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# Glial Control of Synapse Assembly at the *Drosophila* Neuromuscular Junction: A Dissertation

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# Glial control of synapse assembly at the *Drosophila* neuromuscular junction

A Dissertation Presented

By

Kimberly S. Kerr

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Glial control of synapse assembly at the *Drosophila* neuromuscular junction

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## Abstract

Emerging evidence in both vertebrates and invertebrates is redefining glia as active and mobile players in synapse formation, maturation and function. However, the molecular mechanisms through which neurons and glia interact with each other to regulate these processes is not well known. My thesis work begins to understand how glia use secreted factors to modulate synaptic function. We use *Drosophila melanogaster,* a simple and genetically tractable model system, to understand the molecular mechanisms by which glia communicate with neurons at glutamatergic neuromuscular junctions (NMJs). We previously showed that a specific subtype of glia, subperineurial peripheral glia cells (SPGs), establish dynamic transient interactions with synaptic boutons of the NMJ and is required for synaptic growth. I identified a number of potential functional targets of the glial transcription factor, *reverse polarity* (*repo*) using ChIP-chip. I found that one novel target of Repo, Wg, is expressed in SPGs and is regulated by *repo in vivo*. Wnt/Wg signaling plays a pivotal role during synapse development and plasticity, including the coordinated development of the molecular architecture of the synapse. While previous studies demonstrated that Wg is secreted by motor neurons, herein I provide evidence that a significant amount of Wg at the NMJ is additionally provided by glia. I found that Wg derived from SPGs is required for proper GluR distribution and electrophysiological responses at the NMJ. In summary, my results show that Wg expression is

regulated by Repo in SPGs and that glial-derived Wg, together with motor neuron-derived Wg, orchestrate different aspects of synapse development. My thesis work identifies synapse stabilization and/or assembly as a new role for SPGs and demonstrates that glial secreted factors such as Wg regulate synaptic function at the *Drosophila* NMJ.

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## List of Abbreviations

ADNF – activity-dependent neurotrophic factor

BMPs – Bone Morphogenetic Proteins

Brp – Bruchpilot

CAMKII – $Ca^{2+}$/calmodulin-dependent protein kinases II

CSP – Cysteine string protein

Csw – Corkscrew

CNS – Central nervous system

Dlg – Disc-Large

Drpr – Draper

EAATs – Excitatory amino-acid transporters

EJP – Excitatory junction potential

Dfz2 – Frizzled 2 receptor

Fas II – Fasciculin II

Fz – Frizzled receptor

GCM – Glial cells missing

GFP – Green fluorescence protein

GluR – Glutamate receptor

GluRIIA – Glutamate receptor sbunit IIA

HRP – Horseradish proxidase

Mav – Maverick

mEJP – Mini evoked excitatory potential

MN – Motor neuron

NMJ – Neuromuscular junction

PG – Perineurial glial cells

Porc – Porcupine

PSCs – Perisynaptic schwann cells

Repo – Reversed polarity

RGC – Retinal ganglion cells

RNAi – Double stranded RNA interference

S2 – Schneider 2 cells

SPARC – secreted protein, acidic and rich in cysteine

SPGs – Subperineurial glial cells

Tkv – Thickvein

TGF-β −Transforming growth factor beta

TSP – Thrombospondins

Wg – Wingless

Wrg – Wrapping glia

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Raw data of genes identified as Repo binding targets

## Preface

All work described in this thesis was performed at University of Massachusetts Medical School in the lab of Marc Freeman and in collaboration with Vivian Budnik and her lab. In chapter II, Yuly Fuentes-Medel performed the initial experiments to start the project and the quantitative real-time PCR. She also contributed to discussions on experimental design. Romina Barria contributed to the quantitative real-time PCR experiments. Cassandra Brewer and James Ashley performed the electrophysiological recordings. Amy Sheehan cloned the Repo:myc constructs. Marc Freeman and Vivian Budnik supported the work with discussions on designing the experiments, analyzing the data and writing the paper. I designed and performed the experiments, analyzed the data and co-wrote the paper.

# Chapter I:

# Introduction

## Glial cells are the infrastructure of the nervous system

Glial cells, non-neuronal cells, are not simply support cells in the nervous system; they carry out key functions that are fundamental for brain development and function. A few examples are guiding neurons to their appropriate targets, clearing neurotransmitters, maintaining trophic support and maintaining proper ionic balance (Booth et al., 2000; Danbolt, 2001; Gilmour et al., 2002; Schousboe et al., 2004; Xiong and Montell, 1995). Additionally they are critical for regulating the overall health of the nervous system. They isolate and protect the nervous system, maintain homeostasis, and act as immune cells during injury, infection and disease (Abbott, 2005; Hanisch and Kettenmann, 2007; Kreutzberg, 1996; MacDonald et al., 2006; Ransohoff and Perry, 2009; Robitaille, 1998; Schousboe et al., 2004).

The principal types of glial cell types in the mammalian nervous system are astrocytes, oligodendrocytes, microglia, $NG2^+$ cells, and Schwann cells (Pfrieger, 2009). In the CNS, astrocytes are best known for their role in preventing neurotoxicity by regulating synaptic neurotransmitter concentrations such as glutamate (Bacci et al., 1999). Oligodendrocytes form myelin to wrap axons for more efficient propagation of action potentials through saltatory conduction (Pfrieger, 2009). $NG2^+$ cells remain a mysterious cell type, but have recently

been shown to be precursors for oligodendrocytes, and are thought to replenish their populations in the mature brain (Dawson et al., 2000; Dawson et al., 2003; Lin et al., 2005; Zhu et al., 2008). Microglia, are the primary immune cell type in the brain whose functions include eliminating injured neurons to prevent inflammation in the brain (Pfrieger, 2009). Finally, Schwann cells play the combined role of astrocytes and oligodendrocytes in the peripheral nervous system (PNS) by regulating neurotransmitter release and myelinating axons (Haydon, 2001; Robitaille, 1998).

**Vertebrate glia regulate synapse formation and function**

The heart of neuronal communication is the synapse. Everything from movement to cognitive function depends upon the accurate flow of information through our neural networks. The main gate keeper that regulates this intercellular communication is the synapse. This specialized cell junction is formed when the presynaptic terminal of an axon contacts a postsynaptic target, such as a neuron or muscle. The presynaptic neuron transforms the electrical signal of an action potential into a chemical signal by releasing synaptic vesicles containing neurotransmitter onto the postsynaptic target. In order for synaptic transmission to occur, there must be precise coordination between the presynapse and the postsynapse.

Both inductive factors and chemical signals help recruit postsynaptic proteins and neurotransmitter receptors to stabilize the postsynaptic side of the connection (Garner et al., 2006). One cell type critical for synaptic function is the glial cell. The most well-known role of the glial cell is regulating neurotransmitter concentrations to prevent neurotoxicity; however, emerging evidence demonstrates that glia-derived signals are important for synapse formation and synaptic plasticity, but the mechanisms by which glia modulate synapse biology remain poorly defined (Stellwagen and Malenka, 2006; Ullian et al., 2004).

Glial secreted factors have emerged as key regulators of synapse formation, maturation, efficacy, and plasticity (Pfrieger and Barres, 1997; Ullian et al., 2004; Ullian et al., 2001) in both the CNS and PNS. For example, purified CNS neurons, retinal ganglion cells (RGCs), produced more functional synapses when cultured with astrocytes rather than without (Pfrieger and Barres, 1997). Also, RGC cultures without astrocytes had very little spontaneous synaptic activity. Conversely, synaptic activity was enhanced when either an astrocyte feeder layer or astrocyte-conditioned medium was present in the RGC cultures. One secreted factor isolated from astrocyte-conditioned media, thrombospondin (TSP), was shown to be necessary and sufficient for increased synapse number *in vitro,* and the number of excitatory synapses were found to be decreased in TSP null mutant mice (Christopherson et al., 2005). α2βδ-1 was recently identified as the TSP neuronal receptor and appears to be essential for

synaptogenesis *in vitro* and *in vivo* (Eroglu et al., 2009). Additionally, Kucukdereli et al. (2011) showed that hevin, an astrocytic secreted protein, promoted presence of excitatory synapses in the CNS. Yet, co-culturing both secreted astrocytic proteins, acidic and rich in cysteine (SPARC) and hevin in retinal ganglion cells (RGCs) resulted in a decrease in synapses. Interestingly, SPARC did not act as a negative synaptogenic regulator to TSP. This suggests that astrocytes use different molecular pathways to modulate synaptogenesis.

Other glial-derived factors modulate the efficiency of synapse function. For example, apolipoprotein E-containing particles are also secreted by astrocytes and have been shown to enhance synapse efficacy (Goritz et al., 2005). Glial-secreted activities have even been identified that specifically modulate the insertion of postsynaptic AMPA receptors, thereby promoting the functional maturation and probably the plasticity of synapses (Christopherson et al., 2005). Another study demonstrated that the astrocyte secreted factor, activity-dependent neurotrophic factor (ADNF), acted directly on neurons to regulate the synapse morphology by increasing NMDA receptors (Blondel et al., 2000). Finally, integrin-mediated pathways in astrocytes can induce synaptogenesis via local contact (Hama et al., 2004).

Schwann cells ensheath motor axons in the PNS. The most terminal Schwann cell located at the neuromuscular junction (NMJ) where the motor neuron

contacts the muscle is called the perisynaptic Schwann cell (PSC). PSCs play a role in synapse formation, activity, and maintenance. Motor axons are able to find their targets in the absence of Schwann cells but are unable to maintain stable synapses (Feng and Ko, 2007). In spinal motor neuron cultures, Schwann cells increase the number of excitatory glutamatergic synapses. Xenopus motor neuron cultures have demonstrated that factors in Schwann cell conditioned medium increase synapses between motor neurons and muscle by increasing the level of agrin (Peng et al., 2003). Agrin plays a role in synapse maturation by aggregating and maintaining the expression of postsynaptic acetylcholine receptors (Ullian et al., 2004). *In vivo* studies have demonstrated that PSCs are required to maintain the NMJ. For example, G-protein coupled receptors in PSCs can modulate the activity of motor neurons at the NMJ (Feng and Ko, 2007). When PSCs were ablated from *Xenopus* NMJs, using complement-mediated cell lysis, a reduction in synapse formation, and a retraction in existing synapses were observed (Reddy et al., 2003).

Glia are not only involved in synapse formation, but also synapse elimination. Astrocytes and microglia express secreted proteins in the complement cascade, such as C1q and C3. These proteins become activated during disease and play roles in synapse elimination (Cahoy et al., 2008; Levi-Strauss and Mallat, 1987; Stephan et al., 2012; Veerhuis et al., 1999). Many neurodegenerative diseases result in a loss of neurons. Understanding how to limit neuronal destruction and

promote synaptogenesis could lead to the development of therapies to these diseases. Changes in glial cell physiology or gene expression have been observed in many neurodegenerative diseases, including Alzheimer's disease, spinocerebellar ataxia, amyotrophic lateral sclerosis, Huntington's disease and Parkinson's disease (De Ferrari and Moon, 2006). Whether glia play a supportive role in degenerative disease by enhancing synapse stabilization, or a destructive role, perhaps inappropriately eliminating synapses, remains unclear. By understanding precisely how glial-secreted factors contribute to synapse formation, function and maintenance, we predict that we will significantly contribute to the design of therapeutic targets for treatment of these devastating neurodegenerative diseases.

My studies demonstrated the level of complexity by which glia act as a liaison between the pre- and the postsynapse and argue that this neuron-glia signaling is critical for specific steps in synapse formation, maturation, and function. However, the study of glial cell biology and its influence on neuronal function is just in its infancy, and the molecular mechanisms underlying its function in synapse formation are largely unknown. This places a genetically tractable system such as *Drosophila* in a unique position to rapidly dissect the signaling pathways underlying glia-neuron communication and their impact on brain development and health.

**Invertebrate glia as a model to study fundamental aspects of glial cell biology**

Several invertebrate glial subtypes have been identified that are similar to vertebrate glia regarding function (Edenfeld et al., 2005; Freeman and Doherty, 2006; Ito et al., 1995; Logan and Freeman, 2007; Stork et al., 2012). The three principal types of invertebrate glia are based on their position in the brain, and are (from deepest to most superficial) neuropil-associated, cortex-associated and surface-associated glia. The three subtypes of neuropil-associated glia are astrocytes, ensheathing glia, and wrapping glia (Doherty et al., 2009). Astrocytic glia densely infiltrate the neuropil and have a similar tufted morphology to vertebrate astrocytes (Doherty et al., 2009). They have been shown to maintain neurotransmitter homeostasis by the expression of excitatory amino acid transporter, dEAAT1, a glutamate transporter (Rival et al., 2006). Ensheathing glia surround parts of the neuropil and act as phagocytes by responding to injury to engulf neuronal debris (Awasaki et al., 2008; Doherty et al., 2009). Wrapping glia (WrG), most similar to nonmylinating Schwann cells, closely associate with and wrap axons. Cortex glia surround the neuronal cell bodies and are thought to provide trophic support to neurons(Doherty et al., 2009). Surface-associated glia are found on the surface of the entire nervous system, with perineurial glia (PG) being the outermost (and likely non-continuous) layer (Awasaki et al., 2008; Doherty et al., 2009; Stork et al., 2008). Subperineurial glia (SPGs) lay directly beneath PGs, and they form a sheath-like structure with one another, sealed by

pleated septate junctions, that acts as the blood brain barrier (Abbott, 2005; Auld et al., 1995; Awasaki et al., 2008; Baumgartner et al., 1996; Schwabe et al., 2005). There are a number of interesting functional similarities between invertebrate and vertebrate glia, such as regulating synaptic neurotransmitter levels (e.g., glutamate), regulating ionic homeostasis, and insulating nerves to propagate action potentials (Freeman and Doherty, 2006; Leiserson et al., 2000; Rival et al., 2006; Rival et al., 2004; Yuan and Ganetzky, 1999). These morphological and functional similarities of *Drosophila* glia to vertebrate glia imply that *Drosophila* will be an excellent system in which to study glial biology and that results from such studies will provide important insights into the development and function of vertebrate glia.

**Genetics of glial cell fate specification in *Drosophila***

During development a key regulator that determines the fate of undifferentiated cells called neuroblasts into neurons and glia is the transcription factor *glial cells missing* (*gcm*) whose mammalian homolog is GCMa/GCM1 (Badenhorst, 2001; Jones et al., 1995; Kania et al., 1995; Mao et al., 2012). In a *gcm* mutant, the progeny of neuroblasts that are normally specified to become glia fail to differentiate into glia, which ultimately results in lethality. Conversely, the overexpression of GCM resulted in the conversion of neurons to glial cells, demonstrating that *gcm* acts as a binary switch for the differentiation of neurons and glia.

