2012-01-27

Role of Glia in Sculpting Synaptic Connections at the Drosophila Neuromuscular Junction: A Dissertation

Yuly F. Fuentes Medel
University of Massachusetts Medical School

ROLE OF GLIA IN SCULPTING SYNAPTIC CONNECTIONS AT THE DROSOPHILA NEUROMUSCULAR JUNCTION

A Dissertation Presented

By

Yuly Fuentes

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biological Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

January 27th 2012

Program in Neuroscience

ROLE OF GLIA IN SCULPTING SYNAPTIC CONNECTIONS AT THE DROSOPHILA NEUROMUSCULAR JUNCTION

A Dissertation Presented
By
Yuly Fuentes

The signatures of the Dissertation Defense Committee signify
completion and approval as to style and content of the Dissertation.

_____
Vivian Budnik, Ph.D. Thesis Advisor

_____
Marc Freeman, Ph.D. Thesis Advisor

_____
Eric Baehrecke, Ph.D. Member of Committee

_____
Patrick Emery, Ph.D. Member of Committee

_____
Hong-Sheng Li, Ph.D. Member of Committee

_____
Rob Jackson, Ph.D. Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets
the requirements of the dissertation committee.

_____
Michael Francis, Ph.D. Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies
that the student has met all graduation requirements of the school.

_____
Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

Program in Neuroscience
January, 27th 2012

ACKNOWLEDGMENTS

I would like to offer my sincerest gratitude to both of my thesis advisors, Dr. Vivian Budnik and Dr. Marc Freeman, for letting me work the project of my dreams. Thank you for your honesty, support and friendship. Thank you for giving me the freedom to make science happen.

I would like to thank the members of my Dissertation Defense Committee, Dr. Michael Francis, Dr. Eric Baehrecke, Dr. Hong-Sheng Li, Dr. Patrick Emery and Dr. Rob Jackson for a fun discussion, encouragement and attention to detail.

I would like to give thanks to the faculty of the Neurobiology Department at UMass Medical School for supporting my scientific initiatives outside the United States. In particular, I would like to thank Dr. Scott Waddell for making me believe I could do things I did not know I was able to do. I would also like to thank Dr. Steven Reppert for his caring feedback and support on my career development. I would also like to thank all the members of the Budnik and the Freeman laboratories, both past and present, for their contributions to my thesis research and my development as a scientist. They have been my family during these years and I owe gratitude to each of them in many different ways.

On a personal level, I would like to thank my husband Ricardo, for supporting each step of this endeavor, also to my family, my mother and father, Teresa and Luis, for letting me be. Lastly, I would also like to thank all my friends who have made my graduate studies a fun experience.

ABSTRACT

Emerging evidence in both vertebrates and invertebrates is redefining glia as active players in the development and integrity of the nervous system. The formation of functional neuronal circuits requires the precise addition of new synapses. Mounting evidence implicates glial function in synapse remodeling and formation. However, the precise molecular mechanisms governing these functions are poorly understood. My thesis work begins to define the molecular mechanisms by which glia communicate with neurons at the *Drosophila* neuromuscular junction (NMJ).

During development glia play a critical role in remodeling neuronal circuits in the CNS. In order to understand how glia remodel synapses, I manipulated a key component of the glial engulfment machinery, Draper. I found that during normal NMJ growth presynaptic boutons constantly shed membranes or debris. However, a loss of Draper resulted in an accumulation of debris and ghost boutons, which inhibited synaptic growth. I found that glia use the Draper pathway to engulf these excess membranes to sculpt synapses. Surprisingly, I found that muscle cells function as phagocytic cells as well by eliminating immature synaptic ghost boutons. This demonstrates that the combined efforts of glia and muscle are required for the addition of synapses and proper growth.

My work establishes that glia play a crucial role in synapse development at the NMJ and suggests that there are other glial-derived molecules that regulate synapse function. I identified one glial derived molecule critical for the

development of the NMJ, a TGF-β ligand called Maverick. Presynaptically, Maverick regulates the activation of BMP pathway confirmed by reducing the transcription of the known target gene Trio. Postsynaptically, it regulates the transcription of *Glass bottom boat* (*Gbb*) in the muscle suggesting that glia modulate the function of Gbb and consequently the activation of the BMP retrograde pathway at NMJ. Surprisingly, I also found that glial Maverick regulates the transcription of *Shaker* potassium channel, suggesting that glia potentially could regulate muscle excitability and consequently modulate synaptic transmission. Future work will elucidate such hypothesis.

My work has demonstrated two novel roles for glia at the NMJ. First is that glia engulfing activity is important for proper synaptic growth. Second is that the secretion of glial-derived molecules are required to orchestrate synaptic development. This further supports that glia are critical active players in maintaining a functional nervous system.

TABLE OF CONTENT

LIST OF FIGURES

FIGURES IN CHAPTER I

FIGURES IN CHAPTER II

FIGURES IN CHAPTER III

LIST OF ABBREVIATIONS

BMPs – Bone Morphogenetic Proteins

Brp – Bruchpilot

ChR2 – Channelrhodopsin-2

CSP- Cysteine string protein

CNS – Central nervous system

DLG – Disc-Large

Daw – Dawdle

Fas II – Fasciculin II

EAATs – Excitatory amino-acid transporters

Gbb – Glass bottom boat

GluR – Glutamate receptor

MAD – Mother Against Dpp

Mav – Maverick

MYO- Myoglianin

MN – Motorneuron

NMJ – Neuromuscular Junction

PSCs – Perisynaptic Schwann cells

P-Mad – phosphorylated-MAD

+RT – with reverse transcriptase

-RT – without reverse transcriptase

TGF-β –Transforming Growth Factor beta

Tkv -Thickvein

TTX – Tetrodotoxin

Wit- wishful thinking

Wg – Wingless

CHAPTER I

General Introduction

Basic aspects of neural circuit assembly

The nervous system is the most complex tissue in mammals and is comprised of millions of neurons and glia. Groups of neurons are assembled into circuits that, with the help of glial modulation, dynamically govern brain function and behavior. The basic unit of neuron→neuron communication is a specialized cell-cell junction termed the synapse. The presynaptic cell, when stimulated, releases chemical neurotransmitters at the synapse onto the postsynaptic cell. This process, termed synaptic transmission, results in the activation of specific receptors on the postsynaptic cell and ultimately postsynaptic cell firing. This process occurs in a timescale of milliseconds, allows for the sequential activation of a circuit of cells, and its molecular basis and regulation has been a focus of study for many years.

The assembly of neural circuits during development occurs in three discrete steps. First, axons and dendrites sprout from newly born neurons and extend to their appropriate target fields. Second, upon finding the appropriate pre- or postsynaptic target cell, neurons initiate the process of synapse formation (synaptogenesis). This process is often exuberant, and leads to the production of an excessive number of synapses. Finally, through activity-dependent mechanisms neural circuits are refined—excess axons, dendrites, and synapses are pruned, and functional connections are maintained.

Each of the above steps in neural circuit formation involves an amazing synchronization of cellular and molecular events. During the period of axon

pathfinding, the cell is investing an incredible amount of energy to extend the length of the axon, while the growth cone is carefully following chemical cues to reach the appropriate target (Bashaw and Klein, 2010; Evans and Bashaw, 2010). At the initiation of synaptogenesis, a number of cell adhesion molecules such as Fas II, are responsible solidifying connections, while at the same time scaffolding proteins such as DLG, the homologous protein of mammalian PSD-95, bring the appropriate receptors and ion channels to the membrane (Guan et al., 1996). The coordination of this event in particular has been a major focus of study and excellent progress has been made toward understanding the molecular basis of synapse assembly. Interestingly, neural activity itself is a key regulator of circuit refinement, which allows experience or changing environmental conditions to shape circuit development of plasticity. As an example, the role of activity in regulating growth of the neuromuscular junction is discussed in detail below.

The molecular pathways that modulate neural circuit refinement in the context of synaptic and neurite pruning remain poorly defined. How are specific synapses, axons, or dendrites selected for elimination? Once chosen, how do they undergo programmed self-destruction? After degradation, how is neuronal debris removed from the nervous system? What would be the consequence of failure of debris clearance? Our lack of knowledge on these topics is quite remarkable since refinement mechanisms are thought to play critical roles in the

functional maturation of neural circuits and complex brain functions such as behavior or learning and memory.

Mammalian glial cells in synapse formation

For years, glial cells were thought to provide simple physical and trophic support for neurons. However, recent studies support the notion that glial cells are critical regulators of nervous system development and function, particularly with respect to synapse development and plasticity.

In the mammalian central nervous system (CNS) there are four major glial subtypes, microglia, oligodendrocytes, $NG2^+$ glia, and astrocytes. Microglia are the major immune cell type in the CNS, responding to trauma, engulfing dead cells or degenerating axons, and generally overseeing brain health (Saijo and Glass, 2011). Oligodendrocytes are the major wrapping glial subtype in the CNS, known to ensheath the nerves, and in many cases form myelin sheaths that dramatically enhance nerve conduction velocity (Fancy et al., 2011). $NG2^+$ cells remain a mysterious pool of glia found throughout brain, whose function is not well understood, but appear to act as precursors for oligodendrocytes in the mature CNS (Richardson et al., 2011). Finally, astrocytes are intimately associated with synapses in the brain and appear to play critical roles in synaptic signaling in the recently described tripartite synapse (Halassa and Haydon, 2010; Zhang and Barres, 2010).

In the mammalian peripheral nervous system there is one primary glial subtype termed Schwann cells. Schwann cells ensheath axon bundles, in many cases myelinate axons, and support the survival of long axons. At the neuromuscular junction (NMJ) Schwann cells are tightly associated with the presynaptic motor neuron endplate, covering it entirely (Brill et al., 2011). The most-distal Schwann cell which is associated with the NMJ, termed the perisynaptic (or terminal) Schwann cell, is thought to be a specialized Schwann cell that modulates NMJ growth and synaptic physiology(Griffin and Thompson, 2008; Todd et al., 2010).

Astrocytes appear to be critically important for the formation of synaptic contacts during neural circuit assembly (Eroglu and Barres, 2010). For example, when retinal ganglion cells (RGCs) are cultured *in vitro* on their own they form very few synapses. However, when RGCs are grown beneath an astrocyte feeding layer, the amount of mature synapses increases dramatically. Since in these cultures astrocytes and neurons had no physical contact, and it was found that astrocyte-conditioned medium was sufficient to promote synapse formation, these studies argued that soluble glial-derived factors promote formation of mature synapses (Pfrieger and Barres, 1997; Nagler et al., 2001; Ullian et al., 2001). Fractionation of astrocyte conditioned medium ultimately revealed thrombospondins (TSPs) as key glial derived synaptogenic factors (Christopherson et al., 2005). Interestingly, while TSPs were found to induce morphologically normal synapses, TSP-induced synapses were post-synaptically

silent, lacking their normal AMPA receptor-mediated response (Christopherson et al., 2005). Subsequent studies of astrocyte-expressed matricellular proteins led to the identification of Hevin and SPARC, which regulate the formation of synapses *in vivo*. Hevin promotes the formation of morphologically and functionally mature synapses, while SPARC acts as a negative regulator of Hevin function (Kucukdereli et al., 2011). Together, these studies provided direct evidence supporting the notion that astrocyte-secreted molecules can promote synapse formation. However, key questions regarding how these or other molecules modulate synapse formation and plasticity *in vivo* await clarification.

Neurite pruning in neural circuit refinement

Axonal pruning has been used as an excellent model to study the cellular and molecular biology of neural circuit refinement. Axon pruning can occur through either retraction, where short axonal projections are simply resorbed by the parent arbor, or through wholesale degeneration (degenerative pruning), where a portion of the axon degenerates and is cleared from the nervous system (Luo and O'Leary, 2005). A well studied example of axonal pruning during mammalian nervous system development is the elimination of subsets of axons in layer 5 of the neocortex (O'Leary and Koester, 1993; Portera-Cailliau et al., 2005). Similarly, *Drosophila* mushroom body gamma neuron axons undergo pruning at metamorphosis (Watts et al., 2003; Freeman, 2006). In this latter case, elimination of the larval-specific axonal arbors and growth of new adult-specific

projection axons are thought to be essential for proper circuit connectivity to be achieved in the mature adult brain. Pruning events are not limited to entire axons. Pruning can also occur at the level of individual synapses in mammals by morphological and molecular criteria, thereby leading to the elimination of very specific synaptic connections (Eroglu and Barres, 2010; Chung and Barres, 2011).

Roles for glia in axonal and synaptic pruning

The majority of neurons in the mammalian nervous system initially generate excessive axonal and dendritic projections, and synapses, and subsequently eliminate exuberant neural connections through developmental pruning (Luo and O'Leary, 2005). Pruning of circuits through resorption of excessive neural projections is not thought to require glial cells, but this has not been thoroughly investigated. On the other hand, glial cells are important cellular players in degenerative pruning, which entails two steps. First, axons or dendrites degenerate, producing neural debris. Next, glial cells engulf neural debris to clear it from the CNS. Such clearance is thought to be critical to suppress inflammatory responses in the nervous system, and auto-immunity (Barres, 2008). A clear cellular role for glial cells in engulfing pruned axons and dendrites was first shown in *Drosophila* (Watts et al., 2003; Freeman, 2006). Surprisingly, which CNS cells engulf pruned neural material in the mammalian brain has remained a mystery. However recent work has shown that microglial cells likely

engulf pruned synaptic material in the CNS (Paolicelli et al., 2011), and it is likely they are also responsible for engulfing degenerating axons and dendrites. Whether engulfment plays an active role in promoting axonal, dendritic, or synaptic degeneration/pruning (i.e. telling which parts of the neuron to die) remains unclear. However, engulfing microglia appear to actively promote the apoptotic death of a large number of Purkinje neurons during development (Marin-Teva et al., 2004), raising the possibility that glia might be instructive in sculpting connectivity through directed elimination of specific neural connections.

The perisynpatic Schwann cell appears to play a critical role in the elimination of exuberant motorneuron inputs during NMJ development. Briefly, early in development muscle fibers are poly-innervated by multiple motorneurons (MNs). During refinement of muscle fields a single MN maintains an input, and additional MN axons undergo a distal-to-proximal retraction. During this retraction MN axons shed membrane-enclosed “axosomes” at the distal ends, which are pinched off and engulfed by surrounding Schwann cells (Bishop et al., 2004). These observations form the basis for the suggestion that Schwann cells may in fact cause axosome shedding and are the driving force behind “loser” MN process retraction, rather than the MN itself.

The role of Schwann cells in forming, maintaining, and repairing motor neuron terminals

The study of vertebrate perisynaptic Schwann cells (PSCs) has contributed in important ways to understand the key physiological functions of glial cells on synaptic growth/modification. Anatomically, PSCs cover the entire MN terminal and PSC membrane extensions appear to lead MN outgrowth during normal development (Reddy et al., 2003). While initial formation of synapses does not appear to require PSCs (Kullberg et al., 1977; Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999), acute ablation of PSCs in frog strongly suppressed MN outgrowth at the NMJ and resulted in the retraction of MN processes approximately one week after PSC ablation (Feng et al., 2005).

After denervation of a single NMJ, local MNs in the area will sprout processes and re-innervate the vacant motor endplate (Lu and Lichtman, 2007). However, PSCs appear essential for this event, and in fact prefigure all MN process growth—it appears the MNs will only grow toward vacant endplates when PSCs provide a so-called “bridge” (Love and Thompson, 1999). Interestingly, PSC outgrowth to form the bridge after denervation appears to be an activity-dependent process, requiring both pre- and postsynaptic signaling (Love and Thompson, 1999; Love et al., 2003). Together these data suggest that Schwann cell processes are essential for MN outgrowth and stabilization. However the molecular pathways involved in these events remain to be identified.

The *Drosophila* NMJ as a new model to study neuron-glia signaling

The *Drosophila* NMJ has been used as model to study synaptic function since the mid 1970's, when investigators from USA and later from Russia started describing its basic physiological and pharmacological properties (Jan and Jan, 1976; Magazanik and Vyskocil, 1979). A major advantage of this preparation is its experimental accessibility with respect to the synaptic function: the *Drosophila* NMJ can be easily analyzed morphologically and electrophysiologically. Work over the last twenty years has shown that the molecular constituents of the mammalian glutamatergic central synapse and *Drosophila* NMJ are well conserved (Koh et al., 2000), although NMJ synapses of course represent connections between a presynaptic motorneuron and a muscle cell rather than two neurons. The *Drosophila* NMJ has the important advantage of being a powerful genetic system to investigate glutamatergic synapse function and has thus been an avenue for the discovery of new molecules involved in the development, physiology, and plasticity of synapses.

Anatomy of the *Drosophila* NMJ

In 1982 Jan and Jan reported that antibodies against Horse Radish Peroxidase (HRP) recognize neuronal tissue in *Drosophila*. HRP stains have been a great tool for the analysis of NMJ morphology since it is specific for the presynaptic compartment (See Figure 1-1,B). Additional presynaptic markers include a number of synaptic vesicle associated proteins such as cysteine string protein

(CSP) (Dawson-Scully et al., 2007) (See Figure 1-1,E) or synaptotagmin (Yoshihara and Littleton, 2002). The presynaptic side of boutons contains the sites for neurotransmitter release (active zones) and these are arranged along the NMJ. Active zones can be detected with the monoclonal antibody nc82 (See Figure 1-1,D) that it is believed to recognize the protein Bruchpilot (Wagh et al., 2006).

Postsynaptic marker proteins are clustered to specific locations within the subsynaptic reticulum (SSR), and include DLG (Guan et al., 1996), Fas II (Thomas et al., 1997), and the Shaker potassium channels (Tejedor et al., 1997). While DLG is found both at the pre- and postsynaptic membranes, it appears to be most abundant postsynaptically (Lahey et al., 1994) (See Figure 1-1,B). Glutamate Receptors (GluRs) are organized into clusters directly apposed to Brp^{+} active zones (See Figure 1-1,D) presumably to ensure an efficient synaptic release (Qin et al., 2005).

In contrast to vertebrate NMJs, at the glutamatergic larval NMJ, terminal glial cells do not appear to cap the entire synaptic arbor. Instead, NMJ arbors are buried within the muscle surface, which wraps around the boutons (Banerjee et al., 2006; Parker and Auld, 2006). Glial cells were previously reported to be absent from the NMJ, terminating at the point at which the MN enters the muscle. However, I show in Chapter 2 of this thesis that this is incorrect and that *Drosophila* glial cells dynamically associate with the NMJ.

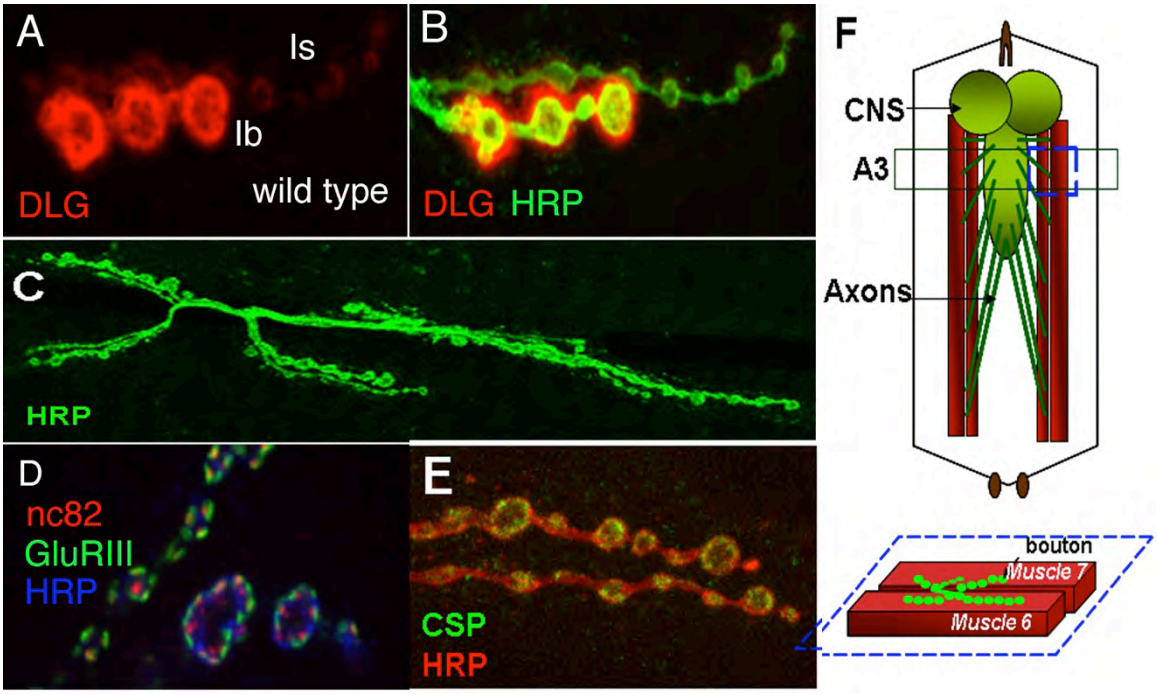


Figure 1-1: Third instar larval NMJs synaptic markers. Wild type preparations labeled with (A) anti-DLG (red), (B) co-label anti-HRP (green) with DLG (green), (C) an entire muscle 6 and 7 wild type branch stained with HPR (green) (D) anti-HRP (blue), anti-GluRIII (red), and nc82 (green) and (E) Cysteine string protein (CSP) (green) co-label with HRP (red). (F) cartoon of body wall muscle 6/7 (red) anatomy with relation to central nervous system (green) below a representation of synaptic outgrowth shown in C

Development of the *Drosophila* NMJ

During embryonic development motorneurons begin to extend their axons to a precise target muscle cell. By late embryonic development, synapses have formed. During the larval stage they begin to differentiate into characteristic presynaptic structures (synaptic boutons), which increase in size and number in relation to muscle growth (Budnik, 1996). During larval stages the NMJ (MN and postsynaptic muscle cell) undergoes a remarkable amount of growth. In only ~3.5 days it increases in volume ~100-fold. Such dramatic expansion of the NMJ requires rapid and efficient growth, and has made the *Drosophila* NMJ a very appealing model for developmental synaptic plasticity (Griffith and Budnik, 2006; Ruiz-Canada and Budnik, 2006).

Glial cells that wrap the peripheral nerves derive largely from CNS precursors. In addition to the axons navigating out of the CNS, a group of glial cells migrate along with the axons and wrap the axon bundles (See Figure 1-2,B) (Sepp et al., 2001; Edenfeld et al., 2007). Glial cells do not appear to contact the NMJs before the first larvae stage, and ablation of peripheral glia does not inhibit MNs from finding the appropriate target cells (Sepp et al., 2000), indicating that the MN growth cone can find its postsynaptic target in the absence of glia.

The role of neural activity in *Drosophila* NMJ growth

Previous studies using intact third instars larvae have demonstrated that as the larva develops in size, new synaptic boutons are added through the process of budding (Zito et al., 1999). Many of the new synaptic boutons are added at the end of a NMJ branch, where a bud forms, is stabilized, and matures into a new bouton (Torroja et al., 1999; Zito et al., 1999). In postsynpatic muscle cells, the subsynaptic reticulum (SSR) increases in size and complexity to accommodate these increases in bouton number and size (Guan et al., 1996).

The Budnik laboratory has used the *Drosophila* NMJ extensively to explore the molecular mechanisms of synaptic growth. In particular, we have focused on live-imaging of growing NMJs while manipulating various aspects of synaptic activity. For example, using an open fillet preparation we can directly image synapses in live preparations while exposing the NMJ to treatments such as potassium pulses (for massive depolarization), or directly voltage clamping and stimulating nerves electrophysiologically. Conveniently, the very same preparation can be fixed and stained for synaptic molecular markers to correlate molecular changes with changes in cellular morphology or physiology (Ataman et al., 2008). In chapter one, I will describe a genetic approach to stimulate synaptic activity by expressing the light activated channel Channel Rhodopsin 2 (Zhang and Oertner, 2007), and explore both how activation of the MN affects axon stability and how glial control NMJ morphogenesis (Fuentes-Medel et al., 2009).

Subtypes of *Drosophila* glia in the peripheral nerve

Drosophila peripheral glia form a multi-layered sheath around the peripheral nerves consisting of three glial subtypes: wrapping glia, which are directly associated with axonal tracks; subperineurial glia, which form the blood brain barrier around the entire peripheral nerve; perineural glia, which lay at the surface of the nerve and are believed to secrete the neural lamella, a protective carbohydrate sheath that encapsulates the peripheral nerve (see Figure 1-2) (Stork et al., 2008).

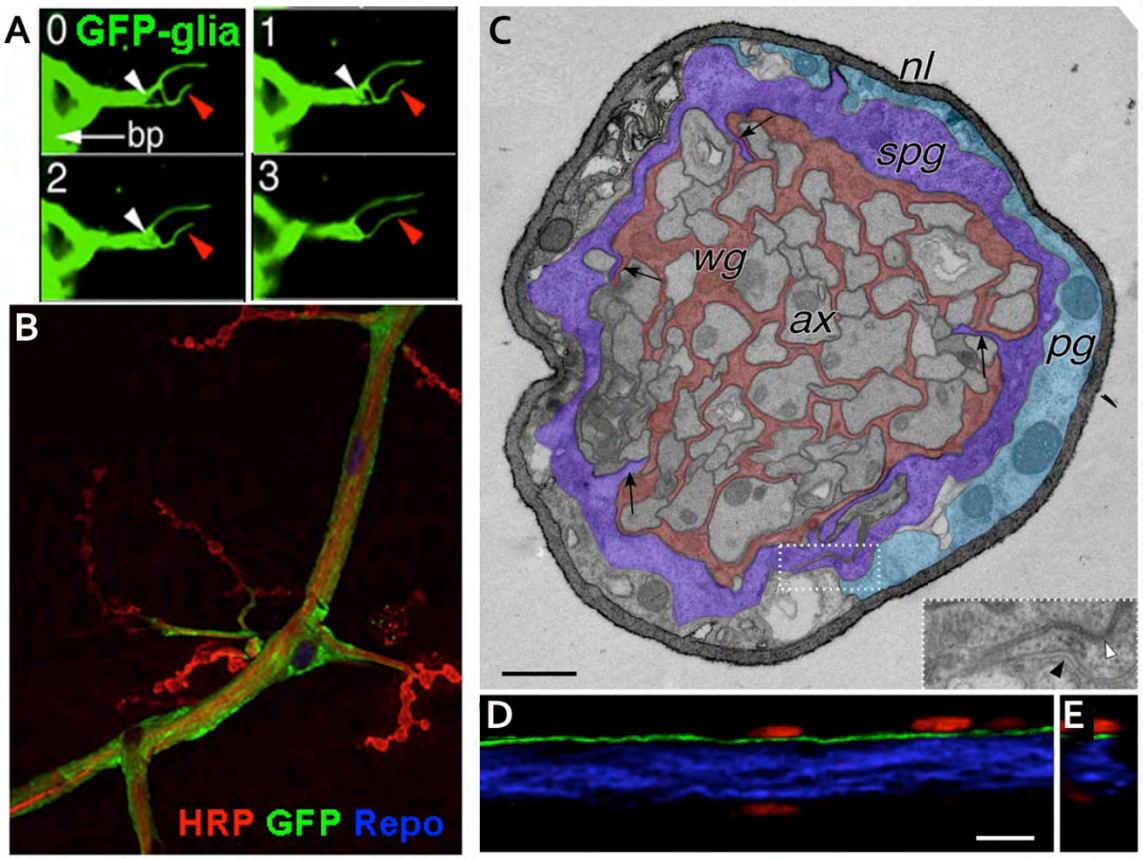


Figure 1-2

Figure 1-2: Glial cells in the larval nervous system of Drosophila.

(A) Time-lapse imaging of glial extensions in a preparation expressing mCD8-GFP in glial cells over the course of 6 min. 0,1,2,3 represent time points. bp= NMJ branch point. White arrowheads: lamellipodia like extensions. Red arrowheads: gliopods. (B) close magnification of a third instar larvae peripheral nerve and synatic boutons stained with HRP (red), Glial-GFP (green) and Repo (blue). (C) Electron micrograph of a third larval instar peripheral nerve, (nl) neural lamella, (pn) perineurial glial (highlighted by light blue shading), (spg) subperineurial glia, arrows, the cell is labeled in blue (D) Third instar larval nerves were stained for HRP (blue), Repo expression (red), and Dlg:GFP expression (green) under the control of the SPG:Gal4 driver. Scale bar, 1 **u**m. (E) orthogonal view of nerve from (Stork et al., 2008)

Drosophila glia sculpt the nervous system

During *Drosophila* nervous system remodeling glial cells play a critical role in the reorganization of neural circuits. The *Drosophila* mushroom body gamma neurons have been used as an excellent model to explore neuron-glia interactions during pruning events. During axonal pruning at metamorphosis glial cells have been shown to invade the dorsal and medial lobes of the mushroom body, axons subsequently fragment, and then axonal debris is rapidly cleared from the CNS through glial engulfment activity (Awasaki and Ito, 2004). Axonal fragments within invading glial cell types have been identified in electron microscopic studies that employ genetically encoded markers for axons or glia (Watts et al., 2003), and genetically inhibiting glial endocytosis during metamorphosis has been shown to block the clearance of the dorsal and medial branches of the mushroom body (Awasaki and Ito, 2004). It has been proposed that engulfing glia may play an active role in axonal destruction based on this latter observation, however this idea remains controversial and has not been rigorously tested. Consistent with a pro-degenerative role for glia in *Drosophila*, a recent study by Keller *et al.* revealed in a model for progressive synaptic retraction that peripheral glia actively release the TNFα-like molecule Eiger, which activates MN-expressed Wengen to initiate caspase-dependent degradation of MN terminals (Keller et al., 2011).

A direct role for mammalian astrocytes or microglia in developmental axon pruning has not been demonstrated, but appears highly likely based on the fact

that these cell types clear axonal debris from the mammalian CNS in many other contexts (Aldskogius and Kozlova, 1998; Davalos et al., 2005). For example, at later developmental stages glia selectively eliminate subpopulations of neurons through their phagocytic activity (Marin-Teva et al., 2004). Very recently microglia have also been shown to engulf synapses that are eliminated through developmental pruning in the mouse CA1 striatum radiatum. Interestingly, suppressing microglial engulfing activity leads to an accumulation of $PSD95^{+}$ puncta, and a delay in the maturation of neural circuits, suggesting that glial pruning of synaptic material is essential for final maturation of circuit function (Paolicelli et al., 2011).

The Draper signaling pathway mediates glial engulfment of neuronal debris

Draper, the *Drosophila* orthologs of the engulfment receptor *C. elegans* CED-1, has been shown to be a central component of the glial engulfment machinery (Freeman et al., 2003a). In *C. elegans* CED-1 is required in engulfing cells for the phagocytosis of cell corpses and it is thought to act as a recognition receptor for cues presented by cells undergoing apoptotic death (Hamon et al., 2006). CED-1 engagement with an unidentified ligand on the engulfment target is thought to initiate downstream signaling through the PTB domain coiled-coil protein Ced-6 (Reddien and Horvitz, 2004) and subsequent corpse engulfment.

