Glia/Axon connections in the peripheral nerve of *Drosophila* melanogaster

by

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ABSTRACT

The *Drosophila* nervous system is protected by blood barriers composed of a layer of subperineurial glia, tightly bound together by septate junctions. Previous studies have shown the structure of septate junctions have high similarity to paranodal junctions in vertebrates. Paranodal junctions are formed between glia and axons in the myelinating glia that flank the Nodes of Ranvier. Although the process of myelination does not exist in *Drosophila*, there is evidence that a similar structure may be formed at the distal end of the *Drosophila* peripheral nerve. Study of the conserved subunits between septate junctions and paranodal junctions will help direct future studies on how glial cells regulate the barrier formation in the nervous system. Thus, we have focused on the degree of glia-axon contact and the formation of septate junctions between subperineurial glial cells and the axon membrane in larval and adult nerves and at the neuromuscular junction (NMJ). We demonstrate that GFP reconstitution across synaptic partners (GRASP) can be utilized to show that glial cells are in contact with axon membranes. Glia surround nerves in both adult and larva; however, the boutons of the adult NMJ are partially covered by glia, while there is not a consistent presence of glia in the larval NMJ. Septate junctions and the subperineurial glia stop at the distal end of motor axons but do not extend into either the larval or adult NMJ. We found the loss of septate junction components in axon does not lead a significant impact on larval locomotion speed and adult proboscis extension response.

LAY SUMMARY

Glial cells are important cells in the nervous system of all animals. Glia cells protect the neurons, which carry the signals throughout the body. Glia cells construct a wall against harmful environments and from pathogens such as bacteria and viruses by forming a barrier to separate the nervous system from the blood and the environment. This thesis studied how glial cells and the neurons interact in a developing animal and in the adult. When the two cells touched a new signal was generated and the sites where this signal was made investigated. This thesis also analyzed where the barriers created by the glia were found and if these barriers corresponded ot the contact points between the glia and the neurons.

PREFACE

This dissertation is original, unpublished, independent work by the author, J. Meshkani.

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LIST OF ABBREVIATIONS

PNS	peripheral nervous system
CNS	central nervous system
ECM	extracellular matrix
NRG	neuregulin
MS	Multiple Sclerosis
NrxIV	Neurexin IV
IG	immunoglobulin
NMJ	neuromuscular junction
GFP	green fluorescent protein
RFP	red fluorescent protein
Repo	reverse polarity
RNAi	ribonucleic acid interference
UAS	upstream activating sequence
VNC	ventral nerve cord
HRP	horseradish peroxidase (axon marker)
Cont	Contactin
SJ	Septate junction
PPG	Peripheral perisynaptic glia
PG	Perineurial glia
SPG	Subperineurial glia
WG	Wrapping glia
GRASP	GFP Reconstitution Across Synaptic Partners assay
BNB	Blood nerve barrier
BBB	Blood brain barrier
Cora	Coracle
AX	Axon
ALS	Amyotrophic lateral sclerosis
DLG	Disc large
MCR	Macroglobulin Complement Related
Scrib	Scribble
PFA	Paraformaldehyde
TEM	Transmission electron microscopy
um	Micron

ACKNOWLEDGEMENTS

Thank you to my supervisors, Dr. Michael Gordon and Dr. Vanessa Auld, for providing me roots to explore my curiosity within biological science. Thank you to my lab members and my committee members.

DEDICATION

To my dear wife Zohreh

and

To my family who supported me all the way

1 INTRODUCTION

While neurons are considered to be critical to nervous system function, glia also play an essential role in maintenance and support of neurons in both central and peripheral networks. To ensure glia function, precise communication is essential between neurons and glia. In the current project, we studied the role of cell-cell contacts and junction formation between neurons and glia in the peripheral nerve in *Drosophila* melanogaster in adult and larval stages.

1.1 OVERVIEW

Glia play many roles in maintaining the nervous system, including insulating the neurons and axons and creating physical barriers, which protect and maintain the microenvironment of the nervous system. Barriers are formed by glial cells at many levels, from induction or the formation of blood-brain or blood-nerve barriers or barriers to subdivide regions of axons and establishment of barriers that separate individual axons Therefore, the study of the structure and function of glial barriers can facilitate a better understanding of diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Although the molecular structure of glial barriers has been well characterized, the interaction between glia and axons in forming and maintaining the barriers is still being elucidated in both vertebrate and invertebrate. Based on previous work on glia-axonal barriers in vertebrates, the current project focused on the study of glia-axon communication and cell-cell junction formation in *Drosophila* peripheral nerves.

1.2 THE VERTEBRATE PERIPHERAL NERVOUS SYSTEM

A common feature in most nervous systems is the presence of supportive glial cells. In vertebrates, glial cells are crucial regulators of development, function and continued health of the nervous system. Several subtypes of glial cells are found in the vertebrate nervous system. Three

major types of glia have been classified in the central nervous system (CNS) of vertebrates including astrocytes, oligodendrocytes and microglia. The supporting glial cells of the peripheral nervous system (PNS) are enteric glia and Schwann cells, which included both myelinating and non-myelinating subtypes.

1.2.1 Schwann cells of the vertebrate peripheral nerve

In vertebrates, peripheral axons are found in both myelinated and unmyelinated fibers within the center or core of the peripheral nerve. Schwann cells are the glial of the peripheral nervous system and fall into two subtypes: non-myelinating and myelinating Schwann cells. Axons that are myelinated have a large caliber, while small caliber axons are wrapped by non-myelinating Schwann cells (Jessen and Mirsky, 2005). The non-myelinating Schwann cells ensheath bundles of axons such as C-fibers and form Remak fibers (Purves et al., 2004).

Myelinating Schwann cells wrap individual large caliber axons of both motor and sensory neurons (Verkhratsky et al., 1991). Individual Schwann cells myelinate a single axon unlike the CNS oligodendrocytes, which myelinate multiple axons. The myelin sheath is composed of multiple Schwann cell membranes that combine to create an insulating layer of lipid membrane around the motor neuron axons. The concentric layers of myelin allow action potentials to travel along the fibers at a rapid rate, due to the insulation created by the myelin sheath. Loss of the myelin sheath results in a block or reduction action potential propagation, which causes muscle weakness and paralysis (Burns, 2008).

1.2.2 Nodes of Ranvier and the paranodal junction

In the vertebrate PNS, action potentials are rapidly transmitted between each myelinated segment and are regenerated at the nodes of Ranvier (Fig 1.1) (Burns, 2008). Nodes of Ranvier are characterized by the accumulation of voltage-gated Na^+ channels in an axon domain flanked

by the myelin loops created by the Schwann cell. The myelin loops attach to the axonal membrane in the flanking paranode domains on each side of a node of Ranvier (Thaxton et al., 2010). This paranodal region has a specific junction called the paranodal junction, which functions to prevent the lateral movement of voltage-gated Na^+ channels away from the node into myelinated regions. When paranodal junctions are disrupted, Na^+ channels are distributed along the length of the axon, which leads to a reduction of action potential velocity along the nerve (Rosenbluth, 2009). The paranodal junction is also necessary to limit voltage-gated K^+ channels away from the Node and retain these channels under the myelin sheath (Arancibia-Carcamo and Attwell, 2014). Furthermore, the paranodal junction and the myelin sheath determine the shape of the node of Ranvier by sealing the myelin loop to the axons (Denisenko-Nehrbass et al., 2002). Paranodal junctions are characterized by a linear array of electron dense septa that extend around the circumference of the axon (Banerjee et al., 2006b). While it is clear that paranodal junctions act a barrier to prevent the lateral movement of ion channels within the axon membrane, it is less clear that these junctions act as a permeability barrier (Shroff et al., 2011; Nualart-Marti et al., 2013). As will be outlined below, vertebrate paranodal junctions are highly conserved with septate junctions found in invertebrate glia, which have been well characterized in *Drosophila*. Thus, the genetic capabilities of *Drosophila* can be used to understand the function and molecular mechanisms that underlie the paranodal junction formation.

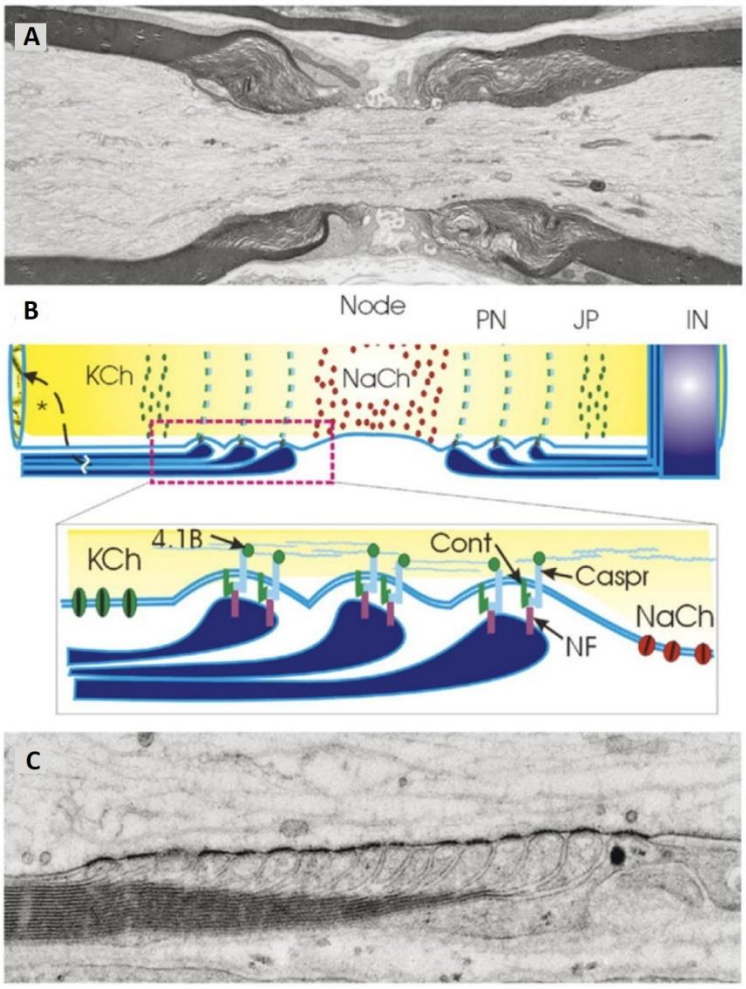

Figure 1.1 Electron micrograph of an orthogonal section from spinal dorsal root nerve of rat.
(A) An electron microscopic image of a node of Ranvier, the unmyelinated domain flanked by a series of myelin loops. (B) In the node of Ranvier, three axonal domains are defined including the node domain containing the voltage-gated Na^+ channels, the paranode (PN) region, the juxtaparanodal (JP) domain containing voltage-gated K^+ channels and the myelinated internode (IN). Cont = contactin; NF = neurofascin 155; Caspr= contactin-associated protein; 4.1B protein; KCh = K^+channel; NaCh = Na^+ channel. The myelinated loops are attached to the axon via the intercellular heterotrimer complex of paranodal junction contains Cont, Caspr and NF, and 4.1B protein localized inside of the cell membrane. (C) The dense paranodal junctions between each paranodal loop and axon (Fields, 2008).

1.3 THE *DROSOPHILA* PERIPHERAL NERVOUS SYSTEM

Drosophila glia also have a wide range of functions in the nervous system; glial cells developmentally and functionally contribute to the completion of neuronal connectivity, form the

blood-brain and blood-nerve barriers and control synaptic function, all characteristics conserved with vertebrate glia (Granderath and Klambt, 1999; Parker and Auld, 2006). The entire length of each peripheral nerve is encased in a number of glial cells from the CNS (ventral nerve cord) and along the nerve trunk to the neuromuscular junctions (NMJs). Four subtypes of glia have been identified in *Drosophila* peripheral nerves, including wrapping glia (WG), subperineurial glia (SPG), perineurial glia (PG) and peripheral perisynaptic glia (PPG), though this last class is only found in adults. The WG are found within the core and wrap the axons, the SPG form the next layer and are the site of the blood-nerve barrier and the PG form the outer layer closely associated with the overlaying neural lamella (Fig 1.2) (Stork et al., 2008; Xie and Auld, 2011). As the motor axons branch away from the main nerve, the distribution of each glial subtype changes as the axon terminates at the NMJ, particularly in the adult. The distribution and association of these glial subtypes within each motor axon branch is not well established. It clear SPG and PG appear to ensheath each motor axon up to the axon terminus at the NMJs (Fuentes-Medel et al., 2009; Brink et al., 2012). While in the adult the distribution of the SPG, the PG and the PPG, which is contained within the NMJ proper (Strauss et al., 2015), has not been well established.

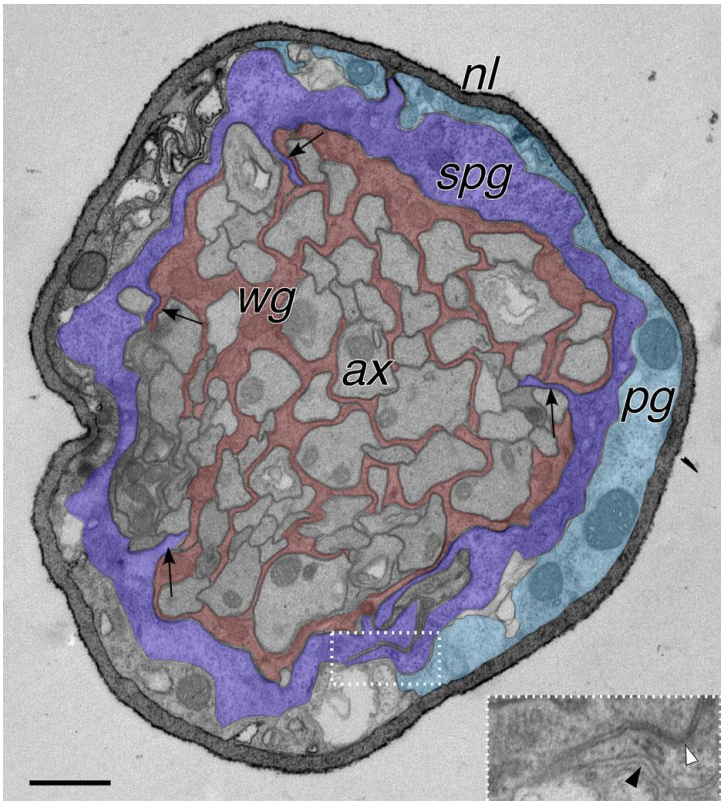

Figure 1.2 Transverse section of *Drosophila* peripheral nerve.
Electron microscopic image provided from the peripheral nerve of a 3rd instar larva. Three types of glia are positioned in the profile of *Drosophila* peripheral nerve. The neural lamella (nl) surrounds glia layers, perineurial glial cells (pg) under the lamella establish the outermost layer, subperineurial glial cells (spg) form medial layer, and the innermost layer is formed by wrapping glial cells (wg) which directly covers the axons (ax). One SPG encircles the fascicle, while forms a short process toward the profile of axon (arrows). SJs are formed in SPG (boxed area and magnification; SJs and SJs free cell-cell contact pointed by white and black arrowheads respectively). Scale bar 1um (Stork et al., 2008).