GCM mutants were one of the earliest examples in the fly to show that glial cells are required for the proper development and function of the nervous system. In addition, other downstream transcription factors regulated by *gcm* that determine glial cell fate have been identified such as *repo*, *pnt*, *tramtrack* and *loco* (Badenhorst, 2001; Campbell et al., 1994; Giesen et al., 1997; Granderath et al., 2000; Granderath et al., 1999; Halter et al., 1995; Klaes et al., 1994; Klambt and Goodman, 1991; Xiong et al., 1994; Yuasa et al., 2003). Repo, for example, is a homeodomain protein that is not involved in the early differentiation of glial cells but is required for the late expression of glial genes such as EAATs (Rival et al., 2006). One exciting new candidate I identified in my work is Wingless (Wg), a key regulator in synapse formation at the NMJ.

**The *Drosophila* NMJ is a powerful *in vivo* system to study neuron-glia communication at synapses**

During *Drosophila* embryogenesis, motor neuron axons migrate and extend to their target muscles to form rudimentary synapses (Budnik et al., 2006). These motor neurons innervate the muscles in the larval body wall in a stereotypical way that allows us to perform a detailed analysis of the same synapse across different larvae.

During larval development, these synapses elaborate into branched structures called arbors that contain numerous presynaptic varicosities called boutons. Each bouton contains several active zones, which are sites of neurotransmitter release. *Drosophila* NMJ synapses are thought to be molecularly similar to vertebrate central synapses because they both release the same excitatory neurotransmitter, glutamate (Prokop et al., 2006), and have other components that are homologous to vertebrates such as scaffolding proteins (Dlg/PSD95), cell adhesion molecules (Fas II), and protein kinases (CAMKII) (Koh et al., 2000). There is a vast collection of markers for both the pre- and postsynapse such as: HRP, presynaptic membranes; Brp, active zones; CSP, presynaptic vesicles; Dlg, a marker of postsynaptic specializations; and postsynaptic glutamate receptors (GluRs). The combination of the above markers and tools to drive expression of transgenes in a cell specific manner (motor neuron, glia, and muscle specific) at the NMJ allows us to perform *in vivo* functional analysis.

Over the course of 4 days, *Drosophila* larvae increase ~100-fold in size. In order to maintain synaptic efficacy, motor neurons need to keep pace with the rapidly increasing surface area of the muscle and therefore add ~10-fold new synaptic contacts in this relatively short developmental time frame (Gorczyca et al., 1993; Griffith et al., 2006). This dramatic example of rapid synapse addition makes this system an ideal developmental setting in which to study synaptic growth and plasticity. The addition of new synaptic contacts at the NMJ is modulated by

activity and requires extensive reciprocal communication between pre- and postsynaptic cells (Ataman et al., 2008; Griffith et al., 2006; Marqués et al., 2006). Traditionally, work with this system has focused on signaling pathways mediating neuron-muscle communication. However, recent work from our laboratory, coupled with my preliminary data, argue strongly that *Drosophila* glia also play key roles in regulating synaptic growth at the NMJ (Fuentes-Medel et al., 2009). Here I will use the powerful array of molecular genetic tools available in *Drosophila* to determine precisely how glial secreted factors modulate NMJ synapse formation.

### *Drosophila* glia have dynamic interactions at the NMJ

Stork et al. (2008) morphologically defined three distinct subtypes of glia that associate with the nerves through which motor neurons project to body wall muscle targets: wrapping, subperineurial (SPGs), and perineurial glia (Stork et al., 2008). Wrapping glia are directly associated with motor neuron axons and often parse axons into specific bundles. SPGs form a layer around the entire nerve. Most work regarding SPGs has revolved around its role during embryonic development (Parker and Auld, 2006). Previous studies have focused on the non-developmental role of SPG in blood-brain barrier formation (Banerjee and Bhat, 2008; Stork et al., 2008). Moreover, SPGs, were shown to regulate glutamate levels through a cysteine/glutamate transporter that ultimately affect postsynaptic glutamate receptor expression (Augustin et al., 2007).

Initial characterization of glia at the *Drosophila* NMJ led to the conclusion that glial membranes failed to invade the NMJ and associate with most synaptic boutons (Banerjee et al., 2006; Sepp et al., 2000). Therefore the role of glial subtypes in synapse formation has not been explored until recently. Fuentes-Medel et al. (2009) demonstrated that both glia and muscle work in concert to regulate synaptic growth by engulfing synaptic debris. They used live preparations to reveal that the glial subtype SPGs in fact extend dynamic, transient processes deeply into the NMJ, interacting with boutons localized even at distal regions of the motor neuron arbor (Fuentes-Medel et al., 2009). Intriguingly, Brink et al. (2012) used live imaging as well to support these findings and in addition show that not just subperineurial but perineurial processes as well interact with the NMJ. They showed a direct correlation between glial NMJ coverage and synaptic growth using temperature and a *highwire* mutant.

While it has been known for awhile that Axotactin, a secreted factor expressed in longitudinal glia in the CNS, regulates neuronal excitability at the NMJ, the possibility that secreted factors from other glial subtypes regulate synapses has remained a mystery (Yuan and Ganetzky, 1999). Excitingly, Fuentes-Medel et al. (2012) recently identified the first *Drosophila* glial secreted factor, Maverick, a TGFβ ligand, as a modulator of synaptic growth at the NMJ via the Gbb-dependent retrograde pathway in muscle. My thesis work has identified another

glial secreted factor, Wingless (Wg), which demonstrates that *Drosophila* glia is an ideal model system to identify potential candidates.

**Wg signaling modulates synaptic growth**

Wingless/Int (Wnt) is a secreted glycoprotein known to be an important regulator of embryonic patterning, but recent evidence also supports a central role for Wnt signaling in synapse formation in both vertebrates and invertebrates (Hall et al., 2000; Packard et al., 2002). Disruptions in the Wnt pathway can lead to a multitude of neurodegenerative diseases (Yang, 2012). Also, many studies have demonstrated the role of Wnt in synapse formation and growth (Cuitino et al., 2010; Henriquez and Salinas, 2012). For example, in the mouse cerebellum, granular rosettes form by mossy fibres, making multiple synapses on granule cells. In a mutant mouse lacking Wnt-7, there is a delay in synapse formation due to the lack of Wnt7, a retrograde signal from granule cells onto mossy fibres, which prevents the clustering of the presynaptic proteins Synapsin I (Hall et al., 2000). In the mouse spinal cord, lateral motor neurons innervate the limb muscles. Wnt3 secreted from lateral motor neurons increases the number of axon branches and the size of the growth cone in sensory neurons that express neurotrophin 3 but not in sensory neurons that express nerve growth factor (Krylova et al., 2002).

Interestingly, recent work has shown that activity-dependent release of Wg (i.e., fly Wnt) at the *Drosophila* NMJ plays a critical role in synapse formation (Ataman et al., 2008; Mathew et al., 2005; Packard et al., 2002). Packard et al. (2002) demonstrated that Wg is critical for synapse formation by using a temperature sensitive *wg* mutant ($wg^{ts}$) to block Wg secretion during the 3$^{rd}$ instar larval stage (when 50% of new boutons are formed) (Gorczyca et al., 1993; Packard et al., 2002). This bypassed the requirement for Wg in patterning during embryonic development and allowed for the study of the role of Wg in synapse formation. When Wg secretion was suppressed, the rate at which new boutons were added was dramatically decreased. A partial loss of Wg function resulted in the formation of boutons with disrupted pre- and postsynaptic specializations, while boutons that formed in the absence of Wg completely lacked active zones and postsynaptic specializations. Moreover, loss of Wg function dramatically affected organization of postsynaptic GluRs (Packard et al., 2002). These exciting data reveal a fundamental role for Wg in synapse formation; however, the source(s) of synaptic Wg remain an open question.

**Wg Pathway (secretion/Fz pathway)**

Little is known about the Wg secretion pathway. Wg is post-translationally modified by the endoplasmic reticulum protein, porcupine (porc) (Logan and Nusse, 2004). Although Porc is thus far only known to modify Wg, skinny hedgehog, a different member of the O-acyltransferase superfamily, is known to

modify hedgehog in a similar way (Coudreuse and Korswagen, 2007). Although a general mechanism is used for the secretion of Wg, the molecules involved in the secretion process give specificity to Wg. Subsequently, Wntless/Evenness Interrupted/Sprinter (Evi), a transmembrane domain protein, transports Wg through the Golgi to the plasma membrane for secretion via an endosome (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Neither the Hedgehog (Hh) pathway nor the JAK/STAT pathway require Evi for secretion (Bartscherer et al., 2006). Then the retromer complex retrieves Evi and recycles it back to the golgi to be used again and prevents it from being degraded by the lysosome (Belenkaya et al., 2008; Eaton, 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). The retromer complex is not specific to Wg secretion. Interestingly, a novel Wnt, WntD, does not require Porc or Evi for secretion (Ching et al., 2008).

How Wg is secreted is still unclear. It has been proposed that lipid linked morphogens like Wg bind to lipoprotein particles to facilitate long range signaling by either traveling through tissue or across the extracellular matrix (Panakova et al., 2005). Lipoproteins are a monolayer of phospholipids that surround a core containing cholesterol and triglycerides, similar to low-density lipoprotein (LDL). These lipoproteins contain apolipoproteins, molecules known to carry hydrophobic ligands, like Wg and Hh, across cells. Interestingly, LDL receptor proteins (LRPs) are known to function as coreceptors for both Wg and Hh

(Eaton, 2006). Alternatively, Wg could be secreted in a multimeric form, similar to Hh (Miller, 2002) or in an exosome (Greco et al., 2001). It has been shown in *Drosophila* wing imaginal discs, that glycophosphatidylinositol (GPI) lipid rafts form vesicles containing Wg that are capable of traveling long distances (Greco et al., 2001). How Wg is secreted from the motor neuron is still unclear, but recent studies are beginning to shed light on the mechanism. Work from the Budnik Lab has shown that Evi, in an exosome-like vesicle, is required to transport Wg across the synapse of the *Drosophila* NMJ (Koles et al., 2012; Korkut et al., 2009).

A key step in Wg signaling at the NMJ is the activation of DFz2, a seven transmembrane receptor, which is localized both presynaptically on the motor neuron endings and postsynaptically on the muscle (Speese and Budnik, 2007). Presynaptically, DFz2 is thought to be activated via Wg autocrine signaling to activate a divergent canonical pathway that modulates the cytoskeleton to regulate synaptic expansion (Franco et al., 2004; Miech et al., 2008; Speese and Budnik, 2007). Postsynaptically, Wg activates the Frizzled Nuclear Import (FNI) pathway. In this pathway, postsynaptic DFz2 is endocytosed and translocated to the periphery of the nucleus. Then the C-terminal intracellular domain of DFz2 (DFz2-C) is cleaved and associates with RNA granules between the inner and outer nuclear membrane. It is thought that these DFz2-C RNA-associated granules exit the nucleus and return to the synapse for local translation. Wg and DFz2 are thus critical regulators of synapse formation and growth, however, a

number of intriguing questions remain regarding NMJ Wg/DFz2 signaling. For example, what is the precise source(s) of Wg *in vivo*?

Wg expression was observed in presynaptic motor neurons by antibody staining and a *wg* reporter line, and defects in synaptic growth (i.e., bouton number) were significantly rescued in a $wg^{ts}$ mutant by Wg expression in motor neurons (Packard et al., 2002). These data argue that motor neurons serve as one source for Wg release at the NMJ, and this model is directly supported by my data. Nevertheless, it is important to note that potential roles for glia in the release/modulation of NMJ Wg signaling have not been assayed. My work shows that glia do, in fact, act as a critical source for Wg during NMJ growth.

**Dissertation overview**

Neuron glia communication is essential for the overall function of the nervous system. The flow of information between these two cell types is critical for everything from axon guidance, trophic support, and ion balance to the regulation of neurotransmitters. However, the molecular mechanisms to which neurons and glia interact with each other to regulate these processes are not well understood. My thesis work focuses on understanding the role of how glia use secreted factors to modulate synaptic function. In chapter II of this thesis, I describe use of chromatin immunoprecipitation with Affymetrix whole genome tiling arrays (ChIP-chip) to identify a number of potential functional targets of the glial transcription factor, *reverse polarity* (*repo*). Next, I show that one novel target of Repo, Wg, is

expressed in SPGs and is regulated by *repo in vivo*. Finally, I demonstrate that Wg derived from SPGs is required for proper GluR distribution and electrophysiological responses at the NMJ. My thesis work identifies synapse stabilization and/or growth as a new role for SPGs and demonstrates that glial secreted factors such as Wg regulate synaptic function at the *Drosophila* NMJ.

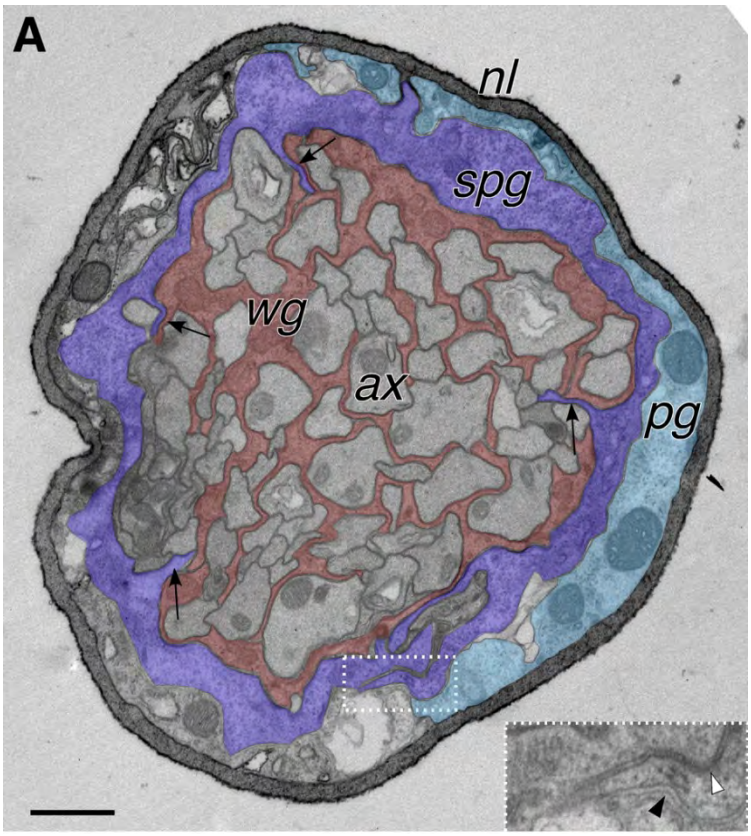


**Figure 1-1. The *Drosophila* larval peripheral nervous system glia.**

Electron micrograph of a third larval instar peripheral nerve, neural lamella (nl), perineurial glial (pg) in light blue, subperineurial glia (spg) in purple, wrapping glia (wg) in pink and axons (ax) in grey. Scale bar, 1 um.

