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Glial remodeling during metamorphosis influences the stabilization of motor neuron branches in *Drosophila*

Sarita Hebbar [1], Joyce J. Fernandes *

Department of Zoology, and Center for Neuroscience, Miami University, Oxford, OH 45056, USA

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ABSTRACT

Motor neurons that innervate the dorsal longitudinal (flight) muscles, DLMs, make multiple points of contact along the length of fibers. The stereotypy of the innervation lies in the number of contact points (CPs) made by each motor neuron and is established as a consequence of pruning that occurs during metamorphosis. Coincident with the onset of pruning is the arrival of glial processes that eventually ensheath persistent branches. To test a possible role for glia during pruning, the development of adult-specific glial ensheathment was disrupted using a targeted expression of dominant negative *shibire*. Such a manipulation resulted in fewer contact points at the DLM fibers. The development of innervation was examined during metamorphosis, specifically to test if the reduction was a consequence of increased pruning. We quantified the number of branches displaying discontinuities in their membrane, an indicator of the level of pruning. Disrupting the formation of glial ensheathment resulted in a two-fold increase in the discontinuities, indicating that pruning is enhanced. Thus glial–neuronal interactions, specifically during pruning are important for the patterning of adult innervation. Our studies also suggest that FasII plays a role in mediating this communication. At the end of the pruning phase, FasII localizes to glia, which envelops each of the stabilized contact points. When glial FasII levels are increased using the Gal4/UAS system of targeted expression, pruning of secondary branches is enhanced. Our results indicate that glia regulate pruning of secondary branches by influencing the balance between stabilization and pruning. This was confirmed by an observed rescue of the innervation phenotype of FasII hypomorphs by over expressing FasII in glia.

Introduction

Just about a decade ago, it was recognized that glia played more than just a passive role and that at the synapse, they are intimately involved in regulating neuronal activity and synapse strength (Araque et al., 1999). This led to consideration of glia as an important modulatory component of a tripartite synapse, and the bidirectional communication between neurons and glia is now known to include a variety of molecules such as ions, neurotransmitters, signaling molecules and cell adhesion molecules (Fields and Stevens-Graham, 2002). Recent work from vertebrate models has begun to unravel the notion that proper brain function is a result of co-ordinated activity in networks of glia and neurons (Bezzi and Volterra, 2001; Perea et al., 2009). Studies in invertebrate models such as *Drosophila*, *Manduca* and *Caenorhabditis* have also revealed a range of roles that glia play in nervous system development by regulating processes such as axonal pathfinding, nerve fasciculation, neuronal cell death, and remodeling of neuronal connections (reviewed in Oland and Tolbert, 2003; Edenfeld et al., 2005; Freeman, 2006; Freeman and Doherty, 2006), many of which have similarities with vertebrates as well.

In addition to their role at mature synapses, formation of synapses in the CNS and PNS are also influenced by glia (reviewed in Ullian et al., 2004b; Bolton and Eroglu, 2009; Pfrieger, 2009). In vertebrates, astrocyte derived soluble factors are important during the formation of functional synapses (Pfrieger and Barres, 1997; Ullian et al., 2001; Nagler et al., 2001). Immature astrocytes express thrombospondins that work along with other astrocyte derived signals to facilitate synapse assembly in the CNS (Christopherson et al., 2005). The neuronal receptor for thrombospondin has recently been identified (Eroglu et al., 2009). Similar to the role of astrocytes, in vitro studies have shown that Schwann cells promote development of synapses between spinal neurons and are necessary for synaptic activity (Ullian et al., 2004a). In vivo and in vitro studies of the frog neuromuscular junction have shown that glia play important roles in the maintenance of synapse structure and function as well for growth of synapses (Peng et al., 2003; Reddy et al., 2003; Cao and Ko, 2007). A recent study has implicated TGF β1 derived from Schwann cells to promote synaptogenesis in frog as well as mammalian neuromuscular junctions (Feng and Ko, 2008).

Relevant to the current study is the importance of glia in sculpting existing connections. For instance, experimentally induced retraction

* Corresponding author. Fax: +1 513 529 6900.
E-mail address: fernanjj@muohio.edu (J.J. Fernandes).
[1] Current address: National Center for Biological Sciences, Bangalore, India.

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of Bergman glia in the cerebellum decreases activity dependent elimination of climbing fiber synapses (Iino et al., 2001). The remodeling of insect nervous systems during metamorphosis also has instances of glial-dependent axon branch removal. One such example has been demonstrated during pruning of axonal branches in the mushroom bodies of *Drosophila* (Awasaki and Ito, 2004; Watts et al., 2004). In this case, glia actively eliminate axonal branches by engulfment, in a process that is dependent on the apoptotic cell engulfment genes draper and ced-6 (Awasaki et al., 2008). In an injury model using the adult *Drosophila* antennal nerve, it has been shown that glia are activated for engulfment, as a result of axon injury (MacDonald et al., 2006). Most recently, glial involvement in sculpting of Drosophila NMJs has been shown to involve Draper-mediated clearance of synaptic debris (Fuentes-Medel et al., 2009). This bears similarity to the vertebrate NMJ, where Schwann cells clear away axosomes that are shed from retracting axons during the process of synapse elimination (Bishop et al., 2004).

Here we present our investigations of the role of glia during axonal pruning of motor axons that innervate adult thoracic flight muscles in Drosophila. The Dorsal Longitudinal flight Muscles (DLMs) are innervated by motor axons that make multiple contacts along the length of the muscle fibers (Hebbar and Fernandes, 2004). The number of contact points (CPs) is stereotypic, and the stereotypy arises during metamorphosis as a consequence of pruning. More than 70% of adult-specific motor neuron branches are eliminated. We have shown that an earlier expression of FasII in a subset of branches initiates their stabilization (Hebbar and Fernandes, 2005). We also observed that at the end of the pruning phase, in addition to being expressed in the stabilized CP, FasII is also expressed in a glial compartment. This led us to investigate a role for glia during the patterning of DLM innervation. We followed glia during the patterning of adult innervation and demonstrate that the ensheathment is remodeled during metamorphosis. Interestingly, glia retract along the nerve-trunk at a time when adult specific axonal outgrowth and arbor formation is occurring. Later, they extend and arrive at the second order axonal branches just before the onset of morphologically detectable pruning. When the development of glial processes was disrupted during metamorphosis using *shibire^{ts}*, the DLM innervation profile was altered. Specifically, fewer CPs were displayed on the muscle fibers. We followed the fate of second order neuronal branches as a result of this experimental manipulation, and found an increase in the number of axonal branches displaying membrane discontinuities, which is indicative of enhanced pruning. Thus, glia play a protective role during the patterning of DLM innervation and our results suggest that neuron–glia interactions may affect the balance between stabilization and pruning. We also show that FasII is a likely player in these interactions. Interestingly, this protective role for glia in patterning DLM innervation is distinct from the situation of axonal pruning reported for the mushroom bodies where glial engulfment of neurons brings about their pruning (Awasaki and Ito, 2004; Watts et al., 2004).

Materials and methods

Fly strains

Oregon R raised on standard Drosophila food at 25 °C was used as the wild-type strain. To visualize glial processes, we expressed GFP using a glial specific Gal4 driver, repo-Gal4. This driver is able to drive reporter gene expression in all post mitotic PNS and CNS glia during embryogenesis and has no expression in neurons (Sepp et al., 2001). repo-Gal4 (second chromosome insert, source: B. Jones, NYU) was recombined with UAS-2xEGFP (source: H. Keshishian, Yale University) to generate homozygous repo-Gal4; UAS-2xEGFP. FasII over-expression in glia was achieved by crossing UAS-FasII (source: G. Davis, USCF) to +;repo-Gal4;UAS-2xEGFP. Rescue experiments were carried out in male progeny of the cross involving FasII^{e76}; UAS FasII with repo-Gal4; UAS-2xEGFP.

