Perineurial glia regulate morphology of the Drosophila nervous system

by

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ABSTRACT

The nervous system is surrounded by neural lamella composed of large glycoproteins including perlecan, collagen and laminin, which bind to underlying perineurial glial cells. The function of perineurial glia and their interaction with the neural lamella is just beginning to be elucidated. Previous studies have demonstrated that integrin is critical for glial wrapping in both vertebrates and *Drosophila*. Therefore, we have focused on perineurial glia and the role of laminin, an integrin ligand, and basigin, a transmembrane protein known to interact with integrin.

Laminin is a heterotrimer composed of an alpha (LanA or Wb), beta (LanB1) and gamma (LanB2) subunit and we demonstrate that LanB2 loss in glia results in accumulation of LanB1 leading to distended ER, ER stress and glial swelling in addition to decreased larval locomotion and lethality. Loss of LanB2 in wrapping glia affected glial ensheathment of axons but surprisingly not larval locomotion. We found that Tango1, a protein thought to exclusively mediate collagen secretion, is also important for laminin secretion in glia via a collagen-independent mechanism. We conclude that it is the loss of one laminin subunit that leads to deleterious consequences through the accumulation of the remaining subunits.

Basigin is a highly conserved transmembrane protein involved in cancer metastasis, however its developmental roles are just beginning to be elucidated. We show that basigin is specifically expressed in perineurial glia where it is present in a complex with integrin. Basigin knockdown resulted in a shortened ventral nerve cord, ruffles in the peripheral nerves as well as a collapsed actin cytoskeleton and a redistribution of myosin motors in perineurial glia. We examined the domains within

basigin that are required for association with integrin and also examined the effect of basigin knockdown on integrin-associated proteins.

Together, the results in this thesis highlight the role of perineurial glia in secreting laminin, facilitating larval locomotion and regulating both central and peripheral nervous system morphology in *Drosophila*. Due to the conserved structure and function of glia between vertebrates and *Drosophila*, these results will help direct future research on how perineurial glia regulate nervous system development.

PREFACE

Chapter 2: **"Laminin accumulation disrupts glial morphology and larval locomotion"**
Work in this chapter was accepted to the Journal of Neuroscience on December 9, 2015 and was published on January 27, 2016.
Title: Accumulation of laminin monomers in Drosophila glia leads to glial ER stress and disrupted larval locomotion
Authors: Lindsay M Petley-Ragan, Evan L Ardiel, Catharine H Rankin, and Vanessa J Auld
For this publication, I contributed to all aspects of the manuscript including study design, data collection, analysis and interpretation. Evan Ardiel wrote the script used for converting larval tracking raw data to numerical excel data. Vanessa Auld contributed to study conception and design, data interpretation, writing, and editing of the manuscript.

Chapter 3: **"Basigin regulates actin and nervous system morphology"**
Work in this chapter has produced a manuscript in preparation as:
Title: Basigin in perineurial glia regulates Drosophila nervous system morphology
Authors: Lindsay M Petley-Ragan, Mriga Das, Vanessa J Auld
For this manuscript, I contributed to all aspect of the manuscript including study design, data collection, analysis and interpretation. Mriga Das conducted the proximity ligation assay experiments. Vanessa Auld contributed to study conception and design, data interpretation, writing, and editing of the manuscript.

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PNS	peripheral nervous system
CNS	central nervous system
ECM	extracellular matrix
NRG	neuregulin
TGF-β	transforming growth factor β
MS	Multiple Sclerosis
DNA	deoxyribonucleic acid
Gcm	glial cells missing
FGF	fibroblast growth factor
RNA	ribonucleic acid
EGF	epidermal growth factor
nrxIV	neurexin IV
MMP	matrix metalloproteinase
LE	laminin EGF-like
LG	laminin globular
LanB1	laminin B1 (*Drosophila* laminin beta subunit)
LanB2	laminin B2 (*Drosophila* gamma subunit)
LanA	laminin A (*Drosophila* alpha subunit)
Wb	wing blister (*Drosophila* alpha subunit)
ER	endoplasmic reticulum
COPII	coat protein complex II
βPS	β protein subunit of Drosophila integrin
Bsg	basigin
EMMPRIN	extracellular matrix metalloproteinase inducer
MCT1	monocarboxylate transporter 1
Ig	immunoglobulin
NMJ	neuromuscular junction
GFP	green fluorescent protein
RFP	red fluorescent protein
repo	reverse polarity
RNAi	ribonucleic acid interference
UAS	upstream activating sequence
VNC	ventral nerve cord
TEM	transmission electron microscopy
PLA	proximity ligation assay
Mys	myospheroid (*Drosophila* integrin β subunit)
MLCII	myosin light chain II
HRP	horseradish peroxidase (axon marker)

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First of all, thank you to my parents for teaching me to wonder. Thank you to my supervisor, Dr. Vanessa Auld, for providing me with solid scientific roots and for letting me explore my passions within science. Thank you to my committee members and lab members for all of your time, help and ideas along the way. Thank you to my fellow graduate students for bushwacking through biology with me; you are the ultraviolet light to my GFP structure. Specifically, thank you to Dr. Stephanie Ellis and Michael Fairchild for helping inspire me in the beginning, you made me realize now was the time to jump right in, release my genuine excitement about biology and ponder those scientific questions!

I've had the incredible fortune of being surrounded by brilliant, caring and colourful characters during my time in graduate school who have kept me balanced and continued to nudge me in the right direction every day. Thank you to Thomas Bevan for inspiring me to dream big. Thank you to Josh Taylor for helping me find my soul whenever I lose it. Thank you to Julia Meyer-MacLeod for teaching me more than I ever thought you could learn from friendship. Thank you to Mike Istchenko for popping in every now and then and making sure no one was taking life too seriously. Thank you to Pablo Lopez-Caballos for making me believe in magic. Thank you to Elise Barber for consistently stunning me with what is possible. Thank you to Victoria Jakobson for riding the rollercoaster with me since the beginning and thank you to my sister, Arianne, for being the best Yin to my Yang I could have ever asked for. You can do anything you want! And finally, thank you to Kelsey Pinch for reminding me how grateful I am and that no person is an island.

While my passion for research has been questionable at times, my fascination in nature and cell biology has always remained the most grounding part of my life.

Tubby wubby was a fly
Tubby wubby had to die
Tubby wubby was near and dear to me
And here's the story why.

To the brilliant women in my life,

Especially Nancy Ragan, Joan Hamilton, Paula Mathews and Pat Petley

1 INTRODUCTION

The most defining feature of any nervous system in multicellular organisms is the ability of neurons to receive, process and transfer information via electrical signals. During the past few decades, another type of cell in the nervous system, glial cells have been found to be increasingly important for a variety of processes during nervous system development and throughout the lifespan of an organism (Ndubaku and de Bellard, 2008). The nervous system is considered one of the earliest adaptations in animal evolution separating sponges from cnidarians – such as hydras and jellyfishes – which have a basic nerve net (Holland, 2003). Concentrated clusters of neurons eventually evolved into nerve cords in bilateral animals, forming a ventral nerve cord in invertebrates and a dorsal nerve cord accompanied by a notochord in chordates. Interestingly, molecular genetic studies have revealed a striking conservation in the expression patterns and functions of genes involved in neural development across the animal kingdom (Holland, 2003).

A common ancestor of all bilateral animals possessed glial-like cells, which are believed to have evolved to aid the nervous system and function in structural support, by-product removal, phagocytic needs, developmental programming and circuit modulation (Hartline, 2011). It appears that once glia evolved in a certain phyletic line, they continued to develop new functions now apparent in the multi-functionality of glial cells in complex animals today (Hartline, 2011). Most glia and neurons derive from the embryonic germ layer known as the neuroectoderm and the proportion of glia in the nervous system seems to be correlated with animal size and behavior complexity, with the most complex animals having brains composed of almost 90% glial cells (Ndubaku

and de Bellard, 2008). Emerging research shows that glia can do more than simply support neurons and have an active role in brain function and information processing (Allen and Barres, 2009). In fact, recent findings suggest that glial cells are a critical player in dealing with stress, determining intelligence, and musical abilities (Bengtsson et al., 2005; Han et al., 2013; Woodbury-Farina, 2014). Although the role of glial cells as a regulator and modulator of neuron function is being increasingly appreciated, the molecular pathways and mechanisms by which glia accomplish these functions are still being elucidated. Research aimed at revealing the conserved mechanisms and functions of glial cells across the animal kingdom, such as that presented in this thesis, will continue to highlight how glial cells have evolved from a simple neuronal support cell to an important player in complex human behaviour. This thesis will compare the function of glial cells in the vertebrate and *Drosophila* nervous system, with an emphasis on glia in the peripheral nervous system (PNS).

1.1 THE VERTEBRATE NERVOUS SYSTEM

In vertebrates, there are four main categories of glial cells: oligodendrocytes, Schwann cells, astrocytes and microglia. Oligodendrocytes are present in the central nervous system (CNS), namely the brain and spinal cord, where they insulate axons. Oligodendrocytes extend multiple processes and wrap surrounding axons with a thick, multi-layered membranous sheet of myelin (Voyvodic, 1989). Myelin is essential to the efficient transfer of neuronal signals along an axon and a variety of human diseases are caused by ineffective myelination; they will be discussed in section 1.1.4. Individual oligodendrocytes will wrap a section of an axon about 150-200 μm in length with the exposed areas between these sections being called the nodes of Ranvier (Butt and

Ransom, 1989). Nodes of Ranvier are distinguished by a high concentration of voltage-gated Na^+ channels causing localized depolarization within the node and the continuation of the electric signal down the axon resulting in a process called saltatory conduction (Waxman and Ritchie, 1993). Similarly, in the PNS, Schwann cells myelinate large caliber axons with Nodes of Ranvier separating myelinated sections. Distinct from oligodendrocytes that can myelinate multiple axons, Schwann cells will only wrap one section of one axon. Furthermore, a subtype of Schwann cell, called non-myelinating Schwann cells, do not wrap an individual axon multiple times at all, but instead engulf bundles of small caliber axons simultaneously without the formation of myelin to form Remak bundles (Jessen and Mirsky, 2005). The third type of glia, astrocytes, are present in the CNS with multiple subtypes having various functions including triggering the formation of the blood-brain barrier, regulating synaptic neurotransmitter concentrations and modulating synaptic structure and plasticity (Sofroniew and Vinters, 2010). Finally, microglia are found within the CNS and act as nervous system-specific macrophages that remove neuronal debris and are activated in response to neuronal injury. The development of these four glial subtypes is distinct resulting in the differentiation of four unique morphological and functional glial cell types (Freeman and Doherty, 2006).

1.1.1 Peripheral nerve structure

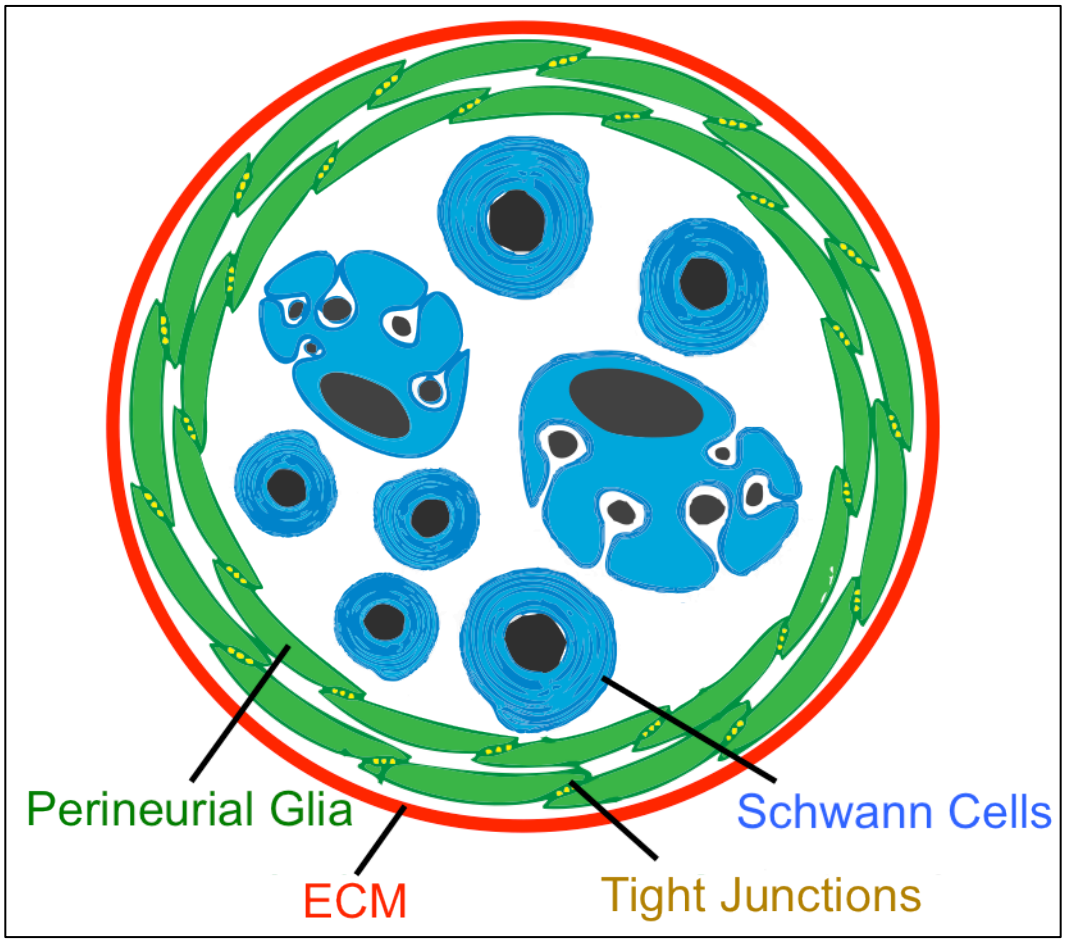


Figure 1.1. Schematic view of a cross-section of a vertebrate peripheral nerve. Perineurial glia (green) form tight junctions (yellow) and are surrounded by an extracellular matrix (red). Large diameter axons are surrounded by myelinating Schwann cells and small diameter axons are surrounded by non-myelinating Schwann (blue) cells which engulf multiple axons. (adapted from Rodrigues et al., 2011).

In the vertebrate peripheral nerve above (Fig. 1.1), large caliber axons are myelinated by Schwann cells and smaller caliber axons are engulfed by non-myelinating Schwann cells (Jessen and Mirsky, 2005). Schwann cells are eventually bundled into fascicles and surrounded by the perineurium and epineurium, connective tissue sheaths consisting of extracellular matrix (ECM) components and many flattened fibroblast-like cells called perineurial glia. The perineurium is essential for protecting Schwann cells

from ionic flux, toxins and infection; however, its origin and role during development are just beginning to be understood (Kucenas et al., 2008). Surrounding the entire peripheral nerve is the epineurium, another basement membrane, which bundles the fascicles of axons together with the arteries and veins (Rodrigues et al., 2011).

1.1.2 Schwann cell development

Schwann cells originate from neural crest cells, a small group of cells that break away from the dorsal part of the neural tube as it closes. Neural crest cells can give rise to neurons, glia, pigment cells, cartilage and smooth muscle (Le Douarin and Smith, 1988) (Weston, 1991). Neural crest cells can be already committed to a particular fate, while others are bi- or multi-potent (Jessen and Mirsky, 2005). A sub-population of neural crest cells already expresses P_0, a myelin protein restricted to the Schwann cell lineage (Jessen and Mirsky, 1998; Lemke, 1988) and these neural crest cells will differentiate first into Schwann cell precursors (mouse E12/13, rat E14/15), then immature Schwann cells (mouse E15/rat E17) and finally, form myelinating and non-myelinating Schwann cells (Fig 1.2) (Jessen and Mirsky, 2005). The neural crest cells differentiate into Schwann cell precursors as they migrate away from the dorsal part of the neural tube and meet axons growing out of the ventral part of the neural tube (Jessen and Mirsky, 1998). A series of external and internal cues are required at specific time points as neural crest cells transition into mature Schwann cells. The transcription factor Sox-10 is expressed in the earliest migrating neural crest cells where it allows for their specification between neuronal and glial fate and continues to be functional as Schwann cells differentiate (Jessen and Mirsky, 2002; Kuspert et al., 2012).

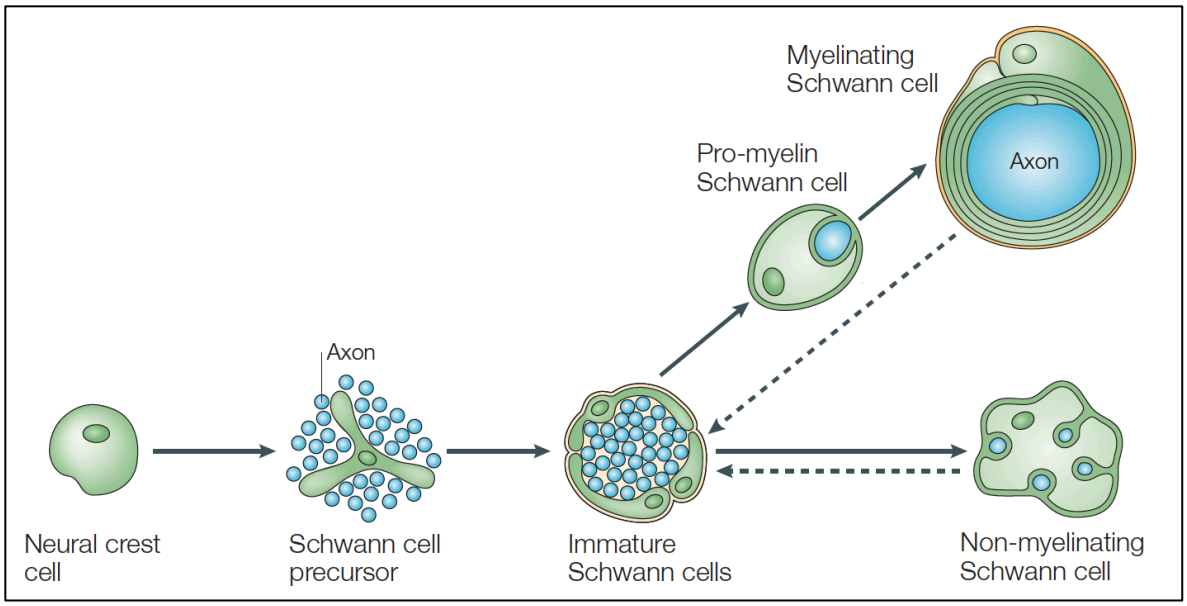


Figure 1.2. The Schwann cell lineage. Schematic illustration of the main cell types and developmental transitions involved in Schwann cell development. Dashed arrows indicated the reversibility of the transition from immature Schwann cells to myelinating and non-myelinating Schwann cells. (Jessen and Mirsky, 2005).

Axons growing out of the ventral part of the neural tube express the transmembrane protein neuregulin-1 beta (NRG1 beta), which is sufficient and necessary for the survival of Schwann cell precursors *in vitro* and may be responsible for their differentiation into immature Schwann cells (Dong et al., 1995; Riethmacher et al., 1997). Mice deficient for NRG or the NRG receptor, ErbB3, have remarkably few Schwann cell precursors at early stages of nerve development (Meyer and Birchmeier, 1995; Riethmacher et al., 1997). Schwann cell precursors migrate along peripheral axons and bundle them into one fascicle (Gilmour et al., 2002; Raphael et al., 2010). This process is guided by attractive and repulsive axonal cues, most notably the Slit/Robo system in which Slit-2 and its Robo receptors, Robo-1 and Robo-2, are highly expressed in Schwann cells and regulate their migration *in vitro* via Ca^{2+}-dependent signaling (Wang et al., 2013). Early Schwann cell survival, maturation and migration is controlled

by neuronally derived signals and, in turn, Schwann cells regulate neuronal number, axonal caliber, distribution of ion channels and neurofilament phosphorylation in myelinated axons (Mirsky et al., 2002). One gene important for the survival and differentiation of Schwann cell precursors is the transcription factor Pax-3. Pax-3 is highly expressed in early precursors and maintained throughout differentiation in non-myelinating Schwann cells in adult mice, where it acts to block expression of Oct-6, Krox-20 and myelin basic protein (MBP), important physiological regulators of myelination (Doddrell et al., 2012). In healthy mice, Oct-6 is expressed in all Schwann cells in late embryogenesis and the early postnatal period, with highest expression in pro-myelinating Schwann cells while Krox-20 is selectively expressed in Schwann cells destined to myelinate (Arroyo et al., 1998; Blanchard et al., 1996). Oct-6 is thought to induce Krox-20 expression required for myelination and in return, Krox-20 downregulates Oct-6 in myelinating cells. Finally, Krox-20 blocks NRG-beta-induced proliferation of Schwann cells, thus linking together changes in expression pattern throughout Schwann cell differentiation with changes in responsiveness to neuronally-derived survival cues (Jessen and Mirsky, 2002). Many other molecules also demonstrate an autocrine survival loop in mature Schwann cells including insulin-like growth factors, platelet-derived growth factor-BB, and neurotrophin 3, which together with laminin support long-term Schwann cell survival – even in the absence of axons (Mirsky et al., 2002).

Immature Schwann cells differentiate into both myelinating and non-myelinating Schwann cells and the signals that cause some to begin myelination and some to form Remak bundles are slowly being revealed (Jessen and Mirsky, 2005). First, immature

Schwann cells send processes into bundles of axons and begin the process of radial sorting. Radial sorting involves larger diameter axons establishing a 1:1 association with individual Schwann cells while smaller diameter axons are surrounded by non-myelinating Schwann cells. While radial sorting is only beginning to be understood, it is known that interactions between laminins present in the basal lamina and integrins on the surface of Schwann cells are involved in this process (Woodhoo and Sommer, 2008; Yang et al., 2005; Petersen et al., 2015). Other factors involved in this process include NRG, neurotrophin 8 and brain-derived neurotrophic factor (BDNF) (Meintanis et al., 2001; Yamauchi et al., 2004). During this time, Schwann cell numbers are tightly regulated by axonal factors such as NRG1, transforming growth factor-β (TGF-β) and p75 neurotrophin receptor (Jessen and Mirsky, 2005). The same signaling pathways that maintain immature Schwann cell survival suppress the beginning of myelination until Krox-20 is expressed and cyclic AMP levels are elevated (Jessen and Mirsky, 2005). Myelination of axons by Schwann cells is consistent with oligodendroglial myelination in the CNS, namely Sox-10 is critical in both glial subtypes for the upregulation of myelin-associated proteins such as MBP (LeBlanc et al., 2006; Li et al., 2007; Srinivasan et al., 2012).

1.1.3 Perineurium development

Early studies were unclear of the origin of perineurial glia (Joseph et al., 2004). However, studies in both zebrafish and mice have confirmed that perineurial glia are CNS-derived and are essential at the motor exit point at the CNS/PNS border for the proper development of motor nerves (Clark et al., 2014; Kucenas et al., 2008; Lewis and Kucenas, 2014). Additionally, it has been determined that Sox-10, described above for its

role during Schwann cell differentiation, also plays a role in perineurium and perineurial glia development by directly activating the expression of Desert Hedgehog (Dhh), a member of the Hedgehog family of signaling molecules (Kuspert et al., 2012). In Dhh-knockout mice, the perineurium is abnormally thin, the basement membrane surrounding Schwann cells is discontinuous and the tight junctions between perineurial glia are immature (Mirsky et al., 2002; Parmantier et al., 1999). While it is known that Dhh is secreted from Schwann cells, the process by which Dhh activates perineurial glia and results in the organization and maintenance of the perineurium remains unknown (Jessen and Mirsky, 2005).

1.1.4 Glia and disease

Glial cells can play a role in diseases associated with a variety of tissues including the brain, muscles and immune system. In the brain, specific glial cells are important for correct neuronal migration, synapse formation and learning and defects in these processes due to developmental or environmental disturbances have been implicated in neurological diseases such Autism Spectrum Disorder (ASD) (Zeidan-Chulia et al., 2014). Glia of the CNS and PNS are also susceptible to immune-mediated diseases such as Multiple Sclerosis (MS) and Guillain-Barré-Syndrome (GBS) which result in glial inflammation and demyelination (Kamm and Zettl, 2012). Muscular dystrophies are a class of diseases that can be caused by mutations in a variety of genes, affecting both peripheral nerve and muscle integrity. For example, the most frequent muscular dystrophy in Europe, called Merosin Deficient Congenital Muscular Dystrophy (MDC1A), is characterized by a mutation in laminin-2 (merosin) that results in loss of myelination, slower nerve conduction velocity, white matter lesions in the CNS and muscular dystrophy (Masaki

and Matsumura, 2010). Similarly, Charcot-Marie-Tooth is an inherited disease characterized by peripheral neuropathies, defects in Schwann cell myelination and eventual degeneration of nerves resulting in muscle weakness and atrophy in the extremities (Brennan et al., 2015; Ekins et al., 2015). The variety of genetic mutations associated with Charcot-Marie-Tooth disease include genes related to intracellular trafficking, myelin structure and mitochondrial fusion; however, the mechanisms through which each mutation results in Charcot-Marie-Tooth disease remain to be determined (Bucci et al., 2012). Other diseases involving glia include leprosy, caused by infection by the bacteria *Mycobacterium leprae*, which has a unique affinity for Schwann cells and results in decreased Schwann cell proliferation and demyelination among other symptoms (Singh et al., 1997). Of note, defective laminin signaling has been associated with all of the above demyelinating diseases except those associated with the immune system (Feltri and Wrabetz, 2005). Homozygous mutations in human LAMB2 gene cause autosomal recessive Pierson syndrome, characterized by nephrotic and neurological abnormalities in the first decade of life. However, how laminin mutations result in these phenotypes is still unknown (Matejas et al., 2010). Overproliferation or hyperactivity of glial cells can also result in human diseases. Neurofibromatosis is characterized by benign tumours composed of nervous tissue, sometimes specifically Schwann cells (Schwannomatosis) (Hilton and Hanemann, 2014). Gliomas are neoplasms originating from glial tissue within the brain and can be benign or malignant depending on the biology of the tumour. Animal models exist to help researchers study diseases such as those described here in a more controlled manner with the hope of using that we can use the knowledge acquired

through animal studies to benefit the understanding of healthy human glial biology, and if possible, to help foster ideas for novel therapies for the glial-related diseases.

1.2 THE DROSOPHILA NERVOUS SYSTEM

Based on functional and molecular criteria, the nervous system is highly conserved between mammals and *Drosophila*; therefore studying nervous system development *in vivo* in *Drosophila* can help address many fundamental questions about

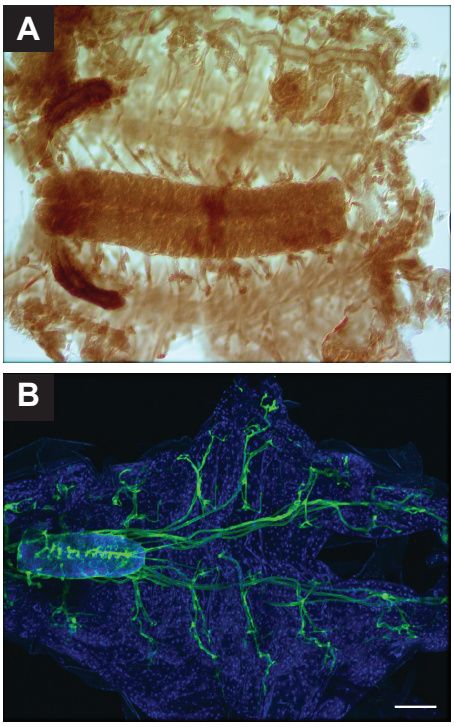


Figure 1.3. Drosophila nervous system developmental stages. (A) Stage 17 embryo fillet imaged at 20x magnification and stained for perineurial glia using RAB (brown). (B) Second instar larva fillet stained using DAPI to label nuclei (blue) and perineurial glia are in green. Scale bar is 100 µm. (C) Third instar larva CNS and ventral nerve cord with peripheral nerves stained for LanB2 (red) and perineurial glia in green. Scale bar is 100 µm.

human nervous system development as well as glial and neuronal cell biology. For example, *Drosophila* research has helped us understand complicated concepts in glial biology such as the mechanisms mediating reciprocal trophic support between neurons and glia, glial pathways for recycling synaptic neurotransmitters, and the mechanisms of glioma progression (Jeibmann et al., 2015; Stork et al., 2014). The larval *Drosophila* nervous system includes the CNS, comprised of the ventral nerve cord and central brain

lobes and the PNS, comprised of the peripheral nerves and neuromuscular junctions (Fig. 1.3).

