Development ePress. Posted online 10 March 2015
 | Development (2015) 142, 1-10 doi:10.1242/dev.116616

RESEARCH ARTICLE

Axonal wrapping in the *Drosophila* PNS is controlled by glia-derived neuregulin homolog Vein

Till Matzat, Florian Sieglitz*, Rita Kottmeier*, Felix Babatz, Daniel Engelen and Christian Klämbt‡

ABSTRACT

Efficient neuronal conductance requires that axons are insulated by glial cells. For this, glial membranes need to wrap around axons. Invertebrates show a relatively simple extension of glial membranes around the axons, resembling Remak fibers formed by Schwann cells in the mammalian peripheral nervous system. To unravel the molecular pathways underlying differentiation of glial cells that provide axonal wrapping, we are using the genetically amenable *Drosophila* model. At the end of larval life, the wrapping glia differentiates into very large cells, spanning more than 1 mm of axonal length. The extension around axonal membranes is not influenced by the caliber of the axon or its modality. Using cell type-specific gene knockdown we show that the extension of glial membranes around the axons is regulated by an autocrine activation of the EGF receptor through the neuregulin homolog Vein. This resembles the molecular mechanism employed during cell-autonomous reactivation of glial differentiation after injury in mammals. We further demonstrate that Vein, produced by the wrapping glia, also regulates the formation of septate junctions in the abutting subperineurial glia. Moreover, the wrapping glia indirectly controls the proliferation of the perineurial glia. Thus, the wrapping glia appears center stage to orchestrate the development of the different glial cell layers in a peripheral nerve.

KEY WORDS: *Drosophila*, Wrapping glia, Neuregulin, Subperineurial glia, Septate junction, Neuron-glia interaction

INTRODUCTION

Neuronal function requires the transmission of electric signals. It has long been known that glial cells insulate axons to ensure fast and reliable transmission of action potentials. In vertebrates, fast saltatory conductance is possible due to myelin-forming glial cells. Long segments of the axons are tightly insulated by several glial wraps, leaving free only the nodes of Ranvier (Nave and Trapp, 2008; Poliak and Peles, 2003; Sherman and Brophy, 2005). In addition, glial cells support the integrity of the axons (Fünfschilling et al., 2012; Nave, 2010). In the mammalian peripheral nervous system (PNS), the activation of the EGF receptor by axonally provided neuregulin 1 (Nrg1) type III ensures the correct differentiation of Schwann cells (Michailov et al., 2004; Taveggia et al., 2005). However, following injury, remyelination is not controlled by axonally derived Nrg1. Instead, denervated Schwann cells transiently express Nrg1 type I to promote Schwann cell differentiation and remyelination in an autocrine/paracrine manner (Stassart et al., 2013).

Institut für Neurobiologie, Universität Münster, Badestr. 9, Münster D-48149, Germany.
*These authors contributed equally to this work

‡Author for correspondence (klaembt@uni-muenster.de)

Received 13 August 2014; Accepted 12 February 2015

In *Drosophila*, the organization of the segmentally organized peripheral nerves is remarkably conserved compared with vertebrates. In all abdominal segments, axons of 42-44 sensory and 34-36 motoneurons connect the body wall with the central nervous system (CNS) (Bodmer and Jan, 1987; Bodmer et al., 1989; Landgraf and Thor, 2006; Mahr and Aberle, 2006; Merritt and Whitington, 1995; Sink and Whitington, 1991). The different abdominal nerves (A1-A8) vary in length. The A2 nerve reaches ~500 µm in length and is thus six times shorter than the A8 nerve, which is ~3000 µm in length. Sensory and motoaxons are accompanied by three different glial cell types: perineurial glia, subperineurial glia (SPG) and wrapping glia (Stork et al., 2008).

The wrapping glial cells of *Drosophila* resemble the non-myelinating Schwann cells that form the so-called Remak fibers in mammals (Nave and Salzer, 2006; Rodrigues et al., 2011). The wrapping glial cells do not divide during larval stages but rather grow to a considerable size. In the third larval instar stage, the wrapping glial cells have engulfed all axons but never form multiple membrane wraps as observed in the vertebrate myelin (Stork et al., 2008). The lineage and identity of all peripheral glial cells found in the larval PNS has been recently described (von Hilchen et al., 2013). Along every abdominal nerve, only three to four wrapping glial cells are found. The SPG is also generated during early embryogenesis. These cells do not divide once they are born and persist until adult stages (Awasaki et al., 2008; Stork et al., 2008; Unhavaithaya and Orr-Weaver, 2012; von Hilchen et al., 2013). The SPG forms the blood-brain barrier by establishing intensive cell-cell contacts characterized by pleated septate junctions (pSJ). These cell junctions are structurally and molecularly related to the septate-like junctions at the paranodes in vertebrates (Banerjee and Bhat, 2007; Banerjee et al., 2006; Baumgartner et al., 1996; Peles et al., 1997; Stork et al., 2008). In all abdominal nerves, only four to five subperineurial glial cells can be identified. The perineurial glial cells, the function of which is currently unknown, cover all nerves. These cells are able to divide throughout development and their number reflects the length of the respective peripheral nerve. How this number is adjusted and which mechanisms orchestrate the differentiation of wrapping and subperineurial glial cells is currently unknown.

In the present study, we first provide a thorough transmission electron microscopy (TEM) analysis of the developing peripheral nerves. The wrapping glia stretches out during larval stages to reach a length of up to 1 mm. We then show that wrapping glial cell differentiation is controlled by activation of the EGF receptor through the *Drosophila* neuregulin homolog encoded by *vein*. Furthermore, cell type-specific knockdown experiments demonstrate a cell-autonomous requirement of Vein during glial differentiation. Vein is not required in neurons but rather in wrapping glial cells in order to induce efficient axonal wrapping. This resembles the mode of EGF receptor activation seen during regenerative myelination in the mammalian nervous system and sheds light on the evolution of glial differentiation.

Development (2015) 142, 1-10 doi:10.1242/dev.116616

RESULTS

Ultrastructural analysis of wrapping glia development

The relatively constant number of wrapping and subperineurial glial cells in each abdominal nerve in relation to the enormous growth of the larva points towards an extreme hypertrophic growth and differentiation potential of these cells (von Hilchen et al., 2013; and see Fig. 1). To address the molecular mechanisms underlying the differentiation of the wrapping glia, we first performed a careful ultrastructural analysis of the abdominal nerves. The exact differentiation of the wrapping glial cells can only be analyzed by electron microscopy. In order to obtain quantitative data on the nerve ultrastructure, analysis has to occur on identified nerves at stereotyped positions (Fig. 1A). Therefore, we developed a whole-mount embedding procedure, which enables us to determine the identity of each nerve and the exact position along the nerve (supplementary material Fig. S1; and see Materials and Methods). In general, nerves A1 to A7 appeared very similar. We counted an average of 78 axon profiles in each of these nerves (n=47 nerves), which corresponds well to the previously counted numbers of moto- and sensory neurons (Bodmer and Jan, 1987; Bodmer et al., 1989; Landgraf and Thor, 2006; Mahr and Aberle, 2006; Merritt and Whitington, 1995; Sink and Whitington, 1991). By contrast, the A8 nerve is different, as it is made up by ∼110 axons (n=18 nerves).

Wrapping is constant along the nerve

To determine the extent of glial wrapping of axons, we defined a wrapping index, which indicates the percentage of individually wrapped axons or axon bundles in relation to the total number of axons (see Material and Methods). Our ultrastructural analysis demonstrates that, in wild type, the wrapping index is ∼20%. The wrapping is relatively constant at 2 µm, 50 µm, 200 µm or 400 µm distance from the CNS, suggesting that, at third-instar stage, the wrapping of axons occurs independently of the position along the anterior-posterior axis (Fig. 1A; supplementary material Fig. S1).

Interestingly, we found that tracheal cells are located within the nerve close to the CNS but are not found in the nerve 200 µm or more away from the CNS (supplementary material Fig. S1D,E, arrows). Tracheal cells establish extensive autocellular pSJ; however, they do not form pSJ with the abutting subperineurial glial cells, which also form autocellular pSJ (supplementary material Fig. S2A-C). Tracheal cells are always found in the perineurial glial layer, often covered by processes of the SPG (supplementary material Fig. S2A, green shading). The SPG forms spot-adherens junctions with the tracheal cells (supplementary material Fig. S2D). Similar junctions can be seen between all glial cells (supplementary material Fig. S2E,F).

Wrapping glia differentiation is a continuous process

During embryonic development the wrapping glia does not engulf individual axons (Stork et al., 2008). However, in third-instar larvae all axons are either wrapped individually or in smaller clusters (Fig. 2; Stork et al., 2008). To better dissect the process of axon wrapping, we performed a detailed temporal analysis concentrating on A1-A7 nerves (Fig. 2). These studies demonstrate that the differentiation of the wrapping glia begins at early larval stage and proceeds until puparium formation, whereas the number of axonal profiles appears to be constant during larval stages (Fig. 2). Within all abdominal nerves, different axon sizes can be recognized (Fig. 2A-F), but preferential wrapping of larger caliber axons, as described for vertebrates, cannot be noted (Nave and Trapp, 2008). We also failed to detect statistically significant distributions of stereotyped axon fascicle sizes (n=49, data not shown). To discriminate whether motoaxons are wrapped differently than sensory axons, we expressed a *UAS-CD2HRP* construct specifically in motoneurons employing the *OK371-Gal4* driver (Dubois et al., 2001; Mahr and Aberle, 2006; Watts et al., 2004). Following a 3,3′-diaminobenzidine (DAB) staining we detected the precipitate by its strong contrast in the TEM (Fig. 2G). This analysis revealed 29-32 motoaxons, which corresponds well to the number of motoneurons determined in previous reports (n=4) (Landgraf and Thor, 2006; Sink and Whitington, 1991). Motoaxons run preferentially in common fascicles, but we did not detect prominent differences in axon size distributions compared with sensory neurons. In summary, these findings indicate that wrapping is a progressive process.

