In the article “Predetermined embryonic glial cells form the distinct glial sheaths of the Drosophila peripheral nervous system” we combined our expertise to identify glial cells of the embryonic peripheral nervous system on a single cell resolution with the possibility to genetically label cells using Flybow. We show that all 12 embryonic peripheral glial cells (ePG) per abdominal hemisegment persist into larval (and even adult) stages and differentially contribute to the three distinct glial layers surrounding peripheral nerves. Repetitive labelings of the same cell further revealed that layer affiliation, morphological expansion, and control of proliferation are predetermined and subject to an intrinsic differentiation program. Interestingly, wrapping and subperineurial glia undergo enormous hypertrophy in response to larval growth and elongation of peripheral nerves, while perineurial glia respond to the same environmental changes with hyperplasia. Increase in cell number from embryo (12 cells per hemisegment) to third instar (up to 50 cells per hemisegment) is the result of proliferation of a single ePG that serves as transient progenitor and only contributes to the outermost perineurial glial layer.

Keywords: peripheral glia, peripheral nervous system, Flybow, hypertrophy, hyperplasia

*Correspondence to: Benjamin Altenhein; Email: balt@uni-mainz.de
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Tracing cells throughout development
Insights into single glial cell differentiation

Christian von Hilchen and Benjamin Altenhein*
Institute of Genetics; University of Mainz; Mainz, Germany

Introduction

One of the beauties of neurobiological research in Drosophila is the combination of early development and stem cell biology with functional neurobiology and physiology on a nearly single cell resolution. Previous work on embryonic neural stem cells, so called neuroblasts (NB), and their lineages revealed a precise description of each NB and the progeny at the end of embryogenesis.1-3 Most NBs both in the embryonic brain as well as the ventral nerve cord give rise to neurons. Only six of the 30 NBs per abdominal hemisegment of the embryo generate mixed lineages of neurons and glial cells. In our group we focused on development, specification, and migration of these glial cells in the past. Using a set of molecular markers we are able to identify almost every single glial cell in the central and peripheral nervous system individually.4,5 This enabled us to precisely describe the origin, migration behavior, and final pattern of embryonic peripheral glia (ePG). Despite this knowledge, we were unable to link these ePG to the three different layers surrounding larval peripheral nerves as were described by other groups.6,7 The Flybow method however, allowed us to close this gap.8 We labeled all ePG as single cell Flybow recombination events, identified the cells at late embryonic stages to third instar (up to 50 cells per hemisegment) is the result of proliferation of a single ePG that serves as transient progenitor and only contributes to the outermost perineurial glial layer.

Predetermined Glial Cell Fate

As already mentioned above, our previous work on embryonic peripheral glial cells (ePG) suggested a predetermined cell fate of each ePG with respect to origin, marker gene expression, migratory

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behavior, and final positioning at the end of embryogenesis.\textsuperscript{5} None of these descriptive levels however, allowed us to link any of the ePGs to the three glial layers surrounding peripheral nerves of late third instar larvae. These layers comprise the innermost wrapping glia, the tightly sealing subperineurial glia, and the outermost perineurial glia.\textsuperscript{6,7} Though it was also described that both wrapping and subperineurial glia are addressable with markers (e.g., enhancer-trap Gal4 fly strains nrx2-Gal4 and moody-Gal4, respectively) and each show a constant number of 3–4 cells per abdominal hemisegment at larval stages, it was not evident which ePG becomes a wrapping or subperineurial glia. Marker gene expression is not comparable between embryonic and larval stages, because their expression is variable at least to some extent and might change during development. The fact that the amount of glial cells per peripheral nerve increases enormously during larval stages was complicating matters too, as it was entirely unclear where these additional cells would come from and to which layer they would contribute. With the Flybow system\textsuperscript{8} we were able to unravel these mysteries. Our precise knowledge on positioning of the 12 ePGs as prerequisite, we were able to identify single labeled cells in the PNS and trace these cells into third instar larvae. One example of such a Flybow embryo with labeled glial cells is given in Figure 1. Recombination of the Flybow 1.1 cassette in ePG12 (abdominal segment A2) and ePG11 (segment A4) resulted in a change from EGFP to mCherry expression (Fig. 1A and B). Note that recombination might also result in inversion of the entire Flybow cassette and hence change from cd8-EGFP to cd8-Cerulean expression. Since our filter settings did not allow the detection of this fluorophore, cd8-Cerulean labeled cells appear unstained as can be seen for ePG10 and 11 in segment A3 (Fig. 1B, encircled area). Repetitive labelings of all 12 ePG showed that peripheral glia are indeed predetermined with respect to layer affiliation and association/wrapping of nerve branches including morphology and cellular extension. The reproducible morphology and association with distinct peripheral nerve branches is shown for ePG5 as an example (Fig. 1C–H). In addition, proliferation and mitotic control are also predetermined. Only one of the 12 ePG per hemisegment re-enters mitosis during larval development and generates all additional cells in the PNS. These were astonishing findings, because along each peripheral nerve in the most posterior segments A7 and A8, up to 80 additional

