerve root. As these tracts develop, the glia differentiate to ensheath and support the axon bundles (Jacobs and Goodman, 1989).

Although we are beginning to understand something about the structure and function of these glia, we know little about where they come from and how they are specified. For example, to what extent do these identified glial cells arise from the same lineages that generate neurons? And, if some or all of them are independently generated, at what stage are the fates of glial versus neuronal precursor cells differentially specified? For one of the classes of glia, the midline glia, these questions are beginning to be answered. The 3 pairs of midline glia in each segment arise from the midline of the developing CNS, a strip of mesectoderm that is developmentally quite distinct from the lateral neuroepithelium that generates the bilateral plates of neuroblasts (NBs); the mesectoderm also gives rise to several neuronal precursor cells, including the median NB and several midline precursors (Thomas et al., 1988; Crews et al., 1988; Rothberg et al., 1988; Jacobs and Goodman, 1989).

However, the origin of the longitudinal glia has been the source of speculation for some years. Bastiani and Goodman (1986; and personal communication) discovered these glia in the grasshopper embryo and observed their migration and division; they speculated that these glia arise from a transient, lateral NB-like cell. Doe and Goodman (1985) observed a transient NB that appeared in the lateral part of the neuroepithelium in the grasshopper embryo and speculated that this cell might be Bastiani and Goodman's putative glioblast (GB). More recently, Doe et al. (1988) examined the expression of the *fushi tarazu* (*ftz*) gene during Drosophila neurogenesis. Using a P element containing the *ftz* promoter in front of the *lacZ* reporter gene, they followed the expression of β-galactosidase as a marker for *ftz* expression in a subset of neuronal precursor cells and progeny. Under these conditions, β-galactosidase is also expressed in a lateral, NB-like cell that divides once to generate 2 cells, which migrate medially and appear to be glial cells. In these transformant embryos, the β-galactosidase disappeared before the ultimate fate of this pair of cells could be determined; expression of the *ftz* protein was even weaker and more transient in these cells, appearing at a very low level in the putative glial precursor and being immunologically undetectable in its 2 progeny.

To resolve this issue of the origin and lineage of the longitudinal glia, we have used a P element construct containing a 130 bp sequence from the *ftz* gene in front of the *lacZ* reporter gene. Some transformants carrying this construct direct the expression of the *lacZ* gene specifically in the longitudinal glia, and the β-galactosidase is present from precursor cell to differentiated progeny. This expression is much stronger, lasts longer, and is more specific for the glial lineage than for previous constructs; thus it has allowed us to unambiguously observe the lineage, migration, and morphogenesis of the longitudinal glia. Using this molecular lineage marker, we show that 1 of the "neuroblasts" in each hemisegment is in fact a GB. In contrast to NBs, which typically divide asymmetrically to produce chains of ganglion mother cells, this GB divides once symmetrically to produce 2 glial cells, which leave the NB layer and migrate medially; they then further divide to generate the 6 longitudinal glial cells.

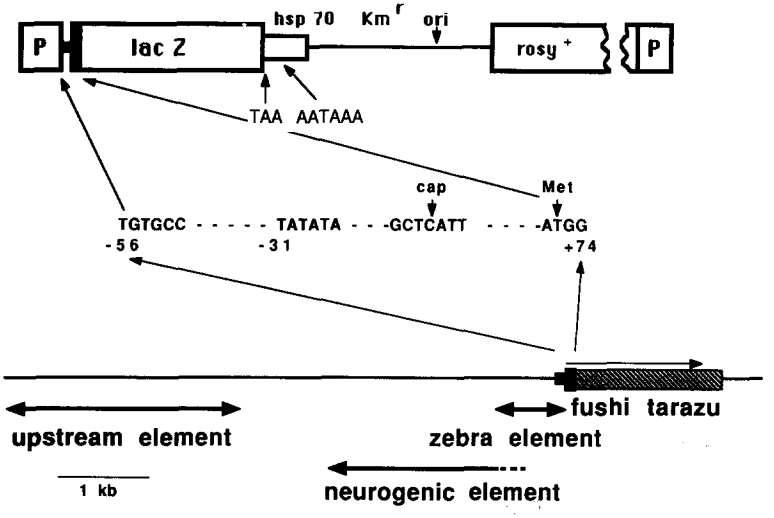

Figure 1. Schematic Diagram of P Element Construct Used as a Lineage Marker for the Longitudinal Glia