1.2.1 Embryonic glial development

All glia in *Drosophila*, with the exception of the midline glia, express the early transcription factors glial cells missing (gcm) and reversed polarity (repo) (Jones et al., 1995; Lee and Jones, 2005; Xiong et al., 1994). A DNA binding protein regulated by the dorsoventral patterning genes, gcm activates transcription of repo and other genes involved in glial differentiation (Kim et al., 2015; Miller et al., 1998). Gcm is first expressed during embryonic stage 10 but fades by embryonic stage 14, whereas once repo is expressed, it continues expression throughout the life cycle of the *Drosophila* (Chotard et al., 2005; Stork et al., 2012). In *gcm* mutants, glial cells are transformed into neuronal cell types and, conversely, when *gcm* is overexpressed, ectopic glial cells develop (Klambt et al., 2001). Most glia arise from neuroblasts in the ventral nerve cord which contains about 25-30 glia per hemisegment (Stork et al., 2012). However, the midline glia derive from a set of mesectodermal precursors and are present along the midline of the ventral nerve cord where they associate with and are critical for the proper formation of the two horizontal commissure axon tracts (Jacobs, 2000; Tear, 1999). Glia in the PNS can arise from two sources: the neural stem cells along the lateral edge of the CNS and from sensory organ precursors in the ectoderm (Parker and Auld, 2006; Schmidt et al., 1997). Glia then extend cytoplasmic processes and migrate along sensory axons while the sensory axons use the glia to direct their movement (Sepp and Auld, 2003; Sepp et al., 2000). At the CNS/PNS boundary, glia dictate the entry point of sensory axons into the CNS and the exit of motor neurons out of the CNS, similar to in

vertebrates (Kucenas et al., 2008; Sepp et al., 2000, 2001). Nearly all glia are born in the CNS and those that ensheath nerves in the PNS migrate out of the CNS along motor axon tracts during midembryonic development (Banerjee and Bhat, 2008). Glial cells migrate until glial- and neuronally-derived cues trigger glial differentiation. Studies in the *Drosophila* eye disc have shown two FGF-like ligands, glial-derived Pyramus and neuronally-derived Thisbe, are required to initiate glial differentiation which triggers glial cells to switch from migrating to wrapping around axons (Franzdottir et al., 2009). Notch also plays a role in both migration and differentiation as it is required for normal migration of peripheral glia in *Drosophila* embryos as well as negatively regulating gcm, thereby affecting gliogenesis in the *Drosophila* PNS (Edenfeld et al., 2007; Van De Bor and Giangrande, 2001; Binari et al., 2013). Another important factor in glial differentiation is crooked neck (Crn), a splicing factor that interacts with the RNA-binding protein, held out wing (How), to promote splicing of septate junction-specific genes (Edenfeld et al., 2006; Lo and Frasch, 1997; Zaffran et al., 1997). The vertebrate homologue of How is Quaking, which is also required for glial differentiation and myelination (Sidman et al., 1964). By embryonic stage 17 (~17 hr after egg laying), glial ensheathment completely surrounds peripheral nerves and the septate junctions are complete, forming the blood-brain barrier (Banerjee et al., 2006). Septate junctions formed between *Drosophila* glial membranes are homologous to those in vertebrates that are present in the glial regions flanking a node of Ranvier and show remarkable functional similarities (Bhat, 2003) (Banerjee and Bhat, 2008). At the end of embryogenesis, the *Drosophila* nervous system includes the CNS, comprised of the ventral nerve cord and brain lobes and the PNS, comprised of the peripheral nerves and

the neuromuscular junctions. As in humans, *Drosophila* glia are subdivided based on morphology, function and location. For the purposes of this thesis, I will briefly summarize glia found in the CNS and expand on glia found in the PNS and their counterparts in the vertebrate nervous system (Fig. 1.1) (Parker and Auld, 2006).

1.2.2 Larval glial subtypes in the CNS

Most experiments in this thesis examine the function and morphology of *Drosophila* glial cells at the 3rd instar larval stage (Fig 1.3). At this time the *Drosophila* CNS contains three types of CNS-specific glia, as well as three types of glia that are found throughout the CNS and PNS (Fig 1.4). Specific to the CNS, there are the cortex glia, the ensheathing glia and the astrocyte-like/reticular glia (Stork et al., 2012). The cortex glia form a network that tightly surrounds neuronal cell bodies outside the neuropil and contacts other glial subtypes (Dumstrei et al., 2003; Pereanu et al., 2005). One cortex glial cell can ensheath dozens of neuronal cell bodies to supply trophic and metabolic support for the neurons (Stork et al., 2012). During larval stages, when neuroblasts are dividing, cortex glia form chambers around the neuroblasts and newly generated neurons. Mutations causing a lack of cortex glial processes within in the neuropil result in neuronal degeneration (Buchanan and Benzer, 1993; Hartenstein, 2011). Also, within the neuropil, astrocyte-like/reticular glia closely associate with the synaptic neuropil. The close contact with synapses and star-like morphology of these glia are reminiscent of astrocytes in the vertebrate CNS and these astrocyte-like/reticular glia have an important role in synaptic transmission by taking up neurotransmitters from the synaptic cleft (Grosjean et al., 2008; Jackson and Haydon, 2008; Rival et al., 2004). The final type of glial cell specific to the CNS is the ensheathing glia, which encircle the neuropile and

respond to axonal injury by clearing neuronal debris by phagocytosis (Doherty et al., 2009).

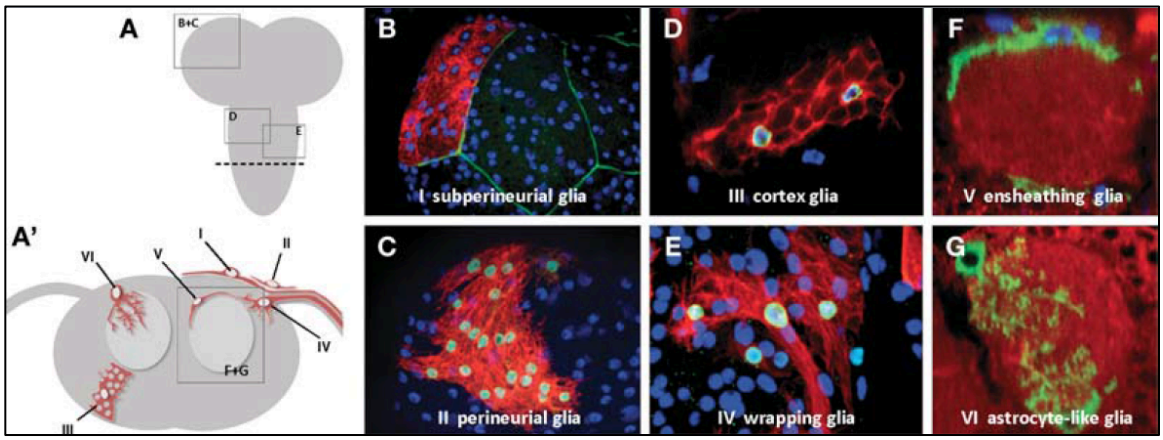


Figure 1.4. Glial subtypes in the *Drosophila* CNS. (A) Schematic of the CNS of a third-instar larva with two brain lobes and the ventral nerve cord shown in gray. The dashed line indicates the plan of cross section shown in panel A'. (A') Gray depicts the cortex, the region of cell bodies and the light gray depicts the synaptic neuropil with one peripheral nerve exiting the ventral nerve cord on each side. Subtypes of glia are highlighted as a single cell in red and numbered I-VI corresponding to the images in B-G. (B-G) Confocal images of glial subtype morphology highlighted using different "flip-out" strategies. Glial nuclei are visualized by staining for Repo (blue) and glial membranes are shown with the use GAL4-driven UAS-mCD8-Cherry (B-E) or UAS-mCD8-GFP (F,G). (B) Subperineurial glia in red and NrxIV-GFP in green which highlights the cell borders of subperineurial glia. (C-E) Cortex glia, perineurial glia and wrapping glia in red with nuclear β-galactosidase in green. (F,G) Ensheathing glia and astrocyte-like glia/reticular glia in green and stained for HRP in red to label neuronal membranes. Note that in B, a single subperineurial glial cell is labeled and all the Repo-positive nuclei correspond to other, deeper located glial cells. (Stork et al., 2012).

1.2.3 Comparing Drosophila and vertebrate peripheral nerve structure

The structure of the *Drosophila* PNS is similar to that of vertebrates (Fig. 1.5) in that axons are insulated by multiple layers of glial membrane and surrounded by a basement membrane. Axons in *Drosophila* are directly engulfed by wrapping glia similar to how non-myelinating Schwann cells engulf multiple axons simultaneously. The subperineurial glia form a layer around wrapping glia containing septate junctions

resulting in the blood-brain barrier similar to the barrier formed by astrocytes and endothelial cells of blood vessels in vertebrates (Abbott et al., 2006). Finally, perineurial glia surround the subperineurial glia and a basement membrane called the neural lamella surrounds the entire nervous system. The perineurial glia help form the neural lamella with which it is adjacent to. This relationship is similar to the close association between the perineurium and perineurial glia in vertebrates (Rodrigues et al., 2011).

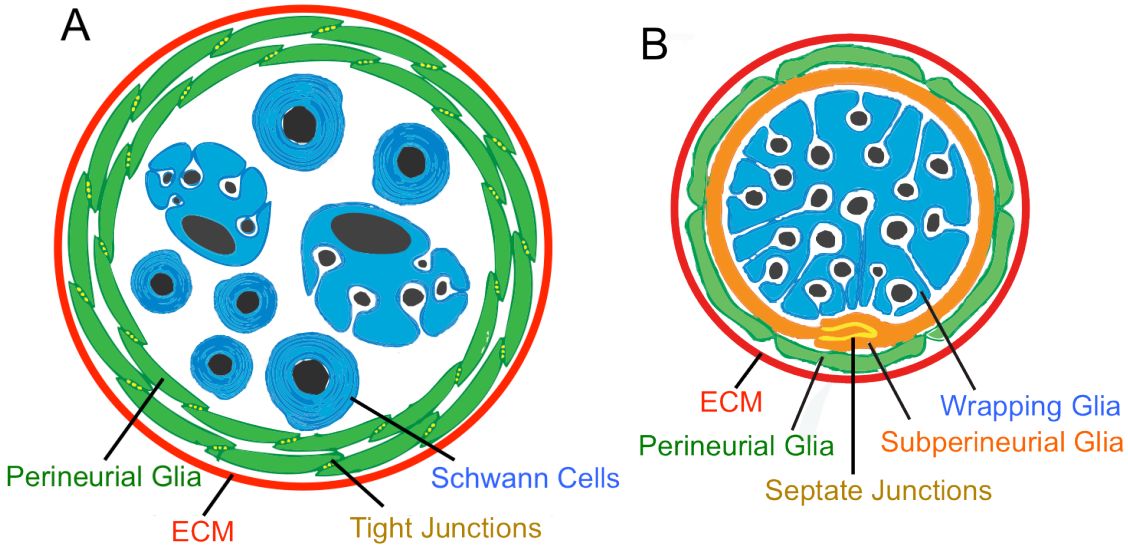


Figure 1.5. Schematic view of cross-sections of a *Drosophila* peripheral nerve and vertebrate peripheral nerve. (A) Perineurial glia form tight junctions and are surrounded by the epineurium. Large diameter axons are surrounded by myelinating Schwann cells and small diameter axons are surrounded by non-myelinating Schwann cells which engulf multiple axons. **(B)** *Drosophila* axons directly interact with wrapping glia (blue). Subperineurial glia (orange) form septate junctions (yellow) and perineurial glia (green) are surrounded by the neural lamella (red). (Adapted from Rodrigues et al., 2011).

1.2.4 Wrapping glia

Wrapping glia arise during late embryogenesis and have a similar location to ensheathing glia within the ventral nerve cord; however, wrapping glia also send out processes to the PNS (Stork et al., 2012). This glial subtype bears the most resemblance

to the non-myelinating Schwann cells in vertebrates (Nave and Salzer, 2006; Nave and Trapp, 2008). Wrapping glia begin to send out processes into the PNS during early larval stages and by late larval stages, although they do not proliferate during this time, they fully ensheathe all axons and axon bundles and their cell bodies can span more than 1mm of axonal length (Hartenstein, 2011; Matzat et al., 2015; von Hilchen et al., 2013). Wrapping glia secrete Vein, a homolog of mammalian neuregulin that binds to the EGF receptor on their surface, allowing for the autocrine activation and regulation of wrapping glial process extension around axons (Matzat et al., 2015).

1.2.5 Subperineurial glia

The subperineurial glia are large cells that surround the cortex glia in the CNS and the wrapping glia in the PNS. During embryogenesis, the subperineurial glia are in contact with axons in the PNS; however as the wrapping glia extend processes and surround individual axons, the subperineurial contact with axons decreases. Between embryogenesis and larval stages, subperineurial glia do not duplicate, but they undergo enormous hypertrophy in correlation to the length of the peripheral nerve and form a blood-nerve barrier just proximal to the neuromuscular junction (Brink et al., 2012; von Hilchen et al., 2013). Subperineurial glia establish intercellular septate junctions with one another to form the structural basis of the blood-brain barrier in Drosophila (Carlson et al., 2000; Pichon and Treherne, 1973; Treherne, 1962). The formation of septate junctions has been found to be regulated by Vein, which is secreted by wrapping glia (Matzat et al., 2015). Septate junctions appear as large disc shapes in the CNS and as a long thin line in the PNS (Stork et al., 2008). Core components of the septate junction in subperineurial glia include neurexin-IV (nrxIV), contactin and neuroglian, all of which

are required for proper functioning of the blood-brain barrier (Banerjee et al., 2006; Baumgartner et al., 1996).

1.2.6 Perineurial glia

The perineurial glia are located just below the neural lamella and surround the entire *Drosophila* nervous system, including peripheral nerves (Fig. 1.6). The origin of the perineurial glia is still debated and was originally thought to be mesodermal since *Drosophila* mutants lacking mesodermal derivatives were absent of perineurial glia (Edwards et al., 1993). Perineurial glia are made locally on the surface of the CNS and do not involve the gcm transcription factor central to most other glial cells differentiation; however, they do express repo throughout development (Awasaki et al., 2008). Perineurial glia arise during late embryogenesis and replicate substantially during larval stages as first instar larvae have only a few perineurial glial cells, but by the third instar larval stage they have completely covered the ventral nerve cord and peripheral nerves (Awasaki et al., 2008; Stork et al., 2008; von Hilchen et al., 2013). By knocking down Cyclin A specifically in perineurial glia to inhibit mitosis, it was confirmed that most glia along the peripheral nerves are perineurial glia (von Hilchen et al., 2013). They extend beyond the subperineurial glia into the neuromuscular junction where they contact synapses (Brink et al., 2012). The function of the perineurial glia is just beginning to be elucidated and the persistence of some perineurial glia into the adult fly brain suggests that they could become more essential later during metamorphosis (Hartenstein, 2011; von Hilchen et al., 2013). Recently, the perineurial glia have been demonstrated to secrete matrix metalloproteinases into the neural lamella, thereby resulting in regulating the length of the ventral nerve cord (Meyer et al., 2014). The perineurial glia are closely

associated with the neural lamella and degradation of the neural lamella by overexpression of MMP2 results in reduced perineurial glial wrapping and delicate peripheral nerves (Xie and Auld, 2011). Thus, understanding the relationship between the perineurial glia and the neural lamella would help determine the function of the perineurial glia as well as the importance of the neural lamella.

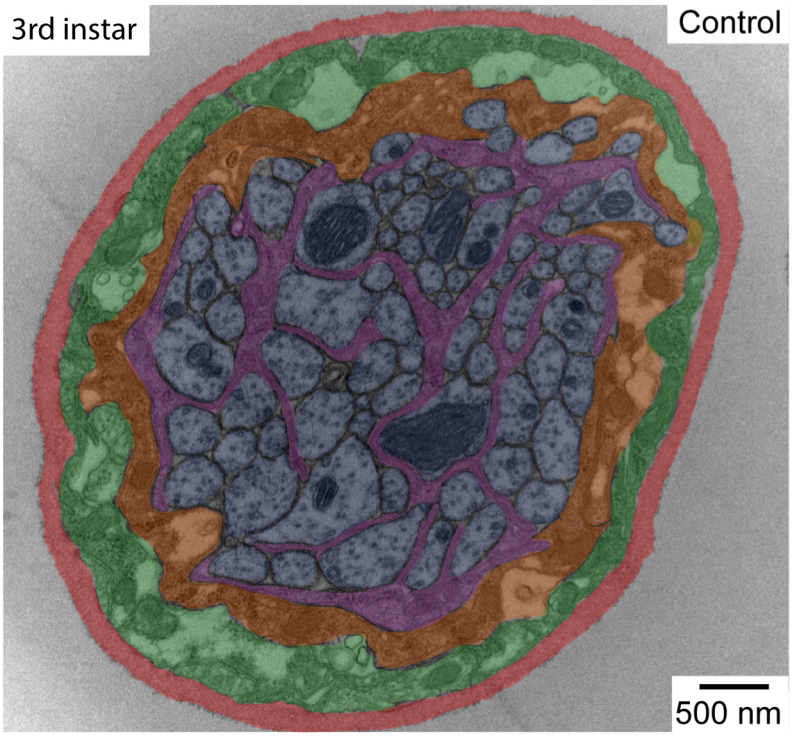


Figure 1.6 Electron microscopic image of peripheral *Drosophila* 3rd instar nerve. Axons (blue) in Drosophila peripheral nerves are surrounded by wrapping glia (purple), subperineurial glia (orange), perineurial glia (green) and a basement membrane called the neural lamella (red).

1.2.7 Neural Lamella

The neural lamella is a continuous dense network of ECM components that surrounds the entire nervous system from embryonic stage 16 onwards (Stork et al., 2008). The neural lamella is composed of ECM components secreted by three different cell types: hemocytes, fat body cells and glia (Olofsson and Page, 2005; Pastor-Pareja and Xu, 2011; Xie and Auld, 2011). Finally, the neural lamella is essential for normal

nervous system development, as in its absence, the nervous system contains major morphological defects (Martinek et al., 2008). Understanding the deposition and organization of the neural lamella in *Drosophila* would provide valuable insight into the role of the neural lamella during *Drosophila* PNS development and how the neural lamella maintains the shape of the overall nervous system. In order to study the function and importance of the neural lamella, it is critical to separate and understand the role of the individual extracellular matrix components that together form the complex and multi-functional neural lamella.

1.3 EXTRACELLULAR MATRIX INTERACTIONS

Basement membranes were first described surrounding muscle tissues and they are now known to surround nearly all tissues. First identified in the late 1970s, basement membranes contain laminin, collagen, nidogen and perlecan, but the specific interactions between these components and their importance in basement membrane assembly, strength and function are still being revealed today (Yurchenco, 2011). For example, it was recently determined that different compositions of ECM components result in stiff or soft basement membranes and that the stiffness regulates glial migration in both Drosophila and mammalian glioma models (Kim et al., 2015). It is becoming increasingly apparent that each basement membrane is a unique composition of ECM proteins and that each ECM component plays a distinct critical role in the overall basement membrane function. In Drosophila, the ECM is evolutionarily conserved and consists of two laminins, perlecan, two collagens, nidogen and SPARC (Hynes and Zhao, 2000). Here, I will focus on a few key components and processes related to ECM-glial adhesion and development in vertebrates and *Drosophila*.

1.3.1 Laminin in the ECM

All laminins are heterotrimers composed of one α subunit, one β subunit and one γ subunit. In mammals, there are five α subunits, three β subunits and three γ subunits that can combine to make 16 different laminin isoforms (Hohenester and Yurchenco, 2013). In *Drosophila*, there are 2 α subunits (Wing Blister/Wb and Laminin A/LanA), 1 β subunit (LanB1) and 1 γ subunit (LanB2); therefore, there are only 2 unique isoforms (Urbano et al., 2009). The expression of specific laminin isoforms is regulated spatially and temporally throughout development and each laminin isoform can preferentially bind specific receptors. All laminin heterotrimers share repeats of laminin EGF-like domains (LE domains) followed by α-helical domains wound together to form a coiled-coil structure and 5 homologous globular domains (LG domains) responsible for binding receptors. The C-terminal of the vertebrate α subunit contains the 5 LG domains whereas the β and γ subunits are shorter, are involved in the formation of the coiled-coil domain and interact with the N-terminal of the α subunit resulting in the self-assembly of laminin into a matrix (Fig. 1.7) (Durbeej, 2010; Hamill et al., 2009).

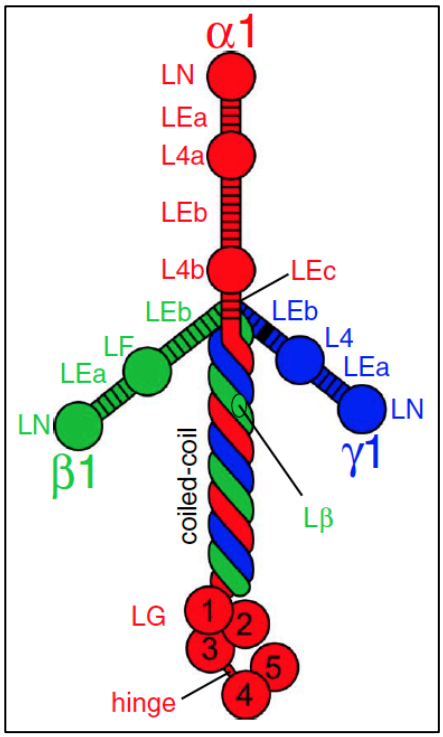


Figure 1.7. Schematic structure of laminin-111 as a representative model of a laminin heterotrimer with the α subunit in red, β subunit in green and γ subunit in blue. LN: laminin N-terminal domain, LE: laminin epidermal-growth-factor-like domain, LG: laminin globular domain, Lβ: laminin β-knob, present only in β chains and interrupts the coiled coil of β chains (Durbeej, 2010).

Many domains are conserved between vertebrate and *Drosophila* laminins including the EGF-like domains, the coiled-coil domain and the globular domains in the C-terminal of the α subunits (Henchcliffe et al., 1993). N-linked glycosylation of the laminin subunits affects their activity and function, however N-linked glycosylation is not necessary for laminin assembly and secretion (Matsui et al., 1995). Once secreted, laminin undergoes further physiological maturation including proteolytic cleavage between LG3 and LG4 domains that can substantially affect its function within the basement membrane and have important cellular and behavioural effects (Rousselle and Beck, 2013). Additionally, glycolipids such as galactosyl-sulfatide are expressed by Schwann cells and anchor laminin to the glial surface, thus initiating basement membrane assembly and enabling receptor signaling (Li et al., 2005). Laminin is deposited in a specific mesh pattern on the surface of Schwann cells in contrast to the organized fibril pattern in lung tissue. While various laminin deposition patterns are thought to be

regulated via laminin-receptor interactions and affect cell migration and polarity, it is difficult to study the importance of laminin deposition patterns since the mechanism of basic laminin secretion and deposition remains unknown (Hamill et al., 2009).

1.3.2 Secretion of ECM components

As basement membranes surround many tissues, multiple types of cells are responsible for the secretion of ECM components. Cells across species and tissues synthesize ECM components, like most secreted proteins, in the endoplasmic reticulum (ER) and they are then transported to the Golgi apparatus for post-translational modifications before being secreted extracellularly. Schwann cells are one of the major contributors of neural ECM proteins, which enable Schwann cells to ensheath and myelinate axons (Singh et al., 1997). One major difference between most ECM components and other secreted proteins is the size of the protein. Collagens are a 300-400 nm rod-like structure and laminins are over 100 nm in length while ER-to-Golgi transport vesicles are only 60-90nm in diameter (Malhotra and Erlmann, 2011; Rousselle and Beck, 2013). This poses the question of how large ECM components are transported from the ER to Golgi and eventually out of the cell. Recently, it was found that at ER exit sites, Tango1 and cTAGE5 are responsible for the synthesis of larger ER-to-Golgi trafficking vesicles containing collagen (Fig. 1.8) (Malhotra and Erlmann, 2011; Saito et al., 2009).

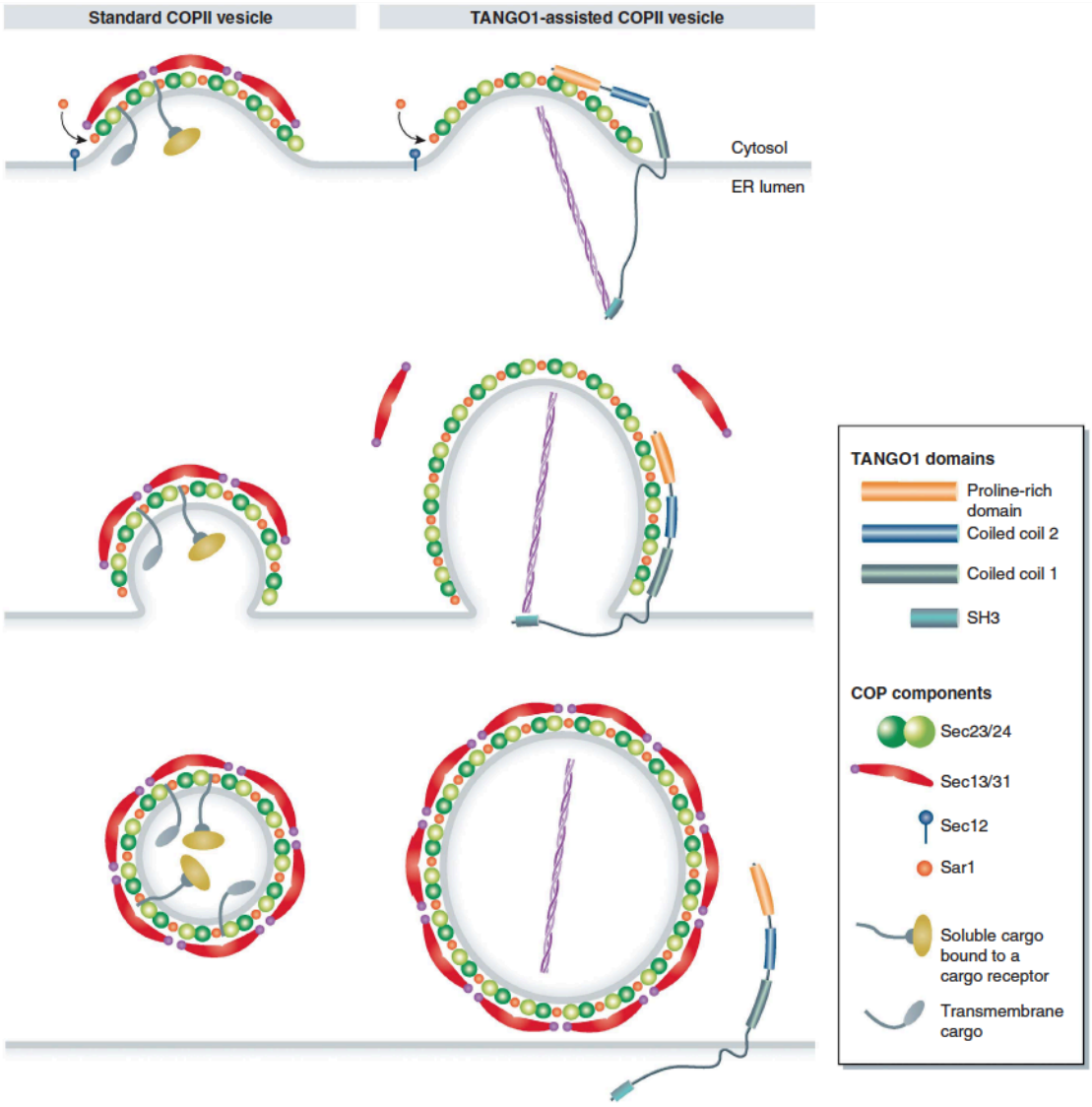


Figure 1.8. Comparison of normal and TANGO1-assisted formation of COPII carriers. TANGO1 is not required for general protein secretion and therefore, has no role in the biogenesis of a generic COPII vesicle. TANGO1 binds Collagen VII through its SH3 domain and Sec23/24 through its PRD. In this mode, the Sec13/31 dimer cannot bind Sec23/24 and the completion of the Collagen VII-containing COPII carrier is delayed. The carrier thus continues to grow in size. Release of Collagen VII triggers separation of TANGO1's PRD from the Sec23/24. Sec13/31 can then bind Sec23/24 to generate a mega COPII carrier (Malhotra and Erlmann, 2011).

Standard sized trafficking vesicles are formed by the recruitment and assembly of COPII coats at the ER, which are responsible for regulating the scission of 60-90 nm COPII vesicles (Fig. 1.8). First, Sec12 in the ER membrane recruits the GTPase Sar1 to

the membrane, which binds the cytoplasmic dimeric Sec23/Sec24 complex and together they form the first layer of the COPII coat (Bi et al., 2002). Sec23 binds Sar1 while Sec24 is thought to be key for the selection and binding of transmembrane cargoes (Bi et al., 2002; Wendeler et al., 2007). The binding of the outer COPII coat, consisting of Sec 13 and Sec 31 regulates the GAP activity of Sec23 on Sar1 and thus controls the completion of the COPII vesicle biogenesis after which scission of the vesicle takes place (Bi et al., 2007). Tango1 contains a SH3 domain that binds collagen and a proline-rich domain (PRD) that binds Sec23/24 effectively preventing the binding of Sec13/31 to Sec23/24 when collagen is located within a vesicle at ER exit sites (Saito et al., 2011). This is thought to delay the binding of the outer COPII coat and allow the ER-to-Golgi vesicle to become large enough to fit collagen monomers. While the mechanism of laminin secretion and of other ECM components has not been thoroughly investigated, the role of Tango1 in collagen secretion has been studied *in vitro*, in mice and in *Drosophila* (Lerner et al., 2013; Saito et al., 2009; Wilson et al., 2011). In *Drosophila* follicle cells, it was found that expression of Tango1-RNAi prevented the secretion of both collagen and laminin, but the question remains as to whether laminin and Tango1 interact directly or indirectly, potentially via collagen (Lerner et al., 2013).

Laminin-specific secretion has been studied mostly *in vitro,* where it was determined that the β and γ subunits bind each other before binding the α subunit to form a laminin heterotrimer that is subsequently secreted. Without the other, neither the β or γ subunit can bind the α subunit individually, and binding to the α subunit was found to be necessary for effective secretion. Thus, if a cell lacks one of the β or γ subunits, the other will accumulate intracellularly while the α subunit is secreted (Yurchenco and Cheng,

1994). This model of laminin secretion has been supported by studies in *C. elegans*, which show intracellular β subunit accumulation in body wall muscles and intestine when the α subunit was mutated (Huang et al., 2003; Kao et al., 2006). However, the physiological and cellular effects of accumulated laminin subunits have not been determined nor has the mechanism of laminin secretion from the ER.

