ABSTRACT

GLIAL CELL REMODELING DURING TERMINAL NERVE TRUNK FORMATION IN
DROSOPHILA MELANOGASTER

by Matthew E. Siefert

During Drosophila development, remodeling of the larval nervous system is necessary for adult specific behavior. During metamorphosis abdominal peripheral nerves fuse to form a terminal nerve trunk (TNT). We propose that glial cells are required for this fusion to occur. Here four glial layers that ensheath Drosophila peripheral nerves are analyzed. The inner most layer, wrapping glia, and outermost layer, neural lamella, are not present during TNT formation, while the perineurial and subperineurial glial layers persist. Glial cells increases 3 fold during early pupation and ~75% of these glia are perineurial glia. Induction of perineurial glial cell death via the cell death gene reaper, resulted in abnormal TNT formation, suggesting that perineurial glial cells play a role in the fusion process. The characterization of Drosophila glial layers during development will allow for future functional studies and could lead to a model system used to study gliopathies.
Tables of Contents

1. INTRODUCTION ............................................................................................................. 1
  1.1 Nervous system remodeling .......................................................................................... 1
  1.2 Glia assist in nervous system remodeling: vertebrate and invertebrate ...................... 2
  1.3 Classes of Glia ............................................................................................................... 4
  1.4 Development of Drosophila peripheral glia ................................................................. 5
  1.5 Scope of Thesis ............................................................................................................ 6
2. MATERIALS AND METHODS ........................................................................................ 7
  2.1 Analysis of glial ensheathment during metamorphosis .............................................. 7
  2.2 Proliferation Analysis .................................................................................................. 10
  2.3 Ultrastructural Analysis .............................................................................................. 12
  2.4 Induction of cell death via activation of the cell death gene reaper ............................ 13
3. RESULTS ......................................................................................................................... 15
  3.1 Qualitative analysis of glial ensheathment along abdominal nerves: light microscopy .... 15
  3.2 Qualitative analysis of glial ensheathment along abdominal nerves: electron microscopy 16
  3.3 Quantitative analysis of glial nuclei ............................................................................ 17
  3.4 Perineurial glial cell proliferation ................................................................................. 19
  3.6 Quantitative analysis of axon profiles within the A3 peripheral nerve: electron microscopy ................................................................. 21
4. DISCUSSION ................................................................................................................. 50
  4.1 Formation of the adult terminal nerve trunk ............................................................... 50
  4.2 Absence of the neural lamella during TNT formation ................................................ 51
  4.3 Expansion of perineurial glia before TNT formation ................................................... 52
  4.4 SJs can be seen throughout pupal development ......................................................... 52
  4.5 Absence of wrapping glia during TNT formation ....................................................... 52
5. CONCLUSION ................................................................................................................. 54
6. FUTURE DIRECTIONS ................................................................................................. 55
7. APPENDIX 1 ............................................................................................................... 56
  7.1 Proliferation revealed by PH3 staining ........................................................................ 56
  7.2 BrdU Incorporation via feeding .................................................................................. 56
8. REFERENCES ............................................................................................................... 58
List of Tables

Table 1: Identified glial layers that ensheath the peripheral nerves of *Drosophila*.

Table 2: Combinations of primary and secondary antibodies used for the experiments described in this thesis.

Table 3: The number of glial nuclei along peripheral abdominal nerves A3, A4 and A5 at the larval, 12hAPF, and 24h APF stage.

Table 4: Glial cell distribution along peripheral nerves prior to TNT formation.

Table 5: Repo positive and perineurial glial nuclei along TNT.

Table 6: c527 glial nuclei along peripheral nerves during early pupal development.

Table 7: Proliferation of perineurial glia visualized by *in vitro* BrdU labeling.

Table 8: Glial nuclei are significantly reduced at 24hr APF after activation of the cell death gene *reaper*.

Table 9: Glial nuclei are significantly reduced at 48hr APF after activation of the cell death gene *reaper*.

Table 10: Glial nuclei at the adult stage after activation of the cell death gene *reaper* along the TNT.

Table 11: Ultrastructural analysis of axons diameters within peripheral nerve A3 throughout metamorphosis.

Table 12: Summary of glia ensheathment, EM analysis, and number of nerves fused/unfused throughout pupal development.
List of Figures

Figure 1: Remodeling of the nervous system throughout the *Drosophila* life cycle.

Figure 2: TNT formation during *Drosophila* metamorphosis.

Figure 3: Wrapping glia do not ensheathe individual axons at the 1st instar larval stage.

Figure 4: Schematic representation of regions analyzed along unfused nerves in the larva and 24h, 48h, 72h APF, and the adult TNT.

Figure 5: Heat shock protocol to induce cell death in perineurial glial.

Figure 6: Glial ensheathment of peripheral nerves and *repo* positive nuclei throughout development as detected by *repo*-EGFP.

Figure 7: The neural lamella visualized by viking-GFP.

Figure 8: Perineurial glia ensheathment throughout metamorphosis.

Figure 9: Subperineurial glial ensheathment and nuclei along peripheral nerves throughout metamorphosis.

Figure 10: Wrapping glia ensheathment during metamorphosis.

Figure 11: Ultrastructural analysis of 24h APF peripheral nerves.

Figure 12: Ultrastructural analysis of 48h APF peripheral nerves and TNT.

Figure 13: Ultrastructural analysis of adult peripheral nerves and TNT.

Figure 14: Perineurial glial nuclei along abdominal peripheral nerves and along the TNT.

Figure 15: Defects in TNT formation in the adult after activation of *reaper* from 0h APF to 24h APF.

Figure 16: Axon profiles within peripheral nerves fascicles during *Drosophila* development.

Figure 17: Working model of TNT formation.
Acknowledgements

I would like to first thank my advisor, Dr. Joyce Fernandes for her support throughout my time in her lab. I appreciate her guidance as well as the freedom allowed to pursue my own research interests.

I would next like to thank Dr. Richard Edelmann and Matt Duley for their technical assistance in the use of the confocal and transmission electron microscope. The training I have received from the center for advanced microscopy and imaging at Miami University has been invaluable to my work.

Next I would like to thank the members of my graduate committee, Dr. Lori Isaacson and Dr. Kathleen Killian. I greatly appreciate their scientific input throughout my research endeavors.

I will always be thankful for my colleagues in the Fernandes Lab: Dr. Soumya Banerjee and Kumar Vishal. Soumya was always there to help when I needed assistance genetic crosses while Kumar was the BrdU guru and helped me with my experiments on multiple occasions. I would also like to thank the undergraduates that I worked with over the years especially Tarah Fallah and Jack Wilber. I am also grateful for the Zoology department, especially Joni Robinson and Dr. Doug Meikle.

Lastly I would like to thank my family and friends for not only being supportive but also patient during my time as a graduate student, primarily my wife, Jill. Without her support this would not have been possible.
1. INTRODUCTION

Organisms such as zebrafish, mice, and Drosophila are used as model systems to study the basis of biological processes which in turn help in understanding corresponding processes in humans. Drosophila is used as a popular invertebrate model organism due to its short generation time, relative ease of maintenance, having a fully sequenced genome, and the availability of tools for genetic manipulations. In the present study, the process of glial ensheathment has been investigated in the context of Drosophila nervous system remodeling that occurs during metamorphosis.

In humans there are many diseases in which defects in glial function result in debilitating neurodegenerative diseases such as Alzheimer’s disease, ALS, and Parkinson’s disease (reviewed in Ciechanover and Brundin, 2003). One specific peripheral nervous system disease is Guillain-Barre Syndrome (GBS). GBS is characterized by an autoimmune response causing damage to the myelin which weakens extremities and later results in muscle failure in the respiratory system causing asphyxiation. If untreated, mortality rates are as high as 60%. If treatment is successful, ensheathment is restored over the course of a few weeks (Reeves and Swenson, 2008). Using simple model systems such as Drosophila, it may be possible to develop a disease model for neurodegenerative diseases such as GBS.

1.1 Nervous system remodeling

During the formation of the nervous system, the establishment of appropriate pre- and post-synaptic connections is an important process. During embryonic development excessive pre- and post-synaptic connections are created and during the postnatal period, many connections are removed and others are reorganized such that the final mature pattern is generated. This involves elimination of excess axons, dendrites, or even synapses (reviewed in Kantor and Kolodkin, 2003). As a result of such remodeling, new functions such as motor coordination are established (Beyeler et al., 2008).

Remodeling of the nervous system is not restricted to the post-natal period but also occurs throughout the lifecycle. In mammals, skeletal fibers are innervated at birth by multiple motor neurons. It is thought that trophic factors produced by the target become limited, which in turn drives the elimination of all but one motor neuron. As a result, a single muscle fiber
becomes innervated by only one motor neuron (Kuffler et al., 1977; Balice-Gordon and Lichman, 1990).

Holometabolous insects, such as the moth, *Manduca*, and the fruit fly, *Drosophila*, have been used as models to study nervous system remodeling during metamorphosis. Through the combined involvement of neurogenesis, respecification, and cell death, a morphologically and functionally distinct nervous system is created to accommodate new behaviors exclusive to the adult.

1.2 Glia assist in nervous system remodeling: vertebrate and invertebrate

The presence of glial cells within the nervous system has been known for well over a century but only within the past few decades have their functions been revealed. At first, it was believed that glial cells played a passive role and their primary function was to act as glue to hold other cells and structures within the nervous system in place (reviewed in Kettenmann and Ransom, 2005). However, it is now understood that glial cells play a role in neuronal proliferation and survival, (Xiong and Montell, 1995; Booth et al., 2000, Lemke, 2001, axonal growth (Booth et al., 2000; Gilmour et al., 2002), and are shown to ensheathment and maintenance of synapses at the neuromuscular junction (Barres and Raff, 1999; Auld and Robitaille, 2003). Glial cells also perform the primary immune response within the nervous system by phagocytizing dying cells or other pathogens that may cause damage to the surrounding neural tissues (Freeman et al., 2003; Awasaki and Ito, 2006).

In the mammalian central nervous system (CNS) the oxytocinergic system, located in the paraventricular and supraoptic nuclei of the hypothalamus, is a well-studied model to analyze glial and neuronal remodeling. It is known that oxytocin (OT), a neurohormone, is released from nerve endings in the neurohypophysis to facilitate limbic systems functions (review in Richard et al., 1991). During periods of enhanced or long term release of OT, glial wrapping of OT somata and dendrites is reduced which allows for increased synapse generation (Theodosis et al., 2004). This retraction of glial processes occurred as early as a few hours after induction of a stimulus (Montagnese et al., 1987; Langle et al., 2003). Interestingly, once stimulation has ceased, glial cells will once again wrap surrounding OT somata so they are not in direct contact (Langle et al., 2003).
The role of glial cells has also been studied after periods of injury. Experimentally induced strokes in mouse models resulted in structural changes in the surrounding regions of the damaged brain tissue (Reinecke et al., 2003). One important function of glial cells within the nervous system is to reuptake glutamate at synapses. This maintains appropriate glutamate concentrations to avoid excitotoxicity (Danbolt, 2001). When a stroke occurs, cells within the damaged area will die. This, in turn, will reduce glutamate reuptake by glia, potentially resulting in high levels of glutamate at the synapse. However it was found that glial cells contralateral to the stroke site increased glutamate reuptake, suggesting glial cells within the intact region of the brain remodel to aid in recovery (Takatsuru et al., 2013).