Temperature shift regimens

It has been previously described that over-expression of a dominant negative temperature sensitive allele of shibire (Kitamoto, 2002), shi^{ts}, specifically in glia (using repo-Gal4) can block glial infiltration into the mushroom bodies (Awasaki and Ito, 2004). The temperature sensitive allele allows temporal control of the disruption of glial endocytosis, so that it can be induced specifically during metamorphosis. Flies of the repo-Gal4; UAS-2xEGFP genotype were crossed to UAS-shi^{ts} and the crosses were maintained at 18 °C. Four heat pulse regimens were applied (see Table 1). In the first regimen, third instar larvae were raised at the restrictive temperature (31 °C) until adulthood. These adults were then processed for immunocytochemistry. In the second regimen, larvae were heat pulsed for 12 h at 31 °C. The third regimen included a heat pulse from the larval stage up to 22 h at 31 °C and for the fourth heat pulse, third instar larvae were heat pulsed for 26 h at 31 °C. At the end of the heat pulse, age of the animals was approximately 24 h APF and 31 h APF respectively (APF = after puparium formation). Control animals were aged based on head eversion, DLM innervation and muscle profiles (Fernandes and VijayRaghavan, 1993). Controls included repo-Gal4; UAS-2xEGFP that were subjected to the same heat pulse regimes.

Immunocytochemistry

The general protocol followed was as described previously (Hebbar and Fernandes, 2004). Tissues were fixed with 4% paraformaldehyde (Ted Pella, Inc, CA). 10% donkey serum in 0.1% BSA and 0.3% Triton-X buffered saline was used as a blocking solution prior to primary antibody application. The following primary antibodies were used: anti-HRP (1:200, raised in goat, source: Jackson ImmunoResearch Laboratories, Inc, PA), MAb1D4 (1:3 mouse anti-transmembrane FasII, source: (Hybridoma Bank, Iowa), MAb 8D12 (1:25 mouse anti repo, source: Hybridoma Bank), anti GFP (1:200, raised in rabbit, source: Molecular Probes, OR). A cocktail of the following secondary antibodies were used: Alexa Fluor 488 Donkey anti-rabbit, Alexa Fluor 555 Donkey anti-mouse and Alexa Fluor 660 Donkey anti-goat (all at 1:200; Molecular Probes).

Image acquisition

All immunostained tissues were visualized using an Olympus FV500 Confocal Microscope. Fluorescent dyes were excited using Ar 488 and HeNe 543 nm lasers. Optical Sections of 1 μm thickness (for pupal preps) and 1–3 μm (for adult preps) were taken and stacked using Fluoview Software to obtain a 2D projection. Image panels were prepared using Adobe Photoshop® 6.0 (Adobe Systems Incorporated, CA).

Data analysis

All morphometric measurements were carried out on 2D projections using Image-Pro Plus 4.5 (Media Cybernetics®, MD). In the pupal stages, our analysis focused on primary branches that innervate

Table 1
Repo-positive nuclei increase in numbers during metamorphosis.

Age (h APF)	Number of repo-positive nuclei
Larva[a]	14.8 ± 1.0
10 h	30.2 ± 5.9
18 h	56.5 ± 6.6
24 h	76.8 ± 6.0
38 h	101.8 ± 4.5

[a] Includes a region with the three larval muscle scaffolds for the DLMs.

dorsal muscle, DLM a. DLMs a and b are innervated by one motor neuron, MN5 (Ikeda and Koenig, 1988).The axon of MN5 divides into two to three longitudinal branches (each defined as a primary branch). These include the anterior (a), medial (b) and posterior (c) branch (Ikeda et al., 1980). Secondary branches are transverse outgrowths off a primary branch. All values represent mean ± S.E.M. Basic statistical functions such as mean, standard error of mean and two tailed Student's *t*-test were performed using Microsoft Excel. Chi Square test was performed and interpreted using Minitab program (Minitab Inc, State College, PA).

Results

The innervation pattern of the DLMs has been described as multi-terminal, a terminology used in the insect literature to describe multiple contacts made by a motor neuron along the length of its target fiber (Hoyle, 1983). This is in contrast to the "single terminal" pattern seen in larval fibers (Johansen et al., 1989), where an arbor is elaborated by a motor neuron at the site of nerve entry. Innervation patterns of adult muscles in *Drosophila* arise from the remodeling of their larval counterparts during metamorphosis (Currie and Bate, 1991; Fernandes and VijayRaghavan, 1993; *reviewed in* Fernandes and Keshishian, 1999). In the case of the DLMs, at the onset of the metamorphic period, larval NMJs retract and soon after, adult specific longitudinal or primary branches grow along the persistent larval muscles that serve as templates for the DLM fibers. This is followed by the development of transverse second order branches. Adult myogenesis occurs simultaneously with the development of innervation, and as nascent fibers make an appearance, higher order branches elaborate on the muscle surface. By the end of the first day of metamorphosis, 24 h APF, six DLM fibers have formed and are innervated by an exuberance of second order branches and their higher order arbors (Fernandes et al., 1991; Fernandes and VijayRaghavan, 1993) Between 24 and 38 h APF, more than 75% of second order branches are pruned and this results in the generation of a stereotypic number of secondary branches (Hebbar and Fernandes, 2004). These stabilized branches bear the higher order arbor, which in the adult is observed as a multi-terminal innervation pattern. The cell adhesion molecule, FasII is expressed in a subset of branches during the elaboration phase (18–24 h APF), and, is important for branch stabilization (Hebbar and Fernandes, 2005). By 38 h APF, when the adult complement of contact points is established, FasII is also seen in the glial ensheathment. The glial expression of FasII prompted us to examine glial remodeling during metamorphosis, and to test the hypothesis that they are involved in shaping DLM innervation.

Glial processes closely associate with nerve branches during remodeling

As a first step towards understanding how glia may contribute to patterning of DLM innervation, we examined the development of glial ensheathment of the Posterior Dorsal Mesothoracic Nerve (PDMN), which innervates the adult DLMs. In the adult, each DLM fiber is innervated by a single motor neuron, which makes multiple contacts along the length of the fiber (Hebbar and Fernandes, 2004). The terminal arbor is present distal to these "contact points" (CPs). We examined glial ensheathment of the PDMN as informed by GFP fluorescence in a repo-Gal4/UAS-EGFP line. The PDMN and its branches are well ensheathed and so are the branches of motor axons all the way up to the contact points (Fig. 1B). A closer examination revealed that not all higher order branches of a CP are ensheathed (Figs. 1C, D). Thus, some branches that bear boutons are devoid of glial ensheathment, comparable to the larval NMJ (Sepp et al., 2000). Glial wrapping of the larval mesothoracic nerve which is remodeled to the PDMN is similar (Fig. 2A); many boutons at the larval neuromuscular junctions are not wrapped by repo-positive glia. In order to delineate glial remodeling events during metamorphosis, we examined ensheathment at specific stages in relation to the development of innervation (Fig. 2).

By 10 h APF larval NMJs have retracted, the nerve trunk remains closely associated with each of the three DLM larval templates (Fig.