1.3.3 Ventral nerve cord condensation in Drosophila

Although the condensation of the *Drosophila* nerve cord is commonly observed by scientists staging embryos, the process by which this happens is poorly understood. During embryogenesis, the ventral nerve cord is approximately 80% of the length of the embryo at stage 15 and decreases to around 60% embryo length at stage 17 (Olofsson and Page, 2005). This condensation is known to rely on the migration of hemocytes, which begin to surround and migrate along the ventral nerve cord by stage 12 (Olofsson and Page, 2005; Tepass et al., 1994). Hemocytes are known to secrete ECM components such as Collagen IV and Peroxidasin and inhibiting their migration is associated with a loss of these ECM components throughout the embryo including in the neural lamella around the ventral nerve cord (Fessler and Fessler, 1989; Olofsson and Page, 2005). Additionally, mutations in both Collagen IV and its integrin receptors also result in a lack of ventral nerve condensation indicating a role for the neural lamella in condensation (Borchiellini et al., 1996; Brown, 1994; Wright, 1960). However the mechanism through which the neural lamella contributes to ventral nerve cord condensation is unknown. It has been hypothesized that collagen and perlecan generate opposing forces on the glial cells that ensheathe the nervous system, and together regulate the appropriate size and shape of the nervous system (Pastor-Pareja and Xu, 2011). Both collagen and perlecan bind integrin,

but the mechanism through which these different ECM components act through the same receptor to regulate different signaling pathways remains to be elucidated (Broadie et al., 2011). Blocking hemocyte migration along the ventral nerve cord also inhibits neural development within the CNS during embryogenesis likely due to loss of ECM deposition (Evans et al., 2010). Glial cells have also been implicated in ventral nerve cord condensation as mutations that reduce glial number also result in a failure of ventral nerve cord condensation (Campbell et al., 1994). Glycuronyltransferase mutations in hemocytes that result in an elongated ventral nerve cord and shorter peripheral nerves also reduce expression of the important glial transcription factor repo (Pandey et al., 2011). Additionally, perineurial glia in *Drosophila* have been shown to express ECM receptors and secrete matrix metalloproteinases necessary for the proper condensation of the ventral nerve cord (Meyer et al., 2014; Xie and Auld, 2011). Glial cells in vertebrates also secrete ECM components into the surrounding basement membrane during development highlighting the conserved function of peripheral glia (Colognato and Tzvetanova, 2011). The roles of glia as both contributors to and as binding partners of the surrounding basement membrane during nervous system development place glial cells as important regulators of the overall structure of the nervous system.

1.3.4 ECM and glial development

Glial cells contribute ECM proteins to form basement membranes, which in turn is an important regulator of glial signaling pathways, contributing to a variety of developmental processes including progenitor proliferation, axonal pathfinding, synaptic function and glial differentiation (Broadie et al., 2011). Neural progenitor polarity is a key feature of asymmetric division and may be affected by the polarity of the overlying

ECM in *Drosophila* (Broadie et al., 2011; Mirouse et al., 2009; Schneider et al., 2006). Furthermore, the quiescence stage that neuroblasts enter near the end of embryogenesis is maintained by glycoproteins secreted by glial cells and requires perlecan to be re-activated (Datta, 1999; Ebens et al., 1993; Voigt et al., 2002). Many ECM proteins and their receptors are implicated in axonal pathfinding in *Drosophila* and vertebrates, both directly and indirectly via defective glial development and migration. Laminin is known to promote axon outgrowth and trigger cell spreading in cultured neuronal cell lines (Takagi et al., 1996; Takagi et al., 1998), and knockout of laminin γ1 leads to defects in neuronal migration *in vivo*, likely through integrin signaling (Chen et al., 2009). Consistently, integrin mutations cause a significant increase in navigational errors by axonal outgrowths – specifically in pioneer neurons, but not in follower neurons (Huang et al., 2007). Taken together with the finding that changes in ECM composition alters glial migration and that glial cells maintain reciprocal interactions with neurons throughout development (Kim et al., 2015; Oland and Tolbert, 2011), these findings highlight the role of the ECM as a regulator of both proper neuronal and glial development.

The ECM is also becoming increasingly appreciated for its role in altering synaptic structures. For example, in *Drosophila*, perlecan has been shown to promote the accumulation of extracellular Wingless/Wnt (Wg) proteins and mutations in perlecan result in overproduction of synaptic boutons at neuromuscular junctions (Kamimura et al., 2013). Glial interactions with the ECM are also important for appropriate glial migration and differentiation, as demonstrated by the aberrant migration and distribution of glia after integrin knockdown in the *Drosophila* eye disc (Xie et al., 2014). Finally, the

ECM has been demonstrated to play a critical role during glial wrapping in both vertebrates and *Drosophila* and this will be discussed further in the next section (McKee et al., 2012; Xie and Auld, 2011).

1.3.5 ECM and glial wrapping

In vertebrates, the process of glial wrapping occurs once glial cells have differentiated into Schwann cells and oligodendrocytes, and although the molecular mechanisms underlying this process are only beginning to be elucidated, several studies have implicated the ECM and its receptors as being important regulators (Colognato and Tzvetanova, 2011). The process by which *Drosophila* glia wrap axons is most similar to that of non-myelinating Schwann cells for a variety of reasons. Firstly, Schwann cells secrete and assemble their own ECM, while oligodendrocytes have only been shown to secrete low levels of ECM components. Secondly, Schwann cells interact with a classic basement membrane whereas oligodendrocytes have only "brief encounters" with cell-associated ECM found in the developing CNS. Finally, non-myelinating Schwann cells engulf multiple axons simultaneously forming Remak bundles, similar to *Drosophila* wrapping glia (Sherman and Brophy, 2005). The first step in wrapping by Schwann cells is radial sorting, a process highly dependent on interactions between Schwann cell adhesion receptors and ECM components, particularly laminins (Colognato and Tzvetanova, 2011). Many recent studies using mice engineered to lack individual laminin subunits have highlighted the role of laminins in both radial sorting and wrapping, which will be discussed further in Chapter 2 (Fig 1.9) (Chen and Strickland, 2003; McKee et al., 2012; Nakagawa et al., 2001; Wallquist et al., 2005; Yang et al., 2005; Yu et al., 2005).

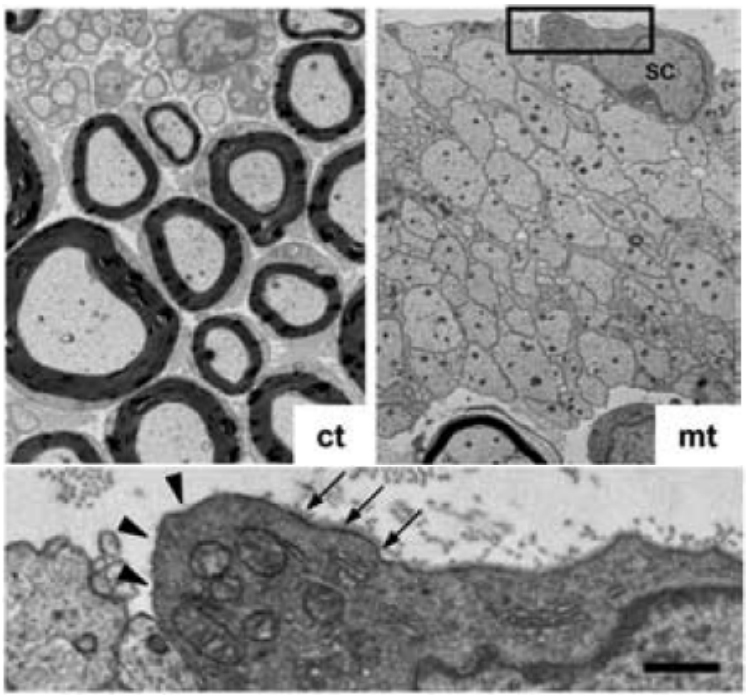


Figure 1.9. Schwann cells lacking laminin γ1 expression show severe hypomyelination. Electron micrographs of 28 sciatic nerves show that mutant nerves have large bundles of unsorted axons with some Schwann cells located outside (SC). Higher magnification of the boxed region (below) shows that the mutant Schwann cell lacks a continuous basal lamina (compare fuzzy materials indicated by arrows and denuded areas indicated by arrowheads) and does not extend processes between axons (Yu et al., 2005).

In Schwann cells, the roles of two main laminin receptors have been investigated: dystroglycan and integrins. Dystroglycan is part of a multimeric transmembrane protein complex and can bind multiple ECM components, however in Schwann cells, it is often studied mainly as a laminin-receptor due to over-lapping phenotypes between laminin- and dystroglycan- deficiencies (Colognato and Tzvetanova, 2011). Dystroglycan was first identified for its role in providing a physical link between the ECM and the cytoskeleton in muscle tissue, but it also plays a role in Schwann cell myelination (Masaki and Matsumura, 2010). Dystroglycan has been found to be expressed following radial sorting has been accomplished during Schwann cell development, being present most densely in early myelinating Schwann cells (Masaki et al., 2002). This is consistent with the finding that Schwann cells lacking dystroglycan undergo mostly normal radial sorting and proliferation with defects only in Schwann cell myelin ultrastructure (Saito et al., 2003). Therefore, the role of dystroglycan is critical in later developmental stages, whereas the

role of integrin as a laminin-receptor is more central to earlier Schwann cell radial sorting, a process more similar to how *Drosophila* glia wrap peripheral axons.

Integrins are heterodimeric receptors that can bind multiple types of ECM components depending on the specificity of the integrin α and β subunits. In vertebrates, there are currently 18 α subunits and 8 β subunits discovered which together can form 24 integrin heterodimer receptors (Hynes, 2004). In contrast, *Drosophila* only possess 5 α and 2 β subunits, and only two integrin heterodimers are observed in the peripheral nerves: α2βPS and α3βPS (Brown, 2000; Xie and Auld, 2011). By downregulation of the βPS integrin subunit in *Drosophila* glia, integrins were found to play a role in the development and maintenance of peripheral glial layers as well as the migration of glia and photoreceptor axons in the eye disc (Xie and Auld, 2011; Xie et al., 2014). Consistently, in vertebrates, integrins have been implemented in the proliferation, survival and differentiation of oligodendrocytes and radial sorting of Schwann cells (Feltri et al., 2002; O'Meara et al., 2011). Two specific laminin isoforms in vertebrates have been shown to be essential for radial sorting, both of which associate specifically with integrins (Nakagawa et al., 2001; Sonnenberg et al., 1991; Yang et al., 2005). Consistently, Schwann cells lacking integrins, but not dystroglycan, also show severe radial sorting defects (Feltri et al., 2002; Saito et al., 2003). However, mutations in laminins cause more severe Schwann cell defects in early development than integrin mutations, such as decreased Schwann cell proliferation (Feltri et al., 2002; Yang et al., 2005; Yu et al., 2005). Altogether these studies suggest that laminin mutations cause defects via another mechanism than preventing integrin and dystroglycan signaling such as having receptor-independent functions or through signaling via a still undetermined

laminin receptor that contributes to Schwann cell development, radial sorting and wrapping. It would be likely that any protein affecting laminin binding to glial cells would interact genetically with integrin or dystroglycan, and demonstrate some structural indicators of ECM-binding potential. For these reasons, we began to investigate basigin as a potential ECM-binding protein and examined its role in glial cells.

1.3.6 Basigin and the ECM

We first became interested in basigin as a potential laminin receptor due to its biochemical structure and ability to interact genetically to integrin (Curtin et al., 2005; Reed et al., 2004). Basigin/EMMPRIN/CD147 is a member of the basigin/embigin/neuroplastin family of glycoproteins and is composed of a single transmembrane domain, a short intracellular domain and two glycosylated extracellular immunoglobulin-like domains (Biswas et al., 1995; Miyauchi et al., 1991). Immunoglobulin domains consist of a two-layer sandwich of 7 to 9 antiparallel β-strands arranged into two β-sheets and are often involved in protein-protein and protein-ligand interactions (Bork et al., 1994; Williams and Barclay, 1988). In humans, there are currently four basigin alternative splicing variants, of which basigin-2 is the most predominant (Belton et al., 2008). Crystal structure analysis has revealed that basigin-2 likely forms homo-dimers with itself as well as hetero-dimers with basigin-3 (Liao et al., 2011). Furthermore, basigin forms heterodimers with other proteins including monocarboxylate transporter 1 (MCT1), integrin-β1 and cyclophilin and this dimerization may be critical for its function (Kirk et al., 2000; Yu et al., 2008). Basigin is the only member of the basigin/embigin/neuroplastin family in *Drosophila* and its structure is highly conserved between humans and *Drosophila* with two extracellular Ig domains, a

transmembrane domain and a short intracellular domain (Fig. 1.10) (Besse et al., 2007; Reed et al., 2004).

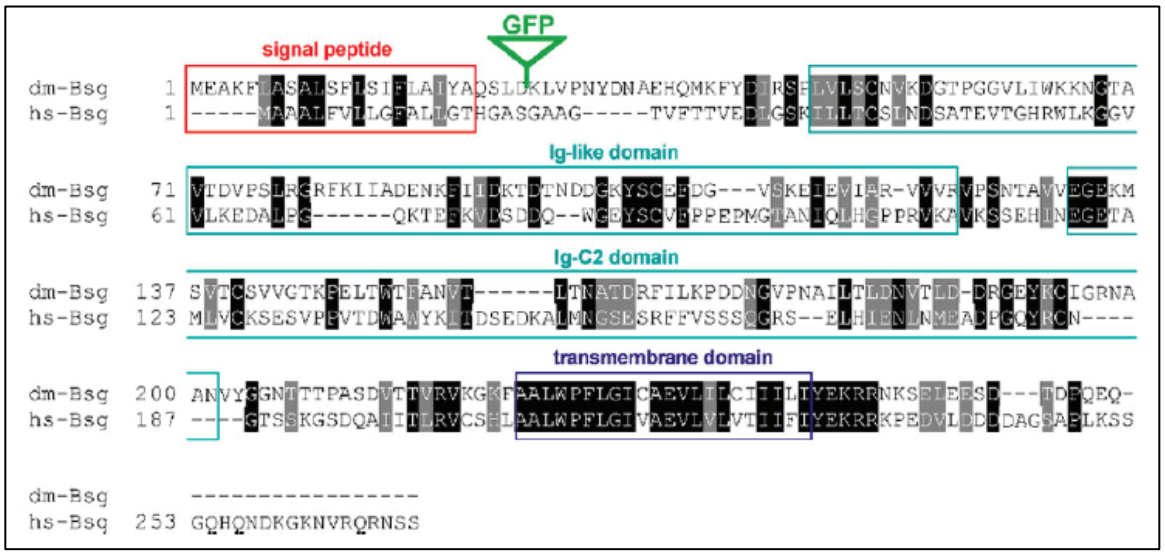


Figure 1.10. Alignment of *Drosophila melanogaster* and human Bsg proteins. The green triangle indicates the location of a GFP tag. IG:immunoglobulin. Note the highly conserved transmembrane domain and amino acids just intracellular of the transmembrane domain. Black bars represent conserved residues and gray bars represent similar residues (Besse et al., 2007).

In *Drosophila*, basigin has been shown to interact with integrin, mediate neuron-glia interactions and affect neuromuscular junction architecture via interactions with the actin cytoskeleton (Besse et al., 2007; Curtin et al., 2005; Curtin et al., 2007; Reed et al., 2004). These studies highlight a role for basigin in the nervous system, but its role in central and peripheral glia has not been investigated in any organism. Studies in vertebrates have focused mainly on the involvement of basigin in inducing matrix metalloproteinases (MMPs) and mediating metastasis in almost every type of cancer and therefore basigin has thus been termed a cancer-related biomarker (Li et al., 2009; Xiong et al., 2014). Due to its significant role in cancer, how basigin contributes to the development and survival of healthy tissues, including glia, is only beginning to be

appreciated. For example, basigin is now known to play a role in vertebrate erythrocytes, cardiovascular systems, nervous systems and T cells of the immune system (Agrawal et al., 2013; Joghetaei et al., 2013; Kanyenda et al., 2014; Seizer et al., 2014; Spring et al., 1997). Finally, due to its ability to induce MMPs, regulate ECM degradation and interact with integrin, basigin has been clearly demonstrated to modulate tissue remodeling by fibroblasts and endothelial cells (Huet et al., 2008). The *Drosophila* nervous system undergoes extensive remodeling during embryogenesis, larval stages and pupation and we hypothesize that basigin may be a significant player throughout this process.

1.4 THESIS QUESTIONS

Originating from previous studies in our lab focusing on the role of integrin in *Drosophila* glia in the PNS, we were interested in proteins that interact with and potentially bind to integrin. One well-studied integrin ligand applicable to glial wrapping in vertebrates is laminin; thus, we decided to investigate its role in *Drosophila* glia in the PNS. We were originally interested in whether laminin knockdown in glia would phenocopy the integrin phenotype. While this was not the case, likely due to laminin contribution to the neural lamella by hemocytes and fat bodies, we instead were able to examine the intracellular effects of individual laminin subunit knockdown in peripheral glia and began investigating the process of laminin secretion. Similarly, we had initially set out to study the role of basigin in glia due to its ability to interact with integrin and to affect the ECM. We were able to examine the relationship between basigin and integrin as well as the phenotype associated with basigin knockdown in glia. Therefore, the general question directing my studies was: What are the morphological and physiological effects of disrupting glial-ECM interactions in *Drosophila* peripheral glia?

2 LAMININ ACCUMULATION DISRUPTS GLIAL MORPHOLOGY AND LARVAL LOCOMOTION

2.1 OVERVIEW

The nervous system is surrounded by an extracellular matrix composed of large glycoproteins including perlecan, collagens and laminins. Glial cells in many organisms secrete laminin, a large heterotrimeric protein consisting of an alpha, beta and gamma subunit. Prior studies have found that loss of laminin subunits from vertebrate Schwann cells results in loss of myelination and neuropathies, results attributed to loss of laminin-receptor signaling. We demonstrate that loss of the laminin gamma subunit (LanB2) in the peripheral glia of *Drosophila melanogaster* results in the disruption of glial morphology due to disruption of laminin secretion. Specifically knockdown of LanB2 in peripheral glia results in accumulation of the beta subunit (LanB1) leading to distended ER, ER stress and glial swelling. The physiological consequences of disrupting laminin secretion in glia included decreased larval locomotion and ultimately, lethality. Loss of the gamma subunit from wrapping glia resulted in a disruption in the glial ensheathment of axons but surprisingly did not affect animal locomotion. We found that Tango1, a protein thought to exclusively mediate collagen secretion, is also important for laminin secretion in glia via a collagen-independent mechanism. However loss of secretion of the laminin trimer does not disrupt animal locomotion, rather it is the loss of one subunit that leads to deleterious consequences through the accumulation of the remaining subunits.

2.2 INTRODUCTION

Basement membranes are composed of large glycoproteins that form an extracellular matrix (ECM) around organs to provide them with structural and functional support. Laminin, a conserved component of basement membranes, is a heterotrimer composed of an alpha, a beta and a gamma subunit. Vertebrates have multiple genes for all three laminin subunits resulting in 18 different laminin isoforms. Mutations in individual laminin subunits can cause hereditary diseases including congenital muscular dystrophy type IA (OMIM #607855) characterized by peripheral dysmyelination and brain defects and Pierson syndrome (OMIN #609049) characterized by renal failure, vision loss and muscular and neurological defects (Durbeej, 2010; Feltri and Wrabetz, 2005). Laminin is essential in peripheral nervous system (PNS) development for proper sorting and myelination of axons by glia known as Schwann cells (Yu et al., 2009a; Yu et al., 2009b). Individually, deficiencies in alpha laminin subunits disrupt radial sorting of axons and impair myelination by Schwann cells while combined deficiencies result in a total lack of radial sorting and myelination (Wallquist et al., 2005; Yang et al., 2005). Furthermore disruption of all laminin isoforms in Schwann cells results in decreased Schwann cell radial sorting, myelination, differentiation and survival (Yu et al., 2005; Yu et al., 2007). Vertebrate research has focused on a lack of laminin-receptor signaling as the underlying cause of dysmyelination and neurological defects (Feltri et al., 2002; Yu et al., 2007). Studies in other tissues have focused on receptor-independent effects of laminin loss. For example, studies done in kidney showed that mutations in beta laminin triggered endoplasmic reticulum (ER) stress potentially due to defective secretion of remaining subunits (Chen et al., 2011; Chen et al., 2013). This led us to the hypothesis that

disruption to laminin secretion may contribute to the dysmyelination and neurological defects associated with laminin mutations.

Laminin trimerization occurs in the endoplasmic reticulum (ER) where the beta and gamma subunits bind together first and requires alpha subunit incorporation to form a functional laminin heterotrimer prior to secretion (Yurchenco et al., 1997). The beta-gamma dimer is retained in the ER and requires alpha subunit incorporation to drive secretion. If either the beta or gamma subunit is missing, the other accumulates intracellularly and the alpha subunit is secreted alone (Yurchenco et al., 1997). This model of laminin secretion was supported by studies in *C. elegans*, which showed intracellular beta laminin accumulation in body wall muscles and intestine when alpha laminin was mutated (Huang et al., 2003; Kao et al., 2006). However, the physiological and cellular effects of accumulated laminin subunits has not been determined nor has the mechanism of laminin secretion.

In *Drosophila*, there are 2 alpha (Wb and LanA), 1 beta (LanB1) and 1 gamma (LanB2) subunits. Studies in embryos found that mutations in LanB1 result in embryonic lethality, absent basement membranes and accumulation of other ECM proteins in mutant tissues (Urbano et al., 2009; Wolfstetter and Holz, 2012). The embryonic lethality of laminin mutations is likely because many tissues secrete laminin during development, including hemocytes and fat bodies, both of which contribute to the ECM surrounding tissues such as the nervous system (Bunt et al., 2010; Pastor-Pareja and Xu, 2011). Muscle cells also secrete laminin and secretion of LanA regulates larval neuromuscular junction (NMJ) growth (Tsai et al., 2012). In this study we investigated the cellular and

physiological consequences of laminin knockdown in peripheral glia and examined if changes to the glia led to disruption in animal locomotion and survival.

We found that glial knockdown of the laminin subunits with a focus on the gamma subunit, LanB2, resulted in glial swelling and accumulation of the remaining beta subunit (LanB1) in the ER. The knockdown of LanB2 in all glia and the perineurial glia led to ER stress, decreased larval locomotion and eventual larval death. Loss of LanB2 in the wrapping glia disrupted axon ensheathment but did not disrupt larval locomotion. Furthermore, we determined that Tango1, a mediator of collagen secretion from the ER (Malhotra and Erlmann, 2011), also mediates laminin secretion via a collagen-independent mechanism. Together, our results provide evidence for receptor-independent mechanisms through which a loss of laminin in glia affects glial structure and function and ultimately, we provide a new perspective on the cause of peripheral neuropathies associated with laminin mutations.

2.3 MATERIALS AND METHODS

2.3.1 Fly strains and genetics

The following fly strains used in this study were obtained from the Bloomington stock center: *repo-GAL4* (Sepp et al., 2001); *nrv2-GAL4* (Sun et al., 1999); UAS-mCD8::GFP (Lee and Luo, 1999); UAS-Dicer2 (Dietzl et al., 2007); UAS-KDEL::GFP; UAS-LanB1$^{EP\text{-}600}$; UAS-hid; UAS-rpr; Viking::GFP (Morin et al., 2001); Df(2L)Exel7032L; UAS-CD8::RFP; UAS-GFP. *46F*-GAL4 (Xie and Auld, 2011) was obtained from the Kyoto stock center. The *xbp1>dsRed* (Ryoo et al., 2013) line was a gift from Dr. Don Ryoo. The RNAi lines were from VDRC: LanA-RNAi (GD6022), LanB1-RNAi (GD13179), LanB2-RNAi (GD2394), Tango1-RNAi (GD956);

Bloomington: LanA-RNAi (JF02908). All TEM, confocal and projection images are of third instar larvae nerves and experiments were carried out at 29°C with UAS-Dicer2. All larval tracking experiments were carried out at 25°C with controls expressing UAS-Dicer2 and UAS-mCD8::GFP in glia. Larvae of either sex were used in all experiments.

2.2.2 Immunolabeling and image analysis

Dissection and fixation for immunofluorescence was performed as described previously (Sepp et al., 2000). For immunolabeling, larvae were fixed in 4% formaldehyde for 15-30 minutes. The following primary antibodies were used: rabbit anti-LanB1 (1:500, Abcam, Cambridge, MA), rabbit anti-LanB2 (1:500, Abcam, Cambridge, MA), rabbit anti-HRP (1:500, Jackson ImmunoResearch, West Grove, PA). Secondary antibodies were goat anti-mouse or goat anti-rabbit conjugated with Alexa 488, Alexa568 or Alexa647 (1:300, Invitrogen, Toronto, Canada).

High magnification fluorescent images were obtained with a DeltaVision (Applied Precision, Mississauga, Canada) using a 60X oil immersion objective (NA 1.4) at 0.2 μm steps. Stacks were deconvolved (SoftWorx, Toronto, Canada) using a measured PSF using 0.2 μm fluorescent beads (Invitrogen, Toronto, Canada) in Vectashield (Vector Laboratories, Burlington, Canada). Low magnification images were taken using a 20X water immersion lens (NA 0.95) on a Leica Confocal. Images were compiled using Photoshop and Illustrator CS4. For transmission electron microscopy (TEM) analysis, larval brains were fixed in 4% formaldehyde and 3% glutaraldehyde, rinsed in 0.1 M PIPES, post-fixed in 1% osmium tetroxide, embedded in 1:1 acetonitrile:Spurr resin and polymerized in Spurr's resin. Thin sections (50 nm) were obtained with a Leica

ultramicrotome and analyzed with a FEI Tecnai TEM operating at an accelerating voltage of 80 kV.

2.3.3 Larval tracking

Larval tracking was performed using an adapted multi-worm tracker and script (Swierczek et al., 2011). Each tracking session included 5-30 larvae placed on 100 mm diameter apple juice plates, tapped to elicit movement, and tracked for 30 seconds. The instantaneous speed of all larvae at 15 seconds was measured and differences analyzed using a one-way ANOVA plus Tukey's post hoc test.

2.3.4 Morphological quantification

Nerve diameters in Figure 6 were measured at the widest point along nerves within 300 μm of exit from the ventral nerve cord and analyzed using a one-way ANOVA and Tukey's post hoc test. In Figure 7, nerves with at least one section in which no wrapping glial processes were observed using *nrv2>GFP* were defined as discontinuous. Nerves with at least one membrane-bound swelling as large as a wrapping glial nucleus were counted as nerves with vacuole-like structures. Unpaired t-tests were used to calculate p-values between controls and experimental averages.

2.4 RESULTS

2.4.1 Knockdown of laminin in glia results in severe morphological defects and death

In order to study the role of glial-derived laminin in PNS nerves, we used glial-specific expression of RNAi to knockdown individual laminin subunits in *Drosophila* glia and examined the resulting glial morphology and function in larval stages. Since *Drosophila* glia express laminin but neither perlecan nor collagen-IV (Xie and Auld,

2011), the study of laminin secretion is not affected by the secretion of other major ECM proteins from this tissue. The PNS of the *Drosophila* third instar larva is composed of three distinct glial layers that surround motor and sensory axons (Fig. 2.1H) (Stork et al., 2008)). The innermost layer, the wrapping glia (Fig. 2. 1H, purple), are similar to non-myelinating Schwann cells in that they directly contact and ensheath axons. The middle layer, the subperineurial glia (Fig. 2.1H, orange), encircle the wrapping glia and form the blood-brain barrier via septate junctions. The outermost layer, the perineurial glia (Fig. 2.1H, green), is surrounded by a basement membrane composed of an extensive ECM, or neural lamella (Fig. 2.1H, red). While the overlying ECM contains perlecan, laminin, and collagen IV, glia do not secrete perlecan or collagen IV (Stork et al., 2008; Xie and Auld, 2011). We tested whether the perineurial glia were contributing laminin to the ECM using GAL4 drivers to express membrane tagged fluorescent markers (mCD8::GFP) in the perineurial glia (*46F-GAL4*) and assayed for the presence of laminin subunits in peripheral nerves. Immunolabeling confirmed the presence of the beta subunit, LanB1, and the gamma subunit, LanB2, in the ECM surrounding the perineurial glia (Fig. 2.1F,G). This is similar to vertebrates where axon fascicles of peripheral nerves are surrounded by a layer of ECM containing collagen, nidogen, perlecan and laminin (Yurchenco, 2011).

To test the role of laminin in peripheral glia we used RNAi transgenes to knock down laminin expression in all glia using *repo-GAL4*. Unfortunately, quantification of laminin deposition in the neural lamella by glia is not possible as hemocytes and fat bodies contribute extensively to the ECM surrounding peripheral nerves (Bunt et al., 2010; Pastor-Pareja and Xu, 2011). To overcome this limitation we used a range of RNAi lines

to knockdown the alpha subunit, LanA, or the LanB1 and LanB2 subunits. We used two LanA-RNAi lines, one of which (GD6022) has been previously used (Tsai et al., 2012) and individual LanB1 and LanB2 lines. All larvae analyzed demonstrated a peripheral nerve phenotype (LanA-RNAi, n=25; LanB1-RNAi, n=30, LanB2-RNAi, n=75). The peripheral nerve phenotypes resulting from knockdown of LanA included wider peripheral nerves characterized by glial swelling and small vacuoles (Fig. 2.1B,C). Knockdown of LanB1 or LanB2 also resulted in peripheral nerve phenotypes in all larvae analyzed that were similar but stronger and consisted of severe glial swelling and large vacuoles in addition to larval death (Fig. 2.1D,E; Table 1.1). None of the RNAi constructs significantly impacted axon morphology at this resolution (Fig. 2.1A"-E"), the apparent difference in size is due to the large swelling and a difference in scale. Swollen areas or vacuoles were never observed in control larvae (*repo-GAL4* with *UAS-Dicer2* and *UAS-mCD8::GFP,* n=170). Similar observations with four different RNAi constructs against three separate laminin subunits suggest that the phenotypes are unlikely due to off-target effects of the RNAi constructs. The most penetrant phenotype was seen with LanB2-RNAi, and we used this line for all subsequent experiments. To further characterize the ultra-structure of the swollen nerves, we analyzed peripheral nerves of control and *repo>LanB2-RNAi* larvae using transmission electron microscopy (TEM)(Fig. 2.1H,I). All LanB2-RNAi larvae (n=8) had peripheral nerve glia with large vacuoles, extensive ER and increased width, phenotypes not observed in any control larvae (n=12). The majority of the swelling was due to the increased size of the perineurial glia, which contained multiple vacuole-like structures (Fig. 2.1I). Note the

scale difference between control (500 nm) and LanB2 knockdown (2 µm) due to the increased diameter and size of the outermost layer of perineurial glia.