The importance of glia in the nervous system is not exclusive to the CNS. Studies done in the amphibian, *Bufo marinus*, analyzing glial cells at the location of motor nerve terminal branches, revealed constant and active remodeling of the peripheral nervous system (Macleod et al., 2001). In this study time lapse imaging and dye injections were used to observe both motor neuron branching and their ensheathment by glial cells. Twenty four nerve terminal branches were observed after dye injections. Of these twenty four, two branches displayed a region where a glial cell process extended further than the nerve branch, while two other terminal branches had no glial ensheathment present. Over time, the two terminal branches which had a glial process continued to grow outward toward where the end of the glial process was located. Meanwhile, the other two branches which had no glial process present retracted their branches. These observations were done over a period of less than an hour and indicate glial cells can directly influence remodeling of synapses (Macleod et al., 2001).

Research of *Manduca* and *Drosophila* nervous systems has demonstrated that glial cells have a direct role in the process of nervous system remodeling. For instance, during *Manduca* olfactory development, peripheral axons from developing sensory organs will extend toward and integrate with the brain. The ingrowing sensory axons cause glial cell proliferation within the brain which will then aid in axon path finding for the other sensory axons attempting to integrate with the brain (Rossler et al., 1999; Oland and Tolbert, 2011).

The *Drosophila* olfactory system is comprised of an olfactory lobe located in the brain and the antennae which act as the sensory organ in the periphery (Jhaveri and Rodrigues, 2002; Sen et al., 2005). During olfactory development, neurons within the olfactory system become ensheathed by glia during pupation (Jhaveri et al., 2000; Jhaveri and Rodrigues, 2002; Sens et
al., 2005). Near the end of metamorphosis day 1 (20h APF), axons from the antennae extend towards the brain. Once the axon integrates with the brain, peripheral glial cells will begin to migrate and proliferate along the axon in the direction in which the axon originated (Sen et al., 2005). Halfway through the second day (36h APF), olfactory axonal tracks have been ensheathed by peripheral glia (Banerjee et al., 2006a).

Mushroom bodies in *Drosophila* are necessary for learning and memory in the adult (reviewed in Heuer et al., 2012). Within the MB there is a subset of neurons, gamma-neurons, which forms two distinct axon branches, the dorsal and medial branch (Lee et al., 2000). After the initiation of metamorphosis, axons of the gamma-neurons must be remodeled to accommodate specific behaviors such as olfactory memory in the developed adult. Axon pruning occurs via degeneration and is carried out by the surrounding glial cells (Awasaki and Ito 2004).

In the peripheral nervous system, glia are known to pattern the development of adult innervation at the NMJs. The Posterior Dorsal Mesothoracic Nerve (PDMN) of the thorax of *Drosophila* becomes de-ensheathed during metamorphosis. During this time, adult axonal outgrowth and arbor pruning occurs, and glia migrate along the PDMN to ensheath the nerve up to the secondary axonal arbors. Disruption of glial ensheathment during this process leads to decreased contact points made by the motor neurons (Hebbar and Fernandes, 2010).

### 1.3 Classes of Glia

In vertebrates, four glial types have been classified: astrocytes, microglia, oligodendrocytes, and Schwann cells (reviewed in Freeman and Doherty, 2006). Astrocytes act as cellular conduits for nutrients between blood vessels and axons and they are present at synapses aiding in neurotransmitter reuptake (Santello and Volterra, 2008). Microglia are the primary immune response cells within the nervous system and help break down damaged cells or pathogens via phagocytosis. Oligodendrocytes aid in the myelination (ensheathing) of nerves which improves signal transduction velocity, and are only found within CNS. Schwann cells, however, are located within the peripheral nervous system (PNS) and function similarly to oligodendrocytes.

In an invertebrate system, specifically *Drosophila*, vertebrate glial cell counterparts have been identified based on their morphology and function. The four classes of CNS glia present in *Drosophila* are cortex, neuropil, surface, and peripheral glia. Cortex glia are morphologically
similar to astrocytes and are come into contact with neurons. Neuropil glia are similar to oligodendrocytes and aid in ensheathing axons to form nerve fascicles. *Drosophila* do not have a distinct glial cell for immune function and it has been found that cortex, neuropil, and surface glia perform immune like functions (Sonnenfeld and Jacobs, 1995; Freeman et al., 2003). Lastly, within the PNS, peripheral glia ensheathe peripheral nerves and are generated within the CNS (reviewed in Freeman and Doherty, 2006).

*Drosophila* peripheral nerves are surrounded by 3 glial layers and an extracellular matrix. The outer most layer the neural lamella (NL) is the extracellular matrix, the perineurial glia (PG) present just below the lamella, the subperineurial glia (SPG), and lastly the wrapping glia (WG) (Stork et al., 2008; Table 1). SPG ensheathe nerve fascicles and form autocellular junctions known as septate junctions (SJs; Banerjee et al., 2006b; Banerjee and Bhat., 2007; Stork et al., 2008). SJs create a barrier for ions and molecules which allows for regulation of ion concentrations as well as the diffusion of other molecules (Baumgartner et al., 1996; Banerjee and Bhat, 2007). The combination of ensheathment by the NL, PG, and WG along peripheral nerves and the formation of SJs by the SPG create the blood-nerve barrier (BNB).

### 1.4 Development of *Drosophila* peripheral glia

Glial cells originate during embryonic development and are required for proper axonal ensheathment of the PNS and the CNS. During early embryonic development, peripheral glia arise in the ventral nerve cord (VNC) from neuroglioblasts which express a gene called *glial cell missing (gcm)*. This gene is responsible for altering glial cell fates from their neuronal precursors (Hosoya et al., 1995). *Gcm* is also known to activate a downstream target known as *reversed polarity (repo)* which is used as a nuclear glial cell marker (excluding midline glia) within the CNS and PNS (Halter et al., 1995). Ensheathment of nerve bundles begins as soon as glial cells exit the CNS. Peripheral glia extend their processes along the tracts while the cell bodies migrate behind the extension. The glial cell that exits first is responsible for locating the proper migration path and has distinct filopodia. It is believed that the remaining migrating peripheral glia will adhere to the leading glial cell (Sepp and Auld, 2003). In the late embryo (stage 16-17, 13-22hrs) the peripheral nerves are ensheathed by peripheral glial cells and have stereotypic positions along each peripheral nerve (von Hilchen et al., 2008).
1.5 Scope of Thesis

The *Drosophila* nervous system is remodeled throughout the lifecycle (Figure 1). The nervous system of the fruit fly is composed of the brain and the ventral nerve cord (VNC; Skeath and Thor, 2003). The VNC can further be divided into the abdominal and thoracic regions and each of these regions have nerves that project to the body wall. In the larva, 3 pairs of thoracic nerves (T1-T3) and 8 pair of abdominal nerves (A1-A8), exit the VNC. Work from our lab has shown that in the adult, A1-A3 directly exit from the ventral ganglion as they did in the larva. However, abdominal nerves A4-A8 fuse together to form the TNT. This transition is initiated just after day 1 of metamorphosis (28h APF) and is completed by the end of day 2 (48h APF; Figure 2). Our goal is to understand the role of glia during TNT formation. Here, we analyze glia before (12h APF, 24h APF), during (28h APF), and after (48h APF, 72h APF, Adult) formation of the TNT.

The studies described in this thesis are motivated by the need to understand what happens to glial ensheathment during the process of nerve fusion to create the TNT. A necessary prerequisite is to understand ensheathment at the third instar larval stage. Although glial ensheathment has been visualized using *repo*:GAL4::UAS:tau-GFP at the embryonic and larval stages (Banerjee et al., 2006a), only recently has it been reported that distinct layers ensheath the nerves (Stork et al., 2008). Glial ensheathment during 1st to 3rd instar larval stages at the ultrastructure level were also reported (Figure 3).

This current study describes glial ensheathment during the post larval period, a topic that has not been studied previously. In the context of TNT formation, the following questions were posed:

1. What is the fate of the four ensheathing layers during the four day period of metamorphosis?
2. Do glial cells increase or decrease in number as the TNT is formed?
3. What are the mechanisms by which individually ensheathed nerves fuse to form the TNT?
2. MATERIALS AND METHODS

2.1 Analysis of glial ensheathment during metamorphosis

**Targeted expression:** In order to observe glial ensheathment of abdominal peripheral nerves the GAL4/UAS system was utilized. This two-part system utilizes a yeast transcription factor, GAL4, and a target upstream activation sequence (UAS) which are incorporated into the *Drosophila* genome producing two transgenic lines. The GAL4 driver line is tissue specific and is only expressed in the cells of interest. Once the GAL4 driver line is crossed with the UAS line, the activator protein GAL4 can bind to UAS and activate a reporter gene located directly downstream of the UAS region (Brand and Perrimon, 1993). In this study, we used layer-specific GAL4 drivers identified in peripheral glia to drive GFP expression. This allows detection and analysis of individual glial layers throughout the *Drosophila* life cycle.

**Fly strains:**

*General glial marker:* To analyze glial ensheathment along abdominal nerves, *repo:*GAL4::UAS-EGFP was used as a driver to express cytoplasmic GFP in all glial cells (Table 2). As a result, the collective extent of glial ensheathment can be visualized (fly line source: H. Keshishian, Yale University).

*Layer specific GAL4 drivers:* c527:GAL4, *moody:*GAL4, and *nervana:*GAL4 were used to express membrane bound GFP in the perineurial, subperineurial, and wrapping glia, respectively (Table 2). The neural lamella was labeled using a gene trap, *Viking*-GFP (fly line source: C Klämbt, University of Münster).

**Reporter Lines:** To visualize glial cell ensheathment the respective GAL4 line was crossed with a membrane bound reporter line, UASmcd8GF, to create the following homozygous lines: c527:GAL4::UASmcd8GFP, *moody:*GAL4::UASmcd8GFP, *nervana:*GAL4::UASmcd8GFP (Table 2). To visualize glial nuclei, GAL4 lines were crossed with a nuclear bound reporter line, UASnlsGFP. Perineurial glia were visualized using the fly line c527:GAL4::UASnlsGFP, subperineurial glia using *moody:*GAL4::UASnlsGFP, and wrapping glia using *nervana2:*GAL4::UASnlsGFP (reporter lines source: Bloomington Stock Center).
Staging and Dissection: Larval dissections were done at the wandering third instar stage. White prepupa (0h APF) were selected and placed in a petri dish with moist filter paper. They were aged at 25\(^0\)C until the following stages: 4h, 6h, 8h, 12h, 24h, 48h APF, and adult. All samples were dissected in insect saline.

1. **Larva:** Wandering 3\(^{rd}\) instar larva were dissected in petri dishes containing a thin layer of Sylgard. Larva were cut along the dorsal midline and filleted open using dissecting pins. Fat bodies and trachea were removed to expose both the CNS and the peripheral nerves.

2. **Pupa:** 4h and 6h APF pupa were dissected by first cutting the anterior and posterior end of the animal and then along the dorsal midline prior to removing the pupal case. Then samples were prefixed in 4% paraformaldehyde for approximately 5 minutes at room temperature. Then they were filleted and pinned in Sylgard dishes, and the pupal case was removed. Once again, fat bodies and trachea were removed to expose the nervous system.

**Modification for BrdU labeling:** Animals were filleted with the pupal case attached, organs were removed to expose the CNS and nerves, exposed to BrdU for 2 hours at room temperature, and then fixed and prepared for immunolabeling.

For 12h, 24h, 48h, and 72h APF dissections, the pupal case was peeled off using forceps. The exposed pupa was then cut anteriorly and posteriorly as described above, then cut along the dorsal midline. Animals were filleted, pinned, and organs removed to expose the CNS and peripheral nerves.

