UNDERSTANDING THE MOLECULAR MECHANISMS UNDERLYING GLIA-GLIA COMMUNICATION IN THE DROSOPHILA PERIPHERAL NERVE

by

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Abstract

Development and maintenance of the peripheral nervous system (PNS) relies on glial cells that insulate and protect axons. In invertebrates, wrapping glia isolate axons into separate bundles similar to non-myelinating Schwann cells (NMSCs) in vertebrate Remak bundles. The mechanisms by which NMSCs communicate with each other remains unknown. In this thesis, we focused on identifying and characterizing proteins required for glia-glia communication in non-myelinating classes of glia using the Drosophila peripheral nerve as our model.

We show that Innexin1 (Inx1) and Innexin2 (Inx2) based gap junctions (GJs) exist between two peripheral glial layers, the subperineurial glia (SPG) and wrapping glia (WG). WG survival is dependent on its communication with the SPG and is mediated by a channel rather than adhesive function of Inx2. Inx2 GJs mediate calcium pulses exclusively in the SPG and WG survival is not dependent on Ca\(^{2+}\) and inositol 1,4,5-trisphosphate (IP3). Therefore, we find that GJs mediate glia-glia communication to ensure the survival of WG through an unknown mechanism.

We next tested the role of scaffolding complexes in mediating glia-glia communication and screened for the role of the PSD95-Dlg-ZO1 (PDZ) family of proteins. We identified a role for Dlg5, a membrane-associated guanyl kinase protein, in peripheral glia. Loss of Dlg5 results in glial disruptions, including loss of septate junction formation and axonal ensheathment. Dlg5 has multiple roles identified in other systems including trafficking of cadherins. However, in glia the loss of Dlg5 did not affect cadherin localization to spot adherens junctions (SAJs). Therefore, we find that Dlg5 plays a novel role in peripheral glial development.
SAJs were previously identified in the *Drosophila* peripheral glia, but the composition and function of this complex had not been characterized. We find that classical cadherins associate with catenins in the peripheral nerve, and loss of DE-Cad but not DN-Cad leads to disruptions in glial morphology. However, loss of DE-Cad does not affect SAJ assembly, suggesting a redundancy with DN-Cad in peripheral glia.

Taken together this thesis provides novel insights and proposed models by which glia communicate in the PNS and will help direct future work in NMSCs in all animals.
Lay Summary

Efficient delivery of signals from the brain to different regions in the body requires proper insulation of neurons by glial cells. This task requires glia-glia communication to ensure a stable glial wrap, but how glia communicate with each other is not well known. I examined the role of three types of proteins in glial communication. The first protein Innexin2, is a protein that forms channels between cells for direct communication, the second is Dlg5, a protein that facilitates protein-protein interactions. Lastly, I examined cell-cell adhesion molecules called cadherins. I found that all three proteins are required for glial insulation of neurons. Furthermore, Inx2 is required for glial survival whereas Dlg5 and cadherins are involved in the formation of a sealing structure, the septate junction that protects nerves from the surrounding blood. I have therefore identified new mechanisms by which glia communicate and have highlighted their importance in nerve function.
Preface

Chapter 2: “Gap junction-mediated communication between different glial cells is required for glial survival and axon ensheathment in the peripheral nervous system.”

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Title: Gap junction-mediated communication between different glial cells is required for glial survival and axon ensheathment in the peripheral nervous system.

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For this publication, I contributed to the research design, data collection, analysis, writing the manuscript. TEM images were provided by Till Matzat and mutants were generated by Xiaojun Xie. Vanessa J Auld contributed to research design, data interpretation, writing the manuscript.

All authors contributed to manuscript editing.

Chapter 3: “A screen for key PDZ proteins identified a role for Dlg5 in peripheral glia development”

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For this manuscript, I contributed to the research design, data collection, analysis, writing and editing of the manuscript. TEM images were provided by Till Matzat. Vanessa J Auld contributed to the design, interpretation, writing and editing of the manuscript.
Chapter 4: “Cadherins in the Drosophila peripheral nerve”

Work in this chapter is a continuing effort to characterize the role of cadherins in peripheral glia development.

For this chapter, I contributed to research design, data collection, analysis, writing and editing. TEM images were provided by Till Matzat. Vanessa J Auld contributed to research design, data interpretation, writing and editing.
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<th>Description</th>
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<tbody>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>Aka</td>
<td>Anakonda</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl</td>
</tr>
<tr>
<td>Arm</td>
<td>Armadillo (Drosophila beta-catenin)</td>
</tr>
<tr>
<td>ATPα</td>
<td>Na/K ATPase alpha subunit</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BNB</td>
<td>Blood-nerve barrier</td>
</tr>
<tr>
<td>CadN2</td>
<td>Drosophila N-Cadherin2</td>
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<tr>
<td>CED-1</td>
<td>Cell death abnormality protein 1</td>
</tr>
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<td>CMTX</td>
<td>Charcot-Marie Tooth disease</td>
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<td>Drosophila E-Cadherin</td>
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<td>DGC</td>
<td>Dystrophin-glycoprotein complex</td>
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ECM Extracellular matrix
ER Endoplasmic reticulum
Fas3 Fasciclin3
Gcm Glial cells missing
GJ Gap junction
Gli Gliotactin
GFP Green fluorescent protein
GUK Guanyl kinase
HRP Horseradish peroxidase
IMA Inner mesaxon
Inx Innexin
IP3 Inositol 1,4,5-trisphosphate
JAM Junctional adhesion molecules
Kune Kune-kune
Lac Lachesin
LamG Laminin globular domain
LanB1 Laminin B1
LanB2 Laminin B2
MAGI Membrane associated guanyl kinase, WW and PDZ domain-containing protein 1
MAGUK Membrane associated guanyl kinase
MAG Myelin associated glycoprotein
Mcr Macroglobulin complement-related
MEGF10 Multiple epidermal growth factor domains 10
MMP1  Matrix metalloproteinase 1
N-Cad  N-cadherin
NMJ    Neuromuscular junction
NMSC   Non-myelinating Schwann cell
Nrg-1  Neuregulin-1
Nrg    Neuroglian
NrxIV  Neurexin IV
Nrv2   Nervana 2
Ogre   Optic ganglion reduced
OMA    Outer mesaxon
OPC    Oligodendrocyte precursor
PATJ   Pals1-associated tight junction protein
Panx   Pannexin
PCCD   Primitive classic cadherin domain
PDZ    PSD95/Discs large/Zona occludens 1
PG     Perineurial glia
PLA    Proximity ligation assay
PMLD   Pelizaeus-Merzbacher-like disease
PN     Paranode
PNL    Paranodal loop
PNS    Peripheral nervous system
PP4    Protein phosphatase 4
PSC    Perisynaptic Schwann cell
<table>
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<tr>
<td>pSJ</td>
<td>Pleated septate junction</td>
</tr>
<tr>
<td>repo</td>
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</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
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<td>Spot adherens junction</td>
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<tr>
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Dedication

To my parents for making many sacrifices so I could pursue my passion for research

To my brothers for always advising me and being such good role models

To Rodrigo for always reminding me to have faith in myself
Chapter 1: Introduction

The nervous system is composed of two major cell types: neurons and glia. For decades scientists focused on neurons whereas the role of glial cells remained relatively underappreciated. Glial cells not only outnumber neurons in the human brain but are also involved in almost all neural functions (Ndubaku and de Bellard, 2008). Work over the last two decades has shown that rather than playing passive roles, glia act as master regulators of many aspects of nervous system development, function and disease (Barres, 2008, Stork et al., 2012). For instance, glial cells, once thought to primarily support neurons, are now known to be active participants in synaptic plasticity (Todd et al., 2006). Although the range of glial functions known to us today is much broader than in the past, they only partially represent the true potential of glial cells in the nervous system. Despite our progress, the underlying molecular mechanisms and pathways required to accomplish glial functions are yet to be fully understood. For example, glial cells in the peripheral nervous system (PNS) are known to ensheath axons however, how glial cells interact and communicate with each other to initiate axonal ensheathment as well as maintain the glial sheath around the axons remains largely unknown.

Work in this thesis will focus on the importance of glia-glia communication and adhesion in the Drosophila peripheral nervous system (PNS). This thesis will also discuss how these mechanisms might differ or remain conserved in vertebrate glial cells.

1.1 Vertebrate and Drosophila glia

The vertebrate nervous system is composed of four major glial cell types: astrocytes, oligodendrocytes, Schwann cells and microglia. Astrocytes are the most abundant glial cells of the vertebrate central nervous system (CNS) and can be divided into three types, the
protoplasmic, fibrous and radial astrocytes. Together these astrocytes perform diverse functions such as the buffering of extracellular ions, metabolism and clearance of neurotransmitters from synaptic clefts, production of neurotrophic factors and regulation of the blood-brain barrier (Broux et al., 2015, Sofroniew and Vinters, 2010). Another type of glial cell in the CNS is the oligodendrocyte. These cells are the myelin forming cells of the CNS and extend multiple processes that insulate axonal segments and are required for a high nerve conduction velocity (Kettenmann and Verkhratsky, 2013). Apart from the myelinating oligodendrocytes, there are two other populations of oligodendrocytes in the CNS, NG2 glia (also referred to as adult oligodendrocyte precursors (OPCs) or polydendrocytes) and satellite or perineuronal oligodendrocytes. NG-2 glia are considered a major glial subtype (Nishiyama, 2007, Nishiyama et al., 1999). The NG2 glia not only proliferate and differentiate into mature oligodendrocytes but also perform diverse functions. These glial cells interact with neurons and influence synaptic activity, they support and regulate the blood-brain barrier, coordinate myelination and angiogenesis and can mediate changes in behaviour and body weight (Birey et al., 2017).

Satellite oligodendrocytes were referred to as non-myelinating oligodendrocytes, until a recent study found that these cells myelinate axons of varying small diameters (Battefeld et al., 2016). The same study showed that satellite oligodendrocytes are connected to the astrocyte syncytia and respond to action potentials by buffering $K^+$ (Battefeld et al., 2016). Apart from myelination, studies in the past have shown that satellite glia might also play a role in remyelination (Ludwin, 1979).

In the PNS, axons are insulated by the Schwann cells. However, unlike oligodendrocytes each Schwann cell can only wrap one axonal segment at a time. Moreover, Schwann cells can be divided into different classes: non-myelinating Schwann cells and myelinating Schwann cells.
As their name suggests, the myelinating Schwann cells form myelin sheaths and usually wrap large caliber axons. Non-myelinating Schwann cells, on the other hand, do not generate myelin. Instead, these cells engulf several small-caliber axons such as C-fibers, forming the so-called Remak Bundle (Griffin and Thompson, 2008) (Fig. 1.1A). Moreover, non-myelinating Schwann cells at the neuromuscular junction (NMJ) called the perisynaptic Schwann cells (PSCs) perform astrocyte-like functions (Auld and Robitaille, 2003). PSCs ensheath perisynaptic nerve terminals and respond to changes in neurotransmitter release and denervation. These cells help re-establish synapses during reinnervation and can secrete or respond to several trophic factors (Auld and Robitaille, 2003).

Lastly, microglia are the immune cells of the CNS. These cells continuously send out and retract their protrusions to scan the surrounding domains for lesions. Upon sensing an injury they undergo ‘microglial activation’, cluster around the injured area and act as phagocytes that remove apoptotic debris (Kettenmann and Verkhratsky, 2013). Therefore, astrocytes, oligodendrocytes, and microglia play important and diverse roles in the vertebrate CNS, whereas Schwann cells play similar roles to the myelinating and non-myelinating oligodendrocytes and astrocytes in the PNS.

The fruit fly nervous system shares many common features with its mammalian counterpart, with glial numbers being the major difference between the two. Although the mammalian brain has far more glia than *Drosophila*, the fruit fly nervous system has a wide array of glial subtypes that share morphological and functional similarities to mammalian glia (Freeman, 2015, Freeman and Doherty, 2006). The *Drosophila* larval nervous system includes a CNS, consisting of the central brain lobes and ventral nerve cord (vertebrate spinal cord equivalent) and a PNS that includes peripheral nerves and neuromuscular junctions and sensory
neurons. In the *Drosophila* CNS, glial cells are divided into three main classes: Neuropile glia, Cortex glia and Surface glia. The neuropile glia cell body lies in the synaptic neuropil while it extends its processes to ensheath axon fascicles similar to oligodendrocytes (Freeman and Doherty, 2006). Neuropile glia can be subdivided into ensheathing glia, which extend processes along the outer surface of the neuropil to isolate axons, and the astrocyte-like glia that are involved in neurotransmitter recycling. Ensheathing glia are also known to be involved in clearing of neuronal debris via phagocytosis similar to microglia (Stork et al., 2012). Moreover, astrocyte-like glia not only perform similar functions as mammalian astrocytes but also have a star-like appearance similar to their mammalian counterparts (Stork et al., 2012). Cortex glia lie within the cortex where they are in close contact with neuronal cell bodies (Pereanu et al., 2005, Dumstrei et al., 2003). Each cortex glial cell is capable of ensheathing dozens of neuronal cell bodies. This glial subtype also provides metabolic and trophic supports for neurons (Stork et al., 2012). Lastly, surface glia surround the *Drosophila* CNS and can be further divided into outer Perineurial glia (PG) and inner Subperineurial glia (SPG). The PG cells along with the SPG cells that form elaborate septate junctions (SJs) regulate the blood-brain-barrier (BBB) (Freeman and Doherty, 2006). However, the exact contribution of the PG is currently unknown. These glial cells also take up sugars from the hemolymph and process it to alanine and lactate that are required for the survival of neurons (Volkenhoff et al., 2015).

The *Drosophila* peripheral nerve is ensheathed by three glial layers. Similar to the CNS, the PG form the outermost layer and contact the overlying ECM. The middle layer is created by the SPG, which create the blood-nerve barrier through septate junctions. The final central layer is created by wrapping glia (WG) which ensheath individual or bundles of axons (Figure 1.1B). *Drosophila* wrapping or ensheathing glia do not myelinate their associated axons. Instead they
function similar to non-myelinating Schwann cells or non-myelinating oligodendrocytes. The organization of the multiple glial layers around the axon in Drosophila nerves, however, resembles the morphology of the vertebrate myelin sheath. Furthermore, key components required for formation of myelin-axon junctions (paranodal junctions) in mammals are also found in Drosophila glia in the septate junctions (Baumgartner et al., 1996, Bhat et al., 2001). Therefore, despite the differences between Drosophila and mammalian glia, many functions and molecular components between the two are conserved (Freeman and Doherty, 2006, Rodrigues et al., 2011, Matzat et al., 2015, Stork et al., 2012). Moreover, the wide range of genetic tools available in Drosophila make it an excellent model to answer basic questions about development of glia in all animals. The following section will compare the development and interactions of vertebrate and Drosophila glia in peripheral nerves.

1.1.1 Schwann cell development and Schwann cell-axon interactions

Glial cells of the vertebrate PNS, the Schwann cells, originate from neural crest cells. Neural crest cells segregate from the tips of the neural fold as it fuses to form the neural tube. These cells migrate, proliferate and differentiate to form a wide variety of cells such as neurons, glia, pigment cells, cartilage and smooth muscle (Le Douarin and Smith, 1988, Weston, 1991). The generation of the Schwann cells is preceded by the production of two cell types: Schwann cell precursors (SCPs) and immature Schwann cells. Both of these cell types intimately associate with axons unlike neural crest cells. The SCPs make this association during early stages (E14-15 rat, E12-13 mice) and generate immature Schwann cells during later stages (E17-birth rat, E13-15 mice) (Jessen and Mirsky, 2005). The intimate association of SCPs with axons is essential as these axons secrete Neuregulin-1 (Nrg1), a potent factor for survival and proliferation of SCPs
(Jessen and Mirsky, 2005). In time, SCPs stop migrating to generate immature Schwann cells that ultimately diverge into two mature Schwann cell populations.

The postnatal fate of the immature Schwann cells is determined by the type of axons they associate with. A process known as radial sorting precedes the ensheathment step and continues postnatally (Monk et al., 2015). Radial sorting can be divided in stages. During the first stage, axons are grouped into bundles and surrounded by a common basal lamina which is deposited by a group of 3-8 Schwann cells. This stage is followed by the projection of Schwann cell processes into the axon bundles to sort the large caliber axons destined to be myelinated away from the small caliber axons that eventually reside in Remak bundles (Monk et al., 2015). The amount of Nrg1-type III presented by the axon determines the fate of the Schwann cell ensheathing it. Large caliber axons express higher levels of Nrg1-type III and therefore instruct Schwann cells to take on a myelinating fate, whereas low levels of Nrg1-type III associated with small caliber axons instruct a non-myelinating fate (Monk et al., 2015, Taveggia et al., 2005). During the pro-myelin stage, each Schwann cell associates with a single large diameter axon forming a 1:1 relationship. The process of axon sorting has been extensively studied and the ECM components, including laminin (Yu et al., 2005) and β1 integrin (Feltri et al., 2002), are known to be required for axon sorting and subsequent myelination. The role of the ECM in axon sorting and myelination will be discussed in detail in Section 1.1.4.

In summary, there are two types of glial cells that directly ensheath axons in the vertebrate peripheral nerve. The large caliber axons are wrapped by the myelinating Schwann cells which form a multilayered sheath whereas, non-myelinating Schwann cells engulf several small caliber axons to form Remak bundles. The entire nerve bundle is eventually wrapped by tight junction forming perineurial glia and the extracellular matrix (ECM).
1.1.2 **Embryonic glia development and glia-axon interactions in Drosophila**

In *Drosophila* three types of glial cells ensheath peripheral axons (Fig. 1.1B). The embryonic ventral nerve cord contains 30 neuroectoderm derived neuroblasts in each hemisegment (Stork et al., 2012) that give rise to most glial cells. All glial cells except the midline glia express the transcription factors glial cell missing (Gcm) and reversed polarity (repo) (Lee and Jones, 2005, Jones et al., 1995). Glial cells in the developing ventral nerve cord express Gcm as early as stages 10-11, but Gcm expression fades by embryonic stage 14. In comparison, Repo is expressed by glial cells shortly after Gcm, at approximately stage 11 (Campbell et al., 1994, Halter et al., 1995) and continues to be expressed throughout the *Drosophila* life cycle (Stork et al., 2012). Unlike glia in the CNS, which are born close to where they are needed, peripheral glia migrate over long distances from their birth site. During *Drosophila* embryogenesis, peripheral glia proliferate in the CNS until stage 13. During stage 13 they arrange into a cone shape at the CNS/PNS border. By the end of stage 13, the peripheral glial array loosens and the peripheral glia migrate out along the pioneering motor nerves that have preceded them into the periphery (Sepp et al., 2001, Sepp and Auld, 2003, Banerjee et al., 2006b). Glia migrate as a train of cells along the pre-established motor axon tracts and absence of these tracts blocks migration (Sepp et al., 2001). Around the same time, pioneering sensory axons born in the periphery extend their growth cones toward the CNS making contact with the glia exiting the CNS (Sepp et al., 2001). By embryonic stage 16, peripheral glial migration along nerve tracts is complete and their processes fully ensheath major axons tracts (Banerjee et al., 2006b). The embryonic glial sheath transitions to a three-layered sheath after embryogenesis during the 1st larval instar (Fig. 1.2).
The WG starts ensheathing individual peripheral axons at 1st larval instar and this process is very similar to the formation of Remak bundles by the non-myelinating Schwann cells (Stork et al., 2008). During this early larval stage, some SPG cells still directly contact axons (Stork et al., 2008) (Fig. 1.2A). The direct contact between axons and SPG cells is progressively lost during the 3rd larval instar stage as the WG cells complete ensheathment of individual axons or small bundles (Fig. 1.2B). SJs forming the blood-nerve barrier are present between apposing subperineurial membranes that encircle the entire nerve bundle. These SJs are homologous to the mammalian paranodal junctions (PJs) formed at regions flanking the nodes of Ranvier in myelinating glia (Banerjee and Bhat, 2008, Bhat, 2003). The SPG are surrounded by the outermost glial layer called the PG (Stork et al., 2008), which itself is covered by the neural lamella, an extracellular matrix. The specific function of PG still remains unclear (von Hilchen et al., 2013). Unlike the polyploid WG and SPG that can no longer divide by the larval stages, the PG originate from a single embryonic glial cell that undergoes mitosis throughout development (von Hilchen et al., 2013). Therefore, the outermost PG, the intermediate SPG and innermost WG constitute the three distinct layers that ensheath axons in Drosophila (Fig. 1.2). Therefore, Drosophila peripheral glia form multi-layered membrane sheaths around axons that are morphologically similar to the myelin sheath in vertebrates. In addition, the molecular mechanisms that control axonal wrapping are similar in vertebrates and Drosophila (Rodrigues et al., 2011, Matzat et al., 2015). For example, the mechanism by which glial cells differentiate after axonal injury in vertebrates resembles that of WG differentiation in Drosophila (Matzat et al., 2015). In vertebrates, activation of EGF receptors in SCs by axon-derived Nrg1-type I leads to SC differentiation and remyelination of axons (Stassart et al., 2013). Similarly, WG
differentiation in *Drosophila* is facilitated by the homologue of Nrg, Vein, which is essential for EGF mediated axonal ensheathment (Matzat et al., 2015).

1.1.3 **Glia-axon interactions during axonal injury and degeneration**

Glia-axon interactions during axonal injury and degeneration play a major role in normal nerve function but are also essential for nerve repair. The degenerative process that occurs in injured axons is called Wallerian degeneration (Waller, 1850, Wang et al., 2012). Upon peripheral nerve injury, Schwann cells first begin to shed their myelin, and then proliferate, produce cytokines/trophic factors and phagocytose the detached myelin debris. Removing the myelin debris is essential as they secrete molecules that inhibit axon growth (Huang et al., 2005). Moreover, the trophic factors and cytokines secreted by the Schwann cell recruits immune cells, macrophages, into the site of injury (Shen et al., 2000). These recruited macrophages then take on the primary role of phagocytosing debris. Time course analyses in zebrafish show that Schwann cells phagocytose distal axonal fragments of degenerating axons (Xiao et al., 2015) and the process of clearing myelin debris is mediated by phagocytic TAM (Tyro3, Axl and Mertk) receptors (Brosius Lutz et al., 2017). Similar roles are performed by astrocytes and microglia in the CNS. Astrocytes contribute to the refinement of neural circuits in the developing CNS by engulfing neuronal debris. Moreover, engulfment of synapses and apoptotic cells by astrocytes is also mediated by a phagocytic receptor MEGF10, as well as Mertk (Chung et al., 2013, Iram et al., 2016).

Interestingly, extensive work in *C. elegans* and *Drosophila* were instrumental in deciphering the phagocytic role of astrocytes in vertebrates (Reddien and Horvitz, 2004, Tasdemir-Yilmaz and Freeman, 2014). Indeed, the *C. elegans* receptor CED-1 and its *Drosophila* orthologue Draper have well conserved functions with their mammalian orthologue MEGF10. Draper signals...
through a Src family kinase cascade to remodel the cytoskeleton and leads to engulfment of cellular debris (Ziegenfuss et al., 2008). Both Draper and MEGF10 are required by glial cells for the clearance of apoptotic cells and axon pruning (Awasaki et al., 2006, Tasdemir-Yilmaz and Freeman, 2014, Irham et al., 2016). Moreover, several studies have shown that Draper is not only involved in pruning and apoptosis but is also required for the clearance of neuronal debris in the injured adult brain (Winfree et al., 2017, Musashe et al., 2016, Purice et al., 2016). However, the role of MEGF10 in glia responding to injury has not yet been assessed. Furthermore, it was shown that Drosophila glia undergo expansion in response to axonal injury (MacDonald et al., 2006). The mechanism underlying this expansion of glial membrane is not entirely understood and it is postulated that the expansion might represent glial hypertrophy or redistribution of glial processes toward the site of injury (Logan and Freeman, 2007). Recent studies have shown that axonal injury activates PP4, Rho GTPase Rac1 (Winfree et al., 2017) and MMP1 (Purice et al., 2017). These proteins are required for cytoskeletal remodeling and infiltration of glial cells into the site of injury. Interestingly the process of glial engulfment has parallels to axonal ensheathment in the peripheral nerve since both processes involve expansion/extension of glial membranes and engulfment/wrapping. Therefore, it is possible that mechanisms underlying both processes are similar. Proper axonal ensheathment however, does not solely rely on glia-axon interactions. The following sections will discuss the importance as well as similarities and differences between glial-ECM interactions in the vertebrate and Drosophila peripheral nerve.

1.1.4 Schwann cell-ECM interactions

One of the first steps required for the process of glial wrapping is the secretion of the ECM, including its components such as laminins by Schwann cells. Once the Schwann cells
establish their ECM, the process of radial sorting relies on interactions between receptors expressed by the Schwann cell and ECM components such as laminins. Laminins are heterotrimeric composed of one alpha, one beta and one gamma subunit. There are 16 different isoforms of laminin in vertebrates that arise from different combinations of five alpha, three beta and three gamma subunits (Hohenester and Yurchenco, 2013). Laminins perform three overlapping functions: 1) they provide structure to the basement membrane (Yurchenco and Wadsworth, 2004); 2) they interact with cell surface receptors to present attachment sites (Henry and Campbell, 1996) and 3) they act as ligands for receptors on cells to regulate cell survival and behavior.

Several studies have highlighted the role of laminins in the process of radial sorting which involves the extension of glial processes around axons (Feltri et al., 2002, Chen and Strickland, 2003, Wallquist et al., 2002, Yang et al., 2005). Laminins have two main receptors; integrins and dystroglycans. Integrins are heterodimeric receptors composed of alpha and beta subunits. In vertebrates, there are 18 alpha and 8 beta subunits, which can give rise to 24 types of integrin receptors (Hynes, 2004). Depending on the specificity of the alpha and beta subunits, the integrin receptors can bind several types of ECM components. In vertebrate Schwann cells, integrins (α6β1, α7β1, α6β4, α3β1) bind two specific laminin isoforms required for radial sorting (Nakagawa et al., 2001, Sonnenberg et al., 1991, Yang et al., 2005). Moreover, the absence of integrins in Schwann cells arrests radial sorting at early stages (Feltri et al., 2002, Berti et al., 2011). Therefore, laminin interacts with integrin to control at least some aspects of Schwann cell development.

Dystroglycan consists of two subunits (alpha and beta) and was first identified as an integral component of the dystrophin-glycoprotein complex (DGC), a multimeric complex
isolated from skeletal muscle membranes (Ervasti et al., 1991, Ibraghimov-Beskrovnaya et al., 1992). In muscle tissue, dystroglycan was identified as a physical link between the ECM and the cytoskeleton (Masaki and Matsumura, 2010). In Schwann cells however, it is often studied as a laminin receptor due to similar phenotypes observed between laminin and dystroglycan mutants (Colognato and Tzvetanova, 2011). Unlike integrins, dystroglycans play a critical role during later developmental stages as Schwann cells lacking dystroglycan undergo normal radial sorting and proliferation (Feltri et al., 2002) but sorting only arrests during later stages. Therefore, dystroglycan is required at a later step than integrins during the process of axon sorting (Berti et al., 2011). Although lack of integrins in Schwann cells leads to radial sorting defects, loss of laminins not only lead to radial sorting defects but also cause more severe phenotypes in early Schwann cell development such as reduced Schwann cell proliferation (Feltri et al., 2002, Saito et al., 2003). These results suggest that laminins can not only signal through integrins and dystroglycan but could possibly have a receptor-independent function. It is also possible that laminins interact with other unknown receptors. Apart from its role in myelinating Schwann cells, laminins also play an important role in the development of non-myelinating Schwann cells (Yu et al., 2009). Laminin deficient mice not only lack Remak bundles, but also display insensitivity to heat stimuli. Moreover, loss of laminin contributes to the pathogenesis of many neuropathies such as merosin-deficient congenital muscular dystrophy (Masaki and Matsumura, 2010), Charcot-Marie Tooth 4F (Brennan et al., 2015), neurofibromatosis (Hilton and Hanemann, 2014) and leprosy (Singh et al., 1997). Many laminin receptors such as integrins are not only expressed by myelinating SCs but also by the NMSCs (Feltri et al., 1994, Niessen et al., 1994, Masaki et al., 2000, Previtali et al., 2003a, Previtali et al., 2003b) and it is likely that a loss of laminin signaling in NMSCs also contributes to these neuropathies. Therefore, Schwann cell-
ECM interactions are required for the development of both myelinating and non-myelinating SCs and play important roles in axonal ensheathment.

1.1.5 Glia-ECM interactions in the Drosophila larval peripheral nerve

Most of the components that make up the ECM in the vertebrate peripheral nerve have direct counterparts in Drosophila. For example, two laminin isoforms are found in Drosophila, which are composed of a combination of two alpha subunits (Wing blister/Wb and Laminin A/LanA), one beta subunit (Laminin B1/LanB1) and one gamma subunit (Laminin B2/LanB2) (Urbano et al., 2009). Knockdown of LanB1 and LanB2 in glial cells results in glial swellings, due to LanB2 or LanB1 accumulation in the endoplasmic reticulum (ER) (Petley-Ragan et al., 2016). However, similar to vertebrate laminin, laminins in Drosophila glia are also essential for ensheathment of axons (Petley-Ragan et al., 2016). Knockdown of LanB2 in glia leads to loss of glial wrapping around axons and to gaps between the wrapping glia processes. Whether the loss of glial wrap in LanB2 knockdowns is a consequence of receptor mediated signaling or remains to be elucidated. There are two integrin heterodimers in Drosophila peripheral nerves; α2βPS and α3βPS (Keereweer et al., 2012, Brown et al., 2000). Interestingly, similar to the results observed with the LanB2 knockdown, integrin knockdown in the wrapping glia also leads to lack of wrapping glia process extension around axons or axons bundles (Keereweer et al., 2012), suggesting that integrin may bind laminin either between axons and WG or between WG and SPG. Apart from the lack of wrapping glia extension, integrin knockdown also leads to loss of PG wrapping around axons (Xie and Auld, 2011). Since the loss of laminin secretion from glia leads to different glial phenotypes, such as glial swelling, it is possible that the lack of ensheathment by the PG in integrin knockdown larvae is not laminin mediated. However, it has
also been suggested that similar phenotypes were not observed in the laminin and integrin knockdown as laminins are not only secreted by glia but can also be provided by other cells in *Drosophila*. The fat body and hemocytes also contribute to the deposition of ECM components in the basement membrane surrounding the nervous system (Bunt et al., 2010, Pastor-Pareja and Xu, 2011). Glial cells in the *Drosophila* optic stalk are also known to rely on integrin mediated signaling for their migration and differentiation (Xie et al., 2014). Therefore, laminin and integrin perform similar functions in *Drosophila* axonal ensheathment compared to their vertebrate counterparts in SC-ECM signaling.

### 1.2 Cellular junction in peripheral glia

Vertebrates and invertebrates have both developed several types of cellular junctions that allow cell-cell interactions and these types of junctions are very important in glia. The major types of cellular junctions in vertebrate tissues include focal adhesions, adherens junctions (AJs), desmosomes, hemidesmosomes, gap junctions (GJs) and tight junctions (TJs). Although AJs and GJs are expressed in most animal tissues, desmosomes, hemidesmosomes and tight junctions appear to be absent in *Drosophila* (Tepass and Hartenstein, 1994). *Drosophila* on the other hand possess, a different kind of occluding junction called the septate junction (SJ). Moreover, SJ-like structures called paranodal junctions are present in the myelinated CNS and PNS axons, at the paranodes flanking the Nodes of Ranvier and form between myelinating glia and the underlying axon. In comparison to myelinating Schwann cells, the identity and function of glial adhesion complexes in non-myelinating Schwann cells and glial cells of the *Drosophila* peripheral nerve remain unknown. Glia-glia and glia-ECM interactions in the peripheral nerve rely on these junctions and will be discussed in the following sections.
1.2.1 Adherens junctions

Adherens junctions, as the name suggests are required for basic cell-cell adhesion. These junctions link membrane and cytoskeletal components at discrete regions. In epithelial cells the localization of the molecular components of the AJ are critical for processes such as cell polarization, where the location of the AJ defines an apico/basolateral axis (Nejsum and Nelson, 2007). During tissue morphogenesis, AJs link the cytoskeletons of adjacent cells to enable coordinated movements (Lecuit and Yap, 2015, Harris and Tepass, 2010). Therefore, mislocalization or loss of function of AJ components can lead to loss of polarity, uncontrolled migration and proliferation, as seen during tumor development (Berx and van Roy, 2009, Kaplan et al., 2009). AJs consist of two main adhesive components: the cadherin/catenin and nectin/afadin complexes. The role of the classical cadherins, E-Cadherin and N-Cadherin, alone will be covered in the scope of this thesis. Classical cadherins were the first family of adhesion components found at the AJ and consist of type I single-pass membrane glycoproteins that mediate cell adhesion in a calcium dependent manner. Vertebrate type I classical cadherins are composed of five extracellular cadherin repeats and catenin-binding cytoplasmic tail (Oda et al., 2005, Hulpiau and van Roy, 2009). The homophilic interactions between the extracellular domains of identical cadherins which are located on the membranes of neighbouring cells results in formation of cell-cell junctions. The cytoplasmic domains of cadherins mediate signaling activities required for adhesion via their association with three types of catenins; beta-catenin also known as Armadillo (arm) in *Drosophila*, alpha-catenin and p120-catenin (Ozawa et al., 1989). While beta-catenin binds directly to cadherins, it also contains binding sites for alpha-catenin (Aberle et al., 1996). Alpha-catenin in turn regulate actin dynamics and can bind actin
directly in vitro (Rimm et al., 1995, Desai et al., 2013). In contrast to alpha-catenin, p120-catenin does not bind actin directly, but instead regulates actin dynamics through its ability to inhibit the small GTPase, Rho (Anastasiadis et al., 2000). Moreover, in vertebrates, p120 regulates the level of cadherins at the membrane by antagonizing endocytosis (Davis et al., 2003, Xiao et al., 2003, Xiao et al., 2005). Therefore, the cadherin/catenin complex gives rise to the AJ complex in both vertebrates and Drosophila.

E-Cadherin and N-Cadherin mediate glia-glia interactions in the peripheral nerve

Unlike epithelial cells, where adherens junctions are formed between different cells, in myelinating Schwann cells, E-Cadherin form adherens junctions between membranes synthesized by the same Schwann cell (Fannon et al., 1995). Since these adherens structures form between the same contiguous Schwann cell membrane, adherens junctions in the myelinating peripheral nerve are called autotypic adherens junctions. E-Cadherin does not mediate adhesion between neighbouring Schwann cells or between Schwann cells and axons (Fannon et al., 1995). Regions of compact myelin and axons are devoid of E-Cadherin and it is exclusively expressed in non-compact regions of the myelin sheath which include, the outer and inner mesaxon, the Schmidt-Lanterman incisures and the paranodes flanking the nodes of Ranvier (Fannon et al., 1995) (Fig. 1.3).

Studies have shown that E-Cadherin is necessary for a properly formed outer mesaxon and that the maintenance of the Schmidt-Lanterman incisures relies on the interaction between E-Cadherin and p120 catenin (Young et al., 2002, Tricaud et al., 2005). The interaction between E-cadherin and p120 is essential for the regulation of Rho GTPase which may organize the cytoskeleton (Tricaud et al., 2005). Since the Schmidt-Lanterman incisures are important for the...
transport of metabolites across the myelin sheath (Balice-Gordon et al., 1998, Ghabriel and Allt, 1981), disrupting the AJs leads to loss of communication between the myelin layers. Moreover, knocking down p120 leads to hypomyelination suggesting that E-Cad might indirectly be required for myelination of axons (Perrin-Tricaud et al., 2007). In addition to its structural role, E-Cadherin also performs a signaling function that promotes axonal Nrg1 signaling necessary for myelin formation in vitro (Basak et al., 2015). The role of E-Cadherin in non-myelinating Schwann cells is unknown.

Apart from E-Cadherin, another classical cadherin called N-Cadherin is also expressed by myelinating Schwann cells. In vitro studies using Schwann cells co-cultured with dorsal root ganglia (DRG) neurons, show that N-Cadherin is required for early interactions between Schwann cells and axons, where N-Cadherin accumulates at the tips of migrating Schwann cells and directs their growth along the axon (Wanner and Wood, 2002). Furthermore, N-Cadherin also mediates Schwann cell-Schwann cell contacts (Wanner and Wood, 2002, Corell et al., 2010). Interestingly, a more recent study has shown that N-Cadherin is expressed in the cytoplasm of the non-myelinating Schwann cells and is concentrated at the Schwann cell-axon contacts as well as between the membranes of the same non-myelinating Schwann cell (autotypic junctions) (Corell et al., 2010). The presence of N-Cadherin in the cytoplasm suggests that N-Cadherin must be trafficked to the membrane. However, the identity of the pathway by which N-Cad is trafficked to the non-myelinating Schwann cell membrane is yet to be determined. Furthermore, less intense N-Cadherin was observed in the outer leaflet of the myelin sheath, however it is not clear whether N-Cadherin forms Schwann cell-axon or Schwann cell-Schwann cell contacts in this context (Corell et al., 2010). Therefore, both E-Cadherin and N-Cadherin are required for glia-glia adhesion in the vertebrate peripheral nerve, whereas N-Cadherin may also
mediate glial-axonal adhesion requires during earlier stages of development. These studies however, only provide a minor understanding of AJs in the peripheral nerve and a lot of questions still remain unanswered. In particular, the distribution of E-Cadherin in the non-myelinating Schwann cells has not yet been described. Many questions, such as whether both classical cadherins play overlapping/redundant roles in the non-myelinating Schwann cells and whether E-Cadherin like N-Cadherin is also able to form glial-axonal contacts with small caliber axons, remain unanswered.