(Top) The P element construct contains a truncated *ftz* promoter fused to the *lacZ* gene after its ATG codon. The stop codon and the polyadenylation signal is provided from the *hsp70* gene. The *ftz* sequence used in this construct includes 56 bp upstream of its transcriptional initiation site, 70 bp of 5′ untranslated leader, and 4 bp from the protein coding sequence (including the ATG). In addition to the *ftz* 5′ sequences, the *lacZ* coding region, and the *hsp70* 3′ sequences, the vector also contains a kanamycin resistance gene (Km^r), an origin of replication (ori), and the rosy gene ($rosy^+$) to mark successful P element insertion. A summary of the overall organization of the 5′ flanking sequences responsible for normal *ftz* expression is shown at the bottom of the figure (see Hiromi et al., 1985).

Results and Discussion

P Element Construct and Recurrent Glial Expression Motif

The *ftz* gene has two different patterns of expression during embryonic development. It is initially expressed as a pair-rule gene during segmentation. After its initial transient expression in seven stripes at the cellular blastoderm stage, *ftz* is transiently expressed in a segmentally repeated subset of cells during neurogenesis (Carroll and Scott, 1985; Hiromi et al., 1985), including identified neuronal precursor cells, neuronal progeny, and a putative glial lineage (Doe et al., 1988). Previous experiments identified the upstream sequences required for appropriate *ftz* expression during segmentation and neurogenesis (Hiromi et al., 1985). The functional unit of the *ftz* gene lies within an approximately 10 kb sequence. The *ftz/lacZ* fusion genes (in which the 5′ flanking sequence of the *ftz* gene is fused to the reporter *lacZ* gene) have previously been used to identify three *cis*-acting control elements in the 5′ flanking region: the zebra element, which confers the striped blastoderm pattern; the neurogenic element, which is involved in the CNS expression; and the upstream element, which has an enhancer-like effect on the expression of the striped pattern (Hiromi et al., 1985).

For separate experiments, one of us (Y. H.) constructed a series of P elements containing short fragments from the promoters of three different genes (the *P* promoter, the *ftz* promoter, and the *hsp70* promoter). These act as relatively "neutral" promoters in front of the *lacZ* coding region for an "enhancer trap" mutagenesis to detect developmentally interesting nervous system transcriptional enhancers (Hiromi and Goodman, unpublished data; the enhancer trap method has been described by O'Kane and Gehring [1987]). One of these P elements, the FZ construct, contains a 130 bp sequence from the 5′ end of the *ftz* gene that includes 56 bp of flanking sequences with the TATAA box and 74 bp of leader sequence (Figure 1). This 130 bp sequence does not appear to be completely neutral. Rather, in a small percentage of transformant lines, as described below, it directs the cell-specific expression of this reporter gene in the longitudinal glia from precursor cell to differentiated progeny.

Using the construction shown in Figure 1, we generated 284 independent transformant lines. Of these lines, about 20% demonstrated β-galactosidase expression within the CNS (Hiromi, unpublished data). We were intrigued to find 14 transformant lines (about 5% of the total lines) that showed a common pattern of β-galactosidase expression in the laterally located GB. Many of these lines also had various levels of β-galactosidase expression in other patterns, which varied from line to line. For the reasons outlined below, we consider the expression in the GB to be conferred by the *ftz* promoter sequence utilized in this construct, rather than by chromosomal position effects. First, we determined the cytological location of the P element insertion for six third chromosomal insertion lines and found them all to

