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Predetermined embryonic glial cells form the distinct glial sheaths of the *Drosophila* peripheral nervous system

Christian M. von Hilchen¹, Álvaro E. Bustos¹, Angela Giangrande², Gerhard M. Technau¹ and Benjamin Altenhein^{1,*}

SUMMARY

One of the numerous functions of glial cells in *Drosophila* is the ensheathment of neurons to isolate them from the potassium-rich haemolymph, thereby establishing the blood-brain barrier. Peripheral nerves of flies are surrounded by three distinct glial cell types. Although all embryonic peripheral glia (ePG) have been identified on a single-cell level, their contribution to the three glial sheaths is not known. We used the Flybow system to label and identify each individual ePG in the living embryo and followed them into third instar larva. We demonstrate that all ePG persist until the end of larval development and some even to adulthood. We uncover the origin of all three glial sheaths and describe the larval differentiation of each peripheral glial cell in detail. Interestingly, just one ePG (ePG2) exhibits mitotic activity during larval stages, giving rise to up to 30 glial cells along a single peripheral nerve tract forming the outermost perineurial layer. The unique mitotic ability of ePG2 and the layer affiliation of additional cells were confirmed by *in vivo* ablation experiments and layer-specific block of cell cycle progression. The number of cells generated by this glial progenitor and hence the control of perineurial hyperplasia correlate with the length of the abdominal nerves. By contrast, the wrapping and subperineurial glia layers show enormous hypertrophy in response to larval growth. This characterisation of the embryonic origin and development of each glial sheath will facilitate functional studies, as they can now be addressed distinctively and genetically manipulated in the embryo.

KEY WORDS: Flybow, Cell tracing, Glial sheaths, Peripheral nervous system, Hyperplasia, Hypertrophy, Cell-specific mitotic abilities, *Drosophila*

INTRODUCTION

During the development of higher metazoan animals, the maintenance and functionality of the nervous system depend on a precise interplay between neurons and glial cells. Whereas neurons are known to perceive, transmit and integrate information encoded as electrical impulses, glial cells have been shown to accomplish multiple tasks in both vertebrates (reviewed by Barres, 2008) and invertebrates (reviewed by Parker and Auld, 2006; Stork et al., 2012). One pivotal role of glial cells in *Drosophila* is the correct insulation of neurons. In the peripheral nervous system (PNS) this is achieved by three distinct glial layers surrounding the peripheral nerves (Banerjee et al., 2006; Stork et al., 2008) (reviewed by Rodrigues et al., 2011). The innermost layer is formed by wrapping glia, which progressively separate the axons of the 30 motoneurons and 42 sensory neurons of each abdominal hemisegment (hs), projecting together along the main peripheral nerve tracts. At the early larval stage, groups of axons are enwrapped by these glial cells, similar to the Remak bundles formed by Schwann cells in the vertebrate PNS, and, later in development, the peripheral axons of *Drosophila* become individually separated and isolated. The second layer is built by subperineurial glia surrounding the entire nerve bundle. They represent the key component of the blood-brain barrier

in *Drosophila* and are tightly connected to each other via pleated septate junctions (Bainton et al., 2005; Schwabe et al., 2005; Stork et al., 2008). The outermost glial sheath is established by perineurial glia (Stork et al., 2008), for which a mesodermal origin has been proposed (Edwards et al., 1993). At present, the precise function of this glial sheath is unclear, beside being a barrier for macromolecules (Stork et al., 2008).

Formation of the glial sheaths starts during embryogenesis. Most of the embryonic peripheral glia (ePG) arise from neural stem cells (neuroblasts) of the central nervous system (CNS) (Schmidt et al., 1997). After birth, ePG migrate out of the CNS to their final positions along the peripheral nerves in order to subsequently ensheath the axons along all parts of the PNS. Recently, we showed that a fixed number of 12 ePG are born in abdominal hs A2-A7 (von Hilchen et al., 2008). Each ePG can individually be identified by its origin, the expression of a specific set of markers, and its stereotypic migration pattern that results in a characteristic positioning at the end of embryogenesis. However, a clear assignment between embryonic and larval peripheral glia has not been accomplished to date.

Here we have used the Flybow system (Hadjieconomou et al., 2011) to label each ePG individually, and followed their development until third instar. We reveal the embryonic origin of all three glial layers and describe the larval differentiation of each peripheral glial cell. Wrapping and subperineurial glia maintain a constant number from embryo to late L3 larvae and undergo enormous hypertrophy that correlates to the length of the peripheral nerves. By contrast, the perineurial sheath originates from a single ePG (ePG2) that remains mitotically active throughout larval development. It represents a transient precursor that produces cells in accordance with the length of the peripheral nerve. This unique ability was further confirmed by *in vivo* ablation experiments and

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cell-specific block of mitosis. In addition, we provide examples to show that at least some ePG survive metamorphosis and persist until adulthood.

MATERIALS AND METHODS

Fly stocks and genetics

The following fly strains were used: wild-type OregonR, *nrv2-Gal4* (Sun et al., 1999), *repo-Gal4* (Sepp et al., 2001), *Gli-Gal4* (Sepp and Auld, 1999), *moody-Gal4* (Schwabe et al., 2005), *46F-Gal4* (Xie and Auld, 2011), *Mz97-Gal4* (Ito et al., 1995), UAS-*gap::GFP* [Bloomington Drosophila Stock Center (BDSC)], UAS-stinger-GFP (nGFP) (Barolo et al., 2000), UAS-*CycA*-RNAi (Vienna Drosophila RNAi Center, 32421GD) and UAS-*Rbf* (Du et al., 1996).

For *in vivo* ablation of ePG2, the *Mz97-Gal4* insertion was combined with UAS-nGFP and *repo-Gal4*. For single-cell tracing via the Flybow system, *repo-Gal4* (either recombined with nGFP on the third chromosome or alone) was combined with heat shock mFlp5 and crossed to homozygous UAS-FB1.1 flies (Hadjieconomou et al., 2011). Whenever required, GFP-tagged balancer chromosomes (BDSC) were used for identification of genotypes.

Generation of anti-Repo antibodies

repo full-length cDNA was cloned into pQE31 (Qiagen) in frame with 6×His at the N-terminus and expressed in *E. coli* strain SG13009 (Qiagen). Purification of recombinant protein was performed using the NiNTA kit (Qiagen). Protein was injected into guinea pigs by Pineda Antibody Service (Berlin, Germany), followed by two booster injections after 30 days. Animals were sacrificed, serum collected and the IgG fraction isolated. Specificity of the antibody was controlled *in vivo* by immunohistochemistry of wild-type and *glial cells missing* mutant embryos as well as *in vitro* on western blots (data not shown).

Immunohistochemistry

Embryos were fixed as described (Rogulja-Ortmann et al., 2007). Second and third instar larvae (L2/L3) were opened along the dorsal midline and flattened to a silicon ground using minuten fine pins. Larvae were fixed for 45 minutes in 4% formaldehyde in PBS and incubated for at least 1 hour in PBS containing 0.5% Triton X-100 and 10% milk powder. Stained larvae were scanned with a Leica TCS SP5 confocal microscope. For complex staining patterns of L3 larvae, an overhead transparency was fixed to the computer monitor to draw labelled cells stepwise throughout the stack. Primary antibodies were mouse anti-Fas2 (1D4; 1:10) and mouse anti-22C10 (1:10), both from the Developmental Studies Hybridoma Bank (Iowa City, USA), mouse anti-GFP (Promega; 1:500), rabbit anti-NrxIV (1:500; gift from C. Klämbt, University of Münster, Germany), guinea pig anti-Repo (1:1000) and mouse anti-phospho-Histone H3 (Cell Signaling Technology; 1:1000).

Secondary antibodies were Cy3-, Cy5- or FITC-conjugated (all from donkey; Jackson ImmunoResearch; 1:250). For imaging, a Leica TCS SPII or SP5 confocal microscope was used. Images were processed using Leica Confocal Software and Adobe Photoshop.

Labelling single peripheral glial cells using Flybow

Overnight egg collections from *hs-mFlp5/CyO.Dfdl-EYFP; repo-Gal4* (nGFP) crossed with UAS-FB1.1 were heat shocked for 80 minutes at 37°C in a water bath followed by 1 hour incubation at 18°C. Then, embryos were kept at 25°C for ~4-5 hours to enable development until embryonic stage 16-17. Documentation of cell labelling events was carried out *in vivo* with a Leica DM 5500B fluorescence microscope connected to a Leica DFC 350 FX camera. Only recombination events that led to expression of mCherry were analysed. Embryos with labelled glial cells were then individually transferred to breeding vessels containing Instant *Drosophila* Medium BLUE, FORMULA 4-24 (Carolina Biological Supply, Burlington, NC, USA) and cultivated at 25°C until L3 stage. Finally, animals were dissected as described above and recorded on a Leica TCS SP5 confocal microscope. If required, additional antibody staining for Repo, NrxIV or 22C10 was performed. Some individuals were kept for documentation of glial labelling in the imago.

Ablation of single peripheral glial cells in the embryo

UV ablation of single ePG was performed as described (Soustelle et al., 2008). In one *hs* ePG2 was irradiated for 45 seconds using a UV power of

2 mW. Larvae were transferred to breeding vessels containing standard fly food and kept at 25°C until they reached L3. Dissection, antibody staining and documentation were performed as described above.

RESULTS

Extension of peripheral nerves and the pattern of associated glia during larval development

During embryogenesis of *Drosophila*, a fixed number of 12 ePG per *hs* arise from known central neuroblasts and sensory organ precursors in the trunk. They display individual identities reflected in the expression of specific marker genes. In addition, *in vivo* recordings revealed that each ePG migrates along peripheral nerve tracts in a stereotypical fashion, resulting in a characteristic positioning in the PNS at the end of embryogenesis (von Hilchen et al., 2008) (Fig. 1A,A'). During larval stages the number of glial cells along each peripheral nerve increases and these cells contribute to three different glial layers or sheaths around the nerves (Stork et al., 2008). However, to date it has not been elucidated whether (all) ePG survive during larval development, from which progenitors additional cells derive, and to which glial sheath they contribute.

Regarding the overall morphology of the PNS, no gross changes between the embryonic and late third instar (L3) stages are recognisable, as all nerve tracts established during embryogenesis are well preserved (Fig. 1). The main difference is an the enormous elongation of peripheral nerves, in particular the section proximal to the region where segmental nerves (SNa-SNd) separate from intersegmental nerves (ISN) (Fig. 1B). This extreme longitudinal extension is accompanied by massive growth of the larval body to preserve the integrity of neuromuscular as well as sensory connectivity. The corresponding region in the embryo is significantly shorter (Fig. 1A,B). Hence, we call this region the nerve extension region (NER). During larval development, growth of the NER gradually increases from anterior to posterior abdominal *hs* (the peripheral nerves of *hs* A8 and A9 fasciculate together). For instance, the NER of A7 increases ~50-fold in length during larval development (compare Fig. 1A,B).

Another difference between embryo and larva is in the increasing number of glial nuclei along the NER, apparently correlating with the total length of this section (Fig. 1D). In the embryo and L1 larva three ePG (ePG1-ePG3) always populate the NER of *hs* A2-A7, whereas their number progressively increases up to five cells in L2 and up to a median of 23 cells along A7 in L3 larvae ($n=11$ *hs*, Fig. 1D). Even more glial nuclei can be observed in A8/A9, with a median of 80 nuclei ($n=17$ *hs*, Fig. 1D).

In contrast to the deviating numbers of glial nuclei along the NER, a constant number is found in the more distal part of the peripheral nerves, henceforth called the muscle field area (MFA) (Fig. 1A,B). In the embryo, nine ePG (ePG4-ePG12) are present in the MFA (Fig. 1A'). In L3 the same number of glial nuclei (median of nine, $n=13$ larvae) can be detected in this region (Fig. 1C). The constant number of glial cells in the MFA supports the idea of a one-to-one relation between embryonic and larval cells. The increasing number of glial nuclei along the NER raises the question of the correlation between embryonic and larval identities and of the origin of these additional cells.