Surprisingly, in comparison to our understating of AJs in the vertebrate peripheral nerve, not much is known in *Drosophila*. A single gene coding for E-Cadherin (DE-Cad) (Oda et al., 1994) and two genes coding for N-Cadherin (DN-Cad: CadN1 and CadN2)(Prakash et al., 2005, Iwai et al., 1997) are found in *Drosophila* (Fig. 1.4). Unlike the vertebrate classical cadherins, DE-Cad contains seven extracellular cadherin repeats followed by a primitive classical cadherin proteolytic site domain (PCPS, previously termed the non-chordate domain), an EGF-like and a laminin globular domain (LamG) (Oda et al., 1994) (Fig. 1.4). These three extra motifs together constitute the primitive classic cadherin domain (PCCD) (Oda and Tsukita, 1999). *Drosophila* CadN1 contains 16 extracellular cadherin repeats as well as the PCCD with two LamG domains (Iwai et al., 1997)(Fig. 1.4). On the other hand, CadN2 has only six extracellular repeats and does not exhibit adhesion activity (Yonekura et al., 2007). Nevertheless, the intracellular domains of both DE-Cad and DN-Cad bear a strong resemblance to vertebrate cadherins and despite the fact that the extracellular regions show significant differences in domain organization, both vertebrate and *Drosophila* cadherins share similarities in biological functions (Harris and Tepass, 2010, Nishimura and Takeichi, 2009, Takeichi, 2007). Although the roles of DE-Cad in epithelial tissues and that of DN-Cad in neural development have been studied, the
role of cadherins in *Drosophila* peripheral glia remains unknown. A previous study has identified spot adherens-like junctions between the different peripheral glial layers in *Drosophila* (Matzat et al., 2015) (Fig. 1.5). The role of these junctions, whether adhesive or not and what these junctions are comprised of remains unknown. Since the non-myelinating Schwann cells are similar to wrapping glia in *Drosophila*, it is possible that cadherins in the wrapping glia might function similarly to N-Cadherin in non-myelinating Schwann cells. This hypothesis has however, not yet been tested and is explored in Chapter 4 of this thesis.

### 1.2.2 Tight junctions and septate junctions

**Tight junctions**

In the vertebrate nervous system, tight junctions (TJs) in the perineurial cells form a selective diffusion barrier between the endoneurium and the epineurium (Thomas, 1963, Pummi et al., 2004). Apart from the perineurial cells, autotypic TJs also form in non-compacted regions of myelin and have been observed using transmission and freeze fracture electron microscopy (Mugnaini and Schnapp, 1974, Sandri et al., 1977, Tetzlaff, 1978, Tetzlaff, 1982). TJ strand-like structures form a circumferential belt and occur along incisures on both sides of a single unrolled flat Schwann cell (Poliak et al., 2002) (Fig. 1.6A). It is thought that when a Schwann cell is rolled around the axon, the TJ strand on one side of the Schwann cell pairs with the strand on its other side (Miyamoto et al., 2005)(Fig. 1.6A). Although the function of autotypic TJs is not entirely clear, they were proposed to have two main functions. TJs are considered important for adhesion, to function as a mechanical link and as a permeability barrier isolating the extracellular space surrounding the myelin sheath from the intramyelinic space between the lamellae (Mugnaini and Schnapp, 1974). The molecular composition of TJs in the peripheral nerve is not
as well established as in epithelial cells. In epithelial cells, TJs are composed of a mixture of adhesion proteins such as Zona occludens (ZO) (Stevenson et al., 1986), claudins ((Furuse et al., 1998, Morita et al., 1999), occludin (Furuse et al., 1993) and junctional adhesion molecules (JAMs) (Martin-Padura et al., 1998). Zona occludens are scaffolding proteins of the membrane associated guanyl kinase (MAGUK) family. Claudins are integral membrane proteins that have four transmembrane domains and are found at TJs. Members of the claudin family possess a PDZ-binding motif that allows them to interact with PSD95/Discs large/Zona occludens 1 (PDZ) proteins such as ZO1 and ZO2 at the TJ (Fanning et al., 1998, Itoh et al., 1999). These interactions lead to the formation of large macromolecular complexes that facilitate the localization of proteins to the membrane (Itoh et al., 1999, Fanning et al., 1998). Occludin, similar to claudins, is an integral membrane protein with four transmembrane domains and can directly interact with proteins of the ZO family via their C-terminal domain (Furuse et al., 1993). Members of the JAM superfamily, have an extracellular domain with two Ig-like domains, a single transmembrane domain and cytoplasmic tail (Martin-Padura et al., 1998). Dimerization of the JAM extracellular domains allow for both homophilic and heterophilic interactions. Moreover, similar to claudins and occludin, all members of the JAM family except JAM-L interact with scaffolding proteins such as ZO-1 via a PDZ-binding motif in their C-terminal domain (Bazzoni et al., 2000, Ebnet et al., 2001). In contrast to the epithelial TJs, the molecular composition of the TJs in Schwann cells still remains incomplete. So far, few members of the claudin family including Claudin-2 and Claudin-5 have been observed at the Schmidt-Lanterman incisures in adult rat sciatic nerves (Poliak et al., 2002). Interestingly, a study in human Schwann cells showed that apart from Claudin-2 and Claudin-5, Claduin-1 and Claudin-3 were also expressed at the Schmidt-Lanterman incisures. Moreover, Claudin-3 is also present in the
mesaxons in humans Schwann cells. The biological significance of these differences between humans and rodents remains to be elucidated. Moreover, earlier studies showed that JAMs were not expressed in the rodent peripheral nerve (Poliak et al., 2002), however JAM-C was later observed in regions of non-compact myelin (Kikuchi et al., 2010). Occludin is also expressed in the peripheral nerve but its expression is restricted to the perineurium and is not found in myelinating Schwann cells (Nagaoka et al., 1999). Autotypic TJs in rodents are also composed of Claudin-19 and tricellulin (TRIC), a novel tricellular TJ protein, with both expressed in regions of non-compact myelin (Miyamoto et al., 2005, Kikuchi et al., 2010). Claudin-19 is integral to the formation of TJs in rodent nerves, and mice deficient for Claudin-19 lacked autotypic TJs (Miyamoto et al., 2005) (Fig. 1.6B,C). When subjected to behavioural tests that evaluate PNS functions such as the rotarod test, where mice are put on a rotating rod and how long they remain on the rod is measured, the Claudin-19 deficient mice fell off quicker than wild type mice. Therefore, mice lacking TJs in Schwann cells suffer from peripheral neuropathies and it has been proposed that the lack of electro-physiological “sealing” function in Schwann cells leads to these behavioural abnormalities (Miyamoto et al., 2005). Additionally, most TJ components are either PDZ proteins themselves (MAGUKs like ZO-1) or have PDZ binding motifs in their C-terminal domains that allow them to interact with PDZ proteins (Poliak et al., 2002). These scaffolding proteins can potentially contribute to the formation of macromolecular complexes. Furthermore, PDZ proteins such as MUPP1, PATJ, MAGI and Par3 have been found to associate with TJ components in non-compact regions in Schwann cells (Poliak et al., 2002). These PDZ proteins are proposed to maintain the cyto-architecture of myelinating Schwann cells (Poliak et al., 2002) but their function at these junctions have not been fully described. Moreover, the function of PDZ proteins may extend beyond TJs as some PDZ proteins (ZO-1
and MAGI) are also present at AJs in myelinating Schwann cells (Ide et al., 1999, Nishimura et al., 2000). In comparison, TJ components are yet to be identified in the non-myelinating Schwann cells and nothing is known about their interactions with junctional proteins in these cells. The first step towards understanding the interactions between junctional components and PDZ proteins would be identify components of these complexes. Furthermore, in comparison to myelinating Schwann cells, far less is known about the glia-glia interactions between non-myelinating Schwann cells and whether autotypic TJs are present in these cells. For these reasons, we conducted a genetic screen to identify PDZ proteins required in Drosophila glial cells. The results from the screen will be described in Chapter 3 of this thesis.

**Septate junctions**

The “sealing” function in invertebrates is carried out by the septate junctions. Septate junctions are composed of different molecular components than those in TJs, however like TJs, these junctions form permeability barriers in the peripheral nerve. In the peripheral nerve SJs form between SPG and exhibit prominent ladder-like structures in electron micrographs (Banerjee et al., 2006b, Banerjee et al., 2006c, Bellen et al., 1998) (Fig. 1.7B). The blood-nerve barrier (BNB) formed by the SJ components expressed in the SPG, protects axons from changes in the concentration of ions in the extracellular environment (Auld et al., 1995). SJs can be divided into two different classes based on their morphology; pleated SJs and smooth SJs. Pleated SJs are found in ectoderm-derived tissues including glial cells and will be referred to as SJs through the thesis. SJs are composed of two groups of proteins, the core components that are necessary for barrier function and those that localize to the SJ but are not part of the main complex. Epithelial SJ are composed of a core protein complex some of which includes the
proteins Neurexin IV (Nrx IV), Coracle (Cora), Contactin (Cont), Varicose (Vari), Macroglobulin complement protein (Mcr), Neuroglian (Nrg) and the Na\(^+\)/K\(^+\) ATPase (Baumgartner et al., 1996, Genova and Fehon, 2003, Hortsch and Margolis, 2003, Paul et al., 2003, Batz et al., 2014, Hall et al., 2014, Bachmann et al., 2008, Moyer and Jacobs, 2008, Faivre-Sarrailh et al., 2004). SJ associated proteins are Disc large 1 (Dlg1), Scribbled (Scrib), Fasciclin 3 (Fas3), Lachesin (Lac), Sinuous (Sinu) and Kune–kune (Kune) (Woods and Bryant, 1991, Bilder, 2004, Wu et al., 2004, Nelson et al., 2010). Apart from these components, two proteins, Gliotactin (Gli) and Anakonda (Aka; aka Bark-beetle) have been identified as tricellular junctional proteins are found at the convergence of three cells or three SJ (Schulte et al., 2003, Byri et al., 2015, Hildebrandt et al., 2015). Many components of the epithelial SJs are also found in the peripheral nerve and have similar functions. For example, it has been show that the core components NrxIV, a member of the Neurexin family (Peles et al., 1997) and its intracellular adapter proteins, Coracle, Contactin an Ig domain protein (Faivre-Sarrailh et al., 2004) and Neuroglian (Bieber et al., 1989) are co-expressed in glial cells and are required for the formation of glia-glia SJs (Banerjee et al., 2006b) (Fig. 1.7A,C-C’). Moreover, their loss leads to the disruption of the BNB (Banerjee et al., 2006b). Furthermore, loss of Neuroglian also increases the intercellular spacing between glial layers, suggesting that Neuroglian, in addition to its role in SJ formation, is required for glia-glia adhesion (Banerjee et al., 2006b). Although SJs and vertebrate TJ s serve similar functions, the vertebrate homologues of Neurexin IV, Contactin, Neuroglian and Coracle are present at the vertebrate axo-glial junction called the paranodal junction (PJ) rather than TJ s (Fig. 1.8). The paranodal junction, as the name suggests, forms in the paranodal region flanking the nodes of Ranvier where the myelinating Schwann cells are in close contact with the axon. At this site, Neurofascin 155 (Neuroglian homologue) present in
myelin loops binds an axonal complex that consists of Caspr (Neurexin IV homologue) and Contactin. This complex is connected to the cytoskeleton via the interaction of Caspr and Protein Band 4.1 (Coracle homologue) (Fig. 1.8). This axo-glial interaction creates a SJ-like junction that organizes the axon into unique molecular domains such that the electrical activity at the nodes of Ranvier (distribution of Na⁺ channels) is separated from the internodal region (distribution of K⁺ channels)(Rosenbluth et al., 1995, Wiley and Ellisman, 1980). Therefore, SJ and SJ-like junctions (paranodal junctions) are required for both glia-glia and glia-axonal interactions that are necessary for the ensheathment of axons. In epithelial cells, many PDZ proteins are part of signaling complexes that dictate the localization of junctional proteins and ultimately the polarity of the cell (Bilder, 2001). Polarity in a myelinating cell is set up by autotypic junctions between the membranes of the Schwann cell which divide the cell into areas of non-compact and compact myelin. If and how non-myelinated Drosophila glial cells are polarized and which junctional components, including PDZ proteins play a part in this process remain unknown.

1.2.3 Gap junctions

Cell-cell communication is essential for a variety of biological processes such as development, differentiation, establishment of a neural or immune response, and mediating cell mobility (Maeda and Tsukihara, 2011). One of the ways by which direct cell-cell communication can occur is via gap junctions (GJs). There are three families of proteins required for forming junctions that facilitate cell-cell communication by allowing passage of ions and small molecules. They are the vertebrate connexins (Cx) and pannexins (Panx) and the invertebrate innexins (Inx). All three have comparable membrane topology and are four-pass transmembrane
proteins, with intracellular amino- (NH2) and carboxyl- (COOH) termini (Phelan, 2005) (Fig. 1.9A-C). There are 21 connexins and 3 pannexins in humans compared to 8 Drosophila innexins. Pannexins predominantly form only hemichannels due to the glycosylation of the extracellular loop that blocks association between hemichannels in adjacent cells (Boassa et al., 2007). In contrast, both connexins and innexins form homomeric or heteromeric hemichannels (Fig. 1.9G) (Holcroft et al., 2013, Evans et al., 2006, Bennett et al., 2003, Bruzzone et al., 1996, Phelan, 2005, Stebbings et al., 2000, Saez and Leybaert, 2014), that are capable of associating with hemichannels in apposing cell membranes to form gap junction channels (Fig. 1.9D-G). Thus, these channels link the membranes of two apposing cells together and permit rapid signaling over large population of cells. Typically, gap junctions function by forming intercellular channels between coupled cells. However, these proteins can also act as cell adhesion molecules (Elias et al., 2007). This is clearly evident in the developing rat neocortex, where Cx26 and Cx43 mediate the interaction between radial glial fibers and migrating neurons (Elias et al., 2007). In this study Cx mutants were able to form channels but not adhere and these failed to rescue Cx-RNAi induced radial migration defects, suggesting that the adhesive property was crucial for the migration process. Another study showed that the CT-domain of Cx43 was essential for neuronal migration in the mouse neocortex and CT-truncated forms of Cx43 failed to rescue neuronal migration defects (Cina et al., 2009). Some gap junction proteins can also function as hemichannels, allowing communication between intracellular and extracellular compartments. These channels therefore allow the release of autocrine and paracrine signaling molecules such as ATP and glutamate to the extracellular milieu (Saez et al., 2010, Belousov et al., 2017). Many Cx and Panx proteins perform hemichannel functions (Orellana, 2016, Giaume et al., 2013).
comparison, some leech and *C. elegans* innexins form hemichannels but evidence for Inx hemichannel function in *Drosophila* is yet to be found (Chuang et al., 2007, Bao et al., 2007).

**Gap junctions in glia-glia communication**

Myelinating cells in both the CNS (oligodendrocytes) and PNS (Schwann cells) express connexins. Schwann cells predominantly express Cx32 and Cx29 while oligodendrocytes express connexins 32, 29 and 47 ((Menichella et al., 2006, Dermietzel et al., 1991, Micevych and Abelson, 1991, Scherer et al., 1995, Li et al., 1997, Altevogt et al., 2002, Nagy et al., 2003, Odermatt et al., 2003, Kleopa et al., 2004). In myelinating Schwann cells, Cx32 is localized to non-compact myelin, with highest Cx32 in the paranodes and incisures (Bergoffen et al., 1993).

The location of Cx32 in non-compact regions of myelin suggests that Cx32 forms autotypic GJs between the membrane, providing a radial diffusion pathway (Fig. 1.10A). This hypothesis was tested by injecting dyes into live myelinating Schwann cells. It was shown that only low molecular mass dyes diffuse across the myelin sheath (Balice-Gordon et al., 1998). A radial pathway of diffusion across the myelin layers provides a shortcut to diffusion that is much faster than a circumferential pathway (Balice-Gordon et al., 1998). The radial diffusion pathway is not disrupted in Cx32 deficient mice (suggesting the involvement of other connexins) (Balice-Gordon et al., 1998). Nevertheless, Cx32 is essential for maintaining the functional and structural integrity of myelin as these mice develop a peripheral demyelinating neuropathy called X-linked Charcot-Marie Tooth disease (CMTX) (Scherer and Kleopa, 2012). Cx32 is also localized between the layers of myelin in oligodendrocytes but these GJs are less conspicuous than those in Schwann cells. Unlike Cx32, Cx29 is primarily expressed in the adaxonal membrane (apposing the axonal membrane), in the juxtaparanodal region of small myelinated fibers.
Moreover, Cx29 does not form GJ plaques or functional GJs (Ahn et al., 2008, Altevogt et al., 2002). It has been speculated that Cx29 forms hemichannels on the adaxonal membrane of both oligodendrocytes and myelinating Schwann cells (Kamasawa et al., 2005) (Fig. 1.10A). The distribution of connexins in non-myelinating Schwann is mostly unknown. Cx32 immunolabeling has been observed in non-myelinating Schwann cells (Scherer et al., 1995) (Fig. 1.10B). However, Cx32-immunoreactivity in the non-myelinated fiber was significantly reduced and the appearance was more punctate and discontinuous compared to that in myelinated fibers (Scherer et al., 1995). Earlier studies suggest that at least adjacent non-myelinating Schwann cells in culture are dye-coupled (Konishi, 1990), suggesting that GJs must be present between non-myelinating Schwann cells. The functional significance of these GJs however, remains unclear. Gap junctions also play an important role in astrocyte-astrocyte (A-A) coupling, forming a glial syncytium in the CNS connected by Cx43 and Cx30 (Scemes, 2000, Nagy and Rash, 2000, Dermietzel et al., 2000) (Fig. 1.11A,B). Gap junctions mediate calcium and inositol trisphosphate (IP3) transmission between a large group of astrocytes allowing long range signaling. In the presence of a stimulus, many channels and receptors in astrocytes allow for increases in internal calcium (MacVicar, 1984, Higashi et al., 2001). For example, the spatial buffering of K+ is mediated by the K+ channels and transporters located at the perisynaptic processes of astrocytes (Kofuji et al., 2002, Neusch et al., 2006, Walz and Hertz, 1982, Walz and Hertz, 1984). High extracellular K+ leads to increases in cytosolic calcium in astrocytes. Calcium and/or IP3 travel to adjacent astrocytes through gap junctions or via purinergic signaling that involve Pannexin 1 hemichannels (Suadicani et al., 2006, Scemes and Giaume, 2006). Unlike astrocytes, which are coupled to each other by gap junctions, Oligodendrocyte-Oligodendrocyte (O-O) coupling occurs in vitro (Massa et al., 1984, Gonatas et al., 1982) and, if they exist in vivo,
are extremely rare (Mugnaini, 1986). Interestingly, coupling between astrocytes and oligodendrocytes has been observed with Cx32/Cx30 and Cx47/Cx43 forming functional channels between astrocytes and oligodendrocytes (Orthmann-Murphy et al., 2007) (Fig. 1.11A,C). This study also found that Cx32/Cx30 and Cx47/Cx43 exhibited different permeabilities to dyes and had distinct voltage-gating properties (Orthmann-Murphy et al., 2007). Moreover, it was shown that when Cx47 is mutated it can no longer pair with Cx43 and this loss of functional Cx47/43 coupling might be the cause of Pelizaeus-Merzbacher-like disease (PMLD), a disease with a severe demyelinating phenotype (Orthmann-Murphy et al., 2007). Therefore, GJ mediated glia-glia communication has been described in the vertebrate literature. Hence, we began to test whether glia-glia communication between the different peripheral glial layers in Drosophila are also GJ mediated. This work will be described in Chapter 2 of this thesis.

1.3  Gap junctions in Drosophila

1.3.1  Innexins form connexin-like gap junction channels

The eight members of the *Drosophila* innexin family are Optic ganglion reduced (Ogre or Inx1), Inx2, Inx3, Inx4, Inx5, Inx6, Inx7 and ShakB (Bauer et al., 2005). Due to their analogous functions to connexins these proteins were named invertebrate-connexins or innexins. *In situ* hybridization studies show expression patterns for three innexins in glia; Ogre, Inx2, and Inx3 (Stebbings et al., 2002). My thesis will mostly focus on the role of Inx2 and its interaction with Inx1.

Inx2 mRNA is found expressed throughout the life of the fly, from as early as developing ovaries to late larval, pupal and adult stages (Stebbings et al., 2002). Studies show that Inx2 not
only forms homotypic channels (Stebbings et al., 2000) but also associates with other innexins such as Inx3 (Knieps et al., 2007, Stebbings et al., 2000) and Inx4 (Jacobs et al., 2000) to form heteromeric and heterotypic channels, respectively when expressed in *Xenopus* oocytes. Moreover, Inx2 is shown to form voltage-sensitive homotypic channels in oocytes (Fig. 1.12C) (Holcroft et al., 2013, Stebbings et al., 2002). Conversely, Inx1 does not form homotypic channels (Fig. 1.12B) and relies on another innexin to partner with (Holcroft et al., 2013) and it was demonstrated that if one oocyte expresses Inx1 and the other Inx2, the pair was not electrically coupled (Fig. 1.12D) however, co-expression of Inx1 and Inx2 RNA in paired *Xenopus* oocytes leads to formation of functional channels (Fig. 1.12E). These channels, unlike Inx2 homotypic channels, do not show a sensitivity of voltage (Holcroft et al., 2013).

Furthermore, while Inx2 can form non-junctional hemichannels when expressed in single oocytes, Inx1 only forms channels when co-expressed with Inx2 (Holcroft et al., 2013) (Fig. 1.12). These findings suggest that Inx1 is very likely to form heterotypic (Inx2-Inx1/2)/heteromeric (Inx1/2-Inx1/2) GJs with Inx2 in glial cells *in vivo*.

Very little is known about what passes through gap junction channels. In *Drosophila*, it has been demonstrated that Innexin 2 forms intercellular channels that transport GDP-<sub>L</sub>-fucose between wing disc epithelia (Ayukawa et al., 2012). In the *Drosophila* adult eye visual system, an Inx2 based network of gap junctions are required for the transportation of histamine metabolites between perisynaptic glia and photoreceptor neurons (Chaturvedi et al., 2014). There is also evidence that a leech (*Hirudo*) innexin, *Hm*-Inx2 is expressed in CNS glial cells (Dykes et al., 2004). The calcium waves in these glia cells trigger the release of ATP through *Hm*-Inx2 hemichannels that consequently leads to microglial migration towards the site of nerve injury (Samuels et al., 2010).
1.3.2 **Innexin 2 in glial cells of Drosophila CNS**

While *in situ* hybridization studies have shown expression patterns in glia for three innexins; *inx1*, *inx2*, and *inx3*, (Stebbings et al., 2002) recent immunofluorescence labeling data has shown that Inx1 and Inx2 co-localize in the *Drosophila* CNS (Holcroft et al., 2013). In particular, it was demonstrated that both innexins are expressed in repo-positive glial cells of the CNS rather than elav-positive neurons with the most overlap in the ventral ganglion midline (Holcroft et al., 2013). Furthermore, RNAi knockdown of Inx2 in a glial specific manner using the pan-glial driver, *repo-GAL4*, lead to a pronounced reduction in size of the developing CNS and these flies fail to eclose as adults (Holcroft et al., 2013). Knockdown of Inx1 also results in a decrease in the size of the larval CNS. In addition, this reduction is accompanied with behavioral defects including the inability to fly, circling behavior, and uncoordinated grooming behavior. All these behaviors are hallmarks of locomotor and sensorimotor defects. Furthermore, these flies unlike Inx2 knockdown flies, die a few days post-eclosion. This data suggests that Inx1 and Inx2 are required in glial cells for the development of the larval CNS. These results were consistent with another study by Spéder and Brand 2014, which showed that Inx1 and Inx2 form heteromeric channels in the subperineurial glia (SPG) of the CNS. This conclusion was based on the observation that Inx2 staining is lost after knockdown of Inx1 in glia and vice versa. Moreover, it was demonstrated that these heteromeric channels are required for synchronized calcium oscillations in the SPG cells which result in insulin secretion required for the reactivation of quiescent neural stem cells in the CNS (Speder and Brand, 2014). RNAi-mediated knockdown of both Inx1 and Inx2 individually, leads to the inactivation of quiescent neural stem cells in the CNS. The inactivation of neural stem cells results in a reduction of size in larval brain.
lobes due to lack of neural stem cell proliferation (Speder and Brand, 2014). Protein fusions of Inx1 and Inx2 (GFP-Inx1 and RFP-Inx2) that interfere with the folding of the N-terminus were expressed in glia cells were found to act as dominant negatives which phenocopied the reduced brain lobe phenotype. Since the N-terminus is essential for proper channel function (Oshima et al., 2016), it was concluded that Inx1 and Inx2 form heteromeric channels in the SPG of the CNS. This study demonstrated that calcium oscillations in the SPG of the CNS were uncoordinated in Inx1 mutants, however the properties of the calcium oscillations were not addressed in a loss of Inx2 background. In comparison to the CNS, almost nothing is known about gap junctions in the Drosophila PNS. Which innexins, if any, are expressed in glial cells of the peripheral nerve and whether they mediate glia-glia communication has yet to be elucidated. For this reason, in Chapter 2 of this thesis, we tested if innexins are expressed in the Drosophila peripheral nerve and determine their role, if any, in the PNS.

1.3.3 Innexin 2 as a potential adhesive protein

In Drosophila embryonic epithelial cells, Inx2 is detected along the apico-basal axis where it interacts with the adherens junction (AJ) components DE-Cadherin (DE-Cad) and Armadillo, as well as the septate junction (SJ) component Coracle (Bauer et al., 2004). These interactions suggest that Inx2 might play a role in cell-cell adhesion and polarity of epithelial cells. Moreover, embryos lacking both maternal and zygotic Inx2 fail to form cuticle and result in cell death implying that Inx2 is required for epithelial morphogenesis (Bauer et al., 2004). Perhaps Inx2 also associates with the SJ in the glia and Inx2 could be required for glial adhesion and polarity. Inx2 is also known to be required for proper foregut morphogenesis and inx2 mutant flies show feeding defects (Bauer et al., 2002).
Other work suggests that Inx2 is required for the transcriptional activation of *hedgehog*, *wingless* and *Delta* which are required for folding and invagination of the proventriculus (Lechner et al., 2007). Inx2 also plays an important role in the survival of germ cells and formation of the egg chamber via regulation by DE-Cad (Mukai et al., 2011). However, the molecular mechanisms underlying both these functions remain elusive. It is hypothesized that either Inx2 directly interacts with DE-Cad to increase its accumulation or it allows signaling between the germ cells and prefollicular cells (Mukai et al., 2011). It is not known whether Inx2 has a channel dependent or channel-independent adhesive role in glia.

1.3.4 Innexin 2 might interact with scaffolding proteins

The localization and activity of gap junctions must be highly regulated. The regulation of channel forming proteins, such as connexins by phosphorylation usually requires the formation of a multi-protein complex. Connexins are known to interact with a wide range of proteins including, enzymes, cytoskeletal proteins, tight junction and adherens junction associated proteins. Most of these protein-protein interactions take place via the different PDZ (postsynaptic density-95/discs large/zona occludens-1) and SH (Src Homology) domains present in the C-terminal domain of connexins (Giepmans, 2004). Innexins have a topology similar to connexins including intracellular N- and C-terminal domains. The carboxyl tails of *Drosophila* innexins show the highest sequence variability.

The C-terminal domain of Inx2 is 80 amino acids long. Based on the presence of the consensus sequence, X-y-X-y, where ‘X’ represents any amino acid and ‘y’ represents a hydrophobic amino acid it has been predicted that Inx2 has a putative class II PDZ domain binding motif. Similarly, the carboxyl tails of connexins possess PDZ domain binding motifs, as
well as putative phosphorylation sites. For example, Cx43 is phosphorylated by several kinases such as MAPK, v-Src and PKC (Cottrell et al., 2003, Lin et al., 2001, Saez et al., 1997, Lampe et al., 2000) predominantly on residues present in the C-terminal domain. Gap junctions are potential targets for posttranslational modifications and could possibly act as scaffolding proteins e.g. as part of a signalosome. Indeed, several studies have shown that connexins can interact with a variety of proteins including: kinase and phosphatase signaling enzymes (Doble et al., 2000, Swenson et al., 1990, Giepmans et al., 2003), tight junction associated proteins like ZO-1 and Occludin (Giepmans and Moolenaar, 1998, Kausalya et al., 2001, Nagaoka et al., 1999), adherens junctions associated proteins such as N-cadherin (Xu et al., 2001) as well as cytoskeletal components e.g. F-actin (Yamane et al., 1999). Therefore, the C-terminal in vertebrate gap junctions is required for numerous roles in different tissues and deletions in this domain can lead to loss of function. Moreover, in glial cells, a Cx43 C-terminal truncation (Δ244–382) results in reduced cell migration (Bates et al., 2007, Cina et al., 2009). It is not known if the C-terminal domain of Inx2 and other intracellular domains are required for function in glia.

1.4 Thesis questions

The larval segmental nerves in Drosophila are insulated by three glial layers, including the wrapping glia (WG) which are the morphological equivalent of non-myelinating Schwann cells. The protein(s) required for these three glial sublayers to communicate with each other in order to insulate the nerve is not entirely understood. So far, septate junction components have been identified in the Drosophila peripheral glia (Banerjee et al., 2006b, Baumgartner et al., 1996). However, not much is known about gap junctions and glial-adhesion complexes in these
cells. Freeze fracture studies show that gap junctions are present in insect glia (Lane, 1982) and recent data suggests that they play an important role in Drosophila CNS glia (Holcroft et al., 2013, Speder and Brand, 2014). However, these proteins have not yet been characterized in peripheral glia. **Chapter 2** of this thesis will highlight the role of Drosophila gap junctions in glial cells of the PNS.

The glial adhesion/scaffolding complexes in the Drosophila PNS and their components are also yet to be explored. A number of PDZ encoding proteins scaffold tight junction components as well as other proteins at the paranodal junction in the vertebrate peripheral nerve. Moreover, connexins are linked to protein scaffolds by PDZ proteins via the PDZ-binding motifs in their C-terminal domains. However, not much is known about scaffolding complexes, including whether gap junction components are part of protein scaffolds in Drosophila peripheral glia. **Chapter 3** of this thesis will include results from a genetic screen conducted to identify PDZ encoding proteins with potential roles in the Drosophila glia, with the focus of this chapter being on the characterization of the PDZ domain protein, Discs Large 5 (Dlg5).

Apart from gap junctions and scaffolding complexes, recent studies using electron microscopy (EM) have identified potential spot adherens junctions between the wrapping glia (WG) and subperineurial glia (SPG) as well as between subperineurial glia and perineurial glia (PG) (Matzat et al., 2015). The function and molecular composition of these junctions however, is completely unknown. **Chapter 4** of this thesis will focus on classical cadherins and their involvement in the formation of potential glial-adhesion complexes, in the Drosophila peripheral nerve.
The following questions were addressed in:

Chapter 2:

1. **We hypothesized that Inx1 and Inx2 are expressed in peripheral glia.**
   - Is Inx1 and Inx2 expression restricted to one or more glial layers?

2. **We hypothesized that calcium pulses are mediated by Inx1 and Inx2 gap junctions in the peripheral nerve.**
   - Are calcium pulses present in the peripheral nerves and does knocking down Inx1 and Inx2 in glial cells block or affect the properties of these pulses?

3. **We hypothesized that Inx1 and Inx2 gap junction mediated glia-glia communication in the peripheral nerve is required for axonal ensheathment.**
   - Does the loss of Inx1 and Inx2 signaling affect the morphology and physiology of the animal?

4. **We hypothesized that Inx1 and Inx2 form gap junction channels.**
   - Can Inx1 and Inx2 form channels that allow diffusion of ions or small metabolites or do they play an adhesive role?
   - Are Inx1 and Inx2 perform independent functions in the peripheral glia?

Chapter 3:

1. **We hypothesized that PDZ proteins are required in the peripheral glia.**
   - Which PDZ proteins play a role in glial development and maintenance?

2. **We hypothesized that Dlg5 (identified in the PDZ screen) mediates glia-glia interactions in the peripheral nerve.**
   - Is Dlg5 expression restricted to a particular domain in the peripheral glia?
- Does glial Dlg5 interact with known partners in other cells?
- What are the cellular consequences of Dlg5 knockdown in the peripheral nerve?
- Does Dlg5 localize cadherins and initiate glia-glia adhesion?

3. **We hypothesized that a PDZ protein is required for the scaffolding of Inx2.**
- Does knockdown of a particular PDZ protein affect Inx2 localization?

Chapter 4:

1. **We hypothesized that classical cadherins and catenins are expressed in the larval peripheral nerve.**
- What does the expression pattern of DE-Cadherin and DN-Cadherin look like in the *Drosophila* peripheral nerve? Do they form adherens-like belts or have a punctate expression, indicative of spot adherens junctions?
- Which catenins are expressed and co-localize with the classical cadherins?

