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Morphogenesis and proliferation of the larval brain glia in *Drosophila*

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Abstract

Glial cells subserve a number of essential functions during development and function of the *Drosophila* brain, including the control of neuroblast proliferation, neuronal positioning and axonal pathfinding. Three major classes of glial cells have been identified. Surface glia surround the brain externally. Neuropile glia ensheath the neuropile and form septa within the neuropile that define distinct neuropile compartments. Cortex glia form a scaffold around neuronal cell bodies in the cortex. In this paper we have used global glial markers and GFP-labeled clones to describe the morphology, development and proliferation pattern of the three types of glial cells in the larval brain. We show that both surface glia and cortex glia contribute to the glial layer surrounding the brain. Cortex glia also form a significant part of the glial layer surrounding the neuropile. Glial cell numbers increase slowly during the first half of larval development but show a rapid incline in the third larval instar. This increase results from mitosis of differentiated glia, but, more significantly, from the proliferation of neuroblasts.

Keywords: Glia; Brain; *Drosophila*; Larva; Proliferation

Introduction

Brain development in *Drosophila* encompasses three phases. In the embryo, a stereotyped set of neuroblasts generates small lineages of primary neurons and glial cells that differentiate into the functional larval brain. Primary neurons extend neurites that assemble in a highly ordered fashion into distinct neuropile compartments (Younossi-Hartenstein et al., 2003). During the larval period, neuroblasts resume their proliferative activity and produce large lineages of secondary neurons. Secondary neurons form bundles of axons that cross the brain cortex and invade the neuropile but do not differentiate any further and instead remain unbranched secondary axon tracts (Dumstrei et al., 2003a). During the third phase of brain development that takes place in the pupa, secondary neurons form dendritic and axonal branches that become integrated with the remodeled neurites of primary neurons and form the adult brain neuropile.

Throughout brain development, glial cell processes form a close-meshed scaffold around neuroblasts, neuronal cell bodies and neurites. Glial cells fall into three classes: (i) surface (or subperineurial) glia that extend sheath-like processes to wrap around the entire brain; (ii) cortex glia that encapsulate neuronal somata and neuroblasts; and (iii) neuropile glia that are located at the cortex–neuropile interface and form a sheath around the neuropile compartments, as well as major tracts of neurites (Cantera, 1993; Hoyle, 1986; Ito et al., 1994; Saint Marie et al., 1984). Surface glial cells, interconnected by septate junctions and covered by a thick basement membrane, act as the blood–brain barrier (Lane, 1982). Developmentally, surface glia appear to produce signals required for neuroblast proliferation (Ebens et al., 1993). Cortex glia fulfill important trophic roles for neuronal cell bodies (Hoyle et al., 1986). In the larval brain, the meshwork of cortex glial processes ("trophospongium") is required for stabilizing the position of neurons in the cortex and for extension of secondary axon tracts (Dumstrei et al., 2003b). Neuropile and surface glia play numerous roles in axon pathfinding and targeting (Hidalgo, 2003; Pielage and Klambt, 2001; Sepp and Auld,

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2003; Sepp et al., 2001). Specialized neuropile glial cells in the eye disc and optic lobe act as intermediary targets for ingrowing retinal axons (Poeck et al., 2001; Rangarajan et al., 1999; Suh et al., 2002; Tayler and Garrity, 2003). Glial septa formed by neuropile glia are essential to establish and stabilize neuropile compartments, such as the glomeruli formed by neurites of olfactory receptors and interneurons in the antennal lobe (Oland and Tolbert, 2003; Tolbert and Oland, 1990).

The glial cells of the early larval brain (primary glia) arise from a small number of neuroblasts (or, better, neuro-glioblasts) that are active during the embryonic period. Precursors of neuropile glia form a tight cluster (BPLG in Hartenstein et al., 1998) of approximately 20 cells at the base of the brain primordium. During late embryogenesis, these cells spread out dorsally along the inner surface of the extending neuropile founder axons. Precursors of surface glia (approximately 25–30 in the hatching larva) and cortex glia (approximately 10) also originate in circumspect clusters from which they migrate outward to populate the entire brain (Hartenstein et al., 1998). Anecdotal evidence suggests that all three classes of glial cells increase during the larval period. Recent advances have been made in understanding origin, proliferation and migration of defined subsets of glial cells (e.g., Aigouy et al., 2004; Van De Bor and Giangrande, 2002). However, the time course of larval glial proliferation is unknown, and many important details of glial architecture and morphogenesis have not been described. In this paper we have analyzed GFP-labeled clones to allow us to describe the morphology, development and proliferation pattern of the three types of glial cells in the larval brain. We show that two types of glial cells, surface glia and cortex glia, contribute to the glial layer surrounding the brain. Cortex glia also form a significant part of the glial layer surrounding the neuropile. Glial cell numbers increase slowly during the first half of larval development but show a rapid incline in the third larval instar. This increase results from mitosis of differentiated glia, but, more significantly, from the proliferation of neuroblasts.

Material and methods

Markers

The neuropile of the larval brain was labeled with a monoclonal antibody against the Syntaxin protein (Developmental Studies Hybridoma Bank 8C3). Primary and secondary neurons were labeled with a monoclonal antibody against Elav (Developmental Studies Hybridoma Bank 9F8A9), and glial cells with a monoclonal antibody against Repo (Developmental Studies Hybridoma Bank 8D12). Cells undergoing mitosis were labeled using a monoclonal antibody against the phospho-histone H3 protein (Upstate Cell Signaling Solutions 06-570). Glial cells were also labeled by Nrv2-Gal4;UAS-GFP (Sun et al., 1999), *gc*m-Gal4 (kindly provided by Dr. L. Zipursky) or using a Gal4 line generated in an enhancer trap screen (Bloomington Stock 3733; Manseau et al., 1997). Other antibody markers used were anti-beta-galactosidase (in rabbit; Cappel), anti-Serpent (kindly provided by Dr. D. Hoshizaki) and anti-Peroxidasin (Tepass et al., 1994).

Clonal analysis

We used the FLP/FRT technique to induce labeled clones in early larval brains (Ito et al., 1997). Briefly, in one series of experiments, a UAS-GFP construct containing an FRT flanked flip-out cassette was driven by elav-Gal4, which is expressed in neurons, as well as secondary neuroblasts (in the larval brain). Beside these two chromosomes, the stock contains an hs-FLP construct that allows one to induce the flip-out event, leading to a GFP-expressing cell, by applying short, 30- to 40-min heat pulses. A second series of clone inductions made use of hs-Flp and a construct that contained an actin promoter, followed by a flip-out cassette and Tau-lacZ (Ward and Skeath, 2000). Both approaches yielded glial clones at the same frequency and general structure.