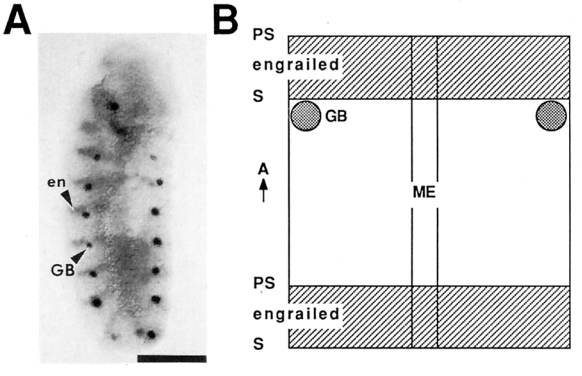

Figure 2. Location of the Glioblast within the Neuroblast Plate

In the 6 hr embryo (stage 11) in (A), the segmentally repeated GB is located immediately posterior to the stripe of cells expressing *engrailed* (en), whose posterior borders mark the segment boundary. Anterior is at top, ventral at left. The GB location is shown schematically in (B) from a ventral view. The mesectodermal cells (ME) mark the ventral midline. Segmental (S) and parasegmental (PS) boundaries are indicated by the *engrailed* stripe. Bar, 100 μm.

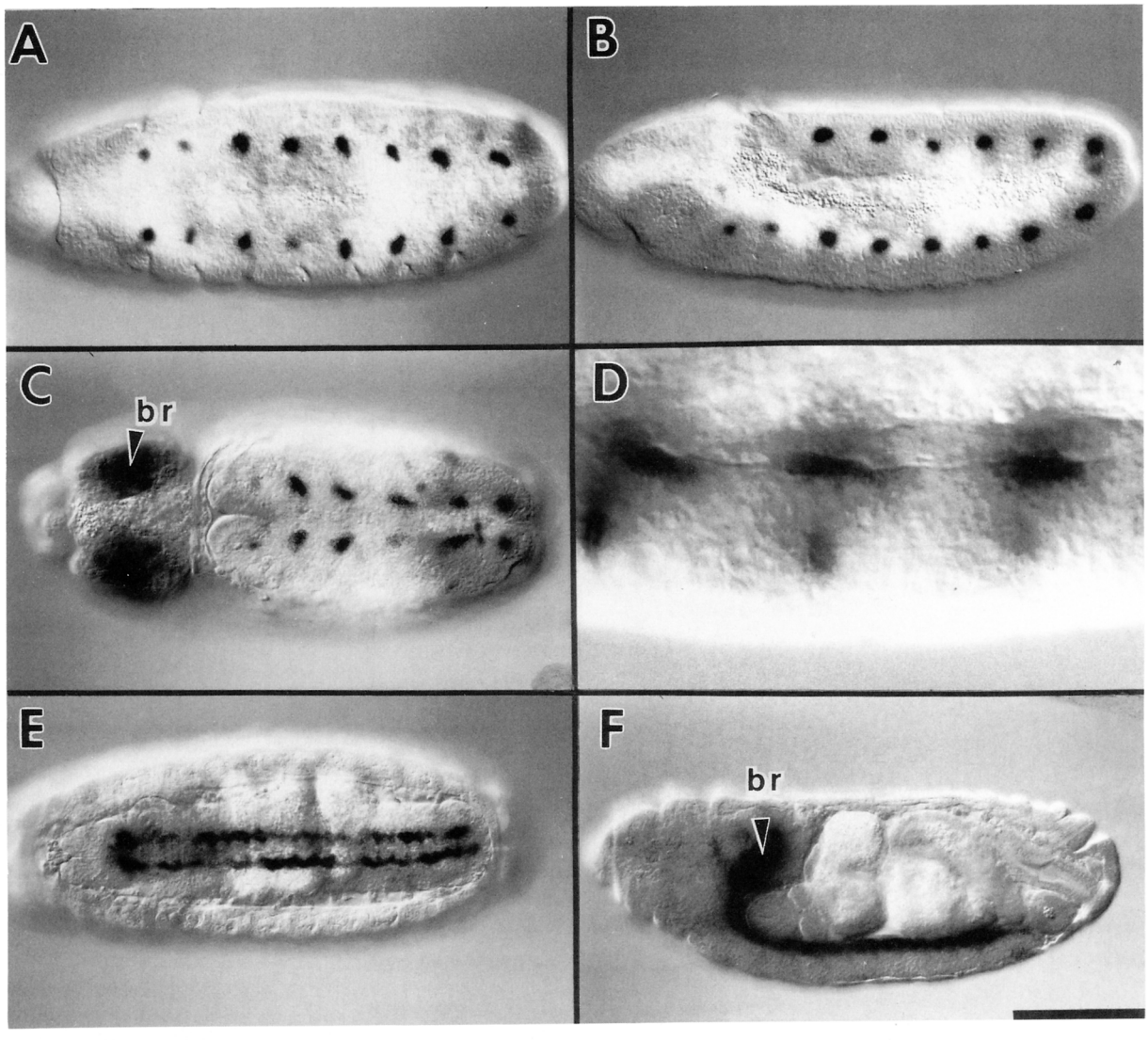

Figure 3. Migration and Differentiation of the Glioblast Progeny

The GB lineage in embryos at 6 (A and B), 7.5 (C and D), and 12 (E and F) hr of development (stages 11, 12, and 15, respectively) stained for β-galactosidase activity is shown; ventral views are on the left, and saggital views are on the right. At 6 hr, the GB has just divided symmetrically. During germ band retraction (C and D), the GB progeny migrate anteriorly into the next anterior segment (across the posterior boundary of the *engrailed* stripe marking the segment border) and medially to the location of the presumptive longitudinal tract. At their medial location, the glia divide and elongate before axonogenesis to form a bilaterally represented longitudinal column of cells. Developing brain glia (br) are indicated. Bar, (A–C, E, and F) 100 μm; (D) 20 μm.