Identification of individual peripheral glia using molecular markers

In the past, different marker lines were used to label the perineurial, subperineurial or wrapping glia in the larval PNS (Stork et al., 2008; Xie and Auld, 2011) (reviewed by Rodrigues et al., 2011). Here, we

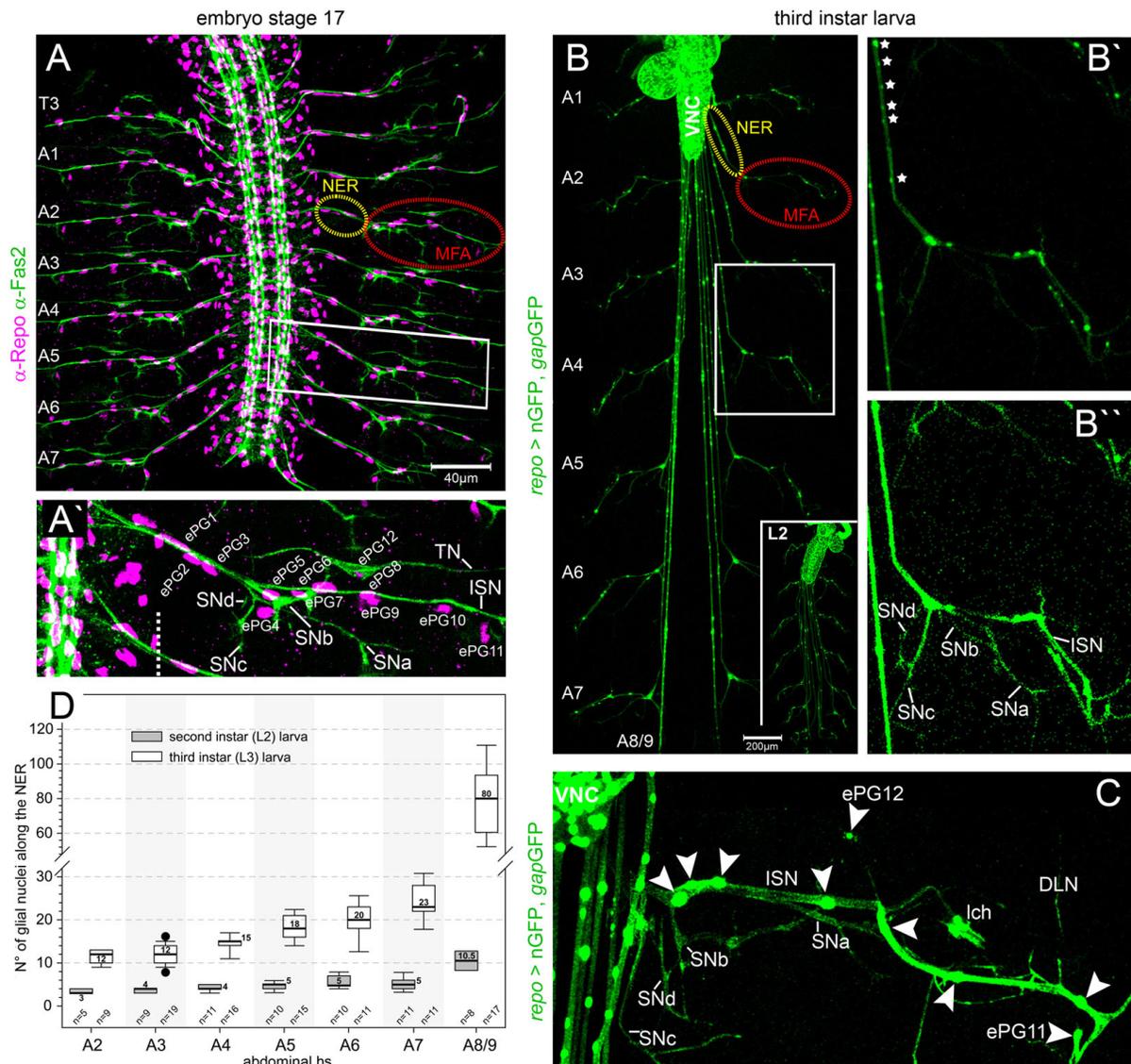


Fig. 1. Extension of peripheral nerves and the increasing number of glial nuclei along the NER during *Drosophila* larval development. (A,A') Confocal stacks of a dissected wild-type stage 17 embryo stained for the glial marker Reversed polarity (Repo, magenta) and the neuronal cell-adhesion molecule Fasciclin 2 (Fas2, green). (A) Pattern of glial nuclei in the embryonic CNS and PNS (segments T3-A7). In hemisegment (hs) A2 (right side) the nerve extension region (NER) is marked by a yellow dotted circle and the muscle field area (MFA) by a red dotted circle. (A') Higher magnification of the boxed area in A; 12 embryonic peripheral glia (ePG1-ePG12) occupy characteristic positions along peripheral nerve tracts. Three (ePG1-ePG3) are located in the NER and nine in the MFA (ePG4-ePG12). These are associated with the segmental nerve (SN; ePG4), the intersegmental nerve (ISN; ePG5-ePG10), the Fas2-negative dorsal longitudinal nerve (DLN; ePG11) and the transversal nerve (TN; ePG12). Dotted line indicates the edge of the ventral nerve cord (VNC). (B-C) Confocal stacks of a dissected L3 larva carrying *repo-Gal4>UAS-nuclear-GFP* (nGFP) and *UAS-gap::GFP* transgenes (endogenous signal). Anterior is up. (B) Overview of an L3 nervous system showing extended peripheral nerve tracts (compare the distance to the posterior rim of the VNC). In particular, the length of the NER (marked in A2 on the right by the yellow dotted circle) increases enormously along with an increasing number of glial nuclei in this region. (B') As an example, already six peripheral glia nuclei (white stars) can be detected in this magnified part of the NER from B. (B'') The intensity of the green channel was amplified to better visualise the fine branches of the segmental nerve (SNa-SNd) in the MFA (red dotted circle in B). For comparison, an L2 nervous system is shown at the same scale (inset, bottom right in B). (C) As in the embryo, nine peripheral glial cells exist in the MFA at L3 (arrowheads), two of which can be identified as ePG11 and ePG12 due to their exposed position. (D) Quantification of the number of PG nuclei along the NER of hs A2-A8/9 in L2 and L3. Boxes encompass the 25th and 75th percentiles, bold horizontal lines indicate the median, whiskers indicate the 10th and 90th percentiles, and black circles the 5th and 95th percentile outliers; total numbers of analysed hs are indicated (n).

compared some of these markers between embryonic and larval stages on a single-cell level in order to follow individual ePG throughout development (Fig. 2; supplementary material Fig. S1, Movie 1).

The enhancer construct *nrv2-Gal4*, a marker commonly used for the innermost wrapping glia (Stork et al., 2008; Sun et al., 1999),

drives expression in three glial cells per embryonic abdominal hs with slight variability (Fig. 2A, $n=101$ hs/eight embryos). Based on position, these *nrv2-Gal4*-positive ePG can be identified as ePG1, ePG5 and ePG9. The same number of *nrv2-Gal4*-positive cells can be detected in L3 larvae at comparable positions. This suggests that ePG1 in the NER, ePG5 at the branching point of the SN, and ePG9

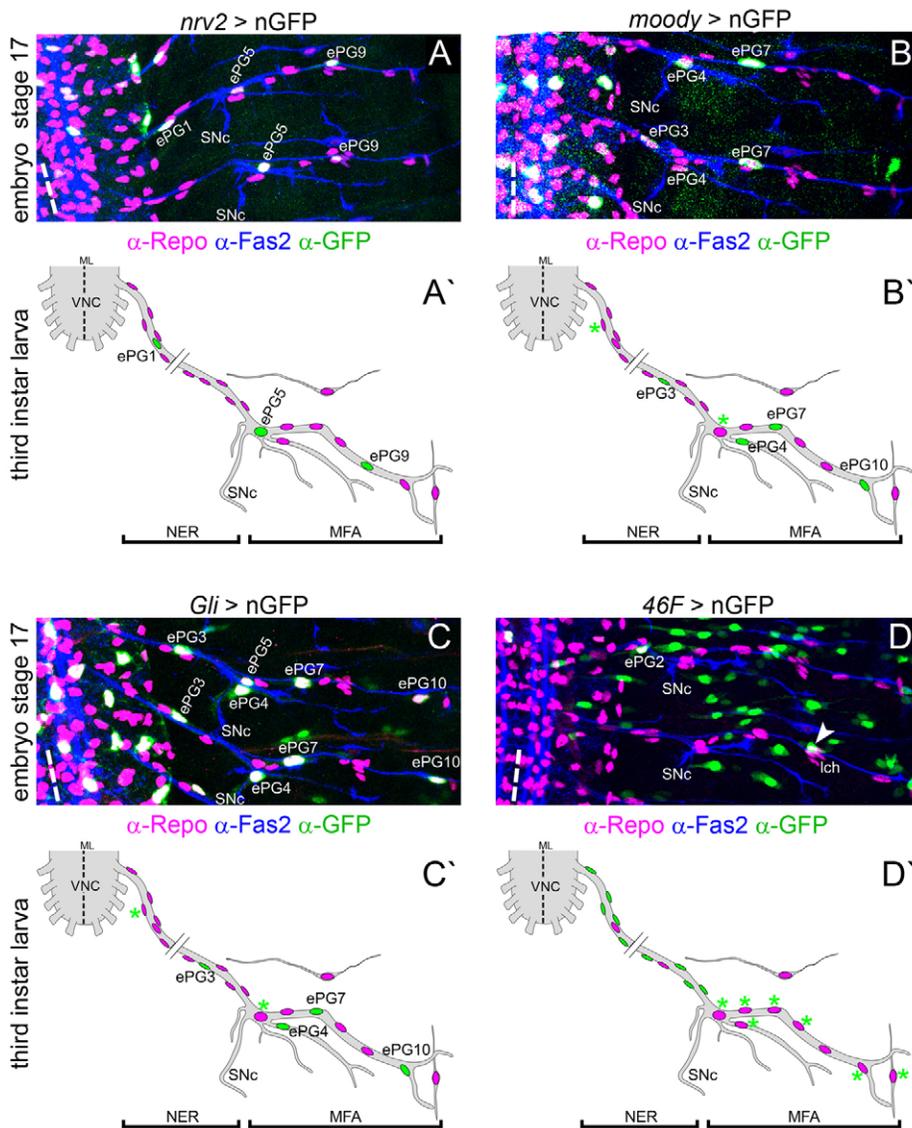


Fig. 2. Marker lines to trace individual ePG up to L3. (A–D) Confocal stacks of stage 17 *Drosophila* embryos (showing two abdominal hs each) of the indicated marker strains, stained for Repo (magenta), Fas2 (blue) and GFP (green). (A'–D') Larval expression patterns of marker lines presented as schematics of one abdominal hs in L3 showing the subset of peripheral glia labelled by each marker line (green); it is not practicable to illustrate all ePG of one hs in a maximum projection of the original scans. Anterior is up. ML, midline (dotted line). (A) In the embryo, *nrv2*-Gal4 drives expression in ePG1, ePG5 and ePG9. (A') The same glial cells are *nrv2* positive in L3. (B) In the embryo, the subperineurial glia marker *moody*-Gal4 is expressed by ePG3, ePG4 and ePG7. (B') In L3, ePG3 is constantly *moody*-Gal4 positive, as is frequently a second glial cell along the NER (asterisk). In the MFA, ePG4, ePG7, ePG10 and in rare cases ePG5 (asterisk) are *moody* positive. See also supplementary material Fig. S1 and Movie 1. (C) *Gli*-Gal4 is expressed in ePG3, ePG4, ePG7 and ePG10 in the embryo. Sporadically, ePG1, ePG5 and ePG9 also show reporter expression. (C') Along the NER of L3, ePG3 and often a second PG (asterisk) are marked by *Gli*-Gal4. In the MFA, ePG4, ePG7 and ePG10 are labelled. Rarely, ePG5 appears positive as well (asterisk). (D) The perineurial marker strain *46F*-Gal4 sporadically labels ePG2 in the NER and ePG4 and ePG10 in the MFA of stage 17 embryos. The arrowhead marks a presumptive GFP-positive macrophage lying above the *46F*-Gal4-negative ligament cells of the lateral chordotonal organ (lch). (D') All except for two peripheral glia along the NER of L3 are *46F*-Gal4 positive. In the MFA almost all glia can be labelled by this marker line (asterisks), except for ePG9. SNc, segmental nerve c; VNC, ventral nerve cord.

more distal at the ISN persist until late larval stages to build the wrapping glia layer (Fig. 2A').

For labelling of subperineurial glia, several marker strains exist, of which *moody*-Gal4 and *Gli*-Gal4 are described here. Both subperineurial markers drive expression in three ePG in the embryo, which are ePG3 in the NER and ePG4 and ePG7 in the MFA (Fig. 2B, $n=39$ hs/four embryos; 2C, $n=37$ hs/four embryos). Expression of *moody*-Gal4 starts late during embryonic development and often not all three ePG show a detectable signal at early stage 17. By contrast, *Gli*-Gal4 is also expressed in ePG10, and occasionally in ePG1, ePG5 or ePG9. In the larva both Gal4 lines constantly drive expression in ePG3 and, in around half of the hs, also in a second cell along the NER ($n=40$ hs/four larvae). In the MFA, three *moody*- and *Gli*-positive glia can consistently be recognised at the position of ePG4, ePG7 and ePG10 (Fig. 2B', $n=28$ hs/five larvae; 2C', $n=14$ hs/three larvae). In rare cases (8%), ePG5 is labelled in addition.

For labelling of perineurial glia along the peripheral nerves, the enhancer-trap line *46F*-Gal4 has recently been described (Xie and Auld, 2011). Analysis on a single-cell level revealed faint expression of *46F*-Gal4 in stage 17 embryos (suggesting a late onset

of Gal4 expression), which was restricted to ePG2 in the NER and to ePG4 and ePG10 in the MFA (Fig. 2D, $n=115$ hs/nine embryos).

Remarkably, in L3 all glial cells along the NER are *46F*-Gal4 positive (except two, presumably ePG1 and ePG3) (Fig. 2D', $n=22$ hs/four larvae). In the MFA, expression is detected in all glial cells in a variable fashion, except for ePG9, which never expresses *46F*-Gal4 (Fig. 2D', $n=27$ hs/six larvae).

