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ORIGINAL ARTICLE

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Glial patterning during postembryonic development of central neuropiles in the brain of the honeybee

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Abstract Glial cells are involved in several functions during the development of the nervous system. To understand potential glial contributions to neuropile formation, we examined the cellular pattern of glia during the development of the mushroom body, antennal lobe and central complex in the brain of the honeybee. Using an antibody against the glial-specific repo-protein of *Drosophila*, the location of the glial somata was detected in the larval and pupal brain of the bee. In the early larva, a continuous layer of glial cell bodies defines the boundaries of all growing neuropiles. Initially, the neuropiles develop in the absence of any intrinsic glial somata. In a secondary process, glial cells migrate into defined locations in the neuropiles. The corresponding increase in the number of neuropile-associated glial cells is most likely due to massive immigrations of glial cells from the cell body rind using neuronal fibres as guidance cues. The combined data from the three brain regions suggest that glial cells can prepattern the neuropilar boundaries.

Key words *Repo* · Glial migration · Central complex · Mushroom body · Antennal lobe

Introduction

The development of the nervous system involves the birth of neuronal and glial cells, directed cellular migration and outgrowth of processes. One of the functions of glial cells during embryogenesis is the construction of guidance structures for migrating neurons or outgrowing axons. In embryonic vertebrates, radially arranged processes of specialized early astrocytic glial cells provide guiding rails for the migration of differentiating neurons to their final destination in various brain areas (Rakic 1971, 1972; for reviews, Rakic 1990; Hatten 1990; O'Rourke 1996).

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Mechanisms of glial axon guidance appear remarkably similar in both vertebrates and insects. For example, in the early mouse forebrain, a population of primitive glial cells forms a median bridge-like structure. This "sling" guides the first callosal axons which form the anterior commissure to the contralateral side of the brain (Silver et al. 1982). The formation of the primary preoral brain commissures in *Drosophila* also occurs in close association with an interhemispheric glial cell bridge (Therianos et al. 1995). In the embryonic brain of the grasshopper, an initial axonal bridge across the midline is established by axonal navigation along glial borders (Boyan et al. 1995). Moreover, insect glial cells have been described to support the interganglionic migration of a special class of neurons (Cantera et al. 1995) and as guidance cues for photoreceptor axons (Perez and Steller 1996). Ablation of glia in the embryo disrupts the formation of commissural and longitudinal pathways in the ventral nerve cord (Bastiani and Goodman 1986; Jacobs and Goodman 1989; Klämbt et al. 1991; Hosoya et al. 1995; Jones et al. 1995; Hidalgo et al. 1995).

Apart from their guidance functions, glial cells support neuronal survival and differentiation by the release of growth factors (e.g. Woodward et al. 1992). Similar conclusions have been derived from the study of glial-deficient mutants in *Drosophila*. In the absence of glial cells in the mutant *gcm*, peripheral bipolar dendrite neurons show an abnormal morphology of their process pattern (Jones et al. 1995). Recent investigations of the *Drosophila* gene *repo*, which codes for a transcription factor expressed in glial cells (Campbell et al. 1994; Halter et al. 1995; Xiong et al. 1994), and the mutant *drop-dead* (Buchanan and Benzer 1993), which is deficient in glial morphology, provide further evidence that glial cells may be required for maintenance of the structural integrity of the nervous system.

Finally, boundaries of glial-derived extracellular matrix (ECM) play an important role in the patterning of the central nervous system (CNS), for example the rhombomere boundaries of the hindbrain (for review, Steindler 1993). In the olfactory neuropile of *Manduca*,

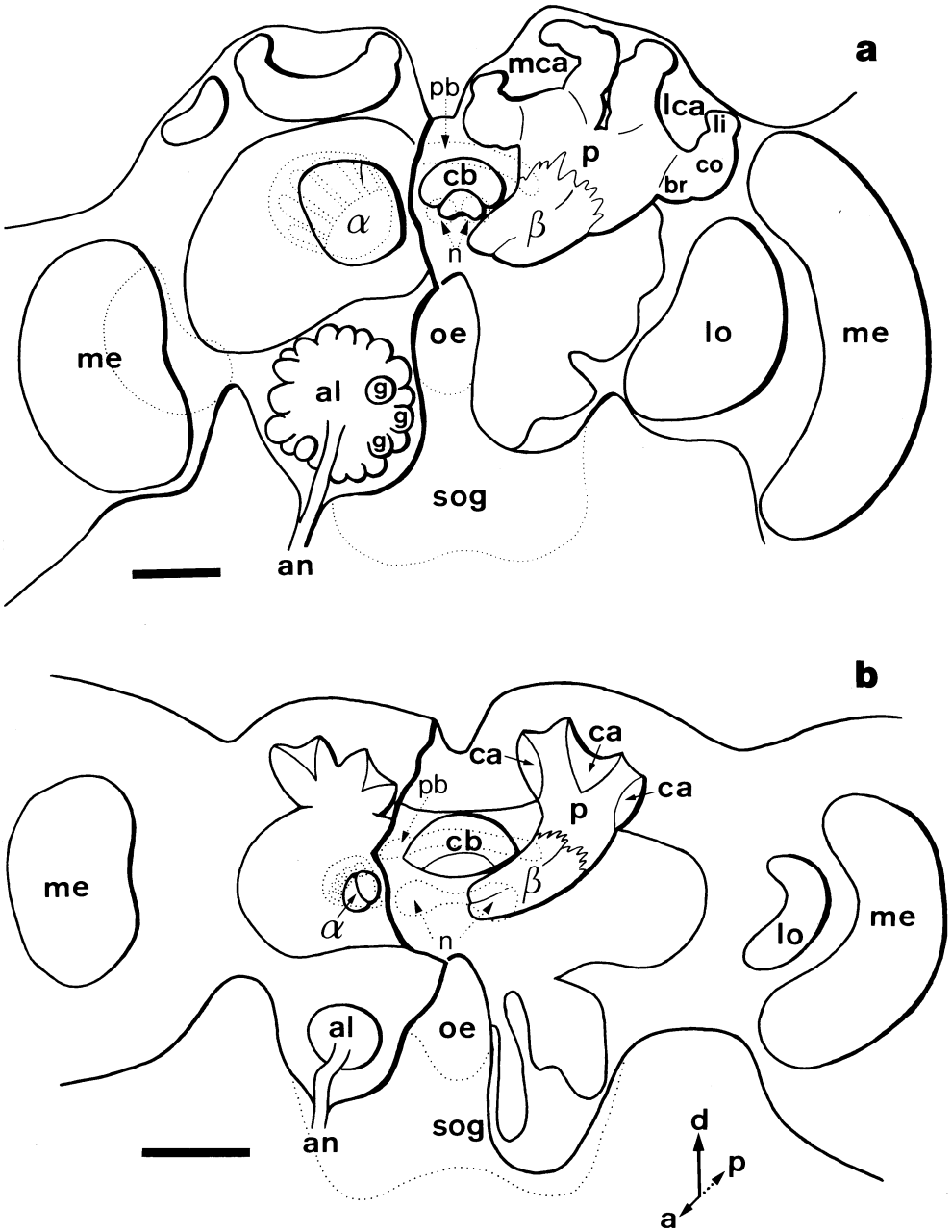

Fig. 1a, b Schematic drawings of an adult and larval bee brain in a frontal view. The *left* hemispheres indicate anterior and the *right* hemispheres more posterior portions of the brain. **a** Adult brain. The central complex is composed of the central body (*cb*), the more posterior situated two noduli (*n*) and protocerebral bridge (*pb*). The paired mushroom bodies consist of a medial (*mca*) and a lateral calyx (*lca*), a pedunculus (*p*), an α- (α) and a β-lobe (β). The calyces are divided into lip (*li*), collar (*co*) and basal ring (*br*). *Dots* outline the pedunculus sectioned close to the α-lobe which contains separated fibre bundles projecting into the α- and the β-lobe. In the more posterior drawn right hemisphere, the branching fibre bundles appear as finger-like boundaries between pedunculus and β-lobe. The neuropile of the antennal lobes (*al*), organized in glomeruli (*g*), receives input from the antennal nerve (*an*). **b** Corresponding scheme of a brain at larval stage L5. In the central complex the noduli (*n*) are interconnected with each other. The dorsal regions of the mushroom bodies comprise calyx developmental zones (*ca*). The α-lobe (α) is divided longitudinally into a median and lateral half. The antennal lobe neuropile (*al*) appears as a spherical shell (*oe* oesophagus, *sog* suboesophageal ganglion, *me* medulla, *lo* lobula, *scale bars* 200 μm). Coordinate axes indicate anterior (*a*), posterior (*p*) and dorsal (*d*) directions

glial cells are involved in the induction of the glomeruli (Oland and Tolbert 1996) and in the secretion of an extracellular molecule restricting the glomerular boundaries (Krull et al. 1994a,b).