# Chapter II:

# Glial control of synapse assembly at the *Drosophila* neuromuscular junction

By

Kimberly S. Kerr, Yuly Fuentes-Medel, Cassandra Brewer, Romina Barria,

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**Abstract**

Glial cells, the infrastructure of the nervous system, carry out key functions that are fundamental for brain development and function. The molecular mechanisms by which glia and neurons interact with each other to regulate these processes such as synapse formation, maturation and function, are poorly defined. In D*rosophila*, *reversed polarity* (*repo*) is essential for the late stages of glial fate and function. We performed a chromatin immunoprecipitation along with an Affymetrix *Drosophila* v2.0 tiling array (ChIP-chip) to identify transcriptional targets of Repo that play a role in glial function and neuron-glia signaling. Within the collection, we identified and confirmed *in vivo* several previously known glial genes. Excitingly, we identified a novel Repo target, *wingless* (*wg*), a secreted morphogen that regulates synaptic growth at the *Drosophila* larval neuromuscular junction (NMJ). We provide evidence that subperineurial glial cells (SPGs) secrete Wg to regulate glutamate receptor cluster formation and the evoked electrophysiological response at the NMJ. We identify synapse stabilization as a new role for SPGs and demonstrate that glial secreted factors such as Wg regulate synaptic function at the *Drosophila* NMJ.

## Introduction

Glial cells are intimately associated with neurons throughout the life cycle of complex metazoans and exert significant control over neuronal development and function. During nervous system morphogenesis glia modulate diverse neurogenic events including neuroblast proliferation (Ebens et al., 1993), axonal outgrowth and fasciculation (Booth et al., 2000; Gilmour et al., 2002), and the formation and maturation of synapses (Barres and Raff, 1999). In the mature nervous system, glia ensheath axons, associate closely with synapses, buffer ions/pH and neurotransmitters, and are thought to modulate synaptic function (Fields and Stevens-Graham, 2002). Despite the widespread importance of glia in neuronal development, remarkably little is known about the molecular basis of glia-neuron signaling in any organism.

Early aspects of glial cell fate specification are well-defined in *Drosophila*. The vast majority of newly born glia express the transcription factor encoded by the *glial cells missing* (*gcm*) gene (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Gcm is the earliest known marker of glial cell fate in *Drosophila* and appears to be necessary and sufficient to induce the glial developmental program in the embryonic nervous system: *gcm* loss-of-function mutant embryos lack most glia, and reciprocally, overexpression of Gcm is sufficient to induce many neurons to become glia. However, Gcm appears to be a context-dependent cell fate molecule, rather than specifying glial fates *per se*. For example, at pupal

stages Gcm is also required to specify neuronal fate (Van De Bor et al., 2002), and outside the nervous system Gcm has key roles in the development of hemocytes (Bernardoni et al., 1997) and apodemal cells (Kammerer and Giangrande, 2001).

Within the nervous system Gcm activates the expression of a number of key glial genes including *reversed polarity* (*repo*) (Akiyama et al., 1996). Repo is a homeodomain-containing transcription factor that, in contrast to Gcm, is only expressed in the nervous system. Repo is the most widely used marker for glial cells in *Drosophila* and appears to be critical for both maturation of glial cell fate and active repression of neuronal fate (Xiong et al., 1994; Yuasa et al., 2003). Null alleles of *repo* cause embryonic lethality, pointing to a critical role for glia in animal survival in *Drosophila*. Interestingly, most glia appear to be specified in *repo* mutants, although they exhibit defects in ventral nerve cord condensation and slightly disorganized CNS glia (Campbell et al., 1994; Halter et al., 1995). The most notable defect in *repo* mutants is the failure to activate a number of key glial genes including the EAAT1 and EAAT2 transporters which mediate reuptake of the neurotransmitter glutamate (Rival et al., 2006), and the regulator of G protein signaling *locomotion defective* (*loco*), a key mediator of blood-brain barrier formation (Granderath et al., 1999). Loss of Repo function also leads to dramatic changes in neuronal physiology in the retina, where depolarization of the photoreceptor field in the electroretingram is completely reversed (Xiong and

Montell, 1995). Together these observations argue that Repo, while not critical for early glial cell fate specification, plays a more specific role in activating programs essential for late steps in glial fate and neuron-glia signaling.

We reasoned the identification of direct targets of Repo would shed significant light on the signaling pathways activated during glial cell differentiation. In this study we used chromatin immunoprecipitation with Repo to identify a large collection of potential direct targets of Repo. This collection contains several previously known glial genes, and we confirm Repo-dependent *in vivo* regulation of an additional subset. Intriguingly, we find that the *wingless* (*wg*) gene, which encodes a *Drosophila* Wnt molecule that can potently modulate synaptic growth, is also potential direct target of Repo. Using the *Drosophila* neuromuscular junction (NMJ) we demonstrate that Repo can modulate Wg levels *in vivo* and that glial-released Wg is a critical regulator of NMJ synaptic development and physiology.

## Results

### Reversed polarity (Repo) regulates multiple genes involved in neuron-glia signaling and specification of glial fate

We sought to identify novel potential transcriptional targets for Repo and explore their *in vivo* roles in glial cell development and function. We therefore generated two constructs designed for expression of either an amino- or carboxy-terminally

Myc-tagged versions of Repo (Myc:Repo and Repo:Myc, respectively) in *Drosophila* S2 cells. We transfected S2 cells with either Myc:Repo or Repo::Myc, performed chromatin immunoprecipitations (ChIP) using anti-Myc antibodies, and Repo-bound genomic regions were then identified by hybridization of isolates to *Drosophila* v2.0 tiling arrays (Affymetrix). Genomic regions exhibiting significant binding in ChIP assays were identified using the Model-based tiling array (MAT) algorithm (Johnson et al., 2006) for both Myc:Repo and Repo:Myc separately. Then data sets were cross-compared, and only those loci found to be significantly enriched in both experiments were selected. This approach led to the identification of 2041 loci exhibiting significant binding by Repo (Supplementary Table 1).

Analysis of Repo bound genomic fragments led to the identification of 16 known glial genes including *loco*, *pointed*, *EAAT1, Glutamine synthetase 2*, *akap200*, *distalless*, *gliotactin*, and *dead ringer/retained* (Table 1) . Each of these genes exhibited significant binding in either 5', intronic, and/or 3' regions. Examples are listed in Figure 1, thus representing potential direct targets for Repo-dependent transcriptional activation *in vivo*. Consistent with this, one of the genes, *EAAT1*, has already been shown to be regulated *in vivo* by Repo (Soustelle et al., 2002).

**Repo regulates Wg expression in peripheral glia and neurons and glia are *in vivo* sources for NMJ Wg**

Interestingly, in the above analysis we also found genes not previously associated with glia, such as members of the Wnt family (Table 2). Wnt-1/Wingless (Wg) is known to be released by motor neuron terminals at the larval NMJ and to regulate the development of both pre- and postsynaptic compartments through DFrizzled2 (DFz2) receptors localized at both sites (Ataman et al., 2008; Mathew et al., 2005; Miech et al., 2008; Packard et al., 2002). In the absence of Wnt signaling the number of synaptic boutons is reduced, glutamate receptor (GluR) subunits become distributed in abnormally broad clusters (Speese et al., 2012) , and a subset of boutons (ghost boutons) lack postsynaptic proteins, postsynaptic structures, and presynaptic active zones (Ataman et al., 2006; Packard et al., 2002).

Given our previous studies suggesting that NMJ glia function is required for normal NMJ development (Fuentes-Medel et al., 2009), we used anti-Wg antibodies to label larval body wall muscle preparations and examined the segmental nerves, where peripheral glial cell bodies are localized. In these preparations we also expressed mCD8-GFP in nearly all glia by using the Repo-Gal4 driver to label glial membranes. Endogenous Wg signal was found throughout glial cell bodies, but was excluded from glial cell nuclei (Fig. 2A). In addition, we examined whether overexpressing Repo in glia upregulates *wg*

transcript. In these experiments, dissected 3rd instar larval segmental nerves were used for RNA extraction, as peripheral glial cell bodies are the only cell bodies found at these nerves. Quantitative PCR revealed that overexpressing Repo in peripheral glia, using rl82-Gal4 resulted in an increase in *wg* transcript (Fig. 2B, C).

Similarly, overexpressing Repo in peripheral glia resulted in an increase in Wg protein levels at the NMJ (Fig. 2D, E, J). The opposite effect, a reduction in Wg protein level, was observed in a hypomorphic *repo* allele, $repo^{1}$, over a deficiency of the *repo* locus ($repo^{1}/Df$), as well as by down regulating Repo in glia (Fig. 2F, G, J). Combined with the ChIP and quantitative PCR, these data strongly suggest that Repo regulates *wg* gene expression. Further, these observations raise the possibility that a pool of NMJ Wg is derived from glial cells. Nevertheless, NMJ Wg was also derived from neurons, as expressing Wg-RNAi in neurons also led to a decrease in the intensity of the endogenous Wg signal at the NMJ (Fig. 2J).

**Subperineurial glia can deliver Wg to the NMJ**

If glial-Wg contributes directly to the NMJ Wg protein pool, then glia should be able to secrete Wg. Expressing Wg-GFP in glia, and examining the localization of the Wg-GFP label at the NMJ tested this hypothesis. Repo Gal4- drives Gal4 expression in nearly all glial cell types, and as previously demonstrated, some of

the peripheral glia extend membrane extensions associated with proximal regions of the NMJ (Fuentes-Medel et al., 2009). Consistent with this, upon driving a mCD8-GFP reporter using the Repo-Gal4 driver, we observed GFP-labeled glial membrane extensions that associated with proximal regions of the NMJ (Fig. 3A; arrowheads). Notably, expressing Wg-GFP with the Repo-Gal4 driver resulted in Wg-GFP signal being localized to all synaptic boutons of the NMJ (Fig. 3F). Given that glial membrane extensions are associated with few synaptic boutons at proximal NMJ regions (Fuentes-Medel et al., 2009), the finding that expressing Wg-GFP in glia results in distribution of transgenic Wg-GFP throughout the NMJ provides evidence that peripheral glia are able to deliver Wg to the entire NMJ.

Several glial cell types are present in peripheral nerves (Stork et al., 2008). In particular, the “perineurial” glia establish the brain-blood barrier, and are organized into an outer layer that completely wraps segmental nerves. Underneath the perineurial glial cell layer is the “subperineurial” glial cell layer, which also surround the nerves, but that additionally extends inward membrane projections that associate with axon bundles (Stork et al., 2008). Finally, the “wrapping” glia are localized underneath the subperineurial glial cells and wrap discrete axon bundles within the nerve (Stork et al., 2008).

Several Gal4 strains that express Gal4 in different peripheral glial subtypes have been isolated (Stork et al., 2012). Similar to rl82-Gal4 (Fuentes-Medel et al., 2009) (Fig. 3B), expressing mCD8-GFP with the subperineurial Gal4 driver, Moody-Gal4 (Schwabe et al., 2005), resulted in the presence of glial membrane extensions associated with the proximal region of the NMJ (Fig. 3C; arrowheads), although this SPG-Gal4 had additional expression in tracheal cells (Fig. 3C; arrow). As with Repo-Gal4, expressing Wg-GFP using rl82- or Moody-Gal4 resulted in Wg-GFP localization in all synaptic boutons of the NMJ (Fig. 3G, H); consistent with the idea that subperineurial glia can release Wg.

Examination of PG-Gal4 (Awasaki et al., 2008; Hayashi et al., 2002), which expresses Gal4 in perineurial glial cells, revealed that membrane extension from perineurial glia also reached the proximal region of the NMJ and became associated with a few boutons (Fig. 3D; arrowheads). However, unlike subperineurial glia, driving Wg-GFP in perineurial glia did not result in Wg-GFP label at the NMJ (Fig.3I), suggesting that perineurial glia are unable to secrete Wg. We also examined the distribution of wrapping glial membranes by driving mCD8-GFP with Nervana-Gal4 (Nrv-Gal4) (Sun et al., 1999). Although bright mCD8-GFP signal was observed in the segmental nerves (Fig. 3E1) no GFP positive membrane extensions associated with the NMJ were observed (Fig. 3E2). Consistently, no Wg-GFP label was observed at the NMJ when driven with Nrv-Gal4 (Fig. 3J). Together, these results provide compelling evidence that

subperineurial, but not perineurial or wrapping glia, can secrete Wg. Further they suggest that an NMJ Wg protein pool is derived from subperineurial glial cells.

**Subperineurial glia function is required for normal glutamate receptor cluster formation**

The finding that subperineurial glia can deliver Wg to the NMJ raised the possibility that glia are responsible for some of the structural and functional phenotypes observed upon interfering with Wnt signaling. Consistent with this view, *repo*$^{1}$, *repo*$^{1}$*/Df* or expressing Repo-RNAi in glia with rl82-Gal4 resulted in an increase in the size of Glutamate receptor IIA (GluRIIA) clusters (Fig. 4A-D). However, in contrast to Wnt signaling mutants, *repo* mutants or Repo-RNAi expression in glia did not change NMJ size, as determined by labeling body wall muscle preparations with anti-HRP antibodies (Jan, 1982) and counting the number of synaptic boutons at the third instar larval stage (Fig. 4E-H).

To determine if the above phenotype in GluR clustering was due to Wg function in subperineurial glia, we examined NMJ size and organization of GluRIIA clusters upon selectively down regulating Wg in subperineurial glia. Unlike the *wg*$^{1}$ hypomorph, or expressing Wg-RNAi in neurons with C380-Gal4, in which a small but significant decrease in bouton number was observed (Fig. 5A, C, G), no changes in bouton number was apparent upon expressing Wg-RNAi in subperineurial glia (Fig. 5B, G). However, down regulating Wg in these glia led to a substantial increase in the size of GluRIIA clusters (Fig. 5D, E,H), mimicking

the *repo* mutant phenotype. To determine if the increase in GluRIIA cluster size/intensity was exclusively derived from Wg function in glia, we also down regulated Wg in neurons with C380-Gal4. Notably, expressing Wg-RNAi in neurons also led to a significant increase in the size/intensity of GluRIIA clusters (Fig. 5E, G). Thus, Wg operates in both glia and neurons for normal organization of GluRIIA clusters, and the function of Wg in each of these cell types alone is not sufficient for normal development of GluRIIA clusters.

To obtain further evidence for a role of subperineurial glia Wg in the formation of normal GluR clusters, we down regulated Porcupine (Porc), an endoplasmic reticulum (ER) resident protein required for post-translational Wg modifications that are essential for Wg exit from the ER (Kadowaki et al., 1996; Tanaka et al., 2002; van den Heuvel et al., 1993). Down regulating Porc with rl82-Gal4 resulted in a significant increase in the size/intensity of GluRIIA clusters (Fig. 5I), reinforcing the notion that Wg secretion by glia is required for normal GluRIIA clustering. Combined with the Repo data, these results provide strong evidence that glia and neurons collaborate during certain aspects of NMJ development. Nevertheless, the function of Wg in neurons and glia is distinct. While neuronal Wg is required both to establish normal NMJ size and GluRIIA clustering, glial Wg regulate GluRIIA clustering but does not appear to influence NMJ size.