Immunohistochemistry and histology

The anti-Syntaxin, anti-Elav and anti-Repo antibodies were diluted 1:10-fold. Anti-phospho-histone H3 was diluted 1:1000-fold. Anti-serpent and anti-peroxidasin were diluted 1:200. Anti-beta-galactosidase was diluted 1:5000. Secondary antibodies were Alexa546-conjugated anti-rabbit immunoglobulin (Ig, Molecular Probes A11010) and anti-mouse Ig (Molecular Probes A11031) used at a 1:500 dilution. For antibody labeling, standard procedures were followed (e.g., Ashburner, 1989). Staged *Drosophila* dissected brains were viewed as whole mounts in the confocal microscope (Bio-Rad MRC 1024ES microscope with Radiance 2000 using Bio-Rad Lasersharp 2000 version 5.2 build 824 software; 40× oil lens). Complete series of optical sections were taken at 2-mm intervals for at least five specimens per stage. Some preparations were counterstained with the nuclear marker Sytox green (Molecular Probes; added at a concentration of 1:10,000 to one of the final washing steps before mounting). For histology and electron microscopy, larval brains were dissected and fixed in 2% glutaraldehyde in PBS for 20 min, followed by a postfixation for 30 min in a mixture of 1% osmium tetroxide and 2% glutaraldehyde in 0.15 M cacodylate buffer (on ice). Specimens were washed several times in PBS and dehydrated in graded ethanol and acetone (all steps on ice). Preparations were left overnight in a 1:1 mixture of Epon and acetone and then for 5–10 h in unpolymerized Epon. They were transferred to molds,

oriented and placed at 60°C for 24 h to permit polymerization of the Epon. Blocks were sectioned (0.1 μm). Sections were mounted on net grids (Ted Pella) and treated with uranyl acetate and lead citrate.

BrdU incorporation

BrdU was dissolved in 40% ethanol (1 mg/100 μL) and mixed with fly medium to a final concentration of 1 mg/ml. Larvae were placed in vials with BrdU containing food for 5 h, at which point the brains were either dissected and fixed immediately in 4% formaldehyde or placed in normal fly medium until they reached the desired stage. After dissection and fixation for 20 min the brains were washed 3 × 15 min in PBT and incubated in 2 M HCl for 45 min to denature BrdU-labeled DNA. The brains were washed 3 × 15 min and antibody stained according to standard procedures (e.g., Ashburner, 1989).

Generation of three-dimensional models

A series of optical sections were volume rendered using the Voltex visualization module of Amira 3.0 (Mercury Computer Systems, Inc.). The surface-rendered neuropile compartments were brought into registration with each data set by warping (Pereanu and Hartenstein, 2004).

Results

Glial cells form a double-layered blood–brain barrier at the brain surface

The larval brain is surrounded by two closely apposed layers of glial processes formed by the surface glia and cortex glia (Fig. 1). Surface glial cells form the outer layer and consist of extremely flattened cells that lack processes growing radially into the brain cortex (Figs. 1A, D, F, G, H, I). By contrast, cortex glia span the thickness of the cortex, forming multiple lamelliform processes that form sheaths around neuronal cell bodies (Figs. 2A–F). The most superficial cortex glial processes are the ones that are apposed to the inner surface of surface glia (Fig. 1D). This peripheral cortex glial sheath forms around hatching or during the early larval period; it is not apparent in the embryo (Figs. 3A and B). At the time when neuroblasts reappear in the late larva the glial double layer completely surrounds these stem cells (Figs. 1B and C). Prominent septate junctions strengthen the contact in between glial layers and between cortex glia and neurons (Fig. 1E). These junctions, along with the basement membrane deposited on the surface glia (Fig. 1E), form the blood–brain barrier of the insect brain (Carlson et al., 2000; Lane and Swales, 1978, 1979).

It has been reported that the *Drosophila* embryonic nervous system is surrounded by a mesodermally derived layer of voluminous, electron-dense perineurial cells (Edwards et al., 1993). Such a layer was not observed in subsequent studies in which the nervous system was dissected out of the animal (e.g., Ito et al., 1994). In this study a perineurial layer was also absent (Figs. 1J–L). Thus, the repo-positive, clearly neuroectodermally derived surface glial cells form the outermost layer (Figs. 4A and B). We speculate that the cells forming the so-called perineurial layer in embryos are hemocytes that are closely associated with the brain and ventral nerve cord in embryos (Figs. 4C–E; Tepass et al., 1994; see Discussion).

Cortex glia compartmentalize the brain cortex

Lamelliform processes of cortex glial cells pervade the brain cortex, forming a scaffold around neurons and neuroblasts (Figs. 2A–F). These processes vary in thickness; at most places they measure less than 0.1 μm (Fig. 2G). Glial and neuronal membranes are so closely attached that an intercellular cleft is not discernible electron microscopically at most positions (Figs. 2G–I). Septate junctions can be seen at places where membranes diverge (Fig. 2H). Cortex glial processes have been shown to play an essential role in stabilizing the position of neuronal cell bodies during larval development (Dumstrei et al., 2003b). They are also in contact with secondary axons growing into the neuropile. Thus, as neuroblasts resume their proliferative activity during the third instar, they produce lineages of secondary neurons whose axons form tight bundles of extremely thin fibers (secondary axon tracts) growing towards the neuropile. Secondary axon tracts extend in contact with cortex glial processes (Fig. 2I) in all sections observed with the electron microscope.

Cortex glial cells are highly branched and lamellated cells whose processes undergo extensive rearrangements during development. Cortex glia differentiate in the stage 16 embryo as elongated, radially oriented cells spanning the distance between neuropile and brain surface (Figs. 3A–D). Subsequently lateral processes are formed, leading up to the three-dimensional, honey-combed structure revealed by the larval clones shown in Fig. 3. Shortly after hatching these processes are still modest, forming relatively large chambers that enclose multiple primary neurons (Fig. 3E). At subsequent stages, process density increases, so that by the second instar (Fig. 3F) each primary neuron is completely enclosed by cortex glia. Beginning with the third instar, dividing neuroblasts produce secondary neurons that form an outer cortex of increasing thickness around the inner layer of primary neurons. Initially, cortex glial processes build large chambers that enclose individual neuroblasts and their progeny (Figs. 3G and H). As the cortex increases in thickness, these chambers become subdivided by the addition of glial processes. Subdivisions start out deep in the cortex, where the oldest neurons are located (Fig. 3H). This process continues throughout metamorphosis (Fig. 3I) until every secondary neuron is individually wrapped by cortex glial processes (Fig. 3J).