Comparison of marker expression already indicates that ePG persist until larval stages and contribute to predetermined layers. However, we cannot rule out the possibility that marker expression might be transient and therefore labelled cells in the embryo and in late larvae might be of different identities. In order to more precisely follow all ePG individually throughout larval stages and confirm persisting marker gene expression, we next used the Flybow technique to label single glial cells in the embryonic PNS and analyse their layer affiliation and morphology in L3.

The origin, proliferation behaviour and differentiation of glia cell types in the larval NER

The Flybow technique provides the possibility to genetically and continuously label cell clones or single cells (Hadjiceconomou et al.,

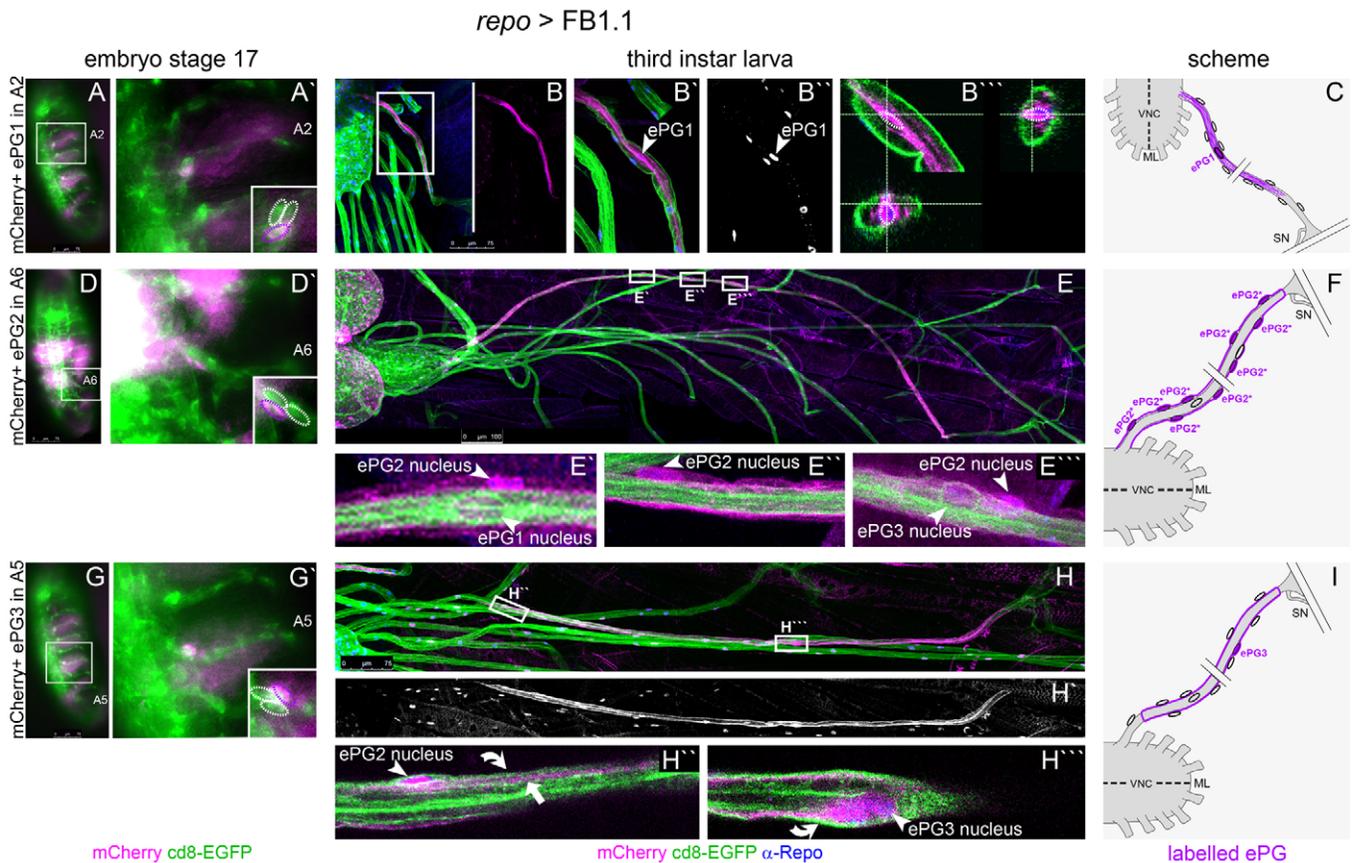


Fig. 3. Flybow labelling reveals the layer affiliation and the differential control of hyperplasia and hypertrophy for ePG1-3 along the NER. (A,A',D,D',G,G') Maximum projections of confocal stacks showing mCherry-labelled ePG1, ePG2 and ePG3, respectively, in living *Drosophila* embryos at stage 17 in an overview (A,D,G) and (boxed areas) at higher magnification (A',D',G'). cd8-EGFP-positive (white dotted circles) and mCherry-positive (magenta dotted circle) ePG in the NER are marked. Anterior is up. (B-B''') In L3, ePG1 extends almost along the entire NER, enveloping the axon bundles within the nerve. (B) Maximum projection; on the right mCherry signal is shown alone. (B',B'') Magnified section of the boxed area in B (B'), with Repo staining shown alone (B''). The nucleus of ePG1 lies within the fascicle (arrowhead). (B''') Transverse/sagittal section; dotted circles mark the position of the ePG1 nucleus. (C) Illustration of ePG1 morphology in L3. (E-E''') ePG2 establishes the outermost perineurial sheath along the NER in L3. All except two nuclei are mCherry positive, indicating that all of these derive from ePG2. Anterior is to the left. (E) Maximum projection. (E'-E''') Enlarged views of the boxed areas in E presenting separations of selected focal planes showing the mCherry-positive ePG2 and the mCherry-negative ePG1 lying within the fascicle (E') and ePG3 positioned between the wrapping and perineurial sheath (E''). (F) Representation of the perineurial glial sheath and the numerous nuclei derived from ePG2. (H-H''') ePG3 forms the subperineurial sheath that almost completely surrounds the NER in L3. (H,H') Maximum projection. Anterior to the left. Although only ePG3 is labelled by mCherry (the entire cell is visible), other glial nuclei also appear mCherry positive due to a strong Repo signal bleeding through. (H') mCherry alone for better visualisation of ePG3 hypertrophy. (H'',H''') Higher magnification (selected focal planes) of the indicated areas in H showing cd8-EGFP-positive membranes of ePG2 (bent arrow) covering both the ePG3 membrane (H'', arrow) and nucleus (H'''). (I) Illustration of ePG3 morphology. ML, ventral midline; VNC, ventral nerve cord; SN, segmental nerve.

2011) and to follow their development. We used the Flybow1.1 construct to label single ePG, identify them *in vivo* based on their characteristic position at the end of embryogenesis and follow these cells throughout larval stages. As mentioned above, the NER shows striking differences between embryo and larva with respect to glial proliferation/hyperplasia and growth/hypertrophy (both depending on the length of the nerve).

The proximal-most ePG in the NER (ePG1) is positive for *nrv2-Gal4*, indicating that this cell contributes to the wrapping glial layer in late larval stages. Single-cell labellings of ePG1 at late stage 17 ($n=6$) revealed that this cell does not divide and resulted in a proximal wrapping glia in L3 being the only glia located within the fascicle enveloping axons along the entire NER (Fig. 3A-C). Particularly in posterior hs, this goes along with the enormous hypertrophy of this cell, with an expansion of as much as 1 mm or more in length (supplementary material Fig. S2, Movie 2).

Flybow labelling of ePG2 ($n=8$) shows that this glial cell gives rise to the outermost perineurial layer, surrounding the complete NER. Furthermore, this cell appears to be the precursor of all additional glial nuclei arising during larval development in the NER (Fig. 3D-F). This hyperplasia is inferred from the observation that embryonic ePG2 labelling always resulted in mCherry labelling of all glial nuclei in the NER except for two. One mCherry-negative nucleus can clearly be assigned as ePG1 by its position and characteristic morphology (Fig. 3E'). The second mCherry-negative glial cell lies between the wrapping and perineurial layer, suggesting that this cell is part of the subperineurial sheath (Fig. 3E''').

Labelling of ePG3 ($n=3$) revealed that this cell does not divide and forms the subperineurial layer within the NER (Fig. 3G-I). Like ePG1, ePG3 undergoes enormous hypertrophy to ensheath and seal the major part of the nerve bundles along the NER. The most proximal part of the NER is not covered by ePG3 protrusions but is

surrounded by the membrane extensions of a subperineurial glia of the ventral nerve cord (VNC) (supplementary material Fig. S3). The nucleus of this cell might also lie in the periphery, resulting in two (instead of one) *moody*-Gal4-positive or *Gli*-Gal4-positive subperineurial glia along the NER (Fig. 2B',C').

Taken together, ePG1, ePG2 and ePG3 differentially respond to larval growth (hyperplasia versus hypertrophy) and form distinct layers along the NER.

Origin and unique mitotic activity of perineurial glia along the NER

Ablation experiments were performed to confirm that additional glial nuclei along the NER of larvae originate from ePG2. High-energy UV irradiation was used to eliminate ePG2 in the embryo, a method previously used to ablate glial cells in the developing fly wing (Aigouy et al., 2008; Aigouy et al., 2004).

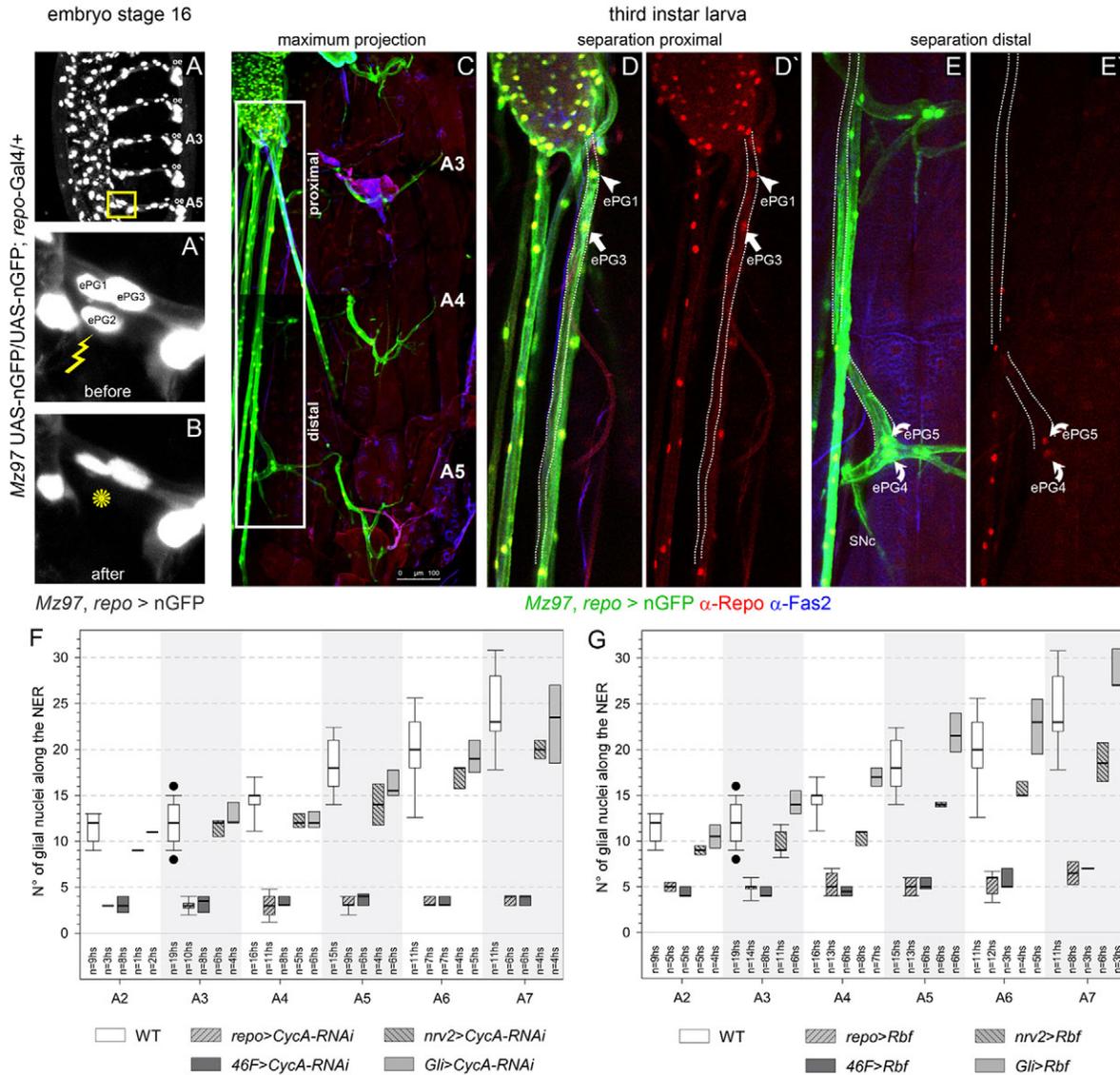


Fig. 4. Ablation experiments and subgroup-specific block of mitosis determine that all additional glial nuclei along the NER in larva belong to the perineurial glia and arise from ePG2. (A) Maximum projection of endogenous nGFP in the living *Drosophila* embryo driven by *repo*-Gal4 to visualise glial nuclei and by *Mz97*-Gal4 for clear identification of abdominal hs due to expression in oenocytes (oe). (A') Higher magnification of the boxed area in A showing the NER of hs A5. In this hs, ePG2 can be recognised as the most posterior glial cell. For ablation, ePG2 was UV irradiated (yellow flash). (B) This results in a loss of ePG2 (asterisk), whereas the remaining glial cells along the NER are unaffected. (C-E') The same animal after dissection at L3 stage, showing endogenous nGFP (green), anti-Repo (red) and anti-Fas2 (blue) staining. Since nerves in A4 and A5 are closely attached to each other (boxed region in C), nerve A5 is highlighted by dotted lines in magnifications (D,E; Repo staining alone in D',E'). Only two remaining glial cells (ePG1, ePG3) are found along the proximal part of the NER in A5 (D,D'). The distal part of the NER is completely free of glial nuclei. In the MFA, glial number is unaltered (E,E'; the most proximal ePG4 and ePG5 are marked). In all images anterior is up. (F,G) Quantification of the results obtained from pan-glial or subgroup-specific knockdown of *CycA* (F) and ectopic expression of *UAS-Rbf* (G). Boxplots (explanation as Fig. 1D) show the numbers of PG nuclei along the NER of the hs A2-A7. For each hs non-parametric statistics were performed (Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's multiple pairwise comparison test), consistently demonstrating a significant decrease in glial number compared with wild type ($P < 0.05$) only when using either pan-glial (*repo*>) or perineurial glia (*46F*>) specific driver lines. For each genotype at least four larvae were analysed. Numbers of evaluated hs are given beneath boxplots.