2. **We hypothesized that classical cadherins are required for glia-glia adhesion in the larval peripheral nerve.**
- Is axonal ensheathment by glial cells disrupted by knocking down classical cadherins and catenins in the peripheral nerve?
- Do cadherins play redundant roles in the peripheral nerve?
FIGURES – CHAPTER ONE:

**Figure 1.1** Schematic representation of a cross-section of a vertebrate and Drosophila peripheral nerves

In the vertebrate peripheral nerve large caliber axons are insulated by myelinating Schwann cells (green) whereas the non-myelinating Schwann engulf small caliber axons (green) (A). Tight junctions are formed between the perineurial glia (blue) which are surrounded by an extracellular matrix (grey, A). (B) In the Drosophila peripheral nerve, axons are directly engulfed by the wrapping glia (green), followed by the subperineurial glia (purple) and perineurial glia (blue). Septate junctions (black) are formed when the subperineurial membranes meet. The entire nerve bundle is surrounded by an extracellular matrix (grey) (Adapted from Rodrigues et al., 2011 with permission from Cellular and Molecular Life Sciences).
Axonal ensheathment is a gradual process and is complete by 3rd larval instar. Transmission electron micrograph (TEM) cross-sections of a peripheral nerve from (A) 1st instar larvae compared to (B) 3rd instar larvae. Wrapping glia (pink, wg) have not completely surrounded axons (ax) at the 1st larval instar and these axons still contact the subperineurial glia (purple, spg). Axons in 3rd instar larvae are completely surrounded by wrapping glia. Perineurial glia (cyan, png) are present in the 1st instar and expand to surround each nerve. The side of the septate junction is highlighted in the dashed white box in (B). Scale bars: 1µm
Reprinted from Stork et al., 2008, doi: https://doi.org/10.1523/JNEUROSCI.4367-07.2008
**Figure 1.3.** Electron micrographs and schematic representation of a peripheral myelinated nerve showing the distribution of E-Cad labeling (black arrows) with immuno-gold observed in electron dense areas seen in electron micrographs taken from different regions of non-compact myelin including the (A) Schmidt Lanterman incisures, (B) Paranodal loops and (C) the outer mesaxon (Reprinted from Fannon et al., 1995, http://jcb.rupress.org/content/129/1/189.long, doi: 10.1083/jcb.129.1.189). (D) Schematic representation of a myelinated peripheral nerve showing the location of E-Cad based autotypic adherens junctions in the Schmidt Lanterman incisures, paranodal loops and mesaxons (Adapted from Poliak et al., 2002, doi: 10.1083/jcb.200207050).
**Figure 1.4.** Comparison of domain structures of vertebrate and Drosophila classical cadherins. The *Drosophila* classical cadherins, DE-Cad, DN-Cad and DN-Cad2 contain the primitive classical cadherin domain (red, PCD), Laminin globular domains (yellow, LamG), EGF like domains (green) and more extracellular cadherin domains (orange) compared to their vertebrate counterparts.
**Figure 1.5.** Spot adherens junctions form between different glial layers of the Drosophila peripheral nerve

(A) TEM cross-section of a larval peripheral nerve. Spot adherens junctions can be seen as electron dense regions between the wrapping glia and subperineurial glia (upper inset, yellow arrowhead) as well as between the subperineurial and perineurial glia layers (lower inset, yellow arrowhead). (B) A high resolution image showing a spot adherens junction formed by two glial membranes (Reprinted from Matzat et al., 2015, doi: 10.1242/dev.116616, http://dev.biologists.org/content/142/7/1336.long#skip-link).
Figure 1.6. Tight junction (TJ) components in the vertebrate peripheral nerve and the loss of autotypic TJs in Claudin 19-mutant mice

(A) Schematic representation of a myelinated peripheral axon. The panel of the left shows an unrolled Schwann cell, revealing regions of compact myelin (asterisks, grey), the outer mesaxon (OMA), inner mesaxon (IMA), the Schmidt-Lanterman incisures (SLI) and the paranode (PN). TJ strand like structures are found on either side (on top and under/far side) of the unrolled Schwann cell as well as along the incisures. This circumferential belt of TJ components are represented by the dotted (far side/underneath the Schwann cell) and continuous green lines (this side/on top of the Schwann cell). When the Schwann cell is rolled around the axon (right panel), the TJ components along the dotted lines pair with those along the continuous line to form TJ structures. The TJ structures form in a spiral manner at the PN and SLI whereas they form two parallel lines along the outer (1) and inner (2) mesaxon. (B) A TEM image showing two kissing points of TJs (arrowheads) near the outer mesaxon (asterisk) in wild type myelinated axons. (C) Mice that are deficient in the TJ component Claudin 19 lack these kissing points resulting in intercellular gaps between the Schwann cell membranes (arrow). Reprinted from Miyamoto et al., 2005, doi: 10.1083/jcb.200501154.
Figure 1.7. Pleated septate junctions are formed by the subperineurial glia in the Drosophila larval nerve

(A) Schematic representation of a cross-section of a Drosophila larval peripheral nerve. The subperineurial glia (SPG, green) membrane meet to form pleated septate junctions (pSJs). The box represents the region shown in (B). (B) A TEM image showing the ladder-like structure of pSJs formed by SPG membranes. (C-C’) NeurexinIV (NrxIV) (green) is found within the SJ forming SPG (blue) in a wild type larval peripheral nerve. The schematic (A) was adapted from Rodrigues et al., 2011 and reprinted with permission from Cellular and Molecular Life Sciences and the TEM image (B) was taken from Matzat et al., 2015, doi: 10.1242/dev.116616, http://dev.biologists.org/content/142/7/1336.long#skip-link.
Figure 1.8. Structure and molecular composition of the vertebrate paranodal axo-glial junction (A) TEM image of paranodal junctions found between the paranodal loops (PNL) and the underlying axon. (B) Schematic representation of interactions that occur between the glial (paranodal loop; Neurofascin 155) and axonal components (Caspr, Contactin, Protein 4.1B) to give rise to the paranodal junction. The TEM image was adapted from Spiegel and Peles, 2002 and reprinted with permission from Molecular Membrane Biology.
Figure 1.9. Gap junctions allow cell-cell communication and adhesion
There are three gap junction protein families; the vertebrate (A) Pannexins (Panx) and (B) Connexin (Cx) families and the invertebrate (C) Innexin (Inx) family. All three share a similar membrane topology. (D-E) Members of the Panx, Cx and Inx families oligomerize to form a hemichannel which is localized to the membrane. (F) Hemichannels from apposing membranes pair to either form gap junction channels that allow direct cell-cell communication via passage of small metabolites and ions or perform channel independent roles, mediating cell-cell adhesion. (G) Eight identical innexins can oligomerize to form homomeric hemichannels. Two different innexins can oligomerize to form heteromeric hemichannels. Two hemichannels of similar composition form homotypic channels whereas two hemichannels of different compositions form heterotypic channels. The extracellular loop of Panxs are glycosylated (A) and prevent most Panxs from forming gap junction channels. Adapted from Bosco et al., 2011 and reprinted with permission from Physiological Reviews.
Figure 1.10. Connexins are expressed in the peripheral nerve and form gap channel channels
(A) Connexin 32 (Cx32, green) is expressed at regions of non-compact myelin including the
Schmidt-Lanterma incisure and paranodal loops, forming a pathway that allows passage of
metabolites between the Schwann cell nucleus and the underlying axon. Cx29 hemichannels
(Cx29, light blue) from adjacent to the axonal membrane. Adult rat cervical sympathetic trunk
immunolabeled with (B) Cx32 and (B’) myelin associated glycoprotein (MAG). Cx32 is not only
present in MAG expressing myelinating Schwann cells but is also found in non-myelinating
Schwann cells with low levels of MAG (black arrows). Adapted from Kleopa et al., 2011,
doi:10.1523/JNEUROSCI.4824-11.2011 (A), Scherer et al., 1995 (B,B’)
http://www.jneurosci.org/content/15/12/8281.long
**Figure 1.11.** Gap junctions form between different glial cells in the CNS

(A) Cx47 is expressed in the oligodendrocyte (O) cell body and processes and pairs with both Cx43 and Cx30 in astrocytes (A) to form majority of the O/A gap junctions. Both Cx47 and Cx30 form O/O gap junctions and A/A gap junctions are formed by Cx43 and Cx30. (B) TEM image of a gap junction between two astrocytes (A/A) and (C) between an oligodendrocyte and an astrocyte (O/A). Adapted from Kleopa et al., 2011, doi:10.1523/JNEUROSCI.4824-11.2011. (A), Peters et al., 1990 with permission from Oxford University Press (B) and Mugnami et al., 1986 with permission from Elsevier (C).
**Figure 1.12.** Expression of Innexin 1 and Innexin 2 in the Drosophila CNS and differences in their junctional properties

(A) Innexin 1 (Inx1, red) and Innexin 2 (Inx2, green) overlap in regions of larval brain lobe and the ventral nerve cord. (B-E) Representative traces from dual voltage clamp electrophysiology experiments used to record channel activity in paired Xenopus oocytes. (B) Inx1 cannot form homotypic channels. (C) Inx2 forms voltage sensitive homotypic channels. (D) Inx1 does not form heterotypic channels with Inx2 (E) Co-expression of Inx1 and Inx2 in both cells results in the formation of functional channels. Images were taken from Holcroft et al., 2013, http://jcs.biologists.org/content/126/17/3823.long, doi: 10.1242/jcs.117994.
Chapter 2: Gap junction-mediated communication between different glial cells is required for glial survival and axon ensheathment in the peripheral nervous system

2.1 Synopsis

Axons in the Drosophila peripheral nerve are ensheathed by three glial layers which insulate neuronal signals and set the blood-nerve barrier. Achieving these tasks requires glia-glia communication; the mechanisms that mediate this communication however, remain unknown. Here we present evidence showing that heteromeric gap junction (GJ) channels consisting of Innexin 1 (Inx1) and Innexin 2 (Inx2) are present between the subperinuclear glia (SPG) and wrapping glia (WG). We show that calcium pulses in the SPG are mediated by Inx2 and loss of Inx2 but not Inx1 in the SPG leads to fragmentation and apoptosis-independent death of the neighbouring WG. However, altering calcium levels in the larva does not affect WG survival. Moreover, WG survival is dependent on a GJ channel role rather than an adhesive role for Inx2. Therefore, GJs in the SPG not only facilitate calcium, but also mediate SPG-WG communication, to maintain WG survival and ensheathment.

2.2 Introduction

Glial cells are required for the development and function of neurons. In the peripheral nervous system (PNS) glia ensheath axons, providing a tight insulation to neuronal signals, and participate in the formation of the blood-nerve barrier (BNB) (Carlson and Saintmarie, 1990).
Two types of Schwann cells, myelinating and non-myelinating insulate vertebrate PNS axons. The former makes a multilayered myelin sheath around large caliber axons whereas the latter directly ensheath small caliber axons. Myelinating Schwann cells (SC) have been the focus of vertebrate literature and dye diffusion studies in the myelin sheath found that low molecular mass compounds can diffuse between the inner and perinuclear SC cytoplasm via gap junctions (Cina et al., 2007). The role of gap junctions in non-myelinating Schwann cells (NMSC) however remains unknown.

In Drosophila, the role of axonal ensheathment is performed by three glial cells: perineurial glia (PG), subperineurial glia (SPG) and wrapping glia (WG). The WG start ensheathing individual peripheral axons at 1st larval instar similar to vertebrate NMSCs in Remak bundles. The SPG still contact axons until the 3rd larval instar stage at which WG completely ensheath individual axons. Septate junctions, forming the BNB are present between apposing SPG membranes that encircle the entire nerve bundle. The outermost layer is formed by the PG, which is covered by the neural lamella (Stork et al., 2008). Therefore, in Drosophila, the outermost PG, the intermediate SPG and innermost WG are in close proximity to each other and constitute the three distinct layers that ensheath the nerves. The mechanism underlying communication between these glial layers is however unknown. Since different layers of the myelin sheath are coupled by gap junctions, we hypothesized that glia-glia communication in the Drosophila PNS might also rely on gap junctions.

Gap junctions in invertebrates are composed of innexins and eight innexin genes have been identified in Drosophila (Bauer et al., 2005). A gap junction channel is formed when a hemichannel pairs with a hemichannel from an apposing cell membrane. These channels facilitate direct cell-cell communication by allowing ions and small molecules to pass through
them. In addition, connexins are known to also function as cell adhesion proteins in a gap junction independent manner (Bruzzone et al., 1996, Kumar and Gilula, 1996, Phelan et al., 1998, Elias et al., 2007). Studies of the three-dimensional structure of invertebrate Innexin-based gap junctions revealed that each hemichannel in the C. elegans Inx6 channel is comprised of eight subunits (Oshima et al., 2013, Oshima et al., 2016). It is therefore likely that the innexin hemichannels in Drosophila contain eight subunits. On the contrary, all known models of vertebrate connexin-based channels show that six subunits form a hemichannel (Yeager and Harris, 2007). Although innexins and connexins do not share sequence similarity, they perform similar functions (Skerrett and Williams, 2016). For example, propagation of calcium pulses in astrocytes is primarily facilitated by connexins (Scemes and Giaume, 2006). Similarly, Speder and Brand (2014) found that calcium oscillations in SPG of the Drosophila CNS are gap junction mediated. However, the role of gap junctions in the PNS and whether they might be involved in peripheral glia-glia communication remains unknown.

To determine whether GJs mediate glia-glia communication we set out to identify GJ proteins that are present and required in the peripheral glia. We found that Inx1 and Inx2 are expressed at the SPG-WG boundary and form GJs that mediate dye transfer from the SPG to the WG. We show that calcium pulses in the SPG are mediated by Inx2 based gap junctions. Moreover, knockdown of Inx2 but not Inx1 in the SPG, leads to reduced axonal ensheathment, death of the WG and reduced larval locomotion. However, we found that in the WG, loss of both Inx1 and Inx2 lead to reduced axonal ensheathment and in the case of Inx2, WG death. Since Inx1 cannot form homomeric channels, our results indicate that Inx1 pairs with Inx2 in the WG. We further show that Inx2 function as a GJ channel role rather than as an adhesion protein is necessary for WG survival. However, WG survival is not dependent on IP3 and Ca^{2+}. Therefore,
we propose that Inx2 hemichannels in the SPG couple with Inx1/Inx2 hemichannels in the WG to facilitate SPG-WG communication required for the survival and function of the WG.

2.3 Material and methods

2.3.1 Fly strains and genetics

The following fly strains were used in this study: repo-GAL4 (Sepp et al., 2001); Nrv2-GAL4 (Sun et al., 1999), 46F-GAL4 (Xie and Auld, 2011); Gli-GAL4 (Sepp and Auld, 1999); SPG-GAL4 (Schwabe et al., 2005); UAS-mCD8::GFP (Lee and Luo, 1999); UAS-Dicer2 (Dietzl et al., 2007); UAS-mCD8::RFP (gift from Elizabeth Davis, Princeton University); UAS-RFP::Inx2 (Speder and Brand, 2014), UAS-p35 (Bloomington); UAS-GCaMP6S (Bloomington) and UAS-GCaMP3 (Tian et al., 2009). The following GFP protein-trap insertions were used: Nrv2::GFP, Jupiter::GFP and NrxIV::GFP ((Morin et al., 2001) (Kelso et al., 2004) (Buszczak et al., 2007). The following Inx2-RNAi lines were used: JF02446 (Transgenic RNAi Project, TRiP), Bloomington), KK111067 (Vienna Drosophila Resource Center, VDRC) and 4590R-3 (National Institute of Genetics, NIG). The Inx1-RNAi line was GD3264 (VDRC). The following Inx3-RNAi lines were used: GD14965 (VDRC) and HM05245 (TRiP, Bloomington). The Inx7-RNAi line used was: KK112684 (VDRC). The IP3-receptor RNAis used were: 1063-R2 (NIG), JF01957, HMC03351, GLC01786 (TRiP, Bloomington), the plc21C RNAi lines used were: HMS00600, JF0120, JF01211 (TRiP, Bloomington). The Gaq RNAis used were JF02464, HMJ30300 and UAS-SERCA.S495P.tdTomato, 58973 (TRiP, Bloomington). All RNAi experiments were carried out at 25°C without Dicer2 in the background unless specified. All controls were crossed to w^{118}. The Inx2/ΔI/ mutant was generated for this work.
2.3.2 Generation of Inx2 null mutant and transgenic lines

The Inx2 null mutant Inx2[Δ1] was generated as a result of an imprecise excision of the Inx2NP6052 P element inserted in the 5′UTR of the Inx2 gene, using standard transposase mediated excision. PCR amplification (with the following flanking primers: 1700-1A: 5’-GACTGGCTTTGCTTGTTC; 43-2S: 5’-GGTCTACGCAAAGTGGTTAT) and sequencing confirmed a deletion of 3.3 kb (breakpoints 6998886 & 7001806: Dmel R6.14), which includes the entire Inx2 gene but did not disrupt the coding sequence of the overlapping Inx7 gene.

2.3.3 Immunolabeling and image analysis

Larvae were dissected and fixed for immunolabeling using previously described methods (Sepp et al., 2000). The following primary antibodies were used in this study: guinea pig anti-Inx2 (1:500), (Smendziuk et al., 2015); rabbit anti-Inx1 (1:50), (Bauer et al., 2004); rabbit anti-HRP (1:500, Jackson ImmunoResearch, West Grove, PA); mouse anti-Futsch/22C10 (1:1000, DSHB); rabbit anti-p35 (1:1000, Novus Biologicals, Oakville, Ca); rabbit anti-Dcp1 (1:1000, Cell Signaling Technology). The following secondary antibodies were used at a 1:300 dilution: goat anti-mouse Alexa 488, Alexa 568 and Alexa 647; goat anti-rabbit Alexa 568 and goat anti-guinea pig Alexa 647 (Molecular Probes). DAPI (1:1000, Invitrogen) was used to stain nuclei. Images were obtained using Delta Vision Spectris (Applied Precision/GE Healthcare) using a 60x oil immersion objective (NA 1.4). An image was captured every 0.2 µm and the resulting stacks were deconvolved (SoftWorx, Applied Precision/GE Healthcare) using a point spread function measured with 0.2 µm beads conjugated to Alexa dyes (Molecular Probes) and mounted in Vectashield (Vector Laboratories, Burlington, Canada). Orthogonal sections were generated using SoftWorx. A single z-slice, conveying the information relevant to the experiment, was
chosen from each z-stack and images were compiled using Adobe Photoshop and Adobe Illustrator CC. For transmission electron microscopy analysis larvae were dissected and prepared using previously described methods (Matzat et al., 2015).

2.3.4 Larval tracking

Larvae were tracked using a multi-worm tracker and script (Petley-Ragan et al., 2016). During each session 5-6 larvae were placed on 100 mm diameter apple juice plate. A gentle tap was applied to the plate to elicit movement and the larvae were tracked for 25 seconds. The speed of all larvae at 15 seconds was obtained and significance between the speeds of control and RNAi expressing larvae were analyzed using a one-way ANOVA. This was replicated twice to obtain speeds from a minimum of 25 larvae from each genotype.

2.3.5 In vivo calcium imaging

UAS-GCaMP6S or UAS-GCaMP3 were expressed in the SPG and WG using Gli-GAL4 and Nrv2-GAL4 respectively. Third instar larvae were anesthetized using isoflurane for 4 mins, on average. Each larva to be anesthetized was placed in a 50ml tube containing a Kimwipe soaked with 300µl of isoflurane. The larva was removed from the tube when visible movement had ceased (approximately 2-4mins). Each larva was placed ventral side up on a prepared agarose slide and gently pressed with a 18x18 mm coverslip and tape to reduce movement. GCaMP6S and GCaMP3 fluorescence was imaged using a Leica SP5 II laser scanning confocal microscope with a tandem scanner and HyD detector. Image stacks of the posterior region of the ventral nerve cord and peripheral nerves were collected using a 25X water objective (NA 0.95). 3D projections of the stacks were acquired using the Leica Application Suite Advanced
Fluorescence (Leica AF software). Each image was acquired at a speed of 8,000 lines per second with a line average of 4 resulting in a collection time of 131 ms per frame at a resolution of 512 x 512 pixels. The pinhole was opened to 2.5-4.5 Airy units (AU). The total z-steps taken for each stack were set to around 25-52 steps. ROIs were manually selected and the mean fluorescence intensity was measured using the Leica AF software.

### 2.3.6 Dye transfer assay

Intact wandering 3rd instar larvae were inverted and bathed in an artificial hemolymph using previously described methods (Brink et al., 2009). Calcein Red-Orange AM (C-3487) was added to the artificial hemolymph at a final concentration of 5µM and the larval preparation was bathed in freshly prepared dye for 40 min. Excess dye was removed with three quick washes with artificial hemolymph. The larva was mounted and imaged using previously described methods (Brink et al., 2009).

### 2.4 Results

#### 2.4.1 Innexin 1 and Innexin 2 are expressed in glial cells of the larval peripheral nerve

To test for the presence of innexins in the peripheral glia, we first used an RNAi approach to knockdown those innexins known to be expressed in Drosophila glia (Inx1, 2, 3, 7)(Stebbings et al., 2002, Ostrowski et al., 2008). RNAi-mediated knockdown in all glia using the pan glial driver, repo-GAL4, generated peripheral glia phenotypes for only Inx1 and 2 (Table 2.1). Pan glial knockdown of Inx3 (n=14 larvae, TRiP HM05245; n=4 larvae, VDRC GD14965) and Inx7 (n=4 larvae, VDRC KK112684) did not affect glial or nerve morphology (data not shown). We thus concentrated on these two innexins and examined the distribution of Inx1 and
Inx2 in the glia of 3rd instar peripheral nerves. At this stage, peripheral nerves contain three glial layers, the innermost wrapping glia (WG) that wrap the axons, intermediate subperineurial glia (SPG) that form the blood-nerve barrier and the outermost perineurial glia (PG). Several markers can be used to visualize the individual glial layers and a combination of these glial markers were used in conjunction with antibodies specific to Inx1 (Bauer et al., 2004) and Inx2 (Smendziuk et al., 2015). We first assayed the distribution of Inx2 in nerves where the PG were labeled with Jupiter::GFP along with SPG labeled with SPG-GAL4 driving the expression of UAS-mCD8::RFP (Fig. 2.1A). Inx2 immunolabeling was observed as puncta that associated with the PG as well as the SPG membranes (Fig. 2.1A and B, arrowheads). Inx2 puncta were also observed at the center of the nerve where the axons and WG normally reside (Fig. 2.1A and B, arrowheads). The presence of Inx2 puncta in the individual layers can be seen more clearly in the cross sections (Fig. 2.1B-B'''', arrowheads). To test if Inx2 is expressed between the SPG and WG layers, we expressed mCD8::RFP in the SPG and used Nervana 2 endogenously tagged with GFP (Nrv2::GFP) to label the WG. These nerves were assayed for association of Inx2 puncta with the labeled SPG and WG membranes. We observed lines of Inx2 puncta (Inx2 plaques) along the SPG (Fig. 2.1C,D,D''', white arrowheads) and WG boundary (Fig. 2.1D'''). The prominent Inx2 labeling at the SPG-WG boundary suggests that Inx2 gap junctions are present between the SPG and WG (Fig. 2.1C, D-D''', white arrowheads). Apart from the SPG-WG boundary, Inx2 puncta were also observed in the center of the nerve within the WG membrane labeled with Nrv2::GFP (Fig. 2.1C, yellow arrowhead). These Inx2 plaques most likely represent WG to WG gap junctions between the extensive glial membrane processes these cells form. We also analyzed the distribution of Inx1 in peripheral nerves where the SPG and WG were labeled with mCD8::RFP and Nrv2::GFP, respectively. Similar to Inx2 labeling, Inx1
was prominent in the SPG-WG boundary (Fig. 2.1E, F-F'', white arrowheads). These findings provide strong evidence that Inx2 is present between all three peripheral glial layers and is highly expressed along with Inx1 at the SPG-WG boundary as well as within the WG at larval stages. To test if Inx1 and Inx2 are present in the same gap junction plaque and potentially forming heteromeric channels, we analyzed the distribution of both Inx1 and Inx2 in the peripheral nerve. We found that Inx1 and Inx2 puncta co-localize along the SPG-WG boundary (Fig. 2.1G, H-H’’, white arrowheads) as well as within the WG membrane (Fig. 2.1G, yellow arrowhead) suggesting that Inx1 and Inx2 are constituents of the same gap junction plaque.

2.4.2 Gap junctions form between the SPG and WG

To determine if gap junction channels form between the SPG and the WG, we tested for dye transfer from the SPG into the WG using a Calcein dye assay used to test for gap junction mediated cell-cell transfer (Elzarrad et al., 2008, Chen et al., 2016). Wandering third instar larvae with the WG labeled using Nrv2::GFP were inverted and live imaged using our previously established protocol (Brink et al., 2009). This preparation keeps the nervous system intact including the SPG generated blood-brain and blood-nerve barrier. Calcein red-orange AM is a cell-permeant dye, which is intrinsically fluorescent and retained within live cells due to AM ester hydrolysis by intracellular esterases (Chen et al., 2016, Elzarrad et al., 2008). As the WG are protected from the circulating hemolymph by the blood-nerve barrier created by the SPG, the presence of fluorescent dye in the WG is indicative transfer of dye from the SPG to the WG through gap junctions. Using this approach, we observed dye transfer from the SPG into the WG in 71 % nerves (n=4 larvae) (Fig. 2.2A-A’’, yellow arrowheads). The presence of Calcein labeling was not due to the ability of the dye to pass through the blood-nerve barrier septate
junctions as we never observed dye within the lumen of the trachea (Fig. 2.2B-B’) (n=4 larvae), suggesting that Calcein does not pass through a functional septate junction-barrier. Therefore, our results suggest that gap junction channels are present between the SPG and the WG.

2.4.3 Inx2 based gap junctions mediate calcium pulses in the peripheral SPG

Synchronous calcium oscillations in the SPG of the CNS are dependent on heteromeric Inx1/Inx2 gap junctions (Speder and Brand, 2014), but whether gap junctions are also involved in propagation of calcium pulses in peripheral glia had not been previously tested. We tested if there were measureable Ca$^{2+}$ signals within the peripheral glia using GFP Calcium indicators GCaMP3 (Tian et al., 2009) and GCaMP6S (Chen et al., 2013). GCaMP6S was expressed in the SPG alone (Gli>GCaMP6S) and imaged in live intact 3rd instar larvae. In controls we observed changes in Ca$^{2+}$ levels in the SPG that were initiated in the ventral nerve cord (VNC) and then descended down the SPG in some but not all peripheral nerves (n=9 larvae) (Fig 2.3A,A’, Movie 2.1 https://drive.google.com/open?id=189VGlrQHZ-OBpxYiKv8UpW4_AMnPGJM7). Calcium pulses were observed in 49.3% of nerves, particularly in the nerves of abdominal segments A6-A8 at the posterior end of the ventral nerve cord. There was a range of responses where some nerves had no detectable changes in Ca$^{2+}$, others pulsed infrequently, and other pulsed multiple times. We never observed a wave travel the entire distance of any given peripheral nerve along the nerve extension region (NER) into the muscle field area (MFA). However, our ability to detect calcium in distal regions of the nerve is likely limited by the thin (approximately 0.5µm) layer of the SPG surrounding the nerve. We then tested if Inx2-based gap junctions are required for the calcium pulses in the SPG. Calcium pulses were not observed in any peripheral nerves with Inx2 knockdown in the SPG (Gli>Inx2-RNAi, GCaMP6S) (n=5
labeled larvae) (Fig 2.3B,B’, Movie 2.2 https://drive.google.com/open?id=189VGlrQHZ-OBpxYiKv8UpW4_AMnPGJM7). This suggests that Inx2-based gap junction channels mediate calcium signals in the SPG of peripheral nerves. Since Inx1 and Inx2 form heteromeric channels in the SPG of the CNS (Speder and Brand, 2014), we tested the role of Inx1 in mediating the peripheral Ca\(^{2+}\) pulses by knocking down Inx1. Knock down of Inx1 in the SPG (Gli>GCaMP6S, Inx1-RNAi) did not disrupt the calcium pulses in the peripheral nerves (n=5 larvae) (data not shown) and we observed calcium pulses in a pattern and frequency similar to control larvae (Gli>GCaMP6S). Moreover, calcium pulses were observed even when Dicer2 was expressed with the Inx1-RNAi (Gli>GCaMP6S, Inx1-RNAi, Dicer2(n=5 larvae) (data not shown). This suggests that calcium signals in peripheral SPG cells rely on Inx2- but not Inx1-based gap junctions. We then used the same imaging conditions to record calcium in the peripheral WG by expressing GCaMP3 alone (Nrv2>GCaMP3) as well as with Inx2-RNAi (Nrv2>GCaMP3, Inx2-RNAi). Calcium pulses were observed in WG of 41% of the peripheral nerves in control larvae (n=5 larvae) (Fig 2.3C,C’, Movie 2.3 https://drive.google.com/open?id=189VGlrQHZ-OBpxYiKv8UpW4_AMnPGJM7). The number of nerves, the frequency and pattern of calcium pulses in the WG were similar to our observations with the SPG. Unlike the SPG, which forms a very thin membrane around each nerve, each WG extends several processes to wrap multiple axons making it possible to detect changes in calcium not just near the VNC but also in more distal regions of the nerve including the NER but not the MFA. However, these calcium pulses were not eliminated with knockdown of Inx2 (Nrv2>GCaMP3, Inx2-RNAi) (n= 4 larvae) (Fig 2.3D,D’, Movie 2.4 https://drive.google.com/open?id=189VGlrQHZ-OBpxYiKv8UpW4_AMnPGJM7). Therefore, although calcium pulses are present in the WG, the calcium signals in the WG are not Inx2
dependent. Furthermore, knockdown of Inx1 in the WG, even in the presence of Dicer2
(Nrv2>GCaMP3, Inx1-RNAi, Dicer2), did not disrupt calcium signals in the WG (n=5 larvae).
This implies that Inx1 is not necessary to mediate calcium changes in the WG or the SPG. We
were not able to determine if the calcium pulses were mediated by WG to WG gap junctions or
through SPG to WG gap junctions. We have however, determined that loss of Inx2 disrupts the
propagation of Ca\textsuperscript{2+} pulses along the SPG but not the WG in larval peripheral nerves.

2.4.4 Innexin1 and Innexin2 are required in glial cells of the Drosophila larval PNS

To determine the role of Inx2 in the larval peripheral glia, we were unable to use a
Inx2[Δ1] deletion mutant that we generated due to the embryonic lethality (data not shown) and
the inability to generate frequent somatic clones in the polyploid SPG and WG. Therefore we
knocked down Inx2 in all glia using repo-GAL4 paired with Inx2-RNAi lines, and tested for
changes in glial morphology using a fluorescently tagged membrane marker (mCD8::GFP). Inx2
knockdown experiments were carried out using three independent Inx2-RNAi lines with or
without Dicer2 to increase the effectiveness of the RNAi (Table 2.1). All Inx2-RNAi lines with
Dicer2 were lethal at early larval stages suggesting that Inx2 is specifically required in glial cells
during the early stages of larval development but not during embryogenesis. Without Dicer2 two
RNAi lines, Inx2-RNAi (TRiP) and Inx2-RNAi (VDRC), resulted in a few larvae surviving to
the third instar stage. These larvae had an overall reduction in body size, including smaller brain
lobes similar to CNS phenotypes observed previously (Holcroft et al., 2013). The peripheral
nerves were also thinner compared to controls. We observed fragmented glial membranes along
the length of 67% of nerves (n=3 larvae) with the TRiP RNAi (Fig. 2.4C, D) and 75% of nerves
in the single larvae that survived with the VDRC RNAi (data not shown). The remaining glial
fragments were present only within the core of the nerve and glial membranes were not observed in the outer circumference (Fig. 2.4 C’,D’) compared to the control (Fig. 2.4A’,B’). Control larvae (repo> mCD8::GFP) did not show any glial defects (n=4 larvae) (Fig. 2.4A,A’ and B,B’).

To better characterize the fragmented membrane phenotypes (Fig. 2.4C, D), we analyzed a third RNAi line, Inx2-RNAi (NIG), which was less efficient than the others. repo> Inx2-RNAi (NIG) larvae raised at 25°C were viable and morphologically normal. However, at 29°C, we observed glial membrane aggregates in 49% of nerves (n=5 larvae) (Fig. 2.4G, H). None of the control nerves (repo>mCD8::RFP) (n=5 larvae) showed this fragmented glia phenotype at 29°C (Fig. 2.4E, F). Outer glial membranes were still present in the repo> Inx2-RNAi (NIG) larvae but glial fragments were observed within the core of the nerve (Fig. 2.4G’,H’) compared to control larvae (Fig. 2.4E’,F’). For all three RNAi lines, the glial membrane aggregates were observed in the interior of the nerves where WG are present suggesting that knockdown of Inx2 in all glia led to defects in WG morphology.

We next test the role of Inx1 in peripheral glia. Knockdown of Inx1 in all glia led to smaller brain lobes as observed in previous studies (Holcroft et al., 2013, Speder and Brand, 2014). Furthermore, pan glial knockdown of Inx1 (repo> Inx1-RNAi) resulted in peripheral glia swelling in 32% of nerves (n= 12 larvae) (Fig. 2.4K-H’) compared to control larvae (repo>mCD8::RFP), which had no swelling (n=8 larvae)(Fig. 2.4I-J). However, the Inx1 loss of function phenotypes were different from those generated with the Inx2 RNAi, particularly in that Inx2 knockdown did not lead to the glial swellings observed with Inx1 knockdown. To further investigate the role of Inx1 in peripheral glia, we knocked down Inx1 in individual glial layers.

To test the role of Inx1 in the SPG, we used Gli-GAL4 to drive RNAi with mCD8::RFP to mark the SPG membranes and Nrv2::GFP to mark the WG membranes (Nrv2::GFP, ...
Gli>mCD8::RFP). Inx1 knockdown in SPG (Nrv2::GFP, Gli>Dicer2, Inx1-RNAi) did not affect the morphology of the SPG or the neighbouring WG, even in the presence of Dicer2 (n=6 larvae)(Fig. 2.5C-D’; Fig. 2.6I) and the morphology of these glial layers was similar to control larvae (Nrv2::GFP, Gli>mCD8::RFP, Dicer2)(n=6 larvae)(Fig. 2.5A-C’; Fig. 2.6I). To test the role of Inx1 in the WG, we knocked down Inx1 using the Nrv2-GAL4 driver and mCD8::GFP to mark the membranes (Nrv2>Inx1-RNAi, mCD8::GFP). Inx1 knockdown at 29°C in the WG lead to WG defects, with less WG strands in 85% of nerves (n=5 larvae) (Fig. 2.6I). Control nerves (Nrv2>mCD8::GFP) did not show any WG defects (n=3 larvae). Co-expressing Dicer2 with the Inx1-RNAi at 25°C (Nrv2>mCD8::GFP, Inx1-RNAi, Dicer2) enhanced the severity of the WG phenotype and resulted in 86% of nerves with reduced and discontinuous WG strands, where neighboring WG processes failed to meet (n=5 larvae)(Fig. 2.5G-H’; Fig. 6I). Control larvae expressing Dicer2 at 25°C alone (Nrv2>mCD8::GFP, Dicer2) did not have any WG defects (n=6 larvae)(Fig. 2.5E-F’; Fig. 2.6I). To test the role of Inx1 in the outermost perineurial glia, we used the 46F-GAL4 driver to knockdown Inx1 (46F>mCD8::RFP, Inx1-RNAi). Control nerves (46F>mCD8::RFP) did not show defects (n=4 larvae)(Fig. 2.5I-J’) and Inx1 knockdown did not affect the morphology of the PG (n=5 larvae)(Fig. 2.5K-L’). The Inx1 knockdown results taken together suggest that Inx1 is required in the WG but not the PG and SPG in the PNS. Moreover, knockdown of Inx1 in each of the three glial layers alone (Fig. 2.5C,G,K) did not result in glial swellings suggesting that this phenotype might be due to the loss of Inx1 in at least two different glial layers. We then wanted to determine the function of Inx2 in the peripheral glia.
2.4.5  **Innexin2 knockdown in the SPG affects WG morphology**

To determine if Inx2 is specifically required in one or all three glial layers, we knocked down Inx2 in individual glial layers using the most efficient Inx2-RNAi (TRiP) line. To test the role of Inx2 in the SPG, we used Gli-GAL4 to drive RNAi with mCD8::RFP to mark the SPG membranes and Nrv2::GFP to mark the WG membranes (Nrv2::GFP, Gli> Inx2-RNAi, mCD8::RFP). Knockdown of Inx2 had no effect on the morphology of the SPG membrane (Fig. 2.6C). Surprisingly the knockdown of Inx2 in the SPG did affect the morphology of the WG, where we observed a range of WG phenotypes (Fig. 2.6C-D’,I). 67.1% of the nerves had either fewer strands of WG membrane or a single, discontinuous WG membrane (Fig. 2.6I). WG aggregates similar to those seen in the pan glial knockdown of Inx2 were observed in 4.5% of nerves (n=11 larvae) (Fig. 2.6C-D’,I). Control larvae (Nrv2::GFP, Gli>mCD8::RFP) did not show any WG defects (n=5 larvae) (Fig. 2.6A-B’,I). To further test if the SPG were affected, we looked at the distribution of a SJ protein Nrv2.1 as the SJ domain is integral to the function of the SPG in blood-nerve barrier formation (Baumgartner et al., 1996). Normal WG morphology (Fig. 2.7A,A’’) and Nrv2.1 distribution (Fig. 2.7A,A’) was observed in all the control nerves (Nrv2::GFP, Gli) (n=4 larvae). Inx2 knockdown in the SPG (Nrv2::GFP, Gli> Inx2-RNAi) resulted in fragmentation of the WG (Fig. 2.7B,B’’) but did not disrupt the distribution or localization of the SJ (Fig. 2.7B,B’) (n=6 larvae). Thus, the loss of Inx2 from the SPG had little or no effect on the SPG itself but rather has a cell non-autonomous effect on the WG.

2.4.6  **Innexin2 knockdown in WG only affects WG morphology**

To analyze the role of Inx2 in the WG, we knocked down Inx2 using the Nrv2-GAL4 driver and mCD8::RFP to mark the membranes (Nrv2> Inx2-RNAi, mCD8::RFP). We observed
single strands of WG processes in 71.4% of nerves (n=9 larvae) (Fig. 2.6G-H’,I) compared to the multiple strands normally observed in the WG (Fig. 2.6A,B). Membrane aggregates in the WG were observed in 28.6% of nerves (n=9 larvae), while control larvae (Nrv2>mCD8::RFP) (Fig. 2.6E-F’,I) did not show any glial defects (n=6 larvae). To determine if the loss of Inx2 in the WG affected the morphology of the SPG layer, we analyzed the distribution of NrxIV::GFP in control (NrxIV::GFP, Nrv2>mCD8::RFP)(Fig. 2.8A-A’’) and WG knock down larvae (NrxIV::GFP, Nrv2>mCD8::RFP, Inx2-RNAi)(Fig. 2.8B-B’’) and looked for SPG defects. Several, continuous WG strands were observed in control nerves (Fig. 2.8A,A’’) and NrxIV::GFP was correctly localized to the SJ domain (Fig. 2.8A,A’) (n=6 larvae). Loss of Inx2 in the WG resulted in fragmentation of the WG (Fig. 2.8B,B’’) but did not affect the distribution or levels of NrxIV::GFP and the overall SPG morphology (Fig. 2.8B,B’) (n=6 larvae). To test the role in the outer most perineurial glia, we used the 46F-Gal4 driver to knockdown Inx2 and assayed the WG using Nrv2::GFP (Nrv2::GFP, 46F>mCD8::RFP). We did not observe any WG or PG phenotypes (n=6 larvae, data not shown), even when we used Dicer2 to increase the effectiveness of the RNAi knockdown (Nrv2::GFP, 46F>Inx2-RNAi, Dicer2). Our results point to a clear role for Inx2 within the WG and overall suggest that Inx2 gap junctions provide a critical cell-cell communication between the SPG and WG that is required for WG integrity.

2.4.7 Innexin2 knockdown in WG processes and ensheathment of axons

We next wanted to characterize the phenotypes we observed within the WG when Inx2 was knocked down in the SPG. Using both fluorescence and transmission electron microscopy, a range of phenotypes were observed the WG as a result of Inx2 knockdown in the SPG (Gli> Inx2-RNAi) compared to control larvae (Gli-GAL4) (Fig. 2.9). At the ultrastructural level,
we found that the overall structure of the nerve was intact in both control (Fig. 2.9A’’) and $Gli>\text{Inx2-RNAi}$ larvae (Fig. 2.9B’’-E’’). Moreover, SPG morphology appeared normal with SJs present along the SPG layer as expected (Fig. 2.9A’’-E’’, magenta arrowheads). In the control nerves, WG strands in the light microscope images (Fig. 2.9A,A’) correspond to the processes of a single WG as they ensheath individual or bundles of axons, which is clearly observed in the TEM images (Fig. 2.9A’’). WG in $Gli>\text{Inx2-RNAi}$ nerves were able to extend along the center of the nerve but had reduced numbers of strands around some but not all axons in some segments of the nerve (Fig. 2.9C) whereas in other nerves WG failed to ensheath the majority of the axons and appeared as a single process though the core of the nerve (Fig. 2.9B, D, E). At the ultrastructural level, less WG processes were observed in the $Gli>\text{Inx2-RNAi}$ nerves and the extent of axonal ensheathment was reduced compared to the control (Fig. 2.9B’’- E’’). The aggregates of WG membrane ranged in appearance from small regions with remnants of membrane (Fig. 2.9E), to regions where physical breaks appeared along the WG membrane strands (Fig. 2.9B).