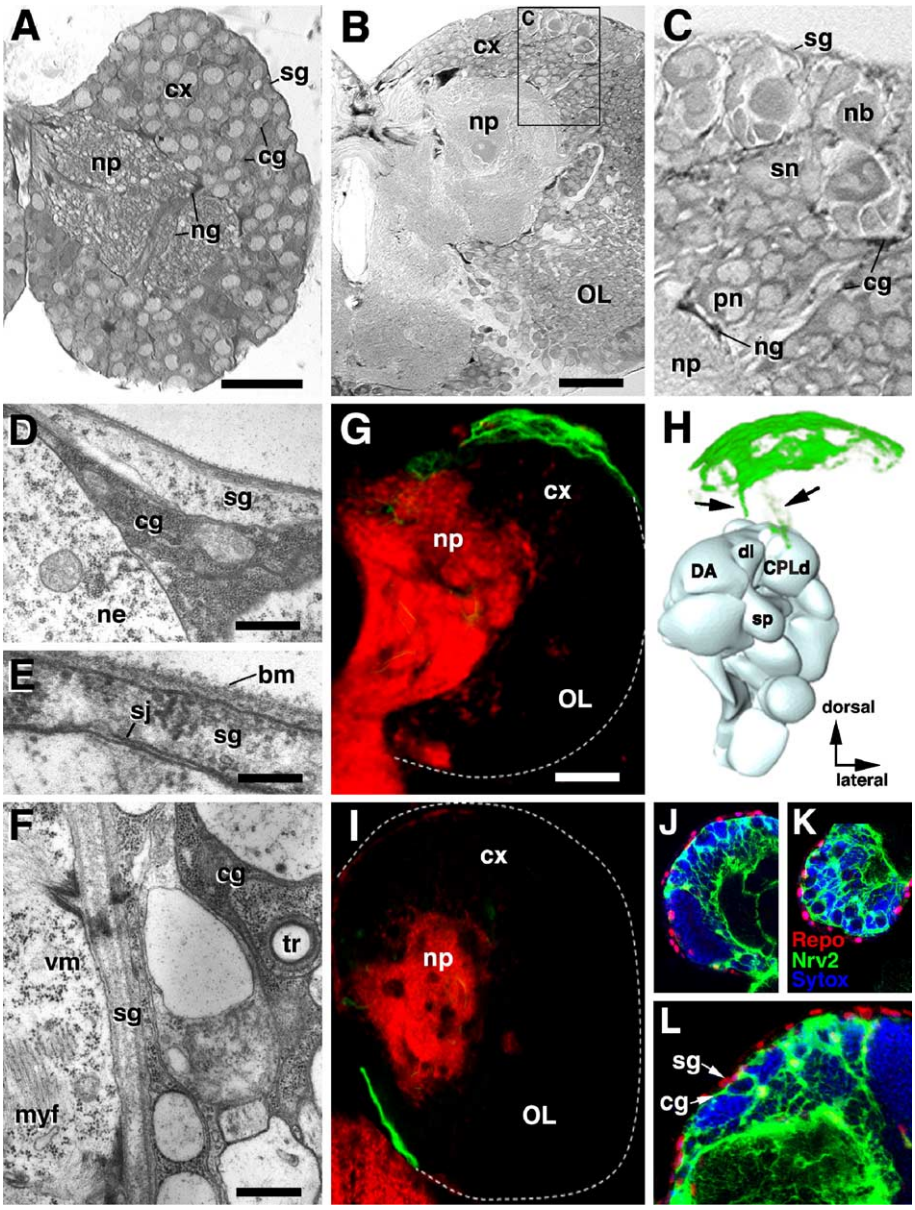

Fig. 1. Structure of the surface glia. (A–C) Larval brain stained with methylene blue/toluidine blue. (A) Horizontal section of first instar (lateral to the right; anterior to the top). (B–C) Frontal section of early third instar (lateral to the right; dorsal to the top). Glial cells (cg, cortex glia; ng, neuropile glia; sg, surface glia) appear more darkly stained than neuronal cell bodies of the cortex (cx). Surface glia form a smooth layer covering the brain. Together with cortex glia it envelops the secondary lineages (nb, neuroblast; sn, secondary neuron) that form at the brain surface during the third larval instar (B and C). (D–F) TEM sections of the glial sheath at the brain surface (first instar). Underneath the relatively electron-translucent surface glia (sg) appears a second layer formed by the surface lamella of the electron-dense cortex glia (cg). Cortex glia, rather than surface glia, contacts neurons (ne) and neuroblasts of the cortex at most locations. Panel E shows basement membrane (bm) and septate junctions (sj) formed by surface glial cell. In panel F, the glial sheath separating the brain (to the right) from the foregut (to the left) is illustrated. The smooth surface glial layer (sg) is closely attached to the visceral muscle (vm), containing myofilaments (myf) that surrounds the foregut. Note the profile of tracheole (tr) enclosed within cortex glia (cg). (G–I) GFP-labeled clones of surface glia at late third larval instar. Panels G and I show *Z*-projections of frontal confocal sections of brain hemispheres (lateral to the right, dorsal to the top) in which glial clone appears in green, neuropile (np, labeled with anti-Syntaxin antibody) in red. Panel H shows a volume rendering of the corresponding clone (G), superimposed upon a digital model of the neuropile compartments (gray). Clones were induced at the early first instar and consist of 2–4 cells. Note irregular horizontal processes (arrows) that emanate from clone boundaries. (J–L) Confocal sections of an early larval brain hemisphere (J and L) and ventral nerve cord (K) triple labeled with anti-Repo (red), nrv2-GFP (green) and Sytox (blue). Note that the superficial glial layer is formed by surface glia (sg; also called subperineurial glia) and cortex glia (cg). No additional, Repo-negative (and Sytox-positive) cells are located external to the surface glia, demonstrating that the so-called perineurial layer does not exist in dissected larval brains. Other abbreviations: CPLd, centro-posterior lateral compartment; DA, dorso-anterior compartment; dl, dorsal lobe; OL, optic lobe; pn, primary neuron; sp, spur. Scale bars: 10 μm (A); 25 μm (B); 0.5 μm (D); 0.25 μm (E); 1 μm (F); and 25 μm (G–J).