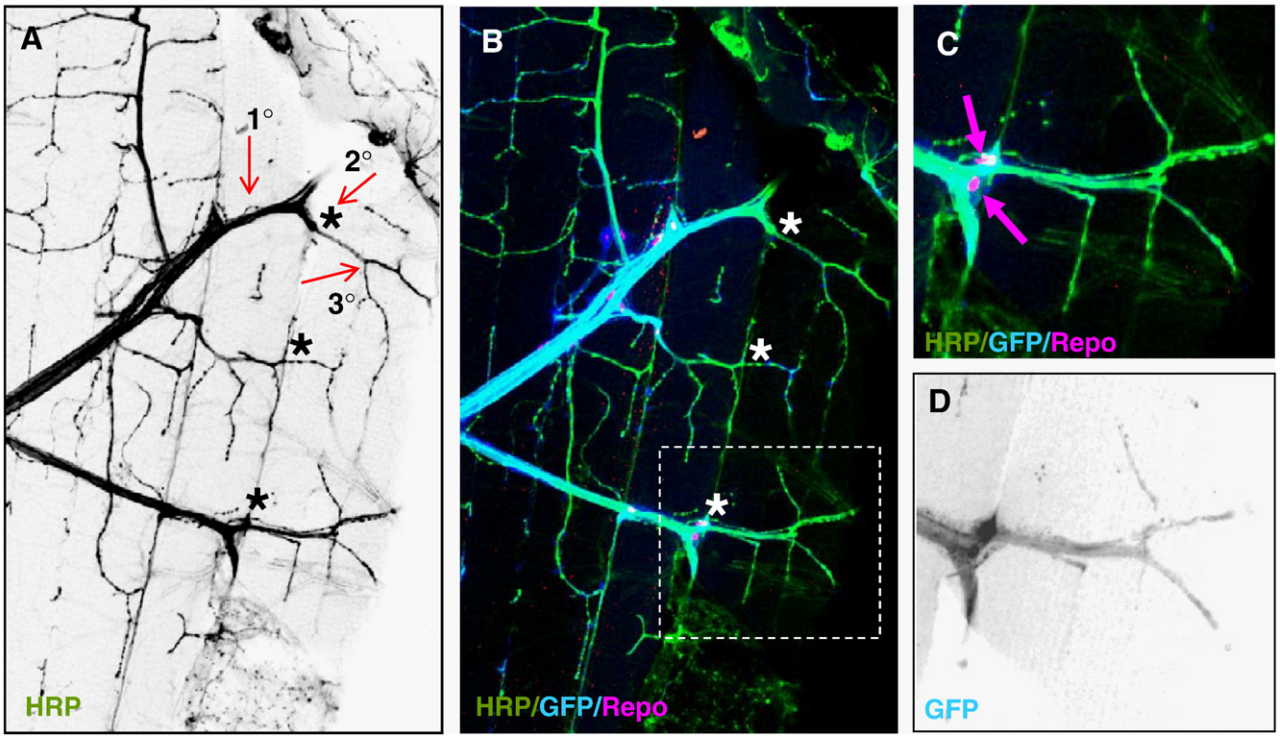

Fig. 1. Glial ensheathment of DLM motor neurons visualized using repo-Gal4; UAS-2xEGFP. (A) DLM innervation as revealed by anti-HRP. (*) represents a contact point (second-order branch), from which tertiary and higher-order branches are elaborated. (B) Three-channel labeling for innervation (HRP, green) glial ensheathment (GFP, blue), and glial nuclei (repo, red). The boxed region is magnified in (C) showing all three channels (arrows point to glial nuclei) and in (D) showing the GFP channel, representing glial ensheathment was used.

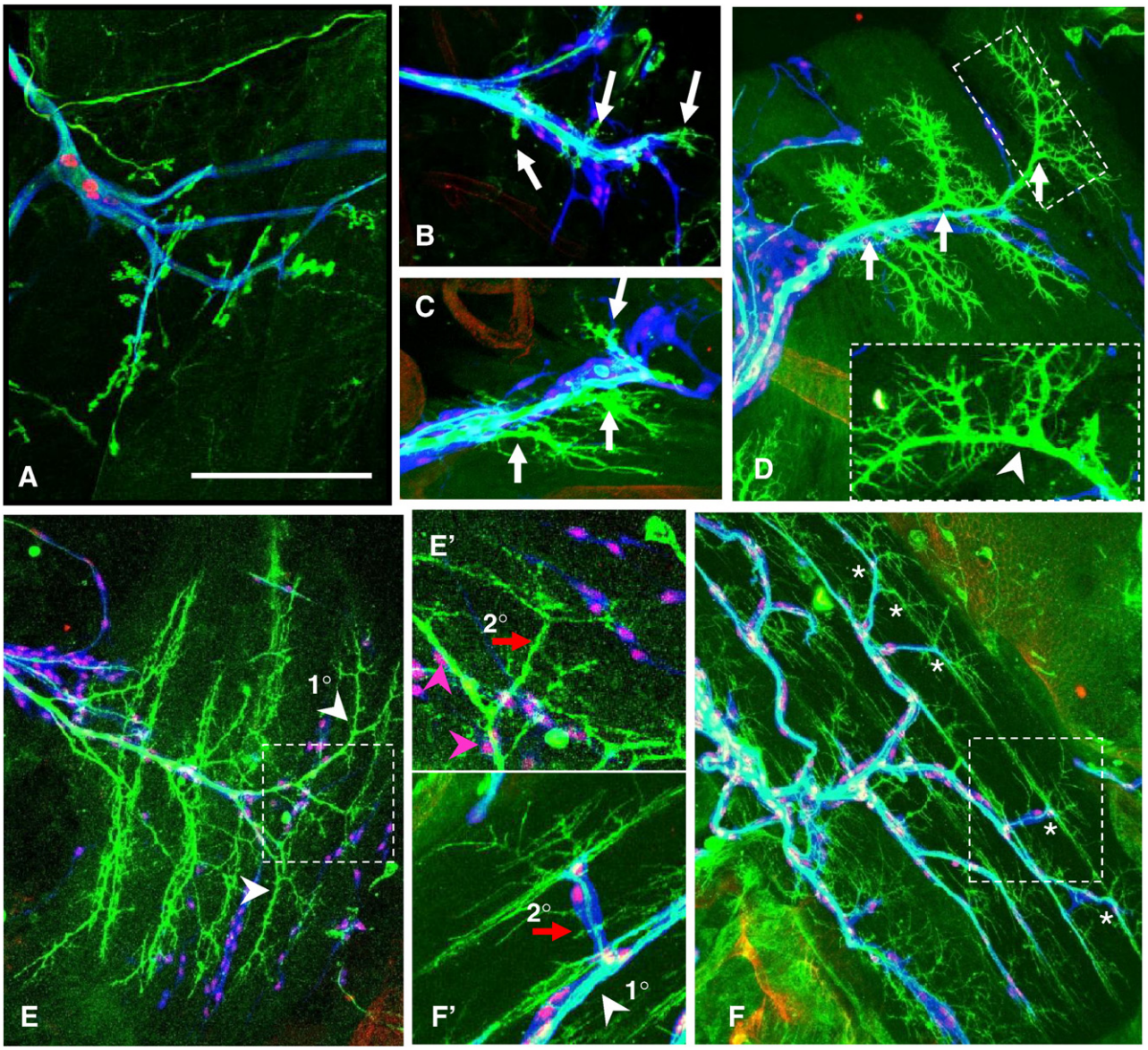

Fig. 2. Glial remodeling associated with the developing DLM innervation during metamorphosis. Glial remodeling associated with the developing DLM innervation during metamorphosis. Glial processes (blue) have been visualized in a repo-Gal4; UAS-2xEGFP strain by labeling with anti-GFP. Innervation is visualized with anti-HRP (green) and glial nuclei (red) have been visualized with anti-repo. (A) Larva: Main nerve trunk is ensheathed; terminals are not. (B) 10 h APF: The larval nerve is sheathed. Arrows indicate retracting terminals at the persistent larval muscles that serve as templates for the DLMs. (C) 12 h APF: Adult-specific neurite outgrowth is seen from the three points (arrows) that correspond to (B). (D) 18 h APF: The nerve is unsheathed. Boxed region is represented in the inset. Arrowhead indicates a primary branch that is devoid of glial ensheathment. (E) 24 h APF: repo-positive nuclei are dispersed along the nerve trunk in the periphery. The primary branches (arrowheads) are not fully sheathed. The boxed area is represented in (E′), where pink arrowheads show repo-positive nuclei along the primary branch. Arrow shows an unsheathed secondary branch. (F) 38 h APF: The adult pattern of innervation and ensheathment. Asterisks (*) mark the five contact points on the most dorsal fiber, DLMa. Boxed area is represented in (F′), where the arrow shows a sheathed secondary branch and the arrowhead points to the sheathed primary branch. Bar represents 10 μm in all panels except E′ and F′.