We are interested in understanding a potential glial contribution to the postembryonic development and maintenance of olfactory pathways in the honeybee. Using osmium ethyl gallate staining of adult tissue, we found antennal lobe and mushroom body neuropiles ensheathed and interspersed by glia (Hähnlein and Bicker 1996). In many regions, the distribution pattern of the neuropile intrinsic glia seems to provide an internal compartmentation of the neuropile.

In this study, we traced the cellular location of glial somata during neuropile development in the larval and pupal brain of the bee using an antibody against the glial-specific nuclear repo-protein of *Drosophila*. We focused on the postembryonic formation of the antennal lobe and mushroom body neuropile, including the bilateral symmetrical neuropile of the central complex, a major interhemispheric link in the median protocerebrum. The establishment of the glial pattern during neuropile development was characterized by two features: the appearance of initially continuous layers of glial cell bodies defining the boundaries of the developing neuropiles and an increase in numbers of associated glial cells during subsequent neuropile growth. The increase in glial cell numbers is most likely due to massive immigrations of glial cells from the cell body rind using neuronal fibres as guidance cues.

Materials and methods

Experimental animals

Histological staining was performed on brain tissue of worker honeybees (*Apis mellifera carnica*) obtained from colonies at our institute. Adult bees were caught at the entrance of their hive. Larvae and pupae were taken from a hive kept in a flight room (24–26°C; 60–70% humidity) under a 14-h light: 10-h dark cycle. Experimental animals were staged according to Eichmüller (1994) and Thompson (1978). Larval development takes about 5–6 days from larval hatching (L1) to the 5–6th larval day (L5). After the prepupal stages, pupal development is completed within 9 days from pupal ecdysis (P1) to adult emergence (P9).

Immunocytochemistry on frozen sections

The experimental animals were immobilized by cooling at 4°C and were then decapitated. Brains were removed from the head capsule, fixed in 4% carbonate-buffered formaline (pH 6.9) for 2 h at 4°C, rinsed in TRIS-buffered saline, 0.1 M, pH 7.4 (TBS) and stored in 30% sucrose / TBS overnight. The tissue was then embedded and quickly frozen in Tissue Freezing Medium (Jung). Frontal sections (6–10 μm) were cut with a cryostat and mounted on poly-D-lysine coated slides. The frontal plane was defined in accordance with the body axis (Mobbs 1985). For the following immunocytochemical procedure, sections were rinsed in TBS and incubated with a rabbit polyclonal antiserum against the glial repo-protein (Halter et al. 1995) diluted 1:250 in TBS / 1% normal goat serum / 0.1% Triton-X-100 / 0.1% sodium azide overnight at 4°C. The primary antibody was visualized by a Vectastain Kit (Vector Laboratories) according to the instructions of the manufacturer using 3,3'-diaminobenzidine (DAB; Sigma) as chromogen.

DAPI staining of cell nuclei

After the immunocytochemical procedure sections were rinsed in TBS and incubated for 20 min with DAPI staining solution. To prepare the staining solution, a stock solution of 0.01% 4', 6-diamidino-2-phenylindole (Sigma) in distilled water was diluted 1:99 in methanol. After washing in TBS sections were mounted in glycerol. Nuclear staining was examined under the UV fluorescence filter combination U1 of a Reichert-Jung microscope.

Glial cell counts

The numbers of glial cells were quantified on serial sections by counting the repo-immunoreactive (repo-IR) nuclei. Two animals per developmental stage were evaluated. Cell counts were corrected for double split-cell counts according to Abercrombie (1946) by consideration of the respective average nuclear diameter and section thickness.

Results

Central complex

The unpaired central complex, consisting of the bean-shaped central body, the protocerebral bridge and two spherical noduli, bridges the dorso-median parts of the protocerebrum (Fig. 1a). The central body is separated into an upper and lower division. Its fan-shaped appearance is caused by the highly ordered innervation of several groups of interneurons (Williams 1975; Strausfeld 1976; Homberg 1985).

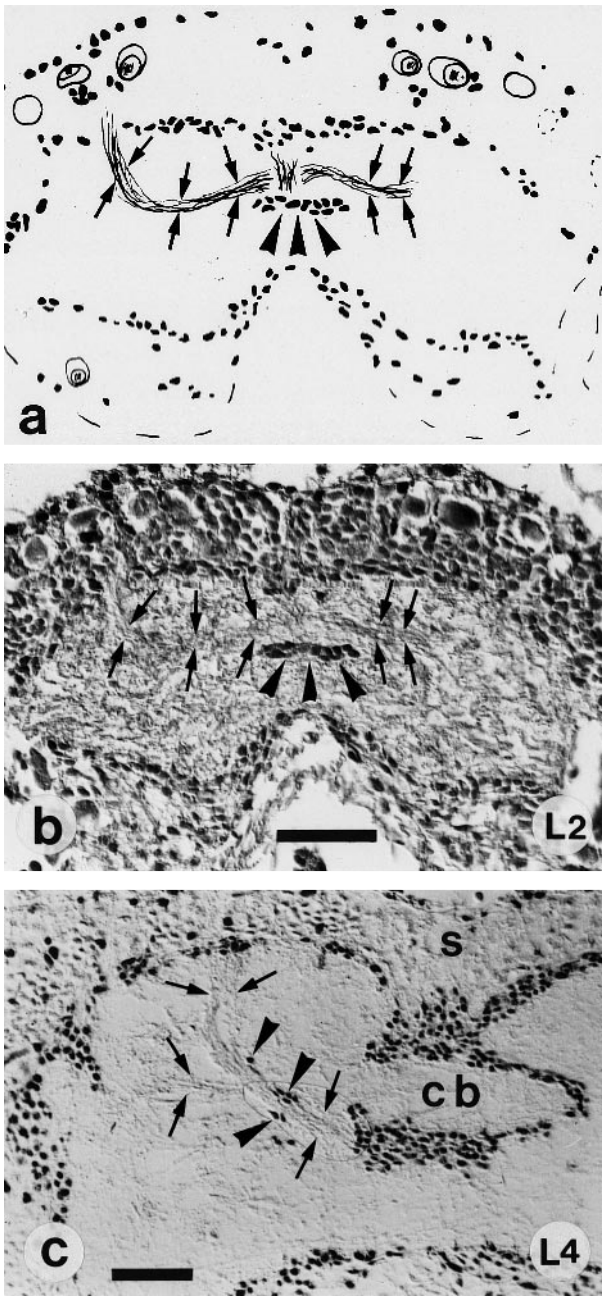

Fig. 2a–c Early development of the central complex. **a, b** Drawing (**a**) and micrograph (**b**) of the protocerebrum at larval stage L2. The cell body rind contains neuronal somata, a few large neuroblasts (*open spheres*) and repo-immunoreactive (repo-IR) nuclei of some glial cells. The neuropile is surrounded by a layer of glial cells, indicated in the drawing by their *dark nuclei*. Two axonal fibre bundles project from the cell body rind into the median region of the neuropile forming a commissural structure (*arrows*). *Arrowheads* point to a cluster of glial nuclei associated with the commissural fibre bundle. To expose the immunoreactivity in the central cluster of glial cells, the section required prolonged diaminobenzidine (DAB) incubation resulting in unspecific background staining of neuropile and soma rind. **c** Central brain region at larval stage L3/4 sectioned in a slightly oblique frontal plane. This reveals the course of two fibre bundles extending from the cell body rind (*arrows*) into the central body neuropile (*cb*). *Arrowheads* indicate glial nuclei in close association with the fibre bundles (*Scale bars* 100 μm)