1.3.1 Wrapping glia

Wrapping glia (WG) form the inner layer of the peripheral nerve and directly wrap both motor and sensory axons (Fig 1.2) (Stork et al., 2008). WG do not replicate or divide to match the expansion in nerve length as an animal increase in size from embryonic to the larval stage. Rather the WG and the SPG described below undergo hypertrophy (von Hilchen et al., 2013; Matzat et al., 2015). WG develop later starting at the beginning of larval stages, with the extensive wrapping of peripheral axons only being complete in the 3rd instar larval stages

(Matzat et al., 2015). The process of WG extension around axons is regulated by an autonomous regulatory mechanism via secretion of Vein, a ligand for the EGF receptor (Matzat et al., 2015). Vein is a homolog of the vertebrate neuregulin, which in the vertebrate PNS is required for differentiation of Schwann cells, and plays a role in neuronal development (Vartanian et al., 1999). WG cell membranes surround the axon profile, thus they bear similarity to the non-myelinating Schwann cells (Nave and Salzer, 2006; Nave and Trapp, 2008). However, WG do not extend beyond the main nerve and thus do not wrap the motor axons as they branch off the nerve towards the NMJs (Brink et al., 2012). This leads to the question whether the next glial layer, formed by the SPG, takes on the wrapping role for the motor axon prior to the NMJs.

1.3.2 Subperineurial glia

Subperineurial glia (SPG) are large protective cells that entirely cover the surface of the CNS and ensheath each peripheral nerve. The SPG in insects form the blood-brain barrier in the CNS and the blood-nerve barrier in the PNS (Banerjee et al., 2006b) and as such are formed early in nervous system development within the embryo. In the peripheral nerve the SPG form a thin glia layer positioned in the middle of the three glia layers (Fig 1.2) (Stork et al., 2008). The blood permeability barrier is created by septate junctions formed within each SPG wrap and between neighboring SPG (Banerjee et al., 2006b). A defective the blood barrier leads to paralysis due to exposure of the underlying neural tissue to the high K^+ hemolymph (Deligiannaki et al., 2015). As noted above, the SPG do not divide to match the increasing length of the PNS during the larval stages, instead these cells increase cell size (von Hilchen et al., 2013). This means that each peripheral nerve is encased by two to three SPG, which extend for hundreds of microns to encase the nerve. Each SPG extends a process to cover the motor axons as they leave the main peripheral nerve and the SPG appear to terminate at the axon terminus

proximal to the NMJs. However, the distribution of the septate junction domain and the SPG membrane has not been clearly determined in either larval or adult motor axons.

1.3.3 Perineurial glia

Perineurial glial (PG) form the outermost layer of glia in peripheral nerves and contact the overlaying neural lamella (Fig 1.2) (Stork et al., 2008). PG replicate substantially during larval stages and by the 3rd instar larval stage hundreds of PG have completely covered the ventral nerve cord and peripheral nerves (Awasaki et al., 2008; Stork et al., 2008; von Hilchen et al., 2013). PG do extend beyond the main nerve to cover each motor axon as they branch off towards the NMJs (Brink et al., 2012). However, the role of this class of glia is not well established, though PG may contribute to regulation of the neural lamella by secretion of matrix metalloproteinases (Meyer et al., 2014).

1.3.4 Peripheral presynaptic glia

Peripheral perisynaptic glia (PPG) are the fourth type of peripheral glia and only recently identified (Strauss et al., 2015). PPG are distributed along the terminus of each motor axon and extended into the NMJs. PPG participate in forming tripartite synapses in the adult NMJs which are important in motor function. The tripartite synapses are formed in the adult NMJs but the presence of PPG is not detected in larval stages (Strauss et al., 2015). The relationship of PPG and the other glia that ensheath the most distal part of the motor axon has not been well characterized and whether PPG express septate junction proteins and create a blood-nerve barrier at the NMJ is also not known.

1.3.5 Neural lamella

Around each peripheral nerve is a layer of extracellular matrix (ECM) called the neural lamella (NL). The ECM components identified around the peripheral nerve include laminin,

collagen, and perlecan (Stork et al., 2008; Xie and Auld, 2011; Petley-Ragan et al., 2016). These extracellular matrix components are formed around the perineurial glia as a continuous dense network (Fig 1.2) (Stork et al., 2008). The components found in the NL are secreted by glia, hemocytes and fat body cells (Olofsson and Page, 2005; Pastor-Pareja and Xu, 2011; Xie and Auld, 2011). The NL plays a critical role in the development of neuronal tissues and maintenance of nervous system shape (Martinek et al., 2008).

1.3.6 Comparing *Drosophila* and vertebrate peripheral nerve structure

In general, both vertebrate and invertebrate nerves are composed of similar structures. Vertebrate nerves contain multiple fascicles surrounded by a layer of perineurial cells. Each fascicle is remarkably similar to the *Drosophila* peripheral nerve, with both encased in a layer of small perineurial cells. The perineurial layers of both are encased by a dense extracellular matrix, which contains a conserved complement of proteins, including collagen, elastin, proteoglycans, perlecan and laminin (Stork et al., 2008; Xie and Auld, 2011; Petley-Ragan et al., 2016). Within each vertebrate fascicle and the *Drosophila* peripheral nerve, the axons are ensheathed by wrapping glia. While *Drosophila* does not have myelinated axons, the wrapping glia are similar the non-myelinating Schwann cells. In both glia types, axons are encased by a single glial wrap and action potentials are propagated uniformly along the length of the axon. While there is not direct cellular homolog to the *Drosophila* SPG, the main component of the SPG, the septate junction is highly conserved with the paranodal junction found in the vertebrate peripheral glia at the nodes of Ranvier. The structure of the peripheral nerves and the presence of the paranodal junction raises the question as to where the permeability barriers are created and if these barriers are conserved.

1.4 NERVE BARRIERS

The barriers that separate the brain or nerves from the circulating hemolymph or blood are key to animal viability in both invertebrates and vertebrates. Neuronal tissues need barriers to be protected against variations in solutes found in blood, and pathogens. In vertebrates, the existence of the brain barrier was confirmed when a dye injected into the body failed to enter the brain (Ehrlich, 1885). In 1900, the selective physiological blood barrier was described as a wall between the circulation and the cerebro-spinal fluid (CSF). Blood barriers limit the flux of hydrophilic molecules in the nervous system, while lipophilic molecules and gas molecules such as O2 and CO2 can diffuse through the barrier (Grieb et al., 1985). The blood barrier acts as a checkpoint against immune cells and pathogens (Coisne and Engelhardt, 2011; Greenwood et al., 2011). Active transporter and endocytotic systems mediate uptake of nutrients such as amino acids and glucose, and large molecules such as insulin and iron transferrin respectively (Pardridge et al., 1985; Zhang and Pardridge, 2001). Blood barriers also keep the nervous system separate from plasma neurotransmitters in plasma and insulates the nervous system neurotransmitters pool (Abbott et al., 2006; Bernacki et al., 2008). In vertebrates, plasma contains many macromolecules such as albumin, prothrombin and plasminogen that are excluded from the nervous system to prevent damage and apoptosis (Gingrich et al., 2000; Gingrich and Traynelis, 2000). The blood barrier in the CNS and PNS are called the blood brain barrier (BBB) and the blood nerve barrier (BNB), respectively.

1.4.1 Blood nerve barrier in vertebrate

As outlined above, each fascicle of the vertebrate peripheral nerve is encased in a layer of perinerial cells that create the perineurium. The perineurium barrier is covered by a basement membrane (Carvey et al., 2009), similar to the *Drosophila* neural lamella. Electron microscopic

analysis revealed the space between perineurium cells is filled by junctional complexes of tight junctions (TJs) strands, which form the blood-nerve barrier (Reale, 1975). TJs prevent the diffusion of solutes across the perineurium barrier which protects the microenvironment in the interior of each fascicle (Hawkins and Davis, 2005). Injury of peripheral nerves causes a decrease in TJs, which is thought to lead to increased permeability of the BNB to fluorescent dyes (Echeverry et al., 2011). Lack of Schwann cell–derived Desert hedgehog signals causes disruption of the perineurium barrier via reduction of TJs, which in turn leads to penetration of Evans blue albumin into nerve fascicles (Parmantier et al., 1999). Therefore, the site of the vertebrate BNB within the TJs of the perineurial cells resides within a different cell type than the *Drosophila* BNB, which is created by the septate junction within the SPG.

1.4.2 Paranodal junctions

Another potential blood barrier within the vertebrate peripheral nerve resides within the paranodal junction. The paranodal junction is found on each side of a node of Ranvier within the loops of Schwann cells at the edge of the myelin sheath. These loops are attached to the axon by paranodal junctions, which appear as an array of transverse bands with a ladder-like array when view using electron microscopy (Rosenbluth et al., 1995). Whether the paranodal junction can also serve as a solute and pathogen barrier similar the blood-brain barrier is a point of discussion. It has been suggested that the transverse bands of paranodal junctions form a barrier, which protects the myelinated domain of the axon from the extracellular environment. However, the paranodal barrier is far from tight, allowing a slow diffusion from the extracellular space into the intermodal myelinated region of the axon (Hirano et al., 1969; Rosenbluth et al., 2006). Dye penetration assays have indicated movement of 3kD and 70kD dextran tracers through the

paranodal junction by passive diffusion. Thus, water soluble material as large as ~12–16 nm in diameter can penetrate myelinated domain via the paranodal pathway (Mierzwa et al., 2010).

However, paranodal junctions may form other types of barriers, including barriers to the movement of membrane proteins. The loss of the paranodal junction leads to the spread of the sodium channels away from the node along the axon and the mislocalization of the voltage-gated K^+ channels away from the paranode and juxtaparanode domains. Loss of paranodal junctions leads to axon dysmyelinating and leads to progressive neurological impairment (Rosenbluth et al., 2008). Thus, paranodal junctions act as an adhesion junction required for maintaining myelin-axon contact and this junction also form a physical barrier to limit the spread of voltage-gated ion channels, thus ultimately maintaining the integrity of the node of Ranvier (Lyons and Talbot, 2008).

1.4.2.1 Paranodal junction components

Many of the molecular components of the paranodal junctions are well established and at the core of the paranodal junction is a complex of three proteins Caspr/Paranodin, Contactin (Bennett et al., 2003) and Neurofascin 155 (NF-155) (Faivre-Sarrailh and Devaux, 2013). In this complex of Caspr and Cont are expressed in the axon and these bind to NF-155 which is expressed in the Schwann cells in the myelin loops (Faivre-Sarrailh and Devaux, 2013). NF-155 is an IG domain transmembrane protein expressed in myelinating Schwann cells at the paranode (Thaxton et al., 2010). NF-155 binds to an IG domain GPI linked protein, Cont. Cont is in a complex with the transmembrane protein Caspr, a member of the Neurexin family of proteins with LamG domains. In addition, a range of intracellular scaffolding proteins has been identified at the paranode including: 4.1 Protein (Buttermore et al., 2011), hDlg/Sap97 (Gregorc et al., 2005) and hScrib1 (Bilder et al., 2000). Casper, in particular, is known to recruit protein 4.1B

(Poliak et al., 2001; Gollan et al., 2002). However, how other intracellular scaffolding proteins are recruited to the paranode and the identity of other paranodal proteins is not known. Caspr is required for localization of Cont, and proper formation of paranodal junctions (Bhat et al., 2001). Lack of either Casper or Cont in null mutants, leads to the disappearance of the paranodal junctions and loss of contact between the myelin loops and the axon (Bhat et al., 2001; Boyle et al., 2001).

1.4.3 *Drosophila* blood nerve barrier

While the permeability function of the vertebrate paranode is unknown, it is very clear that the *Drosophila* homolog, the septate junction, is the site of the BBB and BNB. The *Drosophila* BNB is a highly selective permeability barrier and is formed in the SPG. The main goal of the SPG in both the CNS and PNS is to protect the underlying nervous system from the high potassium hemolymph, which bathes the entire body cavity (Silies et al., 2007; Stork et al., 2008). The SPG establish the permeability barrier through septate junctions (SJs) (Auld et al., 1995; Baumgartner et al., 1995; Stork et al., 2008). SJs are found in a wide range of other epithelial derived tissues, where they are found in the apical portion of the lateral membrane immediately below the adherens junctions (Hortsch and Margolis, 2003). SJs appear as a ladder-like structure under electron microscopy due to a series of intracellular septa or strands which are located between neighboring cells (Fig 1.3) (Fristrom, 1982; Tepass et al., 2001; Banerjee et al., 2006b). By the last stage of embryogenesis (embryonic stage 17), the SPG surround the entire CNS and the peripheral nerves, and SJs are completed form the BNB (Banerjee et al., 2006b). As the composition and morphology of *Drosophila* SJs are conserved with vertebrate paranodal junctions, the study of SJs in *Drosophila* is directly relevant to our understanding of the formation and function of paranodal junctions.

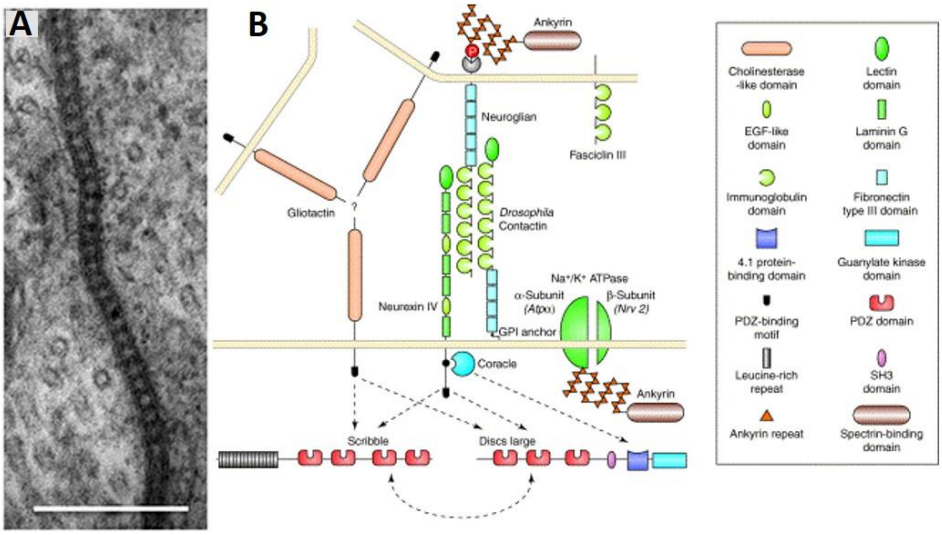

Figure 1.3 Schematic of the protein complex involved in *Drosophila* SJs establishment.
(A) The electron microscopic image of the septate junction between two SPG cells (Modified from Limmer et al., 2014). (B) The SJs components are provided by two adjacent cell membranes, which act as a partner for each other. The adhesion junction is formed by a series of proteins including Fasciclin III, Neurexin IV, Coracle and the two Ig-domain cell-adhesion molecules (CAMs) Neuroglian (NRG) and Dcontactin. Gliotactin, Scribble and Disc Large are other proteins that interfere in this structure. The Na^+/K^+ ATPase is an essential component in the organization of SJs which anchored to the plasma membrane by ankyrins (Modified from Hortsch and Margolis, 2003).