2B, arrows), and glia continue to ensheath the nerve trunk. At 12 h APF, new adult specific outgrowth begins to elaborate along the persistent larval muscles (Fig. 2C), which by this time have initiated a process of dedifferentiation (Fernandes et al., 1991; Fernandes and VijayRaghavan, 1993). The glial sheath is still visible around the nerve trunk, but the primary branches are mostly devoid of glial ensheathment. By 18 h APF, primary branches have extended along the developing muscle and the transverse second order branches are elaborating higher order branches over the developing muscle surface (Hebbar and Fernandes, 2004); Fig. 2D). At this stage, absence of glial ensheathment is more obvious (Fig. 2D, inset); 60% of preparations examined displayed a bare nerve trunk in the region of the DLM muscles. The observed ensheathment actually corresponds to groupings of sensory neurons that run parallel to the nerve trunk. By 24 h APF, the six DLMs are formed (Fernandes et al., 1991), the primary branches have increased in length. At this time, we have seen that glial ensheathment advances along the PDMN, but does not extensively ensheath the primary or secondary branches (Figs. 2E, E′). By 38 h APF, pruning of exuberant axonal branches is completed and the stabilized second order branches are incorporated into the innervation pattern as the stereotypic number of contact points (CPs) and their arbors (Hebbar and Fernandes, 2004). At this time, glia are seen to completely ensheath all primary branches, and "terminate" at the contact points, just where the terminal arbor grows out onto the muscle fiber (Figs. 2F, F′). This pattern of glial ensheathment resembles what seen in the adult.

We also followed glial nuclei using anti-repo (Table 1), which provided an additional perspective of glial remodeling. Repo-positive glial nuclei can be detected all along the glial ensheathment in larval, adult and pupal stages. At the 18 h APF stage, much of the glial sheath

is present in a ventral location, and correspondingly, a large number of glial nuclei are revealed in that location. A two-fold increase in repo-positive nuclei is observed at this stage (Table 1), as compared to 10 h APF at which time the nerve is sheathed and presumably displays the larval component. By 24 h APF, glial nuclei are seen along the main nerve-trunk (Fig. 2E), but the ensheathment has not fully developed as evidenced by the lack of extensive repo driven EGFP labeling. Glial nuclei can be seen along primary nerve branches but not along the second order branches (Fig. 2E′). By 38 h APF, many more nuclei are seen along the nerve trunk (Table 1), and primary branches (Fig. 2F). Unlike the prior stage of 24 h, glial nuclei can now be seen along second order branches (2F′), and consistently, the repo >EFGP signal is much stronger.

Disruption of glial membrane endocytosis results in a reduced number of terminal arbors

To test if glia are important for patterning the adult innervation, we expressed a temperature sensitive allele of *shibire* (Kitamoto, 2002), *shi^{ts}*, in glia. *shibire* encodes dynamin, which is involved in a number of membrane related events (Praefcke and McMahon, 2004) including endocytosis. This tool has been previously used for testing the role of glia in pruning of mushroom body axons (Awasaki and Ito, 2004); driving *shi^{ts}* expression in glia resulted in blocking process outgrowth, presumably a result of blocking membrane endocytosis.

The temperature sensitive allele allows temporal control of the disruption of membrane functions in glia for specific time periods during metamorphosis. First, we examined adults that emerged when pupae were heat pulsed for the entire duration of metamorphosis (4 days; Table 2). Animals of the genotype repo-Gal4; UAS-2xEGFP were used as controls since they exhibited no differences in the innervation pattern as compared to the wild-type strain (Oregon R), when raised at the restrictive temperature (Table 3; Fig. 3A1). In these controls, the most dorsal DLM fiber, DLMa, exhibits 4.9 ± 0.22 contact points (CPs). In heat pulsed repo-Gal4/UAS-shi^{ts}; UAS-2xEGFP animals (experimental), the number of stereotypic CPs is 3.7 ± 0.23 (Table 3; Fig. 3B1); this reduction is statistically significant ($p<0.0025$). The range of CPs displayed by the two genotypes is also different (Table 3, Fig. 3C); the control animals exhibit 4–6 CPs with the majority, 62.5%, displaying the stereotypic 5 CPs. By contrast, the experimental animals displayed 3–5 CPs with only 11% displaying 5 CPs and the majority (88.9%) displaying less than 5 CPs. It is interesting to note that the outcome of a block in glial membrane endocytosis is not that glial ensheathment is completely absent but it is not as extensive as in the controls (Fig. 3-A2 and B2).

Alterations in glial ensheathment during metamorphosis enhances pruning of motor neuronal branches

Having determined that the adult innervation profiles are altered as a result of disrupting glial membrane endocytosis, we next focused on the pupal stages to examine motor neuron remodeling as well as glial ensheathment. Three heat pulse regimens were carried out. One group of pupae was heat pulsed from the late third larval instar until 12 h APF, when adult specific outgrowths are growing along the muscle surface (Regimen 2, Table 2). Our observations indicate that there are no gross defects in outgrowth of longitudinal branches or in secondary branch outgrowth (data not shown). In order to disrupt glial ensheathment during branch elaboration and stabilization, two other groups of animals were heat pulsed (Table 2). One group received a heat pulse from the late third larval instar until 22 h APF (Regimen 3; corresponds to 24 h of development at 25 °C) and another group received a heat pulse from the late third larval instar until 26 h APF (Regimen 4; corresponds to 31 h of development at 25 °C). Innervation and ensheathment was examined at the corresponding endpoints (24 and 31 h). At 24 h APF, we observed no gross morphological changes in innervation pattern, as determined by quantifying the total number of secondary branches on DLMa (Table 4). A subset of second order branches have higher order branches/arbors; experimental animals displayed 8.4 ± 0.6 of these on the most dorsal muscle, DLMa, which was not significantly different from control animals (9 ± 0.9; $p=0.6$).

By 31 h APF, in the controls, glia have advanced along the PDMN, migrated further to completely ensheath the primary branches and are beginning to ensheath some secondary branches (Fig. 4A). We have previously established that at this time, pruning of axonal branches is underway (Hebbar and Fernandes, 2004). To obtain independent confirmation of pruning, we quantified second order branches that have arbors. There are 7.85 ± 0.4 secondary branches on DLMa in the control group, reflecting a 13% reduction in secondary branches as compared to that at 24 h APF (9.0 ± 0.9, Table 4). In experimental animals, there are significantly fewer second order branches with arbors as compared with controls (5.6 ± 0.5; $p<0.01$), representing a 33% reduction. How does this correlate with glial ensheathment? We examined the extent to which the developing innervation was ensheathed, and established three categories—PDMN and primary branches are ensheathed, only the PDMN is ensheathed, PDMN is not ensheathed (Table 5). Only 21% of experimental animals displayed ensheathment of the PDMN as well as primary branches; by contrast 89% of control animals displayed this phenotype. Thus, glial advancement is slowed down as a result of targeting expression of shi^{ts}. Unlike in controls, where glial ensheathment is seen at the base of second order branches or contact points (Figs. 4A′ and 2F′), this is not the case in experimental animals (Fig. 4C′).