**Wnt signaling is required both in glia and neurons for proper NMJ function**

Previous studies suggest that an increase in size and intensity of GluR clusters upon disrupting Wg signaling at the NMJ is reflected by a change in neurotransmission, particularly in an increase in the amplitude of miniature excitatory junctional potentials (mEJPs) (Speese et al., 2012). To determine if interfering with Wg function in glia also mimicked this phenotype, we recorded synaptic potentials by expressing Wg-RNAi with rl82-Gal4. Notably, abnormalities in NMJ function were also observed upon interfering with Repo function in the same subset of glia (SPGs). We found that the amplitude of mEJPs was significantly increased when Wg-RNAi was expressed in glia with the rl82-Gal4 driver (Fig. 6A, C). As expected from the similar effects of disrupting Wg in glia or neurons for normal GluRIIA cluster formation, mEJP amplitude was also increased upon down regulating Wg in neurons (Fig. 6A, C). Although both glial and neuronal Wg were required for normal mEJP amplitude, other aspects of neurotransmission were affected differentially. Down regulating Wg in glia led to a marked increase in mEJP frequency, but the opposite was true when Wg was down regulated in neurons (Fig. 6A, D). In addition, while expressing Wg-RNAi in glia elicited a significant decrease in the amplitude of nerve evoked EJPs, EJP amplitude was unchanged when Wg was down regulated in neurons (Fig. 6A, E). Nevertheless, quantal content was decreased either by down regulating Wg in glia or neurons. Thus, Wg functions both in glia and neurons to regulate synaptic strength.

## Discussion

In the past decade glial cells have emerged as important regulators of neural circuit assembly and function. In particular, glia have been shown to exert significant control over synapse formation, growth, and plasticity, but glial-derived factors capable of regulating neural development and physiology *in vivo* are only beginning to be defined. By initiating the discovery of transcriptional targets of the glial factor Repo, we have identified the *Drosophila* Wnt molecule Wingless (Wg) as a glial-derived synaptogenic factor. We showed that Repo can bind the *wg* locus in cultured cells and that Repo can regulate synaptic levels of Wg *in vivo*. We further showed that glial Wg is an important regulator of synapse formation and physiology as downregulation of glial-derived Wg at the NMJ leads to defects in the assembly of glutamate receptors, increases in spontaneous mini-EJPs, and a dramatic decrease in nerve-evoked EJPs. Wg is thus a novel pro-synaptogenic molecule released by glia that potently modulates both assembly of post-synaptic structures, and electrophysiological responses at the synapse.

### Repo regulates a broad class of genes involved in neuron-glia signaling

The diversity of genes directly activated by Repo—a critical regulator of all lateral glial cell development in *Drosophila*—had not been previously explored. Several of the genes that we identified as potential direct Repo targets in our ChIP studies govern fundamental aspects of glial development or function. For

example, known targets were identified that actively promote glial cell fate specification (e.g., *pointed*, *distalless*), blood-brain barrier formation (e.g., *gliotactin*, *loco*, *coracle, Nrv1*), engulfment activity (e.g., *dCed-6*), neurotransmitter metabolism (e.g., *EAAT1*, *Gs2*), ionic homeostasis (e.g., *fray*), and neuron-glia signaling during nervous system morphogenesis (e.g., *Pvr*). Given their broad roles in glial cell biology, our work supports the notion that Repo transcriptionally regulates a broad class of genes that modulate many phases of glial cell development. For example, Pointed is a key glial factor that both activates glial fate and inhibits neuronal fate at very early developmental stages. Likewise, Repo appears to regulate Gliotactin, Coracle, and Nrv1, which are molecules essential for formation of the pleated septate junction-based blood-brain barrier at mid- to late embryogenesis in *Drosophila*. Reciprocally, EAAT1 and GS2 are activated late in the embryonic glial program and are critical for recycling of neurotransmitters at the synapse.

While the majority of candidates in our collection of potential Repo targets remain to be verified, we predict that two major classes of genes will emerge from this data set. First, we would expect to identify additional novel molecules that function in glia to promote glial fate, morphogenesis or function. Second, since Repo has been shown to actively suppress the neuronal program, we speculate that a number of neuronal genes identified in our data set might be targets for negative regulation by Repo.

**Glia to synapse signaling through Wingless/Wnt**

Mammalian excitatory glutamatergic synapse formation is modulated by multiple glial-derived factors including Thrombospondins (Tsps) (Christopherson et al., 2005), Hevin/Sparc interactions (Kucukdereli et al., 2011), and glypicans 4 and 6 (Allen et al., 2012). These factors appear to be essential for initial synapse formation and (with the exception of Tsps) can also promote postsynaptic differentiation through membrane insertion and clustering of AMPA receptors (Kucukdereli et al., 2011)(Allen et al., 2012). Here we identified Wg as a regulatory target of Repo and define Wg as a novel glial-derived factor essential for synapse assembly and function *in vivo*.

Wg/Wnt signaling potently modulates the coordinated assembly of both pre and post synaptic structures at the *Drosophila* NMJ. Loss of Wg, or its receptor dFrizzled2 (dFz2), leads to a dramatic decrease in synaptic boutons and disrupted clustering of post-synaptic glutamate receptors. Interestingly, we find that loss of glial-derived Wg can account for some, but not all of these phenotypes. For example, while depletion of glial-derived Wg disrupted clustering of post-synaptic glutamate receptors, it had no effect on the formation of synaptic boutons. By contrast, depletion of neuronal Wg led to defects in both glutamate receptor clustering, as well as bouton formation. These data argue that while both glial and neuronal Wg are capable of modulating the assembly of glutamate receptor complexes, only neuronal Wg regulates bouton growth.

Glial and neuronal Wg both appear to have important, but remarkably different roles in modulating synaptic physiology at the *Drosophila* NMJ. Loss of glial Wg resulted in an increased size and frequency of miniature excitatory junctional potentials (mEJPs) and a strong decrease in nerve-evoked responses. Loss of neuronal Wg led to a similar increase in the size of mEJPs. However, in contrast to depletion of glial-Wg, loss of neuronal Wg resulted in a decreased frequency of mEJPs and no change in nerve-evoked responses at the NMJ, but both ultimately decreased quantal content.

Since GluRs are critical for regulating neurotransmission, these results support the notion that there is an increase in GluRs. Recently, it was demonstrated that downregulation of the postsynaptic Frizzled Nuclear Import (FNI) pathway increased GluRs as well (Speese et al., 2012). This suggests that Wg from both glia and neurons act in concert via the FNI pathway to stabilize the synapse by regulating GluRs. Precisely how Wg derived from glia versus neurons might differentially modulate synaptic physiology is not clear, but these observations suggest there are in fact key qualitative (or quantitative) differences in Wg signals from glial versus neuronal sources. Such a mechanism would allow for glia to modulate specific aspects of NMJ physiology independently from neuronal Wg, perhaps in an activity-dependent manner.

## Material and Methods

### *Fly Strains and Constructs*

Flies were raised on standard *Drosophila* media at 25°C except where indicated. Experiments including controls performed with RNAi lines were raised at 29°C. The following fly strains were used in this study: wild type (Canton-S); rL82-Gal4 (Sepp and Auld, 1999), Repo-Gal4 (gift from B. Jones), C380-Gal4 (Budnik, 1996), OK6-Gal4 (Marques et al., 2002), SPG-Gal4 (gift from R. Bainton) (Schwabe et al., 2005), PG-Gal4 (NP6293-Gal4) (Awasaki et al., 2008; Hayashi et al., 2002), Nrv2-gal4 (Bloomington stock # 6800 ) (Sun et al., 1999), UAS-Wg-RNAi (stock # 13351 & 13352; Vienna *Drosophila* RNAi Center), UAS-Porc-RNAi (stock # 47864; Vienna *Drosophila* RNAi Center), UAS-Repo-RNAi (stock # 10424; Vienna Drosophila RNAi Center), $wg^{1}cn^{1}$ (Bloomington # 2987), $repo^{1}$ (Bloomington stock # 4162), $w^{1118}$; $ry^{506}$ P[$ry^{+t7.2}$=PZ]$repo^{03702}$/TM3, $ry^{RK}$ $Sb^{1}$ $Ser^{1}$ (Bloomington stock # 11604), UAS-mCD8-GFP (Bloomington stock # 5137 and # 5130)

### *Repo Constructs*

UAS-myc::Repo: 1.8 KB fragment of Repo from GH05443 was cloned into NT UAS-myc using Bgl/Xho sites.

UAS-Repo::myc: 1.8 KB fragment from GH05443 was cloned into CT UAS-myc Bgl/SpeI sites.

*Immunolabeling, antibody source and concentration*

Third instar *Drosophila* larvae were dissected in calcium free saline and fixed for 10 min with Bouin's solution unless otherwise noted (Budnik, 1996). Primary antibodies were used at the following dilutions; rabbit anti-DLG 1:20,000 (Koh et al., 1999); mouse anti-DLG 1:500 (clone 4F3, Developmental Studies Hybridoma Bank, DSHB);mouse anti-GFP 1:200 (Molecular Probes); anti-GluRIIA 1:3 (DSHB); FITC or Texas red-conjugated a-HRP 1:200 (Jackson Immunoresearch). Secondary antibodies conjugated to FITC, Texas Red, or Cy5 (Jackson Immunoresearch) were used at a concentration of 1:200.

*Image Quantification*

Samples were imaged using an Intelligent Imaging Innovations Everest spinning disc confocal system using a PlanApo 63x 1.4na oil lens. Different genotypes were processed simultaneously and imaged using identical confocal acquisition parameters for comparison. Fluorescence signal intensity was quantified by volumetric measurements of confocal stacks using Volocity 6.0 Software (Improvision). Quantification performed based on previously published protocol (Ataman et al., 2008). For measurement of synaptic intensity, single boutons were selected from muscles 6 and 7, abdominal segment 3 and analyzed as three- dimensional volumes in Volocity. The immunostained region around the boutons was segmented using intensity thresholding with the subtraction of

background intensity. Total intensity was defined as the combined total of pre- and postsynaptic intensity divided by the bouton volume. Presynaptic intensity was measured by calculating the volume occupied by the immunostaining of interest that overlapped with the volume occupied by the anti-HRP immunostaining (presynaptic bouton volume) and measuring the total intensity within that volume. Postsynaptic intensity was measured by calculating the total volume of the immunostaining of interest in which the presynaptic immunostaining was subtracted out resulting in the postsynaptic volume. For measurement of synaptic volumes, the immunostained region of interest around the boutons was segmented using intensity thresholding. The volume was measured and divided by the bouton volume as determined by the immunostaining occupied by anti-HRP (presynaptic bouton volume). All samples and controls were normalized to wild type.

*Semi-quantitative 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), Wingless (Assay ID Dm01803387_m1) and Gapdh (Assay ID Dm01841185_m1) as a housekeeping

control 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 protocol was 95 °C for 10 min followed by 40 cycles of 95°C for 15 sec and 60 °C for 1 min. 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.

*Electrophysiology*

All experiments were performed using the previously published protocol (Ashley et al., 2005). Third instar larvae were dissected in HL3 saline containing 0.3 mM $CaCl_2$. The brains were removed after carefully cutting the segmental nerves proximal to the ventral ganglion. During the electrophysiological recordings larvae were continuously superfused with HL3 saline containing 0.5 mM $CaCl_2$. All recordings were performed at M6 in segment A3. mEJPs were continuously recorded for 4 mins and the average mEJP frequency in Hz was calculated by dividing the total number of mEJP events by 240 seconds. Applying a 1 ms suprathreshold stimulus at a rate of 0.3 pulses per second to the segmental nerve through a suction electrode generated evoked EJPs. eEJPs were recorded from a sharp electrode filled with 3M KCl impaled into M6. eEJP amplitudes were averaged from a total of 4 mins of recording for each larvae.

HL3 saline: (NaCl 70 mM, KCl 5 mM, $MgCl_2$-$6H_2O$ 20 mM, $CaCl_2$-$2H_2O$ **x** mM (as indicated), $NaHCO_3$ 10 mM, Sucrose 115 mM, Trehalose 5 mM, HEPES 5 mM) pH to 7.2 with NaOH

*S2 cell transfections*

Insect Schneider S2 cells were seeded into T-75 $cm^2$ flasks ($1 \times 10^6$ cells/ml for 10ml) and transfected with Effectene from QIAGEN according to the manufacturer's protocol.

*Formaldehyde Cross-linking of Chromatin*

S2 cells were harvested and fixed with 1/10 volume of 11% Formaldehyde Solution then quenched with 1/20 volume of 2.5 M glycine. Cells were pelleted at 1,100g at 4°C. Cells were resuspended in phosphate buffer solution (PBS) and cell concentration was measured. Aliquots contained $5 \times 10^7$ to $1 \times 10^8$. Pellets were then stored at -80°C.

*Preparation of Magnetic Beads*

100 µL of Dynabeads were washed and incubated in Block Solution. Beads were incubated overnight at 4°C in Block Solution with 10 µg of antibody.

*Chromatin Isolation*

Cells were resuspended in Lysis Buffer 1. Then cells were incubated in Lysis Buffer 1 for 10 min at 4°C then pelleted. Cells were resuspended in lysis buffer 2. Then cells were incubated in Lysis Buffer 2 for 5 min at 4°C then pelleted. Cells were resuspended in Lysis Buffer 3 and transferred cells to eppendorf tubes for sonication using a Bioruptor. Cells were sonicated on high power, 30 sec on, 30 sec off, 15 min X 2. Samples were consolidated and 1/10 volume of 10% Triton X-100 was added. Samples were centrifuged at 20,000g for 10 min at 4°C to pellet debris. Supernatent was stored at -20°C.

*Chromatin Immunoprecipitation*

Cell lysate was precleared the added to the antibody/magnetic bead mix and incubated overnight at 4°C on rotator. Beads with sample were collected using Dynal MPC as described above. Samples were washed with Wash Buffer (RIPA) several times then once with 1 mL TE + 50 mM NaCl. Samples were eluted with elution buffer and incubated 65°C heat block for 30 min. Samples were centrifuged at 16,000g for 1 min supernatant was collected.

*Crosslink Reversal*

The sample (eluted DNA/Protein) was incubated at 65°C for a minimum of 6 hrs.

*Purification of DNA*

TE was added to each sample. Then treated with RNAse A (0.2 mg/mL final concentration) and incubated at 37°C for 2 hrs. Next samples were treated with Proteinase K (0.2 µg/mL final concentration) incubated at 55°C for 2 hrs. Then standard phenol chloroform extraction was performed using Heavy Phase Lock gel tube (follow instructions provided by 5 Prime). DNA was precipitated with NaCl (200 mM final concentration) and glycogen (30 µg). Then pelleted and washed with 80% ethanol. Samples were resuspended in 10 mM Tris-HCl, pH 8.0 then analyzed using a Bioanalyzer

*Reagents*

Formaldehyde Solution: 50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1mM EDTA, 0.5 mM EGTA, and 11% Formaldehyde. Lysis Buffer 1: 50 mM HEPES-KOH, pH 7.5,140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100,1X protease inhibitor cocktail. Lysis Buffer 2: 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1X protease inhibitor cocktail. Lysis Buffer 3:10 mM Tris-HCl, pH 8.0, 100 mM NaCl,1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% *N*-lauroylsarcosine.1X protease inhibitor cocktail. Blocking Solution: 1X PBS, 0.5 % BSA. Wash Buffer (RIPA): 50 mM HEPES-KOH, pH 7.5, 500 mM LiCl,1 mM EDTA, 1.0% NP-40, 0.7% Na-Deoxycholate

*Drosophila* tiling array

Probes for GeneChip *Drosophila* Tiling Array 2.0 (Affymetrix) were made according to the protocol provided by Affymetrix. Experiments were performed using a previously published protocol (Menet et al., 2010).