1.4.3.1 Septate junction components

A set of proteins have been identified which are involved in forming *Drosophila* septate junctions in both glia and other epithelial tissues (Fig 1.3) (Hortsch and Margolis, 2003). The intercellular strands of SJs are composed of *Drosophila* Contactin (DCont), Neurexin IV (NrxIV) and Neuroglian (Nrg), which are conserved with the paranodal junction complex of cont, Caspr and NF-155 respectively. Neurexin IV (NrxIV) was the first member of the Neurexin family isolated in non-mammalian species. The NrxIV gene encodes two isoforms that are generated by differential splicing: the Nrx-IV^{exon3} isoform is expressed in septate junction-forming tissues such as glia; and the Nrx-IV^{exon4} isoform is expressed by neurons (Rodrigues et al., 2012). In the PNS, the Nrx-IV^{exon3} isoform is colocalized with Coracle (Cora) in the septate

junction domain, and forms a cis complex with Cont. NrxIV is the homolog of Caspr, which is a core component of the paranodal junction. *Drosophila* Cora belongs to the cytoplasmic Protein 4.1 genes superfamily and is a homolog of Band 4.1 (Fehon et al., 1997). Cora is located at the cytoplasmic face of SJs where it binds and is necessary for localization of NrxIV to the SJ (Lamb et al., 1998). The *Drosophila* homolog of vertebrate Contactin (*Drosophila* Cont), co-localizes with NrxIV in SJs. Cont, a GPI-anchored membrane associated glycoprotein, is expressed in both epithelium and glia, and plays an important role in the organization and maintenance of SJs (Faivre-Sarrailh et al., 2004). Neuroglian (Nrg) belongs to the L1-type family (Bieber et al., 1989; Hortsch, 2000), and is the homolog of the vertebrate NF-155 (Bieber et al., 1989). Nrg, which is co-localized with NrxIV and Cont, has a critical role in the formation of SJs (Genova and Fehon, 2003; Schulte et al., 2003). Other septate junction proteins include an IG domain protein Lachesin, Mcr (Macroglobulin Complement Related) and both subunits of the Na/K ATPase (α subunit: ATPα; β subunit: nervana2). The Na/K ATPase pump participates in the formation or maintenance of the diffusion barrier in a pump independent manner (Paul et al., 2003). The Na^+/ K^+ ATPase with Cora, NrxIV and Nrg constitute an interdependent complex. Loss of any one of these proteins leads to the disruption of the entire SJ suggesting that this complex is independent of the assembly and function (Lamb et al., 1998; Genova and Fehon, 2003; Paul et al., 2003; Faivre-Sarrailh et al., 2004; Llimargas et al., 2004; Moyer and Jacobs, 2008). Proper localization of components in the core of septate junction is required for the functioning of barrier (Baumgartner et al., 1996; Banerjee et al., 2006a). Lack of any one of the components of the core complex results in a loss of both intercellular septa and the permeability barriers.

Other related proteins that are found at the SJ domain are the tumor suppressor components, including scribbled (scrib) and discs-large (dlg), though the binding partners of these proteins at the SJ are not known. Dlg is required to construct SJs via localization of Cora, Fasciclin III and Nrg in epithelia (Woods et al., 1996), though the role of Dlg in peripheral glia has not been determined. Varicose, is another scaffolding protein localized at septate junctions in both epithelia and glia. This protein is involved in the assembly of the SJ, and localization of essential components such as NrxIV (Moyer and Jacobs, 2008). A range of tetraspanin proteins, including the *Drosophila* homolog of claudins is also found at the SJ domain, though the function of these proteins is not clear as the loss of these proteins does not disrupt the formation of septa (Wu et al., 2004; Nelson et al., 2010).

1.4.4 Are Septate junctions and Paranodal junctions conserved?

The protein complex core of *Drosophila* SJs morphologically shares homologous features with mammalian paranodal junctions. Therefore, vertebrate axo-glial paranodal junctions may share their evolutionary origin with invertebrate SJs (Faivre-Sarrailh et al., 2004). NrxIV and Nrg are respectively homologous for Caspr and NF-155 found in paranodal junctions. Molecular interactions between NrxIV, Cont and Nrg are likely to be involved in the organization and lateral positioning of the junctional strands, similar to that observed at the vertebrate paranodal junctions. However, one large different remains in that the paranodal junction is an axo-glial junction while the SJ is a glia-glia junction formed between SPG. The question arises is there an axo-glial junction in *Drosophila* that is mediated by components of the SJ complex. One line of evidence to suggest this might be the case is the observation of septa formed between the glial wrap at the motor axon terminus prior to the NMJ (Fig 1.5) (Keller et al., 2011). In this region SPG processes are known to extend along the axon and stop prior the NMJ synapses (Fuentes-

Medel et al., 2009; Brink et al., 2012). The SJ domain labeled with NrxIV endogenously tagged with GFP also extends into the axon terminus and there appears to be a BNB created in this region (Fig 1.4) (Brink et al., 2012). Prior studies indicated SJs form between axons and glial cells (Banerjee and Bhat, 2008). However, whether NrxIV is involved with other SJ components in creating an axo-glial junction at this point is unknown in either larvae or adults. It is also unknown the extent of SPG to axon contact near the NMJ. It is the aim of this thesis to address these questions.

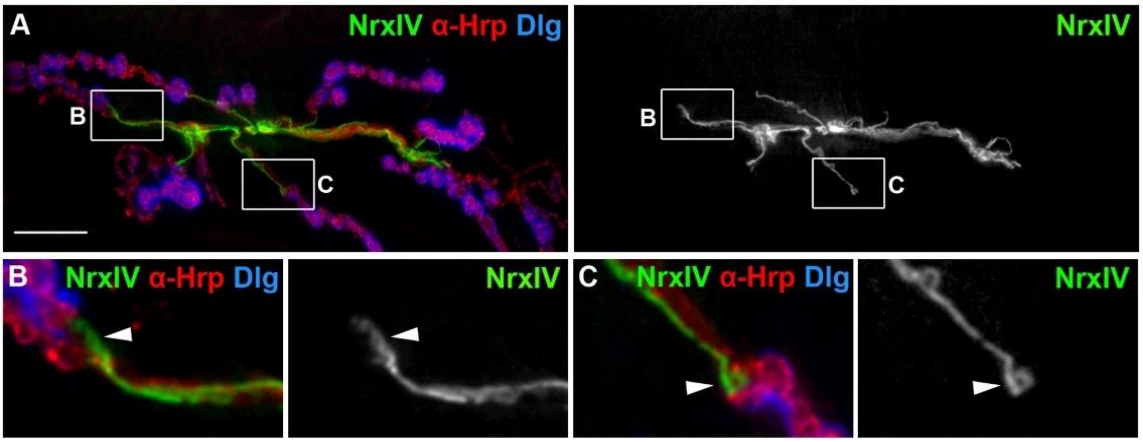

Figure 1.4 Septate junction strands formed between the glial wrap at the motor axon terminus.
A fixed NMJ from a W3 larva, the septate junctions were labeled by using NrxIV-GFP (green), the axons with anti-HRP and the post-synaptic SSR with anti-Dlg (blue). The corresponding grayscale panel shows the NrxIV::GFP distribution. (B-C) Magnified regions boxed in panel A were digitally scaled 400% with corresponding grayscale panel shows the NrxIV-GFP distribution. The presence of NrxIV terminates prior to branching NMJ, and form blunt or tapered ends (B; arrowhead) or bulb-like structure (C; arrowhead). Scale bar is 15 µm (Modified from Brink et al., 2012).

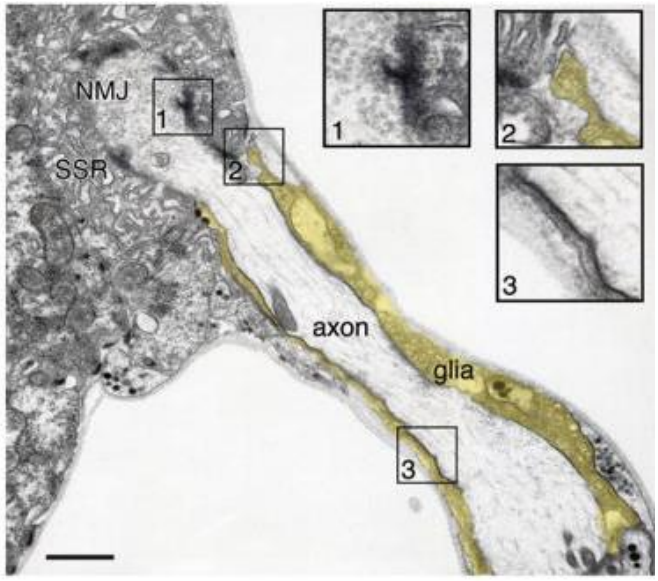

Figure 1.5 Glia reside in close proximity to motoneurons.
Electron microscopic image of a *Drosophila* 3rd motoneuron axon extended towards muscle 12. Glia (pseudo-colored yellow) ensheath the axon, and the postsynaptic SSR surround the terminal NMJ bouton. The magnified boxed regions (1-3) indicated an active zone with an accumulation of synaptic vesicles, the connection between glia and muscle, and septate junctions between glia and neurons, respectively. Scale bar, 500 nm (Modified from Keller et al., 2011).

1.5 THESIS QUESTIONS

We assumed the glia layers must be in direct contact with their associated axons. We hypothesized SJs are established between the SPG and axon membrane at the distal end of an axon to form a proper BNB. Although identification of SJs between glia and axons was not possible in our experiments, the distal region of the nerve provides an opportunity for studying the extent of glial-axon contact and the distribution of SJs in these regions. We thus aimed to examine whether the SPG and NrxIV form a paranodal-like structure at the motor axon terminus.

In this project, we designed experiments to ask three questions: (1) do glia layers directly contact peripheral axons? (2) What is the pattern of NrxIV expression along the peripheral nerve

and at the NMJ? (3) Does disruption of SJs by knockdown of SJ components result in abnormal behavior?

2 GLIA/AXON CONTACT AND SJ LOCALIZATION ALONG THE *DROSOPHILA* PERIPHERAL NERVE

2.1 Overview

The SPG ensheath the peripheral nerve and extend processes along each motor axon as they extend towards the NMJs. It is unknown whether the SPG processes are attached to the axon at the distal end of the motor axon before the transition to boutons and synapses at the NMJ. It is also unknown if the SPG create a sealed end through the formation of a septate junction between the axon and the glial membrane similar to the paranodal junction at the nodes of Ranvier. Our goal was to determine the correlation between SJs, axon-glia contact and the presence of the SPG processes at the distal end of motor axons in the peripheral nerves of adults and larvae.

2.2 INTRODUCTION

In the BNB, the SJ components are provided by two cell membranes that partner with each other. Thus, imaging of the core components such as Cont, Nrg or NrxIV can provide a way to determine the localization of SJs. As an aim in the current project, we examined the degree of glial-axon contact at the axon terminus and examined if the SJ domain is formed at the axon terminus between axon and glial membranes.

We followed the distribution of NrxIV endogenously tagged with GFP (NrxIV::GFP) as a marker of SJs in the *Drosophila* PNS. The Neurexin family are a family of structural proteins, first categorized as the synaptic receptor of the black widow spider venom (Sudhof, 2008). The Neurexin proteins contain a large extracellular domain, which is involved in forming cell-cell contacts via protein-protein connections. The Neurexin family is divided into two groups: Neurexin 1-3, which are found at synapses in the CNS; and Caspr/Paranodin (homologous of

NrxIV in *Drosophila*), which are found in paranodal and septate junctions respectively (Rios et al., 2003). In vertebrates, Caspr is expressed in axons with Cont, which together form a complex with NF-155 expressed in the glia to establish the core of the paranodal complex. In *Drosophila*, a homologous complex is formed by NrxIV, Cont and Nrg. While the formation of paranodal complex has been detected between cells of the same type – i.e. glia to glia and epithelia to epithelia – it is unknown whether this complex appears between axons and glia. Both NrxIV and Nrg are expressed in axons as well as glia (Snow et al., 1989; Baumgartner et al., 1996), suggesting that the formation of an axon-glia junction is possible. In addition, analysis of the distal end of the motor axon just prior to the NMJs and muscle surface has indicated the presence of septate-like structures between the axon and glia in the 3rd larva (Fig 1.4, Fig 1.5) (Keller et al., 2011; Brink et al., 2012; Till Matzat; personal communication 2017). The degree of axon/glia contact and the distribution of SJ protein complexes has not been determined in the adult NMJs and the transition from SPG to the peripheral perisynaptic glia (PPG) is not known. Therefore, it is possible a SJ does form between the axons and glia at the distal end of each motor axon. As the first test of this hypothesis, we wanted to determine the degree of contact between glia and axon membranes in adults and larvae along with the distribution of the SJ protein with respect to the processes of the SPG in the larvae and the transition between the SPG and PPG in the adult.

2.3 MATERIALS AND METHODS

2.3.1 Fly strains and genetics

Table 2.1 strains were used in the experiment

Genotype	Reference
Gli-Gal4	(Sepp and Auld, 1999)
UAS-mCD8::RFP	(Mellert and Truman, 2012)
NrxIV::GFP	(Edenfeld et al. 2006)
OK6-Gal4	(Sanyal, 2009)
Repo-LexA	(Neukomm et al., 2014)
UAS-CD4::spGFP1-10	(Gordon and Scott, 2009)
LexAop-CD4::spGFP11	(Gordon and Scott, 2009)
E49-Gal4	(Gordon and Scott, 2009)
UAS-mCD8::DsRed	(Ye et al., 2007)

Flies were kept at standard conditions at 25 °C and 70% relative humidity.

2.3.2 Immunolabeling and image analysis

Staining and imaging were performed as described previously (Wang et al., 2004). Adult brains containing the E49 motor nerve were dissected from 3-10 day old adults and fixed in 4% Paraformaldehyde (PFA) for 20 min at room temperature (18-25°C). The adult abdominal region or the whole body of 3rd instar larva was dissected in 1X PBS, and fixed in 4% Paraformaldehyde (PFA) for 20 min at room temperature (18-25°C). Samples were washed three times in 1% PBST (PBS plus 0.1% Triton-X100) and blocked either for 1 hour at room temperature (RT) or overnight at 4°C with 2% normal goat serum. The samples are immunolabeled with primary antibodies for 2 hours at RT or overnight at 4°C. The primary antibodies were used at the following dilutions: mouse anti-GFP (1:100; Sigma), rabbit anti-HRP (1:200; Jackson Immuno 123-005-021), rat anti-DsRed (1:500; Sigma), mouse anti-Dlg (1:100; NICHD, University of Iowa) and rabbit anti-NrxIV (1;1000; Baumgartner et al., 1996). The samples are rinsed three times, incubated with goat anti-mouse, goat anti-rat or goat anti-rabbit conjugated secondary antibodies Alexa488, Alexa568 or Alexa647 (1:300, Invitrogen, Toronto, Canada) before being mounted onto a slide with Vectashield (Vector labs) and imaged. The fluorescent images were obtained from a DeltaVision microscope (Applied Precision,

Mississauga, Ontario) with a 60X oil immersion objective (NA 1.4) at 0.2 μm steps (Fig 2.1, 2.3-2.9). High and low magnification fluorescent images of the labeled tissues were obtained using a 63X oil immersion objective and a 25X water immersion lens on a Leica Confocal at 0.2 μm steps (Fig 2.2).

2.3.3 Morphological quantification

Images were deconvolved (SoftWorx, Toronto, Canada) using a measured PSF using 0.2 μm fluorescent beads (Invitrogen, Toronto, Canada) in Vectashield (Vector Laboratories, Burlington, Canada) via SoftWorx 2.0 Software to create side projections. Stacks were analyzed by ImageJ (Schneider et al., 2012) and compiled in Adobe Photoshop/Illustrator CS3.