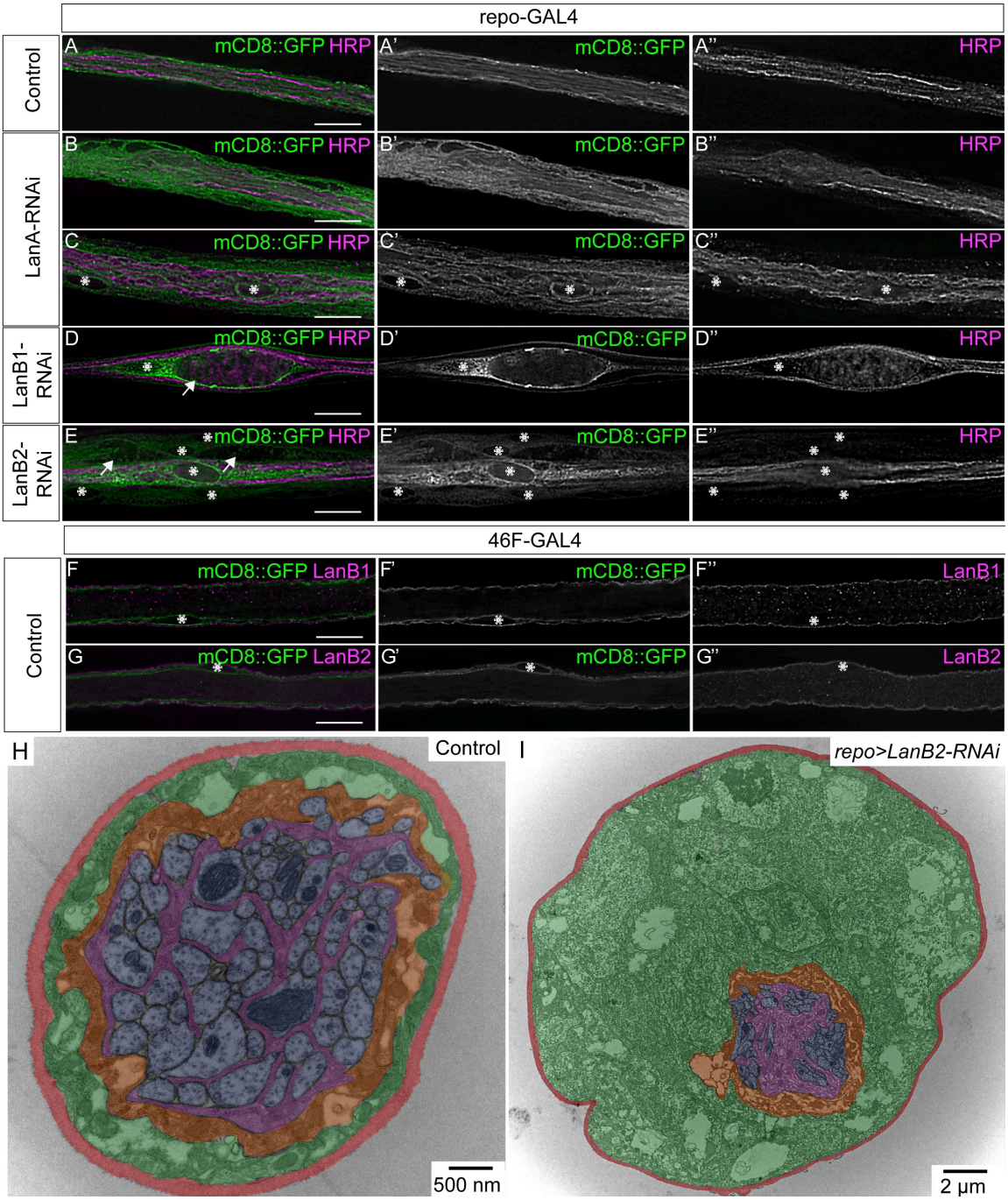


Figure 2.1. Laminin subunit knockdown results in peripheral glial swelling. **(A-E)** Longitudinal sections of control peripheral nerves with glial membranes labeled with mCD8::GFP (green) and axons immunolabeled using anti-HRP antibody (magenta). Asterisks mark nuclei and scale bars are 15 µm. RNAi against LanA (C,D), LanB1 (D), or LanB2 (E) resulted in wider nerves and vacuole-like structures (arrows). **(F,G)** Control

perineurial glial membranes were labeled with mCD8::GFP (green) and the ECM immunolabeled using anti-LanB2 or anti-LanB1 antibody (magenta). Laminin is found predominantly exterior to the perineurial glia. **(H, I)** TEM images of peripheral nerve sections from control (*repo-GAL4*) (H) or *repo>LanB2-RNAi* (I) larvae. False coloured to show ECM (red), perineurial glia (green), subperineurial glia (orange), wrapping glia (magenta) and axons (blue). Control nerves have a thin layer of perineurial glia (H) while LanB2 knockdown generates severely swollen perineurial glia with vacuole-like structures (I). Scale bars are 0.5 μm (H) and 2 μm (I).

We found that expression of LanB2-RNAi using the perineurial glia driver 46F-GAL4 also resulted in glial swelling and vacuoles (Fig. 2.2E,F). The phenotypes were observed in all larvae (n=80) compared to controls where this phenotype was never observed (n=80). LanB1-RNAi was expressed in the perineurial glia using 46F-GAL4 and showed a similar phenotype to *46F>LanB2-RNAi* but with a lower penetrance (Fig. 2.2 H). Though we observed more severe phenotypes using *repo-GAL4* than *46F-GAL4* (Fig. 2.2C,D), the knockdown of LanB2 was lethal using both drivers, with lethality occurring in late 3rd instar larval stages and pupae respectively (Table 1.1). It is possible that glial layers other than the perineurial glia contribute to the more severe phenotypes seen using *repo-GAL4* or that *repo-GAL4* is stronger and expressed earlier. Overall these results demonstrate that glial cells, and specifically perineurial glial cells, express laminin and that glia are severely affected when LanB2 is knocked down.

Table 1.1 Summary of survival and mobility of genotypes

GENOTYPE	MOBILITY	LETHALITY
repo-GAL4	++	Viable
repo>LanB2-RNAi	-	Lethal (3rd instar)
repo>Tango1-RNAi	++	Lethal (pupal)
repo>LanB2-RNAi, Df(LanB1)	-	Lethal (3rd instar)
repo>LanB1	++	Viable
46F-GAL4	++	Viable
46F>LanB2-RNAi	+	Lethal (pupal)
46F>Tango1-RNAi	++	Viable
46F>hid,rpr	-*	Lethal (early larval)
nrv2-GAL4	++	Viable
nrv2>LanB2-RNAi	++	Viable

nrv2>hid,rpr	-	Lethal (pupal)

++ denotes average speed not significantly different than controls, + denotes average speed significantly slower than controls, - denotes average speed less than 1.5 mm/s
* GAL80ts present, temperature shifted to 29^{o}C for 24 hours

2.4.2 LanB2 knockdown results in intracellular accumulation of LanB1

In vitro studies have shown that the absence of either the beta or gamma subunit prevents their dimerization and results in the intracellular accumulation of the unpaired monomeric subunit while the alpha subunit is secreted alone (Yurchenco et al., 1997). We hypothesized that the swollen areas of the glia when LanB2 is knocked down may contain accumulated LanB1 protein. Expression of LanB2-RNAi using either *repo-GAL4* or *46F-GAL4* resulted in large accumulations of LanB1 within the interior of the peripheral glia (Fig. 2.2C,D,F,G). Similarly, expression of LanB1-RNAi with 46F-GAL4 lead to accumulation of LanB2 within peripheral glia (Fig. 2.2H). Accumulations were found in concentrated GFP-labeled deposits (Fig. 2.2D,F) as well as clustered puncta within the cytosol (Fig. 2.2G,H). Accumulations were observed in all LanB2-RNAi larvae (n=35) but never observed in controls (n=35). Rather, controls displayed the normal concentration of LanB1 along the exterior of the nerve within the neural lamella (Fig. 2.2B,E). LanB1 accumulations were observed in glia within both the CNS and PNS and coincided with the swollen regions in the peripheral nerves (Fig. 2.2C).

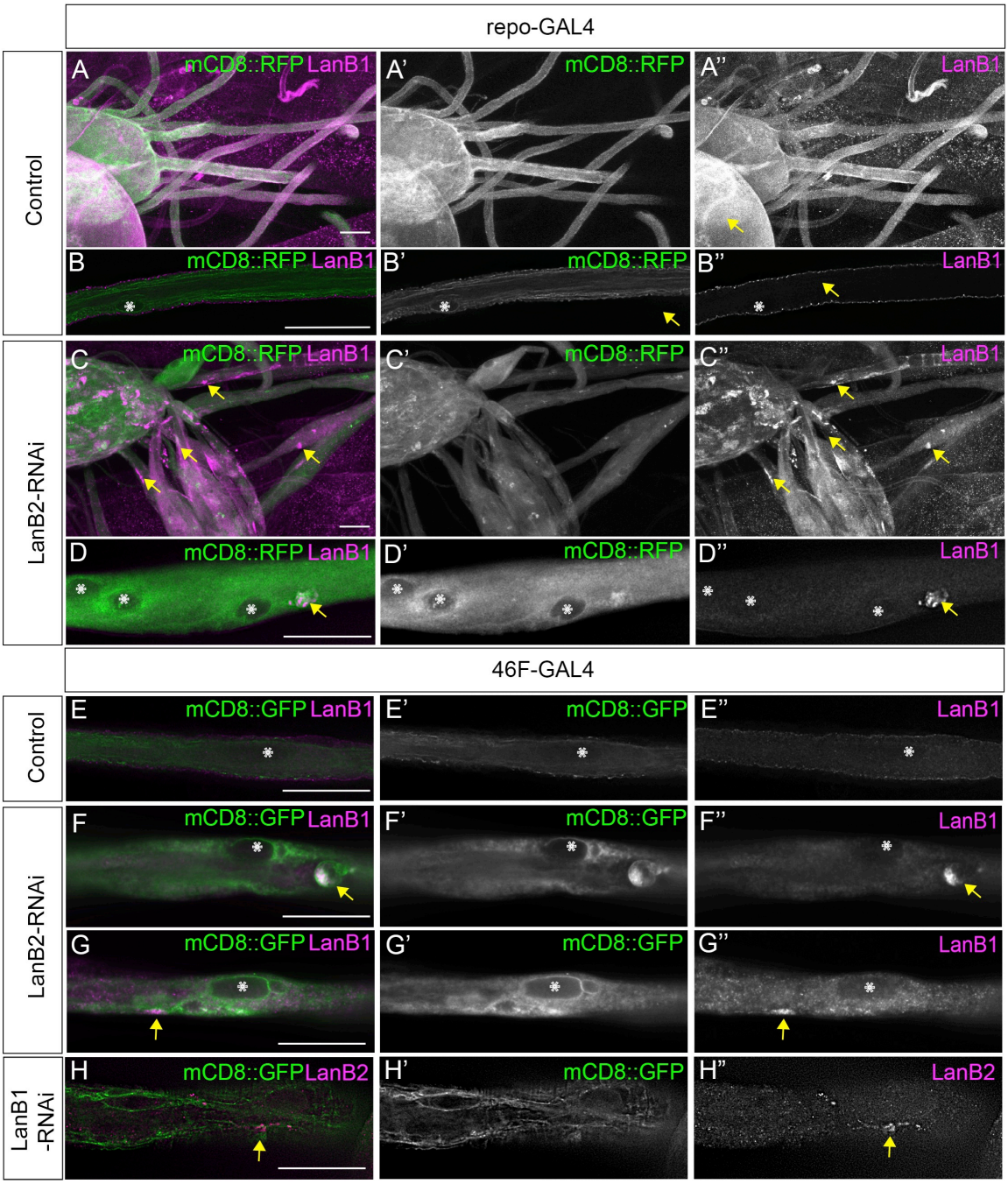


Figure 2.2. LanB2 knockdown results in LanB1 accumulation in glia. (A-D) Peripheral nerves of control (A,B) and *repo>LanB2-RNAi* larvae (C, D). Glia were labeled with mCD8::RFP (green) and immunolabeled for anti-LanB1 (magenta). (A, C) Low magnification images of peripheral nerves and ventral nerve cord. (B, D) Higher magnification of individual peripheral nerves in longitudinal sections. (E-G) Longitudinal sections of peripheral nerves in control (E) and *46F>LanB2-RNAi* larvae (F, G). Glia were labeled with mCD8::GFP (green) and immunolabeled with anti-LanB1 antibody (magenta). Asterisks mark nuclei and arrows mark LanB1 accumulations. Scale bars are 30 μm.

2.4.3 LanB2 knockdown results in increased ER and ER stress

A prediction of the laminin secretion model from *in vitro* studies is that LanB1accumulations are likely to be primarily unbound LanB1 monomers (Yurchenco et al., 1997), which in turn may lead to changes to the endoplasmic reticulum (ER). Our ultrastructural analysis found that knockdown of LanB2 in *Drosophila* glia resulted in areas of increased ribosomes and expanded ER in many nerves (Fig. 2.3 B, B"). To determine whether the accumulated LanB1 was localized to the expanded ER, we co-expressed LanB2-RNAi and a GFP-tagged ER marker, KDEL::GFP, in perineurial glia and then immunolabeled for LanB1. In control larvae (n=15), KDEL::GFP was evenly distributed throughout perineurial glia and LanB1 was found in the ECM (Fig. 2.3 C). After LanB2 knockdown we observed GFP-positive aggregates in the CNS and PNS that co-localized with LanB1 in all mutant larvae (n=10) (Fig. 2.3 D). The presence of KDEL::GFP-positive aggregates within perineurial glia was similar to the areas of expanded ER we observed ultrastructurally. The localization of LanB1 within these aggregates suggested unbound LanB1 accumulated in the ER leading to ER expansion and possibility could lead to ER stress. To test this, we used an ER stress marker where dsRed is placed under the control of the promoter for XBP1 (Ryoo et al., 2013). ER stress, the unfolded protein response and high protein secretory load increases transcription from the XBP1 promoter and an increase in the expression of dsRed. When expressed in all glia using *repo-Gal4*, we found that glial cells exhibit almost no XBP1 transcription under control conditions (Fig. 2.3 E). However upon LanB2 knockdown in glia, XBP1 activation via dsRed expression was visible (Fig. 2.3 F). Our results suggest

that the loss of LanB2 leads to accumulation of LanB1within the ER and results in ER stress.

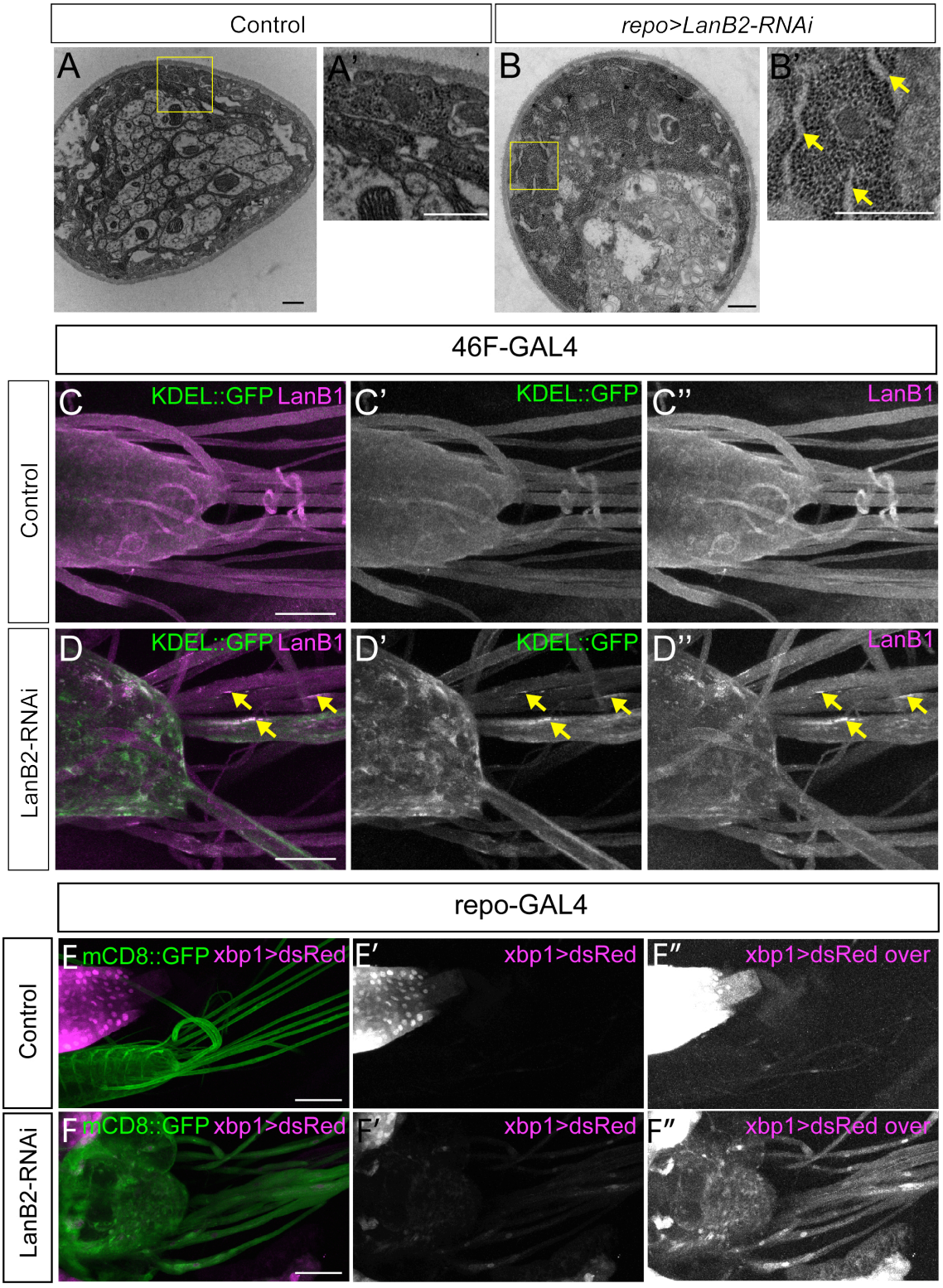
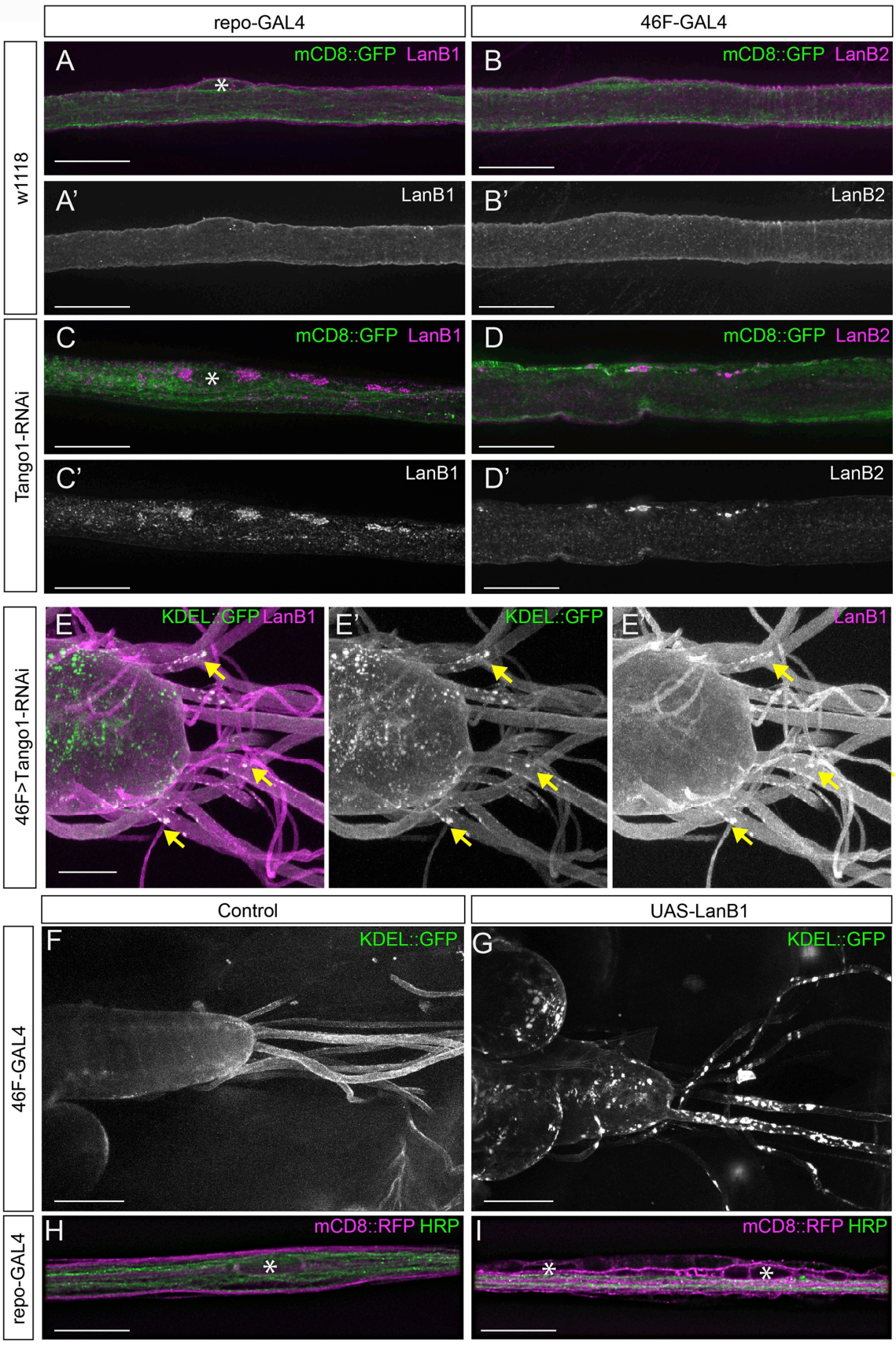


Figure 2.3. Loss of LanB2 leads to increased ER, LanB1 aggregates and ER stress. **(A,B)** TEM images of peripheral nerve section. (A) Control nerves show the normal distribution of ribosomes in perineurial glia. (B) *repo>LanB2-RNAi* generated swollen perineurial glia with increased density of ribosomes and areas of distended ER. Yellow boxes are shown digitally magnified in A' and B' and yellow arrows point to areas of distended ER. Scale bars in all panels are 500 nm. **(C-D)** The ventral nerve cord and peripheral nerves of larvae where the perineurial glia ER was labeled using *46F>KDEL::GFP* (green) and immunolabeled with anti-LanB1 antibody (magenta). (C) Distribution of KDEL::GFP and LanB1 is uniform and diffuse in control nerves. Co-localization of LanB1 accumulations and ER aggregates was observed in *46F>LanB2-RNAi* (D) (yellow arrows). Scale bars are 50 μm. **(E-F)** Ventral nerve cord and peripheral nerves were labeled using *repo>mCD8::GFP* and xbp1 expression was visualized using the *xbp1>dsRed* expression reporter. Control, *repo-GAL4* (E) had weak levels of dsRed in the glia (the bright area is the esophagus) while *repo>LanB2-RNAi* (F) showed increased dsRed expression. The red channel was overexposed equally in E'' and F'' to show differences in xbp1 expression. Scale bars are 100 μm.

We hypothesized that if accumulation of LanB1 results in ER accumulation, then overexpression of LanB1 in perineurial glia using a UAS-LanB1 (an EP insertion in LanB1, *LanB1[EP-600]*, that drives LanB1 expression (de Celis and Molnar, 2010) would result in ER aggregates. Consistently, we found that the expression pattern of KDEL-GFP in *46F>LanB1* larvae demonstrated that the ER was increased and aggregated compared to control larvae (Fig. 2.4 F,G), very similar to the phenotype seen after LanB2 knockdown in perineurial glia. Finally, when LanB1 was overexpressed in all glia using repo-GAL4, glia in all larvae (n=10) demonstrated vacuoles similar to those observed with LanB2 knockdown (Fig. 2.4 H,I).


repo-GAL4
46F-GAL4
w1118
Tango1-RNAi
46F>Tango1-RNAi
Control
UAS-LanB1
46F-GAL4
repo-GAL4
A
mCD8::GFP LanB1
B
mCD8::GFP LanB2
A'
LanB1
B'
LanB2
C
mCD8::GFP LanB1
D
mCD8::GFP LanB2
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E'
KDEL::GFP
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H
mCD8::RFP HRP
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Figure 2.4. Knock down of Tango1 leads to accumulations of LanB1 and LanB2
(A-D) High magnification of control *repo-GAL4* (A) and *46F-GAL4* (B) peripheral nerves with glial membranes labeled with mCD8::GFP (green) and immunolabeled with LanB1 (A, magenta) or LanB2 (B, magenta). *repo>Tango1-RNAi* (C) and *46F>Tango1-RNAi* peripheral nerves demonsrate accumulations of LanB1 and LanB2 when Tango1 is knocked down (arrows). Nuclei are marked by asterisks and scale bars are 20 μm. **(E)** The ventral nerve cord and peripheral nerves of larvae where the perineurial glia driver *46F-GAL4* drove the expression of an ER marker KDEL::GFP (green) and Tango1-RNAi. Co-localization of LanB1 accumulations (magenta) and ER aggregates was observed (yellow arrows). Scale bars are 50 μm. **(F,G)** Low magnification of control CNS and nerves with *46F-GAL4* driving KDEL::GFP (F) and after overexpression of LanB1 using UAS-LanB1$^{EP\text{-}600}$ resulting in ER aggregates. Scale bars are 100 μm. **(H,I)** High magnification of control nerves with glial membrane labeled with mCD8::RFP (magenta) and axons immunolabeled using anti-HRP antibody (green) (H). Nerves with overexpression of LanB1 in glia, *repo>LanB1$^{EP\text{-}600}$*, demonstrated vacuoles (I). Nuclei are marked by asterisks and scale bars are 15 μm.

2.4.4 Tango1 mediates laminin secretion via a collagen-independent mechanism

Most proteins are secreted from the ER to Golgi via small COPII vesicles (50-60nm), however ECM components such as collagen and laminin are too large to be secreted in these vesicles. While the mechanism of laminin secretion remains unknown, collagen secretion has been investigated and a transmembrane ER protein, Tango1, has been found to mediate secretion of many types of collagen (Wilson et al., 2011). By binding to collagen and simultaneously delaying COPII vesicle budding from ER exit sites, Tango1 accomodates the budding of larger COPII vesicles containing collagen from the ER (Malhotra and Erlmann, 2011; Saito et al., 2009). We hypothesized that an analogous mechanism might be used by glia to secrete laminin. Using a RNAi known to specifically target Tango1 (Lerner et al., 2013), we found that expression of Tango1-RNAi in all glia, and perineurial glia specifically, resulted in intracellular LanB2 and LanB1 accumulations (Fig. 2.4 A-D). The LanB1 accumulations co-localized with GFP-labeled ER aggregates identified with KDEL::GFP (Fig. 2.4 E). We screened other members of the Tango family (Tango6, Tango7, Tango5, Tango9, and Tango14) (Bard et

al., 2006) by expressing RNAi constructs against each in glia (data not shown) and found that only Tango1-RNAi affected laminin secretion. Previous models of Tango1 function suggest that laminin accumulates passively in COPII vesicles by following Tango-1 mediated secretion of collagen (Lerner et al., 2013). In Drosophila, collagen-IV is not observed within the peripheral glia (Fig. 2.5 A,B) (Xie and Auld, 2011), and is deposited by circulating hemocytes and fat bodies (Bunt et al., 2010; Pastor-Pareja and Xu, 2011). We examined the effect of Tango1 knockdown on viking::GFP, the *Drosophila* homologue of Collagen IV endogenously lagged with GFP (Morin et al., 2001). Tango1-RNAi expressed in perineurial glia affected laminin secretion with accumulation of LanB2 within the glial cell but had no impact on the external localization of viking::GFP in the PNS nor did we observe any accumulations of viking::GFP within the cytosol of the peripheral glia (Fig. 2.5 C,D). Our results confirm that viking::GFP is not secreted by glia and suggests that Tango1 is capable of mediating laminin secretion independent of collagen. The results also suggest two possibilities: that Tango1 and laminin may bind directly to trigger large vesicle formation, or that Tango1 may have a more ubiquitous role in large vesicle formation than previously thought.