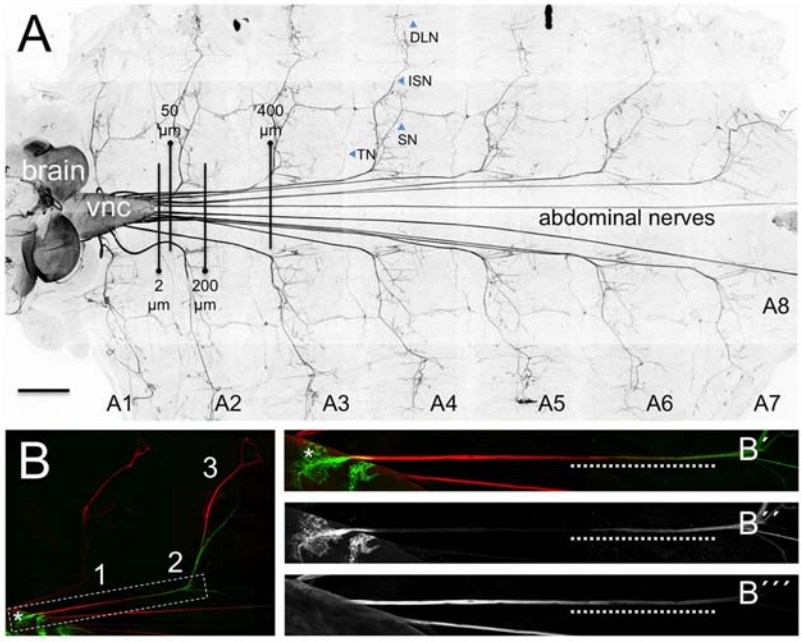

Fig. 1. The larval nervous system. (A) Filet preparation of a third-instar larva stained for HRP illustrating the abdominal nerves (A1-A8) connecting the ventral nerve cord (vnc) to the muscle field area. The two brain lobes are indicated (brain). The black lines indicate the distances from the vnc at which the sections were taken for ultrastructural analysis. The nerve extension region is defined as the part of the axon from the vnc to the muscle field area (von Hilchen et al., 2013). DLN, dorsal longitudinal nerve; ISN, intersegmental nerve; SN, segmental nerve; TN, transversal nerve. Scale bar: 200 µm. (B) To assess the morphology of the wrapping glia we used a modified flip-out system (*nrv2-Gal4, UAS-flp; UAS>CD2>CD8::GFP*), in which cells either express CD2 or GFP. Three peripheral wrapping glial cells (1-3) are labeled in red, green and red, respectively. The boxed area is shown in higher magnification in B′-B‴. The wrapping glial cells 1 and 2 intermingle for a stretch of ∼200 µm (dotted white line). The asterisk in B′ denotes the fanned-out morphology of a wrapping glial cell in the CNS.

Development (2015) 142, 1-10 doi:10.1242/dev.116616

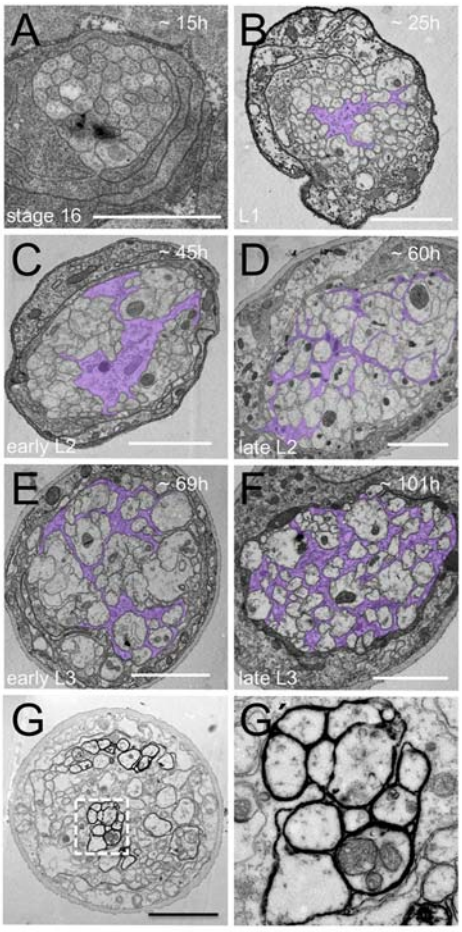

Fig. 2. Progressive wrapping of peripheral axons during larval development. (A-F) Electron micrographs of cross-sections taken from peripheral nerves at different time points of development. The wrapping glial cell is indicated by purple shading. Panel A shows only a part of a segmental nerve of an embryo. (G) Cross-section through an abdominal nerve, with all motoaxons visualized by Ni-intensified DAB staining using the following genotype: *OK371-Gal4 UAS-CD2HRP*. DAB precipitate is electron-dense and can be recognized by the dark staining. 31 DAB-positive motoaxons are organized in distinct fascicles. The boxed area is shown in higher magnification in G′. (G′) Motoaxon fascicle. Scale bars: 2 µm.

Glial wrapping is autonomously controlled by EGF receptor activation in wrapping glia

To test the molecular determinants controlling glial differentiation, we followed a candidate approach. We first tested the role of the EGF receptor (EGFR) in regulating glial wrapping in *Drosophila*. To circumvent the early requirements of EGF receptor signaling (e.g. during the formation of the blood-brain barrier), we first utilized the temperature-sensitive mutant $egfr^{tsla}$ (Kumar et al., 1998). However, when we placed $egfr^{tsla}$ mutants at the restrictive temperature in the first or second larval instar stage, no third-instar larvae were obtained. We then expressed a dominant negative form of the EGF receptor in all glial cells using the *repo-Gal4* driver, and restricted the expression time to larval stages using the TARGET system (McGuire et al., 2003). The inhibition of EGF receptor signaling from first instar larval stage onwards resulted in a significantly reduced wrapping index (Table 1), and many axons were wrapped in abnormally large fascicles (Fig. 3A,B; Fig. 4). Thus, the EGF receptor needs to be activated during early larval development to allow normal glial wrapping.

We then tested whether activation of the EGF receptor is required within the wrapping glial cells or whether the EGF receptor acts in a non-cell-autonomous way to control the differentiation of the wrapping glia. Therefore, we reduced EGF receptor activation specifically in the wrapping glia by expressing $UAS\text{-}EGFR^{DN}$ using the *nrv2-Gal4* driver. This regime also resulted in a reduction of the wrapping index (Fig. 3A,C and Fig. 4; Table 1) but did not alter the number of wrapping glial cells along the nerve (n=5 animals, counts of $nrv2{\gg}lamGFP{\gg}EGFR^{DN}$-positive nuclei in 10 nerves per animal). By contrast, the expression of a dominant negative EGF receptor specifically in the SPG did not cause any abnormal wrapping phenotypes (Table 1). Similar results were obtained following expression of $EGF\ receptor^{dsRNA}$ (Fig. 3D).

The findings described above suggest a direct relationship between EGF receptor activation and wrapping glial membrane extension. To further validate these results, we performed gain-of-function experiments. Whereas the panglial expression of activated EGF receptor (λtop) results in the induction of cell proliferation in peripheral nerves (Read et al., 2009; Witte et al., 2009), the expression of activated EGF receptor only in the wrapping glia does not alter wrapping glial cell number (n=5 animals, counts of $nrv2{\gg}lamGFP{\gg}EGFR^{\lambda top}$-positive nuclei in ten nerves per animal). In addition, we determined the ultrastructure and analyzed nine nerves of four animals. The wrapping index of these nerves was grossly normal (Table 1). In two specimens, however, we noted a dramatic increase of glial membrane production, which was never observed in control animals (Fig. 3E,E′). In conclusion, the EGF receptor is autonomously required in wrapping glial cells to instruct glial wrapping around axons.

The neuregulin homolog Vein cell-autonomously controls wrapping glial differentiation

The *Drosophila* EGF receptor can be activated by four different ligands: Spitz, Keren, Gurken and Vein. As *spitz* null mutants die during early embryogenesis, we suppressed the *spitz* gene function by RNA interference (RNAi) in either neuronal or glial cell types. Suppression of *spitz* function in neurons (using the *elav-Gal4* driver) resulted in a normal wrapping phenotype (Table 1). Moreover, neuronal expression of secreted Spitz, which is known to be a potent activator of the EGF receptor, also does not influence glial wrapping (Table 1). Suppression of *spitz* function in glial cells (using the *repo-Gal4* driver) also does not affect glial differentiation (Table 1). However, when secreted Spitz is expressed by glial cells, axonal wrapping is promoted (Table 1).

As *Keren* and *gurken* mutants are viable and do not show behavioral deficits (Brown et al., 2007; Reich and Shilo, 2002; Schüpbach, 1987), we then focused our analysis on *vein* mutants. *vein* encodes the fly homolog of neuregulin 1, which regulates the ensheathment of peripheral axons in mammals (Michailov et al., 2004). The allelic combination $vein^{RY}/vein^{L6}$ represents the null situation and results in lethality during early pupal stages (Donaldson et al., 2004; Schnepp et al., 1996; Yarnitzky et al., 1997).

In $vein^{RY}/vein^{L6}$ mutant third-instar larvae, we noted a prominent reduction of the wrapping index (Fig. 3A,F; Fig. 4; Table 1). Overexpression of *vein*, which is known as a weak activating EGF receptor ligand (Schnepp et al., 1996), in either neurons or glial cells did not significantly change the wrapping index (Table 1). To test whether Vein is provided by neurons or glial cells we performed cell type-specific RNAi-mediated gene knockdown experiments (Table 1). Suppression of *vein* function specifically in neurons did not cause any abnormal phenotype in peripheral nerves. By contrast, when we silenced *vein* specifically in all glial cells we observed a

Development (2015) 142, 1-10 doi:10.1242/dev.116616

Table 1. Summary of all specimens quantified following electron microscopy analysis

Genotype	mv (%)	s.d. (%)	Median (%)	Animals	Nerves	*t*-test	*P*
w^{1118}	19	7	18	9	27	C	
$vein^{RY}/vein^{L6}$	11	6	9	7	31	2.4×10^{-4}%	***
$nrv2\gg w^{1118}$	19	6	18	8	34	C	
$nrv2\gg EGFR^{DN}$	16	7	16	10	26	4.90%	*
$nrv2\gg\lambda top$	17	9	14	4	9	33%	n.s.
$nrv2\gg vein^{RNAi}$	8	3	9	5	29	1×10^{-9}%	***
$moody\gg w^{1118}$	16	4	16	5	27	C	
$moody\gg EGFR^{DN}$	14	9	11	5	18	40%	n.s.
$moody\gg\lambda top$	13	6	11	5	18	5.27%	n.s.
$moody\gg vein^{RNAi}$	16	8	16	4	11	92%	n.s.
$repo\gg w^{1118}$	15	6	14	6	26	C	
$repo\gg\lambda top$	11	5	10	5	20	1.3%	*
$repo\gg vein^{RNAi}$	10	6	11	5	12	2.89%	*
$repo\gg spitz^{RNAi}$	16	6	16	5	26	56%	n.s.
$repo\gg spitz^{secreted}$	20	5	22	4	9	3.19%	*
$repo\gg vein$	18	7	17	6	23	17%	n.s.
$elav\gg w^{1118}$	18	7	15	6	24	C	
$elav\gg spitz^{RNAi}$	16	8	14	5	21	54%	n.s.
$elav\gg vein^{RNAi}$	16	4	16	5	16	43%	n.s.
$elav\gg spitz^{secreted}$	19	6	16	4	18	64%	n.s.
$elav\gg vein$	19	6	18	7	25	41%	n.s.
$repo\gg w^{1118};tubG80^{ts}$	16	9	13	3	12	C	
$repo\gg EGFR^{DN};tubG80^{ts}$	10	3	9	11	24	1.2%	*
$repo\gg\lambda top;tubG80^{ts}$	22	7	18	2	3	31%	n.s.