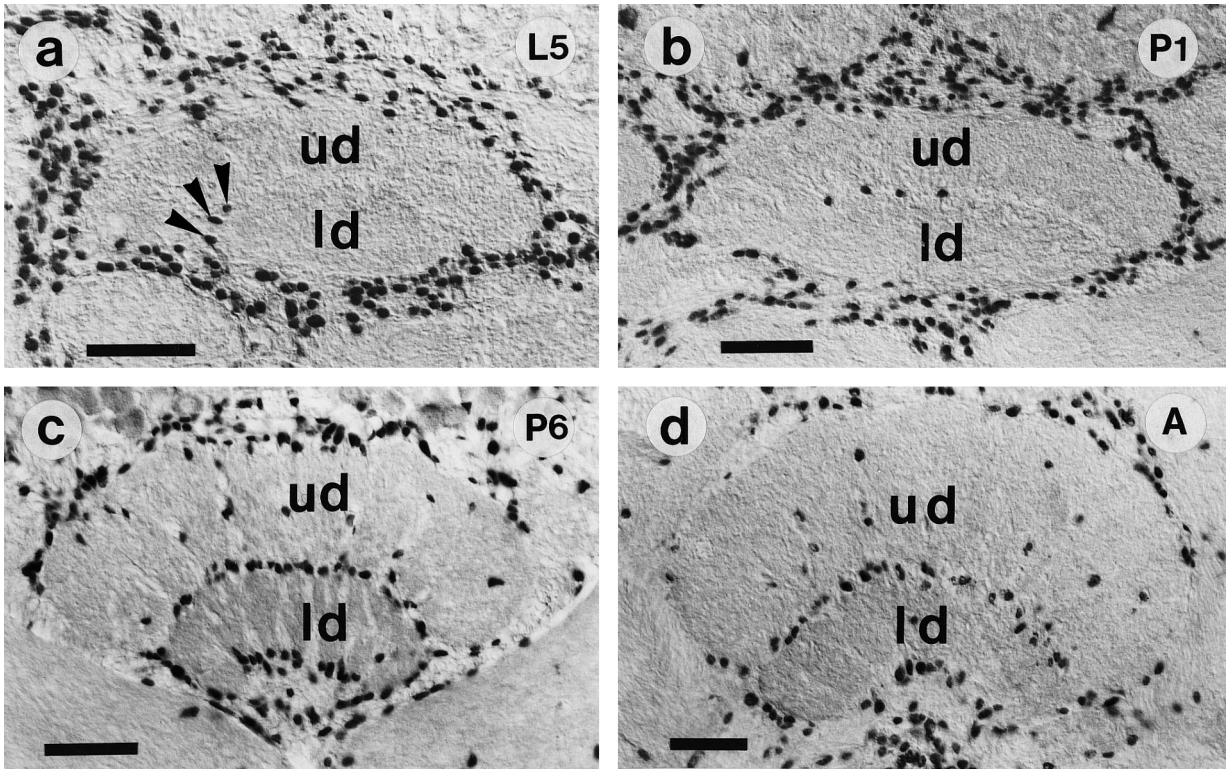

Fig. 3a–d Glial cells of the central body. **a** Central body at larval stage L5. The neuropile shows an internal structuring into an upper (*ud*) and lower division (*ld*). *Arrowheads* point to glial nuclei located in the lateral regions of the boundary between the two divisions. **b** Pupal stage P1. Glial nuclei are now also located in the median region of the boundary between the upper (*ud*) and lower (*ld*) division. **c** Pupal stage P6 and **d** adult stage. Upper (*ud*) and lower division (*ld*) are clearly separated by a dense layer of glial nuclei. The neuropilar divisions are interspersed by the nuclei of glial cells (*Scale bars* 100 μm)

Larval development

Shortly after larval hatching, the protocerebral neuropile appears as a uniform unstructured mass. It is lined by a layer of glial nuclei separating the neuropile from an external cell body rind which contains large neuroblasts, smaller neuronal progeny and some glial cells with IR nuclei (Fig. 2a, b). At larval stage L2, two symmetrical bundles of axonal processes project from the dorsal cell body rind to the middle of the protocerebral neuropile where they come in contact with each other forming an axonal bridge (Fig. 2a, b). A cluster of glial nuclei is associated with the ventral side of this median commissural bundle (Fig. 2a,b). Based on haematoxylin-stained sections of the early larva, Panov (1959) has described a similar arrangement of axonal bundles and glial somata.

At L4, the central body can be clearly discerned from the surrounding protocerebral neuropile as a dense neuropilar arch bordered by multiple layers of glial nuclei (Fig. 2c). In each brain hemisphere, an additional fibre bundle extends from the cell body rind into the central body neuropile. Glial somata attach to all four medially directed axonal bundles (Fig. 2c).

Conspicuously high amounts of glial nuclei are found in the region where the dorso-median glial sheath of the central body is in contact with the cell body rind (Fig. 2c). Large numbers of glial nuclei are also accumulated in two blocks at the posterior region of the concave ventral central body surface (Fig. 2c). Presumably, these glial clusters establish the boundaries of the developing noduli.

At the last larval stage (L5), all main parts of the central complex, central body (Fig. 3a), protocerebral bridge (Fig. 4a) and noduli (Fig. 4c) are clearly visible. Each component is entirely surrounded by a layer of glial nuclei. The two spherical noduli are interconnected by a fibrous bridge (Fig. 4c). During L5, the volume of the central body neuropile increases and attains its characteristic partition into an upper and lower division (Fig. 3a). Moreover, single glial nuclei appear in lateral regions of the border zone between the upper and lower division (Fig. 3a). However, until pupal ecdysis, the neuropiles of the central body's upper and lower division, the protocerebral bridge and the noduli remain devoid of glial nuclei.

Pupal development

During pupal life, the expanding central body of the pupa acquires the characteristic internal fan-shaped architecture and, in parallel, the neuropile intrinsic glial pattern. At the beginning of the pupal phase (P1), glial nuclei become visible at the median part of the boundary region between the upper and lower division (Fig. 3b) and, from stages P2/3 on, glial cell nuclei form a contin-