3. **Adults:** Adults were pinned to the Sylgard dish through the head in order to remove all appendages (legs and wings). After cutting dorsally, the animal was filleted open. Forceps were used to remove internal organs in the abdomen as well as thoracic muscles that may interfere with visualization of the abdominal nerves exiting the CNS.

Dissected samples were fixed in 4% paraformaldehyde for 30 minutes at room temperature, and washed thoroughly with 0.3% PBT-X, prior to immunocytochemistry.
**Immunohistochemistry:** Samples were blocked with 10% donkey serum in 1% Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO) and 0.3% Triton-X buffered saline solution for 1 hour at room temperature (RT). Incubation with primary antibodies was done overnight at 4°C. The following primary antibodies were used: GFP (1:1000, raised in rabbit to visualize glial ensheathment along peripheral nerves, Abcam, Cambridge, ENG), anti-repo (1:50, raised in mouse to visualize all glial nuclei along peripheral nerves), anti-HRP (1:200, raised in goat to visualize an animal’s innervation pattern, Abcam, Cambridge, ENG). Following exposure to primary antibodies, samples were washed with 0.3% PBT-X for 2 hours and then blocked for one additional hour (Table 2). The samples were exposed to secondary antibodies at RT for 4 hours. The following secondary antibodies were used: Alexa Fluor 488 Donkey anti-rabbit (1:400), Alexa Fluor 555 Donkey anti-goat (1:400), and Alexa Fluor 647 Donkey anti-mouse 647 (1:100). Samples were mounted on Frostbrand Superfrost microscope slides using vectashield (Vector Laboratories, Burlingame, CA).

**Data Analysis:**

*Qualitative Analysis:* Analysis of glial ensheathment was done using images taken on an Olympus Fluoview 500 confocal microscope. The peripheral nerves of larval, pupal (12h, 24h, 48h APF), and adult samples were examined to determine if specific glial layers were present during the lifecycle of the organism. The use of a membrane bound GFP reporter allowed visualization of individual glial layers. Thus, if GFP was present along peripheral nerves it would indicate the presence of a particular glial layer. If GFP was absent along peripheral nerves, it would indicate that particular layer is absent. HRP was used to detect abdominal nerves in the periphery.

*Quantitative Analysis:* Glial nuclei counts were analyzed along abdominal peripheral nerves A3-A5 and obtained from confocal images. This was done to compare a nerve that did not fuse into the TNT (A3) to nerves that did fuse (A4 and A5). Two different analyses were conducted: (1) The total number of repo positive nuclei, perineurial nuclei, subperineurial nuclei, and wrapping glia nuclei were quantified along segmental nerves prior to the nerve entering the body wall (Figure 4). (2) Glial nuclei were analyzed along abdominal peripheral nerves A3-A5 within a 50
µm distance to determine per unit length. A scaled 50 µm bar was placed at locations proximal, medial, and distal to where the nerve exited the CNS. This analysis was done to determine if nuclei were evenly distributed along the peripheral nerves (Figure 4).

After formation of the TNT at 48h, 72h APF, and adult, nerves have fused into the trunk while others remain unfused. To analyze glial nuclei, we focused on specific regions of the TNT. A4, A5 and A6 exit the terminal nerve trunk to innervate their respective muscle field. Regions along the TNT between peripheral nerves exit points were quantified (Figure 4). In this case, the region of the TNT between the CNS and the location of nerve A4 exiting the trunk would be analyzed. Analysis also occurred between the regions after A4 exited and before A5 exited the trunk. Quantification of glia along nerves at their point of exit from the CNS to the muscle field was also done. Unfused nerves were analyzed similarly to previous pupal stages (Figure 4). 

Note: This analysis was not done for the subperineurial and wrapping glia layers because there were only 1-2 nuclei along each nerve.

**Image Acquisition:** Samples were visualized using the Olympus FV500 confocal microscope. Alexa Fluor dyes were excited using Ar 488, HeNe 543, and HeNe 647 lasers. Images were taken as 1-3 µm optical sections and later stacked via Fluoview Software which resulted in 2D images that were used to quantify repo positive glia, c527 nuclei, and proliferation.

### 2.2 Proliferation Analysis

**Fly strain:** Proliferation was quantified along peripheral nerves of c527:GAL4::UASnlsGFP flies (Table 2).

**In vitro BrdU labeling to detect glial proliferation:** A previously described protocol (Truman and Bate, 1988) was modified for our BrdU labeling experiments. After dissections, filleted samples were exposed to BrdU at 15 mg/ml in Leibovitz medium for 2 hours at 25⁰C with slight agitation via a multi-purpose rotator (Thermo Fischer Scientific, Florence, KY). Samples were then fixed using Carnoy’s fixative for 30 minutes at 25⁰C followed by three rehydration steps of 5 minutes with 70%, 50%, and 30% ethanol. Samples were then rinsed in 0.5% PBS-TX and kept in 0.5% PBS-TX until they were labeled with antibodies.
**Immunohistochemistry:** Samples were triple labeled with primary antibodies that included goat anti-GFP (1:200; Abcam, Cambridge, ENG), mouse anti-BrdU (1:200; Developmental Studies Hybridoma Bank, University of Iowa, Iowa), and rabbit anti-repo (1:10000; Technau Lab, Mainz, Germany). The following secondary antibodies were used (Alexa Fluor): donkey anti-goat 488 (1:400), donkey anti-mouse (555), donkey anti-rabbit (647). Prior to the application of primary antibodies, samples were blocked in 10% donkey serum in TBS-BSA (0.1% BSA and 0.3% Triton-x) for 1 hour at room temperature. **Modification for BrdU labeling:** Goat anti-GFP and rabbit anti-repo primary and secondary antibodies were applied prior to the HCl treatment the same as previously described (Table 2).

**Image Acquisition:** As previously described in 2.1.

**Data Analysis:** 4h, 6h and 8h APF pupa were used to analyze glial cell proliferation along abdominal nerves A3-A5. c527 nuclei were quantified using a nuclear GFP reporter. BrdU positive nuclei were also quantified along with the total number of repo positive cells along the peripheral nerves. Data were expressed as the percentage of proliferating c527 nuclei. Averages, standard error and standard deviation were calculated using Microsoft Excel. Two tailed t-tests were calculated using Excel to determine statistical differences between control and experimental groups. A significance value of 0.05 was used.
2.3 Ultrastructural Analysis

**Fly Strains:** w^{1118} flies were grown at 25°C and were used to analyze glial ensheathment during *Drosophila* development.

**Staging and Dissection:** White prepupa (0h APF) were selected and placed in a petri dish with moist filter paper. They were aged at 25°C until the following stages: 24h, 48h APF, and Adult. Larval dissections were done at the wandering third instar stage. Samples were dissected as previously described.

**TEM sample preparation:**

*Fixation:* Samples were staged and dissected as previously described. Dissected samples were first fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in a 0.05M sodium cacodylate buffer solution (Electron Microscopy Sciences, Hatfield, PA) overnight at 4°C. Samples were washed throughout the day using 0.05M sodium cacodylate buffer solution and then placed at 4°C for an overnight wash. Secondary fixation was done using 1% osmium tetroxide (OsO₄; Electron Microscopy Sciences, Hatfield, PA) for 3 hours at room temperature followed by four washes of 20 minutes each using 0.05M sodium cacodylate. Tertiary fixation was done using 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) overnight at room temperature by four washes of ddH₂O at room temperature. Samples were dehydrated at room temperature using varying concentrations of acetone diluted with EM grade ddH₂O.

*Dehydration:* Samples were dehydrated using varying ratios of acetone to water. The first four dehydration concentrations consisting of 25%, 50%, 75%, and 95% acetone solutions lasted 30 minutes. These were followed by three, one hour dehydration steps of 100% acetone solution.

*Embedding:* Once completely dehydrated, samples were then infiltrated with resin. Spurr’s Firm was used at varying concentrations diluted with acetone at room temperature. Initial infiltration was done in a 1:3 ratio of resin to acetone for 3 hours, followed by 1:1 and 3:1 ratios of resin to solvent for 3 hours each. Lastly, two infiltrations steps of 100% resin were done for 3 hours each. Samples were then placed in embedding trays until the resin blocks solidified.
**Microtomy:** Thick sections 1.65 and 2 \( \mu \)m in thickness were cut using a glass knife. Once an area of interest was identified, ultrathin sections were created for TEM analysis. Ultrathin sections 60-75 nm were cut using a diamond knife (Diatome, Hatfield, PA), transferred to 300 mesh grids using the perfect loop tool (Electron Microscopy Sciences, Hatfield, PA), dried with filter paper, then processed for post section staining.

**Post section staining:** Grids were placed section side down onto droplets of 1% Uranyl Acetate for 10 minutes at room temperature, rinsed by dipping into ddH\(_2\)O repeatedly for 30 seconds and then dried with filter paper. For the second post section stain, grids were again placed sample side down onto droplets of lead citrate for 10 minutes at room temperature. Droplets were surrounded by a ring of sodium hydroxide pellets to reduce sample exposure to carbon dioxide. Grids were first rinsed dropwise with NaOH for 30 seconds, dipped into ddH\(_2\)O repeatedly for 30 seconds, and lastly dried on filter paper.

**Image Acquisition:** Grids were examined using the JEOL-1200 Transmission Electron Microscope at 60kv accelerating voltage with a 100\( \mu \)m aperture. Digital images were taken with an 11 megapixel, bottom mounted SIA-L9C digital camera.

**Data Analysis:** Ultrastructure images were qualitatively analyzed to assess individual glial ensheathment along abdominal peripheral nerves during metamorphosis. Image Pro Plus software was used to measure average diameters of axons profiles within abdominal peripheral nerves to determine diameters of sensory and motor neurons (see Figure 16).

### 2.4 Induction of cell death via activation of the cell death gene reaper

**Fly lines:** Homozygous c527-GAL4::GAL80 flies were crossed with homozygous \( rpr::UAS\text{mcd8GFP} \) flies resulting in c527-GAL4::\( rpr::\text{GAL80;UASmcd8GFP} \) flies (Table 2). Activation of cell death only occurred in c527 glial cells.

**Staging and Dissections:** As previously described in section 2.1.
**Induction of cell death:** Cell death activation was temporally controlled using the GAL4/GAL80 system (McGuire et al., 2003). The fly cross was grown at 18°C through larval stages to inhibit the activation of *reaper*. To activate *reaper* during the pupal period, 0h APF samples were placed in 29°C incubator. A 19hr real time exposure at 29°C equals 24hrs at 25°C(Figure 5). Control animals did not have the *rpr* gene but were still subjected to the temperature regiments.

**Immunohistochemistry:** As previously described in section 2.1.

**Image Acquisition:** Samples were visualized using the Olympus FV500 confocal microscope. Alexa Fluor dyes were excited using Ar 488, HeNe 543, and HeNe 647 lasers. Images were taken as 1-3 µm optical sections and later stacked via Fluoview Software which resulted in 2D images that were used to analyze glial ensheathment and glial nuclei.

**Data Analysis:** *Repo* positive glial cells were quantified along abdominal peripheral nerves at 24h APF. At 48h APF and adult stages glial nuclei were quantified along the TNT, nerves that branch out of the TNT, as well as along nerves that have not fused with the TNT. Because our approach involved a membrane bound GFP, c527 nuclei could not be visualized.
3. RESULTS

3.1 Qualitative analysis of glial ensheathment along abdominal nerves: light microscopy

Using repo:GAL4::UAS-EGFP to visualize all peripheral glia, we were able to observe glial ensheathment of abdominal segmental nerves. The analysis revealed that ensheathment of persists throughout pupal development and into the adult stage. GFP expression along segmental nerves could be detected at the larval, 12h APF, 24h APF, 48h APF, and at the adult stage (Figure 6A-E).