Each peripheral nerve in $Drosophila$ larvae is ensheathed by three WG cells (von Hilchen et al., 2013). The first WG ensheathes axons in the nerve extension region (NER)(as the nerve extends from the ventral nerve cord), the second WG nucleus is present at the branching point of the segmental nerve and the third WG wraps the axons in the muscle field area (MFA)(von Hilchen et al., 2013)(Fig. 2.10A). The WG aggregates were most frequently observed at the end of the nerve extension region, which is wrapped by both the first and second WG (Fig. 2.10B). Overall, we observed a gradient of phenotype severity where less severe phenotypes (reduced WG strands or single strands) were observed in regions closer to the ventral nerve cord compared to complete disruption of the WG membranes observed at the distal end of the nerve.
extension region. Therefore, the loss of Inx2 gap junctions between the SPG and the WG result in a range WG defects suggesting that communication between the two glial layers (SPG and WG) is required for normal WG morphology and axonal ensheathment. Knockdown of Inx2 however, does not result in the loss of SJs further confirming that the signal mediated by Inx2 affects the WG but not the SPG.

2.4.8 Innexin2 is required in the SPG for the survival of WG

The aggregation of the WG membranes suggested that the WG were dying when Inx2 was knocked down in the SPG. We visualized the WG nuclei using DAPI labeling and did not observe shrunken or pyknotic nuclei indicative of apoptosis (Kroemer et al., 2009). Rather we observed morphologies consistent with necrosis including uniform deterioration of nuclei, and disorganized membranes (Zheng et al., 2014, Kroemer et al., 2009). To confirm if the WG aggregates in Nrv2>lnt-2-RNAi larvae are a result of apoptotic cell death, we blocked apoptosis in the WG by expressing p35, which is a baculoviral protein that blocks caspase activation (Hay et al., 1994). All control larvae expressing p35 in the WG did not display any WG defects (Fig. 2.10C-C’)(n=5 larvae). WG aggregates were observed even when p35 was co-expressed with Inx2-RNAi (n = 16 larvae, Fig. 2.10D-D’). The presence of WG aggregates in these larvae suggests that the WG aggregates are not a result of apoptosis-mediated cell death. We further tested if the WG aggregates in Gli>lnt-2-RNAi and Nrv2>lnt-2-RNAi larvae were positive for Drosophila caspase-1 (Dcp-1), an effector caspase in apoptosis, using a cleaved Dcp-1 antibody (Song et al., 1997). We did not detect Dcp-1 in WG nuclei in nerves of both Gli>lnt-2-RNAi and Nrv2>lnt-2-RNAi (n=14; data not shown). This result further confirms that the WG are not dying due to apoptosis and suggested that loss of Inx2 in the SPG and WG leads to WG necrosis.
2.4.9 Knockdown of Innexin 2 in the SPG leads to defects in locomotion

As the WG ensheath and insulate axons, we next tested whether the death of WG affected the motility of 3rd instar larvae compared to controls. A Multiworm tracker system (Petley-Ragan et al., 2016) was used to determine the speed at which control larvae moved compared to Inx2-RNAi larvae. We found that larvae with knockdown of Inx2 in the SPG (Nrv2::GFP, Gli>Inx2-RNAi) (n= 27) moved at a significantly slower speed than control larvae (n= 59) (Nrv2::GFP, Gli-Gal4) (Fig. 2.10E). However, there was not a significant difference between the speed of larvae with knockdown of Inx2 in the WG (Nrv2>Inx2-RNAi) (n= 39) and control (w1118, Nrv2-Gal4) larvae (n=112) (Fig. 2.10F). Larvae of both genotypes (Gli>Inx2-RNAi and Nrv2>Inx2-RNAi) fail to eclose and are lethal at pupal stages, suggesting that while larval locomotion was not affected by the loss of the WG, the continued expression of Inx2 in the WG is necessary for survival. We do not think the lack of locomotion defects with Nrv2>Inx2-RNAi larvae is due to a lack of penetrance of the Inx2 phenotype as 100% of nerves showed a WG defect, with single WG processes in 71.4% of the nerves and 28.6% of the nerves with WG aggregates, under these conditions (Fig. 2.6I). Rather loss of WG processes appears not to affect larval locomotion, which mirrors previous observations (Petley-Ragan et al., 2016).

The reduction in larval locomotion with Inx2 knockdown in the SPG might reflect a role for other gap junctions within the SPG. For instance, Inx2 forms a heteromeric gap junction in the CNS SPG with Inx1 (Speder and Brand, 2014). In the CNS, Inx2/Inx1 gap junctions in the SPG play a role in neurogenesis and loss of Inx2 in the SPG leads to smaller brain lobes (Speder and Brand, 2014). To test for any role of Inx1, we knocked down Inx1 in the SPG and tested larval locomotion. We found that Nrv2::GFP, Gli>Inx1-RNAi larvae (n= 37) had significantly
slower speeds than control larvae (n= 59) (*w1118, Nrv2::GFP, Gli-GAL4*) (Fig. 2.10E). However, knockdown of Inx1 in the SPG does not affect WG morphology (n=6 larvae) (Fig. 5C, D) (Fig. 2.6I). Knockdown of Inx1 in WG (*Nrv2> Inx1-RNAi*) resulted in subtle WG defects but did not result in WG aggregates (n=5 larvae) (Fig. 2.6I) and had no effect on locomotion (n=57) compared to control (*w1118, Nrv2-Gal4*) larvae (n=112, Fig. 2.10F). Therefore, Inx1 and Inx2 loss in the SPG both affect larval locomotion, which might reflect a secondary effect of loss of neurogenesis in the CNS or a role for Inx1-Inx2 heteromeric gap junction communication in the peripheral SPG. Overall our results suggest that Inx2 may have additional functions in the SPG, likely within the CNS SPG, independent of Inx2-mediated SPG-WG gap junction communication.

### 2.4.10 Survival of the WG is not dependent on inositol 1,4,5-trisphosphate (IP3)

Innexin 2 gap junction channels have been previously shown to mediate calcium waves in many tissues, including glial cells of the *Drosophila* larval CNS, in the *Drosophila* embryo epidermis in response to wounding, in the larval wing imaginal disc as well as in follicle cells to specify border cell fate during oogenesis (Sahu et al., 2017, Razzell et al., 2013, Restrepo and Basler, 2016, Speder and Brand, 2014). Therefore, Inx2 has a well established role in mediating different functions through calcium and we wanted to test if the survival of the WG depends also relies on the regulation of calcium by Inx2.

A rise in intracellular calcium can either result from an influx of extracellular calcium or release of calcium from internal stores. In astrocytes intercellular calcium waves propagate via direct diffusion of IP3 or calcium through the gap junction channel or via extracellular ATP induced Ca$^{2+}$ release (Scemes and Giaume, 2006). Both myelinating and non-myelinating SCs
respond to the application of extracellular ATP by increasing intracellular calcium as a result of P2Y$_2$ and P2Y$_1$ receptors respectively (Mayer et al., 1998). However, since *Drosophila* lack ATP-activated P2X and P2Y receptors (Schulz and Schoneberg, 2003, Fountain and Burnstock, 2009), calcium signaling in the peripheral glial must be mediated by an ATP-independent mechanism. To test if the survival of the WG is dependent on IP3 signaling in the SPG, we knocked down the IP3 receptor and other pathway components in the SPG (*Nrv2::GFP, Gli>RNAi*) using multiple RNAi lines (Table 2.2). The IP3 receptor RNAi line (NIG 1063-R2) was validated by previous studies and results in an efficient knockdown (Restrepo and Basler, 2016, Agrawal et al., 2009). Expressing the NIG 1063-R2 line, as well as two IP3 receptor RNAi lines, in the SPG did not affect the morphology of the WG (Table 2.2). In confirmation of these results, knockdown of the alpha subunit of the heteromeric G protein as well as PLCβ involved in IP3 signaling (Rhee, 2001, Berridge and Irvine, 1984) in the SPG did not result in a WG defect (Table 2.2). This suggests that the survival of the WG is not mediated by IP3 generated in the SPG and points to another mechanism mediated by Inx2 that is necessary for WG survival.

### 2.4.11 Survival of the WG is independent of calcium signaling in the SPG

We hypothesized that if Ca$^{2+}$ from the SPG was required in WG for survival and axon ensheathment, increasing the levels of cytosolic calcium in the WG could in part rescue the WG phenotype in *Gli> Inx2-RNAi* larvae. The sarco-endoplasmic reticular Ca$^{2+}$-ATPase pump (SERCA) normally pumps cytosolic Ca$^{2+}$ into the endoplasmic reticulum (ER) and maintains the concentration of calcium in the cytosol and the ER. Changing SERCA levels reduces Ca$^{2+}$ uptake into the ER and thereby leads to a prolonged increase in the level of cytosolic Ca$^{2+}$ (Banerjee et al., 2006a). To manipulate SERCA activity, we used a dominant negative mutant
allele \((Kum^{170})\) for the SERCA gene (Banerjee et al., 2006a) and introduced the heterozygous mutant in the \(Gli>\text{Inx2-RNAi}\) larvae \((Kum^{170}/+, Nrv2::GFP, Gli>\text{Inx2-RNAi})\). The \(Kum^{170}\) allele is a single base pair substitution in the hinge region of SERCA, which affects ATP binding or the conformational state of the molecule (Sanyal et al., 2005). Moreover, a single copy of \(Kum^{170}\) is sufficient to slow down the rate of \(\text{Ca}^{2+}\) entry into intracellular stores, thus leading to a rise in cytosolic \(\text{Ca}^{2+}\) (Banerjee et al., 2006a). We assayed the effects on the WG morphology using \(Nrv2::GFP\) and observed that increasing cytosolic \(\text{Ca}^{2+}\) levels in the WG did not alter the penetrance of the WG phenotypes. Specifically, we observed single or discontinuous WG strands and membrane aggregates in 49% (\(\text{mean} = 4.0 \pm 1.7\) nerves per larvae) and 6% of the nerves respectively \((n=11\) larvae) (Table 2.2). A comparable percentage of single or discontinuous WG strands \((67%; \text{mean} = 3.6 \pm 0.7\) nerves per larvae\)) and membrane aggregates \((4.5%)\) were observed in the \(Gli>\text{Inx2-RNAi}\) nerves \((n=11)\) (Fig. 2.6I). When the degree of single or discontinuous WG strands was quantified there was not a significant difference between the two genotypes. In comparison, introducing Dicer2 to increase the efficiency of \(\text{Inx2 knockdown in the SPG (Dicer2, Gli>\text{Inx2-RNAi}) lead to a comparable percentage of single or discontinuous strands (47%; \text{mean} = 3.2 \pm 1.3) but a significantly higher percentage of membrane aggregates (34.2%) (Fig. 2.6I). Therefore, the presence of the \(Kum^{170}\) allele did not change the \(\text{Inx2-mediated WG phenotypes; it neither suppressed nor enhanced these phenotypes. Given that the Kum}^{170}\) allele would alter SERCA function in both the SPG and WG, we next tested the effect of changing SERCA function specifically in the WG. We used a mutant form of SERCA \((\text{SERCA.S495P})\) that acts as a dominant gain of function mutant leading to ionic leakage and depletion of stored \(\text{Ca}^{2+}\) levels (Kaneko et al., 2014). Expression of the \(\text{SERCA.S495P}\) protein in the WG using \(Nrv2-GAL4\) did not lead to any WG phenotypes (Table 2.2). Taken altogether our
results suggest that misregulation of cytosolic calcium levels is not the mechanism that underlies the disruption of the WG when Inx2 is knocked down.

**2.4.12 Innexin2 mediates WG survival by forming gap junction channels and not via SPG-WG adhesion**

Changes to Ca\(^{2+}\) and IP3 levels did not lead to the disruption of the WG suggesting that other mechanisms underlie the disruption of the WG in the absence of Inx2. Inx2 could mediate WG morphology or survival by forming a gap junction channel or as a cell adhesion protein, given that gap junctions can either function as pores between cells or cell adhesion proteins in a channel independent manner (Bruzzone et al., 1996, Kumar and Gilula, 1996, Phelan et al., 1998, Elias et al., 2007). Thus, Inx2 might mediate cell adhesion between the SPG and WG in the peripheral nerve in a channel independent manner. In this context loss of cell-cell adhesion may disrupt the ability of WG to maintain contact with the SPG. To test whether Inx2 acts as a channel or as an adhesive protein, we utilized a dominant negative transgene (RFP::Inx2). This Inx2 transgene is tagged at the N-terminus and leads to a loss of Inx2 function (Speder and Brand, 2014, Oshima et al., 2016, Nakagawa et al., 2010, Sahu et al., 2017), likely by interfering with the folding of the N-terminal domain which is essential for the formation of a functional channel (Speder and Brand, 2014, Oshima et al., 2016, Nakagawa et al., 2010). We expressed RFP::Inx2 in the SPG and looked for changes in WG morphology using Nrv2::GFP (Nrv2::GFP, Gli>RFP::Inx2). Expression of the dominant negative RFP::Inx2 in the SPG resulted in a range of WG phenotypes, similar to those seen in the SPG-specific knockdown of Inx2, in 77% of the nerves (n=7 larvae) (Fig. 2.11D-L’). Control larvae did not have any WG phenotypes (Nrv2::GFP, Gli>mCD8RFP)(n=6 larvae)(Fig. 2.11A-C’). WG aggregates were observed in
11% of the nerves in Nrv2::GFP, Gli>RFP::Inx2 larvae (Fig. 2.11F,F’) while 100% of the nerves had discontinuous strands (Fig. 2.11I,I’) or reduced numbers of strands (Fig. 2.11L,L). RFP labeled Inx2 puncta were observed along the SPG-WG boundary in the Nrv2::GFP, Gli >RFP::Inx2 nerves (Fig. 2.11D-E’, M,N, G-H’,Q,R and J-K’, U,V) and co-labeled with an anti-Inx2 (Fig. 2.11M,P and Q,T) and Inx1 (Fig. 2.11U,X) antibodies suggesting that RFP::Inx2 was localized to the membrane and integrated into complexes with existing Inx2 and Inx1. The RFP::Inx2 puncta in the SPG were localized adjacent to the remnants of the Nrv2::GFP WG membrane (Fig. 2.11M, Q, U; white arrowheads). Since the RFP::Inx2 colocalized with endogenous Inx2 and Inx1 gap junctions at the SPG-WG boundary, the WG membrane aggregates observed in these nerves are likely not due to loss of SPG-WG adhesion but likely due to the inability to form gap junction channels. Overall our data suggest that it is the formation of Inx2 gap junction channels between the SPG and WG that is necessary for the continued survival of the WG.

2.5 Discussion

Our study shows that gap junction signaling between two different glial cells in the Drosophila larval peripheral nervous system (PNS), the subperineurial glia (SPG) and wrapping glia (WG) is mediated primarily by Innexin 2 (Inx2) where Inx2 gap junctions are necessary for WG survival. Inx2 gap junctions mediate calcium pulses within the SPG however, the survival of the WG is not due to changes in IP3 signaling or changes to the cytosolic levels of Ca^{2+} in the WG.
2.5.1 Gap junctions in the peripheral nerve

The presence and requirement for gap junctions in myelinating Schwann cells (SCs) of the vertebrate PNS is well established. Dye transfer experiments showed that apposing membranes of the myelin sheath are coupled by autotypic gap junctions to mediate rapid intercellular communication over the myelin layers (Balice-Gordon et al., 1998, Nualart-Marti et al., 2013). In comparison, very little is known about gap junctions in non-myelinating SCs apart from a few studies which imply that gap junctions are also present in these cells (Konishi, 1990, Vega et al., 2003). We show that in Drosophila, at least two gap junction proteins, Inx1 and Inx2, are expressed in all the peripheral glial layers, with a clear role for Inx2 in WG function and survival. When the other innexins were tested, only Inx1 knockdown resulted in a peripheral glial phenotype. Inx1 is thought to complex with Inx2 and RNAi knockdown of Inx1 or Inx2 in all glia leads to the reduction in the larval CNS (ventral nerve cord and brain lobes)(Holcroft et al., 2013, Speder and Brand, 2014). However, the loss of Inx1 in all three glial layers resulted in qualitatively different phenotype in the PNS compared to knockdown of Inx2, and Inx1 knockdown did not disrupt the Ca\(^{2+}\) pulses in the SPG. *In vitro* studies using Xenopus oocytes found that Inx1 does not form homomeric hemichannels or channels and must pair with other innexins to form functional gap junctions (Holcroft et al., 2013). Since Inx1 does not from homotypic channels, when a pan-glial knockdown of Inx2 is performed, functional channels cannot form between the SPG and WG (Fig. 2.12A). Conversely Inx2 is able to form homotypic gap junctions albeit with altered properties (Holcroft et al., 2013). These results explain why Inx2 (but not Inx1) knockdown in all glial layers leads to lethality or WG defects. We hypothesize that Inx1 and Inx2 form heteromeric channels in both the SPG and the WG, and in the absence of Inx1, Inx2 can form Inx2 homotypic channels between the SPG and the WG (Fig.
The differences between the channel properties of Inx2 homotypic versus Inx1/Inx2 heterotypic channels (Holcroft et al., 2013), could explain the glial swellings seen in the pan glial knockdown of Inx1. Interestingly, SPG-specific knockdown of Inx1 did not affect either SPG or WG morphology, suggesting that Inx1 does not play a necessary role in the SPG. Therefore, in our model we proposed that Inx2 can form homomeric channels in the SPG (Fig. 2.12C). On the other hand, Inx1 knockdown in the WG with Dicer2, leads to WG defects suggesting that Inx1 plays a role in the WG. However, these WG defects were not as severe as Inx2 knockdown and did not result in dying WG aggregates. In our model we propose that Inx1 forms heteromeric channels with Inx2 in the WG (Fig. 2.12D) and where Inx2 or Inx1/Inx2 in the SPG layer could couple with Inx1/Inx2 heteromeric channels in the WG, to mediate a signal that is essential for WG survival (Fig. 2.12C). The idea that the gap junctions in the WG are likely composed of Inx1/Inx2 heteromers is further supported by observations that Inx1 and Inx2 co-localized in this glia. The severe WG defects in the Inx2 knockdown compared to Inx1 knockdown can be explained by the observation that Inx1 cannot form channels (Holcroft et al., 2013). Thus, in the absence of Inx2 in either the SPG or WG, gap junction channels do not form at all between the SPG and the WG (Fig. 2.12E,F).

It is also possible that Inx1 can form gap junctions with other innexins in the PG and SPG. Thus, the lack of a morphological phenotype with Inx2 knockdown in the PG and SPG layers may be due to a redundant role with another innexin in these outer glial layers. However, this other innexin is unlikely to be Inx3 or Inx7 as knockdown did not affect peripheral glial or nerve morphology and knockdown of Inx3 in all glia does not lead to a reduced VNC or brain lobes in larvae (Holcroft et al., 2013). Thus, our data points to Inx1/Inx2 heteromeric channels
between the SPG and WG, mediating signals between the SPG and the WG and possibly Inx2 autotypic junctions between SPG-SPG.

2.5.2 **Innexin2 mediates communication between two glial layers: SPG and WG**

Knockdown of Inx2 specifically in WG phenocopied the pan-glial knockdown of Inx2, resulting in membrane aggregates and WG death. WG were also disrupted when Inx2 was knocked down in the SPG. As the SPG are in contact with and form a glial wrap around the underlying WG this suggests that gap junctions are required for communication or adhesion between these cells. Moreover, the transfer of Calcein dye from the SPG to the WG confirms the presence of gap junctions between the two layers. Loss of Inx2 led to a range of phenotypes in the WG from loss of the glial wrap to progressive loss of membrane and finally cell membrane remnants indicative of cell death, which we confirmed was not due to apoptosis. Consistent with prior work in the vertebrate CNS, which identified gap junction coupling between oligodendrocytes and astrocytes (Orthmann-Murphy et al., 2008), we found that Inx2 mediates WG survival through the formation of channels between the SPG and the WG and not via SPG-WG adhesion. Moreover, it is unlikely that Inx2 mediates adhesion given that loss of integrin mediated adhesion within the WG leads to loss of WG-SPG contact and axon ensheathment, and yet these WG survive for the most part (Xie and Auld, 2011).

2.5.3 **Innexin2 mediates communication necessary for WG survival**

Our working model is that Inx2 forms channels to link the SPG and WG and that this linkage is necessary for WG survival. Yet the nature of the signal or mechanisms necessary for WG survival is not certain. Dye transfer and diffusion of glucose analogues between non-
myelinating SCs (Konishi, 1990, Vega et al., 2003), which overlap with large areas of contiguous membrane suggest extensive gap junction communication. However, whether calcium propagates between non-myelinating SCs via gap junctions still remains unknown. We found that Ca\(^{2+}\) pulses are present in both the SPG and WG. Moreover, Inx2 is necessary for the propagation of Ca\(^{2+}\) pulses along the SPG. We were unable to determine if the Ca\(^{2+}\) pulse passes from the SPG into the WG or rather represents pulses that travel between SPG to SPG exclusively. However, given the need for Inx2 in both cell types, it is likely that Ca\(^{2+}\) passes between SPG and WG as well as within the SPG along the length of the peripheral nerve. While Inx2 clearly facilitates calcium pulses in the SPG, it is unlikely that calcium itself mediates the survival of the WG given our results.

Another possible cause of WG death might be due to loss of ion buffering. Vertebrate glial gap junctions facilitate spatial buffering of ions via redistribution of K\(^{+}\) through a glial syncytium (Scemes and Spray, 2012). Thus, it is possible that Inx2 might also facilitate K\(^{+}\) buffering and that its loss might disrupt the movement of ions from the WG to the SPG resulting in an increase in K\(^{+}\) within the periaxonal space. Leiserson et al. 2011 showed that K\(^{+}\) is released along the length of the axon following an action potential and is siphoned by the Na-K-Cl cotransporter (NCC69) in the SPG. However, the loss of NCC69 leads to swelling of the peripheral nerve due to increased extracellular K\(^{+}\) and fluid buildup in the periaxonal space and although ensheathment was also reduced, WG remained intact and do not die (Leiserson et al., 2011). Therefore, we do not think that the WG necrosis observed in Inx2 knockdown larvae is due to an increase in K\(^{+}\). Similarly, if cytosolic Ca\(^{2+}\) levels play a key role in the WG necrosis, changing Ca\(^{2+}\) levels in the WG with either the SERCA mutant (Kum\(^{170}\)) or the SERCA.S495P transgene would have enhanced the necrosis, which we did not observe. Additionally, loss of the
IP3 receptor and blocking IP3 production in the SPG also did not generate a WG phenotype. Therefore, the loss of the WG in the Inx2 knockdown is not due to lack of ionic homeostasis and is not mediated by Ca^{2+} or IP3 in the peripheral glia.

As gap junctions are permeable to a wide range of metabolites and small ions such as ATP, IP3, Ca^{2+}, cAMP and sugars, there are multiple possible signals that could pass through the gap junction between the SPG and WG. It has been speculated that gap junctions are involved in the transfer of the sugar trehalose between the PG and SPG in the CNS (Volkenhoff et al., 2015). Yet Inx2 gap junctions do not mediate sugar transfer between glia (Volkenhoff et al., 2017) and we were unable to phenocopy the loss of WG when trehalose transporters were knocked down in the SPG (data not shown). It is possible that Inx2 gap junctions in the peripheral SPG mediate transfer of unidentified metabolites to the WG and that the absence of this leads to WG necrosis.

In summary, our study is the first to show that gap junctions are required between two different glial cells (SPG and WG) unlike studies in the Drosophila CNS glia, which describe gap junction coupling among the same type of glial cells (SPG in the CNS) and in SCs, which demonstrate the presence of autotypic gap junctions between different layers of the same cell. Furthermore, in the vertebrate CNS, although gap junction coupling has been observed between two different glial cells (oligodendrocytes and astrocytes), the type of signal or signals that permeate these gap junctions remains unresolved (Tress et al., 2012). We show that Inx2 gap junctions mediate calcium pulses in the SPG but can also mediate other signal(s) from one glial cell (SPG) to another (WG) that facilitate glial (WG) survival and axonal ensheathment. Although Ca^{2+} transients have been observed in non-myelinating SCs, whether Ca^{2+} pulses between neighbouring non-myelinating SCs are mediated by gap junctions is yet to be determined. Therefore, our study provides a mechanism by which two different glial cells in the
peripheral nerve communicate with each other and emphasizes on the importance of glia-glia communication for sustaining normal glial morphology and physiology.
**Table 2.1** Summary of Inx RNAi phenotypes.

Each Inx RNAi was expressed in all glia using the pan glial driver *repo-GAL4*. Knockdown experiments were conducted in the presence or absence of Dicer2 at the temperatures indicated. Peripheral nerves were screened for glial and nerve defects.
<table>
<thead>
<tr>
<th>Driver</th>
<th>Gene(s)</th>
<th>Transgenic Lines (n=larvae)</th>
<th>Wrapping glia phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gli-GAL4</td>
<td><em>Inx2-RNAi, Kum170/+</em></td>
<td>RNAi TRiP JF02446 (BL-29306), (BL-26700) (n=11)</td>
<td>49% larvae single or discontinuous strands Mean = 3.6 nerves per larvae 6% larvae membrane aggregates</td>
</tr>
<tr>
<td>Gli-GAL4</td>
<td><em>Kum170/+</em></td>
<td>(BL-26700) (n=5)</td>
<td>Wild type</td>
</tr>
<tr>
<td>Nrv2-GAL4</td>
<td>SERCA</td>
<td>SERCA.S495P.tdTomato (BL-58973) (n=12)</td>
<td>Wild type</td>
</tr>
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</table>

Table 2.2: Knockdown or overexpression of genes involved in IP3 mediated calcium release. Peripheral nerves were screened for wrapping glia defects. Experiments were conducted at 25°C. RNAi-mediated knockdown of genes required for IP3-mediated Ca\(^{2+}\) release, including the IP3 receptor (*itp3-r83A*), phospholipase C (*plc21C*) and the alpha subunit of the heteromeric G protein (*Ga\(q\)*) did not affect the morphology of the wrapping glia. Changing cytosolic Ca\(^{2+}\) levels with mutations in the Sarco-Endoplasmic Reticulum Ca\(^{2+}\) ATPase pump (SERCA) did not have any effect on the wrapping glia morphology and did not rescue or enhance the *Inx2-RNAi* phenotypes.
FIGURES – CHAPTER 2:

Perineurial glia and Subperineurial glia

Subperineurial glia and Wrapping glia

Gi-GAL4

Subperineurial glia and Wrapping glia

Wrapping glia (WG)

Nrv2::GFP
**Figure 2.1.** Innexin 1 and Innexin 2 are expressed in the larval peripheral nerve

A, B-B'': Longitudinal section (A) or cross section (B-B'') of a control peripheral nerve with the perineurial glia (PG) membrane and subperineurial glia (SPG) membranes labeled with Jupiter::GFP (green) and mCD8::RFP (white) respectively. Inx2 immunolabelling (magenta) identified puncta (yellow arrowheads) present in the PG and SPG, and within the interior of the nerve where the wrapping glia (WG) are present. The yellow line in A indicates the region from which cross sections B-B'' were taken.

C-D, E-F: Longitudinal sections of control peripheral nerves with Inx2 and Inx1 immunolabelling (magenta), respectively in the subperineurial glia and wrapping glia with corresponding cartoons of nerve cross-sections. SPG membrane are labeled with mCD8::RFP (blue, C-D, E-F), and WG membranes labeled with Nrv2::GFP (green, C-D, E-F). The yellow boxes were digitally magnified (200x) and shown in D-D'' and F-F'' respectively. White arrowheads in C-D and E-F indicate the Inx2 and Inx1 puncta respectively in the SPG. Inx2 puncta in the WG are indicated by the yellow arrowhead (C). Both Inx2 and Inx1 expression puncta are observed in the SPG-WG boundary (C, D'-D'' and E-F-F'', white arrowheads). G-H: Inx1 green) and Inx2 (magenta) form plaques along the SPG-WG boundary (G, H-H'', white arrowheads) and within the WG membrane (G, yellow arrowhead).

Scale bars: 15µm.

**Figure 2.2.** Gap junctions mediate dye transfer between the subperineurial glia and wrapping glia.

A-B: Calcein dye transfer past the SPG into the WG in a 3rd instar peripheral nerve and exclusion of the dye by the trachea. The WG membrane was labeled with Nrv2::GFP (green, A,A''). The Calcein dye (red, A,A') is observed within the WG (yellow arrowheads, A-A'') whereas the dye is excluded by the trachea (yellow arrowheads, B-B''). Scale bars: 15µm.
Figure 2.3. Calcium pulses are present in peripheral nerves and are mediated by Inx2 in the subperineurial glia in control 3rd instar larvae.

A: Control peripheral nerves with GCaMP6S expressing subperineurial glia (Gli>GCaMP6S).

A': Graph showing the change in mean fluorescence intensity of GCaMP6S over time (seconds) in different peripheral nerves, indicated by the regions of interest (ROIs) shown in (A). Calcium pulses were present in some but not all nerves (ROIs: 1, 12-14, 16; green, purple, orange, light blue and blue, respectively).

B: GCaMP6S expression in larvae lacking Inx2 in the subperineurial glia (Gli>Inx2-RNAi, GCaMP6S).

B': Graph showing that the mean fluorescence intensity of GCaMP6S in the peripheral nerves indicated by the ROIs shown in (B) does not change when Inx2 is knocked down in the subperineurial glia.

C: Control peripheral nerves with GCaMP3 expressing wrapping glia (w1118, Nrv2>GCaMP3).

C': Graph showing the change in mean fluorescence intensity of GCaMP3 over time (seconds) in different peripheral nerves, indicated by the regions of interest (ROIs) shown in (C). Calcium pulses were present in some but not all peripheral nerves (ROIs: 10, 12-15, 17; navy blue, purple, orange, light blue, pink and blue respectively).

D: GCaMP3 expression in larvae lacking Inx2 in the wrapping glia (Nrv2>Inx2-RNAi, GCaMP3).

D': Graph showing that the mean fluorescence intensity of GCaMP3 in the peripheral nerves indicated by the ROIs shown in (D), changes even when Inx2 is knocked down in the wrapping glia. The ROI 18 (grey, A, B) and ROIs 18, 16 (grey, C, D) were manually placed in a region where peripheral nerves were absent and hence represents the basal level of the GCaMP6S and GCaMP3 signal, respectively. Scale bars: 25 µm
Figure 2.4. Knockdown of Inx2 in all glia leads to fragmentation of the inner glial membrane, whereas knockdown of Inx1 leads to glial swellings.

A-D: repo-GAL4 crossed to w[1118] (control) or Inx2-RNAi (TRiP) at 25°C.

E-H: repo-GAL4 crossed to w[1118] (control) or Inx2-RNAi (NIG) at 29°C.

(A, E, I) Longitudinal sections of control peripheral nerves in larvae with repo-GAL4 driving mCD8::GFP (green) to label the glial membrane and axons labeled with anti-HRP or anti-22C10 (magenta). The yellow lines indicate the region from which the cross sections were taken. The axons (magenta) are present in the center of the nerve and completely surrounded by the glial membrane (green) in the control peripheral nerve.

(C, G) Longitudinal sections of repo> Inx2-TRiP RNAi and repo> Inx2-NIG RNAi, raised at 25°C and 29°C respectively, with the glial membrane labeled with mCD8::GFP (green) and axons labeled with anti-HRP (magenta). The peripheral nerves in repo> Inx2-RNAi (TRiP) (C) are thinner compared to control nerves (A) and disrupted glial membranes are present along the length of the nerve (arrows, C, D). Glial membrane aggregates are more prominent within the interior of the nerve in repo> Inx2-RNAi (NIG) nerves (G, H). The yellow lines indicate the region from which the cross sections were taken. The glial membrane (green) does not completely surround axons which are present in the center of the nerve (C’, G’) with remnants of the glial membrane present in the repo> Inx2-RNAi (TRiP) peripheral nerve (arrowheads, C’, D’). The outer glial membranes (green) still surround the nerve bundle in repo> Inx2-RNAi (NIG) nerves but only a part of the glial membrane remains in the center of the nerve, without completely ensheathing the axons (magenta, arrowheads, G’, H’).

(K) Longitudinal section of repo> Inx1-RNAi raised at 25°C with repo-GAL4 driving mCD8::GFP (green) to label the glial membrane and axons labeled with anti-22C10 (magenta). Swellings between the different glial membranes (green) are indicated by the yellow arrows (K, H). The yellow lines indicate the region from which the cross sections were taken. A schematic representation of the labeled glial layers (green) and axons (magenta) in control nerves (A, E, I), Inx2 knockdown nerves (C, G) and Inx1 knockdown nerves (K) are shown and include representative changes in the circumference of the entire nerve for the corresponding genotypes.

Scale bars: 15μm.
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**Figure 2.5.** Knockdown of Inx1 in the wrapping glia leads to subtle wrapping glia defects but its knockdown in the other glial layers does not affect glial morphology.

**A-D:** Control (A) and Gli>\textit{Inx1}-RNAi (C) peripheral nerves with SPG and WG membranes labeled with mCD8::RFP (magenta) and Nrv2::GFP (green), respectively. The yellow lines indicate the region from which the cross sections were taken. The WG (green) in the Gli>\textit{Inx1}-RNAi nerve (C) similar to the control nerve (A), extends processes along the entire length of the nerve and the thin SPG membrane (magenta) surrounds the WG (A’,C’). WG membranes extend their processes (B, B’, D,D’).

**E-H:** Control (E) and Nrv2>\textit{Inx1}-RNAi (G) peripheral nerves with WG membranes labeled with mCD8::RFP (green) and axons immunolabeled with 22C10 (magenta). The yellow lines indicate the region from which the cross sections were taken. Several strands of WG wrap (green) around axons (magenta) in the control peripheral nerve (E, E’) and cross sections indicate that WG membrane surrounds axons (magenta). A subtle loss of WG strands (green) is observed in Nrv2>\textit{Inx1}-RNAi nerves (G,G’, yellow arrowhead) and the WG membrane does not fully wrap around axons in some regions of the nerve (magenta).

**I-L:** Control (I) and 46F>\textit{Inx1}-RNAi (K) peripheral nerves with PG membranes labeled with mCD8::RFP (green) and axons labeled with 22C10 (magenta), respectively. The yellow lines indicate the region from which the cross sections were taken. PG (green) and axons (magenta) in both control (I) and 46F>\textit{Inx1}-RNAi (K) look normal. A schematic representation of the labeled glial layers in control nerves and Inx1 knockdown nerves are also shown. Scale bars: 15µm.
Figure 2.6. Knockdown of Inx2 individually in the subperineurial glia and wrapping glia leads to fragmentation of the wrapping glia.

A-D: Control (A) and Gli>lnx2-RNAi (C) peripheral nerves with SPG and WG membranes labeled with mCD8::RFP (magenta) and Nrv2::GFP (green), respectively. The yellow lines indicate the region from which the cross sections were taken. The WG (green) in the control nerve (A) extends processes along the entire length of the nerve and the thin SPG membrane (magenta) surrounds the WG (A'). WG membranes wrap around the peripheral axons (A, A'). WG membrane aggregates (green, C) are present along the length of the nerve in Gli>lnx2-RNAi. Remnants of the WG membrane (green) are found in the center of the nerve (C', arrowhead) and do not wrap around axons (magenta) in Gli>lnx2-RNAi.

E-H: Control (E) and Nrv2>lnx2-RNAi (G) peripheral nerves with WG membranes labeled with mCD8::RFP (green) and axons immunolabeled with 22C10 (magenta). The yellow lines indicate the region from which the cross sections were taken. Several strands of WG wrap (green) around axons (magenta) in the control peripheral nerve (E, E') and cross sections indicate that WG membrane surrounds axons (magenta). Loss of WG strands in Nrv2>lnx2-RNAi, with only a single WG strand (green) present in the peripheral nerve (G,G'). WG membrane in the Nrv2>lnx2-RNAi nerve is only present at the center with an uneven morphology and does not wrap around axons (magenta).

I: Comparison of the WG phenotypes observed when Inx2 and Inx1 are individually knocked down in the SPG and the WG. The WG phenotypes were divided into three categories: wild type WG (grey), less, discontinuous WG strands (green) and WG aggregates (magenta). The percentage of nerves that fall under the three categories of WG phenotypes were as follows: w1118, Nrv2::GFP,Gli>mCD8::RFP, 100% wild type, n=5, Gli>lnx2-RNAi, 28.5% wild type, 67% less, discontinuous strands, 4.5% WG aggregates, n=11, Gli>lnx1-RNAi, 100% wild type, n=6, Dicer2,Nrv2::GFP,Gli>mCD8::RFP 100% wild type, Dicer2,Gli>lnx2-RNAi, 18.4% wild type, 47.4% less, discontinuous strands, 34.2% WG aggregates, n=11, Dicer2,Gli>lnx1-RNAi, 100% wild type, n=5, w1118, Nrv2>mCD8::RFP, 100% wild type, n=6, Nrv2>lnx2-RNAi, 71.4% less, discontinuous strands, 28.6% WG aggregates n=9, Nrv2>lnx1-RNAi, 15% wild type, 85% less, discontinuous strands, 0% WG aggregates n=5, Dicer2, Nrv2>mCD8::RFP, 100% wild type, n=6, Dicer2, Nrv2>lnx2-RNAi, 77.3% less, discontinuous strands, 22.7% WG aggregates n=9, Dicer2, Nrv2>lnx1-RNAi, 14% wild type, 86% less, discontinuous strands, 0% WG aggregates n=5.
Figure 2.7. Knockdown of Inx2 individually in the subperineurial glia leads to fragmentation of the wrapping glia but does not affect SJ morphology.