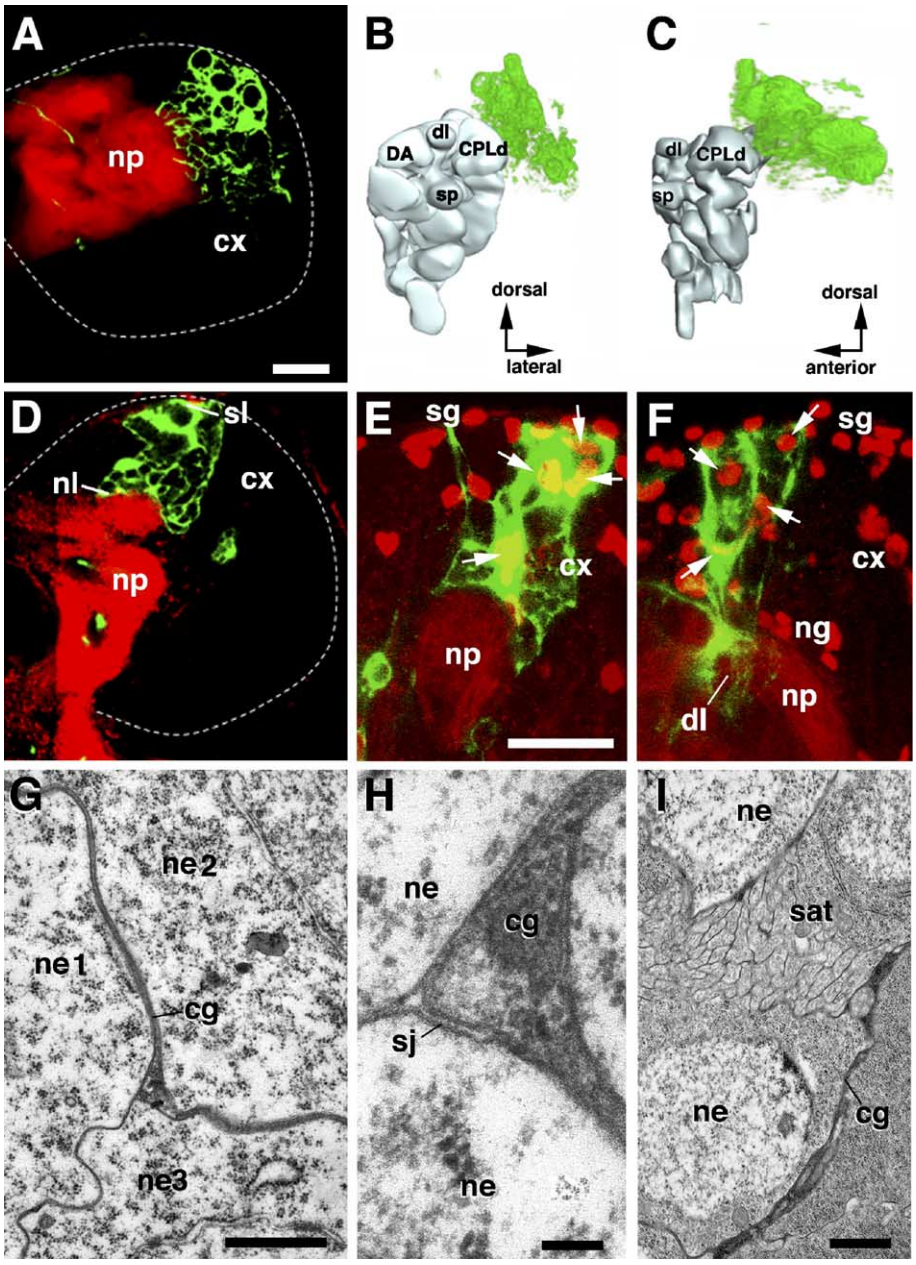

Fig. 2. Structure of cortex glia. (A–F) GFP-labeled clones of surface glia at late third larval instar. Panels A and D show *Z*-projections of frontal confocal sections of brain hemispheres (lateral to the right, dorsal to the top) in which the glial clone appears in green, neuropile (np, labeled with anti-Syntaxin antibody) in red. Panels B and C are volume renderings of the clone shown in panel A (B: frontal view, C: lateral view) superimposed upon a digital model of the neuropile compartments (gray). Panels E and F are *Z*-projections of two GFP-positive cortex glial clones (green) double-labeled with anti-Repo (red). Faint background staining of neuropile occurs with this antibody. Clones were induced at the early first instar and consist of between 2 and 4 cells. Cortex glial cells extend from the brain surface (sl, surface lamella) to the neuropile (nl, neuropile lamellae). Note spongy texture of cortex glia; "holes" represent chambers enclosing neuroblasts (near brain surface) and neurons. (G–I) TEM sections of the brain cortex (G and H: first larval instar; I: third instar), showing glial sheaths in between neurons. At the first instar, cortex glial sheaths are not yet completed between all neurons; ne1 and ne2 in panel G are separated by glial lamella, whereas ne1 and ne3 are not. Small septate junctions (sj) are formed between neurons and cortex glia (H). Bundles of tightly packed, thin secondary axons (sat) travel alongside cortex glia (I). Other abbreviations: CA, centro-anterior compartment; CPLd, centro-posterior lateral compartment; CX, calyx; DA, dorso-anterior compartment; dl, dorsal lobe; sp, spur. Scale bars: 25 μm (A–F); 0.5 μm (G); 0.1 μm (H); and 1 μm (I).

Neuropile glia and cortex glia compartmentalize the neuropile

A prominent glial sheath can be seen between the deep cortex, formed mostly by cell bodies of primary neurons, and the neuropile, consisting of primary neuronal axons and dendrites (Figs. 1A–C and 5D, E). In the dorsal and lateral part of the brain, the neuropile sheath is formed by basal processes of cortex glia (Fig. 2D). In more basal and medial regions of the brain, the neuropile sheath is formed by "dedicated" neuropile glial cells, defined by the location of their cell body and nucleus right adjacent

to the neuropile (Ito et al., 1994). Neuropile glial cells are irregularly shaped, flattened cells with cell bodies located at the cortex–neuropile interface (Figs. 5A–C). Some neuropile glial cell bodies and nuclei are sunken deep into the neuropile (Fig. 5C). Beside the sheath around the neuropile, processes of neuropile glia also penetrate into the center of the neuropile, forming septa that subdivide the neuropile into discrete compartments (Figs. 5F and 6A). Both cortex–neuropile boundary and inter-compartmental septa are not impermeable barriers but leave openings where secondary axon tracts can enter the neuropile ("neuropile portals") or where individual neuronal processes can cross from one compartment into another (Figs. 5E–F).

The brain is tracheated by the cerebral branch of the second thoracic tracheal primordium entering from dorsomedially (Manning and Krasnow, 1993). During larval development this stem tracheae grow and form branches of increasing density. Tracheal branches and tracheoles (the finest terminal branches of tracheae) are always seen in contact with the neuropile glial processes around or within the neuropile (Fig. 5E). In some instances, glia completely enwrap the tracheole. More typically, tracheoles are sandwiched in between glia on one side and neuronal processes on the other. Given the time course of glial and tracheal development (glia coming first) it seems plausible that glial cells may act as a guiding scaffold for tracheal growth.

Glial proliferation occurs by division of glial cells and neuroblasts

All three classes of glial cells increase significantly between early and late larval stages. Whereas glial proliferation is rather slow during the first half of larval life, it increases dramatically during the third instar. Overall, glial cell numbers (counted from staged preparation of Nrv2-Gal4;UAS-GFP brains double labeled with anti-Repo; Figs. 6A–C) increases from 33 to 109 for surface glia, from 11 to 164 for cortex glia and from 17 to 90 for neuropile glia (Fig. 6D).

The overall increase in glial cell number is at least in part due to the mitotic divisions of glial cells. Thus, feeding BrdU to larvae at different stages results in clusters of labeled cells that include all three types of glial cells (Figs. 7A–C). The BrdU labeling by itself did not allow us to discern whether the increase in glial number occurs by mitosis of glial cells themselves, or by continued formation of glial cells by secondary neuroblasts. To address this issue, we double-labeled Nrv2-Gal4;UAS-GFP brains with the mitosis marker anti-phospho-histone. Double-labeled cells do occur at a frequency of 0.75% (10 brain counted; Fig. 7D), demonstrating that at least some glial cells are added