Expression of viking-GFP (neural lamella) could be detected during the third instar larva stage along abdominal segmental nerves (Figure 7A). At 12h APF patchy GFP expression could be detected (Figure 7B). At 24h (Figure 7C, n=8) and 48h APF (data not shown, n=8), no GFP expression was seen, suggesting that the layer is reorganized. At the adult stage (n=7), GFP expression was detected along all the abdominal nerves (Figure 7D). Taken together, these data suggest that the ECM is removed from peripheral nerves during TNT formation and is later re-deposited sometime between 48h APF and the adult stage.

Perineurial ensheathment was present during the larval stage (Figure 8A), throughout pupal development (Figure 8B-D), and into the adult as detected by the presence of the GFP label (Figure 8E). Since the NL was absent between 24h and 48h APF, the perineurial glial layer is the outer most layer during this period.

Subperineurial ensheathment followed a similar pattern to that of the perineurial layer. GFP expression was seen at the larval, 12h, 24h, 48h (not shown), and adult stages (Figure 9A-D).

GFP expression in the wrapping glia was detected at larval and 12h APF stages (Figure 10A,B), but not at 24h APF (Figure 10C). At 48h APF, GFP expression was still not detected (data not shown, n=7). At the adult stage, expression was detected, suggesting that this layer, like the neural lamella, is reorganized (Figure 10D).
3.2 Qualitative analysis of glial ensheathment along abdominal nerves: electron microscopy

**24h APF:** Immunohistochemistry data suggested that the NL and the WG were no longer present along peripheral nerves. Ultrastructural analysis of peripheral nerves confirmed that the NL was absent. In the larval stage, the NL appeared as a dark band completely ensheathing the nerve, whereas at 24h APF, this structure was absent. The WG layer was still present but did not ensheath individual axons like it did at the third instar larval stage. Instead, it appeared retracted. Ensheathment by the other two layers, PG and SPG, was not different from the larval stage. The PG still ensheath the entire nerve fascicle and was the outer most layer due to the absence of the NL. Multiple PG were seen surrounding the same area of the nerve. The SPG remained beneath the PG and was identified by autocellular SJs (Figure 11).

**48h APF:** Ensheathment of unfused peripheral nerves (A1-A3) at 48h APF resembled what is seen at 24h APF. Absence of the NL, presence of PG/SPG, and a retracted WG layer confirming the light microscopy observations. PG are now the outermost layer and can be seen to completely ensheath nerve fascicles while the SPG is beneath it. In cross sections of the TNT, multiple PG are ensheathing the TNT as it exits the CNS, while the SPG are just below and can be identified by their SJs. The NL was absent, and WG did not individually wrap axons (Figure 12).

**Adult:** By the adult stage, all layers are easily detectable. The NL and could be seen completely surrounding each nerve and the TNT. PG continued to maintain their sheath but occasionally in unfused nerves PG did not completely surround the nerves allowing the NL to come into contact with the SPG. WG were seen ensheathing individual axons within the nerve separating them from one another. Surrounding the entire TNT were the NL and the PG. PG ensheathment along the TNT appeared thinner than along individual nerves. Analysis of the SPG suggests that once individual nerves fuse into the TNT the SPG maintained their sheath around each nerve bundle separating them from each other within the TNT. Finally, WG appeared to ensheath individual axons within the TNT (Figure 13).
3.3 Quantitative analysis of glial nuclei

Glial nuclei increase during pupal development

At the third instar larval stage, the anterior abdominal nerves (A1-A3) are shorter than the posterior nerves (A4-A8). The number of glial nuclei visualized using anti-repo accordingly varied. An average of 14.75 ± 0.7 nuclei were found along A3 nerves, 16.1 ± 0.3 nuclei along A4 nerves, and 19.9 ± 0.9 nuclei along A5 nerves. (Table 3; Figure 6A’)

At 12h APF, there was a significant increase in the number of nuclei along A3, A4 and A5 (Table 3; p < 0.05). A 2-fold or greater increase was observed along abdominal nerves A3, A4, and A5 (Table 3; Figure 7B’). At 24h APF, an increase of 3 fold or greater was seen which was statistically significant (p < 0.05; Table 3; Figure 6C’).

Perineurial Glia increase in number during early pupal development

Quantitative analysis of perineurial glia at the wandering third instar larval stage revealed that this subgroup makes up a significant proportion of the total glial nuclei. They represent 76.1% to 77.4% of the total glial cell population along segmental nerves A3 through A5 (Table 3). There are 11.3 ± 0.7 nuclei along A3, 12.25 ± 0.4 nuclei along A4 and 15.4 ± 2.5 along A5 (Table 3; Figure 14A). This is in agreement with a recent study (von Hilchen et al., 2013; published while this thesis was being written).

To determine if the perineurial glia were as abundant during the pupal stages, the number of nuclei were quantified at 12h APF and 24h APF. At 12h APF, there are 24.2±1.3 nuclei along A3, 30.8±1.7 nuclei along A4 and 36.3±3.4 nuclei along A5 (Table 3; Figure 14B). This represents a greater than 2-fold increase (p < 0.05) when compared to the larva. Between 12h APF and 24h APF, the number of perineurial glial cells continued to increase. The number of nuclei increased to 35.5±4.2 along A3, to 46.5±1.1 along A4, and to 51.6±1.3 along A5 (p < 0.05; Table 3; Figure 14C).

As in the larva, perineurial glia represent a majority of glial cells present along segmental nerves during early pupal development. At 12h APF, the proportion of perineurial glia along A3 (77.1%) remained similar to the larval stage (76.6%). Along A4 and A5, they account for 82.8% and 85.2% respectively, of the total glia. These proportions were maintained at 24h APF (Table 3).
Sub perineurial glia and wrapping glia do not increase during early pupal development

Analysis of moody-Gal4::UASnlsGFP at the third larval instar stage revealed 2 SPG nuclei along each of the three abdominal nerves analyzed (A3-A5). There was no significant difference in the number of SPG at 12h APF and 24h APF when compared to the larva (Table 3).

Analysis of nervana2::GAL4::UASnlsGFP revealed 1 wrapping glia nucleus along A3-A5 at the third larval instar stage. Thus, only 1 cell is responsible for ensheathing individual axons along an entire abdominal nerve. At 12h APF, 2 wrapping glia nuclei were present. (p < 0.05; Table 3). Wrapping glial nuclei were not analyzed at 48h APF.

Distribution of glial nuclei along abdominal nerves

The distribution of glial nuclei along 50 µm segments along each nerve was analyzed. There were 2.5 ± 0.42 repo-positive nuclei along A3, 2.4 ± 0.34 repo-positive nuclei along A4, and 2.3 ± 0.4 repo-positive nuclei along A5 (Table 4). These data were not significantly different (p > 0.05), indicating that the distribution of repo nuclei along larval nerves is uniform between nerves regardless of their lengths. Interestingly, a variable density of glial nuclei per unit length is seen across nerves at 12h APF and 24h APF, which is distinct from what is observed in the larva. At 12h APF, the number of glial nuclei per unit length increases (p < 0.05) for each nerve examined (A3-A5) compared to the larva, indicating an increased density of glial nuclei, which is consistent with the >2 fold increase in number of glial nuclei. At 24h APF, the number of glia per unit length decreases significantly (p < 0.05) along A3 and A4, when compared to 12h APF. In light of the increase in total number of glia (Table 3), this suggests a possible lengthening of the nerves. Along the A5 nerve, the number of glia per unit length remains similar to what is observed at 12h APF. In light of the significant increase in total number of glia, this is consistent with shortening of nerves.

Perineurial glial nuclei are evenly distributed along abdominal nerves.

As with repo-positive nuclei, the distribution of PG along larval nerves A3, A4, and A5 was significantly different (Table 4). Analysis of 50 µm segments along each nerve revealed a distribution ranging from 1.8 ± 0.4 to 1.9±0.2 nuclei. The distribution was similar across the three nerves examined at the larval stage. At 12h APF, the distribution of glial nuclei significantly increased along peripheral nerves when compared to the larval stage of
development. (p < 0.05). Although glial nuclei increased, the distribution along A3 and A4 were not significantly different at 12h APF, while the distribution along nerve A5 was significantly less when compared to A3 and A4 (p < 0.05). Be consistent with spacing.

At 24h APF there was a significant decrease in nuclei per 50 µm along A3 and A4 when compared to 12h APF, while the distribution along A5 remains the same. When comparing nerves at 12h APF, the nuclei distribution along A3 was significantly less when compared to A4 and A5 (Table 4; p < 0.05). As seen for distribution of total glia, we observed an increase from larva to 12h APF, and a decrease from 12h APF to 24h APF (Table 4).

**Perineurial glial represent a smaller portion of glial nuclei after TNT formation.**

The TNT represents a fusion of 5 pairs of abdominal nerves. To innervate their respective muscle fields, abdominal peripheral nerves A4-A7 defasciculate from the TNT at specific locations along the A/P axis. In order to analyze the distribution of glial nuclei, the total number of glial nuclei were counted in successive segments prior to a defasciculation (Figure 4), and compared with the presence of perineurial glia as detected with c527-Gal4:nlsGFP. At 48h APF, the number of c527-positive nuclei made up a smaller proportion of the total glia present in all regions of the TNT that were analyzed (Table 5).

Only 18-21.5% of all glia were perineurial glia; this is in contrast to >74% of total glia being PG, at 24h APF. At 72h APF, the number of c527-positive nuclei glia in three successive segments of the TNT decreased significantly (p < 0.05), and this trend continued into the adult stage. Consistent with this observation, the proportion of PG nuclei within the pool of total glial nuclei (detected by anti-repo) also decreased.

**3.4 Perineurial glial cell proliferation**

**Glial cell proliferation can be detected along abdominal segmental nerves**

There is a >2-fold increase in the number of PG nuclei at 12h APF. By examining earlier stages, we determined that between larval and 6h APF, there was a 1.3 fold or greater increase in the number of repo positive glial nuclei along abdominal segmental nerves A3-A5. This increase was statistically significant (p < 0.05; Table 6). Between 6h and 8h APF, the number of repo positive glial nuclei increased another 1.5 fold (p < 0.05) along A3-A5 (Table 6). This trend is seen for c527 positive nuclei as well (Table 6). To determine if this increase occurs by
proliferation, incorporation of BrDU was monitored in vitro. Analysis of pupae that were dissected at 4h APF and incubated in BrDU for two hours, revealed substantial proliferation of c527 positive nuclei (Table 7) along A3-A5. 16-20% of c527 positive glial nuclei were determined to be proliferative. Analysis of pupae that were dissected at 6h APF and incubated in BrDU for two hours, revealed that fewer PG nuclei were BrDU positive. The proportion of c527 glial undergoing proliferation is less than at the 4h APF stage, the range being from 6-7% in A3 and A4. A higher proportion of c527 glia (15%) showed proliferation in A5.