In the mushroom bodies (Lee et al., 2000; Awasaki and Ito, 2004), the presence of axon branches with membrane discontinuities has been used as an indicator of pruning. Upon close examination of the secondary branches in the wild-type at 31 h APF, we observed that there are some branches which are intact while others exhibit discontinuous membranes as if in the process of "break-down". In our case, these discontinuities span the entire length of a secondary branch or a part of its higher-order arbor (Fig. 5); we have not observed any such signs of break down at the level of the primary branches. In order to determine if this ongoing process of break-down is also disrupted in the heat pulsed animals, we quantified the number

Table 3
Innervation phenotypes resulting from experimental manipulations of glia.

Genotype	No. of CPs (mean ± SEM)	Range of CPs	<5 CPs %	5 CPs %	>5 CPs %
Oregon R	5.2 ± 0.12 (13)	4–6	7.7	69.3	23
+; repo>2xEGFP (heat pulsed)	4.9 ± 0.22 (8)	4–6	25	62.5	12.5
+; repo>2xEGFP/UAS-shi^{ts} (heat pulsed)	3.7 ± 0.23 (9)**	3–5	88.9	11.1	0
+/y; repo>FasII; 2xEGFP/+	4.6 ± 0.17 (19)$^{+}$	4–6	52.6	32.6	15.8
FasII^{e76}/y; repo>FasII; 2xEGFP/+	4.9 ± 0.30 (15)	3–6	26.7	53.3	20
FasII^{e76}; UAS-FasII; +	5.6 ± 0.21(13)$^{+}$	4–6	15.3	7.7	77

$^{+}p<0.05$ comparison with Oregon R. **$p<0.0025$ comparison with +; repo-Gal4; UAS-2xEGFP (heat pulsed).
Number in parenthesis represents sample size.

Table 2
Heat pulse regimes applied to +; repo-Gal4/+; UAS-2xEGFP/UAS-shi^{ts}.

	Grown at 18 °C (permissive temperature)	Shifted to 31 °C (restrictive temperature)	Age (equivalent to 25 °C)
1.	Embryo—3rd instar larva	Adulthood	Adults
2.	Embryo—3rd instar larva	12 h	14 h APF
3.	Embryo—3rd instar larva	22 h	24 h APF
4.	Embryo—3rd instar larva	26 h	31 h APF

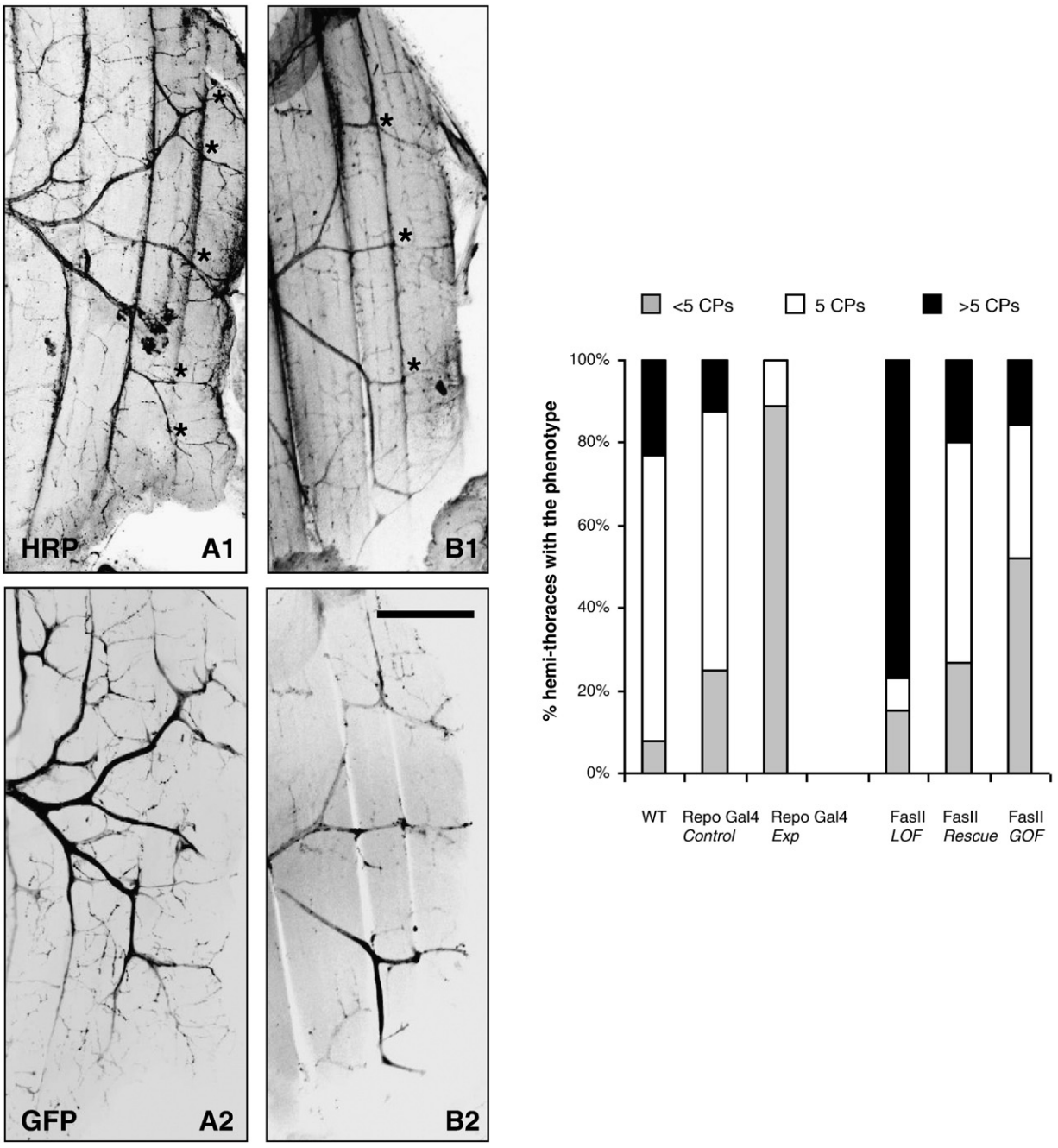

Fig. 3. The adult innervation pattern is disrupted as a result of repo driven shi^{ts} expression. (A and B) Hemithoracic preparations of repo-Gal4; UAS-2xEGFP (Control-A) and repo-Gal4/UAS-shi^{ts}; UAS-2xEGFP (Experimental-B) adults subjected to heat pulses (regimen 1). The preparations have been double labeled with anti-HRP to visualize innervation (A1 and B1) and anti GFP (A2 and B2) to visualize glial ensheathment. Heat pulses in the experimental animals result in a decrease in the extent of glial ensheathment (B2), and a reduced number of CPs/terminal arbors (asterisks). (C) A stack represents the percentage of hemithoraces within a genotype exhibiting either less than 5 (gray), 5 (white) and more than 5 (black) CPs. * indicates a significant difference in the distribution as compared to heat pulsed control, repo-Gal4 ($p<0.025$) and + indicates a significant difference in the distribution as compared to wild type control, Oregon R ($p<0.025$). Bar indicates 10 μm.

of secondary branches exhibiting membrane discontinuities at 31 h APF. In control animals, 30.7% of secondary branches are in the process of breakdown and there are on an average, 5.42 ± 0.36 intact branches (Table 4). By contrast, in heat pulsed animals, 41% of secondary branches exhibit membrane discontinuities and there are only 3.2 ± 0.35 intact secondary branches (Table 4). The number of intact secondary branches at 31 h APF is reduced in comparison to the wild-type ($p<0.0005$).