be different. Thus, the expression is not due to insertion into a small number of hot spots that can induce this pattern. Second, a high frequency of transformants exhibiting this expression pattern was observed only with the FZ construct and not with other enhancer trap constructs (Hiromi, unpublished data). Third, this pattern is a part of the normal expression pattern of the *ftz* gene (Doe et al., 1988). Thus, it appears that the 128 bp sequence of the *ftz* gene contains most, if not all, of the information required for cell-specific expression in the GB, which can be visualized by insertion into various genomic positions that allow high levels of expression. We have used these lines to examine the lineage, migration, and morphogenesis of one class of nonneuronal support cells: the longitudinal glia.

The transformant line used in this study is called F263, and its insert is located on the third chromosome.

Lineage, Migration, and Morphogenesis of Glioblast Progeny

Expression of β-galactosidase in the GB of transformant Drosophila is first detectable at 6 hr of development (Figure 2A; Figures 3A and 3B; Figure 4A), coincident with the onset of detectable *ftz* expression in the GBs in wild-type Drosophila. The GB is a large, NB-like cell that enlarges in the same layer as, and adjacent to, the other NBs. The GB is located in the lateral-most row of NBs and at the anterior margin of the segment, just behind the segmental border. This location is immediately adjacent to the posterior boundary of the epidermal stripe