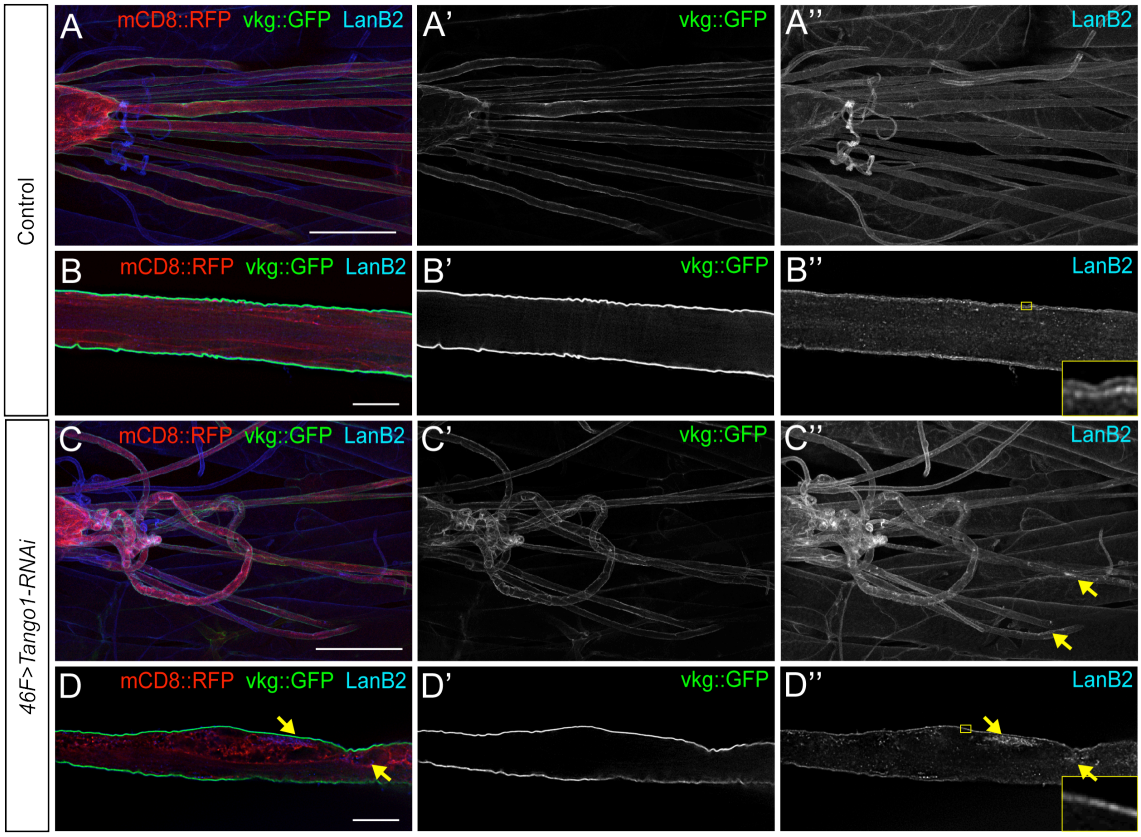

Figure 2.5. Tango1 mediates laminin secretion via a collagen-independent mechanism. (A) Low magnification of control ventral nerve cord and peripheral nerves in larvae with *46F-GAL4* driving mCD8::RFP in perineurial glia (red), collagen-IV endogenously tagged with GFP, Viking::GFP (green), and immunolabeled for LanB2 (blue). Scale bar is 100 μm. **(B)** High magnification of nerve section in A. Scale bar is 15 μm. Digitally magnified section of ECM in inset shows two distinct layers of LanB2. Viking::GFP is not observed in the interior of the nerve. **(C)** Low magnification of ventral nerve cord and peripheral nerves in *46F>Tango1-RNAi* larvae with the perineurial glia membrane labeled with mCD8::RFP (red), Viking::GFP (green) and immunolabeled for LanB2 (blue). Loss of Tango1 lead to accumulations of LanB2 (yellow arrows) but not Viking::GFP. Scale bar is 100 μm. **(D)** High magnification of a nerve in C. Scale bar is 15 μm. Digitally magnified section of ECM in inset shows one layer of LanB2. Viking::GFP was not observed in the interior of the nerve while LanB2 was observed in intracellular accumulations (yellow arrows).

Overall we observed that knockdown of Tango1 had differential effects on peripheral nerves compared to LanB2-RNAi. Tango1-RNAi was lethal only using *repo-GAL4* (not with *46F-GAL4*) and did not result in glial swelling using either GAL4 driver (Table 1.1; Fig. 2.6 E). Knockdown of LanB2 had a more substantial effect on glial structure than

knockdown of Tango1, possibly because the Tango1-RNAi transgene may be less effective. Alternatively it is likely that laminin subunits are able to successfully form the laminin heterotrimer in the absence of Tango1, but not LanB2, suggesting that it is the unbound LanB1 in glia expressing LanB2-RNAi that resulted in the more severe consequences.

2.4.5 Reduction of LanB1 rescues swelling due to LanB2 knockdown

Although knockdown of both LanB2 and Tango1 result in accumulation of LanB1 intracellularly, our results and previous studies suggest that knockdown of LanB2 resulted in the retention of unbound LanB1 monomers while knockdown of Tango1 resulted in the retention of the entire laminin heterotrimer (Lerner et al., 2013; Yurchenco et al., 1997). We noted that knockdown of LanB2, but not Tango1, caused in glial swelling suggesting that unbound LanB1 within the ER may be more deleterious than accumulation of laminin heterotrimers (Fig. 2.6 A,B,D). To quantify this difference, the diameters of peripheral nerves were measured at the widest point within 300 μm of the ventral nerve cord (Fig. 2.6 E). The maximum nerve diameters in control larvae (*repo>mCD8::RFP*) measured on average 10.42 μm (n=72). Expression of Tango1-RNAi did not result in significantly swollen nerves, with an average diameter of 11.73 μm (n=74). In contrast, expression of LanB2-RNAi resulted in nerves that were significantly wider than controls and had an average maximum width of 20.23 μm (n=44). To further distinguish if this swelling was due to an intracellular accumulation of unpaired LanB1 monomers, we compared widths of *repo>LanB2-RNAi* nerves in a hetero-deficient LanB1 background (*Df(2L)Exel7032L/+*) to widths of *repo>LanB2-RNAi* expression alone. We found that reducing the gene dose of LanB1 by half resulted

in significantly less swollen nerves with an average width of 16.13 μm (n=45). Together with the lack of swelling due to Tango1-RNAi, our findings suggest that external depletion of glial-derived laminin heterotrimers and subsequent reduced laminin signaling does not initiate glial swelling. Rather the reduced swelling seen after the simultaneous reduction of LanB1 and LanB2 suggests that the swelling is due to the accumulation of unbound intracellular LanB1.

2.4.6 LanB2, but not Tango1, knockdown in glia disrupted larval movement

The lethality associated with LanB2 knockdown implies that the unbound LanB1 monomers accumulating in glia are not only affecting glial morphology, but also glial function. To determine if the morphological defects caused by LanB2 knockdown in glia were associated with significant physiological defects, we assayed larval locomotion. The speeds of 3rd instar larvae over a period of 15 seconds were recorded using an adapted "multi worm tracker" system (Swierczek et al., 2011). This system records and quantifies the movements of multiple larvae simultaneously over a defined period of time. Control larvae (*repo-GAL4*) moved at an average speed of 3.69 mm/s (Fig. 2.6 F).

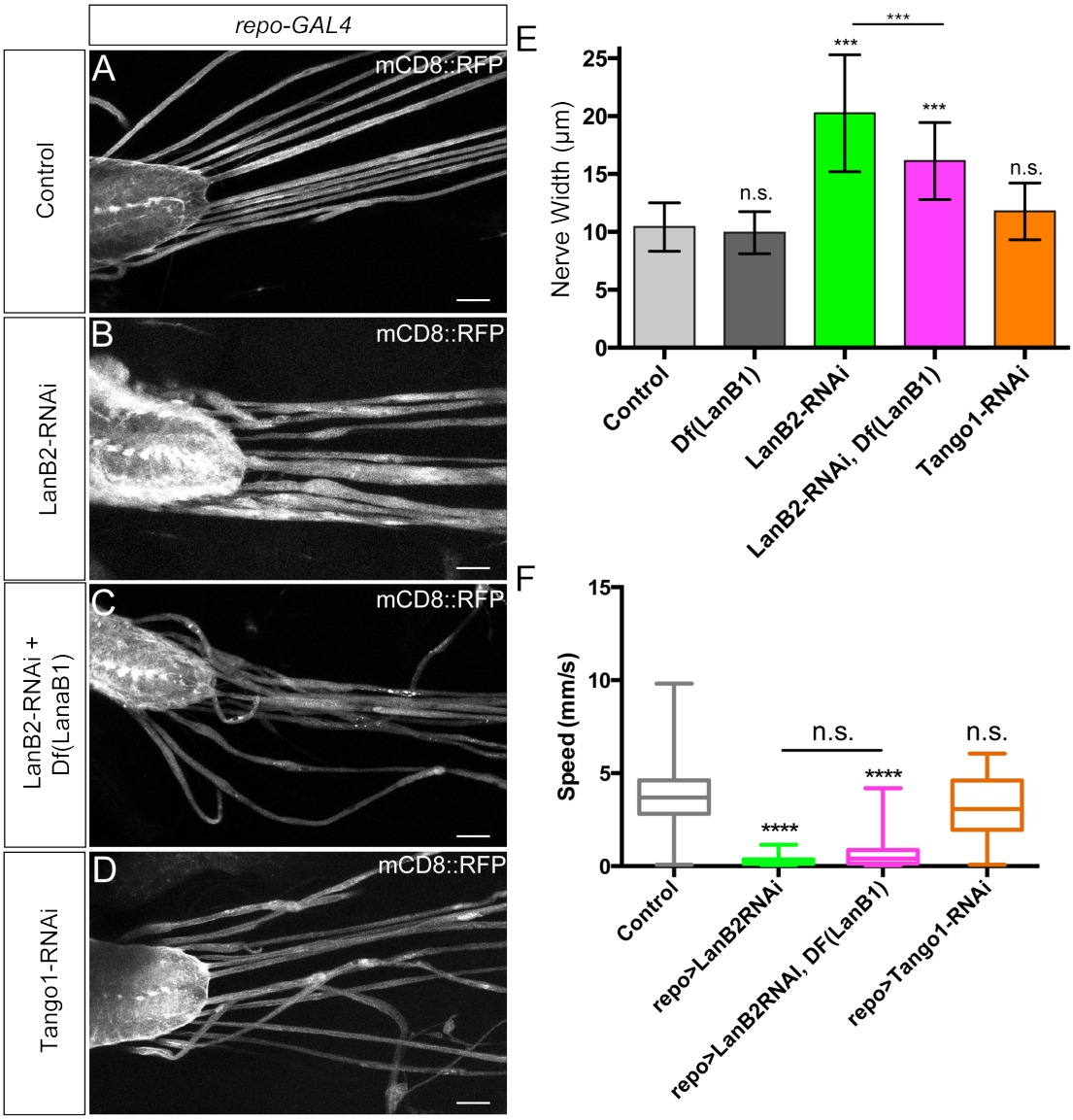

Figure 2.6. LanB2 but not Tango1 knockdown generates glial swelling and locomotion defects. (A-D) The ventral nerve cord and peripheral nerves with glial membranes labeled with mCD8::RFP. Control *repo-GAL4* (A), *repo>LanB2-RNAi* (B), *repo>LanB2-RNAi* heterozygous for a LanB1 deficiency (C), and *repo>Tango1-RNAi* (D). Scale bars are 50 µm. **(E)** The mean width of the widest section of peripheral nerves in control, *repo-GAL4*, versus *repo>RNAi* lines shown in A-D. Mean widths were: controls (10.42 ± 2.09 µm, n=72), Df(LanB1)/+ heterozygotes (9.94 ± 1.82 µm, n=42, p=0.923), *repo>LanB2-RNAi* (20.23 ± 5.04 µm, n=44, p<0.0001), *repo>LanB2-RNAi, Df(LanB1)/+* (16.13 ± 3.33 µm, n=45, p<0.0001), *repo>Tango1-RNAi* (11.73 ± 2.45 µm, n=74, p=0.054). Differences in mean width was analyzed using a one-way ANOVA and Tukey's post hoc test and significance compared to control shown for each experimental (N.S. – not significant, *** p<0.001). Means widths between *repo>LanB2-RNAi* and *repo>LanB2-RNAi, Df(LanB1)/+* were significantly different (p<0.001). **(F)** Average instantaneous larval speed of control (*repo-GAL4*) versus *repo*>RNAi lines. For each

box and whisker plot the box represents the 1st and 3rd percentiles, the middle line the median and the whiskers are the minimum and maximum. Larval speeds: control (3.69 ± 1.73 mm/s, n=118), *repo>LanB2-RNAi* (0.32 ± 0.26 mm/s, n=35), *repo>LanB2-RNAi,Df(LanB1)/+* (0.74 ± 0.96 mm/s, n=56), *repo>Tango1-RNAi* (3.16 ± 1.55 mm/s, n=34), repo>LanB1 (3.44 ± 1.33 mm/s, n=43). Knockdown of LanB2 alone ($p<0.0001$) and in Df(LanB1)/+ heterozygotes ($p<0.0001$) resulted in larvae that were significantly slower than controls while knockdown of Tango1 ($p=0.2117$) or overexpression of LanB1 ($p=0.996$) did not. The difference between LanB2-RNAi alone and in a Df(LanB1)/+ heterozygous background was not significant ($p=0.518$).

Using this system, we found that *repo>LanB2-RNAi* larvae were almost immobile with an average speed of 0.32 mm/s. Prior to tracking, all larvae were confirmed to be alive, exhibited feeding behavior and responded to touch by rolling or bending. However, *repo>Tango1-RNAi* larvae were not significantly slower than controls and had an average speed of 3.16 mm/s (Fig. 2.6 F). We also recorded the instantaneous speed of *repo>LanB2-RNAi* larvae in a *LanB1* hetero-deficient background. While these larvae had an improved average speed of 0.74 mm/s, this was not significantly different from *repo>LanB2-RNAi* larvae (Fig. 2.6 F) and both genotypes were lethal in late third instar stages (Table 1.1). Although reducing the gene dose of LanB1 by half significantly reduced the degree of glial swelling, it was not sufficient to significantly rescue larval locomotion, and glial function is likely still disrupted even with a reduction in LanB1. This may suggest that reducing LanB2 and LanB1 levels in glia can have detrimental effects on other organelles than solely the ER.

2.4.7 Effects of LanB2 knockdown on perineurial and wrapping glia.

As with *repo-GAL4* driven knockdown of LanB2, knockdown of LanB2 in the perineurial glia using *46F-GAL4* also results in lethality. While the function of the perineurial glia is not known, expression of two initiators of apoptosis, *hid* and *rpr*, under UAS/GAL4 control with *46F-GAL4* resulted in early larval lethality (Table 1.1). We

utilized a temperature sensitive GAL80 to control expression and when shifted to 29^{o}C for 24 hours, *46F>hid,rpr* larvae survived to third instar but were completely immobile. This finding suggests that perineurial glia are critical for nervous system function and the effects of LanB2 knockdown in this sub-type of glia was tested using the "multi-worm tracker" system. The average speed of *46F>LanB2-RNAi* larvae was 2.60 mm/s, which is significantly lower than the average speed of controls (*46F-GAL4*), 3.73 mm/s (Fig. 2.7 A). Although significantly slower than controls, *46F>LanB2-RNAi* larvae were still mobile, unlike *repo>LanB2-RNAi* larvae. This is likely due to LanB2-RNAi expression in all glia as well as the earlier expression of *repo-GAL4* beginning in embryogenesis, whereas *46F-GAL4* is expressed in perineurial glia, which arise in the last embryonic stages (von Hilchen et al., 2013). It may be that laminin is expressed by other glia and loss of LanB2 in these other layers contributes to the immobility seen in *repo>LanB2-RNAi* larvae. Wrapping glia directly contact and ensheath axons making them an ideal candidate for affecting signal transmission and larval movement. Ultrastructural analysis of *repo>LanB2-RNAi* nerves using TEM revealed that the wrapping glia had severe morphological defects including vacuole-like structures and a lack of processes extending around axons (Fig. 2.7 C,F). To determine whether this was cell-autonomous or an indirect effect on wrapping glia, we expressed LanB2-RNAi using the wrapping glia driver, *nrv2-GAL4*, and analyzed wrapping glial morphology by co-expression of cytosolic GFP. We observed vacuole-like structures near the wrapping glia nuclei and a lack of processes extending between nuclei (compare Fig. 2.7 D,E and Fig. 2.7 G,H), however accumulations of LanB1 within wrapping glia could not be detected (data not shown). Normally wrapping glia extend processes to wrap around and along each axon

with processes from neighbouring wrapping glia overlapping to form a continuous glial cover along the length of the nerve (Fig. 2.7 C, E, J). This extensive network of wrapping glial processes was reduced when LanB2 was knocked down in the wrapping glia. For quantification, disruption was defined by measuring the processes from different wrapping glia that did not overlap resulting in gaps in the glial coverage or discontinuous processes along the length of the axon (Fig. 2.7 F,H,J). The morphological defects observed with LanB2-RNAi were quantified by comparing the percentage of nerves in which vacuole-like structures were observed and the percentage of nerves in which wrapping glial processes were discontinuous (Fig. 2.7 I). We observed a significant increase in both vacuole-like structures and breaks or discontinuous wrapping glial processes compared to controls (*nrv2-GAL4*). These results clearly demonstrate that LanB2 knockdown specifically in wrapping glia has a cell-autonomous effect on glial morphology. To determine if the morphological defects were physiologically significant, the average speeds of control (*nrv2-GAL4*) and *nrv2>LanB2-RNAi* larvae were measured using the "multi-worm tracker" system. Surprisingly, we found that there was no significant difference in speed between control and *nrv2>LanB2-RNAi* larvae (Fig. 2.7 B), however we cannot exclude a more subtle effect on locomotion. Furthermore, we observed that knockdown of LanB2-RNAi in wrapping glia resulted in viable adults (Table 1.1). To verify that wrapping glia were indeed important for locomotion and larval survival, we expressed the initiators of apoptosis, *hid* and *rpr,* using *nrv2-GAL4* and found that the larvae were significantly slower (Fig. 2.7 B) and died during pupal stages (Table 1.1). These results suggest that either the loss of *nrv2-GAL4* expressing cells at sites other than the PNS are critical or that the wrapping glia maintain an essential

function even with the LanB2-RNAi induced morphological defects. Overall these results suggest that loss of LanB2 leads to a disruption in wrapping glia morphology, including loss of the glial wrap and gaps between neighbouring wrapping glial cells but that these defects do not disrupt peripheral nerve function.

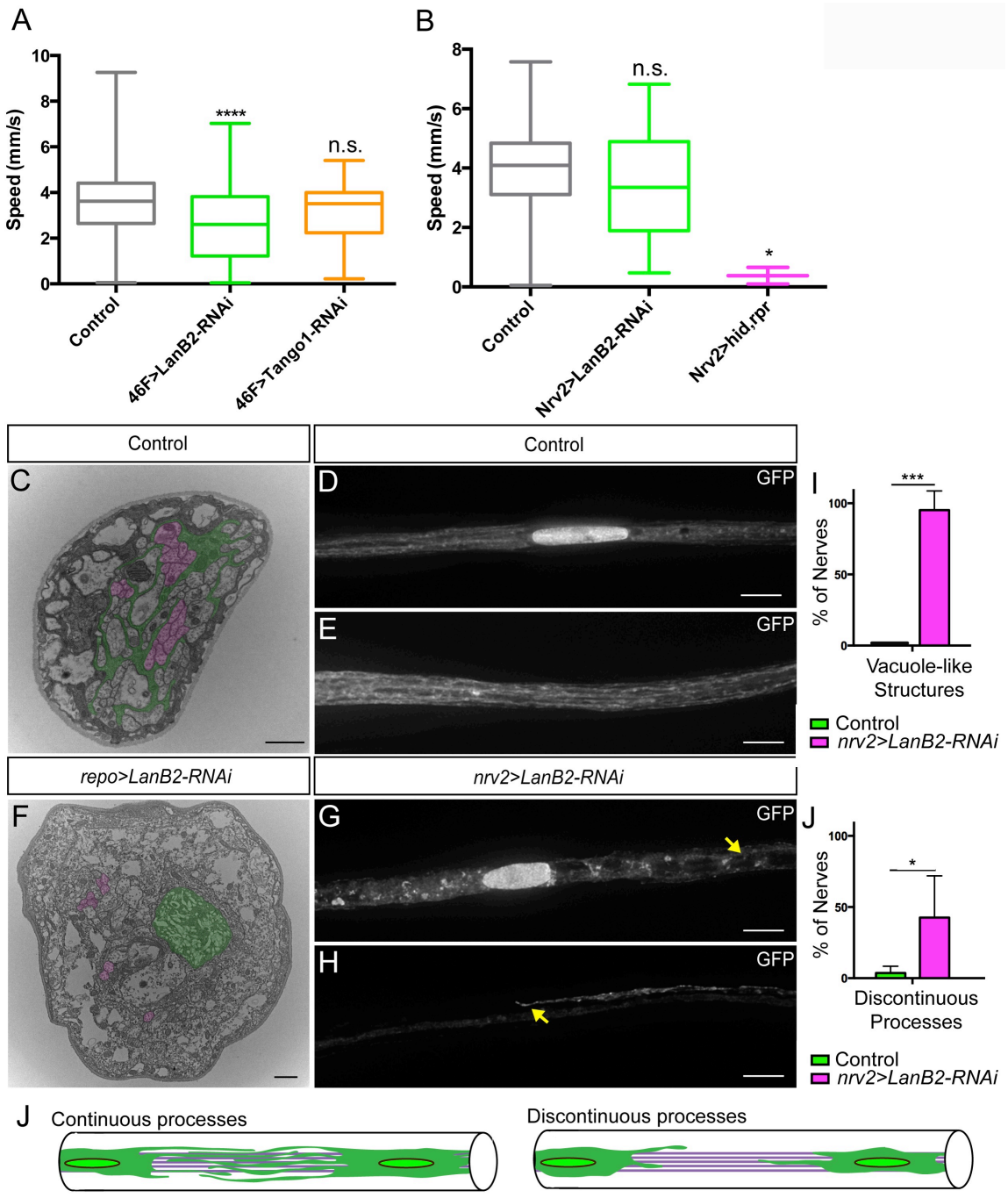

Figure 2.7. Effects of LanB2 knockdown on perineurial and wrapping glia.

(A) Average instantaneous larval speed of control (*46F-GAL4,* grey) and *46F*>RNAi lines. Larval speeds were: control (3.73 ± 1.79 mm/s, n=116), *46F>LanB2-RNAi* (2.60 ± 1.65 mm/s, n=58), *46F>Tango1-RNAi* (3.13 ± 1.40 mm/s, n=22). Knockdown of Tango1 (p=0.281) did not significantly affect speed. Knockdown of LanB2 resulted in significantly slower larvae (p=0.0002). For each box and whisker plot the box represents the 1st and 3rd percentiles, the middle line the median and the whiskers are the minimum and maximum.
(B) Average instantaneous larval speed of control (*nrv2-GAL4)*, *nrv2>LanB2-RNAi* and *nrv2>hid,rpr* lines. There was no significant difference between controls (3.87 ± 1.64 mm/s, n=74) and *nrv2>LanB2-RNAi* (3.42 ± 1.78 mm/s, n=50, p=0.310). There was a significant difference between controls and *nrv2>hid,rpr* (0.38 ± 0.40 mm/s, n=2, p=0.013).
(C,F) TEM of peripheral nerve sections from control *repo-GAL4* and *repo>LanB2-RNAi* nerves. Wrapping glia were false coloured green and a selection of axons were false coloured magenta. Control wrapping glia extended processes around axons (C) while wrapping glia in *repo>LanB2-RNAi* nerves did not (F). Scale bars are 1 μm.
(D,E) Projection images of control peripheral nerves with *nrv2-GAL4* driving GFP expression in wrapping glia near nuclei (D) and between nuclei (E) demonstrate the extensive wrapping glial processes within the core of the nerve. Scale bars are 15 μm.
(G,H) Projection images of peripheral nerves in *nrv2>LanB2-RNAi* larvae, near a nucleus (G) and between nuclei (H) demonstrate vacuole-like structures (arrow, G) and discontinuous processes (arrow, H) that illustrate the failure of neighbouring wrapping glia to connect and ensheath the peripheral axons. Scale bars are 15 μm.
(I) Quantification of the wrapping glial morphological defects in control *nrv2-GAL4* versus *nrv2>LanB2-RNAi* nerves. Control nerves (n=55) had significantly less vacuole-like structures compared to *nrv2>LanB2-RNAi* nerves (n=47)($p<0.001$). Controls nerves had no evidence of discontinuous processes (n=47) while a significantly higher percentage of *nrv2>LanB2-RNAi* nerves did (n=21)($p<0.001$). Error bars are standard deviation.
(J) Diagram depicting continuous processes in control nerves seen by overlapping glial processes (green) between two wrapping glial nuclei (green circle) covering axons (grey). Discontinuous processes are defined as glial processes that do not over lap between two wrapping glial nuclei.

2.5 DISCUSSION

The role of laminin and the ECM in glial wrapping has been investigated in vertebrate systems by examining the gross morphological and molecular changes induced by knocking out individual laminin subunits in glia. A decrease in glial wrapping, proliferation and survival has been attributed to a lack of laminin binding to various receptors including integrins, dystroglycan and syndecans, however, it was found that

knockout of laminin resulted in more severe phenotypes than the additive phenotypes of multiple receptor knockout studies (Yu et al., 2005). In this paper, we present evidence that eliminating one laminin subunit causes cellular changes that go beyond the loss of receptor-ligand signaling and contribute to glial mutant phenotypes.

We have demonstrated that glial cells expressing RNAi against three different laminin subunits result in morphological defects. Knockdown of the laminin gamma subunit, LanB2, results in accumulated LanB1 in the ER, distended ER leading to ER stress with disrupted intracellular morphology including vacuole-like structures. In laminin assembly, the beta-gamma dimer is formed followe by alpha chain recruitment where trimer formation is necessary for transport through the Golgi and secretion (Morita et al., 1985; Peters et al., 1985). Studies *in vitro* and in *C. elegans* found intracellular accumulation of the gamma laminin subunit in the absence of the beta subunit (Huang et al., 2003; Kao et al., 2006; Yurchenco et al., 1997). However the subcellular localization and physiological consequences of the intracellular laminin accumulations was not determined. The results of the ultrastructural and fluorescent analysis of knock down of the gamma subunit (LanB2) in all glia (*repo>LanB2-RNAi)* extend this line of study and found that glia had beta subunit (LanB1) accumulations, ER expansion and higher levels of overall ER stress. We found that glial swelling due to LanB2 knockdown was correlated with excess LanB1 in the ER as we could partially rescue the swollen glial phenotype by introducing a heterozygous LanB1 deficiency in the background which would likely halve the amount of LanB1 available for translation. The partial rescue in glial swelling due to LanB1 hetero-deficiency supports the hypothesis that unbound LanB1 is acting as a misfolded protein in the ER to induce the unfolded protein response

and ER stress. In further support we found overexpression of LanB1 alone resulted in ER aggregates and vacuoles in glia. Knockdown of Tango1, a protein known to mediate ER exit of collagen (Malhotra and Erlmann, 2011), also resulted in LanB1 accumulations in the ER. Previously, Tango1 knockdown was shown to result in laminin retention in *Drosophila* ovary follicle cells, however the subcellular localization of laminin was not demonstrated (Lerner et al., 2013). Our results refine these observations as both LanB2 and Tango1 knockdown result in LanB1 accumulations specifically in the expanded ER. However, while LanB2-RNAi lead to severe glial swelling and locomotion defects, expression of Tango1-RNAi did not. In the case of Tango1-RNAi, this is possibly due to Tango1 knockdown inhibiting secretion of assembled laminin heterotrimers not inhibition of binding between laminin subunits. Together, our findings suggest that the structural and functional glial defects observed after LanB2 knockdown are due to excess amounts of unbound LanB1 monomers in the ER causing ER stress. Alternatively, the increased LanB1 and ribosomal content in these areas may indicate an elevated level of translation potentially due to glia sensing inadequate amounts of external laminin or a dominant negative effect of laminin A subunit alone in the ECM.

Previously, Tango1 has been implicated primarily in collagen secretion (Malhotra and Erlmann, 2011). We also show that Tango1 is capable of mediating laminin secretion in the absence of collagen IV in perineurial glia. Our findings imply that there may be a Tango1-specific binding site on laminin or that Tango1 may play a broader role in protein secretion than previously thought. Together, our findings suggest that the glial defects observed after LanB2 knockdown are due to excess amounts of unbound LanB1 monomers in the ER causing ER stress. Alternatively, the increased LanB1 and

ribosomal content in these areas may indicate increased translation due to glia sensing inadequate amounts of external laminin.

The glial defects we observed are likely due to disruption of laminin secretion but it is possible that the defects we observed were in part due to loss of laminin from the extracellular space and loss of ECM receptor signaling such as integrin signaling. We favour the first explanation as blocking the secretion of the laminin trimer by Tango1-RNAi did not result in glial swelling suggesting that the reduction in laminin secretion per say did not lead to glia disruption. Loss of integrins and MMP-mediated degradation of the surrounding ECM does not lead to glial swelling or formation of vacuoles rather triggers a loss of perineurial glia wrapping of the axon (Xie and Auld, 2011). In *Drosophila*, other cell types contribute to the deposition of ECM proteins in the basement membrane surrounding the nervous system, including the fat bodies and hemocytes (Bunt et al., 2010; Pastor-Pareja and Xu, 2011) explaining why the loss of laminin secretion from glia in Tango-1 does not lead to the same phenotypes as integrin knockdown.

This raises the interesting question of why *Drosophila* glia secrete laminin at all and why not other ECM proteins? Similarly, why do *Drosophila* wrapping glia, which do not contact a basement membrane, seem to express laminin? Our experimental results determined that wrapping glia were morphologically affected by LanB2 knockdown, however accumulations of LanB1 within wrapping glia were not seen, implying that wrapping glia express low levels of laminin or that there may be amounts of laminin between axons and wrapping glia or between wrapping and subperineurial glia undetectable by current antibodies. No laminin antibodies at this time are sensitive enough to detect a small amount of laminin within peripheral nerves. The presence of

laminin interior to the nerve is supported by previous experiments which determined that integrin knockdown in wrapping glia resulted in a lack of process extension (Xie and Auld, 2011), similar to that seen after LanB2 knockdown, suggesting that integrin may bind to laminin present between axons and wrapping glia or between wrapping and subperineurial glia. We found that although LanB2 knockdown in wrapping glia resulted in morphological defects including decreased wrapping, third instar larvae exhibited no mobility defects and even survived to adulthood and this is also true of integrin knockdown in the wrapping glia (data not shown). The surprising finding that full contact between wrapping glia and peripheral axons is unnecessary for locomotion is consistent with mutants in the Na-K-Cl co-transporter, Ncc69. *Ncc69* mutant larvae have significant osmotic swelling that inhibits direct contact between axons and the wrapping glia and yet these larvae had normal action potential activity and survived to adult stages (Leiserson et al., 2011). Apoptosis-induced death of the wrapping glia did however result in a total lack of mobility and lethality indicating that either the wrapping glia or other *nrv2-GAL4* expressing cells are essential to survival and locomotion. Our findings suggest that partial contact between wrapping glia and axons or between the wrapping glia and the overlying subperineurial glia maybe sufficient for proper locomotion and survival.