The different genotypes are indicated. The wrapping index was calculated per animal in percent by dividing the number of wrapped axon clusters through the total number of axons. mv, mean value of wrapping index; s.d., standard deviation; *P*, significance level; C, control; animals, number of section animals per genotype; nerves, total number of sectioned nerves per genotype. Levels of significance: ***$P\leq0.001$%, **$P\leq0.01$%, *$P\leq0.05$%.

reduced wrapping index, similar to *vein* loss of function mutants (Fig. 3F,G; Table 1). This suggests that the EGF receptor is not activated by a neuronally derived signal but rather by a glia-derived signal. To identify the relevant glial cell type, we suppressed *vein* function either in the wrapping glia or in the SPG, which directly abuts the wrapping glia. Surprisingly, silencing of *vein* specifically in wrapping glial cells strongly affected axonal wrapping, indicating that an autocrine signaling mechanism is orchestrating wrapping glial differentiation (Fig. 3A,H; Fig. 4).

To further verify that Vein needs to be expressed by the wrapping glia in order to induce the axonal wrapping, we initiated cell type-specific rescue experiments. However, the basal activity of the UAS-vein construct is so high that it rescues the lethality of $vein^{dddL6}/vein^{dddRY}$ transheterozygous flies, which precludes further genetic analysis.

The EGF receptor is expressed in peripheral glial cells

The loss-of-function experiments mentioned above suggest that the EGF receptor is expressed by wrapping glial cells. Anti-EGF

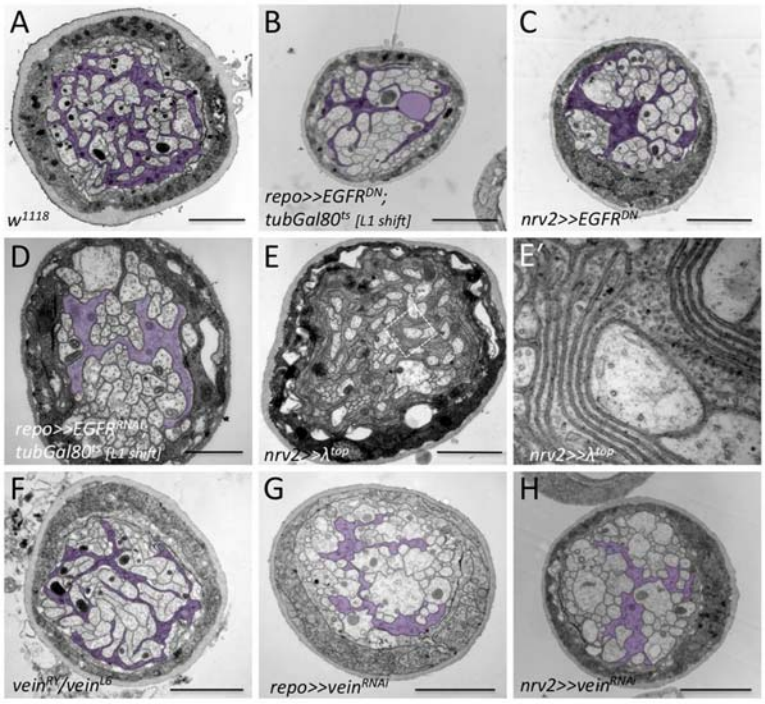

Fig. 3. The EGF receptor is required in wrapping glial cells. Electron micrographs of cross-sections taken from peripheral nerves of third-instar larvae at 400 µm distance from the CNS. The wrapping glial cell is indicated by shading (except for E). For quantification see Fig. 4. (A) Control nerve of a w^{1118} animal. (B) Panglial expression of a dominant negative EGF receptor from early first-instar stage onwards was achieved using $tub\text{-}Gal80^{ts}$ and a temperature shift 24 h after egg laying. The wrapping index is reduced (see Table 1 or Fig. 4 for quantification). (C) Upon expression of a dominant negative EGF receptor only in the wrapping glia using *nrv2-Gal4*, the wrapping index is also reduced. (D) Panglial expression of $EGFR^{RNAi}$ from early first-instar stage onwards was achieved using $tub\text{-}Gal80^{ts}$ and a temperature shift 24 h after egg laying. (E) Upon expression of an activated EGF receptor (λ*top*) specifically in the wrapping glia (using *nrv2-Gal4*), the wrapping index is not changed, but occasionally we noted an extreme increase in the formation of glial membranes. The boxed area is shown in higher magnification in E′. (E′) Note the different glial cell membranes abutting the axons. (F) Nerve of a $vein^{RY}/vein^{L6}$ larva. The wrapping index is reduced by 50%. (G) Panglial suppression of *vein* expression using *repo-Gal4* reduces the wrapping index. (H) A similar reduction of the wrapping index is seen upon suppression of *vein* expression only in the wrapping glia. Scale bars: 2 µm in A-E,F,G; 1 µm in H.

Development (2015) 142, 1-10 doi:10.1242/dev.116616

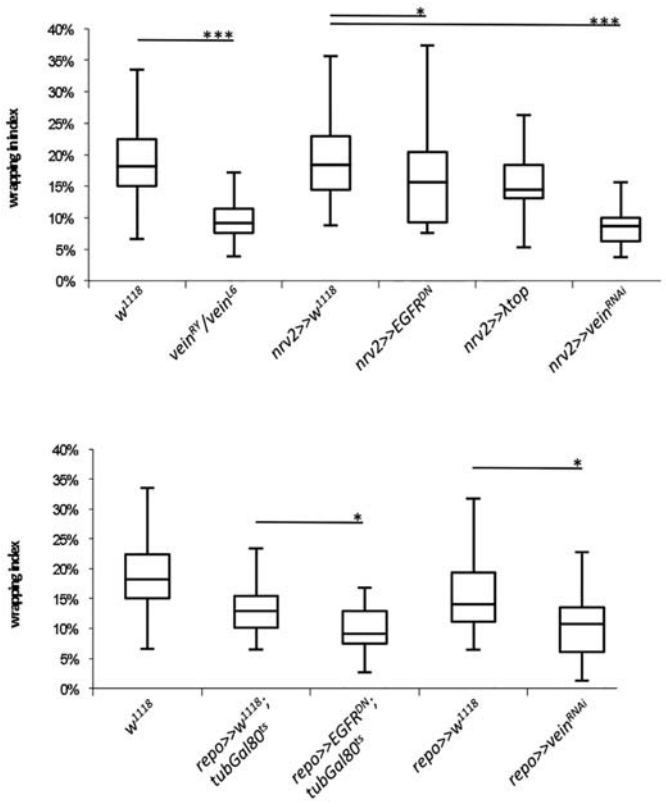

Fig. 4. Quantification of wrapping data. Quantification of the results shown in Fig. 3. The number of nerves use for each genotype is shown in Table 1. Levels of significance: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

receptor antibodies had been generated but did not allow a faithful localization of the protein in larval tissues. Instead, we utilized the recently developed MiMIC technology, which provides an elegant approach to generate endogenously tagged protein variants (Venken et al., 2011). We generated an isoform-specific Cherry-EGF receptor fusion (Fig. 5A; and see Materials and Methods), which is expressed from the endogenous locus and which revealed significant expression in peripheral glial cells (Fig. 5B). The strongest expression was noted in the surface glia but consistent expression was also noted in the wrapping glia (Fig. 5B′). To identify cells expressing the EGF receptor ligand Vein, we utilized a Gal4 enhancer trap insertion, showing weak but consistent expression in the wrapping glia and strong expression in tracheal cells (Fig. 5C). Interestingly, *spitz* expression, as visualized by a Gal4 enhancer trap line, also appeared prominent in wrapping glial cells (Fig. 5D). In conclusion, these findings support the notion that axonal ensheathment is controlled in an auto- or paracrine fashion by Vein secreted from wrapping glial cells.

The wrapping glia induces formation of perineurial glial cells

During the experiments described here we noted that modulation of EGF receptor activity in the wrapping glial cells also influenced the development of the outer glial cell layers that constitute the blood-brain barrier. Most prominently, the number of perineurial glial cells found on every abdominal nerve is affected. In wild-type animals, ~15-30 glial cells associate with the nerve extension region of the A2-A7 nerves (see Fig. 1 for definition of nerve extension region), and ~100 glial cells are found in the nerve extension region of the A8 nerve (Fig. 6C).

In control animals, which carry a copy of the *nrv2-Gal4* element, glial number is generally reduced. Between 10 and 20 glial cells localize to each abdominal nerve extension region (A2-A7, for A8, >60 glial cells were counted) (Fig. 6; also see von Hilchen et al., 2013). Interestingly, a linear increase in glial cell number is noted at nerves A2-A7. As the length of abdominal nerves also increases linearly, within every additional 500 µm nerve length about three extra perineurial glial cells are found. The only exception to this is the A8 nerve, which contains more than 60 perineurial glial cells (Fig. 6C).

Upon expression of a dominant negative EGF receptor specifically in wrapping glial cells, we did not note a reduction of wrapping glial cell number. However, we found a robust reduction in perineurial glial cell number, suggesting that wrapping glia-derived signals control the proliferation of the perineurial glia during larval stages. This, however, does not explain why the perineurial glial cell number is extraordinarily higher along the A8 nerve. As the A8 nerve is significantly larger in length and diameter compared with all other abdominal nerves, and harbors ~110 instead of 78 axons, it might well be that perineurial cell number is regulated by the increase of the nerve surface.

To directly test whether the nerve surface size regulates perineurial glial cell number, we looked for alternative ways to increase the size of the nerve diameter. The serine/threonine kinase Fray is expressed by the subperineurial glial cells and is required to regulate the extracellular volume in peripheral nerves via a conserved co-transporter mechanism (Leiserson et al., 2011, 2000). Upon glial-specific *fray* knockdown, peripheral nerves show prominent swellings and thus show a local increase of the nerve surface. Consistent with the idea that nerve surface takes part in the regulation of perineurial glial cell number, we always detected a significantly increased number of perineurial glial cells that are characterized by Apontic expression in the bulged nerve areas (supplementary material Fig. S3).

The wrapping glia induces blood-brain barrier development

In addition to this, we noted effects of the wrapping glia on the development of the SPG. In wild type, the subperineurial glial cells form autocellular contacts with pronounced septate junctions: The length of this autocellular overlap (ACO), which contains septate junctions, is ~4.7 µm (Fig. 7A,B; n=39). Interestingly, *vein* mutant subperineurial glial cells show a highly significant reduction in the length of the ACO (Fig. 7A-C; n=44). Similar results were obtained when we silenced *vein* specifically in all glial cells (Fig. 7A,B,D; n=37). To determine which glial cell type is required for this effect, we silenced *vein* only in the SPG or in the wrapping glia using *moody-Gal4* or *nrv2-Gal4*, respectively. Suppression of *vein* in the SPG does not affect the length of the ACO, whereas reduction of *vein* specifically in the wrapping glial cells results in a significant reduction of the length of the ACO (Fig. 7A,B,E; n=37 and n=35). To further corroborate these findings, we modulated the activity of the EGF receptor by either expressing a dominant negative or a constitutive active version. Whereas suppression of EGF receptor activity in the SPG results in a reduction of the ACO length, as seen in *vein* mutants (Fig. 7A,B,F; n=30), activation of the EGF receptor specifically in the SPG results in a prominent increase of ACO length, which is also observed following panglial expression of the activated EGF receptor (Fig. 7A,B,G,H; n=33 and n=32).