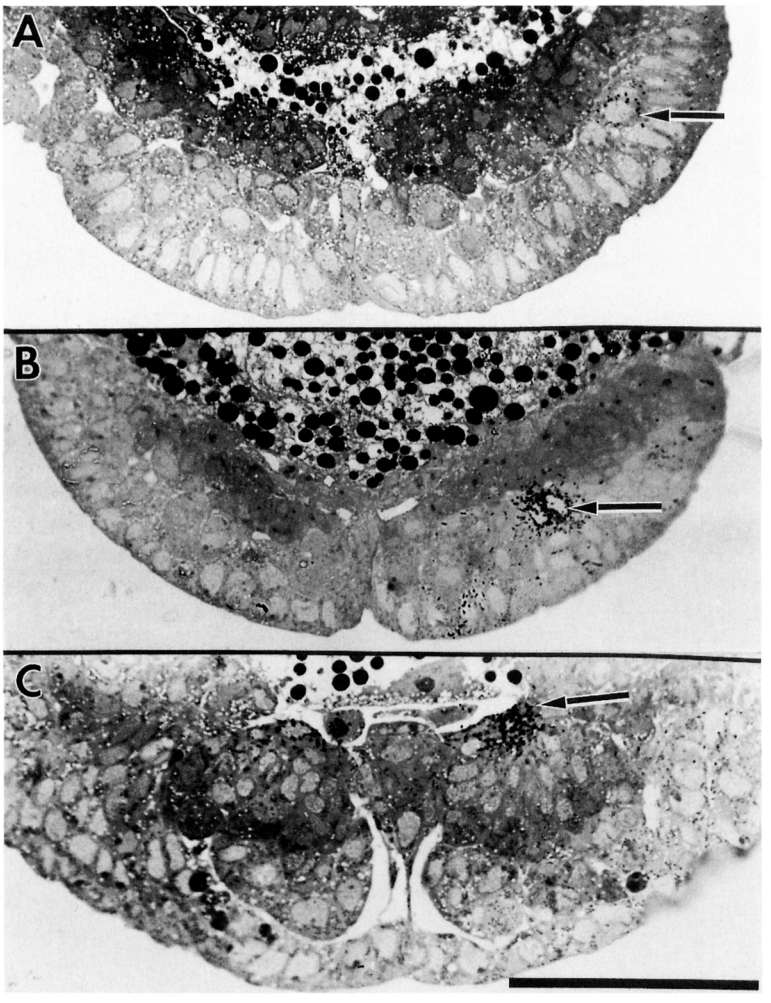

Figure 4. The Glioblast, Its Progeny, and Their Migration in Cross-Section

β-Galactosidase activity can be visualized as a perinuclear deposition of needle-shaped crystals 5–15 μm long (arrows). At 6 hr of development, the GB is located at the lateral margin of the ventral NB plate (A). During germ band retraction, the 2 GB progeny migrate medially over the dorsal surface of the NB plate (B). Progeny of these 2 cells form the longitudinal glia located over the lateral neuropil and longitudinal tract. (C) NB and GB cells are counterstained lightly relative to differentiating neurons and mesoderm. Bar, (A and B) 50 μm; (C) 40 μm.

of cells expressing *engrailed*; this border is a marker of the segment boundary (Figure 2). In total, there are 28 GBs in the ventral nerve cord, 2 (1 on each side) in each of the 14 body segments. There may also be GBs in the brain, but the pattern of β-galactosidase expression in the brain is more complex and has not yet been analyzed at the single cell level.

Very soon after β-galactosidase expression is detectable, the GB divides once symmetrically (Figure 4B). This is in marked contrast to the neighboring and morphologically indistinguishable NBs in the same layer, which divide repeatedly in an asymmetric fashion to generate a chain of smaller ganglion mother cells. The 2 GB progeny then leave the NB layer as they begin to migrate just under the inner basement membrane separating the outer neuroepithelium from the inner mesoderm. The 2 GB progeny migrate predominantly in a medial direction, although also in part anteriorly across the segment border (crossing the posterior boundary of the *engrailed* stripe that marks the segment border), from 6.5 to 8.5 hr of development (Figures 3C and 3D; Figure 4B). Germ band shortening begins during this migration period and is accompanied by major changes in the cellular organization of the CNS.

As the GB progeny migrate medially, they remain at the inner surface of the neuroepithelium as it continues to thicken from cell proliferation (Figure 4B). As the GB progeny cross the segment boundary, they enter the next anterior segment and finish their migration at the medial location where the longitudinal axon tracts will form (12 μm from the midline), posterior to the future position of the anterior commissure. During migration, the 2 GB progeny produce a cytoplasmic "leading process" at their forward edge, which indicates the path of movement (see Figure 3C). In a saggital view at this stage, the GB progeny have elongated over less than half the length of the segment (Figure 3D).

Upon completion of their migration, the GB progeny divide to produce a total of 6 longitudinal glia per hemisegment. We do not yet know the precise pattern of divisions by which these 2 postmigratory precursor cells generate 6 glial progeny. The 6 glial cells elongate as 3 pairs of interdigitated cigar-shaped cells, which traverse the length of each segment, from immediately be-

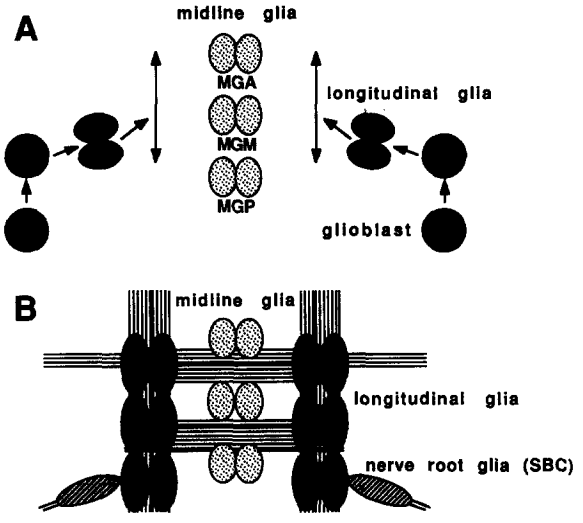

Figure 5. Schematic Summary of the Longitudinal Glial Lineage and the Organization of Glia in Early Embryonic CNS Development