2.4 RESULTS

2.4.1 Glia are directly in contact with axon membrane

As a first step, we looked for the overall connection between axonal membrane and glia along the peripheral nerve and at the distal end of the motor axon in both larvae and adults. To determine whether glia directly contact axons, we used the GRASP (GFP reconstitution across synaptic partners) assay. GRASP was developed as a trans-synaptic expression system to label an individual synapse based on the proximity of the two plasma membranes, which are separated by less than 100 nm of extracellular space (Ahmari and Smith, 2002; Feinberg et al., 2007). GRASP utilizes complementary fragments of a green fluorescent protein (GFP) fused with transmembrane proteins, so that when the two membranes are in close proximity (likely making contact) space is bridged by the tagged transmembrane proteins to reconstitute the whole GFP. GFP contains 11 beta strands of amino acids where the first 214 amino acids of GFP constitute beta strands 1-10 (spGFP1-10), and the last 16 residues constitute beta strand 11 (spGFP11). Each split-GFP can be connected to the cell membrane by tethering to a membrane protein such

as CD4, which allows for expression throughout the cell membrane or to a specific membrane protein to allow for expression in a cell subdomain such as the synapse (Feinberg et al., 2008). Each portion, spGFP1-10 and spGFP11, is stable, soluble and non-fluorescent. GFP is reconstituted and assembled into a fluorescent form when the two cell membranes are sufficiently close and the proteins can bridge the intercellular gap and thus GRASP has been used a measure of cell-cell contact (Feinberg et al., 2008; Gordon and Scott, 2009). We used two expression systems GAL4/UAS and lexA/LexAop to express the two split of GFP (spGFP) components in the axons and glial cells respectively. This double expression system allows the tissue specific expression of two Reporters (Potter and Luo, 2011). For expression in the glia, we used the Repo-LexA driver. Repo is, a glial specific homeodomain protein required for glial differentiation and the expression of late glial markers and is expressed in all glia (Yuasa et al., 2003). The Repo-LexA/LexAop is a reliable system for targeting expression in the majority of glial cells, including all the peripheral glia (Awasaki and Lee, 2011). For expression in motor axons, we used the OK6-GAL4 driver, which is expressed in all motor neurons in the larva and adult stages (Sanyal, 2009). To determine the connection between glia and axons in the peripheral nerve, the spGFP1-10 fused to mCD4 under the control of the GAL4 UAS sequence (UAS-mCD4::spGFP1-10) was expressed in combination with a DsRed-tagged membrane marker (UAS-mCD8::DsRed) on the axonal membrane using OK6-GAL4. The other GRASP component, spGFP11 fused to mCD4 under the control of the LexA target sequence (LexAop-mCD4::spGFP11) was expressed on the glia membrane using Repo-LexA.

We analyzed a range of nerves and NMJs in the 3rd larvae and adults (Fig 2.1-2.4) for the presence of GRASP using an antibody specific for the reconstituted GFP (Gordon and Scott, 2009). We used DsRed and anti-HRP immunolabeling to detect the peripheral axons. At this

stage, a positive GRASP signal was observed along the peripheral and close to the axons tagged with DsRed in both larvae and adults (Fig 2.1-2.2). Positive GFP signals indicative of GRASP were observed along each nerve and colocalized with the axons tagged with DsRed (Fig 2.1 A and C, 2.2 A).

In all tested nerves (n=20 larvae, n=20 adults), continuous GRASP signals were detected where glia entirely cover the axons. The positive GRASP indicated glia are directly in contact with axon membrane in these regions and suggests the absence of a secreted basal lamina. Some GRASP positive signals were detected away from the axons in the core of the peripheral nerve, which may represent background (Fig 2.1 A, arrows). In transverse optical sections of both adult and larval nerves, the axons at the core of each nerve were surrounded by GRASP signals (Fig 2.1 B and D, 2.2 C). The abdominal nerves contain several axons thus several axon profiles were detected in each transverse section (Fig 2.1 B and D). We observed a similar pattern of GRASP positive connections in the adult E49 motor nerves (Fig 2.2). A pair of E49 neuron cell bodies are positioned in the adult brain (Fig 2.2 A, black arrows). The positive GRASP signal was observed along the E49 nerve close to the distal end of axons tagged with DsRed (Fig 2.2 A-B, white arrows). In transverse sections, a green layer surrounded one E49 axon, which represents the binding site between glia and axon membranes (Fig 2.2 C). These positive GRASP signals likely represent the wrapping glia layers that are known to be in contact with axon membrane (Stork et al., 2008; Matzat et al., 2015). However, it is still possible that the SPG also make axon contact within the inner layer of the nerve to form the connection with the axonal membrane. Overall, our results confirm that GRASP can be used to detect contact between the peripheral glia and the axon membrane.

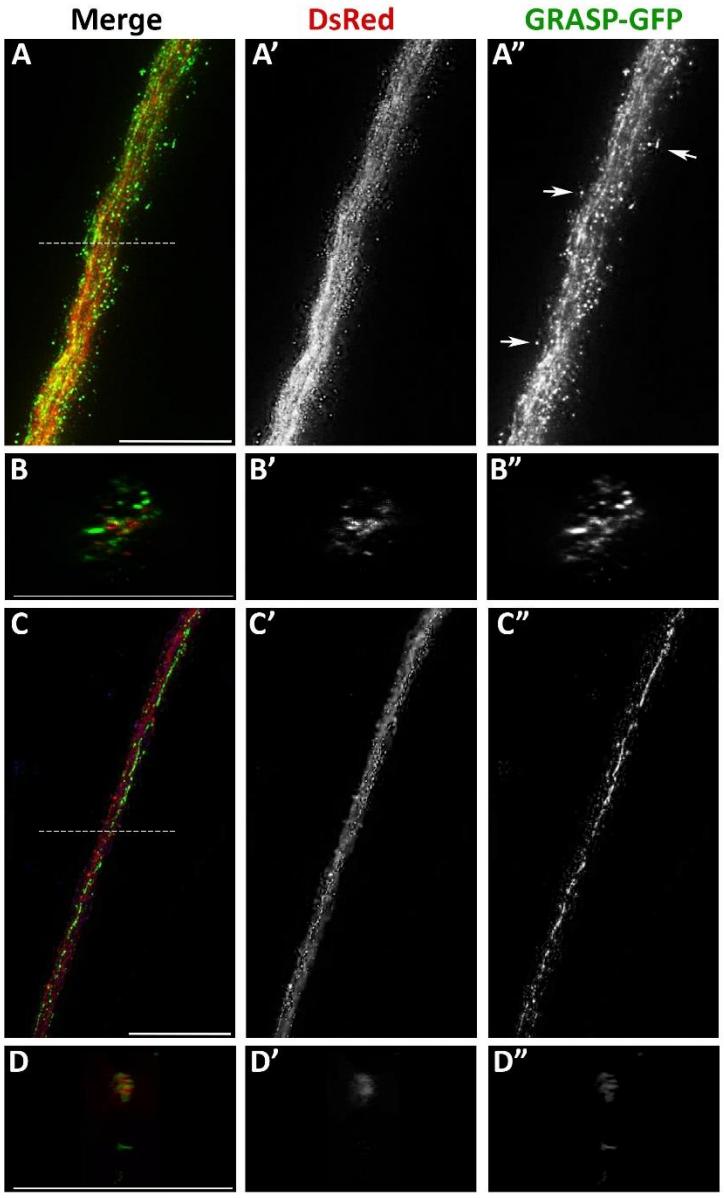

Figure 2.1 Glia are directly in contact with axon membrane.
The axons and glia-axon connection domains labeled using mCD8:DsRed and mCD4::GFP (green) respectively in the fixed peripheral nerve from a 3rd larva (A, B) and the abdominal nerve from an adult (B, D). splitGFP1-10 was expressed in axons using OK6-Gal4 (*OK6-Gal4>mCD4::spGFP1-10*), and splitGFP11 was expressed in glia using Repo-LexA (*Repo-lexA>mCD4::spGFP11*). A 2D projection of the entire stack is shown in each panel. (A, C) The expression of GFP indicated colocalization with DsRed along the length of the nerve. (A, C) The dashed lines indicate the position, which the sections were taken for ultrastructure analysis. Transverse sections of the peripheral nerves in larva (B), and (D) in the adult. GRASP signal is concentrated in the glia surrounding the multiple axons of the larval (B) and adult nerve (D). (A) Several spots detected with a high level of GRASP signal away from the larval nerve (arrows). Scale bars are 15 um.

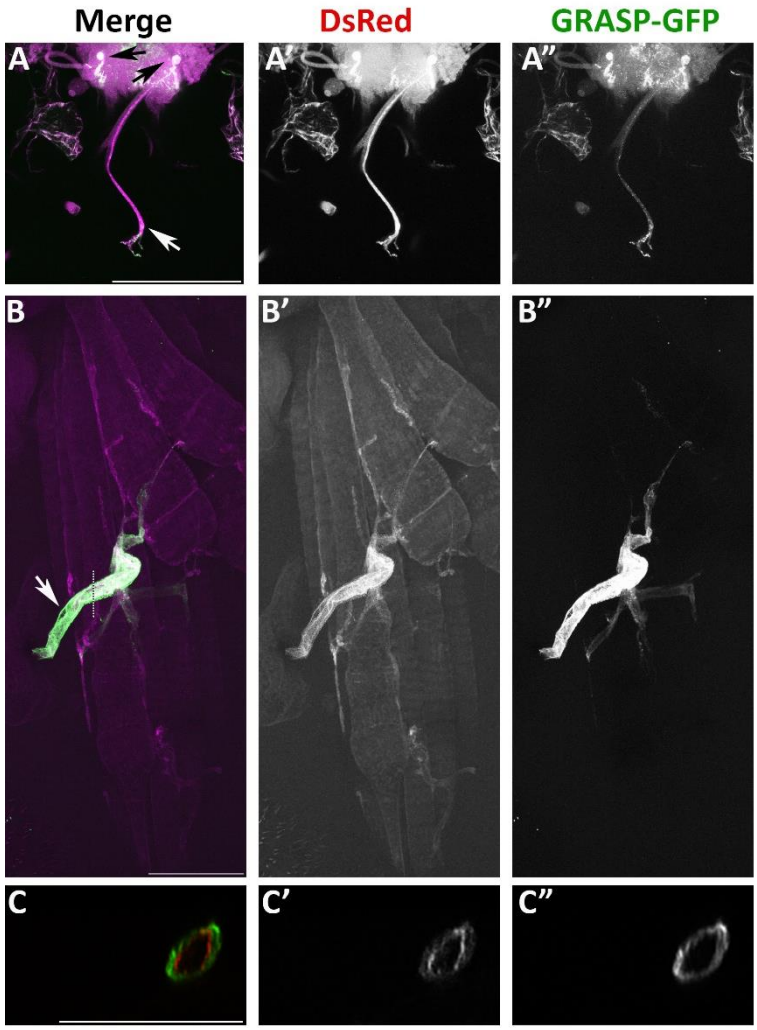

Figure 2.2 The peripheral axon is surrounded by glia.
The axons and glia-axon connection domains labeled using mCD8::DsRed and mCD4::GFP (green) respectively in the fixed E49 motor nerve (white arrows) from an adult. splitGFP1-10 was expressed in axons using E49-Gal4 (*E49-Gal4>mCD4::spGFP1-10*), and splitGFP11 was expressed in glia using Repo-LexA (*Repo-lexA>mCD4::spGFP11*). A 2D projection of the entire stack is shown in each panel. (A) The position of E49 neuron cell bodies indicated in the brain (black arrows). (A, B) The expression of GFP indicated colocalization with DsRed along the length of the nerve (white arrows). (B) The dashed line indicates the position, which the section was taken for analysis. (C) Transverse section of the E49 motor nerve in the adult. GRASP signal is concentrated in the glia surrounding an axon of adult nerve. The glia-axon binding site is indicated as a ring, which entirely covered the axon. Scale bars are 25 (A) and 15 um (B, C).

2.4.2 Glia/axon contact extend into NMJs in adult but not in larval stage

We next wanted to determine the degree of glia-axon contact at the distal end of the motor axon as it extends into the muscle to form the NMJ. The axons and boutons of the NMJs were tagged with membrane bound DsRed. In the adult NMJs, we observed a clear GRASP signaling that extended into the NMJs including regions that covered individual boutons and the synaptic regions of all tested adult NMJs (n=30) (Fig 2.3, arrows). The GRASP signals were concentrated on one side of NMJs, which seems to not be in the synaptic zone of boutons (Fig 2.3, arrows). A part of several branches of NMJs were not covered by GRASP signaling or had reduced levels of GRASP (Fig 2.3, arrowheads).

Conversely, in larva, the GRASP signal mostly stopped at the point where the axon transitioned into forming boutons and synapses at the NMJ (n=20) (Fig 2.4 A-B, arrows). However, in some larval NMJs, the GRASP signals extended within the NMJs but only into the proximal regions and did not extend to the end of the boutons (Fig 2.4 C-D, 2.9 A-B, arrowheads).

Our observations in the main body of each peripheral nerve indicate that GRASP is an effective assay to detect glia-axon contact, as the connections between the wrapping glia or subperineurial glia and axons are well established (Stork et al., 2008). Our results also indicate the glia-axon contact terminates in the distal end of the motor axons in the larva to a region just prior to the boutons and synapses of the NMJs. However, in the adult, the degree of glia-axon contact matches the glia extension into the adult NMJ. This difference between larval and adult glia-axon contact likely represents the presence of the PPG in the adult NMJs and the lack of extensive coverage of the larval NMJs by glial membranes (Strauss et al., 2015).

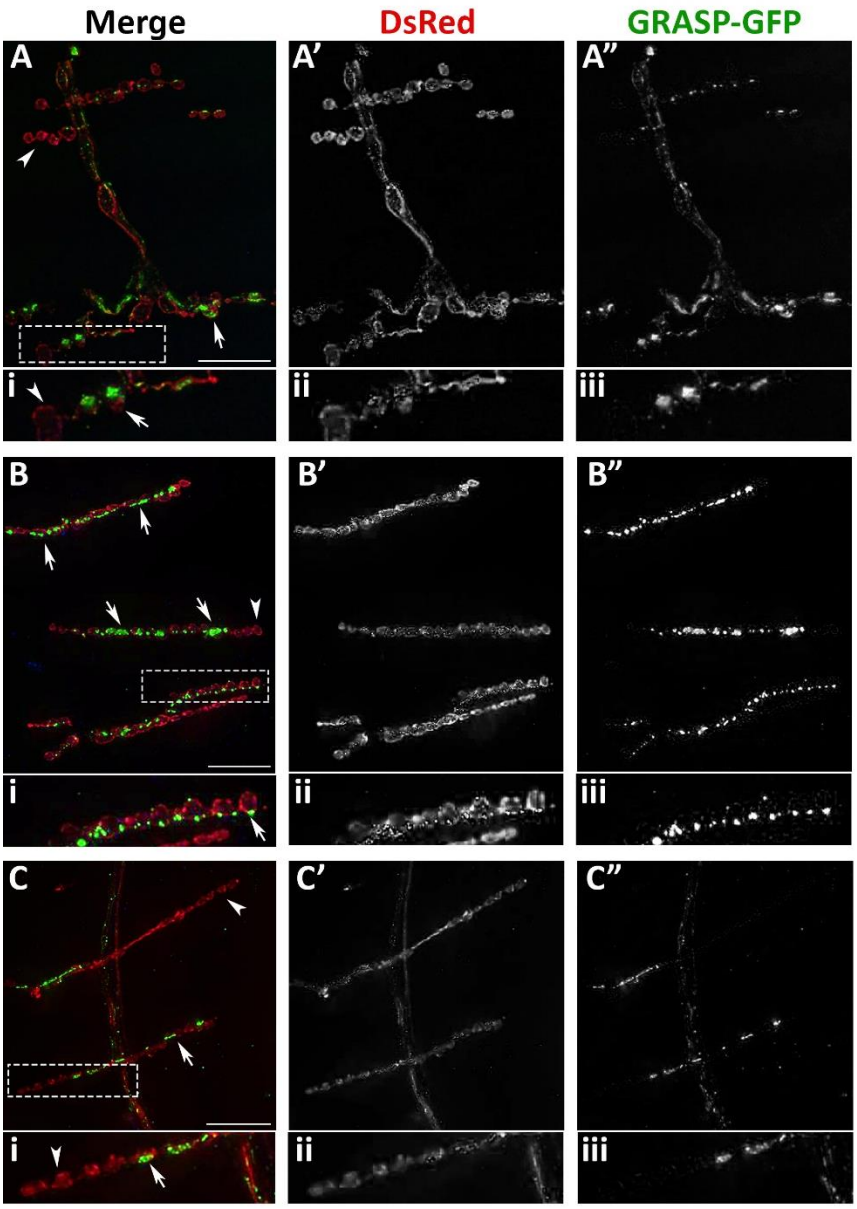

Figure 2.3 Glia extend into the NMJ in adults.
Peripheral axons and glia-axon connection labeled using mCD8::DsRed and GRASP (green) respectively. Split GFP1-10 was expressed in motor neurons using OK6-Gal4 (*OK6-Gal4>mCD4::spGFP1-10*, and splitGFP11 was expressed in glia using Repo-lexA (*Repo-LexA>mCD4::spGFP11)*. A 2D projection of the entire stack is shown in each panel. The boxed regions in panels A-C were digitally scaled 400% and the corresponding grayscale panels show the GRASP distribution with respect to the boutons. The expression of GFP colocalized with DsRed along the NMJ and extended into the end of most of adult NMJs observed (arrows) but not detected in some NMJs (arrow heads). The GFP signal was concentrated on one side of boutons (arrows). Scale bars are 15 um.