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# Figures

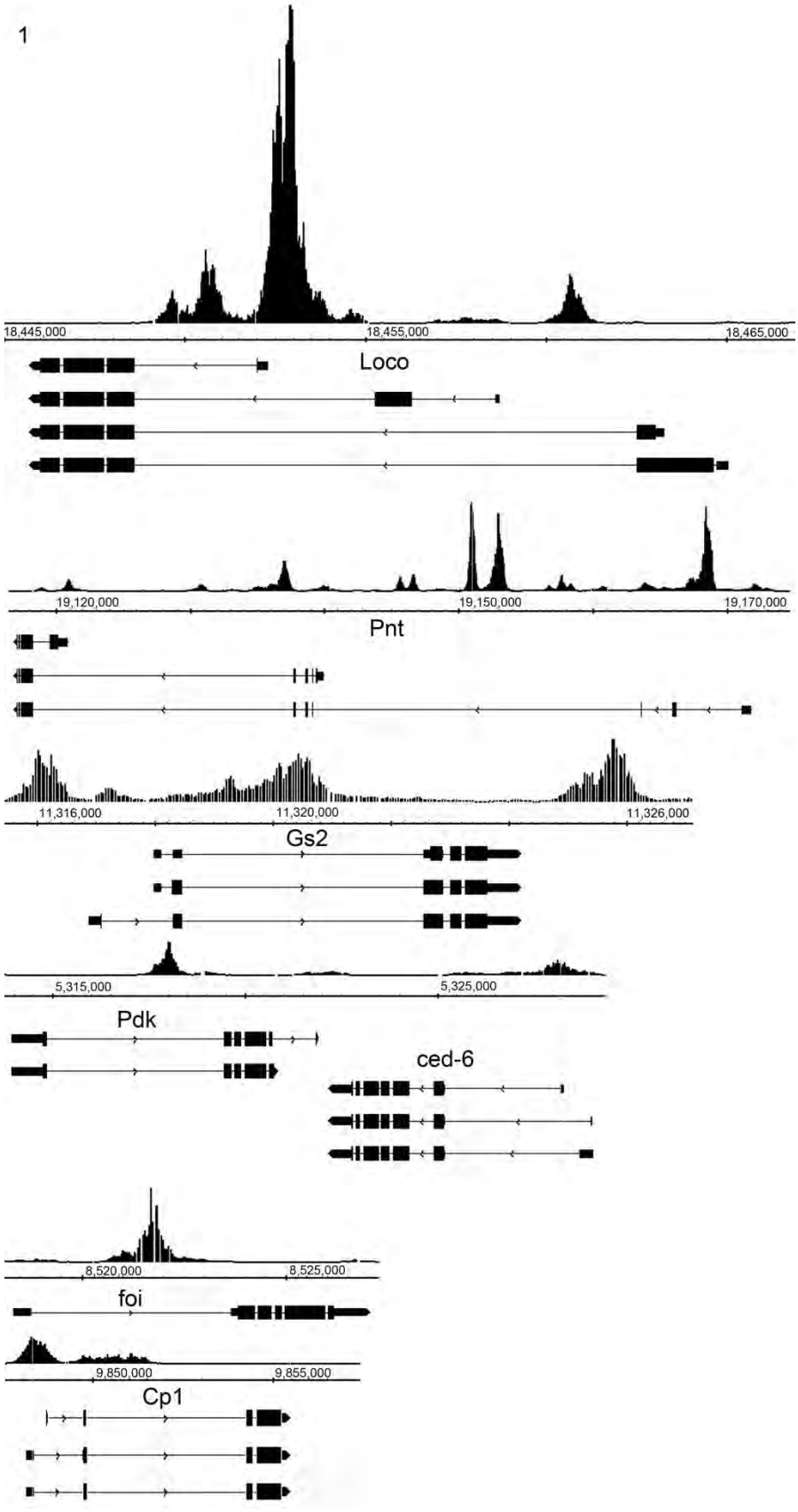
1
18,445,000
18,455,000
18,465,000
Loco
19,120,000
19,150,000
19,170,000
Pnt
11,316,000
11,320,000
11,326,000
Gs2
5,315,000
5,325,000
Pdk
ced-6
8,520,000
8,525,000
foi
9,850,000
9,855,000
Cp1

**Figure 2-1. Repo binds to several glial genes.** Tiling array data from two independent Repo ChIP tiling experiments (myc::Repo and Repo::myc). Data were analyzed using the model-based analysis of tiling arrays (MAT) algorithm (Johnson et al., 2006) and converted to a linear scale to be viewed using Affymetrix's Integrated Genome Browser. For each gene the genomic location and isoforms are shown. The Y-axes is the MAT score that represents the probability of DNA binding. Each peak represents the probability of Repo binding to that region within the genome. The Y-axes are the same for all genes.

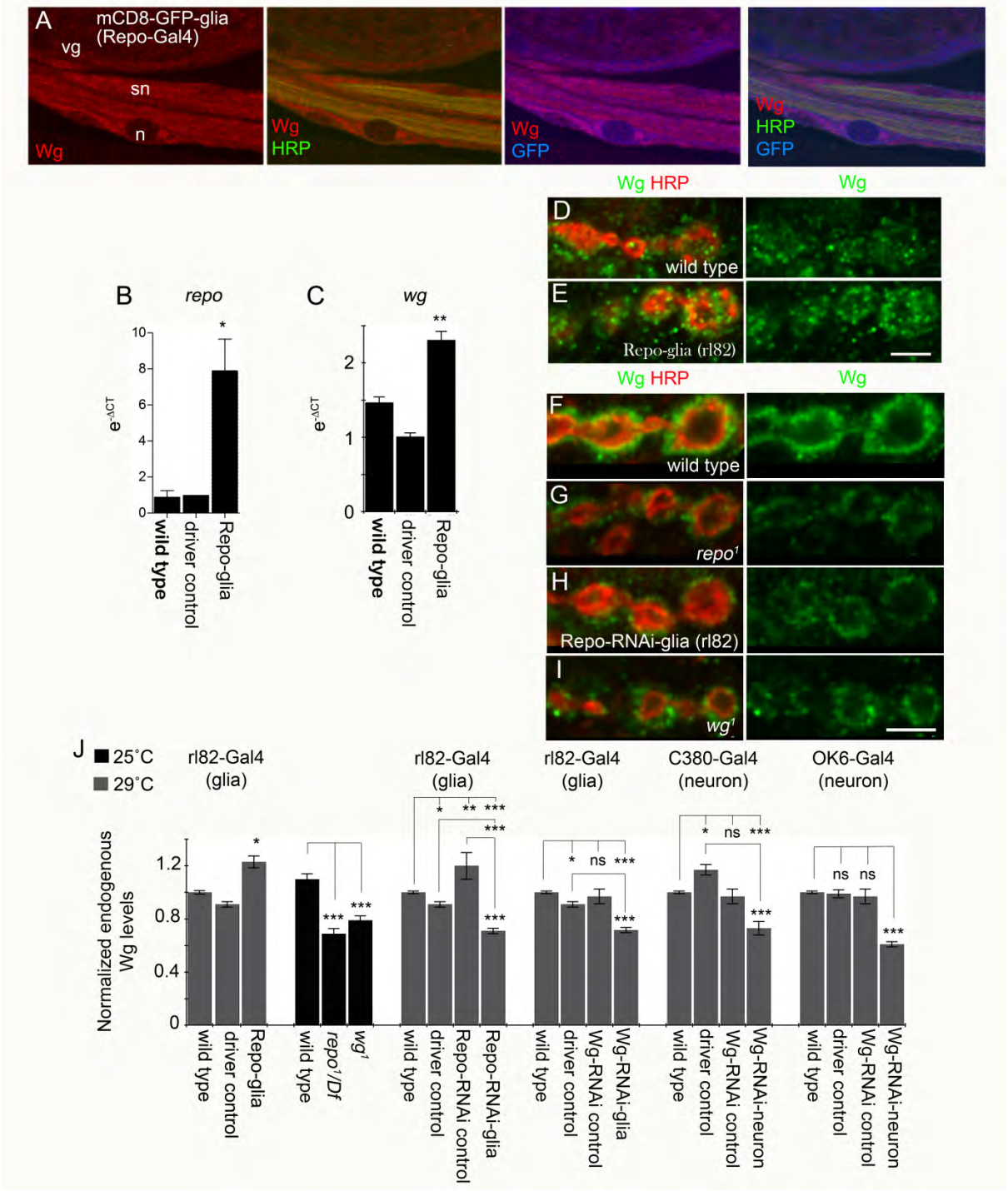
A
mCD8-GFP-glia
(Repo-Gal4)
vg
sn
n
Wg
Wg
HRP
Wg
GFP
Wg
HRP
GFP
B
repo
C
wg
wild type
driver control
Repo-glia
Wg HRP
Wg
D
wild type
E
Repo-glia (rl82)
F
wild type
G
repo1
H
Repo-RNAi-glia (rl82)
I
wg1
J
25°C
29°C
rl82-Gal4
(glia)
C380-Gal4
(neuron)
OK6-Gal4
(neuron)
Normalized endogenous
Wg levels
wild type
driver control
Repo-glia
repo1/Df
wg1
Repo-RNAi control
Repo-RNAi-glia
Wg-RNAi control
Wg-RNAi-glia
Wg-RNAi-neuron
ns

**Figure 2-2. Repo regulation of Wg in peripheral glia. (A)** 3rd instar larval segmental nerves expressing mCD8-GFP in glia and labeled with anti-HRP,anti-GFP, and anti-Wg. **(B)** Real time PCR from larval segmental nerve showing repo transcripts are increased in nerve glia when Repo is overexpressed in peripheral glia. Transcript fold changes were determined using the delta-delta Ct method. **(C)** delta-delta Ct values from real time PCR from larval segmental nerve showing wg transcripts are increased in nerve glia when repo is overexpressed in peripheral glia. **(D,E)** Confocal images of 3rd instar larval NMJ branches in preparations double labeled with anti-HRP and anti-Wg in **(D)** wild type controls and **(E)** in peripheral glia overexpressing Repo. **(F-I)** Confocal images of 3rd instar NMJ branches in preparations double labeled with anti-HRP and anti-Wg in **(F)** wild type controls, **(G)** *repo*$^{1}$ mutants, **(H)** peripheral glia expressing Repo-RNAi and **(I)** *wg*$^{1}$ mutants. **(J)** Quantification of total Wg signal intensity divided by bouton volume in each of the indicated genotypes normalized to wild type. Grey bars indicate experiments performed at 29˚C. Black bars indicate experiments performed at 25˚C. Error bars represent SEM. (*** = $p<0.001$, ** = $p\leq0.01$, * = $p\leq0.05$)

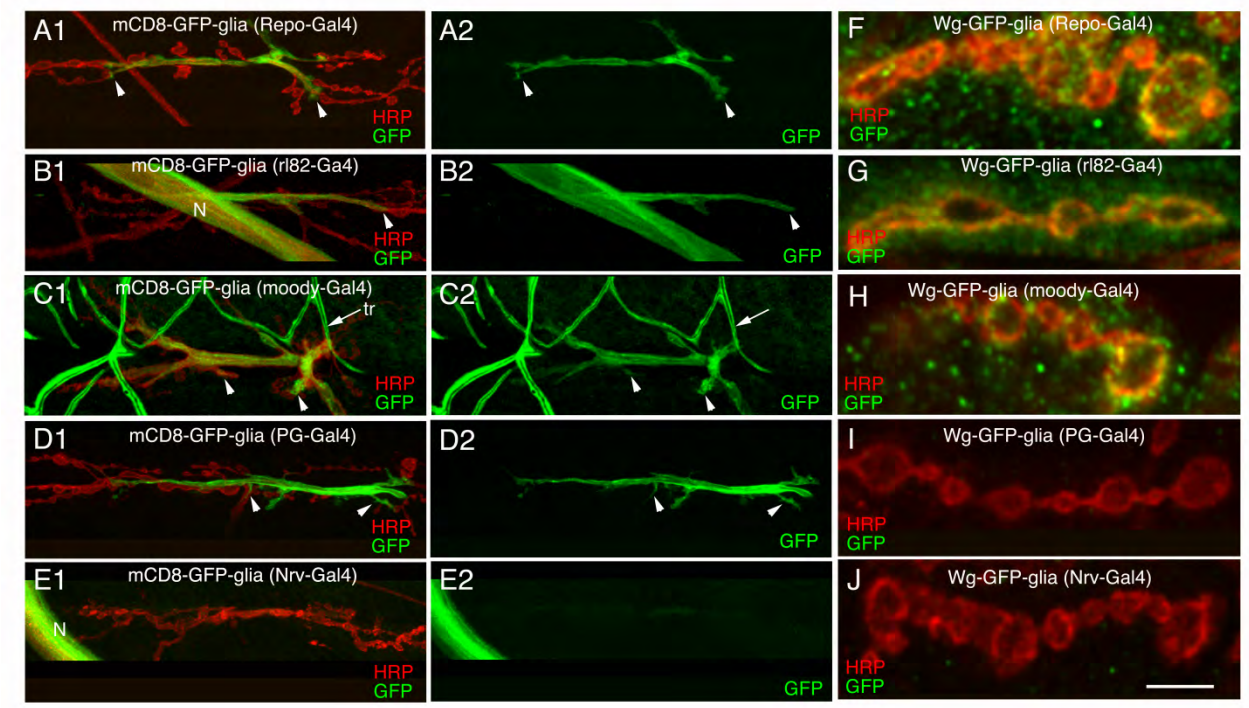


**Figure 2-3. Subperineurial glial membranes invade the NMJ and secrete Wg to the synapse. (A1-E1)** Confocal images of 3rd instar NMJs in preparations double labeled with anti-HRP and **(A2-E2)** anti-GFP and with anti-GFP alone. **(A)** all glia expressing mCD8-GFP, **(B)** subperineurial glia expressing mCD8-GFP, **(C)** subperineurial glia expressing mCD8-GFP, **(D)** perineurial expressing mCD8-GFP, and **(E)** wrapping glia expressing mCD8-GFP. **(F)** all glia expressing Wg::GFP, **(G)** subperineurial glia expressing Wg::GFP, **(H)** subperineurial glia expressing Wg::GFP, **(I)** perineurial expressing Wg::GFP, and **(J)** wrapping glia expressing Wg::GFP.