Larval survival and mobility were also affected by knockdown of LanB2 in perineurial glia, a glial subtype that does not directly contact the axons. Perineurial glia are born late in embryogenesis, divide and surround the CNS and PNS during larval stages (von Hilchen et al., 2013), and have been implicated in ECM remodeling during nervous system growth (Meyer et al., 2014; Stork et al., 2008). Here, we demonstrate that

perineurial glia secrete laminin, but not collagen IV, and contribute to larval mobility. The decreased mobility seen in *46F>LanB2-RNAi* larvae provides support for the role of perineurial glia and the ECM in structurally supporting the larval nervous system as these glia do not contact either neuronal cell bodies or axons.

Ultimately, our findings support an expansion to the view that loss of receptor-ligand signaling is the primary cause of glial morphological and physiological defects due to loss of laminins. We found that LanB2 knockdown resulted in unbound LanB1 monomers, glial ER expansion, mobility defects and decreased glial wrapping. Due to the conserved structure and function of glia across the animal kingdom, it is likely that Schwann cells lacking one subunit of laminin or with a mutation in one subunit affecting laminin trimerization could also demonstrate morphological and functional defects due to the accumulation of unbound laminin subunits and ER stress. This could have significant implications as ER stress is important in the pathogenesis of various diseases affecting myelination in the CNS and PNS (Lin and Popko, 2009). This hypothesis has not yet been investigated in vertebrate glia, but it may provide insight into the reasons decreased glial wrapping, proliferation and survival are seen in vertebrates after knock out or with inherited mutations in laminin subunits. Schwann cell-specific deletion of *Lamc1* (the gamma-1 subunit) leads to a loss of alpha and beta subunit expression and disruption of basement membrane secretion (Yang et al., 2005; Yu et al., 2005). Loss of gamma-1 leads to defects in radial sorting and myelination by myelinating Schwann cells as well as loss of proliferation and blocked differentiation in the premyelination stage (Chen and Strickland, 2003; Wallquist et al., 2005; Yang et al., 2005; Yu et al., 2005). Similarly loss of gamma-1 leads to the complete absence of non-myelinating Schwann cells and the loss

of Remak bundles (Yu et al., 2009b). The wrapping glia of *Drosophila* are most similar to non-myelinating Schwann cells and in these cells loss of the gamma subunit lead to wrapping defects and ER stress. Whether the loss of the vertebrate gamma-1 subunit leads to ER stress to result in the loss of non-myelinating Schwann cells has not been determined. However our findings may represent a broader trend in the cellular response to unequal expression of related subunits that together comprise a multimeric protein such as laminin. Future studies should focus on the effects of ER stress due to mutations in individual subunits of laminin in Schwann cells and other tissues.

3 BASIGIN REGULATES ACTIN AND NERVOUS SYSTEM MORPHOLOGY

3.1 OVERVIEW

The *Drosophila* larval nervous system is surrounded by an outer layer of perineurial glia that are in turn covered by a basement membrane, called the neural lamella. The function of perineurial glia and their interaction with the neural lamella is just beginning to be elucidated. We found that a transmembrane Ig domain protein, basigin/CD147/EMMPRIN, is highly expressed in perineurial glia surrounding the central and peripheral nervous systems of third instar *Drosophila* larvae. The structure of basigin as well as the structure and function of glia are highly conserved between vertebrates and *Drosophila* making it an ideal model organism in which to investigate basigin function. Much of the research on basigin has focused on its role in cancer. However other studies have found that basigin is also involved in a wide range of developmental processes. Here, we investigate a developmental role for basigin in *Drosophila* glia. We show that basigin is specifically expressed in perineurial glia where it is in a complex with integrin. Knocking down basigin in perineurial glia using RNAi results in a significantly shorter ventral nerve cord and ruffles in the peripheral nervous system. We examine the domains within basigin that are required for association with integrin and the effect of basigin knockdown on integrin-associated proteins. We found that basigin knockdown results in a collapsed actin cytoskeleton in perineurial glia and a redistribution of myosin motors within the glia.

3.2 INTRODUCTION

The nervous system is surrounded by large glycoproteins that make up the ECM and regulate its size and shape during development (Pastor-Pareja and Xu, 2011). ECM components including perlecan, collagen and laminin bind to receptors on the surface of glia to maintain the structure and function of glia, which in turn insulate, protect and support neurons and their axons. The integrin heterodimer is expressed by glia and mediates a range of glial processes including glial wrapping and axon sorting in vertebrates (Feltri et al., 2002). However studies in vertebrates have suggested that there remain other unidentified glial receptors that may play a role in regulating glial structure and function in the PNS (Yu et al., 2005). In Drosophila, integrin-based focal adhesion also mediates glial ensheathment of motor and sensory axons (Xie and Auld, 2011), however the role of other ECM receptors has not been determined. The structure and function of glial cells and many of their molecular processes are conserved between vertebrates and *Drosophila* (Stork et al., 2008) making *Drosophila* an excellent model in which to investigate new molecular players in glial function.

Basigin (Bsg/CD147/EMMPRIN) is a transmembrane protein and a member of the immunoglobulin superfamily that is highly conserved between vertebrates and *Drosophila* (Biswas et al., 1995; Reed et al., 2004). In mammals, there are three related Bsg genes, *basigin*, *neuroplastin* and *embigin*, whereas in flies there is only one, *basigin*. The *Drosophila* Bsg protein has two extracellular Ig domains, a transmembrane domain and a short intracellular domain. It shares 26% identity and 34% chemical similarity to mammalian Bsg, while the transmembrane domain is 80% identical (Curtin et al., 2005). The intracellular domain contains a sequence of 5 amino acids, YEKRR, that are

completely conserved between human, mouse and *Drosophila* Bsg (Munro et al., 2010). Crystal structure analysis and chemical cross-linking of membrane fractions has determined that Bsg homodimerizes *cis*- and *trans*-cellularly and that dimerization may be critical for its function (Cui et al., 2012; Fadool and Linser, 1996; Yoshida et al., 2000; Yu et al., 2008). In hepatoma cells, Bsg can also form a heterodimer with integrin via binding between the extracellular membrane-proximal domain of Bsg and the MIDAS motif of integrin β1 (Li et al., 2012). Other functions of Bsg include mediating invadopodia formation and metastasis by matrix metalloproteinase (MMP)-induced remodeling of the ECM, chaperoning monocarboxylate transporters (MCTs) to the membrane, and mediating chemotaxis and inflammation by binding cyclophilin A (Grass et al., 2012; Kirk et al., 2000; Sun and Hemler, 2001; Yurchenko et al., 2002). In *Drosophila*, Bsg genetically interacts with integrin during dorsal closure and restricts synaptic bouton size via organization of cortical actin cytoskeleton (Besse et al., 2007; Reed et al., 2004). Additionally, basigin has been shown to interact with integrin to mediate photoreceptor development and neuron-glia interactions in the optic lamina of the brain (Curtin et al., 2005; Curtin et al., 2007; Fadool and Linser, 1996). However, the role of Bsg during vertebrate or *Drosophila* glial sheath development has not been explored.

Here, we investigate the role of Bsg in *Drosophila* glia during development and in particular, in its interaction with integrin and maintenance of glial morphology in the PNS. At the 3rd instar larval stage, the entire *Drosophila* nervous system is surrounded by an outer perineurial glia layer that contacts the ECM. The subperineurial glia, which contain septate junctions forming a blood-brain barrier, are found internal to the

perineurial glia. The central nervous system (CNS) is composed of neuronal cell bodies and axons that are surrounded by multiple glial subtypes including cortex or astrocyte-like glia and ensheathing or wrapping glia which directly contact neurons and their axons. In the PNS, motor and sensory axons are within the center of the nerve and are ensheathed by wrapping glia. We found that Bsg was expressed specifically in perineurial glia, where it co-localized with integrin. A proximity ligation assay (PLA) found that basigin and integrin associate closely and the association of Bsg and integrin was mediated through the extracellular domain of Bsg. Knockdown of Bsg in the perineurial glia using RNAi resulted in a shorter ventral nerve cord (VNC) and a significant increase in ruffles in the perineurial glia. Finally, we examined the effect of Bsg knockdown on integrin-associated proteins and found that the actin cytoskeleton of perineurial glia was collapsed. Together, our results provide strong evidence that Bsg interacts with integrin and plays an important role in regulating and maintaining the morphology of the perineurial glia via the actin cytoskeleton.

3.3 MATERIALS AND METHODS

3.3.1 Fly strains and genetics

The following fly strains were used in this study: Gli-Gal4 (Sepp and Auld, 1999); Trol::GFP, Viking::GFP, Bsg::GFP, nrv2::GFP, ILK::GFP, talin::GFP (Morin et al., 2001), UAS-mCD8::RFP (gift from Dr. Elizabeth Davis). The UAS-lifeact::GFP line (Riedl et al., 2008) was a gift from Dr. Guy Tanentzapf and the GFP-tagged Bsg mutant constructs were gifts from Dr. Anne Ephrussi (Besse et al., 2007). The following fly strains were from the Bloomington Stock Center: NrxIV::GFP, UAS-GFP, *repo-GAL4* (Sepp et al., 2001); *nrv2-GAL4* (Sun et al., 1999); UAS-mCD8::GFP (Lee and Luo,

1999); UAS-Dicer2 (Dietzl et al., 2007). Pax::YFP and *46F-GAL4* (Xie and Auld, 2011) were obtained from the Kyoto DGRC. Bsg-RNAi lines (GD989, GD15718) were from VDRC. Mys-RNAi (1560R-1) was from Fly Stocks of National Institute of Genetics in Japan. All TEM and fluorescent images are of 3rd instar larvae and experiments were carried out at 29°C with UAS-Dicer2. All larval tracking experiments were carried out at 25°C with controls expressing UAS-Dicer2 and UAS-mCD8::RFP.

3.3.2 Immunolabeling and image analysis

Dissection and fixation for immunofluorescence was performed as described previously (Sepp et al., 2000). For immunolabeling, larvae were fixed in 4% formaldehyde for 15-30 minutes. The following primary antibodies were used: rabbit anti-HRP (1:500, Jackson ImmunoResearch, West Grove, PA), rabbit anti-zipper (1:2500, gift from Dr. Ken Prehoda), rabbit anti-GFP (1:500, Life Technologies, CA, USA), anti-MLCII (Cell Signaling Technologies, MA, USA), mouse anti-βPS (1:10, CF.6G11) (Brower et al., 1984). Secondary antibodies were goat anti-mouse or goat anti-rabbit conjugated with Alexa488, Alexa568 or Alexa647 (1:300, Invitrogen, Toronto, Canada). High magnification fluorescent images were obtained with a DeltaVision (Applied Precision, Mississauga, Ontario) using a 60X oil immersion objective (NA 1.4) at 0.2 μm steps. Stacks were deconvolved (SoftWorx, Toronto, Canada) using a measured PSF using 0.2 μm fluorescent beads (Invitrogen, Toronto, Canada) in Vectashield (Vector Laboratories, Burlington, Canada). Low magnification images were taken using a 20X water immersion lens (NA 0.95) on a Leica Confocal. Images were compiled using Photoshop and Illustrator CS4. For transmission electron microscopy (TEM) analysis, larval brains were fixed in 4% formaldehyde and 3%

glutaraldehyde, rinsed in 0.1 M PIPES, post-fixed in 1% osmium tetroxide, embedded in 1:1 acetonitrile:Spurr resin and polymerized in Spurr resin. Thin sections (50 nm) were obtained with a Leica ultramicrotome and analyzed with a FEI Tecnai TEM operating at an accelerating voltage of 80 kV.

3.3.3 Larval Tracking

Larval tracking was performed using an adapted multi-worm tracker and script (Swierczek et al., 2011). Each tracking session included 5-30 larvae placed on 100 mm diameter apple juice plates, tapped to elicit movement, and tracked for 30 seconds. The instantaneous speed of all larvae at 15 seconds was measured and differences analyzed using a one-way ANOVA plus Tukey's post hoc test.

3.3.4 Morphological quantification

Larval VNCs were measured using Leica Application Suite Advanced Fluorescence software. Nerves with ruffles were defined as a nerve with at least one 100 μm section demonstrating ruffles. Unpaired t-tests were used to calculate p-values between controls and experimental averages.

3.3.5 Proximity Ligation Assay

PLA used Duolink® In Situ reagents and protocol adapted from (Wang et al., 2015) by doubling reaction volumes of PLA probe, ligation and amplification mixes and double incubation times. Humid chambers were placed on horizontal shaker within 37°C incubator. Larval fillets mounted in VECTASHIELD®.

3.4 RESULTS

3.4.1 Basigin is expressed in the central and peripheral nervous systems

In order to determine which cell populations within the central and peripheral nervous system express basigin, we examined the expression pattern of an endogenous basigin-GFP fusion protein (Morin et al., 2001; Reed et al., 2004). In 3rd instar larvae, Bsg::GFP is highly expressed at the NMJ, in the CNS neuropile, in axons exiting the neuropile and in glial cells along the surface of both the CNS and PNS (Fig. 3.1A). Higher magnification of the peripheral nerves revealed that Bsg appeared to be expressed only in peripheral glia, not axons (Fig. 3.1B). Bsg::GFP fluorescence surrounding the CNS and along peripheral nerves was eliminated following expression of Bsg-RNAi using the pan-glial driver repo-GAL4 (Fig. 3.1C,D). However Bsg::GFP fluorescence at the NMJ, in the CNS neuropile and axons exiting the neuropile remained, consistent with previous studies on Bsg at NMJs and neuron synapses as well as within glia of the *Drosophila* nervous system (Besse et al., 2007). There was no Bsg::GFP fluorescence seen along peripheral nerves after Bsg knockdown in glia, indicating that within peripheral nerves, basigin is only expressed by glia (Fig. 3.1D). Previous studies have identified specific glial-glial and glial-axonal interactions at the CNS/PNS transition zone in both vertebrates and *Drosophila* (Sepp et al., 2001; Smith et al., 2014). Using nrv2-GAL4 to drive a RFP-tagged membrane marker (UAS-mCD8::RFP) specifically in ensheathing and wrapping glia, we were able to closely examine the CNS/PNS transition zone. We found that Bsg::GFP was highly expressed in axons interior to the CNS and ensheathed by *nrv2>mCD8::RFP* ensheathing glia as the axons exit the neuropile and approach the CNS/PNS transition zone (Fig. 3.1F). However, as the axons extend into the

PNS, the axonal Bsg::GFP fluorescence diminishes and Bsg::GFP expression remains solely in the glia surrounding the CNS and PNS (Fig. 3.1E,F). While Bsg has a wide range of expression, our study focused on the role of Bsg in the perineurial glia to determine the role of Bsg in mediating the overall morphology and function of larval nervous system.

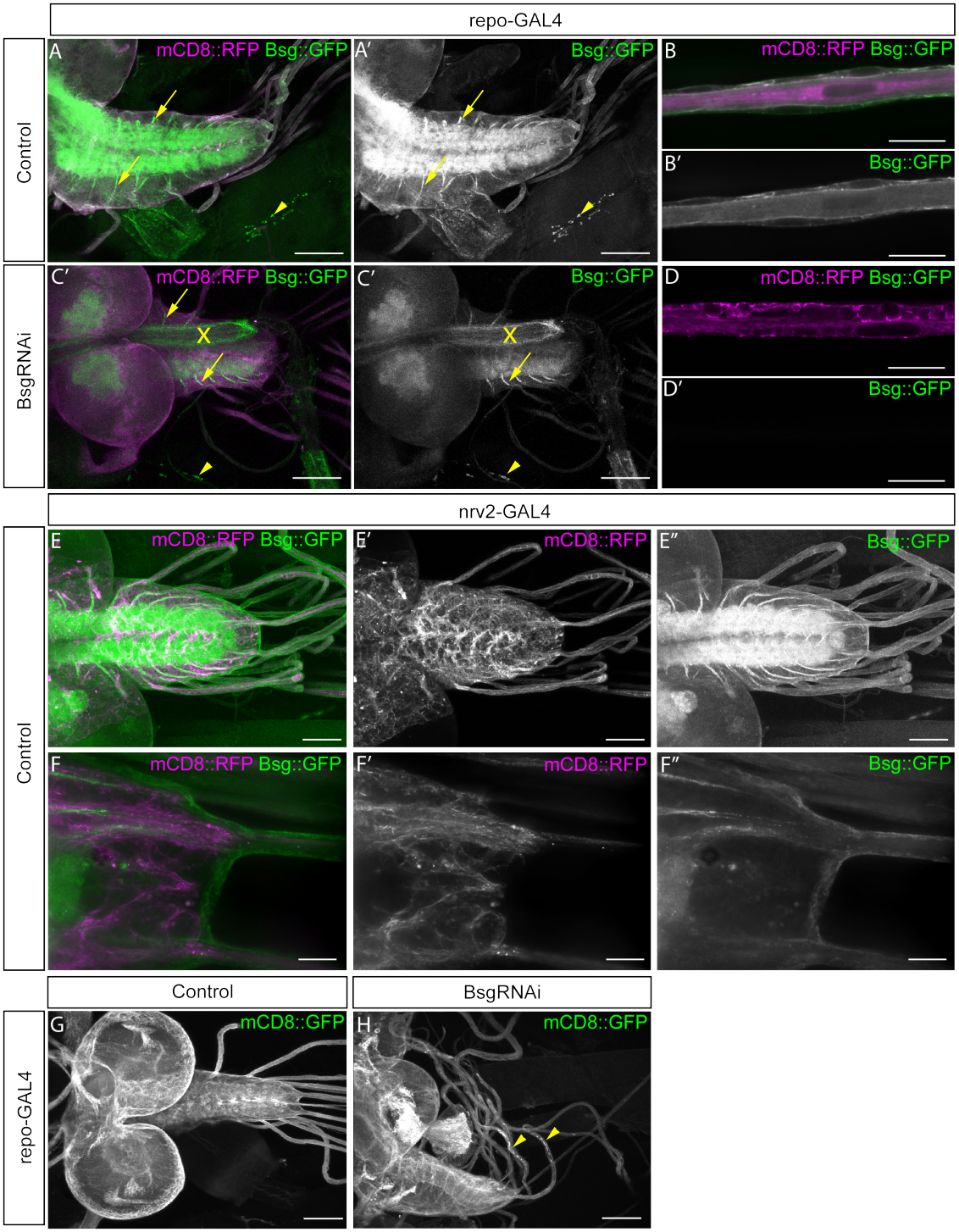

Figure 3.1. Basigin is expressed in the central and peripheral nervous systems. **(A,B)** Low and high magnification of control CNS and peripheral nerves of 3rd instar larvae with repo-GAL4 driving mCD8::RFP (magenta) in glia and endogenous Bsg::GFP (GREEN). Scale bars are 100 μm (A) and 15 μm (B). **(C,D)** Low and high magnification of CNS and peripheral nerves of 3rd instar *repo>Bsg-RNAi* larvae with glia (magenta) and Bsg::GFP (green). Bsg::GFP signal is absent from glia but still present in neurons in the neuropil, in axons exiting the CNS (arrows) and at the NMJ (arrowheads). The X

identifies the esophagus, which should be disregarded as it is irrelevant to these studies. Scale bars are 100 μm (C) and 15 μm (D). **(E)** Control CNS and peripheral nerves with nrv2-GAL4 driving mCD8::RFP (magenta) in wrapping and ensheathing glia and endogenous Bsg::GFP (green). Scale bar is 50 μm. **(F)** High magnification of the CNS/PNS transition zone. In the CNS, wrapping and ensheathing glia surround axons, of which a subset highly express Bsg::GFP until they exit the CNS. Bsg::GFP is also expressed in glia surrounding the CNS. In the PNS, mCD8::RFP is present in wrapping glia and Bsg::GFP expression is no longer present in axons, but only in glia surrounding axons. Scale bar is 15 μm. **(G,H)** Control CNS and peripheral nerves with repo-GAL4 expressing mCD8::GFP (G) and CNS and peripheral nerves of *repo>Bsg-RNAi* larva (H) showing morphological defects to the ventral nerve cord as well as fragments of glial membrane in peripheral nerves (arrowheads). Scale bars are 100 μm.

3.4.2 Glial knockdown of basigin results in morphological defects in the CNS and PNS

In order to determine the role of basigin in *Drosophila* glia, we expressed Bsg-RNAi and a fluorescently-tagged membrane marker (mCD8::GFP) using *repo-GAL4* to examine the effect of basigin knockdown on glial morphology. The *repo>Bsg-RNAi* larvae survived to the 3rd instar larval stage, but died during pupal stages. Compared to control larvae, we found morphological defects in both the CNS and PNS (Fig. 3.1G,H). The VNC was condensed and there were sections of peripheral nerves with extensive membrane ruffles in perineurial glia and fragmented pieces of glial membrane within the interior of the nerve (Fig. 3.2A-D). The peripheral nerve in the 3rd instar larva contains three glial layers: the inner most wrapping glia, the intermediate subperineurial glia and the outer most perineurial glia, surrounded by an ECM called the neural lamella. Peripheral nerves in control larvae are generally smooth in appearance with compact, flat glial layers and no portions containing ruffles (Fig. 3.2A). After Bsg knockdown in glia, a significantly higher percentage, 45.7% (n=108), of peripheral nerves demonstrated at least a 100 μm section with ruffles compared to only 2.8% (n=137) of control nerves (*repo>mCD8::RFP, dicer2*) (Fig. 3.2A,B,G). The location of the ruffles along the exterior of the nerve suggests that they likely consist of perineurial glia. Perineurial glia

also surround the CNS of the Drosophila nervous system, consisting of the brain lobes and VNC. In control larvae, the VNC was on average 434.0 μm ± 54.5 μm (n=19) and the VNC of *repo>Bsg-RNAi* larvae were significantly shorter with an average length of 354.6 μm ± 52.3 μm (n=23) (Fig. 3.2H). The ruffles in the PNS may represent condensation in the PNS similar to the condensation of the VNC in the CNS. The location of the fragmented glial membrane along the interior of the nerve (Fig. 3.1H) suggested that the wrapping glia were affected. Therefore, we used nrv2::GFP, a fusion protein endogenously expressed in wrapping glia but not subperineurial glia or perineurial glia in the PNS to assess the morphology of the wrapping glia in controls and after Bsg knockdown in glia (Fig. 3.2C,D). We confirmed that wrapping glia are fragmented after Bsg knockdown in glia. To confirm that it was specifically wrapping glia that were fragmented following Bsg knockdown and not subperineurial glia, we expressed a fluorescently-tagged core septate junction protein, NrxIV::GFP in controls and *repo>Bsg-RNAi* larvae and determined that the blood-brain barrier is structurally unaffected by Bsg knockdown and extends along the entire length of peripheral nerves confirming that the subperineurial glia are not fragmented like the wrapping glia (Fig. 3.2E,F). Furthermore, we immunolabelled axons in the PNS using an anti-HRP antibody and determined that Bsg knockdown in glia does not affect axonal fasciculation or morphology (Fig. 3.2C-F).

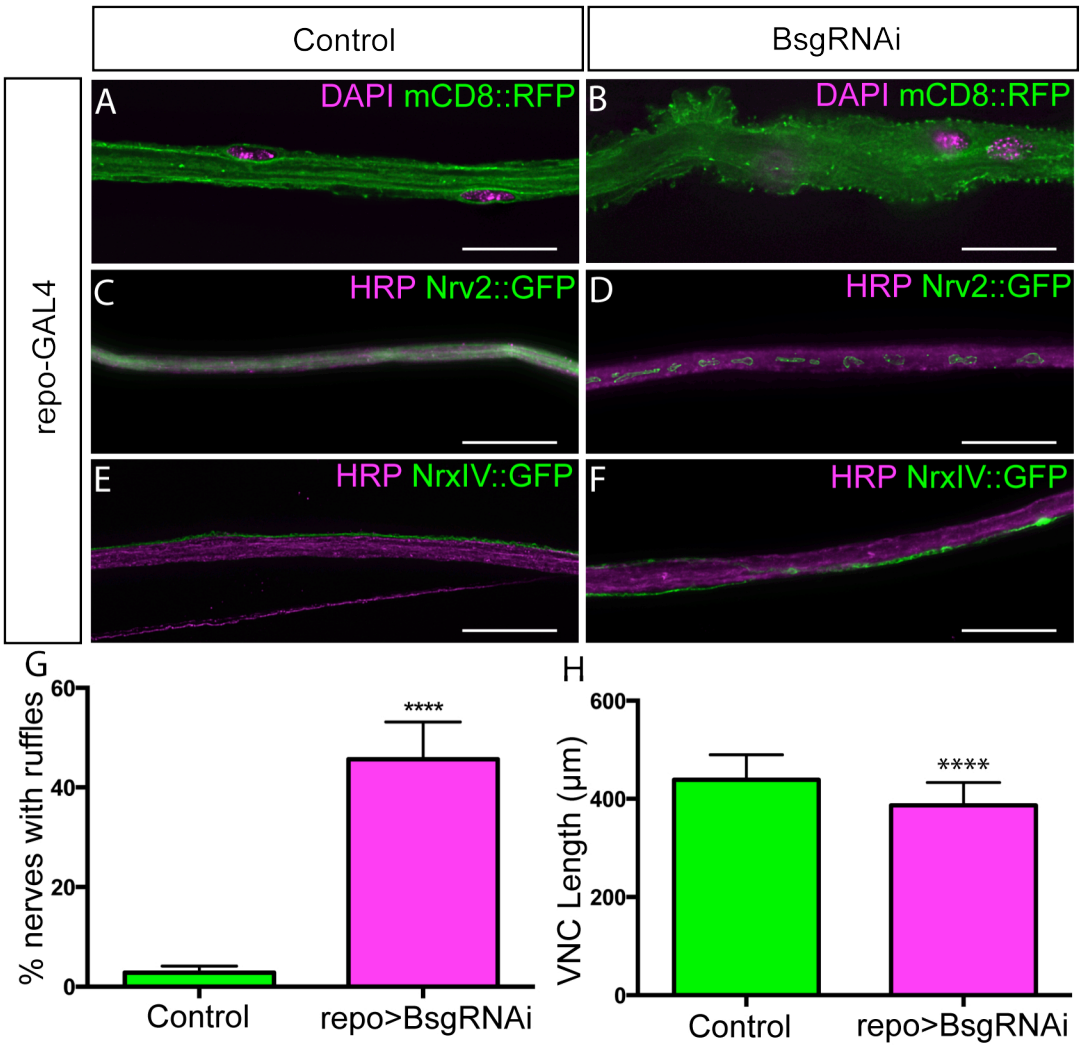

Figure 3.2 Basigin knockdown in all glia affects nervous system morphology. **(A-F)** High magnification of control and *repo>Bsg-RNAi* peripheral nerves. Scale bars are 15 µm. (A,B) Nerves with repo-GAL4 driving mCD8::RFP (green) and nuclei labeled with DAPI (magenta) show ruffles in the glial membrane. (C,D) Nerves expressing endogenous nrv2::GFP (green) in wrapping glia and HRP labeling axons (magenta) show fragmented wrapping glia and unaffected axons. (E,F) Nerves expressing endogenous NrxIV::GFP, a marker of septate junctions in subperineurial glia, and HRP labeling axons (magenta) show unaffected morphology of septate junctions and axons. **(G)** Quantification of percent of nerves with ruffles in controls (2.8 ± 1.3%, n=137) and repo>Bsg-RNAi (45.7 ± 7.5%, n=108, $p<0.0001$). **(H)** Quantification of VNC length in controls (434.0 ± 54.5 µm, n=19) and *repo>Bsg-RNAi* (354.6 ± 52.3 µm, n=23, $p<0.0001$).

Finally, we confirmed our findings using electron microscopy, as control wrapping glia surround axons with extensive processes (n=12) (Fig. 3.3 A), and in

repo>Bsg-RNAi peripheral nerves, wrapping glia do not extend processes but subperineurial and axon structure is unaffected (n=8) (Fig. 3.3 B). Our results suggest that basigin plays a significant role in glia during the development and maintenance of the *Drosophila* nervous system, and is important for *Drosophila* survival during pupal stages.

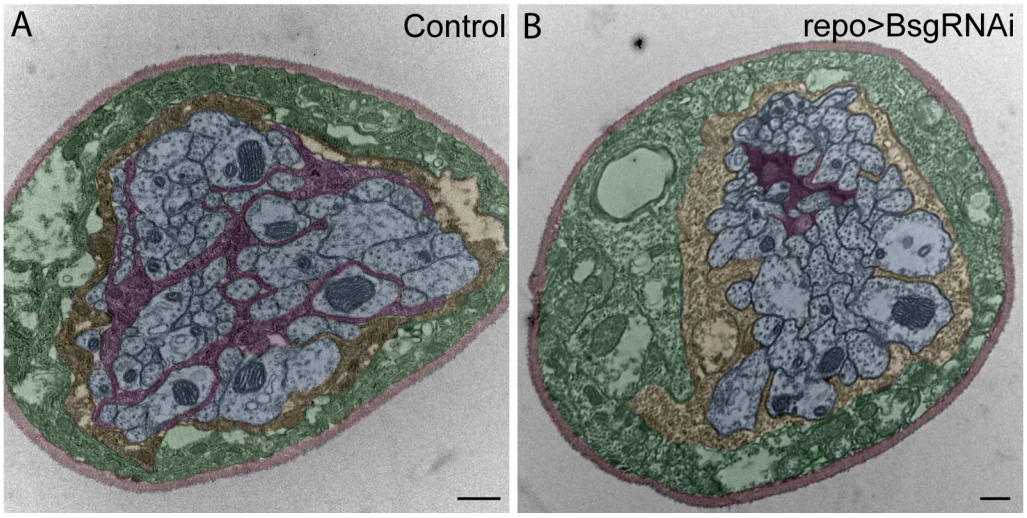

Figure 3.3 Basigin knockdown in all glia affects nervous system morphology. TEM images of peripheral nerve sections from control (A) and *repo>Bsg-RNAi* (B,C) larvae. False coloured to show axons (blue), wrapping glia (purple), subperineurial glia (yellow), perineurial glia (green) and the neural lamella (red). Control nerves have wrapping glia that engulf most axons while Bsg knockdown results in wrapping glia that do not extend processes around axons. Bsg knockdown also results in nerves that are not round, indicative of ruffles. Scale bars are 500 μm.