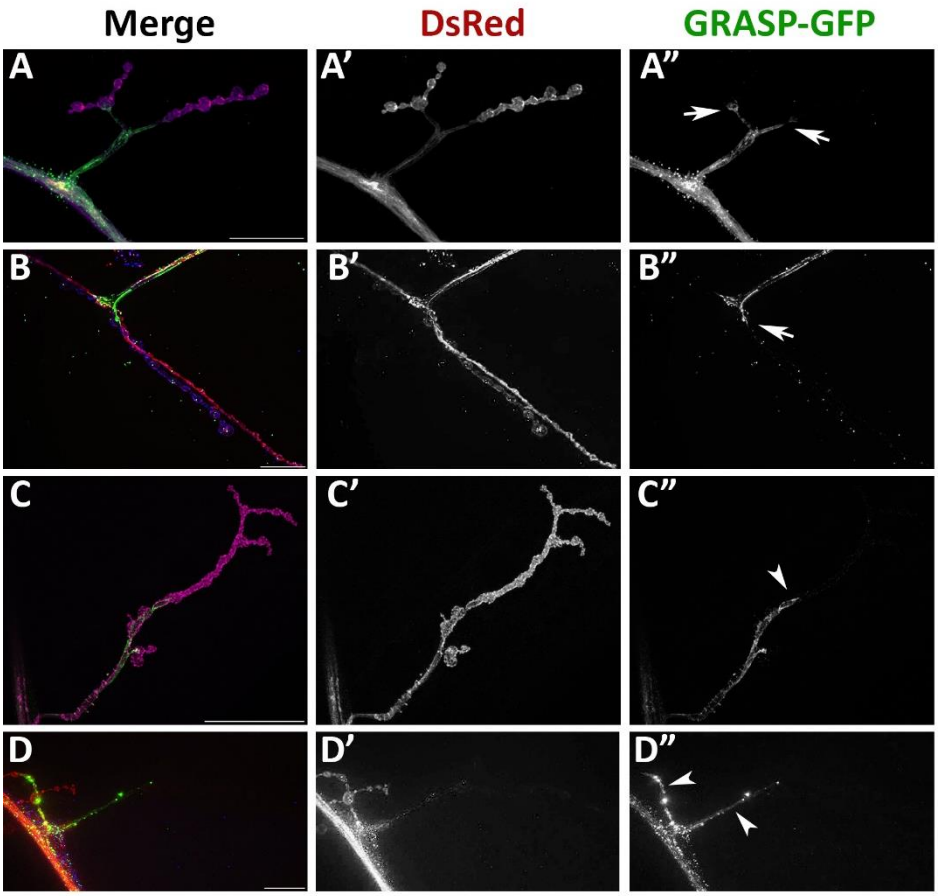

Figure 2.4 Glia do not extend into NMJs in larvae.
Peripheral axons and glia-axon connections labeled using mCD8::DsRed and GRASP (green) respectively in 3rd larva. SplitGFP1-10 was expressed in axons using OK6-Gal4 (*OK6-Gal4>mCD4::spGFP1-10*), and splitGFP11 was expressed in glia using Repo-lexA (Repo-LexA>mCD4::spGFP11). A 2D projection of the entire stack is shown in each panel. The grayscale panels show the GRASP distribution and bouton localization. The expression of GRASP-GFP was colocalized with DsRed along the axon but not in the NMJ. (A, B) GFP expression (arrows) stopped at the distal end of the axons prior to branching into the NMJ. (C, D) Glial processes occasionally extended into the proximal region of the NMJs (arrowhead) but never along the entire length of the NMJs. Scale bars are 15 um.

2.4.3 SJs are not find in the NMJs in both adult and larval stage

Our results confirm prior observations that glial membranes contact motor axons prior they branch into the larval NMJs and continue into the NMJ in adults (Brink et al., 2012). However, the distribution of the septate junction proteins within these two tissues is not well established. To test for the distribution of SJ proteins, we used the SJ core protein Neurexin IV

endogenously tagged with GFP (NrxIV::GFP). We used the anti-HRP antibody to immunolabel the axons and boutons of the NMJs in combination with anti-disc large (anti-Dlg) to visualize post-synaptic membranes of the boutons. In both the larval and adult PNS, SJs appeared as a continuous line along the length of each SPG within the main peripheral nerves (Fig 2.5, arrows). This pattern represents the SJ that form between edges of a single SPG. Circles or loops of NrxIV were observed where two SPGs overlap (Fig 2.5 A,C, arrowheads) and likely correspond to the SJ formed between neighboring glia to create the blood-nerve barrier. This pattern confirmed prior observations of others on the distribution of NrxIV and the SJ domain within the SPG of larval nerves (Brink et al., 2012), and identified that the adult nerve shares a similar structure. In both adult and larval nerves, we observed that the line of NrxIV and the SJ extended along each motor axon as it branched off the main nerve, but expression of SJ terminated at the distal end of each motor axon prior to the formation of the synaptic boutons at the NMJ (Fig 2.6, arrows). This result suggests that SJs form ends that circle or seal the glial to the axon before axon branching into the NMJs (Brink et al., 2012). In the larval stage, in one of 20 tested NMJs, an occasional spot of Nrx::GFP expression was detected within the NMJ, which was separated from the main SJ (Fig 2.6, B, arrowhead).

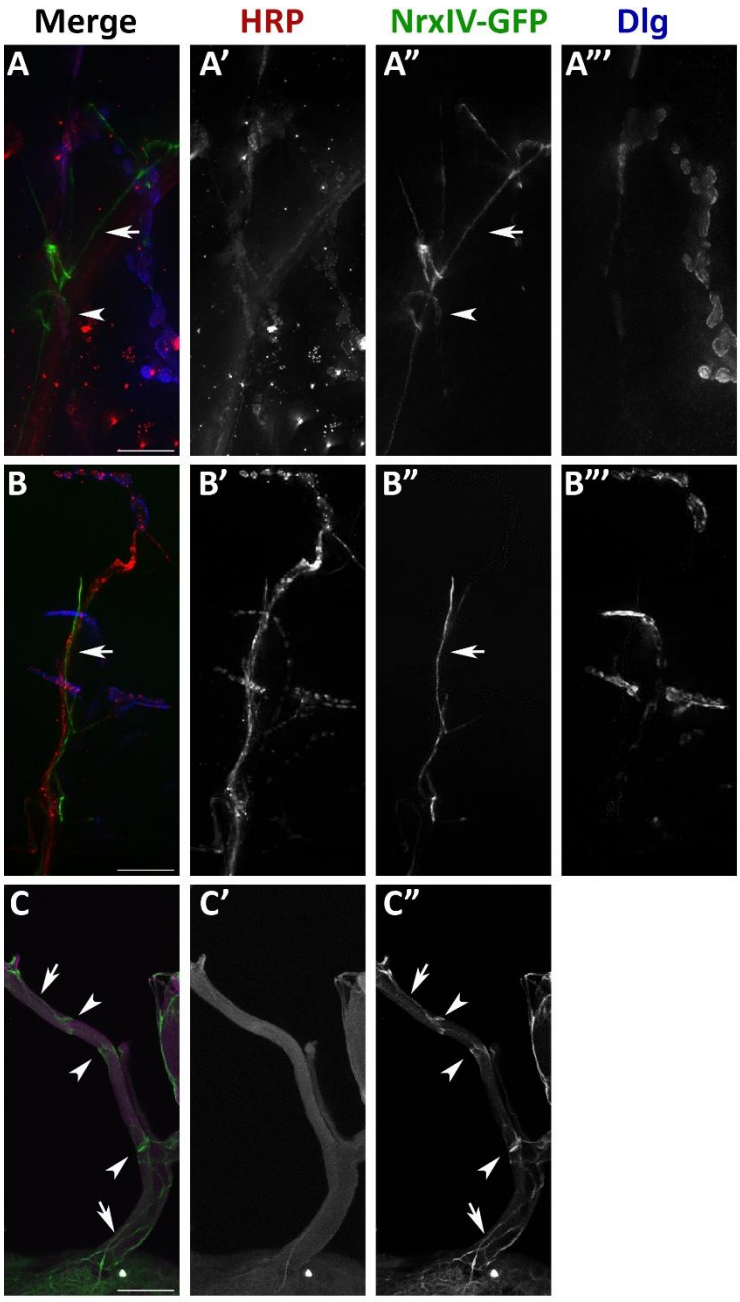

Figure 2.5 NrxIV is expressed in two patterns of linear and circular lines along the axon. Fixed nerves and distal region of the axons labeled with NrxIV::GFP (green), axon membranes labeled using anti-HRP, (A, B) and the post-synaptic SSR labeled using anti-Dlg (blue) in third instar larva. The presented nerves in A, B and C panels are the larval body, adult abdominal and adult nerve close to the brain respectively. A 2D projection of the entire stack is shown in each panel. The corresponding grayscale panels show the NrxIV and bouton distribution. NrxIV::GFP is distributed in linear (arrows) and circular lines (arrowheads) along the axon. Scale bars are 25 um.

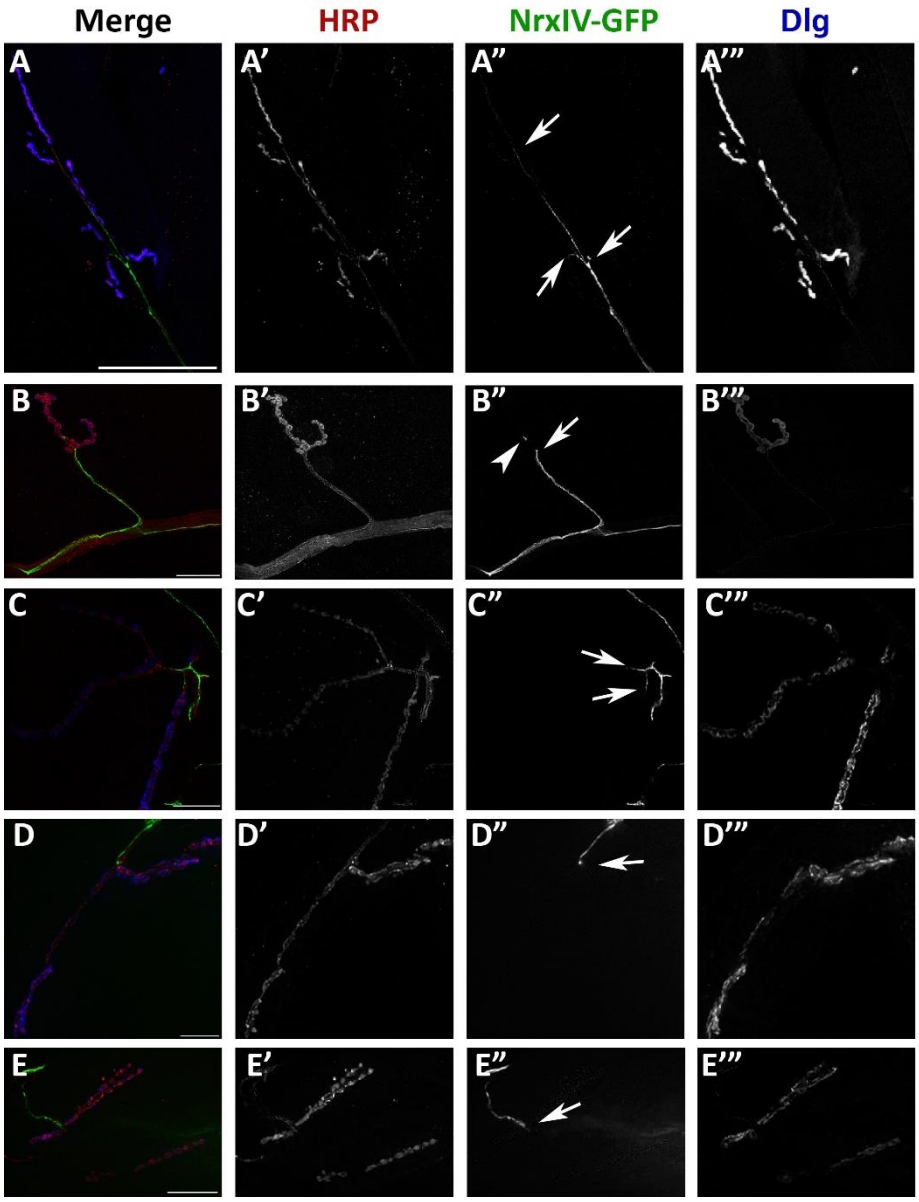

Figure 2.6 NrxIV expression stops at the distal end of motor axons.
Fixed NMJs and distal region of the axons labeled with NrxIV::GFP (green), axon membranes labeled using anti-HRP, and the post-synaptic SSR labeled using anti-Dlg (blue) in 3rd instar larva (A-B) and adult (C-E). A 2D projection of the entire stack is shown in each panel. The corresponding grayscale panels show the NrxIV and bouton distribution. NrxIV::GFP expression stopped at the distal end of the axon prior to branching into NMJs (arrows). (B) Occasionally NrxIV::GFP positive signals were observed within the NMJ (arrowhead) separated from the main SJ. Scale bars are 15 um.

2.4.4 SJs are confined in the SPG in the distal end of the motor axon

As the SPG form the BNB, we wanted to test for the distribution of the SPG membranes and the SJ domain at the distal end of the motor axon. To visualize the SJs we used NrxIV::GFP. To visualize the SPG, we used a SPG specific GAL4 driver, Gli-GAL4, and drove the expression of a membrane bound RFP using UAS-CD8::RFP. In addition, the boutons of the NMJs were detected using immunolabeling to discs large (Dlg), or using anti-HRP. The SPG progress and NrxIV expression indicated colocalization with axon membranes along the nerve (Fig 2.7, A-C). The result of the abdominal nerves of the adult PNS was similar to larva (Fig 2.7, D-F). In the both adult and larval, SPG processes were not detected in the NMJs in that the RFP tagged membrane stopped short of the first boutons and were limited to the distal end of each motor axon (Fig 2.7, A-F). Similarly, the NrxIV and SJ domain was limited to the same distribution as the SPG (Fig 2.7, A-F). Of interest was the differences observed in the distal end of the motor axon in adults and larvae. In the larval stage, the SPG continued right up to the distal end of each motor axon stopping just prior to the formation of synaptic boutons (n=30) (Fig 2.7, A-C, arrows). Whereas in the adult, a gap was observed between the end of the SPG plus SJs expression and the distal end of the axon before the formation of the first bouton in the NMJs (Fig 2.7, D-E, asterisks) (n=30). This gap at the end of the adult motor axon might be the last part of the distal motor axon, which is covered by the PPG.