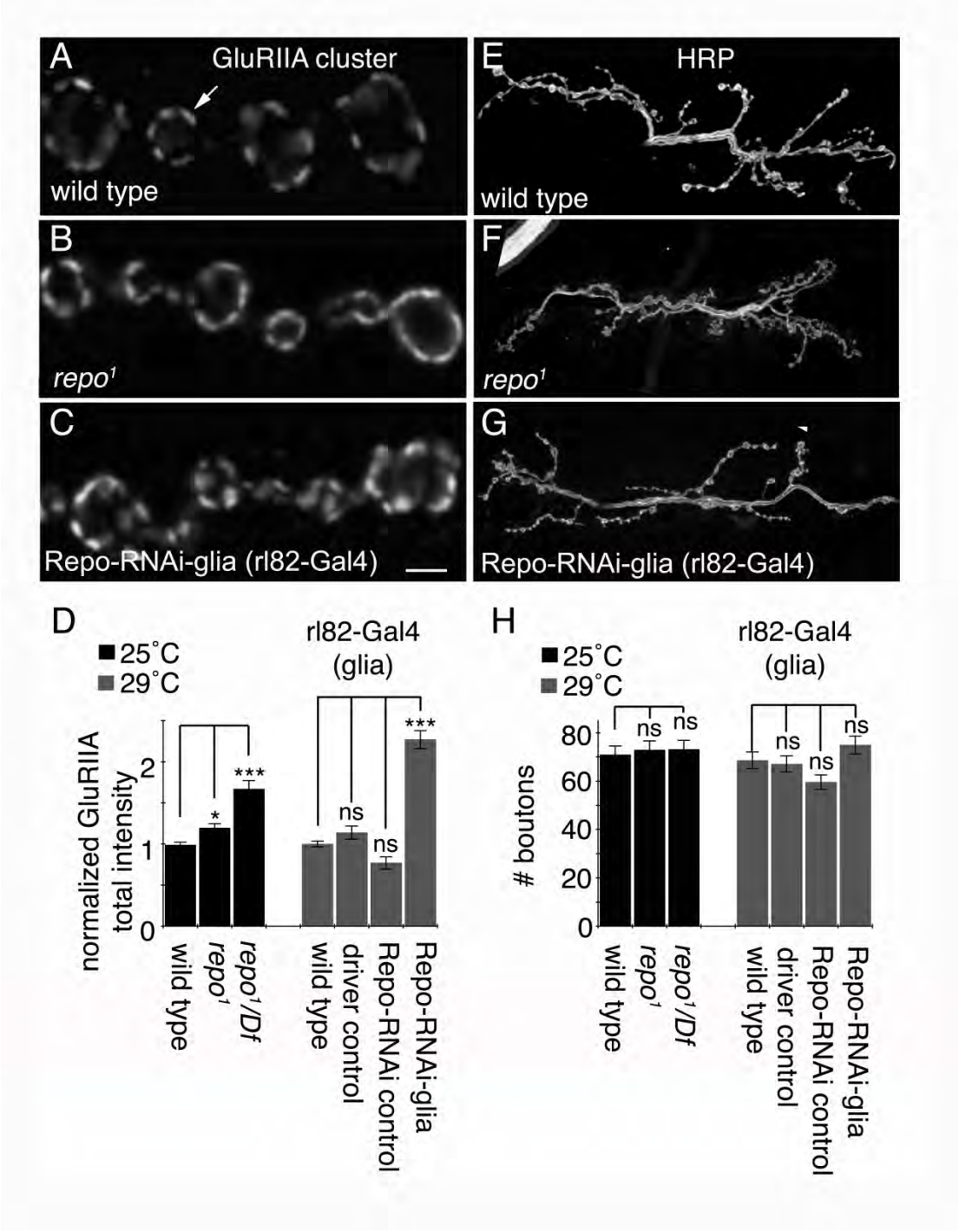
A
GluRIIA cluster
wild type
B
repo¹
C
Repo-RNAi-glia (rl82-Gal4)
E
HRP
wild type
F
repo¹
G
Repo-RNAi-glia (rl82-Gal4)
D
25˚C
29˚C
rl82-Gal4 (glia)
normalized GluRIIA total intensity
0
1
2
*
***
ns
ns
***
wild type
repo¹
repo¹/Df
wild type
driver control
Repo-RNAi control
Repo-RNAi-glia
H
25˚C
29˚C
rl82-Gal4 (glia)
# boutons
0
20
40
60
80
ns
ns
ns
ns
ns
wild type
repo¹
repo¹/Df
wild type
driver control
Repo-RNAi control
Repo-RNAi-glia

**Figure 2-4. Subperineurial glia are required for normal GluRIIA distribution.** **(A-C)** Confocal images of 3rd instar NMJ branches in preparations double labeled with anti-GluRIIA in **(A)** wild type showing normal punctate clusters and **(B)** *repo*$^{1}$ mutants and **(C)** SPGs expressing Repo-RNAi showing diffuse distribution of GluRIIA. **(D)** Quantification of total GluRIIA signal intensity divided by bouton volume in each of the indicated genotypes normalized to wild type. **(E-G)** Confocal images of 3rd instar larval NMJs labeled with anti-HRP in **(E)** wild type, **(F)** *repo*$^{1}$ mutants and **(G)** SPGs expressing Repo-RNAi showing no change in bouton growth. **(H)** Quantification of total bouton number for each of the indicated genotypes. Grey bars indicate experiments performed at 29˚C. Black bars indicate experiments performed at 25˚C. Error bars represent SEM. (*** = $p<0.001$, ** = $p\leq0.01$, * = $p\leq0.05$)

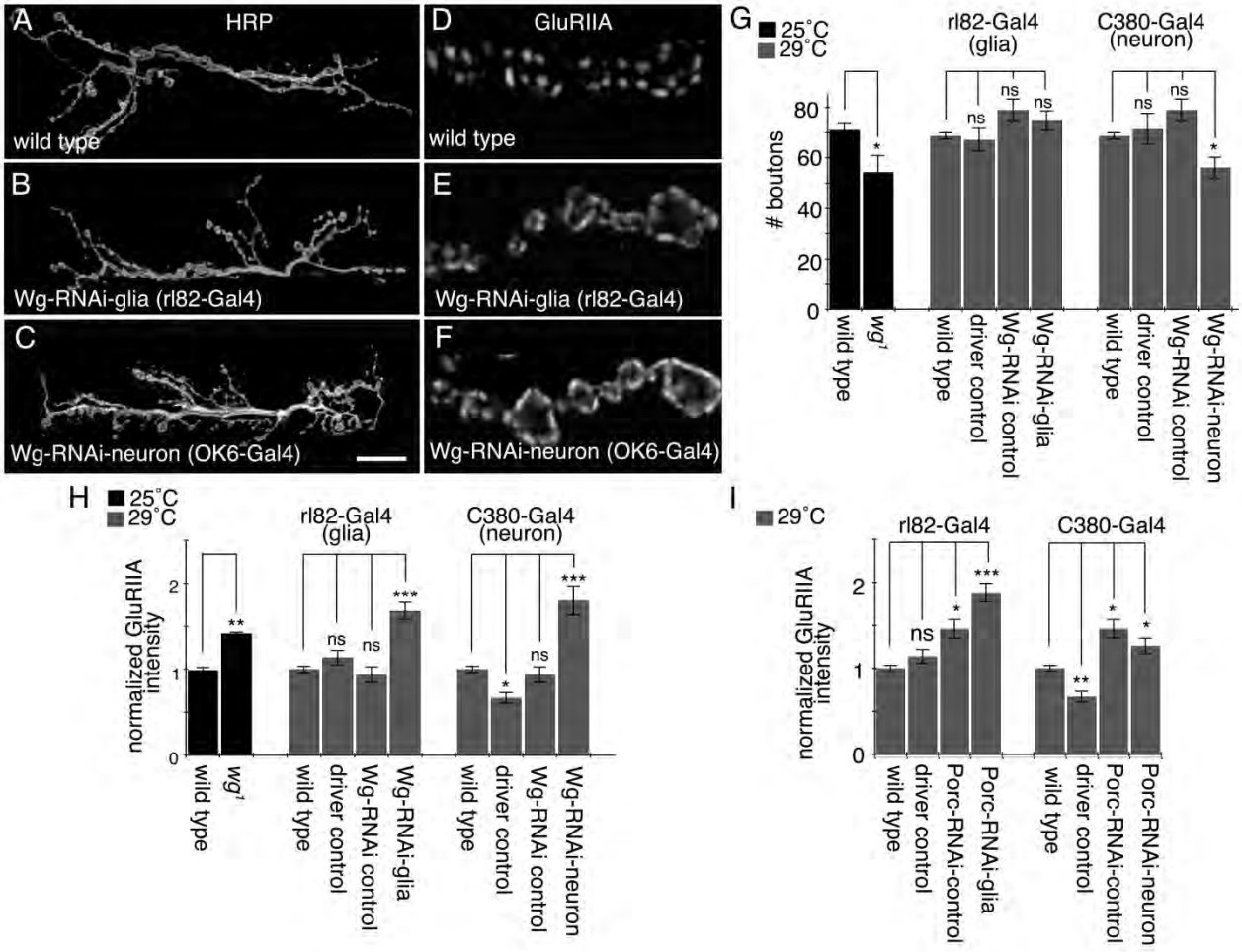


**Figure 2-5. The Wg pathway in both SPGs and motor neurons regulate glutamate receptors. (A-C)** Confocal images of 3rd instar larval NMJs labeled with anti-HRP in **(A)** wild type, **(B)** SPGs expressing Wg-RNAi and **(C)** motor neurons expressing Wg-RNAi. **(D-F)** Confocal images of 3rd instar NMJ branches in preparations double labeled with anti-GluRIIA in **(D)** wild type showing normal punctate clusters and **(E)** SPGs expressing Wg-RNAi and **(F)** motor neurons expressing Wg-RNAi showing diffuse distribution of GluRIIA. **(G)**

Quantification of total bouton number for each of the indicated genotypes. **(H-I)** Quantification of total GluRIIA signal intensity divided by bouton volume in each of the indicated genotypes normalized to wild type. Grey bars indicate experiments performed at 29˚C. Black bars indicate experiments performed at 25˚C. Error bars represent SEM. (*** = $p<0.001$, ** = $p\leq0.01$, * = $p\leq0.05$)

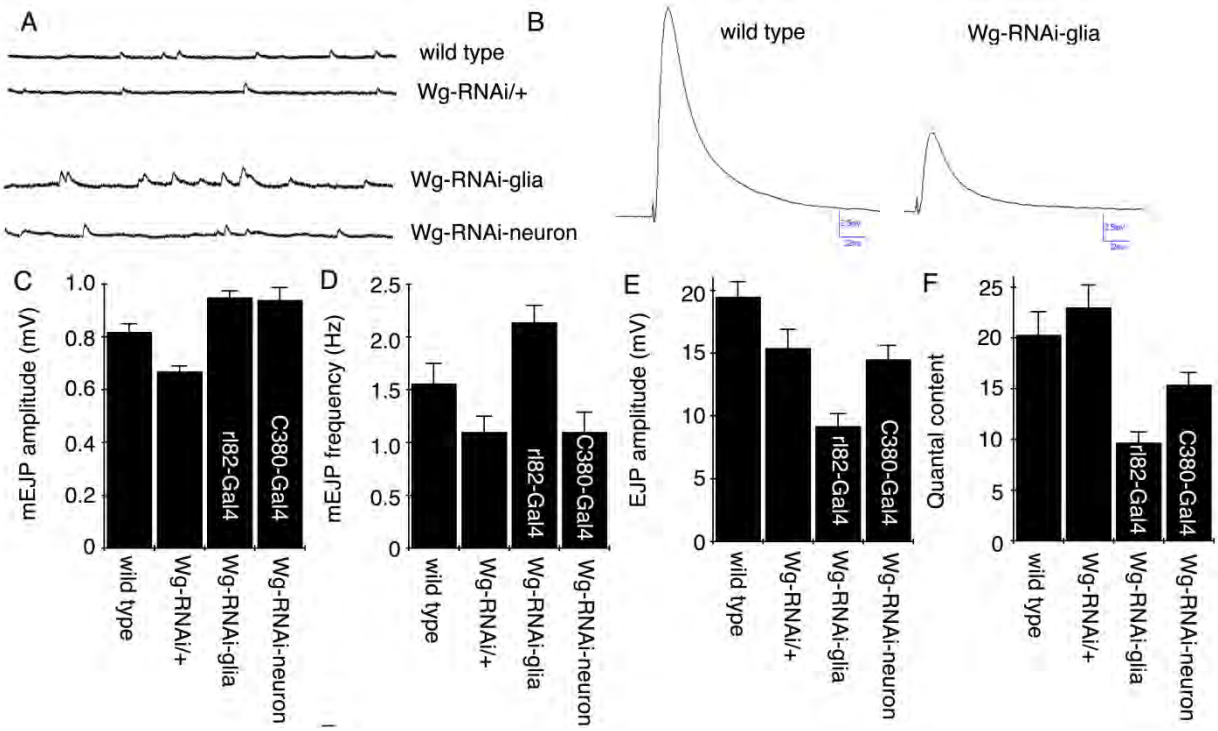


**Figure 2-6. Electrophysiological analyses of Wg knock down in SPGs and motor neurons. (A)** Representative traces of mEJPs wild type controls and Wg-RNAi expressed in SPGs or motor neurons. **(B)** Representative traces of evoke EJPs wild type and Wg-RNAi expressed in SPGs. **(C-F)** Histograms showing mean ± SEM of **(C)** mEJP amplitude, **(D)** mEJP frequency, **(E)** evoked EJP amplitude, and **(F)** quantal content. Calibration: (**B**) 2.5mV, 32ms. Error bars represent SEM. (*** = $p<0.001$, ** = $p\leq0.01$, * = $p\leq0.05$)

# Tables

| Gene | CG# | Chromosome | Genomic start | Genomic end | Mat score | pval | Number of significant peaks clustered | Region type |
|---|---|---|---|---|---|---|---|---|
| nrv2 | CG9261 | chr2L | 6791414 | 6792691 | 221.406 | 6.92 E-07 | 1 | RNA |
| Gs2 | CG1743 | chrX | 11318547 | 11321209 | 151.411 | 3.30 E-06 | 1 | RNA |
| Cp1 | CG6692 | chr2R | 9847653 | 9849186 | 142.594 | 4.30 E-06 | 1 | RNA |
| ced-6 | CG11804 | chr2R | 5327414 | 5329168 | 141.175 | 4.40 E-06 | 1 | RNA |
| Aly | CG1943; CG1101 | chr3R | 2902998 | 2904188 | 135.639 | 5.11 E-06 | 1 | intergenic |
| Dll | CG3629; CG3650 | chr2R | 20722250 | 20723262 | 135.639 | 5.24 E-06 | 1 | intergenic |
| CAP | CG1291; CG18408 | chr2R | 6193597 | 6194776 | 132.954 | 5.61 E-06 | 1 | RNA |
| Akap200 | CG1338; CG31894 | chr2L | 8425151 | 8426995 | 123.965 | 7.29 E-06 | 1 | RNA |
| Sap-r | CG1554; CG12070 | chr3R | 26712901 | 26714343 | 86.487 | 2.78 E-05 | 1 | RNA |
| Eaat1 | CG3747 | chr2L | 9338599 | 9339770 | 80.640 | 3.61 E-05 | 1 | RNA |
| Gli | CG3903; CG3793 | chr2L | 15763229 | 15764265 | 75.189 | 4.67 E-05 | 1 | intergenic |
| retn | CG4019; CG5403 | chr2R | 19502332 | 19503482 | 62.802 | 8.57 E-05 | 1 | intergenic |