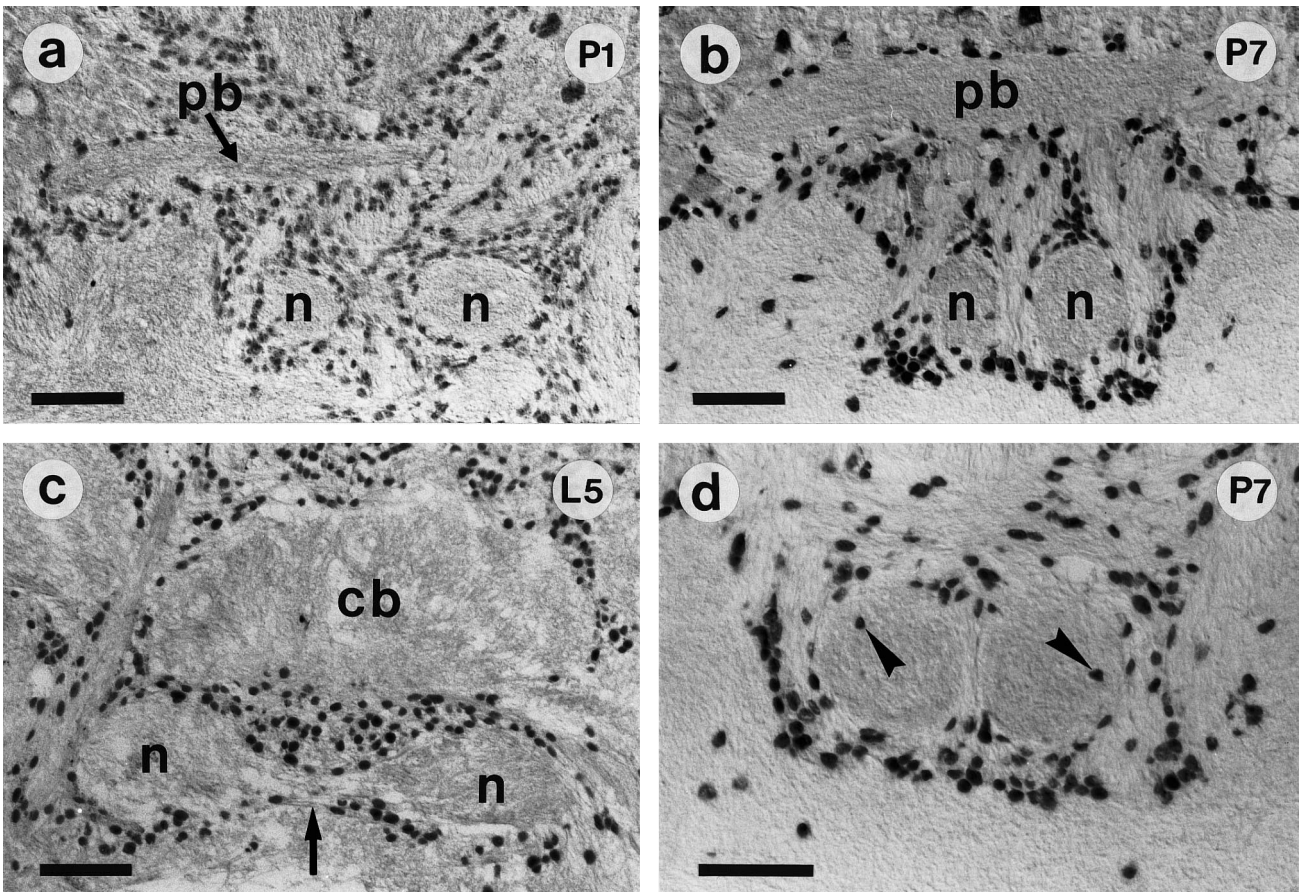

Fig. 4a–d Development of protocerebral bridge and noduli. **a** Protocerebral bridge (*pb*) and noduli (*n*) at pupal stage P1 surrounded by a layer of glial nuclei. **b** Protocerebral bridge (*pb*) at P7 devoid of glial nuclei (*n* noduli). **c** Central body (*cb*) and noduli (*n*) at larval stage L5. The noduli, interconnected by a fibrous bridge (*arrow*), are surrounded by a layer of glial nuclei. Their neuropile lacks glial nuclei. **d** Pupal stage P7. The separate noduli are interspersed by single glial nuclei (*arrowheads*). *Scale bars* 100 μm

uous border layer separating the two divisions. Furthermore, glial nuclei become visible within the upper and lower parts. However, the pattern of glial nuclei is not related to the fan-shaped neuropilar architecture. As evident from the comparison of Fig. 3c and d, the glial distribution pattern of the central body is finished approximately by pupal stage P6.

Whereas the neuropile of the protocerebral bridge remains completely devoid of glial nuclei throughout pupal development and adulthood (Fig. 4b), the noduli are interspersed by glial nuclei (Fig. 4d) immediately from the beginning of the pupal period.

Quantification of glial numbers during the development of central body and noduli

To examine the increase in glial cells during pupal development, we counted the number of repo-IR nuclei in serial sections of the central complex. This count included the glial cells interspersing upper and lower division of the central body, the noduli (Fig. 5a) and glial cells forming the boundary layer between the two divisions of the central body (Fig. 5b).

At the beginning of pupal development, there are no glial nuclei present in the upper and lower division. Within the upper division, the first glial somata appear at stage P3. During the next four pupal stages, the number of glial cells within the upper division shows a steady increase which levels off from P7 to adult emergence. Within the lower division, the first few glial nuclei appear at P4 and increase to approximately ten cells at adult emergence. Thus, the increase in central body intrinsic glial cell numbers is delayed with respect to neuropile development.

Compared to the central body, the noduli are already interspersed by glial cells immediately from the beginning of pupal development with only slight increases of glial cell numbers during the first half of the pupal period. The establishment of a glial boundary layer between upper and lower divisions of the central body is reflected in a steady increase from a few single glial cells at P1 to about 225 cells in the adult central body (Fig. 5b).

Mushroom body

Each of the mushroom bodies consists of two cup-like calyces connected to an α-lobe and a β-lobe via a pedun-

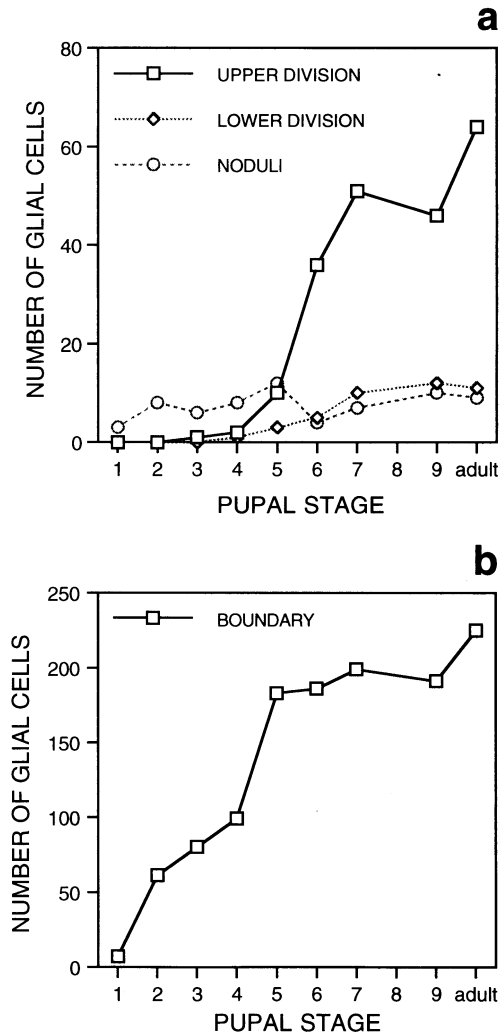

Fig. 5a, b Numbers of glial cells in distinct parts of the central complex during pupal development. **a** Number of glial cells interspersing upper and lower division of the central body and noduli. **b** Increase of glial cell numbers during the formation of the boundary between the upper and lower division of the central body

culus (Mobbs 1982; Fig. 1a). Shape and internal structure are largely determined by the branching pattern of the intrinsic Kenyon cells. Their somata are mainly packed within the calycal cups. The fibres of these interneurons project from their dendritic calycal input areas in a parallel arrangement through the pedunculus and finally branch into the α- and β-lobes (Mobbs 1982). According to the anatomical location and innervation by sensory modalities, the calycal neuropiles can be further subdivided into lip, collar and basal ring (Figs. 1a, 6i).

Larval development

The first signs of mushroom body development appear slightly later than the beginning of central complex formation. At larval stages L3/4, two distinct somata blocks can be discerned within the dorsal cell body rind of the two brain hemispheres (Fig. 6a,b). As described by Panov (1957), each of them consists of a central cluster of large dividing somata, laterally bordered by a thin layer of smaller neuronal Kenyon cell bodies. The repo-antibody marks the nuclei of a few glial somata adjacent to the Kenyon cell bodies (Fig. 6a) The Kenyon cells of each soma group direct a peduncle-like axonal bundle through the cell body rind into the protocerebral neuropile which is separated from the surrounding somata of the rind by a layer of glial nuclei (Fig. 6a,b). Inside the protocerebral neuropile, very few glial nuclei are associated with the bundles of Kenyon cell axons (Fig. 6b).

Up to the end of larval development, the number of Kenyon cells increases and the main features of the mushroom body neuropile emerge (Fig. 1b). A ring of calycal neuropile surrounded by a layer of glial nuclei differentiates around the dorsal part of each pedunculus (Fig. 1b). During neuropilar growth the basal regions of the calycal rings fuse. The bundles of Kenyon cell axons now extend deeper into the protocerebral neuropile to form the α- and the β-lobes (Fig. 1b). Whereas, the calycal developmental zones have always been surrounded by a thick layer of glial nuclei, the pedunculus and lobes are accompanied by glia somewhat later.

Pupal development

The pupal phase of mushroom body development is characterized by a further increase of Kenyon cell numbers. The intrinsic glial pattern is established in parallel to the growth of the neuropile.

Calyces. After pupal ecdysis, the calycal neuropile initially expands as an unstructured mass devoid of internal glial somata. Except for the entrance regions of the Kenyon cell axons, the future calycal bottoms, the neuropile is separated from the cell body mass by a dense cover of glial nuclei (Fig. 6c). A few glial cell bodies become detectable at the border between pedunculus and calycal region. Their number increases during the first pupal stages, such that from P4/5 on the pedunculus becomes separated from the calycal neuropile (Fig. 6e,f).

From pupal stage P3 on, the neuropilar compartments of both calycal rings are separated by the inwards movement of neuronal and glial cell bodies. At pupal stages P5/6, the formation of the characteristic calycal compartments of lip, collar and basal ring is completed. Some glial nuclei are positioned at the border between basal ring and collar. From P6, glial nuclei interspersing the collar compartment and others at the calycal bottom are visible (Fig. 6e).