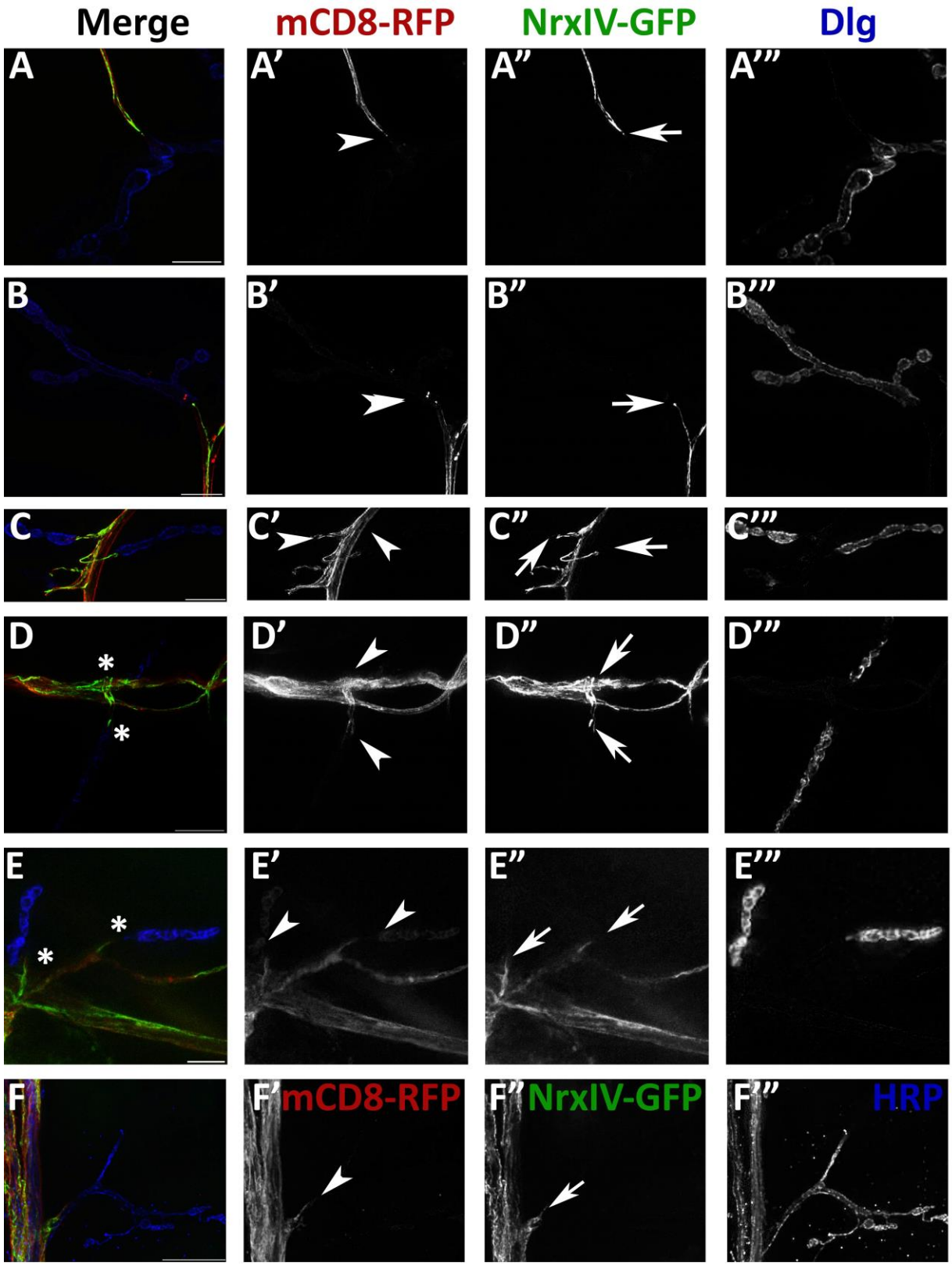

Figure 2.7 Septate junctions are confined to the SPG at the distal end of motor axons.

(A-E) Fixed NMJs and the distal region of the axons where the SPG membranes were labeled using mCD8::RFP, and the septate junctions labeled using NrxIV::GFP (green). The NMJs were labeled using anti-Dlg (blue) in larva and adult (D-E) or using anti-HRP (blue) in adult (F). A 2D projection of the entire stack is shown in each panel. The corresponding grayscale panels show the distribution of SPG and NrxIV and the bouton localization. NrxIV::GFP was colocalized to the SPG membrane RFP along with the motor axons but in the NMJs. NrxIV::GFP expression (arrows) and SPG processes (arrowheads) both stopped at the distal end of the motor axons prior to branching into NMJs. (D-E) In adults, the SPG membrane stopped far short of the NMJ (creating a bare region of axon without RFP, asterisks). Scale bars are 15 um.

We did occasionally observe the presence of NrxIV::GFP within the more proximal region of the larval NMJ, (Fig 2.8 A-E) (n=10 of 30 NMJs). These regions were likely remnants of SJ (arrows) or SPG (arrowheads), as connections were not observed between the spots and the main body of the SJ. These regions were never observed in the adult NMJs (n=20). Overall, we observed, as previously described, the SJs were confined to the SPG, and neither SPG nor SJs are presented in the larval NMJ. We determined that this was true of the adult NMJ as well.

We compared the length of the GRASP signaling with SJs presence along the larval NMJs (Fig 2.9). The GRASP experiment indicated glia extended into the proximal region of several larval NMJs but the presence of SJ was limited only in the distal end of an axon (Fig 2.9). Thus, SPG progress is terminated prior to the NMJ.

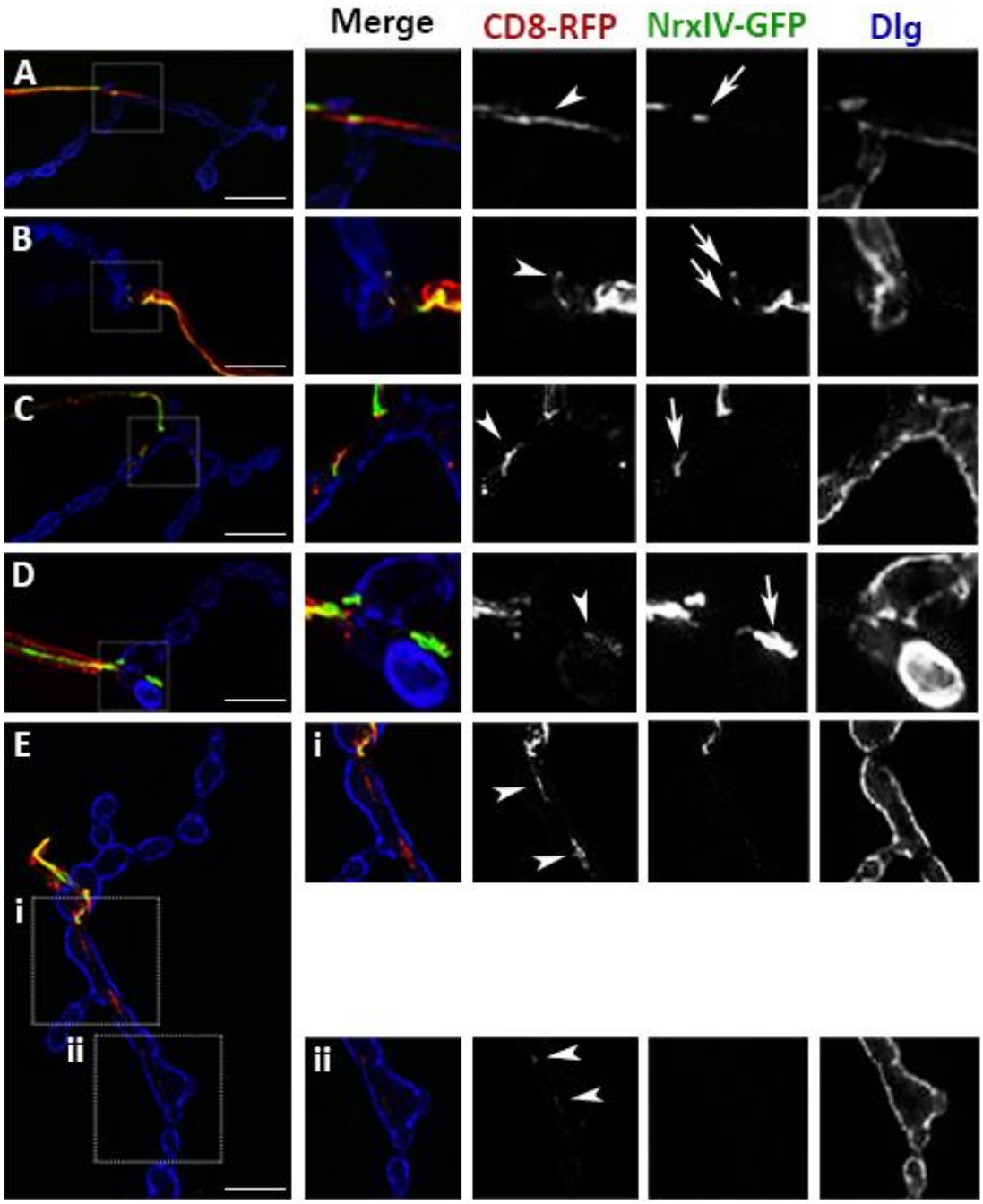

Figure 2.8 Septate junction, Nrx::IV and SPG processes were occasionally detected within the larval NMJs.
(A-E) Fixed NMJs and distal region of the axons where the glial membranes were labeled using mCD8::RFP, the septate junctions labeled using NrxIV::GFP (green), and the boutons labeled using anti-Dlg (blue). A 2D projection of the entire stack is shown in each panel. The boxed regions in panels A-E were digitally scaled 400% and the corresponding grayscale panels show SPG, NrxIV and bouton localization. NrxIV::GFP colocalized with RFP along with the motor axon but not into NMJs. NrxIV::GFP expression (arrows) and SPG processes (arrowheads) were occasionally detected at the proximal region of several NMJs. Scale bars are 15 um.

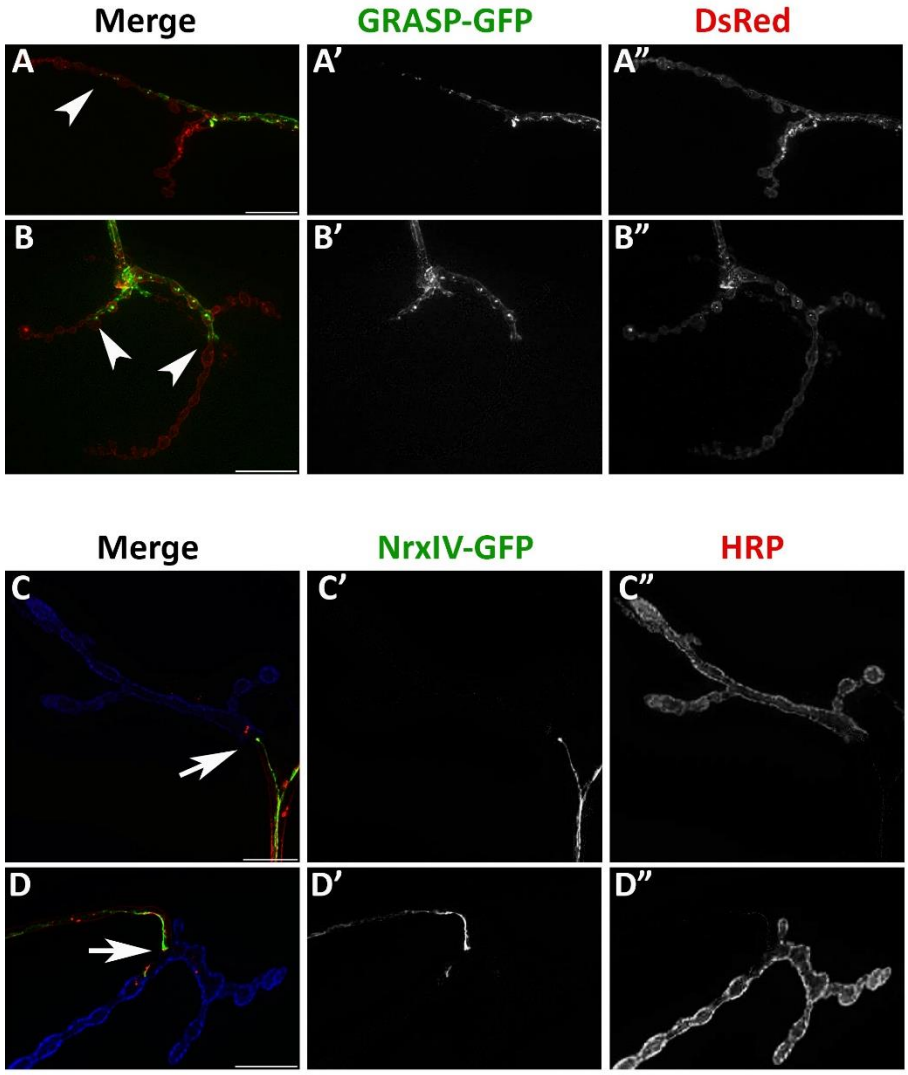

Figure 2.9 Glia occasionally extend within the larval NMJ but Septate Junctions do not.
(A, B) Peripheral axons and glia-axon connections labeled using mCD8::DsRed and GRASP (green) respectively in 3rd larva. SplitGFP1-10 was expressed in axons using OK6-Gal4 (*OK6-Gal4>mCD4::spGFP1-10*), and splitGFP11 was expressed in glia using Repo-lexA (Repo-LexA>mCD4::spGFP11). (C, D) Fixed NMJs and distal region of the axons where the glial membranes were labeled using mCD8::RFP, the septate junctions labeled using NrxIV::GFP (green), and the boutons labeled using anti-Dlg (blue). A 2D projection of the entire stack is shown in each panel. The grayscale panels show the SPG, NrxIV and GRASP distribution and the boutons localization. (A, B) The expression of GRASP-GFP was colocalized with DsRed along the axon and occasionally within the proximal region of NMJ (arrow heads). NrxIV::GFP colocalized with RFP along the motor axon but not into NMJs. NrxIV::GFP expression (arrows) stopped at the distal end of the motor axons prior to branching into NMJs, or occasionally detected at the proximal region of several NMJs. Scale bars are 15 um.

2.5 DISCUSSION

Our goal was to determine the degree of contact between glia and axon membranes in adults and larvae along with the distribution of the SJs and the SPG in peripheral nerves and at the distal end of the motor axon in adult and larva. We determined that GRASP provided a reliable assay for the degree of contact between axon membranes and glia in the *Drosophila* peripheral nerve of adults and larvae. We observed glia-axon contact stopped short of the NMJs in the larva, but was present at consistent levels within the adult NMJs. Although in this experiment we did not determine which glia layer contacts the motor axons. It is likely that the contact in the peripheral nerve is mediated by the WG or to some smaller extent the SPG and the motor axons. Prior research has found that each WG of the larval peripheral nerves extends processes around bundles or single axons (Matzat et al., 2015). It is less clear if the same classes of glia are present in the adult peripheral nerves. It is known that the WG do not extend beyond the main nerve trunk to ensheath the motor axons as they branch towards the NMJs (Brink et al., 2012). In this region, the SPG are present and extend processes up to the larval NMJs but stop in the area of the first bouton formation (Fuentes-Medel et al., 2009; Brink et al., 2012). However, in several larval NMJs, the extension of GRASP signaling was detected in branched regions which were not synchronized with SJs expression stop point in the distal end of the axon. Finally, in the adult NMJs, observed GRASP signal is likely due to the PPG-axon contact as this unique glial type has been previously shown to extensively cover the adult NMJs (Strauss et al., 2015). So while the adult NMJs are covered by an extensive glial layer, the larval NMJs are not and it seems the larval NMJs are exposed to circulating hemolymph and not ensheathed.