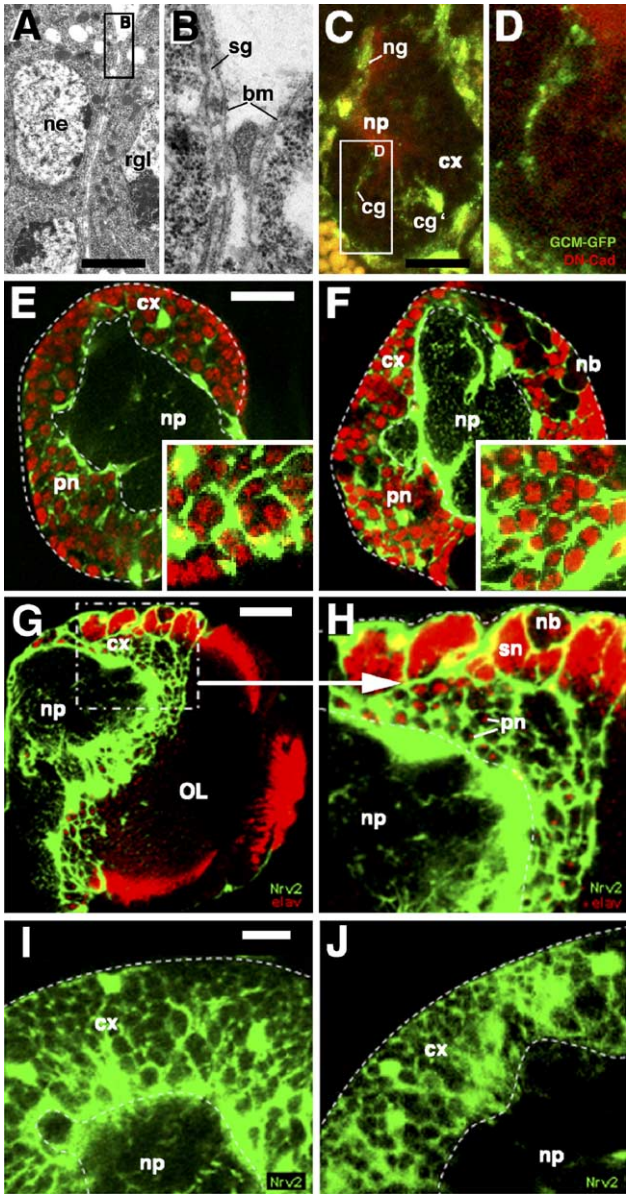

Fig. 3. Morphogenesis of cortex glia. (A–D) Stage 16 embryo. Panels A and B show TEM sections of dorso-medial embryonic brain surface, demonstrating that only surface glia (sg) are present and surface lamella of cortex glia (see Fig. 1) are absent. Panels C and D show *Z*-projections of serial horizontal confocal sections of embryonic brain hemisphere (anterior to the top, lateral to the right). Glial cells are labeled green by GFP reporter construct activated by the gcm-Gal4 driver. Neuropile is labeled with anti-DN cadherin antibody. Cortex glia (cg) are represented by slender, radial cells extending throughout the cortex (cx) from the neuropile (np) to the brain surface. (E–H) Confocal sections (frontal plane) of brain hemispheres of first instar (E), second instar (F), late third instar (G and H), 3-day pupa (I) and adult (J). Glial cells are labeled (green) by GFP reporter activated by the nrv2 driver. Neurons are labeled red (in E–H) by anti-Elav antibody. By the first instar, cortex glia have formed meshwork of lamelliform processes that form incomplete sheaths around primary neurons (pn; see inset). By the second instar (F) each primary neuron is completely enclosed by cortex glia (see inset in F). Near the brain surface, neuroblasts (nb) and their progeny (sn) are enclosed within large glial chambers (G and H). These chambers become subdivided by the addition of glial processes during metamorphosis (I) so that in the adult cortex every neuron is individually wrapped by a glial sheath (J). Other abbreviations: ng, neuropile glia; OL, optic lobe; rgl, ring gland. Scale bars: 2 μm (A); 20 μm (C); 10 μm (E, F); 25 μm (G); and 10 μm (I, J).

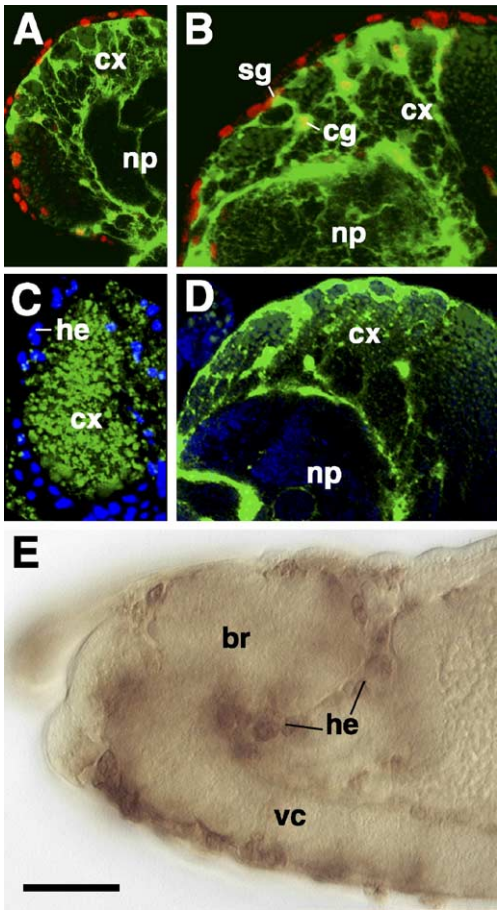

Fig. 4. The glial surface layer. (A and B) Confocal sections of early larval (A) and late larval (B) brain double-labeled with anti-Repo (red) and nrv2-Gal4 (green). The surface glial layer is formed by both nrv2-negative surface glia and nrv2-positive cortex glia. (C and D) Confocal sections of embryonic brain (C) and larval brain (D) labeled with hemocyte marker anti-Serpent (blue) and nrv2-Gal4 (green). Hemocytes form a dense population around the embryonic brain (C) but are absent in dissected larval brains (D). (E) Lateral view of embryo (stage 15, anterior to the left, dorsal up) labeled with the hemocyte marker anti-Peroxidasin (brown). Note the close association of hemocytes with brain (br) and ventral nerve cord (vc). Other abbreviations: cg, cortex glia; cx, cortex; np, neuropile; sg, surface glia. Scale bar: 20 μm.

by mitosis of glial cells. However, the low frequency of anti-phospho-histone-positive glial cells, as well as the finding that glial cells labeled by clonal induction were almost always in close contact with neural lineages (Figs. 7E and F), indicates that the bulk of added glial cells stems from the proliferation of secondary neuro-glioblasts located at the brain surface. This conclusion is further supported by the characteristics of the glial growth curve, which remains very shallow during early larval life when neuroblasts are mitotically quiescent, and becomes steep during the third instar when neuroblasts divide.

Glia of the optic lobe

The above description of glial proliferation and morphogenesis focused on the central brain. For the optic lobe, whose proliferative and morphogenetic history differs significantly from that of the central brain, a classification of glia has been provided for the adult (Eule et al., 1995) based primarily on glial cell position (whether they are in the lamina, medulla, lobula or a chiasm in between) and on what structure the glial process contacts. The glial cells defined for the adult can be easily identified based on these same criteria in the larval brain. Similar to the central brain, three main classes of glia exist in the larval optic lobe. The layer of surface glia covering the central brain continues uninterruptedly over the optic lobe (Fig. 8C). Cortex glia whose processes wrap around neuronal cell bodies, called satellite glia in the optic lobe, are scattered throughout the emerging cortices of the lamina, medulla and lobula complex. It appears that optic lobe cortex glial processes ensheath individual neuronal precursors from the beginning, rather than forming larger chambers enclosing multiple neurons as in the central brain (Figs. 8A–C). Neuropile glial cells fall into multiple subsets with diverse morphology and function (e.g., epithelial glia, marginal glia, giant glia; Fig. 8D) and are described in detail in previous studies (Tix et al., 1997; Winberg et al., 1992).

Discussion

Structural aspects of glial cells of the larval brain

Our results largely confirm previous reports on the structure and classification of glial cells in the insect nervous system. Two novel observations reported in this paper deserve some attention, the contribution of surface glia and cortex glia to the bilayered glial envelope surrounding the brain, and the formation of the neuropile glial sheath by cortex glia.

The bilayered structure of the glial envelope at the brain surface has been investigated in a number of morphological and experimental studies. An outer layer of voluminous, electron-dense perineurial cells was distinguished from an inner layer of flattened, much less electron-dense subperineurial cells. Perineurial cells were discounted as glial cells by several authors because of their suspected or proven mesodermal origin, whereas other authors included them into the class of “true” glial cells (for a discussion, see Edwards et al., 1993). Perineurial cells could not be detected in several other studies of the embryonic and larval nervous system of *Drosophila*, including the present paper, even though molecular markers for glial cells (e.g., Repo expression) were used in combination with a variety of morphological techniques. In these studies, the outermost layer surrounding the brain or ventral nerve cord in late embryos or larvae was identified as surface glia, or subperineurial glia, because cells were flat, electron translucent and Repo positive (Ito et al., 1994).