**Table 2-1. Glial genes identified as binding targets of Repo in this study.**

| Gene | CG# | Chromosome | Genomic start | Genomic end | Mat score | pval | Number of significant peaks clustered | Region type |
|---|---|---|---|---|---|---|---|---|
| pan | CG34403 | chr4 | 89540 | 91431 | 614.003 | 6.30 E-09 | 1 | RNA |
| slmb | CG3412 | chr3R | 16951000 | 16952925 | 528.477 | 1.32 E-08 | 1 | RNA |
| sgg | CG2621 | chrX | 2540992 | 2542331 | 174.164 | 1.90 E-06 | 1 | RNA |
| arr | CG5912 | chr2R | 9366142 | 9367442 | 165.670 | 2.32 E-06 | 1 | RNA |
| drl | CG17348; CG31797 | chr2L | 19249055 | 19250063 | 123.965 | 7.41 E-06 | 1 | intergenic |
| Wnt4 | CG4698; CG31909 | chr2L | 7258000 | 7259166 | 83.096 | 3.21 E-05 | 1 | RNA |
| wg | CG4698; CG31909; CG4889 | chr2L | 7287972 | 7288918 | 67.356 | 6.78 E-05 | 1 | intergenic |
| fz | CG1769; CG13482 | chr3L | 14297628 | 14298584 | 67.356 | 6.78 E-05 | 1 | RNA |

**Table 2-2. Genes in the Wg/Wnt pathway identified as binding targets of Repo in this study.**

# Chapter III:

# General Discussion

In this thesis I have explored the role of glial secreted factors on synapse formation. I performed the first chromatin immunoprecipitation with the transcription factor Repo using Affymetrix whole genome tiling arrays (ChIP-chip) in S2 cells and identified a large collection of potential transcriptional targets. I found many previously described glial genes, and some are known to be regulated by *repo in vivo*. I tested several more by RNA *in situ* to determine that *repo* regulates these genes by comparing wild type and *repo* mutants. I identified the well-known morphogen, *Wg*, as a possible novel target of Repo. I showed that Wg is expressed in SPGs, and Repo is required for proper expression of Wg *in vivo*. Interestingly, I found that only SPGs can secrete Wg at the synapse, despite the fact that PGs are also present at the NMJ. Finally, I demonstrated that glial-derived Wg is required for proper GluR distribution and electrophysiological responses at the NMJ. These data provide exciting new insights into the mechanisms by which glia regulate synaptic function.

## Transcriptional regulation in glia by Repo

The precise communication between synapses is critical for the overall health of the brain and the nervous system. Glia are responsible for an array of functions required to maintain the connections and information flow between synapses. Precisely how glia are programmed to aid in neuron-glia communication events is

not well known. In *Drosophila*, *reversed polarity* (*repo*) encodes a glial specific transcription factor required for the specification of late expressed glial genes, and maturation of the embryonic CNS to a functional state (Halter et al., 1995; Lee and Jones, 2005; Xiong et al., 1994; Yuasa et al., 2003). We reasoned that by identifying targets of Repo we could provide new insights into how this molecule promotes late steps in glial differentiation and efficient neuron-glia signaling for nervous system function.

In order to identify Repo targets we took a genome-wide approach and performed ChIP-chip in S2 cells to identify which genes are potential direct binding targets of Repo. We found several glial genes that Repo binds, which suggest that many previously identified glial genes are direct transcriptional targets of Repo (Freeman et al., 2003; Mandalaywala et al., 2008; Yuasa et al., 2003). A few examples were *locomotion defects* (*Loco), pointed (pnt), Glutamine synthetase 2 (Gs2)* and *Ced-6*. *loco* is in the regulator of G-protein signaling (RGS) family. In *loco* mutants, glial cell differentiation defects lead to the failure of pleated septate junction formation in SPGs, and in turn, defects in blood-brain barrier formation and neuronal ensheathment (Granderath et al., 1999). *pnt* belongs in the ETS transcription factor family and encodes for two proteins: Pnt-P1 expressed in longitudinal glia and Pnt-P2 expressed in midline glia. In *pnt* mutants, axon patterning formation is disrupted (Klambt, 1993). Glutamine synthetase is required for the breakdown of glutamate within glia after uptake, which serves to reduce glutamate levels at the synapse (Caizzi et al., 1990). In

*Gs2* mutants neurotransmitter metabolism is disrupted, leading to defects in flight stability (Nair et al., 2007). Finally, d*Ced-6* was first identified as a cell corpse engulfment gene in *C. elegans* (Ellis et al., 1991). d*Ced-6* mutants give rise to defects in development pruning and the clearance of injury induced degenerating axons (Awasaki et al., 2006; Doherty et al., 2009; Ziegenfuss et al., 2008). Our findings further support the notion that Repo is required for the activation of late glial genes that are involved in a variety of glial functions such as cell fate specification, neurotransmitter metabolism, engulfment, etc. that are necessary for a healthy nervous system.

The ChIP-chip approach has allowed us to identify a number of new genes that might have key roles in glial function. In order to obtain enough DNA to perform the ChIP-chip, we used S2 cells, a stable cell line. However, one caveat to this is that S2 cells are known to be a mixed population but are primarily derived from macrophages (Schneider, 1972). This means that the genes identified in the ChIP-chip might not be *bona fide* functional targets of Repo. Different genomic binding sites may be available or unavailable for Repo to bind to in S2 cells versus glia depending on the state of the chromatin. This may mean that we identify false targets but also means that we may not identify potential targets as well because the chromatin in S2 cells was not in an open state to allow Repo to bind to the DNA. A better way to determine functional targets of Repo would be to perform ChIP-chip *in vivo* using whole flies. This would allow us to find glial specific targets of Repo but not to discriminate the roles of these targets in

different glial subtypes. For example, both PGs and SPGs are present at the NMJ. My work has shown that Wg in SPGs stabilizes synapses. Although Wg is not secreted from PGs into the NMJ, it is possible that Wg is expressed in PGs and regulated by Repo for a different function in the CNS. A ChIP-chip performed in a subtype specific manner would be needed to address this question. We have created transgenic flies with UAS-Repo myc-tagged at the amino and carboxyl ends, respectively. These stocks might allow us to identify genes bound to Repo in a cell type specific manner.

Finally, my work shows that Repo has many functional targets. Additionally, Repo likely regulates many genes and controls many aspects of glial function. It is also possible that Repo acts as a negative regulator to some genes—as Repo is known to actively suppress neuronal fate (Granderath et al., 2000). My studies identified functional targets of Repo, but whether or not they are positive or negative regulators cannot be determined. Further genetic and biochemical studies need to be performed to determine whether Repo could positively or negatively regulate any of the identified functional targets. Ultimately, these studies would allow us to gain a molecular foothold on glial transcriptional mechanisms that non-autonomously modulate synapse structure.

**SPGs secrete Wg at the NMJ**

Exactly which glial cell type interacts with the synaptic field in *Drosophila* and what roles they may play in NMJ development is not clear. Both SPGs and PGs dynamically invade the NMJ (Brink et al., 2012; Fuentes-Medel et al., 2009). SPGs regulate synaptic growth by clearing synaptic debris and through the secreted factor Maverick, a TGFβ ligand, via the Gbb-dependent retrograde pathway in muscle (Fuentes-Medel et al., 2012; Fuentes-Medel et al., 2009). From our ChIP-chip analysis we found Wg to be a possible binding target of Repo. We also found that synaptic Wg levels were decreased in *repo* mutants (Table 3-1). Since Wg is known to play a role in synapse formation at the NMJ, we explored the possibility that the source of Wg was from glia. Surprisingly, we found that glial-Wg did not affect synaptic growth but disrupted the distribution of GluR and synapse electrophysiology. My work demonstrates synapse stabilization as another role for SPGs besides synaptic growth.

Although the role of PGs is not known, both my work and Brink et al. (2012) demonstrated that PGs are present at the NMJ. Brink et al. (2012) also showed that the extent to which PG processes extend into the NMJ is dependent on either temperature or neuronal activity. PG processes also had similar morphologies to SPGs (Brink et al., 2012). We wanted to know which glial subtype could secrete Wg to the NMJ. We found that knock down of Wg or Repo specifically in SPGs decreased synaptic Wg levels (Table 3-1). Therefore we concluded that SPGs but not PGs could secrete Wg to the synapse. Although this does not exclude the possibility that PGs can regulate synapses at the NMJ,

Brink et al. (2012) noted that they did not observe PGs clearing synaptic debris. Hence, my work and that of others provides strong evidence that SPGs are capable of modulating synapse formation and stabilization (Fuentes-Medel et al., 2009).

Interestingly, Brink et al. (2012) observed that PGs had a greater area of coverage over the NMJ than SPGs. We always observed a uniform distribution of Wg::GFP at the synapse despite the fact that SPGs processes are so transient and variable. It is possible that SPGs membranes may increase coverage over the NMJ when they secrete factors. Alternatively, it is possible that glia to glia signaling between SPGs and PGs occurs. In vertebrates, the perineurium acts as a mechanical barrier to protect Schwann cells and the encased nerve (Parmantier et al., 1999). It is possible that PGs play a similar role in *Drosophila* by acting as a scaffold for SPGs. Both the PI3K pathway and neurotransmitter mediated signaling pathways in SPGs have been shown to promote PGs growth (Lavery et al., 2007; Yager et al., 2001). This suggests that SPGs do communicate with PGs. However, the PG membrane dynamics at the NMJ have not been characterized in relation to SPG membranes or in the presence of secreted factors from SPGs. One way to test this would be to use live imaging when both glial subtype membranes are labeled and look at the membrane dynamics in the presence and absence of when a secreted factor such as Wg is expressed from SPGs. If the membranes correlated with each other, this would then provide evidence to suggest that there is SPGs to PGs communication.

How SPG coverage over the NMJ is regulated is unknown. It is possible that temperature and synaptic activity are the determining factors, similar to PGs (Brink et al., 2012). Alternatively, glutamate levels could be the critical factor, while increased coverage due to an increase in temperature or synaptic activity is merely a consequence of that. One study showed that GluR clustering at the NMJ was regulated by cysteine/glutamate transporters (xCTs) expressed in PGs (Augustin et al., 2007). It has also been shown in the rat hippocampus that only 57% of synapses are covered by astrocytes, but synapses that frequently release glutamate have a higher percent of coverage (Ventura and Harris, 1999). Only one third of the perimeter of these synapses were in contact with astrocytic processes, suggesting that glia dynamically regulate synapses and that the synapses do not need to be fully ensheathed to be regulated (Ventura and Harris, 1999). This data suggests that SPGs at the NMJ could act in similar way to astrocytes by extending out glial processes only during increases in synaptic activity or glutamate levels to regulate synaptic function.

SPGs form the blood-brain barrier, which protects and regulates the chemical composition within the nervous system. (Auld et al., 1995; Banerjee et al., 2010; Banerjee et al., 2006; Stork et al., 2008). Recent studies established a role for SPGs in synaptic growth at the *Drosophila* NMJ (Fuentes-Medel et al., 2012; Fuentes-Medel et al., 2009). My work defined an additional novel role for SPGs as a modulator of synapse assembly. Knock down of Wg or Repo in SPGs decreased overall synaptic Wg levels (Table 3-1). Additionally, knockdown of Wg

in SPGs disrupted both the distribution of GluRs and electrophysiological response at the NMJ (Table 3-1). Together this data demonstrates that SPGs play more than a structural role. They are dynamic and actively send and respond to signals that are critical for synapse function.

**Glial Wg regulates synapse formation**

Synaptic growth is dependent on neuronal activity and is critical to maintain the functional integrity of neurons. Glia contribute to the establishment of functional connections in neurons in many ways, including guiding axons to targets, secreting synaptogenic molecules and enhancing neurotransmission to stabilize synapses (Bastiani and Goodman, 1986; Cao and Ko, 2007; Pfrieger and Barres, 1997). Our ChIP-chip data identified a number of growth factors that could play a role in neuron-glia communication.

We identified one secreted factor, Wg, which has been known to play a role in synapse growth and formation at the NMJ in an activity dependent manner (Ataman et al., 2008; Packard et al., 2002). We wanted to determine if Wg was an as yet undiscovered way that *Drosophila* glia can contribute to synapse formation. In a *wg* mutant, synaptic growth was decreased, and there was a disruption in pre- and postsynaptic specializations such as GluR distribution (Packard et al., 2002) (Table 3-1). The synaptic growth defect was rescued when Wg was expressed in motor neurons, suggesting that neuronal Wg is capable of supplying Wg when needed but does not define the Wg source. Our data

demonstrated that one source of Wg at the NMJ comes from neurons. However, we also showed that glial cells act as a second source of Wg at the NMJ. This raises the question as to why two sources of Wg would be needed for synapse formation at the NMJ.

We observed functional differences between glial and neuronal Wg. First, neuronal Wg is required for both synaptic growth and normal GluRIIA distribution, but glial Wg is only required for normal GluRIIA distribution (Table 3-1). This data suggests that glial Wg is required for postsynaptic stabilization but not synaptic growth. However, it is possible that the glial knockdown of Wg was not as robust as neuronal knockdown of Wg. Also, synaptic growth could be less sensitive to Wg levels than postsynaptic stabilization. Therefore, we might see a decrease in synaptic growth if we used Dicer-RNAi to enhance the RNAi knockdown or used a stronger glial driver. It is possible that neuronal Wg signals back to itself to regulate synaptic growth. DFz2 is thought to be activated presynaptically via Wg autocrine signaling to activate a divergent canonical pathway, which modulates the cytoskeleton to regulate synaptic expansion (Franco et al., 2004; Miech et al., 2008). Conversely, it is presumed that the receptor for the presynaptic Wg pathway is DFz2, though knockdown of DFz2 in motor neurons would be needed to determine if it phenocopies knockdown of neuronal Wg. Additionally, Fz and Fz2 have been shown to act redundantly in *Drosophila* (Chen and Struhl, 1999). The possibility that Fz and not Fz2 is the presynaptic receptor of the divergent canonical receptor has not been tested.