Taken together, our results suggest that lack of glial advancement to envelope second order branches results in enhanced and perhaps accelerated pruning during the 24–38 h period, resulting in a reduced complement of secondary branches.

Targeting FasII to glia influences development of the adult innervation pattern

Our studies thus far suggest that glia influence the patterning of DLM innervation. A possible candidate that may mediate the glia–neuron interaction is the cell adhesion molecule, FasII. We have previously demonstrated that at 38 h APF, FasII is expressed along axons and in an additional compartment, most likely the surrounding peripheral glia (Hebbar and Fernandes, 2005). Does this localization of FasII along glial processes have any bearing on their role in pruning/stabilization? To address this question, we examined the effects of altering FasII levels in glia.

Table 4
Targeted expression of shi^{ts} in glia affects pruning of second order branches.

Morphology	Repo>2xEGFP	Repo>2xEGFP/UAS-shi^{ts} (heat pulsed)
24 h APF		
Total number of secondary branches	41±3 (5)	30±3.4 (6); $p=0.9$
# Secondary branches with arbors	9.0±0.9 (4)	8.4±0.6 (5); $p=0.6$
31 h APF		
# Secondary branches with arbors	7.85±0.4 (7)	5.6±0.54* (16)
# Intact secondary branches with arbors	5.4±0.36 (7)	3.28±0.35* (7)

Number in parenthesis represents sample size. Values represent mean±SEM.
* $p<0.01$ as compared to controls.

Table 5
Glial ensheathment at 31 h APF as detected using Repo>2xEGFP.

Genotype	Absence of glial ensheathment along the nerve	Glial ensheathment along nerve trunk alone	Glial ensheathment along nerve trunk and primary branches
Repo>2xEGFP ($n=9$)	11%	0%	89%
Heat pulsed Repo>shi^{ts} ($n=14$)	29%	50%	21%

Using the repo-Gal4 driver, we over-expressed FasII in glia and examined the effect on DLM innervation. In the adult thorax, FasII is usually absent from the innervating neurons, muscle or glia (Hebbar and Fernandes, 2005). Targeted over-expression with repo-Gal4 results in the localization of FasII in adult glial processes (data not shown), confirming over-expression of the protein. In adults where FasII is expressed under the control of repo-Gal4 (repo-Gal4/+; UAS-FasII/+), the average number of CPs is 4.6±0.17 (Table 3), a

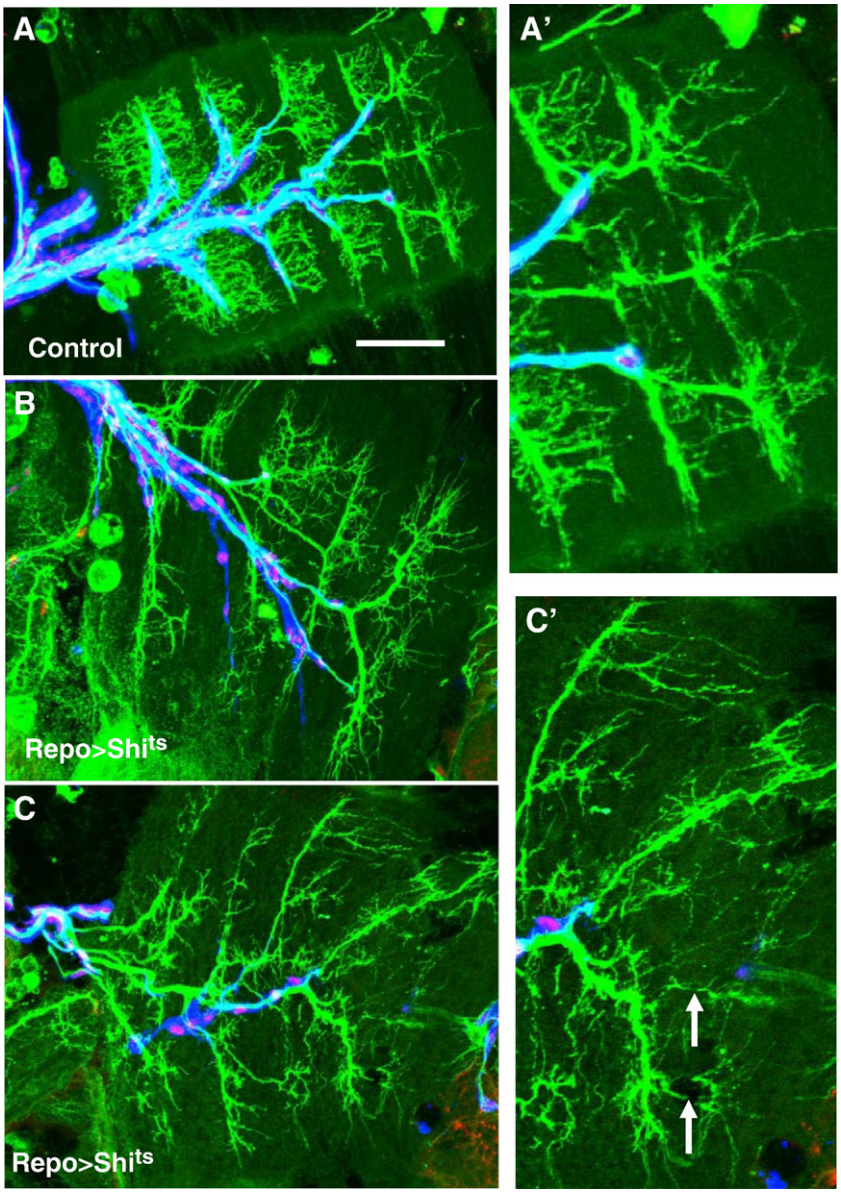

Fig. 4. Effect of glial expression of shi^{ts} on development of the DLM innervation pattern at 31 h APF. Effect of glial expression of shi^{ts} on development of the DLM innervation pattern at 31 h APF Glial processes (blue) have been visualized in a repo-Gal4; UAS-2xEGFP background by labeling with anti-GFP. Innervation is visualized with anti-HRP (green) and glial nuclei (red) have been visualized with anti-repo (A) repo-Gal4; UAS-2xEGPF (B, C) repo-Gal4/UAS-shi^{ts}; UAS-2xEGFP. (A′, C′) Higher magnification of the dorsal most muscle pair (DLMs a and b) from A and C respectively. Arrows (C′) indicate examples of discontinuities in secondary branches.