3.5 Induction of perineurial glial cell death prior to TNT formation

To test the role of PG in TNT formation, the cell death gene reaper was targeted to PG during the first day of metamorphosis, when the bulk of cells are generated, and animals were dissected at 24h, 48h and adult stages. At 24h APF, the number of repo-positive nuclei seen along A3-A5 were significantly reduced (P < 0.05), and represented a 25.9%, 16.1%, and a 13.5% reduction along A3, A4 and A5, respectively (Table 8). TNT formation has not yet begun at this stage, and morphological differences between experimental and control groups were not detected at this time (Figure 15). At 48h APF, there were no significant differences in the number of repo positive nuclei in successive segments of the terminal nerve trunk prior to the defasciculations (Table 9). However, all animals displayed improperly formed TNTs; after A5 branches out, the main trunk bifurcated into two distinct terminal trunks composed of A8, A7, and A6 (Figure 15). This phenotype was only seen once in the control group (~11%).

In the adult stage, similar to the 48h APF phenotype, experimental animals lacked proper fusion after A5 branches from the TNT, resulting in bifurcation into two separate terminal trunks (100% of cases). There was no significant difference (p > 0.05) between control and experimental animals in the TNT segment before A4 branches out. However, there are more nuclei in experimental animals in the next successive segment (p < 0.05, Table 10). When compared with controls. This amounted to approximately 43% more repo positive nuclei.
3.6 Quantitative analysis of axon profiles within the A3 peripheral nerve: electron microscopy

During the formation of the adult motor system, the axons of respecified motor neurons and ingrowing sensory neurons must incorporate themselves into the peripheral nerves, a process that may be aided by remodeling of the wrapping glial layer. The diameters of the axon profiles within the A3 nerve were determined to assess whether they are sensory or motor. It has been reported that the larger axons within a nerve profile are motor axons (Howlett et al., 2008; Lin et al., 2011). Based on axon diameter, two distinct types of profiles could be easily identified, 1) a group with large diameter axons, with diameters ranging from 890 to 910 nm, which were presumably motor neurons, and 2) a group of smaller diameter axons, ranging from 360 to 390 nm. The large and small axon profiles had significantly different axon diameters. (p > 0.5; Table 11; Figure 16). However, throughout development, the axon diameters were not significantly different within the same group. For instance, the diameter of the large axon profiles at 24h APF was not significantly different when compared to the large axon profiles at 48h APF and the adult stage. This was also the case for the small axon profiles (Table 11).
Table 1: Identified glial layers that ensheathe the peripheral nerves of *Drosophila* (source: Stork et al., 2008)

<table>
<thead>
<tr>
<th>Peripheral Glia</th>
<th>Strain</th>
<th>Labels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural lamella (NL)</td>
<td>Viking-GFP</td>
<td>Collagen trap</td>
</tr>
<tr>
<td>Perineurial Glial layer (PG)</td>
<td>c527-GAL4</td>
<td>Unknown</td>
</tr>
<tr>
<td>Subperineurial Glial layer (SPG)</td>
<td>Moody-GAL4</td>
<td>GPCR, labels septate junction</td>
</tr>
<tr>
<td>Wrapping Glia (WG)</td>
<td>Nervana2-GAL4</td>
<td>Na+/K+ Pump</td>
</tr>
<tr>
<td>Strains used</td>
<td>Primary Antibodies</td>
<td>Secondary Antibodies</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td><strong>Repo-EGFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>Viking-GFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>c527::GAL4::UASmcd8GFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>c527::GAL4::UASnlsGFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>c527::GAL4::UASnlsGFP</strong></td>
<td>Goat anti-GFP</td>
<td>Donkey anti-goat 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-BrdU</td>
<td>Donkey anti-mouse 555</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-repo</td>
<td>Donkey anti-rabbit 647</td>
</tr>
<tr>
<td><strong>Moody::GAL4::UASmcd8GFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>Moody::GAL4::UASnlsGFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>Nervana2::GAL4::UASmcd8GFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>Nervana2::GAL4::UASmcd8GFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-mouse 647</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td><strong>c527::reaper::GAL80::UASmcd8GFP</strong></td>
<td>Rabbit anti-GFP</td>
<td>Donkey anti-rabbit 488</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-repo</td>
<td>Donkey anti-goat 555</td>
</tr>
<tr>
<td></td>
<td>Goat anti-HRP</td>
<td>Donkey anti-mouse 647</td>
</tr>
</tbody>
</table>
Table 3: The number of glial nuclei along peripheral abdominal nerves A3, A4 and A5 at the larval, 12hAPF, and 24h APF stage.

<table>
<thead>
<tr>
<th></th>
<th>A3 Nerve</th>
<th>A4 Nerve</th>
<th>A5 Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Larva:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo</td>
<td>14.75±0.7 (8)</td>
<td>16.1±0.3 (8)</td>
<td>19.9±0.9 (7)+</td>
</tr>
<tr>
<td>Perineurial Glia</td>
<td>11.3 ± 0.7 (8)</td>
<td>12.25 ± 0.4 (8)</td>
<td>15.4 ± 2.5 (7)</td>
</tr>
<tr>
<td>Subperineurial Glia</td>
<td>2 ± 0 (7)</td>
<td>2 ± 0 (9)</td>
<td>2.14 ± 0.14 (7)</td>
</tr>
<tr>
<td>Wrapping Glia</td>
<td>1 ± 0 (9)</td>
<td>1.11 ± 0.11 (9)</td>
<td>1.11 ± 0.11 (9)</td>
</tr>
<tr>
<td>% Perineurial</td>
<td>76.6%</td>
<td>76.1%</td>
<td>77.4%</td>
</tr>
<tr>
<td><strong>12h APF:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo</td>
<td>31.4±1.9 (9)*</td>
<td>37.2±1.5 (9)*+</td>
<td>42.6±4.1 (7)*+</td>
</tr>
<tr>
<td>Perineurial Glia</td>
<td>24.2 ± 1.3 (9)*</td>
<td>30.8 ± 1.76 (9)*+</td>
<td>36.3± 3.4 (7)*+</td>
</tr>
<tr>
<td>Subperineurial glia</td>
<td>1.83 ± 0.16 (6)</td>
<td>1.83 ± 0.16 (6)</td>
<td>1.83 ± 0.16 (6)</td>
</tr>
<tr>
<td>Wrapping Glia</td>
<td>2 ± 0 (8)</td>
<td>2 ± 0 (8)</td>
<td>2 ± 0 (8)</td>
</tr>
<tr>
<td>% Perineurial</td>
<td>77.1%</td>
<td>82.8%</td>
<td>85.2%</td>
</tr>
<tr>
<td><strong>24h APF:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo</td>
<td>48 ± 3.6 (n=8)*</td>
<td>54.5 ± 2.5 (10)*+</td>
<td>62.1 ± 1.4 (9)*+</td>
</tr>
<tr>
<td>Perineurial Glia</td>
<td>35.5 ± 4.2 (9)*</td>
<td>46.5 ± 1.1 (10)*+</td>
<td>51.6 ± 1.3 (9)*+</td>
</tr>
<tr>
<td>Subperineurial glia</td>
<td>1.88 ± 0.13 (9)</td>
<td>2.11 ± .13 (9)</td>
<td>2.16 ± 0.16 (6)</td>
</tr>
<tr>
<td>Wrapping Glia</td>
<td>Not Determined</td>
<td>Not Determined</td>
<td>Not Determined</td>
</tr>
<tr>
<td>% Perineurial</td>
<td>74%</td>
<td>85.3%</td>
<td>83.1%</td>
</tr>
</tbody>
</table>

() represents sample size, * denotes significant difference in the number of glial cells along the same nerve when compared to the development stage before it. (p < 0.05). + denotes a significant difference between peripheral nerves at the same developmental stage (p < 0.05).
Table 4: Glial cell distribution along peripheral nerves prior to TNT formation. Glial nuclei were quantified within a 50 µm distance in regions proximal, medial, and distal to the nerves exit point from the CNS.

<table>
<thead>
<tr>
<th></th>
<th>A3 Nerve</th>
<th>A4 Nerve</th>
<th>A5 Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Larva</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo positive glia:</td>
<td>2.5 ± 0.42 (8)</td>
<td>2.4 ± 0.34 (8)</td>
<td>2.3 ± 0.4 (8)</td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>1.8 ± 0.4 (8)</td>
<td>1.9 ± 0.2 (8)</td>
<td>1.9 ± 0.2 (8)</td>
</tr>
<tr>
<td><strong>12h APF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo positive glia:</td>
<td>12.5 ± 0.5 (9)*</td>
<td>11.9 ± 0.7 (8)*</td>
<td>9.4 ± 0.8 (7)*+</td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>8.9 ± 0.7 (9)*</td>
<td>9.2 ± 0.7 (8)*</td>
<td>7.6 ± 0.5 (7)*</td>
</tr>
<tr>
<td><strong>24h APF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo positive glia:</td>
<td>6.2 ± 0.22 (15)*</td>
<td>8.4 ± 0.05 (13)*+</td>
<td>9.5 ± 0.7 (9)+</td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>4.3 ± 0.05 (15)*</td>
<td>7.1 ± 0.09 (13)*+</td>
<td>7.9 ± 0.6 (9)</td>
</tr>
</tbody>
</table>

() represents sample size, * denotes significant difference in the number of glial cells along the same nerve when compared to the development stage before it. (p < 0.05). + denotes a significant difference between peripheral nerves at the same developmental stage (p < 0.05).
Table 5: Repo positive and Perineurial glial nuclei along TNT. Glial nuclei were quantified along specific regions of the TNT (refer to Figure 4). As development progresses through late pupal stages and into the adult there is a significant reduction in the proportion of perineurial glial cells present.

<table>
<thead>
<tr>
<th></th>
<th>Before A4 Nerve Branch</th>
<th>Before A5 Nerve Branch</th>
<th>Before A6 Nerve Branch</th>
</tr>
</thead>
<tbody>
<tr>
<td>48h APF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo positive glia:</td>
<td>34.8 ± 2.8 (13)</td>
<td>44.6 ± 2.8 (13)+</td>
<td>49.3 ± 1.6 (11)+</td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>7.2 ± 1.3 (13)</td>
<td>9.4 ± 1.5 (10)+</td>
<td>9.3 ± 0.7 (11)</td>
</tr>
<tr>
<td>% Perineurial</td>
<td>20.5%</td>
<td>21.5%</td>
<td>18.8%</td>
</tr>
<tr>
<td>72h APF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo positive glia:</td>
<td>30 ± 1.8 (8)</td>
<td>30.75 ± 1.7 (8)*</td>
<td>41.75 ± 4.3 (8)*</td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>3 ± 0.6 (8)*</td>
<td>4.25 ± 0.8 (8)*</td>
<td>5.8 ± 0.9 (8)*</td>
</tr>
<tr>
<td>% Perineurial</td>
<td>10%</td>
<td>13.8%</td>
<td>13.6%</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo positive glia:</td>
<td>32.4 ± 3.2 (8)</td>
<td>34.8 ± 3 (8)</td>
<td>38.8 ± 4 (8)</td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>3.25 ± 0.6 (8)</td>
<td>2.25 ± 0.2 (8)*</td>
<td>3 ± 0.7 (9)*</td>
</tr>
<tr>
<td>% Perineurial</td>
<td>10%</td>
<td>6.5%</td>
<td>7.7%</td>
</tr>
</tbody>
</table>

() represents sample size. * denotes significant difference in the number of glial cells along the same nerve when compared to the development stage before it. (p < 0.05). + denotes a significant difference between peripheral nerves at the same developmental stage (p < 0.05).
Table 6: c527 glial nuclei along peripheral nerves during early pupal development.