3.4.3 Basigin functions in the perineurial glia

The localization of basigin surrounding the CNS and PNS suggested that basigin was present in the perineurial glia. To test the localization and function of basigin in the perineurial glia, we used a perineurial glial-specific driver (*46F-GAL4*) (Xie and Auld, 2011) and expressed Bsg-RNAi in the background of Bsg::GFP. We found that similar to pan-glial knockdown, Bsg::GFP expression surrounding the CNS and PNS was eliminated using 46F-GAL4 (Fig. 3.4 A-D). Bsg::GFP fluorescence remained in the

neuropile, in axons exiting the neuropile and at the neuromuscular junctions (NMJ) (Fig. 3A,B). We found that Bsg knockdown using *46F-GAL4* was sufficient to cause significant shortening of the VNC (Fig. 3.4 E). Control larvae had an average VNC length of 502.1 ± 50.0 μm (n=17) while *46F>Bsg-RNAi* larvae had an average VNC length of 406.5 ± 45.0 μm (n=14). Additionally, we found that knocking down Bsg resulted in a significant increase in the percent of nerves demonstrating at least a 100 μm section with ruffles (Fig. 3.4 C,D,F). Only 5.4% (n=201) of control larvae demonstrated ruffles, while 45.8% (n=141) of *46F>Bsg-RNAi* nerves were ruffled. Thus, much of the phenotype associated with Bsg knockdown in all glia can be attributed to the lack of Bsg specifically in perineurial glia.

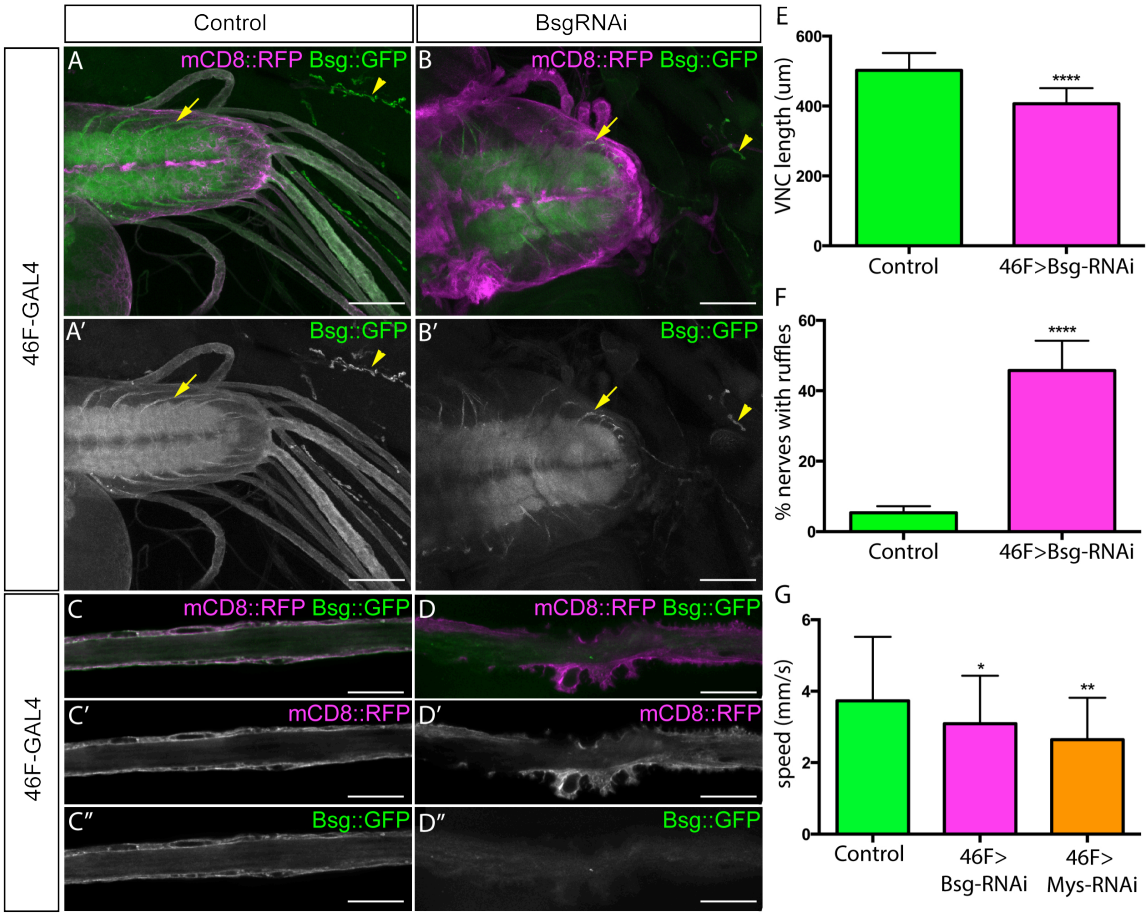

Figure 3.4. Basigin knockdown in perineurial glia affects nervous system

morphology. (A-D) Low and high magnification of endogenous Bsg::GFP (green) and 46F-GAL4 driving mCD8::RFP (magenta) in control (A,C) and *46F>Bsg-RNAi* (B,D) CNS and peripheral nerves. Arrowheads mark Bsg::GFP in axons leaving VNC and arrows mark Bsg::GFP in NMJs. Scale bars are 100 μm (A,B) and 15 μm (C-J). **(E)** Quantification of VNC length in controls (502.1 μm ± 50.0 μm, n=17) and *46F>Bsg-RNAi* (406.5 μm ± 45.0 μm, n=14, p<0.0001). **(F)** Quantification of percent of nerves with ruffles in controls (5.4 ± 1.9%, n=201) and *46F>Bsg-RNAi* (45.8 ± 8.4%, n=xx, p<0.0001). **(G)** Quantification of instantaneous speed of 3rd instar larvae for controls (3.7 ± 1.8 mm/s, n=116), *46F>Bsg-RNAi* (3.1 ± 1.3 mm/s, n=77, p<0.0001) and *46F>mys-RNAi* (2.6 ± 1.2 mm/s, n=28, p=0.0033).

However, when we tested if knockdown of basigin in perineurial glia was sufficient to cause the fragmentation of wrapping glia seen in *repo>Bsg-RNAi* larvae, we found that the expression pattern of nrv2::GFP in the background of *46F>Bsg-RNAi* did not phenocopy the pan-glial knockdown of Bsg as expected (Fig. 3.4 A,B). When we expressed Bsg-RNAi in perineurial glia in a background heterozygous for a Bsg deficiency, we found that the perineurial glia appeared more severely affected, with ruffles as well as kinks in the peripheral nerves (Fig. 3.5 C), thus confirming that the RNAi is decreasing the amount of Bsg in perineurial glia. However, even in a Bsg deficient heterozygous background, we found that the wrapping glia were unaffected. This suggested that perhaps Bsg is present in small quantities in the subperineurial glia or wrapping glia that they were not detected using endogenous Bsg::GFP. However, when we expressed Bsg-RNAi individually in subperineurial glia using *Gli-GAL4* and in wrapping glia using *nrv2-GAL4*, the wrapping glia appeared morphologically unaffected (Fig. 3.5 D-G). This difference is likely due to the earlier expression of repo-GAL4 compared to the subtype-specific glial drivers. Finally, we wanted to determine if the effects of Bsg knockdown in glia on the morphology of the CNS and PNS affected the function of the nervous system. To test this, we examined the mobility of 3rd instar larvae using an adapted multi-worm tracker (Petley-Ragan et al., unpublished). We found that

the average speed of larvae was significantly reduced from 3.7 ± 1.8 mm/s (n=116) in control larvae to 3.1 ± 1.3 mm/s (n=77) after Bsg knockdown using 46F-GAL4 (Fig. 3.4 G). Bsg has been previously demonstrated to interact with integrin in *Drosophila* (Curtin et al., 2005; Curtin et al., 2007; Reed et al., 2004) and integrin knockdown in perineurial glia also results in decreased mobility (Fig. 3.4 M). Integrin knockdown in perineurial glia results in severe morphological defects including a lack of perineurial glial process extension around peripheral nerves (Xie and Auld, 2011). Our results demonstrate that Bsg is also a critical regulator of perineurial glial morphology.

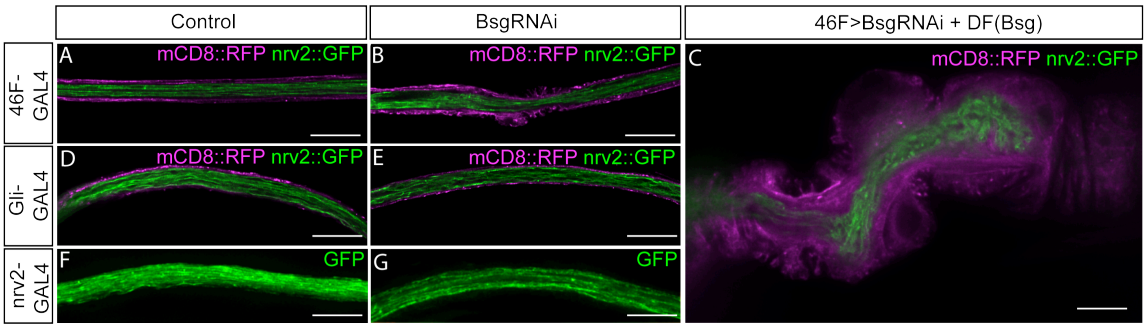

Figure 3.5 Basigin knockdown in perineurial glia
(A,B) Peripheral nerves with endogenous nrv2::GFP (green) and 46F-GAL4 driving mCD8::RFP (magenta) in control (A) and *46F>Bsg-RNAi* (B) larvae. **(C)** Knockdown of Bsg in perineurial glia in a background heterozygous for a deficiency of Bsg resulted in more severe disruption of perineurial glia (magenta), however wrapping glia were unaffected (green) **(D,E)** Peripheral nerves with endogenous nrv2::GFP (green) and Gli-GAL4 driving mCD8::RFP (magenta) in control (D) and *Gli>Bsg-RNAi* (E) larvae. **(F,G)** Peripheral nerves with nrv2-GAL4 driving GFP (green) in control (F) and *nrv2>Bsg-RNAi* (G) larvae. Scale bars are 15μm.

3.4.4 Basigin and integrin bind at the surface of perineurial glia

We hypothesize that Bsg is interacting with integrin to maintain the structure of the perineurial glia and thus of the overall nervous system. Using immunolabeling of the common beta subunit, βPS/myospheroid, we found that Bsg::GFP and integrin co-localize in perineurial glia in the PNS (Fig. 3.6 A). To confirm that this co-localization is functionally relevant, we performed a proximity ligation assay (PLA) that detects protein

interactions within 40nm in fixed tissues (Soderberg et al., 2006). We observed a positive PLA reaction between Bsg::GFP and integrin using anti-GFP and anti-βPS antibodies that recognized the respective epitopes on the extracellular side of the glial membrane (Fig. 3.6 B). Negative controls where one antibody was absent failed to show positive PLA reactions (Fig. 3.6 C,D). The positive PLA reaction indicated that Bsg and integrin are located within the same complex and likely bind directly on the surface of perineurial glia as well as between perineurial and subperineurial glia (Fig. 3.6 A,B). Crystal structure analysis of Bsg in hepatoma cells has indicated the extracellular membrane-proximal domain of Bsg binds integrin in cis conformation (Li et al., 2012; Xiong et al., 2014). However the variety of Bsg functions in multiple different tissues encouraged us to determine which domains within the Bsg structure are responsible for interacting with integrin in *Drosophila* glial cells. To answer this, we expressed a range mutant Bsg constructs tagged with GFP (Besse et al., 2007) using 46F-GAL4 and performed PLA using anti-GFP and anti-βPS antibodies. The control construct UAS-Bsg::GFP, when expressed in the perineurial glia had a positive PLA reaction with βPS integrin (Fig. 3.6 E). Specific residues (KRR) in the intracellular membrane-proximal domain of Bsg have been demonstrated to be important for NMJ structure in Drosophila (Besse et al., 2007), however we found that when these residues were mutated to NGG (Bsg::GFP-NGG), there was still a positive PLA reaction between integrin and Bsg (Fig. 3.6 F). The transmembrane domain of Bsg is highly conserved and contains a charged glutamate residue which has been shown to be critical for its binding to some transmembrane proteins such as monocarboxylate transporters in COS cells and *Xenopus* ooctyes (Manoharan et al., 2006). The Bsg::GFP-CD2 mutant, a chimeric Bsg construct

composed of two Ig domains of Bsg fused to the transmembrane and cytoplasmic domains of rat CD2, still had a positive PLA with integrin (Fig. 3.6 G). Finally, we determined that removing the short C-terminal intracellular domain of Bsg (Bsg::GFP-Δintra) did not affect the PLA interaction with integrin (Fig. 3.6 H). Together, these findings demonstrate that the extracellular portion of Bsg contains the critical sites for binding with integrin or that multiple domains within Bsg are able to independently maintain interactions with integrin.

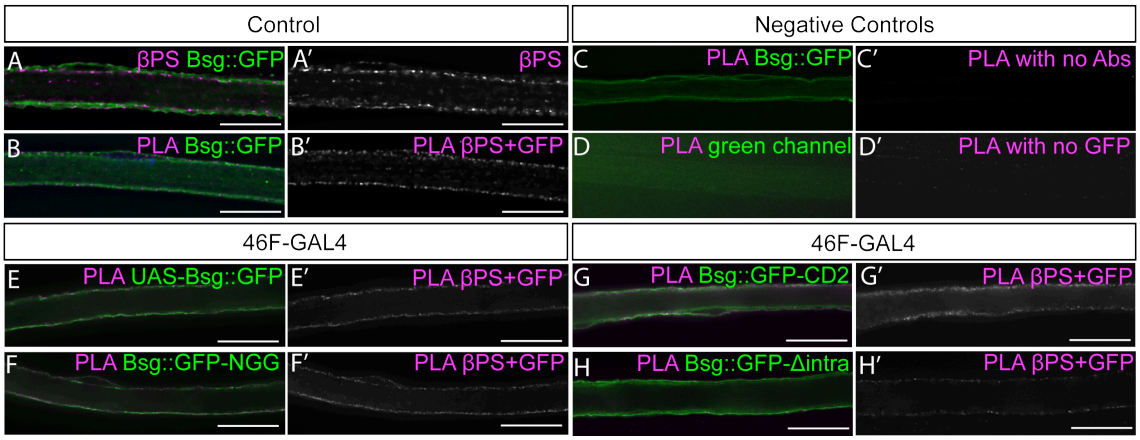

Figure 3.6 Basigin associates with integrin in perineurial glia
High magnification of peripheral nerves. Scale bars are 15 μm. **(A)** Endogenous Bsg::GFP (green) in nerve immunolabeled using anti-βPS antibody showing the distribution of the β integrin subunit (magenta). **(B)** Bsg::GFP in nerve (green), immunolabeled using anti-βPS showing co-localization of Bsg and integrin at the surface of perineurial glia as well as between perineurial glia and subperineurial glia. **(C)** Bsg::GFP in nerve, performed PLA without primary antibodies and does not result in positive PLA reaction (magenta). **(D)** Nerve with no Bsg::GFP, performed PLA, does not result in positive PLA reaction (magenta). **(E-H)** Nerve with 46F-GAL4 driving expression of UAS-Bsg::GFP (E), UAS-Bsg::GFP-NGG (conserved residues KRR mutated to NGG in C-terminal domain) (F), UAS-Bsg::GFP-CD2 (Bsg extracellular domain fused to CD2 transmembrane domain) (G), UAS-Bsg::GFP-Δintra (Bsg without C-terminal intracellular domain) (H), performed PLA using anti-βPS and anti-GFP antibodies, all experiments resulted in positive PLA reactions (magenta).

3.4.5 Actin cytoskeleton is severely affected by Bsg knockdown in perineurial glia

Given that basigin associated with integrin in perineurial glia, we wanted to determine if basigin knockdown affected focal adhesion complex components or the

actomyosin network. We first tested the distribution of focal adhesion complex components in control perineurial glia and in *46F>Bsg-RNAi* larvae. We immunolabeled control and *46F>Bsg-RNAi* nerves using anti-βPS in a background with talin endogenously tagged with GFP (talin::GFP). We observed in controls that talin::GFP and βPS co-localized at the interface between perineurial glia and subperineurial glia as well as on the surface of perineurial glia adjacent to the neural lamella and that Bsg knockdown did not affect the localization of either talin::GFP or βPS (Fig. 3.7A,B). We observed an endogenously GFP-tagged integrin-linked kinase construct (ILK::GFP) in the background of controls and *46F>Bsg-RNAi* larvae and found that it is localized at the border between perineurial glia and subperineurial glia and this does not change upon Bsg knock down (Fig. 3.7C,D). This finding indicates that focal adhesions are present between perineurial and subperineurial glia and that they are not affected by Bsg knock down. We also examined the localization of paxillin, an actin-regulating adaptor protein that is part of the integrin adhesion complex using an endogenous paxillin tagged with YFP (Pax::YFP) (Deakin and Turner, 2008; Turner et al., 1990). In controls, paxillin was located in the perineurial glia below the plasma membrane adjacent to the neural lamella (Fig. 3.7 E). In *46F>Bsg-RNAi* larvae, Pax::YFP maintained its localization near the plasma membrane, however was present at the base of ruffles (Fig. 3.7 F).

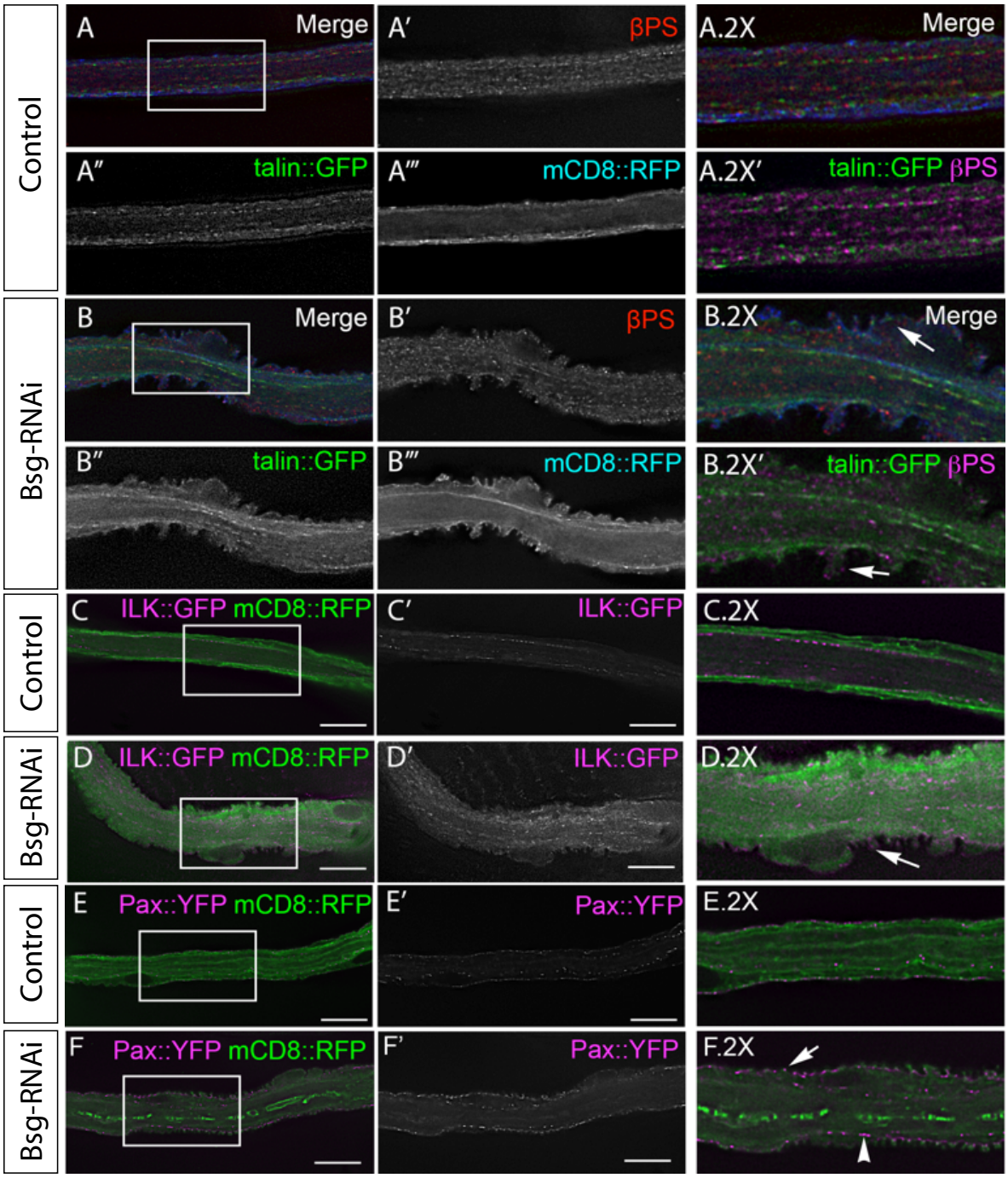

Figure 3.7. Integrin-associated protein localization after Bsg knockdown. (A,B) 46F-GAL4 driving mCD8::RFP (blue) with endogenous talin::GFP (green) and immunolabeled for myospheroid (red) in control (A) and *46F>Bsg-RNAi* (B). Boxes in panel A and B are shown at 2X magnification to the right. **(C,D)** 46F-GAL4 driving mCD8::RFP (green) with endogenous ILK::GFP or (magenta) in controls (C) and *46F>Bsg-RNAi* (D). Boxes in panel C and D are shown at 2X magnification to the right. **(E,F)** repo-GAL4 driving mCD8::GFP (green) with endogenous Pax::YFP (magenta) in

controls (E) and *repo>Bsg-RNAi* (F). Boxes in panel D and F are shown at 2X magnification to the right. Arrows point to ruffles and scale bars are 15 μm.

Next, we examined the localization of proteins involved in the actomyosin network that provide structural support to the perineurial glia. Actin filaments can provide rigidity and myosin motors use ATP hydrolysis to pull on the actin filaments to generate contractile tension (Munjal and Lecuit, 2014). We immunolabeled control nerves for myosin light chain II (MLCII) and myosin heavy chain (zipper) and found that both were concentrated in perineurial glia, specifically near the external membrane contacting the neural lamella (Fig. 3.8 A-D). Due to the involvement of non-muscle myosins in generating cortical tension and mediating cell shape changes and cytokinesis (Munjal and Lecuit, 2014), this observation suggests that perineurial glia can play a role in maintaining the overall structure of the *Drosophila* nervous system. When Bsg was knocked down in perineurial glia using *46F>Bsg-RNAi*, MLCII and zipper were both localized to the tips of the ruffles (Fig. 3.8 B2X and D2X). Since myosin motors exert their function by pulling on actin filaments, we wanted to determine if the perineurial glia ruffles also contained actin filaments. To do this, a UAS-lifeact::GFP construct was expressed in perineurial glia to visualize F-actin without interfering with actin dynamics (Riedl et al., 2008). In controls (46F-GAL4), actin filaments extended along the length of perineurial glia on either side of the nerve when viewed in a longitudinal section (Fig. 3.8 E). However, after Bsg knockdown, the actin cytoskeleton appeared collapsed or condensed and was no longer entirely surrounding the nerve (Fig. 3.8 F,H). In *46F>Bsg-RNAi* larvae, we never observed a lack of perineurial glial membrane along any portion of a nerve leading us to conclude that the lack of the actin cytoskeleton on one side of the nerve is not due to absence of perineurial glia wrapping the nerve, but due to a specific

loss of actin cytoskeleton in a portion of the perineurial glia. We observed no disrupted actin in controls (n=10), while all *46F>Bsg-RNAi* larvae (n=10) demonstrated about 50% nerves with actin disruptions. This is similar to the percentage of nerves with ruffles (Fig. 3.2G), however whether the collapsed actin cytoskeleton is the cause of the ruffles remains to be determined. Of note, there was often no lifeact::GFP present in ruffles indicating that myosin motors, localized to ruffles (Fig. 3.8A-D) are no longer in contact with the actin cytoskeleton. In portions of peripheral nerve where ruffles were visible using lifeact::GFP, the actin cytoskeleton also appeared concentrated or condensed (Fig. 3.8 G,I).

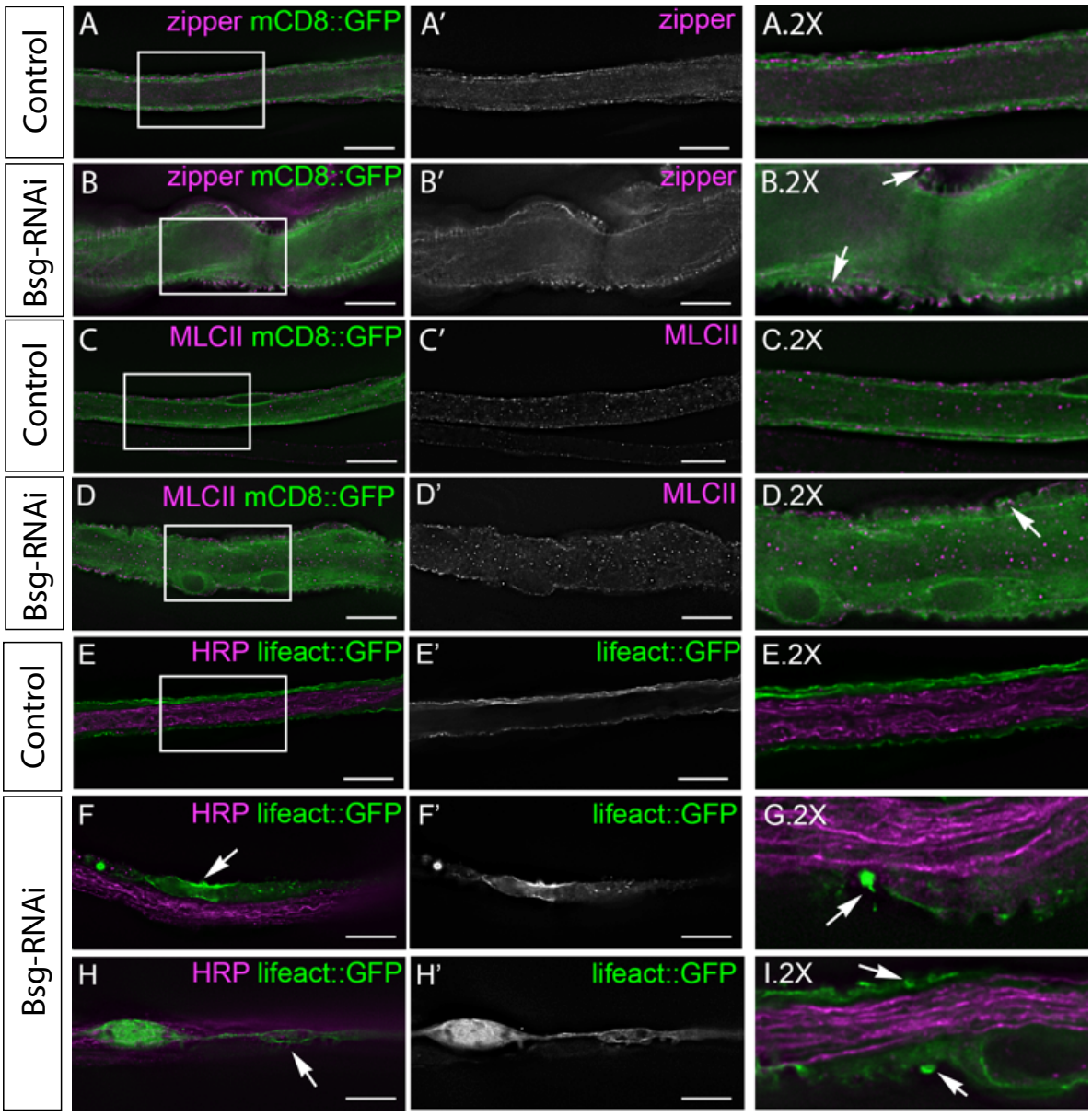

Figure 3.8. Actin-associated protein localization after Bsg knockdown. (A,B) 46F-GAL4 driving mCD8::GFP (green) immunolabeled for zipper (magenta) in control (A) and *46F>Bsg-RNAi* (B). Boxes are shown at 2X magnification at right. **(C,D)** 46F-GAL4 driving mCD8::GFP (green) and immunolabeled for myosin light chain II (magenta) in controls (C) and *46F>Bsg-RNAi* (D). Arrows point to ruffles. **(E-I)** 46F-GAL4 driving lifeact::GFP (green) and immunolabeled for HRP (magenta) in controls (E) and *46F>Bsg-RNAi* (F-I). Arrows point to disrupted actin. Scale bars are 15 μm.

Together, our analysis of integrin- and actin-associated proteins in perineurial glia in controls and after basigin knockdown indicate that components of the actomyosin network are highly expressed in perineurial glia compared to other glial layers and that

this network is likely involved in regulating contraction of the perineurial glia that surround not only peripheral nerves, but also the entire CNS. Furthermore, we found that the actomyosin network was affected by Bsg knockdown in perineurial glia. However, the localization of focal adhesion complex components was not.