Second, we observed differences in synaptic activity between glial and neuronal Wg. Our electrophysiology data showed that loss of Wg from motor neurons resulted in an increase in mEJP amplitude while the frequency of mEJPs and evoked EJPs were unchanged, resulting in an overall decrease in quantal content (Table 3-1). This would indicate that there is an increased amount of neurotransmitter packed into each vesicle, which could increase the amount of overall postsynaptic GluRs. However, loss of glial Wg increased both the amplitude and frequency of mEJPs and decreased evoked EJPs, resulting in an overall decrease in quantal content (Table 3-1). Interestingly, only glial Wg affected the evoked EJP. This suggests a decreased response from the muscle could be due to a presynaptic defect in presynaptic release or a decrease in muscle excitability.

GluRs are an essential component required for neurotransmission at the *Drosophila* NMJ synapse. Disruptions in genes such as *bazooka* and *aPKC* that play a role in the postsynapse stabilization cause an increase in GluRs (Ramachandran et al., 2009; Ruiz-Canada et al., 2004). Our results showed that the loss of Wg in either motor neurons or glia resulted in the same phenotype (Table 3-1).

Recently, it was demonstrated that downregulation of the postsynaptic Frizzled Nuclear Import (FNI) pathway increased GluRs as well (Speese et al., 2012). Additionally, it was shown that the FNI pathway exports mRNA transcripts of postsynaptic proteins out of the nucleus, presumably to the synapse for local

translation. This suggests that postsynaptic Wg via the FNI pathway transcriptionally regulates genes required for proper formation of the postsynapse. Previous studies demonstrated that retrograde communication between the muscles to motor neurons via the TGF-β/BMP pathway (for example) is critical for the proper coordinated development between the pre- and postsynapse (Packard et al., 2003). Recent work from Fuentes-Medel et al. demonstrated that secreted glial factors such as the TGF-β ligand, Maverick, regulates this retrograde pathway. This opens the possibility that the Wg pathway could indirectly regulate GluRs. It is possible that the muscle, through a retrograde pathway such as the TGF-β pathway, senses the presynaptic release defect due to a loss of Wg and upregulates GluRs. One way to test this would be to look for changes in phosphorylated Mad (p-Mad) when Wg is downregulated. One would predict to see an upregulation in p-Mad, which would indicate activation of the retrograde pathway. However, a recent study demonstrated that the phosphorylation and dephosphorylation of Mad regulated both the BMP and Wg pathways (Eivers et al., 2011). This leaves the possibility that unphosphorylated Mad could be regulating Wg while p-Mad regulates TGF-β/BMP. Additionally, looking for changes in Wg levels in a Maverick background would determine whether this pathway regulates Wg as well.

Synaptic growth and Wg secretion are activity-dependent (Ataman et al., 2008). If neuronal Wg acts pre- and postsynaptically then it is possible that during times of rapid growth an additional source of Wg is needed, which is supplied by glia.

Glia are capable of sensing neuronal activity, thus it is feasible that glial cells secrete Wg based on the amount of neuronal activity (Haydon, 2001). We could test if the release of glial Wg is upregulated by the induction of neuronal activity by using a photoconvertable version of Wg expressed in glia (Ataman et al., 2008; Lund et al., 2011). Additionally, we could determine if the transcription of Wg is regulated by neuronal activity by using an *in vivo* transcriptional read out in SPGs.

Alternatively, glial Wg could play a completely different role than neuronal Wg. We observed that loss of glial Wg resulted in a decrease in evoked EJP, suggesting that there is a defect in presynaptic vesicle release. The caveat to immunohistochemistry is that it does not indicate whether or not the increased GluRs observed were either functional or at the cell surface. It is possible that the postsynapse is trying to compensate for the decrease in neuronal activity by producing more GluRs, but they are not on the surface of the postsynapse, rendering them nonfunctional. We could address this question by looking at GluR immunostaining in nonpermeabilized conditions in a glial Wg-RNAi background. Axotactin, a secreted factor expressed in longitudinal glia in the CNS regulates neuronal excitability at the NMJ (Yuan and Ganetzky, 1999). Yuan and Ganetzky hypothesized that Axotactin was required for the expression or localization of proteins required for action potential propagation, such as ion channels. It is possible that Wg expressed in SPGs plays a similar role to regulate neuronal activity. The Wg pathway has been shown to regulate

intracellular $Ca^{2+}$ release (Li et al., 2005). The loss of glial Wg could decrease the amount of intracellular $Ca^{2+}$ and result in a presynaptic defect such as vesicle release. Increases in $Ca^{2+}$ in astrocytes at hippocampal synapses has been shown to increase neurotransmitter release (Perea and Araque, 2007). Additionally, astrocytes released glutamate to activate metabotropic glutamate receptors in a $Ca^{2+}$-dependent manner. We could manipulate $Ca^{2+}$ levels in a glial Wg-RNAi background to determine if the decreased evoked EJP is rescued.

My work has defined the *in vivo* functional role of a novel glial-secreted factor (Wg) and determined that one-way neuron-glia signaling events modulate synapse formation and function. I have identified that the Wg is secreted from both glia and neurons, yet each has a different function at the synapse. Surprisingly, knock down of Porc in glia resulted in the same phenotype as knock down of Wg (increase in GluRs) but not in neurons. Porc post-translationally modifies Wg so that it can be secreted out of the cell. This suggests that molecules in the secretion process can give specificity to Wg. Porc could be required for glial Wg secretion but not for neuronal Wg. One way to test this would be to establish if synaptic Wg levels are decreased in a glial or neuronal Porc RNAi background. Another way would be to perform tissue specific rescue of Porc in a *porc* mutant. It is also possible that Porc regulates GluRs but in a Wg-independent manner. Korkut et al. (2009) demonstrated that Evi is required for the secretion of neuronal Wg. Further studies are needed to determine if Evi is expressed and functions in glia. As mentioned above, another way Wg could

be different is that glial and neuronal Wg activate different downstream Fz receptors or that one source of Wg requires a different co-receptor. Alternatively, proper development of the pre- and postsynapse could be dependent on overall synaptic Wg levels, with the observed differences due to the tools used (RNAi, Gal4 drivers, etc.), or certain aspects of synapse formation are more sensitive to Wg levels than others. One way to test this would be to distinguish whether the observed phenotypes were rescued when Wg was expressed in glia in a neuronal Wg-RNAi background and vice versa. A caveat to this would be that if the expression of Wg in glia provides too much Wg, and rescue does not occur. We would need to determine if overexpression of Wg in glia or neurons increases GluRs. Finally, location could be critical for the activation of glial or neuronal Wg. Although glial Wg reaches the synapse, it is possible that glial Wg primarily regulates the NMJ by acting on the neuronal cell bodies in the CNS. Another possibility along those lines is that glial Wg could act along the entire segmental nerve while neuronal Wg only acts on the bouton.

The differences between glial and neuronal Wg remain to be explored, but these experiments have helped to unravel some of the complexities of glial control of NMJ synapse formation and provide important insights into how glia and motor neurons use a single secreted factor, Wg, to coordinate the formation of a functional synapse.

**The importance of glia in the nervous system**

Rudolf Virchow first identified glia in 1856, and almost 100 years later Holger Hyden made a functional connection between them (Villegas et al., 2003). Over the past 50 years, our knowledge of glia has increased exponentially. We know that glia are not simply support cells and that there are several types of glia with specific roles that are critical for the health of a functioning nervous system. Yet, even today a nervous system is often defined as a network of specialized cells called neurons. However, some neurodegenerative diseases (amyotrophic lateral sclerosis, spinocerebellar ataxia, Huntington's disease, multiple sclerosis, etc.) are caused by malfunctions in glia (Barres, 2008). We know that neurons are important, but without glia neurons could not find their way to targets, regulate synaptic neurotransmitter levels, maintain ionic homeostasis or protect themselves from toxins and disease-carrying organisms, in other words, ultimately survive. We realize that what we know about glia is just the tip of the iceberg.

Over the past eight years, our laboratory has made a number of significant contributions to the field of glial biology. We have helped identify and characterize several glial subtypes in *Drosophila* that are functionally homologous to vertebrate glia, such as ensheathing glia, astrocytes and SPGs, which support the notion that glia are evolutionary conserved between vertebrates and invertebrates (Doherty et al., 2009; Freeman and Doherty, 2006; Fuentes-Medel et al., 2012; Fuentes-Medel et al., 2009; Logan and Freeman, 2007). We have established an injury assay to study the molecular mechanisms

behind axon degeneration and glial engulfment of axonal debris (MacDonald et al., 2006). Also, we have identified key components in signaling pathways, such as Drpr, Shark, Crk/Mbc/dCed-12, Rac-1 and Csw, which regulate glial engulfment during injury (Logan et al., 2012; MacDonald et al., 2006; Ziegenfuss et al., 2008; Ziegenfuss et al., 2012). Moreover, in the PNS we identified molecules such as Drpr and Mav and characterized SPGs as the glial subtype that regulates synaptic growth and sculpts synaptic activity at the NMJ (Fuentes-Medel et al., 2012; Fuentes-Medel et al., 2009). Additionally, my work has identified a novel glial-secreted factor, Wg, and provided a molecular foundation to understanding glial control of synapse formation. Finally, by identifying functional targets of Repo my work will help us understand how glial transcriptional mechanisms non-autonomously modulate glial function. In conclusion, our laboratory has helped to define *Drosophila* as a powerful model system to understand the underlying mysteries in glial biology.

In the field of *Drosophila* glial biology, the next big question at the NMJ would be to determine the mechanism that glia use to sense synaptic growth and modulate synaptic function. Is there a relationship between glial membrane coverage at the NMJ and neuronal activity? Also, is there a correlation between glial membrane coverage at the NMJ and the presence of glial secreted factors, or are they independent events? There are several techniques available and proven to be able to manipulate neuronal activity at the NMJ, such as activity paradigms or channel rhodopsin (Ataman et al., 2008; Olsen and Keshishian, 2012) that can

be combined with *in vivo* imaging and genetic tools to address these questions. Alternatively, glia could sense synaptic changes by sensing $Ca^{2+}$ (Harrisingh et al., 2007) or glutamate levels (Augustin et al., 2007), which could be manipulated in conjunction with *in vivo* imaging and a variety of genetic tools. Another interesting question would be to elucidate the function of PGs at the NMJ. Are they active partners in synapse formation and function, or are they a structural support for SPGs? Live imaging of SPGs while ablating PGs either genetically or using laser ablation could begin to address this question.

The identification of numerous functional targets of Repo from our ChIP-chip studies in S2 cells leads to the exciting possibility of identifying novel glial genes. However, performing the ChIP-chip *in vivo* in each glial subtype using transgenic flies that have a UAS-myc:repo would allow us identify novel glial genes in each subtype and novel roles of previously known glial genes. For example, a way to solidify the role of SPGs in NMJ synapse modulation would be to find secreted factors only expressed in SPGs. Alternatively, we could find secreted factors solely expressed in PGs that regulate NMJ synapses or signaling molecules involved in PGs to SPGs communication. Finally, these experiments would allow us to find candidates in other glial functions such as glial engulfment of injured axons or synaptogenesis in the CNS. Glia play a role in many complex functions such as development, learning and memory, and sleep, but the molecular mechanisms are poorly understood (Molofsky et al., 2012) (Havekes et al., 2012).

My work has helped to establish *Drosophila* as a model system to study the role of glia in synapse formation and function. The vertebrate field has only identified a few glia secreted factors out of hundreds of identified secreted factors across different cell types. This suggests that there are many more glial secreted factors that regulate synapses, which emphasizes how little we know about the complexities of the nervous system. My work has identified one novel secreted factor, Wg. The strength of using *Drosophila* as a system to study glial biology is the vast array of genetic tools and ability to perform forward and reverse *in vivo* genetic screens quickly. It would be possible to screen hundreds of secreted factors using RNAi and look for synapse defects at the NMJ. These studies in *Drosophila* will help to elucidate the molecules involved in these complex functions and hopefully will lead to identifying treatments and therapeutics for neurodegenerative diseases.

| Genotype | Wg levels | GluR volume | mEJP amplitude | mEJP frequency | EJP amplitude | Quantal content |
|---|---|---|---|---|---|---|
| *repo*$^{1}$ | ↓ | ↑ | – | – | – | – |
| *repo*$^{1}$*/Df* | ↓ | ↑ | – | – | – | – |
| *Repo-RNAi-all glia (lethal)* | n/a | n/a | n/a | n/a | n/a | n/a |
| *Repo-RNAi-SPG* | ↓ | ↑ | – | – | – | – |
| *Repo-SPG* | ↑ | – | – | – | – | – |
| *wg*$^{ts}$ | ↓ | ↑ | – | – | – | – |
| *wg*$^{1}$ | ↓ | ↑ | – | – | – | – |
| *Wg-RNAi-SPG* | ↓ | ↑ | ↑ | ↑ | ↓ | ↓ |
| *Wg-RNAi-neuron* | ↓ | ↑ | ↑ | = | = | ↓ |
| *PorcRNAi-SPG* | – | ↑ | – | – | – | – |
| *PorcRNAi-neuron* | – | = | – | – | – | – |

**Table 3-1. A summary of phenotypes when *repo*, *wg* or *porc* is manipulated.** The symbols in the table represent the following: increase (↓), decrease (↑), no change (=), and not done (–).

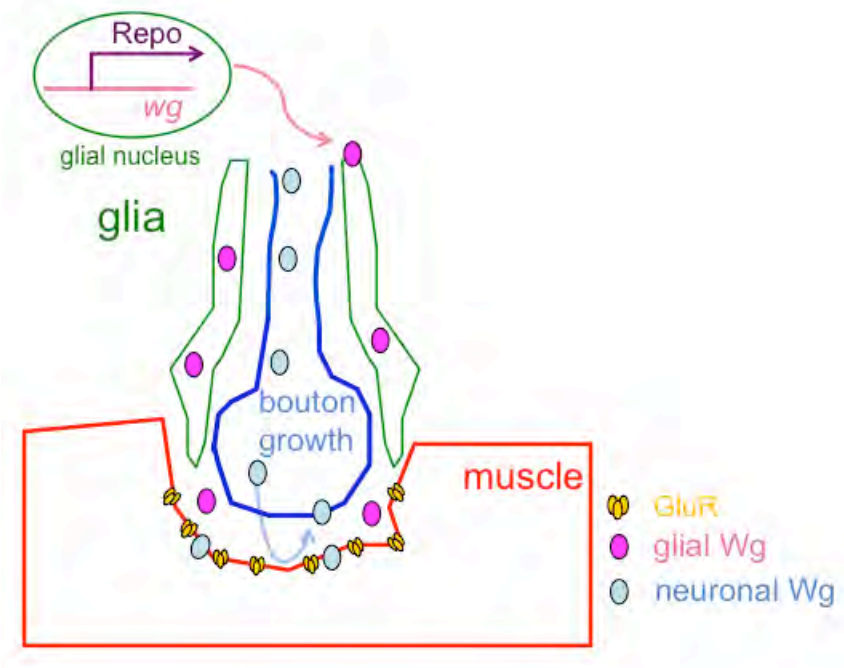


**Figure 3-1. A model for glial regulation of Wg at the synapse.**

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