<table>
<thead>
<tr>
<th></th>
<th>A3 Nerve</th>
<th>A4 Nerve</th>
<th>A5 Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Larva</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo</td>
<td>14.9 ± 0.6 (10)</td>
<td>17.7 ± 0.7 (11)</td>
<td>20.25 ± 0.8 (8)</td>
</tr>
<tr>
<td>Perineurial Glia</td>
<td>10 ± 0.7 (8)</td>
<td>10.1 ± 0.7 (7)</td>
<td>14 ± 2.5 (8)</td>
</tr>
<tr>
<td>% c527</td>
<td>67.1%</td>
<td>57.1%</td>
<td>69.1%</td>
</tr>
<tr>
<td><strong>6h APF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo</td>
<td>21.6 ± 1.2 (7)*</td>
<td>23.9 ± 1.8 (7)*</td>
<td>28.14 ± 2.3 (7)*+</td>
</tr>
<tr>
<td>Perineurial Glia</td>
<td>17.5 ± 1.02 (7)*</td>
<td>19.6 ± 1.5 (7)*</td>
<td>24.3 ± 2.2 (7)*+</td>
</tr>
<tr>
<td>% c527</td>
<td>81.1%</td>
<td>80.1%</td>
<td>86.3%</td>
</tr>
<tr>
<td><strong>8h APF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repo</td>
<td>32.3 ± 5.4 (4)*</td>
<td>39.4 ± 2 (8)*</td>
<td>41.3 ±1.3 (7)*</td>
</tr>
<tr>
<td>Perineurial Glia</td>
<td>26.4 ± 1.1 (7)*</td>
<td>31.9 ± 1.7 (8)*</td>
<td>35.6 ± 2.4 (7)*</td>
</tr>
<tr>
<td>% c527</td>
<td>82%</td>
<td>81%</td>
<td>86.2%</td>
</tr>
</tbody>
</table>

() represents sample size, * denotes significant difference in the number of glial cells along the same nerve when compared to the development stage before it. (p < 0.05). + denotes a significant difference between peripheral nerves at the same developmental stage (p < 0.05).
Table 7: Proliferation of c527 glia visualized by in vitro BrdU labeling. Samples were exposed to BrdU for 2 hours at room temperature. The age of the animal is indicated at time of dissection, in this case 4h and 6h APF.

<table>
<thead>
<tr>
<th></th>
<th>A3 Nerve</th>
<th>A4 Nerve</th>
<th>A5 Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4h (+2h) APF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>18.2 ± 1.2 (9)</td>
<td>21.4 ± 1.3 (8)</td>
<td>23.9 ± 1.8 (7)</td>
</tr>
<tr>
<td>BrdU(+) Perineurial glia:</td>
<td>3.6 ± 0.5 (9)</td>
<td>3.5 ± 0.2 (8)</td>
<td>4.8 ± 0.65 (7)</td>
</tr>
<tr>
<td>% Proliferation</td>
<td>20.1%</td>
<td>16.4%</td>
<td>20%</td>
</tr>
<tr>
<td><strong>6h (+2) APF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perineurial glia:</td>
<td>24.4 ± 1.2 (7)</td>
<td>26.9 ± 1.3 (8)</td>
<td>35 ± 1.0 (7)</td>
</tr>
<tr>
<td>BrdU(+) Perineurial glia:</td>
<td>1.7 ± 0.5 (7)</td>
<td>1.8 ± 0.5 (8)</td>
<td>5.3 ± 0.8 (7)</td>
</tr>
<tr>
<td>% Proliferation</td>
<td>7%</td>
<td>6.7%</td>
<td>15%</td>
</tr>
</tbody>
</table>

() represents sample size
Table 8: Glial nuclei are significantly reduced at 24hr APF after activation of the cell death gene *reaper*. Experimental and Control groups received a heat shock from 0h to 24h APF. Control group does not have the *rpr* gene.

<table>
<thead>
<tr>
<th></th>
<th>A3 Nerve</th>
<th>A4 Nerve</th>
<th>A5 Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td>49.6±2.8 (8)</td>
<td>56.9±1.6 (8)</td>
<td>65±1.2 (7)</td>
</tr>
<tr>
<td>Experimental:</td>
<td>36.8±1.6 (8)*</td>
<td>47.7±1.8 (11)*</td>
<td>56.2±2.2 (9)*</td>
</tr>
<tr>
<td>Percent Change:</td>
<td>25.8% Reduction</td>
<td>16.2% Reduction</td>
<td>13.5% Reduction</td>
</tr>
</tbody>
</table>

* denotes significant difference between control and experimental groups. (p < 0.05)

() represents sample size.
Table 9: **Glial nuclei are not significantly reduced at 48hr APF after activation of the cell death gene reaper.** Experimental and Control groups received a heat shock from 0h to 24h APF. In experimental animals the region before A6 branches out of the TNT did not form so data was not collected.

<table>
<thead>
<tr>
<th></th>
<th>Before A4 Branch</th>
<th>Before A5 Branch</th>
<th>Before A6 Branch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td>34.8±1.6 (9)</td>
<td>44.8±2.2 (9)</td>
<td>49.7±1.01 (7)</td>
</tr>
<tr>
<td>Experimental:</td>
<td>32.3±1.2 (8)</td>
<td>42.8±3.1 (8)</td>
<td>Did not form</td>
</tr>
<tr>
<td>Percent Change:</td>
<td>Not Significant</td>
<td>Not Significant</td>
<td>N/A</td>
</tr>
</tbody>
</table>

() represents sample size, * denotes significant difference between control and experimental groups (p < 0.05)
Table 10: Glial nuclei at the Adult stage after activation of the cell death gene *reaper* along the TNT. Experimental and Control groups received a heat shock from 0h to 24h APF. In experimental animals the TNT bifurcated early (before A6 branched from the TNT).

<table>
<thead>
<tr>
<th></th>
<th>Before A4 Branch</th>
<th>Before A5 Branch</th>
<th>Before A6 Branch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td>33.1±2.6 (8)</td>
<td>35.7±1.7 (7)</td>
<td>42.8±3.4 (6)</td>
</tr>
<tr>
<td>Experimental:</td>
<td>34±2.8 (8)</td>
<td>47.1±3.05 (7)*</td>
<td>Did not form</td>
</tr>
<tr>
<td>Percent Change</td>
<td>Not Significant</td>
<td>32% Increase</td>
<td>N/A</td>
</tr>
</tbody>
</table>

() indicates sample size, * denotes significant difference between experimental and control groups.

Table 11: Ultrastructural analysis of axons diameters within peripheral nerve A3 throughout metamorphosis. There was no significant difference in axon diameters between the developmental stages.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Small Axons Average Diameter</th>
<th>Large Axons Average Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h APF</td>
<td>0.39 ± 0.0014 (2)</td>
<td>0.89 ± 0.007 (2)+</td>
</tr>
<tr>
<td>48h APF</td>
<td>0.36 ± 0.03 (3)</td>
<td>0.89 ± 0.09 (3)+</td>
</tr>
<tr>
<td>Adult</td>
<td>0.38 ± 0.04 (2)</td>
<td>0.91 ± 0.06 (2)+</td>
</tr>
</tbody>
</table>