We propose that perineurial cells, which were already shown to be mesodermal in origin, are hemocytes. Hemo-

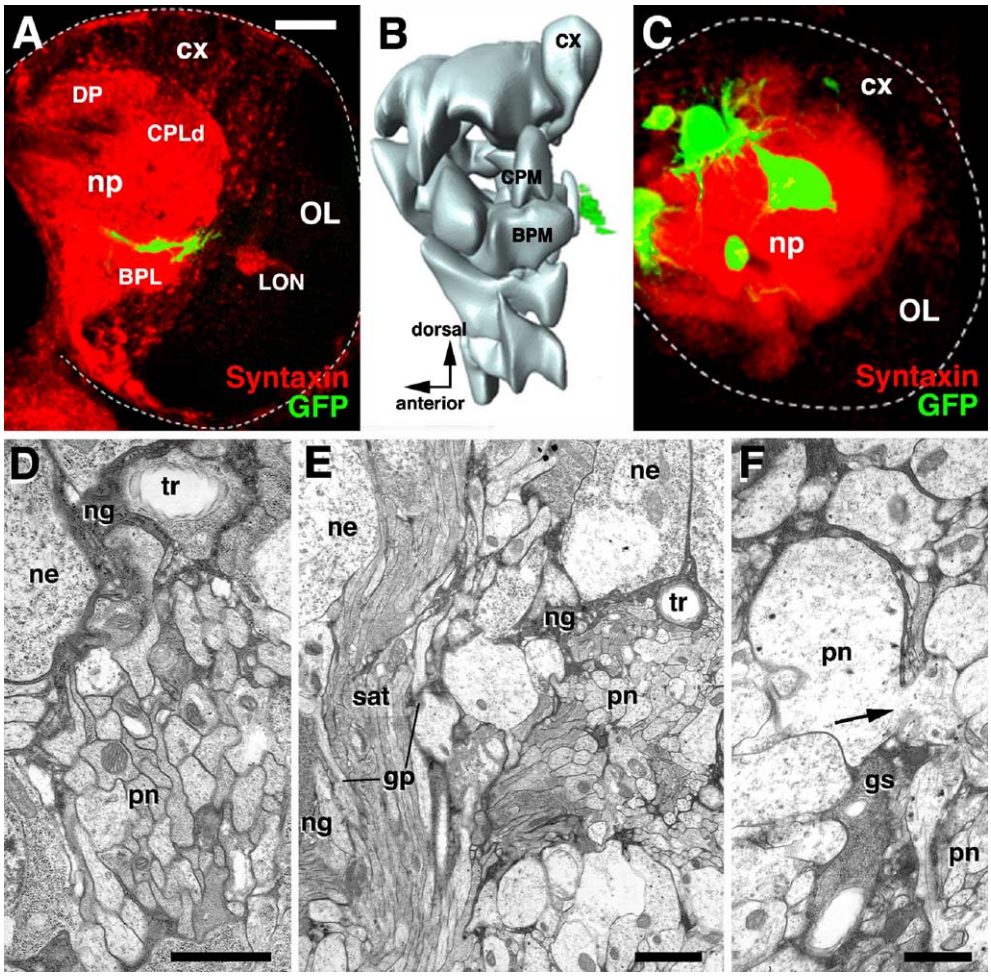

Fig. 5. Structure of neuropile glia. (A–C) GFP-labeled clones of neuropile glia at late third larval instar. Panels A and C show *Z*-projections of frontal confocal sections of brain hemispheres (lateral to the right, dorsal to the top) in which glial clone appears in green, neuropile (np, labeled with anti-Syntaxin antibody) in red. Panel B is a volume rendering of the clone shown in A (lateral view) superimposed upon a digital model of the neuropile compartments (gray). Neuropile glia are associated with part of the surface of the neuropile and the septa in between neuropile classes. At least two subclasses of neuropile glia, represented in A/B and C, can be distinguished. Shown in A/B is a type of slender, flattened cells that are stretched out along the neuropile surface and project lamelliform processes in between compartments. The type of neuropile glia illustrated in panel C consists of more rounded, multipolar cells that project multiple, finger-like processes into the neuropile. (D–F) TEM sections of larval brains (D, F: first instar; E: third instar) at cortex–neuropile boundary. Panel D illustrates prominent, electron-dense neuropile glial sheath separating neuronal somata (ne) from bundles of neurites (pn) that constitute the neuropile. Tracheae and tracheoles (tr) penetrating the neuropile are always associated with glial sheaths. Panel E illustrates tract formed by secondary axons (sat) penetrating the neuropile glial sheath through a glial portal (gp). Panel F shows glial septum (gs) between neighboring neuropile compartments (to the left and the right of the panel, respectively). Inter-compartmental glial septa represent a barrier for most neurites, although some branches (see arrow) pass through. Other abbreviations: BPM, baso-posterior medial compartment; CPM, centro-posterior medial compartment; CX, calyx. Scale bars: 25 μm (A–C); 1 μm (D–F).

cytes are motile cells that act as macrophages (Tepass et al., 1994) but also appear to contribute to the basement membrane around tissues that they (transiently) attach to. In the late embryo, the nervous system is surrounded by a dense population of hemocytes, although clearly not all parts of its surface are covered by this cell type. Hemocytes show all of the characteristics described and illustrated for perineurial cells in the *Drosophila* embryo (Edwards et al., 1993) and in some of the other adult insect systems investigated (Hoyle, 1986). The identity of perineurial cells with hemocytes would explain why in larval brains, which are dissected out of the animal so that all hemolymph and hemocytes are removed, this cell type is no longer attached to the brain surface. If further analyses confirm that hemocytes are indeed identical with perineurial glial cells, we suggest to no longer use the latter term since hemocytes represent a well-defined class by themselves and, more importantly, have only transient contacts with the nervous system.

The development of the neuropile glial sheath has not been described in much detail in previous papers. These papers (e.g., Hartenstein et al., 1998; Ito et al., 1994) usually imply that a distinct subpopulation of cells, the neuropile glia, is responsible for generating the neuropile sheath. Given that at the onset of morphogenesis (stages 13/14) neuropile glial cells are all concentrated on one side of the nascent neuropile (i.e., ventral for the ventral nerve cord, medial for the brain; Hartenstein et al., 1998), this would