Drosophila glial cells have long been known to be the primary engulfing cell type in the nervous system by EM studies (Sonnenfeld and Jacobs, 1995) .

Previous work from the Freeman laboratory demonstrated that nearly all glia express Draper, and that neuronal cell corpses accumulate in *draper* mutant embryos (Freeman et al., 2003a). More recently, Draper has been shown to be required for the clearance of severed axons undergoing Wallerian degeneration (MacDonald et al., 2006), and the timely removal of developmentally pruned mushroom body axons by glia (Awasaki et al., 2006; Hoopfer et al., 2006). Draper, like CED-1, appears to signal through dCed-6 to activate engulfment (Doherty et al., 2009).

The Draper/CED-1 signaling pathway appears to be well-conserved in mammals. The mammalian genome contains two potential Draper orthologs, MEGF10 and Jedi. The mammalian receptor MEGF10 has been shown to partially rescue Ced-1 phenotype in C. elegans (Hamon et al., 2006). Moreover, MEGF10 and Jedi are highly expressed in satellite glial cells in dorsal root ganglia as they are engulfing neuronal cell corpses, and knockdown of MEGF10 or Jedi inhibits engulfment of cell corpses in mammalian cell culture (Wu et al., 2009).

TGF-β signaling pathways regulate NMJ growth

The coordinated growth of both pre- and post-synaptic compartments at the NMJ is critical for maintenance of synaptic signaling. Balancing the growth of these two compartments is accomplished by both anterograde (neuron→muscle) and retrograde (muscle→neuron) signaling pathways. A key regulator of retrograde

signaling in *Drosophila* is the Bone Morphogenetic Protein (BMP) family of signaling molecules and their receptors (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003; Rawson et al., 2003).

Expansion of presynaptic terminals is regulated by the release of the *Drosophila* BMP ligand Glass Bottom Boat (Gbb) from the growing muscle cells. Gbb binds to the type I BMP receptors Thick veins (Tkv) or Saxophone (Sax) and type II BMP receptor, Wishful thinking (Wit), at presynaptic terminals (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003; Rawson et al., 2003). Ligand-dependent activation of type I and type II BMP receptors leads to phosphorylation of the intracellular transcription factor Receptor Activated Smad (R-Smad), which in *Drosophila* is called Mothers Against Dpp (Mad). Phosphorylated-R-Smad then binds to a co-Smad termed Medea, thereby forming a transcriptionally active complex (Mad/Medea). The Mad/Medea complex is imported into the nucleus where it regulates the transcription of BMP target genes including *trio* (Moustakas and Heldin, 2009). Consistent with this model, mutations in *tkv*, *sax*, *wit* and *gbb* result in NMJs with reduced numbers of synaptic boutons, a decrease in the size of evoked responses after electrophysiological stimulation of MNs, and decreased signals in embryonic motorneuron nuclei when they are stained with antibodies that cross-react with phosphorylated-MAD (P-Mad) (Marques et al., 2002; McCabe et al., 2003; Rawson et al., 2003). Retrograde signaling to the nucleus also requires retrograde axonal transport in MNs, since genetic blockade of retrograde axonal

transport also reduces P-Mad signals in MNs. The mechanism by which the translocation of P-Mad into the nucleus is translated into changes in synaptic growth and function remains unclear despite intense research in this area. However, Ball and colleagues have recently demonstrated that TRIO transcription is controlled by the retrograde BMP pathway, and together with Rac, is involved in presynaptic growth and regulation of neurotransmitter release (Ball et al., 2010).

Although BMPs have been best studied for their role as retrograde regulators, several BMP receptors and P-Mad are also found in *Drosophila* larval muscles (Dudu et al., 2006), suggesting that BMP regulation is more complex, and likely to control a variety of synaptic mechanisms beyond retrograde control. Indeed, recent studies have uncovered a role for the type I Activin-type BMP receptor, Baboon (Babo), in larval muscles in controlling the transcription of Gbb (Ellis et al., 2010a). Although the exact source of the BMP ligand in this case, Dawdle, is not clear, these experiments suggest that BMP signaling in the muscles themselves might regulate the retrograde activity of BMPs.

Significance of the work in this thesis

Despite the widespread observation that glial cells are closely associated with synapses in both the CNS and PNS, we know very little about the cellular and molecular roles for glial cells in synaptic growth and function. A central question regarding glial function is whether or not glial cells actively regulate synaptic

growth and circuit formation, or if they simply support neuronal growth and allow circuits to form. (The correct answer is probably both.)

In the second chapter of this thesis, I have explored how glial engulfment activity modulates the growth of synaptic fields. Briefly, I show that growing NMJ synaptic fields shed an amazing amount of presynaptic debris. Glial cells invade the NMJ, and engulf this debris (in collaboration with muscles) using the Draper signaling pathway. By maintaining the NMJ in this way, glial clear out presynaptic debris which, if left in the synaptic field, severely retards new synapse addition. My work therefore shows that maintenance of the NMJ is a critical function for glia, and is essential for normal synaptic growth.

In the third chapter of this thesis, I have addressed the question of whether or not *Drosophila* glial cells secrete factors that actively modulate synaptic growth. I show that peripheral glia secrete the TGF-β molecule Maverick, which acts on muscles to stimulate presynaptic changes in gene expression, and the addition of new boutons. In addition, I identified new targets of postsynaptic TGF-β signaling downstream of glial Maverick including Gbb and the TGF-β inhibitor molecule DAD. Remarkably, in the absence of glial Maverick I observe reduced transcription of *Shaker*. Thus, glial-derived signaling molecules are indeed critical is for the coordinated growth of the postsynaptic muscle and the motorneuron at the NMJ.

CHAPTER II

Glia and muscle sculpt neuromuscular arbors by engulfing destabilized synaptic boutons and shed presynaptic debris

Yuly Fuentes-Medel, Mary A. Logan, James Ashley, Bulent Ataman, Vivian Budnik*, and Marc R. Freeman*

CONTRIBUTION SUMMARY

All work described in this thesis was performed at University of Massachusetts Medical School in the lab of Marc Freeman and Vivian Budnik. In chapter II, Mary Logan made the Drpr rescue lines and contributed to analyze experiments and review the manuscript; James Ashley collaborate to perform live imaging experiments and review the manuscript; Bulent Ataman provided data to support the disappearance of ghost boutons. Marc Freeman and Vivian Budnik supported the work by discussions to design the experiments, analyzing the data and writing the paper. I designed and performed the experiments, analyzed the data and co-wrote the paper.

The following work is reprinted from the Plos Biology article of the same name published in August 2009.

ABSTRACT

Synapse remodeling is an extremely dynamic process, often regulated by neural activity. Here we show during activity-dependent synaptic growth at the *Drosophila* NMJ many immature synaptic boutons fail to form stable post-synaptic contacts, are selectively shed from the parent arbor, and degenerate or disappear from the NMJ. Surprisingly, we also observe the widespread appearance of presynaptically-derived “debris” during normal synaptic growth. The shedding of both immature boutons and presynaptic debris is enhanced by high-frequency stimulation of motorneurons, indicating that their formation is modulated by neural activity. Interestingly, we find that glia dynamically invade the NMJ and, working together with muscles cells, phagocytose shed presynaptic material. Suppressing engulfment activity in glia or muscle by disrupting the Draper/Ced-6 pathway results in a dramatic accumulation of presynaptic debris, and synaptic growth in turn is severely compromised. Thus actively growing NMJ arbors appear to constitutively generate an excessive number of immature boutons, eliminate those that are not stabilized through a shedding process, and normal synaptic expansion requires the continuous clearance of this material by both glia and muscle cells.

INTRODUCTION

The wiring of the nervous system, from initial axon sprouting to the formation of specific synaptic connections, represents one of the most dramatic and precise examples of directed cellular outgrowth. Developing axons navigate sometimes tortuous routes as they seek out the appropriate target cells. Once in their target area, interactions between axons and their potential targets are extremely dynamic, attempts are made to identify appropriate postsynaptic partners, and initial synaptic contacts are established (Walsh and Lichtman, 2003; Lohmann and Bonhoeffer, 2008)(reviewed in (Lu et al., 2009)). A next critical step in the formation of functional neural circuits is the remodeling of initial patterns of connectivity. To facilitate the elaboration and refinement of developing neural circuits synaptic partners often remain highly responsive to their environment and add or eliminate synaptic connections (Luo and O'Leary, 2005; Alvarez and Sabatini, 2007), frequently in an activity-dependent fashion, presumably to fine-tune connectivity to specific activity patterns.

After the axons have found their partners, two distinct mechanisms can drive the developmental reorganization of synaptic connectivity: intercellular competition between cells for common targets (reviewed in (Luo and O'Leary, 2005; Alvarez and Sabatini, 2007)), and the addition/elimination of synapses within a single arbor in response to the physiological demands of the signaling unit (Balice-Gordon et al., 1990; Gorczyca et al., 1993; Schuster et al., 1996). The former mechanism dictates the circuit "wiring diagram" by defining precisely

which subsets of cells will communicate through synaptic contacts; while the latter, in contrast, modulates the strength of connectivity between specific pre- and post-synaptic cells after circuits are assembled.

Early in nervous system development an excessive number of axonal projections and synaptic connections are initially established. What then ensues is cell-cell competition between neurons innervating the same target for limiting target-derived cues or sites of innervation during synaptogenesis. Appropriate synaptic contacts are then strengthened and exuberant processes are destabilized and eliminated through activity-dependent mechanisms (Luo and O'Leary, 2005; O'Leary and McLaughlin, 2005). For example, at the mammalian neuromuscular junction (NMJ) muscles are initially innervated by more than one motor input. However, through a process of intercellular competition for motor endplates, all but one motor input are eliminated, with the "losers" retracting wholesale from the motor endplate (Walsh and Lichtman, 2003). Likewise, at the retinotectal projection in frogs, retinal axons initially establish a rough topographic map with substantial overlap between branches. However, these local synaptic terminals ultimately compete for target space and through activity-dependent modulation of synapse stabilization the spatial map of synaptic inputs is ultimately refined to a highly selective subset of inputs (Cline, 1991).

In the intercellular competition model the elimination of exuberant inputs (the "losers") can entail large-scale elimination of axon branches, and perhaps smaller scale pruning of individual synaptic contacts. During axon and synaptic

pruning in mammals and *Drosophila* entire axon branches are destabilized, degenerate, and are then cleared from the CNS by engulfing cell types (reviewed in (Luo and O'Leary, 2005)). Similarly, recent work has shown that excessive motorneuron inputs at the mammalian NMJ also become destabilized, detach from the motor endplate, and undergo axosome shedding. In this process local Schwann cells processively engulf motorneuron terminals in a distal to proximal direction and constitute the force that drives retraction bulbs toward the parent arbor during input elimination (Bishop et al., 2004). Ultimately, this mechanism results in a reduction of the total number of cells supplying synaptic input to the target cell.

In the second and mechanistically distinct mode of synapse remodeling, individual synaptic contacts are added or removed from a single arbor to strengthen or weaken synaptic input to the target cell. Such changes are generally elicited by changes in the target size or neural activity. For example, *Drosophila* motorneurons have established synaptic contacts with specific embryonic muscle cells by the end of embryogenesis (Johansen et al., 1989). At subsequent larval stages individual arbors, along with the target muscle itself, grow in size ~100-fold (Gorczyca et al., 1993; Schuster et al., 1996). This coordinate increase in muscle size and synaptic contacts at motorneuron terminals serves to increase synaptic input from the motorneuron as needed to drive activation of the expanding muscle fiber. Similar mechanisms appear in place to modulate the balance of neural input versus target cell size in mammals:

at the mammalian adult bulbocavernous muscle, testosterone manipulation lead to increases or decreases in muscle size, and these changes were accompanied by respective expansion or shrinkage of the postsynaptic region of the NMJ, respectively (Balice-Gordon et al., 1990).

Here we explore the *in vivo* dynamics of synaptic expansion in motorneuron arbors at the *Drosophila* NMJ. We show in live preparations that the addition of new synapses during normal synaptic growth entails a large amount of shedding of presynaptic membranes in the form of small debris and a subpopulation of undifferentiated synaptic boutons (ghost boutons) which failed to mature. This process is distinct from intercellular competition, as none of the motorneuron terminals are eliminated. Rather, this mechanism appears to regulate the final size of the terminal arbor. We find that the formation of presynaptic debris (this report) and ghost boutons (Ataman et al., 2008) are modulated by neural activity, as acute stimulation of motor inputs leads to increased appearance of these structures. Intriguingly, presynaptic debris and the subpopulation of ghost boutons that become detached from the parent arbor appear to be actively cleared from the NMJ as they disappear over developmental time. We show that glia dynamically invade the NMJ and phagocytose presynaptically shed debris, and that ghost boutons are engulfed or degraded primarily by muscle cells. Loss of phagocytic function in glia or muscle cells through manipulating the Draper signaling pathway (a key engulfment signaling pathway) results in an accumulation of presynaptic debris or ghost

boutons at the NMJ and a severe reduction in NMJ expansion, indicating that continuous clearance of shed presynaptic debris and/or ghost boutons is essential for normal synaptic growth. Thus glia and muscles work together to sculpt connectivity at developing NMJ arbors, clearing multiple types of shed presynaptic structures that are inhibitory to the formation of new synaptic boutons.

RESULTS

The larval NMJ sheds presynaptic membranes

In insects, α-HRP antibodies cross-react with neuron-specific membrane antigens (Jan and Jan, 1982) likely by binding to carbohydrate moieties present in a number of neuronal membrane proteins, including the cell adhesion molecules Fasciclin (Fas) I and II (Snow et al., 1987). Consistently, at the *Drosophila* larval NMJ α–HRP antibodies labeled the entire presynaptic arbor (Fig. 2-1A1). However, we also noticed the presence of HRP-immunoreactive puncta at the postsynaptic junctional region, beyond the presynaptic membrane (Fig. 2-1A1, 2 arrows). These puncta also labeled with antibodies to FasII and did not appear to be connected to the presynaptic arbor (Fig. 2-1A3, 4). We wondered whether the HRP and FasII-positive postsynaptic staining might correspond to postsynaptic muscle structures, or whether the puncta might be derived from the presynaptic arbor. To distinguish between these possibilities, we expressed a membrane tethered GFP (UAS-mCD8-GFP) in motorneurons using the motoneuron-specific Gal4 driver OK6-Gal4 (Aberle et al., 2002). We found that the postsynaptic HRP puncta were exactly colocalized with the presynaptically derived GFP signal (Fig. 2-1D, arrow), suggesting that the HRP puncta are likely membrane fragments derived from presynaptic boutons. The presynaptically derived mCD8-GFP puncta were also observed by imaging through the cuticle of intact (undissected) larvae using a spinning disk confocal

microscope, indicating that they are naturally occurring and not an artifact of the dissection or sample preparation (Fig. 2-1E, arrows).

The nature of the presynaptically derived puncta was examined using a number of synaptic markers. Cysteine string protein (CSP) and Synapsin (Syn) are presynaptic vesicle proteins that associate with the readily releasable and the reserve pool of synaptic vesicles respectively (Ranjan et al., 1998; Akbergenova and Bykhovskaia, 2007). We found that the postsynaptic HRP puncta colocalized with CSP (Fig. 2-1B, arrows and inset), but not with Syn immunoreactivity (Fig. 2-1C). The presence of CSP in the HRP puncta further validates the idea that these puncta are presynaptic in origin. Labeling with antibodies against the active zone marker Bruchpilot (Brp) did not reveal immunoreactivity at the postsynaptic HRP-positive puncta (not shown). Together these results suggest that during NMJ development the motorneuron sheds membrane fragments (here referred to as presynaptic debris). Based on the presence of CSP but not Syn, the absence of Brp and the presence of FasII, we propose that presynaptic debris might arise from the perisynaptic bouton region.

Studies in many systems have suggested that the state of a mature synapse is the result of a dynamic equilibrium between growth and retraction (Wilson and Deschenes, 2005). Therefore, to determine what conditions lead to the shedding of presynaptic debris, we attempted to perturb this equilibrium by inducing activity-dependent synaptic growth (Ataman et al., 2008). Previous studies at the larval NMJ show that an acute increase in activity induces a *de*

novo formation of new synaptic boutons. In particular, spaced cycles of stimulation, consisting of either K^+-induced depolarization, high frequency nerve stimulation, or light gating of neuronally expressed channelrhodopsin-2 (ChR2), induces rapid structural changes at the NMJ. These changes include an increase in the number and length of dynamic presynaptic filopodia (synaptopods) and the number of undifferentiated boutons (ghost boutons) containing synaptic vesicles but lacking active zones and postsynaptic proteins (Ataman et al., 2008). Imaging of intact larvae also showed that synaptopods and ghost boutons were naturally occurring structures observed even in unstimulated preparations albeit at low frequency (Ataman et al., 2008).

In our experiments we expressed ChR2 in motorneurons using OK6-Gal4 and stimulated the motorneurons of intact larvae with 5 cycles of spaced light stimulation as previously described (Ataman et al., 2008). Body wall muscles were then dissected either 30 min or 18 hrs after the stimulation was complete and labeled with anti-HRP. As a control, we used unstimulated larvae expressing ChR2 in motorneurons but not subjected to the light pulses. Notably, we found that the total area occupied by particles of presynaptic debris around the NMJ was significantly increased 30 min after the end of spaced stimulation (Fig. 2-1I), indicating that acute stimulation of neural activity resulted in an increase in presynaptic debris at the NMJ. Allowing NMJs to recover for 18 hr after stimulation resulted in debris returning to wild type levels (Fig. 2-1I), suggesting the presence of an active mechanism to eliminate presynaptic debris from the

NMJ. We conclude that presynaptic debris are normally present at the NMJ and conditions that lead to synaptic growth result in a transient increase in the amount of presynaptic debris, thus shedding of debris is associated with NMJ growth.

We also conducted time-lapse imaging of identified NMJs from live intact larvae expressing ChR2 in motorneurons using C380-Gal4 (Budnik et al., 1996). These larvae also contained fluorescent markers that allowed us to simultaneously image the pre- and the postsynaptic compartment. In particular, these larvae expressed UAS-mRFP in motorneurons to visualize the presynaptic NMJ arbor and mCD8-GFP::Sh in muscles using the myosin heavy chain (MHC) promoter (Zito et al., 1999) to visualize the postsynaptic NMJ region. In the MHC-mCD8-GFP::Sh transgene, the GFP is fused to the last ~150 C-terminal amino acids of the Shaker K^+ channel isoform containing a Discs-Large (DLG) PDZ binding site, and thus it is targeted to the postsynaptic region allowing its visualization *in vivo* (Zito et al., 1999). These larvae were subjected to spaced stimulation with light as above, and the same NMJ imaged for 5-15 min at different intervals. Between imaging intervals larvae were returned to the food. As previously reported (Ataman et al., 2008), we found that ghost boutons were present and some of these became stabilized and recruited postsynaptic label. However, we also observed that many of these ghost boutons did not recruit postsynaptic label and disappeared over time (Fig. 2-2A arrow and inset in right panel).

The presence of presynaptic debris in normal animals, the enhancement of presynaptic debris deposition upon spaced stimulation, and the elimination of some of the newly generated ghost boutons after spaced stimulation suggest that NMJ development involves the continuous shedding of certain presynaptic membrane compartments. Furthermore, the lack of accumulation of these components over developmental time, suggest that they may be actively removed from the NMJ.

To determine if presynaptic debris might originate from the breakdown of ghost boutons that failed to become stabilized and disappeared, we followed the fate of ghost boutons that became detached from the presynaptic arbor and presynaptic debris. In these experiments, identified NMJs from larvae expressing ChR2 and mCD8-GFP in motorneurons were repeatedly imaged through the cuticle as above following spaced stimulation. We found that on several occasions, as ghost boutons detached, debris appeared in the position of the ghost bouton stalk and around the ghost bouton, suggesting that ghost boutons can degenerate directly into presynaptic debris (e.g., Fig. 2-2B, C; ghost boutons are marked by white arrows and debris by black arrowheads). In some samples we were able to directly image the disintegration of ghost boutons into smaller fragments (Suppl. Movie 1). However, in other cases, stalks simply disappeared without leaving debris, and detached ghost boutons became smaller and vanished from the NMJ without leaving any obvious debris (Fig. 2-2D, E white arrows). Interestingly, not all presynaptic debris appeared to derive from ghost

boutons and their stalks — we also observed the appearance and disappearance of presynaptic debris at NMJ regions devoid of ghost boutons (Fig. 2-2E black and red arrowheads), suggesting that presynaptic debris can be generated independently from ghost boutons. In summary, presynaptic debris can apparently arise directly from the breakdown of ghost boutons, or, alternatively may be derived directly from the presynaptic arbor without participation of ghost boutons.

Local engulfing cells clear shed presynaptic material from the NMJ

The very low levels of presynaptic debris and ghost boutons observed here in unstimulated larvae and the removal of the extra debris formed upon stimulation, suggested that as NMJs develop, presynaptic membrane debris and disconnected ghost boutons are actively cleared from the NMJ. Signal transduction mechanisms mediating the engulfment of neuronal debris are beginning to be elucidated (Logan and Freeman, 2007). Most prominent, the engulfment receptor Draper (Drpr; Ced-1 in *C-elegans*) is involved in the engulfment of neuronal cell corpses during programmed cell death, the pruning of mushroom body neuron arbors during fly metamorphosis, and in the phagocytosis of injured axons in the fly olfactory system (Freeman et al., 2003b; Awasaki et al., 2006; Hoopfer et al., 2006; MacDonald et al., 2006). We therefore used *draper* mutants as a tool to block the activity of local engulfing cell types and assayed the effects of loss of Draper function on clearance of shed

presynaptic debris and disconnected ghost boutons from the larval NMJ. Strikingly, we found that *draper* mutant NMJs were highly abnormal, with the presence of unusually large and irregularly shaped boutons and with a marked reduction in the number of glutamatergic type Ib boutons (Fig. 2-3A, B, F). Close examination of the NMJs in these mutants revealed that there was also a dramatic increase in the amount of presynaptic debris (Fig. 2-3C-E arrows, H) and number of ghost boutons (Fig. 2-3E arrowheads, G). Interestingly, we also found that third instar *draper* mutant larvae had reduced larval motility in behavioral assays (Supplementary Figure 2-1), suggesting that the accumulation of presynaptically shed material may adversely affect neuromuscular function. Thus, in the absence of Draper function NMJs develop abnormally and presynaptic debris and ghost boutons accumulate at high levels. These observations suggest that an engulfing cell type might invade, or be a resident component of, the NMJ, and phagocytose shed presynaptic material.

Draper is expressed in muscle and glia and glial cells establish transient interactions with the NMJ

In the fly nervous system Draper is expressed in glia where it has crucial roles in engulfment activity (Freeman et al., 2003b; Awasaki et al., 2006; Hoopfer et al., 2006; MacDonald et al., 2006). To determine if Draper was also present in glial cells at the NMJ, we used α–Draper antibodies (Freeman et al., 2003b). Surprisingly, in addition to its localization in peripheral glia that wrap around

motor nerves (Fig. 2-4A), we found that Draper immunoreactivity was present at the postsynaptic region of every synaptic bouton in colocalization with the *Drosophila* PSD-95 homolog Discs-Large (DLG) (Fig. 2-4C). This immunoreactivity was specific to Draper, as it was virtually eliminated in *draper* null mutants (Fig. 2-4B, D).

The above observation was surprising, since in contrast to vertebrate NMJs, where terminal Schwann cells completely cover the NMJ (Feng et al., 2005), at the glutamatergic *Drosophila* larval NMJ terminal glia have not been reported to cap the synaptic arbor (Sepp et al., 2000; Banerjee et al., 2006). Instead, NMJ arbors are buried within the muscle surface, which wraps around the boutons forming a layered system of membranes, the subsynaptic reticulum (SSR) (Jia et al., 1993; Guan et al., 1996). Previous studies have suggested that at the larval NMJ peripheral glia ensheath the segmental nerve, but for the most part, their membranes terminate at the axon branch point or at the first synaptic bouton closest to the branch point (Sepp et al., 2000). The presence of Draper surrounding the entire NMJ led us to re-examine the organization of glial cell membranes at the NMJ and their relationship to synaptic boutons. For these experiments we expressed a membrane tethered GFP (mCD8-GFP) in peripheral glia, using Gliotactin-Gal4 (Gli-Gal4), and HRP-labeled NMJs from abdominal segments 3 and 4 were systematically examined in fixed preparations. We found that in the majority of cases glial membranes deeply invaded the NMJ (Fig. 2-5), presumably invading the space between the presynaptic motorneuron

terminal and the SSR. Some NMJs (2-40% on average depending on the specific NMJ), particularly those innervating dorsal muscles, appeared completely covered by glial membranes (Fig. 2-5A, E; covered NMJs). A majority (80-100%) of NMJs were associated with lamellipodia-like glial extensions that contacted several boutons (Fig. 2-5A-C, E). Glia also extended thin filopodia-like processes that contacted synaptic boutons at the same NMJ branch or that exited the branch and interacted with synaptic boutons from a different NMJ branch (Fig. 2-5A5, B5). Glial membrane processes were also observed in association with muscle regions around the NMJ that were completely devoid of synaptic boutons (Fig. 2-5A4, C4-5). A small percentage (~7%) of glial extensions had an elliptical appearance and terminated in bulbous structures of variable size (Fig. 2-5D4-5, E). These bulbous structures sometimes surrounded a synaptic bouton (Fig. 2-5D5 arrowhead). In some NMJs (11-33%) glial membranes did not invade the NMJ and muscle, and terminated at the nerve branch-point before synaptic boutons (Fig. 2-5B1-3; blunt ended).

Interestingly, the pattern of glial extensions was not stereotypic and showed a high degree of variability among segments and identified muscles from different individuals. This observation suggests that the glial processes are likely to extend and retract in a dynamic fashion. This possibility was examined by live imaging preparations expressing mCD8-GFP in peripheral glia with Gliotactin-Gal4. We found that glial processes were indeed at the NMJ, and extended or retracted within a period of minutes (Suppl. Movie 2). These observations

indicate that glial cells at the larval NMJ have previously unappreciated dynamics, and that they establish multiple transient associations with the NMJ. However, our studies of Draper localization at the NMJ demonstrated that Draper is present at every NMJ and surrounding each synaptic bouton (Fig. 2-4C). Thus, the extension of glial membranes is unlikely to account for Draper localization at the entire NMJ raising the possibility that muscles might also contribute to NMJ Draper localization.

In *draper* mutants, there were some changes in the distribution and frequency of glial extensions. Glial extensions that covered the entire NMJ (covered NMJs) were absent or drastically reduced in frequency, and there were also changes in the distribution and frequency of gliobulbs (Supplementary Figure 2). In contrast, there was a strong increase in the frequency of blunted projections (i.e. those that end close to the nerve branch point and do not interact with synaptic boutons), and a normal level of lamellipodia-like extensions). These observations suggest that in the absence of Draper function some glial membranes do not extend properly into the NMJ. Thus positive signaling through Draper, perhaps in response to cues released by presynaptic debris, may directly regulate a subset of glial membrane movements at the NMJ.

Both glia and muscle cells act as phagocytes and clear presynaptic debris from the NMJ

To address the possibility that Draper might function both in glia and muscle to sculpt the NMJ we selectively expressed a Draper-RNAi designed to knockdown all Draper isoforms (e.g. Fig. 2-8A) in glia or muscles using cell-specific Gal4 strains. RNAi knockdown of Draper in either muscle or glia resulted in a reduction in the number of synaptic boutons which was not significantly different from the *draper* null mutant (Fig. 2-6E). This indicates that the removal of Draper from either cell type is sufficient to interfere with NMJ growth. Remarkably, however, downregulating Draper in muscle versus glia had a different consequence for the deposition of presynaptic debris and the appearance of detached ghost boutons. RNAi knockdown of Draper in glia resulted in an increase in presynaptic debris to an extent similar to the *draper* null mutant (Fig. 2-6C, G). However, no significant increase in the number of detached ghost boutons was observed (Fig. 2-6F). If glial extensions are primarily involved in engulfing presynaptic debris, we predicted that we should find HRP positive debris within the glial extensions. We found that this was indeed the case. We found several instances in which glial terminals formed bulb-like structures that contained anti-HRP immunoreactive puncta within (Fig. 2-6D, arrowheads).

In contrast, downregulating Draper in muscle resulted in an increase in the number of ghost boutons (Fig. 2-6B, F), but the level of presynaptic debris was

similar to wild type (Fig. 2-6B, G). Expressing Draper RNAi in motorneurons did not affect the number of boutons, ghost boutons, or the levels of presynaptic debris (2- 6E-G). These results support the idea that Draper functions both in muscle and glia, and that the function of Draper in each cell has some degree of specialization. While glial Draper appears to function in removing presynaptic debris, muscle Draper appears to remove ghost boutons fated for elimination. Importantly, these observations also provide the first evidence that muscle cells fulfill a phagocytic function at the NMJ.

Downregulation of Ced-6 mimics cell-specific Draper phenotypes at the NMJ

Previous studies have shown that the PTB-domain protein dCed-6 functions downstream of Draper (Awasaki et al., 2006). Therefore, we used RNAi knockdown of dCed-6 in muscle or glia acts as a second approach to blocking glial and muscle engulfment activity. As in *draper* mutants, downregulating dCed-6 in either muscle or peripheral glia resulted in significant decrease in the number of synaptic boutons (Fig. 2-7D). In contrast, no effect was observed when dCed-6-RNAi was expressed in motorneurons (Fig. 2-7D). Similar to Draper RNAi knockdown, expressing dCed-6-RNAi in muscles or glia had differential consequences for the appearance of presynaptic debris versus ghost boutons. Decreased levels of dCed-6 in muscles led to an increase in the number of ghost boutons, but had no influence in the deposition of presynaptic debris (Fig. 2-7B, E, F). Downregulating dCed-6 in glia, on the other hand, led to

a significant increase in presynaptic debris deposition, but the number of ghost boutons remained unaltered (Fig. 2-7C, E, F). These results are consistent with the notion that dCed-6 functions downstream of Draper during the development of the NMJ. Further, they support the model that both muscle and glia contribute differentially to the clearance of debris versus ghost boutons at the NMJ.