3.5 DISCUSSION

Previous research on Bsg has focused on its role in cancer metastasis leaving the natural biological function of Bsg underappreciated (Xiong et al., 2014). The structure of Bsg is highly conserved between vertebrates and *Drosophila* and thus, our findings on the developmental role of Bsg in *Drosophila* glia should help guide future studies especially since glia are highly conserved both in structure and function (Biswas et al., 1995; Reed et al., 2004; Stork et al., 2008). Bsg is known to be expressed in vertebrate glia and is associated with glioma progression both by mediating metastasis via MMP activation and by regulating lactate transport via association with monocarboxylate transporters (Miranda-Goncalves et al., 2013; Riethdorf et al., 2006; Tian et al., 2013). However, Bsg is also expressed in a number of healthy organ systems including the nervous system (Muramatsu and Miyauchi, 2003; Xiong et al., 2014). In *Drosophila*, Bsg is expressed throughout embryogenesis and larval stages and is associated with a variety of nervous system processes including regulating the actin cytoskeleton in photoreceptor cells and NMJ boutons as well as affecting neuron-glia interactions (Besse et al., 2007; Curtin et al., 2005; Curtin et al., 2007). In vertebrates, an interaction between Bsg and integrin is required for embryonic stem cell differentiation and Bsg reduction in colorectal cancer affects integrin function (Ozeki et al., 2015; Sangboonruang et al., 2014). In *Drosophila*, Bsg had been found to interact genetically and co-localize with integrin. However our

PLA results show the first evidence that Bsg and integrin likely bind in *Drosophila*, specifically in perineurial glial cells (Curtin et al., 2005; Reed et al., 2004). Here, we provide solid evidence for the interaction of Bsg with integrin and its ability to regulate the actin cytoskeleton and morphology of perineurial glia. Our PLA results suggest that Bsg and integrin associate closely and likely bind on the surface of perineurial glia, and while the function of this association remains unknown, we have determined that Bsg knockdown does not affect the binding of integrin to either perlecan or collagen in the neural lamella. We show that after Bsg knockdown in all glia or specifically perineurial glia, we observed a shorter VNC and ruffles in the PNS, both of which suggest that the overall *Drosophila* nervous system has constricted. One possibility for this constriction is that the glia are unable to withstand the constricting forces of the surrounding neural lamella. Previous experiments have demonstrated that collagen IV in the neural lamella exerts a constricting force on the *Drosophila* nervous system while perlecan opposes the constricting force of collagen (Pastor-Pareja and Xu, 2011). The similar VNC constriction seen in perlecan mutants and after Bsg knockdown in glia suggests that Bsg and perlecan are involved in the same signaling pathway and may bind directly. Another possible mechanism through which Bsg knockdown results in constricted glia and nervous system morphology is via Bsg regulating the actomyosin network. Myosin motors function by pulling on actin filaments to generate contractile forces (Munjal and Lecuit, 2014), and the presence of both MLCII and zipper at high levels specifically in perineurial glia suggest that this glial layer is capable of generating contractile forces that could affect *Drosophila* nervous system morphology. We found that both MLCII and zipper were specifically localized to the tips of perineurial glial ruffles following Bsg

knockdown. Whether MLCII and zipper actively translocate to ruffle tips or if this is a consequence of simply being present below the plasma membrane in perineurial glia remains unknown. It is important to note that while MLCII and zipper localized to the ruffles, the actin cytoskeleton was not present in the ruffles and was collapsed or condensed in perineurial glia to the extent that the actin cytoskeleton was sometimes not visible on both sides of the axons in perineurial glia. It is possible that Bsg may regulate the actin cytoskeleton independently of integrin due to the interesting finding that all other actin-associated proteins maintained their location near the membrane after Bsg knockdown. In both controls and *46F>Bsg-RNAi* larvae, integrin and talin were located between glial layers as well as in ruffles and paxillin was located at the base of the ruffles. While we have previously determined that integrin forms adhesion complexes between glial layers and that integrin knockdown results in perineurial glia that are unable to fully wrap subperineurial glia (Xie and Auld, 2011), we never observed ruffles following integrin knockdown nor did we observe lack of perineurial glial wrapping following Bsg knockdown, implying that Bsg and integrin have unique functions in perineurial glia. In control peripheral nerves, Bsg was localized in perineurial glial membrane contacting the neural lamella as well as contacting subperineurial glia and Bsg may have a different function depending on where it is located within perineurial glia. The commonly studied function of Bsg in activating MMPs is unlikely responsible for the phenotypes seen here for two reasons. Firstly, we did not observe any phenotype associated with increased MMP activation (Xie and Auld, 2011) following overexpression of Bsg in perineurial glia using the UAS-Bsg::GFP construct. Furthermore, previous studies found that induced expression of MMP inhibitors in glia

resulted in an elongated VNC (Meyer et al., 2014), which is opposite from the VNC constriction we saw after Bsg knockdown. Furthermore, we did not observe any phenotype associated with overexpression of Bsg using the UAS-Bsg::GFP construct. Interestingly, we saw a ruffled neural lamella associated with VNC constriction, whereas others have observed a ruffled neural lamella associated with VNC elongation indicating that the length of the VNC and the morphology of the peripheral nerves may not be related (Meyer et al., 2014). However, the glial actin cytoskeleton has not been analyzed in these genetic backgrounds and understanding the intracellular effects of integrin knockdown, ECM modification and MMP inhibition on glial cells would provide valuable insight into the causes of the VNC size changes and perineurial glial morphological changes.

An analysis of which domains within the Bsg protein are responsible for regulating VNC length, perineurial glia ruffle formation, and the actin cytoskeleton would provide insight into other Bsg-binding proteins. In this study, we determined that Bsg is likely interacting with integrin via its extracellular domain, which is consistent with a mutational analysis that found that its extracellular portion and transmembrane domain are essential for Bsg function in the *Drosophila* visual system where it co-localizes with integrin (Curtin et al., 2005; Munro et al., 2010). Finally, the pupal lethality associated with Bsg-RNAi expression in perineurial glia suggests that Bsg also plays a critical role during pupal metamorphosis when the nervous system is undergoing severe morphological changes (Omoto et al., 2015). Whether Bsg function during pupal stages is consistent with its function during development remains to be determined. Similarly, Bsg has recently been demonstrated to affect vertebrate glial process

outgrowth (Carney et al., 2014; Goudriaan et al., 2014; Heller et al., 2003), and our results provide guidance for examining how Bsg mediates natural developmental functions in both *Drosophila* as well as vertebrate glia.

4 DISCUSSION

The ECM is becoming increasingly appreciated as a highly organized and specifically composed player in a variety of processes during development, maintenance and function of tissues throughout the lifetime of an animal. Current topics of interest range from the role of the ECM in mechanotransduction, the interaction of the ECM with growth factors, implications of ECM remodelling and the complications of ECM assembly and basement membrane organization (Bonnans et al., 2014; Cichon and Radisky, 2014; Humphrey et al., 2014; Mouw et al., 2014). These topics highlight the breadth of research on the ECM and the vast number of questions still remaining to be solved. The experiments in this thesis are relevant to topics such as ECM secretion and assembly, ECM remodeling and glial-ECM adhesion, specifically perineurial glial-ECM interactions.

At the time that we initiated our studies: i) The understanding of laminin secretion was limited. Therefore we sought inspiration from experiments elucidating the mechanism of collagen secretion (Saito et al., 2009). ii) The function of the perineurial glia in *Drosophila* was undetermined and the identification of perineurial glia in vertebrates was relatively new (Kucenas et al., 2008; Stork et al., 2008). iii) The role of basigin in glial development was just beginning to be appreciated (Curtin et al., 2007; Munro et al., 2010).

In Chapter 2 of this thesis, we describe the effect on glial cells of knocking down individual laminin subunits that together compose the laminin heterotrimer. We show that by eliminating either the beta subunit, LanB1 or gamma subunit, LanB2 from glial cells, the other subunit accumulates intracellularly. We further show that unbound

laminin subunits accumulate within the ER and result in swollen glial cells with large vacuoles and overall wider nerves. We suggest that demyelinating and muscular dystrophy diseases associated with laminin mutations such as Charcot-Marie-Tooth disease may not be solely due to a lack of extracellular laminin forming a matrix network and binding to laminin receptors, but also due to overwhelming ER stress and defects in laminin trafficking and the secretory pathway in glial cells. In support of this, many genes involved in cell trafficking also result in Charcot-Marie-Tooth disease (Bucci et al., 2012). As we found that knockdown of laminin affected secretion of the other subunits, this may represent an alternative hypothesis for how laminin mutations result in glial diseases. Similarly, ER stress has been linked to a number of other demyelinating disorders including Multiple Sclerosis (Lin and Popko, 2009). We demonstrate that Tango1 is involved in laminin secretion in *Drosophila* glia via a collagen-independent mechanism that counters the hypothesis that laminin and collagen are secreted together (Lerner et al., 2013). Through our findings, we have established that the perineurial glia are critical to the development of the larval nervous system.

In Chapter 3 of this thesis, we describe the effect on glial cells of knocking down basigin. We show that basigin functions mainly in the perineurial glia and is involved in maintaining the overall morphology of the nervous system. This is likely a consequence of basigin regulating the glial actin cytoskeleton as a lack of basigin resulted in a collapsed actin cytoskeleton in glia and ultimately, a shorter CNS and compacted peripheral nerves. Furthermore, we demonstrate that basigin is located in a complex with integrin and may bind integrin directly in *cis*. We were not able to determine particular amino acids within basigin were responsible for this interaction, however we hypothesize

that a domain within the extracellular portion of basigin interacts with integrin to maintain the structure of the glial actin cytoskeleton.

This thesis highlights the role of glial cells in interacting with the ECM and the importance and complexities of regulating glial-ECM interactions during development for the proper functioning of a nervous system. Our studies conducted in *Drosophila* glial identified new mechanisms underlying laminin secretion, glial wrapping, and basigin binding partners, all of which are can be applied to research in vertebrates due to the conserved nature of glial dynamics as well as the conserved structure of laminin and basigin proteins.

4.1 ROLE OF LAMININ IN THE NERVOUS SYSTEM

Chapter 2 represents our investigation into the consequences of individual laminin subunit knockdown on glia. Here, I discuss certain caveats that we encountered in our studies and ways to resolve these in the future to continue progress in this field. The main caveats and unanswered questions related to the study of laminin knockdown are: i) Function of laminin in interior portions of the nerves. ii) Early lethality associated with laminin mutations. iii) Identity of the large vacuoles seen in glia after laminin knockdown. iv) The deposition of laminin around the nervous system by cell types other than glial cells. v) The mechanism through which Tango1 enables laminin secretion.

4.1.1 Function of laminin in interior portions of peripheral nerves

One question that was unable to be answered during my studies was whether there is a functional amount of laminin present between glial layers and around axons in the *Drosophila* peripheral nerve. This was impossible to investigate due to the lack of a sensitive enough laminin antibody. The laminin antibodies used in this thesis were

inconsistent and difficult to store and endogenously expressed GFP-tagged laminin subunits have only recently been available. We hypothesize that laminin is present in the interior portion of peripheral nerves due to the presence of integrin in this area, however it is difficult to assess whether the integrin is present in glial cells or on axons (Xie and Auld, 2011). Furthermore, we have shown that integrin and talin co-localize both at the surface of perineurial glia as well as between glial layers. However other integrin-associated proteins, such as ILK, are only present between glial layers and paxillin is only present under the plasma membrane contacting the neural lamella in perineurial glia. Therefore, the identification of laminin between glial layers would help us in determining the specific function of integrin and integrin-associated proteins at different locations within perineurial glia. Recently, we acquired endogenously-tagged LanA::GFP and LanB1::GFP, so the function and secretion of small amounts of laminin will be easier to study in the future. When we examined the localization of these fusion proteins in peripheral nerves, we found that there was laminin along the interior portions of peripheral nerves (Fig. 4.1). The implications of small amounts of laminin around axons where there is no basement membrane would shift the focus of Schwann cell-derived laminin studies from focusing on laminin within basement membranes to the role of laminin alone. There is a possibility that laminin may have a unique function when found on its own than in a basement membrane. The laminin observed within the interior portion of the peripheral nerve did not form a basement membrane around axons, instead it was found inconsistently throughout the nerve, perhaps in areas of focal adhesion between axons and glia or between glial layers (Fig 4.1A-D). Additionally, there were portions of peripheral nerves with significantly more laminin (Compare Fig. 4.1 A and B

as well as 4.1 C and D). This suggests that laminin may be transiently secreted at specific developmental stages or specifically secreted in certain nerves.

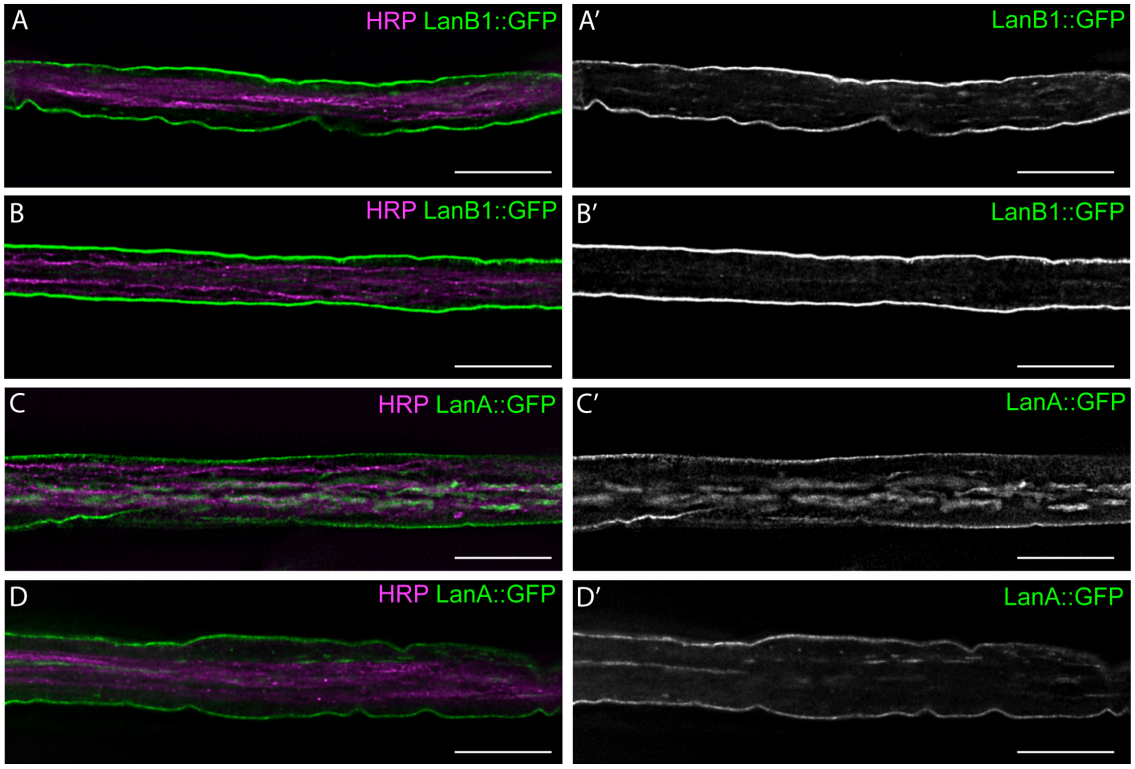

Figure 4.1 Fluorescently-tagged endogenous laminin localization
(A,B) Peripheral nerve with endogenous LanB1::GFP (green) and axons immunolabeled using HRP (magenta). **(C,D)** Peripheral nerve with endogenous LanA::GFP (green) and axons immunolabeled using HRP (magenta). Scale bars are 15 μm.

4.1.2 Early lethality associated with laminin mutations

Another difficulty with studying laminin is that it is expressed by many tissues throughout development, therefore mutations that seriously affect the ability of laminin to be secreted or to form a heterotrimer would also affect basement membrane formation during embryogenesis. Previous studies have found that null mutations in LanB1 are embryonic lethal making the investigation of glial cells in the 3rd instar larva peripheral nerves impossible (Urbano et al., 2009). We attempted to perform MARCM analysis of LanB1 null clones in perineurial glia. Unfortunately, we were unable to form any mutant

clones even though we were able to form control clones suggesting that perineurial glia homozygous for LanB1 null mutations undergo apoptosis. Clonal analysis using hypomorphic mutations that are not lethal to an individual cell would provide insight into whether laminin mutations affect glial cells in a cell autonomous or cell non-autonomous way. We predict that glial cells would be affected cell autonomously due to the severe implications of inhibiting laminin secretion from the ER. However the presence of laminin from other glial cells external to the mutant clones may alleviate some of the consequences associated with laminin mutations.

4.1.3 Identity of large vacuoles seen in glia after laminin knockdown

Large vacuoles such as those described in Chapter 2 are often seen when ECM secretion is inhibited. However the cause of the vacuoles and any specific markers have not yet been identified (Komazaki et al., 1995). It is possible that the vacuoles are a consequence of apoptosis. Indicators suggest that glial cells lacking LanB1 or LanB2 subunits likely undergo apoptosis since glial morphology is severely disrupted and larval mobility is significantly affected and ultimately, larval lethality suggests that the glial cells are not functioning to insulate and protect neurons. The question of whether the glial cells are undergoing apoptosis can be answered by performing TUNEL labeling and furthermore, the morphological defects associated with apoptosis can be differentiated from those specifically associated with LanB2 knockdown or accumulation of LanB1 by expressing an inhibitor of apoptosis in glia. We can do this in *Drosophila* by taking advantage of a UAS construct fused with bacterial DNA for an inhibitor of apoptosis, p35. This will prevent the glial cells from beginning the process of apoptosis and would allow us to determine if the large vacuoles seen after LanB1 and LanB2 knockdown are

the result of glial cells undergoing apoptosis or due to other reasons associated with laminin knockdown.

4.1.4 Deposition of laminin around the nervous system by other cell types

Glial cells, in addition to fat bodies and hemocytes, contribute laminin to the neural lamella surrounding the CNS and PNS in *Drosophila* (Olofsson and Page, 2005; Pastor-Pareja and Xu, 2011). Firstly, this makes studying the role of glial-derived laminin difficult because the lack of glial-derived laminin surrounding the nervous system is likely compensated for by fat body and hemocyte secretion of laminin. Fat bodies and hemocytes are known to also contribute collagen and perlecan to basement membranes such as the neural lamella, raising the question of why glial cells secrete only laminin? We have shown in Chapter 2 that glial cells do not secrete collagen in *Drosophila* and we have similarly observed that glial cells also do not secrete perlecan. Since laminin is uniquely required for basement membrane initiation and is responsible for recruiting other ECM proteins to assemble basement membranes, it is possible that glial cells secrete laminin in order to initiate the organization of the neural lamella but then rely on hemocytes and fat bodies to contribute the remaining neural lamella ECM components (Hohenester and Yurchenco, 2013; Sasaki et al., 2004; Urbano et al., 2009). Interestingly, laminin has also been identified as the key component of basement membrane assembly in cultured Schwann cells (McKee et al., 2007), suggesting that the mechanism of basement membrane assembly and organization around glial cells is conserved between *Drosophila* and vertebrates. Recent studies have examined the ultrastructure of human basement membranes and found that they are asymmetrically organized, whereby the two surfaces have different compositional, biomechanical and

cell adhesion properties. Furthermore, studies have found that the cell side is characterized by being twice as stiff with a high abundance of laminin while the extracellular side has a different protein composition (Halfter et al., 2013). During our studies, we found that laminin was sometimes observed in two separate lines in the neural lamella, providing support for the hypothesis that there is specific organization within the neural lamella and that laminin may play multiple roles within one basement membrane (Fig. 2.5 B''). Ultrastructural studies using electron microscopy and immuno-gold antibodies against laminin or anti-GFP antibodies in a LanA::GFP or LanB1::GFP background would provide insight into where within the neural lamella and peripheral nerves laminin is found.

4.1.5 Mechanism through which Tango1 enables laminin secretion

Tango1 was first identified for its role in Collagen VII secretion whereby an SH3 domain of Tango1 directly binds Collagen VII and incorporates it into large secretory vesicles leaving the ER (Saito et al., 2009). When it was found that Tango1 also affects the secretion of laminin from follicle cells in *Drosophila*, it was hypothesized that laminin simply was secreted in the same vesicles as collagen (Lerner et al., 2013). However our finding that Tango1 is required for laminin secretion in a collagen-independent mechanism suggests that Tango1 may be able to bind directly to laminin. To answer this question, biochemical analysis using immunoprecipitation would determine whether Tango1 and laminin can bind directly. Further analysis would include a mutational analysis of laminin to determine which specific domain is critical for binding to Tango1. We hypothesize that if laminin and Tango1 bind directly, it is most

likely through the α subunit of laminin since only the α subunit is able to be secreted independently, suggesting it may contain a Tango1-binding site (Yurchenco et al., 1997).

4.2 ROLE OF BASIGIN IN THE DROSOPHILA NERVOUS SYSTEM

Chapter 3 focused on the interaction between basigin and integrin and how it affects glial-ECM adhesion, the actin cytoskeleton and nervous system morphology. Here, I discuss caveats associated with our studies and ways to resolve these in the future to continue progress in this field. The main caveats and unanswered questions related to the study of basigin in glial cells are: i) Understanding how basigin knockdown affects wrapping glia. ii) Role of basigin at the CNS/PNS transition zone. iii) Pupal lethality associated with basigin knockdown in glia. iv) Identity of basigin domain responsible for its interaction with integrin. v) Link between basigin and the actin cytoskeleton.

4.2.1 Understanding how basigin knockdown affects wrapping glia

The first phenotype we identified due to basigin knockdown in *Drosophila* glia was fragmented wrapping glia along the interior portion of peripheral nerves. After we determined that basigin was uniquely expressed in perineurial glia in the PNS, we hypothesized that basigin knockdown in perineurial glia would result in fragmented wrapping glia. Interactions between the wrapping glia and the perineurial glia have been previously recorded, for example it was recently noted that wrapping glial expression of the neuregulin homolog, Vein, cell autonomously activates the EGF receptor on wrapping glia, which regulates the formation of the septate junctions in subperineurial and proliferation of the perineurial glia (Matzat et al., 2015). However, after numerous efforts, we did not link basigin knockdown individually in perineurial, subperineurial or wrapping glia to fragmentation of the wrapping glia. Furthermore, when we expressed

initiators of apoptosis using UAS-hid and UAS-rpr constructs in perineurial glia, we observed that although the perineurial glia did not fully wrap subperineurial glia and were discontinuous along peripheral nerves, the morphology of the wrapping glia was still unaffected (data not shown). These results suggest that basigin may be required in a class of glia within the CNS, or that repo-GAL4 is expressed earlier than the other glial layer-specific drivers indicating that basigin is required within glia during embryogenesis. To differentiate between these two possibilities, future experiments would make use of the temperature-sensitive GAL4 inhibitor, tubGAL80^{ts} and inhibit expression of Bsg-RNAi in glia until larval stages. Additionally, specific expression of Bsg-RNAi in CNS glial subtypes using GAL4 drivers identified in Stork et al., 2012 would determine if basigin is required within the CNS for the healthy development and survival of wrapping glia.

4.2.2 Role of basigin at the CNS/PNS transition zone

During our studies, we observed that endogenous Bsg::GFP was highly expressed in the CNS neuropil, and interestingly in motor axons leaving the neuropil until they reached the CNS/PNS transition zone, after which Bsg::GFP was no longer present in axons, and only seen in the perineurial glia. The CNS/PNS transition zone marks the boundary between the CNS and PNS and is the location of boundary cap cells, which can give rise to both neurons and glia (Zujovic et al., 2011). In both vertebrates and *Drosophila*, these cells are critical for the proper guidance of motor axons out of the CNS, however our understanding of the exact molecules and molecular mechanisms involved is fragmentary (Bravo-Ambrosio and Kaprielian, 2011). Peripheral glia have been shown to also regulate axon guidance through this zone in *Drosophila* and Krox20, a master regulatory gene for Schwann cell myelination, has been shown to be required for

the maintenance of the CNS/PNS transition zone in vertebrates (Coulpier et al., 2011; Sepp et al., 2001). The abrupt change in expression pattern of basigin at the CNS/PNS transition zone leads to the interesting question of what is the function of basigin in axons leaving the CNS and does the presence of basigin in perineurial glia, which arise on the surface of the CNS at this location, also play a role in regulating the maintenance of the CNS/PNS transition zone (Awasaki et al., 2008). The first steps in studying these questions would be clearly examining the expression pattern of basigin in neurons and glia throughout development and determining the effects of basigin knockdown in motor neurons. Additionally, since basigin is able to form heterodimers and co-localizes with a variety of transmembrane proteins such as monocarboxylate transporters and integrins(Mannowetz et al., 2012; Yu et al., 2008), using PLA to determine which proteins are found in a complex with basigin within the CNS/PNS transition zone would provide valuable insight into the function of basigin in this area.

4.2.3 Pupal lethality associated with basigin knockdown in glia

An analysis of basigin expression during *Drosophila* development should also include pupal stages as I hypothesize that basigin plays a major role in perineurial glial function during this time for three reasons. Firstly, basigin knockdown in glial cells does not affect viability at the 3rd instar larval stage. However *Drosophila* die during pupal stages which is when the nervous system undergoes dynamic remodeling. Secondly, it has been demonstrated recently that perineurial glia are essential for this process and secrete proteases to remodel the neural lamella surrounding the nervous system (Meyer et al., 2014). Finally, given that basigin has been well studied for its role in inducing MMPs in a variety of tissues, it is tempting to hypothesize that basigin may have an additional

function than interacting with integrin and regulating the actin cytoskeleton in perineurial glia during pupal stages and that the lethality associated with basigin knockdown in glia is also related to the ability of basigin to induce MMPs during *Drosophila* pupation (Xiong et al., 2014). To test this, dissection of control pupae and those with basigin-null glia should be examined for their nervous system morphology and function and neural lamella structure. To differentiate between the function of basigin during larval and pupal stages, we could use tubGAL80^{ts} to inhibit expression of Bsg-RNAi until pupal stages to prevent the morphological defects caused by basigin knockdown during early development from indirectly affecting nervous system remodeling during pupal stages.

4.2.4 Identity of basigin domain responsible for its interaction with integrin

Basigin is a multifunctional protein and it is possible that within one cell type, it plays a variety of roles and may have multiple binding partners. To differentiate between the effect of basigin in binding integrin and potentially inducing MMPs in glial cells, it would be beneficial to develop basigin mutant constructs that only impact one of the functions of basigin. This would require knowledge of which domains within *Drosophila* basigin are responsible for its role in binding to integrin and inducing MMPs. Our studies attempted to answer this question by expressing previously made basigin mutant constructs in perineurial glia and determining if they maintained their ability to bind to integrin. All of the mutant constructs we expressed affected the transmembrane or intracellular domain of basigin and demonstrated a positive PLA signal with integrin leading us to conclude that the domain within basigin responsible for integrin interaction is present in the extracellular domain. This is consistent with findings in malignant hepatoma cells that found that the extracellular membrane-proximal domain of basigin

bound to the MIDAS motif of integrin (Li et al., 2012). We would first need to confirm that the integrin-binding function of this domain is conserved between vertebrates and *Drosophila* before implementing a mutant basigin construct lacking the extracellular domain to study the specific function of basigin-integrin association in perineurial glia.

4.2.5 Link between basigin and the actin cytoskeleton

We demonstrated that Bsg binds integrin in perineurial glia, both on the surface adjacent to the neural lamella as well as between glial layers, and that the extracellular domain of Bsg is sufficient for this binding. However the function of this interaction is unknown. It is possible that Bsg affects the link between integrin and the actin cytoskeleton or that Bsg directly contacts the actin cytoskeleton in perineurial glia. We determined that perineurial glia specifically express higher levels of myosins (MLCII and zipper) than other glial layers in the peripheral nervous system (Fig. 3.7 D,F), indicating that they may be uniquely responsible for maintaining the tight glial layers seen in controls. We found that after Bsg knockdown in perineurial glia, the actin cytoskeleton was collapsed. However the myosins still appeared normally localized suggesting that Bsg knockdown affected the link between actin and myosin. It would be interesting to investigate whether expressing Bsg-RNAi in perineurial glia in backgrounds heterozygous for mutations in actin- or myosin-associated proteins would rescue or further exacerbate the phenotypes described here; namely perineurial glial ruffles and shortening of the VNC. There is also a possibility that the VNC shortening and the peripheral nerve ruffles are not due to the same function of Bsg, since previous studies have found VNC lengthening associated with peripheral nerve ruffles (Meyer et al., 2014). These questions could be addressed by expressing the mutant UAS-Bsg::GFP

constructs used in Fig. 3.6 in a Bsg knockdown background. This would be possible by knocking down endogenously-tagged Bsg::Cherry using Cherry-RNAi in perineurial glia and rescuing with the various UAS-Bsg::GFP constructs. This would allow us to determine which domains within Bsg are associated with VNC shortening and which domains are associated with actin cytoskeleton defects. Finally, disrupting the actin-cytoskeleton directly via expressing RNAi against actin-associated proteins would help determine if disruptions in the actin cytoskeleton directly result in VNC shortening.

4.3 CONCLUSIONS

These experiments represent just the beginning of our understanding of laminin secretion and basigin function in glial cells. Overall, we have highlighted the importance of glial-ECM interactions in the development and maintenance of a healthy nervous system and also highlighted the advantages of using *Drosophila melanogaster* as a model organism. The questions investigated in this thesis could not have been studied in vertebrates due to the lack of background knowledge concerning laminin secretion or basigin function in glia. But the use of *Drosophila* allowed us to explore new fields of cell biology in the hopes of having our hypotheses tested in vertebrates in the near future. But perhaps the most rewarding part of basic biological research is the ability and challenge to raise new interesting questions such as: How does Tango1 mediate laminin secretion? Why do glial cells secrete laminin but not other ECM components? How does basigin regulate the actin cytoskeleton in glial cells? And how many different functions does basigin have during nervous system development? These questions will further stimulate scientists to continue to investigate the complex relationship between glial cells and their associated basement membranes.

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