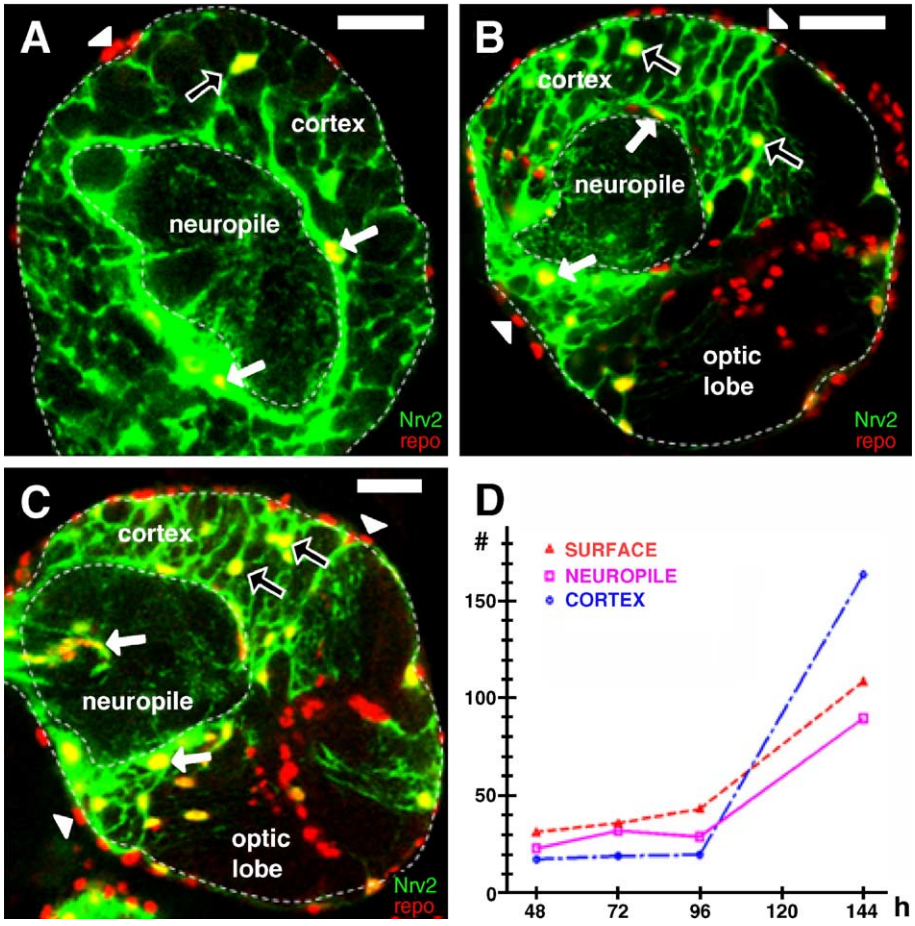

Fig. 6. Glial proliferation: increase in cell number. (A–C) Confocal sections (frontal plane) of larval brain hemispheres (A: 48 h, first instar; B: 96 h, early third instar; C: 144 h, late third instar) in which glial nuclei are labeled with anti-Repo antibody (red) and glial cytoplasm with nrv2-GFP (green). Arrowheads point at representative surface glial nuclei; open arrows at cortex glia, solid arrows at neuropile glia. (D) Plot of glial cell number against time (in hours after egg laying). Five brain hemispheres were counted at each stage. Abbreviations: cx, cortex; np, neuropile; OL, optic lobe. Scale bars: 10 μm (A); 25 μm (B, C).

imply that these cells migrate around to lateral and dorsal positions. Although this paper does not specifically address the formation of the neuropile glial sheath at a high level of resolution, it is clear that in the brain cortex glia, rather than neuropile glia, generate much of the neuropile sheath at lateral levels. This finding suggests the possibility that both types of glial cells, neuropile and cortex, may not represent such different cell types after all. Future studies into molecular determinants of glial development may shed more light on this issue.

Proliferation of brain glia during the larval period

All three classes of brain glial cells multiply during the larval period. The increase is strongest for cortex glia (15-fold), followed by neuropile glia (5-fold) and surface glia (3-fold). These data match the findings of Prokop and Technau (1994) and Awad and Truman (1997) who showed that midline glia (a subpopulation of neuropile glia) increased by a factor of approximately five during the larval period. Interestingly, in the abdominal neuromeres of the ventral nerve cord which grow only insubstantially in *Drosophila melanogaster*, cortex glia and surface glia do not appear to proliferate, although they undergo several rounds of endoreplication (Prokop and Technau, 1994). By contrast, abdominal surface glia multiply by a factor of approximately five in *Drosophila hydei*, suggesting that endoreplication, followed by increased cell size (hypertrophy), and mitosis, resulting in more cells (hyperplasia), are two possible strategies employed by glia to accommodate for nervous system growth.

The increase in glial number can occur by mitosis of differentiated glial cells, by proliferation of dedicated glial progenitors or by proliferation of “regular” neuroblasts that produce both neurons and glia. The origin of glia in the embryo from both glial progenitors and from neuroblasts has been reported and mapped in detail for the ventral nerve cord and some sensory organs (Bossing et al., 1996; Jacobs et al., 1989; Klambt and Goodman, 1991; Schmidt et al., 1997; Van De Bor et al., 2000). Our findings suggest that in the larval brain, dividing neuroblasts also account for a substantial part of the glial cell increase. On the other hand, mitosis of morphologically differentiated glia seems to occur in the cortex glia described in this study, and in

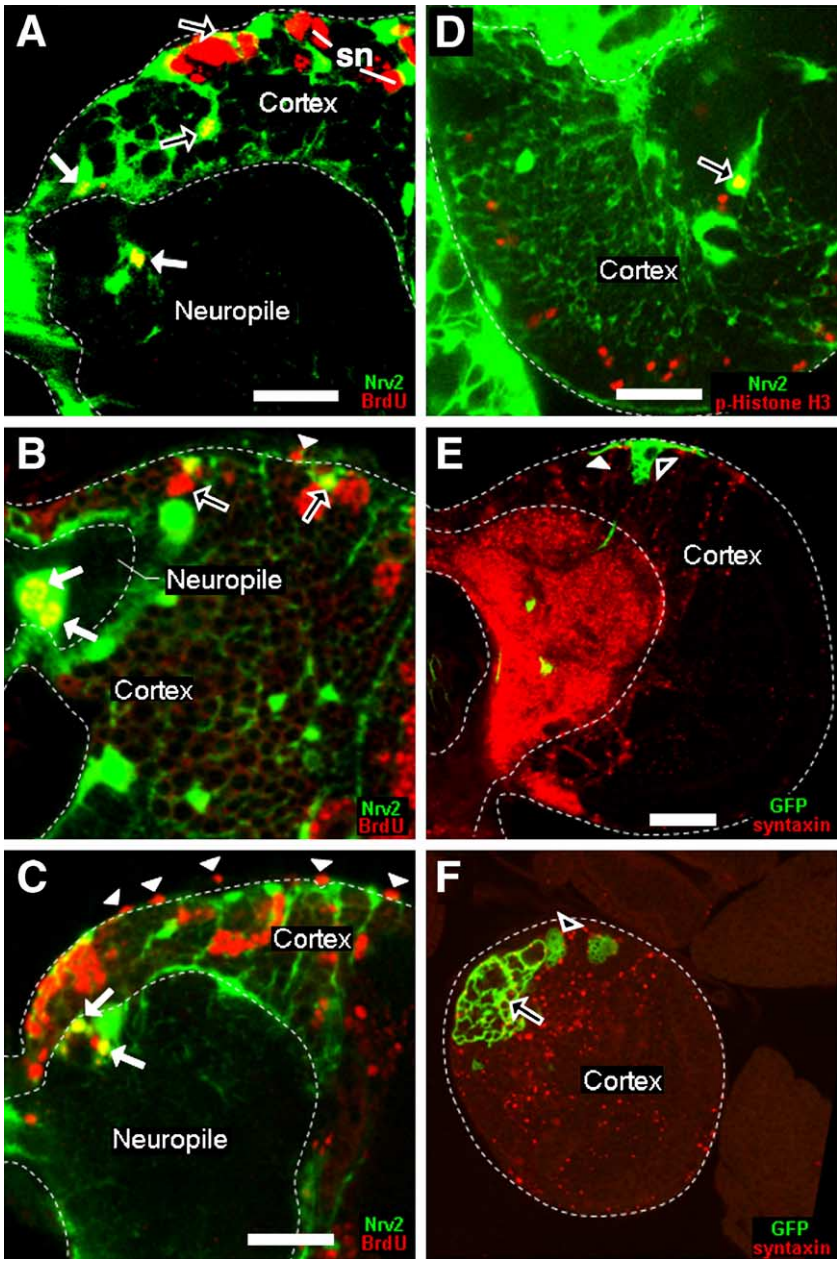

Fig. 7. Glial proliferation: origin of added glial cells. (A–C) Confocal sections (frontal plane, dorsal to the top, lateral to the right) of third larval instar brains. Larvae had been fed BrdU containing medium for 12 h prior to dissection. BrdU incorporation appears in secondary neural lineages (sn), as well as in all three classes of glial cells (arrowheads: surface glia; open arrows: cortex glia; solid arrows: neuropile glia). (D) Labeling of mitotic cells by anti-phospho-histone H3 (red) and glial cells (nrv2-GFP, green) in third larval instar brain. Arrow indicates mitotic glial cell. (E and F) GFP-labeled clones of secondary lineages (neuroblasts indicated by open arrowheads) with adjacent glial cells in third larval instar brains. Panel E shows surface glia (solid arrowhead) forming part of secondary lineage. In panel F, cortex glia (open arrow) is located directly adjacent to neuroblast. Scale bars: 20 μm (A, B); 25 μm (C, D, E, F).

neuropile glia in the ventral nerve cord (Awad and Truman, 1997; Prokop and Technau, 1994). Although unlikely, one cannot entirely exclude the possibility that the mitotic glial cells that were interpreted as differentiated (i.e., having profuse lamelliform processes) were instead rounded glial precursors intermingled with differentiated glial cells. Mitosis of morphologically differentiated glial cells is also found in the vertebrate nervous system (Reznikov et al., 1981; Sturrock and McRae, 1980; Stevenson and Yoon, 1981).