The calyces of an adult bee are entirely lined by glial cell bodies with predominantly flattened or spindle-shaped nuclei (Fig. 6f). A layer of glial nuclei separates the pedunculus from the calycal neuropile. However, within the calycal neuropile, only the compartmental border between collar and basal ring is marked by single glial nuclei. Whereas the collar is interspersed by glial

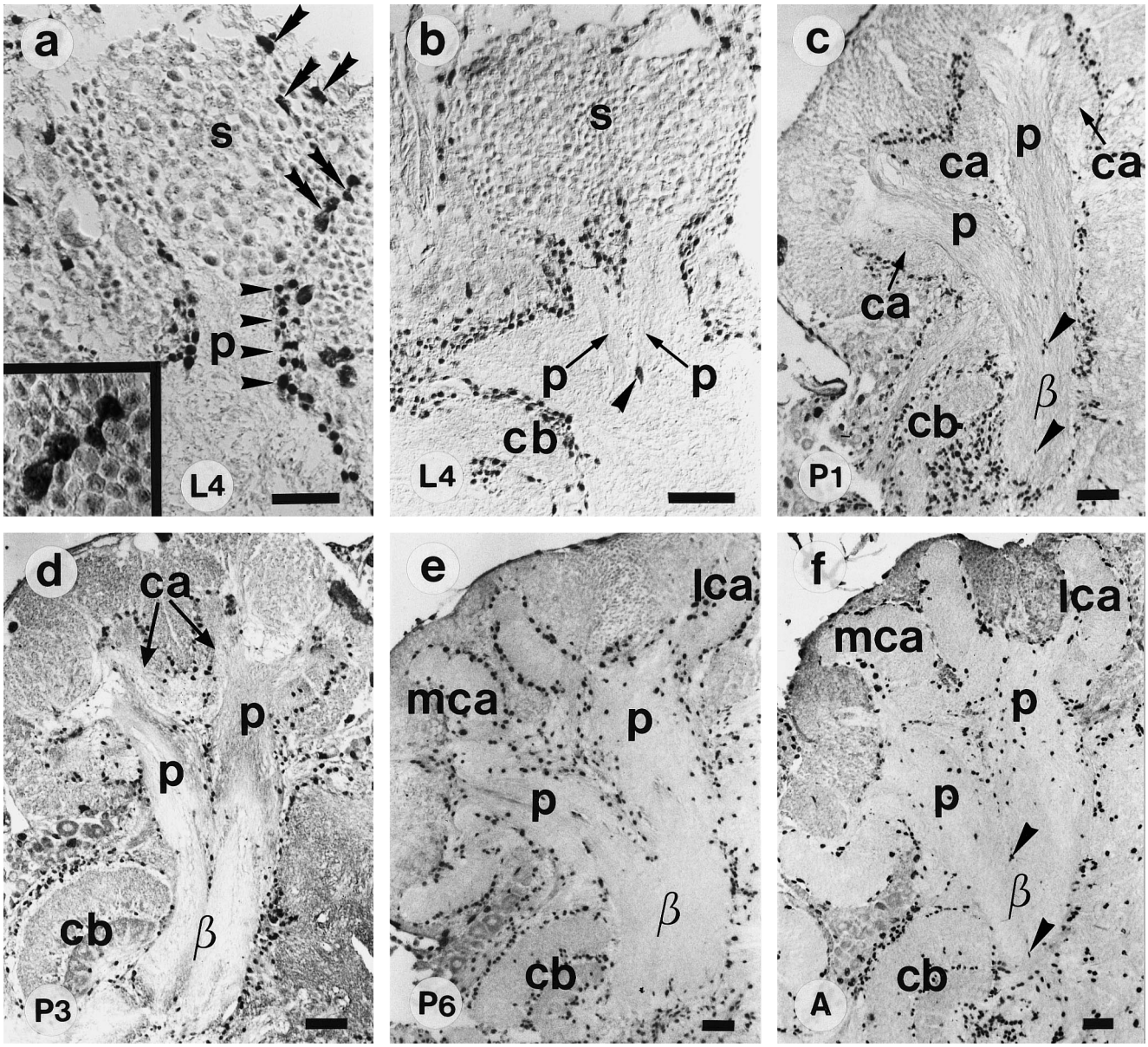

Fig. 6a–f Development of the mushroom body. **a** Section through calyx developmental region at larval stage L4. A soma cluster (*s*) consisting of repo-negative neuroblasts and Kenyon cells is shown. *Double arrowheads* point to the repo-IR nuclei of adjacent glial cells. *Inset* at high magnification shows that some of them are in mitosis. Kenyon cells extend processes into the protocerebral neuropile forming a pedunculus (*p*) which is separated from the surrounding cell bodies by a layer of glial nuclei (*arrowheads*). **b** Larval stage L4. Two peduncle-like fibre strands (*p*) extend from the dorsal cell body rind (*s*) into the neuropile. Within the cell body rind the pedunculi are bordered by a dense layer of glial nuclei. Within the neuropile there are only a few glial nuclei (*arrowhead*). **c** Pupal stage P1. The peduncular projections (*p*) run into the β-lobe (*β*). The dorsal ends of the pedunculus are surrounded by rings of developing calycal neuropile (*ca*). Except for the future calycal bottom, the mushroom body neuropile is entirely surrounded by a thick layer of glial nuclei. A few glial cell nuclei (*arrowheads*) mark the division of the β-lobe into a dorsal and ventral part. **d** Pupal stage P3. The calycal developmental zones (*ca*) are separated from each other by Kenyon cell bodies and repo-IR glia. A thin layer of glial nuclei surrounds the entire mushroom body neuropile except for the calycal bottom. (*β* β-lobe). **e** At pupal stage P6, the nuclei of the neuropile-associated glial cells already show a similar distribution pattern to that in the adult brain. **f** The whole mushroom body, including the bottoms of median (*mca*) and lateral (*lca*) calyces, is surrounded by a layer of glial nuclei. Nuclei of glial cells are located at the boundary between calycal neuropile and pedunculus (*p*). *Arrowheads* point to glial nuclei which are arranged along the longitudinal midline of the β-lobe (*cb* central body, *scale bars* 100 μm, inset **a** 270 μm)

cells with irregular nuclei, the basal ring and lip neuropile do not contain glial nuclei.

Pedunculus and lobes. Separation of the axonal projections into the α- and β-lobes requires a rearrangement of the axonal fibre bundles at the branching sites (Mobbs 1982). In frontal sections of the peduncular branching region, the separated axon bundles appear as finger-like structures (Fig. 1). At the end of larval development, there are hardly any glial nuclei located in this region.