Together, these data suggest that wrapping glia-derived Vein not only regulates the degree of differentiation in the wrapping glia but also directly influences the length of the septate junction containing ACO in the SPG.

Development (2015) 142, 1-10 doi:10.1242/dev.116616

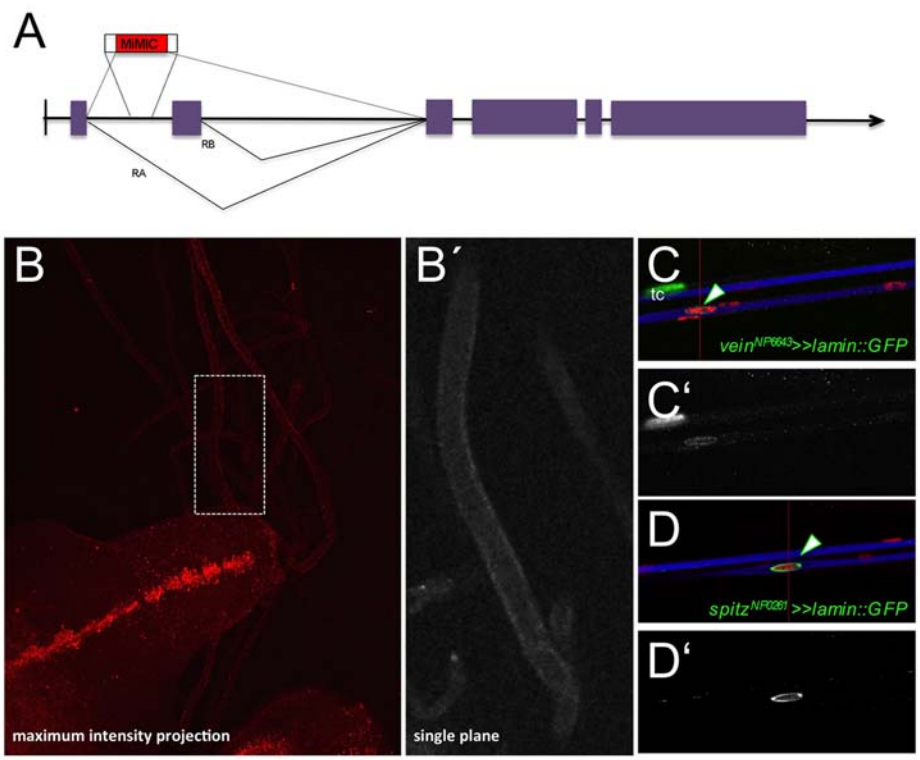

Fig. 5. Expression of EGF receptor and its ligands in peripheral glial cells. (A) Schematic representation of the *egf receptor* locus. The integration position of a MiMIC transposon is indicated. Only one of the two isoforms is targeted by the MiMIC insertion. (B,B′) Following exchange of the integration cassette to a cherry exon trap cassette, we noted prominent expression of the EGF receptor in subperineurial glial cells. Reduced amounts were detected in the wrapping glial cells. The boxed area is shown in higher magnification in B′. (C,C′) To deduce the expression of *vein* we utilized the Gal4 enhancer trap insertion *NP6643* into the *vein* promoter region to drive LaminGFP expression. Weak expression is seen in the wrapping glia (arrowhead) and strong expression is noted in tracheal cells (tc). (D,D′) To deduce the expression of *spitz* we utilized the Gal4 enhancer trap insertion *NP0261* into the *spitz* promoter region to drive LaminGFP expression. Prominent expression is seen in the wrapping glial cells (arrowhead).

DISCUSSION

In the present study, we first demonstrated that the degree of wrapping glial differentiation in peripheral nerves is controlled by the EGF receptor. Interestingly, the activating ligand Vein is not produced by axons but rather generated by the wrapping glia itself. Subsequently, Vein generated by the wrapping glia is also able to influence the differentiation of the subperineurial glia. Lastly, the wrapping glia also regulates the proliferation of the perineurial glial cells outer surface of the nerve in a presumably indirect way. In conclusion, our data show that the wrapping glial cell appears center stage, organizing the development of peripheral nerves in *Drosophila*.

The development of glial cells in *Drosophila* is initiated during embryonic stages. Although the early definition of glial fate is well understood in *Drosophila* (Ragone et al., 2003; Stork et al., 2012), the molecular cues responsible for the acquisition of the wrapping glial cell fate are presently unknown. The allocation of wrapping glial cell fate must be determined very early, as the number of wrapping glial cells accompanying the segmental nerves is already specified at late embryonic stages, and their number does not change during larval stages (von Hilchen et al., 2013). Only the perineurial glia is able to divide during larval development, which in part might be regulated by the surface area of the respective nerve. Abutting the perineurial glial cells are the SPG that establish the blood-brain barrier. Surprisingly, we found that the wrapping glia is crucial for the normal differentiation of the SPG. We show that these cells express relatively high levels of the EGF receptor and directly relate the level of EGF receptor activation to the extent of ACO that contains septate junctions. As the degree of septate junction formation can determine the strength of the blocking of paracellular diffusion (Schwabe et al., 2005; Stork et al., 2008), it is likely that an increase in axonal wrapping will increase the tightness of the paracellular diffusion seal between the subperineurial glial cells.

It has been already noted earlier that expression of Gliotactin is absent in *egfr* and *vein* mutants but is expressed in *spitz* mutants (Sepp and Auld, 2003b). Likewise, it was shown that expression of Neuroglian is dramatically reduced in *egfr* mutants (Sepp and Auld, 2003b). As Gliotactin is an important constituent of tricellular septate junctions and Neuroglian is a core component of general septate junctions (Genova and Fehon, 2003; Oshima and Fehon, 2011; Schulte et al., 2003), this corroborates the pivotal function of the EGF receptor in organizing septate junctions in the SPG. A possible link between EGF receptor activation and septate junction formation might either stem from a direct transcriptional activation or it could be due to changes in alternative splicing (Edenfeld et al., 2006; Oshima and Fehon, 2011). Cell type-specific splicing of *neurexinIV*, encoding another core component of septate junctions, is brought about by the splicing factor How, the activity of which can be regulated through phosphorylation by Raf/MAP kinase (Nir et al., 2012; Rodrigues et al., 2012).

Interestingly, septate-like junctions are also found at paranodal junctions of myelinated nerves in mammals. In the last years, several septate junction components have been molecularly identified and were found to be remarkably conserved (Faivre-Sarrailh et al., 2004; Peles et al., 1997). Quite similarly, loss of the How homolog quaking (Qk) results in glial differentiation defects in mice (Ebersole et al., 1996; Edenfeld et al., 2006; Rodrigues et al., 2012; Sidman et al., 1964).

In *Drosophila*, the wrapping glial cells originate from progenitor cells of the CNS, and as soon as they are specified, they start to migrate out into the periphery, in part using the same molecular machinery that also directs the navigation of axonal growth cones (Edenfeld et al., 2007; von Hilchen et al., 2008, 2010; Sepp and Auld, 2003a; Silies and Klämbt, 2010). At the beginning of larval life, the wrapping glial cells start to grow and extend enormous processes, reaching more than 1 mm in length. Following and in parallel to this stretch growth, axonal membranes are engulfed. Throughout our analysis we found no wrapping preference for either small or large axon calibers or for sensory or motor axons. Rather, all axons appear to have the same probability to become individually wrapped.

Development (2015) 142, 1-10 doi:10.1242/dev.116616

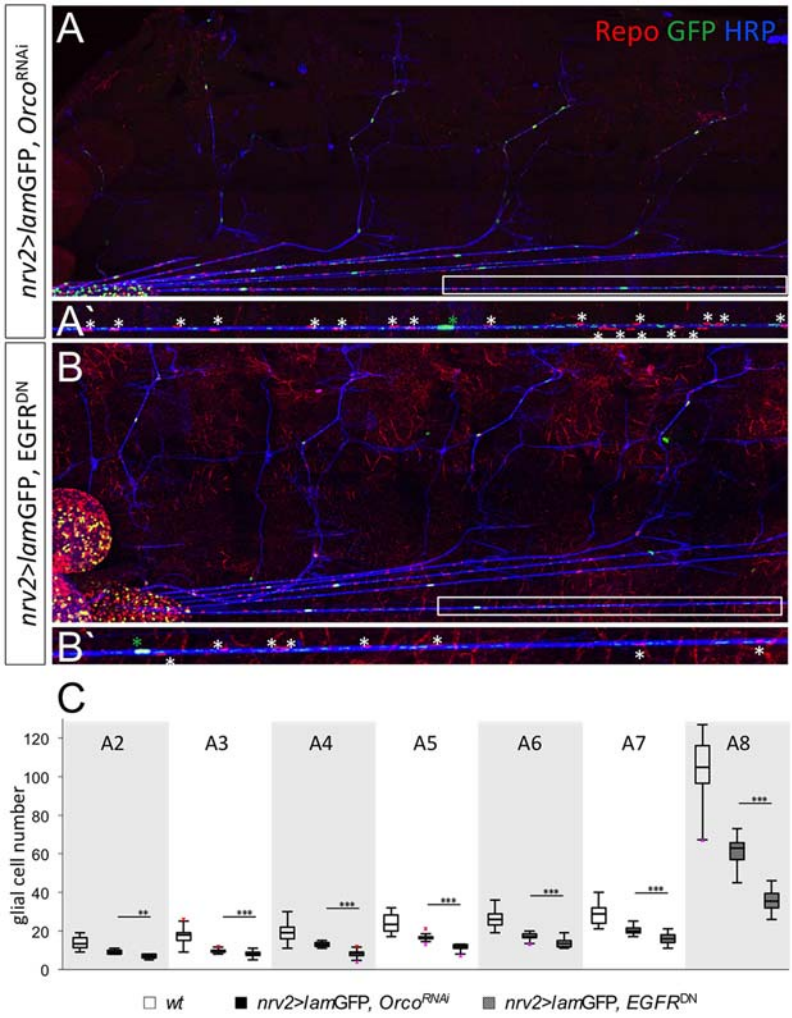

Fig. 6. Differentiation of the wrapping glia affects the perineurial glia. (A) Control larva expressing dsRNA directed against the *orco* gene. The number of peripheral glial cell types was quantified in filet preparations stained for the presence of Repo-expressing glial cell nuclei (red), axonal membranes (blue) and wrapping glia (*nrv2-Gal4 UAS-lamGFP*, green). The boxed area is shown in higher magnification in A′. Glial nuclei are indicated by white asterisks. The wrapping glial nucleus is indicated by a green asterisk. (B) Larva expressing a dominant negative EGF receptor specifically in the wrapping glia. Note a reduction of the number of glial cells along the nerve. The boxed area is shown in higher magnification in B′. Glial nuclei are indicated by white asterisks. The wrapping glial nucleus is indicated by a green asterisk. (C) Quantification of glial nucleus counting. Note that in wild-type larvae the number of glial cells is higher than in animals harboring an *nrv2-Gal4* insertion. Genotypes are indicated. $n>9$ nerves. Levels of significance: $^{***}P\leq0.001$, $^{**}P\leq0.01$, $^{*}P\leq0.05$.