**A-B:** Control (A) and *Gli> Inx2-RNAi* (B) peripheral nerves with WG membranes labeled with Nrv2::GFP (green) and the SJ domain labeled with a Nrv2.1 antibody (magenta). The WG (green) in the control nerve (A”) extends processes along the entire length of the nerve and the SJ domain (magenta) is continuous along the length of the nerve (A’, white arrowheads). WG membrane aggregates (green, B, yellow arrowheads) are present along the length of the nerve in *Gli> Inx2-RNAi*. SJ morphology is not affected (magenta, B’, white arrowheads) in *Gli> Inx2-RNAi*. 
Figure 2.8. Knockdown of Inx2 individually in the wrapping glia leads to fragmentation of the wrapping glia but does not affect SJ morphology.
A-B: Control (A) and Nrv2>lnx2-RNAi (B) peripheral nerves with WG membranes labeled with mCD8::RFP (green) and the SJ domain labeled with NrxIV::GFP (magenta). The WG (green) in the control nerve (A**) extends processes along the entire length of the nerve and the SJ domain (magenta) is continuous along the length of the nerve (A’, white arrowheads). WG membrane aggregates (green, B, yellow arrowheads) are present along the length of the nerve in Nrv2>lnx2-RNAi. SJ morphology is not affected (magenta, B’, white arrowheads) in Nrv2>lnx2-RNAi.
**Figure 2.9.** Knockdown of Inx2 in the subperineurial glia results in a range of wrapping glia phenotypes.
Control (A) and *Gli> Inx2-RNAi* (B-E) peripheral nerves with subperineurial glia and wrapping glia membranes labeled with mCD8::RFP and Nrv2::GFP respectively. Ultrastructural analysis of control (A**) peripheral nerves and nerves lacking Inx2 in the subperineurial glia (*Gli> Inx2-RNAi*, B***-E***). A, A*: Several wrapping glia strands (green) are present and extend along the length of the control peripheral nerve. (A**) Cross section of a control peripheral nerve showing that the wrapping glia (false coloured in green) extend their processes and wrap around several axons. B-E: Loss of Inx2 in the subperineurial glia results in a range of wrapping glia phenotypes, with nerves showing either wrapping glia aggregates (B), less WG strands (C), discontinuous wrapping strands (D) or wrapping glia strands that are about to fragment (E). B***-E***: Representative TEM sections taken from peripheral nerves in *Gli> Inx2-RNAi* larvae, correspond to the wrapping glia phenotypes seen in (B-E). Septate junctions are indicated in the TEM sections taken from peripheral nerves in control and *Gli> Inx2-RNAi* larvae (A***-E***, magenta arrowheads). Scale bars: 15µm, 1 µm
**Figure 2.10.** Inx2 knockdown in the subperineurial glia affects larval locomotion and leads to apoptosis-independent death of wrapping glia.

Schematic representation of a peripheral nerve in a control 3rd instar larva (A) and a larva in which Inx2 is knocked down in either the SPG or WG layers (B). **A,B:** There are three WG present in each peripheral nerve (WG1, WG2 and WG3). The nerve extension region (NER) is ensheathed by WG1 and WG2 while WG3 wraps the nerve in the muscle field area (MFA). **B:** Knockdown of Inx2 in the SPG or the WG results in discontinuous or single WG strands closer to the ventral nerve cord (VNC) while the WG aggregates were mostly observed towards the end of the NER.

**C,D:** *Nrv2>*p35 control (C-C’’) and *Nrv2>*Inx2-RNAi,*p35 (D-D’’) peripheral nerves with WG membranes labeled with mCD8::RFP (green) and anti-p35 labeling (C’,D’, magenta). In control nerves, several wrapping glia processes are extended along the length of the nerve (C, C’’) compared to *Nrv2>*Inx2-RNAi, p35 nerves in which WG aggregates (D, D’’) are present even though the apoptosis pathway is blocked by p35.

**E:** The average instantaneous speed of locomotion in control (*Nrv2::GFP,Gli-GAL4*, black) compared to Inx2 knockdown (*Nrv2::GFP,Gli>*Inx2-RNAi, magenta) and Inx1 knockdown (*Nrv2::GFP,Gli>*Inx1-RNAi, green) in subperineurial glia. The average instantaneous speed with the standard deviation are shown. The mean speeds: *Nrv2::GFP,Gli-GAL4*, 3.633 ± 1.479mm/sec, n=59 (control); *Nrv2::GFP,Gli>*Inx2-RNA, 2.545 ± 1.034mm/sec, n=27; *Nrv2::GFP,Gli>*Inx1-RNA, 2.441 ± 0.9512mm/sec, n=37. Differences in average instantaneous speed were analyzed using a one-way ANOVA and Tukey’s post hoc test and the significance is compared to the control for each experiment. Knockdown of Inx2 and Inx1 in the subperineurial glia result in significant reduction in speed of larval locomotion (*** p=0.0008, **** p<0.0001 respectively). **F:** The average instantaneous speed of locomotion in control (*Nrv2>*mCD8::RFP, black) compared to Inx2 knockdown (*Nrv2>*Inx2-RNAi, magenta) and Inx1 knockdown in wrapping glia (*Nrv2>*Inx1-RNAi, green). The mean plus standard deviation are shown. The mean speeds: *Nrv2>*mCD8::RFP, 3.118 ± 1.205mm/sec, n=112 (control); *Nrv2>*Inx2-RNAi, 2.881 ± 1.003mm/sec, n=39; *Nrv2>*Inx1-RNAi, 3.121 ± 1.437mm/sec, n=57. Differences in average instantaneous speed were analyzed using a one-way ANOVA and Tukey’s post hoc test and the significance is compared to the control for each experiment (ns, not significant). There was no significant difference between control and both Inx2 and Inx1 RNAi larvae.
**Figure 2.11.** Expressing a dominant negative mutant that disrupts Inx2 channel function specifically in the subperineurial glia leads to fragmentation of the wrapping glia

**A:** Control peripheral nerves with SPG and WG membranes labeled with mCD8::RFP (magenta) and Nrv2::GFP (green), respectively. **D,G,J:** Gli>RFP::Inx2 peripheral nerves with the dominant negative Inx2 mutant (RFP::Inx2, magenta) being driven specifically in the SPG and the WG membranes labeled with Nrv2::GFP (green). The yellow lines indicate the region from which the cross sections were taken. The WG membrane (green) in the control nerve (A) extends its processes along the entire length of the nerve and the thin SPG membrane (magenta) surrounds the WG (A’). WG membrane aggregates (green, yellow arrowheads) are present along the length of some nerves in Gli>RFP::Inx2 larvae (F) whereas others have discontinuous WG membranes (I) or a single WG strand (L). The Inx2 dominant negative protein (magenta) is localized to the SPG-WG boundary in the Gli>RFP::Inx2 larvae (D-E’, G-H’, J-K’, white arrowheads). Remnants of the WG membrane (green) are found in the center of the nerve (F’, I’ and L’ yellow arrowheads) in Gli>RFP::Inx2 nerves. The white dashed boxes were digitally magnified (200x) and shown in M-P, Q-T and U-X respectively. The RFP labeled Inx2 puncta (N,R and V, white arrowheads) co-localize with the antibody labeled Inx2 puncta (P,T, and X, white arrowheads) in Gli>RFP::Inx2 peripheral nerves at the SPG-WG boundary (M-X). Scale bars: 15µm.
**SPG**

A. Most severe phenotype

B. Severe but different phenotype

C. Wild type

D. Least severe phenotype

E. Severe phenotype

F. Severe phenotype

**WG**

Inx1 homotypic channels (not possible)

- Inx2 KD in all glia --> WG aggregates, lethal

Inx2 homotypic channels

- Inx1 KD in all layers --> glial swellings

Inx2-Inx1/Inx2 heterotypic channels

- Inx1 KD in SPG --> Wild type SPG & WG
  Inx1 is not necessary in the SPG

Inx1/Inx2-Inx2 heterotypic channels

- Inx1 KD in WG --> Less WG strands Inx1 is required in the WG

Inx1/Inx2-Inx1 heterotypic channels (not possible)

- Inx2 KD in WG --> WG aggregates, death
  Inx2 is required in the WG

Inx1-Inx2/Inx1 heterotypic channels (not possible)

- Inx2 KD in SPG --> WG aggregates, death
  Inx2 is required in the SPG
**Figure 2.12.** Inx2 hemichannels in the SPG couple with Inx1/Inx2 hemichannels in the WG to mediate a signal essential for WG survival

**A:** Pan glial knockdown of Inx2 results in the most severe phenotypes including WG aggregates and larval lethality. In the absence of Inx2, no gap junctions form between the SPG and the WG as Inx1 (green) cannot form homotypic channels.

**B:** Pan glial knockdown of Inx1 results in glial swellings. In the absence of Inx1, Inx2 (magenta) forms homotypic channels that have different properties from Inx1/Inx2 heterotypic channels.

**C:** Knockdown of Inx1 in the SPG results in Inx2 hemichannels (magenta) in the SPG that couple with Inx1/Inx2 hemichannels (green/magenta) in the WG. This does not affect both SPG and WG morphology.

**D:** Knockdown of Inx1 in the WG results in Inx1/Inx2 hemichannels (green/magenta) in the SPG that couple with Inx2 hemichannels (magenta) in the WG. This results in less WG strands and suggests a role for Inx1 in the WG.

**E:** When Inx2 (magenta) is knocked down in the WG, the gap junctions between SPG and WG cannot form because Inx1 (green) cannot form homomeric hemichannels. This results in WG aggregation and death.

**F:** When Inx2 (magenta) is knocked down in the SPG, the gap junctions between SPG and WG cannot form because Inx1 (green) cannot form homomeric hemichannels. This results in WG aggregation and death.
Chapter 3: A screen for key PDZ proteins identified a role of Dlg5 in peripheral glia development

3.1 Synopsis

Glial cells in the peripheral nerve wrap axons to insulate them and ensure efficient conduction of neuronal signals. Glia-glia interactions such as adhesion need to occur to enable efficient ensheathment of axons. However, not much is known about how glial cells adhere and communicate with each other. In the myelin sheath, it is proposed that the autotypic tight junctions and adherens junctions form glia-glia complexes that stabilize the glia sheath in myelinating glia, yet the role of adhesion junctions in non-myelinating glia of vertebrates or invertebrates has not been clearly established. Many components of adhering junctions contain PDZ (PSD95, Dlg, ZO1) domains or are recruited to these junctions by PDZ binding motifs. To test for the role of PDZ domain proteins in glial sheath formation, we carried out an RNAi screen to knockdown each of the 66 predicted PDZ domain proteins in the peripheral glia. We identified seven PDZ proteins required in glial cells of the larval peripheral nerve and in particular identified a role for the MAGUK protein Dlg5. Dlg5 function has been implicated with adherens junction formation and scaffolding a range of signaling complexes but the role of Dlg5 in glia has not been determined. Loss of Dlg5 lead to a disruption of ensheathment of axons by the wrapping glia as well as a disruption of the septate junctions/blood-nerve barrier created by the subperineurial glia.
3.2 Introduction

A critical function of glia is to wrap and ensheath axons in both the central and peripheral nervous systems. In vertebrate peripheral nerves, myelinating Schwann cells enable rapid and efficient transduction of electrical signals by forming the myelin sheath to ensheath and insulate large caliber axons. While there are extensive studies identified the protein complexes that mediate myelin sheath formation and stabilization, almost nothing is known about the adherent junctions that mediate sheath formation in non-myelinating Schwann cells, yet this class of glia is essential to protect and support small caliber axons such as C-fibers that transmit nociceptive information. Disruption of the glial sheath in non-myelinating Schwann cells leads to degeneration of sensory neurons and insensitivity to heat and cold pain (Chen et al., 2003). Although studies have identified many components of adherent junctions in the myelin sheath, the entire composition of proteins present or interacting with in non-myelinating glia is largely unknown. Many components of adherent junctions such as tight junctions (TJ) are either PDZ (PSD-95, Dlg, ZO1) proteins or contains motifs that allow them to interact with PDZ proteins. PDZ domains are 80-90 amino acids and mediate protein-protein interaction by binding to short three amino acid PDZ binding motifs normally found at C-terminus of many proteins (Harris and Lim, 2001, Subbaiah et al., 2011). Moreover, many PDZ proteins often possess different combinations of protein interaction domains and the membrane associated guanylate kinases (MAGUKs) are an example of this. Apart from PDZ domains, MAGUK proteins possess a SH3 and GUK domain, which like the PDZ domain form protein-protein interactions. PDZ proteins, including MAGUKs, regulate processes such as cell polarity, adhesion, migration and signaling through their ability to scaffold proteins to specific regions in the cell.
We use the *Drosophila* peripheral nerve to study the molecular mechanisms that underlie glial sheath formation in non-myelinating glia. In the *Drosophila* peripheral nerve, axons are ensheathed by three glial layers. The innermost glia layer, the wrapping glia (WG) resembles the non-myelinating Schwann cells and extend their processes around individual or bundles of motor and sensory axons. The WG layer is surrounded by the subperineurial glia (SPG) which form septate junctions (SJ) between the glial layers to establish the blood-nerve barrier. Lastly, the outermost glial layer, the perineurial glia (PG) wrap around the nerve and the entire nerve is surrounded by an extracellular matrix (ECM) to form the neural lamella. Adhesion and signaling between these different glial layers must require glia-glia interactions, however the proteins required for these processes remain largely unknown. It is likely that PDZ proteins might play a role in mediating glia-glia junctions similar to the role in myelinating glia. Acquiring a better understanding of PDZ proteins and their interactions at glia-glia junctions requires that we first identify those PDZ proteins that function in non-myelinating glia. The human genome codes for 250 predicted PDZ proteins (Tonikian et al., 2008). In comparison, there are 66 PDZ proteins in *Drosophila* and in this study, we targeted the PDZ-encoding proteins using RNAi knockdown specifically in peripheral glia. The morphology of the glia of the larval peripheral nerve was assessed and we identified seven PDZ proteins whose knockdown lead to changes in glial morphology. We identified Varicose (Vari, vertebrate PALS1) where Vari function is similar to its known function in the embryonic epithelia in septate junction (SJ) formation. We also identified Stardust (Sdt, vertebrate PALS2) where Sdt might have non-canonical partners in the peripheral nerve. We further identified Dlg5 and characterized the distribution of Dlg5 and the cellular effects of Dlg5 knockdown in glial cells. Loss of Dlg5 affected both wrapping glia and subperineurial glia morphology. In contrast to the role of vertebrate Dlg5, *Drosophila* Dlg5 did
not appear to be involved in the scaffolding of cadherins in the peripheral glia. However, we found that unlike cadherin localization in epithelial cells which are highly concentrated and polarized in belts of adherens junctions, cadherins in glial cells are rarely observed in concentrated belt-like regions, making it difficult to distinguish between correctly localized and mislocalized cadherins. Nevertheless, we show that cadherins were still expressed, targeted to the membrane and formed spot adherens junctions in Dlg5 knockdown nerves. We did observe that Dlg5 knockdown affected SJ morphology and was required for the localization of the scaffolding protein Scribble (Scrib) to the SJ.

3.3 Material and methods

3.3.1 Fly strains and genetics

The following fly strains were used in this study: repo-GAL4 (Sepp et al., 2001); Nrv2-GAL4 (Sun et al., 1999); 46F-GAL4 (Xie and Auld, 2011); Gli-GAL4 (Sepp and Auld, 1999); UAS-mCD8::GFP (Lee and Luo, 1999); UAS-Dicer2 (Dietzl et al., 2007); UAS-mCD8::RFP (gift from Elizabeth Davis, Princeton University); UAS-Dlg5-GFP (30927, Bloomington) Dlg5::GFP (Sarov et al., 2016). The following GFP protein-trap insertions were used: Nrv2::GFP (Morin et al., 2001), NrxIV::GFP (Buszczak et al., 2007). The following Dlg5-RNAi lines were used: GL01260 (TRiP, Transgenic RNAi project, Bloomington) and GD16339, GD11943, KK104086 (Vienna Drosophila resource center, VDRC). The Sdt RNAi primarily used was the HMS01652 (TRiP, Bloomington). All RNAi experiments carried out after the initial screen were carried out at 25°C without Dicer2 in the background unless specified.
3.3.2  *In vivo* RNAi knockdown of PDZ genes

RNAi lines were obtained from TRiP and VDRC stock centers. Table 3.1 provides the list of RNAi lines used in the screen. *repo>mCD8::RFP/TM6,Tb* virgin flies were collected and crossed with males from each UAS-RNAi line at 29°C. As a control, *repo>mCD8::RFP/TM6,Tb* virgin flies were crossed with *w^{1118}* males and raised at 29°C. A minimum of 6 larvae were dissected from each cross and stained with anti-22C10, to label the peripheral axons. The morphology of the glial membranes were visualized using UAS-mcD::RFP.

3.3.3  Immunolabeling and image analysis

The peripheral nervous system of 3rd instar, wandering larvae were dissected and fixed for immunolabeling using previously described methods (Sepp et al., 2000). The following primary antibodies were used in this study: guinea pig anti-Inx2 (1:500), (Smendziuk et al., 2015); mouse anti-Futsch/22C10 (1:1000, DSHB); rabbit anti-GFP (1:600, Life Technology); mouse anti-GFP (1:300, Novus Biologicals), rabbit anti-Cherry (1:300, Abcam), mouse anti-βps (1:50, CF.6G11) (Brower et al., 1984), rabbit anti-GM130 (1:600, Abcam), guinea pig anti-HRS (1:500), rabbit anti-Rab11 (1:8000) (Tanaka and Nakamura, 2008), guinea pig anti-vps26 (1:1000, gift from Hugo Bellen), rabbit anti-Rab7 (1:2000) (Tanaka and Nakamura, 2008), rabbit anti-Rbsn 5 (1:6000) (Tanaka and Nakamura, 2008), rabbit anti-Nrv2.1 (1:1000, Abcam). The following secondary antibodies were used at a 1:300 dilution: goat anti-mouse Alexa 488, Alexa 568 and Alexa 647; goat anti- rabbit Alexa 568 and goat anti- guinea pig Alexa 647 (Molecular Probes). DAPI (1:1000, Invitrogen) was used to stain nuclei. Images were acquired with a Delta Vision Spectris compound microscope (Applied Precision/GE Healthcare, Mississauga, Ontario) using a 60x oil immersion objective (NA 1.4). An image was captured every 0.2 µm and the
resulting stacks were deconvolved (SoftWorx, Toronto, Canada) using a point spread function measured with 0.2 µm beads conjugated to Alexa dyes (Molecular Probes) and mounted in Vectashield® (Vector Laboratories, Burlington, Canada). Cross sections were generated using SoftWorx. A single z-slice, conveying the information relevant to the experiment, was chosen from the z-stack and images were compiled using Adobe Photoshop and Adobe Illustrator CC. For transmission electron microscopy analysis larvae were dissected and prepared using previously described methods (Matzat et al., 2015).

3.4 Results

3.4.1 RNAi induced knockdown of PDZ proteins in glial cells

There are only 66 predicted PDZ genes in the Drosophila genome (Aranjuez et al., 2012, Bilder, 2001), making Drosophila a simpler to identify those PDZ proteins involved in glial adhesion or signaling via formation of scaffolding complexes. To understand the role of PDZ proteins in mediating glia-glia communication, we utilized the peripheral nerve of the Drosophila larvae. To address the role of PDZ proteins in peripheral glia, we used a pan-glial driver repo-GAL4 to express UAS-PDZ-RNAi lines while co-expressing UAS-mCD8::RFP to label the glial membranes. Therefore, we scored morphological defects in the RFP labeled glial membranes when each PDZ protein was knocked down using UAS-RNAi lines that targeted each PDZ gene (Table 3.1). Axons were labeled using anti-22C10 to reveal any axonal defects. When available, multiple RNAi lines were used to target each PDZ gene in all three glial layers. For the 66 genes, 150 individual UAS-RNAi lines were tested for abnormal glial phenotypes in the PNS (Table 3.1). As a control repo> mCD8::RFP flies were crossed to w^{118} flies (Fig. 3.1.A-A’). We classified PDZ genes as positive hits (Table 3.1, yellow) if their knockdown using
multiple RNAi lines resulted in lethality or glial phenotypes. Seven PDZ proteins; Discs Large 1 (Dlg1), Scribbled (Scrib), Varicose (Vari), Stardust (Sdt), Discs Large 5 homolog (Dlg5), Locomotion defects (Loco), and a Rho-type guanine nucleotide exchange factor (RhoGEF2) were identified as positive hits with potential functions in glial cells (Table 3.1, Fig. 3.1B-G). The screen resulted in a range of phenotypes such as glial swelling as seen in the glial knockdown of RhoGEF2 and Loco (Fig. 3.1C-C’’,F-F’’). Knockdown of Sdt, Dlg1 and Scrib, all known for their roles in epithelial polarity, resulted in abnormal axonal wrapping (Fig. 3.1B-B’’,D-D’’,E-E’’), whereas Vari knockdown in glial cells was lethal (Table 3.1). Abnormalities in axonal wrapping, specifically by the inner wrapping glial membrane were observed in Dlg5 knockdown larvae (Fig. 3.1G-G’). Overall, we have identified a group of conserved PDZ proteins that may play necessary roles in peripheral glia development.

3.4.2 Varicose is required for axonal ensheathment and its loss affects septate junction morphology in the larval peripheral nerve

The majority of the identified candidates from the screen are scaffolding proteins that have well established roles in maintaining polarity or cell adhesion in epithelial cells. The role of two of the identified candidates, Dlg1 and Scribble in maintaining basal polarity has been widely studied in epithelial tissue. Their role in peripheral glia however, is yet to be fully understood and is currently been investigated by our group (Gilbert M; unpublished data). We therefore performed additional tests on Vari and Sdt. Vari (vertebrate PALS2) is a MAGUK protein known to play a role in maturation of septate junctions (SJs) in Drosophila epithelia as well as tracheal morphogenesis (Wu et al., 2007). Although previous studies have shown that Vari is expressed in embryonic peripheral glia, its role in these cells has not been fully characterized.
We found that loss of Vari in glial cells (repo>Vari-RNAi) (Table 3.1) leads to lethality at larval stages likely due to the disruption of the SJs. SJs are formed between apposing membranes of the SPG that encircle the nerve. These junctions form the blood-nerve barrier thereby preventing paracellular diffusion and protecting axons from the high K⁺ concentration in the hemolymph. Many proteins including Neurexin IV (NrxIV), Neuroglian (Nrg), Nervana 2 (Nrv2), Coracle (Cora), Sinuous (Sinu), Megatrachea (Mega) and Contactin (Cont) are required for the formation of the SJ in the peripheral nerve (Baumgartner et al., 1996, Stork et al., 2008). We therefore used a key SJ component NrxIV (endogenously tagged with GFP) to test if the morphology of the SJs was disrupted in peripheral nerves. We knocked down Vari specifically in the SPG (NrxIV::GFP, Gli>mCD8::RFP, Vari-RNAi) and the NrxIV::GFP and SJ integrity was severely disrupted in 73.4% of nerves with Vari knockdown (Fig. 3.2G-I’’)(n=4 larvae) compared to 0% of controls (n=5 larvae)(NrxIV::GFP, Gli>mCD8::RFP)(Fig. 3.2A-C’’). Vari knockdown in the SPG lead to abnormal glial morphology (Fig. 3.3C’’) with glial swellings observed in all nerves (Fig. 3.3C’’)(n= 5 larvae). Moreover, WG defects and defasciculated axons were observed in 13% of the nerves and correlated with regions where the SPG membranes were extremely swollen (Fig. 3.3C’ and C’’’). In comparison, none of these abnormal phenotypes were observed in the control nerves (Fig. 3.3A-A’’’)(n= 5 larvae). Varicose is therefore not only required for axonal wrapping but its loss also affects SJ morphology in third instar larvae.

3.4.3 Loss of Sdt leads to glial defects

Sdt (vertebrate PALS1) encodes a MAGUK protein and is a core component of the Crumbs protein complex present in both invertebrate and human epithelial and photoreceptor
cells. The Crumbs complex is comprised of four core proteins: Crumbs (Crb), Stardust (Sdt) and PATJ (Pals1-associated tight junction protein) and Lin7 and together this complex is essential for maintenance of apicobasal polarity of epithelia (Bachmann et al., 2001). We found that knockdown of Sdt in glial cells (repo>Sdt-RNAi) resulted in abnormalities in both inner and outer glial morphology in 32.5% of the nerves (n=5 larvae) (Fig. 3.1B-B’’). However, pan-glial knockdown of Patj, a protein that Sdt interacts with in epithelial tissues, using multiple RNAi lines did not result in any glial or axonal defects (Table 3.2). This suggests that while Sdt is required for axonal wrapping, establishment of the glia wrap is independent of Patj. Since the localization of Sdt and Patj in epithelial cells relies on Crumbs (Crb), we looked for Crb expression using Crb endogenously tagged with GFP (Crumbs::GFP-C) (Huang et al., 2009). We found that Crb is not expressed in glial cells in the Drosophila PNS (data not shown). Additionally, Sdt is known to be recruited by Bazooka (Baz) in Drosophila epithelial cells (Krahn et al., 2010). However, although Sdt-RNAi in glial cells leads to defects in axonal wrapping, repo>Baz-RNAi larvae have normal peripheral glial morphology (Table 3.2). These results taken together suggest that unlike its role in epithelial cells, Sdt plays a Crumbs, Patj and Baz independent role in glial cells. These results also imply that polarity is set up differently in glial cells compared to epithelia. Sdt knockdown in the SPG did not affect the morphology of the SJs (NrxIV::GFP, Gli>mCD8::RFP, Sdt-RNAi)(Fig 2D-F’’)(n=6 larvae). Furthermore, Sdt knockdown in the SPG also did not affect the SPG membranes or Nrv2::GFP labeled WG membranes (Nrv2::GFP, Gli>mCD8::RFP, Sdt-RNAi)(Fig 3B-B’’)(n=8 larvae) and were similar to their controls (Fig 3.3A-A’’’)(n= 5 larvae). Moreover, the axonal morphology of larvae lacking Sdt in the SPG (Fig. 3.3B’’’) was also similar to control larvae (Fig. 3.3A’’’). Therefore, the lack of axonal ensheathment phenotypes observed with the pan-glial knockdown of Sdt.
might be due to a lack of Sdt in the WG. The mechanism by which Sdt controls axonal ensheathment, however, still remains unknown.

### 3.4.4 Dlg5 is expressed in glial cells of the larval peripheral nerve

Given the extensive literature regarding the role of Dlg5 in cell polarity and cell-cell adhesion and the fact that Dlg5 has not been studied in glial cells, we decided to focus on Dlg5. Dlg5 is a MAGUK protein with a coiled-coil domain at the amino terminus, four PDZ domains, an SH3 and GUK domains. Knockdown of Dlg5 in all glial cells using *repo-GAL4* resulted in lack of axonal ensheathment by the WG (Fig. 3.1G). To further characterize the effects of Dlg5 knockdown on the peripheral glia, we knocked down Dlg5 in individual glial layers using GAL4 drivers specific to each of the three glial layers. *46F-GAL4* was used to drive RNAi expression specifically in the PG layer. We found that even though Dlg5 is most likely present in the PG (Fig 3.4A), knockdown of Dlg5 in the PG (*46F>mCD8::RFP,Dlg5-RNAi*) (n=5 larvae) does not affect the morphology of the PG layer (Fig. 3.4C-D’’) and was similar to control nerves (*w1118, 46F>mCD8::RFP*, Fig 3.4A-B’’) (n=7 larvae). We then used *Gli-GAL4* to label the intermediate SPG layer with RFP while assaying the WG morphology using Nrv2 endogenously tagged with GFP (*Nrv2::GFP, Gli>mCD8::RFP*). The controls did not show any defects in both the SPG and WG membranes (*w1118, Nrv2::GFP, Gli>mCD8::RFP*) (Fig. 3.4E-F’’) (n=6 larvae). In larvae expressing Dlg5 RNAi (*Nrv2::GFP, Gli>mCD8::RFP, Dlg5-RNAi*) (n=6 larvae) the SPG membrane morphology was disrupted in 17% of nerves, with breaks/gaps in the membrane which were occasionally accompanied by swellings (Fig. 3.4G,G’ and H,H’). The WG membrane however, was not affected by Dlg5 knockdown in the SPG (Fig. 3.4G’’, H’’). Furthermore, knockdown of Dlg5 in the WG using *Nrv2-GAL4* resulted in less WG strands and
discontinuous WG strands in 49% of nerves (Nrv2>mCD8::RFP,Dlg5-RNAi, Fig. 3.4K,K’’) (n=5 larvae) compared to the control nerves with multiple, continuous WG strands insulating the axons (w1118, Nrv2>mCD8::RFP)(Fig. 3.4I-J’’)( n=6 larvae). Dlg5 knockdown in the WG however, did not affect axonal morphology (Fig. 3.4K’,L’) which was similar to control nerves (Fig. 3.4I,J’). Similar WG phenotypes were observed in 50% of nerves, with the pan-glial knockdown of Dlg5 where Nrv2::GFP was used to label the WG (Nrv2::GFP, repo> Dlg5-RNAi) (data not shown)(n=5 larvae). This suggests that Dlg5 is required specifically in both the SPG and the WG but not in the PG layer.

3.4.5 Dlg5 does not localize to the gap junction and focal adhesion complexes in the peripheral nerve

The disruption of the SPG and WG with loss of Dlg5 suggested a role of Dlg5 in these layers. Given the role of Dlg5 in a wide range of cellular processes including signaling, membrane protein trafficking (Nechiporuk et al., 2013, Nechiporuk et al., 2007, Liu et al., 2017, Kwan et al., 2016, Reilly et al., 2015, Wang et al., 2014) we next tested the distribution of Dlg5 and the effects on a range of membrane protein complexes. We began by examining the distribution of Dlg5 in the glial layers of the peripheral nerve in 3rd instar Drosophila larvae. Dlg5 expression was observed in the peripheral nerve using Dlg5 endogenously tagged with GFP (Dlg5::GFP)(Fig. 3.5A-D’’) while peripheral glial layers were labeled with mCD8::RFP(Fig. 3.5A,A’ and B,B’) and the axons with anti-Futsch/22C10 (Fig. 3.5C,C’ and D,D’). Dlg5 puncta were observed throughout the peripheral nerve, with puncta present within the glial cells (Fig. 3.5A-B’’). Some Dlg5 puncta might also be present in the axons (Fig. 3.5C-D’’) however, knockdown of Dlg5 using the pan-neuronal driver elav-GAL4, did not affect either glial or
axonal morphology (n=6 larvae, data not shown). Given the disruption of WG processes with Dlg5-RNAi, we tested for colocalization with Dlg5 with junctional proteins known to also affect WG morphology, gap junctions and the integrin complex. Loss of the gap junction protein Inx2 also leads to less or discontinuous wrapping glia strands (Chapter 2). Dlg5::GFP nerves were assayed for co-localization with Inx2. However, we did not observe any co-localization between the Dlg5::GFP and Inx2 puncta, suggesting that Dlg5 does not localize to the same signaling complex as the gap junctions in the larval peripheral nerve (Fig. 3.5E-F’’). We looked for co-localization between Dlg5 and the beta subunit of integrin (βPS, myospheroid (Mys) as knockdown of βPS results in failure to wrap axons including loss of wrapping glia processes (Xie and Auld, 2011). However, we found that Dlg5 puncta (Dlg5::GFP) did not co-localize with immunolabeled βPS puncta or stripes (Fig. 3.5G-H’’), suggesting that Dlg5 is not present in the integrin complexes found in the peripheral nerve. Furthermore, Dlg5 knockdown in glial cells did not affect either Inx2 and Inx2 was localized at the SPG-WG these nerves (data not shown). The localization of βPS was also not affected by Dlg5 knockdown in glia (data not shown). These results further support the conclusion that Dlg5 plays no role in gap junction or integrin localization.

### 3.4.6 Dlg5 co-localizes with N-Cadherin and associates with some Vps26 positive puncta

Based on previous studies that demonstrate the role of Dlg5 in the localization of cadherins to the plasma membrane (Reilly et al., 2015, Wang et al., 2014), we next tested if Dlg5 interacts with cadherins and is required for their localization to the glial membrane. As a first step, we tested for co-localization between Dlg5 and the two classical *Drosophila* cadherins; E-Cadherin (DE-Cad) and N-Cadherin (DN-Cad). The Dlg5 pattern was assayed using Dlg5::GFP,
whereas the distribution of both DN-Cad (Fig. 3.5I-J’’) and DE-Cad (Fig. 3.5K-L’’) was observed using DN-Cad and DE-Cad tagged with mCherry. DE-Cad::mCherry puncta did not co-localize with Dlg5::GFP puncta (Fig. 3.5K-L’’). However, we found that some but not all DN-Cad::mCherry puncta overlapped with Dlg5::GFP in the peripheral nerve (Fig. 3.5I-J’’). This partial overlap between DN-Cad and Dlg5::GFP is consistent to prior work in mouse embryonic fibroblasts, where Dlg5::GFP only overlaps with a subset of N-Cad (Wang et al., 2014). In fibroblasts, Dlg5-positive vesicles also partially overlap with Rab11, a marker for recycling endosomes, as they fuse with cadherin-carrying vesicles on the way to the plasma membrane. To test whether Dlg5 in Drosophila peripheral glia are involved in trafficking pathways, we stained the PNS with a series of vesicle markers including Rab11, Rabsn5 (an early endosomal marker), Rab7 (a late endosomal maker), HRS (a multivesicular body (MVB) marker), Vps26 (a component of the retromer complex), Sec23 (a COPII vesicle marker), G130 (a Golgi marker) as well as syntaxin1A (a t-SNARE protein). Surprisingly none of these subcellular markers, except Vps26, overlapped with Dlg5::GFP puncta (Fig. 3.5M-N’’,O) and, similar to DN-Cad, the Dlg5::GFP puncta only partially overlapped with Vps26 (Fig. 3.5M-N’’). To determine if Vps26 interacts with Dlg5 in a particular region in the nerve, we looked at orthogonal sections of the nerve and compared the outer circumference to the center of the nerve. We found that Dlg5 positive puncta associate with few Vps26 puncta in the outer circumference of the nerve (Fig. 3.5N-N’’). Interestingly, Dlg5 did not co-localize with Rab-11-positive vesicles that recycle cadherins to the cell surface (data not shown). To examine the vesicle-trafficking machinery of DE-Cad and DN-Cad in the peripheral nerve, we looked for co-localization between Rab11 and the cadherins. We wanted to determine if Rab11 vesicles carry DE-Cad and DN-Cad in the peripheral nerve. Larval peripheral nerves expressing either DN-Cad::Cherry or
DE-Cad::Cherry were labeled with Rab11 using an antibody. We found that some but not all Rab11-positive puncta overlap with DN-Cad (Fig. 3.6C-C’’) and DE-Cad (Fig. 3.6D-D’’). This result suggests that only a subset of the cadherins are being recycled in the Rab11-positive endosomes. The cadherin puncta that do not localize with Rab11 might either represent cadherins that are at the SAJ or are newly synthesized cadherins being trafficked to these junctions. We then assayed for co-localization between cadherins and Vps26, a component of the retromer complex and observed Vps26 immunolabeling with both DN-Cad (Fig. 3.6A-A’’) and DE-Cad (Fig. 3.6B-B’’). Therefore, DE-Cad and DN-Cad appear to be trafficked via the retromer complex and potentially recycled to the membrane in Rab11-positive vesicles in peripheral glia. These results together suggest that both Dlg5 and cadherins interact with the retromer complex but only cadherins are present in Rab-11 positive vesicles.

### 3.4.7 Dlg5 might not affect the localization of DN-Cad and DE-Cad in the larval peripheral nerve

One of the known functions of vertebrate Dlg5 is to localize N-Cadherin (N-Cad) to the membrane, form adherens junctions (AJs) required and maintain cell polarity (Wang et al., 2014). Since, *Drosophila* Dlg5 shows 45-54% sequence homology and 26 % identity to human Dlg5, it is possible that their functions might be conserved. Moreover, a study in *Drosophila* shows that Dlg5 is required for the localization of E-Cadherin (DE-Cad) in follicle cells (Reilly et al., 2015). The role of Dlg5 in *Drosophila* glial cells however, remains to be tested. Spot adherens junctions (SAJs) have been identified in electron micrographs of cross-sections of peripheral nerves in 3rd instar *Drosophila* larvae (Matzat et al., 2015). It is likely that these junctions are composed of the classical cadherins DE-Cad and/or DN-Cad. We wanted to
determine if Dlg5 is required for localization of cadherins and formation of these junctions and tested to see if either DN-Cad and DE-Cad are mislocalized or absent when Dlg5 is knocked down in the peripheral glia. We used repo-GAL4 to drive Dlg5-RNAi in all glial cells while assaying the morphology of the WG using Nrv2::GFP and the distribution of DN-Cad and DE-Cad using DN-Cad::mCherry (n=5 larvae) and DE-Cad::mCherry (n=9 larvae), respectively (DN-Cad::mCherry or DE-Cad::mCherry with Nrv2::GFP, repo>Dlg5-RNAi) (Fig. 3.7C-D’’ and G-H’’). We found that with the knockdown of Dlg5, both DN-Cad and DE-Cad were still distributed in belt-like regions (Fig. 3.7C’’ and G’’) similar to control larvae (DN-Cad::mCherry or DE-Cad::mCherry, with Nrv2::GFP, repo-GAL4, n=4 and 8 larvae, respectively) (Fig. 3.7A’’,E’’). These DN-Cad and DE-Cad belts were observed only on average in 1-3 nerves per larva in both repo>Dlg5-RNAi larvae and controls. We also noticed that the DE-Cad belts observed were always present within and coincided with the remaining WG strands (Fig. 3.7G-G’’, H-H’’). Unlike, DE-Cad, DN-Cad did not show a preferential localization to the WG strands (Fig. 3.7D-D’’). Moreover, both DN-Cad and DE-Cad formed puncta that were spread throughout all the nerves and these were still present after Dlg5 knockdown (Fig. 3.7C,C’’, G,G’’). These puncta may represent cadherin-based spot adherens junctions (SAJs). These results suggest that Dlg5 knockdown in the glia might affect cadherin localization. However, given the disruption of the WG morphology in these nerves it is difficult to assess changes in cadherin expression and trafficking. To determine if Dlg5 affects the formation of SAJs, the ultrastructure of SAJs in Dlg5 knockdown nerves were analyzed using transmission electron microscopy (TEM). SAJs were observed in TEM cross-sections of peripheral nerves in both control (w1118, repo>mCD8::RFP) (n=1) (Fig. 3.7I and I’) and Dlg5 knockdown larvae (repo>Dlg5-RNAi) (n=2) (Fig 7J-K’). The presence of SAJs in Dlg5 knockdown further suggests
that Dlg5 is not necessary for the localization of cadherins and formation of the SAJs. We however noticed that the axons were wrapped in unusually large fascicles in the Dlg5 knockdown nerves (Fig. 3.7J and K) compared to the control (Fig. 3.7I) (n=2). Therefore, our results suggest that the lack of proper axonal ensheathment observed in Dlg5 knockdown nerves is not a result of cadherin mislocalization of loss of SAJs.