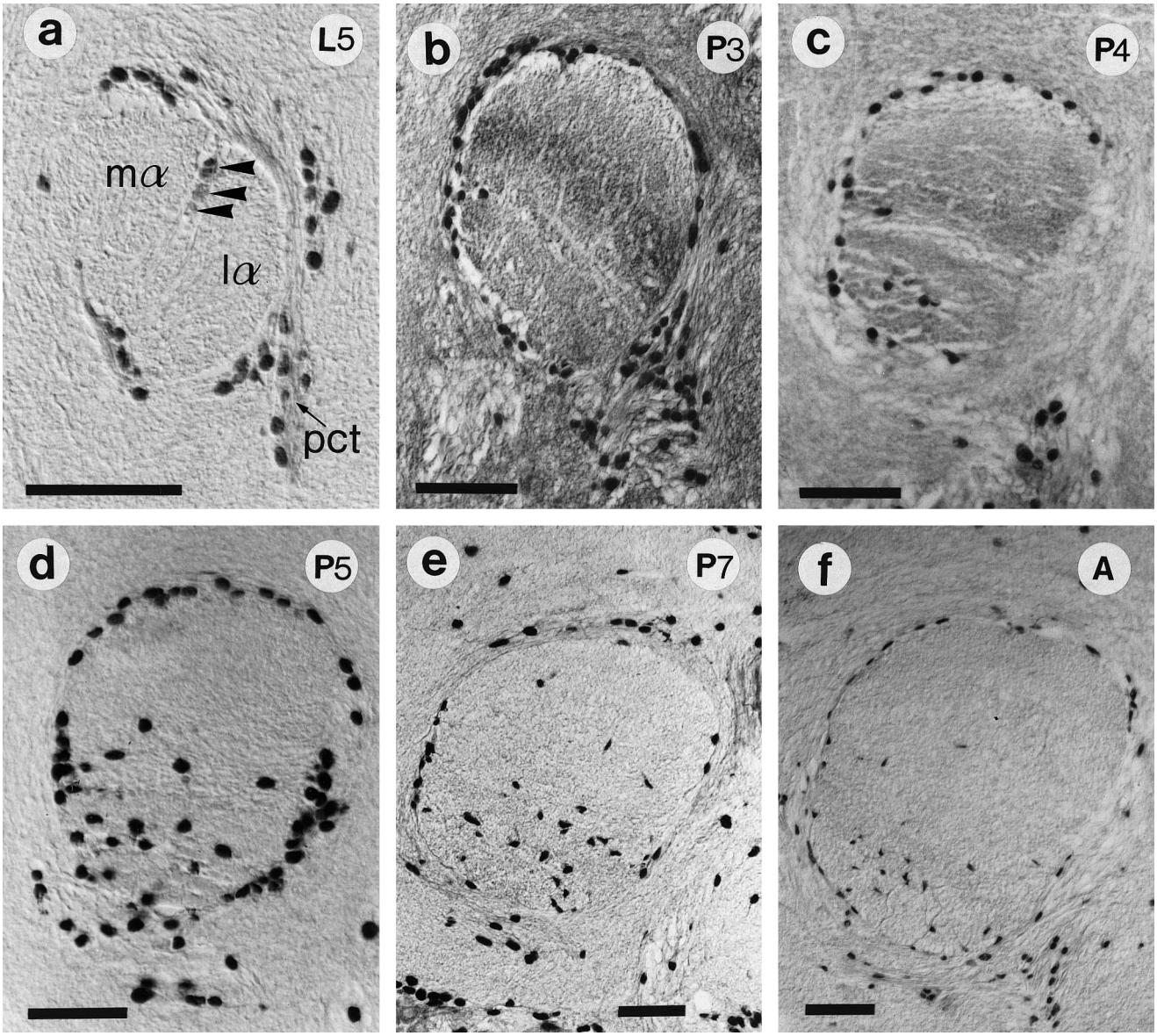

Fig. 7a–f Glial patterning of the α-lobe. **a** Larval stage L5. The α-lobe neuropile shows an internal subdivision into a median (*mα*) and lateral (*lα*) part. Glial nuclei mark the boundary between the α-lobe divisions (*arrowheads*). The protocerebro-calycal tract (*pct*) is escorted by glial cells. **b** Pupal stage P3. The α-lobe is entirely surrounded by the nuclei of glial cells but the neuropile appears nearly free of glial nuclei. **c** Pupal stage P4. First glial nuclei occur within the ventral region. **d** Pupal stage P5 showing considerable increase in glial numbers in the ventral region. **e** Pupal stage P7 and **f** adult stage. Density and distribution of the glial nuclei in the α-lobe at P7 are similar to those of the adult animal. The ventral region is densely interspersed by nuclei of glial cells. Some glial nuclei are scattered throughout the dorsal part. Note the irregular shape of the α-lobe-associated glial nuclei at these developmental stages (*Scale bars* 100 μm)

However, during the pupal phase, glial cell bodies join the distinct bundles of Kenyon cell projections on their way into the two lobes.

In the larval brain, both α- and β-lobes appear longitudinally split into two halves. The internal longitudinal boundaries are associated with glia (Figs. 1b, 7a). The structural bisection of the β-lobe is maintained throughout pupal development up to adult emergence (Fig. 6c–f). In contrast, the internal subdivision of the α-lobe neuropile and related glial boundary disappears completely from pupal stage P2.

During pupal development, the α- and β-lobes acquire their stratified architecture (Mobbs 1982; Rybak and Menzel 1993) which appears in frontal sections as a horizontal banding pattern. During the first pupal stages, the neuropile of the lobes is almost free of glial nuclei (Fig. 7b). In contrast, the protocerebro-calycal tract of the mushroom bodies (Mobbs 1982; Rybak and Menzel 1993) is already densely covered with glial nuclei from the end of larval development (Fig. 7a). From P4, glial nuclei can be detected in the ventral part of the α-lobe (Fig. 7c). During the next pupal stages up to P7, the number of glial nuclei in this region increases considerably, whereas only single nuclei are present in the other

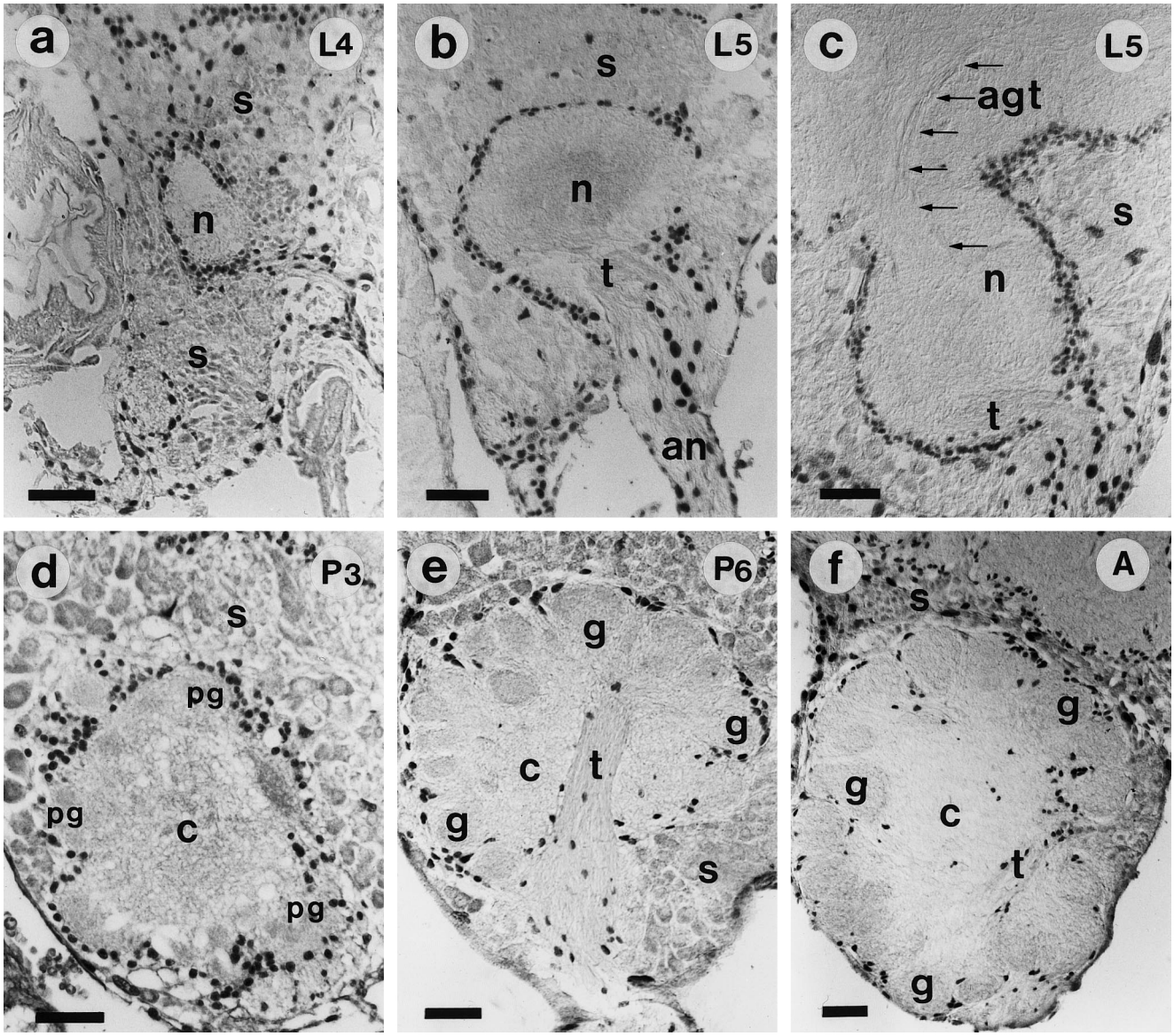

Fig. 8a–f Development of the antennal lobe. **a** Larval stage L4. The antennal lobe consists of a small neuropilar sphere (*n*) which is separated from the surrounding soma rind (*s*) by a layer of glial nuclei. Glial nuclei are also scattered in between the neuronal cell bodies. **b** Larval stage L5. Multiple layers of glial nuclei cover the expanding spherical neuropile (*n*) which is devoid of glial nuclei. The antennal nerve (*an*) is surrounded and interspersed by glial cell nuclei up to its entry into the neuropile. The sensory fibre tracts penetrating into the neuropile (*t*) are completely empty of glial nuclei (*s* soma rind). **c** Larval stage L5, more posterior sectioned than in **b**. *Arrows* indicate the antennoglomerular tract (*agt*) leaving the antennal lobe neuropile (*n*) dorsally (*t* sensory tract, *s* soma rind). **d** Pupal stage P3. The neuropile is divided into a praeglomerular cortex (*pg*) surrounding an unstructured core neuropile (*c*). Glial nuclei cover the outer caps of the praeglomerular complexes. **e** Pupal stage P6 and **f** adult stage. The neuropile is structured into a cortical layer of glomeruli (*g*) surrounding a coarse-textured core (*c*). Whereas the glomeruli are completely devoid of glial nuclei, the core neuropile is interspersed by glial cell nuclei. The sensory fibre tracts of the antennal nerve running inside the neuropile (*t*) contain glial nuclei (*s* soma cluster, *scale bars* 100 μm)

parts of the α-lobe (Fig. 7d, e). Simultaneously with the appearance of the high amount of glial nuclei in the ventral α-lobe, an equivalent high density of glial nuclei is present in the corresponding outer column of the pedunculus (Hähnlein and Bicker 1996). From pupal stage P4 on, single glial nuclei appear scattered throughout the β-lobe neuropile.