Axonal wrapping appears generally regulated by receptor tyrosine kinase signaling. In the developing compound eye, the differentiation of retinal wrapping glial cells is triggered by a sequential activation of the FGF receptor (Franzdóttir et al., 2009). FGF receptor expression is also well documented for *Manduca* glial cells, in which reciprocal communication between neurons and glial cells regulates its activity (Gibson et al., 2012). Interestingly, in *Manduca* it was also shown that the EGF receptor is expressed in a subset of olfactory glial cells. Global inactivation of the EGF receptor using a small inhibitor molecule does not lead to defects in glial cell migration but affects sorting of olfactory receptor axons, which also express the EGF receptor (Gibson and Tolbert, 2006). Similar reports were made regarding the role of the EGF receptor in embryonic *Drosophila* glial cells (Sepp and Auld, 2003b).

Thus, the activation of EGF receptor signaling appears crucial for normal differentiation of the wrapping glia. Based on expression data, we assume that this activation occurs cell-autonomously; however, an indirect mechanism might also be operating. Here, EGF receptor signaling in the CNS cortex glia would have to influence motoneuron differentiation, which in turn affects differentiation of the wrapping glia in the peripheral nerves. This would also have to influence wrapping of sensory axons and therefore appears unlikely.

Surprisingly, we only found a role for the Vein ligand that is normally acting to sustain preset EGF receptor activation (Shilo, 2005). The initial activation of the EGF receptor could be initiated in a ligand-independent manner. The broadly expressed adhesion protein Neuroglian is not only a core component of septate junctions but is also known to activate receptor tyrosine kinase signaling in a ligand-independent manner (Doherty and Walsh, 1996; Donier et al., 2012; García-Alonso et al., 2000; Gibson and Tolbert, 2006; Gibson et al., 2012; Islam et al., 2004; Kristiansen et al., 2005; Nagaraj et al., 2009). Possibly, Neuroglian might thus deliver a first trigger signal to initiate glial differentiation, independent of activating ligands such as Vein or Spitz. As soon as this interaction is set during early development, subsequent glial signaling suffices to sustain the differentiation process of the wrapping glia.

In this study, we have shown that the degree of wrapping glial differentiation in the *Drosophila* PNS crucially depends on the activation of the EGF receptor through an autocrine activation loop involving the neuregulin-like signaling molecule Vein. This is remarkably similar to the mechanism underlying the formation of myelin in the PNS of mammals. Here, the regulation of wrapping intensity is controlled by EGF receptor activation, too (Michailov et al., 2004; Taveggia et al., 2005). However, in contrast to what we have found in *Drosophila*, the activating ligand neuregulin 1 is derived from the axon. Only during injury, loss of axonal contact to Schwann cells triggers expression of neuregulin 1 in denervated Schwann cells, which serves as an autocrine/paracrine signal to

Development (2015) 142, 1-10 doi:10.1242/dev.116616

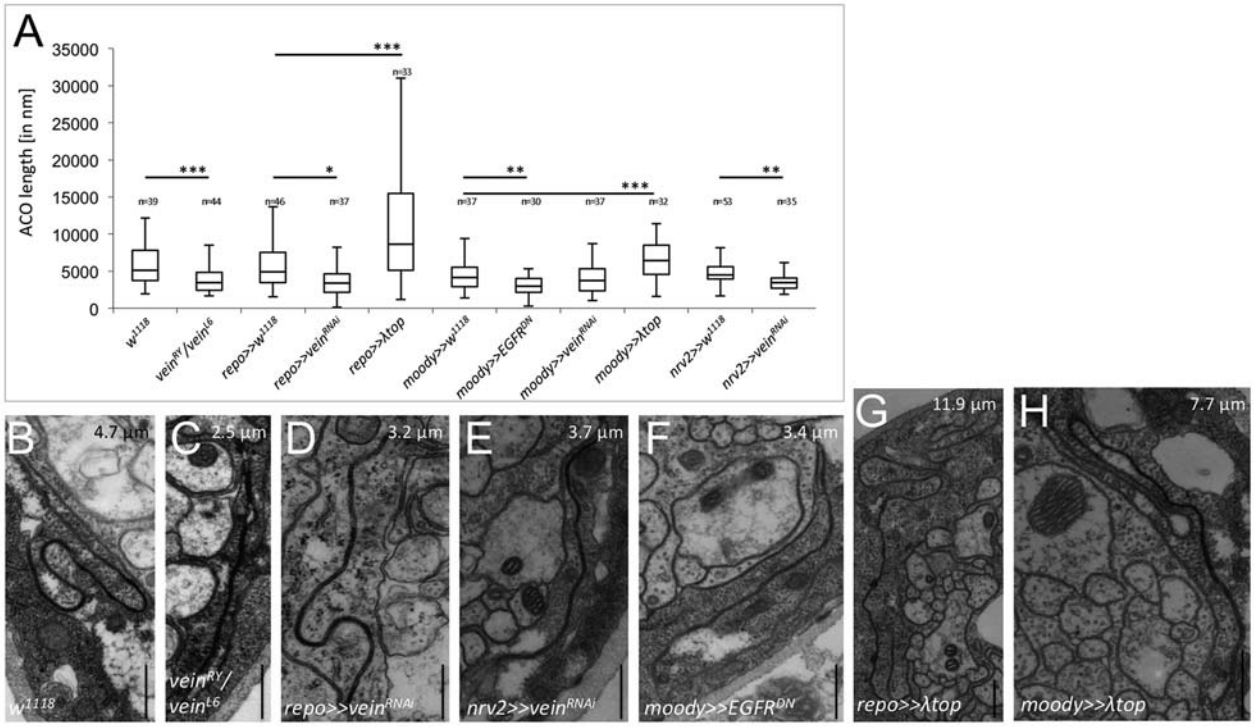

Fig. 7. EGF receptor signaling orchestrates septate junction development. (A) Quantification of the length of the autocellular overlap (ACO) that forms pleated septate junctions. The length is indicated in nm. The different genotypes and the number of cells analyzed by TEM are indicated. Levels of significance: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$. (B-H) Higher magnification images of representative sections. The length of the ACO with septate junctions and the respective genotypes are indicated. Scale bars: 0.5 µm.

promote Schwann cell differentiation and remyelination (Stassart et al., 2013). Thus, this injury model might reveal an evolutionary ancient signaling way of an autocrine activation of axonal ensheathment, which is still used during *Drosophila* development.

MATERIALS AND METHODS

Drosophila work

All *Drosophila* work was conducted according to standard procedures. Flies were kept at 25°C unless indicated otherwise. The following fly strains were used: *UAS-vein2.2* (kindly provided by B. Edgar, Heidelberg, Germany; map position was determined at 94E5), *gliotactin-Gal4* (Auld et al., 1995), *c527-Gal4* and *Mz97-Gal4* (Hummel et al., 2002), *nrv2-Gal4* and *repo-Gal4*; *repo-Gal4* (Lee and Jones, 2005; Sepp and Auld, 1999). These strains allow expression of UAS-based transgenes in glial cells. *OK371-Gal4* allows to express transgenes in glutamatergic motoneurons (Mahr and Aberle, 2006), *repoflp* (Silies et al., 2007), *UAS-λtop* (Queenan et al., 1997), *UAS-EGFR^{DN}*, *UAS-lamGFP* and *UAS-CD2HRP* (Bloomington Stock Center, Bl5364, Bl7376 and Bl8763, respectively).

Single-cell clones were generated using a flip-out approach with flies in the following genotype: *nrv2-Gal4, UAS-flp; UAS>CD2yellow>CD8::GFP* (Wong et al., 2002). The TARGET system (McGuire et al., 2003) was used to induce expression specifically in larval stages. For this, a *tubulin-Gal80^{ts}* (*tub-Gal80^{ts}*) construct (Bloomington Stock Center, Bl7017) was combined with *repo-Gal4* and the corresponding UAS-effector. Animals carrying all genetic elements were kept at the permissive temperature (18°C) until the first-instar stage and were then shifted to the restrictive temperature (29°C). The MiMIC cassette in the insertion *egfr^{Mi02852}*(Bloomington Stock Center, Bl36152) was exchanged with a Cherry exon as described (Venken et al., 2011).

The following UAS-dsRNA flies were used: *UAS-vein^{dsRNA}* [Vienna Drosophila RNAi Center (VDRC) ID 50358]; *UAS-spitz^{dsRNA}* (VDRC ID 3920); *UAS-egfr^{dsRNA}* (TRIP collection Bloomington Stock Center, Bl36770, Bl36773), all obtained from the VDRC, Vienna, Austria. Tubulin–Gal4-driven, ubiquitous expression of *spitz^{dsRNA}* resulted in lethality, whereas expression of *spitz^{dsRNA}* specifically in the developing eye using *GMR-Gal4* resulted in a rough eye phenotype. Other stocks used in this study were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA) or the *Drosophila* Genome Resource Center in Kyoto, Japan.

Immunohistochemistry and electron microscopy analyses

Fixation and treatment of tissues for immunohistochemistry was performed as described (Yuva-Aydemir et al., 2011). Anti-Repo antibodies (Halter et al., 1995; Lee and Jones, 2005) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Other antibodies used were as follows: anti-CD2 (1:1000; Invitrogen, MR6000), anti-GFP (1:1000; Molecular Probes, A11120), anti-Apontic (1:200; Eulenberg and Schuh, 1997) and anti-HRP 649 (1:500; Jackson ImmunoResearch Laboratories, 123-495-021). Labeled specimens were analyzed using a Zeiss 710 LSM, orthogonal sections were taken using the Zen software (Zeiss). For preparations of larval filets, larvae were raised on food containing 0.05% Bromophenol Blue. Third-instar larvae empty their gut a few hours before pupariation (Goldstein et al., 1994). Larvae with empty gut were opened along the dorsal midline and pinned onto sylgard plates. Animals were fixed using Bouin's solution. For electron microscopy analyses larvae were fixed in 4% paraformaldehyde (PFA) and 0.5% glutaraldehyde at room temperature for 2 h. The larvae were then removed from the plastic support and fixed in the same solution at 4°C overnight. Following a fixation in 2% OsO_4 for 1 h on ice and 2% uranyl acetate at room temperature for 30 min, specimens were dehydrated and subsequently embedded in epon using small insect pins. Needles were removed prior to sectioning. Sections were stained with 2% uranyl acetate for 30 min and 0.4% lead citrate for 3 min at room temperature in the dark.