A laterally placed GB in (A) divides symmetrically, and the 2 progeny migrate medially and anteriorly to a position lateral to the midline glia. Later in development (B), the commissural axon tracts are positioned between the pairs of midline glia and the longitudinal axon tracts underlie the longitudinal glia. See text for further discussion. (B) was adapted from Jacobs and Goodman (1989).

hind the anterior commissure to immediately anterior to the anterior commissure of the next posterior segment, leaving a small gap at the level of the anterior commissure (Jacobs and Goodman, 1989). The glia have reached this position by 9.5 hr of development, before the formation of any longitudinal axon tracts.

During neuronal axonogenesis and pathway formation, the longitudinal glia retain their position over the dorsal surface of the CNS, forming a roof over the lateral neuropil and longitudinal tracts of the ventral nerve cord and the brain (Figures 3E and 3F; Figure 4C). The overall development of the longitudinal glia in relationship to the other two classes of glia is summarized in Figure 5. Morphological differentiation of the longitudinal glia is discussed in greater detail in Jacobs and Goodman (1989).

Mutations in *Notch* and Other Neurogenic Genes Lead to Supernumerary Glioblasts

We speculated that the local decision to become a glioblast versus epidermal cell is controlled by the same "neurogenic" genes (Artavanis-Tsakonas, 1988; Campos-Ortega, 1988) as the similar decision of whether to become a neuroblast or an epidermal cell. To test this hypothesis, we examined the production of GBs in embryos mutant for three different neurogenic genes: *Notch*, *mastermind*, and *big brain*. In wild-type Drosophila embryos, the *Notch* gene and other neurogenic genes are involved in the local cell–cell interaction that inhibits cells adjacent to developing NBs from themselves becomes NBs, and as a consequence, many of these cells become epidermal; this is part of a cell–cell

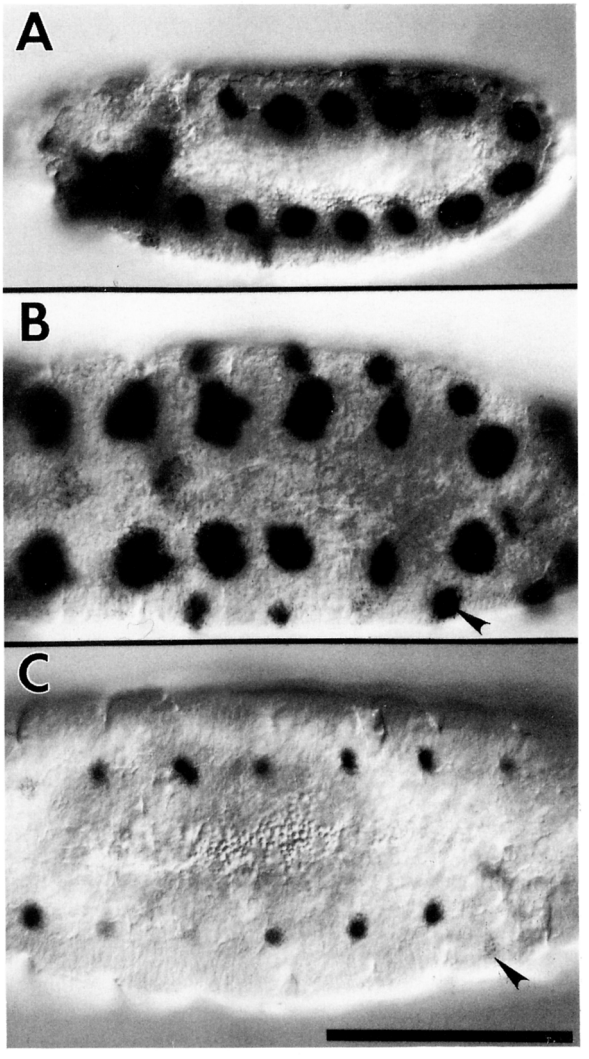

Figure 6. The Neurogenic Mutation *Notch* Causes Overproduction of Glioblasts

In saggital (A) and ventral (B) views at 6 hr of development, a greater than normal number of GBs are seen compared with wild-type (C) embryos. The arrowheads in (B) and (C) point to β-galactosidase expression in a cell outside the developing CNS; this is an artifact often seen in all three enhancer trap constructs (see Results) and is not specific for the 128 bp *ftz* sequence, which specifically labels the GB lineage. Bar, (A) 160 μm; (B and C) 100 μm.