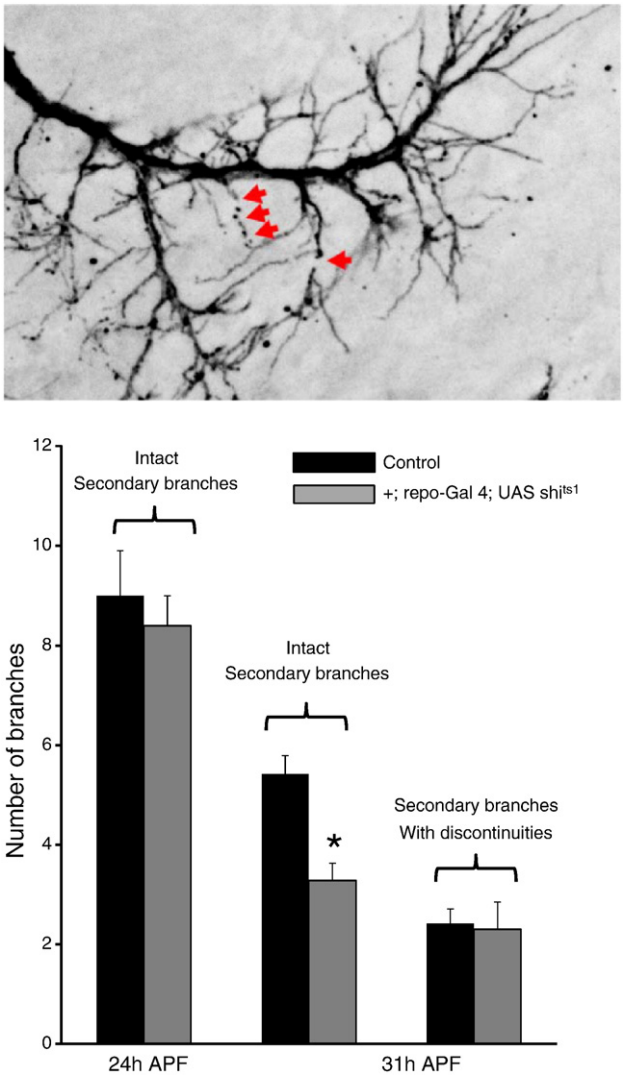

Fig. 5. Pruning of second order branches. Pruning of second order branches. A primary branch (longitudinal) on DLMa and its secondary branches (transverse) visualized with anti-HRP at 31 h APF. At this time pruning is underway and affects secondary branches that are unbranched (three red arrows), or those that bear higher order arbors (red arrow).

reduction which is statistically significant in comparison to controls ($p<0.05$, Table 3). The number of contact points ranges from 4 to 6 CPs with only 32.6% of animals displaying 5 as compared to 69.3% in the wild type (Table 3, Fig. 3C). Thus, over-expressing FasII in glia has an impact on generation of the stereotypic adult innervation.

We had previously described that FasII hypomorphs exhibit an increased number of CPs (Hebbar and Fernandes, 2005), which is due to an enhanced stabilization. Can FasII over-expression in glia rescue the *FasII*^{e76} hypomorphic phenotype? Examination of the adult innervation in *FasII*^{e76}; repo-Gal4/UAS FasII animals revealed that increasing FasII in glia can indeed rescue the hypomorphic phenotype. Whereas *FasII*^{e76}; UAS FasII animals displayed an increased number of CPs, 5.6 ± 0.21 ($p<0.05$ as compared to wild type, Table 3), in FasII^{e76}; repo-Gal4/UAS FasII there are 4.9 ± 0.17 CPs, that is no different than the wild type (Table 3). This rescue is also reflected in the range of CPs displayed. In *FasII*^{e76}; UAS FasII animals, CPs range from 4 to 6 with only 7.7% displaying 5 CPs while a majority, 77% display more than the stereotypic 5 CPs (Table 3 and Fig. 3C). As a result of the rescue, CPs range from 3 to 6 with 53.3% displaying 5 CPs and only 20% display more than 6 CPs. Thus FasII over-expression in glia rescues the hypomorphic phenotype of increased CPs and is comparable to the wild type (Table 3 and Fig. 3C).

Discussion

The stereotypy of DLM innervation lies in the number of times that a motor neuron makes contacts along the length of the muscle fiber. These contact-points (CPs) seen at the adult muscle have their origins in second order branches that are elaborated over the developing muscle surface during the first day of metamorphosis The adult number of contacts is established as a result of axonal pruning that occurs during the following day. By this time the animal is about a third of the way through the pupal period. The patterning of DLM innervation can be divided into four distinct phases (Fernandes et al., 1991; Hebbar and Fernandes, 2004, 2005): retraction of larval processes (6–10 h APF), outgrowth and elaboration (12–24 h APF), during which arbors of secondary branches expand on the muscle surface; pruning and stabilization (24–38 h APF), during which a majority of secondary branches are pruned, while a few are stabilized; arbor expansion (38 h APF-Adult), during which the stabilized secondary branches expand their arbors as the muscle increases in bulk, and begin differentiating into terminal boutons.

To understand how excessive branches are pruned, two aspects must be considered—the selective removal of most branches and the stabilization of those that persist. This paper has examined a role for glia during the pruning/stabilization phase, and we have established the following: (1) Glia undergo a process of remodeling which includes retraction of larval glia, and a subsequent proliferation and ensheathment of developing axons. (2) The mesothoracic nerve remains unsheathed during a period when DLM motor neurons are in the process of maximal branch elaboration. (3) Glia serve a protective role (for subsets of second order branches) during the pruning/stabilization period. (4) Innervation defects seen in FasII hypomorphs can be rescued by targeting a FasII transgene to glia. Key events in the remodeling of glia and in the development of the DLM innervation pattern are summarized in Fig. 6.

Retraction of peripheral glia

At the larval stage, peripheral glia fully ensheath the segmental nerves, their processes often extending into the neuromuscular junction (Sepp et al., 2000). Using repo-Gal4 driven EGFP as a cytoplasmic label, we show that the glial ensheathment undergoes remodeling during metamorphosis. In the mesothorax, NMJs at the persistent larval muscles that serve as scaffolds for the DLMs have mostly retracted by 10 h APF. Even as new adult specific axonal outgrowths are seen, the glial ensheathment is largely intact. Soon after, glial processes recede to the region of the nerve entry point into the muscle field.

The timing of glial retraction is coincident with the expansion of axonal arbors over the developing DLM fibers. It is therefore tempting to speculate that presence of glia may be inhibitory to process outgrowth, and in this regard, the scenario may be similar to vertebrates where it is well understood that the limited capacity of CNS nerve regeneration is due to inhibitory effects of glia (Domeniconi and Filbin, 2005). Another player in the retraction of glial processes is likely to be the adult sensory neurons, which at this time are extending processes into the PDMN, and may require an unsheathed trunk for fasciculation before they can be led towards the CNS. In regard to the signal that may trigger retraction, ecdysteroids are a likely candidate. In holometabolous insects, It is known that retraction of larval NMJs is triggered by ecdysteroids (Weeks, 2003), and it may well be that glial retraction is a later event in the sequence. Another player is likely to be the nascent DLM fiber, which develop as a result of myoblast fusions, a process that occurs simultaneously with the retraction of glia. It is conceivable that the muscle surface provides a signal to promote retraction of glia as well as the outgrowth of axonal arbors.

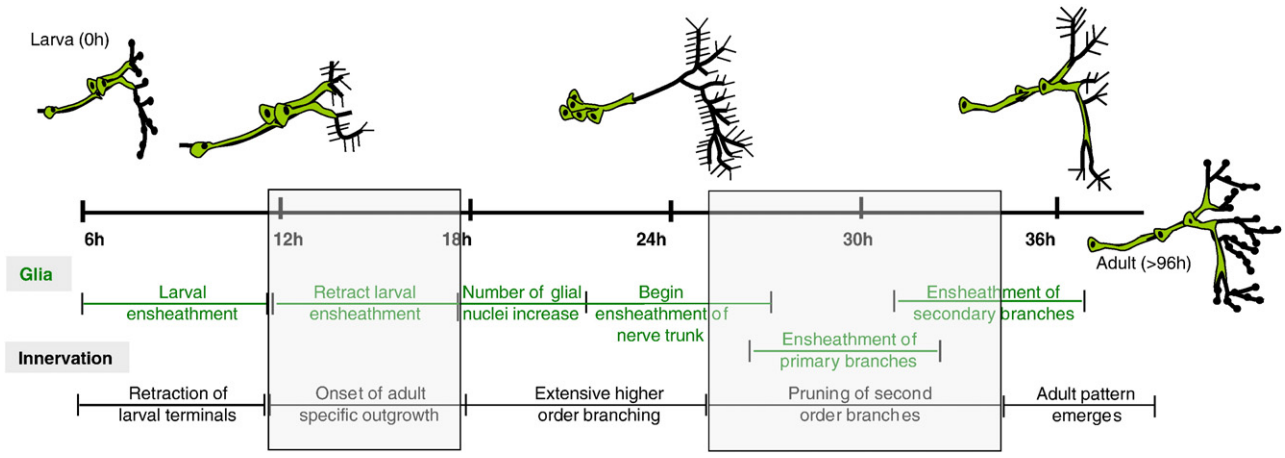

Fig. 6. Glial remodeling and the development of DLM innervation.