Indeed, in 11 out of 14 specimens ablation of ePG2 at embryonic stage 16 resulted in a dramatic reduction of glial nuclei along the corresponding NER in L3 (supplementary material Fig. S4). In four of these just two remaining glial nuclei (ePG1 and ePG3) were found (example shown in Fig. 4). These results are in line with Flybow labelling of ePG2 and confirm that a single mitotically active glial cell (ePG2) is the origin of additional glial nuclei in the NER of L3. The loss of ePG2 and hence the loss of perineurial glia along the NER cannot, or only partially, be compensated for by other glial cells (the origin of which is presently unknown).

In order to confirm that all additional glial cells along the NER are perineurial glia arising from ePG2, we performed subgroup-specific inhibition of mitosis either by knocking down *Cyclin A* (*CycA*) or by ectopic expression of *Retinoblastoma-family protein* (*Rbf*). Larval glia proliferation along the NER can be blocked completely by pan-glial knockdown of *CycA* (*repo>CycA-RNAi*) resulting in a median number of three PG in hs A2-A6 (and four in A7) of L3 larvae. A similar result is observed upon perineurial glia-specific (*46F>CycA-RNAi*) knockdown of *CycA*, whereas *CycA* knockdown in wrapping (*nrv2>CycA-RNAi*) or subperineurial (*Gli>CycA-RNAi*) glia does not affect glial number along the NER significantly (Fig. 4F).

Furthermore, preventing the G1-to-S phase transition by ectopic expression of UAS-*Rbf* (reviewed by Dyson, 1998) in all glial cells (*repo>Rbf*) or exclusively in the perineurial glia (*46F>Rbf*) resulted in a reduction of glial nuclei along the NER similar to *CycA* knockdown (Fig. 4G), with a median of four to six PG per hs. Neither wrapping (*nrv2>Rbf*) nor subperineurial (*Gli>Rbf*) glia expression of *Rbf* affected glial numbers significantly.

We conclude that the perineurial layer is built by progeny of ePG2. Since ePG2 derives from a CNS neuroblast [NB5-6 (Schmidt et al., 1997; von Hilchen et al., 2008)] the perineurial layer is not formed by mesodermal cells as proposed previously (Edwards et al., 1993). Owing to the continuous perineurial sheath formed by the derivatives of ePG2 (Fig. 3E-E'') we were not able to clarify whether ePG2 undergoes complete cytokinesis or just karyokinesis during larval development.

The origin and differentiation of glial cells in the larval MFA

The number of glial nuclei in the muscle field of L3 (see Fig. 1C) as well as marker expression suggest that all ePG in the embryonic MFA (ePG4-ePG12) persist during larval development. We aimed to confirm this using the Flybow technique. Hence, we traced the origin/identities and contribution of each identified ePG to the three distinct glial sheathes in the larval MFA.

Wrapping glia

In addition to a single wrapping glia in the NER (ePG1), two more can be identified in the MFA via Flybow due to their characteristic larval morphology. These cells are ePG5 (Fig. 5A-C, $n=6$) and ePG9 (Fig. 5D-F, $n=1$), both extending within the nerve bundle being surrounded by cd8-EGFP-positive subperineurial and perineurial glia (see transverse section in Fig. 5B,E). ePG5 protrusions extend into the NER, surround all SN branches and reach distally up to the region where sensory axons of the lateral chordotonal organ (lch) fuse with the ISN (Fig. 5B). Fine ePG5 projections even penetrate the lch (Fig. 5B''). Membrane extensions of ePG9 separate the axons along the distal part of the ISN (Fig. 5E-F).

Subperineurial glia

Flybow labellings of ePG4, ePG7 and ePG10 reveal that these cells build the subperineurial sheath along the SN and ISN, each covering discrete nerve branches. ePG4 ($n=5$) exclusively covers the nerve branches of SNa and SNc (Fig. 6A-C). ePG7 (Fig. 6D-F, $n=7$; supplementary material Movie 3) surrounds SNb and SNd, the proximal part of the ISN and the lch in L3. In addition, ePG7 protrusions extend from the ISN anteriorly to come into contact with ePG12 at the transversal nerve (TN) (Fig. 6E,F). Along this connection between ISN and TN, neurons can be detected that express the 22C10 (Futsch – FlyBase) epitope (supplementary material Fig. S5), indicating that ePG7 protrusions ensheath sensory neurons in this area. ePG10 represents subperineurial glia that completely cover the distal part of the ISN (Fig. 6G-I, $n=6$). In addition, ePG10 surrounds the sensory neurons of the dorsal

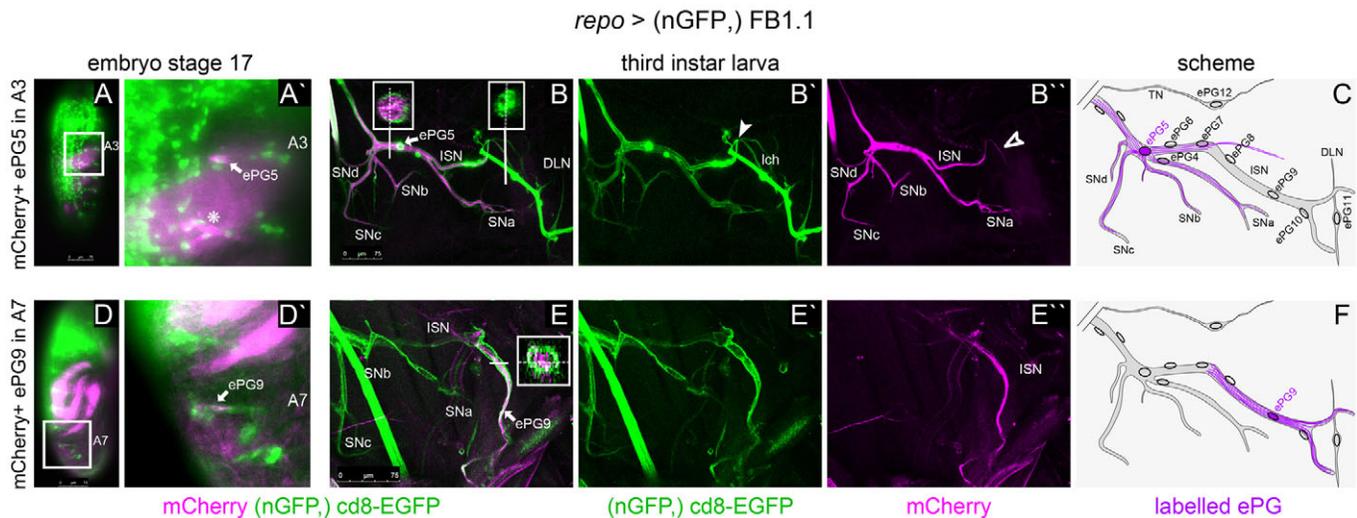


Fig. 5. Origin and morphology of wrapping glia in the MFA. (A,A',D,D') Maximum projections showing the mCherry-labelled wrapping glia ePG5 and ePG9, respectively, in living *Drosophila* embryos at stage 17 in overview (A,D) and at higher magnification (A',D'). Anterior is up. (A') Multiple labelling of ePG can be seen in hs A4 (asterisk) beside ePG5 in A3. These events were not analysed further. (B-B'') In the same individual in L3 stage, ePG5 membranes enwrap axons along the distal part of the NER, all SNs, the proximal part of the ISN and along the lateral chordotonal organ (open arrowhead in B''), which is connected to the ISN (arrowhead in B'). (C) Illustration of ePG5 morphology in L3. (E-E'') ePG9 membranes enwrap axons along the distal part of the ISN. (F) Schematic of ePG9 morphology.

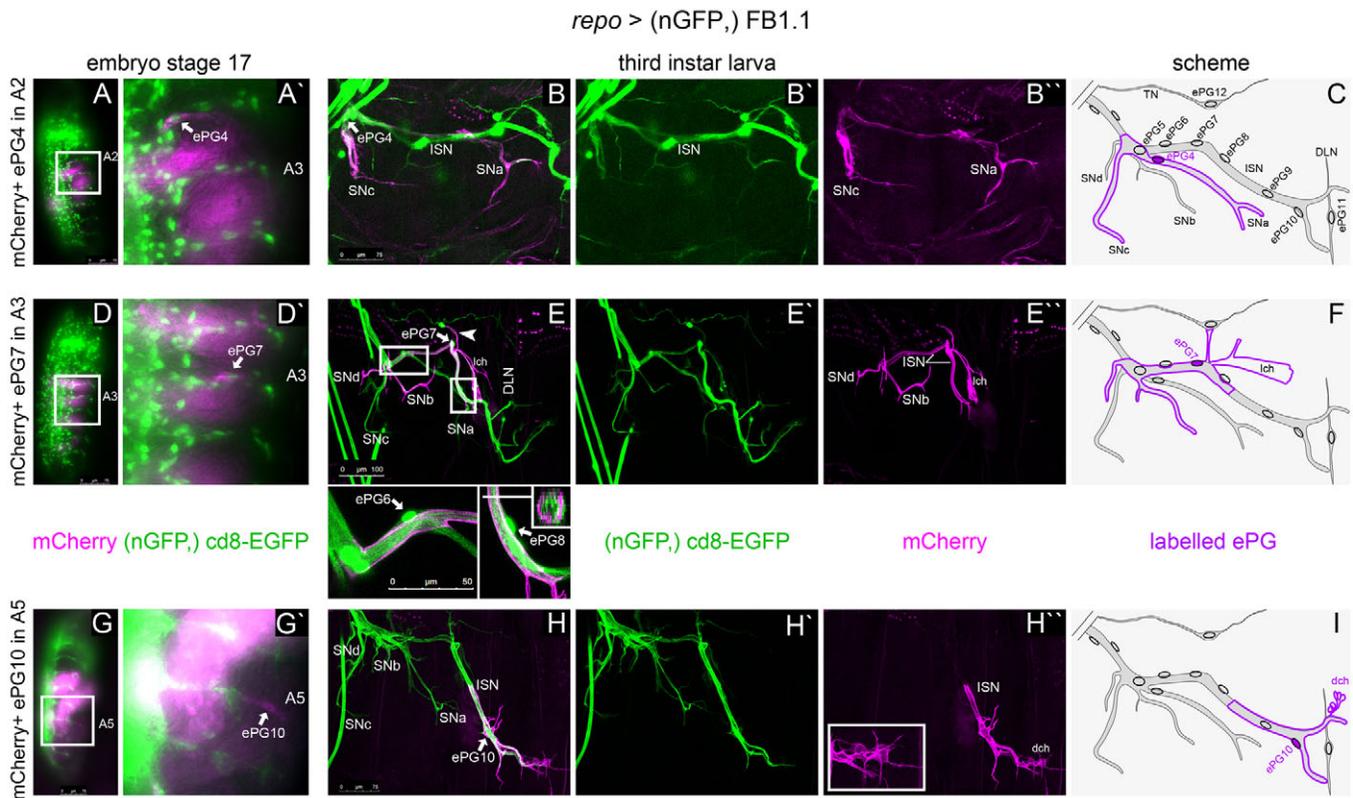


Fig. 6. Origin and morphology of subperineurial glia in the MFA. (A,A',D,D',G,G') Maximum projections illustrating the mCherry-labelled subperineurial glia ePG4, ePG7 and ePG10, respectively, in living *Drosophila* embryos at stage 17 in overview (A,D,G) and at higher magnification (A',D',G'). Anterior is up. (B-B'') In L3, ePG4 seals the entire SNc and SNa. (C) Schematic of ePG4 morphology. (E-E'') Labelling of ePG7 reveals that this glial cell establishes the subperineurial sheath along the SNb and SNd, the proximal part of the ISN and the lch. ePG7 surrounds neurons projecting between the ISN and TN (arrowhead in E). Magnifications of selected focal planes and transverse section (beneath E, boxed regions) clearly demonstrate that ePG6 and ePG8 lie on top of ePG7. (F) Schematic of ePG7 morphology. (H-H'') ePG10 surrounds the distal part of the ISN and the sensory neurons of the dorsal chordotonal organ (dch). (I) Schematic of ePG10 morphology.

chordotonal organ (dch) (Fig. 6H''). This coincides with previous observations made by Han et al. (Han et al., 2011) showing that the sensory neuron ddaC and additional sensory neurons of the dch are ensheathed during embryonic and larval development by glial membranes. These can now be linked to ePG10.