Morphology of the glial nuclei

Mushroom body development is accompanied by changes in the size and shape of glial nuclei which are especially obvious in the α-lobe (Fig. 7). During the end of larval and first half of pupal life, all nuclei appear spherical (Fig. 7a–c) with diameters ranging between 4.2 and 4.4 μm. At pupal stage P5, the average diameter of the glial nuclei increases significantly to approximately 6 μm (Fig. 7d). From pupal stage P7 until adult emergence, the nuclei differentiate into various shapes (Fig. 7e, f).

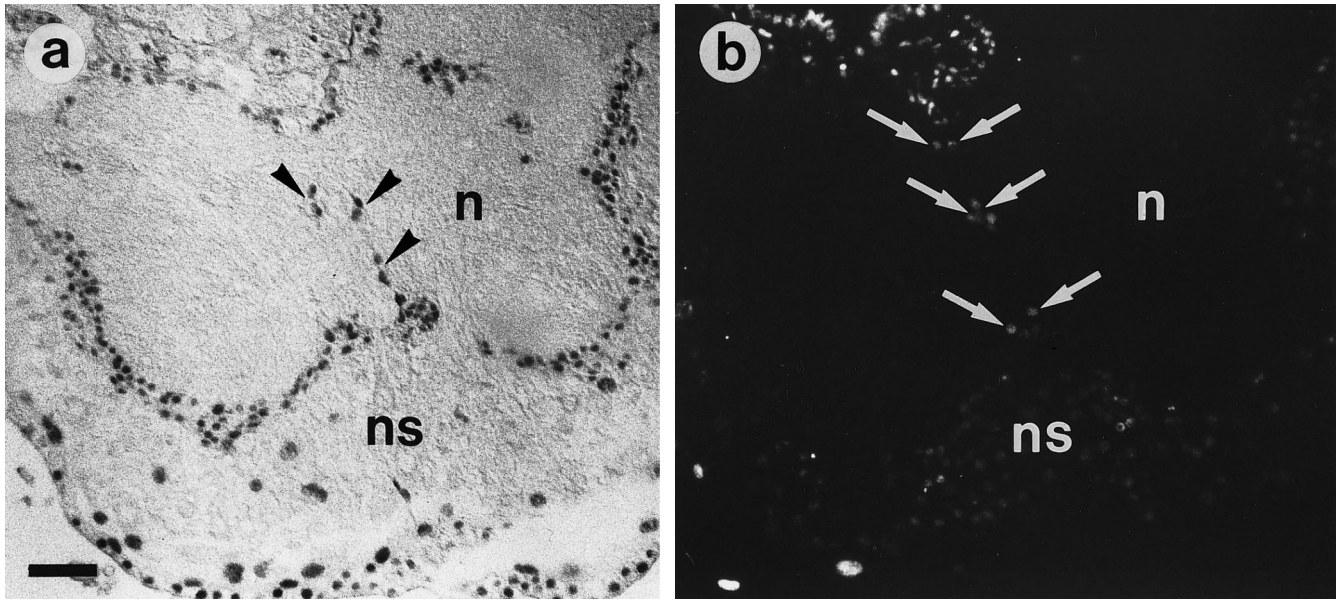

Fig. 9a, b Glial nuclei on a section through the suboesophageal ganglion at larval stage L5. **a** Anti-repo immunocytochemical staining of glial cell nuclei. The neuropile (*n*) is separated from the rind of unstained neuronal somata (*ns*) by a surrounding layer of glial nuclei. *Arrowheads* indicate repo-IR nuclei of glial cells located in the midline-region of the ganglionic neuropile. **b** The same section under UV illumination showing DAPI fluorescence of cellular nuclei. The DAB precipitate in the repo-IR glial cells quenches the DAPI fluorescence of their nuclei. DAPI fluorescence in nuclei along the midline of the neuropile (*n*) reveals repo-negative glial cells (*white arrows*). (*ns* neuronal somata, *scale bar* 100 μm)

Antennal lobe

In the antennal lobe of the adult bee, the synaptic integration between sensory afferents, local interneurons and projection neurons is mainly confined to the spherical glomeruli which surround the central core of coarse-textured neuropile (Mobbs 1985; Arnold et al. 1985; Gascuel and Masson 1991). The principal output of the antennal lobe is carried via the antennoglomerular tracts into the calycal neuropile of the mushroom bodies and into the lateral protocerebrum.

Larval development

Initially, at larval stage L4, the antennal lobe appears as a small neuropilar sphere surrounded by multiple layers of glial nuclei (Fig. 8a). The glial boundary opens at the lateral side of the spherical neuropile to allow for the entrance of neuronal fibres. The neuronal somata are located in a surrounding cell body rind which also contains repo-IR glial somata. The untextured antennal lobe neuropile lacks glial nuclei.

During the last larval stage (L5), the volume of the spherical neuropile increases without visible changes in its internal structure (compare Fig. 8a, b, c). At this stage of development, the sensory axons of the antennal nerve penetrate into the neuropile. Whereas the antennal nerve appears densely interspersed and lined by glial nuclei, its branches inside the neuropile are completely free of glial nuclei (Fig. 8b). During larval stage L5, the projections of the antennoglomerular tracts into the protocerebrum become visible (Fig. 8c). At this early stage these tracts are not escorted by any repo-IR glia (Fig. 8c).

Pupal development

After pupal ecdysis, the initially homogeneously textured neuropile differentiates into glomeruli. This process becomes apparent at pupal stage P2 when the first praeglomeruli arise in the cortex of the neuropile below the glial cover. During the next developmental stage, the number of praeglomeruli increases whilst the surrounding glial layer acquires a scalloped appearance (Fig. 8d). A neuropilar expansion leads to larger distances between the glial cell nuclei and certain nuclei of glial cells become positioned in between the glomerular boundaries. Subsequently, the glial surrounding covers the outer contours of the developing glomeruli (Fig. 8e) whereas glial nuclei can never be detected within the glomerular neuropile. However, from pupal stages P5/6 on, glial nuclei are located within the core neuropile of the antennal lobe (Fig. 8e). From pupal stage P3 on, the first single glial nuclei appear associated with the sensory fibre tracts inside the antennal lobe neuropile. Similar to the antennal nerve, the sensory tracts at P5/P6 are totally interspersed by the nuclei of glial cells (Fig. 8e). Furthermore, glial nuclei start to escort the antennoglomerular tracts leaving the antennal lobe.

At approximately pupal stage P6/7, the formation of the antennal lobe neuropile is nearly completed (Masson and Mustaparta 1990). A comparison of Fig. 8e and f indicates the completion of the adult glial pattern. A layer of mainly flat glial nuclei lines the outer caps of the glo-

meruli. Within the glomeruli glial nuclei are lacking, whereas the core neuropile contains several nuclei. The antennal nerve as well as the sensory fibre tracts running inside the core neuropile are accompanied by many glial cells. Similarly, the antennoglomerular tracts are surrounded and interspersed by glial cell nuclei.