Increase in the number of peripheral glia during metamorphosis

In the embryo, there are 7–8 glial nuclei per hemisegment in the periphery and this number is reported to remain unchanged at the third larval instar (Sepp et al., 2000). In the mesothorax, we find that that number of glia increase at least 10 fold, and may reflect the generation of an adult-specific pool needed for ensheathing the developing PDMN (mesothoracic nerve) which becomes much larger in size as compared to its larval counterpart (King and Wyman, 1980). The increase in size occurs as part of the shift in locomotor control to the thorax during metamorphosis, and an accompanying increase in size of the thoracic ganglion (Truman, 1990). It is known that glia extend processes into the nerve bundle to surround groups of axons (Leiserson et al., 2000; Stork et al., 2008) and during formation of the larger PDMN, many more glia may be required to surround the additional axons in the nerve trunk. At this time we do not know if the observed increase in glia is due to proliferation in the periphery, or if, they arrive from a CNS location. Glia that proliferate in the periphery are usually associated with sensory neurons. Occurrence of glial proliferation in the periphery has been demonstrated along the developing wing margin (Aigouy et al., 2004). It is also known that glia that populate the adult antenna include those that are derived from sensory lineages as well as others that migrate to the periphery from the brain (Sen et al., 2005). In case of glia that ensheath peripheral nerves in the embryo, proliferation occurs at the nerve-exit points and subsequently when axons have left the CNS, glia extend processes and migrate out along the nerve bundle (Sepp et al., 2000).

Glia ensheath the PDMN after neuronal branch elaboration is completed

In larvae, usually a single axon defasiculates from the main nerve trunk and forms an arbor on the muscle surface. At the developing adult DLMs, an anterior and a posterior directed branch extends off the main nerve trunk (PDMN), second order branches emanate from these primary branches and elaborate higher order branches on the muscle surface. The PDMN remains unsheathed for about 10–12 h as the DLM motor neurons extend primary branches along the length of the developing muscle fibers, and as second order branches rapidly expand over the developing muscle surface (Fig. 6). It is only at the end of the first day of metamorphosis when maximal branch outgrowth has occurred, that glia begin to extend along the nerve trunk. The extension of glial processes therefore occurs along a previously laid out neuronal pathway, which is similar to the ensheathment events described for the embryo (Sepp et al., 2000). The initial retraction and subsequent outgrowth of glial processes also supports our previously reported observations that FasII is initially expressed in axons of the nerve trunk and later "expands" to a broader compartment that includes ensheathing glia (Hebbar and Fernandes, 2005).

A role for glia in establishing the stereotypy of DLM innervation: pruning vs. stabilization

About 70% of second order branches elaborated on the surface of the developing DLMs are pruned back during the second day of metamorphosis to reveal the adult number of contact points (Hebbar and Fernandes, 2004). Since glia extend into the periphery after the period of maximal outgrowth, it was of interest to understand if glia bring about pruning or if they are involved in stabilization. The following observations are suggestive of a role in stabilization. When pruning is initiated at the end of the first day of metamorphosis (Hebbar and Fernandes, 2004), glia have not yet ensheathed the primary branches, and are not at all in the vicinity of the second order branches. This rules out a role in bringing about pruning of the DLM axonal arbors. At 38 h APF, when the stabilized branches are evident, glia have wrapped the primary branches and reached the base of the contact points. We favor the possibility that branches destined for stabilization are enveloped by glia, and that in doing so, glia play a protective role during a period when pruning is underway. This is borne out by our observations that when glial process outgrowth is disrupted using shi^{ts}, many more axonal arbors show membrane discontinuities, which is a feature of branches that are being pruned. Consistently, we observe fewer intact secondary branches at 31 h APF, and adults resulting from such manipulations have fewer contact points. This 'protective' role for the glia in neuronal remodeling is novel and differs from the situation in the *Drosophila* mushroom bodies, where glia invade and actively breakdown axon branches (Awasaki and Ito, 2004; Watts et al., 2004), as well as in injury models in which the antennal nerve is crushed (MacDonald et al., 2006). During synapse elimination that occurs post-natally at vertebrate NMJs, glia are seen to clear away axosomes that have been shed by axons in the process of being eliminated (Bishop et al., 2004). More recently, work in *Drosophila* has shown that glia at the larval neuromuscular junction participate in removal of excess immature synaptic boutons as a part of the process of synapse expansion (Fuentes-Medel et al., 2009).

Role of glial–neuronal interactions via FasII during the development of DLM innervation

At 38 h APF, by which time pruning has generated the adult pattern of innervation, stabilized motor axonal branches as well as ensheathing glia express Fasciclin II (Hebbar and Fernandes, 2005). At 24 h APF, which is the onset of pruning (Hebbar and Fernandes, 2004), FasII is expressed in a subset of second order axonal branches (Hebbar and Fernandes, 2005), and at this time, glia are just surrounding primary

branches (Fig. 2E). Our studies show that targeted expression of FasII in glia rescues innervation defects of FasII hypomorphs (Table 4), strongly suggesting that FasII mediates interactions between glia and neurons. We propose that homophillic interactions of transmembrane FasII across glial and axonal membranes lead to stabilization of a subset of second order axonal arbors during the pruning phase. The *Drosophila* Fasciclin isoforms, FasI, II and III are known to engage in homophillic interactions (Snow et al., 1989; Elkins et al., 1990; Grenningloh et al., 1991) that form the basis for axon guidance and target recognition functions which have been well studied in the embryo. Interestingly, in *Manduca*, two distinct FasII isoforms are differentially expressed, a transmembrane form in neurons and a GPI linked isoform in glia (Wright and Copenhaver, 2001; Knittel et al., 2001).

It is very likely that FasII mediates a temporally distinct function during an earlier stage. We have previously shown that FasII is detected in motor axons well before the pruning phase at which point it (16–18 h APF) can be visualized in the glial compartment (Hebbar and Fernandes, 2005). Since adult-specific glial processes advance along a FasII positive nerve tract, it is plausible that homophillic interactions across the compartments facilitate the outgrowth. Such interactions are also known to occur during axonal outgrowth in the *Manduca* embryo (Wright and Copenhaver, 2001).

In conclusion, we have shown for the first time that peripheral glia undergo remodeling during metamorphosis, and that in the meso-thorax this process is necessary for patterning the developing DLM innervation. We show that during the phase of axonal pruning, glia protect subsets of arbors that contribute to the adult arborization pattern. We propose that glia execute this protective role influencing the balance between pruning and stabilization, and that FasII plays an important role in the underlying glial–neuronal interactions. This role is very distinct from the scavenging roles that have been described for glia during expansion of the *Drosophila* larval NMJ and in the case of mushroom body remodeling. Our studies contribute to the already emerging model of a “tripartite NMJ” with the neuron, muscle and glia playing significant and interdependent roles (Araque et al., 1999).

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