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**Figure 1.** Single peripheral glial cells can be identified in Flybow labelings at late embryonic stages and reveal a highly reproducible differentiation in the larval PNS. (A and B) Filet preparation of a Flybow labeled embryo (repo-Gal4 > Flybow 1.1) at stage 16. The ground state of the repo > Flybow 1.1 is pan-glial expression of cd8-EGFP (green). Recombination within the Flybow cassette may result in change from cd8-EGFP to cd8-mCherry (magenta), to pm-mCitrine (blue) or cd8-Cerulean (not detected with our filter settings and thus not visible, encircled area in A3). (C–H) Three independent labelings of ePG5 as an example reveal that not only layer affiliation (in this case the innermost wrapping layer) but also association with individual nerve branches and morphology are predetermined and highly reproducible. Note that the fuzzy magenta staining at the tip of the SNa in G/H is a preparation artifact. A1–A4, abdominal segments 1–4; SNa-SNd, branches of the segmental nerve; ISN, intersegmental nerve.
cells can be found that all derive from this one transient progenitor, ePG2.

In summary, ePG1, ePG5, and ePG9 become wrapping glia, ePG3, ePG4, ePG7, and ePG10 contribute to the subperineurial glia layer and built the blood-nerve-barrier, and ePG2 (and progeny), ePG6 and ePG8 form the outermost perineurial sheath. ePG11 and ePG12 cannot be assigned to any layer as these cells are the only glia surrounding the dorsal longitudinal nerve and the transversal nerve, respectively. No evident correlation, however, is present between embryonic origin and layer affiliation (Fig. 2). Sensory organ precursor (SOP) derived peripheral glial cells ePG4, ePG5 (both from a ventral SOP), and ePG10 (part of a dorsal SOP lineage) contribute to wrapping and subperineurial layers. NB1–3 derived ePG1 and ePG9 become wrapping glia, while ePG3 and ePG7 from the same NB differentiate into subperineurial cells. The perineurial sheath is built by ePG2 (descendant of NB5–6) as well as ePG6 and ePG8 (both generated by NB2–5). A summary of markers, origin, layer affiliation, and association with peripheral nerves is given in Figure 2. A more detailed description of the molecular markers and their expression patterns can be found in the original article. The genetic program underlying cell specification, terminal differentiation, and function of peripheral glial cells is entirely unclear and remains to be shown in the future.

**Function of the Distinct Glial Layers in the PNS**

The specific function of the different layers is not clear for the innermost wrapping and the outer perineurial layers. The pivotal role of subperineurial glia is to establish the blood-nerve-barrier in the PNS by completely sealing the peripheral nerve tracts and isolating them from the potassium rich hemolymph. The tight isolation of the blood-nerve-barrier is achieved by intercellular connection of the subperineurial glia via septate junctions. Thus, these cells express septate junction markers such as Neurexin IV, Contactin, and Neuroglian, as well as the subperineurial marker Moody.6,9

But what about the other two glial layers? The innermost wrapping glia have been shown to progressively separate axons (bundles or individual axons) within a peripheral nerve tract comparable to vertebrate Schwann cells forming either Remak bundles or separating single axons.7,10 Wrapping glia are the only glial cells that have direct contact to the axon at least with respect to those lying deep
inside nerve fascicles. Thus, one possible function of the innermost cells might be support of axons (e.g., trophic, metabolic, homeostasis). Additionally, wrapping glia might speed up conduction velocity due to an increase of membrane resistance.

Almost nothing is known about the function of the outermost perineurial layer. Results of Stork and colleagues describe a potential barrier function of the perineurial glia at least for larger molecules (around 500nm) or pathogens. Our results suggest that perineurial glia ensheathe the peripheral nerve bundle almost completely along the nerve extension region (NER), but rather form a mesh-like and incomplete layer in the muscle field area (MFA). Maybe the outermost layer is involved in regulating the hemolymph composition or required for the generation of the neurolemma / basal membrane? The amount of perineurial glia along the NER is tightly controlled and corresponds to the length of the peripheral nerve. The cells persist metamorphosis as we could show for some Flybow events where animals were allowed to develop into adulthood. Thus, perineurial glia are important for the animal and discovering their precise function will be an interesting task.