Subperineurial glia express the cell-adhesion molecule Neurexin IV (NrxIV), a structural component of septate junctions required for the proper establishment of the blood-brain barrier (Banerjee et al., 2011; Banerjee et al., 2006; Baumgartner et al., 1996; Stork et al., 2008). Antibody staining against NrxIV on Flybow clones of ePG4, ePG7 and ePG10 confirms their subperineurial identity (supplementary material Fig. S6).

Perineurial glia

The outermost perineurial sheath in the MFA is formed by ePG6 and ePG8, which are both clearly positioned on top of the subperineurial layer (see Fig. 6E). Labelling ePG6 (Fig. 7A-C, $n=7$) demonstrates that this cell surrounds the proximal part of the ISN and occasionally a short section of the NER. Moreover, ePG6 extends protrusions that incompletely cover SNa, SNc, occasionally also SNb and SNd, as well as parts of the lch (Fig. 7B''). The larval morphology of ePG8 is shown in Fig. 7D-F ($n=4$). In particular, the distal part of the ISN is covered by ePG8 processes. Occasionally, ePG8 protrusions project proximally along the ISN to some extent. Such projections may even reach and partially cover the SNb (Fig. 7E''). Moreover, fine

protrusions of ePG8 sometimes ensheath parts of the lch (Fig. 7E''). This mesh-like ensheathment by perineurial glia in the MFA appears to be incomplete (see Fig. 7E,E''). A similar morphology has been described in the *Drosophila* brain, where perineurial glia do not form a tight layer but rather a cellular mesh (Hartenstein, 2011).

Peripheral glia ensheathing DLN and TN

The remaining ePG11 and ePG12 each ensheath a distinct nerve tract. ePG11 covers the dorsal longitudinal nerve (DLN) (Fig. 7G-I, $n=6$). ePG12 surrounds at least the distal part of the TN in the MFA (Fig. 7J-L, $n=5$). The TN does not fasciculate with other nerves in the NER but projects alone, entering the VNC dorsally through the dorsal median cells (Luer et al., 1997) (supplementary material Fig. S7). It is not reasonable to classify ePG11 and ePG12 into any of the described layers as each cell forms only a single sheath around their respective nerves. In addition, marker gene expression in these two cells does not allow their allocation to one of the three glial subtypes.

The origin and larval development of each individual ePG and their contribution to the three glial layers is summarised in Table 1.

Tracing VNC glia and ePG to adulthood

We also attempted to trace individual glial cells into the adult. The specimen presented in Fig. 8 shows ePG6 in A5 as an example. It is clearly identifiable due to its position in the embryo (Fig. 8A,A'),

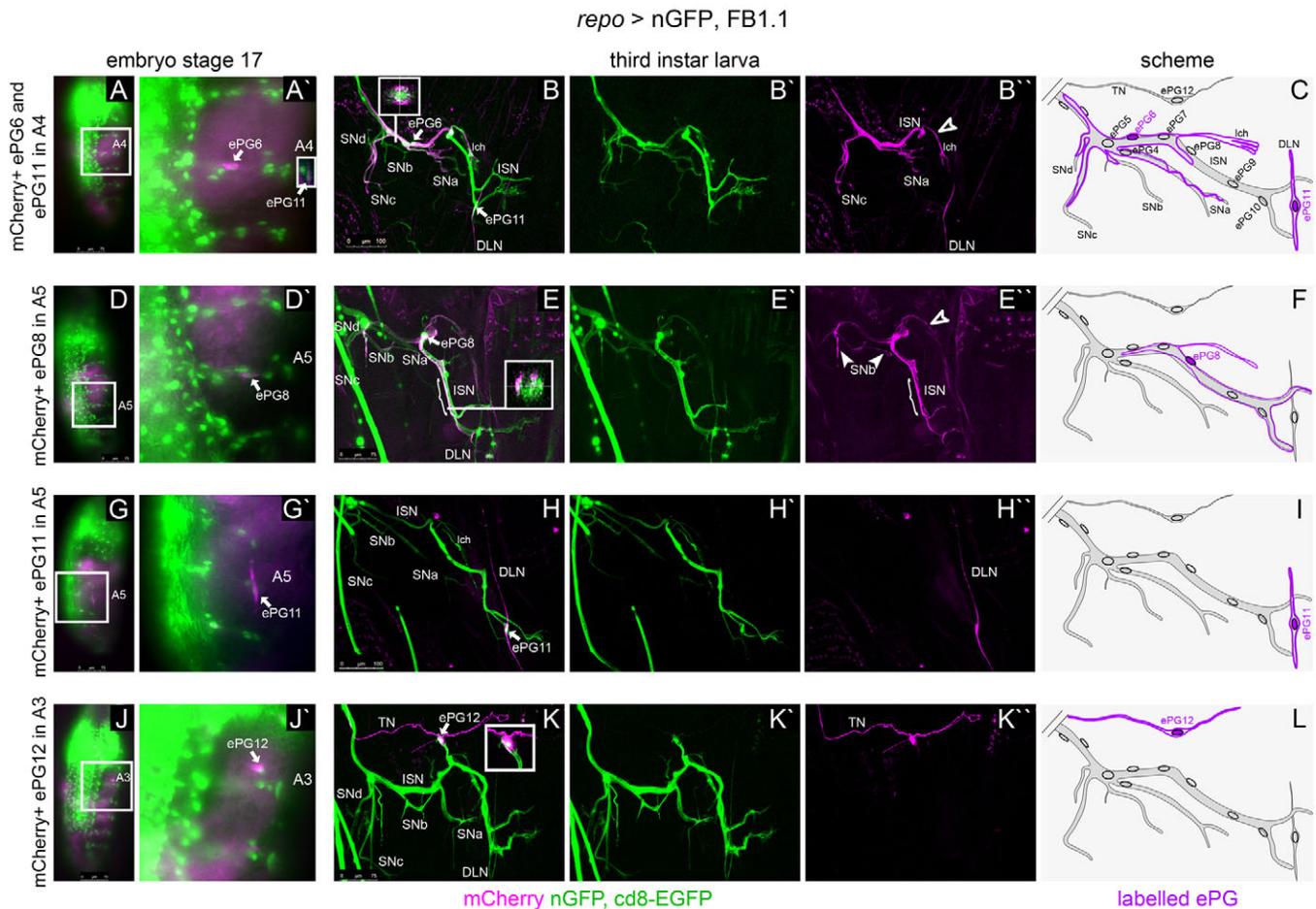


Fig. 7. Origin and morphology of perineurial glia and glia covering DLN and TN in the MFA. (A,A',D,D',G,G',J,J') Maximum projections showing mCherry-labelled ePG6, ePG8, ePG11 and ePG12, respectively, in living *Drosophila* embryos at stage 17 in overview (A,D,G,J) and at higher magnification (A',D',G',J'). Anterior is up. (A-C) Both ePG6 and ePG11 are labelled in hs A4. ePG11 (boxed area in A') is shown in a deeper focal plane. (B-B'') In L3, ePG6 forms the perineurial sheath in the MFA, surrounding the proximal region of the ISN, SNa and SNC, as well as parts of the Lch (open arrowhead in B''). A short section of the distal part of the NER is also covered by ePG6 protrusions. The ensheathment by ePG6 is incomplete, as can be seen in a transverse section (inset in B). (C) Illustration of ePG6 morphology. (E-E'') ePG8 forms the perineurial sheath along the distal part of the MFA, in particular surrounding the distal ISN. ePG8 protrusions can also partially cover the SN (here SNb) and the Lch. The incomplete ensheathment of the ISN is evident in a transverse section (inset in E) within the zone marked by the brackets (E,E''). (F) Schematic of ePG8 morphology. (H-H'') ePG11 is the only glial cell along the DLN. (I) Schematic of ePG11 morphology. (K-K'') The distal part of the TN is covered by ePG12. The inset in K shows at higher magnification the ePG12 soma surrounded by ePG7 protrusions. (L) Schematic of ePG12 morphology.

survives metamorphosis, and can be detected in the adult fly (Fig. 8B) covering the proximal part of the ISN. In the same specimen, a second glial cell is labelled within the VNC. Based on its position in the embryo this cell can be identified as a medialmost cell body glia (MM-CBG) (Ito et al., 1995) (Fig. 8C,C'), which derives from NB6-4 (Dittrich et al., 1997). In the corresponding hs of the imago, the mCherry-labelled MM-CBG can still be detected (Fig. 8D-E'). It exhibits a sponge-like morphology surrounding neuronal somata in the cortex of the adult VNC (for the morphology of this glial subtype in L3, see also supplementary material Movie 4).

These examples show that the Flybow technique, in combination with the *in vivo* identification of individual cells in the late embryo and their known origin, allows identified cells to be traced from the embryonic stem cell to the imago.

DISCUSSION

In this study we used the Flybow system (Hadjieconomou et al., 2011) to trace individual embryonic glial cells up to third instar

larvae and even into the imago. We concentrated on ePG of abdominal hs A2-A7 for several reasons. First, this glial subgroup comprises a defined population of 12 cells (per hs) of known origin and identity (von Hilchen et al., 2008). Second, it was already well described that peripheral nerves are covered by three distinct glial layers formed from inside out by wrapping, subperineurial and perineurial glia, with molecular markers available to distinguish them (Bainton et al., 2005; Stork et al., 2008; Xie and Auld, 2011). However, to date it has not been shown whether and to what extent cell specification of ePG contributes to functional differences and hence to the generation of these glial layers. Finally, the number of ePG increases during larval development in correlation with the length of the peripheral nerve. Yet, to date, neither the origin of these additional cells nor the mechanism that controls the number of glial cells in response to peripheral nerve growth has been deciphered.

Using the Flybow system to label individual ePG in the embryo and subsequently analyse their larval morphology reveals that all 12 ePG survive at least until the third instar larval stage. We further

Table 1. Summary of the origin, marker expression, proliferation and specification of each larval glial sheath

Region	Wrapping glia		Subperineurial glia		Perineurial glia	
	NER	MFA	NER	MFA	NER	MFA
Embryonic peripheral glia	ePG1	ePG5 ¹ and ePG9 ²	ePG3	ePG4 ¹ , ePG7 ² and ePG10 ³	ePG2	ePG6 and ePG8
Origin	NB1-3	vSOP ¹ and NB1-3 ²	NB1-3	vSOP ¹ , NB1-3 ² and dSOP ³	NB5-6	NB2-5
Marker expression		<i>nrv2-Gal4</i>		<i>moody-Gal4</i> and <i>Gli-Gal4</i>	<i>46F-Gal4</i>	<i>cas-Gal4</i>
Proliferation	No	No	No	No	Yes	No

ePG11 and ePG12 are not included as each covers a nerve tract alone and is therefore not classified as wrapping, subperineurial or perineurial glia.

^{1,2,3}Lineage affiliation where cells forming one layer arise from different progenitors.

unravel the embryonic origin of all three glial layers that ensheath the peripheral nerves at late third instar. The results presented in this study show that ePG1 in the NER and ePG5 and ePG9 in the MFA give rise to the larval wrapping glial layer. The subperineurial sheath is formed by ePG3 along the NER and by ePG4, ePG7 and ePG10 in the MFA. Neither wrapping nor subperineurial glia of the PNS proliferate during larval development but show enormous hypertrophy along with an increase in nerve length. With respect to the outermost perineurial sheath, our findings clearly exclude a mesodermal origin of this layer (Edwards et al., 1993), as we show that it is formed by ePG2 (and progenies) along the NER and by ePG6 and ePG8 along the MFA, all three derived from ventral neuroectodermal neuroblasts (NBs).

This detailed knowledge about the embryonic origin of all three glial sheaths will help to decipher their various functions. Our results show that determination of the three glial layers in the PNS takes place already in the embryo, as each layer is constantly formed by a discrete set of ePG. This enables us to answer the question of the origin of these layers, as raised in previous studies (Hartenstein, 2011). Wrapping and subperineurial glia share common progenitors: NB1-3 and a ventral sensory organ precursor (vSOP) (Table 1). Besides, NB1-3 gives rise to additional glial cells in the VNC (Beckervordersandforth et al., 2008). By contrast, perineurial glia have a separate pool of progenitors. Both perineurial glia in the MFA (ePG6 and ePG8) arise from NB2-5, which does not produce any other glial cell

(Schmidt et al., 1997). The perineurial layer of the NER (ePG2) has its origin in NB5-6, which does not produce additional peripheral glia but gives rise to additional glia in the VNC. These belong to the subperineurial glia (DL, VL and MV-SPG) (Beckervordersandforth et al., 2008; Ito et al., 1995). However, classification of VNC glia includes the term ‘subperineurial glia’ for all surface-associated cells. Although we find that some subperineurial glia in the VNC also cover the proximal part of the NER (supplementary material Fig. S3), it still needs to be shown whether surface-associated glia (arising from NB5-6 and other NBs) contribute to the subperineurial and/or the outermost perineurial sheath.