Accumulation of presynaptic debris, ghost boutons, and defects in NMJ growth map to the *draper* gene

The *draper* gene gives rise to three different Draper isoforms, each with a unique combination of intracellular and extracellular domains (Fig. 2-8A). Draper-I bears 15 extracellular EGF repeats, whereas Draper-II and -III only contain 5 (Freeman et al., 2003b). In their intracellular domains, all isoforms contain a potential dCed-6 binding site (NPXY), but the Shark binding site is only present in Draper-I and -II. To determine which of the isoforms might be involved in NMJ development, we first carried out RT-PCR of body wall muscles. Interestingly, we found that Draper-I and III, but not Draper-II were expressed at the neuromuscular system (Fig. 2-8B). Therefore, we carried out rescue experiments by expressing Draper-I or -III in muscles or glia in a *draper* null mutant background.

None of the Draper isoforms completely rescued the decrease in bouton number observed in the *drpr* null (Fig. 2-8C). This is consistent with the observations with cell-specific Draper-RNAi expression, showing that Draper

functions both in muscle and glia, and that downregulating Draper in either cell is sufficient to decrease bouton number to an extent similar to the *draper* null mutant alone. In the case of ghost boutons, expressing Draper-I in glia or Draper-III in muscle completely rescued the mutant phenotype (Fig. 2-8D). However, expressing Draper III in glia or Draper I in muscle also resulted in substantial but incomplete rescue. For the deposition of presynaptic debris, only expressing Drpr-I in glia completely rescued the phenotype, but partial rescue was also observed when Drpr-III was expressed in muscle (Fig. 2-8E). These data provide conclusive evidence that the phenotypes we observe in *draper* null mutant NMJs indeed map to the *draper* gene, and that the phenotypes we observe in *draper* mutants can be significantly rescued by resupplying Draper in glia or muscle cells (Fig. 2-8F). The incomplete rescue of some of the phenotypes by specific isoforms might represent redundant functions by these isoforms, a requirement for multiple isoforms for complete rescue, or simply result from increased Draper expression in transgenic animals.

DISCUSSION

Here we have studied the *in vivo* dynamics of synaptic connectivity between single motor inputs and their target muscle cells. We describe a novel event that occurs during the remodeling of single synaptic arbors during development or activity-induced plasticity: the shedding of presynaptic debris and aborted synaptic boutons that failed to stabilize. This process differs from developmental pruning or intercellular competition during synapse elimination, as in those cases entire nerve terminals are eliminated, thereby changing the wiring diagram of a circuit. Rather, we show that the expansion of an already established synaptic input involves significant production of presynaptic membrane debris and the detachment of undifferentiated synaptic boutons destined for elimination from the main arbor. Both glial and muscle cells act in concert to clear the developing NMJ of this shed presynaptic material, and the suppression of engulfing activity in glial or muscle cells leads to highly disrupted NMJ growth. We propose that this novel mechanism might serve to rapidly adapt the size of a growing synaptic terminal to the changing demands of the target cell by shifting the equilibrium between synapse stabilization and synapse destabilization.

Expanding presynaptic arbors shed membrane debris in an activity-dependent manner

During larval development, the NMJ is continuously increasing the size and number of synaptic boutons. This expansion serves as a compensatory mechanism to preserve synaptic strength, despite the massive growth of muscle cells (Griffith and Budnik, 2006). Our studies provide evidence that normal NMJ growth includes the constitutive shedding of presynaptic membranes. The presynaptic origin of HRP-positive debris was demonstrated by labeling motorneuron membranes with genetically encoded mCD8-GFP, which consistently labeled the debris, by the observation that in some cases ghost boutons that detached from the main arbor disintegrated into debris, and by the finding that the debris also contained presynaptic proteins, such as CSP. Thus, synaptic debris might contain synaptic vesicles or vesicle membrane remnants that failed to be recycled. Interestingly, Brp, an active zone marker (Kittel et al., 2006), was absent from the debris. This might reflect its degradation, or alternatively, the derivation of presynaptic debris from periactive regions of the NMJ. Indeed, FasII, which is localized at periactive zones (Sone et al., 2000) was also present in presynaptic debris.

Acute spaced stimulation of the larval NMJ leads to the formation of dynamically extending and retracting synaptopods, and to the appearance of ghost boutons (Ataman et al., 2008). While some ghost boutons differentiate by acquiring active zones and postsynaptic proteins (Ataman et al., 2008), here we

found that others lost their connection with the presynaptic arbor and were specifically removed. What happens to ghost boutons that detach from the main arbor? In most cases we found that detached ghost boutons rapidly disappeared from the NMJ. Based on our finding that suppressing engulfing action in muscle leads to the accumulation of ghost boutons, we propose that these are engulfed directly by muscle cells (Fig. 2-8F).

In other cases we found that ghost boutons, along with the stalk by which they were initially attached to the main arbor, would degenerate into smaller fragments resembling presynaptic debris. Thus at some level, ghost boutons also appear to be able to disintegrate into presynaptic debris. That presynaptic debris and ghost boutons are unique cellular remnants is also argued by the fact that they are differentially engulfed by glia and muscle cells, respectively (Fig. 2-8F). Nevertheless, the detachment and elimination of ghost boutons we describe represents a simple and newly defined mechanism for the removal of excessive synapses formed by individual innervating motorneuron. This process might also serve as a mechanism for rapid stabilization of new synaptic boutons during, for example, periods of increased synaptic or locomotor activity (Budnik et al., 1990; Steinert et al., 2006; Ataman et al., 2008) (see below).

The functional significance of shedding presynaptic debris remains unclear. Manipulations that promote rapid synaptic growth, such as acute spaced stimulation, lead to an increase in presynaptic debris suggesting that its production is associated with synaptic growth. While some presynaptic debris

appears to be derived from the breakdown of disconnected ghost boutons, we also observed the *de novo* formation of presynaptic debris in the absence of any ghost boutons. Thus, presynaptic debris is likely directly shed by motorneuron endings. Presynaptically shed debris might derive from dynamically extending synaptopods, whose formation is dramatically enhanced by increasing neural activity (Ataman et al., 2008). However, in live preparations demonstrating robust synaptopod growth we have yet to directly observe the formation of debris following synaptopod expansion or retraction (Gorczyca M, Ashley J, Fuentes-Medel, unpublished).

The presence of presynaptic debris might highlight the extremely dynamic nature of synapse addition *in vivo*. Two important mechanisms appear to operate during NMJ expansion. First, the NMJ is shaped by a homeostatic mechanism that maintains synaptic efficacy despite larval muscle growth (Griffith and Budnik, 2006). Second, the NMJ has the ability to respond to acute changes in activity and sensory experience with rapid modifications in synaptic structure and function. Well-fed larvae placed in a substrate devoid of food show an increase in synaptic strength within 30 min (Steinert et al., 2006), and spaced stimulation induces robust synaptic growth within 2 hours (Ataman et al., 2008). It is tempting to speculate that presynaptic shedding is the byproduct of a mechanism designed to ensure rapid and efficient changes in synaptic performance. For example, the initiation of synaptic bouton formation might be a continuous process. This pool of synaptic boutons might reach an immature stage and if not

subsequently stabilized by activity or other signals they might be shed and removed. Such a mechanism would provide a continuous supply of immature boutons ready to stabilize if rapid growth becomes essential.

Synaptic debris and ghost boutons are engulfed by glial and muscle cells

Glial cells have a key role in the removal of axonal debris and neuronal cell corpses from the CNS (Aldskogius and Kozlova, 1998; Logan and Freeman, 2007), but mounting evidence also implicates glial cells in the elimination of synaptic inputs. In mammals microglia rapidly spread along neurites of injured motorneurons and displace synaptic inputs through synaptic stripping (Blinzinger and Kreutzberg, 1968). At the mammalian NMJ, terminal Schwann cells are also active participants in the activity-dependent elimination of exuberant motorneuron inputs by apparently pinching off fragments of retracting terminals (Bishop et al., 2004).

Here we describe a novel mechanism by which glia, through their phagocytic clearance of shed synaptic debris, can sculpt synaptic connectivity within a single arbor and ultimately modulate the growth of nerve terminals. The formation of shed presynaptic material appears to be autonomous and not require the engulfing action of glial cells since presynaptic debris and ghost boutons accumulate at high levels in *draper* mutants. Notably, muscle cells collaborated with glia in the removal of shed presynaptic membranes and thus also helped to sculpt the growing NMJ. These observations provide a new view

on the role of muscle cells in regulating synaptic growth: muscle cells are not simply postsynaptic target cells that give and receive synaptogenic signals; they are also phagocytes at the NMJ and through engulfing shed presynaptic material can help shape synaptic connectivity.

Why has such presynaptic material not been previously described at the well-studied *Drosophila* NMJ? This is likely due to the fact that we have assayed NMJ morphology for the first time in engulfment mutants. Even in wild type a very low level of presynaptic debris (this report) and a small number of ghost boutons (Ataman et al., 2008) is observed. However in *draper* mutants or knockdown animals we observe their dramatic accumulation, which is reminiscent of the process of cell corpse engulfment after apoptotic cell death. Cell corpses are rapidly engulfed during development and thus very few are observed in wild type animals. In contrast, they accumulate at significant levels in animals with reduced cell corpse engulfment activity, such as *C. elegans ced-1* or *ced-6* mutants (Reddien and Horvitz, 2004).

We found that glial cells extended membrane processes that deeply invaded the NMJ. These cellular interactions were highly dynamic, as demonstrated by our time-lapse imaging, and by the high variability in the extent and type of glial membrane projections we found at the NMJ. Some projections were in the form of thin gliopods that associated with boutons within a branch or that extended across branches. Others resembled flat lamellipodia that associated with synaptic boutons or with the muscle. Given the requirement for

glial Draper in the removal of synaptic debris, it is tempting to speculate that glial membranes are continuously and dynamically surveying the NMJ for the presence of synaptic debris, which is then engulfed. Consistent with this notion, we found several examples of glial membranes extending away from the arbor and overlapping with presynaptic debris. We also found that in some cases, HRP positive fragments were found associated bulbous structures formed by the glial projections, suggesting that glia can engulf presynaptic debris. We also observed glial membrane projections that had the form of boutons, sometimes draping over an entire bouton, or extending well beyond the terminal bouton. While the function of these structures remains unclear we envisage at least two potential roles. First, these might represent glial extensions actively engulfing ghost boutons, although this would be predicted to be a rare event since our cell-type specific analyses argue that muscle cells are primarily responsible for clearance of ghost boutons. Second, these extensions, along with the additional types described above that extend beyond axonal arbors into the muscle, could be physically opening up space in the muscle cell for new bouton formation or process extension.

Recognition and clearance of shed presynaptic debris and ghost boutons requires the Draper signaling pathway

Interestingly, we found that in *draper* mutants both disconnected ghost boutons and presynaptic debris accumulated, and this accumulation had a negative effect

on NMJ expansion and bouton morphology. Moreover, synaptic growth appeared to be highly sensitive to both types of shed presynaptic material since the accumulation of either ghost boutons or presynaptic debris (when engulfment activity was blocked in muscles or glia, respectively) led to reductions in bouton growth similar to that seen in *draper* null mutants. As mentioned above, shed material might contain important signaling factors that potently stimulate or inhibit new synapse formation. If, for example, presynaptic debris contains molecules that inhibit synaptogenesis, the accumulation of such material would be expected to negatively regulate synaptic growth. Perhaps a similar type of inappropriate modulation of synaptogenesis by the membrane fragments of pruned terminals also accounts for their rapid clearance from the CNS after degeneration.

Drosophila glial cells also engulf neuronal cell corpses and pruned or degenerating axons. Each of these targets is generated by a unique degenerative molecular cascade: cell corpses are produced by canonical apoptotic cell death pathways (Rogulja-Ortmann et al., 2007), pruned axons undergo degeneration through a ubiquitin proteasome-dependent mechanism (Watts et al., 2003), and severed axons undergo Wallerian degeneration via Wld^{S}-modulated mechanisms (MacDonald et al., 2006). Despite their unique pathways of production, each is engulfed by glia through Draper-dependent mechanisms, implying that these engulfment targets autonomously tag themselves with molecularly similar “eat me” cues. Our observations that mutations in *draper* led to accumulation of presynaptic debris and detached

ghost boutons suggests that these new glial/muscle engulfment targets also produce similar cues for phagocytic cells to promote their destruction. If so, these data argue that all the necessary machinery essential for tagging membrane fragments for engulfment are present in a ghost bouton or fragment of presynaptic membrane. Importantly, while a lack of glial-mediated clearance of several targets has been observed *in vivo*—cell corpses, pruned axons or dendrites, and axons undergoing Wallerian degeneration—almost nothing is known about phenotypic consequences of a lack of glial engulfment function in the nervous system. Here we demonstrate that failure of glia and muscle to clear presynaptically-derived material negatively regulates synaptic growth.

In conclusion our studies demonstrate that the process of synaptic growth includes a significant degree of membrane/synaptic instability, and that growing terminals are constantly sloughing off undifferentiated boutons and fragments of membrane. Our observations demonstrate that growing NMJs generate an excess number of undifferentiated synaptic boutons and that only a fraction becomes stabilized and drive the assembly of the postsynaptic apparatus. Exuberant synapses that have failed to form successful postsynaptic contacts are shed, and cleared from the NMJ by glia and muscle cells. The presence of such a pool ensures a continuous supply of nascent synapses available for use to rapidly increase input into the muscle if dictated by dynamic changes in signaling at the NMJ.

MATERIALS AND METHODS

Drosophila strains and behavioral assays

The following fly strains were used for this study: *draper*$^{\Delta 5}$ and UAS-Draper-RNAi (MacDonald et al., 2006), UAS-dCed-6-RNAi (Awasaki et al., 2006); Repo-Gal4 (a gift from B. Jones), Gli-Gal4 (Sepp and Auld, 1999), OK6-Gal4 (Aberle et al., 2002), C57-Gal4 and C380-Gal4 (Budnik et al., 1996), UAS-mCD8-GFP (Lee and Luo, 1999) UAS-myrRFP (Bloomington Stock Center), MHC-mCD8GFP-Sh (Zito et al., 1999), and UAS-ChR2 (Schroll et al., 2006). UAS-Draper-I and UAS-Draper-III were generated by M.A. Logan and will be described in detail elsewhere (M.A.L. and M.R.F., in preparation). For larval motility assays, larvae were cultured at 25°C, wandering 3rd instar larvae were collected, briefly washed in distilled water, transferred to the center of a square agar plate, and covered with a transparent lid. After 30 seconds, total larval movement was followed for 1 minute under red light conditions, 60% humidity, at 25°C degrees.

Immunolabeling, Live-imaging, and Confocal Microscopy

Third instar *Drosophila* larvae were dissected in calcium free saline (Jan and Jan, 1976) and fixed for 10 minutes with non-alcoholic Bouin's solution unless otherwise noted. Primary antibodies were used at the following dilutions: α-Draper, 1:5000 (Freeman et al., 2003b); rabbit α-Discs-Large, 1:20,000 (Koh et al., 1999); mouse α-Discs-Large, 1:500 (clone 4F3, Developmental Studies

Hybridoma Bank, DSHB); α-CSP, 1:100 (Zinsmaier et al., 1994); α–Synapsin, 1:10 (a gift from E. Buchner; (Klagges et al., 1996); α-Fas II, 1:3000 (Koh et al., 1999); α–GFP, 1:200 (Molecular Probes); nc82 (α-Brp), 1:100 (DSHB); FITC or Texas red-conjugated α-HRP 1:200 (Jackson Immunoresearch). Secondary antibodies conjugated to FITC, Texas Red, or Cy5 (Jackson Immunoresearch) were used at a concentration of 1:200. Samples were imaged using a Zeiss Pascal confocal microscope and analyzed using the Zeiss LSM software package and ImageJ.

To study the organization of glial membranes at the NMJ we fixed larval body wall muscle preparations of controls and *draper* mutants expressing mCD8-GFP in glia using the Gli-Gal4 strain for 15 minutes in 4% paraformaldehyde fix, and double stained the preparations with Texas Red conjugated α-HRP 1:200 (Jackson Immunoresearch) and α–GFP (Molecular Probes). Glial membrane extensions at identified body wall muscle NMJs from abdominal segments A3 and A4 were scored individually as "blunt ended" (glial membranes terminated at the branch point), "covered" (glial membranes completely ensheathed the NMJ), "gliobulbs" (glial extensions terminated in a bulbous structure), "gliopods" (small finger-like glial membrane projections), and lamellipodia (glial membranes formed flat extensions that partially covered the NMJ). The percentage of NMJs containing the above types of glial membranes projections was calculated from 20 hemisegments for controls, and 15 hemisegments for *draper*$^{\Delta 5}$ mutants.

Presynaptic debris was scored from type Ib boutons at muscles 6 and 7, abdominal segment A3. This quantification was performed using images of α-HRP labeled NMJs that were acquired with identical confocal settings, and the amount of debris scored blindly according to a subjective scale of 0-3. Number of NMJs analyzed are 10-12 per sample (from 6 animals). To score presynaptic debris after spaced stimulation, intact larvae expressing channelrhodopsin-2 in motorneurons were subjected to spaced light stimulation as in (Ataman et al., 2008), fixed at 2 hr (1.5 hours stimulation, 30 minutes rest) (N=18 for stimulated samples, N=12 for unstimulated controls), and 18 hr after stimulation (N=6 for stimulated samples, N=6 for unstimulated controls), and stained with α-HRP antibodies. Confocal images of NMJs at muscles 6 and 7 (A2 and A3) were acquired with identical settings, and two 8μm diameter circles at the postsynaptic region of each NMJ branch were selected for analysis using NIH Image software. The number of synaptic boutons and ghost boutons were quantified at muscles 6 and 7 (A3) from preparations double stained with α-HRP and α-DLG (N≥10 NMJs per genotype). Data was represented in histograms as the average±S.E.M. Statistical significance of the data was obtained in pair-wise comparisons using the Student t-test.

Live imagining of larvae was performed on either intact or dissected preps as Ataman et al 2008. Briefly intact larvae were anesthetized using Sevoflurane (Baxter) and the dorsal muscles were then imaged through the cuticle using a 40X 1.2 NA objective on an Improvision spinning disk confocal microscope.

Larvae were examined live by expression of UAS-mCD8GFP in motor neurons (pre-Gal4) or glia (gli-Gal4). Increased activity was induced in these larvae by expression of UAS-Channelrhodopsin2, and exposure to a pulsed 491nm LED paradigm described in Ataman et al 2008 and Figure 1H. Larvae were examined every hour, every four hours, or at 18 hour intervals depending on the experiment. In order to visualize the debris, samples were converted to rainbow gradient color, and then contrast enhanced until the main arbor was saturated, as the debris is much dimmer than the presynaptic membrane.

Live imaging of glia was also performed in dissected preps, as Ataman et al 2008. Briefly, larvae were dissected in low calcium Drosophila saline (Stewart et al 1994), and imaged on a Zeiss Pascal Confocal (Carl Zeiss) using either 25X or 40X water immersion objectives.

RT-PCR

Total RNA was isolated from third instar body wall muscle preparations with Trizol (Invitrogen) and purified using the RNeasy Mini Kit (QIAGEN). First strand cDNA was synthesized using Superscript II (Invitrogen) enzyme and oligo (dT) 12-18 primer (Invitrogen). PCR was performed using the following Draper isoform specific primers to detect expression of Draper-I, Draper-II, or Draper-III: DrprIuECDF (5'-GGGTCCCCTATGTGATATGC-3') and DrprIuECDR (5'-TTGTAGCACTCGCAGCTCTC–3'); DrprIIuF (5'-GAAAATATATAGCAAGATTTTGTTTCC–3') and DrprIIuR (5'-

TTCGTGTTGTCGAAGCACTC–3'); DrprIIIuF (5'-GTCATTAGACTTTTACACAGG c–3') and DrprIIIuR (5'-CTAGCGTATAGAATCAGAC–3'). Plasmids containing the Draper isoforms (pUAST-DraperI, pUAST-DraperII, and pUAST-DraperIII) were used as controls for PCR amplification. PCR program was as follows: denature at 95° for 1 minute, anneal at 56° for 30 seconds, extension at 72° for 30 seconds (30 cycles total). PCR products were run on a 0.8% agarose gel and visualized by ethidium bromide stain.

ACKNOWLEDGEMENTS

We thank Ms. Romina Barria, Dr. Michael Gorzcyca and Ms. Kimberly Kerr for their experimental support and expertise. Special thanks to Dr. Sean Speese for critical discussion on the manuscript. We also thank Dr. Vanessa Auld for kindly sharing fly stocks.

FIGURES

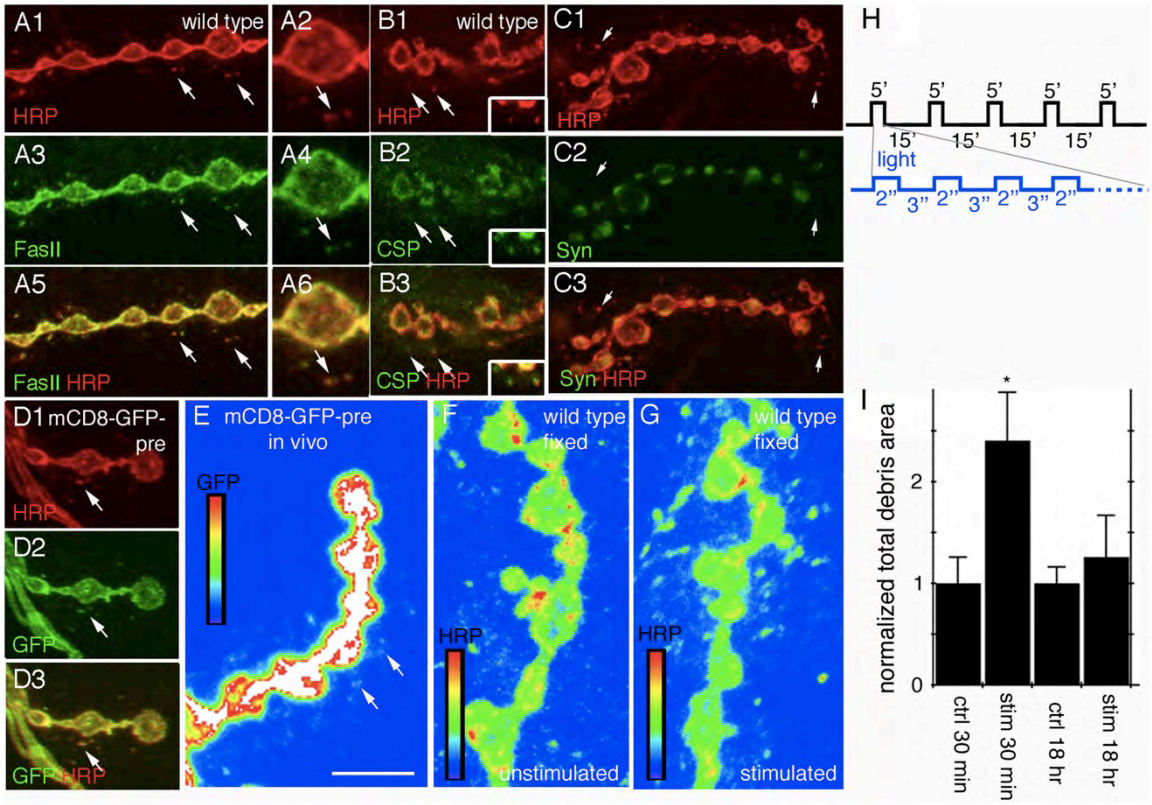


Figure 2-1. Motor axons at the NMJ constitutively shed presynaptic debris in an activity-dependent manner.

(A-D) Third instar Drosophila larvae were fixed and stained with various markers to visualize the morphology of glutamatergic NMJ branches at muscles 6 and 7. All motor neurons labeled with α-HRP (red) and small HRP^{+} puncta were observed adjacent to many NMJ arbors (arrows in A1-D3). These puncta colocalized with the motor neuron marker FasII (green A1-6) and the synaptic

vesicle marker CSP (green in B1-3). Presynaptically-derived HRP^+ debris does not stain for Syn (green in C1-3), a marker for reserve pools of synaptic vesicles. (D,E) UAS-mCD8-GFP was driven in motor neurons with the OK6-Gal4 driver. All HRP^+ puncta were also GFP^+ in fixed samples (arrows D1-3), indicating that the HRP^+ puncta are presynaptically derived. Presynaptically-derived GFP+ debris was also observed in live, intact animals by imaging through the cuticle. (F-I) Unstimulated NMJs display very little or no HRP^+ debris surrounding NMJ arbors (F). Spaced light stimulation of larvae expressing presynaptic channelrhodopsin-2 (H) led to a dramatic increase in the formation of HRP^+ presynaptic debris surrounding the NMJ 2 hours after stimulation ended (G). (I) Quantification of normalized total area of HRP^+ debris.

Calibration scale is 5 µm for (A,C,D,E), and 2.5 µm for (B). n=18,12, 6, 6, respectively for I.

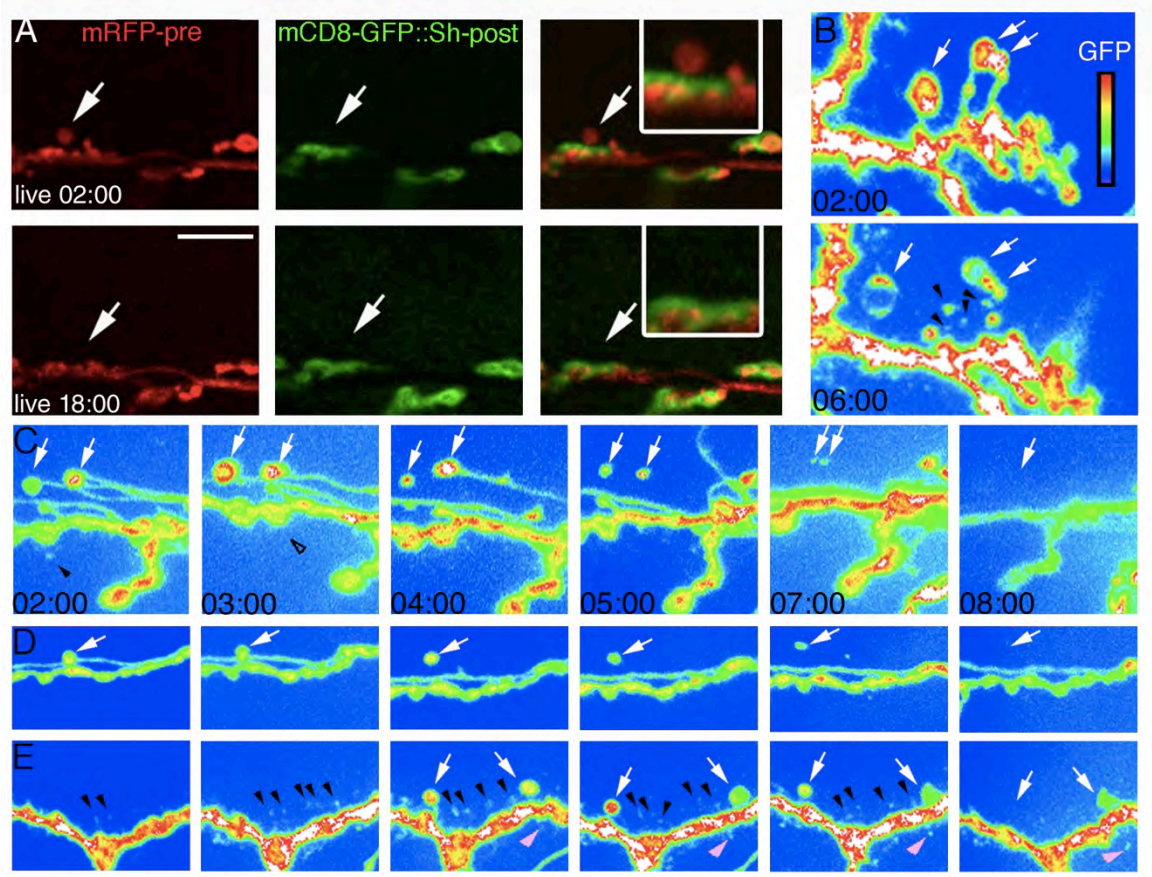


Figure 2-2. NMJs shed ghost boutons that stabilize or disappear.

(A) Example of live imaging of an NMJ through the cuticle of an intact larvae expressing channelrhodopsin-2 and mRFP (red) in motoneurons, and a synaptically targeted mCD8-Shaker-GFP protein (green) in postsynaptic muscles. Motor neurons were stimulated with a spaced blue light paradigm (as in Fig. 2-1) and NMJs were imaged at indicated times. Stimulation led to the formation of a ghost bouton (arrow) that lacked postsynaptic mCD8-Shaker-GFP. Eighteen hours later, the ghost bouton was eliminated.

(B) Live, intact larvae expressing channelrhodopsin-2 and mCD8-GFP in

motor neurons where imaged immediately and 4 hours after spaced light stimulation. White arrows point to ghost boutons observed before and after stimulation. Black arrowheads point to presynaptic debris that formed after stimulation.

(C-E) Live, intact larvae expressing channelrhodopsin-2 and mCD8-GFP in motor neurons where imaged immediately and at 1 hour intervals after spaced light stimulation. In some instances, detached ghost boutons simply became smaller and disappeared leaving debris (arrows in C and D), while detached ghost boutons sometimes simply became smaller and disappeared without leaving any obvious debris (white arrows in E.) Presynaptic debris at NMJ regions devoid of ghost boutons would also appear and then disappear following stimulation (black and pink arrowheads in E).

Calibration scale is 17 µm for (A, C-E), 12 µm for (B), and 9 µm for (A inset). Times correspond to hours from beginning of experiment when preparations were first imaged.

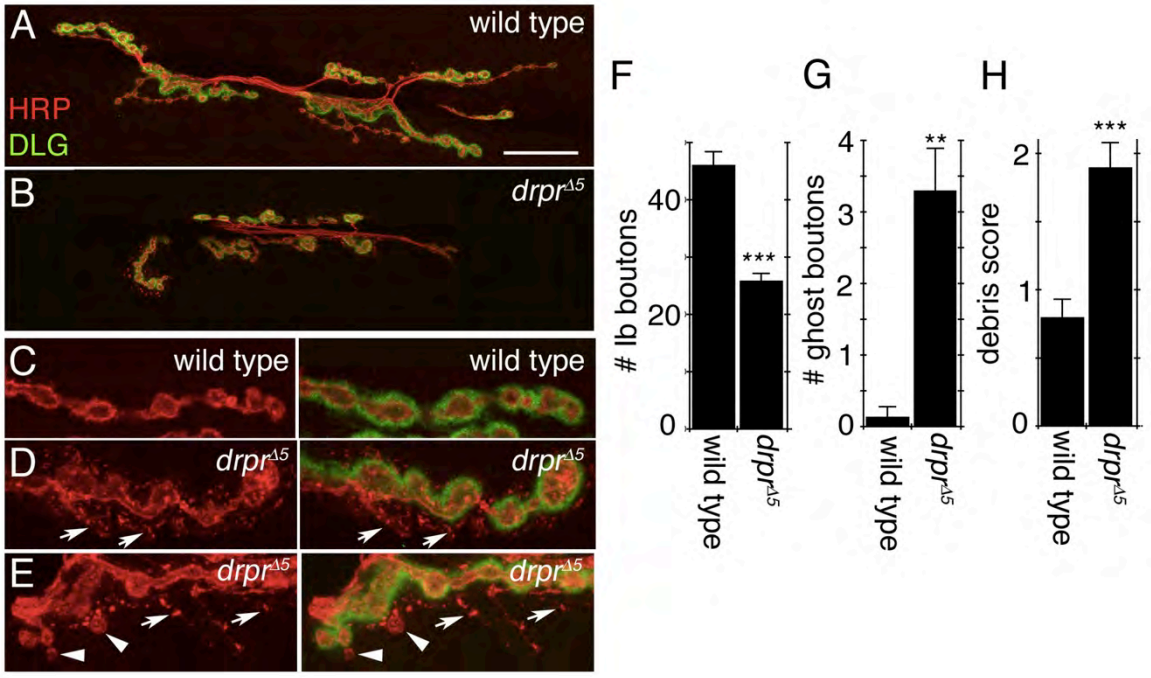


Figure 2-3. *draper* mutant NMJs exhibit reduced synaptic growth and accumulate pruned ghost boutons and presynaptic debris.