() indicates sample size. + denotes significant difference between small and large axon profiles. (p < 0.05).
Table 12: Summary of glia ensheathment, EM analysis, and number of nerves fused/unfused throughout pupal development.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Glial layers</th>
<th>EM</th>
<th>Nerves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larva</td>
<td>All present</td>
<td>NL, PG, SPG ensheath nerve,</td>
<td>8 Unfused Pairs: A1-A8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WG ensheath individual axons</td>
<td></td>
</tr>
<tr>
<td>12h APF</td>
<td>NL begins to break down</td>
<td>N/A</td>
<td>8 Unfused Pairs: A1-A8</td>
</tr>
<tr>
<td></td>
<td>PG, SPG, WG present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h APF</td>
<td>NL and WG absent</td>
<td>NL is absent, WG seen with retracted</td>
<td>8 Unfused Pairs: A1-A8</td>
</tr>
<tr>
<td></td>
<td>PG and SPG present</td>
<td>processes, PG and SPG ensheath nerve</td>
<td></td>
</tr>
<tr>
<td>48h APF</td>
<td>NL and WG absent</td>
<td>NL is absent, WG seen with retracted</td>
<td>3 Unused Pairs: A1-A3</td>
</tr>
<tr>
<td></td>
<td>PG and SPG present</td>
<td>processes, PG and SPG ensheath nerve</td>
<td>5 Fused Pairs: A4-A8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and TNT</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>All present</td>
<td>NL, PG, SPG ensheath nerves and TNT,</td>
<td>3 Unused Pairs: A1-A3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WG ensheath individual axons</td>
<td>5 Fused Pairs: A4-A8</td>
</tr>
</tbody>
</table>
Figure 1: Remodeling of the nervous system throughout the Drosophila life cycle. (A) During late embryonic development, the CNS condenses and nerves must now project a farther distance to reach their respective muscle fields (Truman, 1990). (B) Between the embryonic and larval stage the animal grows over 100 fold in size. Nerves exiting the CNS must also grow extensively in length in order to reach their appropriate muscle fields (C) In the adult, abdominal nerves A4-A8 have fused to form the TNT (red arrow). Abdominal nerves exit either from the CNS (A1-A3), or from the TNT to innervate their respective muscle fields. Images were acquired from samples dissected in our lab by undergraduate Meredith Dorr.
Figure 2: TNT formation during *Drosophila* metamorphosis. Larva: 8 pairs of peripheral nerves (A1-A8) exit the CNS and project toward their respective hemisegment. 28hAPF: TNT formation has begun. Here nerves A6-A8 have fused together while A4 and A5 remain unfused. 48h APF: TNT formation has been completed and nerves A4-A8 have been incorporated into the TNT while A1-A3 remain unfused. Adult: the TNT is seen while nerves A1-A3 are still unfused. Images acquired by an undergraduate from our lab, Camilo Molina. Scale Bar: Larva, 28h APF = 50 µm, 48h APF = 75 µm, adult = 150 µm.
Figure 3: Wrapping glia do not ensheathe individual axons at the 1st instar larval stage. In the 1st instar larval, the NL is the outer most layer and is a dense extracellular matrix. PG do not completely surround the nerve fascicle, while SPG do. WG does not ensheathe individual axons resulting in axons to come into direct contact with the SPG. At the 3rd instar larval stage, the NL is still present as the outer most layer. Multiple PG and SPG surround the entire nerve fascicle. WG ensheathe individual axons and contact between axons and SPG is lost. Color code: black = neural lamella (NL), light blue = perineurial glia (PG), purple = subperineurial glia (SPG), red = wrapping glia (WG), gray = axon.
Figure 4: Schematic representation of regions analyzed along unfused nerves in the larva and 24h, 48h, 72h APF, and the adult TNT. Nerves that project out of the CNS are composed of two different regions. The region that projects towards the muscle field (represented here as black), and the area in which the nerve enters the muscle field (represented here as gray). Glial cells were quantified along the black region of the nerve prior to when the nerve entered the muscle field. At 48h APF, 72h APF, and adult our analysis focused on glial cells along the TNT. We designated these regions based on when nerves exited from the TNT. For instance, regions before peripheral nerves A4 exit the TNT is known as before A4 (indicated by the brackets). The regions before nerves A5 and A6 were also analyzed and are indicated brackets. The extent of the TNT would be at the exit point from the CNS to the point where bifurcation occurs. Here, the TNT begins at the top of the first bracket (designated Before A4) and ends at the bottom of the last bracket (designated Before A7).
Figure 5: Heat shock protocol to induce cell death in perineurial glial. c527:reaper::UASmed8GFP:GAL80 animals were raised at 18°C until they become white pre-pupa. Pre-pupa were collected and placed in a 29°C incubator to activate the cell death gene reaper. Samples were aged to 24h APF in the incubator and then removed and placed back at 18°C. Dissections were then done at 24h APF, 48h APF, and at the adult stage (arrows).
Figure 6: Glial ensheathment of peripheral nerves and repo positive nuclei throughout development as detected by repo-EGFP. GFP reporter is detected via anti-GFP antibodies and labels glial ensheathment (green). The peripheral nerves exiting the CNS are detected with anti-HRP antibody (red). Co-localization of GRP and HRP (yellow) indicate presence of glial ensheathment around peripheral nerves. Glial ensheathment is detected at all stages analyzed indicated by the GFP expression along abdominal peripheral nerves. (A-E) Glial ensheathment of peripheral nerves from wandering third instar stage, 12h APF, 24h APF, 48h APF, and the adult stage. (A’-C’) repo positive glial nuclei (red) located along peripheral nerves at larva, 12h APF, and 24h APF. (D’-E’) repo positive glial nuclei along the TNT. Scale bar for top row=150 µm; bottom row=35 µm.
Figure 7: The neural lamella visualized by viking-GFP. GFP reporter is detected and visualized via anti-GFP antibodies and labels the neural lamella (green). The peripheral nerves exiting the CNS are detected via HRP staining (red). Co-localization of GFP and HRP (yellow) indicates glial ensheathment around peripheral nerves. (A) In the larval stage the neural lamella is present around peripheral nerves. (B) By 12h APF, the neural lamella begins to break down and gaps are seen in the ensheathing layer (white arrows). (C) 24h APF, the neural lamella is almost completely lost along peripheral nerves. (D) Adult: the neural lamella ensheaths the peripheral nerves and the TNT.: scale bar A-C = 150 µm, D = 60 µm.
**Figure 8: Perineurial glia ensheathment throughout metamorphosis.** (A-E). A GFP reporter was used to visualize perineurial glial ensheathment of abdominal peripheral nerves. Glial ensheathment (green) was detected along peripheral nerves throughout metamorphosis. The peripheral nerves are labeled with HRP (red). Co-localization of GFP and HRP (yellow) represent regions in which glial ensheathment is along the peripheral nerves. Scale bar = 150 μm
Figure 9: Subperineurial glial ensheathment and nuclei along peripheral nerves throughout metamorphosis. (A-D). Subperineurial glial ensheathment was detected using a GFP reporter during pupal development (green). Glial cell nuclei were detected using anti-repo (red). Glial nuclei that are labeled GFP and repo may appear yellow. (A-C) GFP was detected in moody nuclei (white arrow heads) (D) GFP was detected indicating glial ensheathment. Scale bar = 35µm
**Figure 10: Wrapping glia ensheathment during metamorphosis.** Glial ensheathment along peripheral nerves is visualized using a membrane bound GFP reporter (green) while peripheral nerves were detected using anti-HRP (red). Co-localization of GFP and HRP (yellow) indicates glial ensheathment around peripheral nerves. (A,B) At the larval and 12h APF stages wrapping glia ensheathment is present along peripheral nerves. WG nuclei were also seen along peripheral nerves (white arrow heads). (C) By 24h APF, GFP expression cannot be detected in wrapping glial cell processes indicating that ensheathment is lost along peripheral nerves (white arrow). (D) GFP is detected again at the adult stage. Scale bar in A,B = 150 µm, C= 35 µm D = 60 µm.
Figure 11: Ultrastructural analysis of 24h APF peripheral nerves. (A) Cross section of abdominal nerve A3 at 24h APF. The NL is not present around the nerve resulting in the PG being the outer most layer. Here multiple PG cells can be seen ensheathing the nerve fascicle. The WG is seen with retracted glial extensions. Individual axons are not ensheathed. (A’) High magnification image from A. SPG layer can be identified by the presence of autocellular spectate junctions (black arrows). (B) Cross section of abdominal nerve A4 at 24h APF. NL is absent, multiple wrapping glia are seen ensheathing the nerve, and WG appears bunched together. (B’) high magnification image of boxed in region in B. SJs (black arrows) can be seen and are used to identify the SPG layer. Scale bar A,B = 5 µm; A’.B’ = 0.5 µm
Figure 12: Ultrastructural analysis of 48h APF peripheral nerves and TNT. (A) Unfused abdominal nerve A3. The NL is still absent during this stage of development. The nerve is ensheathed by multiple PG cells, and it appears that the WG begins to ensheath individual nerves again. (A’) High magnification image of the boxed region in A. SJs are present and are identified within the SPG (black arrow). (B) The TNT at 48h APF. Just like individual nerves, many PG cells can be seen ensheathing the TNT. SJs can also be detected (black arrow; high mag not shown). WG can be seen extending their processes to ensheath individual nerves (white arrows). Scale bar A,B = 5µm; A’ = 0.25 µm.
Figure 13: Ultrastructural analysis of adult peripheral nerves and TNT. (A) Unfused adult abdominal nerve A3. The NL is present and is now the outer most layer. The PG continues to ensheath the nerve fascicle but in some cases not completely (white arrow). This allows the NL to come into direct contact with the SPG. The WG can be seen ensheathing individual axons. (A’) High magnification image of boxed region in A. SJs are present within the SPG (black arrows). (B) The NL ensheaths the TNT. PG are seen surrounding the TNT, but once again there are regions where the SPG contact the NL (white arrows). (B’) High magnification image of box region in B. SJs can be seen in the ensheathing SPG layer. Scale bar A,B = 10µm; A’, B’ = 0.25 µm.
Figure 14: Perineurial glial nuclei along abdominal peripheral nerves and along the TNT. All nuclei are labeled using anti-repo (red) while perineurial cells are labeled with anti-GFP (green). Double label (yellow) indicate that a repo positive glial cell is a perineurial glial cell. Red nuclei without any GFP double label would indicate a non perineurial peripheral glial cell (white arrows). (A-E) repo and PG during the larva, 12h APF, 24h APF, 48h APF, and the adult stage of development. Scale bar = 35 µm.
Figure 15: Defects in TNT formation in the adult after activation of reaper from 0h APF to 24h APF. Control and Experimental groups received a heat shock from 0h APF to 24h APF and then raised at 18°C until emergence from the pupal case. Only the right hand side of the peripheral nerves is shown. (A) In 100% of the control animals, nerves A4, A5, and A6 branch out of the TNT and project toward their respective muscle fields. (B) In the experimental group, bifurcation of the TNT can be seen just after nerve A5 exits the TNT (red arrows). (C) Schematic representation of TNT abnormalities observed after induction of glial cell death. In some experimental animals, A4 did not fuse appropriately with the TNT on one, or both sides (red arrow head). Here, only one side is shown (red arrow head). Scale bar = 75 µm
Figure 16: Axon profiles within peripheral nerves fascicles during *Drosophila* development. (A-D) Cross sections of abdominal peripheral nerves analyzed using Image Pro Plus software; segregated by average axon diameter. Axons labeled green are considered large, while axons labeled red are small. (A) 24h APF abdominal nerve A3. (B) 24h APF abdominal nerve A4. (C) 48h APF abdominal nerve A3. (D) Adult abdominal nerve A3.
Figure 17: Working model of TNT formation. At 12h APF the NL begins to break down, PG and SPG are seen ensheathing the entire fascicle, WG ensheath axons. By 24h APF the NL is absent, PG and SPG still ensheath the nerve, WG is seen with retracted processes. At 28h APF peripheral nerves come into contact with one another and the PG shift to maintain their position as the outer most glial layer while SPG maintain their sheath around their respective nerve fascicles. WG begins to resheath individual axons. In the adult, the NL is present again surrounding the entire TNT along with the PG and SPG. WG resheath individual axons. Ensheathment color code: black = neural lamella, light blue = perineurial glia, purple = subperineurial glia, red = wrapping glia, gray = axons.
4. DISCUSSION

4.1 Formation of the adult terminal nerve trunk

On the first day of metamorphosis the segmental nerve pattern still resembles the larval pattern. On the second day, the formation of the TNT begins when posterior abdominal nerves A6-A8 start to fuse. Over day 3 and 4, additional peripheral nerves, A5 and A4, will also fuse together until the morphology of the segmental nerve pattern becomes adult like. The analysis of four ensheathing layers revealed that the most external and the most internal layers, neural lamella and wrapping glia, are absent prior to the transition from larval like nerve pattern to adult like nerve pattern (Table 12). This occurs at the same time that the PG undergoes expansion, and the SPG remains sheathing peripheral nerve fascicles.

Our studies examining the four ensheathing layers around peripheral nerves during metamorphosis have allowed us to develop a working model of the cellular mechanisms by which the TNT is formed (Figure 17). At 12h APF, NL breakdown begins along peripheral nerves. The PG and SPG surround the entire nerve fascicles, while WG ensheath axons. By 24h APF, the NL is absent along peripheral nerves resulting in PG becoming the outer most layer. The SPG still maintains its thin sheath around the axons. WG retract their extensions and no longer ensheath individual axons. Around 28h APF, when peripheral nerves contact one another, PG remain the outer most layer, while SPG remain sheathing each nerve separately. The WG still has retracted processes and they do not ensheath individual nerves. At 48h APF, the TNT is formed and the NL is still absent, PG and SPG ensheath the entire TNT, and WG can be seen extending their processes out to ensheath axons. Lastly, at the adult stage, the NL, PG, and SPG can be seen encircling the entire TNT, while WG ensheath axons.

Considering the results from ultrastructural and immunohistochemistry studies, we propose another aspect of our model which relates to the organization of peripheral nerves within the TNT at the adult stage. One possible way nerves could be organized within the TNT is that glial ensheathment of the TNT resembles that of individually wrapped nerves. Meaning, the NL, PG, and SPG surround the entire TNT while the WG are responsible for separating nerve fascicles as well as individual axons within the trunk. The other possibility is that the NL and PG surround the entire TNT, while the SPG maintain sheaths only around the nerve fascicles within the TNT. Here, the WG would only separate axons along their respective nerve.
In our model we suggest that SPG not only wraps the entire TNT but also maintains ensheathment around nerve fascicles. Since nerves within the TNT eventually exit to innervate their respective muscle fields, it would be advantageous to preserve SPG ensheathment within the trunk. Prior to TNT formation, PG are represented at a higher proportion than the other layers. However, after the TNT has formed, PG is represented at a much smaller fraction of the total glial cells present. One explanation as to why this may occur could be that SPG increase in number to ensure ensheathment of nerves within the TNT as well as ensheathment of the TNT.

4.2 Absence of the neural lamella during TNT formation

Breakdown of the neural lamella is initiated prior to 12h APF and it is completely removed within the following 12 hours. At 48h APF, ultrastructural analyses revealed that the NL is absent, but that it is re-deposited by the adult stage. Breakdown and reconstruction of the NL has been reported previously in the CNS of the wax moth, *Galleria mellonella* (Osinska, 1981). The process seems to parallel the larval-pupal ecysis and found that the NL reappeared 2-3 days before the pupal-adult ec dysis. It has been shown that ec dysis is activated during *Drosophila* metamorphosis and is required for the motor neuron cell death (Robinow et al., 1993; Truman et al., 1994). Ecdysone may be responsible for the removal of the NL and could potentially be a necessary prerequisite so that during fusion of the TNT, the cellular layers can integrate.