For visualization of HRP-expressing cells, tissues were prefixed for 60 min in 4% PFA at room temperature. Following washes with 0.1 M phosphate buffer (pH 7.2), specimens were treated with 1% $NaBH_4$ for 20 min. Diaminobenzidine (DAB, 0.6%), $(NH_4)_2Ni(SO_4)_2$ and $CoCl_2$

Development (2015) 142, 1-10 doi:10.1242/dev.116616

(0.025% each) and H_2O_2 (0.06%) in 0.1 M phosphate buffer for 1.5 h. Subsequently, tissues were fixed in 4% PFA overnight at 4°C and 1% OsO_4 for 1 h on ice, followed by 30 min 2% uranyl acetate at room temperature, dehydration and embedding using epon. Ultrathin sections were directly imaged with a Zeiss EM900 with a SIS Morada digital camera.

Statistical analysis

In order to quantify the number of axons, axonal profiles were identified by their characteristic cytoplasmic staining, the shape and the presence of central microtubules, and were counted using Fiji with the cell counter plug-in. All axons with at least one direct axonal neighbor were defined as one axon bundle. The number of wrapped individual axons and axon bundles in relation to the total number of axons gives the wrapping index. A wrapping index of 100% implies that every single axon of the nerve is individually wrapped. Counting of wrapped axonal profiles was performed in part double blinded. All nerves that contained less than 76 or more than 82 axons were not included in the statistical analysis, since preparation and fixation artifacts sometimes caused loss of axons or additional axon-like structures. The typical wrapping index of wild-type wandering third-instar larvae is 19%. Significance was determined using a two-tailed Student's *t*-test.

Acknowledgements

We thank B. Edgar and B. Z. Shilo for flies and reagents. We are grateful to B. Risse, D. Zeuschner for help throughout the project, and instructors of the EMBO course on electron microscopy and stereology for advice. General fly stocks were provided by the Bloomington Stock Center. Antibodies were provided by Developmental Studies Hybridoma Bank and Cytoskeleton, Inc. We are thankful to FlyBase for providing information and all members of the Klämbt laboratory for support throughout the project.

Competing interests

The authors declare no competing or financial interests.

Author contributions

T.M. designed and performed most TEM experiments and quantified the data, participated in paper writing. F.S. designed and performed cell biological analysis of nerve development, participated in paper writing. R.K. quantified the number of glial cells along the abdominal nerves in the different genetic backgrounds. F.B. performed the HRP-labeling experiments. D.E. initiated the work and performed first TEM analysis on nerve development. C.K. designed the experiments and wrote the paper.

Funding

This work was supported through grants of the Deutsche Forschungsgemeinschaft (DFG) [SFB629 and SFB1009 to C.K.].

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.116616/-/DC1

References

Auld, V. J., Fetter, R. D., Broadie, K. and Goodman, C. S. (1995). Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in *Drosophila*. *Cell* **81**, 757-767.

Awasaki, T., Lai, S.-L., Ito, K. and Lee, T. (2008). Organization and postembryonic development of glial cells in the adult central brain of *Drosophila*. *J. Neurosci.* **28**, 13742-13753.

Banerjee, S. and Bhat, M. A. (2007). Neuron-glial interactions in blood-brain barrier formation. *Annu. Rev. Neurosci.* **30**, 235-258.

Banerjee, S., Pillai, A. M., Paik, R., Li, J. and Bhat, M. A. (2006). Axonal ensheathment and septate junction formation in the peripheral nervous system of *Drosophila*. *J. Neurosci.* **26**, 3319-3329.

Baumgartner, S., Littleton, J. T., Broadie, K., Bhat, M. A., Harbecke, R., Lengyel, J. A., Chiquet-Ehrismann, R., Prokop, A. and Bellen, H. J. (1996). A *Drosophila* neurexin is required for septate junction and blood-nerve barrier formation and function. *Cell* **87**, 1059-1068.

Bodmer, R. and Jan, Y. N. (1987). Morphological differentiation of the embryonic peripheral neurons in *Drosophila*. *Dev. Genes Evol.* **196**, 69-77.

Bodmer, R., Carretto, R. and Jan, Y. N. (1989). Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron* **3**, 21-32.

Brown, K. E., Kerr, M. and Freeman, M. (2007). The EGFR ligands Spitz and Keren act cooperatively in the *Drosophila* eye. *Dev. Biol.* **307**, 105-113.

Doherty, P. and Walsh, F. S. (1996). CAM-FGF receptor interactions: a model for axonal growth. *Mol. Cell. Neurosci.* **8**, 99-111.

Donaldson, T., Wang, S.-H., Jacobsen, T. L., Schnepp, B., Price, J. and Simcox, A. (2004). Regulation of the *Drosophila* epidermal growth factor-ligand vein is mediated by multiple domains. *Genetics* **167**, 687-698.

Donier, E., Gomez-Sanchez, J. A., Grijota-Martinez, C., Lakomá, J., Baars, S., Garcia-Alonso, L. and Cabedo, H. (2012). L1CAM binds ErbB receptors through Ig-like domains coupling cell adhesion and neuregulin signalling. *PLoS ONE* **7**, e40674.

Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E. and Vincent, J.-P. (2001). Regulated endocytic routing modulates wingless signaling in *Drosophila* embryos. *Cell* **105**, 613-624.

Ebersole, T. A., Chen, Q., Justice, M. J. and Artzt, K. (1996). The quaking gene product necessary in embryogenesis and myelination combines features of RNA binding and signal transduction proteins. *Nat. Genet.* **12**, 260-265.

Edenfeld, G., Volohonsky, G., Krukkert, K., Naffin, E., Lammel, U., Grimm, A., Engelen, D., Reuveny, A., Volk, T. and Klämbt, C. (2006). The splicing factor crooked neck associates with the RNA-binding protein HOW to control glial cell maturation in *Drosophila*. *Neuron* **52**, 969-980.

Edenfeld, G., Altenhein, B., Zierau, A., Cleppien, D., Krukkert, K., Technau, G. and Klämbt, C. (2007). Notch and Numb are required for normal migration of peripheral glia in *Drosophila*. *Dev. Biol.* **301**, 27-37.

Eulenberg, K. G. and Schuh, R. (1997). The tracheae defective gene encodes a bZIP protein that controls tracheal cell movement during *Drosophila* embryogenesis. *EMBO J.* **16**, 7156-7165.

Faivre-Sarrailh, C., Banerjee, S., Li, J., Hortsch, M., Laval, M. and Bhat, M. A. (2004). *Drosophila* contactin, a homolog of vertebrate contactin, is required for septate junction organization and paracellular barrier function. *Development* **131**, 4931-4942.

Franzdóttir, S. R., Engelen, D., Yuva-Aydemir, Y., Schmidt, I., Aho, A. and Klämbt, C. (2009). Switch in FGF signalling initiates glial differentiation in the *Drosophila* eye. *Nature* **460**, 758-761.

Fünfschilling, U., Supplie, L. M., Mahad, D., Boretius, S., Saab, A. S., Edgar, J., Brinkmann, B. G., Kassmann, C. M., Tzvetanova, I. D., Möbius, W. et al. (2012). Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* **485**, 517-521.

García-Alonso, L., Romani, S. and Jiménez, F. (2000). The EGF and FGF receptors mediate neuroglian function to control growth cone decisions during sensory axon guidance in *Drosophila*. *Neuron* **28**, 741-752.

Genova, J. L. and Fehon, R. G. (2003). Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in *Drosophila*. *J. Cell Biol.* **161**, 979-989.

Gibson, N. J. and Tolbert, L. P. (2006). Activation of epidermal growth factor receptor mediates receptor axon sorting and extension in the developing olfactory system of the moth Manduca sexta. *J. Comp. Neurol.* **495**, 554-572.

Gibson, N. J., Tolbert, L. P. and Oland, L. A. (2012). Activation of glial FGFRs is essential in glial migration, proliferation, and survival and in glia-neuron signaling during olfactory system development. *PLoS ONE* **7**, e33828.

Goldstein, L. S. B., Fyrberg, E. A., Wilson, L. and Matsudaira, P. T. (eds) (1994) *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology (Methods in Cell Biology)*, 44. San Diego: Academic Press.

Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A. and Technau, G. M. (1995). The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* **121**, 317-332.

Hummel, T., Attix, S., Gunning, D. and Zipursky, S. L. (2002). Temporal control of glial cell migration in the *Drosophila* eye requires gilgamesh, hedgehog, and eye specification genes. *Neuron* **33**, 193-203.

Islam, R., Kristiansen, L. V., Romani, S., Garcia-Alonso, L. and Hortsch, M. (2004). Activation of EGF receptor kinase by L1-mediated homophilic cell interactions. *Mol. Biol. Cell* **15**, 2003-2012.

Kristiansen, L. V., Velasquez, E., Romani, S., Baars, S., Berezin, V., Bock, E., Hortsch, M. and Garcia-Alonso, L. (2005). Genetic analysis of an overlapping functional requirement for L1- and NCAM-type proteins during sensory axon guidance in *Drosophila*. *Mol. Cell. Neurosci.* **28**, 141-152.

Kumar, J. P., Tio, M., Hsiung, F., Akopyan, S., Gabay, L., Seger, R., Shilo, B. Z. and Moses, K. (1998). Dissecting the roles of the *Drosophila* EGF receptor in eye development and MAP kinase activation. *Development* **125**, 3875-3885.

Landgraf, M. and Thor, S. (2006). Development of *Drosophila* motoneurons: specification and morphology. *Semin. Cell Dev. Biol.* **17**, 3-11.

Lee, B. P. and Jones, B. W. (2005). Transcriptional regulation of the *Drosophila* glial gene repo. *Mech. Dev.* **122**, 849-862.

Leiserson, W. M., Harkins, E. W. and Keshishian, H. (2000). Fray, a *Drosophila* serine/threonine kinase homologous to mammalian PASK, is required for axonal ensheathment. *Neuron* **28**, 793-806.

Leiserson, W. M., Forbush, B. and Keshishian, H. (2011). *Drosophila* glia use a conserved cotransporter mechanism to regulate extracellular volume. *Glia* **59**, 320-332.