interaction and positional mechanism that helps establish the normal density and pattern of NBs. In *Notch* and other neurogenic mutants, there is a failure of this local cell–cell interaction, leading to an overproduction of NBs at the expense of the epidermis. If the neurogenic genes control the GB just as they do the NBs, then we might expect there to be a concomitant overproduction of GBs in *Notch* and other neurogenic mutants. This is in fact exactly what we observe (Figure 6; Figure 7). For example, there is a dramatic overproduction of GBs in embryos mutant in *Notch*, and this hypertrophy of glial blast cells occurs before they divide to produce their glial progeny. Similar results have been obtained with

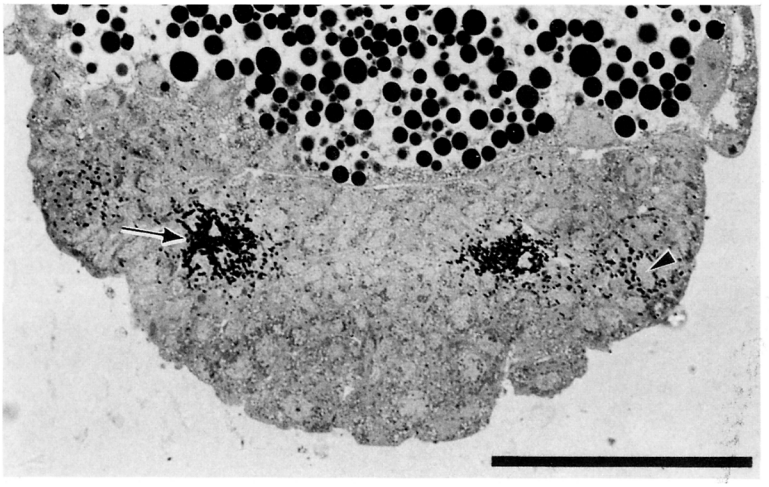

Figure 7. The Overproduction of Glial Cells in a *Notch* Mutant Embryo

β-Galactosidase activity is visualized as deposits of needle-shaped crystals in this cross-section of a 6 hr *Notch* mutant Drosophila embryo (this is a cross-section of the same embryo shown in whole-mount in Figures 6A and 6B). On the left side (arrow) there are 5 labeled cells, and a 6th is in another plane of section. Normally, the single GB divides into 2 GB progeny, which then begin to migrate medially. In this segment of a *Notch* mutant embryo, 6 GB progeny are observed, presumably from an overproduction of 3 GBs. Other segments in this same embryo have different numbers of either GBs or GB progeny, depending upon whether the single GB division has taken place yet in that particular segment, suggesting that anywhere from 2 to 4 GBs are made in different segments. The arrowhead on the right shows an artifact of β-galactosidase activity that is frequently seen in the enhancer trap constructs and is not specific to the construct used in these studies. Bar, 50 μm.

the neurogenic mutants *mastermind* and *big brain* (data not shown). These results indicate that the genetic regulation of local GB density is regulated in the same manner as local NB density.

Is the Glioblast Similar to the Other Neuroblasts?

Until the GB makes its single symmetric division, there is no structural feature that distinguishes it from its neighboring NBs. It is located in a stereotyped position within the plate of 25 or so NBs in each hemisegment (Figure 2B; also see Doe et al., 1988). One hypothesis is that the GB fate, namely, dividing symmetrically to generate 2 GB progeny, which further divide to generate 6 longitudinal glia, is one of the individual fates positionally specified within the array of 25 or so NBs in each hemisegment, that is, that the GB is similar to the other NBs. The only difference we can detect is that the GB happens to generate a specific class of glia rather than a specific family of neurons.