(A) A wild type third instar NMJ at muscles 6/7 visualized with α-HRP (red) and the postsynaptic marker DLG (green). (B) *draper*$^{\Delta 5}$ mutants have disrupted NMJ morphology and a significant reduction in the number of type Ib boutons compared to wild type. (C) The NMJ in wild type animals normally has very little presynaptic debris and ghost boutons are only rarely observed. (D, E) The NMJ in *draper*$^{\Delta 5}$ mutants accumulates large amounts of shed presynaptic debris (arrows) and many ghost boutons (arrowheads). (F-H) Quantification of the number of (F) type Ib boutons, (G) ghost boutons, and (H) presynaptic debris at muscles 6/7. *** $p<0.001$, ** $p\leq0.01$, * $p\leq0.05$. Calibration scale is 25 µm for (A,B), 8 µm for (C,D,E). (n=9 for both wild type and *draper*$^{\Delta 5}$)

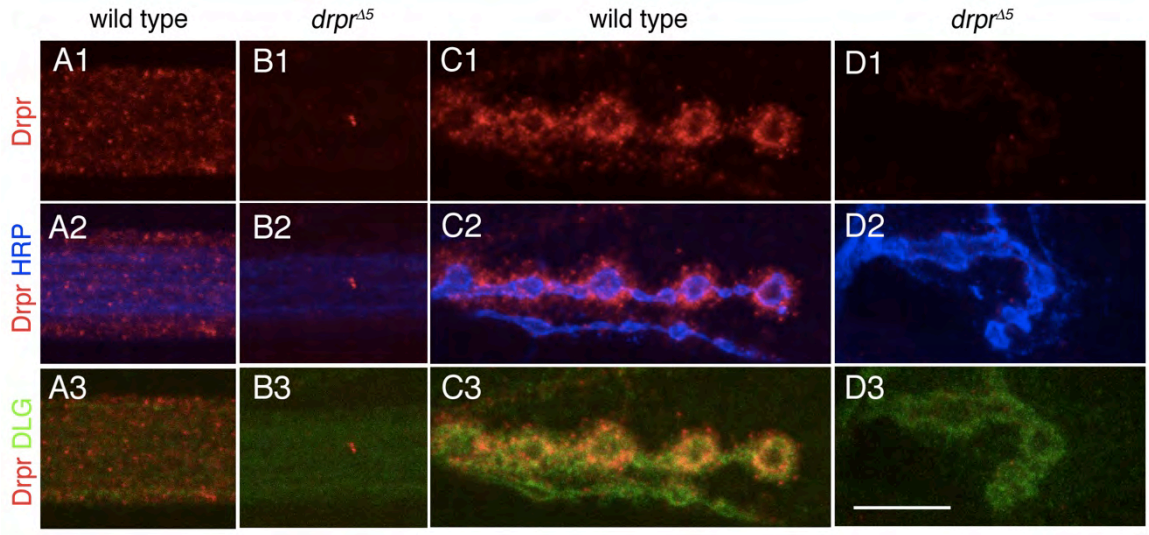


Figure 2-4. Draper is expressed in peripheral glia and in the postsynaptic region of the NMJ.

Wild type and *draper*$^{\Delta 5}$ null mutant third instar larvae were stained with α-Draper (red), α-HRP (blue), and α-DLG antibodies (green). (A1-3) Draper was readily detectable in peripheral glia, which surround the HRP^{+} axons. (B1-3) Draper immunoreactivity is absent from peripheral nerves in *draper*$^{\Delta 5}$ null animals, demonstrating the specificity of α-Draper sera for Draper in the segmental nerves. (C1-3) Draper is present postsynaptically at the NMJ surrounding HRP^{+} presynaptic boutons (C2), and colocalizes with the primarily postsynaptic marker DLG (C3). (D1-3) Draper immunoreactivity is absent from the NMJ in *draper*$^{\Delta 5}$ null animals. Calibration scale is 9.0 µm.

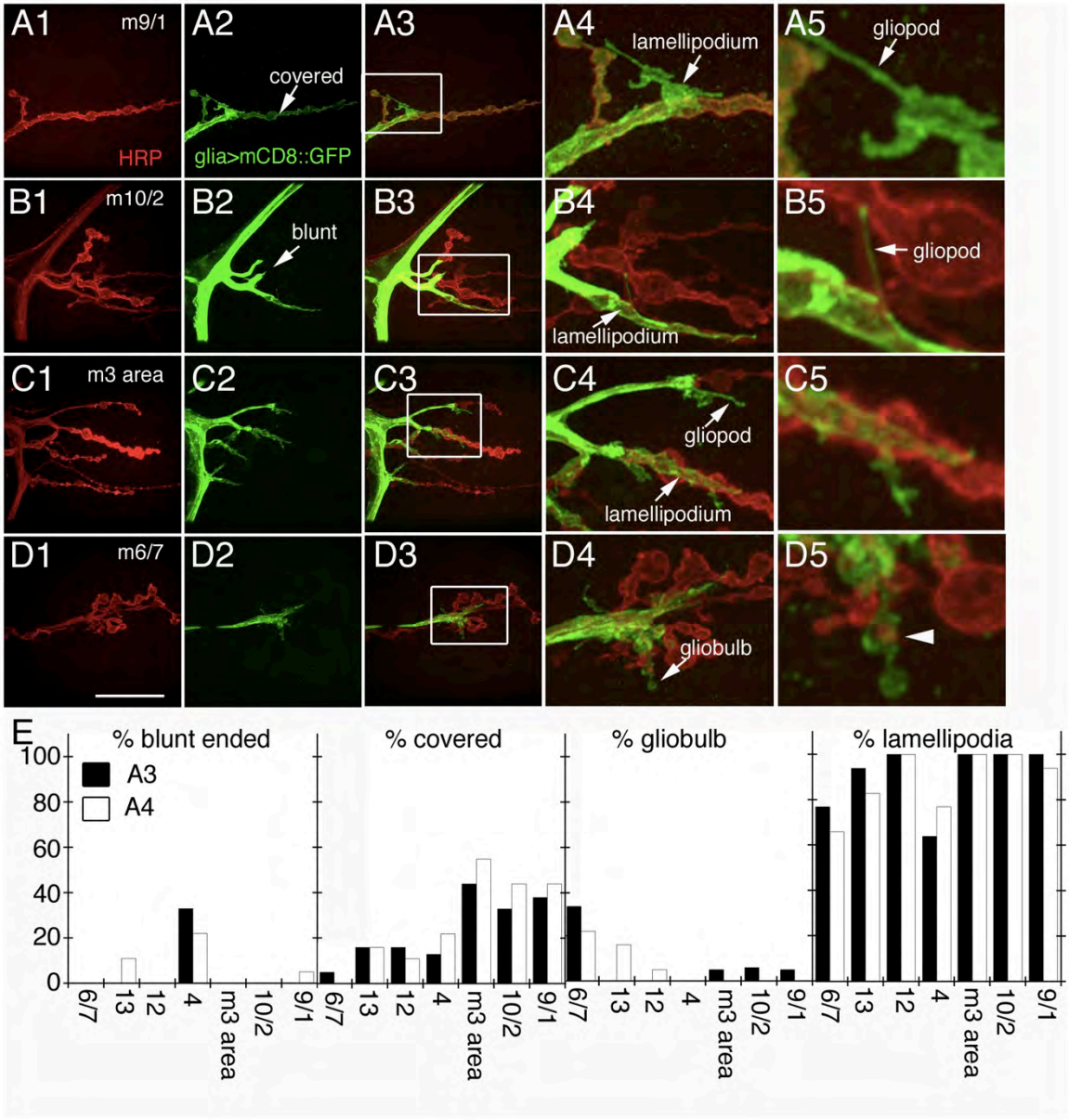


Figure 2-5. Glial cells dynamically invade the larval NMJ and their membrane extensions exhibit diverse morphologies.

Glial processes at the NMJ were observed by expressing mCD8-GFP in glia (with the Gli-Gal4 driver) and staining with α-HRP (red) and α-GFP (green) antibodies. Low magnification views of specific NMJs (identity indicated by the

numbers in the panels) are presented in columns (1-3). Higher magnification views of the boxed regions in column (3) are shown in columns (4) and (5).

(A1-5) In some cases, glial cell processes appear to cover the entire NMJ arbor (covered; arrow in A2). Glial cells could also be found extending lamellipodia-like extensions away from the parent arbor (lamellipodium; arrow in A4), or smaller filopodia-like projections (gliopods; arrow in A5).

(B1-5) In many cases glial cell processes terminated at the branch point where the motor axon entered the muscle field (blunt; arrow in B2). When glial processes invaded the NMJ, gliopods could be found extending from one NMJ branch across to another (arrow in B5).

(C1-5) An example of a gliopod extending into an area devoid of synaptic boutons (arrow in C4), and the extension of a lamellipodium contacting several synaptic boutons as well as a muscle region devoid of boutons (C5).

(D1-5) Glial cellular extensions can take on a spherical shape similar to boutons (gliogbulb; arrow in D4), which sometimes surrounds a synaptic bouton (arrowhead in D5) or are devoid of synaptic boutons.

(E) Quantification of glial projections at the third instar larval NMJ. The identity of muscles scored is indicated on the X-axis. “m3 area” (C1, E) corresponds to NMJs at muscles 3, 19, 20, and 11. n=20 hemisegments assayed.

Calibration scale is 18 µm for (columns 1-3), 9 µm for (4), and 4 µm for (5). n=10 animals.

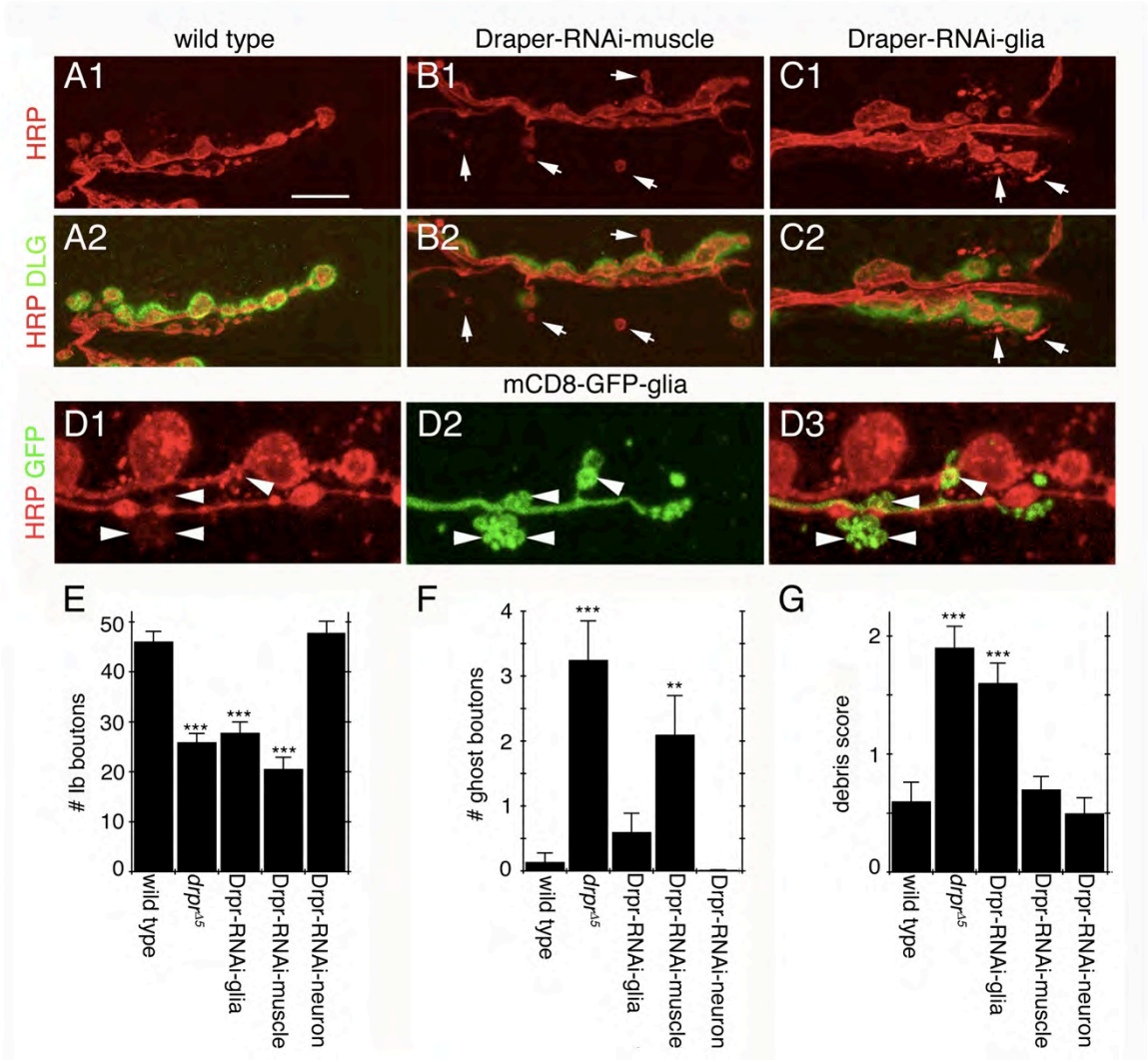


Figure 2-6. Draper function is essential in both glia and muscle cells for clearance of ghost boutons and shed presynaptic debris and for normal synaptic growth.

Draper function was knocked-down by expressing UAS-Draper-RNAi in either muscle (C57-Gal4), glia (repo-Gal4), or motor neurons (OK6-Gal4), and ghost boutons and presynaptic debris were quantified by staining for HRP (red), and the postsynapse was visualized with DLG (green).

(A1-2) Wild type NMJs have very little presynaptic debris and few or no ghost boutons.

(B1-2) Muscle-specific Draper knockdown leads to the accumulation of ghost boutons (arrows), but not of presynaptic debris.

(C1-2) Glial-specific Draper knockdown leads to the accumulation of presynaptic debris (arrows), but not of ghost boutons.

(D) mCD8-GFP (green) was expressed in glia with repo-Gal4 and motor neurons were visualized by staining for HRP (red). Representative images of weak HRP signal detected within glial extensions (arrowheads).

(E) Quantification of number of type Ib synaptic boutons at muscle 6/7 showing that Draper knockdown in glia or muscle cells reduces bouton number to those in *draper*$^{\Delta 5}$ null mutants, while Draper knockdown in motor neurons has no effect.

(F) Quantification of ghost bouton number. Knockdown of Draper in muscle cells, but not glia or motor neurons, leads to the accumulation of ghost boutons at levels equivalent to those found in *draper*$^{\Delta 5}$ null mutants.

(G) Quantification of shed presynaptic debris. Draper knockdown in glial cells, but not muscles or motorneurons, leads to the accumulation of presynaptic debris at levels similar to *draper*$^{\Delta 5}$ null mutants.

*** $p<0.001$, ** $p\leq0.01$, * $p\leq0.05$. error bars represent SEM.

Calibration scale is 12 µm for (A,B), and 3 µm for (D). ($n\geq10$ for each genotype)

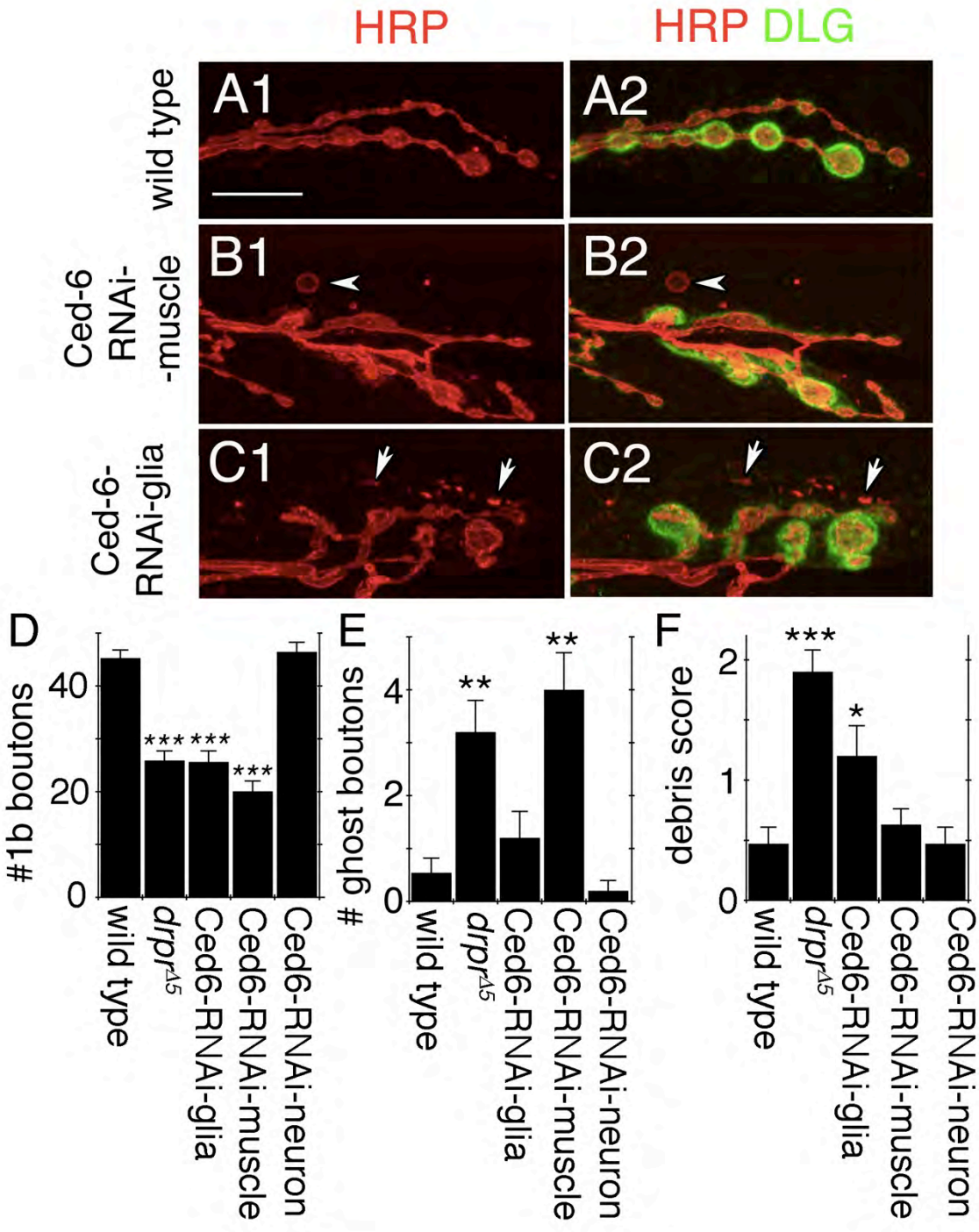

HRP
HRP DLG
wild type
Ced-6 RNAi--muscle
Ced-6-RNAi-glia
A1
A2
B1
B2
C1
C2
D
#1b boutons
40
20
0
***
***
***
wild type
drpr^Δ5
Ced6-RNAi-glia
Ced6-RNAi-muscle
Ced6-RNAi-neuron
E
ghost boutons
4
2
0
**
**
F
debris score
2
1
0
***
*

Figure 2-7. dCed-6, a key component of the Draper signaling pathway, is required for clearance of ghost boutons and presynaptic debris for and normal synaptic growth.

dCed-6 function at the NMJ was assayed by expressing UAS-dCed-6-RNAi in glia, motor neurons, and muscles. Preparations were labeled with the presynaptic marker α-HRP (red) and the postsynaptic marker α-DLG (green).

(A1-2) Wild type NMJs exhibit little or no presynaptic debris and ghost boutons.

(B1-2) Muscle-specific dCed-6 knockdown leads to the accumulation of ghost boutons (arrowheads) but very little presynaptic debris.

(C1-2) Glial-specific dCed-6 knockdown leads to the accumulation of presynaptic debris (arrows) but not ghost boutons.

(D-F) Quantification of the number of (D) type Ib boutons, (E) ghost boutons, and (F) presynaptic debris in control and dCed-6 knockdown backgrounds. dCed-6 function is required in both muscles and glia for (D) normal synaptic growth, in (E) muscles for the clearance of ghost boutons, and (F) in glia for clearance of presynaptic debris.

*** $p<0.001$, ** $p\leq0.01$, * $p\leq0.05$. error bars represent SEM. For D-F, n=12 for wild type, 9 for *drpr*$^{\Delta 5}$, and 13 for dCed-6^{RNAi}. Calibration scale is 12 µm.

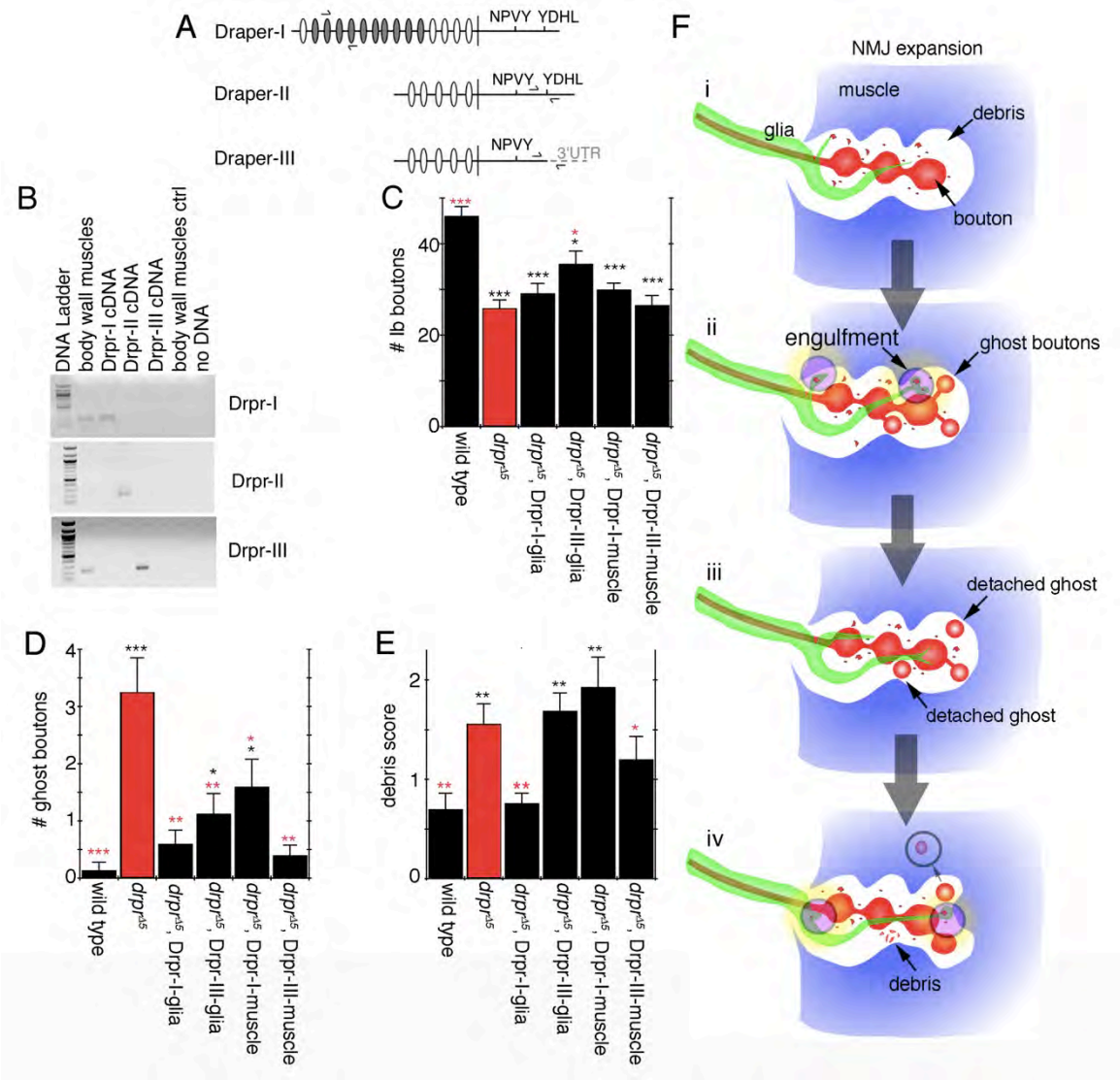


Figure 2-8. Cell type-specific rescue of *draper* mutant phenotypes with alternative Draper receptor isoforms.

(A) Three isoforms of the Draper receptor have been identified in *Drosophila* (Freeman et al., 2003b). We designed isoform-specific primers (arrows) to determine the presence of each unique isoform in larvae. Ovals represent EGF-like repeats in the extracellular domain.

(B) RT-PCR shows that Draper-I and Draper-III are expressed in body wall muscles. cDNAs for each isoform were used as positive controls, along with a minus RT reaction.

(C-E) To assay for the cell-specific function of Draper-I or Draper-III, each isoform was expressed in either glia (with Gli-Gal4) or muscle cells (with C57-Gal4) in *draper*$^{\Delta 5}$ null mutant backgrounds to determine which isoform rescued mutant phenotypes, including (C) decreased bouton number, (D) accumulation of ghost boutons, and (E) accumulation of presynaptic debris. *draper*$^{\Delta 5}$ mutant phenotypes are shown in red bars.

(C) Expression of Draper-III in glia provides a partial rescue of the decrease in type Ib bouton number observed in *draper*$^{\Delta 5}$ mutants.

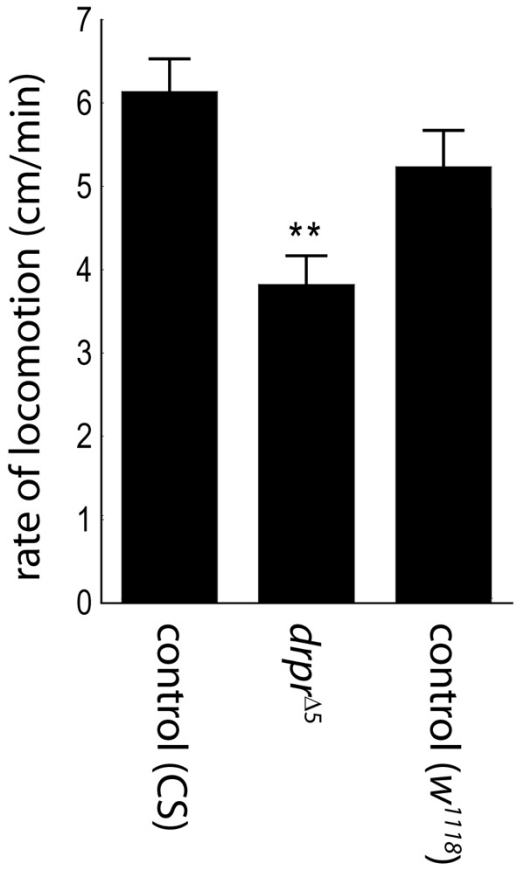
(D) Expression of Draper-I in glia or Draper-III in muscle or glia provides complete rescue of the accumulation of ghost boutons observed in *draper*$^{\Delta 5}$ mutants. Expression of Draper-III in glia or Draper-I in muscle also provides a partial rescue of ghost bouton number.

(E) Expression of Draper-I in glia fully rescues the accumulation of presynaptic debris observed in *draper*$^{\Delta 5}$ mutants. Expression of Draper-III in muscle also provides weak but significant rescue.

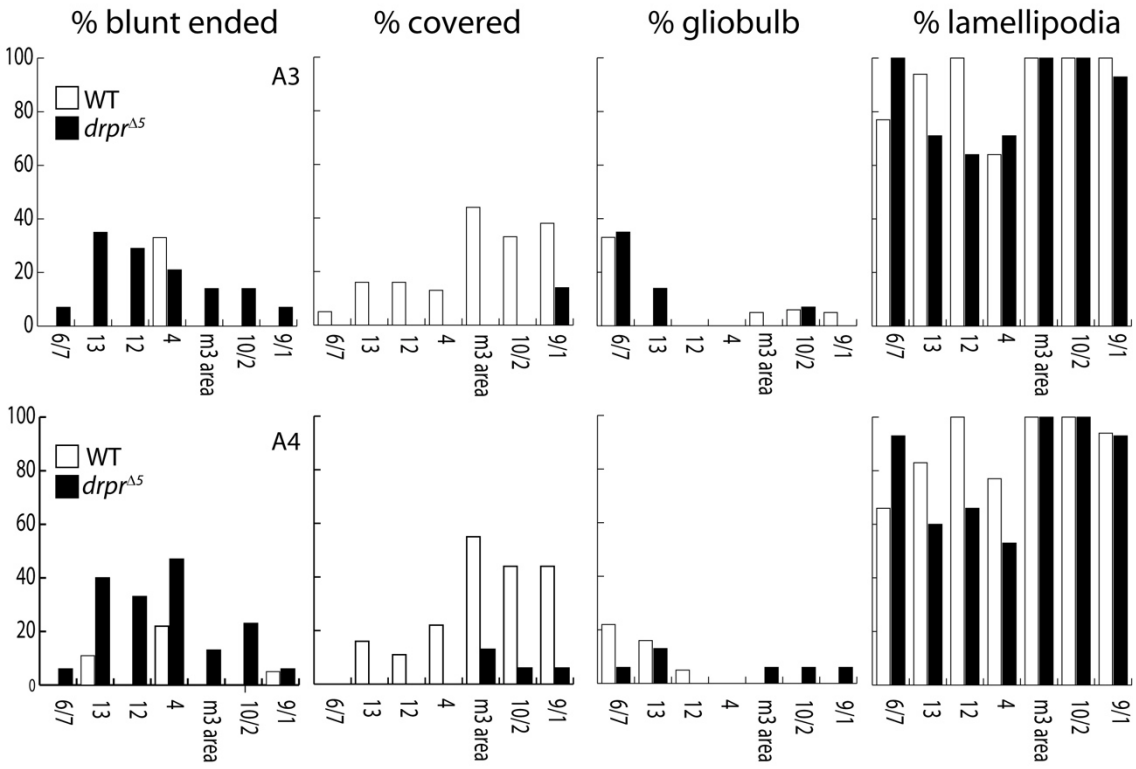
(F) Model for Draper receptor function at the NMJ. i) A motorneuron with an increase in activity or other developmental cues produces ii) more ghost boutons, and an increase in debris which is engulfed by glial extensions. The newly formed ghost boutons will either iii) stabilize or detach from the main arbor.