In contrast to the CNS, where all three major glial classes (surface glia, cortex glia and neuropil glia) undergo proliferation during larval development (Pereanu et al., 2005) (reviewed by Hartenstein, 2011), our cell-tracing results demonstrate that in the PNS just a single cell, ePG2, along the NER divides. Division of this cell during larval stages was confirmed by staining for phosphorylated Histone H3 (pH3) in addition to Repo- and subtype-specific marker staining. Although pH3-positive peripheral glia were only rarely detectable, they always belonged to the perineurial glia ($n=5/17$ larvae, data not shown). Suppression of mitosis, either by knocking down *CycA* or ectopic expression of *Rbf* in all glia (driven by *repo-Gal4*), prevents the generation of additional cells along the NER. This can be phenocopied by perineurial glia-specific (using *46F-Gal4*), but not by subperineurial- or wrapping glia-specific,

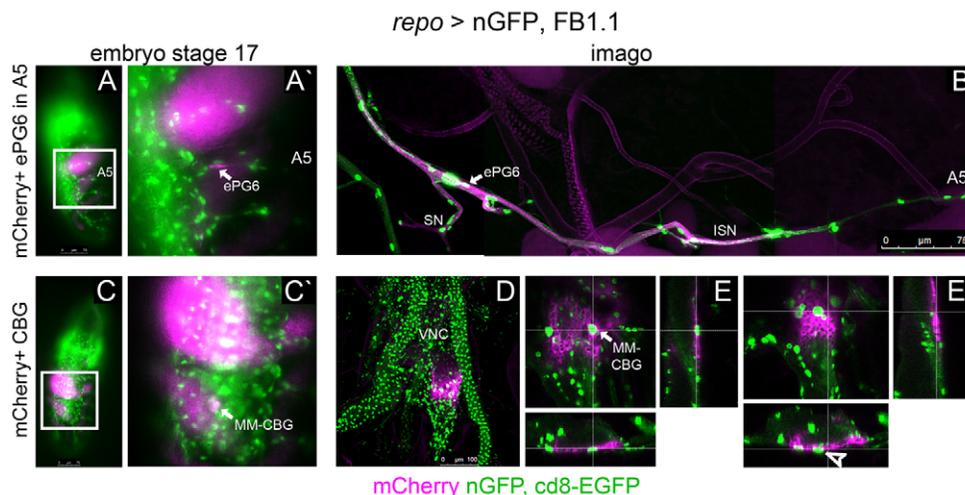


Fig. 8. Tracing glial cells from embryo to imago. (A,A',C,C') Maximum projections of living *Drosophila* embryos at stage 17 presenting mCherry-labelled ePG6 and MM-CBG in the VNC, respectively, in overview (A,C) and at higher magnification (A',C'). Anterior is up. **(B)** ePG6 (nucleus marked by an arrow) is still present in the imago surrounding the proximal part of the ISN. **(D-E')** The MM-CBG persists until adulthood, exhibiting a sponge-like morphology. Individual neuronal somata surrounded by MM-CBG membranes can be seen in the transverse section at the level of the nucleus (E). The nucleus of a surface glia (open arrowhead in E') covering the labelled CBG is shown in a more ventral transverse section.

expression of either transgene. Ablation experiments further confirm the perineurial origin, as embryonic ablation of ePG2 results in a reduction, or frequently even in complete loss, of perineurial glia nuclei along the NER in larva. However, we could not determine which other progenitor for perineurial glia accounts for the additional cells in some of the ePG2 irradiated hs. Block of cell cycle progression in all perineurial glia results in three cells along the NER (as seen in the embryo), indicating that any failure in producing additional perineurial glia cannot be compensated for by other glial subtypes. Alternatively, additional cells in irradiated hs might arise from incomplete damage of ePG2, as irradiation does not always lead to instant cell death.

Another uncertainty remains as to whether perineurial glia undergo cytokinesis or just karyokinesis, as they form a continuous sheath along the NER that does not allow the identification of single cells within. Yet, recent results (Xie and Auld, 2011) support full cytokinesis, as they observe individual perineurial cells being detached from each other as a consequence of Integrin β -subunit knockdown.

A further question concerns the control of perineurial glia proliferation. Apparently, the total number of glial nuclei along the NER is not merely dependent on the overall length of the axonal fibres as *Tubby* larvae with a substantially shorter NER display no significant reduction in glial number (data not shown). Contrary to this finding, it is reported that *brave* (*GlcAT-P*) mutants, which exhibit significantly shorter peripheral nerves, have fewer glia along the nerve while their spacing and distribution appear unaffected (Pandey et al., 2011). Glial cell numbers along the NER increase from anterior segment A1 (with a short NER) to the posteriormost segments A8/A9 (with a ~10 times longer NER), suggesting not a predetermined but rather a dynamic process that probably relies on glia-glia or glia-neuron interactions. A non-autonomous function of peripheral glia in determining perineurial sheath thickness has been shown by Lavery and colleagues (Lavery et al., 2007). According to their model, perineurial growth depends on activation of the Ras pathway within the *Gli*-Gal4-positive subperineurial glia, which is mediated by PI3K and Akt and results in an inhibition of the transcription factor Foxo, which, in turn, is assumed to directly or indirectly suppress the expression of growth factor(s) promoting perineurial cell growth. It remains to be shown whether this mechanism might underlie additional perineurial nuclei arising along the NER. Recent results show that, in the brain, FGF signalling (a known activator of Ras) acts synergistically with InR/Tor to regulate perineurial glia proliferation (Avet-Rochex et al., 2012). Further experiments need to be performed in order to establish whether these signalling cascades control perineurial glia proliferation in the PNS as well.

There is a lack of clarity regarding perineurial glial function. Experimental data indicate that they might contribute to the blood-brain barrier, at least for large molecules of ~500 kDa (Stork et al., 2008). However, perineurial glia apparently play no pivotal role in the formation of a sealed blood-brain barrier, as they do not cover peripheral nerves completely until the late larval stage (Stork et al., 2008). Our Flybow labellings of perineurial glia reveal that the ensheathment provided by these cells in the MFA is even incomplete in L3. However, at least some cells of the larval perineurial layer survive metamorphosis and persist to the adult. Thus, they could become more essential later on during metamorphosis or in the adult in blood-brain barrier formation or by providing mechanical stability to peripheral nerves. In the future, the precise elimination of distinct glial layers will help to unravel the function of the various glia in larvae and in the imago.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

The project was conceived by C.M.v.H., G.M.T. and B.A. Experiments were conducted by C.M.v.H. and A.E.B. Ablations were conceived and performed with the help of A.G. The manuscript was written by C.M.v.H., G.M.T. and B.A.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.093245/-/DC1>

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moody > nGFP

third instar larvae

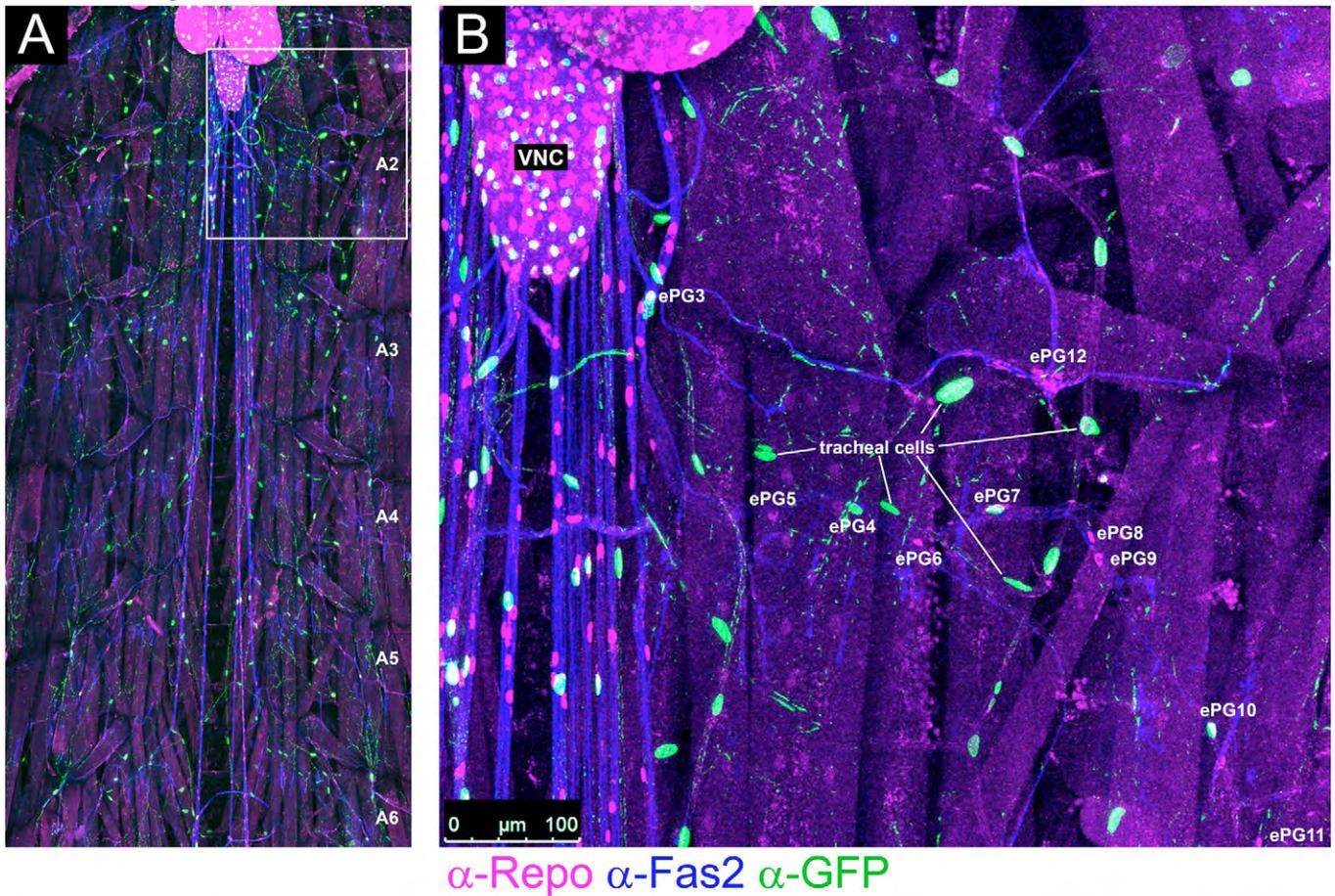


Fig. S1. Expression pattern of *moody*-Gal4 in third instar. Maximum projection illustrating a dissected *moody*-Gal4>nGFP L3 larva stained for Repo (magenta), Fas2 (blue) and GFP (green) in overview (A) and (boxed area) at higher magnification (B). (B) Individual ePG of the MFA of hs A2 are marked. ePG4, ePG7 and ePG10 are GFP positive. In the MFA the Repo staining is not always clearly recognizable in maximum projections as several PG are hidden behind muscles. Along the NER, *moody*-Gal4-expressing ePG3 is indicated. *moody*-Gal4 is additionally expressed in tracheal cells. Anterior is up.

repo > FB1.1

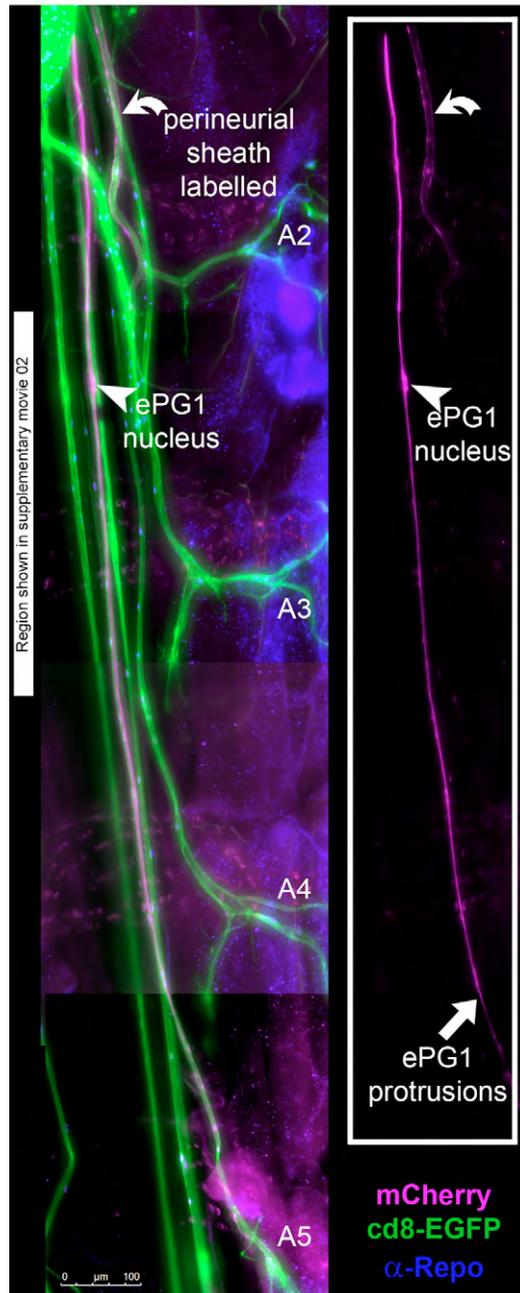


Fig. S2. ePG1 hypertrophy in third instar. Maximum projection showing mCherry-labelled ePG1 in L3 in hs A5. During larval development, ePG1 spreads almost along the entire NER resulting in a total length of more than 1 mm. The ePG1 nucleus is marked by an arrowhead. mCherry signal alone is illustrated in the boxed area. The region presented in Movie 2 is indicated by the white bar on the left side. In addition to ePG1, ePG2 progenies forming the perineurial sheath along the NER in hs A2 are also mCherry labelled (bent arrow). Anterior is up.