Comparative aspects of glia in Drosophila and vertebrates

In the vertebrate CNS, two main classes of glial cell types, astrocytes and oligodendrocytes, can be distinguished. Both glial types are multipolar cells. The processes of astrocytes mainly interact with capillaries and neuronal cell bodies, whereas oligodendrocytes form flat, sheath-like processes around axons that differentiate as the myelin sheath. On the basis of structural and developmental characteristics, parallels can be drawn between the astro-

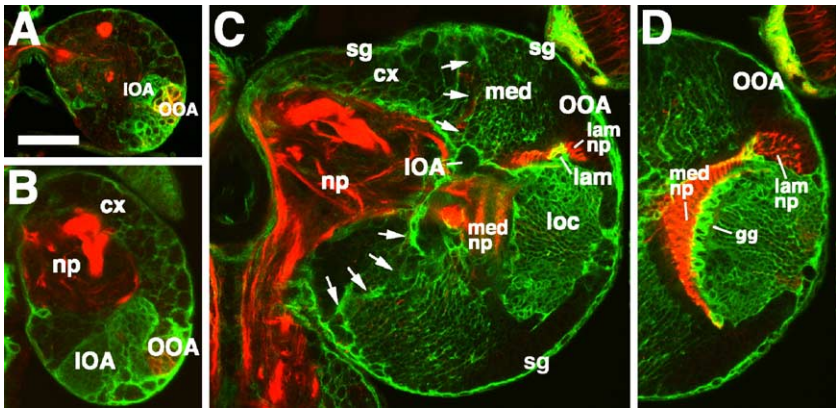

Fig. 8. Glia of the optic lobe. Panels A–D show confocal sections (frontal plane, dorsal to the top, lateral to the right) of a first instar (A), second instar (B) and third instar (C, D) brain where glial cells are labeled by UAS-GFP (green) activated by a Gal4 line (Bloomington 3733) that is expressed by surface and cortex glia in the optic lobe and central brain. Neuropile tracts are labeled with anti-FasII antibody (red). The surface glial layer (sg) continues around the optic lobe primordium (IOA: inner optic anlage; OOA: outer optic anlage) from early onward. Cortex glia (cg) forms a dense meshwork in the optic lobe cortices, the medulla (med), lobula complex (loc) and lamina (lam; no substantial cortex yet at the stage illustrated). Specialized classes of neuropile glia surround the emerging neuropiles; the giant glia (gg; Eule et al., 1995) is indicated explicitly in panel D. Arrows in panel C delineate the boundary between medulla cortex and central brain. Other abbreviations: cx, cortex; np, neuropile. Scale bar: 25 μm (A–D).

cytes of vertebrates and cortex glia in insects, and between oligodendrocytes and neuropile glia. These similarities and their phylogenetic significance will be briefly explored in the following.

Astrocytes and cortex glia represent the most abundant type of glial cell in vertebrates and *Drosophila*. Both are involved in the blood–brain barrier (Rubin and Staddon, 1999; Willis et al., 2004). Cortex glia form the tissue on which tracheae grow. This interaction closely parallels the developmental relationship between astrocytes and blood vessels in vertebrates (Ramsauer et al., 2002). Differentiated cortex glia can undergo mitosis to create other cortex glia. Similarly, differentiated astroglia divide (goldfish optic tectum: Stevenson and Yoon, 1981; dentate gyrus and parietal cortex of mouse: Reznikov et al., 1981). It has been reported that differentiated astrocytes in adult mouse can divide to form neurons (Seri et al., 2001), a characteristic that is seemingly not shared with cortex glia in *Drosophila*.

Beside these structural and functional similarities, it is worthwhile to point out the close parallelism in the development of astrocytes and cortex glia. Astrocytes differentiate throughout the embryonic nervous system from precursors called radial glia. At an early stage, a subset of the epithelial cells that constitute the wall of the neural tube form specialized processes around the bundles of neurites emitted by early differentiating neurons (Berman et al., 1997; Chanas-Sacre et al., 2000; McMahon and McDermott, 2002). These cells are the forerunners of radial glia. Thus, radial glia represent an epithelial cell type that stretches from the ventricular lumen to the outer surface of the neural tube. Radial glia serves as the substrate for the migration of neural precursors from the ventricular surface where they are born to more superficial layers. In anamniotes, radial glia maintain their basic architecture throughout development, forming elongated cells reaching from the ventricle to the surface even in the adult CNS. By contrast, radial glia withdraw their contact to the ventricular surface and form multiple processes, thereby differentiating into star-shaped astrocytes, in amniotes (Mission et al., 1991). This developmental pathway is very similar to the one observed for cortex glia in *Drosophila*. At an early stage (embryonic stages 13–16), cortex glial precursors form spindle shaped, radially oriented cells reaching from the outer brain surface to the neuropile. Subsequently, multiple lateral fiber-like and lamelliform processes are extended.

Drosophila neuropile glia, similar to oligodendroglia in vertebrates, enclose axons. An interesting parallel between neuropile glia and oligodendroglia is seen in development. Oligodendrocytes have a restricted origin in the ventral and (at a later stage) dorsal neural tube (Cai et al., 2005; Thomas et al., 2000; Vallstedt et al., 2005). From there, oligodendrocyte precursors migrate radially into the mantle layer, and then invade the white matter in a ventral to dorsal gradient. In a similar manner, neuropile glia in *Drosophila* also undergo considerable migration (Hartenstein et al., 1998; Jacobs and Goodman, 1989).

Do the similarities between the aforementioned two classes of glial cells in vertebrates and insects imply that these cell types are homologous? At the first glance, this possibility seems rather remote, since glial cells, structurally defined by their processes that form sheaths around neuronal cell bodies or axons, are sparse or even non-existent in most “lower” invertebrate phyla, including coelenterates, flatworms, hemichordates or echinoderms (Radojcic and Pentreath, 1979). In nematodes, phylogenetically close to arthropods according to new trees based on molecular data, a small number of glial cells have been identified (Bird, 1971; Chitwood and Chitwood, 1950). For the rest of the nervous system, instead of glial cells, the epidermis and its basement membrane surround neuronal cell bodies and axon tracts. The same can be said for many other invertebrate groups where glial sheaths are generally

absent, but a close relationship exists between neurites and basal processes of the epidermis (or other epithelia). This suggests that it is fairly unlikely that the bilaterian ancestor had glial cells with elaborate processes enclosing the central nervous system, the neuropile or axon bundles. On the other hand, it is quite possible that the bilaterian ancestor possessed a generalized type of neural support cell, be it part of the nervous system or the overlying epidermis. Starting from such a population of ancestral glial cells, different evolving animal taxa would modify this cell type in response to selective pressures imposed upon the organism to create more specialized glial cells.

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