3.4.8 Dlg5 is required for the formation of the septate junction protein complex in the peripheral nerve

Since Dlg5 knockdown affected SPG morphology, we next tested if the defects in the SPG were affecting the SJ protein complex. We looked for changes in the SJ morphology NrxIV::GFP while knocking down Dlg5 in the SPG (NrxIV::GFP, Gli>mCD8::RFP, Dlg5-RNAi). We found that the NrxIV::GFP labeled SJ strands in 32% nerves were disrupted in the regions corresponding to the disruptions in the SPG membrane (Fig. 3.8G-G’’) (n=12 larvae). The SJ strands in 3.4% of the nerves showed severe defects (data not shown). In comparison, the SJ strands in controls (w1118, NrxIV::GFP, Gli>mCD8::RFP) were continuous (Fig. 3.8E-E’’) (n=12 larvae). Moreover, we knocked down Dlg5 in the PG layer (46F>mCD8::RFP, Dlg5-RNAi) (Fig. 3.8C-C’’) (n=6 larvae) while assaying for changes in SJ morphology using a Nrv2.1 antibody and found that there were no changes to the SJs compared to controls (w1118, 46F>mCD8::RFP) (Fig. 3.8A-B’’) (n=6 larvae). Similarly, knockdown of Dlg5 in the WG lead to the expected WG phenotypes (Fig. 3.8K,K’’and L,L’’) but did not affect the morphology of the NrxIV::GFP labeled SJ strands (NrxIV::GFP, Nrv2>mCD8::RFP, Dlg5-RNAi, Fig 3.8K’) (n=6 larvae). Both WG and SJ morphology were normal in the controls (w1118, NrxIV::GFP, Nrv2>mCD8::RFP, Fig. 3.8I-J’’) (n=6 larvae). Therefore, downregulating Dlg5 not only leads to
lack of axonal ensheathment by the WG but also affects SJ morphology and specifically the formation of the SJ complex.

### 3.4.9 Dlg5 affects the distribution of Scribbled (Scrib) in the larval peripheral nerve

To understand the mechanism by which Dlg5 is able to carry out its functions, we looked for other known targets and pathways in which Dlg5 might be involved. We were particularly interested in Scribbled (Scrib) as this scaffolding protein plays a role in trafficking and is localized to the SJ domain along with Dlg1 (Woods et al., 1996, Bilder and Perrimon, 2000). Scrib also regulates different aspects of endosomal trafficking, including internalization of BMP type 1 receptor Thickvein (Tkv) and the sorting of cargos that pass through the retromer pathways (Gui et al., 2016, de Vreede et al., 2014, Verges, 2008). Moreover, mammalian Scrib prevents retromer sorting of lysosomal-destined E-Cad by stabilizing the E-Cad-p120-catenin interaction (Lohia et al., 2012). Therefore, Scrib is a well-known regulator of retromer-dependent endocytic trafficking. Since Dlg5 co-localized with the retromer complex (Fig. 3.4M-N’’), we next tested if Dlg5 associates with Scrib in the peripheral nerve. Using Scrib endogenously tagged with GFP (Scrib::GFP), we followed the distribution of Scrib when Dlg5 was knockdown in the SPG (Scrib::GFP, Gli>mCD8::RFP, Dlg5-RNAi). In control nerves, Scrib is found as a single, continuous strand in along the SJ domain in the SPG of each peripheral nerve (Fig. 3.9A,A’’). Knockdown of Dlg5 leads to disruptions/gaps in 10% of the Scrib labeled strands (Fig. 3.9B,B’’). Severe mislocalization of Scrib was observed in 16.7% of nerves (Fig. 3.9C,C’’) which were usually accompanied with swellings in the SPG membrane (Fig. 3.9C,C’). These results suggest that Scrib is affected upon loss of Dlg5. Whether Dlg5 is required for the
trafficking of Scrib to the SPG membrane or if Scrib is mislocalized as a consequence of the SJ defects in Dlg5 knockdown nerves remains to be tested.

3.5 Discussion

In the human genome, approximately 100 genes are predicted to encode 250 PDZ proteins (Venter et al., 2001, Lander et al., 2001). One of the main features of these PDZ proteins is their ability to direct many developmentally and physiologically important proteins to distinct domains in a cell, making it important to identify and characterize their function. In comparison, *Drosophila* encodes 66 PDZ proteins and makes use of genetic tools that allow us to manipulate these proteins *in vivo*. Moreover, most vertebrate PDZ proteins have a conserved fly counterpart. The importance of PDZ proteins has been recognized in recent years and several genetic screens have identified PDZ proteins that play important roles in cell polarity and well as cell migration. However, the biological significance of PDZ mediated protein interactions in glia is largely unknown. In this study, we screened all PDZ proteins in *Drosophila* and identified seven PDZ proteins that are required in glial cells of the larval peripheral nerve. The majority of the PDZ proteins identified belonged to the MAGUK family, including Dlg1, Dlg5, Vari and Sdt. Previous analyses in *Drosophila* epithelial tissues have identified MAGUK proteins as being important regulators of cell polarity but the role in glia has not been established. Here we show that although Vari plays a conserved role in the peripheral nerve, Sdt might interact with non-canonical partners. We found that Dlg5 is expressed throughout the peripheral nerve and localizes with N-Cad and Vps26 in the peripheral nerve suggesting a role in cadherin trafficking. However, knockdown of Dlg5 did not affect localization of both DN-Cad and DE-Cad or the formation of spot adherens junction. Knock down in the WG did lead to lack of axonal
ensheathment as a result of less and discontinuous WG strands. Similarly, loss of Dlg5 lead to defects in the SPG membrane, accompanied by glial swelling as well as abnormal SJ morphology. The axon morphology does not seem to be affected by Dlg5 knockdown in glia or neurons, suggesting a glia-specific role of Dlg5 in the peripheral nerve.

3.5.1 Sdt plays a non-canonical role in glial cells

Our analysis of PDZ proteins in *Drosophila* glial cells has revealed some PDZ proteins that play a role in epithelial cell polarity are either not expressed in glial cells or do not necessarily have the same function in glia. The differences in the architecture of epithelial cells compared to glia suggest that the way polarity is set up in these cells varies. Our results suggest many components of epithelial polarity complexes including Baz and Patj might not be required in glial cells. Moreover, Crumbs is not expressed in the peripheral nerve (data not shown). This raises the question as to whether Sdt interacts with non-canonical partners to perform similar functions as seen in epithelial cells. It is also possible that Sdt might have an entirely different and novel function in glial cells. Our results showing that Sdt knockdown does not affect SJ morphology suggest that the latter is more likely. SJs are well-defined cell-cell junction in the *Drosophila* peripheral nerve, with known components such as Dlg1 and Scrib that have a well polarized distribution that results in SJ belts along the length of the nerve. In comparison the localization of potential AJ components, DN-Cad and DE-Cad appears to be mostly in the form of SAJs and rarely as a adherens-like zone or belt. Therefore, polarity is not clearly defined in glial cells and it is difficult to be certain when polarity is affected or not. We can however consider changes to the SJ strands as a read out for loss of polarity as two components of the Scrib/Dlg/Lgl polarity complex, Dlg1 and Scrib normally localize to the SJs. Moreover, we
found that pan-glial knockdown of Sdt affects glial morphology, however further tests need to be performed to identify the specific glial layer(s) in which Sdt functions. So far we have shown that glial phenotypes observed in pan-glial Sdt knockdown are not contributed by the loss of Sdt in the SPG layer.

3.5.2 Vari has a conserved function in the larval peripheral nerve

Vari is a MAGUK protein and homolog of PALS2. Its role in the maturation and function of SJs in epithelial tissues has been well described in previous studies (Bachmann et al., 2008, Moyer and Jacobs, 2008). The effect of Vari loss or knockdown in embryonic epithelial tissues, including the epidermis and trachea (Moyer and Jacobs, 2008) as well as post embryonic tissues such as the wing and eye imaginal discs have been well studied (Bachmann et al., 2008). In vitro data has also shown that Vari interacts with NrxIV via its PDZ domain (Bachmann et al., 2008) and co-localization between the Vari and NrxIV has been observed in embryonic glia (Moyer and Jacobs, 2008). However, the effects of Vari downregulation on glial cells in the larval peripheral nerve had not been tested. We show that Vari knockdown in the SPG affects the NrxIV labeled SJs in the larval peripheral nerve. Moreover, loss of Vari in the SPG leads to abnormal SJ morphology, accompanied by swellings in the SPG. This is consistent with the role of Vari in embryonic epithelia where down regulating Vari leads to mislocalization of NrxIV as well SJ defects (Moyer and Jacobs, 2008). The swellings observed in Vari knockdown nerves are more severe than those observed with the knockdown of NrxIV (M. Gilbert, personal communication). Therefore, Vari might have functions that go beyond its role in the formation of the SJ.
3.5.3 Dlg5 is required for the development of glial cells in the peripheral nerve

Studies in mammalian cells show that Dlg5 is required for the trafficking of N-Cad to the membrane and stabilization of the cadherin-catenin complexes in epithelial tissues (Nechiporuk et al., 2007). Moreover, Dlg5 was also observed in Rab11-positive vesicles in this study, further supporting the notion that cadherin trafficking maybe Dlg5 mediated. On the other hand, our study in Drosophila peripheral nerves indicates that Dlg5 might not affect cadherin localization and formation of AJs. To understand why Dlg5 does not affect cadherin trafficking and SAJ maintenance we must consider the differences between AJ structure in the peripheral nerve compared to epithelial cells. SAJs favour anchoring/adhesion functions whereas ZAs which are not observed and possibly do not form in the peripheral nerves, assist in cell-cell movements during tissue morphogenesis (Niessen and Gottardi, 2008). It is possible that there is a greater need for recycling of cadherins in epithelial tissues whereas cadherins do not need to be recycled from SAJs as they form stable complexes that maintain glia-glia adhesion in the peripheral nerve. Moreover, in contrast to vertebrate epithelial cells where Dlg5 assists the delivery of N-Cadherin from the Golgi to the plasma membrane in Rab11 positive secretory vesicles (Nechiporuk et al., 2007), Dlg5 in the peripheral glia did not associate with Rab11 nor any other Rab protein tested. However, co-localization between DN-Cad and Dlg5 suggests that it is possible that these proteins interact. Many studies show that cadherins are constantly turning over and have to be recycled back to the plasma membrane, often in Rab11-positive recycling endosomes (Lock and Stow, 2005, Le et al., 1999, Cadwell et al., 2016). However, there are multiple ways by which a cargo can reach the plasma membrane. A vital component of the endosomal sorting machinery is the retromer, which can mediate either endosome to Golgi or endosome to cell surface retrieval (Seaman, 2012). The retromer is made up of two
subcomplexes: the cargo selective trimer, Vps26, Vps29 and Vps35 and the sorting nexins (Snx) that sort cargo by bending the membrane around them (Rojas et al., 2007, Verges et al., 2007). Our data shows that Dlg5 co-localizes with Vps26, the component required for bringing the cargo selective complex to the endosome (Gokool et al., 2007). Therefore, it is possible that Dlg5 interacts with DN-Cad and assists the retromer complex in mediating either endosome to Golgi or direct endosome to cell surface retrieval of DN-Cad. Moreover, the discontinuities in the WG strands upon knocking down Dlg5 suggested that Dlg5 might mediate WG-WG adhesion. However, loss of Dlg5 did not block DN-Cad localization to the glial membrane nor block the formation of spot adherens junctions in the glia. The possibility that the lack of an effect of Dlg5 knockdown on cadherin localization and the presence of SAJs in these nerves is due to functional redundancy between DE-Cad and DN-Cad, cannot be ruled out. Furthermore, the loss of WG strands and WG discontinuities observed in Dlg5 knockdown nerves might not be due to loss of cadherin based adhesion. Further investigation will be needed to determine the mechanism by which Dlg5 regulates wrapping glia ensheathment in the peripheral nerve.

3.5.4 Dlg5 affects SJ morphology and localization of Scrib polarity protein

Scrib and Dlg1 genetically interact in epithelial cells (Bilder et al., 2000) and immunoprecipitation and yeast two-hybrid studies confirmed that these proteins form a complex (Mathew et al., 2002). Since Dlg1 and Dlg5 are both MAGUK proteins with similar protein-protein interaction domains, it is possible that Dlg5 might also interact with Scrib. Scrib is known regulator of endocytic trafficking in epithelial cells. In Drosophila pupal wings, posterior crossvein (PCV) formation relies on the role of Scrib mediated localization of BMP-type 1 receptor Thickvein (Tkv) to the basolateral region of the PCV cells, where ligand trafficking
takes place (Gui et al., 2016). Moreover, Scrib facilitates the internalization of Tkv in Rab5-postive early endosomes where it is active (Wu et al., 2007). A study in the Drosophila wing imaginal disc epithelia shows that Scrib is required for the sorting cargos that pass through the retromer (de Vreede et al., 2014). Components of the retromer complex genetically interact with Scrib where Scrib is involved in the retromer organization on endosomes. Moreover, Scrib mutants might not lead to a loss or gain of retromer activity but rather result in incorrect sorting of cargo. Furthermore, mammalian Scrib determines whether E-Cad is sent to the lysosome for degradation or recycling through the retromer (Lohia et al., 2012). By weakening the interactions between E-Cad and p120-catenin, Scrib enhances endolysosomal degradation of E-Cad, where in the absence of Scrib, E-Cad is diverted to the Gogli through the retromer (Lohia et al., 2012). Our data shows that Dlg5 knockdown disrupts the SPG membrane and leads to the loss of the core SJ proteins NrxIV in the SPG. Since Dlg5 associates with the retromer protein Vps26, it might assist in the delivery of either a SJ protein or a yet unidentified protein to the SPG membrane. Moreover, whether Dlg5 controls trafficking of Scrib to the SJ domain in SPG or if Scrib regulates the trafficking of other cargos to the SPG membrane remains to be tested.
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Table 3.1 Summary of PDZ protein knockdown in glial cells of the *Drosophila* peripheral nerve

The stock numbers of each UAS-PDZ-RNAi line obtained from the Bloomington (BL) and Vienna Drosophila Research Center (VDRC) are provided. The glial phenotypes observed by expressing each RNAi line in glial cells are described. Candidates were divided into: positive hits (yellow), those tested using only one RNAi line (orange) and those candidates for which two RNAi lines were tested and one resulted in a glial phenotype whereas the other did not (green). The RNAi lines that resulted in glial phenotypes are highlighted in purple.
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Table 3.2 Summary of PDZ proteins that are required for epithelial polarity but are not required in peripheral glia
Stock numbers of RNAi lines used to knockdown Bazooka (Baz) and Patj in the glial cells and their corresponding phenotypes. Knockdown of Baz and Patj does not affect glial morphology.
**Figure 3.1.** PDZ proteins required in glial cells of the peripheral nerve. Longitudinal sections of peripheral from control and RNAi mediated knockdown with the glial membranes labeled with mCD8::RFP (green) and axons immunolabeled with anti-Futsch (22C10; magenta).

A-A’’: Control (w1118) peripheral nerve

B-B’’: repo>Sdt-RNAi peripheral nerve. The repo>Sdt-RNAi peripheral nerves had accumulations of glial membrane in some regions of the nerve indicated by the arrowhead (B’, yellow arrowhead). The morphology of the axons was not affected by the knockdown of Sdt in the peripheral glia (B’’).

C-C’’: repo>Rho-GEF2-RNAi. The glial cells in repo>RhoGEF2-RNAi peripheral nerves were swollen (C’, yellow arrowheads) and these swellings were accompanied by defasciculation of the axons in that region of the nerve (white arrowheads, C’’).

D-D’’: repo>Dlg1-RNAi. Glial membranes in repo>Dlg1-RNAi peripheral nerves were disrupted (D’, yellow arrowhead) compared to control nerves.

E-E’’: repo>Scrib-RNAi. The glial membranes in repo>Scrib-RNAi peripheral nerves were abnormal (E’, yellow arrowhead) compared to control nerves.
**F-F’’**: *repo>*Loco-RNAi. The *repo>*Loco-RNAi peripheral nerves were swollen and vacuole-like structures were observed within the glial membranes (F’, yellow arrowheads) compared to control nerves.

**G-G’’**: *repo>*Dlg5-RNAi. The *repo>*Dlg5-RNAi peripheral nerves had disrupted inner glial membranes (G’, yellow arrowheads). The morphology of axons in these nerves were unaffected (G’’) and looked similar to the control.

Scale bars: 15µm
**Figure 3.2.** Knockdown of Varicose in the SPG affects SJ morphology whereas knockdown of Stardust does not.

Peripheral nerves with the SPG membranes and SJ labeled with mCD8::RFP (magenta) and NrxIV::GFP (green). Schematic representations of a peripheral nerve (green) and sections taken through the XZ (blue) and YZ (magenta) axes are also shown. Scale bars: 15µm

**A-C:** Control peripheral nerves. **A-A’’**: Sections taken through the XZ axis of a control peripheral nerve showing NrxIV labeling (green) was limited to the SJ domain in the SPG membrane (magenta). **B-B’’**: Longitudinal sections of control peripheral nerves showing a continuous SJ strand labeled with NrxIV::GFP along the SPG membrane. **C-C’’**: Sections taken through the YZ axis of a control peripheral nerve showing that NrxIV labeling (green) was limited to the SJ domain and is continuously expressed along the length of the nerve.

**D-F:** *Gli*Sdt-RNAi. **D-D’’**: Sections taken through the XZ axis showing NrxIV labeling (green) was limited to the SJ domain in the SPG membrane (magenta) similar to controls. **E-E’’**: Longitudinal sections showing a continuous SJ strand labeled with NrxIV::GFP along the SPG membrane. **F-F’’**: Sections taken through the YZ axis showing that NrxIV (green) was limited to the SJ domain and continuously expressed along the length of the nerve.

**G-I:** *Gli*Vari-RNAi. **G-G’’**: Sections taken through the XZ axis showing diffuse NrxIV labeling (green) in the SPG membrane (magenta) compared to controls. **H-H’’**: Longitudinal sections showing completely disrupted NrxIV distribution (green) compared to controls. **I-I’’**: Sections taken through the YZ axis showing that NrxIV labeling (green) was spread out and discontinuous compared to controls. Scale bars: 15µm
**Figure 3.3.** Knockdown of Varicose in the SPG affects SPG and WG morphology whereas knockdown of Stardust does not.

**A-B**: Longitudinal sections of peripheral nerves with SPG and WG membranes labeled with mCD8::RFP (magenta) and the SJ domain labeled with Nrv2::GFP (green). Axons were immunolabeled with anti-Futsch (22C10, blue).

**A-A’’’**: Control peripheral nerves. The WG (green) in the control nerve (A’) extended processes along the entire length of the nerve and ensheathed the peripheral axons (blue, A’’’) while the thin SPG membrane (magenta, A’’) surrounded the WG.

**B-B’’’**: Gli>Sdt-RNAi peripheral nerves. The WG (green, B’) in Gli>Sdt-RNAi nerves extended along the length of the nerve and wrapped axons (blue, B’’’) similar to controls. The morphology of the SPG membrane (magenta, B’’) in Gli>Sdt-RNAi nerves was similar to control nerves.

**C-C’’’**: Gli>Vari-RNAi. The WG (green, C’) in Gli>Vari-RNAi nerves extended fewer processes along the length of the nerve compared to control nerve. The axons were defasciculated (blue, C’’’) and the SPG (magenta, C’’) had swellings compared to control nerves.

Scale bars: 15µm
Figure 3.4. Knockdown of Dlg5 in the SPG and the WG leads to disruptions in the SPG membrane and loss of axonal ensheathment.

**A-D:** Control (A) and 46F>Dlg5-RNAi (C) peripheral nerves with the PG membrane labeled with mCD8::RFP (green) and axons immunolabeled with anti-Futsch (22C10, magenta). The PG (green) and axons (magenta) in both the 46F>Dlg5-RNAi nerve (C) and control nerve (A) appeared normal.

**E-H:** Control (E) and Gli>Dlg5-RNAi (G) peripheral nerves with the SPG and WG membranes labeled with mC8::RFP (magenta) and Nrv2::GFP (green), respectively. The WG in both control (E’) and Gli>Dlg5-RNAi (G’) nerves extended several processes along the length of the nerve. The SPG membrane in control nerves (E’’) was continuous along the entire length of the nerves whereas in Gli>Dlg5-RNAi nerves (G’’), the SPG was disrupted with breaks/gaps in the membrane (yellow arrowheads).

**I-L:** Control (I) and Nrv2>Dlg5-RNAi (K) peripheral nerves with the WG membrane labeled with mCD8::RFP (green) and axons labeled with anti-Futsch (22C10, magenta). The WG in control (I’’) nerves extended several processes along the length of the nerve and ensheathed the peripheral axons (I’, magenta). The cross-sections in J-J’ indicate that the WG (green) surrounded the axons (magenta). In comparison, Nrv2>Dlg5-RNAi nerves had fewer WG processes and those present were disrupted (K’, yellow arrowheads), and axons (K’’) were not properly ensheathed. The cross-sections in L-L’’ indicate that the WG processes did not extend around the axons (yellow arrowhead). Scale bars: 15µm
**Figure 3.5.** Dlg5 co-localizes with N-Cad and Vps26 but not with the gap junction and focal adhesion complexes in the peripheral nerve

A-D: Longitudinal sections of peripheral nerves with Dlg5 (A and C) and peripheral glial membranes were labeled with mCD8::RFP (magenta, A') and the axons with anti-Futsch (22C10, magenta, C'). Dlg5 puncta were observed throughout the peripheral nerve, with puncta within the glial cells (A) as well as the axons (C). Cross-sections of the peripheral nerves expressing Dlg5::GFP (B-B'' and D-D'') indicate that Dlg5 (green, B,B'' and C,C'') was expressed in glia (magenta, B,B') as well as the axons (magenta, D,D').

E-F: Longitudinal sections of peripheral nerves labeled with Dlg5 and Inx2 immunolabeled gap junction complexes in the peripheral nerve. (E'', green) in the peripheral nerve. Dlg5 puncta were present throughout the nerve but did not co-localize with Inx2 labeled gap junction plaques (E', magenta).

F-F': Cross-sections of the peripheral nerve showing that Dlg5 (green, F,F'') does not co-localize with Inx2 (magenta, F,F').

G-H: Longitudinal sections of peripheral nerves labeled with Dlg5 and focal adhesion complexes immunolabeled with the beta subunit of integrin, myospheriod (βPS). Dlg5 puncta (green, G'') were observed throughout the nerve but did not co-localize with the βPS puncta or stripes (magenta, G'). H-H': Cross-sections of the peripheral nerve showing that Dlg5 (H,H'', green) does not co-localize with βPS (H,H', magenta).

I-J: Longitudinal sections of peripheral nerves labeled with Dlg5 and DN-Cadherin. DN-Cadherin was labeled using DN-Cad::mCherry (magenta, I') and Dlg5 was labeled with Dlg5::GFP (green, I''). The Dlg5 puncta overlapped with some (yellow arrowheads) but not all DN-Cad puncta in the peripheral nerve. J-J': Cross-sections of the peripheral nerve showing co-localization between Dlg5 (green, J,J'') and DN-Cad puncta (magenta, J,J').

K-L: Longitudinal sections of peripheral nerves labeled with Dlg5 and DE-Cadherin. DE-Cadherin was labeled using DE-Cad::mCherry (magenta, K') and Dlg5 was labeled with Dlg5::GFP (green, K''). The Dlg5 puncta did not overlap with DE-Cad puncta in the peripheral nerve. L-L'': Cross-sections of the peripheral nerve showing that Dlg5 (green, L,L'') and DE-Cad (magenta, L,L') did not co-localize.

M-N: Longitudinal sections of peripheral nerves labeled with Dlg5 and Vps26. The retromer complex was immunolabeled with Vps26 (magenta, M') and Dlg5 was labeled with Dlg5::GFP (green, M''). The Dlg5 puncta (green, M) overlapped with some (yellow arrowheads) but not all Vps26 puncta (magenta, M) in the peripheral nerve. N-N'': Cross-sections of the peripheral nerve showed co-localization between Dlg5 (green, N,N'') and Vps26 puncta (magenta, N,N').

O: Table showing results from co-localization studies between Dlg5 and vesicle/organelle markers. Dlg5 was not observed in immunolabeled Rab5-positive early endosomes, Rab7-positive late endosomes, Rab11-positive recycling endosomes, HRS immunolabeled multivesicular bodies (MVB), GM130 immunolabeled Golgi, Sec23 immunolabeled COPII vesicles and with Syntaxin1A immunolabeled t-SNAREs. Scale bars: 15µm.
Figure 3.6. DN-Cad and DE-Cad associate with the retromer complex and Rab11 in the larval peripheral nerve

**A-B:** Longitudinal sections of peripheral nerves with DN-Cad (magenta, A, A') and DE-Cad (magenta, B, B') labeled with DN-Cad::mCherry and DE-Cad::mCherry, respectively. These nerves were immunolabeled with anti-Vps26 (green, A, A'' and B, B''). Vps26 puncta (yellow arrowheads, A, A'' and B, B'') are expressed throughout the nerve and overlap with DN-Cad (green, A, A') and DE-Cad (green, B, B') belts (white arrowheads).

**C-D:** Longitudinal sections of peripheral nerves with DN-Cad (magenta, C, C') and DE-Cad (magenta, D, D') labeled with DN-Cad::mCherry and DE-Cad::mCherry, respectively. These nerves were immunolabeled with anti-Rab11 (green, C, C'' and D, D''). Rab11 puncta (yellow arrowheads, C, C'' and D, D'') were expressed throughout the nerve and some but not all overlapped with DN-Cad (magenta, C, C') and DE-Cad (magenta, D, D') belts (white arrowheads).

Scale bars: 15μm
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**Additional Images:**

**I, J, K**

**I’, J’, K’**
**Figure 3.7.** Knockdown of Dlg5 does not affect cadherin localization and spot adherens junction assembly.

**A-D:** Control (A) and repo>Dlg5-RNAi (C) peripheral nerves with WG membranes labeled with Nrv2::GFP and DN-Cad labeled with mCherry (NCad::cherry). The WG extended many processes along the length of the nerve in control nerves (green, A,A') whereas a single discontinuous WG strand was observed in repo>Dlg5-RNAi nerves (green, C,C'). DN-Cad puncta were organized in adherens-like belts in both control (magenta, A,A'') and repo>Dlg5-RNAi nerves (C,C''). Cross-sections from control (B-B'') and repo>Dlg5-RNAi (D-D'') nerves showing that the WG processes (green) did not extend in the repo>Dlg5-RNAi nerves (D,D') compared to control nerves (B,B').

**E-H:** Control (E) and repo>Dlg5-RNAi (G) peripheral nerves with WG membranes labeled with Nrv2::GFP and DE-Cad labeled with mCherry (Ecad::cherry). The WG extended many processes along the length of the nerve in control nerves (green, E,E') whereas a single discontinuous WG strand was observed in repo>Dlg5-RNAi nerves (green, G,G'). DE-Cad puncta were organized in adherens-like belts in both control (magenta, E,E'') and repo>Dlg5-RNAi nerves (G,G''). The DE-Cad belt (white arrowheads, G) in repo>Dlg5-RNAi nerves was observed within the remaining WG strand (yellow arrowhead, G). Cross-sections from control (F-F'') and repo>Dlg5-RNAi (H-H'') nerves showing that the WG processes (green) did not extend in the repo>Dlg5-RNAi nerves (H,H') and that DE-Cad (white arrowhead, H,H'') was present within the remaining WG membrane (yellow arrowhead, H,H') compared to control nerves (F,F').

**I-K:** Ultrastructural analysis of control (I) peripheral nerves and nerves lacking Dlg5 in the peripheral glia (repo>Dlg5-RNAi, J,K). I: Representative TEM image of a cross-section of a control peripheral nerve showing that the wrapping glia (false coloured in green) extended their processes and wrapped around several axons, separating them into fascicles. J-K: Loss of Dlg5 in peripheral glia resulted in less axonal wrapping, with larger than normal sized axon fascicles. Spot adherens junctions were observed between the WG (false coloured in green) and the SPG in both control nerves (dashed magenta box, I) and those lacking Dlg5 (dashed magenta box, J-K). Boxed regions in I'--K' are shown at a higher magnification in I'--K''. The electron dense regions between the SPG and WG membranes in TEM images of control (I') and repo>Dlg5-RNAi nerves (J',K') represent SPG-WG spot adherens junctions. Scale bars: 15µm, 1 µm.
**Figure 3.8.** Knockdown of Dlg5 in the SPG but not the PG and WG lead to disruptions in SJ morphology

**A-D:** Control (A) and 46F>Dlg5-RNAi (C) peripheral nerves with the PG membrane labeled with mCD8::RFP (magenta) and SJs immunolabeled with anti-Nrv2.1 (green). The PG (magenta) and SJs (green) in both the 46F>Dlg5-RNAi nerve (C) and control nerve (A) appeared normal.

**E-H:** Control (E) and Gli>Dlg5-RNAi (G) peripheral nerves with the SPG membrane and SJs labeled with mCD8::RFP (magenta) and NrxIV::GFP (green), respectively. The SPG membrane and NrxIV labeled SJs in control nerves (E”) were continuous while the SJ stands in Gli>Dlg5-RNAi (G”) nerves were disrupted in regions corresponding to the disruptions in the SPG membrane (yellow arrowheads).

**I-L:** Control (I) and Nrv2>Dlg5-RNAi (K) peripheral nerves with the WG membrane labeled with mCD8::RFP (magenta) and SJ labeled with NrxIV::GFP (green), respectively. The WG in control (I”) nerves extended several processes along the length of the nerve and ensheathed the peripheral axons (I”, magenta) and had continuous SJ strands (I’, green). The cross-sections of control nerves J-J’ indicate that the WG (green) extended their processes. In comparison, Nrv2>Dlg5-RNAi nerves had less and disrupted WG processes (K”, yellow arrowhead) but the SJs were continuous similar to control nerves (K’). The cross-sections in L-L” indicate that the WG processes did not extend their processes (yellow arrowhead). Scale bars: 15µm
Figure 3.9. Knockdown of Dlg5 in the SPG affects the distribution of Scribbled in the peripheral nerve

A-C: Longitudinal sections of control (A) and Gli>Dlg5-RNAi (B,C) peripheral nerves with the SPG membrane labeled with mCD8::RFP (magenta) and Scrib::GFP (green). Scrib (A,A”) is located to the SJ which extended a single, continuous strand along the SPG membrane (A,A’) in control nerves. In 10% of the Gli>Dlg5-RNAi nerves (n=5), Scrib (green, B,B”) is lost in regions of the nerve where the SPG membrane (B,B’) is disrupted (yellow arrowheads, B-B”). Severe mislocalization of Scrib (green, C,C”), accompanied by swelling of the SPG membrane (magenta, C,C’), was observed in 16.7% of the Gli>Dlg5-RNAi nerves (n=5). Scale bars: 15µm
Chapter 4: Cadherins in the Drosophila peripheral nerve

4.1 Synopsis

The efficient delivery of neuronal signals in the Drosophila peripheral nerve is ensured by three layers of glial cells; the wrapping glia (WG) which directly wraps axons similar to non-myelinating Schwann cells, the subperineurial glia (SPG) which forms the blood-nerve barrier and the outer most layer of perineurial glia (PG). These layers must adhere to each other to set up and maintain an effective glial wrap around the axons. However, the mechanisms underlying glia-glia adhesion in the peripheral nerve is poorly understood. We show that Drosophila E-Cadherin (DE-Cad) and N-Cadherin (DN-Cad) are present in glia and co-localize with catenins. Moreover, DE-Cad and DN-Cad overlap in the peripheral nerve. Furthermore, the loss of DE-Cad but not DN-Cad in all glial has deleterious effects on glial morphology resulting in loss of ensheathment and swelling, while the loss of DN-Cad had no phenotypes. Loss of DE-Cad in the WG resulted in loss of glial wraps, disconnected WG process and membrane fragmentation. Loss of DE-Cad in the SPG disrupted septate junction morphology. However, using transmission electron (TEM) analysis, we observed that spot adherens junctions (SAJs) are still present in the peripheral glia after DE-Cad knockdown, suggesting redundancy between DE-Cad and DN-Cad in glia. In summary, we demonstrate that the cadherin/catenin complex is present in glia and DE-Cad in the WG and SPG is required for axonal ensheathment and septate junction formation.

4.2 Introduction

Glial cells insulate axons to ensure that axonal signals are transmitted in an efficient manner. Saltatory nerve conduction in the vertebrate peripheral nerve is achieved by the myelinating Schwann cells that ensheath large caliber axons (Nave and Trapp, 2008). The
stability of the myelin structure and the regions that flank the Nodes of Ranvier requires that the multiple layers of Schwann cell membrane adhere to each other. This is achieved by various junctional complexes, including the adherens junction. Adherens junctions allow for cell-cell adhesion and link the membrane to the cytoskeleton. Adherens junctions can be divided into spot adherens junction (SAJs) and zonula adherens junctions (ZA). The former is scattered along the lateral membrane of epithelial cells (Drenckhahn and Franz 1986) and at neuronal synapses (Fannon 1996). Unlike the SAJ which is often discontinuous or ‘spot-like’, the ZA is continuous or ‘belt-like’ found in the apical domain of most epithelial tissues (Carein and Gottardi, 2007). During development of tissues, it is postulated that SAJs favour anchoring/adhesion while ZAs enable coordinated cell movements (Carein and Gottardi, 2007). The AJ is composed of cadherins, homophilic adhesion receptors, as well as catenins that link the AJ to the cytoskeletal network. The classical cadherins, E-Cadherin (E-Cad) and N-Cadherin (N-Cad) form complexes with three catenins; α-catenin (α-cat), β-catenin (β-cat) and p120-catenin (p120) to mediate cell-cell adhesion (Harris, 2012). The AJs in Schwann cells differ from the typical epithelial AJ, in that these AJs are autotypic and form between membrane of a single Schwann cells (Fannon et al., 1995). In comparison to myelinating Schwann cells, nothing is known about cell-cell adhesion in the second the non-myelinating Schwann cells (NMSC). NMSCs ensheathe small caliber axons such as C-fibers and sensory axons in single wraps to form Remak bundles. It is not known how the processes of a NMSC adhere to each other and whether adhesion occurs between neighbouring NMSCs. Of the classical cadherins N-Cad is expressed in the NMSCs (Corell et al., 2010) but its function remains unknown. Furthermore, E-Cad expression and function in the NMSCs is yet to be determined in the NMSCs.
In the *Drosophila* peripheral nerve, sensory and motor axons are insulated by three glial layers; the WG, SPG and PG (Stork et al., 2008). The WG resemble the NMSC that form the Remak bundles in vertebrates (Rodrigues et al., 2011). The SPG establishes the blood-nerve barrier by forming septate junctions at SPG-SPG membrane contacts. These structure and composition of these septate junctions are conserved with vertebrate paranodal junctions (Baumgarnter 1996, Peles 1997, Banerjee and Bhat 2008, Banerjee 2006). The PG surround the entire nerve but their function remains unknown. Spot adherens junctions between different glial layers of the *Drosophila* larval peripheral nerve have been previously observed (Matzat et al., 2015). These junctions were either found between two different glial layers (SPG-WG and SPG-PG) or within the WG (WG-WG) (Matzat et al., 2015). It is not known, however, whether these junctions provide a critical function in the three glial layers.

The goal of this study was to identify which classical cadherins are present in the larval peripheral nerve and determine their function. Drosophila has one E-Cad homologue, DE-Cad (encoded by the *shotgun* gene) and two N-Cad homologues, DN-Cad (encoded by the *CadN* gene) and CadN2. We found that both DE-Cad and DN-Cad are expressed in the larval peripheral nerve and that each cadherin associates with α-cat, β-cat (*Drosophila* Armadillo/Arm) and p120-catenin (p120). Moreover, we show that unlike DN-Cad, DE-Cad is required for normal glial morphology as knockdown in all glial cells results in reduced WG strands that are discontinuous and accompanied by swellings and the appearance of vacuole-like structures. Loss of DE-Cad in the SPG disrupted the formation of the septate junction and loss of DE-Cad in the WG disrupted the glial wraps/strands. Knockdown of α-cat and p120 function in the peripheral glia did not disrupt glial morphology and did not result in the same phenotypes observed with the loss of DE-Cad. However, the consequence of beta-catenin knockdown in glial cells remains to
be determined. Using TEM analysis, we continued to detect the presence of spot adherens junctions when DE-Cad was knocked down in all glia. Therefore, our results suggest that DE-Cad and DN-Cad are both present and may have redundant roles in glia.