repo > nGFP, FB1.1

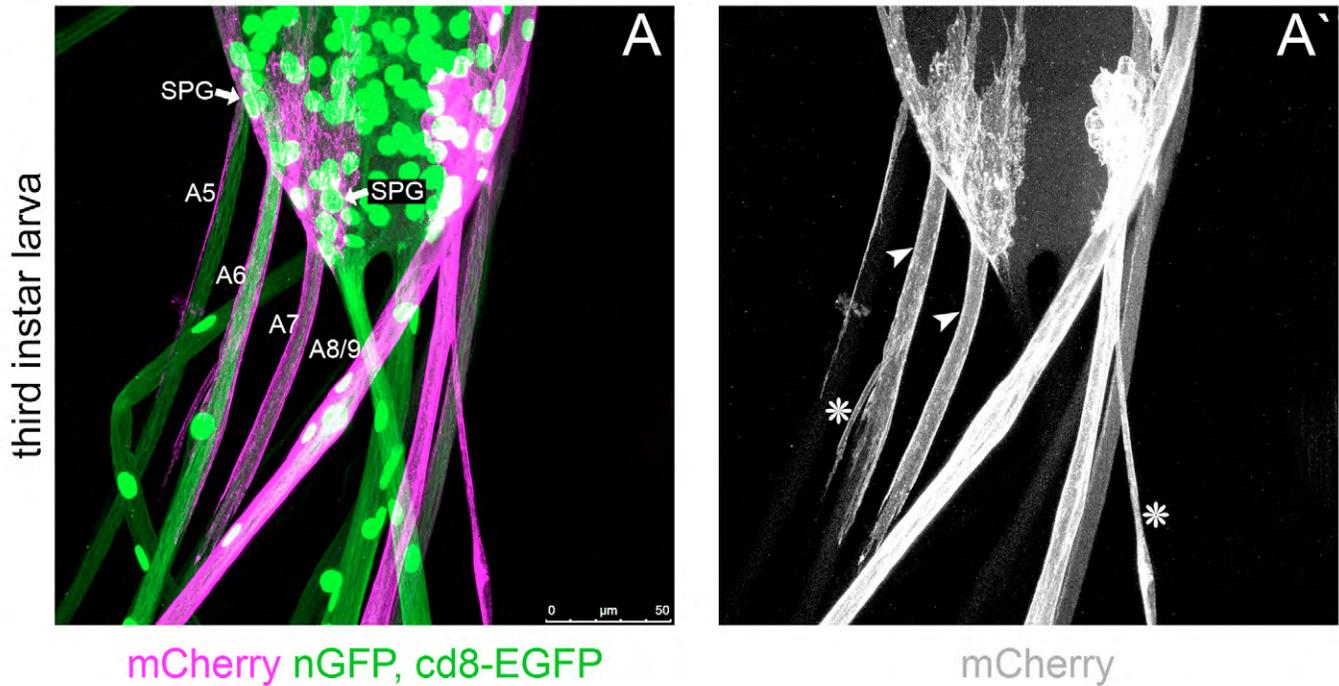


Fig. S3. VNC surface glia cover the proximal part of the NER. (A) Maximum projection showing the posterior tip of the VNC exhibiting several mCherry-labelled glial cells. (A') mCherry alone. Among these are two subperineurial glia of the VNC (nuclei marked by an arrow) that ensheath the proximal part of the peripheral nerve tract in hs A6 and A7 (arrowheads in A'). In addition, glial membranes can be observed covering a structure branching off the peripheral nerve (asterisk). Probably, this carries afferent sensory axons projecting into the VNC. The right-hand side of this animal was not analysed due to multiple labellings within the VNC and the PNS. Anterior is up.

A

Table summarizing the results of ePG2 ablation

specimen	N° of PG nuclei along NER	position of remaining PG along the NER	ablated in hs
larva 01 (B)	5	3 proximal; 2 distal	A5
larva 02 (C)	6	equally distributed but distal part free of nuclei	A5
larva 03	3	proximal located, extending up to middle of the nerve	A5
larva 04 (Fig.4)	2	both proximal	A5
larva 05	4	all distal	A4
larva 06	2	ePG1 proximal; ePG3 distal	A4
larva 07	2-4 precise counting impossible due to close vicinity of A4 and A5 in the proximal section of the NER	all proximal; distal part free of nuclei	A4
larva 08	3	all distal	A4
larva 09 (D)	4	all distal	A4
larva 10	8 - no significant effect	equally distributed	
larva 11	2	both distal	A4
larva 12	2	ePG1 proximal; ePG3 distal	A4
larva 13	15 - no effect	equally distributed	A4
larva 14	17 - no effect	equally distributed	A5

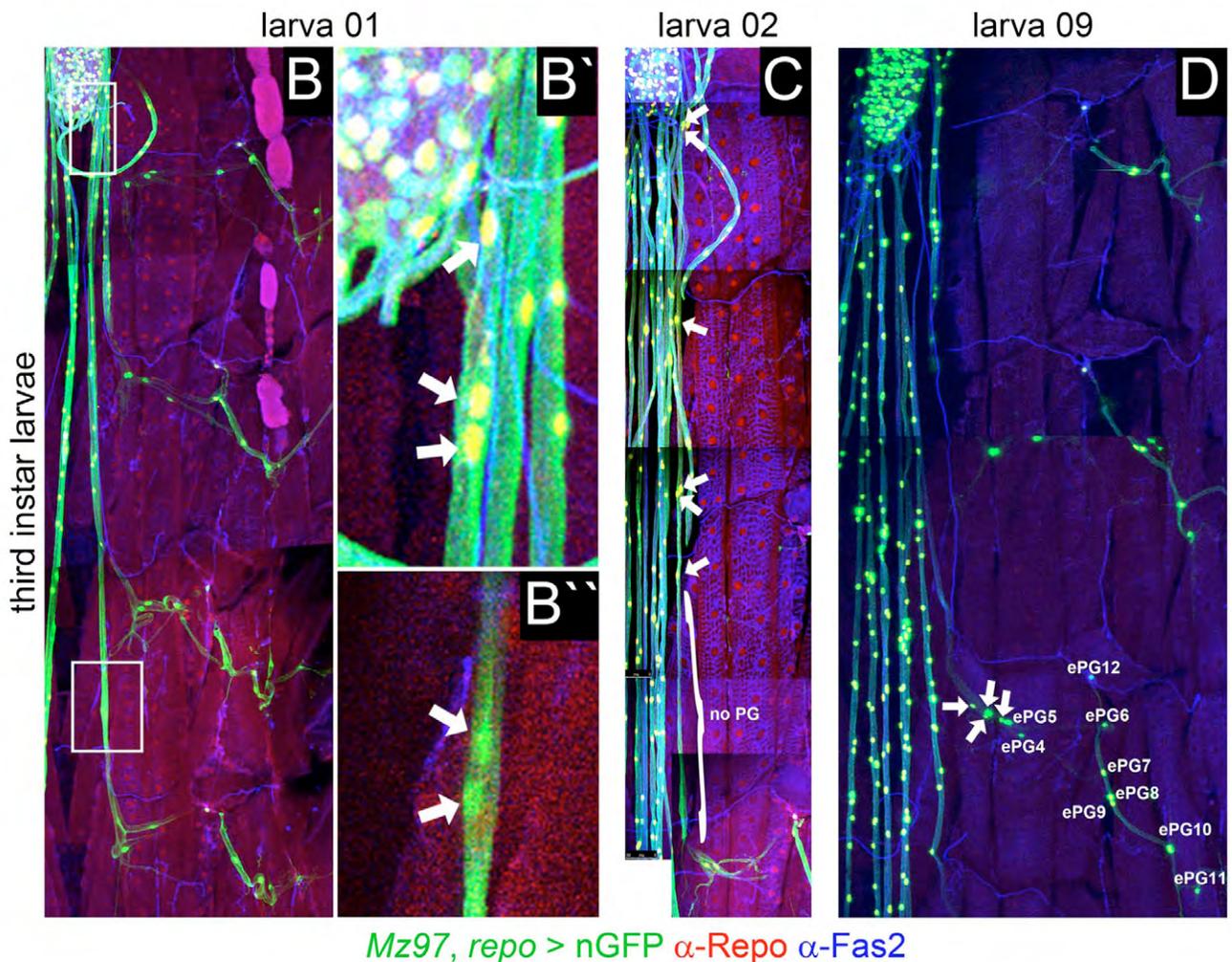
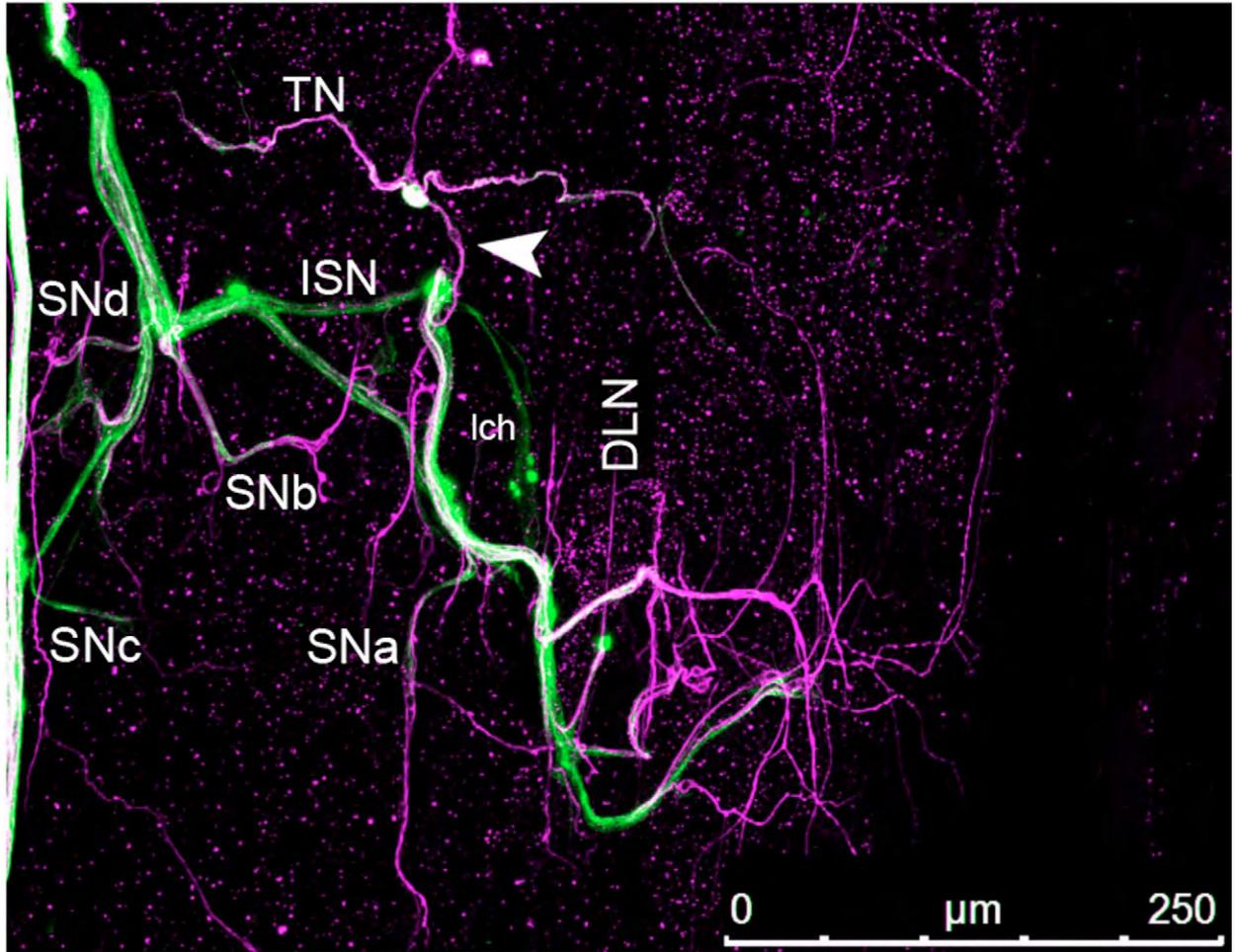


Fig. S4. Phenotypes resulting from embryonic UV irradiation of ePG2. (A) Summary of the number and position of remaining PG along the NER of irradiated hs in L3. Larva 10, 13 and 14 display no significant PG reduction along the NER. (B-D) Position of remaining PG along the NER of three dissected larvae stained for Repo (red) and Fas2 (blue). Anterior is up.

third instar larva

hs A3 shown in Fig.6 E



α -22C10 nGFP, cd8-EGFP

Fig. S5. 22C10-expressing neurons can be detected between ISN and TN. Confocal stacks of the identical hs as presented in Fig. 6E; however, antibody staining against 22C10 (magenta) was performed on this larva subsequently. 22C10-expressing neurons can be seen projecting between ISN and TN (arrowhead). Probably, these are covered by ePG7 protrusions in the corresponding region (arrowhead in Fig. 6E). Anterior is up.