Detached boutons will either iv) degrade into debris or be engulfed by the muscle.

For C-E, *** p<0.001, ** p≤0.01, * p≤0.05. error bars represent SEM. Red asterisk, compared to *draper*$^{\Delta 5}$ mutants; black asterisk, compared to wild type. For C-E, n=9, 9, 8, 8, 8, 8, for genotype as listed left→right, respectively.



Supplementary Figure 2-1. *draper* mutants exhibit reduced larval motility. Wild type controls (*CS* and *w*^{1118}) were compared to *draper*$^{\Delta 5}$ mutant larvae in larval crawling assays (see methods). *draper* mutants show reduced rates of locomotion (p<0.001). error bars represent SEM.



Supplementary Figure 2-2. Changes in glial membrane extensions in *draper* mutants.

Glial membrane extensions in *draper*$^{\Delta 5}$ mutants were compared to controls by labeling membranes with mCD8-GFP (see Figure 5 and methods). A3 and A4 correspond to abdominal segments. The identity of muscles scored is indicated on the X-axis. “m3 area” corresponds to NMJs at muscles 3, 19, 20, and 11. n=15 hemisegments. *draper*$^{\Delta 5}$ mutants showed a dramatic decrease in the number of covered NMJs, a change in the distribution of gliobulbs, and an increase in the number of blunt ended glial projections.

Supplementary Movie 2-1: Active disintegration of ghost boutons into smaller structures and disappearance from the NMJ. Motorneurons were labeled with mCD8-GFP (using *C380-Gal4*), and imaged every 10 seconds for a 5 minute interval. Note that one ghost bouton (center of field of view) splits into two smaller GFP^+ structures, one lingers at the NMJ, while the other shifts its position dramatically and then disappears from the plane of focus. Full analysis of the Z-stack revealed that this particle had moved to a position deep within the muscle cell (not shown), apparently having been engulfed. A 3-dimensional rendering of the Z stack revealed that the presynaptic debris particles imaged remained fully within the Z-series and the changes observed were not the result of specimen drift.

Found at: doi:10.1371/journal.pbio.1000184.s003

Supplemental Movie 2: Glial cells rapidly invade the NMJ *in vivo*

Peripheral glia were labeled with mCD8-GFP (using the Gli-Gal4 driver), and glial dynamics at the NMJ were assayed in living third instar larvae. Total movie length is 6 minutes. Note the extension of gliopods at the distal tip, and spreading of glial membranes at the branch point.

Found at: doi:10.1371/journal.pbio.1000184.s004

CHAPTER III

Coordination of Pre- and Postsynaptic Development by Glia-Derived Maverick

Yuly Fuentes-Medel, Marc Freeman* and Vivian Budnik*

CONTRIBUTION SUMMARY

In chapter III, Amy Sheehan collaborate to make UAS-mav:GFP and UAS-MAV flies; Romina Barria collaborate with her magic dissections and technical expertise's. Marc Freeman and Vivian Budnik supported the work by discussions to design the experiments, to analyze the data and to write the chapter. I designed and performed the experiments, I analyzed the data and I wrote the chapter.

ABSTRACT

The transforming growth factor beta (TGF-β) pathway is critical for the development of the nervous system. Furthermore, *glassbottom boat* (Gbb) is the retrograde signal at the *Drosophila* neuromuscular junction (NMJ). Glia are known to regulate synapse development and modulate synaptic efficacy. However, the molecular pathways involved are poorly understood.

We found that *Drosophila* glia express three TGF-β ligands (Dawdle, Myoglianin and Maverick) and these molecules are important for NMJ development. We used the phosphorylation of MAD as a read out of bone morphogenic protein (BMP) pathway activation. Surprisingly, we found that glial-expressed Maverick regulates the BMP pathway both pre- and post-synaptically, and this ligand regulates the transcription of *glassbottom boat* (*gbb*) in the body wall muscle. This suggests a regulatory loop in which glia affect Gbb in the muscle and as a consequence the activation of the retrograde pathway. Finally loss of glial Maverick decreased the transcription of the *shaker* potassium channel, suggesting the exciting possibility that glia can directly regulate muscle membrane excitability that ultimately will regulate synaptic transmission properties. These data propose a model where glia orchestrate the regulation of retrograde signaling and functional synaptic activation.

INTRODUCTION

Bone Morphogenetic Proteins (BMPs), function in postmitotic neurons to regulate synapse development and plasticity, as well as patterning formation during development (Keshishian and Kim, 2004; Marques, 2005; Krieglstein et al., 2011). For example, in the vertebrate CNS, removal of the BMP inhibitor cordin, enhances paired pulse facilitation and long-term potentiation (LTP), which results in alterations in spatial learning (Sun et al., 2007). At the *Drosophila* larval neuromuscular junction (NMJ) the BMP Glass bottom boat (Gbb) functions as a retrograde signaling mechanism to regulate presynaptic growth (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003; Rawson et al., 2003; Goold and Davis, 2007). In this process, Gbb is released by muscles and binds to the type I BMP receptors, Saxophone (Sax) or Thickveins (Tkv) and to the type II BMP receptor Wishful thinking (Wit) present in presynaptic motorneurons. According to canonical BMP signaling, this leads to phosphorylation of the receptor Smad, Mothers against Dpp (Mad), which becomes competent for binding to the co-Smad Medea (Miyazono et al., 2010). The complex then translocates to the motorneuron nucleus where it activates the transcription of genes involved in synapse development, including the actin regulatory protein TRIO (Ball et al., 2010; Fuentes-Medel and Budnik, 2010). This signaling pathway is required to signal the proliferation of new synaptic boutons in direct correlation with muscle size as the animal grows during the larval period.

While the above pathway was initially documented as a retrograde signaling pathway at the NMJ, recent studies provide evidence for BMP function in postsynaptic muscles (Dudu et al., 2006). Activated Mad (Phospho-Mad; P-Mad) is also observed in muscle, and Tkv receptor has been shown to colocalize with postsynaptic DLG at the NMJ (Dudu et al., 2006; Higashi-Kovtun et al., 2010). In addition, studies suggest that activation of the activin-type type II receptor Baboon (Babo) in muscles regulates the transcription of Gbb (Ellis et al., 2010b). However, the source of the ligand remains unclear.

P-Mad has also been observed at sites of neurotransmitter release or apposed postsynaptic glutamate receptor (GluR) clusters at the larval NMJ, although the pre- or postsynaptic nature of this signal has remained controversial (Dudu et al., 2006; O'Connor-Giles et al., 2008). Here we demonstrate that peripheral glia are a source of a TGF-β superfamily ligand, Maverick (Mav) that regulates synaptic development. We find that Mav is secreted from peripheral glia and that interfering with Mav function in peripheral glia prevents proper development of the NMJ. Further, we show that Mav is required for activation of BMP signaling in both neurons and muscles. Our results are consistent with a model whereby glia-derived Mav regulates the expression of Gbb in muscles, thus modulating the function of the retrograde signal from muscles to neurons to regulate presynaptic growth. In addition, we show that in postsynaptic muscles, glial Mav negatively regulates the expression of Shaker (Sh) K^+ channel. Sh mediated I_A potassium currents, this result suggest that I_A currents could be

affected in the absence of glial maverick, but further investigation will be necessary to support a model where glia molecules to modulate muscle membrane excitability.

RESULTS

A BMP signaling pathway is activated by peripheral glia during synapse development

Glial cells establish transient interactions with synaptic boutons and muscles at the NMJ and the function of glia is crucial for normal NMJ expansion (Fuentes-Medel et al., 2009). However, the signals provided by glia to regulate synaptic growth are unknown. At the *Drosophila* larval NMJ, BMP signaling pathways are activated both in motorneurons and muscles to regulate synaptic development (Keshishian and Kim, 2004; Dudu et al., 2006). We therefore asked whether glia had any role in the regulation of BMP signaling. *In vitro* experiments have suggested that cultured *Xenopus* Schwann cells promote synaptogenesis by releasing TGF-β1, although the mechanisms underlying this process are not well understood (Feng and Ko, 2008). As a first approach to examine if glia regulates TGF-β signaling, we determined if TGF-β ligands were expressed in peripheral glia at the larval stage. To isolate transcripts encoding for TGF-β ligands specifically in peripheral glia, we extracted mRNA from the peripheral nerves of 3rd instar larvae, as cell bodies from peripheral glia are the only cell bodies found within these nerves. Real-time PCR (qPCR) analysis revealed the presence of

several BMP ligands, including Myoglianin (MYO), Dawdle (Daw) and Maverick (Mav) (Fig. 3-1A). In contrast, Activinß (Actß) transcripts were not detected in these preparations (Fig. 3-1A).

To determine if any of the above ligands found in glia could be involved in NMJ development, we expressed Daw-RNAi, Mav-RNAi and MYO-RNAi in peripheral glia using the peripheral glia Gal4 driver rl82-Gal4 (Auld et al., 1995; Fuentes-Medel et al., 2009) and examined the morphology of larval NMJs. Downregulating any of the above genes in peripheral glia resulted in a substantial decrease in NMJ size, as determined by counting the number of synaptic boutons at the 3rd instar larval stage (Fig. 3-1B-F). However, this defect was more pronounced when Daw or Mav was downregulated in peripheral glia (Fig. 3-1B).

Glia-derived Maverick is required for normal activation of the BMP pathway in motorneurons.

A classical read-out of BMP pathway activation is the presence of phosphorylated Mad (P-Mad) (Ross et al., 2001). At the *Drosophila* larval NMJ, BMP activation through P-Mad detection has been documented both in the nuclei of motorneurons (McCabe et al., 2003), as well as at synaptic boutons of the NMJ (Dudu et al., 2006). Thus, we determined if downregulating Daw or Mav in glia resulted in altered P-Mad levels at these sites. Expression of Mad-RNAi in motorneurons led to a drastic decrease in P-Mad immunoreactivity at

motorneuron nuclei, demonstrating that P-Mad immunoreactivity at this site is specific (Fig. 3-2A, B, D). Similarly, P-Mad immunoreactivity levels were significantly decreased in the nuclei of larval motorneurons when Mav-RNAi, but not MYO-RNAi or Daw-RNAi, was expressed in peripheral glia (Fig. 3-2A, C, D). However, this reduction was not as severe as that observed by downregulating Mad in neurons (Fig. 3-2D). This is not surprising as BMP signaling in motorneurons is also known to be activated through the binding of muscle-derived Gbb to the BMP receptors Wit and Sax/Tkv present in motorneurons (Marques et al., 2002; McCabe et al., 2003). These results suggest that glia-derived Mav is at least partially required to activate BMP signaling in motorneurons.

To further test the model that glia-derived Mav is required for P-Mad levels in motorneurons, we also examined the levels of a BMP target gene, Trio (Ball et al., 2010). Trio is a Rac activating protein that contributes to cytoskeletal remodeling during synaptic growth. Previous studies demonstrate that upon activation of motorneuron BMP signaling by muscle Gbb, *trio* transcription is upregulated. (Ball et al., 2010; Fuentes-Medel and Budnik, 2010). Real-time PCR revealed that Trio transcript levels were significantly reduced in total RNA isolated from larval brains when Mav-RNAi was expressed in peripheral glia (Fig. 3-2E). In contrast, the levels of Cyclophilin transcript were unchanged by this manipulation (Fig. 3-2E). Thus, like muscle Gbb, glial Mav is required for normal

NMJ growth, for activating BMP signaling in motorneurons, and for the transcription of a BMP target gene in motorneurons.

Glia-derived Mav is also required for BMP signaling at synaptic boutons

As noted above, activation of BMP signaling is also observed in the form of changes in P-Mad immunoreactivity at the NMJ (Dudu et al., 2006). This immunoreactivity is observed as discrete puncta at synaptic boutons (Dudu et al., 2006) (Fig. 3-3A). Thus, we next determined whether BMP activation at synaptic boutons was also regulated by glia-derived Mav. Notably, downregulating Mav in peripheral glia with two different Mav-RNAi constructs, virtually eliminated P-Mad immunoreactivity at synaptic sites (Fig. 3-3B, J). Quantification of the percentage of boutons containing P-Mad immunoreactivity revealed that just over 5% of the boutons in larvae expressing Mav-RNAi-1 in glia displayed P-Mad labeling at the NMJ. In contrast, downregulating MYO was without effect (Fig. 3-3C, J). A decrease in P-Mad was also observed by downregulation of Daw in glia (Fig. 3-3D), although this effect was much weaker than that observed upon downregulating Mav. Indeed, over 65% of the boutons displayed P-Mad immunoreactivity when Daw was downregulated in glia compared with 5-7% when Mav was downregulated in glia (Fig. 3-3J). To determine if Mav was exclusively required in glia for activation of the BMP pathway at synaptic sites, we also expressed Mav-RNAi in either muscles or motorneurons and examined levels of P-Mad immunoreactivity at the NMJ. No significant change in P-Mad

immunoreactivity was observed by downregulating Mav in either of these cells (Fig. 3-3E, F, J), suggesting that Mav is exclusively required in glia for BMP activation at synaptic sites. Further support for these observations was obtained by examining the effect of overexpressing Mav in glia on P-Mad immunoreactivity levels. This manipulation resulted in an increase in the intensity of the P-Mad puncta at the NMJ (Fig. 3-3G-I, K). Thus, glia derived Mav is required for BMP activation both at motorneuron nuclei and at synaptic boutons, and this signal is required for normal synaptic development.

To determine if Mav function was exclusively required in glia for normal NMJ development, we downregulated Mav in motorneurons or muscles and examined the number of synaptic boutons at the third instar larval stage. In contrast to Mav downregulation in glia, which significantly reduced the number of synaptic boutons, no change in bouton number was observed when Mav was downregulated in motorneurons or muscles (Fig. 3-3L). Thus, Mav appears to be required exclusively in glia for normal NMJ development.

Mav is present in peripheral glia and peripheral glia can release Mav

The requirement of peripheral glia-derived Mav for normal activation of the BMP pathway in motorneuron and synaptic boutons, as well as for proper NMJ development, suggested that Mav is released by peripheral glia. To determine if Mav was present in glia we generated a peptide antibody against Mav. Immunocytochemistry revealed the presence of small immunoreactive puncta

within the segmental nerve sheath, corresponding to glial cell bodies surrounding the axons in this nerve (Fig. 3-4A). This immunoreactivity was specific as it was severely decreased upon downregulating Mav in peripheral glia by RNAi (Fig. 3-4B).To further test the hypothesis that peripheral glia could release Mav, we also generated transgenic flies expressing a GFP-tagged Mav transgene that could be driven in glia by the rl82-Gal4 driver. Expressing Mav-GFP in glia resulted in punctate GFP staining within glial membranes in the peripheral nerves, similar to that observed with endogenous Mav (Fig. 3-4A). This punctate GFP label was most prominent at glial extensions observed at the NMJ (Fig. 3-4D, arrowhead), showing that Mav-GFP is efficiently transported to these glial extensions when expressed in peripheral glia. Notably, bright GFP-positive punctae were observed beyond the glial extensions (Fig. 3-4D), suggesting that Mav-GFP can be released by peripheral glia. Close observation of the Mav-GFP puncta outside the glial membrane extensions, revealed their localization both in close association with synaptic boutons, as wells as with the postsynaptic junctional region of the muscle (Fig. 3-4E, arrows). In contrast, expressing Mav-GFP in neurons, resulted in punctate and diffuse GFP staining within synaptic boutons, but no GFP signal was observed at the postsynaptic region (Fig. 3-4F) showing that Mav-GFP cannot be released by synaptic boutons. Similarly, expressing Mav-GFP in muscles resulted in very dim GFP signal in muscles, but this signal did not localize to the NMJ (Fig. 3-4G). Thus, Mav is present in peripheral glia and peripheral glia are capable of releasing Mav to the NMJ.

Synaptic bouton P-Mad signal is pre- and postsynaptic

The finding that glia can release Mav, and that glia derived Mav is required for BMP signaling at the NMJ, raised the question as to which cells (neurons or muscle) respond to this Mav signal. Reports in the literature appear to be divergent in this regard. In one study, it was found that NMJ P-Mad partially colocalized with the presynaptic active zone marker BRP, while it did not colocalize with DLG, suggesting that the P-Mad signal was presynaptic (O'Connor-Giles et al., 2008). However, DLG is localized at the perisynaptic region (Sone et al., 2000), and therefore it is not expected to colocalize with the postsynaptic density. In another study it was found that in *wit* mutants the P-Mad signal at the NMJ was eliminated (Higashi-Kovtun et al., 2010), which, given the role of Wit in presynaptic motorneurons, led to the conclusion that the P-Mad signal at the NMJ was presynaptic. However, whether Wit is also expressed in muscles is not known. In a third report, NMJ P-Mad immunoreactivity was compared to the localization of a GluR tagged transgene and found to be completely colocalized, which suggested a postsynaptic P-Mad localization (Dudu et al., 2006). However, a comparison with endogenous GluRs was not done in these studies. To address this issue more directly, we first used a strong hypomorphic *mad* mutant, mad^{12}, over a *mad* deficiency chromosome and examined P-Mad labeling at the NMJ. P-Mad immunoreactivity was eliminated in this mutant (Fig. 3-5A, B), providing further evidence for the specificity of the P-

Mad signal at the NMJ. We then downregulated Mad in either motorneurons or muscles by expressing Mad-RNAi and examined the intensity of the P-Mad signal. We found that downregulating Mad either in neurons or muscles resulted in significant decrease in P-Mad signal intensity (Fig. 3-5C, E-G), suggesting that the synaptic P-Mad signal is both pre- and postsynaptic. Furthermore, the number of synaptic boutons was significantly reduced by either downregulating Mad in neurons or muscles (Fig. 3-5D), again suggesting the requirement of Mad function in both cell types.

We also examined the localization of P-Mad signal in comparison with the endogenous localization of GluRIIA and BRP. Confirming previous reports with the GluRIIA transgene, we found that the synaptic P-Mad signal was always present within the boundaries of GluRIIA clusters (Fig. 3-5H, I, M). In contrast, there was only partial colocalization between BRP and P-Mad, and the signals appeared juxtaposed (Fig. 3-5J-L). These results suggest that at least some of the P-Mad signal is both pre- and postsynaptic. However, given that active zones and postsynaptic GluR clusters are apposed to each other in close proximity, light microscopy alone cannot resolve this issue. Nevertheless, the above results showing that downregulating Mad either in muscles or neurons leads to a reduction in the P-Mad signal, are a strong indication that the signal is localized in both cells.

Glia-derived Mav activates Gbb, DAD and Shaker expression in muscles

The above observations suggest that glia derived Mav activates both neuron and muscle BMP signaling. Given the well characterized role of Gbb release by muscle in activating a neuronal BMP cascade involving Wit and Tkv or Sax, a potential model to explain the role of Mav in neurons and muscles is that it regulates the expression of Gbb in muscles. Consistent with this model, quantitative analysis of Gbb transcript in muscle revealed that Gbb mRNA was decreased upon downregulating Mav in glia (Fig. 3-6A). Thus, by regulating Gbb expression in muscle, glia derived Mav may regulate the potency of the retrograde Gbb signal. Moreover, the transcript leveles of the known TGF-β inhibitor Smad, DAD, were also decreased at the BWM, supporting a post-synaptic role for glial Maverick (Fig. 3-6A).

Previous studies have also suggested that TGF-β signaling pathway regulates the expression of K^+ channels (Cameron et al., 1998; Cameron et al., 1999; Zhuang et al., 2012). In *Drosophila*, the *Sh* locus encodes for several Sh isoforms. To determine if Mav could also regulate the expression of Sh in muscles, we carried out real time PCR using primers that recognize all of the multiple Sh transcripts (Ingleby et al., 2009). Mav downregulation in peripheral glia resulted in approximately 60% reduction in Sh transcripts (Fig. 3-6A).

DISCUSSION

Here we have unraveled a TGF-ß signaling pathway that is initiated by glia through the release of Mav, a TGF-ß ligand. This finding is particularly significant, since despite the knowledge that glial cells are required for normal synaptic development, the signals provided by glia during this process are largely unknown. Equally important is the finding that glia-derived Mav regulates BMP signaling both in muscles and motorneurons. Thus, glia might serve as a regulatory hub for coordinating the development of pre-and postsynaptic cells.

The observation that three TGF-β superfamily ligands are expressed in peripheral glia brings new complexity to our understanding of BMP signaling at the NMJ. Indeed, knockdown of each ligand prevented the normal expansion of the NMJ. Although at present we do not know the cellular targets for MYO, Mav or Daw, the release of multiple BMPs may serve as a mechanism to regulate the release of tissue specific signals that impinge on synaptic development. For example, our studies reveal that Mav regulates the production of Gbb by muscle, thus influencing NMJ development by modulating retrograde signaling from muscles to motorneurons. Similarly, it is possible that peripheral glia release multiple BMPs to regulate other (non-synaptic) aspects of development, such as positive regulation of nutrient storage and energy homeostasis (Ballard et al., 2010), modulate immune responses (Clark et al., 2011), or even the transition to metamorphosis (Gibbens et al., 2011). In this regard, the exposure of all organs

to the hemolymph in this animal with open circulatory system, may allow a response by cells that are quite distant from the glia. Furthermore, it is also possible that there could be molecular pathways activated by the TGF-β ligands that function independent of P-Mad (Moustakas and Heldin, 2009) or a different R-Smad. For example, genetic interactions have linked Daw with the activation of the R-smad, Smox (Jensen et al., 2009; Ellis et al., 2010b). Furthermore, Smox transcripts have been found to be expressed in BWM and Smox mutant has decreased growth of NMJs (Ellis et al., 2010b) This pathway could potentially regulate completely different sets of downstream target genes. It has also been reported that *dawdle* mutants have undergrown NMJs. However, rescue experiments suggested that Daw is released from many cellular sources (Ellis et al., 2010b). This is consistent with our finding that Daw is expressed in glia and that downregulation of Daw in glia resulted in underdeveloped NMJs.

Previous studies provided evidence that *MYO* mRNA was expressed both in glia and muscle (Lo and Frasch, 1999). However, its role in these cells has remained unclear. We found that MYO did not affect either nuclear or synaptic P-Mad signal, indicating that there are alternative target tissues or MYO activates an unknown signaling pathway independent of Mad phosphorylation. In contrast, we identified a substantial role for glial Maverick in regulating MAD activation at synaptic boutons. Our results suggest that Maverick secretion from glia, not neurons or muscle, is critical for the normal growth of the NMJ. Finally, the

mechanisms of how these three molecules coordinately regulate NMJ growth remain to be investigated.

Our results suggest that glia-derived Mav regulates the expression of Gbb. Therefore the most simple explanation for our finding that glia derived Mav is required for normal BMP signaling at motorneuron nuclei is that by releasing Mav, glia controls the magnitude of the retrograde signal by Gbb. However, an additional possibility is that besides regulating Gbb expression in muscle, glia-derived Mav regulates the function of Gbb by forming a heterodimer. Such heterodimeric interactions have been observed, for example, in the case of Dpp (Decapentaplegic) and Gbb. The formation of a Gbb-Dpp heterodimer serves to create a gradient of the ligand activity in the wing tissue (Bangi and Wharton, 2006)

Our results make it highly likely that Mav is locally secreted by peripheral glia at the NMJ. First, endogenous Mav was found within peripheral glia at the segmental nerves, and this distribution pattern was mimicked by expressing a Mav-GFP transgene. Notably, expression of Mav-GFP in glia resulted in GFP signal not only within glial membranes, but beyond these membranes, in association with synaptic boutons and in the muscle region close to synaptic boutons. Previous studies demonstrated that peripheral glial membrane extensions are dynamic, extending and retracting processes that became associated with synaptic boutons and muscles (Fuentes-Medel et al., 2009).Thus, it is possible that either glia-derived Mav is released at the NMJ

branchpoint, where most glial membranes terminate, and Mav then diffuses to synaptic boutons and muscles. Alternatively, glial membrane extensions might directly deposit Mav as they interact with boutons or muscles.

Understanding the cellular origin of the synaptic P-Mad was critical to distinguish between models whereby Mav could be activating BMP signaling in muscle, at the pre-synaptic bouton membrane, or both. While the activation of retrograde BMP signaling at the NMJ through Gbb release has been well characterized (McCabe et al., 2003), the finding that BMP pathway components, including receptors, also exist within muscle cells suggest that BMPs might be used bidirectionally. In support of this notion, knocking down MAD in either muscles or motorneurons decreased the levels of P-Mad signal at the NMJ and decreased normal synaptic growth. The finding that glia locally releases Mav to regulate both pre- and postsynaptic BMP signaling strongly suggest that glia serves to integrate and to coordinate pre- and postsynaptic development. Similarly, vertebrate perisynaptic Schwann cells secrete TGF-β1, this ligand regulates the levels of agrin in the presynaptic neuron and increase the acetylcholine receptor clustering in vitro (Feng and Ko, 2008). However, none direct postsynaptic response has been reported.

Bidirectional signaling at the NMJ has also been observed for other secreted factors, such as the Wingless (Wg) pathway. Interestingly, In the case of Wg, different transduction pathways are activated in each cell, and each different pathway has its own specific outcome, which has alternative

implications for synapse development. Furthermore, one future line of investigation will be to understand the regulation of Wg and TGF-β pathways in the muscle cell. Interestingly, the state of phosphorylation of MAD itself has been shown to control the competition between Wg and BMP signaling (Eivers et al., 2011), however none of this is known at the NMJ in *Drosophila*. Moreover, none of the downstream muscle target genes has been described so far. We focused our attention on transcripts that were affected by glial Maverick. Intriguingly we found that glial Maverick regulates the transcription of Shaker channels, raising the possibility that a glial ligand can affect the synaptic potassium currents, and this could have different implications, such as delaying repolarization of the synaptic current, which would lead to different spread of electronic signals in this isopotential muscle.

The results above are a strong indication that glia cells can actively regulate the normal growth of synapses and perhaps orchestrate the communication between presynaptic and postsynaptic cells. In addition this data provides compelling evidence that BMP signaling is more complex than previously anticipated.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used for this study: Gli-Gal4 (rl82) (Sepp and Auld, 1999), Tubulin Gal4 (Lee and Luo, 1999), C57-Gal4 and C380-Gal4 (Budnik et

al., 1996), UAS-mCD8-GFP (Lee and Luo, 1999), UAS-mCD8Cherry (gift from Mary Logan) UAS-*mad-RNAi* Transformant ID: 12635 , *UAS-myo- RNAi* Transformant ID: 33132, UAS-*dawdle-RNAi* Transformant ID: 105309 (Vienna *Drosophila* RNAi Center), UAS-*maverick* RNAi-1,(called R34 and made by Tzumin Lee) UAS-*maverick* RNAi-2, Stock ID 1901R-4 (Fly Stock of National Institute of Genetics, Japan) , we also generated a UAS-*maverick* and a UAS-*mav*erick:GFP. To generate UAS-*maverick* and UAS-*maverick*:GFP transgenic flies, wild–type *maverick*-A cDNAs were obtained, subcloned into a pUAST vector, and injected to w^{1118} flies.

Real Time PCR

Total RNA was isolated from dissected third instar peripheral nerves and extracted with Trizol (Invitrogen) and purified using the RNeasy Micro Kit (QIAGEN) for nerves. First strand cDNA was synthesized using Sensiscript RT (QIAGEN) enzyme with oligo (dT) 12-18 primer (Invitrogen). Real time PCR for nerves was performed using the following Taqman primers to effectively detect expression of Repo (Assay ID Dm02134815_g1), Myoglianin (Assay ID Dm01820708_g1) , Dawdle (Assay ID Dm01814209_g1) Maverick (Assay ID Dm01825561_g1), Activin B (Assay ID Dm01831511_m1) and Gapdh (Assay ID Dm01841185_m1) from Applied biosystems. This procedure was necessary to enhance the specificity and sensitivity of the assay, due to the limited amount of mRNA from peripheral nerves. The real time curves were monitored comparing the –RT controls as negative controls (data not shown). cDNA from wild type

embryos was used as a positive control for the primers. The PCR products were run on a 0.8% agarose gel and visualized by ethidium bromide stain. Data was analyzed via the delta-delta Ct method.

Total RNA was isolated from dissected third instar brains or body wall muscle and extracted with Trizol (Invitrogen) and purified using the RNeasy Mini Kit (QIAGEN). First strand cDNA was synthesized using Supescript III (Invitrogen) enzyme with oligo (dT) 12-18 primer (Invitrogen). The real time PCR for brains was performed using the following primers: Trio (Assay ID Dm01795013_m1), Cyclophilin 1 (Assay ID Dm01813702_m1) and RpL32 (Assay ID Dm02151827_g1) as house keeping control from Applied biosystems. PCR for the body wall muscles was performed using the following primers to detect expression of Dad (Assay ID Dm02134937_m1), Shaker (Assay ID Dm01799618_g1), Gbb (Assay ID Dm01843010_s1), Cyclophilin 1 (Assay ID Dm01813702_m1) and RpL32 (Assay ID Dm02151827_g1) as house keeping control from Applied biosystems. The PCR protocol was 95 °C for 10 min followed by 40 cycles of 95°C for 15 sec and 60 °C for 1 min. Data was analyzed via the delta-delta Ct method.

Immunolabelling and Confocal Microscopy

Third instar *Drosophila* larvae were dissected in calcium free saline (Jan and Jan, 1976) and fixed for 10 minutes with non-alcoholic Bouin's fixative unless

otherwise noted. Brain samples were fixed with 4% paraformaldehyde in phosphate buffer pH 7.4 for 1 hour. Primary antibodies were used at the following dilutions: rabbit α-pMad (1:100) (cell signaling), rat α-Elav (DSHB), mouse α-GluR IIA (1:3) , rabbit α-Discs-Large, 1:20,000 (Koh et al., 1999); mouse α–GFP, 1:200 (Molecular Probes); mouse nc82 (α-Brp), 1:100 (DSHB); FITC or Texas red-conjugated α-HRP 1:200 (Jackson Immunoresearch). Secondary antibodies conjugated to DyLight 488, 594, or 649 (Jackson Immunoresearch) were used at a concentration of 1:200. Samples were imaged using a 3i spinning disc confocal system and analyzed using the Image J software.