**Hypertrophy vs. Hyperplasia**

In general there are two ways to realize tissue growth – increasing cellular volume (hypertrophy) or increasing the amount of cells via proliferation (hyperplasia). For glial cells of the PNS, both mechanisms exist. Innermost wrapping glia and subperineurial glia show enormous hypertrophy, best seen in the NER where they become up to one millimeter in length. In contrast, outermost perineurial glia divide and compensate for nerve elongation in the same area by increasing cell number from one cell in the embryo (ePG2) up to 50 cells or even more in late larval stages. But why do these different glial cells react on larval growth and nerve elongation in such different ways? We think one major reason for the observed hypertrophy of the subperineurial glia is the fact that these cells have to maintain a sealed blood-nerve-barrier. Thus, any disruption of the connectivity of individual glial cells within this sheath during mitosis would cause a leaky barrier. Similar results have been reported for subperineurial glia of the developing brain. A similar reason might account for the hypertrophy of wrapping glia. Although the precise function of this glial subtype is currently unknown (see above), an even contact between these cells and neurons has to be guaranteed. In contrast, perineurial glia undergo extensive proliferation in the NER. These cells have no direct contact to axons and are obviously not essential for the sealing of the PNS. Apparently their function is not linked to a constant cellular connectivity and thus enables this glial subtype to undergo cell proliferation.

**Cell Specific Mitotic Control**

One of the most astonishing results of our work was the finding that only the perineurial glial cells along the NER undergo cell proliferation starting from a single transient precursor, ePG2. The unique ability of ePG2 to re-enter mitosis must on the one hand go along with distinct intrinsic cellular properties. On the other hand the precise number of perineurial cells along a certain nerve depends on the length of this nerve and therefore presumably requires multiple steps of cellular interactions between glia and glia as well as glia and neurons. In this context, Larvery and colleagues (2007) could already show that thickness of the perineurial glial layer is controlled by underlying subperineurial cells. But thickness and cell number do not necessarily correlate. Pandey and colleagues also show that in brave mutants less glial cells are found along shortened nerves, again indicating that cell specific proliferation of perineurial cells is controlled by underlying peripheral nerves. In contrast to these data, we observed the same amount of glial cells in tubby mutant larvae (which are about 30% shorter) compared with wild type animals of the same stage. We cannot interpret this discrepancy, nor do we know the underlying signals for both perineurial specific proliferation and subperineurial/ wrapping glial cell growth. We assume that growing nerves either actively send signals to both wrapping and subperineurial glia or are sensed by these cells. In any way, cells of both glial layers are in intimate contact with neurons and grow along with axonal elongation during larval development. Growing subperineurial glia in turn might send signals to the outermost perineurial cells which in response proliferate. Ablation experiments in addition revealed only a partial compensatory ability by peripheral glial cells. If ePG2 was ablated, the remaining glial cells were able to partially compensate for the loss of this perineurial progenitor in only half of the cases. We were not able to clarify which glial cell generates these (perineurial) cells in the absence of ePG2. We assume that either ePG6 in the MFA or most likely perineurial glial cells of the ventral nerve cord (which have not been described so far) proliferate in the absence of ePG2. It is unlikely that ePG1 or ePG3 compensate for the loss of ePG2, because a general block of mitosis in the perineurial subtype only abolishes the generation of additional perineurial cells completely and results in glial cell numbers comparable to the late embryo. These results indicate that (1) glial cells of the inner layers have no compensatory abilities, (2) proliferation capacity is regulated by intrinsic properties, (3) developmental stimuli such as systemic growth and/or axonal elongation have different effects (hyperplasia vs. hypertrophy) within the same area of a segment and between segments of the same animal. The nature of these stimuli and signal transduction pathways that lead to these different outputs need to be addressed in the future. Perineurial glia of the brain also undergo extensive proliferation during larval development in response to Insulin-like receptor/Target of rapamycin and fibroblast growth factor receptor signaling. Such paracrine signals, however, are unlikely to account for the mitotic control of perineurial glia in the PNS, because cell numbers within the same animal increase from anterior to posterior segments.

**Conclusions/Outlook**

Flybow offers a perfect system to label and trace individual cells throughout development. For our approach on peripheral glial cells, the precise knowledge about individual cell identities was an indispensable prerequisite for successful
identification of the cells in the late embryo and thus the subsequent description in L3 larvae. The knowledge about the origin of each glial layer and the opportunity to manipulate them specifically using either subtype specific Gal4 driver lines or ablation experiments will help to unravel the function of each glial layer, probably even down to the precise function of individual cells. We will extend this work to CNS glia and also follow cells beyond metamorphosis into the adult. In future studies, the possibility to genetically manipulate a distinct subgroup or to perform subgroup specific RNAi-mediated knock-down experiments will further allow to decipher cell–cell interactions and signaling cascades required for specific behavior of the cells, e.g., hyperplasia vs. hypertrophy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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