In *Drosophila*, there are two different collagen IV polypeptides associated with basement membranes (BMs) in the body (Broadie et al., 2011). Here, we analyzed one of these collagen IV poypeptides, viking, which is generated by haemocytes that migrate throughout the body incorporating ECM proteins, such as viking, into BMs (Mirre et al., 1988; Yasothornsrikul et al., 1997; Olofsson and Page, 2005). However, during adult nervous system development it is currently not know if the same mechanisms which result in embryonic ECM deposition play a role in the deposition of the ECM along the TNT and the other peripheral abdominal nerves.

Developmental studies during embryonic development demonstrated that the loss of collagen IV resulted in BMs becoming thinner and more fragile preventing the animals from hatching (Borchiellini et al., 1996). Another study attributed observed effects on bone morphogenic protein (BMP) signaling to loss of viking expression. BMP is a vital signaling protein required during patterning events during development (Hogan, 1996; Kishigami and
Mishina, 2005). These studies highlight the defects that occur during development when viking is lost. However, in our case, throughout *Drosophila* metamorphosis a loss of viking expression appears to be a normal developmental occurrence.

4.3 Expansion of perineurial glia before TNT formation

In the absence of the NL, the PG remains the most external layer and may be the reason why this layer expands more than 3 fold between the third instar larva and 24h APF. Our ultrastructural data at 24 APF shows multiple PG cells ensheathing peripheral nerves. A role for the PG in aiding formation of the blood brain barrier (BBB) by the SPG has been proposed. Large molecules can enter the nerve cord in *gem* mutant embryos, but were unable to do so in a SPG mutant that had the other glial layers intact (Stork et al., 2008). It is possible that PG function in BNB barrier formation similarly to their role in BBB formation.

When PG number is reduced by using the cell death gene *reaper*, there was significant reduction (13-25%) in the number of glial cells present along abdominal peripheral nerves. By 48h APF, developmental defects were already apparent such as early bifurcation of the TNT and failure of some abdominal nerves to fuse into the TNT. By reducing the number of PG present via cell death, it may result in a decreased communication with other glia and cause defects in PG growth or function leading to the defects in TNT formation we report here.

4.4 SJs can be seen throughout pupal development

In this current study, SJs are present at 24h APF, 48h APF, and at the adult stage within peripheral nerves as well as the TNT. Due to the SJs importance for the BNB, it is likely that SJs remain intact throughout TNT formation. SJs can be identified with their stereotypic zipper-like structure and are used to determine the SPG from the other layers. Being able to identify the SPG enables identification of the other glial layers within the peripheral nerve or the TNT.

4.5 Absence of wrapping glia during TNT formation

In this study we demonstrated that WG is absent by 24h APF and ultrastructurally does not ensheathe individual axons within nerve fascicles. This may occur during metamorphosis to allow integration of newly developed sensory neurons into the PNS. Removal of WG may also
allow for other cells such as phagocytes to enter the area directly around axons to remove axons that are no longer required.

During metamorphosis of holometabolous insects, the larval nervous system is remodeled in a way that adult specific neural circuits are made to execute adult behaviors. It is known that motor neurons found in the adult can persist from the larval stage (Levine and Truman 1982; Thorn and Truman, 1989; Bate and Martinez, 1991; Consoulas et al., 2000; Duch and Levine, 2000) while some sensory and interneurons are development anew. As the adult motor system is being built, many changes are occurring within each peripheral nerve such as retraction of motor neurons which will die and the ingrowth of sensory axons. One specific example is the development of mechanoreceptors in the abdomen of the *Drosophila* during metamorphosis (Madhavan and Madhavan, 1980). Division of a sensory organ precursor cell results in the development of the bristles located on the abdomen and their neurons (Gho et al., 1990). The axons of these neurons must then migrate through the PNS to integrate with targets located within the CNS. This would require larval wrapping glial to de-ensheath so that the adult complement of axons can be established.

De-ensheathment of axons during embryonic development has been shown (Stork et al., 2008). Here, as the larval musculature and nervous system develop, WG does not ensheath individual axons within peripheral nerve fascicles. Similar to what we report during metamorphosis, de-ensheathment of WG may be necessary for incorporation of newly generated motor, sensory, and interneurons during embryonic development.
5. CONCLUSION

The current study analyzed four glial layers that ensheathe peripheral nerves throughout the life cycle of the fruit fly. At the 3rd instar larval stage, all four glial layers were present. The NL, PG, and SPG completely ensheathed the nerves while the WG ensheathed individual axons within the nerve fascicle. By 12h APF, the NL began to breakdown while the other glial layers remained, ensheathing abdominal peripheral nerves. At 24h APF, the NL was completely gone resulting in the PG layer being the outermost ensheathing layer. The SPG was still present with intact SJs, while the WG had retracted its processes no longer ensheathing individual axons. At 48h APF, the NL was still absent around peripheral nerves, the PG and SPG remained ensheathing the nerve fascicles. WG extended their processes and begin to ensheathe axons as they did during the 3rd instar larva. Between 48h APF and the adult stage the NL returned and was the outer most layer and the WG completely ensheath individual axons.

Quantitatively it was found that glial cells increased more than 3 fold between the 3rd instar larval stage and 24h APF, a time point just prior to initiation of TNT formation. Also, between this time period, SPG and WG nuclei did not increase in number while PG increased 3 fold. It was also found that PG were represented at a higher proportion than any other peripheral glial subtype. PG represented 75% or more of the total glia present along abdominal peripheral nerves between the 3rd instar larval stage and 24h APF. However, after TNT formation, only 13% of the glia along peripheral nerves were PG, suggesting the increase in PG may be required for TNT formation.

Activation of the cell death gene reaper in PG resulted in a significant decrease in the number of PG nuclei present along peripheral nerves. The decrease seen ranged between 15-30%. Analysis of TNT development at the adult stage revealed that a reduction in PG during pupal development lead to defects in TNT formation. In most cases, early bifurcation of the TNT was seen resulting in the posterior portion of the TNT to be split into two separate trunks. It was also noted that A4, a nerve that typically fused with the TNT, did not fuse in some of the experimental animals.
6. FUTURE DIRECTIONS

One of the major questions we set out to address that has not yet been answered is exactly how individual nerves fuse together to make the TNT. There are many factors that we have not examined. For instance, this project focused strictly on the peripheral glial cells located along peripheral nerves, while there are glial cells located within the CNS. It has been shown that there are glial cells that are positioned at the CNS/PNS border known as exit glia which are responsible for the initial steps in axon path finding out of the CNS (Sepp et al., 2001, Sepp and Auld 2003, Banerjee et al., 2003). These exit glia may also be playing a role in nervous system remodeling. If the exit glia are perineurial glia, induction of cell death will also be influencing their glial cell number.

Another question that still remains unanswered is just how different are nerves that fuse into the TNT and those that remain unfused. Our analysis only revealed that the number of perineurial cells differed between nerves depending on their lengths. However, there must be something different between the nerves in order for some to fuse and others to remain unfused.

Lastly, after TNT, perineurial glia have proportionally fewer glial nuclei compared to time points prior to TNT formation. It may be that either the WG or SPG increase in number. With the introduction of new sensory neurons, more WG may be required to separate individual axons or more SPG may be required to ensheath both individual nerve fascicles within the TNT and the TNT. It might even be that our marker for perineurial glial cells, c527 is no longer expressed post TNT formation.

To address some of these unanswered questions, additional experimentation will need to be done. One study of interest is the prevention of glial cell process extension using DN-UAS ShiTs. Here, prevention of glial process extension will occur during developmental time points when re-ensheathment occurs, such as the re-ensheathment of individual axons by WG after TNT formation. Also, single cell labeling can be done to observe glial processes and cell positioning throughout development. Lastly, understanding glia-axon and glia-glia communication are necessity in order to apply our model to other organisms. The moody gene codes a GPCR and is a component in the signaling pathway involved in SJ formation (Stork et al., 2008). Currently, it is unknown as to what ligand binds this particular GPCR.
7. APPENDIX 1

7.1 Proliferation revealed by PH3 staining

Phosphorylation of histone 3 can be labeled as a marker for proliferation due to its presence in the late G2 phase and the M phase of the cell cycle (Hendzel et al., 1997; Taupin 2007). Analysis at 6h and 8h APF revealed very few labeled cells. More often than not, no proliferation was seen and in rare cases, one or two cells would be PH3 positive along peripheral nerves (data not shown). The limiting factor here is the short window in which PH3 can be stained during the cell cycle. This greatly reduces the change to find proliferating cells at any given point during development. To address this issue, BrdU feeding and in vitro labeling will be more advantageous for two reasons. First, the exposure time will be longer so there will be a greater chance to label proliferative cells. Second, we are able to analyze proliferation over a specific time period. For instance, with feeding, BrdU will label proliferation from the time the larva feeds to the point in which they are dissected, while for in vitro labeling, we expose dissected animals to BrdU for 2 hours.

7.2 BrdU Incorporation via feeding

In order to determine the appropriate amount of BrdU and length to feed larva, 3 different experiments were done.

1. 0.1 mg/ml for 48hrs (hatch to 3rd instar)
2. 0.5 mg/ml for 24hrs (3rd instar to pupa)
3. 1 mg/ml for 6 hours (during 3rd instar)

These specific experiments were chosen based on previously published data which mapped proliferation of cells within the Drosophila CNS during larval, pupal, and adult (Truman and Bate, 1988). Here, our goal was to label glial cells in the PNS and we thus needed to establish a protocol to do so. In the first experiment, robust labeling was detected at the third instar larval period. This was expected because it has been reported that the perineurial glia proliferate during larval development (Leiserson et al., 2000). However, analysis at 12h APF resulted in less BrdU labeling than at the larval stage (Table 1). Between larval and 12h APF there is greater than a 2 fold increase in the number of c527 nuclei along segmental nerves. In theory, the labeled cells seen during the larval stage should also be present at 12h APF in addition to the other cells that proliferated. These data suggest that 0.1 mg/ml BrdU solution was not sufficient in labeling multiple cell divisions thus resulting in fewer cells being BrdU positive at the pupal stage.
One primary drawback to experiment one was the fact that we were labeling proliferation between the 1st and 3rd instar larval stages and our primary goal was to analyze proliferation during pupal development. Therefore, our second experiment was to only feed during the third instar larval stage, a time when proliferation of peripheral glia should be minimal. In order to accommodate the reduction in proliferation we saw at 12h APF and a shorter feeding window, the concentration of BrdU was increased to 0.5 mg/ml. Our results showed a smaller portion of c527 nuclei proliferating during the 3rd instar larval stage when compared to proliferation between the 1st instar and 3rd instar stages. Unfortunately, our results for experiment 2 were similar to that of experiment 1. We saw no significant BrdU incorporation in peripheral glial cells (Table 2). Statistically a greater percentage of BrdU incorporation occurred in experiment 2 as compared to experiment 1 at 12h APF, but the amount of proliferation detected did not account for the overall increase in glial cells.

Due to the potential toxicity from high concentrations of BrdU during feeding, no data was collected from experiment 3. It has been demonstrated that short feeding pulses ranging from 2-8 hours can be sufficient to address high concentrations (Truman and Bate, 1988). However, in our case feeding 1 mg/ml BrdU for 6 hours resulted in larval death. The animals would feed and then die while crawling on the side of the vial (data not shown).
8. REFERENCES


Macleodo GT, Dickens PA, Bennett MR. 2001. Formation and function of synapses with respect to Schwann cells at the end of motor nerve terminal branches on mature amphibian (Bufo marinus) muscle. Journal of Neuroscience. 21(7):2380-92