Fluorescent intensity quantifications

The intensity of p-Mad nuclei was quantified by selecting the middle section of Elav positive staining and collect single confocal section of both Elav and p-Mad in the ventral nerve cord. The nuclei of motorneurons were selected by positive staining of Elav and anatomically location, then intensities of p-Mad were measured by using Image J software. Intensities of nuclear p-Mad were normalized to wild type controls.

In Maverick overexpression, the intensity of synaptic p-Mad punta was quantified by manually selecting the area of p-Mad positive puncta. 10 NMJs were blindly selected from each wild type control and glial Maverick overexpression animals. The data is shown as p-Mad fluorescent intensity.

The number of positive p-Mad boutons was quantified on 1b type boutons. This quantification was performed using images of α-HRP labeled NMJs that were acquired with identical confocal settings, and we quantified the boutons with presence of p-Mad signal. Number of NMJs analyzed were 10-12 per sample. Data was represented in histograms as the percentage of positive p-Mad boutons ±S.E.M. Statistical significance of the data was obtained in pair-wise comparisons using the Student t-test.

To analyze the p-Mad derivation, we used samples where we knocked down Mad in muscle of motorneurons. We used volocity software to detect positive puncta and calculated p-Mad intensities normalized to HRP volume and subsequently normalized to wild type. Statistical significance of the data was obtained in pair-wise comparisons using the Student t-test.

Generation of Maverick antibody.

Maverick antibody was generated against amino acids 371 to 389 of Maverick-PA, which is the following peptide sequence: C-PLTNAQDANFHHDKIDEA-N-amide. The antibody was produced and purified by 21st Century Biochemicals, Inc. using chicken as the host. We fixed larvae with Bouin's fixative for 10 minutes and used the peptide-purified Maverick antibody at a concentration of 1:50.

FIGURES

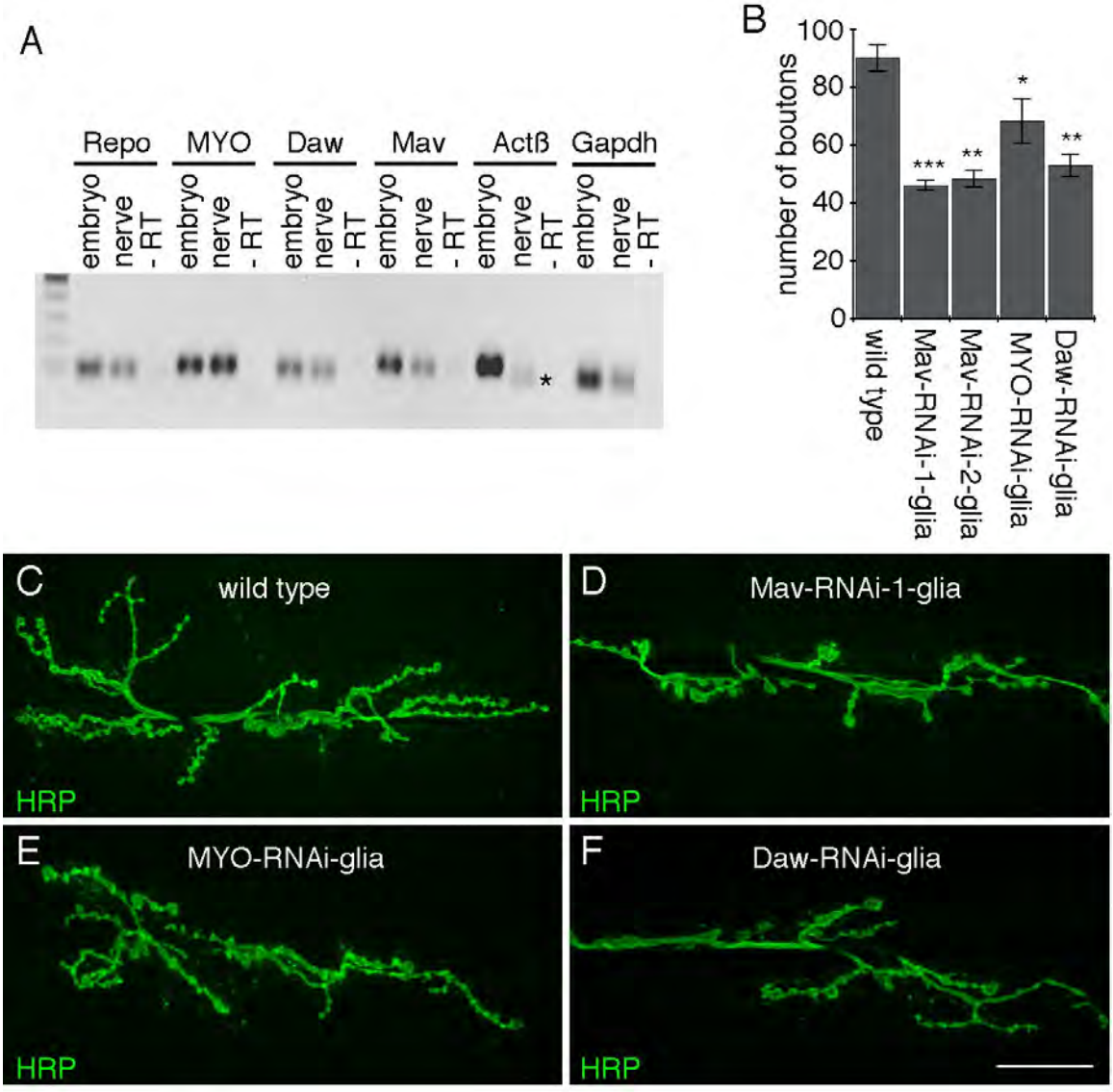
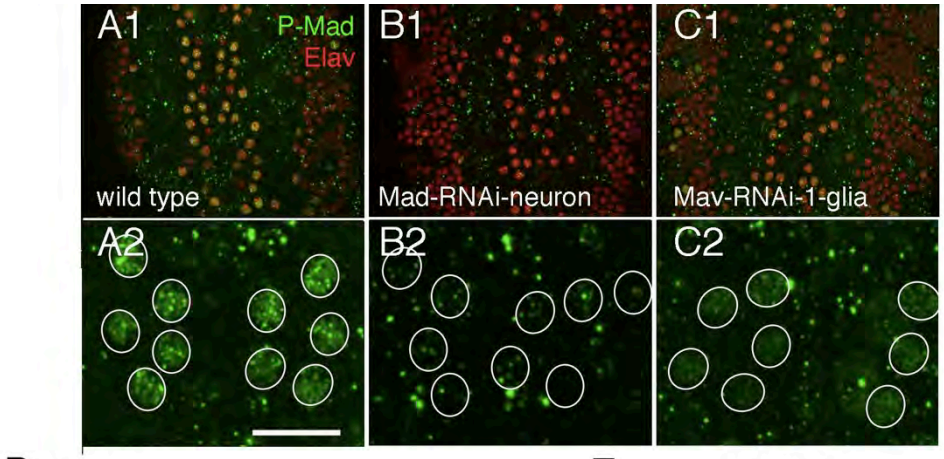
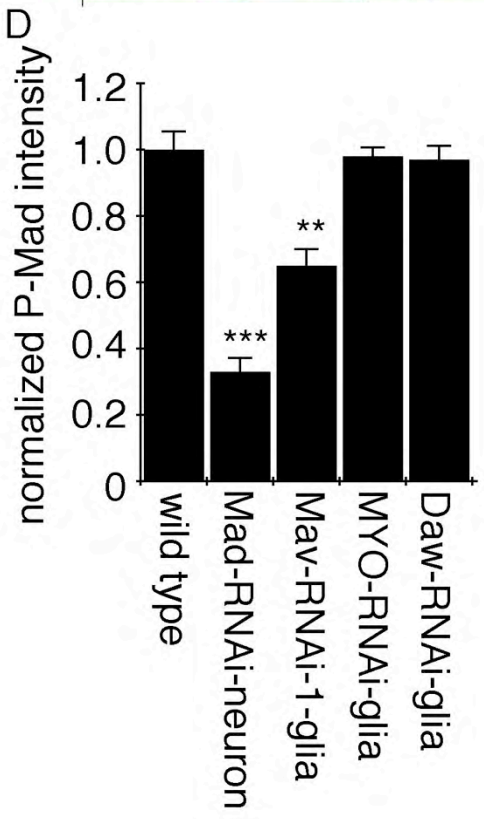

A
Repo
MYO
Daw
Mav
Actß
Gapdh
embryo
nerve
- RT
B
number of boutons
100
80
60
40
20
0
***
**
*
**
wild type
Mav-RNAi-1-glia
Mav-RNAi-2-glia
MYO-RNAi-glia
Daw-RNAi-glia
C
wild type
HRP
D
Mav-RNAi-1-glia
HRP
E
MYO-RNAi-glia
HRP
F
Daw-RNAi-glia
HRP

Figure 3-1. Could glia be the source of TGF-β ligands?

(A) mRNA analysis of transcripts in the peripheral nerves of *Drosophila* larvae. * indicates an unspecific product in the negative control. Embryos were used as positive controls for the primers. (B) Quantification of the number of total number of boutons at muscles 6/7. (C, D, E, F) third instar NMJs at muscles 6/7 visualized with α-HRP (green) *** $p<0.001$, ** $p\leq0.01$, * $p\leq0.05$. Calibration scale is 25 µm for (C, D, E, F).


A1
P-Mad
Elav
wild type
B1
Mad-RNAi-neuron
C1
Mav-RNAi-1-glia
A2
B2
C2


D
normalized P-Mad intensity
1.2
1.0
0.8
0.6
0.4
0.2
0
***
**
wild type
Mad-RNAi-neuron
Mav-RNAi-1-glia
MYO-RNAi-glia
Daw-RNAi-glia

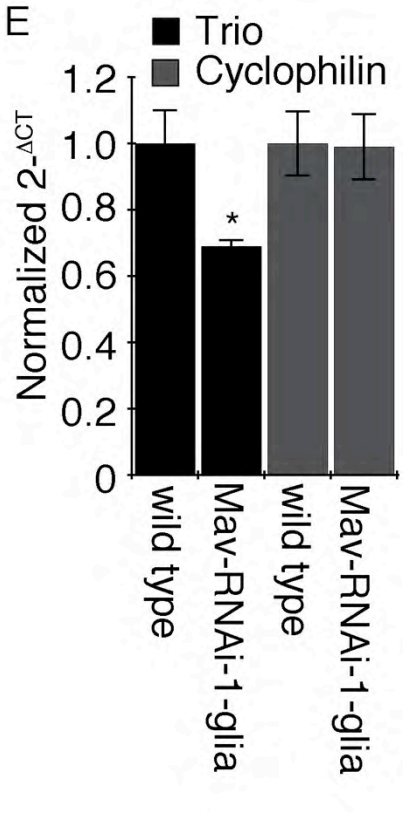

E
Trio
Cyclophilin
Normalized 2-ΔCT
1.2
1.0
0.8
0.6
0.4
0.2
0
*
wild type
Mav-RNAi-1-glia
wild type
Mav-RNAi-1-glia

Figure 3-2. Do glia ligands affect the p-Mad presynaptic activation of TGF-β pathway?

(A1, B1, C1) Ventral nerve cord of third instar larvae brain Elav (red) P-Mad (green) (A2, B2, C2) zoom of motorneuron nuclei stained with a α-p-Mad antibody. Presynaptic *mad*-RNAi was used as a control for p-Mad levels. (D) Quantification of nuclear normalized p-Mad intensity in motorneuron nuclei ,*** $p<0.001$, ** $p\leq0.01$(E) Trio mRNA levels and *cyclophilin* mRNA levels in third instar larval brains. Calibration scale is 10 µm for (A2,B2,C2) and 25 µm for (A1, B1, C1)

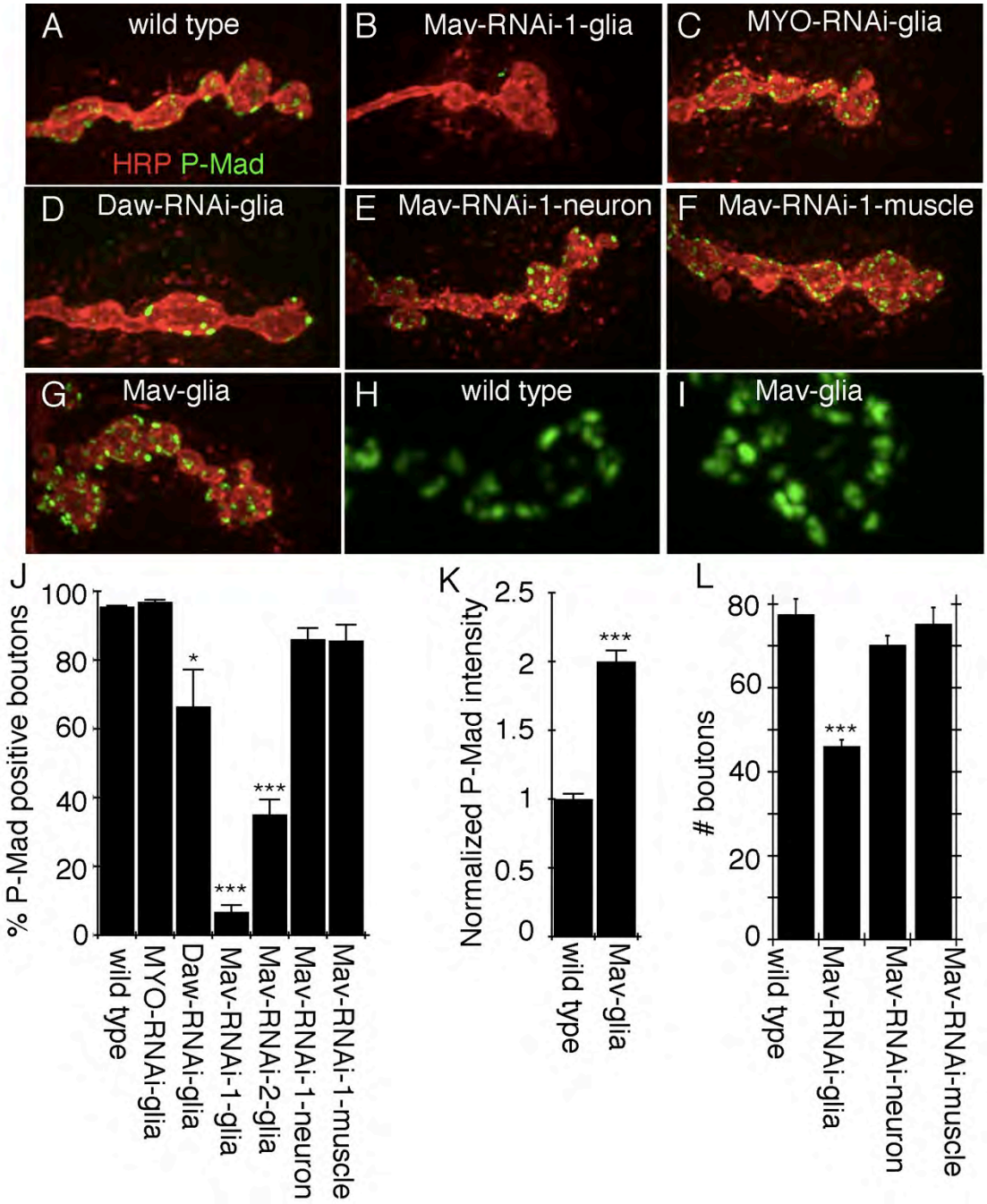

A wild type
HRP P-Mad
B Mav-RNAi-1-glia
C MYO-RNAi-glia
D Daw-RNAi-glia
E Mav-RNAi-1-neuron
F Mav-RNAi-1-muscle
G Mav-glia
H wild type
I Mav-glia
J
% P-Mad positive boutons
100
80
60
40
20
0
*
***
***
wild type
MYO-RNAi-glia
Daw-RNAi-glia
Mav-RNAi-1-glia
Mav-RNAi-2-glia
Mav-RNAi-1-neuron
Mav-RNAi-1-muscle
K
Normalized P-Mad intensity
2.5
2
1.5
1
0.5
0
***
wild type
Mav-glia
L
boutons
80
60
40
20
0
***
wild type
Mav-RNAi-glia
Mav-RNAi-neuron
Mav-RNAi-muscle

Figure 3-3. Glial Maverick regulates activation of synaptic p-Mad

(A) Wild type NMJs stained with α-HRP (red) and α-P-Mad (green) . We knocked down TGF- β ligands in glia: (B) Maverick (Mav) (C) Myoglianin (MYO) (D) Dawdle (Daw). Only glial Maverick knockdown reduced synaptic p-Mad while knock down of Mav in (E) motoneurons or (F) muscles did not affect p-Mad activation. Overexpression of Maverick in glia (G) increases the intensity of p-Mad puncta (I) (J) Quantification of % of p-Mad positive synaptic boutons. (K) Quantification of p-Mad mean intensity. (L) Quantification of the number of total number of boutons at muscles 6/7. *** $p<0.001$, ** $p\leq0.01$, * $p\leq0.05$.

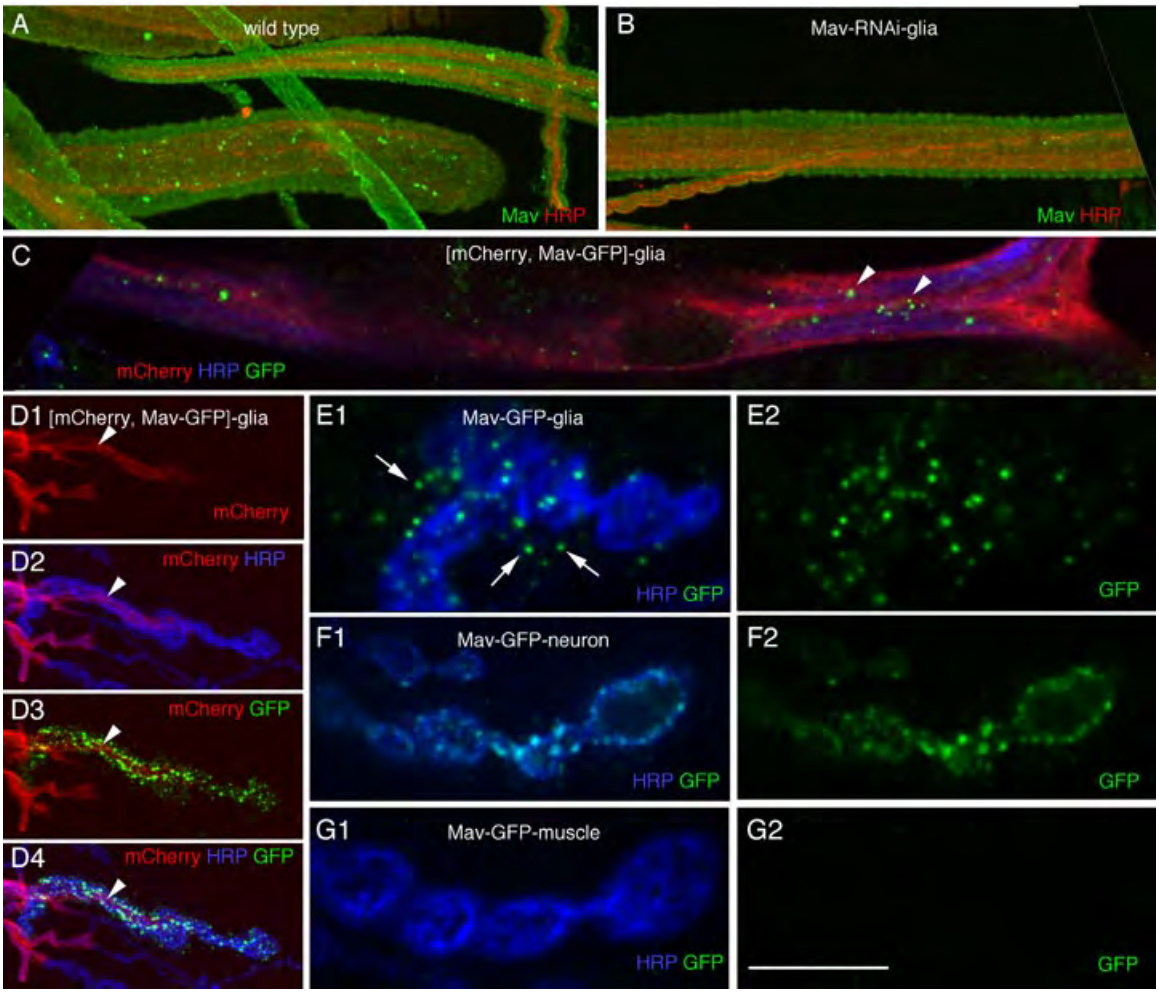


Figure 3-4. Maverick is expressed in peripheral glia cells and is secreted to synapses.

(A) Maverick staining at wild type peripheral nerves and (B) glial *mav*-RNAi. (C) When *mav*-GFP (green) is expressed in peripheral glia (red). (D) Maverick is able to reach the synaptic boutons stained with HRP (blue) and (E) further postsynaptic space when expressed in peripheral glia. In contrast, when Maverick is expressed in motorneurons (F) or muscle (G) it d is not able to leave the terminal, or reach the boutons respectively.

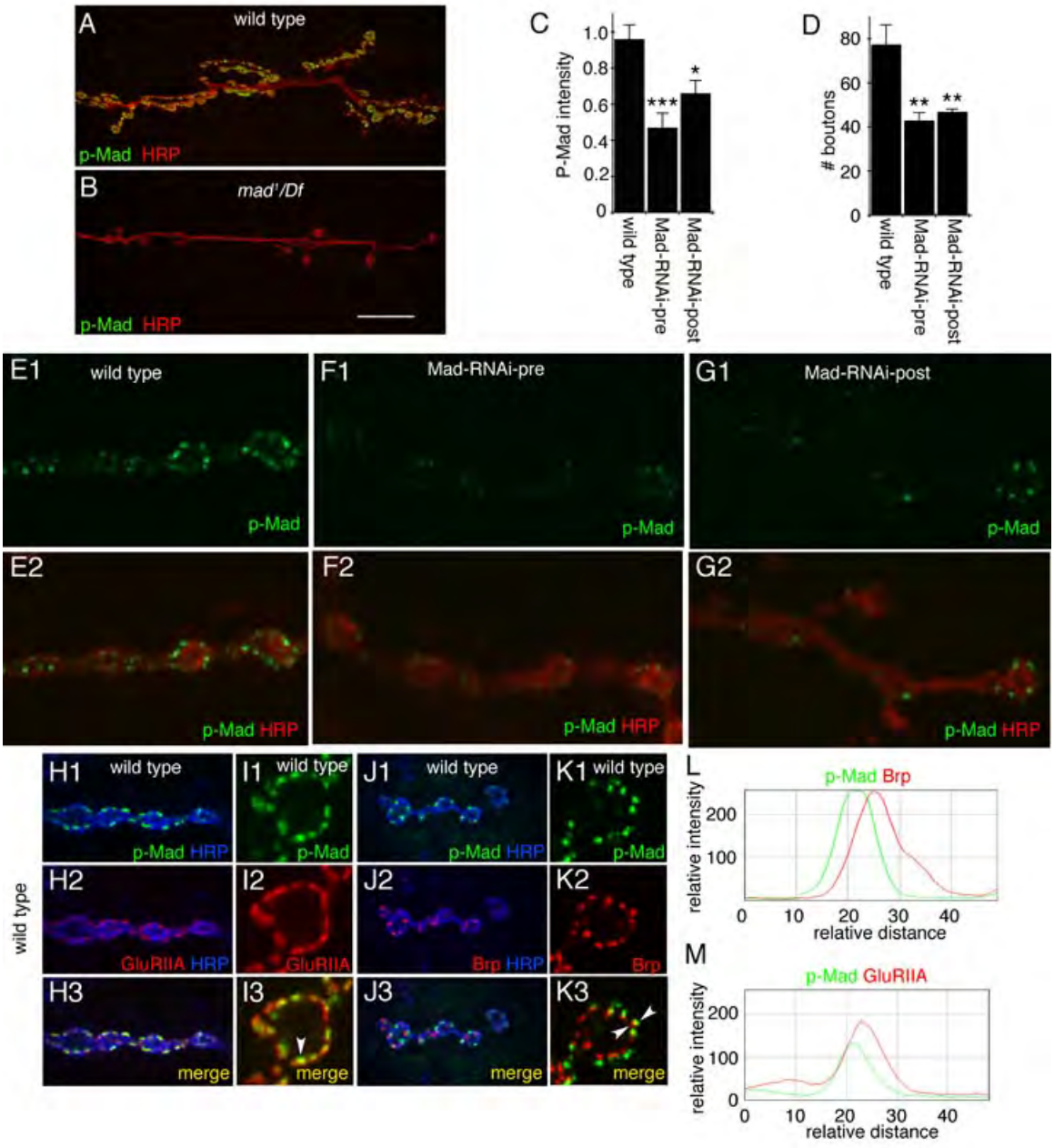

A
wild type
p-Mad HRP
B
mad1/Df
p-Mad HRP
C
P-Mad intensity
1.0
0.8
0.6
0.4
0.2
0
***
*
wild type
Mad-RNAi-pre
Mad-RNAi-post
D
boutons
80
60
40
20
0
**
**
wild type
Mad-RNAi-pre
Mad-RNAi-post
E1
wild type
p-Mad
F1
Mad-RNAi-pre
p-Mad
G1
Mad-RNAi-post
p-Mad
E2
p-Mad HRP
F2
p-Mad HRP
G2
p-Mad HRP
wild type
H1
wild type
p-Mad HRP
I1
wild type
p-Mad
J1
wild type
p-Mad HRP
K1
wild type
p-Mad
H2
GluRIIA HRP
I2
GluRIIA
J2
Brp HRP
K2
Brp
H3
merge
I3
merge
J3
merge
K3
merge
L
p-Mad Brp
relative intensity
200
100
0
10
20
30
40
relative distance
M
p-Mad GluRIIA
relative intensity
200
100
0
10
20
30
40
relative distance

Figure 3-5. Synaptic p-Mad functions pre- and post- synaptically.

(A) The p-Mad staining is specific since (B) in *Mad*^{12} mutants in trans to a deficiency the staining is virtually absent. (C) Normalized p-Mad intensities were calculated by knocking down Mad either in the (F) pre or in the (G) post- synaptic cell. (D) Quantification of the total number of boutons at muscles 6/7. *** $p<0.001$, ** $p\leq0.01$, * $p\leq0.05$. (H1,2,3) p-Mad (green) colocalizes with glutamate receptors IIA (red) (J 1,2, 3) P-Mad (green) is localized juxtapositional to Bruchpilot (red) , (L) Profile of p-Mad and Brp (M) profile of pMad and GluR. Calibration scale is 25 µm for A and B, 3 µm for E, F and G , 7 µm for H and J.

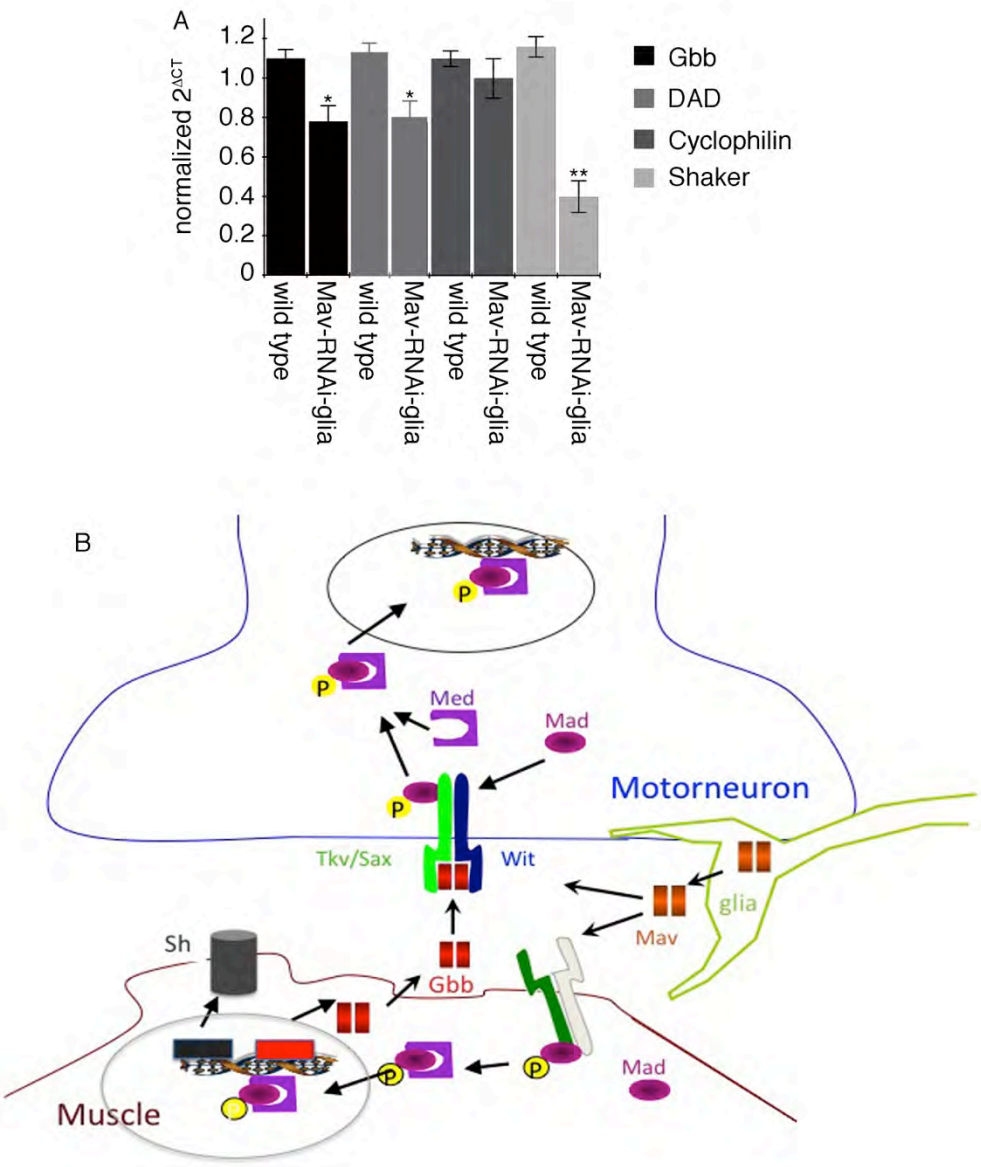

A
normalized 2^ΔCT
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
Mav-RNAi-glia
wild type
Mav-RNAi-glia
wild type
Mav-RNAi-glia
wild type
Mav-RNAi-glia
Gbb
DAD
Cyclophilin
Shaker
B
P
Med
Mad
Motorneuron
Tkv/Sax
Wit
glia
Mav
Sh
Gbb
Mad
Muscle

Fig 3-6. What is the function of maverick?