In the larval PNS, the distribution of the SJs within the SPG both along the nerve and at the distal end of motor axon confirmed prior observations (Brink et al., 2012) as was the failure

to detect the presence of SJs within the NMJs (Brink et al., 2012). The same observations held true in the adult. Both the SPG and SJ stopped prior the formation of the NMJs and with a gap between the glial terminal and the distal end of the axon (i.e. before the formation of the first boutons). The GRASP results confirmed an extensive contact between the glia and motor axon terminus with the NMJ. Therefore, there is a glial presence, likely the PPG, within the adult NMJs. However, given the lack of SJ proteins, there is unlikely to be a permeability barrier at the adult NMJ. The PPG contribute in tripartite neuromuscular synapses, which are essential to make connections between branches of motor axons and muscle surface. The tripartite synapses are thought to directly interact with tracheal vessels to mediate gas exchange and plays a critical role in motor function (Strauss et al., 2015). The PPGs contributes to glutamatergic neuromuscular synapses, and exhibit synaptic activity by inducing calcium transients (Todd et al., 2010; Danjo et al., 2011; Strauss et al., 2015). Thus, PPGs might form a barrier within the NMJ in the absence of the SPG BNB.

In both adult and larval stages, the stop point for SJ expression was just before the point where the motor axon branched into the NMJs to create the synaptic boutons, with a large gap in the adult likely taken up by the presence of the PPG membrane. Similar to the paranodal junction flanking the nodes of Ranvier in vertebrate peripheral nerves, the SJs in the SPG may attach to the axon membrane and form a sealed end at the axon terminus. The sealed end could potentially form a BNB that insulates the axon to block the flow of solutes up the motor axon. Prior work determined that this region blocks the penetration of low molecular weight dyes (Brink et al., 2012) suggesting a presence of a BNB in this region. Similarly electron micrographs of this region point to the presence of septate-like junctions that form between the glia and the axon (Till Matzat, personal communication, 2017). Thus the evidence confirms the

BNB is formed in the distal end of axons, however, more experiments are needed to illustrate the formation of SJs between glia and axon membranes.

3 KNOCKDOWN OF SEPTATE JUNCTION COMPONENTS DOES NOT DISRUPT LARVAL LOCOMOTION OR ADULT FEEDING BEHAVIOR

3.1 OVERVIEW

Motor neurons control voluntary muscle contraction and reflex actions to drive animal locomotion. Disruption of axons or ensheathing glia leads to spasms, muscle weakness and paralysis. In *Drosophila*, lack of the SJ results in disruption of the blood-nerve barrier and paralysis. In the current project, SJ molecules were targeted for a knockdown in peripheral nerves and behavioral changes were studied in both adult and larva. It was expected if the axons partner with glia in forming SJs that knockdown of SJ proteins in axons might lead to disruption of the BNB, which can lead to changes in behavior.

3.2 INTRODUCTION

Adhesive molecules exhibit a critical role in forming barriers in nervous tissues. The disruption of the paranodal junction in the vertebrate peripheral nerve causes the Na^+ channels to become distributed along the length of the axon membrane as they spread away from the paranode. This results in aberrant action potentials and a reduction of conduction velocity leading to locomotion defects and weakness in targeted limbs (Kuwabara, 2007; Sun et al., 2009). Similarly, loss of septate junctions in the *Drosophila* peripheral nerve leads to a loss of cell-cell junction and a disruption of the BNB. Affected glia are not able to insulate the axons from the high potassium hemolymph and axon conduction velocity is reduced, leading to paralysis (Auld et al., 1995; Baumgartner et al., 1996; Deligiannaki et al., 2015). Septate junctions similar to paranodal junctions are comprised of a complex of NrxIV, Nrg and Cont. Lack of any one of these proteins leads to disruption of the BNB. Many proteins have been identified as SJ components in *Drosophila* including NrxIV and the associated scaffolding

protein Cora (Baumgartner et al., 1996; Ganot et al., 2015), both subunits of the Na^+/K^+ ATPase, (Genova and Fehon, 2003; Paul et al., 2003), as well as the associated proteins Dlg and Scrib (Hortsch and Margolis, 2003).

The disruption of the blood-nerve barrier in SJ mutants has been attributed to changes in SJs within the subperineurial glia (SPG) layer. The loss of SJ proteins has not yet investigated for glia-axon communication, especially at the axon terminus for each motor axon. More evidence is needed to confirm the formation of sealed ends formed by SJs in the axon termini. We hypothesize there is an axon-glia SJ formed at the axon terminus. Thus, knockdown of the essential SJ proteins within the axons is predicted to disrupt the BNB and lead to changes in behavior due to the improper conductance of electrical impulses along the axon.

3.3 MATERIALS AND METHODS

3.3.1 Fly strains and genetics

Table 3.1 strains were used in the behavioral assays

Genotype	Reference
VT005008-Gal4	(Pool et al., 2014)
OK6-Gal4	(Sanyal, 2009)
UAS-Dicer2	(Dietzl et al., 2007)
w1118	Bloomington Stock center

3.3.2 Proboscis extention response

In the PER assay, 10 adult flies 3–10 days old with indicated genotypes were kept in starvation condition, on 1% agar at room temperature, for 24-72 hours before testing. Flies were mounted while the backside of thorax attached on glass slide surface via a drop of liquid glue, and legs freely can move in the air and allowed 1-2 hours to recover before testing. A drop of 100 mM Sucrose solution was provided to the foreleg taste receptors to stimulate flies for

proboscis extension and food taking. A web camera was used to monitor movements of the rostrum. In each genotype, the number of flies was calculated with defective PER (Gordon and Scott, 2009). All statistical tests were performed and analyzed using a one-way ANOVA plus Tukey's post hoc test in using GraphPad Prism 6 software.

3.3.3 Larval locomotion tracking

The experiments for larval locomotion tracking were carried out as previously described (Petley-Ragan et al., 2016). Two groups were assayed: one at room temperature, and one at 25°C. For each experiment, 5-30 wandering 3rd instar larva from defined genotype placed on 100 mm diameter apple juice plates. All larva tracked in 30 seconds (Swierczek et al., 2011), and the instantaneous speed of each larva measured at the 15th second. All statistical tests were performed and analyzed using a one-way ANOVA plus Tukey's post hoc test in using GraphPad Prism 6 software.

3.4 RESULTS

3.4.1 Knockdown of SJs components in axon had no effect on normal PER

In *Drosophila* adults, sensory neurons are concentrated in the labella and legs, which act as the detectors of palatable substances such as solutions of various sugars. Sensory response ultimately link to motor neurons that triggers the extension of the proboscis, which contains the rostrum, haustellum and labella that are extended via contraction of muscles positioned in the trunk of proboscis to drive the Proboscis Extension Response (PER) and food taking behavior. The bilateral pair of E49 motor neurons has been identified as the taste circuit neurons in *Drosophila* brain, which drive the PER (Gordon and Scott, 2009). Proboscis extension is directed by 12 pairs of muscles under control of E49 motor neurons (Rajashekhar and Singh, 1994; Gordon and Scott, 2009). We supposed the peripheral glial cells might facilitate propagation of

the action potential in the E49 axons by creating a BNB between axons and glia at the axon terminus. We predicted that the lack of SJ components in the axons would lead to defective PER because of disruption of the BNB.

To test the involvement of motor axons in mediating a paranodal-like SJ between axons and glia, expression of SJ components was inhibited by using RNAi mediated knockdown in the motor axons that underlie the PER. We used RNAi lines for the SJ genes homologous to core paranodal complex, NrxIV, Nrg, Cont, as well as other SJ proteins: Cora, Dlg and Scrib. Moreover, several RNAi lines for each were used to control for any potential off target effects and potentially weak RNAi lines.

Table 3.2 strains were used in the behavioral assays

RNAi	Stock
UAS-NrxIV RNAi 8353	VDRC: 8353
UAS-NrxIV RNAi 108128	VDRC: 108128
UAS-NrxIV RNAi 9039	VDRC: 9039
UAS-NRXIV RNAi 3142	Bloomington Stock Center
UAS-Nrg RNAi 6688	VDRC: 6688
UAS-Nrg RNAi 3151	Bloomington Stock Center
UAS-Cont RNAi 40613	VDRC: 40613
UAS-Cora RNAi 9787	VDRC: 9787
UAS-Cora RNAi 9788	VDRC: 9788
UAS-Cora RNAi 5144	Bloomington Stock Center
UAS-Dlg RNAi 188	Bloomington Stock Center
UAS-Dlg RNAi 41134	VDRC: 41134
UAS-Scribble RNAi 3229	Bl: 29552
UAS-Scribble RNAi 105412	VDRC: 105412

VT5008-Gal4 is a driver that is expressed in the motor neurons that lead to proboscis extension (Gordon and Scott, 2009, Pool et al., 2014). We used the VT5008 driver which is highly specific for these motor neurons paired with SJ RNAi lines to knock down each protein in the PER motor neuron. We used Baboon RNAi as a positive control as knockdown of this

protein leads to significant PER defects (Danielle McEachern; personal communication 2017). We did not observe any defects in PER for any of the SJ RNAi lines (Table 3.3). PER was normal, even when rearing temperatures were raised to 25°C or 29°C to increase GAL4 efficiency (Table 3.3).

Table 3.3 % of adults displaying normal PER in response to 100 mM Sucrose

Genotype	20°C	25°C	29°C
VT5008-Gal4>UAS-Baboon RNAi	0% n=20	0% n=20	0% n=20
VT5008-Gal4>UAS-NrxIV RNAi 8353	100% n=23	100% n=25	100% n=12
VT5008-Gal4>UAS-NrxIV RNAi 108128	100% n=42	100% n=17	100% n=15
VT5008-Gal4>UAS-NrxIV RNAi 9039	100% n=15	ND	ND
VT5008-Gal4>UAS-Nrg RNAi 6688	100% n=31	100% n=22	100% n=15
VT5008-Gal4>UAS-Nrg RNAi 3151	100% n=12	100% n=16	100% n=23
VT5008-Gal4>UAS-Cont RNAi 40613	100% n=23	ND	ND
VT5008-Gal4>UAS-Cora RNAi 5144	100% n=24	100% n=14	ND
VT5008-Gal4>UAS-Cora RNAi 9787	100% n=12	100% n=19	100% n=12
VT5008-Gal4>UAS-Cora RNAi 9788	100% n=16	100% n=23	ND
VT5008-Gal4>UAS-Dlg RNAi 188	100% n=20	ND	100% n=37
VT5008-Gal4>UAS-Dlg RNAi 41134	ND	ND	100% n=11
VT5008-Gal4>UAS-Scribble RNAi 3229	ND	100% n=15	100% n=25
VT5008-Gal4>UAS-Scribble RNAi 105412	ND	100% n=16	100% n=25

"n" = number of adults tested, ND = not done

3.4.2 Knockdown of SJs components is effectless on larval locomotion speed

In the *Drosophila* larval stages, motor neuron control drives larval locomotion where the larva crawls via contraction of abdominal muscles. Locomotion tracking of larva is an assay to monitor behavioral changes that might result from with disruption of the larval nervous system (Caldwell et al., 2003). Our hypothesis for the larval locomotion tracking assay was similar to that for the PER assay. We predicted that lack of each SJ component from the motor axon would result in a reduction of larval locomotion speed. We used the same RNAi lines (Table 3.2) and crossed these to the OK6-Gal4 driver, which drives expression in all peripheral motor neurons.

OK6-Gal4 crossed to w1118 was used as the control. If RNAi mediated knockdown disrupts the BNB at the distal end of the axon, we predicted that the breakdown of the BNB might decrease the action potential conductance in the abdominal nerves leading to loss of locomotion and perhaps paralysis. In this experiment, we measure locomotion speed for individual larva using a worm tracking system (Swierczek et al., 2011). The average of locomotion speed at a defined time was compared between each genotype (OK6-Gal4 X UAS-RNAi) with the control (OK6-Gal4 X w1118). In the first attempt, we used larva raised at room temperature (18℃-21℃), but no significant differences were observed in larval speed between any of the RNAi crosses and the control (Fig 3.1, Table 3.4). For the next experiment, larva raised at 25℃ were used, but again there was no significant difference indicated in the locomotion speed between the RNAi lines and the control (Fig 3.2, Table 3.5).

Overall, these results indicated that RNAi knockdown of SJ components in all motor axons had no effect on larval locomotion and suggested that propagation of electrical impulses along the peripheral nerves was not affected. This suggests that SJs may not be present between glia (SPG) and axons at the axon terminus in peripheral nerves, or that the BNB disruption was not paired with a change in animal behavior.

Table 3.4 Larval locomotion speed in larvae raised at room temperature

n	Genotype	mean	P Value	Result
66	W1118 × OK6-Gal4	3.83		Normal
23	OK6-Gal4>UAS-NRXIV RNAi 8353	2.994	0.0993	ns
52	OK6-Gal4>UAS-NRXIV RNAi 108128	3.138	0.0536	ns
56	OK6-Gal4>UAS-NRXIV RNAi 3142	3.159	0.0637	ns
12	OK6-Gal4>UAS-NRG RNAi 6688	3.03	0.2415	ns
25	OK6-Gal4>UAS-NRG RNAi 3151	4.505	0.1954	ns

“ns” means average speed not significantly different than controls
“n” means number of larvae. Significantly different $P<0.05$
`

Table 3.5 Larval locomotion speed in larvae raised 25°C

n	Genotype	mean	P Value	Result
73	W1118 × OK6-Gal4	3.069		Normal
102	OK6-Gal4>UAS-NRXIV RNAi 8353	2.993	0.7699	ns
47	OK6-Gal4>UAS-NRXIV RNAi 108128	2.971	0.7329	ns
56	OK6-Gal4>UAS-NRG RNAi 6688	3.076	0.9818	ns

"ns" means average speed not significantly different than controls
"n" means number of larvae. Significantly different $P<0.05$