4.3 Materials and methods

4.3.1 Fly strains and genetics

The following fly strains were used in this study: repo-GAL4 (Sepp et al., 2001); Nrv2-GAL4 (Sun et al., 1999), Gli-GAL4 (Sepp and Auld, 1999); UAS-mCD8::GFP (Lee and Luo, 1999); UAS-Dicer2 (Dietzl et al., 2007); UAS-mCD8::RFP (gift from Elizabeth Davis, Princeton University), Nrv2::GFP (Morin et al., 2001), DN-Cad::Cherry (39640, Bloomington), DE-Cad::Cherry (59014, Bloomington), α-cat::GFP (59405, Bloomington). The following DE-Cad-RNAi lines were used: GD14421 (strongest knockdown, VDRC, Vienna Drosophila resource center), GD2659 (weak knockdown, VDRC). The DN-Cad-RNAi lines used were GD161 (VDRC), JF02653 (TRiP, Transgenic RNAi project, Bloomington). The following α-catenin RNAi lines were used: GL00636 (TRiP, Bloomington) and HMS00317 (TRiP, Bloomington). The p120-RNAi line used was: HMC03276 (TRiP, Bloomington. All RNAi experiments were carried out at 25°C without Dicer2 in the background unless specified.

4.3.2 Immunolabeling and image analysis

Wandering third instar larvae were selected, dissected and fixed for immunolabeling according to previously described methods (Sepp et al., 2000). The following primary antibodies were used in this study: guinea pig anti-vps26 (1:1000), (REF); rabbit anti-mcherry (1:300, Abcam); chicken anti-mcherry (1:500, Abcam); rabbit anti-GFP (1:600, Life Technologies);
mouse anti-GFP (1:300, Novus Biologicals) mouse anti-Futsch/22C10 (1:1000, DSHB); mouse anti-Dlg14F3 (1:100, DSHB); mouse anti-Arm (1:50, DSHB); mouse anti-p120cat (1:100, DSHB). The following secondary antibodies were used at a 1:300 dilution: goat anti-mouse Alexa 488, Alexa 568 and Alexa 647; goat anti-rabbit Alexa 488, Alexa 568; goat anti-guinea pig Alexa 647 and goat anti-chicken Alexa 647 (Molecular Probes). DAPI (1:1000, Invitrogen) was used to stain nuclei. The mCherry and GFP signals were enhanced using anti-mCherry and anti-GFP, respectively. Fluorescent images were captured with Delta Vision Spectris (Applied Precision/GE Healthcare) using a 60x oil immersion objective (NA 1.4). The z-step was set at 0.2 µm. The z-slices were combined into stacks which were deconvolved (SoftWorx, Applied Precision/GE Healthcare) using a point spread function measured with 0.2 µm beads conjugated to Alexa dyes (Molecular Probes) and mounted in Vectashield, Vector Laboratories, Burlington, Canada). SoftWorx was used to generate orthogonal sections. A single z-slice, conveying the information relevant to the experiment, was chosen from the z-stack and images were compiled in Adobe Photoshop and Adobe Illustrator CC. Larval dissections and preparation for the transmission electron microscopy analysis larvae were performed using previously described methods (Matzat et al., 2015).

4.4 Results

4.4.1 N-Cadherin and E-Cadherin, are expressed in the larval peripheral nerve

To determine if cadherins form glia-glia adhesion complexes in the Drosophila larval peripheral nerve, we began by looking at whether DN-Cad and DE-Cad are expressed in the 3rd instar larval peripheral nerve. The distribution of both cadherins was examined using DN-Cad and DE-Cad endogenously tagged with mCherry (DN-Cad::mCherry and DE-Cad::mCherry,
respectively). Since the mCherry signal in peripheral nerves of both DN-Cad::mCherry and DE-Cad::mCherry larvae was not easily detectable, the intensity of the signal was strengthened using an mCherry antibody. Moreover, glial membranes and axons were labeled using repo-GAL4 to express UAS-mCD8::GFP (Fig. 4.1C,C and D,D’, G,G’ and H,H’) and anti-22C10 (Fig. 4.1E,E’ and F,F’, I,I’ and J,J’), respectively. DN-Cad puncta were observed throughout the peripheral nerve (Fig. 4.1A, C-D’’ and E-F’’) whereas short DN-Cad, ZA-like belts were occasionally observed in some parts of the nerve (Fig. 4.1A, C’, E’’). The DN-Cad belts were discontinuous and primarily observed in regions of the nerves close to the ventral nerve cord (VNC) (Fig. 4.1A). Interestingly, DN-Cad belts were not present in all nerves in any given larvae. Furthermore, we found that similar to DN-Cad, DE-Cad puncta were present throughout the nerve (Fig. 4.1B, G-J’’) and occasionally formed discontinuous belts in some nerves (Fig. 4.1B, G’, I’’). Unlike the DN-Cad distribution however, the DE-Cad belts were not restricted to regions close to the VNC and were also observed in more distal regions of the nerve (Fig. 4.1B). Interestingly, the punctate expression of DN-Cad and DE-Cad was more prevalent than the cadherin belts and are likely representative of spot adherens junctions (SAJs). Although, both DN-Cad and DE-Cad are expressed in the larval peripheral glia, it was difficult to assess whether these cadherins were also expressed in the axons. Overall, both classical cadherins, DN-Cad and DE-Cad, were expressed in the peripheral nerve, either as puncta or in ZA-like belts.

To determine if DE-Cad and DN-Cad both contribute to the formation of the same structures in the peripheral nerve, we looked for co-localization of DE-Cad with DN-Cad using DE-Cad tagged with GFP (DE-Cad::GFP) and DN-Cad::mCherry. Interestingly, we found that the DE-Cad and DN-Cad ZA-like belts partially colocalize in 77% of the peripheral nerves (n=4) (Fig. 4.1K-K’’ and L-L’’). Furthermore, co-localization between DE-Cad puncta and DN-Cad
puncta was observed in 23% nerves, however we never observed overlap between all DE-Cad and DN-Cad puncta, rather the overlap represented a small number of many puncta observed. This suggests that DE-Cad and DN-Cad might have some overlapping functions in the peripheral nerve in the domain of the ZA-like belts but may also form independent clusters in the SAJs. Overall our results suggest that both DN-Cad and DE-Cad are expressed in distributed puncta or in ZA-like belts and might have some overlapping functions in the peripheral nerve.

4.4.2 Catenins are expressed in the Drosophila larval peripheral nerve

The cytoplasmic domain of cadherins are known to interact directly with the catenins, β-cat (Drosophila Armadillo/Arm) and p120-catenin (p120) and indirectly via Arm with α-cat (Ozawa et al., 1989, Pai et al., 1996). To determine which catenins are expressed in the peripheral nerve and determine if they associate with cadherins, we labeled the peripheral nerves with DN-Cad and DE-Cad using DN-Cad::Cherry and DE-Cad::Cherry respectively, and looked for co-localization with p120, Arm and α-cat. We labeled alpha-catenin (α-cat), p120-catenin (p120) and beta-catenin (Armadillo or Arm) using α-cat tagged with GFP and antibodies raised against p120 and Arm. We found that p120 puncta are expressed throughout the nerve, and these p120 puncta co-localized with the DN-Cad and DE-Cad puncta (Fig. 4.2A’’, B-B’’).

However, p120 did not overlap with the either the DN-cad or DE-cad cadherin belts (Fig 2A’’, B-B’’). In contrast, Arm puncta throughout the nerve but of note only a very few Arm puncta co-localized with DN-cad or DE-cad. Instead, Arm was localized to the ZA-like belts in some parts of the nerve and here Arm co-localized with both DN-Cad and DE-Cad (Fig. 4.2C-C’’, D-D’’). However, we did observe in other nerves belts positive for Arm but not DN-Cad or DE-Cad. Similarly, α-cat puncta were observed throughout the peripheral nerve and these did not appear
to co-localized with either DN-cad or DE-cad. Similar to Arm, α-cat formed belts that co-
localized with DN-Cad and DE-Cad (Fig 2E-E’’, F-F’’). We have therefore shown that p120, α-
cat and Arm are present in the peripheral nerve. However, only α-cat and Arm consistently co-
localized with DN-Cad and DE-Cad in the ZA-like belts and only p120 consistently co-localized
with DN-Cad and DE-Cad in the SAJs puncta.

4.4.3 DE-Cad but not DN-Cad is required for axonal ensheathment of larval peripheral
nerves

We next wanted to examine the function of DN-Cad and DE-Cad in the peripheral nerve.
To assay for abnormalities in glial morphology, repo-GAL4 was used to express RNAi and label
the glial membranes with mCD8::GFP. Moreover, to test for SPG integrity we analyzed the
distribution of a septate junction marker, Discs large 1 (Dlg1). Normal glial morphology and
Dlg1 distribution was observed in all the control nerves (repo>mCD8::GFP, Dicer2) (n= 6
larvae) (Fig. 4.3A-A’’). However, pan-glial knockdown of DE-Cad (repo>DE-Cad, Dicer2)
resulted in disrupted glial membranes and the appearance of vacuole-like structures within the
glial membrane in 71.4% nerves (n= 4 larvae) (Fig. 4.3C,C’). Interestingly, Dlg1 was also
mislocalized in 72% of the affected nerves (n=4 larvae) (Fig. 4.3C,C’’). Surprisingly,
knockdown of DN-Cad (repo>DN-Cad, Dicer2) using multiple RNAi lines did not affect glial
morphology (Fig. 4.3B,B’) and Dlg1 distribution (Fig. 4.3B,B’’). Therefore, we found that DE-
Cad but not DN-Cad, is required for proper ensheathment of axons as well as the integrity of SJ
morphology.
4.4.4 DE-Cad is required in the wrapping glia for axonal ensheathment of larval peripheral nerves

To determine if DE-Cad is required in one or all glial layers, we knocked down DE-Cad in individual glial layers using layer specific GAL4 drivers. Since the pan-glial knockdown of DE-Cad leads to abnormalities in glial cells that lie within the interior of the nerve (Fig. 4.3C'), we began by assaying the role of DE-Cad in the innermost wrapping glia layer. Nrv2-GAL4 was used to label the wrapping glia membranes with mCD8::GFP. Axons were stained with anti-Futsch/22C10 to assay axonal morphology. Control nerves had multiple, continuous wrapping glia strands ensheathing the peripheral axons (Nrv2>mCD8::GFP, Dicer 2) (Fig. 4.3D,D'). In comparison, DE-Cad knockdown in the wrapping glia resulted in less and discontinuous wrapping glia strands in 88.9% nerves (Nrv2>DE-Cad, Dicer2) (n=4 larvae) (Fig. 4.3F,F'). Moreover, axon morphology was not affected by the knockdown of DE-Cad (Fig. 4.3F,F''). Furthermore, as expected and, in agreement with results from the pan-glial knockdown of DN-Cad, knockdown of DN-Cad in the wrapping glia did not affect glial morphology (Fig. 4.3E,E') and the morphology of the axons (Fig. 4.3E,E'') is also comparable to the control (Fig. 4.3D,D''). Therefore, DE-Cad is required in the wrapping glia and is essential for the ensheathment of axons by these glial cells.

4.4.5 DE-Cad is required in the subperineurial glia

The septate junction is formed along the SPG membrane to create a permeability seal along the length of the glia. Since pan-glial knockdown of DE-Cad not only leads to disruptions in the wrapping glia layer but disrupted the SJ domain, we next tested the function of DE-Cad in the SPG layer. To examine the function of DE-Cad in the subperineurial glia (SPG) we used Gli-
**GAL4** to drive RNAi and label the SPG membranes with mCD8::RFP. Moreover, we assayed for SJ formation and morphology using a Dlg1 antibody. The SPG membranes observed in all control nerves were continuous and Dlg1 was correctly localized to the septate junction (Gli>mCD8::RFP, Dicer 2) (n= 6 larvae) (Fig. 4.3G-G’’). In comparison 19.5% nerves had discontinuous SPG membranes when DE-Cad was knocked down in the SPG (Gli>DE-Cad-RNAi, Dicer 2) (Fig. 4.3I,I’’) (n=6). Interestingly, the gaps in the SPG membrane coincided with the loss of Dlg1 (Fig. 4.3I,I’). Moreover, the affected nerves had swellings (Fig. 4.3I), suggestive of a loss of ionic balance (Leiserson et al., 2011). Therefore, DE-Cad is required in the SPG for proper ensheathment and SJ formation.

To determine if the ultrastructure of the peripheral nerve is affected by the loss of DE-Cad in glia, we used transmission electron microscopy to assay for the presence or absence of SAJs in these nerves. We found that SAJs were present in both control (repo>mCD8::RFP) (n=1) (Fig. 4.4A-A’,A’’) and DE-Cad knockdown nerves (repo>DE-Cad-RNAi, Dicer2) (n=1) (Fig. 4.4B-B’). Moreover, we observed that the WG processes in controls nerves were elaborate, with several, thin processes branching out and separating axons (Fig. 4.4A). In comparison, the WG in DE-Cad knockdown nerves had thicker processes (Fig. 4.4B,C). TEM images from both control and DE-Cad knockdown nerves showed that the axons had been separated into fascicles (Fig. 4.4A-C). Therefore, we found that DE-Cad is not necessary for the formation of SAJs, however its loss leads to changes in the morphology of the WG and the SPG. The function of DE-Cad in the PG remains to be determined.
4.4.6 p120- and α- catenins are not required in glial cells of the larval peripheral nerve

To test the role of the different catenin proteins, we used repo-GAL4 to drive catenin specific RNAi and assessed glial morphology. Glial membranes were labeled with mCD8::GFP and the SJs assessed using immunolabeling with a Dlg1 antibody. Interestingly, knockdown of both p120 (repo>p120-RNAi, Dicer2) (n=6) and α-cat (repo>α-cat, Dicer2) (n=6) did not affect glial morphology (Fig. 4.5B, B’ and C, C’) and was similar to control nerves (n=6) (Fig. 4.5A, A’). Both Dlg1 distribution and SJ morphology were normal in controls (Fig. 4.5A, A’’) as well as both p120 and α-cat knockdown nerves (Fig. 4.5B, B’’ and C, C’’). The role of the third catenin protein, Arm, has yet to be determined. Therefore, we have shown that the role of p120 and α-cat in glial cells of the larval peripheral nerve may not be necessary or maybe redundant with other catenin proteins.

4.5 Discussion

Our study shows that DN-Cad and DE-Cad overlap in the Drosophila peripheral nerve and co-localize with catenins. We show that DE-Cad is required in the WG and the SPG for axonal ensheathment and SJ formation, respectively however DN-Cad is not. Moreover, we show that SAJs are still present between peripheral glia when DE-Cad is knocked down, suggesting that DE-Cad and DN-Cad might have redundant roles in glia.

4.5.1 Does DN-Cad play a role in the peripheral nerve?

Our results show that although DN-Cad is expressed in the peripheral nerve, loss of DN-Cad does not affect glial morphology and thus DN-Cad may not be required for the stabilization
of the glia sheath. However, DE-Cad and DN-Cad are expressed in the peripheral glia and their overlap in the adheren-belt like regions and a subset of puncta suggest the DN-Cad may be functionally redundant with DE-Cad. In other words, in the absence of DN-Cad, DE-Cad could potentially fulfill DN-Cad functions. However, it has been shown that in photoreceptor axons the roles of DN-Cad and DE-Cad are not interchangeable and that the loss of DN-Cad cannot be rescued by overexpressing DE-Cad in these cells (Prakash et al 2005). On the other hand, there are two N-Cad loci in *Drosophila*, DN-Cad (CadN) and a duplication known as CadN2 (Prakash et al., 2005). CadN2 is homologous to DN-Cad and shares 72.5% amino acid sequence identity and CadN2 is partially redundant to DN-Cad (Yonekura et al., 2008). Therefore, it is possible that the loss of DN-Cad is compensated by CadN2. However, *in vitro* cell-aggregation assays have shown that CadN2 cannot form homophilic interactions and CadN2 lacks adhesive activity (Yonekura et al., 2008). Therefore, if DN-Cad has an adhesive function in glial cells, CadN2 would not be able to compensate for its loss. However, whether the roles of DN-Cad and DE-Cad are interchangeable in glial cells remains to be determined.

### 4.5.2 Does DE-Cad mediate adhesion between different glial layers in the peripheral nerve?

Adherens junctions have been well studied in epithelial cells and perform two major functions in these cells; these junctions provide cell-cell adhesion and act as a link to the actin cytoskeleton. In Schwann cells, E-Cad forms autotypic adherens junction that adhere membranes of a single myelinating Schwann cell to each other (Fannon et al., 1995). Whether E-Cad is required in the non-myelinating Schwann cells however remains to be determined. Since the vertebrate non-myelinating Schwann cells are remarkably similar to wrapping glia, our study
provides insight into the potential role of cadherins in non-myelinating Schwann cells. Our data suggest that DE-Cad but not DN-Cad is essential for the ensheathment of axons. Moreover, DE-Cad knockdown in the WG results in discontinuities in the WG strands, suggesting that DE-Cad might be required for adhesion between neighbouring WG. Interestingly, we also noticed reduced WG strands implying that DE-Cad might also be required for adhesion between different WG strands. Furthermore, DE-Cad knockdown in the SPG results in gaps in the SPG membrane. It is possible that these gaps represent the inability of the SPG membrane to adhere to itself. It is also likely that cadherins play a role in anchoring the SPG to the WG as the phenotypes we observed with the pan-glial knockdown of DE-Cad knockdown were more severe than those observed with individual knockdown in the WG and SPG. The presence of SAJs between SPG and WG (Matzat et al., 2015) further supports that E-Cad mediated adhesion might occurs between SPG and WG. Whether SAJ in the Drosophila larval nerve are composed of DE-Cad, however has never been tested. We found that SAJs are still present in nerves in which DE-Cad was knocked down in all glia, suggesting a redundancy between DE-Cad and DN-Cad. Alternatively, the presence of SAJs could be due to incomplete loss of DE-Cad using RNAi or it is possible other adhesive molecules might contribute to the SAJ structure. To overcome the incomplete knockdown of DE-Cad one approach is to generate somatic DE-Cad mutant clones in the SPG and WG cells. This approach is not efficient in the WG and SPG which are polyploid as they undergo enormous hypertrophy during early larval growth (von Hilchen 2013). To test for redundancy pan-glial knockdown of DE-Cad could be performed in a mutant that deletes both DN-Cad and DN-Cad2 (N-CadΔ14) to determine if both DE-Cad and DN-Cad contribute to the SAJs.
4.5.3 Does DE-Cad affect glial cell polarity and blood-nerve barrier function?

We found that E-Cad knockdown in glial cells not only affected glial morphology but also lead to the mislocalization of Dlg1, a common marker for the morphology of the septate junction. Dlg1 is a member of the Scribble/Dlg/Lethal giant larvae polarity complex necessary to form the polarity of epithelial cells (Bilder et al., 2000). In the SPG, Dlg and Scrib are confined to the septate junctions in the larval peripheral nerve. If these proteins also establish polarity in glial cells it is possible that the mislocalization of Dlg1 after DE-Cad knockdown reflects a loss of polarity in these cells. Alternatively, the disruption in Dlg1 may reflect the disruption of the SJ itself. The swellings and vacuole like structures observed after DE-Cad knockdown suggests that these cells might have a disrupted blood-nerve barrier and can no longer restrict the flow of extracellular ions (Leiserson et al 2011). Further experiments, including dye uptake experiments need to be conducted to analyze the integrity of the barrier after DE-Cad knockdown.

4.5.4 Does DE-Cad function independently of p120 and α-catenin?

We show that DE-Cad knockdown results in deleterious glial phenotypes as well as the mislocalization of Dlg1 and SJ disruption. However, knockdown of p120 and α-cat did not phenocopy the DE-Cad knockdown phenotypes and both glial and SJ morphology were unaffected. The easiest explanation is that the RNAi reagents utilized were not effective in mediating protein knockdown. This may be the case with the p120-catenin-RNAi which has not been utilized in previous studies, however the alpha-catenin RNAi line HMS00317 has been shown to effectively knockdown alpha-catenin in other studies (Yang et al., 2015, Shipstone, 2015). Alternatively, the lack of phenotypes after p120 or alpha-catenin knockdown may represent different functions for these catenins in the peripheral glia compared to other tissues.
Our study was focused on the role of cadherin-catenin complex in glial cells of third instar larval peripheral nerves. Axonal ensheathment in first and second instar larvae is not complete (Stork et al., 2008) and suggests that the wrapping glia are still in the process of engulfing the axons. In comparison, axons are almost fully insulated by the third larval instar. It is possible that the functions of DE-Cad during the earlier, morphogenetic stages of glial development are dependent on p120 and α-cat.

Previous studies have shown that p120 promotes cell adhesion but is a non-essential component of adherens junctions and that p120 null mutants are viable and do not result in significant changes to adherens junction structure or function in embryos (Myster et al., 2003). Furthermore, a study in Drosophila embryos showed that α-cat is dispensable for the stability of SAJs and actin stabilization at SAJs (Cavey et al., 2008). Rather than stabilizing SAJs, α-cat functions via the actin network to restrict lateral mobility of SAJs in epithelial tissues (Cavey et al., 2008). It is possible that DE-Cad is required to maintain adhesion between the different glial layers in the peripheral nerve and knocking down α-cat does not necessarily affect the stability of DE-Cad already present at the membrane. This is also likely because unlike epithelial sheets that form zonular junctions required for coordinating epithelial sheet movements, glial cells form SAJs that favour anchoring/adhesion (Carien and Gottardi, 2007). Drosophila β-catenin/Arm is also an integral structural component of cadherin-based adherens junction and required for the stability of SAJs (Valenta et al., 2012, Cox et al., 1996). Whether β-cat/Arm knockdown phenocopies the loss of DE-Cad remains to be determined. In conclusion, we propose that p120 and α-cat might have non-essential functions in glial cells of the larval peripheral nerve.
In summary, our study shows that two cadherins, DN-Cad and DE-Cad are present and co-localize with catenins in the *Drosophila* peripheral nerve. We describe a role for DE-Cad in the WG, where DE-Cad is required for axonal ensheathment and in the SPG where DE-Cad facilitates the formation of septate junctions. Furthermore, we find that DN-Cad and DE-Cad may have redundant roles in glia as SAJ are present between the peripheral glia membrane is larvae lacking DE-Cad. A role for cadherins in non-myelinating Schwann cells has not yet been described. Therefore, our study furthers our understanding of cadherins in the peripheral nerve and highlights the importance of DE-Cad in supporting glial morphology and function.
FIGURES – CHAPTER 4

A

VNC

B

VNC

N-Cad::Cherry, repo-GAL4

E-Cad::Cherry, repo-GAL4

C

mCD8::GFP N-Cad

D

G

mCD8::GFP E-Cad

H

C’
mCD8::GFP

D’

G’
mCD8::GFP

H’

C”
N-Cad

D”

G”
E-Cad

H”

glia

E

22C10 N-Cad

F

I

22C10 E-Cad

J

E’
22C10

F’

I’
22C10

J’

axon

E”
N-Cad

F”

I”
E-Cad

J”

N-Cad::Cherry, E-Cad::GFP

K

N-Cad E-Cad

L

N-Cad E-Cad

K’

N-Cad

L’

N-Cad

K”
E-Cad

L”
E-Cad

161
Figure 4.1. DN-Cadherin and DE-Cadherin are expressed in glial cells of the larval peripheral nerve

A-B: Schematic representation of DN-Cad (A) and DE-Cad (B) distribution in a peripheral nerve in a 3rd instar larva. DN-Cad puncta (magenta, A) observed throughout the peripheral nerve and formed ZA-like belts (magenta stripes, A) in regions close to the ventral nerve cord (VNC) as well as in other regions along the length of the nerve. DE-Cad puncta (magenta, B) were expressed throughout the peripheral nerves and formed ZA-like belts (magenta stripes, B) in different regions along the nerve.

C-F: Longitudinal sections of a control peripheral nerve with DN-Cad::mCherry (magenta, C,C'' and E,E''). The glial membranes were labeled with mCD8::GFP (green, C,C') and axons were immunolabeled (green, E,E') using anti-Futsch (22C10). DN-Cad ZA-like belts (magenta, C'' and E'') were observed along the length of the nerve (C and E). Cross-sections taken from the peripheral nerves in C and E, shown in D-D'' and F-F'', respectively show that DN-Cad overlaps with both glia and axons.

G-J: Longitudinal sections of a control peripheral nerve with DE-Cad::mCherry (magenta, G,G'' and I,I''). The glial membranes were labeled with mCD8::GFP (green, G,G') and axons were immunolabeled (green, I,I') using anti-Futsch (22C10). DE-Cad ZA-like belts (magenta, G'' and I'') were observed along the length of the nerve in glial cells (G) and axons (I). Cross-sections taken from the peripheral nerves in G and I, shown in H-H'' and J-J'', respectively show that DE-Cad appears to be present in both glia and axons (yellow arrowheads).

K-L: Longitudinal sections of peripheral nerves with DN-Cad (magenta, K,K' and L,L') and DE-Cad (green, K,K'' and L,L'') labeled with DN-Cad::mCherry and DE-Cad::GFP, respectively. The DN-Cad ZA-like belts (white arrowheads, K,K' and L,L') partially overlap with DE-Cad ZA-like belts (yellow arrowheads, K,K'' and L,L'') in different regions along the length of the nerve. Scale bars: 15µm
**Figure 4.2.** DN-Cad and DE-Cad associate with p120-, alpha- and beta-catenins in the larval peripheral nerve

**A-B:** Longitudinal sections of peripheral nerves with DN-Cad (green, A,A’) and DE-Cad (green, B,B’) labeled with DN-Cad::mCherry and DE-Cad::mCherry, respectively. These nerves were immunolabeled with anti-p120-catenin (magenta, A,A” and B,B”). Some but not all p120-cat
puncta (magenta, A,A’’ and B,B’’) overlap with DN-Cad (green, A,A’’) and DE-Cad (green, B,B’’) puncta (yellow arrowheads). p120 was not observed in the cadherin positive ZA-like belts.

C-D: Longitudinal sections of peripheral nerves with DN-Cad (green, C,C’) and DE-Cad (green, D,D’) labeled with DN-Cad::mCherry and DE-Cad::mCherry, respectively. These nerves were additionally immunolabeled with anti-beta-catenin (Arm) (magenta, C,C’’ and D,D’’). Arm positive puncta were expressed throughout the nerve and formed belts (magenta, C,C’’ and D,D’’)(yellow arrowheads).

E-F: Longitudinal sections of peripheral nerves with DN-Cad (green, E,E’) and DE-Cad (green, F,F’) labeled with DN-Cad::mCherry and DE-Cad::mCherry, respectively. These nerves were additionally labeled with α-cat::GFP (magenta, E,E’’ and F,F’’). The α-cat puncta were expressed throughout the nerve and formed belts (magenta, E,E’’ and F,F’’)(yellow arrowheads). Scale bars: 15µm
Figure 4.3. Knockdown of DE-Cad but not DN-Cad in the WG and SPG leads to loss of axonal ensheathment and affects Dlg1 localization.

A-C: Longitudinal sections of control (A), repo>DN-Cad-RNAi, Dicer2 (B) and repo>DE-Cad-RNAi, Dicer2 (C) peripheral nerves with glial membranes labeled with mCD8::GFP (green). The SJ associated protein Dlg1 was immunolabeled (magenta). The glial membranes continuously extend along the length of control (green, A,A’) and repo>DN-Cad-RNAi, Dicer2 nerves (green, B,B’) whereas disruptions in the glial membranes (yellow arrowheads, C,C’) and vacuole-like structures (white arrowheads, C,C’) were observed in repo>DE-Cad-RNAi, Dicer2 nerves. Dlg1 is located to the SJ which forms a single strand along the length of the both control (magenta, A,A’’) and repo>DN-Cad-RNAi, Dicer2 (magenta, B,B’’) nerves. Knockdown of DE-Cad in the presence of Dicer2, disrupted the SJ marked by Dlg1 (magenta, C,C’’).

D-F: Longitudinal sections of control (D), Nrv2>DN-Cad-RNAi, Dicer2 (E) and Nrv2>DE-Cad-RNAi, Dicer2 (F) peripheral nerves with WG membranes labeled with mCD8::GFP (green) and axons immunolabeled with anti-Futsch (22C10, magenta). The WG membranes continuously extend along the length of control (green, D,D’) and Nrv2>DN-Cad-RNAi, Dicer2 nerves (green, E,E’) whereas discontinuous and disrupted WG membranes (yellow arrowheads, F,F’) were observed in Nrv2>DE-Cad-RNAi, Dicer2 nerves. The axon morphology in both Nrv2>DN-Cad-
RNAi, Dicer2 (magenta, E,E’’) and Nrv2>DE-Cad-RNAi, Dicer2 (magenta, F,F’’) peripheral nerves looked similar to controls (magenta, D,D’’).

G-I: Longitudinal sections of control (G), Gli>DN-Cad-RNAi, Dicer2 (H) and Gli>DE-Cad-RNAi, Dicer2 (I) peripheral nerves with the SPG membrane labeled with mCD8::RFP (magenta) and Dlg1 immunolabeled (green). The SPG membrane is continuous along the length of control (magenta, G,G’) and Gli>DN-Cad-RNAi, Dicer2 nerves (magenta, H,H’) whereas the SPG membrane observed in Gli>DE-Cad-RNAi, Dicer2 nerves was discontinuous and disrupted (yellow arrowheads, I,I’). The localization of Dlg1 was normal in both control (green, G,G’’) and Gli>DN-Cad-RNAi, Dicer2 (green, H,H’’) nerves whereas Dlg1 was lost in regions of the nerve where the SPG membrane was disrupted in Gli>DE-Cad-RNAi, Dicer2 (green, I,I’’) peripheral nerves. Scale bars: 15µm
Figure 4.4. Knockdown of DE-Cad in glial cells does not result in the loss of spot adherens junctions

A-C: Ultrastructural analysis of control (A) peripheral nerves and nerves lacking DE-Cad in the peripheral glia (repo>DE-Cad-RNAi, Dicer2)(B,C). A: Representative TEM image of a cross-section of a control peripheral nerve showing that the wrapping glia (false coloured in green) extend their processes and wrap around several axons, separating them into fascicles. B-C: Loss of DE-Cad in peripheral glia did not severely affect the gross morphology of the WG (false coloured in green) but the WG processes appeared thicker (black double-sided arrowheads, B,C) compared to controls (black double-sided arrowheads, A). Spot adherens junctions were observed between the WG and SPG (dashes magenta box, A) well as between SPG and PG in control nerves (dashed yellow box, A). Spot adherens junctions were also present in nerves with DE-Cad knockdown and were observed between WG processes (dashed magenta box, B) in the representative TEM cross-section. Boxed regions in A and B are shown at a higher magnification in A’ (magenta box), A” (yellow box) and B’ (magenta box), respectively. Scale bars: 1µm
<table>
<thead>
<tr>
<th>repo-GAL4, Dicer2</th>
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<tbody>
<tr>
<td><strong>A</strong> mCD8::GFP Dlg1</td>
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<tr>
<td><strong>A’</strong> mCD8::GFP</td>
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<td><strong>A”</strong> Dlg1</td>
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<td><strong>C’</strong> mCD8::GFP</td>
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<td><strong>C”</strong> Dlg1</td>
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Figure 4.5. Knockdown of p120- and alpha-catenins does not affect glial morphology and Dlg1 localization

A-C: Longitudinal sections of control (A), repo>p120-cat-RNAi, Dicer2 (B) and repo>a-cat-RNAi, Dicer2 (C) peripheral nerves with the glial membranes labeled by repo-GAL4 driving the expression of mCD8::GFP (green, A’, B’ and C’) and Dlg1 immunolabeled (magenta, A”, B” and C”). The glial membranes extend continuously along the length of the nerve in control (green, A, A’), repo>p120-cat-RNAi, Dicer2 (green, B, B’) and repo>a-cat-RNAi, Dicer2 (green, C, C’) nerves. The SJ marked by Dlg1 forms a single, continuous line in control (yellow arrowheads, A, A’), repo>p120-cat-RNAi, Dicer2 (yellow arrowheads, B, B’) and repo>a-cat-RNAi, Dicer2 (yellow arrowheads, C, C’) nerves.

Scale bars: 15µm
Chapter 5: Discussion

The integrity and function of axons in the peripheral nerve rely on glial cells and their ability to correctly organize themselves around axons and separate them into fascicles. This organization must require glia-glia communication. Evidence from studies in the myelinating Schwann cells (SCs) show that reflexive gap junctions and autotypic adhesive junctions play an important role in maintaining the myelin sheath (Balice-Gordon et al., 1998, Tricaud et al., 2005, Young et al., 2002, Miyamoto et al., 2005). The role of these junctions and their components have not been fully elucidated in SCs. Moreover, almost nothing is known about the role of gap junctions and glia adhesion complexes in non-myelinating Schwann cells (NMSCs). *Drosophila* glial cells have comparable functions to vertebrate glia making it a good model to study glia-glia communication.

In the *Drosophila* PNS, three morphologically distinct glial subtypes ensure that axons are properly ensheathed and protected. The overarching goal of our studies was to understand how these glial cells organize themselves and communicate with each other to achieve these essential tasks. Previous studies from our lab have shown that glia-ECM interactions are important for glial development and maintenance (Xie and Auld, 2011, Xie et al., 2014, Petley-Ragan et al., 2016). However, the contribution of gap junctions and other adhesive proteins in the peripheral glia had not been previously tested. One of the goals of this thesis was to investigate the function of gap junctions in peripheral glia. Previous studies had identified two glial gap junction proteins; Innexin1 and Innexin2 in Drosophila, but their function in peripheral glia was unknown. We therefore sought to determine if Inx1 and Inx2 mediate glia-glia communication in the peripheral nerve, with a focus on Inx2. The scaffolding of connexins (Cxs) to the cell membrane is mediated by the interaction of Cx-PDZ binding motifs with PDZ proteins.
such as ZO-1 (Thevenin et al., 2013). Since Inx2 has a predicted Class II PDZ binding motif, we sought to identify PDZ proteins that might interact with Inx2 and facilitate its localization. Although all PDZ proteins were analyzed in a genetic screen, we were unable to identify any PDZ proteins that phenocopied the Inx2 knockdown phenotypes. We however, identified 7 PDZ proteins from this study and the second main goal of this thesis focused on the role of Dlg5. Our decision to focus on Dlg5 was based on two reasons: 1) nothing was known about Dlg5 in glial cells; 2) previous studies had identified a role for vertebrate Dlg5 in mediating cell adhesion via the localization of cadherins. Furthermore, biochemical interaction studies found that Inx2 interacts with adherens junction and septate junction components and we speculated that Dlg5 might mediate interactions between these junctions. At the time that we initiated our studies, it was not known whether cadherin/catenin complexes were even present in the peripheral nerve. Therefore, the third main goal of this thesis was to identify which cadherin/catenin complexes, if any, are present in the peripheral nerve and determine their functions.

In Chapter 2 of this thesis, we describe the role of gap junctions in mediating communication between two different glial cells (SPG and WG). We show that the gap junction between the SPG and WG are composed of Inx1 and Inx2 and that calcium pulses in the SPG are mediated by Inx2 but not Inx1. We further show that eliminating Inx2 in the SPG results in the loss, discontinuities and fragmentation of WG strands, emphasizing the importance of gap junctions in the survival of the WG. Our data shows that the survival of the WG relies on a gap junction channel rather than an adhesive role for Inx2. Moreover, we found that even though calcium pulses are mediated by Inx2 in the SPG, altering the levels of calcium and IP3 does not affect WG survival. We therefore propose a gap junction mediated mechanism by which two different glial cells interact in the peripheral nerve. Through our findings, we highlight the
importance of glia-glia communication in the survival and maintenance of glial cells in the peripheral nerve.

In Chapter 3 of this thesis, we summarize our findings from a RNAi-based genetic screen to identify PDZ proteins required in *Drosophila* peripheral glia. A comprehensive analysis of PDZ proteins resulted in the identification of seven PDZ proteins with important functions in sustaining the morphology of glial cells in the peripheral nerve. These seven PDZ proteins include Discs Large 1 (Dlg1), Scribbled (Scrib), Varicose (Vari), Stardust (Sdt), Discs Large 5 (Dlg5), Locomotion defects (Loco) and Rho-type guanine nucleotide exchange factor 2 (RhoGEF2). We show that Vari is required for axonal ensheathment and eliminating Vari in the SPG affects SJ morphology in the peripheral nerve. We further show that a core component of the Crumbs complex, Sdt plays a non-canonical role in glial cells. Even though most components of the Crumbs complex, including Crumbs is not present in glial cells eliminating Sdt affects glial morphology. The mechanism by which Vari and Sdt function in glial cells however requires further investigation. We show that Dlg5 is expressed throughout the peripheral nerve but does not localize with gap junction and focal adhesion complexes. Our co-localization studies show that Dlg5 co-localizes with DN-cad and Vps26 (a retromer complex component) suggesting a role in trafficking cadherins. However, eliminating Dlg5 in glia does not affect cadherin localization and SAJs are still present between peripheral glial membrane. But eliminating Dlg5 in the WG and the SPG affects axonal ensheathment and SJ morphology, respectively. Therefore, our study has not only identified seven PDZ proteins with important function in glial cells but also highlights the function of Dlg5 in axonal ensheathment and formation of the SJ complex.
In Chapter 4 of this thesis, we identify cadherin/catenin complexes in the Drosophila peripheral nerve. We show that DN-Cad and DE-Cad are expressed as puncta or form adherens-like belts in the peripheral nerve. We further show that DN-Cad and DE-Cad are expressed in overlapping patterns and co-localize with catenins. We demonstrate that eliminating DE-Cad in the WG and the SPG results in less and discontinuous WG strands and affects SJ morphology, respectively. In contrast, eliminating DN-Cad does not affect glial or SJ morphology. Moreover, we show that knocking down DE-Cad does not eliminate SAJs, suggesting a redundant role for DN-Cad in the peripheral glia.