(A) Gbb and DAD transcript are significally reduced at the BWM when maverick is knocked down from glia. Interestingly, the Shaker transcript isoforms are significally reduced while cyclophilin did not changed upon lack of glial Maverick. N=3 independent RNA isolations. (B) Glia Maverick orchestrates synapses, Our model suggest that glial maverick activates the presynaptic BMP pathway and post-synaptic TGF-β pathway that controls the expression of Gbb and that translate in activation of presynaptic retrograde pathway and Shaker potassium channels.

CHAPTER IV

General Discussion

In this thesis I have explored the role of glial cells in the formation and maintenance of synaptic fields. Using the *Drosophila* NMJ I have made several novel discoveries. First, in contrast to what has been previously reported (Sepp et al., 2000), I showed that *Drosophila* glia dynamically invade the NMJ and associate closely with both the presynaptic motorneuron and postsynaptic muscle cell. Second, I have shown that invading glial cells help maintain the NMJ synaptic field by engulfing shed presynaptic debris through the Draper engulfment signaling pathway. Unexpectedly, I found that clearance involved a coordination between glia and a novel engulfing cell type in *Drosophila*, the postsynaptic muscle. That growing presynaptic arbors shed such a significant amount of debris in an activity dependent way was also surprising, and suggests that the integrity of membranes of axon and synapses may not be as stable as expected. Finally, I showed that invading glial cells actively secrete the TGF-β molecule Maverick to promote NMJ growth. Loss of glial Maverick dramatically reduced p-Mad levels at the synapse, led to decreases in muscle cell expression of muscle retrograde ligand Gbb, suggesting that glia modulates retrograde muscle→motorneuron signaling. Together this work provides exciting new insights into how glial cells, using two very different methods of control—the engulfment of debris versus the secretion of growth factors—can modulate both synaptic growth and signaling.

Importance of glial cells in the brain

The brain is capable of processing an enormous amount of information that ultimately controls everything from eye blinking to the cognitive interpretation of our world. Brain anatomy and general functional territories are quite well-described: we know which brain region control the processing of specific sensory, motor, or cognitive information. In many cases our insights have come from identifying neuronal functional loci that are modified in patients with specific cognitive or motor defects.

Despite the fact that the majority of human cells in the brain are glia, most of the studies of brain function have focused on the properties of neurons. In fact, if you search in PubMed for “glia” versus “neuron”, there is approximated 6.7 times more publications which exclude the word glia and include neuron, than those that include glia. Moreover, many of those including “glia” often mention it only in passing. If we really want to understand how our brain works, we have to give greater attention to the mysterious glial cells. It is critical that we aim to understand specific molecular pathways that govern glial development and function, and how those pathways respond to changes on the neuronal circuits integrity or activity. We need to understand how much control glia exert over neurons and circuits, and which pathways mediate these events. In recent research it has become increasingly clear that glial cell populations (e.g. astrocytes) are highly connected through gap junctions, and through these connections can maintain a global level of communication across the brain

space. These findings raise the interesting possibility of glial coordination of broad responses across the brain, the potential existence of glial circuits, and understanding the functional implication in the future should be a major focus of the field.

Rethinking the stability of the neurons—are neurites always fragmenting and glia always eating?

Across the animal kingdom there are numerous examples of developmental pruning of neuronal circuits, and such pruning is a key mechanism allowing for neuronal plasticity during circuit development. Surprisingly few molecules directly involved in the programmed destruction of axons, dendrites, or synapses have been identified (see Introduction). As such we know very little about the molecular program that promotes neurite or synapse destruction. My observation that growing motorneurons at the NMJ are constantly shedding presynaptic debris in an activity-dependent manner raises a number of interesting questions regarding this shedding process and classical pruning events. For example, do signaling pathways that promote the pruning of specific dendritic arbors such as the cytoskeleton-binding protein Mical (Kirilly et al., 2009) also promote the shedding of presynaptic debris? Is the shedding of presynaptic debris more similar to Wallerian degeneration, which is mediated by the neuroprotective Wld^{S} protein (Avery et al., 2009; Coleman and Freeman, 2010) and dSarm (Freeman lab unpublished)? Alternatively, is the shedding of

presynaptic debris governed by a synaptic specific and completely novel genetic program?

It would seem unlikely that the shedding of presynaptic debris is mechanistically related to axosome shedding in mammals (Bishop et al., 2004). During axosome shedding the “loser” motorneurons that ultimately retract from the NMJ shed axosomes, which in some ways may be similar to shed presynaptic debris at the *Drosophila* NMJ. However axosomes are believed to be pinched off by the surrounding Schwann cell as the Schwann cell engulfs the loser (Bishop et al., 2004). Based on my observations it seems unlikely that *Drosophila* glial cells might be functioning in a similar way. First, *Drosophila* glia are only transiently associated with the NMJ, whereas one might expect a more robust association with the NMJ if they were driving the process. Second, the shedding of presynaptic debris appears to be neuron-autonomous and not require glial engulfment of motorneuron terminals since mutations affecting glial engulfing activity (i.e. *draper*, or *dced-6*), rather than leading to less debris instead lead to dramatic increases in axonal debris. In the future these questions could be answered directly by assaying the shedding of presynaptic debris in newly-identified neurite degeneration and pruning mutants.

That motorneurons at the NMJ are apparently so unstable raises the interesting question of whether or not mature neurons in the brain are similarly unstable and potentially fragmenting at all times. Indeed components of the Draper signaling pathway are robustly expressed by a number of subtypes of

glial cells in the adult brain (MacDonald et al., 2006; Doherty et al., 2009), and these glia are capable of engulfing axonal debris (MacDonald et al., 2006). It is noteworthy that when single axons are examined in *draper* mutant backgrounds, one does not find an accumulation of GFP^{+} debris along axons (MAL and MRF, unpublished). This observation might suggest that mature axons are more stable, however to date the status of adult synapses in *draper* mutant backgrounds has not been examined. It would be exciting if it were found that synapses specifically—the site of plasticity in mature neurons—were specifically affected.

If it were true that axons, dendrites, or synapses were continuously fragmenting in the mature brain, this would have profound implications for how we think about normal neuronal stability, and in turn neurodegenerative disease. If mature axons were constantly shedding debris, this would lead to the production of a huge amount of debris that would need to be constantly cleared from the brain. Perhaps this is why Draper and other engulfment genes are robustly expressed by glia under normal conditions (Awasaki et al., 2006; Ziegenfuss et al., 2008). In turn one could envision how defects in glial engulfment activity could lead to the dramatic accumulation of shed neuronal debris, which I have shown has severe negative consequences on new synaptic growth (Fuentes-Medel et al., 2009). Perhaps it might also negatively affect the maintenance of neuronal integrity. Such an explanation for neurodegenerative phenotypes has not been proposed previously, is an exciting new potential defect

in patients which needs to be considered, and would identify glial engulfment signaling as a novel therapeutic target for intervention in neurodegenerative disease.

Potential roles for presynaptic debris shedding in synapse growth and plasticity

The presence of the shed presynaptic debris at the NMJ in *draper* mutants likely causes the inhibition of new synaptic growth during NMJ development. The presynaptic arbor secretes a number of factors such as Wg that signal to the postsynaptic muscle cell to modulate growth. The presence of excess Wg could have a negative effect on Wg signaling, for example over-activation of the dFz2 Wg receptor causes a dominant-negative phenotype at the NMJ (Mathew et al., 2005; Ataman et al., 2006). In addition, shed presynaptic debris could contain inhibitory factors that are normally cleared efficiently by glia and muscle at the NMJ, and if not cleared can suppress synapse addition. Interestingly, work from the Budnik laboratory has found that debris also contains exosome proteins such as evi that are critical for the communication between the pre and postsynaptic cells. A major future goal will be to identify the signaling factors present on shed presynaptic debris that negatively regulate NMJ growth, and definitively demonstrate that shed presynaptic debris in fact causes the reduced NMJ growth observed in *draper* mutants.

How shed presynaptic debris is autonomously tagged for engulfment remains a mystery. That it can be recognized and phagocytosed by Draper-dependent mechanisms argues that presynaptic debris shares some similarities with cell corpses (Manaka et al., 2004), pruned axons (Awasaki et al., 2006), and axonal debris generated through Wallerian degeneration (MacDonald et al., 2006). Phosphatidylserine (PS) has been proposed as a potential “eat me” cue recognized by CED-1 in *C. elegans*, however this remains primarily speculative. A number of potential ligands for the Draper receptor have also been identified (Hashimoto et al., 2009; Kuraishi et al., 2009), but whether these are involved in the clearance of shed presynaptic debris has not been explored. An equally interesting questions pertains to the mechanism by which neural activity increases the amount of shed presynaptic debris. It is possible that this simply reflects an increase in the turnover rate of ghost boutons or other structures at the NMJ, which in turn results in more debris. Alternatively, activity itself might destabilize neuronal membranes. For example, an excessive level of synaptic vesicle release might lead to loss of membrane from the synaptic terminal, which by our criteria would appear as shed presynaptic debris. Finally, it remains possible that the NMJ is unique in the amount of mechanical stress that it imparts on the motorneuron terminal’s synaptic field—NMJ synapses are embedded in the muscle and are violently pulled as the muscle cycles through contractions and relaxations. It is possible that this mechanical stress somehow physically tears debris off the NMJ terminals.

Why would it be beneficial for synapses to be so highly unstable, or shed by the presynaptic motorneurons in such a profligate way? A common theme in the development of neural circuits is the overproduction of axons, dendrites, and synapses, followed by the subsequent elimination of exuberant connections (see Introduction). In this way the circuit has maximized its initial potential for connectivity, has the opportunity to selectively maintain the most productive connections, and can therefore respond rapidly to changes in activity during development. I would anticipate that the shedding of presynaptic debris serves a similar purpose at the NMJ. In a number of cases the NMJ has to respond rapidly—for example in the case of starvation—to generate new synapses for enhanced synaptic growth. Having a reserve pool of synapses that are normally shed would provide a continuous pool of nascent synapses that at any moment could be stabilized to increase synaptic connectivity. Presumably this would be much more efficient that signaling to the nucleus to enhance the production of new synaptic material. In general it seems the nervous system has made the decision that it is easier to overproduce and eliminate, rather than make things fresh.

Glial dynamics at the *Drosophila* NMJ – not really a tripartite synapse?

My work has demonstrated that while glia is indeed found at the *Drosophila* NMJ and regulate its development, this may not be an ideal system in which to study

the properties of the tripartite synapse as it relates to synaptic physiology. In mammalian CNS synapses glia have been proposed to form a close relationship with synapses, with glia acting as a third cell type that can modulate signaling, thereby forming the “tripartite synapse” (Stevens, 2008; Halassa and Haydon, 2010). Our hope was that the *Drosophila* NMJ would be an ideal tissue in which to explore this close relationship, however my work indicates that glial cells are primarily absent from the NMJ, and only enter a small percentage of the time. It is reasonable to assume that if these glia were performing a critical signaling role that we might observe a more close association. That said, serial EM studies in which a 1 mm^2 cube of neural tissue was reconstructed revealed that the vast majority of CNS synapses (at least in this brain region) were not closely associated with astrocytes. Rather, astrocyte membranes were found at some distance. It is therefore possible that a very close physical association of astrocytes with the synapse is not essential for the synapse to be modulated by glia; or perhaps in some brain regions astrocytes are less involved in signaling than was anticipated?

The molecular mechanisms by which glia are attracted to synapses at the NMJ remain poorly defined. One possibility is that they are able to sense the amount of glutamate, and when levels are high the enter the NMJ and buffer glutamate levels. Certainly peripheral glia are known to express channels capable of transporting glutamate like EAATs (Stacey et al., 2010). Alternatively, there could be very interesting differences in membrane composition at synaptic

boutons compared to the axonal tracks. Perhaps peripheral glia prefer to reside primarily on axons, and only enter the NMJ under conditions of physiological stress? Understanding this neuron-muscle-glia dialog, and the signaling pathways that regulate it, will be essential as we further explore the physiological roles for glia at the NMJ.

Newly identified glial growth factors that modulate synaptic growth

Glia are critical for the survival of neurons, they secrete molecules that enhance the formation of neuronal circuits and regulate the development of synaptic connections (Eroglu and Barres, 2010; Kucukdereli et al., 2011). However, the precise molecular mechanisms for these functions are still poorly understood. The transforming growth factor beta (TGF-β) signaling pathway has a variety of functions in the development of the organism such as pattering formation during embryonic development (Eldar et al., 2002; O'Connor et al., 2006). In *Drosophila*, a total of seven TGF-β family ligands have been identified so far. They can be divided in two categories: the BMP subfamily (Dpp, Gbb and Scw) and the TGF-β-related ligand subfamily (dActivin, Myoglianin, Dawdle and Maverick) (Gesualdi and Haerry, 2007; Moustakas and Heldin, 2009). This classification is a result of their ability to activate BMP type receptors or Activin-like receptors and the phylogenetic similarities with their vertebrate homologous (Raftery and Sutherland, 1999). It has been shown that Gbb loss-of-function mutants have

impaired synaptic growth, and Gbb has been identified as a retrograde signal to activate the presynaptic BMP pathway to promote synaptic growth (McCabe et al., 2003). Interestingly, I found that TGF-β-related ligands are expressed in peripheral glia and knocking them down only in these cells results in detrimental effects for NMJ growth. These results open the possibility that the glial TGF-β ligands could have a direct role in modulating the growth of synapses and the function of the retrograde pathway in the NMJ. Supporting a conserved role of these ligands and their functions, vertebrate peripheral Schwann cells have been shown *in vitro* to secrete TGF-β-1, promoting synaptogenesis in cell culture (Feng and Ko, 2008). However, the downstream molecular mechanism is unknown and this has not been demonstrated *in vivo*. The fact that glia could potentially regulate synapses through TGF-β ligands opens a new avenue of investigation, not only for the TGF-β pathway at the NMJ but also the exciting possibility that glia control the growth of synapses with a non-BMP type of ligand.

Glial regulation of BMP retrograde signaling at the NMJ

The TGF-β signaling cascade has several levels of regulation that ultimately lead to the transcription of specific target genes (Weiss et al., 2010). In general, the identity of the ligand, the combination of receptors, and the downstream transcription factors modulate the functional outcome of this pathway (Marquez et al., 2001; Gesualdi and Haerry, 2007). However, independent of what

molecule is participating in a particular tissue, one common event is the phosphorylation of an R-Smad molecule by the tyrosine kinase TGF-β receptor. The MH2 domain of R-smads contains an SSXS motif that is phosphorylated by the type I receptor kinase (Chen et al., 1996; Haerry, 2010). This phosphorylation has been broadly used as a measurement of activation of the TGF-β pathway. At the *Drosophila* NMJ levels of nuclear p-MAD, the R-Smad protein described in this tissue, serve as a read out of retrograde pathway activation. Furthermore, *gbb*, *wit* and *sax* mutants have been shown to have almost no motorneuron nuclear p-Mad signal (Marques et al., 2003) indicating that Gbb is the key regulator for the presynaptic BMP pathway. Interestingly, I found that among the three TGF-β ligands expressed in glia, only Maverick decreased the levels of p-MAD in the motorneuron nuclei. Moreover, BMP and Activin ligands are known to trigger different effects in a concentration- dependent manner, rather than functioning as an on-off switch (Haerry, 2010). Why do glia express more than one ligand? How does the combination of many ligands regulate the activation of the same receptors? And why is there such a complex environment? One possibility could be that Maverick regulates the effectiveness of Gbb at the NMJ by forming a heterodimer with Gbb. Similarly, in the wing tissue, Gbb forms heterodimers with DPP, and that interaction regulates the function of DPP (Bangi and Wharton, 2006). However, my data suggest that the regulation of BMP signaling could be more complex than predicted before. For instance, the lack of motorneuron nuclear MAD activation by Myoglianin or Dawdle ligands suggests

that these ligands might activate different combinations of receptors or interact with other tissues in the larvae. Supporting evidence for this model comes from results from *dawdle* mutants (Ellis et al., 2010b). These animals have defective NMJs, but the rescue experiments indicated a more ubiquitious source of this ligand, instead of a specific source at the NMJ (Ellis et al., 2010b). In addition, I found no changes of nuclear p-Mad when Myoglianin was knocked down in peripheral glia, suggesting that glial *Myoglianin* could be activating a different combination of receptors and R-Smad. For example, in central larval brain, Myoglianin was found to activate the TGF-β Babo receptor and Myo has been suggested to activate Smox, a different R-Smad, instead of MAD, downstream of the same receptor (Awasaki et al., 2011) Intriguingly, *Myoglianin* mutants have been recently generated, but NMJ characterization reminds unknown (Awasaki et al., 2011).

Glia Regulation of postsynaptic signaling at the NMJ

Although, TGF-β receptor expression in *Drosophila* larval muscle has been controversial (Dudu et al., 2006; Ball et al., 2010; Higashi-Kovtun et al., 2010), it is possible that glial ligands could be activating a post-synaptic pathway. Intriguingly, p-MAD colocalizes with muscle overexpressed glutamate receptors (GluR) at synaptic boutons. However, there is still controversy surrounding the origin of this signal since presynaptic receptors are localized at the synaptic

boutons, and this signal could belong to presynaptic MAD activation (O'Connor-Giles et al., 2008). In contrast, there is evidence supporting an active TGF-β pathway in the muscle cell. First, the molecular components of this pathway have been shown to be expressed in the body wall muscle, and secondly, specific muscle overexpression of either DAD, an inhibitory downstream target of TGF-β, or a constitutively active Tkv receptor results in a decrease and increase of synaptic p-Mad levels respectively (Dudu et al., 2006). Moreover, *babo* mutants have decreased number of boutons and decreased levels of transcription of *gbb* (Ellis et al., 2010b) suggesting a functional role of a TGF-β activin-like pathway in the muscle cell. I found that knock down of *maverick* by RNAi only in glia strongly suppresses the activation of MAD at synaptic boutons. Additionally I found that synaptic p-Mad signal is partially reduced if you either knock down MAD in the motorneuron or in the muscle respectively. These results support a model where Maverick is regulating both pre- and post-synaptic TGF-β signaling pathways.

Remarkably, supporting a postsynaptic role of glial Maverick, I found that both *gbb* and *dad* muscle mRNA levels decreased when I knocked down Maverick only in peripheral glia. Whether this is a direct or indirect effect, remains to be tested. Translocation of p-Mad to the nucleus depends on binding to the co-Smad, Medea. I found Medea to be expressed in muscle (data not shown), suggesting that it could be a key regulator in the activation of particular genes. However, its function in the muscle remains unknown. Furthermore, is the

downstream MAD-Medea protein complex binding to Gbb or DAD promoters? Looking at physical interactions of this complex with the corresponding promoters or examining Gbb or DAD reporter genes in the muscle while expressing Maverick RNAi in peripheral glia, would separate a direct or indirect role of glial Maverick ligand in the muscle. Moreover, in contrast with previous publications (Dudu et al., 2006; Higashi-Kovtun et al., 2010) I did not find p-Mad signal at muscle nuclei (data not shown). One explanation for my result is that the muscle p-Mad uses different phosphorylation sites to translocate into nucleus, and thus the antibody does not recognize this p-Mad, or the p-Mad antibody is unable to detect p-Mad in the muscle nuclear environment. This could be due to the fact that muscles are multi-nucleated cells and the P-Mad signal is therefore diluted to levels below the detection limit of the antibody. Nevertheless, my result of muscle specific MAD RNAi demonstrates both decreases in the number of synaptic boutons and p-Mad signal. Finally, to dissect apart the roles of pre and postsynaptic TGF-β pathways, new tools will be needed such as an specific antibody for immuno-electron microscopy for p-MAD to resolve the resolution limitations or a conditional dominant negative MAD overexpression construct to appreciate the significance of having both for the NMJ development.

Neuronal activity and synaptic TGF-β glial regulation

Synaptic growth, presynaptic efficacy and muscle excitability are tightly regulated processes. We know many of the molecular pathways that coordinate each of these processes (Budnik et al., 1990; Zhong et al., 1992), however the synchronization mechanisms needed to ensure proper function of the NMJ are poorly understood. Moreover, the ultimate goal of these synaptic connections is to ensure a sufficient strength of neurotransmission between motorneuron and muscle cell that will allow for coordinated locomotion of the larvae. One critical element affecting synaptic growth is neuronal activity. In general, increase of neuronal activity results in overgrowth of synaptic boutons at the NMJ (Budnik et al., 1990). Moreover, the growth of the NMJ is regulated by anterograde and retrograde pathways (Keshishian and Kim, 2004; Fuentes-Medel and Budnik, 2010). This communication is essential for the coordination between neuronal activity and muscle growth. The Wingless pathway was the first to be described as an anterograde pathway of synapses (MN to Muscle) (Packard et al., 2002). Wingless was shown to be secreted by the motorneuron and coordinate the development of postsynaptic specializations at the *Drosophila* NMJ. Secreted Wg interacts with DFz2 receptors in the muscle (Ataman et al., 2006). However, recent work has shown that Wg also acts in an autocrine manner to directly regulate the microtubule-associated protein Futsch in MN (Miech et al., 2008). Interestingly, the TGF-β retrograde (Muscle to MN) pathway was found to

regulate the GEF Trio (Ball et al., 2010) that also locally regulates the remodeling of microtubules in the synaptic terminal, suggesting a presynaptic synergistic model between Wg and BMP signaling, where both collaborate to increase synaptic growth. Together these data support the possibility that the regulation of Wg and TGF-β pathways at the synapse is more complicated and requires more precise molecular machinery than previously thought. Despite the fact that a TGF-β neuronal ligand has not yet been identified, similar to the NMJ's Wg pathway, my data and other groups' data support the idea that the TGF–β signaling pathway works in both MN and muscle. In the future we will need to understand how muscle manages and regulates both the Wg activated pathway and a TGF-β modulated pathway. One interesting hypothesis is a competitive co-regulation, where MAD competes between the two pathways. This regulation has been observed in other tissues such as the wing (Eivers et al., 2011). However, it has not been explored if similar mechanisms are in place at the NMJ to regulate growth.

It is unclear how glia could coordinate the anterograde and retrograde pathways. One interesting mechanism, since glia appear closely involved in regulation of synapse growth, would be that Wg and retrograde BMP pathways might directly feed back onto glia to inform them of the status of synaptic growth. None of these ideas have been tested at the NMJ. This model would required both that glia also express TGF-β and DFZ receptors and they are able to use their ligands as signals for growth. Assuming that the secretion of Maverick

responds to growth, looking at the levels of secreted Maverick in *gbb* mutant or *Wg* mutant backgrounds would test the idea if it is possible that these ligands are triggers for Maverick secretion. Alternatively, one could overexpress Gbb in the muscle and examine the secretion of Maverick from glia using the Mav:GFP transgenic fly that I generated in my work. Conversely, Wg could be overexpressed from the motorneuron and one could quantify the levels of Maverick GFP signal at the synaptic boutons.

While Wg secretion has been shown to be enhanced upon synaptic activity (Ataman et al., 2008),it is unclear how postsynaptic TGF-β pathways respond to neuronal activity or if Maverick-activated TGF-β signaling is modulated by neuronal activity. My results have shown that glial Maverick is secreted into the synapse, but it remains to be investigated if glial Maverick secretion is regulated by neuronal activity. One possibility is that Maverick secretion is enhanced upon neuronal firing and glia have an upstream molecular pathway able to sense the changes in synaptic strength. Another possibility is that the secretion of Maverick is independent of neuronal activity, and glia modulate the synapses in response to other molecules present in the hemolymph. Such molecules could reflect the nutrient levels around the larva, so if the environment is nutrient-poor the animal can change locomotion speed to save energy. Furthermore, we can use a channel-rhodopsin light-activated

channel (see chapter 2 of this thesis) to induce neuronal activity only in motoneurons and test if this directly induces the secretion of Maverick from glia.

Interestingly, It is highly possible that glia might constantly monitor the levels of neuronal activity. How could glia recognize neuronal activity? One possibility is that glia express glutamate transporters that enable them to sense the levels of glutamate at the synaptic cleft (Stacey et al., 2010) and by doing so, this reuptake could activate an internal glia “sensor” of neuronal activity. Alternatively, glia could sense the lack of neuronal activity. For example, when action potentials are blocked by treating neurons with tetrodotoxin (TTX), mammalian glia secrete TNF alpha (Stellwagen and Malenka, 2006). These data suggest that in fact, glia are able to respond to neuronal activity, but there are no data of the molecular pathways that glia might use to sense neuronal activity.

Finally, why place the control of sculpting neuronal circuits in the hands of glia? Glia are better placed than neurons for a global response. They predict where and how neurotransmission occurs, they communicate across long distances in the brain and they have the molecular machinery to maintain highly metabolic activity. These three characteristics makes them an ideal candidate to orchestrate the plasticity of neuronal circuits, where have the sensors, the communication system and the energy to tune the response of the nervous system. In the future we need to gain insights into questions such as: How do synapses get eliminated or stabilized? What are the molecular components of

synaptic debris? What are the molecular components that glia use to sense neuronal activity? What are the molecular pathways that glia use to regulate synaptic plasticity? Up to today we know that they regulate complex mechanisms such as circadian rhythms, courtship behavior, synaptic plasticity and longevity (Ewer et al., 1992; Buchanan and Benzer, 1993; Jackson, 2011; Kazama et al., 2011; Ng et al., 2011; Seugnet et al., 2011). However, the molecular identification of key molecular components in these processes will not only lead to change the strategic view of how do we think about our brains, but also will open new avenues to design the applications of improvement of human health to a new level. Understanding the logic of these pathways could provide many potential novel targets for drug development for the nervous system.

References

Aberle, H., Haghighi, A. P., Fetter, R. D., McCabe, B. D., Magalhaes, T. R. and Goodman, C. S. (2002) 'wishful thinking encodes a BMP type II receptor that regulates synaptic growth in Drosophila', *Neuron* 33(4): 545-58.

Akbergenova, Y. and Bykhovskaia, M. (2007) 'Synapsin maintains the reserve vesicle pool and spatial segregation of the recycling pool in Drosophila presynaptic boutons', *Brain Res* 1178: 52-64.

Aldskogius, H. and Kozlova, E. N. (1998) 'Central neuron-glial and glial-glial interactions following axon injury', *Prog Neurobiol* 55(1): 1-26.

Alvarez, V. A. and Sabatini, B. L. (2007) 'Anatomical and physiological plasticity of dendritic spines', *Annu Rev Neurosci* 30: 79-97.

Ataman, B., Ashley, J., Gorczyca, D., Gorczyca, M., Mathew, D., Wichmann, C., Sigrist, S. J. and Budnik, V. (2006) 'Nuclear trafficking of Drosophila Frizzled-2 during synapse development requires the PDZ protein dGRIP', *Proc Natl Acad Sci U S A* 103(20): 7841-6.

Ataman, B., Ashley, J., Gorczyca, M., Ramachandran, P., Fouquet, W., Sigrist, S. J. and Budnik, V. (2008) 'Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling', *Neuron* 57(5): 705-18.

Auld, V. J., Fetter, R. D., Broadie, K. and Goodman, C. S. (1995) 'Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in Drosophila', *Cell* 81(5): 757-67.

Avery, M. A., Sheehan, A. E., Kerr, K. S., Wang, J. and Freeman, M. R. (2009) 'Wld S requires Nmnat1 enzymatic activity and N16-VCP interactions to suppress Wallerian degeneration', *J Cell Biol* 184(4): 501-13.

Awasaki, T., Huang, Y., O'Connor, M. B. and Lee, T. (2011) 'Glia instruct developmental neuronal remodeling through TGF-beta signaling', *Nat Neurosci* 14(7): 821-3.

Awasaki, T. and Ito, K. (2004) 'Engulfing action of glial cells is required for programmed axon pruning during Drosophila metamorphosis', *Curr Biol* 14(8): 668-77.

Awasaki, T., Tatsumi, R., Takahashi, K., Arai, K., Nakanishi, Y., Ueda, R. and Ito, K. (2006) 'Essential role of the apoptotic cell engulfment genes draper and ced-6 in programmed axon pruning during Drosophila metamorphosis', *Neuron* 50(6): 855-67.

Balice-Gordon, R. J., Breedlove, S. M., Bernstein, S. and Lichtman, J. W. (1990) 'Neuromuscular junctions shrink and expand as muscle fiber size is manipulated: in vivo observations in the androgen-sensitive bulbocavernosus muscle of mice', *J Neurosci* 10(8): 2660-71.

Ball, R. W., Warren-Paquin, M., Tsurudome, K., Liao, E. H., Elazzouzi, F., Cavanagh, C., An, B. S., Wang, T. T., White, J. H. and Haghighi, A. P. (2010) 'Retrograde BMP signaling controls synaptic growth at the NMJ by regulating trio expression in motor neurons', *Neuron* 66(4): 536-49.

Ballard, S. L., Jarolimova, J. and Wharton, K. A. (2010) 'Gbb/BMP signaling is required to maintain energy homeostasis in Drosophila', *Dev Biol* 337(2): 375-85.

Banerjee, S., Pillai, A. M., Paik, R., Li, J. and Bhat, M. A. (2006) 'Axonal ensheathment and septate junction formation in the peripheral nervous system of Drosophila', *J Neurosci* 26(12): 3319-29.

Bangi, E. and Wharton, K. (2006) 'Dpp and Gbb exhibit different effective ranges in the establishment of the BMP activity gradient critical for Drosophila wing patterning', *Dev Biol* 295(1): 178-93.

Barres, B. A. (2008) 'The mystery and magic of glia: a perspective on their roles in health and disease', *Neuron* 60(3): 430-40.

Bashaw, G. J. and Klein, R. (2010) 'Signaling from axon guidance receptors', *Cold Spring Harb Perspect Biol* 2(5): a001941.

Bishop, D. L., Misgeld, T., Walsh, M. K., Gan, W. B. and Lichtman, J. W. (2004) 'Axon branch removal at developing synapses by axosome shedding', *Neuron* 44(4): 651-61.

Blinzinger, K. and Kreutzberg, G. (1968) 'Displacement of synaptic terminals from regenerating motoneurons by microglial cells', *Z Zellforsch Mikrosk Anat* 85(2): 145-57.

Brill, M. S., Lichtman, J. W., Thompson, W., Zuo, Y. and Misgeld, T. (2011) 'Spatial constraints dictate glial territories at murine neuromuscular junctions', *J Cell Biol* 195(2): 293-305.

Buchanan, R. L. and Benzer, S. (1993) 'Defective glia in the *Drosophila* brain degeneration mutant drop-dead', *Neuron* 10(5): 839-50.