Mahr, A. and Aberle, H. (2006). The expression pattern of the *Drosophila* vesicular glutamate transporter: a marker protein for motoneurons and glutamatergic centers in the brain. *Gene Expr. Patterns* **6**, 299-309.
McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K. and Davis, R. L. (2003). Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* **302**, 1765-1768.
Merritt, D. J. and Whitington, P. M. (1995). Central projections of sensory neurons in the *Drosophila* embryo correlate with sensory modality, soma position, and proneural gene function. *J. Neurosci.* **15**, 1755-1767.
Michailov, G. V., Sereda, M. W., Brinkmann, B. G., Fischer, T. M., Haug, B., Birchmeier, C., Role, L., Lai, C., Schwab, M. H. and Nave, K.-A. (2004). Axonal neuregulin-1 regulates myelin sheath thickness. *Science* **304**, 700-703.
Nagaraj, K., Kristiansen, L. V., Skrzynski, A., Castiella, C., Garcia-Alonso, L. and Hortsch, M. (2009). Pathogenic human L1-CAM mutations reduce the adhesion-dependent activation of EGFR. *Hum. Mol. Genet.* **18**, 3822-3831.
Nave, K.-A. (2010). Myelination and support of axonal integrity by glia. *Nature* **468**, 244-252.
Nave, K.-A. and Salzer, J. L. (2006). Axonal regulation of myelination by neuregulin 1. *Curr. Opin. Neurobiol.* **16**, 492-500.
Nave, K.-A. and Trapp, B. D. (2008). Axon-glial signaling and the glial support of axon function. *Annu. Rev. Neurosci.* **31**, 535-561.
Nir, R., Grossman, R., Paroush, Z. and Volk, T. (2012). Phosphorylation of the *Drosophila melanogaster* RNA-binding protein HOW by MAPK/ERK enhances its dimerization and activity. *PLoS Genet.* **8**, e1002632.
Oshima, K. and Fehon, R. G. (2011). Analysis of protein dynamics within the septate junction reveals a highly stable core protein complex that does not include the basolateral polarity protein Discs large. *J. Cell Sci.* **124**, 2861-2871.
Peles, E., Joho, K., Plowman, G. D. and Schlessinger, J. (1997). Close similarity between *Drosophila* neurexin IV and mammalian Caspr protein suggests a conserved mechanism for cellular interactions. *Cell* **88**, 745-746.
Poliak, S. and Peles, E. (2003). The local differentiation of myelinated axons at nodes of Ranvier. *Nat. Rev. Neurosci.* **4**, 968-980.
Queenan, A. M., Ghabrial, A. and Schüpbach, T. (1997). Ectopic activation of torpedo/Egfr, a Drosophila receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. *Development* **124**, 3871-3880.
Ragone, G., Van de Bor, V., Sorrentino, S., Kammerer, M., Galy, A., Schenck, A., Bernardoni, R., Miller, A. A., Roy, N. and Giangrande, A. (2003). Transcriptional regulation of glial cell specification. *Dev. Biol.* **255**, 138-150.
Read, R. D., Cavenee, W. K., Furnari, F. B. and Thomas, J. B. (2009). A *Drosophila* model for EGFR-Ras and PI3K-dependent human glioma. *PLoS Genet.* **5**, e1000374.
Reich, A. and Shilo, B.-Z. (2002). Keren, a new ligand of the *Drosophila* epidermal growth factor receptor, undergoes two modes of cleavage. *EMBO J.* **21**, 4287-4296.
Rodrigues, F., Schmidt, I. and Klämbt, C. (2011). Comparing peripheral glial cell differentiation in *Drosophila* and vertebrates. *Cell. Mol. Life Sci.* **68**, 55-69.
Rodrigues, F., Thuma, L. and Klämbt, C. (2012). The regulation of glial-specific splicing of Neurexin IV requires HOW and Cdk12 activity. *Development* **139**, 1765-1776.
Schnepp, B., Grumbling, G., Donaldson, T. and Simcox, A. (1996). Vein is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins. *Genes Dev.* **10**, 2302-2313.
Schulte, J., Tepass, U. and Auld, V. J. (2003). Gliotactin, a novel marker of tricellular junctions, is necessary for septate junction development in *Drosophila*. *J. Cell Biol.* **161**, 991-1000.
Schüpbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster*. *Cell* **49**, 699-707.
Schwabe, T., Bainton, R. J., Fetter, R. D., Heberlein, U. and Gaul, U. (2005). GPCR signaling is required for blood-brain barrier formation in *Drosophila*. *Cell* **123**, 133-144.
Sepp, K. J. and Auld, V. J. (1999). Conversion of lacZ enhancer trap lines to Gal4 lines using targeted transposition in *Drosophila melanogaster*. *Genetics* **151**, 1093-1101.
Sepp, K. J. and Auld, V. J. (2003a). RhoA and Rac1 GTPases mediate the dynamic rearrangement of actin in peripheral glia. *Development* **130**, 1825-1835.
Sepp, K. J. and Auld, V. J. (2003b). Reciprocal interactions between neurons and glia are required for *Drosophila* peripheral nervous system development. *J. Neurosci.* **23**, 8221-8230.
Sherman, D. L. and Brophy, P. J. (2005). Mechanisms of axon ensheathment and myelin growth. *Nat. Rev. Neurosci.* **6**, 683-690.
Shilo, B.-Z. (2005). Regulating the dynamics of EGF receptor signaling in space and time. *Development* **132**, 4017-4027.
Sidman, R. L., Dickie, M. M. and Appel, S. H. (1964). Mutant mice (Quaking and Jimpy) with deficient myelination in the central nervous system. *Science* **144**, 309-311.
Silies, M. and Klämbt, C. (2010). APC/C(Fzr/Cdh1)-dependent regulation of cell adhesion controls glial migration in the *Drosophila* PNS. *Nat. Neurosci.* **13**, 1357-1364.
Silies, M., Yuva, Y., Engelen, D., Aho, A., Stork, T. and Klämbt, C. (2007). Glial cell migration in the eye disc. *J. Neurosci.* **27**, 13130-13139.
Sink, H. and Whitington, P. M. (1991). Location and connectivity of abdominal motoneurons in the embryo and larva of *Drosophila melanogaster*. *J. Neurobiol.* **22**, 298-311.
Stassart, R. M., Fledrich, R., Velanac, V., Brinkmann, B. G., Schwab, M. H., Meijer, D., Sereda, M. W. and Nave, K.-A. (2013). A role for Schwann cell-derived neuregulin-1 in remyelination. *Nat. Neurosci.* **16**, 48-54.
Stork, T., Engelen, D., Krudewig, A., Silies, M., Bainton, R. J. and Klämbt, C. (2008). Organization and function of the blood brain barrier in *Drosophila*. *J. Neurosci.* **28**, 587-597.
Stork, T., Bernardos, R. and Freeman, M. R. (2012). Analysis of glial cell development and function in *Drosophila*. *Cold Spring Harb. Protoc.* **2012**, 1-17.
Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shrager, P. et al. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* **47**, 681-694.
Unhavaithaya, Y. and Orr-Weaver, T. L. (2012). Polyploidization of glia in neural development links tissue growth to blood-brain barrier integrity. *Genes Dev.* **26**, 31-36.
Venken, K. J. T., Schulze, K. L., Haelterman, N. A., Pan, H., He, Y., Evans-Holm, M., Carlson, J. W., Levis, R. W., Spradling, A. C., Hoskins, R. A. et al. (2011). MiMIC: a highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes. *Nat. Methods* **8**, 737-743.
von Hilchen, C. M., Beckervordersandforth, R. M., Rickert, C., Technau, G. M. and Altenhein, B. (2008). Identity, origin, and migration of peripheral glial cells in the *Drosophila* embryo. *Mech. Dev.* **125**, 337-352.
von Hilchen, C. M., Hein, I., Technau, G. M. and Altenhein, B. (2010). Netrins guide migration of distinct glial cells in the *Drosophila* embryo. *Development* **137**, 1251-1262.
von Hilchen, C. M., Bustos, Á. E., Giangrande, A., Technau, G. M. and Altenhein, B. (2013). Predetermined embryonic glial cells form the distinct glial sheaths of the *Drosophila* peripheral nervous system. *Development* **140**, 3657-3668.
Watts, R. J., Schuldiner, O., Perrino, J., Larsen, C. and Luo, L. (2004). Glia engulf degenerating axons during developmental axon pruning. *Curr. Biol.* **14**, 678-684.
Witte, H. T., Jeibmann, A., Klämbt, C. and Paulus, W. (2009). Modeling glioma growth and invasion in *Drosophila melanogaster*. *Neoplasia* **11**, 882-888.
Wong, A. M., Wang, J. W. and Axel, R. (2002). Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell* **109**, 229-241.
Yarnitzky, T., Min, L. and Volk, T. (1997). The *Drosophila* neuregulin homolog Vein mediates inductive interactions between myotubes and their epidermal attachment cells. *Genes Dev.* **11**, 2691-2700.
Yuva-Aydemir, Y., Bauke, A.-C. and Klämbt, C. (2011). Spinster controls Dpp signaling during glial migration in the *Drosophila* eye. *J. Neurosci.* **31**, 7005-7015.