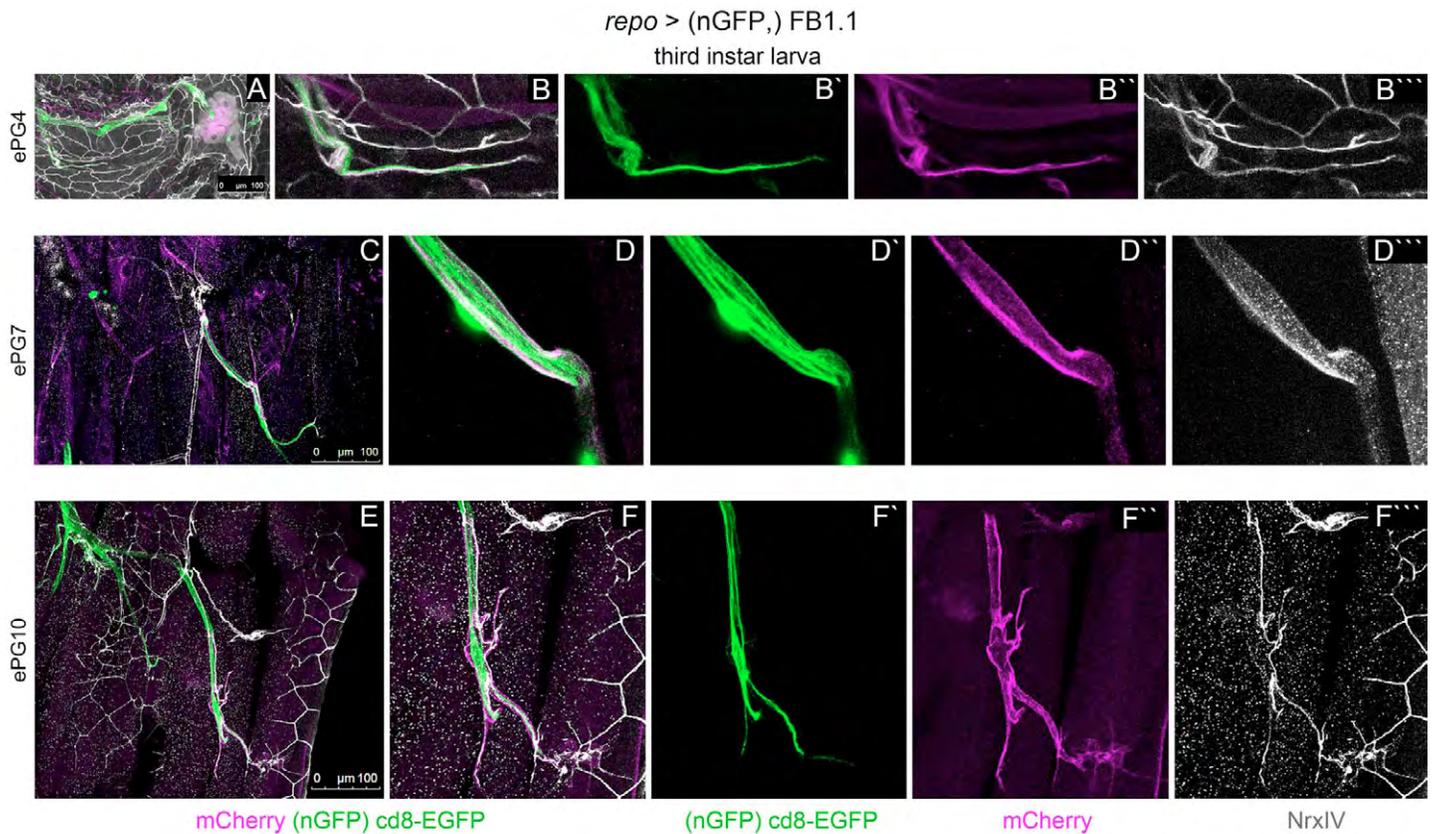


Fig. S6. ePG4, ePG7 and ePG10 express Neurexin IV. (A-F'') Antibody staining against the cell-adhesion molecule Neurexin IV (NrIV) was performed on Flybow-labelled ePG4, ePG7 and ePG10 in L3 to confirm their subperineurial identity. NrIV staining for ePG4 and ePG10 shows the same mCherry-labelled ePG as that presented in Fig. 6B,H. (A,C,E) Maximum projections of triple-labelled subperineurial glia (mCherry, GFP, NrIV). Enlarged views of selected focal planes (B,D,F) are followed by the expression of (nGFP) cd8-EGFP (B',D',F'), mCherry (B'',D'',F'') and NrIV (B''',D''',F''') alone. Anterior is up.

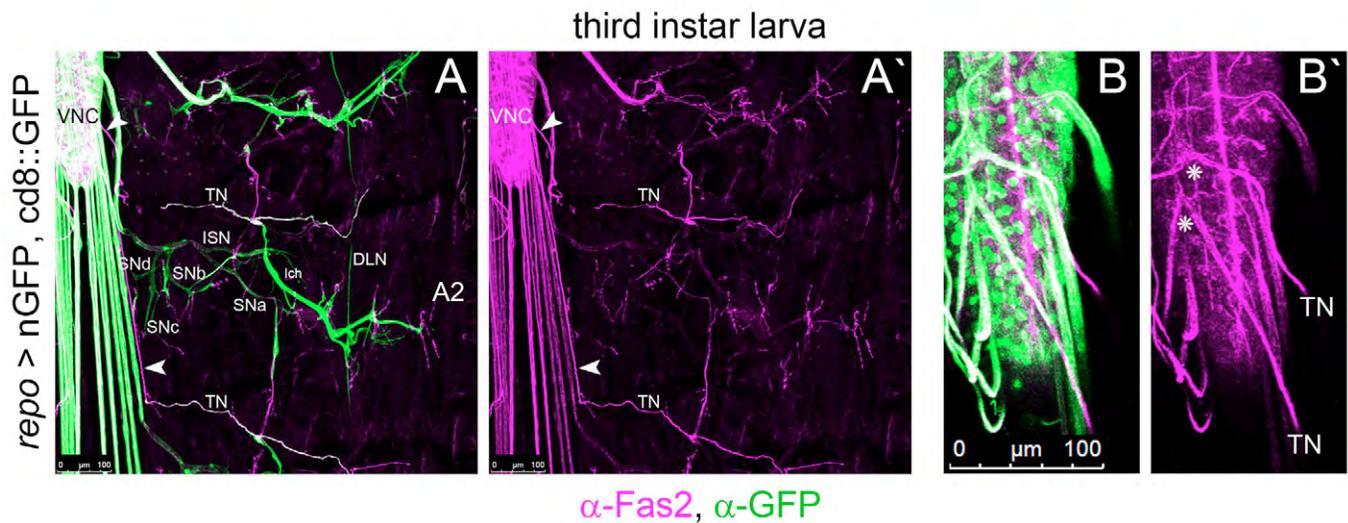


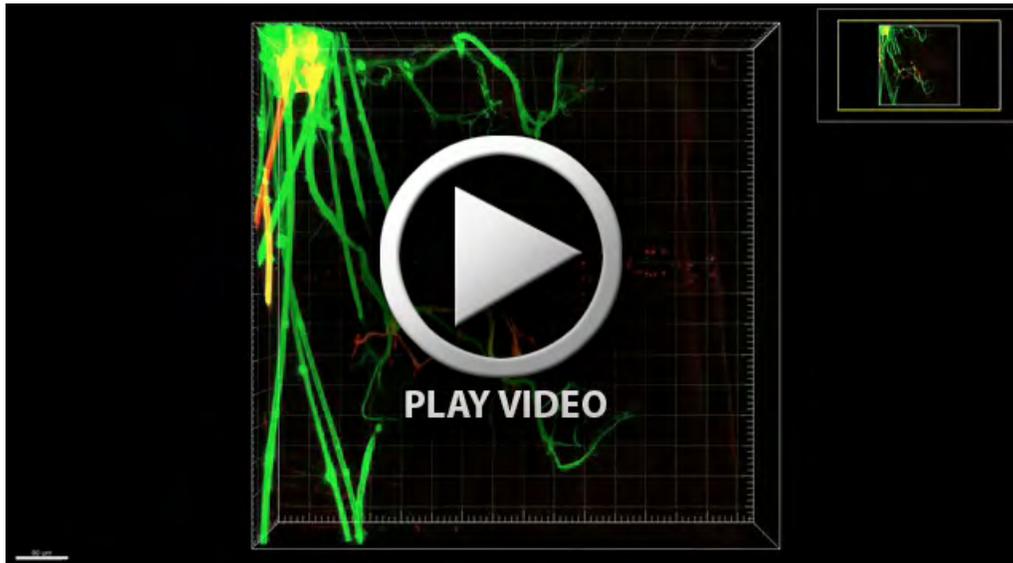
Fig. S7. TN projections enter the VNC separately through the dorsal median cells. Confocal stacks of L3 carrying *repo-Gal4*>UAS-nuclear-GFP (nGFP) and UAS-CD8::GFP transgenes stained for Fas2 (magenta) and GFP (green). (A,A') The overview (A) shows the VNC and a section of the PNS. Main peripheral nerve tracts are labelled in hs A2. Fas2-expressing TN projections can be followed due to their exposed position (arrowheads; A' shows Fas2 staining alone). The TN does not fasciculate with the main peripheral nerve tract along the NER, but enters the VNC at the dorsal midline. (B,B') At higher magnification the Y-shaped entry point of both TNs projecting into the VNC from each side can be seen (asterisks). Anterior is up.



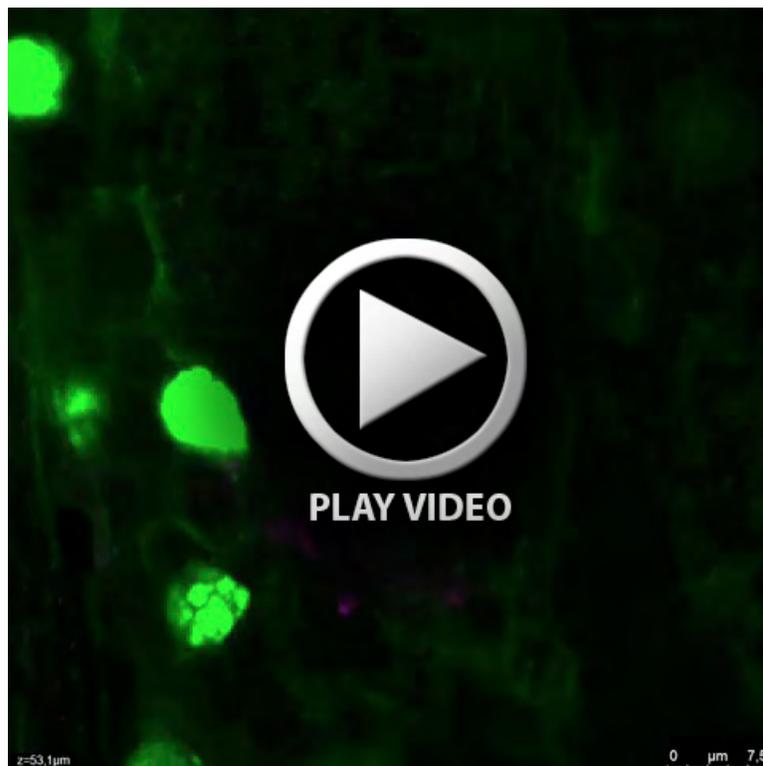
Movie 1. Expression pattern of *moody-Gal4* in third instar. Confocal *z*-stacks of the region designated in Fig. S1. Individual PG in the MFA and *moody-Gal4*-expressing ePG3 are labelled. Anterior is up.



Movie 2. ePG1 protrusions form the innermost wrapping sheath along the NER. Confocal *z*-stacks of the NER region designated in Fig. S2. Due to its morphology, the mCherry-labelled ePG1 can clearly be identified as wrapping glia lying within the fascicle surrounded by cd8-EGFP-positive subperineurial and perineurial glia. Anterior is up.



Movie 3. Three-dimensional reconstruction of ePG7 and PNS morphology. A three-dimensional reconstruction of the mCherry-labelled ePG7 (red) shown in Fig. 6E. It was generated using Imaris software based on confocal stacks (z-step size, 0.8 μm). The reconstruction demonstrates the actual course of the peripheral nerve tracts through the larval tissue. For example, the proximal part of the ISN projects in a more superficial layer (underneath the muscle field in that region) compared with the distal part of the ISN, which lies on top of the muscles. Anterior is up.



Movie 4. Sponge-like morphology of a CBG in the VNC. Confocal z-stacks of an mCherry-labelled CBG (magenta) in the VNC of L3. This glial subtype surrounds neuronal somata in the cortex resulting in a sponge-like morphology. Anterior is up.