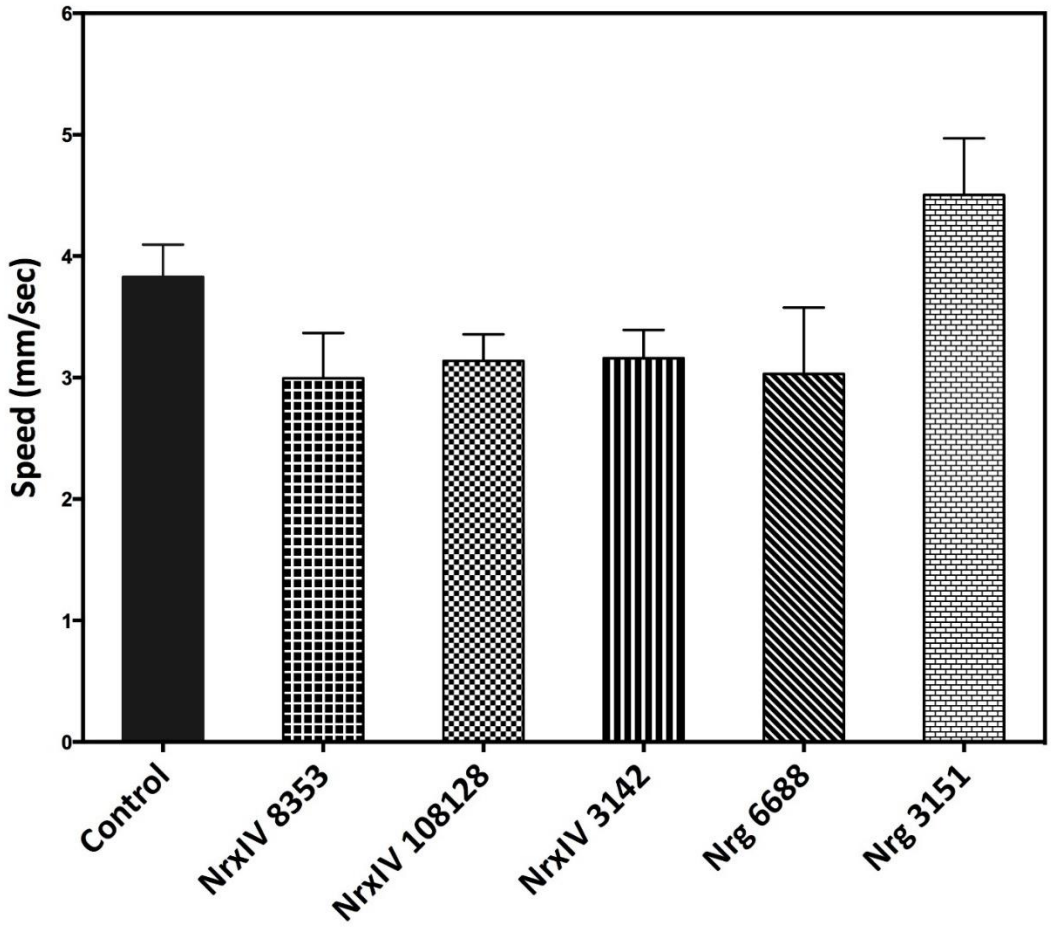

Figure 3.1 Knockdown of SJs components had no impact on larval locomotion speed at room temperature.
The average instantaneous larval speed of control (OK6-GAL4) versus OK6>RNAi lines. Each plot represents 3rd percentiles, whiskers indicate the standard error of the mean, larval speed presented (Table 3.4). Knockdown of each RNAi alone did not result in a significant difference in speed locomotion in comparison with the control.

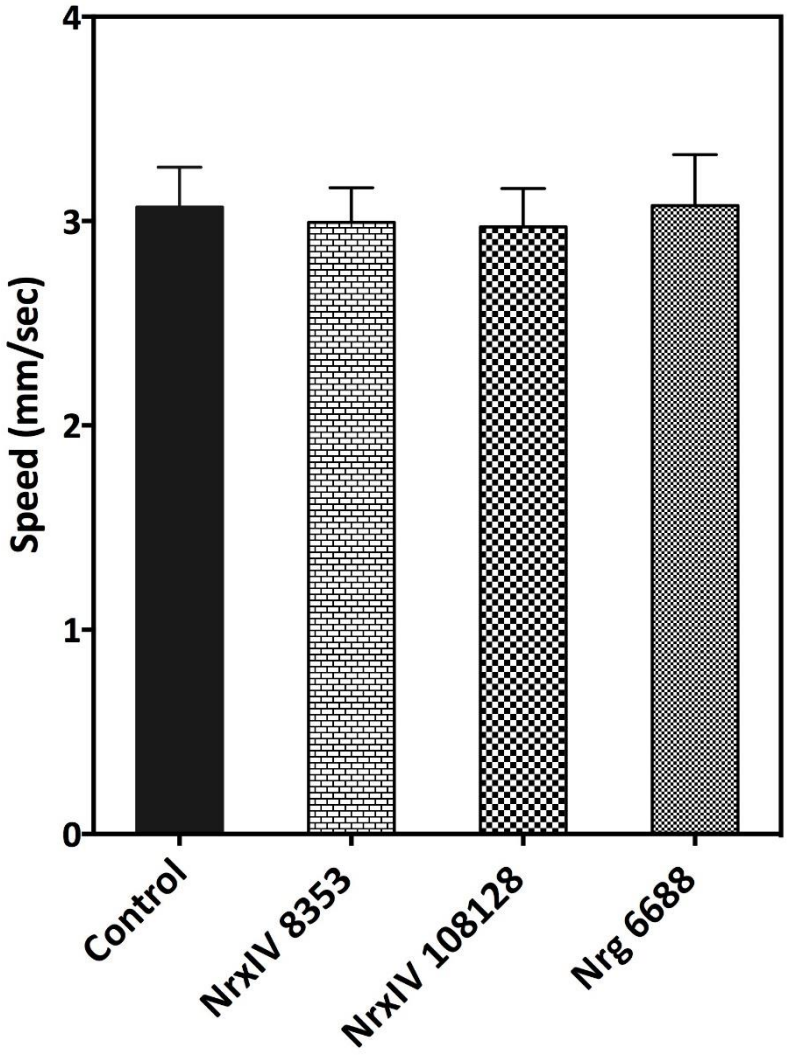

Figure 3.2 Knockdown of SJs components has no impact on larval locomotion speed at 25°C.
The average instantaneous larval speed of control (OK6-GAL4) versus OK6>RNAi lines. Each plot represents 3rd percentiles, whiskers indicate the standard error of the mean(de Vries et al.), larval speed presented (Table 3.5). Knockdown of each RNAi alone did not result in a significant difference in speed locomotion in comparison with the control.

3.5 DISCUSSION

We predicted that a loss of SJ components in the axon may lead to defective PER, if SJs are formed between the SPG and axon membrane specifically at the distal end where it SPG must be sealed to the axon to form a proper BNB. The result of the PER experiments indicated

knockdown of SJ components in the axons had no effect on food taking behavior, even when raised at high temperatures to increase the effectiveness of the RNAi knockdown. Similar when SJ proteins were knockdown in the larval motor axons there was no effect on larval locomotion, even when raised at a higher temperature to increase the effectiveness of the RNAi knockdown. There are a number of possible explanations for these negative results. A simple explanation is that the RNAi lines utilized are not effective or that the GAL4 drivers were not strong enough to result in a sufficient knockdown of the SJ proteins. However, this is unlikely as the OK6-GAL4 has been used extensively to knock down a wide range of proteins (Aberle et al., 2002). Similarly, many of the RNAi lines we used have also been used in other contexts and are highly effective (Deligiannaki et al., 2015; Hall and Ward, 2016).

On the other hand, knockdown of NrxIV in neurons using the elav-Gal4 driver results in late embryonic lethality, where animal complete embryogenesis but fail to hatch (Zweier et al., 2009). However, whether these effects are CNS or PNS mediated is not certain given the ubiquitous neuronal expression of the elav-GAL4 driver. Therefore, while NrxIV might have a clear CNS function, in the PNS NrxIV may not be part of a complex that forms a SJ between the axon and glia. Another possibility is that if SJs are formed between the glia (SPG) and axons, it might be that the disruption of the BNB was not strong enough to disrupt locomotion. Finally, while there appears to be a barrier to dye permeation at the distal end of the motor axon, this barrier may not be formed by SJ or SJ-like components. Many other protein complexes could mediate a glia-axon interaction, including adherens junctions and gap junctions.

4 DISCUSSION

4.1 GLIA/AXON CONTACT AND SEPTATE JUNCTION DISTRIBUTION AT THE PERIPHERAL AXON TERMINI

4.1.1 GRASP as a marker of glia/axon contact

Paranodal and sepate junctions are highly conserved structures that contain a core complex of proteins, Caspr, Contactin, NF1-55 in vertebrates and NeurexinIV, Neuroglian, and Contactin in *Drosophila* (Tepass et al., 2001). Prior studies indicated both Neuroglian and NrxIV are expressed in both neurons and glia in *Drosophila* (Snow et al., 1989; Baumgartner et al., 1996). In the peripheral nerve, NrxIV is a known marker for glial septate junctions (SJs). However, it is unknown whether a septate junction is formed between glia and axon membranes similar to the paranodal junction. We used the GRASP assay to analyze direct connections between glial layers and axon membrans. As a complementary experiment, we tested SJ localization at the distal end of the peripheral nerve. We hypothesized the BNB must be established along the nerve, along each motor axon branch and finally form a parandoal-like loop at the distal end of each motor axon prior to the NMJ.

In the *Drosophila* PNS, axons are surrounded by three subtypes of glia including PG, SPG and WG. Using GRASP, we observed glia-axon contact along the length of the peripheral nerves in both adult and larva. Glia appear to directly contact axon membranes along the nerve, however, our experiments did not distinguish which layer of glia are contacting the axonal membrane. The innermost layer of glia, the wrapping glia (WG) is assumed to be in contact with axon membrane based on prior work and TEM analysis (Fig 1.2) (Stork et al., 2008). However, the SPG layer might also bind to the axonal membrane along and at the distal end of the axon. Although the transverse section of E49 and adult abdominal nerve (Fig 2.1 D, 2.2 C) indicated

similarity with larval nerve (Fig 2.1 B), the electron microscopic image is needed to detect the position of glia layers in profile of adult nerve.

GRASP also indicated that the glia extend processes into the NMJs in the adult, whereas in larva, extension of glia is confined to a region before the motor axon branches into the muscles. Previous studies revealed the glia which extend into the adult NMJs are a new subtype of glia called peripheral perisynaptic glia (PPG) (Strauss et al. 2015). The GRASP results indicated the adult NMJs were not entirely covered by the PPG, and it seems glial process are laid down one side of the boutons but not in the active zone. However, more experiments are needed to analyze localization of glia and active zone in the NMJs. The GRASP assay in combination with anti-Bruchpilot (Wagh et al. 2006) to lable active zones provide a way to distigush glia and active zones in boutons respectively. This experiment can indicate whether NMJs surrounded by PPG or glia partially cover one side of boutons far from the active zones.

4.1.2 Septate junctions are localized along the nerves

The SPG play a critical role and insulate the microenvironment of the PNS from the hemolymph via the formation of the BNB. SJs were detected along the length of adult and larval nerve using the SJ marker Neurxin IV tagged with GFP (NrxIV::GFP) and two patterns of expression were detected: linear and annular. The linear pattern of SJ is formed between two membrane edges of a single SPG, whereas for the circular pattern SJs are formed in at the junction between two SPG as a loop all around the nerve. This result confirmed prior observations that SJs are formed in SPG layer (Banerjee et al., 2006b) but can not support the idea whether SJs exist between glia and axons or not.

The expression of SJs was confined to the end of the motor axon and did not extend into the NMJs in either adult or larva. At the distal end of the motor axon expression of SJs

terminated prior to the axon branching into NMJs and the formation of the synaptic boutons. In particular, the SJ presence was limited to the membrane process of the SPG, thereby the results confirm SJs are confined in SPG in both adult and larval PNS. We found SJ expression terminates prior to branching of the NMJs by forming the blunt or tapered ends. This matches prior observations on the distribution of the SJ and the site of the BNB in motor axons (Brink et al., 2012). Given the distribution of NrxIV, we assume the SPG attached to the axon membrane via SJs at the distal end of the axon, suggesting SJs component could be supplied by both glia and axons. However, we still do not know if the end of the SJ along the distal motor axon is responsible for the formation of a BNB at this point.

The lack of SJ and SPG in the adult NMJ suggests a question: Is this a lack of SJ or SPG or a lack of adequate markers? This suggest that similar to the larval NMJ, there is no BNB within the adult NMJ and that like larva the BNB is formed at the end of the motor axon. It is possible that the PPG can establish a barrier to in the adult NMJ and this would require junctional complexes other than SJ proteins. The PPG can make glial-muscle contact (Strauss et al., 2015) and we have shown that the PPG makes glia-neuron contact via other junctions.

4.1.3 Knockdown of septate junctions components does not disrupt larval locomotion

Previous studies revealed that the BNB is essential for the proper activity of the nerve, as when the BNB is disrupted animals are paralyzed (Auld et al., 1995; Baumgartner et al., 1996; Deligiannaki et al., 2015). We hypothesized knockdown of SJ components in the axons would lead to a disrupted BNB which causes a defective PER and reduction of locomotion speed in adult and larva respectively. However, PER in adults and locomotion speed in larva were normal in knockdown of SJ proteins by RNAi in the motor axons. The result of behavioral experiments indicated that the lack of SJ proteins in the axon had no effect on the function of the nerve. Two

of the SJ components we tested are expressed in neurons, Neuroglian and Neurexin IV (Snow et al., 1989; Baumgartner et al., 1996). Our results suggest that these proteins are not mediating a SJ like complex between the axon and glial membranes. It is possible that at the axon terminus, the glial cells are attached to the axon via another type of junction. Another possibility for the lack of changes in animal behavior is that the loss of the SJ did not lead to a strong enough disruption of the BNB to have a physiological effect. To test for BNB disruption, the behavioral analyses could be paired with assays to test the degree of penetration of fluorescent dyes as used in prior studies (Brink et al., 2012). It is assumed penetration of dye at the distal end of axon could be dependent on the lack of SJ components between axon and glia membranes.

4.2 FUTURE DIRECTIONS

In the current project, we found evidence that indicates glial and axon membranes are attached together via septate junctions. However, while SJs were found to be present at the motor axon terminus we did not find evidence to support that the SJ create BNB at this point through the formation of an axon-glia paranodal like a junction. As outlined above, the fluorescent dye exclusion assay can provide a way to analyze penetration of the BNB in the lack of each septate junction components in the axon and this could be repeated using the RNAi mediated knockdown of SJ proteins in the motor axons. Increasing the efficiency of the RNAi using Dicer2 or increased temperature would be another approach to ensure that the SJ components in the axons were effectively reduced.

To test for the physical presence of an axon-glial septate junction we can adapt the GRASP technique. Specifically, the extracellular domain of NrxIV or Nrg could be tagged with the splitGFP11 sequence. In turn, this can be paired with CD4 tagged with splitGFP1-10

expressed in the glia or axons. We hypothesize that tagged NrxIV would then reconstitute the GFP only when in contact with the splitGFP1-10. Both NrxIV and Nrg are expressed in both neurons and glia and thus we would carry out the reciprocal assays such as expressing tagged NrxIV::splitGFP11 in the axons and then in the glia. Using this approach, we can identify if and where glia bind to the distal end of axons via SJs.

4.3 CONCLUSIONS

This project was an attempt for an understanding of the function of septate junctions in glia-axon communication in the PNS. We have highlighted the expression pattern of septate junctions in the *Drosophila* peripheral nerve in both adult and larva. Septate junctions are established as the linear and circular pattern along the nerves, and finally are stopped at the distal end of the axon. In both adult and larva, septate junctions did not progress into neuromuscular junctions, however a limited extension was detected in several tested larvae. The results indicate glia are directly in contact with axon membrane, thus no connective tissue or extra-cellular matrix exists between glia layers and axon membranes. Although the expression of septate junction components was knockdown during behavioral experiments, not significant results were observed. Either SJs do not mediate axon-glia based BNB or disruption of the BNB in the distal end of axons is not strong enough to cause a difference in behavior. Also, it is possible the used RNAi lines did not target SJ proteins in motor axons. We found the boutons are partially protected by glia in adult neuromuscular junctions but not in larva, however subperineurial glia progress and septate junctions were not detected within neuromuscular junctions. The result indicated subperineurial glia might attach to the axon membrane via septate junctions at the distal end of axon prior to the branching of NMJ.

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