Budnik, V. (1996) 'Synapse maturation and structural plasticity at Drosophila neuromuscular junctions', *Curr Opin Neurobiol* 6(6): 858-67.

Budnik, V., Koh, Y. H., Guan, B., Hartmann, B., Hough, C., Woods, D. and Gorczyca, M. (1996) 'Regulation of synapse structure and function by the Drosophila tumor suppressor gene dlg', *Neuron* 17(4): 627-40.

Budnik, V., Zhong, Y. and Wu, C. F. (1990) 'Morphological plasticity of motor axons in Drosophila mutants with altered excitability', *J Neurosci* 10(11): 3754-68.

Cameron, J. S., Dryer, L. and Dryer, S. E. (1999) 'Regulation of neuronal K(+) currents by target-derived factors: opposing actions of two different isoforms of TGFbeta', *Development* 126(18): 4157-64.

Cameron, J. S., Lhuillier, L., Subramony, P. and Dryer, S. E. (1998) 'Developmental regulation of neuronal K+ channels by target-derived TGF beta in vivo and in vitro', *Neuron* 21(5): 1045-53.

Chen, Y., Lebrun, J. J. and Vale, W. (1996) 'Regulation of transforming growth factor beta- and activin-induced transcription by mammalian Mad proteins', *Proc Natl Acad Sci U S A* 93(23): 12992-7.

Christopherson, K. S., Ullian, E. M., Stokes, C. C., Mullowney, C. E., Hell, J. W., Agah, A., Lawler, J., Mosher, D. F., Bornstein, P. and Barres, B. A. (2005) 'Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis', *Cell* 120(3): 421-33.

Chung, W. S. and Barres, B. A. (2011) 'The role of glial cells in synapse elimination', *Curr Opin Neurobiol*.

Clark, R. I., Woodcock, K. J., Geissmann, F., Trouillet, C. and Dionne, M. S. (2011) 'Multiple TGF-beta superfamily signals modulate the adult Drosophila immune response', *Curr Biol* 21(19): 1672-7.

Cline, H. T. (1991) 'Activity-dependent plasticity in the visual systems of frogs and fish', *Trends Neurosci* 14(3): 104-11.

Coleman, M. P. and Freeman, M. R. (2010) 'Wallerian degeneration, wld(s), and nmnat', *Annu Rev Neurosci* 33: 245-67.

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D. R., Dustin, M. L. and Gan, W. B. (2005) 'ATP mediates rapid microglial response to local brain injury in vivo', *Nat Neurosci* 8(6): 752-8.

Dawson-Scully, K., Lin, Y., Imad, M., Zhang, J., Marin, L., Horne, J. A., Meinertzhagen, I. A., Karunanithi, S., Zinsmaier, K. E. and Atwood, H. L. (2007) 'Morphological and functional effects of altered cysteine string protein at the Drosophila larval neuromuscular junction', *Synapse* 61(1): 1-16.

Doherty, J., Logan, M. A., Tasdemir, O. E. and Freeman, M. R. (2009) 'Ensheathing glia function as phagocytes in the adult Drosophila brain', *J Neurosci* 29(15): 4768-81.

Dudu, V., Bittig, T., Entchev, E., Kicheva, A., Julicher, F. and Gonzalez-Gaitan, M. (2006) 'Postsynaptic mad signaling at the Drosophila neuromuscular junction', *Curr Biol* 16(7): 625-35.

Edenfeld, G., Altenhein, B., Zierau, A., Cleppien, D., Krukkert, K., Technau, G. and Klambt, C. (2007) 'Notch and Numb are required for normal migration of peripheral glia in Drosophila', *Dev Biol* 301(1): 27-37.

Eivers, E., Demagny, H., Choi, R. H. and De Robertis, E. M. (2011) 'Phosphorylation of Mad controls competition between wingless and BMP signaling', *Sci Signal* 4(194): ra68.

Eldar, A., Dorfman, R., Weiss, D., Ashe, H., Shilo, B. Z. and Barkai, N. (2002) 'Robustness of the BMP morphogen gradient in Drosophila embryonic patterning', *Nature* 419(6904): 304-8.

Ellis, J. E., Parker, L., Cho, J. and Arora, K. (2010a) 'Activin signaling functions upstream of Gbb to regulate synaptic growth at the Drosophila neuromuscular junction', *Dev Biol* 24: 24.

Ellis, J. E., Parker, L., Cho, J. and Arora, K. (2010b) 'Activin signaling functions upstream of Gbb to regulate synaptic growth at the Drosophila neuromuscular junction', *Dev Biol* 342(2): 121-33.

Eroglu, C. and Barres, B. A. (2010) 'Regulation of synaptic connectivity by glia', *Nature* 468(7321): 223-31.

Evans, T. A. and Bashaw, G. J. (2010) 'Axon guidance at the midline: of mice and flies', *Curr Opin Neurobiol* 20(1): 79-85.

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M. and Hall, J. C. (1992) 'Expression of the *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms', *J Neurosci* 12(9): 3321-49.

Fancy, S. P., Chan, J. R., Baranzini, S. E., Franklin, R. J. and Rowitch, D. H. (2011) 'Myelin regeneration: a recapitulation of development?', *Annu Rev Neurosci* 34: 21-43.

Feng, Z. and Ko, C. P. (2008) 'Schwann cells promote synaptogenesis at the neuromuscular junction via transforming growth factor-beta1', *J Neurosci* 28(39): 9599-609.

Feng, Z., Koirala, S. and Ko, C. P. (2005) 'Synapse-glia interactions at the vertebrate neuromuscular junction', *Neuroscientist* 11(5): 503-13.

Freeman, M. R. (2006) 'Sculpting the nervous system: glial control of neuronal development', *Curr Opin Neurobiol* 16(1): 119-25.

Freeman, M. R., Delrow, J., Kim, J., Johnson, E. and Doe, C. Q. (2003a) 'Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function', *Neuron* 38(4): 567-80.

Freeman, M. R., Delrow, J., Kim, J., Johnson, E. and Doe, C. Q. (2003b) 'Unwrapping glial biology. Gcm target genes regulating glial development, diversification, and function', *Neuron* 38(4): 567-80.

Fuentes-Medel, Y. and Budnik, V. (2010) 'Menage a Trio during BMP-Mediated Retrograde Signaling at the NMJ', *Neuron* 66(4): 473-5.

Fuentes-Medel, Y., Logan, M. A., Ashley, J., Ataman, B., Budnik, V. and Freeman, M. R. (2009) 'Glia and muscle sculpt neuromuscular arbors by engulfing destabilized synaptic boutons and shed presynaptic debris', *PLoS Biol* 7(8): e1000184.

Gesualdi, S. C. and Haerry, T. E. (2007) 'Distinct signaling of Drosophila Activin/TGF-beta family members', *Fly (Austin)* 1(4): 212-21.

Gibbens, Y. Y., Warren, J. T., Gilbert, L. I. and O'Connor, M. B. (2011) 'Neuroendocrine regulation of Drosophila metamorphosis requires TGFbeta/Activin signaling', *Development* 138(13): 2693-703.

Goold, C. P. and Davis, G. W. (2007) 'The BMP ligand Gbb gates the expression of synaptic homeostasis independent of synaptic growth control', *Neuron* 56(1): 109-23.

Gorczyca, M., Augart, C. and Budnik, V. (1993) 'Insulin-like receptor and insulin-like peptide are localized at neuromuscular junctions in Drosophila', *J Neurosci* 13(9): 3692-704.

Griffin, J. W. and Thompson, W. J. (2008) 'Biology and pathology of nonmyelinating Schwann cells', *Glia* 56(14): 1518-31.

Griffith, L. C. and Budnik, V. (2006) 'Plasticity and second messengers during synapse development', *Int Rev Neurobiol* 75: 237-65.

Guan, B., Hartmann, B., Kho, Y. H., Gorczyca, M. and Budnik, V. (1996) 'The Drosophila tumor suppressor gene, dlg, is involved in structural plasticity at a glutamatergic synapse', *Curr Biol* 6(6): 695-706.

Haerry, T. E. (2010) 'The interaction between two TGF-beta type I receptors plays important roles in ligand binding, SMAD activation, and gradient formation', *Mech Dev* 127(7-8): 358-70.

Halassa, M. M. and Haydon, P. G. (2010) 'Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior', *Annu Rev Physiol* 72: 335-55.

Hamon, Y., Trompier, D., Ma, Z., Venegas, V., Pophillat, M., Mignotte, V., Zhou, Z. and Chimini, G. (2006) 'Cooperation between Engulfment Receptors: The Case of ABCA1 and MEGF10', *PLoS ONE* 1: e120.

Hashimoto, Y., Tabuchi, Y., Sakurai, K., Kutsuna, M., Kurokawa, K., Awasaki, T., Sekimizu, K., Nakanishi, Y. and Shiratsuchi, A. (2009) 'Identification of lipoteichoic acid as a ligand for draper in the phagocytosis of Staphylococcus aureus by Drosophila hemocytes', *J Immunol* 183(11): 7451-60.

Higashi-Kovtun, M. E., Mosca, T. J., Dickman, D. K., Meinertzhagen, I. A. and Schwarz, T. L. (2010) 'Importin-beta11 regulates synaptic phosphorylated mothers against decapentaplegic, and thereby influences synaptic development and function at the Drosophila neuromuscular junction', *J Neurosci* 30(15): 5253-68.

Hoopfer, E. D., McLaughlin, T., Watts, R. J., Schuldiner, O., O'Leary, D. D. and Luo, L. (2006) 'Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning', *Neuron* 50(6): 883-95.

Ingleby, L., Maloney, R., Jepson, J., Horn, R. and Reenan, R. (2009) 'Regulated RNA editing and functional epistasis in Shaker potassium channels', *J Gen Physiol* 133(1): 17-27.

Jackson, F. R. (2011) 'Glial cell modulation of circadian rhythms', *Glia* 59(9): 1341-50.

Jan, L. Y. and Jan, Y. N. (1976) 'Properties of the larval neuromuscular junction in Drosophila melanogaster', *J Physiol* 262(1): 189-214.

Jan, L. Y. and Jan, Y. N. (1982) 'Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos', *Proc Natl Acad Sci U S A* 79(8): 2700-4.

Jensen, P. A., Zheng, X., Lee, T. and O'Connor, M. B. (2009) 'The Drosophila Activin-like ligand Dawdle signals preferentially through one isoform of the Type-I receptor Baboon', *Mech Dev* 126(11-12): 950-7.

Jia, X. X., Gorczyca, M. and Budnik, V. (1993) 'Ultrastructure of neuromuscular junctions in Drosophila: comparison of wild type and mutants with increased excitability', *J Neurobiol* 24(8): 1025-44.

Johansen, J., Halpern, M. E. and Keshishian, H. (1989) 'Axonal guidance and the development of muscle fiber-specific innervation in Drosophila embryos', *J Neurosci* 9(12): 4318-32.

Kazama, H., Yaksi, E. and Wilson, R. I. (2011) 'Cell death triggers olfactory circuit plasticity via glial signaling in *Drosophila*', *J Neurosci* 31(21): 7619-30.

Keller, L. C., Cheng, L., Locke, C. J., Muller, M., Fetter, R. D. and Davis, G. W. (2011) 'Glial-derived prodegenerative signaling in the Drosophila neuromuscular system', *Neuron* 72(5): 760-75.

Keshishian, H. and Kim, Y. S. (2004) 'Orchestrating development and function: retrograde BMP signaling in the Drosophila nervous system', *Trends Neurosci* 27(3): 143-7.

Kirilly, D., Gu, Y., Huang, Y., Wu, Z., Bashirullah, A., Low, B. C., Kolodkin, A. L., Wang, H. and Yu, F. (2009) 'A genetic pathway composed of Sox14 and Mical governs severing of dendrites during pruning', *Nat Neurosci* 12(12): 1497-505.

Kittel, R. J., Wichmann, C., Rasse, T. M., Fouquet, W., Schmidt, M., Schmid, A., Wagh, D. A., Pawlu, C., Kellner, R. R., Willig, K. I. et al. (2006) 'Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release', *Science* 312(5776): 1051-4.

Klagges, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S. and Buchner, E. (1996) 'Invertebrate synapsins: a single gene codes for several isoforms in Drosophila', *J Neurosci* 16(10): 3154-65.

Koh, Y. H., Gramates, L. S. and Budnik, V. (2000) 'Drosophila larval neuromuscular junction: molecular components and mechanisms underlying synaptic plasticity', *Microsc Res Tech* 49(1): 14-25.

Koh, Y. H., Popova, E., Thomas, U., Griffith, L. C. and Budnik, V. (1999) 'Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation', *Cell* 98(3): 353-63.

Krieglstein, K., Zheng, F., Unsicker, K. and Alzheimer, C. (2011) 'More than being protective: functional roles for TGF-beta/activin signaling pathways at central synapses', *Trends Neurosci* 34(8): 421-9.

Kucukdereli, H., Allen, N. J., Lee, A. T., Feng, A., Ozlu, M. I., Conatser, L. M., Chakraborty, C., Workman, G., Weaver, M., Sage, E. H. et al. (2011) 'Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC', *Proc Natl Acad Sci U S A* 108(32): E440-9.

Kullberg, R. W., Lentz, T. L. and Cohen, M. W. (1977) 'Development of the myotomal neuromuscular junction in Xenopus laevis: an electrophysiological and fine-structural study', *Dev Biol* 60(1): 101-29.

Kuraishi, T., Nakagawa, Y., Nagaosa, K., Hashimoto, Y., Ishimoto, T., Moki, T., Fujita, Y., Nakayama, H., Dohmae, N., Shiratsuchi, A. et al. (2009) 'Pretaporter, a Drosophila protein serving as a ligand for Draper in the phagocytosis of apoptotic cells', *EMBO J* 28(24): 3868-78.

Lahey, T., Gorczyca, M., Jia, X. X. and Budnik, V. (1994) 'The Drosophila tumor suppressor gene dlg is required for normal synaptic bouton structure', *Neuron* 13(4): 823-35.

Lee, T. and Luo, L. (1999) 'Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis', *Neuron* 22(3): 451-61.

Lo, P. C. and Frasch, M. (1999) 'Sequence and expression of myoglianin, a novel Drosophila gene of the TGF-beta superfamily', *Mech Dev* 86(1-2): 171-5.

Logan, M. A. and Freeman, M. R. (2007) 'The scoop on the fly brain: glial engulfment functions in Drosophila', *Neuron Glia Biol* 3(1): 63-74.

Lohmann, C. and Bonhoeffer, T. (2008) 'A role for local calcium signaling in rapid synaptic partner selection by dendritic filopodia', *Neuron* 59(2): 253-60.

Love, F. M., Son, Y. J. and Thompson, W. J. (2003) 'Activity alters muscle reinnervation and terminal sprouting by reducing the number of Schwann cell pathways that grow to link synaptic sites', *J Neurobiol* 54(4): 566-76.

Love, F. M. and Thompson, W. J. (1999) 'Glial cells promote muscle reinnervation by responding to activity-dependent postsynaptic signals', *J Neurosci* 19(23): 10390-6.

Lu, B., Wang, K. H. and Nose, A. (2009) 'Molecular mechanisms underlying neural circuit formation', *Curr Opin Neurobiol*.

Lu, J. and Lichtman, J. W. (2007) 'Imaging the neuromuscular junction over the past centuries', *Sheng Li Xue Bao* 59(6): 683-96.

Luo, L. and O'Leary, D. D. (2005) 'Axon retraction and degeneration in development and disease', *Annu Rev Neurosci* 28: 127-56.

MacDonald, J. M., Beach, M. G., Porpiglia, E., Sheehan, A. E., Watts, R. J. and Freeman, M. R. (2006) 'The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons', *Neuron* 50(6): 869-81.

Magazanik, L. G. and Vyskocil, F. (1979) 'Spontaneous junctional currents in Drosophila muscle fibres: effects of temperature, membrane potential and ethanol', *Experientia* 35(2): 213-4.

Manaka, J., Kuraishi, T., Shiratsuchi, A., Nakai, Y., Higashida, H., Henson, P. and Nakanishi, Y. (2004) 'Draper-mediated and phosphatidylserine-independent phagocytosis of apoptotic cells by Drosophila hemocytes/macrophages', *J Biol Chem* 279(46): 48466-76.

Marin-Teva, J. L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N. and Mallat, M. (2004) 'Microglia promote the death of developing Purkinje cells', *Neuron* 41(4): 535-47.

Marques, G. (2005) 'Morphogens and synaptogenesis in Drosophila', *J Neurobiol* 64(4): 417-34.

Marques, G., Bao, H., Haerry, T. E., Shimell, M. J., Duchek, P., Zhang, B. and O'Connor, M. B. (2002) 'The Drosophila BMP Type II Receptor Wishful Thinking Regulates Neuromuscular Synapse Morphology and Function', *Neuron* 33(4): 529-43.

Marques, G., Haerry, T. E., Crotty, M. L., Xue, M., Zhang, B. and O'Connor, M. B. (2003) 'Retrograde Gbb signaling through the Bmp type 2 receptor wishful thinking regulates systemic FMRFa expression in Drosophila', *Development* 130(22): 5457-70.

Marquez, R. M., Singer, M. A., Takaesu, N. T., Waldrip, W. R., Kraytsberg, Y. and Newfeld, S. J. (2001) 'Transgenic analysis of the Smad family of TGF-beta signal transducers in Drosophila melanogaster suggests new roles and new interactions between family members', *Genetics* 157(4): 1639-48.

Mathew, D., Ataman, B., Chen, J., Zhang, Y., Cumberledge, S. and Budnik, V. (2005) 'Wingless signaling at synapses is through cleavage and nuclear import of receptor DFrizzled2', *Science* 310(5752): 1344-7.

McCabe, B. D., Marques, G., Haghighi, A. P., Fetter, R. D., Crotty, M. L., Haerry, T. E., Goodman, C. S. and O'Connor, M. B. (2003) 'The BMP homolog Gbb

provides a retrograde signal that regulates synaptic growth at the Drosophila neuromuscular junction', *Neuron* 39(2): 241-54.

Miech, C., Pauer, H. U., He, X. and Schwarz, T. L. (2008) 'Presynaptic local signaling by a canonical wingless pathway regulates development of the Drosophila neuromuscular junction', *J Neurosci* 28(43): 10875-84.

Miyazono, K., Kamiya, Y. and Morikawa, M. (2010) 'Bone morphogenetic protein receptors and signal transduction', *J Biochem* 147(1): 35-51.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K. F. (1999) 'Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development', *Neuron* 23(2): 273-83.

Moustakas, A. and Heldin, C. H. (2009) 'The regulation of TGFbeta signal transduction', *Development* 136(22): 3699-714.

Nagler, K., Mauch, D. H. and Pfrieger, F. W. (2001) 'Glia-derived signals induce synapse formation in neurones of the rat central nervous system', *J Physiol* 533(Pt 3): 665-79.

Ng, F. S., Tangredi, M. M. and Jackson, F. R. (2011) 'Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner', *Curr Biol* 21(8): 625-34.

O'Connor, M. B., Umulis, D., Othmer, H. G. and Blair, S. S. (2006) 'Shaping BMP morphogen gradients in the Drosophila embryo and pupal wing', *Development* 133(2): 183-93.

O'Connor-Giles, K. M., Ho, L. L. and Ganetzky, B. (2008) 'Nervous wreck interacts with thickveins and the endocytic machinery to attenuate retrograde BMP signaling during synaptic growth', *Neuron* 58(4): 507-18.

O'Leary, D. D. and Koester, S. E. (1993) 'Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex', *Neuron* 10(6): 991-1006.

O'Leary, D. D. and McLaughlin, T. (2005) 'Mechanisms of retinotopic map development: Ephs, ephrins, and spontaneous correlated retinal activity', *Prog Brain Res* 147: 43-65.

Packard, M., Koo, E. S., Gorczyca, M., Sharpe, J., Cumberledge, S. and Budnik, V. (2002) 'The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation', *Cell* 111(3): 319-30.

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L. et al. (2011) 'Synaptic pruning by microglia is necessary for normal brain development', *Science* 333(6048): 1456-8.

Parker, R. J. and Auld, V. J. (2006) 'Roles of glia in the Drosophila nervous system', *Semin Cell Dev Biol* 17(1): 66-77.

Pfrieger, F. W. and Barres, B. A. (1997) 'Synaptic efficacy enhanced by glial cells in vitro', *Science* 277(5332): 1684-7.

Portera-Cailliau, C., Weimer, R. M., De Paola, V., Caroni, P. and Svoboda, K. (2005) 'Diverse modes of axon elaboration in the developing neocortex', *PLoS Biol* 3(8): e272.

Qin, Y., Zhu, Y., Baumgart, J. P., Stornetta, R. L., Seidenman, K., Mack, V., van Aelst, L. and Zhu, J. J. (2005) 'State-dependent Ras signaling and AMPA receptor trafficking', *Genes Dev* 19(17): 2000-15.

Raftery, L. A. and Sutherland, D. J. (1999) 'TGF-beta family signal transduction in Drosophila development: from Mad to Smads', *Dev Biol* 210(2): 251-68.

Ranjan, R., Bronk, P. and Zinsmaier, K. E. (1998) 'Cysteine string protein is required for calcium secretion coupling of evoked neurotransmission in drosophila but not for vesicle recycling', *J Neurosci* 18(3): 956-64.

Rawson, J. M., Lee, M., Kennedy, E. L. and Selleck, S. B. (2003) 'Drosophila neuromuscular synapse assembly and function require the TGF-beta type I receptor saxophone and the transcription factor Mad', *J Neurobiol* 55(2): 134-50.

Reddien, P. W. and Horvitz, H. R. (2004) 'The engulfment process of programmed cell death in caenorhabditis elegans', *Annu Rev Cell Dev Biol* 20: 193-221.

Reddy, L. V., Koirala, S., Sugiura, Y., Herrera, A. A. and Ko, C. P. (2003) 'Glial cells maintain synaptic structure and function and promote development of the neuromuscular junction in vivo', *Neuron* 40(3): 563-80.

Richardson, W. D., Young, K. M., Tripathi, R. B. and McKenzie, I. (2011) 'NG2-glia as multipotent neural stem cells: fact or fantasy?', *Neuron* 70(4): 661-73.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997) 'Severe neuropathies in mice with targeted mutations in the ErbB3 receptor', *Nature* 389(6652): 725-30.

Rogulja-Ortmann, A., Luer, K., Seibert, J., Rickert, C. and Technau, G. M. (2007) 'Programmed cell death in the embryonic central nervous system of Drosophila melanogaster', *Development* 134(1): 105-16.

Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001) 'Twisted gastrulation is a conserved extracellular BMP antagonist', *Nature* 410(6827): 479-83.

Ruiz-Canada, C. and Budnik, V. (2006) 'Introduction on the use of the Drosophila embryonic/larval neuromuscular junction as a model system to study synapse development and function, and a brief summary of pathfinding and target recognition', *Int Rev Neurobiol* 75: 1-31.

Saijo, K. and Glass, C. K. (2011) 'Microglial cell origin and phenotypes in health and disease', *Nat Rev Immunol* 11(11): 775-87.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Voller, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. et al. (2006) 'Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae', *Curr Biol* 16(17): 1741-7.

Schuster, C. M., Davis, G. W., Fetter, R. D. and Goodman, C. S. (1996) 'Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth', *Neuron* 17(4): 641-54.

Sepp, K. J. and Auld, V. J. (1999) 'Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in Drosophila melanogaster', *Genetics* 151(3): 1093-101.

Sepp, K. J., Schulte, J. and Auld, V. J. (2000) 'Developmental dynamics of peripheral glia in Drosophila melanogaster', *Glia* 30(2): 122-33.

Sepp, K. J., Schulte, J. and Auld, V. J. (2001) 'Peripheral glia direct axon guidance across the CNS/PNS transition zone', *Dev Biol* 238(1): 47-63.

Seugnet, L., Suzuki, Y., Merlin, G., Gottschalk, L., Duntley, S. P. and Shaw, P. J. (2011) 'Notch signaling modulates sleep homeostasis and learning after sleep deprivation in *Drosophila*', *Curr Biol* 21(10): 835-40.

Snow, P. M., Patel, N. H., Harrelson, A. L. and Goodman, C. S. (1987) 'Neural-specific carbohydrate moiety shared by many surface glycoproteins in Drosophila and grasshopper embryos', *J Neurosci* 7(12): 4137-44.

Sone, M., Suzuki, E., Hoshino, M., Hou, D., Kuromi, H., Fukata, M., Kuroda, S., Kaibuchi, K., Nabeshima, Y. and Hama, C. (2000) 'Synaptic development is controlled in the periactive zones of Drosophila synapses', *Development* 127(19): 4157-68.

Sonnenfeld, M. J. and Jacobs, J. R. (1995) 'Macrophages and glia participate in the removal of apoptotic neurons from the Drosophila embryonic nervous system', *J Comp Neurol* 359(4): 644-52.

Stacey, S. M., Muraro, N. I., Peco, E., Labbe, A., Thomas, G. B., Baines, R. A. and van Meyel, D. J. (2010) 'Drosophila glial glutamate transporter Eaat1 is regulated by fringe-mediated notch signaling and is essential for larval locomotion', *J Neurosci* 30(43): 14446-57.

Steinert, J. R., Kuromi, H., Hellwig, A., Knirr, M., Wyatt, A. W., Kidokoro, Y. and Schuster, C. M. (2006) 'Experience-dependent formation and recruitment of large vesicles from reserve pool', *Neuron* 50(5): 723-33.

Stellwagen, D. and Malenka, R. C. (2006) 'Synaptic scaling mediated by glial TNF-alpha', *Nature* 440(7087): 1054-9.

Stevens, B. (2008) 'Neuron-astrocyte signaling in the development and plasticity of neural circuits', *Neurosignals* 16(4): 278-88.

Stork, T., Engelen, D., Krudewig, A., Silies, M., Bainton, R. J. and Klambt, C. (2008) 'Organization and function of the blood-brain barrier in Drosophila', *J Neurosci* 28(3): 587-97.

Sun, M., Thomas, M. J., Herder, R., Bofenkamp, M. L., Selleck, S. B. and O'Connor, M. B. (2007) 'Presynaptic contributions of chordin to hippocampal plasticity and spatial learning', *J Neurosci* 27(29): 7740-50.

Tejedor, F. J., Bokhari, A., Rogero, O., Gorczyca, M., Zhang, J., Kim, E., Sheng, M. and Budnik, V. (1997) 'Essential role for dlg in synaptic clustering of Shaker K+ channels in vivo', *J Neurosci* 17(1): 152-9.

Thomas, U., Kim, E., Kuhlendahl, S., Koh, Y. H., Gundelfinger, E. D., Sheng, M., Garner, C. C. and Budnik, V. (1997) 'Synaptic clustering of the cell adhesion molecule fasciclin II by discs-large and its role in the regulation of presynaptic structure', *Neuron* 19(4): 787-99.

Todd, K. J., Darabid, H. and Robitaille, R. (2010) 'Perisynaptic glia discriminate patterns of motor nerve activity and influence plasticity at the neuromuscular junction', *J Neurosci* 30(35): 11870-82.

Torroja, L., Packard, M., Gorczyca, M., White, K. and Budnik, V. (1999) 'The Drosophila beta-amyloid precursor protein homolog promotes synapse differentiation at the neuromuscular junction', *J Neurosci* 19(18): 7793-803.

Ullian, E. M., Sapperstein, S. K., Christopherson, K. S. and Barres, B. A. (2001) 'Control of synapse number by glia', *Science* 291(5504): 657-61.

Wagh, D. A., Rasse, T. M., Asan, E., Hofbauer, A., Schwenkert, I., Durrbeck, H., Buchner, S., Dabauvalle, M. C., Schmidt, M., Qin, G. et al. (2006) 'Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila', *Neuron* 49(6): 833-44.

Walsh, M. K. and Lichtman, J. W. (2003) 'In vivo time-lapse imaging of synaptic takeover associated with naturally occurring synapse elimination', *Neuron* 37(1): 67-73.

Watts, R. J., Hoopfer, E. D. and Luo, L. (2003) 'Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system', *Neuron* 38(6): 871-85.

Weiss, A., Charbonnier, E., Ellertsdottir, E., Tsirigos, A., Wolf, C., Schuh, R., Pyrowolakis, G. and Affolter, M. (2010) 'A conserved activation element in BMP signaling during Drosophila development', *Nat Struct Mol Biol* 17(1): 69-76.

Wilson, M. H. and Deschenes, M. R. (2005) 'The neuromuscular junction: anatomical features and adaptations to various forms of increased, or decreased neuromuscular activity', *Int J Neurosci* 115(6): 803-28.

Woldeyesus, M. T., Britsch, S., Riethmacher, D., Xu, L., Sonnenberg-Riethmacher, E., Abou-Rebyeh, F., Harvey, R., Caroni, P. and Birchmeier, C. (1999) 'Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development', *Genes Dev* 13(19): 2538-48.

Wu, H. H., Bellmunt, E., Scheib, J. L., Venegas, V., Burkert, C., Reichardt, L. F., Zhou, Z., Farinas, I. and Carter, B. D. (2009) 'Glial precursors clear sensory neuron corpses during development via Jedi-1, an engulfment receptor', *Nat Neurosci* 12(12): 1534-41.

Yoshihara, M. and Littleton, J. T. (2002) 'Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release', *Neuron* 36(5): 897-908.

Zhang, Y. and Barres, B. A. (2010) 'Astrocyte heterogeneity: an underappreciated topic in neurobiology', *Curr Opin Neurobiol* 20(5): 588-94.

Zhang, Y. P. and Oertner, T. G. (2007) 'Optical induction of synaptic plasticity using a light-sensitive channel', *Nat Methods* 4(2): 139-41.

Zhong, Y., Budnik, V. and Wu, C. F. (1992) 'Synaptic plasticity in Drosophila memory and hyperexcitable mutants: role of cAMP cascade', *J Neurosci* 12(2): 644-51.

Zhuang, J. L., Wang, C. Y., Zhou, M. H., Duan, K. Z. and Mei, Y. A. (2012) 'TGF-beta1 enhances Kv2.1 potassium channel protein expression and promotes maturation of cerebellar granule neurons', *J Cell Physiol* 227(1): 297-307.

Ziegenfuss, J. S., Biswas, R., Avery, M. A., Hong, K., Sheehan, A. E., Yeung, Y. G., Stanley, E. R. and Freeman, M. R. (2008) 'Draper-dependent glial phagocytic activity is mediated by Src and Syk family kinase signalling', *Nature* 453(7197): 935-9.

Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N. and Benzer, S. (1994) 'Paralysis and early death in cysteine string protein mutants of Drosophila', *Science* 263(5149): 977-80.

Zito, K., Parnas, D., Fetter, R. D., Isacoff, E. Y. and Goodman, C. S. (1999) 'Watching a synapse grow: noninvasive confocal imaging of synaptic growth in Drosophila', *Neuron* 22(4): 719-29.