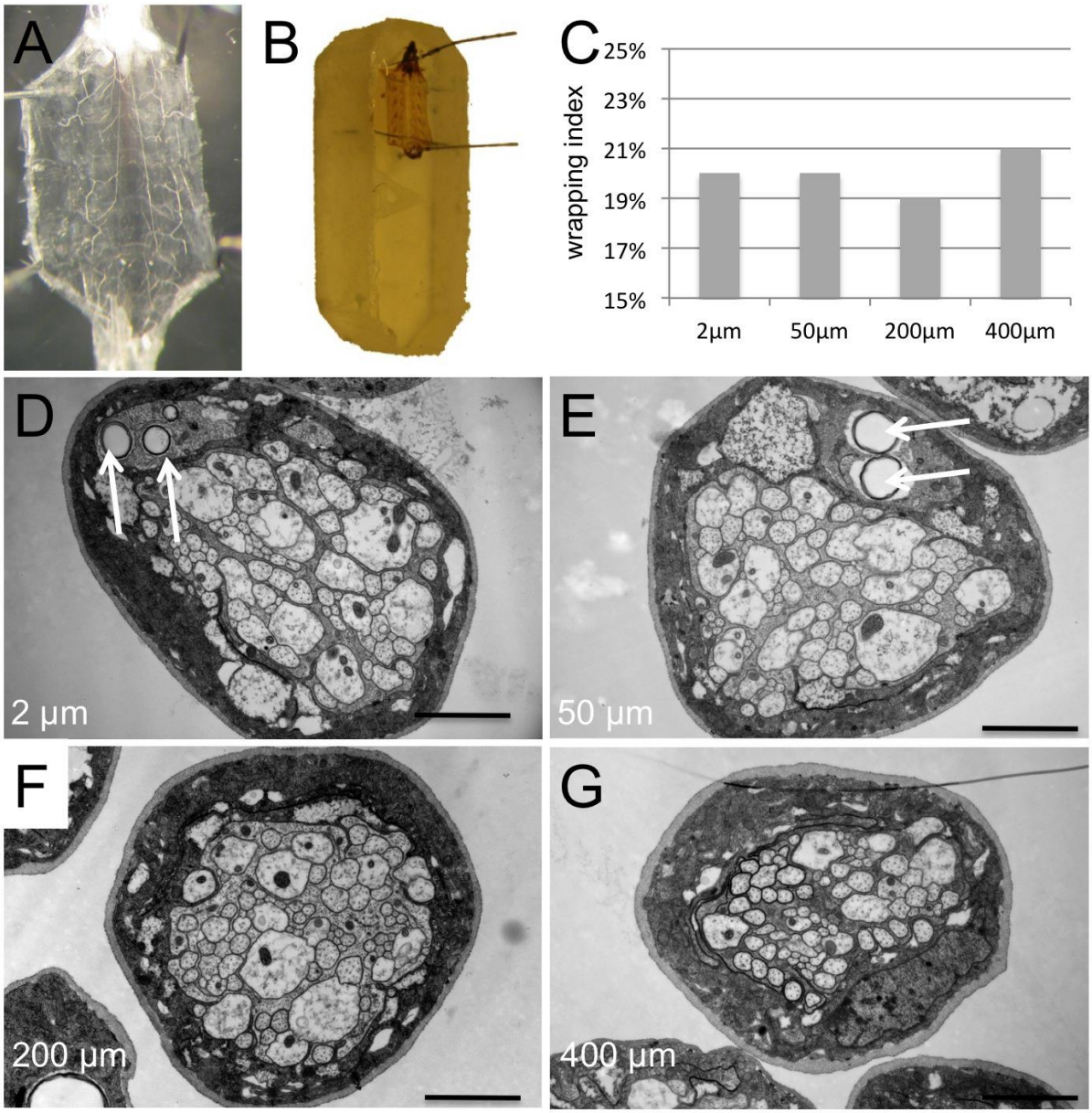

Figure S1

A,B Dissected filet preparations of third instar larvae during prefixation (***A***) and after plastic embedding (***B***). The needles are required to keep the filet in a stereotyped and straight pattern. ***C*** Wrapping index (number of wrapped axon clusters / total number of axons in %) for cross sections taken at 2 µm, 50 µm, 200 µm or 400 µm distance from the CNS along the same peripheral nerve from the same animal. The corresponding images are shown (***D-G***). The scale bar size is 2 µm. The arrows indicate one tracheal cell that forms two tubes.

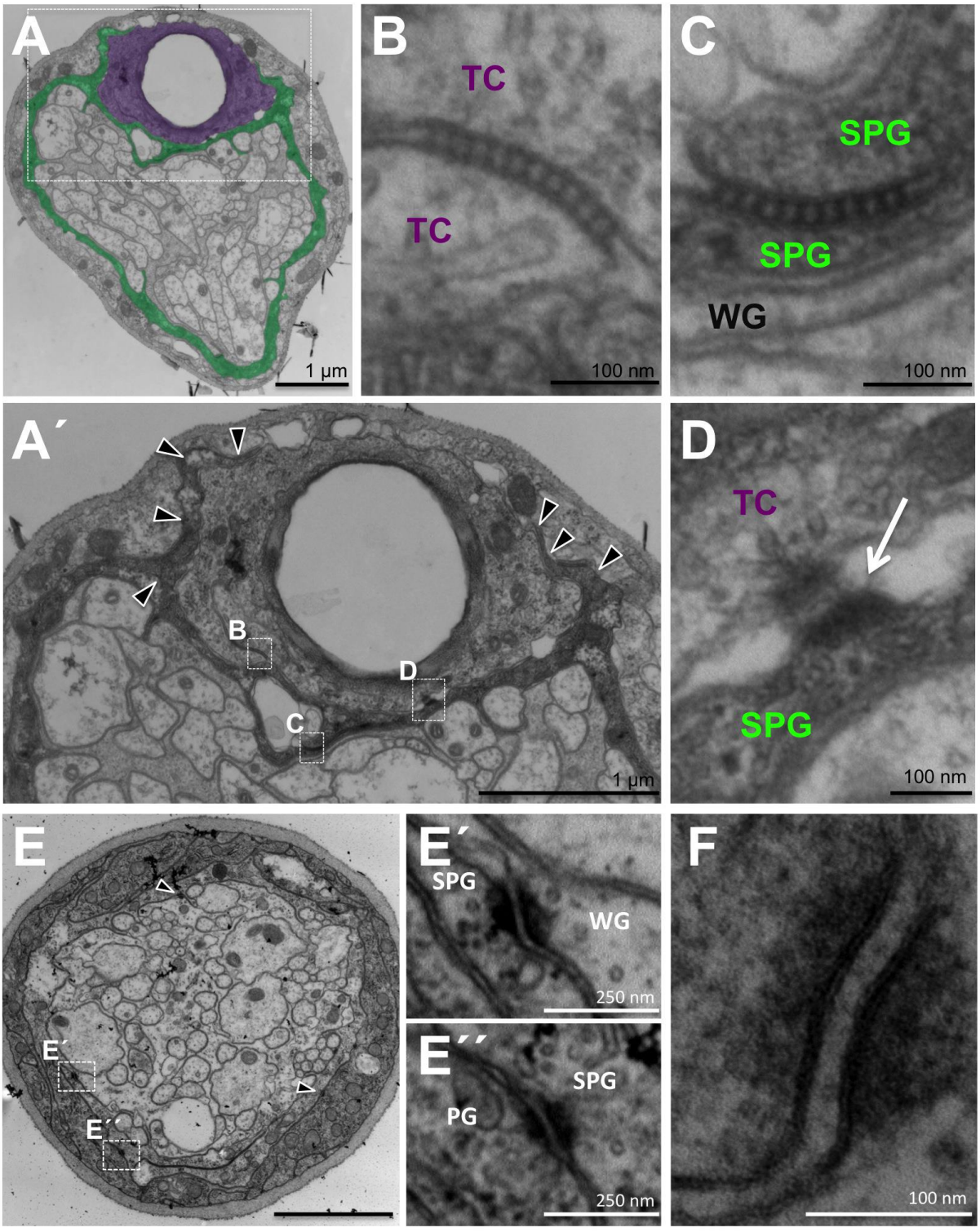

Figure S2

A Tracheal cells are located within the nerve close to the CNS but are not found in the nerve 200 µm or more distant from the CNS. Tracheal cells are always found in

the perineurial glial layer, often covered by processes of the subperineurial glia (green shading). The boxed area is shown in higher magnification in (*A´*). ***A´*** Arrowheads indicate the processes of the subperineurial glia that cover the trachea. The areas boxed in A´are shown in higher magnification in (*B-D*). ***B*** Tracheal cells (TC) establish extensive autocellular pleated septate junctions (pSJ). ***C*** Subperineurial glial cells (SPG) form autocellular pSJ, pSJ are not detected between trachea and subperineurial glia. ***D*** The subperineurial glia forms spot adherens junctions with the tracheal cells. ***E,E´,E´´*** The subperineurial glia forms spot adherens junctions with other glial cells. Boxed areas are shown in higher magnification. ***F*** High resolution image of a glial-glial spot adherens junction. Scale bars are 2 µm, or as indicated.

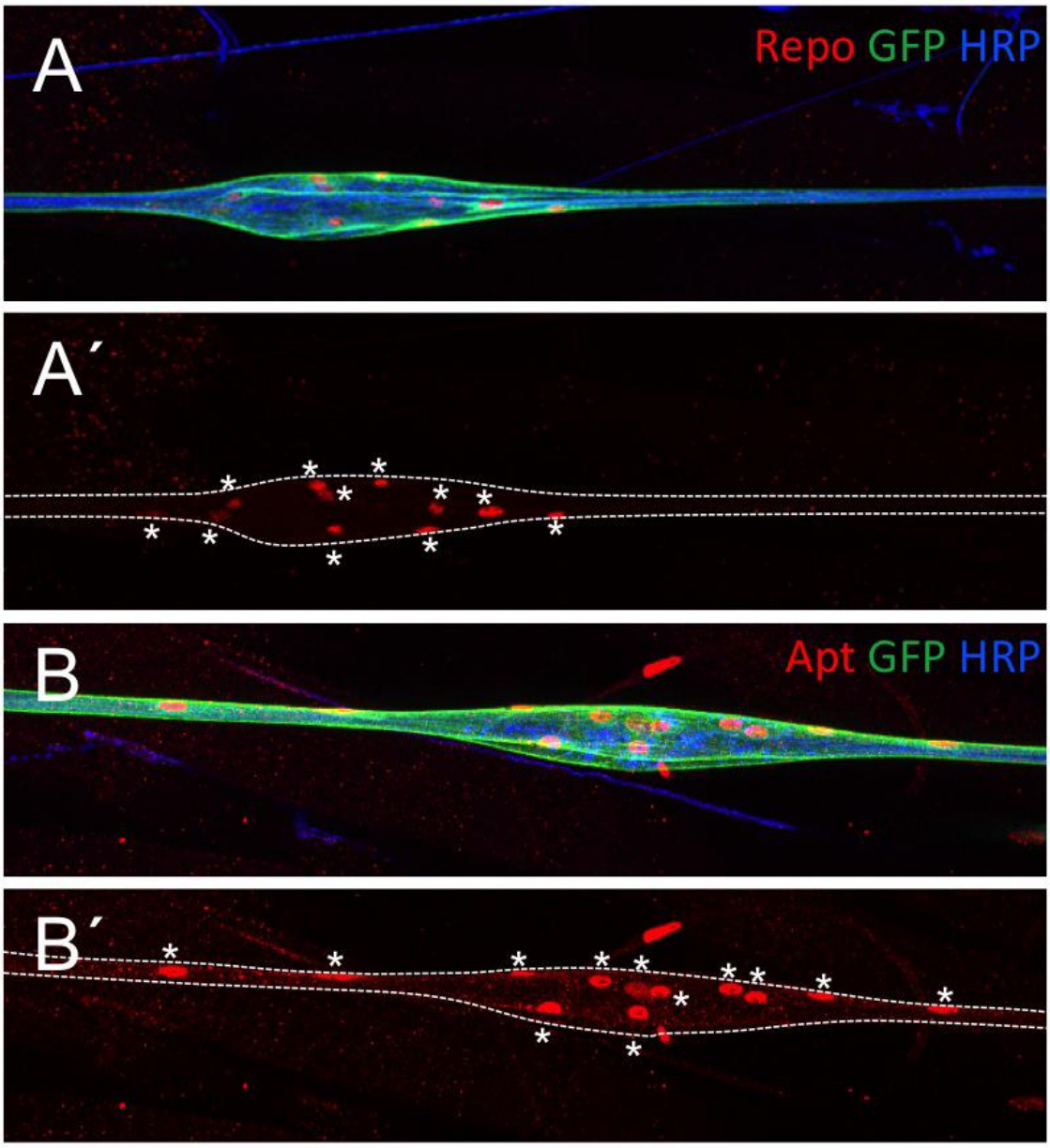

Figure S3

A,A´ Nerve bulge induced by expressing *fray*^{dsRNA} in glial cells. Glial nuclei are in red (Repo staining), glial membranes are in green (*repo>>CD8GFP*) and axonal membranes are in blue. ***B,B´*** Nerve bulge induced by expressing *fray*^{dsRNA} in glial cells. Perineurial glial nuclei are in red (Apontic staining), glial membranes are in green (*repo>>CD8GFP*) and axonal membranes are in blue. Note the increased number of glial nuclei (asterisks) in the bulge area. The dashed line denote the nerve.