In summary, this thesis highlights the importance of glia-glia communication and describes three ways in which axonal ensheathment is regulated by glial cells in the peripheral nerve. Our study is first to show that gap junctions couple two different glial cells in the peripheral nerve. We are the first to characterize the function of Dlg5 in glial cells. Moreover, we are first to provide a description of cadherin/catenin complexes in peripheral glia. Given that NMSCs ensheath axons in a similar manner to Drosophila glia, our study provides insight into potential mechanisms by which gap junctions, Dlg5 and cadherins might mediate wrapping by NMSCs.

5.1 Gap junction mediated glia-glia communication

Chapter 2 represents our efforts in understanding how glial cells communicate with each other in the peripheral nerve and explores the role of gap junctions in this context. In the following sections I will discuss some of the caveats and limitations that were encountered in this study and future directions to resolve these. The main caveats and unanswered questioning related to this study are as follows: 1) Inability to distinguish between innexins in the SPG and
the WG. 2) Determine if dye transfer between SPG and WG can be blocked by manipulating Inx2 function. 3) Determine whether calcium pulses in the SPG and WG arise in response to axonal activity. 4) Identity of the cell death pathway via which WG death occurs after Inx2 knockdown. 5) Identity of the signal mediated by the SPG-WG gap junction.

5.1.1 Inability to distinguish between innexins in the SPG and the WG

In this study, we were able to show that Inx1 and Inx2 are expressed at the SPG-WG using a wide field fluorescence microscope. Although the images from this show that Inx1 and Inx2 form a complex and are part of the same gap junction plaque, we cannot say with confidence, whether the complex is formed between the SPG and WG or within one of these glial layers. One of the factors that limited our ability to distinguish between these possibilities is that the SPG membrane is very thin (∼0.5 µm) and is in very close proximity to the WG membrane. Therefore, the resolution at which we imaged Inx1/Inx2 antibody labeling at the SPG-WG is not sufficient. We propose that using super-resolution microscopy might help achieve a better understanding of which glial layers contribute Inx1, Inx2 or both. Furthermore, using the LexA-based binary expression system, transgenic lines can be used to express Inx2::mRFP in the SPG (Gli-LexA>LexAop-Inx2::mRFP). We can simultaneously label Inx1 in the WG using the GAL4/UAS system (Nrv2-GAL4>UAS-Inx1::GFP). If gap junctions are indeed formed between the SPG and the WG and not just within either of these glial layers, yellow puncta should be observed at the SPG-WG boundary. Furthermore, if the extracellular loops of Inx1 and Inx2 are tagged with GFP and mRFP respectively, the proximity ligation assay (PLA) can be used to assay for interactions between Inx1 in the WG and Inx2 in SPG. This
experiment would further support the results obtained from our dye transfer studies pointing towards a gap junction between the SPG and the WG.

5.1.2 Determine if dye transfer between SPG and WG can be blocked by manipulating Inx2 function

In Chapter 2, we showed that the Calcein-Orange-Red AM (Calcein dye) was taken up the SPG and transferred to the WG. This suggests that a gap junction exists between the SPG and the WG. To confirm that gap junctions exist between the SPG and the WG further experiments must be conducted. In the future, larval preparations can be treated with a gap junction blocker, Carbenoxolone (CBX). CBX has been successfully used to block gap junctions in multiple studies in both vertebrates and invertebrates (Speder and Brand, 2014, Sahu et al., 2017, Connors, 2012, Bao et al., 2007). However, we are limited by how long the live larval preparation can be incubated with CBX. We found that the larvae remain ‘healthy’ and suitable for analysis for a total time of an hour and half. This includes the time required to dissect, incubation with dye and imaging. Since the Calcein dye must pass through multiple glial layers once it is taken up from the hemolymph, the amount of time the larval preparation is incubated with dye cannot be reduced. Therefore, the appropriate concentration of CBX and time required for CBX to work effectively must be determined to be able to perform this experiment while keeping the larvae alive.
5.1.3 Determine whether calcium pulses in the SPG and WG arise in response to axonal activity

Calcium pulses occur in both the SPG and WG in the larval peripheral nerve however, we do not know whether these are spontaneous calcium pulses that originate in the CNS or if they occur in response to axonal activity. Moreover, in Chapter 2 we show that while calcium pulses in the SPG are gap junction mediated. Since gap junctions in SPG of the CNS also mediate calcium pulses, it is likely that the peripheral SPG form a gap junction linked network with SPG in the CNS. The calcium pulses in the WG however, are not gap junction mediated and it is not known whether the WG in PNS are linked to their counterparts in the CNS. Therefore, it is more likely that calcium pulses in the SPG originate in the CNS whereas those in the WG might be triggered locally perhaps by axonal activity. To confirm that calcium pulses in the SPG arise from the CNS and to determine if calcium pulses in WG are a result of axonal activity, we can optogenetically depolarize acetylcholine expressing neurons by using Cha-LexA to express LexAop::Chrimson (Cha-LexA>LexAop::Chrimson). Chrimson is a red light-drivable channel rhodopsin (light gated cation channel) and can be ontogenetically activated by using red light (625nm) (Klapoetke et al., 2014). Changes in the intensity of calcium, by expressing GCAMP in either the SPG (Gli-GAL4>UAS-GCaMP6S) or the WG (Gli-GAL4>UAS-GCaMP3), can be assayed simultaneously with the depolarization of Cha expressing neurons using red light (625nm), which should not interfere with the blue light (430nm) used to excite GCaMP. If calcium pulses in either SPG or WG glia are a result of axonal activity, we expect that depolarization of axons should coincide with the observation of calcium pulses in these glial cells. Furthermore, as a control, acetylcholine releasing cells can be optogenetically silenced by expressing a cyan-gated anion channel rhodopsin (Guillaradia theta anion channel rhodopsin,
GtACR1) (Cha-LexA>LexAop::GtACR1) (Mohammad et al., 2017) while imaging for changes in calcium in the SPG and the WG. We hypothesize that if the calcium pulses occur in response to axonal activity, these pulses should be abolished when neuronal activity is silenced.

5.1.4 Identity of the cell death pathway via which WG death occurs after Inx2 knockdown

We show that knockdown of Inx2 in the SPG and WG leads to fragmentation of the WG membrane, indicative of cell death. To determine the pathway through which the WG are dying, we blocked apoptosis but were unable to rescue WG death. Therefore, we were able to show that the WG do not die via apoptosis. However, we have not yet identified the pathway involved in the death of the WG. Autophagy in Drosophila has been observed in many tissues upon nutrient deprivation (McPhee and Baehrecke, 2009). It is possible that the loss of Inx2 results in the lack of metabolites essential for the survival of the WG and induce autophagy. To test if the WG undergo autophagy, we attempted to use LysoTracker Red. LysoTracker Red probes consist of a red-fluorophore linked to a weak base that is partially protonated at neutral pH. These probes are cell permeable and are specifically retained by acidic organelles. LysoTracker has been used successfully as a marker for autophagy in many Drosophila tissues (DeVorkin and Gorski, 2014). Unfortunately, using LysoTracker in the Drosophila PNS resulted in background noise and red fluorescence signals could not be detected specifically in the peripheral nerve tissue. In the future, we plan to express another autophagy marker, RFP-GFP-tagged Atg8a in the wrapping glial cells while knocking down Inx2 (Nrv2>RFP-GFP-Atg8a, Inx2-RNAi). Atg8a is an autophagy protein tandemly tagged with RFP and GFP, such that the location of Atg8a in the autophagosomal membrane result in yellow puncta (Kimura et al., 2007, Klionsky et al., 2016).
GFP is quenched in the acidic environment of the autolysosome whereas the more stable RFP fluorescence remains resulting in red puncta indicative of flux of the reporter to acidic compartments (Klionsky et al., 2016). If the WG undergo autophagy, we expect to observe more red fluorescent puncta in the Inx2 knock down nerves compared to their controls. To further confirm that the WG undergo autophagy upon loss of Inx2, we will block autophagy by knocking down autophagy genes and assay for the rescue of WG aggregates in these larvae.

5.1.5 **Identity of the signal mediated by the SPG-WG gap junction**

Our observation that calcium pulses in the SPG were present lead us to the hypothesis that calcium or IP3 might be the signal required for survival of the WG. After testing all components of the IP3 and changing intracellular Ca\(^{2+}\) levels, we are confident that neither calcium nor IP3 mediating signalling through the gap junctions is necessary for WG survival. Moreover, we hypothesized that SPG-WG gap junctions might be required for the transfer of sugars such as trehalose and that WG might be dying due to the loss of these sugars. However, when trehalose transporters were knocked down in all glial cells the WG morphology appeared normal. Furthermore, a recent study has shown that Inx2 gap junctions do not mediate transfer of sugars between glia (Volkenhoff et al., 2017). Therefore, the signal transferred from the SPG to the WG remains to be identified. A recent study in the *Drosophila* astrocyte-like glia has recently proposed a model where Inx2 gap junctions are required for the diffusion of glutamine within the glial network (Farca Luna et al., 2017). Recycling of histamines with the *Drosophila* laminar glial network is also mediated by Inx2 gap junctions (Chaturvedi et al., 2014). Therefore, a number of small metabolites and ions could potentially pass through the gap junction and it
would require screening through a number of candidates to determine what goes through the SPG-WG gap junction.

5.2 Role of PDZ proteins in glial cells of the peripheral nerve

Chapter 3 reports our finding from a genetic screen aimed at identifying PDZ proteins that might mediate glia-glia interactions in the peripheral nerve. This study was initially carried out to identify PDZ proteins that regulate the localization of Inx2 to the membrane. However, pan glial knockdown of all the tested PDZ proteins did not phenocopy the loss of Inx2 and did not result in Inx2 mislocalization. However, we did identify Dlg5 as a key regulator of peripheral glial morphology. Surprisingly, we found that knockdown of Dlg5, a known regulator of adherens junction assembly in vertebrates, did not affect the formation of SAJs in Drosophila glia. The caveats and limitations encountered during this study will be discussed in the following sections. In particular I will discuss the following unanswered questions and propose ways to address these in the future: 1) Confirmation that Dlg5 knockdown does not affect cadherin localization. 2) Understanding why knockdown of Dlg5 leads to wrapping glia defects. 3) Understanding how Dlg5 knockdown results in ‘gaps’ in the subperineurial glia.

5.2.1 Confirmation that Dlg5 knockdown does not affect cadherins

In Chapter 3, Dlg5 was one of the PDZ proteins identified as having a role in glial cells of the peripheral nerve. Based on studies that showed a role for Dlg5 in cadherin mediated cell adhesion (Nechiporuk et al., 2007, Liu et al., 2014), we began by looking at whether Dlg5 associates with DN-Cad and DE-Cad. One of the primary limitations in this study was the lack of effective Dlg5, DN-Cad and DE-Cad antibodies. Previous attempts were made by another lab to
generate a *Drosophila* Dlg5 antibody however, they failed at generating an antibody that was effective and specific to Dlg5 (Luo et al., 2016). Moreover, although the DN-Cad and DE-Cad antibodies are effective in epithelial tissues, they do not work well in the peripheral nerve tissue. Therefore, co-localization studies were limited to the use of Dlg5::GFP and cadherins tagged with mCherry. Moreover, the GFP and mCherry fluorescence had to be strengthened by using a GFP and mCherry antibodies, respectively. Even so the Dlg5 labeling was inconsistent, with some nerves expressing bright puncta and others expressing very small faint puncta. We believe that the reasons for these discrepancies in Dlg5 detection were due to the anti-mouse-GFP antibody being less effective than the anti-rabbit-GFP antibody. We looked for co-localization between Dlg5 and a range of subcellular organelle and vesicle markers, including different Rab proteins that localize to specific endosomal compartments. The antibodies available for the detection of Rabs, including the anti-Rab11, anti-Rabsn5 and anti-Rab7 used in this study, were raised in rabbits and Rab proteins are tagged with GFP. Therefore, our co-localization studies between Rab proteins and Dlg5 were limited to the use of anti-mouse-GFP to enhance the Dlg5::GFP signals. We plan to repeat these experiments using an anti-chicken-GFP antibody, to verify whether our initial analysis that Dlg5 is not found in early (Rab-5 positive), late (Rab-7 positive) and recycling endosome (Rab-11 positive) is correct. However, we are certain that DN-Cad and Vps26 co-localize with Dlg5 suggesting that Dlg5 might be involved in the trafficking of DN-Cad through the retromer (Seaman et al., 1998, Gokool et al., 2007). On the contrary, Dlg5 did not localize with DE-Cad and knockdown of Dlg5 did not affect DN-Cad and DE-Cad localization. It is possible that the knockdown of Dlg5 was not efficient as for this experiment a less efficient Dlg5-RNAi (TRiP GL01260) was used as it is the only Dlg5-RNAi on the third chromosome and both DN-Cad::mCherry and DE-Cad::mCherry are on the second chromosome.
The presence of the WG defects in these nerves however, suggest that the Dlg5 knockdown was
efficient. Moreover, Dlg5 knockdown in all glia did not result in the loss of SAJs, further
suggesting that Dlg5 is not necessary for the formation of SAJs. Since Dlg5 localizes with DN-
Cad but not DE-Cad, it is possible that Dlg5 is restricted to the localization of DN-Cad but not
DE-Cad. The reason Dlg5 knockdown might not affect changes in DN-Cad localization or SAJ
formation might be due to redundancies between DN-Cad and DE-Cad, and between CadN2 and
DN-Cad. To test this hypothesis, we would perform Dlg5 knockdown in the absence of DE-Cad
using a double RNAi. The assumption being that DN-Cad won’t localize due to lack of Dlg5 and
DE-Cad would not be present to compensate for the loss of DN-Cad. Given that DE-Cad
knockdown alone leads to the abnormalities in the WG and SPG (Chapter 4), making it difficult
to separate the effect of Dlg5 knockdown from that of DE-Cad knockdown. To overcome this we
could make use of a heterozygous shotgun mutant to reduce the gene dose by 50% to test for a
genetic interaction. If the glia phenotypes are exacerbated by the knockdown or reduction in DE-
Cad along with Dlg5 knockdown it would suggest that they might genetically interact. Another
point of redundancy is the presence of a second DN-Cad protein CadN2. To overcome this
possibility, we could introduce heterozygous mutants for both CadN2 and DE-Cad, to reduce the
gene dosage of both genes by 50%. If Dlg5 is required for DN-Cad localization, knockdown of
Dlg5 in larvae with reduced levels of both CadN2 and DE-Cad should affect SAJ morphology. A
major limitation of this study was our inability to consistently detect cadherins using light
microscopy, making it difficult to determine if cadherins were lost from a particular glial layer.
Therefore, electron microscopy must be used to determine if SAJs are lost when both Dlg5
mediated DN-Cad trafficking and DE-Cad are downregulated.
5.2.2 Understanding why knockdown of Dlg5 leads to wrapping glia defects

The knockdown of Dlg5 in the WG leads to less strands or breaks between strands lead to a discontinuous phenotype. We hypothesized that these WG phenotypes were due to the loss of adhesion between the WG processes. Our rationale for this hypothesis was based on the known role for Dlg5 in mediating cell adhesion by localizing cadherins to the membrane. Studies in mammalian cells have shown that Dlg5 assists the delivery of beta-catenin bound N-Cad to the cell membrane where N-Cad forms adherens junctions required to maintain cell polarity (Nechiporuk et al., 2007, Liu et al., 2014). Although this hypothesis requires further testing, our present data suggests that Dlg5 might not be involved in cadherin localization. Whether Dlg5 might interact with other proteins to mediate adhesion between WG strands and processes however cannot be ruled out. Since, Dlg5 is a MAGUK with at least six protein-protein interaction domains it is likely to scaffold other proteins to carry out its function. A recent study has generated several transgenes carrying different domains of Drosophila Dlg5 (Luo et al., 2016). These transgenes include several truncated forms of Dlg5 including those that lack PDZ1-2 or PDZ3-4PDZ domains, the GUK domain and SH3 domains alone, both GUK and SH3 domains, the coiled-coil and PDZ1-2 domains as well as many other constructs. To obtain a better understanding of Dlg5 function in the wrapping glia, we can determine which domain in Dlg5 is required for wrapping glia ensheathment of axons. To do so, WG phenotypes in Dlg5 knockdown can be rescued by expressing transgenes carrying specific Dlg5 domains in the WG. The domains in Dlg5 that mediate the WG adhesion function should be able to rescue the loss of and discontinuities in the WG strands. By determining which Dlg5 domain(s) is necessary we can narrow down our search for interactors of Dlg5. For example, Dlg5 has 4 PDZ domains and can interact with proteins containing a PDZ-binding motif. If we find that the PDZ function of
Dlg5 is necessary for its function in the wrapping glia, future attempts at identifying interactors of Dlg5 can be confined to proteins with a PDZ binding motif. Alternatively, Dlg5 might be required during development for growth or migration of the WG along the nerve and extension of its processes around axons, rather than maintenance of WG-WG contacts (adhesion). To determine if Dlg5 is required during development, we plan to repress the expression of Dlg5-RNAi until late stages of larval development using temperature sensitive Gal80 and raising the flies at 18°C. The repression of repo-GAL4 activity by Gal80 will be alleviated by switching the cross (Gal80ts, repo>Dlg5-RNAi) to a permissive temperature (29°C) during late larval stages. If Dlg5 is required during development rather than maintenance then, knockdown of Dlg5 during late larval stages should not lead to the loss of WG or discontinuities in the WG. The morphology and extent of glia migration can also be assessed in embryos in which Dlg5 is knocked down in glia and carry a copy of the dlg5 null allele (dlg5^048, repo>Dlg5-RNAi, mCD8GFP) and compared to a control (w^{1118}, repo>mCD8::GFP).

5.2.3 Understanding why knockdown of Dlg5 leads to gaps in the subperineurial glia

Apart from the WG defects observed in Dlg5 knockdown nerves, we also observed gaps in the SPG. Moreover, we show that these gaps in the SPG membrane are accompanied with the loss of the septate junction protein NrxIV. Therefore, it is possible that the ‘gaps’ arise due to loss of Dlg5 mediated trafficking of SJ proteins to the SPG membrane. These SJ proteins are required for the formation of the SJ complex and in its absence the SPG might not be able to form autotypic adhesive contacts. Alternatively, Dlg5 might be required to deliver a yet unidentified cargo to the SPG membrane. Moreover, in Chapter 3, Scrib was not only identified as one of the PDZ proteins required in glial cells but we also found that knockdown of Dlg5
leads to loss of Scrib from the SPG membrane suggesting an interaction between Dlg5 and Scrib. These results combined with the well-known function of Scrib in regulating cargo sorting (Gui et al., 2016, de Vreede et al., 2014, Lohia et al., 2012), have lead us to believe that Scrib might be required for the regulation of the endocytic trafficking pathway. Furthermore, the co-localization of Dlg5 with the retromer complex (Vps26), suggests that Dlg5 and Scrib might be involved in the trafficking of cargos that sort through the retromer complex. Moreover, it is likely that Dlg5 itself is recycled to the cell surface via its interactions with the retromer and scaffolds other proteins to the SPG membrane. To first confirm whether the SJ complex is disrupted by the knockdown of Dlg5 in the SPG, we plan to test the integrity of the blood-brain barrier in Dlg5 knockdown larvae by performing previously used dye permeability assays (Li et al., 2017). In this assay, a fluorescent dye is injected into the larval body cavity and is excluded in the presence of an intact blood-brain barrier. If Dlg5 is required for the formation or maintenance of an intact blood-brain barrier, knockdown of Dlg5 should result in dye diffusion into the brain. We will test whether the localization of other SJ proteins such as Nervana2 (Nrv2) are also affected by Dlg5 knockdown. If so, this suggests that SJ protein localization requires recycling of proteins via the retromer pathway and to test this we plan to knockdown components of the retromer complex, including Vps26 in the SPG and assay for changes in the SPG morphology as well as NrxIV labeled SJ strands. Vps26 is required for recruiting the retromer complex on endosomes (Gokool et al., 2007), therefore knocking down Vps26 should lead to degradation of cargo that was destined to be recycled directly to the cell surface or through the trans-Golgi network (TGN). If Dlg5 does mediate it effects on the SPG via retromer facilitated recycling of proteins, knocking down Vps26 should lead to mislocalization of NrxIV as well as similar SPG phenotypes as those observed in the Dlg5 knockdown nerves. To confirm that the phenotypes observed in the Vps26
knockdown nerves are caused directly by the loss of Dlg5 at the SPG membrane, we can overexpress Dlg5 in the SPG and look for partial rescue of glial phenotypes. The notion being that more Dlg5 will be present at the SPG membrane and will compensate for the lack of Dlg5 recycling. Alternatively, we can screen for different SJ proteins that interact with Dlg5 and use live imaging to observe their movements through the endocytic pathway. A recent study has shown that enhanced GFP (EGFP) can be photo-converted to RFP with 405nm light (Sattarzadeh et al., 2015). Since several proteins, including NrxIV have been tagged with EGFP (Buszczak et al., 2007) the trafficking and dynamics of NrxIV can be followed live and by converting GFP to RFP we will be able to distinguish between mature and nascent NrxIV protein. If NrxIV is recycled to the SJ complex we expect the SJ strands to be positive for both newly synthesized NrxIV (GFP) as well recycled NrxIV (RFP). Furthermore, the trafficking of NrxIV can be followed in Dlg5 knockdown or Vps26 knockdown nerves to look for absence of RFP at the SJ domain.

Next, we plan to identify if there are potential interactions between Scrib and Dlg5. Scrib interacts genetically with Dlg1 in epithelial cells and in vitro Scrib and Dlg1 are found in the same protein complex (Bilder et al., 2000, Mathew et al., 2002). Moreover, Dlg1 is required for the recruitment of Scrib at the neuromuscular junction (NMJ) (Mathew et al., 2002). Since Dlg1 and Dlg5 are both MAGUKs with PDZ, SH3 and GUK domains it is possible that Dlg5 might mediate its effects in the SPG via its interaction with Scrib. Co-immunoprecipitation will be used to determine whether Dlg5 interacts with Scrib in vitro and results from the Co-IP can be confirmed in vivo by using the proximity ligation assay (PLA) which detects proteins that are within less than 40nm. Since a working Dlg5 antibody is not available, we plan to use an anti-GFP antibody to recognize GFP tagged Dlg5. It is possible that Scrib and Dlg5 do not directly
interact and that Scrib regulates the interaction of Dlg5 and a yet unidentified cargo. Scrib is known to localize Crumbs via retromer recycling to the apical membrane in epithelial cells. However, Crumb is not expressed in the larval peripheral nerve (Chapter 3, data not shown) and must not interact with Dlg5. To identify the cargo that interacts with Dlg5 would require co-immunoprecipitation of Dlg5 combined with proteomic mass spectrometry analysis. To determine if Dlg5 is required for recycling of the identified cargo, we could look for the presence of the cargo in LAMP1 labeled lysosomes instead of the SPG membrane. Recycling of many proteins back to the cell surface occurs in Rab11-positive vesicles and loss of Rab11 inhibits tethering and fusion of recycling vesicles of the membrane (Takahashi et al., 2012). If Dlg5 is required for recycling of the identified cargo then blocking Rab11 mediated recycling should phenocopy the loss of Dlg5. Since cadherins might also be recycled via Rab11 it is possible that loss of Rab11 might inhibit cadherin recycling to the membrane. The potential involvement of Dlg5 in cadherin trafficking is discussed in section 5.3.3.

5.3 Cadherins in the peripheral nerve

Chapter 4 represents our efforts at identifying cadherin-catenin complexes and describing their function in the peripheral glia. Previous studies have focused on the role of adherens junctions in epithelial cells. Recently SAJs were identified between the peripheral glia using TEM (Matzat et al., 2015), however nothing is known about their composition and function. To determine if Dlg5 was required for the localization of cadherin in Chapter 3, required that we first define the cadherin/complex in the peripheral nerve. Our study is first to show that DE-Cad and DN-Cad are expressed in overlapping patterns in the peripheral nerve along with catenins. Moreover, we describe a role for DE-Cad in WG and SPG and show that loss of DE-Cad leads to
deleterious glial defect. Here I discuss certain aspects of this study that were not fully addressed and how we plan to address them in the future: 1) Determining whether the formation of ZA-like belts is developmentally regulated; 2) Redundancy between DE-Cad and DN-CAD in the peripheral nerve; 3) Understanding why knockdown of DE-Cad results in glial defects; 4) More extensive analysis of catenin function in the peripheral nerve.

5.3.1 Determining whether the formation of ZA-like belts is developmentally regulated

In Chapter 4, we show that DE-Cad and DN-Cad puncta were prominently expressed in the peripheral nerve and occasionally form ZA-belt like structures. The frequency with which these ZA-like belts were observed was variable and these structures were never observed in all the nerves. The presence of two distinct populations of cadherins might signify differences in their functions, where the diffusely distributed cadherin puncta might allow formation of transient contacts while the ZA-like belts might occur at more stable anchor sites. Alternatively, we think that the presence of cadherin belts vs puncta might represent differences in maturation of the AJ. The hypothesis being that the cadherin puncta represent the early SAJ structure whereas maturation of the SAJ gives rise to the ZA-like belts. We plan to test this hypothesis in the future by quantifying cadherin puncta and ZA-like belts in 2nd instar larvae and comparing it to wandering 3rd instar larvae. We expect to observe more ZA-like belts in wandering 3rd instar larvae compared to 2nd instar larvae. It is also possible that the extent of adhesion might be based on the level of larval locomotion/activity. Increased locomotion in wandering 3rd instar larvae might require increased glia-glia adhesion and interactions with the actin cytoskeleton to support tissue integrity.
5.3.2 Redundancy between DE-Cad and DN-CAD in the peripheral nerve

One of the major limitations in the study described in Chapter 4 is the possible redundancy between DE-Cad and DN-Cad in the peripheral nerve. The partial overlap observed between DE-Cad and DN-Cad in the peripheral nerve further suggests that both cadherins might contribute to the formation of SAJ complexes. Several RNAi lines were used to knockdown DN-Cad in all glia; however, we never observed any effect of DN-Cad knockdown on glial morphology. A previous study in glial cells of the adult Drosophila wing also showed that altering the level of DN-Cad in glial cells does not affect the formation and number of glia-glia SAJs (Kumar et al., 2015). We therefore, think that the loss of DN-Cad in DN-Cad knockdown nerves is compensated by DE-Cad function resulting in normal glial morphology. Moreover, SAJs were present in TEM images of cross-sections of nerve lacking DE-Cad in all glia, suggesting that DN-Cad and DE-Cad have interchangeable functions. However, knocking down DE-Cad in all glia unlike DN-Cad results in glial phenotypes. It is possible that DN-Cad does not fully compensate for the loss of DE-Cad as the morphology of glial cells in the absence of DE-Cad is disrupted. However, the presence of DN-Cad in DE-Cad knockdown nerves is sufficient to maintain the SAJs between glial cells. To determine if DE-Cad and DN-Cad have redundant functions, we plan to overexpress DN-Cad in all glia to test if the glial phenotypes observed in DE-Cad knockdown nerves can be rescued. Moreover, to fully assess the function of cadherins in the peripheral nerve, we plan to use a DN-Cad mutant that deletes both DN-Cad and DN-Cad2 (N-CadΔ14) while knocking down DE-Cad in all glia. Since N-CadΔ14 is homozygous lethal we plan to knockdown DE-Cad in glial cells that are heterozygous for DN-CadΔ14. We hypothesize that reducing the levels of all three cadherins should exacerbate the glial phenotypes observed in the DE-Cad knockdown nerves. As both DN-Cad and DE-Cad interact with beta-catenin/Arm to
stabilize adherens junctions, downregulating Arm should affect both DN-Cad and DE-Cad based cadherin complexes.

5.3.3 **Understanding why knockdown of DE-Cad results in glial defects**

In Chapter 4 we show that DE-Cad knockdown in glia cells has deleterious effects on glial morphology, including glial swelling, presence of vacuole-like structure and lack of axonal ensheathment. We were able to further address the role of DE-Cad in the WG and SPG by performing knockdowns of DE-Cad in those glia layers. Moreover, we showed that the lack of axonal ensheathment was due to loss of DE-Cad in the WG whereas, mild glial swellings were a result of DE-Cad knockdown in the SPG. The occurrence of discontinuous WG strands in DE-Cad knockdown nerves suggests that DE-Cad might play a role in WG-WG adhesion. Surprisingly, WG-WG SAJs were observed in DE-Cad knockdown nerves, suggesting that adhesion between WG strands occurs even in the absence of DE-Cad, possibly via DN-Cad. It is possible that the number of SAJs might have decreased in DE-Cad knockdown nerves. To quantify whether the number of SAJs differ between WG in control and DE-Cad knockdown larvae would require sectioning the entire nerve and reconstructing it. However, our observations as well as those made by a study in glial AJs in the adult wing (Kumar et al., 2015) suggest that we might not see any differences in the number of SAJs due to functional redundancy between DE-Cad and DN-Cad. Furthermore, we did not observe changes in gross morphology of the WG and must consider that while sectioning the peripheral nerves to analyse the WG phenotype using TEM, we might have missed the section where the WG was discontinuous or particularly disrupted. It is possible that we might have captured an intermediate WG phenotype which arise due to loss of transient contacts between the WG rather than the loss of stable SAJs structures. If
this is true then, we should see an exacerbation of the WG defects when DE-Cad is knocked down in larvae that are heterozygous for NCad^{14}. Apart from the WG phenotype, we also observed discontinuities in the SPG membrane, accompanied by a loss of Dlg1 and swellings in the affected region of the nerve. Interestingly, similar phenotypes were observed in Chapter 3 where Dlg5 was knockdown and suggests that Dlg5 and DE-Cad might interact. Therefore, DE-Cad will be considered as one of the candidate cargos that Dlg5 might help deliver to the membrane. Especially, given that DE-Cad knockdown does not lead to the loss of SAJ structures we must confirm whether Dlg5 affects E-Cad localization or not. Moreover, we found that similar to Dlg5, DE-Cad overlaps with Vps26. However, it is possible that they overlap in different endosomal compartments as Dlg5 overlapped with Vps26 in the outer circumference of the nerve whereas overlap between DE-Cad and Vps26 was observed within the core of the nerve. Nevertheless, we plan to use live imaging and photoconvertible EGFP tagged DE-Cad to test whether recycling of DE-Cad is blocked in Dlg5 knockdown nerves. Both DE-Cad and DN-Cad were found in very few Rab11-positive vesicles and many studies have shown that cadherins are recycled in Rab11-positive vesicles in other cells (Lock and Stow, 2005, Cadwell et al., 2016, Le et al., 1999). To confirm our results and determine the pathway through which cadherins are recycled to the membrane, we can knockdown different components in each pathway and look for accumulation of DE-Cad and DN-Cad at different stages of trafficking. For example, if DE-Cad is recycled via the retromer complex, knocking down a component of this complex (Vps26, Vps29 or Vps35) should result in DE-Cad accumulation either in the early endosome or it might get sent to the lysosomes for degradation. Moreover, we can express dominant negative-Rab11 (Rab11 DN) and assay for DE-Cad distribution and glial morphology. Interestingly, Dlg1 and Scrib both normally localize to the SJ domain in the peripheral and their
expression is lost in DE-Cad and Dlg5 knockdown nerves, respectively. Similar to Scrib, loss of Dlg1 from the SPG in DE-Cad knockdown nerves could either be due to SPG defects or a loss of Dlg1 trafficking to the membrane. We plan to test whether NrxIV and other proteins that localize to the SJ domain, including Scrib are affected by the knockdown of DE-Cad. Furthermore, to test if DE-Cad indirectly affects the formation of the SJ complex, the integrity of the BBB, will be tested using a dye assay. Apart from the WG and SPG phenotypes, pan-glial knockdown of DE-Cad also resulted in swellings of the outer glial membrane, possibly the PG as well as vacuole like structures. In the future, we plan to knockdown DE-Cad in the PG and assay for changes in the PG as well as the SPG layer. SAJs are observed between SPG and PG as well as between SPG and WG in TEM images of peripheral nerve cross-sections (Matzat et al., 2015). It will be interesting to see if loss of DE-Cad is one glial layer affects the morphology of its neighbouring glial layer. Moreover, the vacuoles-like structures observed in nerves where DE-Cad was knocked down in all glial cells, have not been characterized. One of the limitations of looking for vacuoles in TEM images is that it is difficult to distinguish between vacuoles and artefacts associated with the processing of the samples. Nevertheless, at the ultrastructural level, we did not notice any differences in the presence or absence of vacuole-like structures between controls and DE-Cad knockdown nerves.

5.3.4 More extensive analysis of catenin function in the peripheral nerve

Our analysis of catenin function in Chapter 4 was limited to use of RNAi-mediated knockdown. Although it possible that alpha-catenin and p120-catenin are dispensable in the peripheral nerve, further experiments need to be conducted to confirm these results. A limited number of RNAi lines were available for the knockdown of alpha-catenin and p120-catenin in
this study. To confirm our knockdown experiments, other RNAi lines targeting alpha-catenin and p120-catenin need to be tested. Moreover, a previous study showed that overexpression of alpha-catenin and p120-catenin leads to partial lethality and segmentation defects in embryos. Overexpression of these catenins affects the localization and activity of Rho1, a protein that is required for the localization of DE-Cad at the AJs in developing epithelial cells (Magie et al., 2002). Whether Rho1 interacts with alpha and p120-catenins in the peripheral nerve was not determined in this study. We can therefore, overexpress alpha-catenin and p120-catenin in glial to determine if they disrupt glial morphology by indirectly affecting cadherin localization. Since p120-catenin null mutants are viable we can also assay for changes in WG morphology in the p120-catenin mutant larvae. Furthermore, the function of beta-catenin/Arm remains to be determined. Multiple RNAi lines can be used to target the knockdown down Arm in all glial cells and the morphology of different glial layers can be assessed. Arm directly binds to the cytoplasmic tail of DE-Cad and can interact with alpha-catenin to regulate changes in the actin cytoskeleton (Pai et al., 1996). Therefore, if DE-Cad mediates changes in the actin cytoskeleton in the peripheral nerve then knocking down Arm should result in similar to those seen in DE-Cad knockdown nerves. Finally, we could visualize changes in actin dynamics with live imaging using Lifeact::GFP (Riedl et al., 2008) in DE-Cad knockdown and Arm knockdown nerves, leading to a better understanding of the role of Arm in the peripheral nerve.

5.4 Conclusions

Overall this work demonstrates the importance of glia-glia communication in maintaining proper glial morphology and function. We illustrate three different ways in which this communication occurs; directly through gap junctions, through PDZ mediated signalling and
lastly via cadherin based adhesion. Most work on gap junctions is performed in cell culture, however we show that gap junctions couple two different glial cells in vivo. Our study is the first to demonstrate a role for Dlg5 in glial cells as well as to provide a description of the cadherin/catenin complex in the peripheral nerve. Ultimately this work has given rise to many big questions such as: What passes through the gap junction between the SPG and WG? Which protein(s) does Dlg5 deliver to the SPG membrane and why? Are classical cadherins required for the formation of SAJs or are they assembled by other adhesive proteins in glial cells? Moreover, the findings from this study might provide clues to how gap junctions, Dlg5 and cadherin might function in NMSCs.
SUMMARY FIGURE CHAPTER 5:

Figure 5.1. Model of Inx2, Dlg5 and E-Cad functions in the subperineurial glia and wrapping glia in the Drosophila peripheral nerve.

A: In a wild type peripheral nerve: 1) Inx2 homotypic gap junctions mediate calcium signaling in the SPG whereas Inx2/Inx1 heterotypic channels allow communication between the SPG and the
In the WG, Dlg5 mediates the ensheathment of axons via an unknown mechanism. Dlg5 is also required in the SPG, where it is necessary for sustaining the SPG and SJ morphology. DE-Cad and DN-Cad are expressed throughout the peripheral nerve and associate with catenins. Pan glial knockdown of Inx2 results in the fragmentation of the WG due to loss of SPG-WG communication via an unknown signal. Additionally, calcium pulses in the SPG are disrupted when Inx2 is knocked down in the SPG. Knockdown of Dlg5 in the WG results in less and discontinuous WG strands, however, DE-Cad and DN-Cad are still present and can form SAJs between the different glial layers. The mechanism by which Dlg5 mediates axonal ensheathment remains to be determined. In the SPG, knockdown of Dlg5 results in gaps in the SPG membrane, accompanied by the loss of SJ proteins. Whether Dlg5 interacts with Scrib or acts alone to traffic SJ proteins or unknown cargos to the SPG membrane remains to be tested. DE-Cad is required in the SPG and WG to sustain the morphology of these glial layers. Knockdown of DE-Cad in the WG results in discontinuities in the WG membrane, however, DN-Cad is still expressed in these nerves and can potentially form SAJs. Additionally, DE-Cad knockdown in the SPG results in phenotypes that were observed in nerves where Dlg5 was knocked down in the SPG suggesting that Dlg5 and DE-Cad might work in the same pathway. Alternately, the SPG phenotypes observed in both Dlg5 and DE-Cad knockdown nerves might be due to the need for Dlg5 and DE-Cad in separate pathways that both maintain SPG and SJ morphology. These hypotheses require further investigation.
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