Anti-repo immunocytochemistry

The preceding results have shown that the antibody against the repo-protein of *Drosophila* also stains glial nuclei within the CNS of developing and adult honeybees. The absence of neuronal cell bodies in the neuropile allows us to test whether all neuropile glial nuclei are labelled. Using the fluorescent nuclear label DAPI in double stainings, we examined whether the antibody reveals the nuclei of all glial cells in larval and pupal neuropiles. This procedure indeed exposes paired cellular nuclei which do not express repo-IR within the suboesophageal ganglion (Fig. 9). Due to their median location, these glial cells may belong to midline glia which in *Drosophila* are also repo-negative (Campbell et al. 1994; Xiong et al. 1994; Halter et al. 1995). In contrast, we do not observe any other repo-negative glial nuclei within the postembryonic central brain neuropiles including the median central complex.

Discussion

Glial phenotype

In this study, we used an antibody against the glial-specific repo-protein of *Drosophila* for a fast and reliable detection of glial cell bodies. This antibody stains the nuclei of neuropilar glial cells in the central brain indicating the soma position. The *repo*-gene of *Drosophila* is thought to be essential for the differentiation and survival of the glial phenotype (Campbell et al. 1994; Xiong et al. 1994; Halter et al. 1995). So far, *repo*-expression has also been found in the embryonic grasshopper (Halter et al. 1995). The expression of *repo* in holo- and hemimetabolous insect species suggests an evolutionary conservation of this glia-specific protein. Nevertheless, the question remains whether all of the neuropile glial cells in the bee express *repo*. For example in *Drosophila*, the midline glia of the ventral nerve cord does not express *repo* (Campbell et al. 1994; Xiong et al. 1994; Halter et al. 1995). Similarly, DAPI staining reveals nuclei in the midline region of the bee's suboesophageal neuromeres which do not stain for *repo* (Fig. 9). However, when we compared DAPI and *repo* staining in the central complex, which covers a considerable amount of the anatomical midline of the brain, and the other two brain neuropiles we found no indication of repo-negative glial cells. Thus, we are confident that the antiserum detects the location of all neuropile glia in the investigated areas.

Both in the developing and mature nervous tissue, glial cell distribution across the neuropilar volume is not homogeneous (Hähnlein and Bicker 1996; Hähnlein et al. 1996). This feature is especially enigmatic in regular neuropilar structures such as the symmetrical central body (Fig. 3c,d) or the columnar organized mushroom body (Fig. 7e,f). At present, we have no functional explanation for this unhomogeneous distribution of glia, but it is evident that glial architecture contributes to the diversity of mushroom body organization. Meanwhile, several reports have also shown that Kenyon cell groups are unique, based on their morphological shapes (Mobbs 1982), spacial distribution of neurochemical markers (Bicker et al. 1988, 1993) and gene expression (Yang et al. 1995).

We are presently lacking a detailed cellular classification of the *repo*-expressing glia. It is conceivable that the regional glial pattern reflects a selective distribution of specialized glial cell types. There is ample evidence that, as in vertebrates, insect glia is also composed of different cellular phenotypes. Using immunocytochemical and reporter gene techniques, Ito et al. (1995) have provided a detailed classification of glial cells in the embryonic and larval ventral nerve cord of *Drosophila*. Thus, the study of glial-specific gene expression and the availability of molecular markers will eventually advance our understanding of glial patterns and functions in the insect nervous system.

Indications of glial cell migrations

A hallmark of the basic building plan of insect ganglia is the separation of the peripheral soma rind versus the central neuropile (Wigglesworth 1959; Lane 1974; Strausfeld 1976). At the beginning of the bee's larval development, besides precursor cells, the rind contains the somata of neurons and glial cells (Fig. 2a, b). In comparison, the neuropilar region comprises only processes of differentiating neurons. Thus, the neuropile is completely devoid of repo-IR glial cell bodies as well as any other somata. However, the onset of central brain neuropile formation is characterized by the appearance of associated repo-IR nuclei. Obviously, at least some of these glial cells have to be immigrants from the cell body rind. Several lines of evidence suggest that glial cells use established neuronal structures as a guiding rail. For instance, at the beginning of formation of the central complex, the nuclei of early neuropile glial cells are located in close association with nerve fibres projecting to the central body neuropile (Fig. 2c). At first these glial nuclei mainly occur near the dorsal initial region of the fibres, but later they spread along the whole fibre path and appear to join the layer surrounding the central body neuropile (Fig. 2c). In a similar way, glial cells can move along the outgrowing Kenyon cell axons from their birth place in the dorsal soma rind to their locations in the mushroom body neuropile (Fig. 6b). A further candidate for a neuronal guiding rail for glial migrations is the protocerebro-calycal tract, stretching from the lateral soma regions to the mushroom

body lobes (Fig. 7a). In the pupal antennal lobe, there are indications that glial cells escorting the three sensory fibre tracts through the core neuropile are immigrants from the antennal nerve. Prior to pupal development, the antennal nerve is already densely interspersed by many glial nuclei, whereas its neuropilar branches are lacking repo-IR nuclei (Fig. 8b). However, at the time when the first glial cell nuclei appear in the sensory tracts, the surrounding core neuropile is virtually empty of glial nuclei. Therefore, we have to assume glial migration from the antennal nerve into the neuropilar tracts.

In the course of neuropilar development the number of glial cells increases continuously (Fig. 5). Unlike in the cell body rind (Fig. 6a), our staining method does not provide indications for dividing glial nuclei within the central neuropiles. Therefore, we have to assume that massive glial immigration from the cell body rind may account for the large increase in neuropile glial cells. However, glial divisions have been observed in the neuropile of a postembryonic lepidopteran brain (Nordlander and Edwards 1969). In the absence of estimates about the velocity of migrating glial cells and the duration of glial mitosis, it is difficult to provide a quantitative assessment of both factors.

Several recent studies describe similar glial migrations in the embryonic enteric nervous system of *Manduca* (Copenhaver 1993) as well as during the postembryonic development of the central (Choi and Benzer 1994) and peripheral (Giangrande 1994) nervous system of *Drosophila*. A common feature of all these different migration events is that glial cells respond to guidance cues of prefigured neuronal pathways.

Glial patterns during postembryonic neuropile development

In the early larva, the neuropilar fibre mass of the brain lacks repo-IR cell bodies and compartmentation into central complex, mushroom body and antennal lobe. The appearance of patterned glial cell configurations within the fibre mass indicates the onset of development of the three characteristic neuropilar complexes. A cluster of glial nuclei associated with an early commissural axon bundle marked the anlage of the future central complex (Fig. 2a,b). Furthermore, the anatomical boundaries of all three examined neuropilar complexes are delineated by continuous layers of glial somata (Figs. 2c, 6c, 8a). In essence, the early appearance of glial cells in close association with the developing neuropiles suggests that glial cells may contribute to neuropile formation.

In the antennal lobe of the sphinx moth *Manduca* (for review, Oland and Tolbert 1996), the arrival of sensory afferents influences the peripheral glial cells to form a border around the assembling protoglomeruli. These glial borders are necessary for the maintenance and stabilization of the protoglomerular structure which serves as a template for the subsequent elaboration of the mature glomeruli (Baumann et al. 1996).

Which role do glial cells play during the formation of the intrinsic structure of the central brain neuropiles? In all three larval brain regions examined, the neuropilar architecture seems to differentiate largely without intrinsic glial somata. In essence, our study which is limited by its focus on glial soma position cannot provide evidence for a glial prepatterning of the neuropilar fine structure.

Nevertheless, the fact that glial cell proliferation proceeds during pupal life (Fig. 5), long after neuronal proliferation has been completed, points towards a role for the glia in the maturation of the neuropile. Moreover, there is evidence that the glia itself undergoes a differentiation process during pupal development. We can demonstrate changes in size and shape of the repo-IR nuclei of the mushroom body glia (Fig. 7) indicating a rearrangement of chromatin structure and gene expression. A morphological differentiation of new glial cell types during metamorphosis has also been described in *Manduca* (Cantera 1993).

In summary, reconstructions of ethyl gallate-stained serial sections of adult tissue (Hähnlein and Bicker 1996), lectin-histochemistry of extracellular matrix (Hähnlein et al. 1996) and examination of the developing glial patterns by repo-immunocytochemistry suggest the following sequence of events of how glial cells shape the neuropilar architecture: Initially, glial cells form continuous boundary layers around the differentiating neuropiles. During the course of refinement of the neuropilar architecture, glial cells migrate along neuronal processes into defined locations in the growing neuropile. Glial cells undergo further differentiation processes and start to elaborate the extracellular matrix (Hähnlein et al. 1996), contributing finally via surface interactions to the maturation and stabilization of the adult neuropile.

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