If the GB is in fact a blast cell similar to the other NBs, then understanding how the fate of individual NBs (or GBs) is positionally determined can be addressed at the level of both classic genetics and biochemical purification of DNA binding proteins. Not only does the P element with the 130 bp sequence from the 5′ end of the *ftz* gene serve as an excellent marker for this one lineage, but the short sequence itself must contain the cell-specific information for the expression of a reporter gene in this single precursor cell. Presumably, the proteins that bind to this 130 bp sequence represent gene products one step closer to the signals that are involved in specifying this unique glial precursor.

Where Do the Other Drosophila Glia Come From?

We previously identified three different classes of early glial cells in the developing CNS that appear to play important roles in the formation of the major CNS axon tracts: the longitudinal glia, the midline glia, and the nerve root glia (Jacobs and Goodman, 1989). Undoubtedly, there are other classes of nonneuronal support cells in the developing Drosophila CNS, including, for example, the glial sheath cells surrounding the neuronal cell bodies (some of these cells have been described in the grasshopper embryo by Doe and Goodman [1985]). Previous studies have shown that the midline glia arise from the mesectoderm cells at the midline of the developing neurogenic region (Thomas et al., 1988; Crews et al., 1988; Jacobs and Goodman, 1989; see also Rothberg et al., 1988). Here we have used a molecular lineage marker to show that the longitudinal glia arise from a lateral GB. But we are still uncertain as to how many other types of glia exist in the developing Drosophila CNS and exactly where they come from. Some of the other nonneuronal cells may arise from other NBs that primarily generate neurons, as indicated by both transplantation (Technau and Campos-Ortega, 1986) and tissue culture experiments (Fredieu and Mahowald, personal communication). Determining the precise lineage and differentiation of the other classes of Drosophila glia may require the type of specific molecular lineage probes described here.

Experimental Procedures

P Element Construction and Germline Transformation

The P element construct FZ was created as follows. First, a truncated *ftz* promoter/*lacZ* fusion gene (a gift of J. Topol and C. Dearolf; Dearolf et al., 1989) was inserted into the polylinker of the transformation vector Carnegie 20 (Rubin and Spradling, 1983). A 0.8 kb fragment containing the *hsp70* trailer and 3′ flanking sequences (Schedl et al., 1978) was cloned into the polylinker of pHSS7 (Seifert et al., 1986). It was then linearized with SalI and inserted into the SalI site located at the 3′ end of the *ftz*/*lacZ* fusion gene, providing the stop codon and the polyadenylation signal (Hiromi et al., 1985).

Germline transformation was performed as described previously (Rubin and Spradling, 1982; Karess and Rubin, 1984). Following injection, 10 independent transformant lines were established. Of these, 4 had P element insertions in the X chromosome. Secondary jumps to autosomes were induced from one of these insertions by providing transposase activity by matings to flies carrying the P[ry$^+$,Δ2-3]99B insertion (Robertson et al., 1988).

Fly Strains

The following alleles of neurogenic genes were used: *Notch 264-39* (Slizynska, 1938), *mastermind 1B*, and *bigbrain 1D5* (both Nusslein-Volhard et al., 1984). The transformant strain used in the characterization of the GP lineage is *F263*, located at 85B on the third chromosome.

β-Galactosidase Histochemistry and Histology

β-Galactosidase histochemistry was performed using modifications of the procedures of Hiromi et al. (1985). Briefly, dechorionated embryos were fixed for 20 min in heptane, equilibrated with 25% glutaraldehyde in cacodylate buffer. Embryos were then devitellinated with a sharp probe into 300 ml of reaction buffer (Simon et al., 1985) to which 10 μl of 8% of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside in dimetyl formamide was added. Embryos were reacted for 6-24 hr at 37°C.

For combined immunolabeling and β-galactosidase histochemistry, embryos were instead fixed over 4% formaldehyde, 0.1% glutaraldehyde. After staining for β-galactosidase activity, they were washed in PBS and processed for immunolabeling by the procedure published by Patel et al. (1987).

Some embryos stained for β-galactosidase activity were embedded in Epon-Araldite after brief treatment in 1% OsO_4 for 10 min. Sections of 0.5 μm thickness were counterstained with basic fuscin. Labeled cells demonstrate perinuclear deposition of blue crystals of measuring to 15 μm in length; these may grow through the cell membrane.

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